STUDIES ON BIOLOGICAL PHOTOSENSITIZATION BY FUROCOUMARINS: PSORALENS

by

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43.	Neet - hair depilatory lotion	White Hall Labs., Inc. New York
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INTRODUCTION

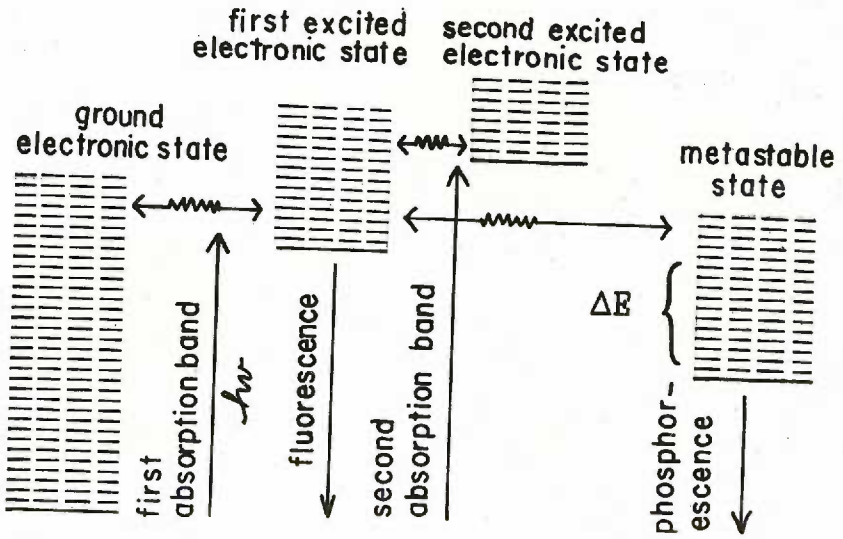
Since ancient times it has been recognized that light plays an important part in certain biological processes. Photosynthesis constitutes the only path by which organized nature has access to solar energy. The effect of ultraviolet light on proteins and the ensuing chemical changes reflected in the alterations of every conceivable property of the proteins (color, viscosity, solubility, isoelectric point, molecular weight, absorption spectra and even immunological specificity) are well known. The effects of light on human beings in terms of vision, synthesis of vitamin D, production of sunburn resulting in erythema, and pigmentation of skin are principal known effects. In chemistry, reactions of many types, e.g., synthesis, decomposition, hydrolysis, oxidation, reduction, polymerization, and isomeric change and free radical formation can be brought about by exposure to suitable light.

After many isolated observations on photosensitive reactions, Grotthus in 1817 and Draper in 1841 recorded their observations which advanced the study of chemical reactions initiated by radiant energy. The first law of photochemistry, known after these workers as Grotthus'-Draper's law, states "that energy must be absorbed in order to induce chemical events. Only light absorbed can cause a reaction; its mere passage through a medium offers no catalytic effect." Studies of the action of ultraviolet radiation on biological

systems is in reality a study of photochemical reaction or reactions. As in all photochemical reactions, the first phase is the capture of a quantum of radiant energy by some atom or molecule in the system. Absorption of light by a molecule results in a conversion of radiant energy into the energy of internal motion within the molecule. This motion may include kinetic changes of the molecule as a whole; vibrations of the atomic nuclei known as bond stretching or bending; and finally, motion of the electrons. The ground state of molecule can be visualized as that state in which the energies of all these motions have their minimum values. The capture of a quantum by a molecule adds a small amount of energy with the result that the energy level of some valence electron is raised, producing what is known as an activated or excited molecule. The duration of the activated state is very short (about 10^{-7} to 10^{-8} seconds). What happens after activation depends not only on the nature of the molecule that has captured the photic quantum, but upon the environment and probability of collision with the other molecules in its vicinity. This can be described in the following way: (a) An activated molecule may lose its energy without undergoing any photochemical reaction. (b) It may dissipate the excitation energy in the form of emission of light, which is generally known as fluorescence. (c) It may lose this increment of energy as heat radiation which results from the increased random rotational and vibrational states of the atoms in the

Figure 1: Schematic Diagram of Molecular Energy Levels

The broken lines represent varying degrees of vibrational excitation superimposed on the electronic energies. Each upward arrow represents the strongest transition within an absorption band. Each downward arrow represents transition within an emission band. The horizontal arrows represent internal conversion transitions.


first excited electronic state
second excited electronic state
ground electronic state
metastable state
first absorption band
hν
fluorescence
second absorption band
ΔE
phosphor-escence

molecule. (d) The activated molecule may be disrupted with the resultant formation of two or more product molecules. (e) It may result in the rupture of chemical bond resulting in the formation of free radical, that is a molecule containing one or more unpaired electrons. By virtue of their high reactivity, the free radical may interact with other molecules in the system in ways that the parent molecule could not. (f) Depending upon the energy of the quantum, the activated molecule may cause the ejection of the electron from an atom and bring about ionization similar to the effects of X-rays, thereby making the molecule a positively charged ion. (g) Many organic molecules, particularly those with conjugated structures, have in addition the ability to radiate after passing through an electronic state intermediate in energy between the normal ground state and the fluorescent state. This type of luminescence, where the emission of energy continues after the irradiation ceases, is known as phosphorescence. Phosphorescent molecules are believed to be in triplet state, that is, a bi-radical in which two electrons have unpaired spins. In biology and chemistry, this state is of great importance, since it is much more reactive chemically than fluorescent state, arising both from its free radical nature and because of its relatively long life. Most of the dyes that sensitize photochemical oxidations have evident phosphorescent properties (Blum[11]). A schematic diagram of molecular energy levels which illustrates these possibilities of energy transfer is given in Figure 1. The multitude electronic energy levels are represented by

horizontal lines. For an ordinary stable molecule, the ground level G and two excited levels F and F1 are singlet states. The metastable level M is presumably a triplet level.

Photodynamic or photosensitization effect: Ultraviolet light has been known to directly effect a number of biological changes including pigmentation of skin (Blum[11]), death of bacteria, inactivation of viruses, production of genetic lethals and other mutations in higher plants and animals (Hollaender[46]), destruction of the activity of isolated enzymes and proteins (McLaren[62]). Moreover, biological systems may be sensitized to light by a variety of organic materials. These include many dyes, such as eosin, methylene blue, acriflavin, acridine, some naturally occurring substances such as free porphyrins, chlorophyll, and some carcinogenic hydrocarbons such as methyl cholanthine (Blum[11], Fowlks[35]). Irradiation of the combined photosensitizer and biological material results in changes in biological activity. However, in the absence of the photosensitizing agent no effect is observed. The phenomenon was discovered by Rabb a little over half a century ago in Professor Von Tappeiner's laboratory in Germany (Blum[11]). He observed that the toxicity of acridine towards paramecia was proportional to the amount of light incident on the biological system. Thinking it to underlie photobiological effects in general, Von Tappeiner called it "photodynamische Erscheinung" or photodynamic action. As defined by Blum and associates(Blum[11], Clare[20]) the term photodynamic action is

meant an oxidation by molecular oxygen brought about in a biological system exposed to radiation through the agency of a fluorescent substance. In accordance with this hypothesis, the photodynamic action may be conceived as follows: the photosensitizing substance acts as a chromophore, absorbing light quanta which is thereby transformed to an excited or activated state. The energy of this activated molecule can be transmitted to a second molecule with which the first may collide. In collision with other molecule of lower energy status, the activated molecule can either bring biological changes or lose its energy in various forms as described earlier. One can represent the reaction in an overall way as follows:

$$P \xrightarrow{h\nu} P^* \text{ (activated molecule)}$$

(i) $P^* + O_2 + S \rightarrow S(OX) + P$

(ii) $P^* \rightarrow S \rightarrow S' + P$

Where P is the photosensitizer and S any substrate, S' a new product and P* is activated state of the molecule, P appears again on the right hand side indicating that the chromophore having released its added energy is reverting to ground state. P* can activate another molecule or substrate; the energy transferred can also bring changes in the biological system without oxidation. The activated photosensitizer may also cause one of the three following effects: (a) chemical combination of photosensitizer with the sensitive cell constituent; (b) indirect excitation of cell constituents which through chemical changes cause inacti-

vation of the vital cellular activities; (c) it can pass its energy on to the cell constituents inside the cell by long range dipole-dipole transfer. However, McLaren [62] and Blum[12] have stressed that many of the effects of ultraviolet light are independent of oxygen (for instance, inactivation of proteins and enzymes where independence of O_2 has been clearly demonstrated). Hence in this thesis, the term "photosensitization" will be used as the more general term covering any observable change in a system containing biological material and photosensitizer which occurs only as a result of irradiation of the mixture.

Photosensitizing compounds: The photosensitizing compound most extensively investigated is methylene blue, although other dyes like fluorescein, eosin, rose bengal, and several others have been shown to induce similar action. Compounds with porphyrin ring, the chlorophylls, hematoporphyrins and flavins of natural origin have been also studied. Several carcinogenic hydrocarbons have also been implicated in photosensitizing action[11]. A new group of naturally occurring compounds, the furocoumarins, more generally known as psoralens, has recently been shown to possess photosensitizing properties (Lerner et al.[59], Fitzpatrick et al.[32], Fowlks et al.[34], Musajo et al.[65], Pathak and Fitzpatrick[72].

Biological changes in photosensitization: From the studies of the effects of ultraviolet on the biological systems, it may be possible to get some idea about the nature of the photosensitized biological re-

Table I Biological materials and effects observed following ultraviolet irradiation

Biological material	Effect noted
Amino acids	
alanine	oxidative decarboxylation
cystine	splitting of -S-S-bond
dihydroxyphenylalanine	oxidation to melanin
histidine	oxidative decarboxylation
methionine	formation of methionine sulfoxide
phenylalanine	oxidation
tyrosine	oxidation - melanization, also oxidative decarboxylation
proline	oxidative decarboxylation
glutathione	-SH group partially oxidized to -S-S-
Proteins	
casein	O_2 absorbed
collagen	becomes partly soluble
insulin	oxidation of aromatic amino acids
-lactoglobulin	oxidation of aromatic amino acids
human albumin	destruction of histidine & tryptophan & denaturation
horse albumin	" " " "
egg albumin	absorption maximum at 2800Å disappears, aldehydes and sulphydryl groups liberated, denaturation and coagulation
serum albumin	change in surface tension, aldehydes liberated, increased UV absorption, coagulation differed
human globin	destruction of histidine, tryptophan & denaturation
horse globin	" " " "
blood plasma	" " " "
gelatin	loss of water swellability, ammonia liberated, increase in electrical conductivity, decrease in viscosity
keratins	intramolecular changes in spacing
lens protein	developed opacity
serum euglobulin	changes in UV absorption
wool	liberation of sulfide ions, H_2S and also loss in strength and extensibility
Enzymes	
chymotrypsin	destruction of histidine and tryptophan residue
hyaluronidase	inactivation
lysozyme	destruction of histidine and tryptophan residues
ptyalin	inactivation
ribonuclease	destruction of histidine and tryptophan residues
rat liver mitochondria	destruction of succinicdehydrogenase, cytochrome, oxidase, acid phosphatase, alkaline phosphatase, apyrase activity, no change in catalase.

Table I (Continued)

yeast mitochondria	destruction of cytochrome oxidase, apyrase activity
deoxyribonucleic acid	depolymerization
bacteriophage	inactivated
viruses (herpes encephalitis, ecto malia, rabbies, typhus, horse-encephalomyelitis)	inactivated
Cellular organisms	
bacteria (E. coli - strains)	killed
spirochetes	killed
trypanosome	killed
neurospora crassia	killed
paramecia	killed
P. caudatum	killed
chick embryo cell culture	inactivated, interference with mitosis
erythrocytes (human, rabbit)	hemolysis
s. aureus	killed
Tissues and organs	
perfused dog lung	histamine-like substance released
chick aminion-urinary bladder	contraction
rootlets	inhibition of growth
Multicelled creatures	
larvae (mosquito, drosophilia melanogaster, ranaesulenta	killed
frog skin	increase of pigment, cellular damage
animal skin (human, guinea pig, rabbit, sheep, mice, rats	erythema, oedema, residual pigmentation

sponses. The following table shows the biochemical changes observed following irradiation in presence of photosensitizer. Table I has been abstracted from the reviews (McLaren[62], Fowlks[35]).

It appears that the photosensitization leads to the oxidation of histidine, tryptophan, tyrosine, methionine and cystine. It also affects the constituents of cells eg., cell membrane, cytoplasm, cytoplasmic organelle (mitochondria, microsomes) and nuclear material.

The most obvious effects produced by light in presence of a photosensitizer in several of these biological systems may therefore be summarized as reactions involving denaturation, rearrangements and sensitization resulting in the damage of cellular functions. Other physical changes not reported in the table are alterations in viscosity, diffusion co-efficient, electrophoretic mobility, dialyzability, absorption spectra, optical rotation, etc.

All these changes reveal that the wavelength of light and hence the energy which has been absorbed in the reacting system has induced some physical or chemical changes in the biological material. It may be of value to consider at this state, the energy associated with a few wavelengths of light. These values were derived mathematically by the relation $E = hv$, where E is the energy in photon called quantum, h is the Planck's constant which is 6.56×10^{-27} erg second and v is the frequency of light per second which is equal to c/λ, where c = velocity of light and is equal to 3.0×10^{10} cm /second, and λ is the wavelength in cen-

timeters. The energy has been calculated in terms of moles, of 6.02×10^{23} (Avogadro number) rather than single molecule and is known as energy per Einstein. The values have been finally converted to calories by means of the relation 10^7 ergs = 1 joule, and 4.18 joules = 1 calorie.

Table II A Energies associated with few wavelengths of light

wavelength (Angstrom)	energy, K cal/mole (the Einstein)
1000	286
2000	142
2537	112
2850	100
3130	91
3600	78.5
4000	71
5000	57
6000	47.6
7000	41
8000	35.7
10000	28.6

In Table II B, some bond dissociation energies, based on the measurements with simple molecules as well as proteins are listed. The values for this table are based on reviews by Szware[86] and McLaren[62] and measurements carried out by Franklin and Lumpkin[38].

Table II B Bond dissociation energies in proteins and peptides

Bond	K. cal/mole	Bond	K. cal/mole
C- C	182	O-H	118
C = C	150	C-H	100
C = O	145	C-S	55
C-N (amide)	98	C-O	90
C-N (peptide)	45	S-S	64
C-N (amine)	66	C-Cl	75
N-H	104	S-H	87

It is evident that energy required for photosensitized biochemical reactions is confined to visible and ultraviolet light and is sufficient to cause displacements of outer electrons in the molecule. It is also evident from these two tables that if allowance is made for rotational and vibrational energy losses, sufficient energy at certain wavelengths is still available to break any single bond in the molecule. This also explains why longer infrared radiation is not effective to produce chemical or photosensitized reactions. The photosensitized reactions are believed to be initiated by a quanta with an energy greater than 95 K cal.

The site of absorption of energy: In spite of great volume of work on the photochemical reactions of amino acids, peptides, proteins and other biological systems, little is known about the site of the absorption of energy. The main process involved in the photochemical or photosensitized reaction is the formation of two radicals by breakage of a peptide bond. Also other reactions such as decomposition of cystine or cysteine, breaking of hydrogen bonds and photooxidation of aromatic chromophores in presence of oxygen eg., tyrosine to dihydroxyphenylalanine, liberation of ammonia from histidine and rupture of the imidazole ring have been observed (Weil et al[97]). Two independent theories have been put forward. The first (McLaren and Finkelstein[63]) is that the light is absorbed by the tyrosine and tryptophan chromophores and is passed through two CH_2 groups to the peptide bond on either side of

the tyrosyl residue. Evidence in favor of this hypothesis has been presented by several workers (Carpenter[17], Mitchell and Rideal[64]) but recent investigations have shown that ultraviolet absorption of proteins is not quantitatively accounted for by their tyrosine, tryptophan, and phenylalanine content. Contribution to the absorption in the same region by the peptide bond has been also shown[62]. The second theory is that the quantum is absorbed at the peptide bond itself (Rideal and Roberts[75]). Evidence in favor of energy being absorbed at the peptide bond is given by a study of Mandl et al.[61] in which quantum yields for several hemipeptides at 2537Å were compared. The quantum yield of peptide bond breaking was found to increase with the number of carbon atoms separating the peptide bond and the phenyl ring. The highest quantum yield was shown by acetylalanine, possessing no aromatic chromophores. West and Miller[101] have proposed a third alternative involving photosensitization mechanism. They postulated that the aromatic ring is a very inefficient sensitizer of the peptide bond. However, since it absorbs considerable radiation at 2500Å and higher wavelengths, it contributes to the photohydrolysis process as well as photolysis of the peptide bond. The mechanism of this hypothesis has been stated as follows:

$$A + h\nu \longrightarrow A_1^* \longrightarrow A_2^*$$
$$A_2^* \longrightarrow A + h\nu' \text{ (fluorescence)}$$
$$A_2^* + B \longrightarrow A_2^*B \longrightarrow A + B^* \text{ sensitization}$$
$$B^* \longrightarrow P+Q \text{ (two products)}$$

where A is the aromatic chromophore which fluoresces, A_1^* and A_2^* are activated states, B is the free -CO-NH-group, A_2^*B is the chromophore collision complex.

It has been suggested that in the photodynamic action of methylene blue on tyrosine, tryptophan, methionine and nicotine may involve a dehydrogenation and subsequent H_2O_2 or organic peroxide formation (Weil et al.[96, 97, 99]). The oxidizing agent can be used in the oxidation of tyrosine or tryptophan leading to the rupture of aromatic nucleus, or in presence of methionine, it may give rise to dehydromethionine and methionine-sulfoxide. The same idea about peroxidatic reaction has been entertained with regard to other radiation effects[2]. But the studies of Fiala[31], Vodrazka and his associates[94, 95], do not support this hypothesis. Their well controlled observations indicated that neither organic peroxide nor hydrogen peroxide appear upon irradiation in presence of a photosensitizer.

It is clear that little can be stated positively about the mechanism of photosensitization. It is not certain where the quantum is absorbed or what the mechanism of the primary process in inactivation or denaturation is and how, if the aromatic chromophores of a protein or polypeptide absorb the incident light, the energy is passed from the site of the absorption to the reactive bond. With this general outline on photosensitization, the object of the subsequent presentation will be to describe the studies related to the photosensitizing action of furocoumarins, more generally known as psoralens. But before presenting the different phases of this study, a brief historical background about how psoralens were recognized as agents inducing photosensitizing ac-

tion in several biological systems will be provided.

Historical aspects of psoralens: Although the first furocoumarin was isolated over a hundred years ago, when in 1834 Kalbrunner isolated bergapten (5-methoxypsoralen) from bergamot oil, the biological importance remained unknown until very recent times. Indeed the plants containing these compounds have been used in certain folk remedies for skin diseases in India and Egypt (Fitzpatrick and Pathak[33]). Some of man's earliest efforts were concerned with attempts to restore the normal skin color to scattered, pigmentless areas considered to be leprosy, although most of which were probably vitiligo. In the Indian sacred book "Atharva Veda" which dates back to 1000 B.C. or earlier, the "cure" of leprosy and leukoderma (vitiligo) has been detailed which involved the use of certain black seeds together with Bringarga (Ellipta prostata), Indra varuni (Colocynth) and turmeric (Curcuma longa). Sunlight was stated to be most essential. In other ancient Indian medical literature such as Astanga Hridaya Samhita by Vagbhata, or "Medicine", a large German encyclopedia of Indo-Iranian studies, reference is made to the use of the plant "Bavachee", a species containing psoralen[9, 102]. In "Bower-Manuscript"[30] which deals with the early remains of Buddhist literature found in Eastern Turkestan, and the studies of medicine in ancient India (about 200 A.D.), the cure of leukoderma with the plant known as "Vasuchika", a form of Bavachee (Psoralea corylifolia) has been distinctly mentioned. In "Sino-Iranica" written by Berthold Laufer[57] a drug called pu-ku-ce (or bu-kut-tsi)identified

as Psoralea-corylifolia by the Maci-collaborator in the Kai Pao Pen Tscao (A.D. 968-976) of the Sung period has been discussed in detail as a treatment for restoring skin pigmentation. The author further comments that the plant named Bwa-ku-ci or Ba-ku-ci popularly but erroneously written as Pu-Ku-Ce is not of Chinese origin but of Indian origin. It resembles the Sanskrit word Va ku-ci or Vasuchika, which is Psoralea-corylifolia. This plant has been used by Hindus in the Ayuvedic system of medicine, and has subsequently been shown to contain several photosensitizing furocoumarins including psoralen.

Another important plant, Ammi-majus (Lin), a wildly-growing weed found in the Nile Valley, had been employed for centuries as a "cure" for leukoderma. Ibn-El Bitar, who lived in 13th century, gave a description of the usefulness of this plant for leukoderma in his famous book "Mofradat El Adwiya" [49]. This plant was used by the Ben-Shoeb, a Berberian tribe in the northwestern African desert. This plant was found to contain several potent photosensitizing compounds, the most important of which is 8-methoxypsoralen.

Many instances of dermatitis following exposure to solar rays in meadows or fields where the skin came into contact with green plants, particularly of Umbelliferae and Rutaceae families, began to appear in literature by 1930 (Blum[11]). Contact with parsnip plant (Pastinaca sativa) or extracts from various parts of these plants followed

by exposure to sunlight or mercury arc radiation were observed to produce marked photosensitized erythemal response. In 1938, Kuske[56] investigated this condition known to dermatologists as phytophotodermatitis, a bullous eruption appearing on the areas of the skin which came in contact with plant species and resulted from exposure to the sun. Working with parsnip, figs and several other species, Kuske believed that the photosensitizing substances responsible for this condition were members of the furocoumarin group. He obtained fairly pure compounds by extraction from oil of bergamot (bergapten), masterwort or peucedenum ostruthium (oxypeucedanin) and figs (psoralen). His observations remained unnoticed until the present.

While it is true, that many of these ancient herbal remedies and researches have remained unnoticed and have suffered considerable decline in usage, at least in the form of the drug itself or their infusions and decoctions, yet it appears that a larger proportion than ever of the medicinal agents in present use are derived from plant substances. It cannot be gainsaid that there is currently in evidence an increasing trend to restudy and reintroduce into medical practice vegetable drugs which had appeared to have gone into decline in medicinal acceptance. The history of psoralens is not an exception to this fact. The modern period of psoralen research began when in 1941, Fahmy and his group at the University of Cairo, Egypt, observed that some Egyptian herb doctors were using a grey green powder called "Atrillal" for the treatment

of vitiligo. He ascertained that this powder was obtained from the fruits of a weed called Ammi majus Linn growing widely along the Nile delta. Fahmy and Abushady[29] in 1947 isolated three crystalline compounds which they believed were the active ingredients of the crude powder. These were identified as furocoumarins and were named after the plant from which they were obtained (Ammi majus Linn): ammoidin, ammidin and majudin. This was an unfortunate selection of names for two of the compounds had already been known. Ammoidin or 8-methoxypsoralen, had been isolated in 1911 from different plant sources (Hans Priess[73], Thoms[88]) and was synthesized in 1933 by Späth[85]. Majudin or 5-methoxypsoralen was a well-known constituent of oil of bergamot and had long been used in the perfume industry.[85] The organic chemists particularly Spath and his associates[38] had not only established the constitution of several naturally occurring furocoumarins, but had succeeded in synthesizing them and characterizing their properties (see also Sethna and Shah[81]). This plant (ammi majus) is a member of parsley family (Umbelliferae) and is known in the United States as bishop's weed. 8-Methoxypsoralen is also known as xanthotoxin and methoxsalen.

The clinical trials of psoralens were initiated by El Mofty[28] and involved the deliberate photosensitization of the skin of patients with vitiligo. The results were encouraging. Soon afterwards, in Europe, particularly in France and England, several dermatologists had started

using 8-methoxypsoralen and other furocoumarins present in Ammi majus for repigmenting the pigmentless skin areas. While early studies with 8-methoxypsoralen used sunlight as radiation source, Musajo et al.[65, 66] demonstrated with an artificial light source the effectiveness of frequencies in the long-wave ultraviolet in producing typical photosensitized responses of erythema followed by pigmentation. In the United States, Lerner, Denton and Fitzpatrick[59] in 1952 initiated studies on 8-methoxypsoralen in treatment of vitiligo and reported the testimonial type of evidence of increased sun tolerance of vitiligo skin and albino skin following controlled treatment. Soon it became apparent that psoralen or perhaps one of its derivatives might possibly be developed as an oral drug to be used to increase the tolerance of human skin to sunlight. Several avenues of research investigations emerged. Fowlks et al.[34] reported the photosensitization effect of furocoumarins on bacteria in presence of long-wave ultraviolet light resulting in lethal effects. Chakraborty et al.[18] observed that out of seventeen natural coumarins which they tested, the furocoumarins including psoralen and imperatorin were most effective antifungal agents. Musajo[66] had mentioned results of Dolcher, Rodighiero and Caporale describing the mutagenic properties of furocoumarins and found 5-methoxypsoralen and psoralen to be almost as effective as the most effective mutagenic agent trypaflavin. It has long been known that people coming in contact with figs, cow parsnips, wild growing parsnips, people engaged in canning

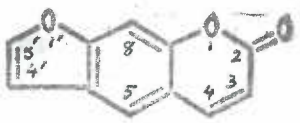
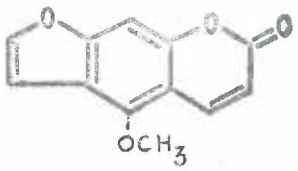
industries processing fruits and vegetables such as carrots, celery, parsley, etc., exhibit photosensitized dermatitis resulting in vesication, erythema followed by residual pigmentation. The author[71] has presented evidence to suggest that phytophotodermatitis due to external contact with many plant species mainly confined to a few families (umbelliferae, rutaceae, mimoceae, leguminosae) is due greatly, if not entirely, to the furocoumarin group of compounds present in these plants. The occurrence of many of these compounds in common edible vegetables particularly in tropical regions of the world where abundant sunshine has constantly augmented the pigmentogenic action from time immemorial, has led this author to consider that olive skin of the Asiatic people, the highly pigmented skin of African natives and other people residing in the tropical region, has been due to two factors: (1) ultraviolet light of the sun, and (2) presence of furocoumarins in the daily foods of these people.

The fact that these compounds are found in various plant materials leads naturally to teleological speculation as to what other biological function they fulfill. Fowlks[36] has suggested that furocoumarins appear to have specific biochemical properties, which may contribute to the survival of certain plant species. He stated that these compounds belong to that group of substances which can inhibit certain plant growth without otherwise harming the plant. Bennett and Bonner[5] isolated Thamnosmin from leaves of desert rue, "Thamnosma montana" because a crude extract of this plant was the best inhibitor found among

the extracts of a number of desert plants surveyed for the property. Thamnosmin was found to be a furocoumarin related to isopsoralen structure. Rodighiero[76] has shown that these furocoumarins (psoralen, 8-methoxypsoralen, etc.) inhibit seed germination, root growth and seedling growth. Thus it is possible that they might have a role as natural growth regulator of certain plants. In this connection it is worthwhile to point out that the amount of these substances in a growing plant like Ammi majus, before appearance of fruits or seeds, is insignificant, but the moment seeds are being formed, one notices high concentration of these substances in leaves, stalks, and the pericarp of the fruit (personal observations). Germination of these seeds (psoralea corylifolia) also takes a long time even under ideal conditions and is observed when the fluorescence intensity and thus the psoralen concentration of seeds drops significantly, indicating that the germination inhibitor has been washed out and the seed is ready to germinate.

The chemistry of psoralens: The psoralens belong to a group of compounds which have been considered as derivatives of coumarin, the furocoumarins. The fusion of a pyrone ring with a benzene nucleus gives rise to a class of heterocyclic compounds known as benzopyrones, of which two distinct types are recognized, (1) benzo-α-pyrones, commonly called coumarins and (2) benzo-γ-pyrones, called chromones, the latter differing from the former only in the position of the carbonyl

TABLE III Distribution of furocoumarins in nature

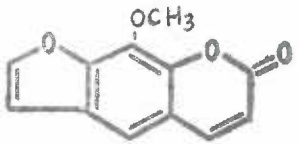
No.	Compound & Structure	Natural sources	Common name	Family	References *
1.	Psoralen (Ficusin) 	Psoralea corylifolia	Babachi	Leguminosae	1, 2, 3
		Ficus carica	Fig	Moraceae	2, 4, 5
		Coronilla glauca		Leguminosae	6, 7
		Phebalium argenteum			8, 9
		Xanthoxylum flavum	(West Indian Satin Wood)	Rutaceae	10
2.	Bergapten or 5-methoxypsoralen 	Ficus carica	Fig	Moraceae	2, 4, 7, 12, 13, 14, 15
		Fagara xanthoxyloides	(Artar prickly ash)	Rutaceae	2, 13, 16
		Skimmia laureola	Neere	Rutaceae	2, 7, 13 18
		Citrus bergamia(Risso)	Bergemot oil	Rutaceae	2, 7, 13 19
		Ruta graveolens	Rue	Rutaceae	7, 14, 15 20, 21, 22
		Citrus limonum	Lemon	Rutaceae	14
		Citrus acida	Lime	Rutaceae	13, 23
		Fagara schinofolia		Rutaceae	24
		Ligusticum acutifolium		Umbelliferae	2, 7, 13
		Ligusticum acutilobum		Umbellifarae	17
		Heracleum sphondylium	cow parsley	Umbellifarae	2, 7, 11 13, 15
			European cow parsnip		
		Heracleum giganteum		Umbelliferae	7, 13, 15

* Reference numbers in this column are listed separately and are detailed at the end of bibliography under the title, "References for Table III: Distribution of furocoumarins in nature."

Table III (Continued)

Heracleum nepalense		Umbellifarae	7, 15
Seseli indicum	Hoegen Celery	Umbellifarae	2, 7, 13, 15, 66
Pastinaca sativa	Garden parsnip	Umbellifarae	7, 15, 25
Heracleum lanatum	Cow parsnip	Umbellifarae	26
Angelica archangelica	Angelica, Engelwurz	Umbellifarae	27, 28
Ammi majus	Bishop's weed	Umbelliferae	15, 29
Pimpinella magna		Umbelliferae	7, 15, [illegible]
Pimpinella saxifraga	Cow Parsnip	Umbelliferae	2, 7, 13 15, 30, 31
Petroselium sativum	Garden Parsley	Umbelliferae	7, 15, 32

3. Xanthotoxin or 8-methoxypsoralen or methoxsalen



Ammi majus	Bishops weed	Umbelliferae	29, 33, 34 35, 61
Angelica archangelica	Engel wurz	Umbelliferae	53
Pastinaca sativa	Garden Parsnip	Umbelliferae	25
Ficus carica	Fig	Moraceae	14
Ruta chalepensis		Rutaceae	2, 7, 13 15, 38
Fagara xanthoxyloides		Rutacae	2, 3, 16, 37
Ruta montana		Rutaceae	7, 15, 39
Aegle marmelos	Bel	Rutaceae	40
Ruta graveolens	Fig	Rutaceae	7, 14 15, 21
Luvanga scandens	Lavanga	Rutaceae	41
Xanthoxylum flavum	West Indian Satin Wood	Rutaceae	10
Ruta bracteosa		Rutaceae	38

Table III (Continued)

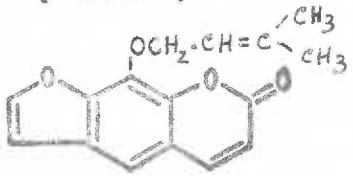
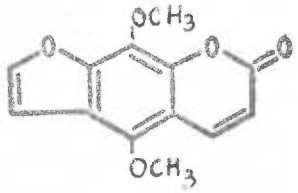
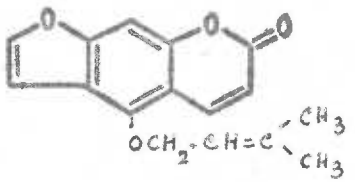
4. Imperatorin (8-isopentenyloxy-psoralen) 	Imperatoria ostruthium	Masterwort hog fannel	Umbelliferae	2, 13, 63
	Angelica glabra		Umbelliferae	2, 13, 40
	Angelica archangelica	Engel wurz	Umbelliferae	2, 13, 36
	Ammi majus		Umbelliferae	34, 35, 61
	Paucedanum ostruthium	masterwort	Umbelliferae	2, 3
	Aegle marmelos	Bel	Rutaceae	13, 49, 62
	Ruta chalepensis		Rutaceae	27
5. Isopimpinellin (5, 8-dimethoxy psoralen) 	Pimpinella saxifraga	Cow parsnip	Umbelliferae	2, 13, 43, 54
	Heracleum sphondylium	Cow parsley	Umbelliferae	2, 13, 44
	Sesli indicum	hog celery	Umbelliferae	2, 13, 66
	Skimmia laureola	neera	Rutaceae	18
	Citrus aurantifolia	West Indian Lime Oil	Rutaceae	46, 13
	Luvanga scandens		Rutaceae	41
	Thamnosma montana		Rutaceae	47
	Fagara oilanthoides		Rutaceae	48
	Heracleum lanatum (var. nipponicum)		Rutaceae	26
6. Isoimperatorin (5-isopentenyloxy-psoralen) 	Peucedenum ostruthium	masterwort	Umbelliferae	2, 3, 13
	Imperatoria ostruthium	masterwort	Umbelliferae	2
	Pastinaca sativa	garden parsnip	Umbelliferae	55

Table III (Continued)

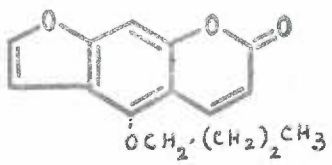
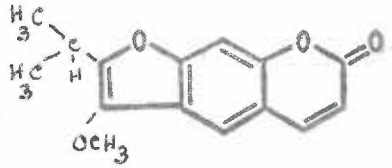
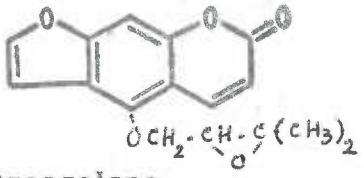
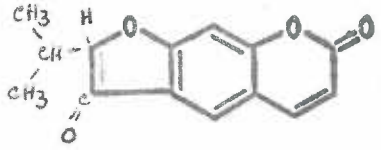
7. Prangenine	Prangos Pabularia		Umbelliferae	56
				
8. Peucedanin (4'methoxy, 5'-isopropyl-psoralen)	Peucedanum officinale	Masterwort	Umbelliferae	2, 57
	Prangos pabularia		Umbelliferae	13
				
9. Oxypeucedanin (5-epoxy isopentenyloxy)	Peucedanum officinale	Masterwort	Umbelliferae	2, 3, 13
	Peucedanum ostruthium	Masterwort	Umbelliferae	13
	Prangos pabularia		Umbelliferae	13
	Imperatoria ostruthuim	Masterwort	Umbelliferae	7
				
10. Oreoselone	Peucedanum officinale	Masterwort	Umbelliferae	3
	Peucedanum oreoselinum			58
				

Table III (Continued)

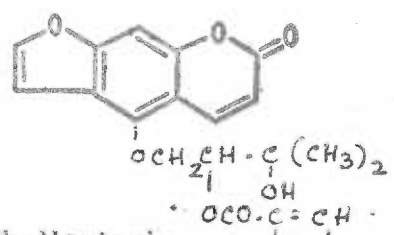
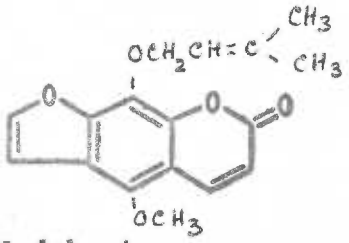
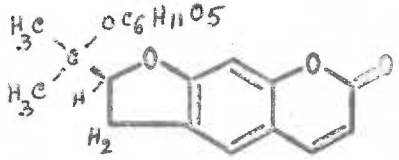
11. Ostruthol 	Peucedanum ostruthium	Masterwort	Umbelliferae	42, 2, 3, 13
12. Phellaptorin (5-methoxy, 8-isopenteny-loxypsoralen) 	Angelica glabra Phellopterus littoralis		Umbelliferae Umbelliferae	3 3
13. Nodakenin (4'-5' dihydro, 5'(-1-glucosoxy-isopropyl) psoralen) 	Peucedanum decursivum		Umbelliferae	45, 13

Table III (Continued)

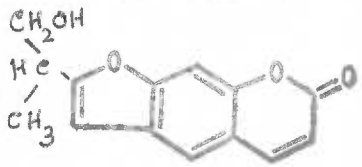
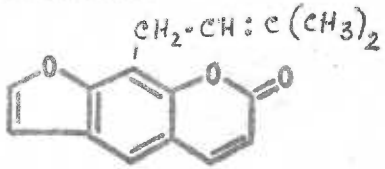
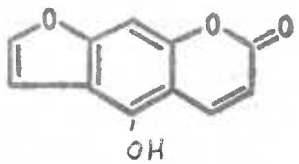
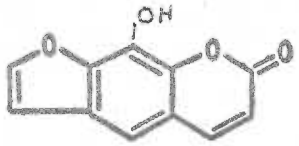
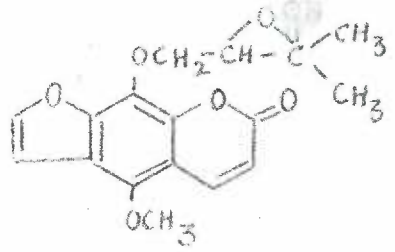
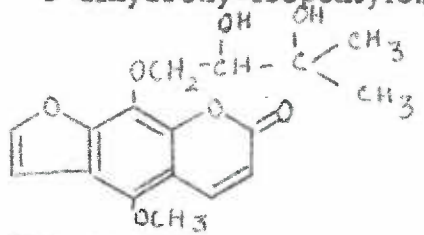
14. Nodakenetin (aglucone of Nodakenin) 	Peucedanum decursivum	Marmesin	Umbelliferae	13, 45 64, 65
15. Psoralidin 	Psoralea corylifolia	Bavachi	Leguminosae	60
16. Bergaptol (5-hydroxypsoralen) 	Citrus bergamia Risso Citrus aurantifolia	Bergamot oil West Indian lime oil	Rutaceae Rutaceae	2, 3 13, 19, 46
17. Xanthotoxol (8-hydroxypsoralen) 	Angelica archangelica	Engelwurz	Umbelliferae	2, 13, 3, 36

Table III (continued)

18.	Byak angelicol (5-methoxy -8-epoxyiso pentenyloxypsoralen)	Angelica glabra	Byakusi (Japanese Ivy)	Umbelliferae	13



19.	Byak angelicin (5-methoxy -8-(2, 3-dihydroxy-isopentyloxy)	Angelica glabra	Byakusi (Japanese Ivy)	Umbelliferae	13



20.	Bergamotin (5-geranyloxy-psoralen	Citrus aurantifolia	Bergamot oil Persian lime	Rutaceae	8, 13

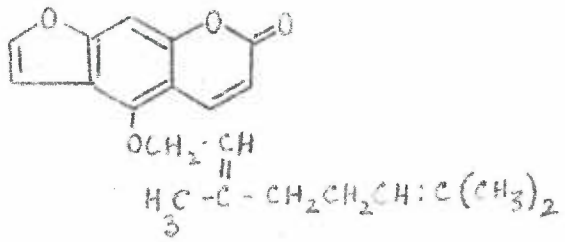


Table III (Continued)

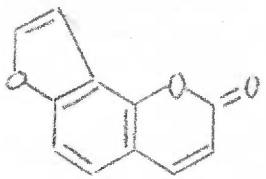
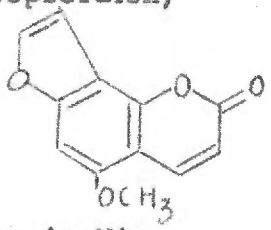
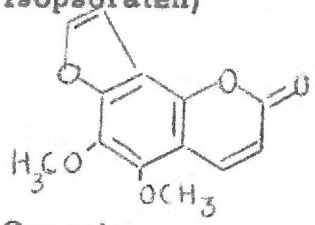
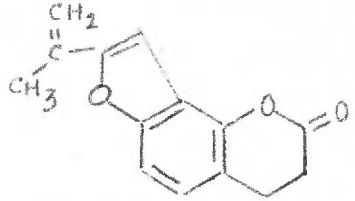
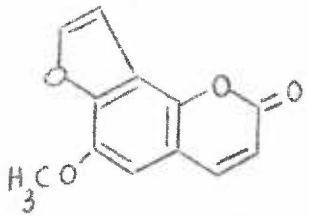
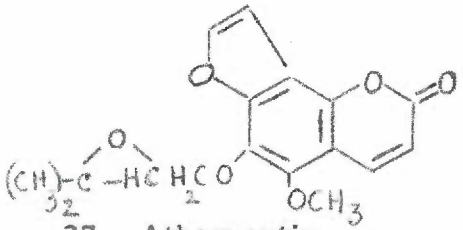
21.	Angelecin (Isopsoralen) 	Psoralea corylifolia	Bavachi	Leguminosae	50, 51, 13
		Angelica glabra		Umbelliferae	13, 52, 53
22.	Isobergapten (5-methoxy-isopsoralen) 	Pimpinella faxifraga	Cow parsnip	Umbelliferae	2, 13, 45, 54
		Heracleum sphondylium	Bibernell	Umbelliferae	2, 13, 43 44, 54
		Heracleum lanatum	Cow parsnip	Umbelliferae	26
		Pimpinella magna		Umbelliferae	7, 9
23.	Pimpinellin (5, -6-dimethoxy-isopsoralen) 	Pimpinella saxifraga	Cow parsnip	Umbelliférae	2, 13, 43
		Heracleum sphondylium	Cow parsley	Umbelliferae	2, 13
		Pimpinella magna		Umbelliferae	7, 9
24.	Oroselon 	Peucedanum oreoselinum		Umbelliferae	58, 59

Table III (Continued)

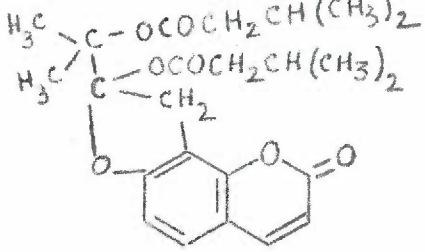
25. Sphondin (6-methoxy-isopsoralen	Pimpinella saxifraga	Cow parsnip	Umbelliferae	30, 31
	Heracleum sphondylium		Umbelliferae	2, 3, 44
	Thamnosma montana		Rutaceae	47
	Heracleum lanatum		Rutaceae	26



26. Thamnosmin	Thamnosma montana		Rutaceae	47

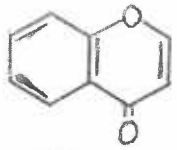


27. Athamentin ((4', 5'-dihydro-5'-(1-hydroxy-isopropyl), 4'-hydroxy diisovaleryl ester))	Athamanta oreoselinum		Umbelliferae	59
	Peucedanum oreoselinum		Umbelliferae	3, 59



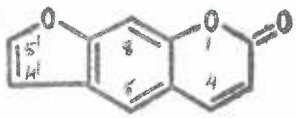
group in the heterocyclic ring as shown below:

Benzo - α - pyrone

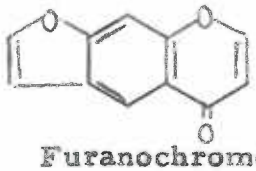


Benzo - γ - pyrone

If the furan ring is built on a suitably substituted coumarin or chromone derivative, it leads to the synthesis of coumarono coumarins or coumaronochromones, more generally known as furocoumarins or furanochromones respectively, the structuresof which are shown below:

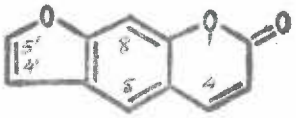


Furocoumarin

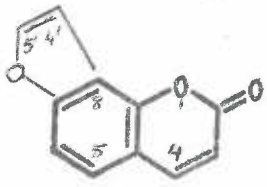


Furanochromone

There are twelve different ways a furan ring can be condensed with coumarin molecule and each of the resulting compounds can become the parent for a family of derivatives. But nature seems to be conservative and it appears that all the naturally occurring furocoumarins so far described turn out to be derivatives of psoralen I or angelicin(isopsoralen) II.



I



II

In Table III, an attempt has been made to tabulate most of the furocoumarins so far isolated from natural sources. The structure, the names of the plants (botanical and common names) and their families have also been included. It can be seen that their distribution in nature

is confined to four or five major plant families. The important genera of plants possessing furocoumarins are Umbelliferae and Rutaceae. Leguminosae and Moraceae have a few but widely used plant species. The occurrence of these compounds in a few plant families does not mean that they are absolutely absent in other closely related families such as Convolvulaceae, Solanaceae, Cesalpenae, etc. Geissman and Hinreiner[39] have revealed the biogenesis of these compounds in these various plant species and have suggested that they owe their formation to certain biochemical processes fundamental to certain genus and families. The point of interest lies in the fact that most of these plant species are confined to tropical and subtropical regions where abundant sunshine plays a major role in the life of various plant species. Klaber[53], Bellringer[4], Goldsmith and Hiller[40], Klauder and Kimmich[51] have published reviews showing several plant species mostly belonging to Umbelliferae, Rutaceae and in a few instances Leguminosae and Mimosae families to cause photosensitized contact dermatitis. Several plant species reported to cause this phytophotodermatitis resulting in skin eruption, erythema, oedema andsubsequently residual pigmentation have been analyzed by some workers interested in this field and have shown to contain furocoumarins, especially 8-methoxypsoralen (xanthotoxin), 5-methoxypsoralen (bergapten) and psoralen. It is not therefore surprising that several species of Rutaceae and Umbelliferae have been implicated in photosensitized dermatitis.

Resumé: The selection of a rational strategy of research in areas of psoralen photosensitization posed a difficult choice between basic and applied approaches. In the early stages of such research investigations, so little was known of the problem that no easy decision could be made between the alternatives of basic research along the lines indicated by fundamental theory of photosensitization or the bold hunch-inspired strikes for immediate practical application to clinical problems. Patient studies with psoralens in the treatment of pigmentary disorders, in chemotherapy, in improving sun tolerance and promoting sun tanning, in prevention of skin cancer through increased pigmentation and increased corneal thickening have revealed potential clinical usefulness of psoralens. Considerable effort has been expended in different laboratories in practical clinical research but it has failed to answer the basic mechanism of their action. Therapeutic and clinical usefulness of psoralen compounds have brought several basic questions yet to be answered. Even though they are relatively non-toxic and are found to occur in many of our edible foods, yet their photosensitizing property poses a significant question to be answered: "Are they harmful in the long range?" Whether long-wave ultraviolet light, in itself biologically innocuous, acquires the capacity to produce skin cancers and cataracts by virtue of the photosensitizing effect of psoralen? As convertors of radiant energy they are capable of inducing chemical and biological changes which may eventually turn out to be harmful. I

leave this question of the ultimate usefulness or dangers of furocoumarins to the more learned and judicious authorities in the field of medicine and biochemistry. As a scientist in part dedicated to the understanding of the mechanism of action of psoralen, I prefer to present some of the facts in the pursuit of the truth. When dealing with the fragmentary material as presented in this chapter and subsequent pages of this thesis, many of the conclusions and tentatives are likely to be erroneous. No one will be happier than myself if they are rectified, for it shall mean a step forward in the clarification of the subject knowledge which we all strive to advance.

Several studies concerned with the biological sensitization to long-wave ultraviolet light induced by psoralen, 8-methoxypsoralen and other related furocoumarins have revealed that (a) human skin is photosensitized following topical or oral administration of the drug (Musajo et al.[65] Fitzpatrick et al.[32, 59] and (b) photosensitized biological response of the skin may be as mild as erythema, followed by augmentation of pigmentation, or as severe as edema, vesication or complete desquamation of the skin depending upon the light-dose, drug-dose relationship (Fitzpatrick et al.[32], Musajo[66], Pathak and Fitzpatrick[72]). In either event the visible biological changes are observed 8 to 24 hours subsequent to irradiation; (c) bacteria are killed (Fowlks et al.[34]); (d) ability of the bacterial cell to oxidize a number of substrates is reduced following 8-methoxypsoralen photosensitization (Oginsky et al.[64]). (e) Applica-

tion of 8-methoxypsoralen to the polar caps of Drosophilia resulted in a greater number of mutations in presence of ultraviolet light[43]. (f) Seed germination and growth of seedlings and roots of vegetables are adversely affected[76]. These observations suggested the possibility that enzyme systems of the bacteria and biological system in general are damaged during illumination either as part or concurrent with the lethal events.

In turn, it would seem reasonable to assume that the observed cutaneous changes resulting from photosensitization are directly related to mortality of individual cells of the skin. While it is quite obvious from the bacterial studies that the individual cells are damaged functionally and are killed (as judged by their inability to reproduce), it is not equally easy to ascertain whether individual cells of the skin have been similarly damaged preceding the observed photosensitized dermatological changes noted above. Studies carried out by a few investigators in the field of psoralen photosensitization are mainly confined to the histological changes in the irradiated skin (Baker[3], Zimmerman[106]. They mainly describe alterations of stratum corneum, formation of stratum lucidum or "modified stratum corneum" followed by increase in melanin pigmentation. They do not reveal biochemical or physical changes at the molecular level which have occurred either intra-cellularly or immediately extra-cellularly preceding and directly related to the observed effects. The question therefore arises: what happens to the cells and

the molecules within the cells when photosensitization occurs?

Of all the multitudinous biochemical processes in the living cell on which its life depends, there is scarcely one which is not due to enzymic function, in fact one can say that there can be no life without enzymes. Any modification of the enzyme pattern or activity may have far reaching consequences for the living organisms. Enzymic activity in a way therefore represents biological activity and is highly dependent on the physical and biochemical integrity of certain regions of the molecule.

Recently, the effects of irradiation of various wavelengths of light on enzyme system has been a favorite subject of a number of investigators (Yost et al.[105], Canzanelli, et al.[16]). The treatment of mitochondria with ultraviolet light resulted in progressive loss of the ability of these particles to oxidize succinate and glutamate as well as to couple efficiently the phosphorylation of ADP (adenosine diphosphate nucleotide) to electron transport. These considerations and previously reported observations that sulphydryl enzymes were damaged in the extract of guinea pig skin as a result of photosensitization with quinine or hematoporphyrin (Repke et al.[74]) and that the succinicdehydrogenase activities of rat liver mitochondria were destroyed following photosensitization with benzypyrene (Graffi et al.[41]) provided sufficient rationale for the present studies reported in this thesis. Enzyme studies were therefore used as sensitive indicators of changes occurring at the molecular level during photosensitization carried out in the presence of psoralens.

Observed cutaneous changes resulting from psoralen photosensitization either intracellularly or immediately extracellularly appear to be directly related to the biochemical and physical changes at the molecular level. Studies carried out by Fowlks et al.[34] concerning the biological photosensitization to long-wave ultraviolet light induced by 8-methoxypsoralen revealed strikingly increased susceptibility of several bacterial species. Not only did they show lethal effects on several bacterial species including gram positive and gram negative strains, but also observed that the ability of the bacterial cell to oxidize a number of substrates was reduced following 8-methoxypsoralen photosensitization (Oginsky et al.[69]). These observations suggested the possibility that enzyme systems of the bacteria are damaged during irradiation either as part of, or concurrent with the lethal events. Life depends on a complex network of chemical reactions brought about by specific enzymes and any modification of the enzyme pattern or activity may result either in temporary or longlasting disorganization of several biochemical functions. Enzyme studies were therefore used as sensitive indicators of changes occurring at the molecular level during photosensitization carried out by the psoralen. Besides demonstrating the inactivation of enzymes such as cytochrome oxidase, succinicdehydrogenase, lacticdehydrogenase, the latter two were used in the major part of this study to explore the mechanism of psoralen photosensitization.

Activating and fluorescent wavelengths of furocoumarins: psoralens and absorption spectra and action spectra of psoralen. The absorption of

ultraviolet radiation by a molecule results in a change in the electronic configuration of that molecule and therefore in a change usually transient and reductive, in the stability of the molecule. The ability of any molecule to absorb ultraviolet radiation of a particular frequency is dependent on the electronic configuration of that molecule. Thus absorptive ability is intimately related to the molecular structure. As stated earlier, to forward any photochemical reaction, a substance, which is called the light absorber or chromophore, must capture quanta. Biological photosensitized reactions are known to result from the absorption of a wide spectrum of radiation. The energy and therefore the wavelength of light absorbed and the biological changes occurring in photosensitized reactions are obviously closely related. The plot of the biological or chemical response as a function of wavelength is called the action spectrum. It is therefore obvious that greatest effectiveness of such responses would be at maxima of absorption and least effectiveness at minima of absorption. The absorption spectrum of a photosensitizing compound thus provides a suggestive pattern in choosing the wavelength for photosensitized reactions. Comparison of such action spectrum with the absorption spectra of various substances has led in numerous instances to the identification of the light absorber. It is well known fact that molecular groupings give rise to important absorptions in ultraviolet region, e.g., unsaturated linkages, aromatic compounds such as tyrosine, tryptophan, histidine and other resonating structures. Thus proteins are favored to

be photochemically active at tyrosine, tryptophan, phenylalanine residues. Several enzymes are known to be altered in vitro by ultraviolet light. Nucleic acids are well known light absorbers in the region 260 mu and 280 mu because of purine and pyrimidine moieties.

The psoralens have been shown to be highly active photosensitizing agents capable of inducing sunburn or erythema and augmenting the skin pigmentation[32, 59, 65, 72]. Furthermore, it will be shown in this thesis that they induce photosensitized inhibition of enzymatic activities such as succinicdehydrogenase &lacticdehydrogenase. Fowlks et al.[34] and Oginsky et al.[69] have demonstrated that these agents are capable of sensitizing bacteria in presence of ultraviolet light. Their fungicidal effect has been also reported. In most of these studies and other studies such as photosensitized contact dermatitis, inhibition of seed germination and ultraviolet carcinogenesis (to be reported in this thesis) long-wave ultraviolet light has been observed by several investigators to be more effective in evoking these biological responses. The precise activating wavelength range which is effective in exhibiting several of these biological responses was not known. The sorting out of which absorbed wavelengths participate in the photosensitizing effect of psoralen or 8-methoxypsoralen is particularly important if the mechanism of psoralen photosensitization is to emerge. Hence the effectiveness of various wavelengths in producing psoralen activation and photosensitization was investigated. The activating and fluorescent wavelengths of several furocoumarin derivatives and related substances were also

studied to correlate the photosensitizing ability of the compound with their molecular structure.

Effects of structural alterations on photosensitizing activity of furocoumarins and coumarins: Studies on the relationship between molecular configuration and the photosensitized erythematous activity of various natural and synthetically prepared furocoumarins following ultraviolet irradiation were carried out to relate the structure of the compound to its biological activity and establish a common denominator of a structure capable of exhibiting photosensitization in biological systems.

Ultraviolet carcinogenesis in albino and pigmented mice receiving furocoumarins: psoralen and 8-methoxypsoralen: Substances such as psoralen and 8-methoxypsoralen, that are capable of absorbing radiant energy and inducing biological changes, can modify and alter the skin responses to ultraviolet irradiation. Skin cancers are etiologically related to the trauma of sunburn repeated over a long period. Augmentation of skin responses to ultraviolet irradiation in presence of psoralens can cause trauma and trigger cancerous growth. This brings an important clinical consideration in the therapeutic uses of psoralens. Becker[3] has reported primary changes in the human skin following 8-methoxypsoralen and 5-methoxypsoralen administration. Stratum corneum showed thickening, increased density and also increased amount of melanin in the stratum corneum and in the basal cell. It was essential to investigate whether such changes induced by psoralens would modify the ultraviolet carcinogenesis.

Furthermore, epidemiological evidence clearly implicates solar radiation as a factor in the induction of human skin cancer (Blum[13, 14], Dorn [25]). In southwest part of the United States, ultraviolet carcinogenesis is a major problem. Among farmers, sailors, and other outdoor workers who are exposed to sunlight over prolonged periods and receive a large amount of ultraviolet radiation, one finds that the incidence of skin cancer of both squamous and basal cell types is quite common (Unna[90], MacDonald[60]). A factor which appears to protect man from carcinoma induced by exposure to solar radiation is melanin pigment, the darkly pigmented peoples having a markedly reduced incidence of carcinoma of the exposed areas (Dorn[25], Roffo[77], Thomson[89]). The generalized human albino living in the tropics almost invariably develops squamous and basal cell carcinoma of the exposed surfaces (Shapiro et al.[82]). Similarly, the fair-skinned Caucasians who easily become sunburned and have a tendency for freckling, have a higher incidence of carcinoma of the exposed areas than the persons who tan readily. The carcinogenicity of ultraviolet irradiation in albino mice has been established by many investigators (Roffo[77], Blum[13, 14], Rusch et al.[79], Kelner et al.[52] and Griffin et al.[42, 43].

The recent widespread uses of psoralens, particularly 8-MOP and psoralen, in augmenting the pigmentation of human skin following irradiation with sunlight and artificial long-wave ultraviolet light, their widely advocated use in increasing sun tolerance and in prevent-

ing sunburning, blistering and peeling , has raised the question whether these photoactive compounds might alter the incidence of ultraviolet carcinogenesis. O'Neal and Griffin[70] initiated studies on 8-methoxypsoralen and its effect on ultraviolet carcinogenesis in albino mice. Their initial report indicated that the final incidence of ear tumors induced by ultraviolet irradiation (15.2 to 17.5x10^8 ergs/cm^2 of short and medium range up to 3200 Å) in mice receiving 8-methoxypsoralen orally (0.5 g/kg diet) was considerably less than that of control mice, evidence suggesting that this photoactive agent had a "protective" effect against ultraviolet carcinogenesis. The extent of protection afforded by this compound appeared to be proportional to its concentration in the diet up to an optimal level of 0.5g/kg diet. They found, however, in the same experiment that intraperitoneal administration of 8-methoxypsoralen (0.4 mg/mouse/day) appeared to increase tumor incidence quite significantly. Whether the discrepant effect of oral and intraperitoneal administration were related to the significant differences in the dose levels or in the route of administration was not resolved. Very recently, when this study was in progress, Griffin et al.[43] extended their studies to the effect of the wavelength upon carcinogenic response in 8-methoxypsoralen treated mice. They investigated the effects of short-wave (principal emission 2537 Å) and long-wave ultraviolet light (Wood's light) following intraperitoneal injection and dietary feeding of 8-methoxypsoralen to albino mice. Mice

receiving 8-MOP in the diet (0.5g per kg diet) did not develop any tumor. They commented that 8-MOP appeared to afford protection from the exposure to 2537 Å. The animals injected intraperitoneally with this drug 0.4mg/mouse/day prior to exposure also did not show any potentiation of carcinogenesis. They thus concluded that 8-MOP administration had no appreciable effect upon short-wave ultraviolet light carcinogenesis. Prolonged exposure of albino mice to long-wave UV light (3200Å) following intraperitoneal injection of 8-MOP (0.4mg/mouse/day) resulted in a rapid and severe erythemal response and a very high tumor incidence in the ears and eyes. Dietary feeding of 8-MOP (0.5g/kg diet) in the animal exposed to long-wave UV light did not show potentiation of carcinogenesis. The control groups that received no drug but only long-wave irradiation did not show any incidence of carcinogenesis. The authors interpreted their observations to suggest that the intake of photosensitizing drugs such as 8-MOP with subsequent exposure to UV light or more specifically to long-wave UV light may accentuate the overall process of formation of keratosis or cancer. It was therefore decided to study the effect of psoralen and 8-methoxypsoralen in albino as well as several pigmented mice strains. The primary purpose was to find out whether these photoactive compounds were carcinogenic or altered the ultraviolet carcinogenesis response by virtue of their biological properties (pigment stimulation, increase the corneum thickening). The role of melanin pigment in ultraviolet carcinogenesis was also evaluated.

MATERIAL AND METHODS

The studies reported in this thesis were conducted primarily for the exploration of the mode of action of psoralen and 8-methoxypsoralen, the two substances which are readily available and have been most investigated clinically. The effects of psoralen and 8-methoxypsoralen were investigated following *in vivo* and *in vitro* irradiation with ultraviolet light. The *in vivo* effect of psoralen photosensitization has been demonstrated by histochemical studies of guinea pig skin. The *in vitro* studies cover the effect of psoralen and 8-methoxypsoralen on rat liver mitochondrial fraction. Specifically their effect upon succinicdehydrogenase activity was investigated. When one considers the vital role which has been assigned to the particulates in the cellular metabolism, it would seem that any damage to this system would result in rather drastic changes in the enzymic activities. Hence, besides investigations of changes in succinic dehydrogenase activity, other enzyme system, eg., cytochrome oxidase, was also studied. While the nature of photosensitizing action of psoralens was being investigated using rat liver mitochondria as a testing agent, it became apparent that substances within the mitochondria, eg., riboflavin, quinones, lipoproteins, cations, etc., could interfere with photosensitization effects. Hence a more simple system, crystalline lacticdehydrogenase, was used. The various methods employed in these enzymic studies have been outlined in the following pages.

Succinic dehydrogenase (SDH) activity of guinea pig skin: Adult albino guinea pigs weighing 600 - 750 grams were used in the major part of this study, although two specific experiments were carried out with black pigmented guinea pigs. The area of the skin on the back of the guinea pig for carrying out photosensitization experiments was prepared as follows: hair on the back of the guinea pig was removed by the aid of an electrically operated hair clipper and a nontoxic, nonirritant, "Neet", a commercially available hair depilatory, was applied liberally. After about five minutes the back of the animal was thoroughly washed under running warm tap water. This treatment enabled us to obtain a smooth skin area about 18x10 cm. It was experimentally ascertained that the hair depilatory contained no photosensitizing compound and that it induced no skin reactions for a period of 48 hours, nor after exposure to long-wave ultraviolet light for more than two hours. The animal was rested for two hours with access to food and water. A total of eight guinea pigs were used. The effect of psoralens was tested in the following two ways: (1) By topical application of 100 ug of the drug (either psoralen or 8-methoxypsoralen) dissolved in 0.2 m.l.of 95% ethanol. With the aid of a micropipette, the compound was applied uniformly in one inch square area of the skin which had been outlined with adhesive tape. Five such areas (1x1") were selected on each animal. Two such areas containing the drug were exposed to ultraviolet light, and one area was kept covered with black paper attached with an adhesive tape

prior to irradiation. The control areas included two one-inch square areas, one of which was exposed to ultraviolet light and the other was kept covered with black paper affixed with an adhesive tape. 0.2 ml.of 95% ethanol without photosensitizer was uniformly applied in each of these areas. One hour after topical application of the drug, the test area and the control area were exposed for 45 minutes to long-wave ultraviolet irradiation at 15 cm. distance. Four albino and one pigmented guinea pigs were exposed to ultraviolet irradiation. The guinea pig was kept supine on a wooden board facing the ultraviolet light source and was immobilized by securing the legs. The optimum time of irradiation under ultraviolet lamp was determined and has been reported earlier[71]. The remaining three guinea pigs treated identically were exposed to bright sunlight in the month of June and early July for one hour. The sunlight exposure time was arbitrarily chosen. (2) The other procedure involved an oral administration of the drug. Psoralen or 8-methoxypsoralen, in a gelatin capsule, was force-fed to the guinea pig using a dose of 10 mg of drug per kg. body weight. The drug was administered 1.5 hours before exposure to the radiant energy. Illumination was performed (under Wood's ultraviolet light) as indicated above for topical application.

After illumination, biopsies of the skin of each animal sufficiently large for histochemical study were taken from the exposed areas at varying times (after 1, 24, and 48 hours post illumination). Specimens were kept in ice-chilled containers before incubation. Before the biopsies

were taken, reflectance readings with green tristimulus filter, which is quite sensitive to detect erythemal response, were also recorded for control areas and photosensitized areas. The method proposed by Daniels and Imbrie[22] was used and has been outlined separately under the study related to the relative photosensitizing activity of furocoumarins.

Determination of succinicdehydrogenase activity: Succinicdehydrogenase activity of these skin biopsy specimens was determined histochemically by a modified method of Rutenberg et al.[80] with the cytochrome system poisoned with 0.1 molar sodium cyanide as recommended by Rosa and Velardo[78]. The method was based on the fact that in the presence of substrate sodium succinate, succinicdehydrogenase reduced colorless tetrazolium salts to highly colored insoluble formazans which precipitated at the sites of enzymatic activity. After thirty minutes incubation at 37°C with the tetrazolium chloride solution (3 ml. of 0.1% 2, 3, 5-triphenyl-2H-tetrazolium chloride, 3 ml. 0.1 M PO_4 buffer pH 7.0, 3 ml. of 0.25 M succinate and 1 ml. of 0.1 M NaCN) the block of the skin, generally 0.6 to 0.8 cm long, 0.2 to 0.3 cm wide and 0.1 cm thick was frozen with dry ice without fixing in formalin. It is essential to note that the control skin biopsy specimens without 8-MOP, either from dark unirradiated area or from UV exposed area) were incubated in the same petridish at the same time along with 8-MOP treated biopsy specimens. Ten ml. of substrate solution was sufficient to cover the biopsy specimens. In some experiments the incubation was extended to 45 minutes in order

to obtain a more intense reaction. The biopsy specimens were periodically turned. Thin sections (30 to 35 microns thick) were cut on the freezing microtome. The slides were inspected promptly and compared for the intensity of color as well as localization of succinicdehydrogenase activity. Photomicrographs of the sections were taken within 12 hours. Dr. W. L. Fowlks, now at the University of Minnesota, also cross evaluated these slides, to confirm the effect of 8-methoxypsoralen or psoralen photosensitization. Most of the sections had an internal control for succinicdehydrogenase activity, since there were bits of muscle and underlying connective tissue rich in activity and showed intense coloration at the end of incubation. Histochemical determination of succinic dehydrogenase in absence of added succinate was also carried out to overrule the other dehydrogenases reducing the tetrazolium salts even in absence of substrate. The site of enzyme activity was intense red coloration. The activity of control biopsy specimens was compared with psoralen treated areas.

Ultraviolet light source and irradiation procedure: All investigations reported in this thesis, unless stated otherwise, were carried out with long-wave ultraviolet light. The principle emission of the lamp was 3654Å band of mercury high pressure arc spectrum. This black light unit model 70 Glo Craft 250 watt (Switzer Brothers, Inc., Cleveland, Ohio) operated on 110 volts line current had a pressed glass "Roundel" filter #41 which failed to transmit all the wavelengths below 3200Å and

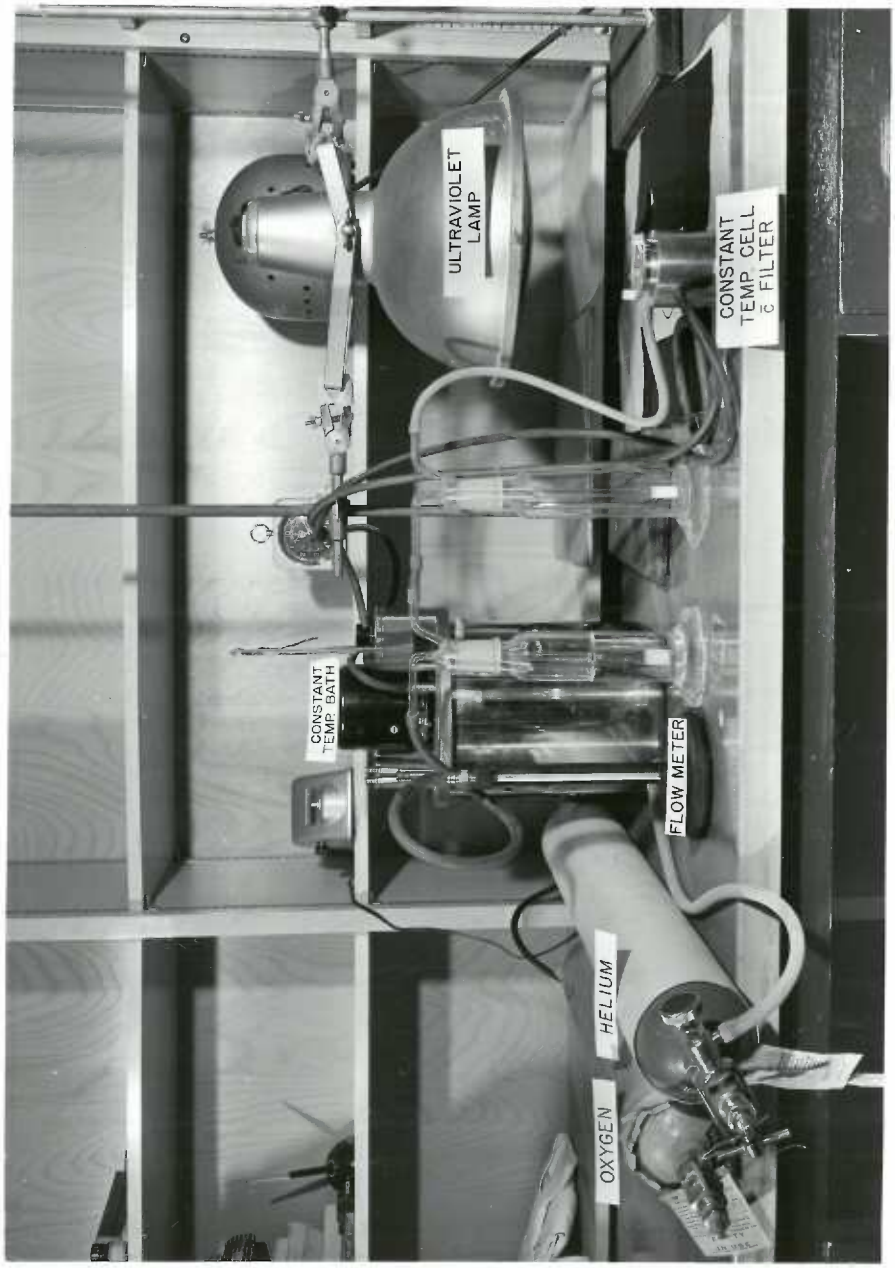
and above 4000Å. With this filter, there was 52-55% light transmission at 3650Å, 25% transmission at 3400Å, and 35% transmission at 3800Å. Guinea pigs were generally irradiated under the central part of this lamp. The lamp had a diameter of nearly 32 cm. and therefore covered sufficient area to place an animal in the center. They were however not left in the same position; every 6th or 7th minute, the wooden board was rotated by 90° angle, so that each area of the back, on an average, got the same amount of light within 45 minutes of irradiation. In enzymic studies to be described in the following paragraph, a specific area under the lamp was selected for irradiating the reaction vessel. A Photovolt photometric instrument survey of the target field 15 cm. from the Wood's filter on the light source showed variation up to 100% in relative intensities between extreme values and up to 20% between adjacent small areas. Therefore a relative intensity map of the light field was plotted using a photometer with a movable probe, and an area which had intensity difference of less than 10% was marked with a 60mm. circle. This circle was the target area used in all irradiation studies involving the effect on enzymic activity. The absolute intensity when measured with uranyl-oxalate actinometer as proposed by Leighton and Forbes[58] was 6×10^5 ergs/cm^2/minute.

Preparation of rat liver mitochondria: Rat liver mitochondria were prepared essentially according to the method of Hogeboom[45]. Fresh livers of young laboratory bred rats were used. Homogenization was performed

in a motor driven glass homogenizer with a teflon pestle. All operations before final incubation with substrate were carried out at 0-2°C in the cold room. Each batch of mitochondria was prepared from the liver of four adult rats. The livers weighed approximately 28 to 36 grams. Liver cell suspension was prepared with isotonic sucrose)0.25 M) as the medium. Isolation of the mitochondria was achieved by means of differential centrifugation. The procedure after isolating nuclear fraction and centrifugation at 9200 r.p.m. (5000 xg) involved centrifugation at high speed. The suspension of mitochondria was transferred to lusteroid tubes and centrifuged for 10 minutes at 20,000 r.p.m. (24000 xg). The procedure of resuspension of the mitochondria, centrifugation at 20,000 r.p.m. and removal of the supernatent was repeated. This final suspension of thrice sedimented mitochondria was used for a few studies. In subsequent experiments, however, the mitochondrial pallet was washed three times with 0.9% sodium chloride solution. Unless stated otherwise; mitochondria within 12 to 72 hours after preparation were generally used for most of these photosensitization studies. A few experiments were purposely carried out with aged mitochondrial preparations. Mitochondria equivalent of one gram of rat liver were regularly suspended in one milliliter of final suspending fluid of 0.9% potassium chloride solution. Deionized distilled water was routinely used in preparing sucrose solution and other reagents.

Illumination procedure for enzyme solution: Psoralen or 8-methoxypsoralen solution was prepared in distilled water and contained 5 mg. of the compound per 100 milliliter of water. A solution of a given sensitizer was kept for no longer than 3 days in low actinic glass flasks. In the early part of mitochondrial succinic dehydrogenase studies, 9 ml. aliquot of the sensitizer solution was mixed with 1 ml. of the final suspension of the mitochondria. A 5 ml. portion of this mixture was illuminated and the remainder kept in the dark to serve as control. However, for studying the effects of glutathione, ethylenediaminetetraacetate (EDTA or versene) and other substances such as cysteine, BAL, etc., on succinic dehydrogenase activity of rat liver mitochondria, and all the experiments related to crystalline lacticdehydrogenase to be described below, only 2.5 ml. aliquots were used for irradiation studies instead of 5 ml.aliquots as stated earlier. The dilution of mitochondrial suspension and the concentration of the sensitizer, however, remained essentially the same. In all these experiments the concentration of psoralen or 8-methoxypsoralen was approximately 45 ug per milliliter ($\pm$ 2 ug) unless stated otherwise. Mitochondrial suspensions with and without psoralens were divided into two equal parts. 2.5 ml. suspensions with and without psoralen were kept in the dark in test tubes covered with aluminum foil. These tubes served as controls. The remaining aliquots (2.5 ml. suspensions with and without psoralens) were irradiated in stainless steel vessels under Wood's long-wave ultraviolet

Figure 2: Ultraviolet Irradiation Unit for Enzyme Studies


ULTRAVIOLET LAMP
CONSTANT TEMP CELL & FILTER
CONSTANT TEMP BATH
FLOW METER
HELIUM
OXYGEN

light. Duplicate runs were carried out for each irradiation experiment. All preparatory and pipetting operations were conducted in a room with dimmed difuse light.

Controlling the temperature: Temperature variation under the lamp were encountered. The temperature after adequate warmup time of 30 minutes was found to be 30°C ± 3°C. A suspension of mitochondria initially warmed to 30°C, then placed for illumination warmed up only a degree or two in 15 minutes. Those suspensions heated to temperatures higher than 35°C would cool off slightly during exposure. Frozen samples of 5 ml. mitochondrial suspensions in 100 ml. beakers when placed under the lamp showed rise in temperature of approximately 8-10°C at the end of 15 to 20 minutes irradiation. To overcome these difficulties, a thermostated apparatus was employed as shown in Figure 2. A stainless steel medicinal cup, 50 ml. capacity, was used as the exposure vessel. It was held in place in the cavity of an aluminum jacket by a shield with a 1.25 inch opening and was sealed against water leakage between the cup and the jacket with a rubber O-ring. An inlet for coolant near the bottom of the jacket and an outlet near the top allowed for circulation of water from a constant temperature bath through the space between the cup and the jacket. The direct contact of the mitochondrial suspension or other enzyme solutions, on the inside of the thin metal cup cooled by direct contact with thermostated water on the outside was sufficient to keep the contents within one degree of the desired temperature during exposure to the ultraviolet source.

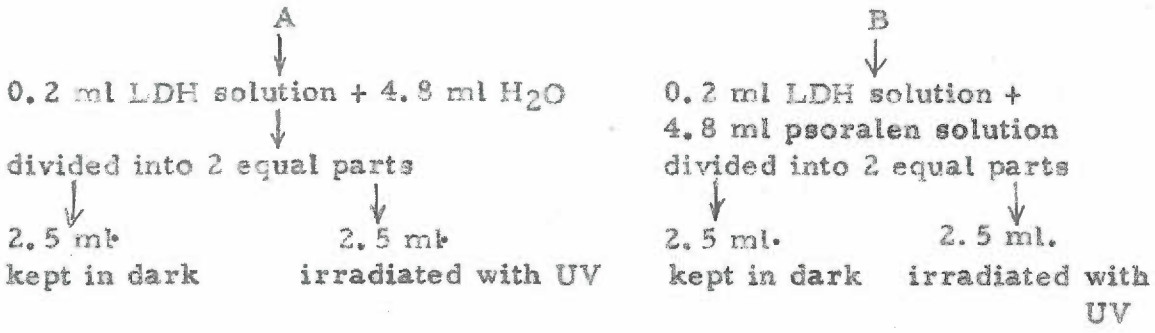
Thus 2.5 milliliter of the mitochondrial suspension with or without photosensitizer was illuminated at one specific area of optimum intensity previously described. The period of illumination was generally 15 minutes, but whenever it was different in specific studies, it has been detailed in the result section.

Assay of succinic dehydrogenase activity of mitochondrial suspension:

The succinicdehydrogenase activity of control and UV irradiated suspensions of rat liver mitochondria was determined by the spectrophotometric method of Slater and Bonner[84]. Immediately after each irradiation, activity determinations simultaneously were carried out for the irradiated specimens and its control specimen. Assays were run on 0.2 ml. aliquots of mitochondrial suspension. Succinicdehydrogenase activity was routinely determined in duplicate or triplicate runs. The assay depended on measuring the reduction rate of $K_3Fe(CN)_6$ in presence of sufficient potassium cyanide to inhibit cytochrome oxidase. Into a standard (1 cm) Beckman silica cell were added 0.3 ml. each of KCN (0.1M previously neutralized to pH 7.0), and $K_3Fe(CN)_6$ solution (0.01M). This was followed by addition of 0.2 ml., 0.2M sodium succinate. Phosphate buffer of pH 7.2 was added to give a final concentration of approximately 0.1M in a total of 3 ml. Buffer solution was used in the reference cell. At zero time 0.2 ml. of the irradiated or unirradiated mitochondrial suspension was added toboth cells, and the optical density at 400 mu was followed as a function of time for at least 10 minutes. The rate of change in optical density was compared against con-

trol runs. (Unirradiated specimen kept in the dark). The optical density readings of each run were plotted. The curves of the control runs (specimens with and without psoralens which were kept in the dark) gave unconditionally a linear drop in optical density. In each experiment, 100% activity refers to the activity of the control specimen kept in the dark. The deviation of the slope of each duplicate run while determining the enzyme activity was found to be not more than +0.05 per minute and hence these figures have not been included in tables.

Studies on lacticdehydrogenase (LDH): Crystalline lactic dehydrogenase was used to study the mechanism of psoralen photosensitization. More often this system will be abbreviated as LDH. Commercially available crystalline lacticdehydrogenase (Sigma Chemical Company) was used. Protein concentration per milliliter of enzyme suspension was determined periodically by measuring the optical density at 280 mu and 260 mu. The protein concentration was calculated by substituting the optical density readings in the following formula: (Kalckar[51])
1.45 OD at 280 mu - 0.74 OD at 260 mu = mg protein/ml. The stock enzyme solutions contained between 20 to 27 mg protein per milliliter. Each time the stock enzyme suspension was diluted with distilled water in such a way that 0.1 ml.of the diluted enzyme solution contained approximately 120 ug $\pm$ 10 ug protein . This amount of enzyme was used each time in 2.5 ml.volume of irradiation solution. Generally a set of four runs were carried out for each experiment as follows:



In most of the studies irradiation of enzyme solution with and without psoralen was carried out for fifteen minutes, but whenever this time has been extended it has been detailed in each run. The enzyme solution was pipetted into irradiation cups and placed in the thermostatically controlled, water jacketed, irradiation unit described under mitochondrial succinic dehydrogenase studies. A specific area of uniform light intensity was selected (see succinicdehydrogenase system) and the irradiation vessel was placed on this area for a definite period. Effects of addition of several compounds in understanding the mechanism of psoralen photosensitization was carried out by adding the specific compound in concentrated form in volume not exceeding 0.2 ml.to obtain the desired concentration in 2.5 ml.enzyme solution. The volume of water or psoralen solution was correspondingly reduced to keep the final volume equal to 2.5 ml. Under these conditions psoralen concentration was a little less than 45 ug/ml.(42-45 ug/ml). Duplicate runs were carried out for each experiment, and in several instances repeated runs were made.

Determination of lacticdehydrogenase activity: Method I: The spectrophotometric method proposed by Neilands[68] was adopted for measuring

the lactic dehydrogenase activity. The measurement involved the determination of the rate of appearance of the absorption peak at 340 mu of reduced diphosphopyridine nucleotide (DPNH). The reaction can be stated as follows:

$$\text{L (+) lactate + DPN} \xrightleftharpoons{\text{Lacticdehydrogenase}} \text{Pyruvate+DPNH+H}^{+}$$

Since the reaction produced one equivalent of acid, the assay was carried out at pH 10. The reagents were prepared exactly as described by Neilands.[68] 0.5M sodium DL lactate was obtained from lactic acid by neutralizing with cautious addition of 5N.NaOH. Any ester formed during neutralization was hydrolyzed by heating at 80°C. The pH of the final solution was adjusted to neutrality. Diphosphopyridine nucleotide (DPN) solution, generally 5 ml.(2×10^{-2}M) was prepared in water, pH was adjusted to 6.0. The solution was stored at 0°C and was consumed within 3 days. The other reagent used was glycine-sodium hydroxide buffer pH10, 0.1M.

Into a 3.5 ml.capacity (1 cm) Beckman silica cells, the following reagents were initially pipetted: 0.2 ml.lactate, 0.1 ml.DPN solution, 2.0 ml.buffer pH 10.0 and 0.6 ml.H_2O. For 100% transmission setting, the blank cuvette contained DPN, buffer, H_2O and 0.1 ml enzyme solution, but no lactate. An electrically operated timer (1'=100 units) was kept ready. At zero time, 0.1 ml.of enzyme solution (either irradiated or unirradiated with and without psoralen) was tipped in through a 0.1 ml.pipette. The change in optical density was measured every 30 unit interval at 340 mu. Δ E at 340 mu was recorded as a

function of time for a period of 3 minutes. The change in optical density readings per minute ($\triangle$OD/min) reported in all the tables related to LDH activity are those for the first 1.5 minutes. The molecular extinction coefficient values of DPNH has been reported to be 6.2×10^3 (68). Assuming this molecular extinction values, the Δ E/minute reading has been converted to millimicromoles of DPNH formed in 3 ml. test volume. Duplicate runs and in great many instances triplicate runs were carried out for measuring the LDH activity. The effect of ultraviolet in presence and absence of psoralen has been compared against the activity of the specimen kept in the dark in each set of experiments. The Δ E values for enzyme solution kept in dark represented the initial activity and was designated as 100% activity. The percent loss of enzyme activity was calculated from this value, following irradiation in presence and absence of psoralen.

Lacticdehydrogenase activity: Method II: Lacticdehydrogenase activity determination in a few selected experiments was also carried out differently, using reduced diphosphopyridine nucleotide (DPNH) in the following system:

$$\text{Pyruvate} + \text{DPNH} \rightleftharpoons \text{lactate} + \text{DPN}$$

The oxidation of added DPNH was observed at 340 mu. Into the cuvette of 1 cm light path and 3.5 ml. capacity, the following reagents were added: 0.1 ml. pyruvate (0.01M), 0.1 ml. DPNH (0.002M, pH 7.5), 1 ml. KH_2PO_4-K_2HPO_4 buffer (0.1M pH 7.4) and water to make up the

to 2.9 ml. At zero time, 0.1 ml enzyme solution was added and optical density values at 30 units interval (1' = 100 units) were recorded for 3 minutes in Beckman DU spectrophotometer. Density changes at 340 mu revealed the rate of oxidation of DPNH. The rate of decrease in optical density readings for the first 1.5 minutes were used to calculate the Δ OD/minute. Results have been expressed in terms of millimicromoles of DPNH oxidized.

Irradiation in gaseous atmosphere other than air: In studying the effect of oxygen during photosensitized inactivation of succinicdehydrogenase in presence of 8-methoxypsoralen, a following procedure was adopted. The pure gases, nitrogen, helium and oxygen were obtained from commercial sources. On the top of the stainless steel cup used for exposing the biological material (mitochondrial suspension or pure enzyme solution), a 3 mm thick, 100 mm diameter, optical quality, fused quartz disk was placed. The tube like short side arm near the top of the steel cup was connected to the gas cylinder by thick rubber tubing. The gas flows were saturated with water and then monitored at 1 liter per minute through the side arm. The gas escaped between the stainless steel cup rim and the quartz disk. The contents of the cup (enzyme system with and without 8-MOP) were flushed with the gas (either nitrogen, oxygen or helium) for 5 minutes prior to irradiation and then subjected to ultraviolet irradiation for a period of 15 minutes without interruption of the gas flow. (See Figure 2).

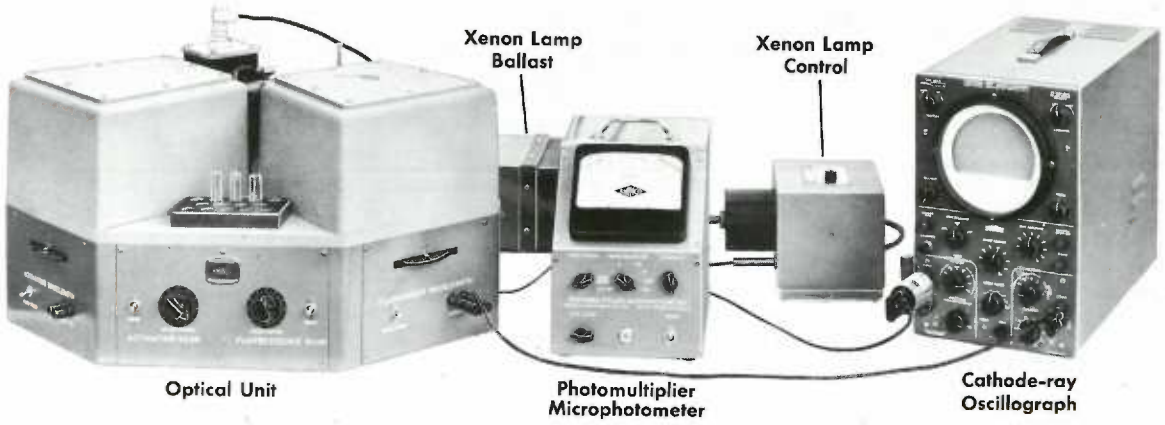
Determination of cytochrome oxidase activity: Rat liver mitochondrial suspensions with and without psoralens were prepared identically as described under the succinicdehydrogenase studies. A macro method as suggested by Cooperstein and Lazarow[21] was adopted to determine cytochrome oxidase activity of rat liver mitochondria. The method is based on the measurement of the rate of enzymic oxidation of reduced cytochrome C. 30 ml. solution of cytochrome C, 1.7×10^{-5} M (molecular weight assumed to be 14,000, and purity 90% when assayed spectrophotometrically) was prepared in 0.03M phosphate buffer pH 7.4. It was reduced by adding 100 ul. of freshly prepared solution of sodium hydrosulfite (1.2M) as outlined by the authors. 2.5 ml. mitochondrial suspensions (irradiated and unirradiated, with and without psoralen) were suitably diluted such that 0.1 ml. of the diluted suspension gave a drop in optical density at 550 mu in the range of 0.070 to 0.100 in one minute. The dry weight determination indicated that 0.1 ml. of this diluted suspension contained approximately 200 to 300 ug of the dried mitochondrial components.

Assay: Into the 3 ml. reduced cytochrome C solution 0.1 ml. of the diluted mitochondrial suspension was added. The reactants were mixed very rapidly and readings of optical density at 550 mu were recorded every 30 units time interval (1 minute = 100 units). Within 3 minutes ($\pm$ 30 seconds) complete oxidation of reduced cyt. C in the control unirradiated specimens was observed. The complete oxidation was further confirmed by adding few grains of $K_3 Fe(CN)_6$. Each time the rate of oxidation of reduced cytochrome C was studied in 4 samples: (1) mito-

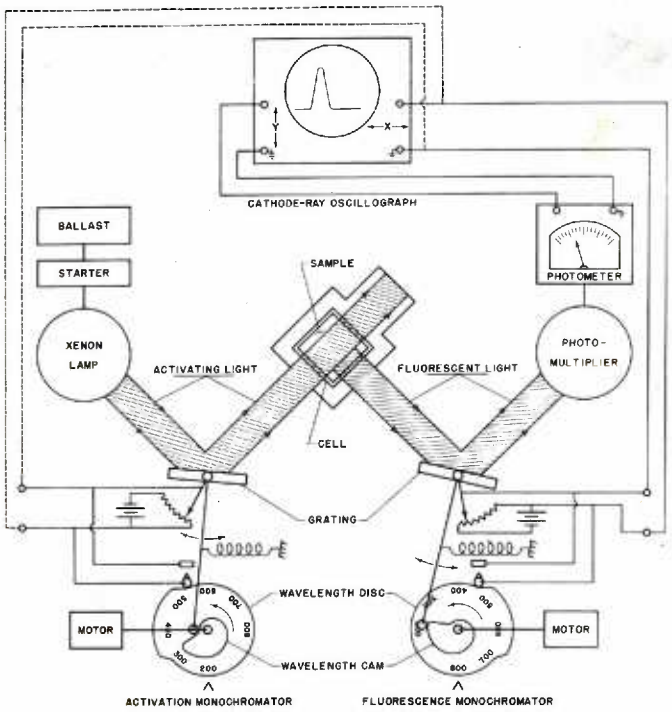
Figure 3a: (Top) Aminco Spectrophotofluorometer

Figure 3b: (Bottom) Schematic Diagram of

Aminco Bowman Spectrophotofluorometer



AMINCO SPECTROPHOTOFLUOROMETER



Schematic of Aminco-Bowman Spectrophotofluorometer.

chondrial suspension kept in dark, (2) UV irradiated specimen without psoralen, (3) unirradiated suspension containing psoralen or 8-methoxypsoralen kept in dark, (4) UV irradiated specimen containing psoralen or 8-methoxypsoralen.

The concentration of oxidized cytochrome C in each case was determined as follows:

$$\frac{do - dt}{1.96 \times 10^{4}} \times \frac{3.1}{1000} \quad \text{which reduces to}$$

$OD \times 1.58 \times 10^{-7}$ moles or = $OD \times 1.58 \times 10^{-4}$ millimoles.

Where do was the initial density reading at 0 time, dt was the optical density reading at any time "t" during the rate determination. 1.96×10^{4} was the difference between molecular extinction coefficients for reduced and oxidized cytochrome C at 550 mu (Horecker & Heppel[106]) 3.1 was the final volume in the reaction cuvettes. All experiments were run at room temperature (approximately 25°C). Duplicate runs were carried out for each determination.

Determination of activating and fluorescent wavelengths of furocoumarins and related compounds: The Aminco-Bowman spectrophotofluorimeter (Model 768) as shown in Figure 3a was used in this study. This instrument permitted the excitation of compounds from a monochromatic Xenon arc light source and measured with a second monochrometer the resulting fluorescence throughout the UV and visible region. As shown in Figure 3b, the light from the Xenon lamp was dispersed by the activating monochromator (grating type) into the monochromatic radiation incident on the sample. Fluorescent light from the sample was dispersed by a

similar monochromator into monochromatic radiation incident on the photomultiplier. The light was there transformed to a weak electrical signal and fed to the photometer where it was amplified. Photometer output was coupled to the vertical axis of the cathode ray oscillograph. The gratings were oscillated by a motor driven cams which were coupled to graduated discs for visual observation and adjustment of wavelengths. The activating monochromator was set at a selected wavelength, the scanning fluorescent monochromator was connected to the oscillograph input. A wavelength vs. intensity diagram(fluorescence spectrum) was observed on the oscilloscope. The maximum activating wavelength and fluorescence peak were thus determined. This was further confirmed by setting the fluorescent monochromator at a wavelength of maximum fluorescence and connecting the oscilloscope input to the scanning activating monochromator. This procedure resolved the major activating peaks. Thus the instrument enabled to measure the wavelength for maximum activation, at the same time measured the wavelength of maximum fluorescence. Photometer sensitivity was controlled by meter multiplier switch and sensitivity knob with meter readings in steps of 1, 1/3, 1/10, 1/30, 1/100, 1/300 and 1/1000. The relative intensity of fluorescent light incident on photomultiplier was the product of meter readings (transmission scale) and the meter intensity multiplication factors (sensitivity from 1 to 1000). The relative percent fluorescence for each compound was therefore calculated from the photometer readings of transmission scale, all values were corrected to 0.01 meter setting. Each compound

was dissolved in 95% ethanol (1 mg/10 ml) and suitably diluted (generally 1 ml to 3 ml) with ethanol. This diluted specimen was used for determining the activation and fluorescent wavelengths. The percent fluorescence readings were for these diluted specimens.

The photosensitizing activity as measured by erythemal response was tested on albino guinea pig skin. The data related to this and enzyme inhibition will be detailed separately under the section of relationship between molecular configuration of furocoumarins and their biological activity. The terms "active" and "inactive" as described in Tables XXII A & B denote the presence or absence of photosensitized erythemal response on a mammalian skin and in a few cases indicate inhibition and lack of inhibition of enzyme activity.

Absorption spectra and action spectra of psoralens: Crystalline lactic dehydrogenase was used as a biological test material. Absorption spectra of psoralen and lactic dehydrogenase was recorded on Beckman DK1 spectrophotometer. The action spectra studies were carried out on Aminco Bowman spectrophotofluorimeter. The Xenon compact arc was the source of high intensity ultraviolet irradiation. The radiation by this lamp as reported by the Hanovia Manufacturing Company was 420 watts for the spectral range of 2000 to 14,000 Å. The percentage of radiant energy from 2100 to 4000 Å was approximately 4.7%, which was equivalent to 19.7 watts output. Silica cells of 3.5 ml capacity were used. The cells slits and photomultiplier slits were removed to obtain a broad beam of light. 2.5 ml lactic dehydrogenase enzyme solution containing approxi-

mately 120 ug ± 10 ug. of protein was used in each case. Enzyme solution and psoralen solution were prepared in deionized distilled water. Psoralen concentration was approximately 48 ug/ml.(= 2.5×10^{-4}M). A specific wavelength extending from 220 mu to 400 mu was selected and the enzyme solution (2.5 ml) with psoralen was irradiated in silica cells for 45 minutes. To determine the effect of ultraviolet irradiation without psoralen, enzyme solution was irradiated at few selected wavelengths, e.g., 240, 280, 290, 360 mu only. The temperature variation during the irradiation period was insignificant. Following irradiation, LDH activity was measured and compared against the control specimen, kept in the dark for the same length of time. Duplicate runs were carried out for each wavelength. Enzyme activity runs were also carried out in duplicates. Studies related to LDH-DPNH complex formation were carried out on Aminco Bowman spectrofluorimeter. Other studies related to LDH, EDTA, Zinc complex determination were carried out on Beckman Recording spectrophotometer model DK.

Relationship between molecular configuration and photosensitizing action of furocoumarins (psoralens): The method of testing the photosensitizing activity of these derivatives has been described by Pathak and Fitzpatrick[72]. Briefly the method consisted of fixing an adhesive tape (25x 7.5 cm) containing ten square "windows" each 2.5 cm^2 on the back of smoothly depilated albino guinea pig. This procedure has been detailed earlier under in vivo effects of psoralens on succinicdehydrogenase activity of guinea pigs. Various amounts usually ranging from 25 ug to

1000 ug per 2.5 cm^2 skin area were applied topically in different windows. Dilutions for each compound were made in such a way that the desired amount was obtained in 0.1 to 0.2 ml. of ethanol. Two standard solutions of psoralen and 8-methoxypsoralen (25 ug per 0.1 ml. ethanol) were also applied in two separate windows each time along with the compound under test. Thirty to forty-five minutes after application, the back of the test animal was irradiated at a fixed distance from a standard long-wave ultraviolet light for forty-five minutes. At the end of 18 hours and 36 hours, the animal was examined for the presence of erythema. Visible intensity of erythema was recorded in conventional + or - signs as shown below and also with Photovolt photoelectric reflectance meter model 610 operated on AC power line equipped with green tristimulus filter as described by Daniels et al[22]. The reflectance of adjacent control area was also measured. The percent reflectance difference between control area and the test area measured the intensity of erythemal response. The erythemal response with red filter was also recorded for each area under test, but the readings showed little correlation with the intensity of erythema and hence have not been presented in tables.

Sign	Erythemal response	Relative intensity
-	no erythema	inactive
±	just perceptible erythema detected by tenderness of skin	weakly active
+	definite erythema of low intensity, pink color	definitely active
++	pink red erythema	quite active
+++	markedly red erythema	very active
++++	deep red, blistering, edema, etc.	highest possible response

Enzyme studies: Crystalline lacticdehydrogenase was used. Various compounds as detailed in Table #3 were dissolved in water as follows: On a microbalance about 1 mg of each compound was weighed and transferred to a graduated tube containing 10 ml. water. The tubes were left in a boiling water bath for an hour and after cooling the volume was brought back to 10 ml. The tubes were then centrifuged and the supernatent containing the dissolved compound was subjected to solubility determination by fluorescence analysis. The degree of fluorescence of each compound was compared against its own standard solution (1 mg/10 ml. of 95% ethanol). The concentration used in each case was therefore varying but was within 40 to 50 ug per ml. This has been detailed in Table XXV. 9.8 ml.of each of these solutions and 0.2 ml.of enzyme solution containing approximately 500 ug protein were mixed and divided into 4 equal parts. Two batches of 2.5 ml each were separately irradiated for 15 minutes under ultraviolet lamp. The remaining two batches of 2.5 ml served as unirradiated control specimens. Following UV irradi-

ation, LDH activity was measured and compared against activities of the control specimens.

Ultraviolet carcinogenesis in albino and pigmented mice receiving furocoumarins: Psoralen and 8-methoxypsoralen

Irradiation unit: An ultraviolet irradiation chamber was constructed with two General Electric UA-3 "Uviarc" lamps mounted adjacently in aluminum reflectors. The design of the UV chamber and the "Uviarc" tubes used was identical to that reported by O'Neal & Griffin[70]. The radiant energy emitted by these lamps covered a wide range of ultraviolet wavelengths extending from 2200 to 4000 Angstrom units. The output for these tubes was as follows: short-wave ultraviolet light (2200 - 2800Å) 10.36 watts, middle ultraviolet light (2800-3200Å) 13.72 watts, and long-wave ultraviolet light (3200-4000 Å) 12.23 watts. In the visible range (4000 to 7600 Å) the output was 25.1 watts. The mice were exposed to this total ultraviolet spectrum at a distance of 50 cm. Intensity of illumination under the lamp in a two square feet area was determined every four weeks by photolysis of uranyl oxalate solutions according to the method of Leighton and Forbes[65]. Quantum yield for the entire UV spectrum was taken as 0.57ϕ. The total energy at the site of irradiation was found to be 2.9 x10^4 ergs/cm^2/second.

Mice strains: Seven different strains of mice were used. Three were Swiss strain albino mice: one strain was from Huntington Farms, Philadelphia; AHF; one was from Napa, California: ANC; and the third was from Jackson Memorial Laboratory, Barharbor, Maine; AJAC.

Four other mice strains were as follows: (1) a black strain C57 Bl/6 with black ears, black eyes and hair; (2) a brown strain C57 Br/cd with black pigmented eyes and deep brown hair; (3) a grey haired strain with pink eyes of genotype a/a, b/b, C/C, D/D and p/p designated as pGJAC, and the fourth strain was of yellow mice C57BL/6AY with yellow orange hair coat and black eyes. All these genetically known strains were obtained from Jackson Memorial Laboratory. The albino mice, the black mice and brown mice were all female. The grey haired mice with pink eyes and yellow mice were of mixed sex.

Irradiation cages: To prevent huddling or movements of the mice during ultraviolet exposures, special cages were fabricated as described by Rusch et al[79] and Griffin et al[42, 43]. The wooden cages 15" x 12-1/2" had 32 small compartments (each compartment 3"x1-1/2"). There was a sliding cover made of wire mesh with big holes. At random the mice from each group were picked and housed in individual compartments and irradiated under ultraviolet light at a fixed distance of 50 cm.

Group selection, administration of drug and irradiation procedure: Mice 8-10 weeks old with comparable average weight within each strain were randomly divided into cage groups of 20 each. There were 50 groups to begin with, but because of early deaths a few groups were abandoned from the study and only observations related to 45 groups have been described. There were 19 groups in albino mice series,

10 in black C57 Bl/6 mice series, 8 in C57 Br/cd brown series, 4 in yellow (pheomelanic C57 Bl/6A^{Y}) series and 4 in pink-eyed grey hair strain pGJAC. Each mouse was tagged for individual identification for the observation of effects of irradiation. Psoralen and 8-methoxypsoralen were administered in two ways: (1) 8-MOP or psoralen was added in a concentration of 0.5g per kilogram of powdered "Purina Laboratory Chow." The diet was then subjected to thorough mixing in a revolving ball mill for 12 hours. Each day's food intake was recorded for each group receiving drugs in the diet. (2) The second method consisted of measured quantities of drug given individually to each mouse by oral feeding from a tuberculin syringe with an attached 18 guage, semicurved metal tube one inch long. It was possible by slowly inserting the bent tube into the mouth to administer 0.05 ml. suspension of either psoralen or 8-methoxypsoralen prepared in 0.5% gum acacia solution. Wood's light examination of the mice after feeding, determined that no spillage of fluorescent compounds had occurred around the mouth. Preliminary tests had indicated that, with oral feeding, 8-MOP and psoralen developed their highest concentration in the blood in 1-1/4 hour. Ultraviolet irradiation was therefore given at this time interval following drug administration. The dose schedule for all the 45 groups is shown below (Table XXVI A.) It included the range of levels used by O'Neal & Griffin[70] and also 0.01 mg., the equivalent of a 20-30 mg. dose of the drugs in man, which

is generally indicated in clinical practice. Administration of the drug was discontinued after 120 days. Changes in temperature during the course of irradiation were minimized by slow circulation of air from a revolving electric fan. To minimize the possibility of photoreactivation (Griffin et al.[42], Kelner and Taft[52]) the animals were housed in a windowless, dark room. Except during the short exposure period, the mice within each group were housed in the aluminum cages over a layer of sawdust. All the groups except the dietary groups receiving 8-MOP or psoralen through diet, were given Purina Laboratory Chow ad libitum.

Ultraviolet dose: Each group received ultraviolet irradiation for six days a week at an average rate of 2.9×10^4 ergs per square cm. per sec. Total ultraviolet irradiation received by Group #1 to 28 was 14.8×10^8 ergs/cm^2 delivered in 850 minutes. Groups #29 to 45 received 8.7×10^8 ergs/cm^2 delivered in about 500 minutes. (Each day irradiation time in these groups had to be decreased because of several deaths after starting the experiment). On an average each group was irradiated for nearly 110 days ($\pm$ 10 days). Following ultraviolet irradiation the observations for tumor incidence were continued for a total of 260 to 280 days.

Evaluation of tumors: Since each mouse was tagged for identification, it was possible to study the gradual neoplastic changes at different time intervals. The mice were examined separately by three observers (M. A. Pathak, T. B. Fitzpatrick and F. Daniels). The last

two observers did not know the identity of the groups until the completion of the experiments. Generally every tenth day mice were examined for neoplastic changes. A five point grading system was used: normal ear, 0; inflammatory response without nodules, 1; suspicious nodules, 2; a definite nodule suggestive of cancer, 3; a definite tumor, 4. Right and left ear were recorded separately for each mouse. A mouse graded 3 or 4 on either ear by at least two observers was recorded as having a tumor. The analysis is based on the number of tumors. The final tumor incidence is expressed as a percentage of the initial number of mice in each group. Statistical analysis of the results was also carried out as follows:

Criterion for comparing any two cage outcomes:

$P_1 - P_2$ is significant at $P = 0.05$ level if,

$$P_1 - P_2 \geqslant \frac{1}{2} N + \frac{1.96}{\sqrt{N}} \sqrt{P_1 + P_2 - \frac{1}{2} P_1^2 - \frac{1}{2} P_2^2}$$

which reduces, when $N = 20$ to

$$0.025 + 0.451 \sqrt{P_1 + P_2 - \frac{1}{2} P_1^2 - P_1 P_2 - \frac{1}{2} P_2^2}$$

Least wholly significant percentage point difference (5% level) = 35 percent, and least wholly significant percentage point difference (1% level) = 40 percent.

RESULTS

Succinicdehydrogenase activity in normal guinea pig skin (control areas) The distribution of succinicdehydrogenase (SDH) activity in the bulb of a hair follicle and sebaceous glands was prominently visible even under low power lens of microscope. The precipitated formazans were intensely colored. In the hair follicle the distribution of SDH appeared to be strongest in the lower portion of the bulb, and in the dermal papilla. The upper bulb showed weak activity. The presence of dehydrogenase activity in the matrix cells was distinctly demonstrable. The sebaceous glands showed considerable amount of formazan deposition indicative of significant dehydrogenase activity. In the epidermis, the reaction was visible in the basal layers and also in the malpighian layer. It was found to decrease in the cells further up in the stratum granulosum and was not detected in the stratum corneum.

Effect of psoralen and 8-methoxypsoralen (topical effect and oral feeding effect): Irradiation of the skin following 8-MOP administration (topical as well as oral effect) resulted in marked reduction in the amount of formazan laid down in the sebaceous glands and in the hair follicles. There was slight, though definite, difference in the results obtained depending upon the mode of application. Illumination following topical application of the drug did not show complete loss of activity in sebaceous glands, although there was complete loss of activity in the hair shaft region. On the other hand, the oral mode of

Figure 4a:

Guinea pig skin x 80. Biopsied immediately after receiving sufficient sunlight to produce a grade 2 erythema. The succinic dehydrogenase activity was demonstrated by treatment of the fresh biopsy in the block by the tetrazolium salt method. Frozen sections, 35 micron, photographed 12 - 14 hours after development. There is some succinic dehydrogenase activity shown by the entire section but high concentration of formazan is found only in the sebaceous glands with somewhat less found in the developing hair shaft just anterior to the bulb.

Figure 4b:

Guinea pig skin section which had been treated topically with 100 micrograms of psoralen per square inch before exposure to sufficient sunlight to produce a grade 2 erythema. There is residual succinic dehydrogenase activity in the sebaceous glands but all other activity had disappeared to a variable depth in the dermal layer. Sections from a similar experiment using 8-methoxypsoralen as sensitizer gave essentially identical results.

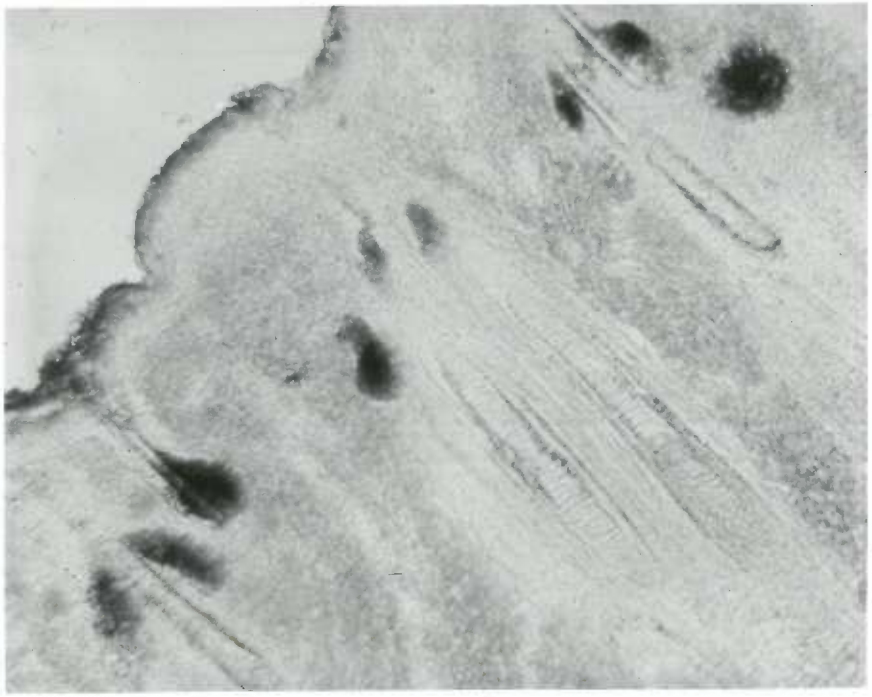


Figure 4c:

Control area without sensitizer similar to Figure 1 except biopsy taken following 45 minutes exposure to the ultraviolet source.

Figure 4d:

Section of guinea pig skin biopsy taken immediately following 45 minutes exposure to the ultraviolet source. The guinea pig had received orally 10 milligrams of 8-methoxypsoralen per kilogram of body weight 1.5 hours before the exposure. Succinic dehydrogenase activity has been completely destroyed to a depth of 1.5 millimeters in all structures. The residual darkness in the sebaceous glands shown in the photomicrograph was a light brown and not the characteristic reddish blue of formazan. When psoralen was used as a sensitizer the sections were essentially identical.

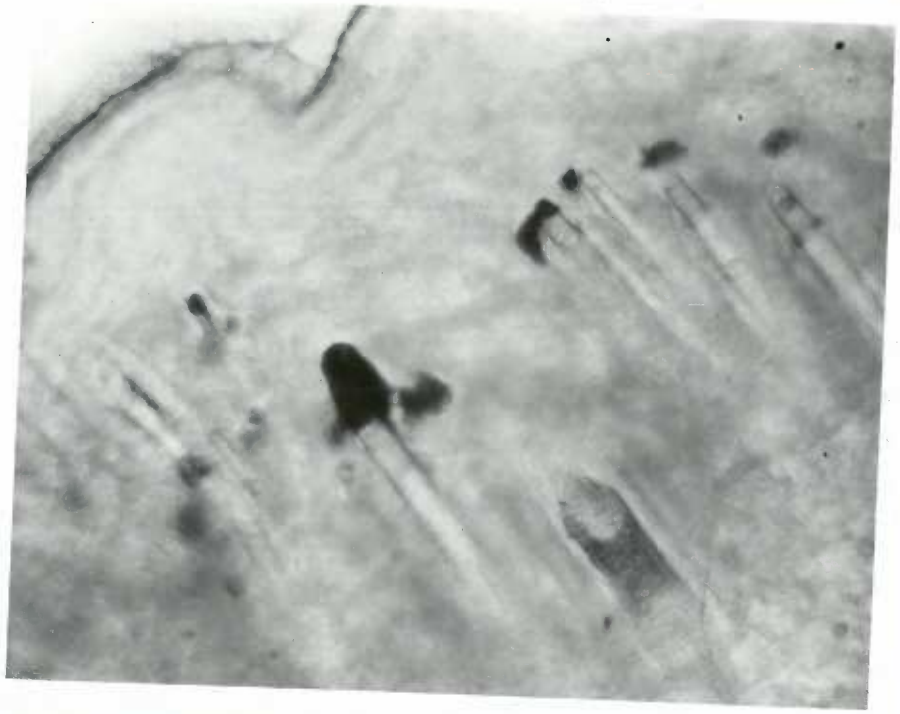


Table IV Effect of UV light on skin succinicdehydrogenase activity following topical application and oral feeding of 8-methoxypsoralen and psoralen

Animal	System		Testing time following UV exposure	Light source	% reflectance difference (green filter)	visible erythema	Observed histochemical changes in "d" and "e" areas
#1 Albino	a. control	dark	after 24 hrs.	Wood's light			Loss of SDH activity in sebaceous glands, hair follicles and surrounding cells
	b. control	UV					
	c. 8-MOP(topical)	dark				+++	
	d. 8-MOP "	UV			10		
	e. 8-MOP "	UV					
#2 Albino	a. control	dark					Loss of SDH activity in basal cell, sebaceous glands, less loss in hair follicles
	b. control	UV	after 48 hrs	Wood's light		+++	
	c. 8-MOP(topical)	dark					
	d. 8-MOP "	UV			8		
	e. 8-MOP "	UV					
#3 Albino	a. control	dark					Significant loss of SDH in hair follicles, sebaceous glands after 24 hrs. In area "d" comparatively less loss of activity
	b. control	UV					
	c. 8-MOP (topical)	dark	after 45 min	sun-light	2	±	
	d. 8-MOP "	UV					
	e. 8-MOP "	UV	24 hrs		12	++++	
#4 Albino	a. control	dark					Very low SDH activity in both areas. Definite destruction of the enzyme activity in sebaceous glands, but hair follicles showed some activity
	b. control	UV					
	c. psoralen(topical)	dark	after 1 hr	sun-light	3	+	
	d. psoralen "	UV					
	e. psoralen "	UV	after 24 hr.		7	+++	

Table IV (Continued)

Animal	System		Testing time following UV exposure	Light source	% reflectance difference (green filter)	visible erythema	Observed histochemical changes in "d" and "e" areas
#5 Light black	a. control	dark					Low activity of SDH in sebaceous glands and hair follicles
	b. control	UV	after				
	c. 8-MOP (topical)	dark		Wood's			
	d,e. 8-MOP "	UV	24 hrs	light	5	++	
#6 Albino	8-MOP (oral-10 mg/kg)	dark	after	Wood's	6	++	Significant destruction of SDH in hair follicles, sebaceous glands and other surrounding areas
		UV	24 hrs	light			
#7 Light black	psoralen (oral-10 mg/kg)	dark	After	Wood's	7	++	Traces of SDH activity in hair follicles, sebaceous glands and in basal cell layer
		UV	24 hrs	light			
#8 Albino	8-MOP (oral-10 mg/kg)	dark	after	Wood's			Significant loss of SDH activity in 48 hour biopsy specimen; 1 hour specimen showed also some loss of activity
		UV	1 hr	light	3	±	
		UV	after 48 hr		8	+++	

administration resulted in almost complete loss of activity in the sebaceous glands while hair shaft was not always so completely affected. Both oral and topical results revealed significant loss of succinic dehydrogenase activity in basal cell, malpighian cell layers. Effect of ultraviolet irradiation following topical application or oral feeding of 8 MOP and psoralen can be stated to result in significant loss of SDH activity. Typical results of these experiments can be seen by examination of the photomicrographs made from typical section of skin as shown in Fig. IV. Table IV summarizes the observations of this study.

Rat liver mitochondrial succinic dehydrogenase and effects of 8-MOP and psoralen following UV irradiation: Observations related to the photosensitized inhibition of SDH activity of guinea pig skin following topical application or oral feeding of 8-MOP and psoralen (in vivo effect) led to study similar effect in an invitro system. Initially, experiments were conducted in which rat liver mitochondria were suspended in phosphate buffer containing psoralens. Following ultraviolet irradiation several such runs indicated that this particular enzyme system was not affected by ultraviolet in the presence of 8-MOP (Fig. 5) or psoralen. Hence dialysis against 0.9% KCl was tried, and to avoid complications, the dialyzed mitochondria were exposed after addition of 8-MOP solution made up in 0.9% KCl. This mitochondrial suspension containing 8-MOP revealed a definite loss of SDH (Figure 5). There was 20 to 25% loss of SDH activity. Mitochondria

Figure 5

Effect of various treatments on succinicdehydrogenase activity of rat liver mitochondria following ultraviolet irradiation ($>$ 3200 Å) in presence of 8-methoxypsoralen.

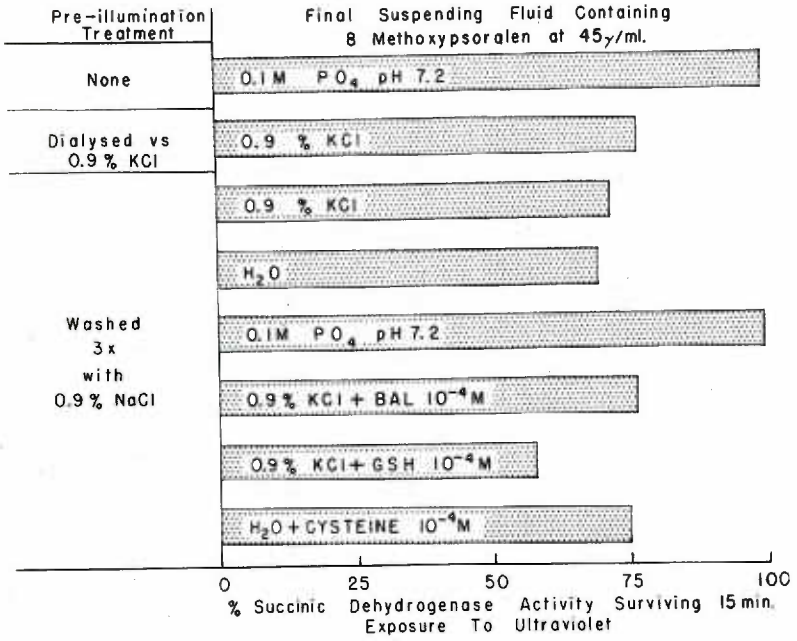

Pre-illumination Treatment
Final Suspending Fluid Containing 8 Methoxypsoralen at 45γ/ml.
None
0.1 M PO4 pH 7.2
Dialysed vs 0.9 % KCl
0.9 % KCl
0.9 % KCl
H2O
Washed 3x with 0.9 % NaCl
0.1 M PO4 pH 7.2
0.9 % KCl + BAL 10-4 M
0.9 % KCl + GSH 10-4 M
H2O + CYSTEINE 10-4 M
0
25
50
75
100
% Succinic Dehydrogenase Activity Surviving 15 min. Exposure To Ultraviolet

Table V Effect of 8-methoxypsoralen and psoralen on succinicdehydrogenase activity of rat liver mitochondria

(A. 8-MOP 45 ug/ml. B. Psoralen 45 ug/ml.)

Mitochondrial SDH activity Δ OD/min

A. 8-MOP effect	I Dark without 8-MOP	II UV 15' without 8-MOP	III Dark with 8-MOP	IV UV 15' with 8-MOP	% loss of enzyme activity in IV
Expt. #					
1.	.033	.033	.034	.027	20
2.	.035	.035	.035	.027	23
3.	.023	.021	.023	.017	26
4.	.019	.018	.019	.0135	29
5.	.022	.020	.020	.0130	39
6.	.022	.021	.022	.0185	14
B. Psoralen effect	Dark without psoralen	UV 15' without psoralen	Dark with psoralen	UV 15' with psoralen	
1.	.0130	.0130	.0130	.0108	17
2.	.0175	.0175	.0175	.0155	14
3.	.0190	.0195	.0195	.0145	24
4.	.0190	.020	.020	.0139	27
5.	.0160	.0160	.0165	.0120	25
6.	.0190	.0185	.0190	.0110	42

dialysed first against 0.9% KCl and then illuminated without 8-MOP showed no loss of activity. It became apparent that the invitro system required some preliminary treatments to make mitochondria susceptible to 8-MOP photosensitization. Only after washing three times with 0.9% NaCl solution by suspending the button of the mitochondria in 5 ml of saline and recentrifuging at 15,000 g for 10 minutes each time, yielded a mitochondrial preparation which when irradiated in presence of 8-MOP showed the loss of SDH activity. This treatment proved to be an effective substitute for dialysis and was less time-consuming. Saline washed mitochondria preparation when irradiated with UV for the same length of time (15' or 30') but without adding any photosensitizer (8-MOP or psoralen) showed no loss of activity. Subsequent experience proved that following saline washing, if mitochondria were suspended in simple distilled water and then irradiated in presence of psoralen or 8-MOP solutions, one could demonstrate the photosensitized inhibition of SDH activity. These initial results are summarized in Fig. 5 and Table V. The observation that dialysis against potassium chloride or washing with saline in addition to the washing that normally accompanies in preparing mitochondria, gave a preparation susceptible to 8-MOP or psoralen photosensitization, strongly suggested that some small molecule was being removed that blocked the photosensitization. While it did not seem likely that sucrose would play this role, nevertheless, an experiment was performed in which sucrose (1×10^{-3}M) and 8-MOP were added to

Table VI Effect of various treatments of rat liver mitochondria on photosensitizing action of 8-methoxypsoralen

Effect of sucrose		Δ OD/10'	% activity	% loss of enzyme activity
1.a. SDH (washed mitochondria free of sucrose)	dark	0.175	100	-
b. SDH	UV	0.170	97	3
c. SDH+8-MOP+sucrose $1x10^{-3}M$	dark	0.170	97	3
d. SDH+8-MOP+sucrose $1x10^{-3}M$	UV 15'	0.130	74.2	25.8
Effect of Phosphate buffer 0.1M, pH 7.2				
2.a. SDH+8-MOP in PO_4 buffer	dark	0.190	100	-
b. " " " "	UV 15'	0.210	110	-
a. SDH+8-MOP in PO_4 buffer	dark	0.210	100	-
b. " " " "	UV 15'	0.205	98	2
a. SDH+8-MOP in PO_4 buffer	dark	0.175	100	-
b. " " " "	UV 15'	0.175	100	-
Effect of washing with NaCl				
3.a. SDH in H_2O	dark	0.060	100	-
b. SDH+8-MOP in H_2O	dark	0.055	92	8
c. SDH in H_2O	UV 15'	0.050	83	17
d. SDH+8-MOP in H_2O	UV 15' dar	0.035	58	42
a. SDH in H_2O	dark	0.070	100	-
b. SDH+8-MOP in H_2O	dark	0.060	86	14
c. SDH in H_2O	UV 15'	0.055	78.6	21.4
d. SDH+8-MOP in H_2O	UV 15'	0.040	57.0	43
Effect of pre-irradiation				
4.a. SDH in KCl	dark	0.350	100	-
b. SDH in KCl. pre-irradiated 15'	dark	0.350	100	-
c. preirradiated SDH+pre-irradiated 8-MOP	dark	0.350	100	-
d. SDH in KCl+8-MOP in KCl	UV 15'	0.270	77	23
Effect of preirradiation				
5.a. SDH in H_2O	dark	0.210	100	-
b. SDH in H_2O preirradiated 15'		0.205	97.6	2.4
c. SDH in H_2O+preirradiated psoralen		0.205	97.6	2.4
d. Preirradiated SDH+preirradiated psoralen 15'		0.205	97.6	2.4

a washed suspension of mitochondria prior to exposure to UV. As expected, the addition of sucrose did not block the photosensitizing action of 8-MOP (Table VI), even when the added sucrose concentration was much higher than in the initial experiments. Subsequently, it was discovered that phosphate buffer (final pH 7.2, 0.1M) was capable to completely block the photosensitized destruction of SDH activity. (Table VI, Figure 5). The additional washing did not seem to provide any complication and seemed to give a delicate preparation susceptible to 8-MOP. The use of sodium chloride washing at least for two times followed by suspension of the mitochondria in distilled water was therefore continued as a routine throughout the other experiments.

Effect of preirradiation of mitochondria and psoralen solution: In order to rule out the formation of a phototoxic compound in mitochondrial suspensions during the course of illumination which could cause the biological damage even in absence of either psoralen or 8-MOP, investigations were carried out to determine the effect of preirradiating mitochondrial suspension and psoralen solutions separately. This was followed by mixing the two together. The SDH activity measurements were carried out and compared against samples kept in the dark without any preirradiation treatment. As can be seen from Table VI, preirradiation of mitochondria with UV light did not result in any loss of activity. Similarly preirradiated psoralen solution did not affect the activity of the enzyme. The results with psoralen as well as 8-MOP show one

Table VII Effect of aging of the mitochondria on the photosensitizing action of 8-methoxypsoralen

Age of mitochondria	Mitochondrial succinicdehydrogenase activity △OD/min.				Photosensitized % loss of enzyme activity
	Dark without 8-MOP	UV 15' without 8-MOP	Dark with 8-MOP	UV 15' with 8-MOP	
Freshly prepared	.033	.033	.032	.028	15
24 hours	.023	.020	.023	.017	26
3 days	.033	.033	.034	.027	20
5 days	.035	.035	.035	.027	23
8 days	.023	.021	.023	.017	26
11 days	.019	.019	.018	.0135	29
13 days	.022	.022	.019	.012	43.2
18 days	.0185	.019	.017	.011	41
25 days	.025	.020	.023	.010	60
44 days	.022	.020	.018	.013	39

important point, that to demonstrate the photosensitization of the biological material, irradiation of a combination of the dual agents, photosansitizer and biological material was essential and no effect was observed in the absence of one or the other of the dual agents.

Effect of aging of the mitochondria: Other procedures that revealed increased susceptibility of the mitochondria to the photosensitization action of furocoumarins was aging of the mitochondria. As shown in Table VII, freshly prepared mitochondria were less susceptible as compared to 1, 3, 5 days old mitochondria. In fact, the response reported in TableVIIwith freshly prepared mitochondria was the optimum response that was observed. Occasionally, no inactivation of the enzyme in presence of 8-MOP or psoralen would be observed with freshly prepared mitochondria. Generally, mitochondria preserved for 8 days at temperature below zero revealed nearly the same degree of susceptibility. However, older preparations (after 10 days) were more vulnerable to 8-MOP photosensitization. As shown in Table VII mitochondrial preparations aged as long as 13 to 20 days lost more than 40% of the succinic dehydrogenase activity following illumination in presence of 8-MOP. Control specimens, aged as long, but kept protected from ultraviolet light, or exposed to ultraviolet for a short period without any photosensitizer, did not lose their enzymic activity.

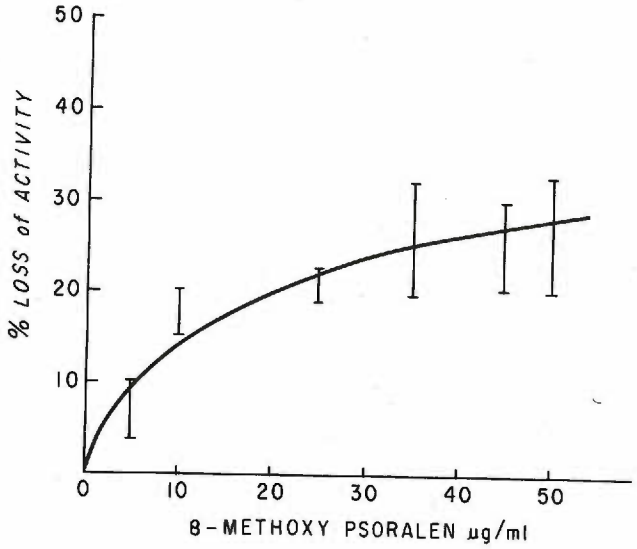
Effect of 8-methoxypsoralen concentration: The standard procedure in which mitochondrial suspension or pure enzyme solution (the investigations related to which will be reported in next section under lactic-

Figure 6:

Photosensitized inhibition of succinicdehydrogenase as a function of 8-methoxypsoralen concentration.

Vertical lines show the range of inhibition observed in triplicate runs.

Photosensitized Inhibition of Succinicdehydrogenase as a Function of 8-Methoxy Psoralen Concentration (Irradiation time: 15 minutes)



dehydrogenase studies) was subjected to irradiation in presence of 45 to 50 ug 8-MOP or psoralen per milliliter of suspension, was not arbitrarily chosen, but had been imposed to a greater extent by the limited aqueous solubility of the compound. Spectrophotofluorimetric determination showed that the maximum solubility of psoralen or 8-MOP in H_2O was in the range of 50 to 55 ug per ml. The relationship between the concentration and photosansitizing action of 8-MOP as measured by the loss of rat liver mitochondrial succinicdehydrogenase activity is shown in Figure 6. The maximum effectiveness was found to be reached at 35 ug/ml 8-MOP concentration. No appreciably greater amount of inhibition of SDH activity was observed after increasing the concentration up to 50 ug/ml. The inhibition curve showed a tendency of a plateau. Based on these observations enzyme studies reported in this thesis were carried out as a routine with an optimum 45 to 47.5 ug 8-MOP or psoralen concentration per ml. unless stated otherwise.

Dose of ultraviolet light: Photochemical reaction depends upon the absorption of light, the greater the absorption, the greater the amount of photochemical reaction to be expected. Although estimations of light absorption in SDH + psoralen system were not carried out, it was assumed that the penetration of such polychromatic light was exponential in character and that with increased irradiation period there was increased absorption of light and consequently a greater probability of activated psoralen molecules coming in contact with enzyme molecules.

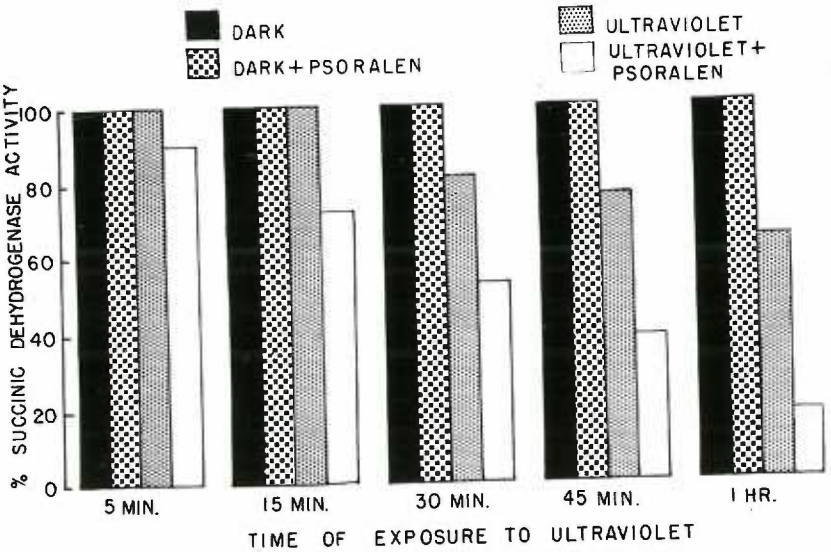
Figure 7

Mitochondrial succinicdehydrogenase activity as a function of time of irradiation with ultraviolet light 3200Å in presence of psoralen or 8-methoxypsoralen.

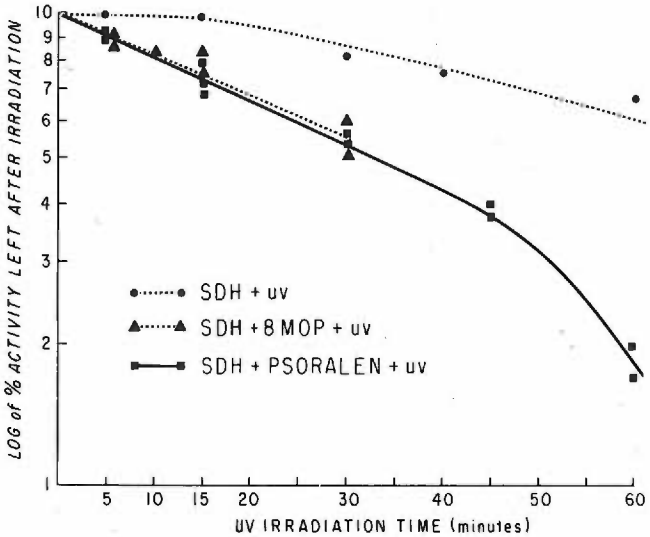
Top: Effect of varying the UV irradiation time in presence of 45 ug. Psoralen.

Bottom: Data when plotted on a semilogarithmic scale. Horizontal axis -- time in minutes.

Vertical axis -- logarithm of percent activity.



Mitochondrial Succinic dehydrogenase Activity as a Function of Time of Irradiation with Ultraviolet Light > 3200 A in Presence of Psoralen or 8-Methoxy Psoralen



The loss of SDH activity of rat liver mitochondria showed the concept of exponential relationship. A straight line was obtained when the logarithm of enzymatic activity was plotted against the dose of UV irradiation, as shown in Figure 7. The data given in Figure 7 is for psoralen as well as 8-methoxypsoralen. There were no significant differences noted when 8-MOP or psoralen were used as sensitizers. However, subsequent experience showed that inhibition with psoralen was slightly more than 8-MOP in a pure enzyme system. Secondary events resulting in the loss of enzyme activity following long-time UV irradiation (for more than 30 minutes) in absence of a photosensitizer complicated the presentation of linear relationship. Even under these conditions, the inhibition of SDH in presence of increasing dose of UV irradiation showed a linear relationship when the data was plotted on a semilogarithmic paper (Figure VII).

Effect of temperature during irradiation: Photodynamic action on sensitive biological systems in case of methylene blue has been found to have a temperature coefficient greater than 1.0 over the range from 0 to 37°C[69]. The temperature coefficients for the 8-MOP photosansitized bactericidal effect, as reported by Oginsky et al,[69] were found to be less than 1.0. It was therefore interesting to study the temperature coefficient of the photosensitized inactivation of SDH. Photosensitization of rat liver mitochondria in presence of 8-MOP was carried out at four temperature ranges: 0-2°C, 8-10°C, 20-21°C, and 28-30°C. As

Table VIII Effect of temperature on the photosensitizing action of 8-methoxypsoralen

Mitochondrial succinicdehydrogenase activity: Δ OD/15 minutes

Expt. #	Temperature	I Dark	II UV 15'	III with 8-MOP Dark	IV with 8-MOP UV 15'	% loss of enzyme activity in IV
1.	0-2°C	.050	.045	.050	.025	50
	30°C	.045	.040	.045	.030	34
2.	0-2°C	.060	.060	.055	.025	60
	30°C	.060	.055		.040	34
3.	7-10°C	.105	.090	.100	.060	43
	20-21°C	.105	.100	.105	.073	30.5
	28-30°C	.070	.070	.080	.054	25

shown in Table VIII it was found that a greater amount of SDH activity was lost as the temperature during exposure to UV was lowered. The temperature coefficients calculated for 8-MOP action were less than 1.0. The Q10 range varied from 0.8 to 0.7.

Role of sulphydryl reagents: The presence of SH groups essential for SDH activity was established by Hopkins et al[47]. The influence of thiol groups in the activity of this dehydrogenase led Hopkins and his coworkers to suggest that thiol group was essential for the activity of SDH. Integrity of sulphydryl groups for succinicdehydrogenase activity has been repeatedly stressed (Dixon and Webb[24], Singer et al.[83]). It seemed possible that photoactivated psoralen might be reacting with essential SH groups of the enzyme molecule and induced inactivation of the enzyme through their oxidation. If such were the case, the presence of sulphydryl reagents such as 2,3-dimercapto-1-propanol (BAL), glutathione or cysteine if provided during the illumination procedure in the system should exert a sparing effect and protect the enzyme. These reagents were tried at 10^{-4}M concentration. Higher concentrations were found to be deleterious to the enzymic activity when kept in dark without any photosensitizer. As shown in Figure 5, neither of these thiol reagents had any protective effect towards the photosensitized inactivation of SDH. By providing excess of these SH groups in the system, it was thought that there was more probability of oxidation of these thiol reagents in psoralen photosensitization than

Table IX Effect of oxidized and reduced glutathione, GSH + $K_4Fe(CN)_6$ GSH + $FeSO_4$ & preirradiation on psoralen photosensitization

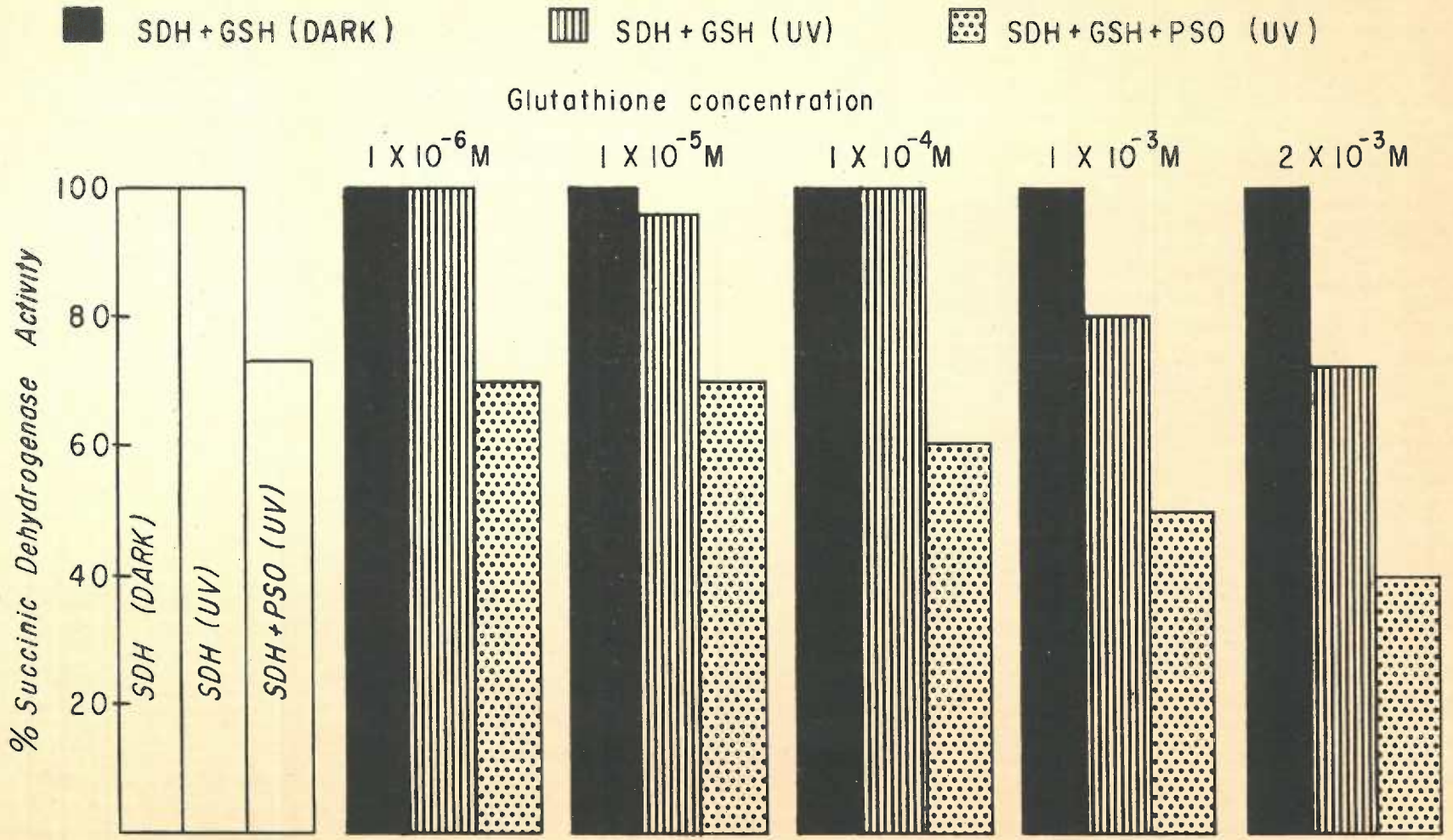
		Succinic dehydrogenase activity			
			Δ OD/10'	% activity	% loss of activity
1.	**Effect of oxidized glutathione**				
a.	SDH + psoralen + G-S-S-G-1x10^{-3}M	dark	.110	100	-
b.	SDH + psoralen	UV 20'	.085	77	23
c.	SDH + psoralen + G-S-S-G 1x10^{-3}M	UV 20'	.065	59	41
d.	SDH + psoralen + G-S-S-G 2x10^{-3}M	UV 20'	.050	45.5	54.5
2.	**Effect of pre-irradiation of psoralen and glutathione solutions**	Preirradiation 30'			
a.	SDH + irradiated psoralen + irradiated GSH 1x10^{-3}M	dark	.210	100	-
b.	SDH + Irradiated psoralen + irradiated GSH 1x10^{-3}M	reirradiated 15'	.125	60	40
3.	**Irradiated and unirradiated glutathione**				
a.	SDH + psoralen + unirradiated GSH 1x10^{-3}M	dark	0.175	100	-
b.	SDH + psoralen + unirradiated GSH 1x10^{-3}M	UV 20'	0.120	68	32
c.	SDH + psoralen + 30' preirradiated GSH 1x10^{-3}M	dark	0.180	100	-
d.	SDH + psoralen + 30' preirradiated GSH 1x10^{-3}M	reirradiated UV 20'	0.110	60	40

Table IX (Continued)

			Succinic dehydrogenase activity		
4.	Effect of $K_4Fe(CN)_6$ & Glutathione		- Δ OD/10'	% activity	% loss of activity
a.	SDH + psoralen	dark	0.175	100	-
b.	SDH + psoralen	UV 15'	0.145	82	18
c.	SDH + psoralen + $K_4Fe(CN)_6$ $1x10^{-3}M$	UV 15'	0.085	47	53
d.	SDH + psoralen + GSH $1x10^{-4}M$	dark	0.170	100	-
e.	SDH + psoralen + GSH $1x10^{-4}M$	UV 15'	0.115	67	33
f.	SDH + psoralen + GSH $1x10^{-4}M$ + $K_4Fe(CN)_6$ $1x10^{-3}M$	UV 15'	0.055	33	67
5.	Effect of Ferrous sulfate				
a.	SDH + psoralen	dark	0.110	100	-
b.	SDH	UV 15'	0.110	100	-
c.	SDH + psoralen	UV 15'	0.080	73	27
d.	SDH + $FeSO_4$ $1x10^{-3}M$	dark	0.100	91	9
e.	SDH + $FeSO_4$ $1x10^{-3}M$	UV 15'	0.100	91	9
f.	SDH + psoralen + $FeSO_4$ $1x10^{-3}M$	UV 15'	0.080	73	27
g.	SDH + psoralen + GSH $1x10^{-4}M$ + $FeSO_4$ $1x10^{-3}M$	dark	0.100	100	-
h.	SDH + psoralen + GSH $1x10^{-4}M$ + $FeSO_4$ $1x10^{-3}M$	UV 15'	0.060	60	40

Figure 8: Effect of Glutathione on Psoralen Photosensitization

EFFECT OF GLUTATHIONE ON PSORALEN PHOTOSENSITIZATION
(Rat liver mitochondrial Succinic dehydrogenase)



the limited number of essential SH group of the enzyme molecule. Thus these agents would exert a sort of protective effect and prevent the inactivation of the enzyme molecules. This hypothesis proved to be untrue. In fact, glutathione was found to increase the sensitivity of the mitochondria to photosensitization. When the concentration of glutathione in the irradiated system containing psoralen was progressively increased by tenfold (from 10^{-6} to 10^{-3}M) it was observed that the increased sensitivity due to the presence of reduced glutathione was proportional to the concentration of the sulphydryl reagent (Figure 8). Incorporation of the oxidized glutathione into SDH+psoralen system had the same effect as noted for the reduced glutathione (Table IX). Glutathione or mixtures of glutathione and psoralen, when irradiated prior to mixing with the mitochondria showed none of the effects noted above when the mixture (mitochondria + irradiated glutathione or mitochondria + irradiated glutathione in presence of psoralen) was left in the dark (Table IX). However this preilluminated material when mixed with the enzyme suspension and subjected to reirradiation, was found to be equally effective in inducing photosensitizing action.

This unprotective effect of glutathione was further investigated in an invivo system using human skin as well as guinea pig skin. The mammalian skin is well known to be rich in sulfur-containing compounds such as cysteine, cystine and methionine. These thiol com-

pounds are present specifically in keratins in the bound form as -S-S- linkages. A simple experiment was performed to show that glutathione did not give any protection against psoralen photosensitization. On the arm of a white-skinned human subject, and on the back of the smooth-skinned albino guinea pig, two areas about 2 cm apart were selected. In one area of 2.5 cm^2, 25 ug of psoralen was applied topically on human skin as well as on guinea pig skin. In another 2.5 cm square area about 770 ug of reduced glutathione dissolved in 0.1 ml H_2O was applied topically on both of the experimental subjects. This was followed by topical application of 25 ug psoralen. On the top of this about 750 ug GSH was further applied. This was followed by UV irradiation. The human skin was exposed for 15 minutes UV irradiation and the guinea pig skin was exposed for 45 minutes UV irradiation at 15 cm distance. Erythemal response was recorded photometrically at the end of 24 and 48 hours. It was of interest to note that both areas showed significant ++++ grade erythemal response. In fact, the skin of the human subject where psoralen and glutathione were applied exhibited a big blister at the end of the 36 hours, whereas psoralen treated area showed no such high degree of response. Guinea pig skin, however, did not show blistering. It is hard to say emphatically that glutathione potentiated psoralen effect, but it was obvious that presence of GSH at the site of psoralen action did not give any protection. The biological damage induced by psoralen in the form of photosensitized erythemal response had oc-

curred even in presence of significant amount of GSH.

The experimental results indicated that glutathione acted as a photosensitizer. If photooxidation was conceived as one of the mechanisms by which glutathione induced its effect (oxidation of -SH groups to -S-S- groups), incorporation of a more readily oxidizable substance such as potassium ferrocyanide or ferrous sulfate would alter the GSH response. It was thought that they would be preferentially oxidized in presence of psoralen and UV irradiation, and prevent the loss of LDH activity. Both of these agents did not prevent psoralen photosensitization. (See Table IX). Neither of them reduced the action of glutathione which was shown earlier to increase the effect of psoralen photosensitization. In fact, $K_4Fe(CN)_6$ acted as a more drastic photosensitizer. Ferrous sulfate was found to be an inhibitor of mitochondrial SDH at $1x10^{-3}$M range in absence of psoralen and UV irradiation. Since the interpretation of SDH activity data was carried out on comparative basis (dark vs. UV), this inhibitory effect of ferrous sulfate on enzyme activity did not complicate the above generalization.

Effect of ethylenediamine tetraacetate: It is possible that exposure of biological material such as mitochondria to ultraviolet light may result in the formation of peroxides (lipide peroxides, H_2O_2 and other organic peroxides). These peroxides may act as oxidative agents to produce the loss of enzyme activity. The cations present, notably Fe++, Co++, Cu++, in the system may also act catalytically in producing peroxides. Succinic dehydrogenase is known to be a metaloprotein and one of its

Table X Effect of ethylenediaminetetraacetate (EDTA) on psoralen photosensitization
(SDH = Mitochondrial succinicdehydrogenase)

Irradiated system		Succinic dehydrogenase activity: Δ OD 10 min.	Succinic dehydrogenase activity: Δ OD per min.	% enzyme activity	% loss enzyme activity
Exp.					
I. a. SDH + psoralen	dark	0.165	0.0165	100	-
b. SDH + psoralen	UV 15'	0.120	0.012	75	25
c. SDH + psoralen + EDTA $1x10^{-3}M$	dark	0.160	0.0160	97	3
d. SDH + psoralen + EDTA $1x10^{-6}M$	UV 15'	0.115	0.0115	70	30
e. SDH + psoralen + EDTA $1x10^{-5}M$	UV 15'	0.115	0.0115	70	30
f. SDH + psoralen + EDTA $1x10^{-4}M$	UV 15'	0.090	0.009	54.5	45.5
g. SDH + psoralen + EDTA $1x10^{-3}M$	UV 15'	0.140	0.014	84.8	15.2
h. SDH + psoralen + EDTA $2x10^{-3}M$	UV 15'	0.165	0.0165	100	-
II. a. SDH + psoralen	dark	0.150	0.0150	100	-
b. SDH + psoralen	UV 15'	0.115	0.0115	77	23
c. SDH + psoralen + EDTA $2x10^{-3}M$	UV 15'	0.165	0.0165	110	-
III. a. SDH + psoralen	dark	0.150	0.015	100	-
b. SDH + psoralen	UV 30'	0.075	0.0075	50	50
c. SDH + psoralen + EDTA $1x10^{-3}M$	UV 30'	0.105	0.0105	70	30
IV a. SDH + psoralen + EDTA $2x10^{-3}M$	dark	0.160	0.0160	100	-
b. SDH + psoralen	UV 30'	0.090	0.009	56	44
c. SDH + psoralen + EDTA $2x10^{-3}M$	UV 30'	0.140	0.0140	87	13
d.					
V. a. SDH + psoralen	dark	0.175	0.0175	100	-
b. SDH + psoralen + EDTA $4x10^{-4}M$	UV 30'	0.100	0.0100	57	43
c. SDH + psoralen + EDTA $2x10^{-3}M$	UV 30'	0.165	0.0165	94.3	5.7

prosthetic group has been confirmed to be iron: Fe++ (Singer et al[83]). A suggestion automatically comes forward that the prosthetic group of the enzyme could be involved in the process of photosensitized inhibition. It is equally possible that cations such as copper, iron, which are part of mitochondrial enzyme systems, are acting as catalytic agents to form peroxides. If such was the case, then substances which can interfere with either peroxide formation or act by virtue of metal complexing properties (chelation) should protect the prosthetic group and prevent the biological effects of psoralen photosensitization. Effect of EDTA was therefore investigated. Incorporation of this substance into the biological system under study prior to illumination revealed a significant protection. (Table X). Initially a concentration of 1×10^{-3}M was arbitrarily chosen. Mitochondrial suspension with psoralen when irradiated in presence of EDTA showed very little loss of enzyme activity. Control runs which were irradiated in presence of psoralen but without any EDTA showed the usual effect of about 25 to 30% loss of SDH activity. The presence of this metal chelating agent versene at 2×10^{-3}M concentration almost completely blocked the photosensitizing action of psoralen. This observation necessitated the study of the effect of variation in EDTA concentration. As shown in Table X, concentrations of EDTA in the range of 1×10^{-6}M to 1×10^{-4}M were ineffective in preventing the photosensitizing action of psoralen, the enzyme SDH was found to be inactivated. The protective

Table XI Effect of Glutathione and EDTA on photosensitization by psoralen

Expt. #	Irradiated system		SDH activity: Δ OD per 10 min	SDH activity: Δ OD per min	% enzyme activity	% loss of enzyme activity
1. a.	SDH	dark	0.220	0.022	100	-
b.	SDH + 8 MOP	UV 15'	0.190	0.019	86	14
c.	SDH + 8 MOP + GSH $1x10^{-3}M$	UV 15'	0.090	0.009	41	59
2. a.	SDH	dark	0.190	0.019	100	-
b.	SDH	UV 15'	0.200	0.020	105	-
c.	SDH + psoralen	UV 15'	0.145	0.0145	76	26
d.	SDH + psoralen + GSH $1x10^{-3}M$	UV 15'	0.060	0.006	31	69
3. a.	SDH + psoralen	dark	0.170	0.0170	100	-
b.	SDH + psoralen + GSH $1x10^{-3}M$	dark	0.165	0.0165	97	3
c.	SDH + psoralen + GSH $1x10^{-3}M$	UV 15'	0.105	0.015	60	40
d.	SDH + psoralen + GSH $1x10^{-3}M$ + EDTA $2x10^{-3}M$	dark	0.170	0.0170	100	-
e.	SDH + psoralen + GSH $1x10^{-3}M$ + EDTA $2x10^{-3}M$	UV 15'	0.165	0.0165	97	3
4. a.	SDH + psoralen + EDTA $1x10^{-3}M$	dark	0.190	0.019	100	-
b.	SDH + psoralen	UV 15'	0.110	0.011	58	42
c.	SDH + psoralen + EDTA $1x10^{-3}M$ + GSH $1x10^{-3}M$	UV 15'	0.160	0.016	84	16
5. a.	SDH + psoralen	dark	0.160	0.016	100	-
b.	SDH + psoralen + EDTA $1x10^{-3}M$	dark	0.160	0.016	100	-
c.	SDH + psoralen	UV 15'	0.125	0.012	75	25
d.	SDH + psoralen + EDTA $1x10^{-3}M$	UV 15'	0.140	0.014	88	12
e.	SDH + psoralen + GSH $1x10^{-3}M$ + EDTA $1x10^{-3}M$	UV 15'	0.170	0.017	100	-

effect of EDTA was apparent at $1x10^{-3}$M concentration and at $2x10^{-3}$M concentration. This chelating molecule was positively shielding the enzyme molecules from being inactivated. By doubling the time of illumination to 30 minutes, the protective effect of EDTA was still significantly noticeable.

Effect of EDTA in presence of glutathione: Earlier it was observed that incorporation of GSH into the irradiation system containing SDH+psoralen showed no protective effect; on the contrary, GSH was observed to be potentiating the effect of psoralen photosensitization. The effect of EDTA was also studied in this system which included SDH, psoralen and glutathione. As shown in Table XI, incorporation of EDTA into this irradiation system revealed a significant protective effect. Provision of EDTA in $1x10^{-3}$M range showed that the photosensitized inhibitory effects of psoralen and glutathione were considerably blocked. The effect of glutathione in presence of psoralen and UV irradiation which was shown to be a sort of potentiating or accelerating effect was almost completely inhibited by incorporating EDTA into the irradiated system.

Effect of molecular oxygen on the photosensitized inhibition of succinicdehydrogenase in presence of 8-MOP: The studies so far revealed that the mechanism of psoralen action did not seem to involve the sulphydryl groups of the succinicdehydrogenase nor oxidative processes as those observed by other workers (Weil et al.[96, 97, 98, 99]). As stated

Table XII Effect of oxygen during photosensitized destruction of succinic dehydrogenase in presence of 8-methoxypsoralen

Biological system		Gas phase	% SDH activity	% loss of activity
a. SDH	dark	air (O_2)	100	-
SDH	UV 15'	air (O_2)	100	-
SDH + 8-MOP	UV 15'	air (O_2)	75, 77	25, 23
b. SDH + 8-MOP	dark	pure O_2	99, 95	1, 5
SDH + 8-MOP	UV 15'	pure O_2	87, 86	13, 14
c. SDH	dark	helium	98, 93	4, 7
SDH + 8-MOP	UV 15'	helium	73, 69, 5	27, 30.5
d. SDH + 8-MOP	dark	nitrogen	96	4
SDH + 8-MOP	UV 15'	nitrogen	70, 76	30, 24

in the section of introduction, the term photodynamic action as proposed by Blum[11, 46], means an oxygen obligate photosensitization of living system where molecular O_2 is believed to take part in the photochemical reaction. The photodynamic action of methylene blue and other dyes such as acryflavin, eosin, etc., have been shown to be oxygen-dependent; in absence of oxygen there was no photodynamic effect. To see whether oxygen was needed to induce photosensitization effect of 8-MOP was further investigated.

A stock mitochondrial suspension with and without 8-MOP was prepared sufficient to complete the duplicate runs for the entire experiment reported in XII. The usual effect of 8-MOP in presence of UV irradiation was ascertained. The gas phase in this case was ordinary air. There was about 25% inhibition of SDH activity (Table XII). In another set using the same stock mitochondrial suspension containing 8-MOP, irradiation was carried out in presence of pure O_2. The results showed that in fact pure oxygen reduced the photosensitizing action of 8-MOP. As compared to the 25% inhibition of SDH when the gas phase was ordinary air, the presence of molecular oxygen consistently diminished the photosensitizing action of 8-MOP. When helium or nitrogen was used as a gas phase during irradiation, 8-MOP was still capable of inducing photosensitized inhibition of SDH. The inhibition was in the range of 25 to 31%. The photosensitization of SDH by 8-MOP thus did not appear to be true photodynamic action as defined by Blum,

Table XIII Effect of psoralen on lacticdehydrogenase activity in presence of ultraviolet light (> 3200Å)

Expt. No.	Biological system		Δ OD/min	Millimicro moles of DPNH	% loss in enzyme activity
1.	a. LDH	dark	0.155	7.44	-
	b. LDH	UV 15'	0.155	7.44	-
	c. LDH + psoralen	dark	0.150	7.20	3
	d. LDH + psoralen	UV 15'	0.135	6.4	13
2.	a. LDH	dark	0.150	7.2	-
	b. LDH	UV 15'	0.150	7.2	-
	c. LDH + psoralen	dark	0.135	6.4	13
	d. LDH + psoralen	UV 15'	0.105	5.0	30
3.	a. LDH	dark	0.195	9.36	-
	b. LDH	UV 30'	0.200	9.6	-
	c. LDH + psoralen	dark	0.185	8.88	5.2
	d. LDH + psoralen	UV 30'	0.135	6.40	31
4.	a. LDH	dark	0.075	3.6	-
	b. LDH	UV 30'	0.080	3.8	-
	c. LDH + psoralen	dark	0.070	3.4	6
	d. LDH + psoralen	UV 30'	0.050	2.4	30
5.	a. LDH	dark	0.085	4.1	-
	b. LDH	UV 30'	0.085	4.1	-
	c. LDH + psoralen	dark	0.085	4.1	-
	d. LDH + psoralen	UV 30'	0.060	2.88	30
6.	a. LDH	dark	0.125	6.0	-
	b. LDH	UV 30'	0.120	5.8	4
	c. LDH + psoralen	dark	0.125	6.0	-
	d. LDH + psoralen	UV 30'	0.090	4.3	28
7.	a. LDH	dark	0.285	13.7	-
	b. LDH	UV 30'	0.266	12.8	5
	c. LDH + psoralen	dark	0.285	13.7	-
	d. LDH + Psoralen	UV 30'	0.200	9.6	30

but represented rather a type of photosensitization not accelerated by molecular O_2 and could take place in absence of this gas. The flushing of gases such as O_2, helium or nitrogen over the surface of the enzyme solution seemed to result in slight inactivation of enzyme in absence of UV irradiation (Table XII) possibly due to surface denaturation.

Effect of psoralen on lacticdehydrogenase activity: When crystalline muscle lacticdehydrogenase solution was irradiated in presence of psoralen, photosensitized inhibition of enzyme was observed. With 15 minutes of UV irradiation, in presence of 45 ug psoralen per ml, definite loss of the enzymatic activity was noted (Table XIII). This system contained no interfering substances such as those reported earlier in mitochondrial suspension. As can be seen from the data, ultraviolet irradiation for a 30 minute period in absence of psoralen did not inhibit enzyme activity, only in presence of psoralen and UV light>3200Å this inhibition was observed.

Effect of psoralen concentration: Experiments similar to inhibition of rat liver mitochondrial succinicdehydrogenase were carried out to determine the most effective psoralen concentration. As stated earlier, solubility of psoralen was not more than 5 mg per 100 ml.H_2O. Hence various dilutions of stock psoralen solution were made to give 6, 12, 24, 36, and 48 ug psoralen per ml H_2O. 2.5 ml lactic dehydrogenase solutions containing psoralen in the above concentrations were irradi-

Figure 9:

Effect of Psoralen concentration on photosensitized inhibition of Lacticdehydrogenase.

The vertical lines indicate the range of inhibition in duplicate or triplicate runs.

Effect of Psoralen Concentration on Photosensitized Inhibition of Lacticdehydrogenase

(uv irradiation: 15 minutes)

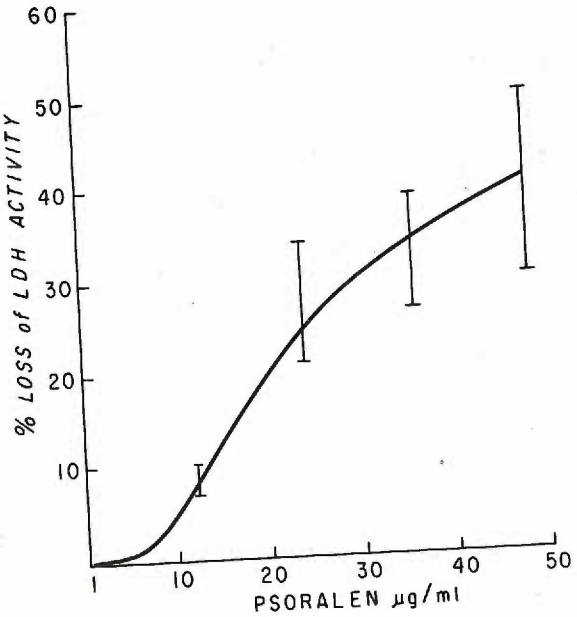


Table XIV Effect of dose of ultraviolet light on psoralen photosensitized inhibition of lacticdehydrogenase

(Psoralen = 46 ug/ml)

			Δ OD/min	Millimicro moles of DPNH	% activity	% loss of activity
a.	LDH	dark	0.105	5.04	100	-
b.	LDH + psoralen	dark	0.105	5.04	100	-
c.	LDH	UV 15'	0.105	5.04	100	-
d.	LDH + psoralen	UV 15'	0.070	3.36	66	34
e.	LDH	UV 30'	0.105	5.04	100	-
f.	LDH + psoralen	UV 30'	0.045	2.16	43	57
g.	LDH	UV 45'	0.100	4.8	95	5
h.	LDH + psoralen	UV 45'	0.030	1.44	28.5	71.5
i.	LDH	UV 60'	0.080	3.84	76	24
j.	LDH + psoralen	UV 60'	0.020	0.96	19	81

ated for 15 minutes. As shown in Figure 9, with the increase in psoralen concentration, there was correspondingly increased inhibition of lactic dehydrogenase. The optimum inhibition was obtained when the solution contained 45 to 48 ug/ml.psoralen. Hence invariably this concentration was used in most of the studies related to psoralen photosensitization.

Effect of dose of ultraviolet light on photosensitized inhibition of lacticdehydrogenase: The enzyme solution with and without psoralen was irradiated under UV light for varying periods. As shown in Table XIV when the dose of UV irradiation was increased progressively from 15 minutes to 1 hour, correspondingly there was increased inhibition of lacticdehydrogenase. When the data was plotted on a semilogarithmic scale, a linear relationship was observed. It was presumed that with the increase in time of UV irradiation, there was proportionally increased absorption of light by the system. These observations are in agreement with the concept of fundamental law of photochemistry.

Effect of thiol reagents: cysteine, glutathione, and 2:3-dimercapto-1-propanol (BAL) on photosensitized inhibition of lacticdehydrogenase by psoralen: Earlier, the effect of these thiol reagents on rat liver mitochondrial succinicdehydrogenase in presence of psoralen was studied. It was observed that they did not provide any protection. In fact, GSH was found to potentiate the effect. Similar studies with crystalline lacticdehydrogenase were carried out. As shown in Table XV, neither

Table XV Effect of cysteine, BAL (2, 3-dimercapto-1-propanol) and glutathione on psoralen photosensitized inhibition of lacticdehydrogenase

Expt. #	Irradiated system		Δ OD/min	Millimicro moles of DPNH	% surviving activity	% loss of activity
1.	Effect of cysteine					
a.	LDH + psoralen	dark	0.285	13.7	100	-
b.	LDH + psoralen	UV 30'	0.200	9.6	70	30
c.	LDH + psoralen + cysteine $1x10^{-3}M$	dark	0.270	13.0	95	5
d.	LDH + psoralen + cysteine $1x10^{-3}M$	UV 30'	0.220	10.5	77	23
2.	Effect of GSH					
a.	LDH + psoralen	dark	0.095	4.6	100	-
b.	LDH + psoralen	UV 30'	0.045	2.2	47	53
c.	LDH + psoralen + GSH $1x10^{-4}M$	dark	0.090	4.3	94	6
d.	LDH + psoralen + GSH $1x10^{-4}M$	UV 30'	0.045	2.2	47	53
3.						
a.	LDH + psoralen	dark	0.135	6.5	100	-
b.	LDH + psoralen	UV 30'	0.080	3.84	59	41
c.	LDH + psoralen + GSH $1x10^{-4}M$	dark	0.130	6.24	96	4
d.	LDH + psoralen + GSH $1x10^{-4}M$	UV 30'	0.065	3.12	48	52
4.						
a.	LDH + psoralen	dark	0.150	7.2	100	-
b.	LDH + psoralen	UV 30'	0.135	6.4	89	11
c.	LDH + psoralen +BAL $1x10^{-4}M$	dark	0.135	6.4	89	11
d.	LDH + psoralen + BAL $1x10^{-4}M$	UV 30'	0.100	4.8	66	34

cysteine and BAL nor glutathione were able to prevent enzyme inhibition. The degree of inhibition by psoralen was the same irrespective whether these SH reagents were present or absent. The thiol groups of lactic dehydrogenase seemed not to be affected by psoralen during the photosensitization.

Effect of metal ions on psoralen photosensitized inhibition of lacticdehydrogenase: The polar side chains of protein molecules lead to interaction with various electrolytes in solution and it is natural that metal ions often have a pronounced effect on the catalytic activity of proteins. It is known that the metal ions have strong ability to form strong complexes with radicals, and polar groups containing O_2, N_2 and sulfur, e.g., carboxyl, imidazole, sulphydryl, terminal NH_2 and terminal carboxyl groups. Another property of metal ions is the ability to act as bridges between protein and certain small molecules. The metal ion may be firmly bound with enzymes, and there are enzymes that have dissociable metal ions too. Although the knowledge of the functional role of some metal ions, e.g., Zn++, Mg++, Mn++, Cu++, etc., as component of metalloenzymes is much less complete, their functional role in catalytic activity of enzymes is beginning to emerge. Recent investigations reported by Vallee and his associates[92, 93], indicate that zinc is a functional component of several dehydrogenases. They have conclusively demonstrated that crystalline rabbit muscle lactic-dehydrogenase contained significant quantities of zinc[92]. Studies involving quantitative emmission spectroscopy, microchemical analysis,

and inhibition studies with metal binding agents, such as sodium diethyldithiocarbamate, 1,10-phenanthroline, sodium azide, sulfide, 8-hydroxyquinoline, etc., have suggested these workers to propose that metals and particularly zinc ions are involved in the catalytic activity of several pyridine nucleotide dependent enzymes and participate in the mechanism of action of dehydrogenases.

Many enzymes are known to lose their activity when the metal ions are removed. The loss is proportional to the metal removed implying the severance of a specific linkage. It is therefore tempting to speculate that the association of metal ions with proteins may stabilize certain helical or folded configurations and thus affect the physical and biological properties. The effect of a few cations was therefore investigated with the hope that by forming chelate compounds or complexes with some specific groups in the enzyme molecule, or just by associating with certain groups, e.g., SH, NH_2, COOH, etc. they would protect the structure and functional group of the enzyme molecule and prevent the photosensitized inhibition of lacticdehydrogenase. It is also known that the electronic configuration of iron (four or five unpaired 3d electrons) permit oxidation and reduction of this ion in solution. Colloidal iron in Fe+++ state, Mg^{++} ions, Cu^{++} ions and Zn^{++} ions mostly in the range of 10^{-4}M concentration were added separately to the system containing lacticdehydrogenase and psoralen. Effect of each ion was investigated separately and before addition, the pH of the salt solutions was checked for neutrality. As usual, an aliquot (2.5ml) was kept in the

Table XVI Effect of metal ions on psoralen photosensitized inhibition of lacticdehydrogenase

			△OD/min	Millimicro-moles of DPNH	% activity	% loss of activity
Effect of Fe+++ ($FeCl_3$ pH 7.0)						
1.	a. LDH + psoralen	dark	0.115	5.52	100	-
	b. LDH + psoralen	UV 15'	0.075	3.60	65	35
	c. LDH + psoralen+$FeCl_3$ $1x10^{-5}M$	dark	0.100	4.8	87	13
	d. LDH + psoralen+$FeCl_3$ $1x10^{-5}M$	UV 15'	0.065	3.12	56.5	43.5
2.	a. LDH + psoralen	dark	0.110	5.30	100	-
	b. LDH + psoralen	UV 15'	0.060	1.92	59.5	35.5
	c. LDH + psoralen+$FeCl_3$ $1x10^{-4}M$	dark	0.105	5.04	95	5
	d. LDH + psoralen+$FeCl_3$ $1x10^{-4}M$	UV 15'	0.030	1.44	27	73
Effect of colloidal Fe $(OH)_3$ pH 7.0						
3.	a. LDH + psoralen	dark	0.130	6.24	100	-
	b. LDH + psoralen	UV 30'	0.038	1.82	30	70
	c. LDH + psoralen+$Fe(OH)_3$ $1x10^{-5}M$	dark	0.115	5.52	88	12
	d. LDH + psoralen+$Fe(OH)_3$ $1x10^{-5}M$	UV 15'	0.085	4.08	65	35
4.	Effect of Magnesium ions ($MgCl_2$) pH7.0					
	a. LDH	dark	0.105	5.04	100	-
	b. LDH + psoralen	dark	0.105	5.04	100	-
	c. LDH + psoralen	UV 15'	0.025	1.20	24	76
	d. LDH + psoralen+$MgCl_2$ $1x10^{-4}M$	dark	0.085	4.08	81	19
	e. LDH + psoralen+$MgCl_2$ $1x10^{-4}M$	UV 15'	0.065	3.12	62	38
	f. LDH + psoralen+$MgCl_2$ $1x10^{04}M$	UV 15'	0.040	1.92	38	62

Table XVI (Continued)

		ΔOD/min	Millimicro-moles of DPNH	% activity	% loss of activity
5. Effect of copper ions ($CuSO_4$)					
a. LDH + psoralen	dark	0.130	6.24	100	-
b. LDH + psoralen	UV 15'	0.080	3.84	61.5	38.5
c. LDH + psoralen+$CuSO_4$ $1x10^{-4}M$	dark	0.120	5.76	92	8
d. LDH + psoralen+$CuSO_4$ $1x10^{-4}M$	UV 15'	0.080	3.84	61.5	38.5
6. Effect of Zinc ions (pH 7.0)					
a. LDH + psoralen	dark	0.125	6.0	100	-
b. LDH + psoralen	UV 15'	0.040	1.92	32	68
c. LDH + psoralen+Zinc acetate $1x10^{-4}M$	dark	0.105	5.04	84	16
d. LDH + psoralen+Zinc acetate $1x10^{-4}M$	UV 15'	0.050	2.40	40	60
g. LDH + psoralen+$ZnCl_2$ $1x10^{-4}M$	dark	0.125	6.0	100	-
h. LDH + psoralen+$ZnCl_2$ $1x10^{-4}M$	UV 15'	0.065	2.16	52	48
i. LDH + psoralen+$ZnSO_4$ $1x10^{-3}M$	dark	0.090	4.32	72	28
j. LDH + psoralen+$ZnSO_4$ $1x10^{-3}M$	UV 15'	0.040	1.92	32	68
7. *					
a. LDH + psoralen	dark	0.210	10.08	100	-
b LDH + psoralen	UV 15'	0.100	4.8	47.6	52.4
c. LDH + psoralen+$ZnCl_2$ $1x10^{-4}M$	dark	0.210	10.08	100	-
d. LDH + psoralen+$ZnCl_2$ $1x10^{-4}M$	UV 15'	0.085	4.08	40.0	60

* Assay system: Pyruvate + DPNH $\rightleftharpoons$ Lactate + DPN

dark to serve as control values (100% activity). Another 2.5 ml was irradiated for 15 minutes. Duplicate runs were carried out for each set.

As shown in Table XVI, no protective effect with any of these ions was observed. The degree of inactivation in presence or absence of Fe^{+++}, Cu^{++}, Mg^{++}, Zn^{++} was of the same magnitude. Earlier, the unprotective effect of Fe^{++} ions ($FeSO_4$) on psoralen photosensitized inhibition of mitochondrial SDH has been described (Table IX).

The activity of original enzyme solutions containing LDH and psoralen only but without any metal ions was found to be always higher than the unirradiated LDH preparations containing metal ions and psoralen. The enzyme was found to be inhibited by these ions at 10^{-4} and 10^{-3}M concentrations. Hence the 100% activity figures represent the initial LDH activity in presence of metal ions, the other values have not been included in the Table. These observations (inhibition of LDH in presence of metal ions without irradiation) are not perplexing. Many of the pyridine nucleotide dependent enzymes are known to be readily inhibited partially with low concentration of metal ions; it may be inhibited, among others, by ions of the very metal which is an integral, functional part of the apoenzyme molecule. The inhibitory effect of metal ions would appear to indicate that the mode and site of binding differ significantly for the enzymatically bound metal and for the added metal ion.

Studies involving reduced diphosphopyridine nucleotide (DPNH) in relation to psoralen photosensitization and inhibition of lacticdehydrogenase: Theorell and Bonnichsen[87] made the interesting discovery that crystalline alcohol dehydrogenase formed a complex in the presence of reduced diphosphopyridine nucleotide, ADH $(DPNH)_2$. The SH group of the enzyme was believed to combine in some form with coenzyme molecule. In view of close similarity in action of alcohol dehydrogenase and lacticdehydrogenase, Chance & Neilands[19] tested LDH for possible complex formation with DPNH. Their results indicated that DPNH also attaches to the apoenzyme. It also happens that reduced coenzyme DPNH has a typical absorption band in the long-wave ultraviolet region with the maximum of the broad peak lying near 340 mu, whereas the oxidized form (DPN) does not show any absorption in the region from 300-420 mu. Psoralen molecule has characteristic absorption in ultraviolet region with three major peaks in the region of 245, 295, 326 mu. The activating wavelengths of fluorescence for this molecule are in two regions: (1) 265 mu and 365 mu. (See further observations in this thesis). It was therefore of interest to study the effect of addition of DPNH in the system containing LDH and psoralen, and to see whether the incorporation of DPNH prevented the photosensitization of psoralen either through the complex formation of coenzyme and apoenzyme or through prevention of access of the activating wavelength which in presence of psoralen bring about the biological damage.

Table XVII A Studies involving reduced diphosphopyridine nucleotide (DPNH) in relation to psoralen photosensitization and inhibition of lacticdehydrogenase.

Assay: Pyruvate + DPNH $\rightleftharpoons$ Lactate + DPN

Exp. #	System		△OD/min	millimicro-moles of DPNH oxidized	% activity	% loss of activity
1.	a. LDH	dark	0.195	9.4	100	-
	b. LDH + psoralen	dark	0.195	9.4	100	-
	c. LDH	UV 15'	0.195	9.4	100	-
	d. LDH + psoralen	UV 15'	0.110	5.2	55	45
2.	a. LDH + psoralen	dark	0.220	10.56	100	-
	b. LDH + psoralen	UV 15'	0.065	3.12	30	70
3.	Effect of DPNH					
	a. LDH	dark	0.200	9.60	100	-
	b. LDH	UV 15'	0.200	9.60	100	-
	c. LDH + psoralen	dark	0.200	9.60	100	-
	d. LDH + psoralen	UV 15'	0.100	4.80	50	50
	e. LDH + psoralen + DPNH $1x10^{-3}M$	dark	0.195	9.40	97.5	2.5
	f. LDH + psoralen + DPNH $1x10^{-3}M$	UV 15'	0.095	4.56	47.5	52.5
	g. LDH + psoralen	UV 15'	0.085	4.08	42.5	57.5
	h. LDH + psoralen + DPNH $1x10^{-3}M$	UV 15'	0.100	4.80	50	50
4.	a. LDH + psoralen + DPNH $2x10^{-3}M$	dark	0.235	11.28	100	-
	b. LDH + psoralen + DPNH $2x10^{-3}M$	UV 15'	0.175	8.40	74	26
	c. LDH + psoralen + DPNH $2x10^{-3}M$	UV 15'	0.170	8.20	72	28
5.	Effect of substrate (pyruvate)					
	a. LDH + psoralen + pyruvate $1x10^{-2}M$	dark	0.140	6.72	100	-
	b " " "	UV 15'	0.070	3.36	50	50
	b. " " "	dark	0.145	6.96	100	-
	d. " " "	UV 15'	0.050	2.40	34.5	65.5

Table XVII B Effect of DPNH (Continued)

	X		Y	
	LDH + Psoralen		LDH + psoralen + DPNH	
	Dark	UV 15'	Dark	UV 15'
1. Δ OD/min	0.125	0.020	0.060	0.040
% activity	100	16	100	66.6
% loss of enzyme activity	-	84	-	33.4
2. Δ OD/minute	0.130	0.040	0.080	0.040
% activity	100	30	100	50
% loss of enzyme activity	-	70	-	50
3. Δ OD/minute	0.135	0.070	0.075	0.040
% activity	100	52	100	53
% loss of enzyme activity	-	48	-	47
4. Δ OD/minute	0.125	0.070	0.045	0.035
% activity	100	56	100	78
% loss of enzyme activity	-	44	-	22
5. Δ OD/minute	0.150	0.055	0.075	0.055
% activity	100	36	100	73
% loss of enzyme activity	-	64	-	27
6. Δ OD/minute	0.100	0.024	0.040	0.040
% activity	100	24	100	100
% loss of enzyme activity	-	76	-	-

Note: Experiment #1, 2, 3 - DPNH 1×10^{-3}M and #4, 5, 6 - DPNH 2×10^{-3}M

The pyridine nucleotide was added in the range of $1x10^{-3}$ and $2x10^{-3}$ M concentration prior to irradiation. Since DPNH was added in the system, lacticdehydrogenase activity measurements of pre and post irradiated specimens were carried out in two ways, one employing pyruvate as the substrate and the other employing lactate as the substrate. In the first instance, additional DPNH, 0.1 ml., 0.002 M was also added in the Beckman silica cells, and the rate of oxidation of DPNH was measured at 340 mu. These observations are reported in Table XVII A. In the other method, excess amount (0.2 ml. of 0.02M) of DPN was added to make the reaction equilibria proceed in favour of pyruvate formation. These observations are reported in Table XVIIB.

As shown in Table XVIIA and XVIIB, the effect of incorporating DPNH into the system does not seem to give appreciable protection to the enzyme against psoralen photosensitization at $1x10^{-3}$M concentration. The degree of inhibition of lacticdehydrogenase in presence of DPNH was practically of the same magnitude as in absence of nucleotide. However, at $2x10^{-3}$M concentration there was definite indication that the DPNH gave protection to the enzyme molecule. There was only 26 to 28% inhibition at this concentration, whereas at $1x10^{-3}$M concentration it was nearly twice that (50 to 57%). (Table XVIIA)
The observations in Table XVIIB require some clarification in interpreting the data. Under column X the change in optical density of control specimens are reported. They did not contain any DPNH, the $\triangle$ OD/min. figures represent the activity of specimens kept in dark

and the activity of specimens after 15' UV illumination. They give the degree of photosensitization in presence of psoralen only. In these cases the reaction went on linearly for nearly 1.75 minutes. In column Y, the rate of change in optical density readings (ΔOD/min) represent apparent LDH activity of enzyme solution containing DPNH. In presence of DPNH, the reaction "Lactate to Pyruvate" proceeded sluggishly, possibly due to the fact that reduced coenzyme binds readily with the enzyme, and does not permit the LDH to reduce the oxidized DPN present in the reaction cuvettes. Hence the Δ OD/min in each case was always less as compared to the control runs where there was no DPNH present. Therefore the percent activity of the UV irradiated specimens has not been compardd against the control runs mentioned in column X, but against the unirradiated system containing LDH, psoralen and DPNH (Column Y). The observations reported in Table XVIIB also indicated that incorporation of DPNH at 2×10^{-3}M concentration gave definite protection to the enzyme molecules against psoralen photosensitization. There was nearly 50% protection. DPNH at 1×10^{-3}M concentration however did not exhibit the same degree of protection, although there was definite indication that the inhibition was less at this concentration.

Effect of substrate: The concept that enzymes act by forming an intermediate enzyme substrate complex is well known. Although (ES) complex is very unstable and shortlived, their existence has been demon-

strated by several workers with different enzyme systems. By providing excess of substrate molecules, the frequency of collision of the enzyme molecules with substrate molecules is known to increase. It is postulated that there are areas on the surface of the enzyme molecule which can attach the substrate as well as product molecules. It was therefore tempting to see the effect of addition of substrate pyruvate into the system consisting of lacticdehydrogenase and psoralen. Whether by providing large number of substrate molecules in the vicinity of enzyme molecules, their association would protect the enzyme molecules from the effect of psoralen photosensitization. Pyruvate at 1×10^{-3} M concentration was added before irradiation. The LDH activity was determined following 15' UV irradiation and compared against control specimens kept in the dark. As shown in Table XVIIA, addition of pyruvate did not alter the usual effect of psoralen photosensitization. The enzyme was found to be inactivated to a significant extent.

Effect of amino acids on photosensitization by psoralen: It is known that proteins irradiated by ultraviolet light undergo denaturation. Very little is known about the mechanism of the primary process in inactivation or denaturation by UV light. McLaren[62] has stated that -Co-NH and S-S bonds are broken during inactivation and it is believed that the main reaction seems to be the breaking of a peptide bond with the formation of two radicals. McLaren and Finkelstein[63] have stated that the light is absorbed by the tyrosine and tryptophan chromophores and

Table XVIII. Effect of amino acids on psoralen photosensitized inhibition of lactic dehydrogenase

Expt. #	System		ΔOD/min.	Millimicro-moles of DPNH	% activity	% loss of activity
1.	Effect of histidine					
	a. LDH + psoralen	dark	0.130	6.24	100	-
	b. LDH + psoralen + histidine $1x10^{-3}M$	dark	0.130	6.24	100	-
	c. LDH + psoralen	UV 15'	0.070	3.36	54	46
	d. LDH + psoralen + histidine $1x10^{-3}M$	UV 15'	0.080	3.84	62	38
	e. LDH + psoralen + histidine $2x10^{-3}M$	UV 15'	0.080	3.84	62	38
	f. LDH + psoralen + histidine $2x10^{-3}M$	UV 15'	0.090	4.32	71	29
2.	Effect of tryptophan					
	a. LDH + psoralen	dark	0.150	7.2	100	-
	b. LDH + psoralen+tryptophan $1x10^{-3}M$	dark	0.150	7.2	100	-
	c. LDH + psoralen	UV 15'	0.070	3.36	47	53
	d. LDH + psoralen+tryptophan $1x10^{-3}M$	UV 15'	0.055	2.64	37	63
	e. LDH + psoralen+tryptophan $2x10^{-3}M$	UV 15'	0.060	2.88	40	60
	f. LDH + psoralen+tryptophan $2x10^{-3}M$	UV 15'	0.100	4.80	67	33
3.	Effect of tyrosine					
	a. LDH + psoralen	dark	0.145	6.96	100	-
	b. LDH + psoralen + tyrosine $1x10^{-3}M$	dark	0.145	6.96	100	-
	c. LDH + psoralen	UV 15'	0.070	3.36	48	52
	d. LDH + psoralen + tyrosine $1x10^{-3}M$	UV 15'	0.080	3.84	55	45
	e. LDH + psoralen + tyrosine $2x10^{3}$ M	UV 18'	0.030	1.44	20	80

Table XVIII. (Continued)

Expt. # System		Δ OD/min	Millimicro-moles of DPNH	% activity	% loss of activity
4. Effect of DL. methionine					
a. LDH + psoralen	dark	0.135	6.48	100	-
b. LDH + psoralen	UV 15'	0.085	4.08	63	37
c. LDH + psoralen+ methionine $1x10^{-3}M$	UV 15'	0.090	4.32	67	33
d. LDH + psoralen + methionine $1x10^{-3}M$	UV 15'	0.080	3.84	59	41
5. Effect of L. cysteine					
a. LDH + psoralen	dark	0.135	6.48	100	-
b. LDH + psoralen	UV 15'	0.095	4.56	70	30
c. LDH + psoralan + cysteine $1x10^{-3}M$	UV 15'	0.080	3.84	59	41
d. LDH + psoralen + cysteine $1x10^{-3}M$	UV 15'	0.080	3.84	59	41
6. Effect of glutathione					
a. LDH + psoralen	dark	0.135	6.48	100	-
b. LDH + psoralen	UV 15'	0.080	3.84	59	41
c. LDH + psoralen + GSH $1x10^{-4}M$	UV 15'	0.065	3.12	48	52

is passed through two CH_2 groups to the peptide bond on either side of the tyrosyl residue. The weight of their evidence points to some definite participation of the aromatic group in the photo reaction.

From studies of the methylene blue photosensitization of amino acids, enzymes, and proteins, Fowlks[35] in his review article on the mechanism of the photodynamic effect has stated that histidine was the most readily altered of all the amino acids, tryptophan was also equally damaged. Oxidation of tyrosine to dihydroxy phenylalanine, breaking of -S-S- bond of cystine, formation of methionine sulfoxides from methionine were some of the major initial changes observed following photosensitization. It was therefore interesting to study the effect of incorporating few aromatic as well as aliphatic amino acids in the biologic system, consisting of a mixture of lacticdehydrogenase and psoralen. Whether presence of free aromatic amino acids such as tyrosine, tryptophan, histidine would block the photosensitizing action of psoralen was investigated.

Prior to irradiation, histidine, tryptophan, tyrosine, methionine, cysteine were added at 10^{-3}M concentration range and the effect of each amino acid was investigated separately. Glutathione concentration was only 1×10^{-4}M. Following 15 minutes irradiation with long-wave ultraviolet lamp, LDH activity was measured in irradiated as well as unirradiated specimens. As indicated in Table XVIII, psoralen photosensitized inhibition of the enzyme activity was present in all cases. No protective effect of any of these amino acids was observed.

Effect of EDTA, FMN, B_{12}, albumin, fibrinogen, quinones and DPN on photosensitization of lacticdehydrogenase by psoralen: Earlier succinicdehydrogenase experiments were reported and it was shown that addition of ethylenediaminetetraacetate (EDTA) in the irradiated system consisting of SDH+psoralen revealed a significant protective effect against psoralen photosensitization. Reduced diphosphopyridine nucleotide (DPNH) also showed some protective effect in case of lacticdehydrogenase inhibition. In order to explore the mechanism of psoralen photosensitization, additional studies were carried out to see whether psoralen action could be blocked by some other means. So far it has been shown that photochemical reactions such as those induced by psoralens, depended upon the absorption of light. Irradiation of biological materials in presence of two or more chromophores (molecules absorbing light) can modify the photochemical reaction to a greater or less extent by virtue of their light-absorbing property or their affinity to bind themselves in some way with the biological material. The effect of far ultraviolet light on mitochondrial oxidative phosphorylation has been studied by Beyer[7] who observed that addition of crystalline bovine serum albumin gave considerable protection against UV induced inhibition of oxidative phosphorylation. The addition of EDTA, however, did not show any protective effect. Barber and Ottolenghi[2], however, reported that UV inhibited succinooxidase system of rat liver mitochondria and EDTA protected it from inactivation. More recently Beyer[8] has reported some further studies with long-wave ultraviolet light and its effect on

Table XIX. Effect of EDTA, FMN, B_{12}, albumin, fibrinogen, quinones and DPN on the inhibition of lacticdehydrogenase in presence of psoralen and UV irradiation

Expt. #	Irradiated system		△ OD/min	Millimicro-moles of DPNH	% activity	% loss of activity
Effect of EDTA $1x10^{-3}M$						
1.	LDH	dark	0.100	4.8	100	-
2.	LDH	UV 15'	0.100	4.8	100	-
	LDH + psoralen	dark	0.100	4.8	100	-
	LDH + psoralen	UV 15'	0.075	3.6	75	25
	LDH + psoralen + EDTA	UV 15'	0.095	4.6	95	5
2.	LDH	dark	0.085	4.08	100	-
	LDH	UV 30'	0.085	4.08	100	-
	LDH + psoralen	dark	0.085	4.08	100	-
	LDH + psoralen	UV 30'	0.035	1.68	41	59
	LDH + psoralen + EDTA	UV 30'	0.085	4.08	100	-
3.	LDH	dark	0.080	3.84	100	-
	LDH	UV 45'	0.080	3.84	100	-
	LDH + psoralen	dark	0.080	3.84	100	-
	LDH + psoralen	UV 45'	0.015	0.72	19	71
	LDH + psoralen + EDTA	UV 45'	0.035	1.68	41	59
4.	LDH	dark	0.100	4.8	100	-
	LDH	UV 1 hr.	0.080	3.84	80	20
	LDH + psoralen	dark	0.100	4.8	100	-
	LDH + psoralen	UV 1 hr	0.010	0.48	10	90
	LDH + psoralen + EDTA	UV 1 hr	0.050	2.40	50	50

Table XIX (Continued)

5. In presence of cysteine $1x10^{-3}M$					
a. LDH + psoralen	dark	0.285	13.68	100	-
LDH + psoralen	UV 30'	0.200	9.60	70	30
LDH + psoralen + cysteine	UV 30'	0.220	10.56	77	23
b. LDH + psoralen + cysteine + EDTA	dark	0.235	11.28	100	-
LDH + psoralen + cysteine + EDTA	UV 30'	0.210	10.08	90	10
c. LDH + psoralen + cysteine + EDTA	dark	0.250	12.0	100	-
LDH + psoralen + cysteine + EDTA	UV 30'	0.250	12.0	100	-
6. In presence of glutathione $1x10^{-4}M$					
a. LDH + psoralen	dark	0.110	5.3	100	-
LDH + psoralen	UV 30'	0.045	2.16	41	59
LDH + psoralen + GSH	dark	0.105	5.04	95	5
LDH + psoralen + GSH	UV 30'	0.045	2.16	41	59
b. LDH + psoralen + GSH + EDTA	dark	0.085	4.1	100	-
LDH + psoralen + GSH + EDTA	UV 30'	0.075	3.6	88	12
c. LDH + psoralen + GSH + EDTA	dark	0.090	4.32	100	-
LDH + psoralen + GSH + EDTA	UV 30'	0.075	3.6	83	17
d. LDH + psoralen + GSH + EDTA	dark	0.085	4.1	100	-
LDH + psoralen + GSH + EDTA	UV 30'	0.080	3.84	94	6
7. Effect of riboflavin monophosphate					
a. LDH + psoralen + FMN $1x10^{-3}M$	dark	0.075	3.6	100	-
LDH + psoralen + FMN $1x10^{-3}M$	UV 15'	0.010	0.48	13.5	86.5
LDH + psoralen + FMN $1x10^{-3}M$	UV 15'	0.020	0.96	27	73
b. LDH + FMN $1x10^{-3}M$	dark	0.195	9.4	100	-
LDH + FMN $1x10^{-3}M$	UV 15'	0.010	0.48	5	95
LDH + psoralen + FMN $1x10^{-3}M$	dark	0.175	8.4	90	10
LDH + psoralen + FMN $1x10^{-3}M$	UV 15'	0.0	0.0	0	100
LDH + psoralen + FMN $1x10^{-3}M$	UV 15'	0.0	0.0	0	100

Table XIX (Continued

8. Effect of albumin					
a. LDH + psoralen	dark	0.165	7.92	100	-
LDH + psoralen	UV 15'	0.135	6.48	81	19
b. LDH + psoralen	dark	0.180	8.64	100	-
LDH + psoralen	UV 15'	0.140	6.72	77	23
c. LDH + psoralen + albumin $1x10^{-5}M$	dark	0.165	7.92	100	-
LDH + psoralen + albumin $1x10^{-5}M$	UV 20'	0.155	7.44	94	6
d. LDH + psoralen + albumin $1x10^{-5}M$	dark	0.145	6.96	100	-
LDH + psoralen + albumin $1x10^{-5}M$	UV 20'	0.145	6.96	100	-
e. LDH + psoralen + albumin $1x10^{-5}M$	dark	0.180	8.64	100	-
LDH + psoralen + albumin $1x10^{-5}M$	UV 30'	0.175	8.40	97	3
9. Effect of fibrinogen					
a. LDH + psoralen	dark	.190	9.12	100	-
LDH + psoralen	UV 30'	.145	6.96	76	24
b. LDH + psoralen + fibrinogen $1x10^{-5}M$	dark	0.195	9.12	100	-
LDH + psoralen + fibrinogen $1x10^{-5}M$	UV 30'	0.195	9.12	100	-
LDH + psoralen + fibrinogen $1x10^{-5}M$	UV 30'	0.195	9.12	100	-
10. Effect of vitamin B_{12}					
a. LDH + psoralen (50 ug/ml)	dark	0.130	6.24	100	-
LDH + psoralen "	UV 30'	0.045	2.16	34	66
LDH + psoralen "	UV 30'	0.035	1.68	27	73
b. LDH + psoralen + B_{12} $1x10^{-4}M$	dark	0.135	6.48	100	-
LDH + psoralen + B_{12} $1x10^{-4}M$	UV 30'	0.100	4.80	77	27
LDH + psoralen + B_{12} $1x10^{-4}M$	UV 30'	0.080	3.84	61	39
c. LDH + psoralen (25 ug/ml)	dark	0.135	6.48	100	-
LDH + psoralen "	UV 30'	0.070	3.36	52	48
d. LDH + psoralen + B_{12} $2x10^{-4}M$	dark	0.135	6.48	100	-
LDH + psoralen + B_{12} $2x10^{-4}M$	UV 30'	0.135	6.48	100	-
e. LDH + psoralen + B_{12} $2x10^{-4}M$	dark	0.120	5.76	100	-
LDH + psoralen + B_{12} $2x10^{-4}M$	UV 30'	0.105	6.96	88	12

Table XIX (Continued)

11. Effect of quinones					
a. LDH + psoralen	dark	0.145	6.96	100	-
LDH + psoralen	UV 15'	0.100	4.80	69	31
b. LDH + psoralen + 2-amino, 1-4-naphthoquinone $1x10^{-3}M$	dark	0.135	6.48	93	7
LDH + psoralen + 2-amino, 1-4-naphthoquinone $1x10^{-3}M$	UV 15'	0.115	5.52	80	20
c. LDH + psoralen + 2-amino, 1-4-naphthoquinone $1x10^{-3}M$	dark	0.140	6.72	100	22
LDH + psoralen + 2 amino, 1-4-naphthoquinone $1x10^{-3}M$	UV 15'	0.105	5.04	75	25
LDH + psoralen + 2-amino, 1-4-naphthoquinone $1x10^{-3}M$	UV 15'	0.100	4.80	71	29
12. a. LDH	dark	0.160	7.7	100	-
LDH	UV 15'	0.160	7.7	100	-
b. LDH + psoralen	dark	0.160	7.7	100	-
LDH + psoralen	UV 15'	0.100	4.8	62.5	37.5
c. LDH + psoralen + 2-methyl-naphthoquinone $1x10^{-3}M$	dark	0.115	5.52	100	-
LDH + psoralen + 2-methyl-naphthoquinone $1x10^{-3}M$	UV 15'	.068	3.26	59	41
d. LDH + psoralen + 2-methyl-naphthoquinone $1x10^{-3}M$	dark	0.115	5.52	100	-
LDH + psoralen + 2-methyl-naphthoquinone $1x10^{-3}M$	UV 15'	0.065	3.12	56.5	43.5
13. a. LDH + psoralen + hydroquinone $1x10^{-3}M$	dark	0.150	7.2	100	-
" " " "	UV 15'	0.160	7.7	106	-
b. " " " "	dark	0.150	7.2	100	-
" " " "	UV 15'	0.165	7.9	110	-
14. Effect of DPN a. LDH + psoralen	dark	0.175	8.4	100	-
b. LDH + psoralen	UV 15'	0.140	6.7	80	20
c. LDH + psoralen+DPN $1x10^{-3}M$	dark	0.175	8.4	100	-
d. LDH + psoralen+DPN $1x10^{-3}M$	UV 15'	0.140	6.7	80	20
e. LDH + psoralen+DPN $1x10^{-4}M$	UV 15'	0.135	6.5	78	22

effect on the enzyme systems of rat liver mitochondria. Ability to oxidise succinate and glutamate and to carry out oxidative phosphorylation was significantly affected. They tested the effect of quinones and observed that vitamin K acetate addition restored the oxidative phosphorylation activity. Flavin mononucleotide and vitamin B_{12} have typical absorption spectra in ultraviolet region. Besides other absorption peaks, these two compounds reveal characteristic peak in the region of 373 and 360 mu respectively. It was therefore interesting to see whether addition of EDTA, crystalline bovine serum albumin, fibrinogen FMN, B_{12} or quinones into the irradiation system consisting of LDH and psoralen in some way or other alter the photosensitizing effect of psoralen.

Effect of EDTA: Irradiation of LDH in presence of psoralen for a period of 15 minutes revealed 25% inhibition of enzyme (Table XIX). When EDTA was added prior to irradiation in the above system, there was only 5% inhibition. Thus the enzyme was found to be significantly protected. This protective effect of EDTA was further investigated. The dosage of UV light was increased, instead of 15 minutes illumination, the time of irradiation was extended to 30, 45, and 60 minutes. As shown in Table XIX, EDTA certainly exhibits a significant protective effect. After 30 minutes illumination, psoralen induced 59% enzyme inhibition, but when EDTA was added, there was no loss of enzyme activity. Psoralen photosensitized inhibition was completely blocked. Likewise when LDH + psoralen was irradiated for 45 min-

utes, 71% destruction of LDH was observed. In presence of EDTA, only 59% inhibition was noted. When the illumination time was extended to one hour, secondary effects complicated the general picture. There was 20% loss of activity just due to UV irradiation (in absence of psoralen). In presence of psoralen, one hour irradiation induced 90% enzyme inhibition. In presence of psoralen and EDTA there was only 50% loss of activity. Even under these conditions of prolonged illumination, when the enzyme was subjected to irradiation for 45 to 60 minutes, the protective effect of EDTA was obvious.

This protective effect of EDTA has been further demonstrated in presence of cysteine and glutathione. In succinicdehydrogenase studies, as well as lacticdehydrogenase studies, it was shown that these SH compounds showed no protective effect. In fact, GSH was found to be more deleterious to SDH and induced greater inhibition as compared to psoralen effect alone. It was therefore interesting to see whether EDTA revealed the same shielding effect against psoralen photosensitization. As shown in Table XIX #5 and 6, incorporation of EDTA in the irradiated system consisting of either LDH + psoralen + cysteine or LDH + psoralen + GSH, definitely showed a significant protective effect. It was therefore interesting to see that photosensitizing effect of psoralen could be blocked by incorporating EDTA in the irradiated system.

Effect of FMN: Riboflavin has been implicated as a photosensitizer. It has absorption peaks in the region of 266,, 373, 445 mu and it was

therefore tempting to see whether the wavelengths which are absorbed by this molecule as well as psoralen molecule would modify the photosensitizing action. As shown in Table XIX #7, FMN addition in the irradiation system consisting of LDH + psoralen, completely destroyed the enzymetic activity. The two sensitizers together in presence of effective wavelengths exhibited a pronounced photosensitization. There was 100% loss of activity. FMN in absence of psoralen also induced a pronounced photochemical action.

Effect of albumin: As shown in Table XIX #8, crystalline bovine serum albumin was found to be a very effective protective agent against photosensitizing action of psoralen. It was interesting to see that albumin exhibited a protective action even after 30 minutes UV irradiation. There was only 3 - 6% loss of activity. Irradiation for 15 and 20 minute periods showed no loss of LDH activity. Thus incorporation of a protein such as albumin in a photosensitive system affords a true protection to the other protein which is highly labile to photosensitization.

Effect of crystalline fibrinogen: Likewise addition of fibrinogen, a protein of molecular weight of 4.4×10^5 also showed a pronounced protective effect (Tble XIX #9). There was no loss of any LDH activity following 30 minutes ultraviolet irradiation.

Effect of vitamin B12 As stated earlier, vitamin B12 was selected because it has a strong absorption peak in the region of 360 mu. B12

molecules would naturally absorb this wavelength and act as a filtering devise against the activation of psoralen molecules. This would result in diminished photosensitizing effect. As shown in Table XIX #10, addition of B_{12} in the system consisting of LDH + 50 ug/ml, psoralen certainly reduced the photosensitizing action of psoralen. In absence of B_{12}, this photosensitizing effect of psoralen was reduced to only 29 to 33%. Further confirmation was made by reducing the concentration of psoralen. In presence of 25 ug/ml psoralen, 48% inhibition of LDH was observed following 30 minutes UV irradiation. Addition of B_{12} at $1x10^{-3}$ concentration showed a complete absence of inhibitory effect of psoralen. It is therefore probable that B_{12} acted as a filter and absorbed considerable amount of light and prevented their access to activate psoralen molecules.

Effect of quinones: If psoralen photosensitization involved oxidation, addition of an agent which can readily accept a proton and get reduced preferentially would exhibit a sparring effect and protect the protein moiety (LDH) from inactivation. 2 amino, 1, 4-naphthoquinone did not show the anticipated protective effect (Table XIX #11). Likewise 2-methyl naphthoquinone was also ineffective (Table XIX #12). In both instances, photosensitization by psoralen was observed. However, hydroquinone exhibited a significant protective effect. No inhibition of lacticdehydrogenase was observed following 15 UV irradiation in presence of psoralen and hydroquinone (Table XIX #13).

Effect of DPN: Incorporation of DPN into the LDH + psoralen system did not block the photosensitized inhibition of LDH by psoralen (Table XIX #14). The oxidised form of diphosphopyridine nucleotide did not reveal any protective effect. Only the reduced form exhibited some protective effect. (Table XVIIA & B). It should be noted that DPN did not show any absorption peak in 340-380 mu whereas reduced DPN (DPNH) showed a characteristic absorption in 340 mu region.

Effect of oxygen on photosensitizing action of lactichdhydrogenase by psoralen: Previous results related to succinicdehydrogenase activity had shown that oxygen was not needed to induce photosensitizing action of psoralen. Addition of cysteine, glutathione and even incorporation of quinones revealed no protective effect on the enzyme molecule when subjected to UV irradiation in presence of psoralen. These observations further supported the above conclusion that the mechanism of psoralen photosensitization did not necessarily depend on presence of oxygen. Further studies were carried out to emphasize the fact that photosensitization by psoralen can be demonstrated in absence of molecular oxygen.

As detailed under succinicdehydrogenase studies, the effect of oxygen was investigated as follows: First, psoralen effect was demonstrated in presence of two gas phases, both containing oxygen. The irradiation of LDH was carried out in presence of ordinary air and in presence of pure oxygen. As shown in Table XX, psoralen manifested its usual photosensitizing effect. In presence of air, as well as oxygen,

Table XX. Effect of oxygen on photosensitization of lacticdehydrogenase in presence of psoralen

Expt. *		Gas phase	ΔOD/min	Millimicro-moles of DPNH	% activity	% loss of Activity
#1						
a. LDH + psoralen	dark	air	0.145	6.96	100	-
b. LDH + psoralen	UV 15'	air	0.095	4.56	65.7	34.3
c. LDH + psoralen	UV 15'	air	0.090	4.32	62	38
d. LDH + psoralen	UV 15'	oxygen	0.090	4.32	62	38
e. LDH + psoralen	UV 15'	oxygen	0.065	3.12	45	55
f. LDH + psoralen	dark	oxygen	0.130	6.25	90	10
g. LDH + psoralen	UV 15'	oxygen	0.065	3.12	45	55
h. LDH + psoralen	UV 15'	oxygen	0.075	3.60	52	48
#2						
a. LDH + psoralen	dark	air	0.105	5.04	100	-
b. LDH + psoralen	UV 15'	air	0.105	5.04	100	-
c. LDH + psoralen	UV 15'	air	0.055	2.64	52	48
d. LDH + psoralen	UV 15'	nitrogen	0.048	2.30	46	54
#3						
a. LDH + psoralen	dark	air	0.100	4.8	100	-
b. LDH + psoralen	UV 15'	air	0.070	3.36	70	30
c. LDH + psoralen	UV 15'	nitrogen	0.055	2.64	55	45
d. LDH + psoralen + EDTA $2x10^{-3}M$	UV 15'	nitrogen	0.095	4.56	95	5
#4						
a. LDH + psoralen	dark	air	0.120	5.76	100	-
b. LDH + psoralen	dark	air	0.120	5.76	100	-
c. LDH + psoralen	UV 15'	air	0.095	4.56	79	21
d. LDH + psoralen	UV 15'	helium	0.080	3.84	66	34
e. LDH + psoralen + EDTA $1x10^{-3}M$	UV 15'	helium	0.110	5.28	91	9

Table XX (Continued)

Expt. # *	Irradiated system		Gas phase	ΔOD/min	Millimicro-moles of DPNH	% activity	% loss of activity
#5							
a.	LDH	dark	air	0.130	6.24	100	-
b.	LDH	UV 30'	air	0.120	5.76	92	8
c.	LDH	UV 30'	nitrogen	0.080	3.84	61.5	38.5
d.	LDH + psoralen	UV 30'	air	0.080	3.84	61.5	38.5
e.	LDH + psoralen	UV 30'	nitrogen	0.015	0.72	11.5	88.5
#6							
a.	LDH	dark	air	0.145	6.96	100	-
b.	LDH	UV 15'	air	0.125	6.0	86.2	13.8
c.	LDH + psoralen	dark	air	0.140	6.72	96.5	3.5
d.	LDH + psoralen	UV 15'	air	0.035	1.68	26.1	75.9
e.	LDH + psoralen	UV 15'	nitrogen	0.020	0.96	13.8	86.2
f.	LDH + psoralen	UV 15'	nitrogen	0.015	0.72	10.3	89.7
g.	LDH + psoralen	dark	oxygen	0.140	6.72	100	-
h.	LDH + psoralan	UV 15'	nitrogen	0.070	3.36	50	50
#7							
a.	LDH	dark	air	0.125	6.0	100	-
b.	LDH + psoralen	dark	air	0.120	5.76	96	4
c.	LDH + psoralen	UV 15'	air	0.025	1.20	20	80
d.	LDH + psoralen	UV 15'	helium	0.045	2.16	36	64

* Expt. #1 to 4 were carried out without any stirring of enzyme solution
Expt. #5 to 7 were carried out with gentle magnetic stirring during illumination

inhibition of LDH was observed. In mitochondrial SDH studies, it was observed that in presence of pure oxygen psoralen induced a decreased photosensitizing effect. In case of LDH, however, no such effect was noted. Flushing of oxygen over the surface of crystalline enzyme solution showed surface denaturation. This inhibitory effect was observed in absence of UV irradiation. Hence the overall psoralen photosensitizing action in presence of pure O_2 was roughly of the same order as observed in case of air (34 to 38%). When irradiation was carried out under nitrogen gas phase, psoralen induced photosensitization to the same extent as in presence of air or pure oxygen. If oxygen was absolutely essential, then presence of nitrogen should have completely blocked the photosensitizing effect with no loss of enzyme activity. Even in presence of helium gas, LDH was inhibited to the same extent that was observed in presence of air. (See Table XX). These observations further supported the earlier conclusion that psoralen action was not dependent on presence of molecular oxygen. These experiments were conducted without any stirring of enzyme solution.

Theeffect of EDTA was investigated just to confirm the earlier findings, this time however the effect of EDTA was investigated in presence of other gas phase: (a) in presence of nitrogen and (b) in presence of helium. In both cases, EDTA revealed the same protective effect, there was very little loss of LDH activity. As shown in Table XX, photosensitizing action of psoralen in presence of nitrogen or helium

Table XXI : Effect of psoralen or 8-methoxypsoralen on rat liver cytochrome oxidase when irradiated with ultraviolet > 3200 Å

		△ OD/min	Millimoles of oxidized cyt. c	% enzyme activity
1.				
a. Mitochondria	dark	0.090	1.42×10^{-5}	100
b. Mitochondria	UV 15'	0.081	1.28×10^{-5}	90
c. Mitochondria+psoralen	dark	0.090	1.42×10^{-5}	100
d. Mitochondria+psoralen	UV 15'	0.065	1.02×10^{-5}	72
2.				
a. Mitochondria	dark	0.080	1.26×10^{-5}	100
b. Mitochondria	UV 15'	0.080	1.26×10^{-5}	100
c. Mitochondria+psoralen	dark	0.080	1.26×10^{-5}	100
d. Mitochondria+psoralen	UV 15'	0.065	1.02×10^{-5}	80
3.				
a. Mitochondria	dark	0.110	1.74×10^{-5}	100
b. Mitochondria	UV 15'	0.115	1.81×10^{-5}	100
c. Mitochondria+psoralen	dark	0.110	1.74×10^{-5}	100
d. Mitochondria+psoralen	UV 15'	0.090	1.42×10^{-5}	81
4.				
a. Mitochondria	dark	0.110	1.74×10^{-5}	100
b. Mitochondria	UV 15'	0.105	1.66×10^{-5}	95
c. Mitochondria+psoralen	dark	0.110	1.74×10^{-5}	100
d. Mitochondria+psoralen	UV 15'	0.080	1.26×10^{-5}	73
5.				
a. Mitochondria	dark	0.040	0.63×10^{-5}	100
b. Mitochondria	UV 15'	0.040	0.63×10^{-5}	100
c. Mitochondria+8-MOP	dark	0.040	0.63×10^{-5}	100
d. Mitochondria+8-MOP	UV 15'	0.030	0.47×10^{-5}	75
6.				
a. Mitochondria	dark	0.045	0.71×10^{-5}	100
b. Mitochondria	UV 15'	0.045	0.71×10^{-5}	100
d. Mitochondria+8-MOP	UV 15'	0.030	0.47×10^{-5}	66
7.				
a. Mitochondria	dark	0.075	1.18×10^{-5}	100
b. Mitochondria	UV 15'	0.080	1.26×10^{-5}	100
c. Mitochondria+8-MOP	dark	0.075	1.18×10^{-5}	100
d. Mitochondria+8-MOP	UV 15'	0.050	0.79×10^{-5}	66

gas phases was significantly blocked by EDTA.

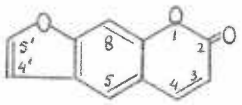
The data in Table XX #5-7 is related to the effect of psoralen on lacticdehydrogenase when the enzyme system was subjected to gentle stirring in the course of illumination. In presence of air, oxygen, nitrogen and helium, the 2.5 ml. mixture of LDH and psoralen was stirred by a magnet stirrer for a period of 15 minutes. This was done with an intention to bring more enzyme molecules in contact with psoralen and UV irradiation. As shown in Table XX #5-7, this gentle stirring resulted in significant photosensitizing effect. The dual effect of stirring and flushing of gas resulted in appreciable loss of enzyme activity even in absence of irradiation. But the result further supported the earlier conclusions, that oxygen was not needed in psoralen photosensitization. The light sensitized photochemical damage can be demonstrated in presence of oxygen as well as in absence of oxygen.

Effects of psoralen or 8-methoxypsoralen on rat liver cytochromeoxidase in presence of ultraviolet light: Table XXI shows that 8-methoxypsoralen and psoralen in presence of UV light inactivated cytochrome oxidase enzyme system of rat liver mitochondria. When protected from light, these two compounds did not exhibit any inhibitory effect. Long-wave ultraviolet irradiation for a period of 15 minutes in absence of either of these photosensitizers was not deleterious to enzyme activity. Only in presence of 8-MOP or psoralen, this important enzyme system was affected by UV irradiation.

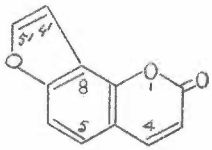
The activating and fluorescent wavelengths of furocoumarins: psoralens, and the absorption spectrum and the action spectrum of psoralen: As stated earlier in Grotthus-Draper's law, the spectral limits of radiation forwarding a particular photochemical process are dependent upon the limits of absorption spectrum of the chromophore. It might be therefore expected too that the effectivenss of any given wavelength within this range would depend upon the amount of absorption corresponding to that wavelength and should follow the absorption spectrum. The effectiveness of various wavelengths in producing psoralen activation and photosensitization was investigated in order to obtain the biological action spectrum of psoralen.

The wavelengths of light which are activating psoralen, 8-methoxypsoralen and other related photosensitizers and exhibiting biological responses were determined in the following two ways: (1) since fluorescence behaviour represents the emission from the activated molecules of absorbed light energy, investigations were carried out to determine the precise activating and fluorescent wavelengths of several furocoumarins. The photosensitizing action of these compounds and their relative activity were determined by testing these compounds on mammalian skin and evaluating the erythemal response elicited by these compounds in presence of long-wave ultraviolet irradiation. In a few instances the photosensitized inhibition of lacticdehydrogenase by psoralen and other compounds was also studied concomitantly. (2) The other approach was to study the action spectrum of psoralen. The spectral map

Table XXIIA Activating and fluorescence wavelengths of furocoumarins and related compounds



Psoralen



Isopsoralen

No.	Compound	Activity erythemal response	Activating wavelengths A	B	C	Fluorescence peak		Percent fluorescence at 0.01 meter setting for A	B	C
1.	Psoralen	Active	270	360		435		1.3	19	
2.	4-methylpsoralen	Active	265	315	360	420		6.7	16.3	61
3.	5',8-dimethylpsoralen	Active	270	375		440		8	12	
4.	4,4'-dimethylpsoralen	Active	270	360		435		7	78	
5.	4,5',8-trimethylpsoralen	Active	270	370		440		14.3	138	
6.	3,4,5',8-tetramethylpsoralen	Active	270	360		430		26	135	
7.	5',4,8-trimethyl-3n-butylpsoralen	Active	275	375		430		32	260	
8.	8-methoxypsoralen (Xanthotoxin)	Active	280	360		360	460	2.2	10	
9.	5-methoxypsoralen (Bergapten)	Active	270 280	350		370 480		2.3 13.3	60	
10.	5',4-dimethyl-8-acetylpsoralen	Active	320	370		430		16	28	
11.	5',4-dimethyl-8-n propylpsoralen	Active	270 400 450	360		430 460 525		34 135 0.6	135	
12.	5',4-dimethyl-8-acetamidopsoralen	Active	275	360	390	450		10.6	61	5.4
13.	5',4-dimethyl-8-acetyl-semicarbazone	Active	360			440		27		
14.	5',8-dimethyl-3,4-benzopsoralen	Active	270	300	355	455		1.2	29	84
15.	5',8-dimethyl-3,4-tetrahydrobenzopsoralen	Active	270	360		430		34	150	
16.	8-bromo-5',4-dimethylpsoralen	Active	275 375			430 460		4.1 31		
17.	Marmesin (5' isopropenol[2"]-4',5'-dihydropsoralen)	Active	270	360		430		34	150	
18.	Anhydro marmesin (5' isopropylpsoralen)	Active	270	365		450		7	57	
19.	2-carboxy-5,6-dihydro-6-(2"-isopropenol)-benzo-[1',2'-b, 4',5'-b'] -difuran	Active	290	340		380		1140	870	
20.	5'-methyl-isopsoralen	Active	280	350	400	460		1.8	35	1.5
21.	4',5'-dihydro-3-bromo-8-methoxypsoralen	Active	295, 550	380		450		57	156	1.1
22.	8-dimethylamine,5',4-dimethylpsoralen	Active	275	320	370	435		21	46	120
23.	5',4-dimethyl-8-aminopsoralen	Inactive	380			530		2.2		
24.	4',5'-dihydro-4-methylpsoralen	Inactive	280	360	545	395		750	1830	19

Table XXII A (Continued)

No.	Compound	Activity erythemal response	Activating wavelengths A	B	C	Fluorescence peak	Percent fluorescence at 0.01 meter setting A	B	C
25.	8-chloro-5',4-dimethylpsoralen	Inactive	370			550	6.0		
26.	4',5'-dihydro-8-methoxypsoralen	Inactive	280 450	365		480 525	0.6	5.3	0.5
27.	4',5'-dihydro-3,5 dibromo-8-methoxypsoralen	Inactive	280 290 300	 375		320 400 470	2.2 8.7 8.0	 15.3	
28.	5-bromo-8-methoxypsoralen	Inactive	280 450	365		480 525	0.6 0.5	5.3	
29.	5,8-dimethoxypsoralen	Inactive	290	360	450	530	1.6	5.6	.005
30.	4',5'-dihydro-5-ethyl-carbamyl-8-methoxypsoralen	Inactive	290 280	360		495 380	33 43	84	
31.	8-hydroxypsoralen	Inactive	350			410	1.5		
32.	4',5'-dihydro, 5',4-dimethylisopsoralen	Inactive	280 535	355		395	760	2400	24
33.	2,9-dimethyl-7H-furo-[3,2-f-][1]-benzopyran-7 one	Inactive	270	360		435	280	1000	
34.	2,9-dimethyl-7-H-oxazolo-[4,5-f-][1]-benzopyran-7-one	Inactive	265 400 450	335		400 450 525	3.4 1.1 0.06	21	
35.	benzoxazole-5-acrylic acid-6-hydroxy-β-7-dimethyl-γ-lactone	Inactive	350	400		410 470	5.6	22	
36.	benzoxazole-5-acrylic acid-2,6-dihydroxy-β-7-dimethyl γ lactone	Inactive	270 265 310	 380		470 490 420	22 27 290	 54	
37.	benzoxazole- 5-acrylic acid -6-hydroxy-β-2-7-trimethyl-γ lactone	Inactive	270 400	310	355	400 470	0.9 54	1.2	14
38.	benzoxazole - 5-acrylic acid - 6-hydroxy-2-isopropyl-β-7-dimethyl-γ-lactone	Inactive	270	310	355	400	1.1	1.8	14
39.	2-carboxy -5, 6-dihydro-8-methoxy-benzo-(1,2-b, 5,4-b')-difuran	Inactive	265	335		430	129	2900	

Table XXII B Activating and fluorescent wavelengths of furocoumarins

Compounds	Total	Activating λ 265-280 mu	Fluorescent λ 420-460 mu	Activating λ 340-380 mu	Fluorescent λ 420-460 mu
Biologically active *	22	16		21	
Biologically inactive *	17	2		2	

* as measured by erythemal response

of the effective wavelengths which produced the photobiological effect in presence of psoralen was determined. Crystalline lacticdehydrogenase was used as a testing material and inhibition of this enzyme in presence of psoralen was investigated at different wavelengths. The results were compared with the absorption spectrum of the chromophore psoralen.

Table XXIIA shows the activating and fluorescent peaks for 39 furocoumarins and related compounds that were tested. It also includes the percent fluorescence for each activating wavelength. Table XXIIB shows the summary of the observations reported under Table XXIIA. Out of 39 compounds that were tested topically on albino guinea pig skin for eliciting photosensitized erythemal response, 22 were found to be active and induced varying degrees of erythemal response. 17 compounds were inactive and showed no biological response even in high concentration range. (For details see Table XXIII).

As indicated in Table XXIIA and XXIIB, the region of activating wavelengths for photosensitizing action of furocoumarins as determined spectrophotofluorometrically was between 265 and 280 mu in the short UV range, and between 340-380 mu in the long ultraviolet range. The fluorescence peaks for these activating wavelengths were observed in the region of 420-460 mu. Only those furocoumarins which induced definite photosensitized erythemal response on mammalian skin showed this region of exciting wavelengths. The inactive furocoumarins which did not exhibit photosensitizing response in general did not show

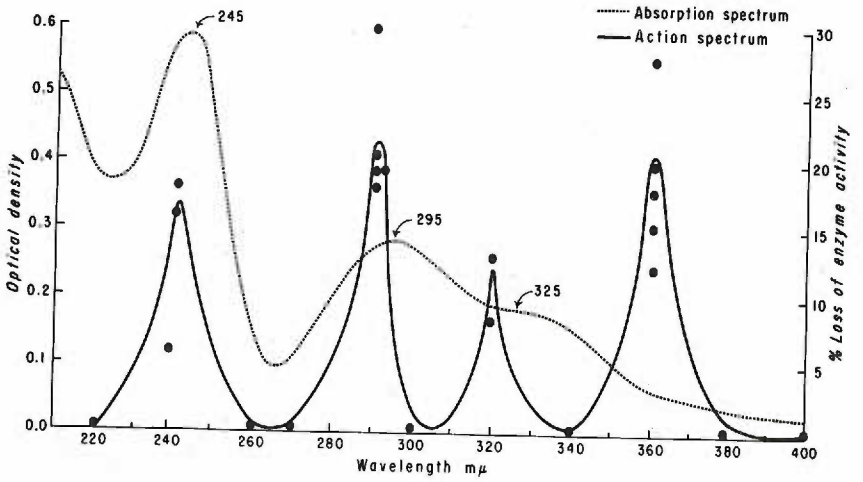
Figure 10: Absorption Spectrum and the Action Spectrum of Psoralen

The absorption spectrum was recorded with Beckman DK1 recording spectrophotometer. (Psoralen concentration approximately 10 ug/3 ml.)

Each point on action spectrum represents a separate run. LDH + psoralen solutions were irradiated at different wavelengths for 45 minutes. Psoralen photosensitized inhibition of LDH was observed at 240, 290, 320 and 360 mu wavelengths. In absence of psoralen, there was no loss of enzyme activity at these wavelengths.

It is interesting to note that the absorption spectrum of psoralen shows no absorption peak in 360 mu region. However, the compound shows some absorption even in low concentration (about 3 ug/ml) at 360 mu. The activating wavelength for maximum fluorescence of psoralen was found to be 360 mu. The wavelength of 360 mu was found to induce psoralen photosensitized inhibition of LDH.

ABSORPTION SPECTRUM AND ACTION SPECTRUM OF PSORALEN
(Photosensitized inhibition of crystalline lacticdehydrogenase)



these specific activating and fluorescent wavelengths. When the activating wavelengths were in the region of 340-380 mu (active compounds), the fluorescence emitted was always greater than the fluorescence emitted at 260-280 mu. The data suggested that 340-380 mu was the major activating wavelength region for these molecules. It is more likely that furocoumarins which show activation peaks in the region of 340 - 380 mu and concomitantly the fluorescent peaks in the region of 420-460 mu can cause photosensitization of skin in this region of long-wave ultraviolet light. The action spectra for these photosensitizing compounds therefore appears to be in the region of 340-380 mu. It may be noted that when some of these compounds were dissolved in water, they showed a shift of about 10 to 15 mu of activating wavelengths.

Absorption spectrum and action spectrum of psoralen: As shown in Figure 10, the absorption spectrum of psoralen has three major peaks: (1) 245 mu, (2) 295 mu, and (3) 325 mu. The solid line shows the action spectrum of psoralen. Ultraviolet irradiation of LDH solution without psoralen at 240, 290, 320, 360 mu wavelengths showed no loss of activity. The degree of inhibition at 290 and 360 mu was more than that observed at 240 or 320 mu. It could not be resolved with the results presented in Figure 10 which specific wavelength (290 or 360 mu) was more effective in inducing inhibition of LDH in presence of psoralen. The attempts were primarily focussed on the relationship between absorption spectrum and the action spectrum of psoralen. These results revealed that psoralen was the chromogen which absorbed light and

and transmitted this energy (either directly or indirectly) to induce photosensitization.

The 360 mu wavelength was found to induce LDH inhibition and yet no absorption peak by psoralen in that region was detected. This perplexing situation, where in absence of absorption peak one found the inhibition of LDH, was further investigated. The activating wavelengths of psoralen were studied. It was observed that psoralen had two activation peaks, (1) at 270 mu and the other at 360 mu. The fluorescence peak for both activating wavelengths was at 435 mu. The Figure #11 shows the pattern of activating and fluorescence peaks of the molecule. As reported earlier in Table XXIIA, the activation at 360 mu was much greater than at 270 mu. In other compounds reported earlier in Table XXIIA, it was stated that the fluorescence emitted by the active compounds was always greater in the region of 340-380 mu activating wavelengths as compared to fluorescence emitted at 265-380 mu. The activating peak at 360 mu for psoralen could very well correspond to the energy of a metastable triplet state for this photosensitizing molecule. Such triplet states are known to be highly reactive.

It is thus clear from Figure 10 that action spectrum of psoralen corresponded very closely with the absorption spectrum of psoralen and in addition there was an activating wavelength of 360 mu which was also reactive and induced photobiological changes.

Relationship between molecular configuration and photosensitizing action of furocoumarins (psoralens): Recent studies on 8-methoxypsoralen or methoxsalen or 8-MOP) and several other furocoumarins have shown that these

compounds evoke changes on mammalian skin manifested by erythema and increased melanogenesis. Musajo and associates[65, 66], have investigated the photosensitizing activity of several furocoumarins following topical application of 25 ug/cm^2 on human skin. Their studies have shown that psoralen was the most active of the naturally occurring furocoumarins. 8-methoxypsoralen, 5-methoxypsoralen, and several other compounds such as imperatorin, isopsoralen, 8-substituted hydroxy, nitro, amino, psoralen derivatives, were either less active or completely inactive. Pathak and Fitzpatrick[72] also carried out similar studies on albino guinea pig skin as well as on human skin, and confirmed these observations. It was additionally shown that coumarin, furan and furochromone derivatives were inactive. They also reported that a linear fusion of furan and coumarin rings, as in the psoralen molecule, was essential for this response, a non-linear structure like isopsoralen had no photosensitizing action. Quite recently Musajo et al[67] reported similar studies on the relationship between chemical constitution and the photodynamic properties in the furocoumarin series. They concluded that the furocoumarin nucleus was essential for activity and that introduction of a methyl group reduced the photodynamic action, the reduction being slight for substitution in the 4, 4' and 5' position but marked in the 3 position. 4'-phenyl substitution was shown by these workers to result in an inactive product. This study covers the effect of methyl and higher alkyl substitution, the effect of hydrogenating the furan ring, and the ac-

Table XXIII. Relative photosensitizing activity of furocoumarins and coumarins
Mean erythemal response after 18 and 36 hours (% reflectance difference)

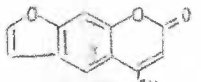
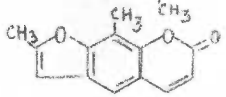
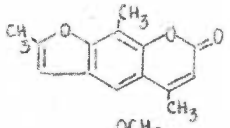
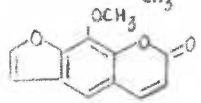
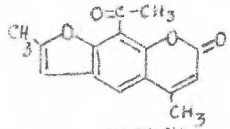
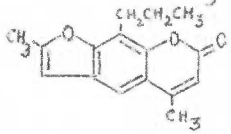
No.	Compound	Structure	Erythema	Micrograms per 2.5 cm^2 skin area									Standard		Relative activity
				5	10	25	50	100	200	250	500	1000	Psoralen 25 ug	8-MOP 25 ug	
1.	Psoralen		Visible	±	++	+++	++++							++	++++
			Green filter	3	6	7	11							5	
2.	4-methylpsoralen		Visible	+	++	+++	++++						+++	++	++++
			Green filter	3	7	11	11						9	7	
3.	5',8-dimethylpsoralen		Visible	±	+	+++	++++						+++	++	++++
			Green filter	3	3	7.5	10						9	7	
4.	4,5',8-trimethyl-psoralen		Visible	±	+	+++	++++						+++	++	++++
			Green filter	2	5	7.5	11						7	6	
5.	8-methoxypsoralen		Visible	-	±	++	+++						+++		+++
			Green filter	1	2	6.5	8						7		
6.	8-acetyl-4,5'-dimethyl-psoralen		Visible			+	+	+		±	±		+++	++	++
			Green filter			5	3.5	3		2	2		8	5	
7.	4,5'-dimethyl-8-n-propylpsoralen		Visible				±	+		++	+	+	+++		++
			Green filter				3	8		9	6	6	9		

Table XXIII (Continued) Mean erythemal response after 18 and 36 hours (% reflectance difference)

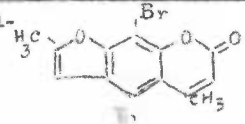
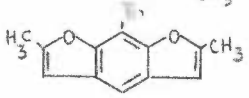
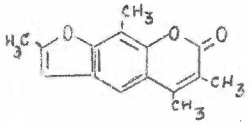
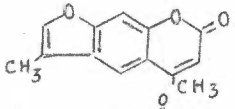
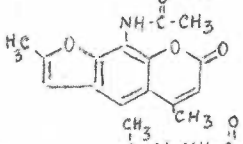
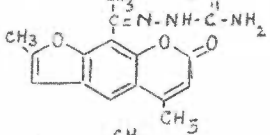
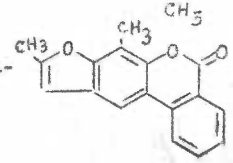
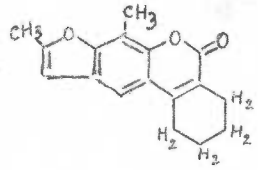
No.	Compound	Structure	Erythema	Micrograms per 2.5 cm^2 skin area							Standard psoralen 25ug	Standard 8-MOP 25ug	Remarks Relative activity
				25	50	100	200	250	500	1000			
8.	8-bromo-4,5'-dimethyl-psoralen		Visible		++	+	+	+	+		+++	++	++
			Green filter		6	2	1	2	3		9	7	
9.	2,6,8-trimethyl-benzo-(1,2-b,5,4-b')-difuran		Visible				-*	±*	+*	++*		++	++ * being liquid, 250,500,750 1000 λ were applied
			Green filter				0	2	4	8		7	
10.	3,4,5',8-tetra-methylpsoralen		Visible		-	+		+	+		+++	++	+
			Green filter		1	2		2	3		7	7	
11.	4,4'-dimethylpsoralen		Visible		±	±	±	±	+		+++	++	+
			Green filter		2	0	0	0	3		10	7	
12.	8-acetamido-4,5'-dimethylpsoralen		Visible		-	-		+	+		+++	++	+
			Green filter		0	0		3	3		9	7	
13.	8-acetyl-4,5'-dimethyl-psoralen semicarbazone		Visible		-	±		+	+		+++	++	+
			Green filter		0	0		3	4		8	7	
14.	3,4-benzo-5',8-dimethyl-psoralen		Visible	±	±	±	±				+++	++	+
			Green filter	3	2	2	3				6.5	5	
15.	3,4-cyclohexeno-5',8-dimethylpsoralen		Visible	±	±	±	+				+++	++	+
			Green filter	2	2	2	2				6.5	5	

Table XXIII (Continued)

Mean erythemal response after 18 and 36 hours (% reflectance difference)

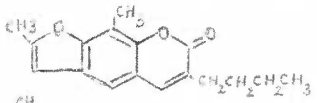
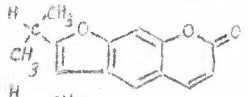
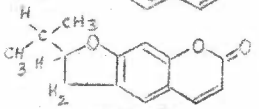
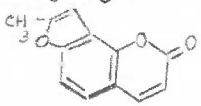
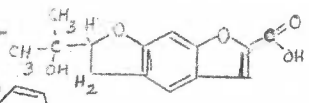
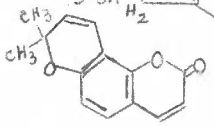
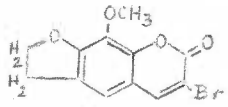
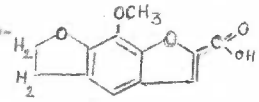
No.	Compound	Structure	Erythema	Micrograms per 2.5 cm^2 skin area							Standard psoralen 25ug	Standard 8-MOP 25ug	Remarks Relative activity
				25	50	100	200	250	500	1000			
16.	3-n-butyl-4,5',8-trimethylpsoralen		Visible	-	±	±		±	±	-	+++	++	+
			Green filter	0	2	2		3	2	0	6	5	
17.	Anhydrosarmesin		Visible		±	±		±			++	+	+
			Green filter		4	1		3			6	2	
18.	Marmesin		Visible		±	±	±	±	-		++	+	+
			Green filter		3	1	3	3	0		6	2	
19.	5'-methylisopsoralen		Visible		-	±		±	±	±		++	+
			Green filter		0	4		3	4	3		7	
20.	2-carboxy-5,6-dihydro-6-(2"-isopropenol)-benzo-1',2'-b,4',5'-b difuran		Visible		+	±	±		±		++	+	+
			Green filter		2	1	1		2		7	4	
21.	Seslin		Visible		-		±	±			++	+	±
			Green filter		0		4	4			6	2	
22.	3-bromo-4',5'-dihydro-8-methoxypsoralen		Visible		-	-		±	±		+++	++	±
			Green filter		0	0		3	3		8	6	
23.	5,6-dihydro-8-methoxy-benzo-(1',2'-b-4',5',b')-difuran-2-carboxylic acid		Visible	-	-	-	-	-	±			+++	±
			Green filter	0	0	2	2	2	6			7	

Table XXIII (Continued)

Mean erythemal response after 18 and 36 hours (% reflectance difference)

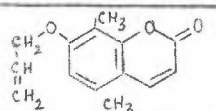
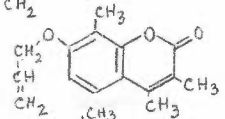
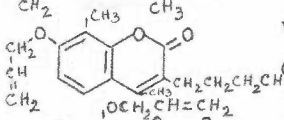
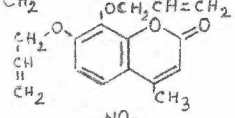
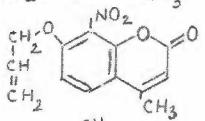
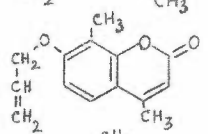
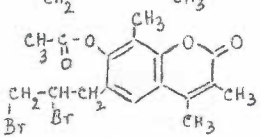
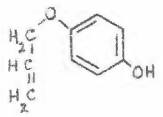
No.	Compound	Structure	Erythema	Micrograms per 2.5 cm^2 skin area							Standard psoralen 25ug	Standard 8-MOP 25ug	Remarks Relative Activity
				25	50	100	200	250	500	1000			
24.	7-allyloxy-8-methyl-coumarin		Visible			-	-	±	±	-		++	+
			Green filter			0	0	4	4	1		5	
25.	7-allyloxy-3,4,8-trimethylcoumarin		Visible	-	-	-		±	±	-		++	±
			Green filter	0	0	3.5		2	0	0		7	
26.	7-allyloxy-3-n-butyl-4,8-dimethyl-coumarin		Visible	-		-	+	+	+			++	+
			Green filter	0		1	4	3	6			7	
27.	7,8-diallyloxy-4-methylcoumarin		Visible			±	-	±	±			++	±
			Green filter			3	0	3	2			6.5	
28.	7-allyloxy-4-methyl-8-nitrocoumarin		Visible	-	-	-	±	++	++			++	+
			Green filter	0	0	0	1	8	9			7.5	
29.	7-allyloxy-4,8-dimethylcoumarin		Visible		-	-		-	±		++	+	±
			Green filter		0	0		0	1		4	2	
30.	7-acetoxy-6-(2',3'-dibromopropyl)-3,4,8-trimethylcoumarin		Visible			-	±	±	±	±	+++	++	+
			Green filter			0	4	4	5	5	7	5	
31.	para allyloxyphenol		Visible	±*	+*								* + being liquid 5 and 25λ were applied
			Green filter	2	2								

Table XXIII (Continued)

Mean erythemal response after 18 and 36 hours (% reflectance difference)

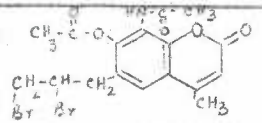
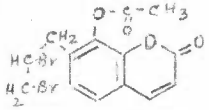
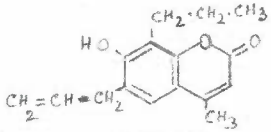
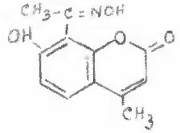
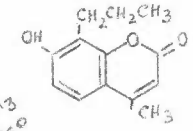
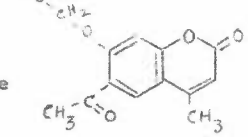
No.	Compound	Structure	Erythema	Micrograms per 2.5 cm^2 skin area							Standard		Relative activity	Remarks
				25	50	100	200	250	500	1000	psoralen	8-MOP		
32.	8-acetamido-7-acetoxy-6-(2',3'-dibromopropyl)-4-methylcoumarin		Visible			-	±	±	±			++	±	
			Green filter			0	2	2	4			5		
33.	8-acetoxy-7-(2',3'-dibromopropyl)-coumarin		Visible		±	±		-	-		+++		+	
			Green filter		4	3		2	0		6			
34.	6-allyl-7-hydroxy-4-methyl-8n-propyl-coumarin		Visible			-	±	±	+			++	±	
			Green filter			0	4	3	6			7		
35.	8-acetyl-7-hydroxy-4-methylcoumarinoxime		Visible				-	±	±			++	±	
			Green filter				0	3	2			7		
36.	7-hydroxy-4-methyl-8-n-propylcoumarin		Visible		-	-			+	±		++	±	
			Green filter		2	1	1		4	3		6		
37.	methyl-6-acetyl-4-methyl-7-coumarinoxyacetate		Visible		-	-	-		-	-		++	±(?)	
			Green filter		1	1	1		1	0		5		

Table XXIV Furocoumarins and coumarins exhibiting no photosensitizing activity

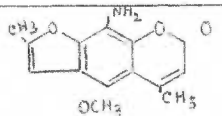
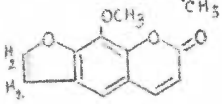
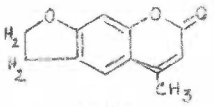
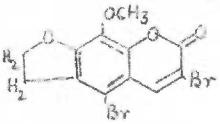
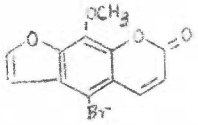
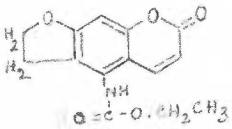
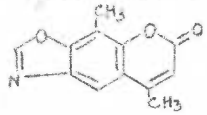
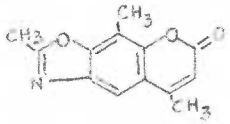
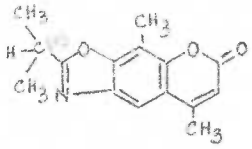
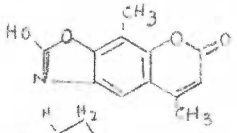
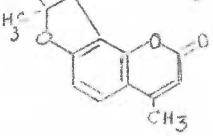
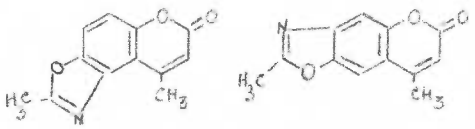
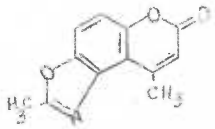
No.	Compound	Structure	Amount applied topically μg / 2.5 cm^2 skin area
38.	8-amino-4, 5'-dimethylpsoralen		50, 100, 250 500, 1000
39.	4', 5'-dihydro-8-methoxypsoralen		50, 100, 250 500, 1000
40.	4', 5'-dihydro-4-methylpsoralen		50, 100, 250 500, 1000
41.	3, 5-dibromo-4', 5'-dihydro-8-methoxypsoralen		50, 100, 250, 500
42.	5-bromo-8-methoxypsoralen		100, 200, 250 500, 1000
43.	4', 5'-dihydro-5-ethylcarbamyl-8-methoxypsoralen		50, 100, 200 250, 500, 1000
44.	β, 7-dimethyl-6-hydroxy-5-benzoxazole acrylic acid, δ-lactone		25, 50, 100, 200 250, 500, 1000
45.	6-hydroxy-β, 2, 7-trimethyl-5-benzoxazole acrylic acid, δ-lactone		25, 50, 100, 200 250, 500, 1000
46.	β, 7-dimethyl-2-isopropyl-6-hydroxy-5-benzofuran-acrylic acid, δ-lactone		50, 100, 200, 250 500, 1000
47.	2, 6-dihydroxy-β, 7-dimethyl-5-benzofuran acrylic acid, δ-lactone		25, 50, 100, 200 250, 500, 1000
48.	4', 5'-dihydro-4, 5'-dimethyl-isopsoralen		50, 100, 250 500, 1000
49.	β, 2-dimethyl-5-hydroxy-4 (or δ) benzofuran acrylic acid, δ-lactone		50, 100, 250 500, 1000
50.	β, 2-dimethyl-5-hydroxy-4-benzosazoleacrylic acid δ-lactone		50, 100, 200, 250 500, 1000

Table XXIV (Continued)

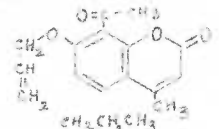
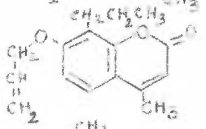
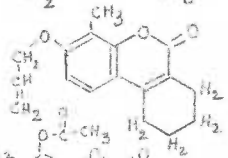
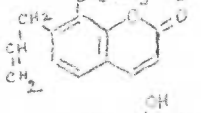
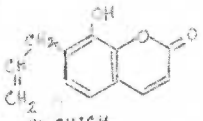
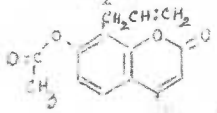
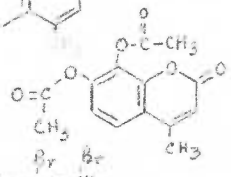
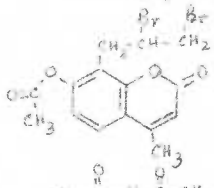
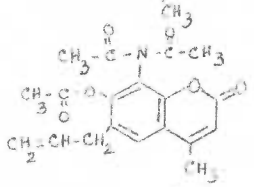
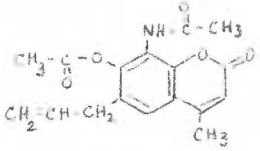
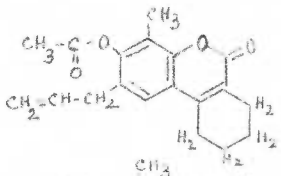
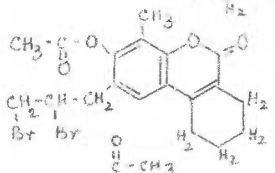
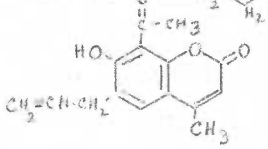
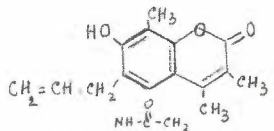
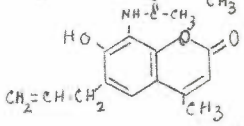
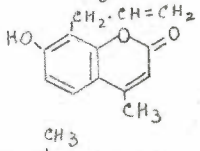
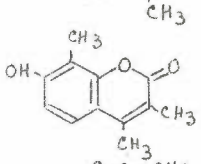
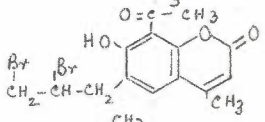
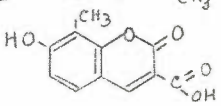
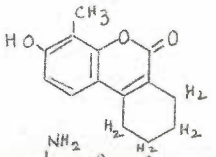
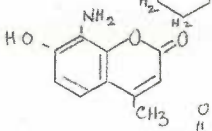
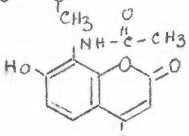
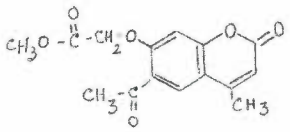
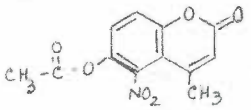
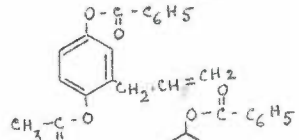
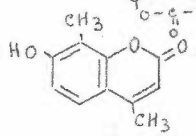
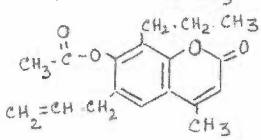
No.	Compound	Structure	Amount applied topically 2.5 cm^2 per skin area
51.	8-acetyl-7-allyloxy-4-methylcoumarin		100, 250, 500 1000
52.	7-allyloxy-4-methyl-8-n propylcoumarin		100, 250, 500 1000
53.	7-allyloxy-3,4-cyclohexeno-8-methylcoumarin		100, 250, 500 1000
54.	8-acetoxy-7- allylcoumarin		100, 200, 250 500, 1000
55.	7-allyl-8-hydroxycoumarin		100, 200, 500 1000
56.	7-acetoxy-8-allylcoumarin		100, 200, 250 500
57.	7,8-diacetoxy-4-methylcoumarin		50, 200, 250 500
58.	7-acetoxy-8-(2', 3'-dibromo-propyl)-4-methylcoumarin		200, 250, 500 1000
59.	7-acetoxy-6-allyl-8-diacetyl-amino-4-methylcoumarin		100, 200, 250 500
60.	8-acetamido-7-acetoxy-6-allyl-4-methylcoumarin		50, 100, 250 500
61.	7-acetoxy-6-allyl-3,4-cyclohexeno-8-methylcoumarin		50, 100, 250 500, 1000
62.	7-acetoxy-3,4-cyclohexeno-6-(2', 3'-dibromo propyl)-8-methylcoumarin		100, 250, 500
63.	8-acetyl-6 allyl-7-hydroxy-4-methylcoumarin		50, 200, 250 500

Table XXIV (Continued)

No.	Compound	Structure	µg Amount applied topically / 2.5 cm² skin area
64.	6-allyl-7-hydroxy-3, 4, 8-trimethylcoumarin		100, 200, 250 500, 1000
65.	8-acetamido-6-allyl-7-hydroxy-4-methylcoumarin		100, 200, 250 500, 1000
66.	8-allyl-7-hydroxy-4-methyl-coumarin		100, 200 250, 500
67.	7-hydroxy-3, 4, 8-trimethyl-coumarin		250, 500, 1000
68.	8-acetyl-6-(2¹, 3¹-dibromopropyl)-7-hydroxy-4-methylcoumarin		100, 200, 250 500, 1000
69.	7-hydroxy-8-methylcoumarin-3-carboxylic acid		100, 200, 500 1000
70.	3, 4-cyclohexeno-7-hydroxy-8-methylcoumarin		50, 200, 250 500, 1000
71.	8-amino-7-hydroxy-4-methyl-coumarin		100, 250, 500
72.	8-acetamido-7-hydroxy-4-methylcoumarin		100, 200, 250 500, 1000
73.	methyl, 6-acetyl-4-methyl-7-coumarinoxyacetate		100, 200, 250 500, 1000
74.	6-acetoxy-4-methyl-5-nitro coumarin		200, 250, 500 1000
75.	2-acetoxy-5-benzyloxy-(2¹-3¹-dibromopropyl)-benzene		100, 250, 500
76.	4-acetoxy-3-allylphenylbenzoate		100, 250, 500
77.	4, 8-dimethyl-7-hydroxycoumarin		50, 100, 200, 500
78.	7-acetoxy-6-allyl-4-methyl-8-n-propylcoumarin		100, 250, 500 1000

tivities of several non furocoumarin compounds including many coumarins. This is an attempt not only to investigate the relationship between molecular configuration and the erythematous activity of various synthetic furocoumarins following ultraviolet irradiation, but also to demonstrate the structure essential for photosensitization response in furocoumarin series. Besides testing the photosensitizing response on mammalian skin, few selected compounds were tested in a biological system consisting of crystalline lacticdehydrogenase. The reason for this investigation was to know whether similar relationship between molecular structure of furocoumarins and inhibition of enzyme activity could also be shown.

In Table XXIII all of the compounds which produced an erythemal response are listed, as closely as possible, in the order of decreasing response. Their activities are tabulated in terms of perceptible erythema (visible intensity) and the photovolt reflection meter readings with a green tristimulus filter. These readings represent percent reflectance difference between the test area and the adjacent control area.

In Table XXIV the compounds which produced no erythema are listed and each entry is followed by the concentrations in μg/ 2.5 cm^2 skin area applied topically for which observations were made.

Effect of group substitution on biological activity: Methyl substitution either at the 4, 5' or 8 positions did not alter the activity of the psoralen molecule. 4-methylpsoralen, 5', 8-dimethyl psoralen and 4, 5', 8-

trimethyl psoralen were found to be as photosensitizing as psoralen (Table XXIII #1, 2, 3, 4). But methyl substitution at the 4' position resulted in considerable loss of activity. 4,4'-dimethylpsoralen was significantly less active than psoralen (Table XXIII #11). Likewise methyl substitution at the 3 position (3, 4, 5', 8- tetramethylpsoralen) was found to mitigate the erythemal response (Table XXIII #10). Several other 3-substituted psoralens, 3,4-benzo-5',8-dimethylpsoralen 3,4-cyclo hexeno-5',8-dimethylpsoralen, and 3-n-butyl-4,5',8-trimethyl psoralen also showed diminished activity (Table XXIII #14, 15, 16).

When the 8-methyl group of the very active 4,5',8-trimethylpsoralen was replaced by an n-propyl substituent, to give 4,5'-dimethyl-8-n-propyl psoralen, the photosensitizing activity dropped markedly, possibly due to the effect of the longer alkyl chain on the solubility of the psoralen molecule (Table XXIII #7). Likewise 5' isopropyl substituted anhydromarmesin (Table XXIII #17) exhibited a weak response. All of the psoralens bearing one or more non-alkyl substituent (e.g. acetamido, acetyl, amino, bromo, methoxy or nitro) were less active than psoralen itself (Table XXIII #6, 8, 12, 13, 22). As reported earlier[72], 8-methoxypsoralen (Table XXIII #5) like 5-methoxypsoralen, was less active than psoralen. In analogy with the effect of hydroxy substitution reported earlier[72], 8-amino substitution (Table XXIV #38) completely eliminated activity.

Effect of hydrogenation at the 4' and 5' positions: Hydrogenation of the 4',5' double bond resulted in almost complete loss of activity. 4'-5'-

dihydro-8-methoxypsoralen, 4'-5'-dihydro-4-methylpsoralen, 4', 5'-dihydro-5-ethyl-carbamyl-8-methoxypsoralen and 3, 5-dibromo-4', 5'-dihydro-8-methoxypsoralen were completely inactive (Table XXIV #39, 40, 43, 41). 3-bromo-4'-5'-dihydro-8-methoxypsoralen showed a very weak response (Table XXIII #22).

Effect of substitution at 3 and 4 positions: Substitutions at 3 and 4 positions showed marked loss of the photosensitizing action. 3, 4, 5', 8-tetramethylpsoralen; 3n-butyl 4, 5', 8-trimethylpsoralen; 3, 4-benzo-5', 8-dimethylpsoralen and 3, 4-cyclohexeno-5', 8-dimethylpsoralen exhibited weak responses (Table XXIII #11, 16, 14, 15).

Activity of oxazolocoumarins: Five oxazolocoumarins (compounds #44-47, 50, Table XXIV) were tested. Although these compounds were quite similar in structure to the furocoumarins, they were all completely inactive.

Activity of difuran derivatives: As shown in Table XXIII #9, 2, 6, 8-trimethyl benzo-(1, 2-b, 5, 4-b') difuran induced a characteristic ++ grade erythema. This compound was available only in a crude form and it is likely that it will show much greater photosensitizing action when purified. This observation indicated that photosensitizing activity was not confined to the furocoumarin nucleus as stated by Musajo et al[67]. Also 2-carboxy-5, 6-dihydro-6-(2'-isopropyl)-benzo 1', 2'-b, 4', 5'b'-difuran and marmesin (Table XXIII #18, 20) showed a weak erythemal response. It appears that a linear tricyclic system of benzene and furan rings also possessed photosensitizing ability. Hydrogenation of

furan ring at the 5 and 6 positions resulted in considerable loss of activity (Table XXIII #20, 23).

Non-linear furocoumarin compounds: As shown in Table XXIV #48, nonlinear furocoumarins were essentially inactive. 4'-5'-dihydro, 4, 5'-dimethyl-isopsoralen was inactive. Seslin, a nonlinear chromanocoumarin, produced a weak response. 5'-methyl isopsoralen showed just a trace of erythemal response (Table XXIII #21, 19). Compound #49, Table XXIV, showed no photosensitizing action.

Studies with coumarin derivatives: Most of the coumarin derivates tested were inactive. Those that were active (compounds #24 to 37, Table XXIII) gave only a weak response only at high concentrations. Compounds 30, 32, 33 are very possibly converted to furocoumarins in vivo. The activity of these 14 coumarin derivatives out of 42 was significantly less than other active furocoumarins reported in Table XXIII. Six compounds with an allyloxy group in the 7 position (24, 25, 26, 27, 28, 29) showed weak but definite activity. Interestingly, p-allyloxy phenol (#31, Table XXIII) showed a definite response. There were a few other derivatives which demonstrated some activity(#34 to 37, Table XXIII). Substitution of methyl, higher alkyl, allyl, hydroxy, nitro, acetyl or acetoxy groups on the coumarin ring did not produce active compounds, (Table XXIV #51-78). But the fact that a few coumarin derivatives elicited photosensitized responses suggested that the furocoumarin structure was not essential for photosensitizing action.

Table XXV Photosensitized inhibition of lactic dehydrogenase by psoralen derivatives

Compound	Concentration ug/ml.	Dark	UV 15'	% loss of LDH activity	Remarks
1. Psoralen	48	0.135	0.100	26	active
2. 8-methoxypsoralen	50	0.110	0.095	14	active
3. 5-methoxypsoralen	55	0.100	0.080	20	active
4. 4-methylpsoralen	49	0.100	0.075	25	active
5. 4, 5'-dimethylpsoralen	45	0.130	0.105	20	active
6. 4, 5', 8-trimethylpsoralen	50	0.120	0.100	17	active
7. 3, 4-benzo-5', 8-dimethylpsoralen	45	0.110	0.095	13.7	active
8. 4', 5'-dihydro-8-methoxypsoralen	40	0.105	0.100	5	weekly active
9. 8-hydroxypsoralen	100	0.110	0.110	0	inactive
10. 5'-methylisopsoralen	70	0.120	0.120	0	inactive
11. 4, 4'-dimethylpsoralen	40	0.115	0.115	0	inactive
12. 3, 4, 8, 5'-tetramethylpsoralen	30	0.110	0.110	0	inactive

Photosensitized inhibition of lacticdehydrogenase by psoralen derivatives: Only twelve compounds were tested for photosensitized inhibition of LDH. Other psoralen derivatives such as 8-acetyl, 5', 4-dimethyl psoralen, 8-acetamido-5', 4-dimethylpsoralen, 4', 5'-dihydro-5-ethylcarbamyl-8-methoxypsoralen, 3, 4-cyclohexano-5'-dimethylpsoralen, 3n-butyl, 4, 5', 8-trimethylpsoralen, 4, 5'-dimethyl-8-n-propylpsoralen were also tried but they were found to be insoluble and hence were not investigated. As shown in Table XXV it can be seen that several of these furocoumarins inhibited LDH in presence of ultraviolet light. They closely followed the activity pattern as demonstrated on guinea pig skin. Although number of observations are needed to quantitate their relative responses as manifested by the degree of inhibition, yet some general observations can be made from the data presented in Table XXV. It can be seen that psoralen and 4-methylpsoralen were most active. 8-methoxypsoralen, 5-methoxypsoralen, 4, 5'-dimethyl psoralen, 4, 5'-8-trimethylpsoralen, 3, 4-benzo-5', 8-dimethylpsoralen were less active. Just as 8-hydroxypsoralen, 5'-methylisopsoralen, 4, 4'-dimethylpsoralen, 3, 4, 8, 5'-tetramethylpsoralen and 4', 5'-dihydro 8-methoxypsoralen were found to be either inactive or showed a very weak response on guinea pig skin, similarly these compounds were found to be either incapable of inducing photosensitized enzyme inhibition or exhibited a very weak action. Thus the relative activity data of enzyme inhibition closely followed the pattern illustrated by determining the erythemal activity of these compounds on guinea pig

skin. It is apparent that this relationship between structure and the photosensitizing activity can be demonstrated in other biological systems also.

Ultraviolet carcinogenesis and effect of 8-methoxypsoralen and psoralen: The present study was undertaken to determine: (a) whether 8-MOP and psoralen (furo .2', 3' 6,7 coumarin) when given in oral form protected or otherwise affected the ultraviolet carcinogenesis in albino and pigmented mice; (b) if the findings of O'Neal and Griffin[70] and Griffin et al.[43] could be explained on the basis of differences between the oral and intraperitoneal route of administration, (since a dose of 0.4 mg/mouse/day injected intraperitoneally to an adult mouse with an average weight of say 25-30 grams corresponds to a dose of nearly 1000 mg per 70 kg body weight as against therapeutically recommended dose of 30 mg/70 kg); the effect of a second more potent furocoumarin psoralen, as compared to 8-methoxypsoralen (Pathak and Fitzpatrick[72], Musajo et al.[65], Fowlks et al.[34], have reported psoralen to be the most potent photo active naturally occurring furocoumarin); (d) the role of melanin pigment in the skin in ultraviolet carcinogenesis.

General observations: Within a week after the beginning of irradiation, both the albino and pigmented mice showed erythema and scaling on the ears. In about three weeks the scale had come loose and ears showed thickening. All types of the pigmented mice showed increased pigmentation of the ears and tail regions. This type of increased melanogenesis was greater in the mice receiving the drugs.

especially in the black and the brown strains. Mice receiving 8-MOP or psoralen at the 0.64 mg level (Group #6, 11, 16, 19, 24, 27) had severe erythema of ears, nose and face regions. These regions were sufficiently burned to be followed by scar formation, loss of hair, and thickening of ears. The control animals showed less severe ultraviolet burns (group #1, 2, 13, 21, 29, 32, 36, 40 and 44). Groups receiving 0.01, 0.04, 0.16 mg of 8-MOP or psoralen orally and the dietary groups (#3-5, 7-10, 12, 14-18, 22-24, 26, 28, 30, 31, 33-35, 37-39, 41-43) showed no appreciable damage of irradiated regions. They did not look in any way different from control ultraviolet or dietary groups. The groups receiving 0.16 mg and 0.64 mg in pigmented mice series showed less damage than the corresponding albino groups. During the first 45 - 100 days, the other observers (TBF, FD) could not differentiate the control groups from groups receiving 0.01, 0.04, 0.16 mg of drugs in albino as well as pigmented series. Since the eye lens absorbs ultraviolet light very strongly, most of these animals became blind within 30 to 45 days after exposure. Cataracts were observed in many animals. The control group as well as groups receiving the drugs were equally affected. Albino mice and pink eyed grey mice suffered eye damages more than the other pigmented mice. In albino series about 5 - 10% of the mice had tumors of the eyelid. There were no tumors of the eyelid in the pigmented groups. The death rate up to the end of irradiation period (120 days) was less than 10% in group #1-28, whereas group #29 to 45 had about 25% deaths in 120 days irradiation period, but after comple-

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Table XXVI A Ultraviolet carcinogenesis: experimental groups and dose schedule

DOSE	MOUSE STRAINS AND GROUP NUMBERS						
	Swiss* albino (AHF)	Brown* C57 Br/cd	Black* C57 Bl/6	Swiss** albino AJAC	Swiss** albino ANC	Yellow** C57 Bl/6A^{Y}	Grey** c̄ pink eyes pG-JAC
Controls (no drug)	1) 2)	13	21) 44)	29	32	36	40
8-methoxypsoralen mg/mouse/day							
oral 0.01	3	14	22	30	33	37	41
oral 0.04	4	15	23				
oral 0.16	5				34	38	42
oral 0.64	6	16	24				
dietary... 1.7	7	17	25) 45)	31	35	39	43
psoralen mg/mouse/day							
oral 0.01	8	18	26				
oral 0.04	9						
oral 0.16	10						
oral 0.64	11	19	27				
dietary .. 1.7	12	20	28				

Note: * Group #1 to 28 were run together (October 1957 - July 1958)

** Group #29 to 45 were run separately (July 1959 - March 1960)

Table XXVI B Incidence of ear tumors in seven strains of mice exposed to ultraviolet irradiation following administration of 8-methoxypsoralen and psoralen (per cent incidence)

Drug and dose		Mouse strains, % tumors						
mg/mouse/day		Swiss albino (AHF)	Brown C57-Br/cd	Black C57-Bl/6	Swiss albino AJAC	Swiss albino ANC	Yellow C57-Bl/6A^{Y}	Grey $\bar{c}$ pink eyes pGJAC
Controls (no drug)		60 70	15	20 0	30	40	0	5
8-methoxypsoralen								
mg/mouse/day								
oral (by tube)	0.01	50	10	5	30	30	0	10
" "	0.04	45	0	5				
" "	0.16	40				45	5	15
" "	0.64	45	0	10				
dietary	1.7	65	25	15 5	35	40	5	10
Psoralen								
mg/mouse/day								
oral (by tube)	0.01	55	0	0				
" "	0.04	65						
" "	0.16	40						
" "	0.64	60	5	15				
dietary	1.7	60	0	5				

tion of the irradiation the animals recovered and lived invariably long enough to reveal tumor development.

Incidence of ear tumors: The final tumor incidences in 45 groups are summarized in Table XXVB In contrast to the findings of O'Neal and Griffin[70], there was no protecting or stimulating effect of 8-methoxypsoralen or psoralen on UV carcinogenesis in albino mice except in groups receiving very high doses of the two drugs. When the tumor incidence of control albino groups was compared with 8-methoxypsoralen or psoralen-treated mice, it was apparent that there were no statistically significant differences among different groups within same strain of mice. Although orally administered 8-MOP groups in albino series, AHF strain showed less incidence of tumors (40 to 50% against control groups 60 to 70%) but the difference was found to be within experimental variation limits (see statistical analysis in Materials & Methods Section). Group #7 which received 8-MOP in the diet showed almost the same incidence of tumors as the control groups. Likewise group #12 which received psoralen through the diet revealed the incidence of tumor very similar to the control group. These observations are further strengthened by the fact that groups #8, 9, 10, and 11 which received psoralen through oral intubation did not show any decreased incidence of skin cancer. The final incidence of tumor was very much like the control groups. Furthermore, when the results of the other two albino strains are compared against their controls (Group #30, 31 vs.29, and Group #33, 34, 35 vs.32) one hardly finds any basis to state that 8-MOP treated

groups are protected against UV carcinogenesis, nor there is an experimental evidence to say that this photosensitizing agent is potentiating UV carcinogenesis in low doses.

The results related to pigmented mice strains are of interest. The incidence of tumors in four pigmented strains of mice ($C57Bl_6$, C57 Br/cd, $C57Bl/6AY^4$ and pGJAc) was significantly much less than in the albino strains. Groups #15, 16, 18, 20, 26, 36, 37 and 44 showed no development of tumors; eight groups had only 5% tumors (#19, 22, 23, 28, 38, 39, 40, 45); four groups had 10 percent tumors (#14, 24, 41 and 43); the rest of the six groups(#13, 17, 21, 25, 27 and 42) had tumor incidences ranging from 15 to 25 percent. The significant variable differentiating the albino and the pigmented strains appeared to be melanin pigment. The presence of melanin pigment more possibly retarded tumor induction and afforded an appreciable protection against ultraviolet carcinogenesis. Administration of 8-methoxypsoralen or psoralen either by way of oral feeding or by dietary intake at these different dose levels did not seem to either potentiate or afford protection to the mice. The incidence of tumor in control groups as well as groups receiving 8-MOP or psoralen was practically the same.

It is thus obvious that when comparisons were made between control groups and groups receiving 8-MOP or psoralen in albino series as well as pigmented strains, the total tumor incidence within a specific mice strain remained close to experimental variation. There was no protection from ultraviolet carcinogenesis.

The rate of tumor induction in albino and pigmented groups was also studied. The measure of tumor induction adopted in these studies was the time elapsed between the first dose of ultraviolet light and the appearance of a tumor. Blum[14] has shown that within a given population of identically treated animals the logarithm of the time to tumor appearance has a normal distribution and therefore tumor induction time in different groups can be compared. The albino mice developed tumors earlier than the four pigmented strains. After starting the ultraviolet irradiation, the control groups in the AHF albino series showed first tumor induction at the end of 90 days. The other two albino strains (AJAC, ANC) showed the appearance of first tumor at the end of 100 days. The control groups in the pigmented mice series which developed tumors showed first tumor induction after 166 days. This prolonged tumor development time difference between albino and pigmented mice was found to be statistically significant. In the albino mice strain AHF, except for group numbers 6 and 11 which received 0.64 mg dose of 8-MOP or psoralen, the rest of the eight groups (#3, 4, 5, 7, 8, 9, 10, 12) which received 8-MOP or psoralen either orally or through the diet, developed tumors after 90-116 days. The other two albino strains (AJAC, ANC) receiving these drugs developed tumors after 110-120 days. As compared to albino mice, the rate of tumor induction in all the four pigmented strains receiving psoralen or 8-methoxypsoralen was very slow. In six pigmented groups(#14, 19, 22, 23, 24, 27) the first tumor induction time was around 147 days. In the rest of groups (including 0.64 mg

dose level groups) the induction time was greater than 166 days. This wide significant difference in induction time (albino vs. pigmented) can be accounted for by the presence of increased melanin pigment following irradiation. It seemed to afford an appreciable protection and delayed the induction of tumor.

The albino groups (#6 and 11) receiving the highest dose of 8-MOP or psoralen, however, did show a shortened induction time, beginning at only 56 to 60 days. This acceleration of cancer induction was not surprising in view of the experimental damage and high degree of hyperplasia associated with administration of such high doses of drug (.64 mg/mouse/day is equivalent to about 1000 mg/70 kg). This dose level certainly accelerated the rate of tumor induction but not the total incidence in the given period of observation. Other dose levels of 8-MOP as well as psoralen (0.01 to 0.16 mg/mouse/day, as well as dietary groups) did not show any acceleration of cancer induction. Most of the tumors induced in these mice with ultraviolet irradiation have been found to be sarcomas or contained sarcoma. The squamous cell and basal cell carcinomas have not been observed (Blum[13, 14], Griffin, et al.[43]). The differences in penetration of the carcinogenic radiation in the mouse skin as compared to human skin accounts for this discrepancy.

DISCUSSION

The inactivation or inhibition effects of enzymes in photosensitization process by psoralens results basically in the obliteration of essential units of the cell's machinery. The destruction of enzyme activities such as succinic dehydrogenase, cytochrome oxidase, lacticdehydrogenase revealed at least qualitatively, the nature of some of the primary events which take place following photosensitization in biological systems. It has been demonstrated that the photosensitizer and the illumination with a specific wavelength of light must be simultaneously present before any effects of these photoactive drugs can be demonstrated. Long-wave ultraviolet light by itself was ineffective and harmless to the biological system. This property would be expected for a complex system with a photoactive component and suggested strongly that a short-lived activated state of psoralen was involved. The ground state of psoralen or 8-MOP molecules is inactive for lack of sufficient energy to induce alterations in enzyme structure. The capture of a quantum (or quanta) by these molecules adds a small increment of energy resulting in an activated or excited molecule. It is the dissipation of this additional energy which seems to alter the biological activities. The activated molecules appear to be short-lived as evidenced by the photochemical inefficiency. The inactivation of an enzyme, ideally speaking, is a one-hit process where a single quantum upon absorption at the proper site should inactivate the enzyme molecule. But it is well known that the enzyme molecules absorb many quanta before they are inactivated.

The degree of inhibition in a few enzyme systems reported in this thesis is undoubtedly low and represents a low photochemical efficiency. It also indicates that it is not a chain reaction where one quantum may set off the reaction of many molecules.

Enzymes are proteins where amino acids are joined to one another in chain like aggregates by means of peptide bonds and cross linked by the primary bonds. The primary bonds that hold the polypeptide chain together are the peptide linkages. The hydrogen bonds give form to the polypeptide chains, e.g., in the form of a helix. The structure of protein is therefore vulnerable to modification by the breaking of these bonds. Loss of enzymic activity may result through the unfolding of this helical structure or by cleavage of peptide linkages. Any minor change in molecular configuration may result in the loss of activity. As discussed in the introductory part of this thesis, sufficient quantum energy is available in the ultraviolet radiation of the wavelengths 3200-3800 Å which are activating psoralen molecule. Johnson et al.[50], have shown that the energy of activation required for denaturation by a general unfolding of the protein molecules is around 25 K. cal. per mole. Undoubtedly the major part of the energy introduced into the protein molecule by a capture of a quantum is lost as heat, increasing the kinetic motion of atoms without modifying their arrangement, but the energy is still sufficient to effect photosensitized denaturation.

The close parallelism between the photosensitized destruction of SDH activity in vivo for guinea pig skin and invitro inhibition of SDH,

LDH, cytochrome oxidase, as well as the observations described by Fowlks et al.[34] and Oginsky et al.[69], concerning the photosensitized bactericidal effect by psoralen and 8-methoxypsoralen, suggested that there may be close link between the two apparently dissimilar processes. The dehydrogenases and cytochrome oxidases as a class of enzymes are rather unique "cogs" in the metabolic machinery providing as they do, energy from metabolic processes. Thus the most immediate and striking effect of psoralen in presence of ultraviolet irradiation appears to be subcellular damage.

The initial results that were presented in Fig. 5 and Table VI revealed that mitochondrial washing was prerequisite to elicit the psoralen response. It was also clear that phosphate buffer acted as a protective agent to the SDH molecules. In this connection it is interesting to note that activation of SDH by phosphate ions present in phosphate buffers has been observed by Singer et al.[83]. TRIS, imidazole or glycylglycine buffers and arsenate showed no activation. The effectiveness of phosphate in exhibiting increased activity has led Slater and Bonner[84] to suggest that phosphate combines with active center of dehydrogenase. Preirradiation of mitochondria as well as of psoralen or 8-methoxypsoralen solution showdd that no phototoxic produtt was produced which was deleterious to enzyme activity. This also demonstrated that the irradiation of the combination of dual agents, photosensitizer and biological material, was essential to effect enzyme inhibition. The increased

susceptibility of mitochondria to 8-MOP photosensitization following storage in a frozen state was possibly due to a change in the state of iron. As believed by Singer et al.[83], it may involve a rearrangement or reorientation of bonds or a change in the valency of the prosthetic group: iron.

While the degree of photosensitized inhibition of SDH or LDH activity was roughly proportional to the concentration of 8-MOP or psoralen, it was not precisely linear. (Fig. 6 and 9). There can be several reasons for this discrepancy. First, it was necessary to investigate this effect only in a limited concentration range since these substances were not very soluble in biological milieu. Secondly, with the progressive increase in the concentration of the sensitizer, the degree of fluorescence increased. The absorbing material at the surface layer may thus act as optical filters. This filtering of the incident light through sequencial layers can mitigate intensitity of light reaching a deeper layer and thus prevent the photosensitized inhibition of enzyme molecules present in inner layers. A gentle stirring of the enzyme solution during the illumination period was carried out to expose enzyme and psoralen molecules to the incident light. Although increased inhibition of enzyme by psoralen was observed by this modification (see Table XX), surface denaturation of the enzyme in absence of psoralen was observed. Finally, it should be noted that mitochondrial suspension and LDH solutions were irradiated in presence of air. Other oxidations due to prolonged irradiation would be expected under these conditions. In spite of these limi-

tations, a linear response was noted.

It is known that crystalline enzymes when exposed to ultraviolet light of specific wavelengths are inactivated. The amount of inactivation is proportional to the dose of light. This relationship is known to be exponential one, i.e., a straight line is obtained if the logarithm of the enzymic activity is plotted against the dose of UV irradiation. Photosensitized inhibition of SDH as well as LDH was found to exhibit this relationship. To plot such data, one must know the amount of light absorbed, since the photochemical or photosensitized reactions can only result from the radiation which is absorbed. The data presented in Fig. 7 and Table XIV does not imply that all the UV light that was incident on the biological system was absorbed quantitatively, nor does it mean that all the light that was absorbed was effective in inducing biological changes. In fact, the quantum efficiency of our system was always very low. Most of the light and hence the energy associated with it appeared to be transferred into heat through collision with other molecules. A major portion of it seemed to be dissipated as fluorescence. By increasing the time of irradiation one has increased the probability of activated psoralen molecules to come in contact with the enzyme molecules which in turn would result in increased photosensitization.

Theeffect of low temperature in potentiating the photosensitizing action of 8-MOP was rather surprising but the findings were in agreement with the data presented by Oginsky et al.[69]. As stated earlier,

these workers characterized the temperature coefficient of 8-MOP photosensitization and found it to be less than one. On the contrary, the temperature coefficient for methylene blue action was found to be greater than one. The 8-MOP photosensitized reaction displaying an overall low temperature coefficient suggested that the thermal reactions which were affecting the photobiological effects had been minimized (loss of energy through vibrational movements and collisions of molecules). This observation is in agreement with the general conception that the temperature coefficient of photochemical processes are on the whole much smaller than the thermal reactions. Furthermore such effect favors the existence of a free radical in psoralen photosensitization which are stabilized at low temperatures.

From the fact that SDH activity of rat liver mitochondria and lactic dehydrogenase was not protected by addition of SH reagents such as cysteine, BAL and glutathione, one is led to the conclusion thatSH groups were not intimately involved in the photochemical steps of this process. In fact, in rat liver mitochondria addition of glutathione increased the degree of photosensitization. This effect was roughly proportional to the concentration of GSH added (Fig. 8). Although there is little published work which indicates the formation of free radical GS° in the presence of UV or a photosensitizer and UV light, a free radical mechanism has been postulated. Inorganic and organic thiol compounds (viz. NaSH, cysteine, glutathione) are known to be oxidized by UV. Bersin[6] as well

as Weiss and associate[100] have studied the oxidation of such thiol reagents. (See also Fava et al.[30], Eldjarn and Pihl[27]). The primary process has been conceived as: $RSH + h\nu \longrightarrow RS^{\circ} + H^{\circ}$.

In aqueous solution one would also expect RS^-

$$RS^- + HOH + h\nu \longrightarrow RS^{\circ} + H^{\circ} + OH^-$$

$$2\,RS^{\circ} \longrightarrow R\text{-}S\text{-}S\text{-}R \quad , \quad RSH + H^{\circ} \longrightarrow RS^{\circ} + H_2$$

$$2\,H^{\circ} \longrightarrow H_2$$

Oxidation of GSH and cysteine by H_2O_2 formation is another possibility: $2\,RSH + O_2 = R\text{-}S\text{-}S\text{-}R + H_2O_2$. Hydrogen peroxide thus produced may further oxidize SH groups. This would then lead to further inhibition of enzyme. Hydrogen peroxide could also be formed by the interaction of the photo-excited metastable triplet state of psoralen with oxygen, leading by electron transfer to the O_2- radical ion and the sequence:

$$P \xrightarrow{h\nu} P^* \xrightarrow{O_2} O_2^- + P$$

$$O_2^- \xrightarrow{+H^+} HO_2 \xrightarrow{+e} HO_2^- \xrightarrow{+H^+} H_2O_2$$

Where P* is an excited psoralen molecule.

The energy of excited P* molecule can be transfered to O_2 and could lead to the formation of an activated O_2 molecule which in turn could give rise to hydrogen peroxide.

A third possibility for the formation of H_2O_2 due to catalysis by metal ions like Fe^{++} Cu^{++}, Zn^{++} etc., may be conceived as follows:

$$P \xrightarrow{hv} P^* \xrightarrow{H_2O} {}^{\bullet}H + {}^{\bullet}OH^- \; ; \; {}^{\bullet}H + {}^{\bullet}H \longrightarrow H_2$$

$$Fe^{++} + {}^{\bullet}OH \longrightarrow Fe^{+++} + OH^-$$

$$Fe^{+++}\,OH^- + {}^{\bullet}OH \longrightarrow Fe^{++} + H_2O_2$$

$${}^{\bullet}OH + {}^{\bullet}OH \longrightarrow H_2O_2$$

The peroxidatic oxidation may also bring inactivation of the enzyme. It can be briefly stated as follows:

$$3H_2O_2 \longrightarrow 2\,HO_2^{\bullet} + 2H_2O, \text{ or } H_2O_2 \xrightarrow{hv} 2{}^{\bullet}OH$$

$$\text{or } Fe^{++} + H_2O_2 \longrightarrow Fe^{+++} + OH^- + {}^{\bullet}OH$$

$${}^{\bullet}OH + H_2O_2 \longrightarrow HO^{\bullet}{}_2 + H_2O$$

$$HO^{\bullet}{}_2 + H_2O_2 \longrightarrow O_2 + H_2O + {}^{\bullet}OH$$

Evidence for a peroxide type reaction can be postulated when one observes the results concerning the effect of EDTA. This metal chelator was found to protect photosensitizing action of psoralens. (Table X, XI, XIX.) EDTA chelation with metal ions present in mitochondria could block peroxide formation and peroxidatic oxidation. Also SDH and LDH have been confirmed to be metalo proteins and respectively have Fe^{++} and Zn^{++} as their prosthetic groups. One is naturally tempted to speculate that versene (EDTA) is shielding the essential metal ions.

There was however no evidence that EDTA formed a complex with protein LDH or its prosthetic group Zn^{++}. The absorption spectra of a mixture of LDH and EDTA solutions did not show any shift. (See Fig. 12, a, b.) Addition of Zn^{++} ions at 1×10^{-4} concentration also did not reveal any change in the absorption spectra of LDH + EDTA mixture.

Furthermore, addition of psoralen to the same mixture did not exhibit any changes in the absorption spectrum or activation fluorescence spectrum. It was apparent that if LDH + psoralen + EDTA complex was indeed formed (with or without zinc) it did not produce a change in the absorption spectrum of the mixture. The spectrum of such a mixture was essentially the same as the sum of the spectra of the separate components. One other fact is clear, that EDTA does not block the effective wavelengths since it has no absorption in 300-400 mu region. Except for the hypothesis that EDTA must be blocking the secondary catalytic effects of metal ions, its effect remains to be clarified.

Furthermore, experimental evidences presented by Blum and Spealman[10], Fiala[31], Vodrazka and his associates[94, 95], do not favor the H_2O_2 hypothesis. Blum[14] has also presented arguments against an H_2O_2 mechanism. The positive free energy ΔF, in the reaction $H_2O + O_2 \rightarrow 2H_2O_2$ does not support in the formation of H_2O_2 but rather it favors the destruction of peroxide (as indicated by arrows) with the evolution of O_2.

In several photosensitized reactions including enzyme inhibition as well, several investigators have failed to demonstrate the formation of H_2O_2 or any other peroxide. An even more cogent argument is that H_2O does not absorb ultraviolet radiation except at wavelengths shorter than those usually included in our biological studies. The same is true in general for oxygen. Moreover, psoralen photosensitization was inde-

pendent of oxygen. Nevertheless, in presence of metal ions, glutathione + O_2 in biological systems, one cannot rule out the formation of H_2O_2 through free radical reactions. It would however result from a secondary effect.

Role of molecular oxygen in psoralen photosensitization: In photosensitization of living cells by organic dyes such as methylene blue, eosin, etc., the basic mechanism has been proposed to be the oxidation of cell components by molecular oxygen and this effect has been called "photodynamic effect." Obviously the reaction will not go in the absence of O_2. However, the observations reported in this thesis related to inhibition of SDH as well as LDH indicated that photosensitization can occur in the absence of oxygen. In presence of O_2 of the air as well as pure O_2, psoralen was found to induce photosensitized inhibition. When O_2 was replaced by nitrogen and helium, inhibition of these two enzymes could still be demonstrated. Oginsky et al.[69] also investigated the effect of molecular O_2 in 8-MOP photosensitization. Suspensions of S. aureus and two strains of E. coli were irradiated in presence of O_2, nitrogen, or helium, with two photosensitizers: (1) 8-MOP in presence of long-wave ultraviolet light; (2) methylene blue in presence of visible light. They observed that presence of molecular O_2 markedly accelerated the death rate with methylene blue, whereas no such effect was observed with 8-MOP. In fact, the presence of molecular O_2 consistently retarded the lethal action of 8-MOP as compared to nitrogen or helium.

It is thus clear that the mechanism of 8-MOP or psoralen does not meet the criteria of photodynamic action as defined by Blum[11, 14]. On these gounds it was preferred to designate the action of psoralen as photosensitization. It is also clear that the mechanism of psoralen action does not involve the SH groups of SDH or LDH enzymes, nor oxidative processes such as those demonstrated by Blum[11], and Weil et al.[96-99]. It is interesting to note that free radicals are more long-lived in absence of oxygen. The low temperature favors their stabilization. The oxygen independent action of psoralen therefore favors a free radical hypothesis.

A very interesting hypothesis postulated in the mechanism of psoralen photosensitization is the formation of a free radical. The free radical may be of psoralen itself, or the activated psoralen molecule may initiate the formation of another free radical as conceived earlier. It may also arise by excitation with 360 mu in a protein molecule. It is possible for a photoactive molecule like psoralen to undergo a metastable triplet state upon irradiation and form a very active biradical which will have two unpaired electrons associated with its electron spins. Allen and Ingram[1] have very recently shown the existence of free radicals in albumin, insulin and melanin at low temperatures following their exposure to ultraviolet irradiation with 360 mu wavelength. The electron spin resonance absorption lines determined by these investigators clearly illustrates that proteins can form free radicals at 360 mu. The effect of albumin and fibrinogen was investigated and it was shown that

addition of these two proteins separately to LDH + psoralen mixture completely blocked and protected the photosensitized inhibition of LDH. The activating wavelengths for maximum fluorescence of psoralen were found in the region of 360 mu. The same wavelength was found by Allen and Ingram[1] to induce free radical formation in albumin and other proteins. It is also now recognized that proteins act as semiconductors and through resonance can transfer electrons very rapidly. The excitation of psoralen by 360 mu may cause a free radical formation in protein. It is also possible that psoralen may act as a stabilizer for free radical formed in proteins. In absence of albumin or fibrinogen, the free radicals, which are characterized by their very high chemical reactivity, are damaging the structure of protein (in our case lacticdehydrogenase and succinic dehydrogenase). Incorporation of albumin or fibrinogen thus shielded LDH molecules from inactivation. LDH concentration used was approximately 3×10^{-4}M. Albumin and fibrinogen were added at 1×10^{-5}M. Both these proteins might have quenched the free radical formation in LDH.

Quinones are known to act as trapping agents for free radicals. They also act as electron acceptors. The effect of addition of quinones on this system (LDH + psoralen) was investigated to test whether free radical mechanism of psoralen could be blocked. Neither of the two quinones, viz. 2-amino, 1,4-naphthoquinone and 2-methyl-1,4-naphthoquinone prevented psoralen photosensitization. These negative re-

sults still do not rule out the free radical hypothesis. It may be that these electron acceptors are not in direct contact with the free radical. The free radical is not absolutely free, but is influenced by the other dipoles in the molecule of which it is a part and hence such electron acceptors may fail to act as protective agents.

The studies related to the addition of DPNH and DPN further clarified the mechanism of psoralen action. If the prosthetic group of this enzyme was the target of psoralen effect, it would seem reasonable to expect a significant protection of LDH and consequently no loss or a very little loss of enzyme activity should follow. Both DPN and DPNH were added in quite large amounts ($1x10^{-3}M$), but both did not alter the inhibitory effect of this sensitizer. It was thought that DPN:LDH complex or DPNH:LDH complex might prevent the inactivation effects of psoralen. Only at higher concentrations ($2x10^{-3}M$), addition of DPNH exhibited some definite protective effect. This indicated that DPNH acted as an optical filter and blocked the effective wavelengths that activated psoralen molecule. Very recently Winer et al.[103, 104] reported the activation and fluorescent spectra of heart muscle LDH-DPNH complex. DPNH has been shown to have activation wavelength of 340 mu and fluoresence peak at 465-470 mu. When DPNH was added to the dehydrogenase solution, the wavelength of maximal emission was found to shift to about 445 mu with relative increase in intensity of fluorescence.

Likewise, effects with heart muscle LDH, beef liver glutamic dehy-

drogenase, horse liver alcohol dehydrogenase and yeast alcohol dehydrogenase, have been observed by several other workers (Boyer and Theorell[15], Winer and Schwert,[103, 104], Duysens and Kronenberg[26]). Similar studies were therefore carried out to see whether muscle lacticdehydrogenase DPNH complex was formed in our system and whether in presence of psoralen there was any shift in the activation and fluorescent wavelengths of LDH + DPNH + psoralen mexture. Intensification and about 10 mu shift in fluorescent wavelengths of the LDH - DPNH complex were observed. DPNH activation and fluorescent peaks were 345:465 respectively. LDH+DPNH showed 345/455 peaks. Following addition of psoralen there was no further shift of activation and fluorescent wavelengths. (This was investigated at low temperature at about 10-12°C). Only intensification of fluorescent peaks was observed which was, however, not additive. It should be known that psoralen + LDH mixture also showed an activation peak at 345 mu $\pm$ 5u and fluorescence peak at 455 mu $\pm$ 5 mu. When DPNH was added to LDH + psoralen mixture, the resultant fluorescent should have been additive. For several low concentrations of DPNH and psoralen mixture there was an increase in intensity of fluorescence when LDH was added but the fluorescence was not additive and proportional to the concentration of 3 components. This indicated that dissipation of absorbed energy as fluorescence (a form of loss of energy) was not occurring. The absorbed energy was retained in the system which was either transferred di-

rectly to the protein and altered its activity or induced a free radical formation. It is tempting to speculate that prosthetic group of LDH also could contribute to the trapping of energy. The energy at 340-360 mu having been absorbed by psoralen and LDH-DPNH complex, could be also causing the damage to biological activity of this protein.

The addition of various substances such as metal ions, substrate pyruvate, various amino acids, flavin mononucleotide, B_{12}, etc., to the photosensitized LDH system also yielded some interesting results. Addition of metal ions such as Fe^{++}, Cu^{++}, Zn^{++}, Mg^{++} had no effect on the system. Exogenous addition of metal ions was investigated with a hope that they would act as sparing agents and protect the metalic prosthetic group of LDH. But none of these metal ions protected the functional part of the apoenzyme molecule. From these, however, one cannot conclude that metal prosthetic group was not involved in psoralen photosensitization.

The amino acids, tyrosine, tryptophan, histidine, cysteine, methionine and glutathione when incorporated into the irradiation system containing LDH + psoralen, did not exhibit any protective effect. These negative results do not necessarily mean that aromatic amino acids of enzyme molecules were not attacked during psoralen photosensitization. Such experiments reveal one interesting point, that molecules which do not absorb in the effective wavelength region will not block the psoralen response. Most of these amino acids in low concentrations do not show any absorption in 320-380 mu region and hence do not affect psoralen photosensitization. This is further confirmed by the fact that vitamin B_{12},

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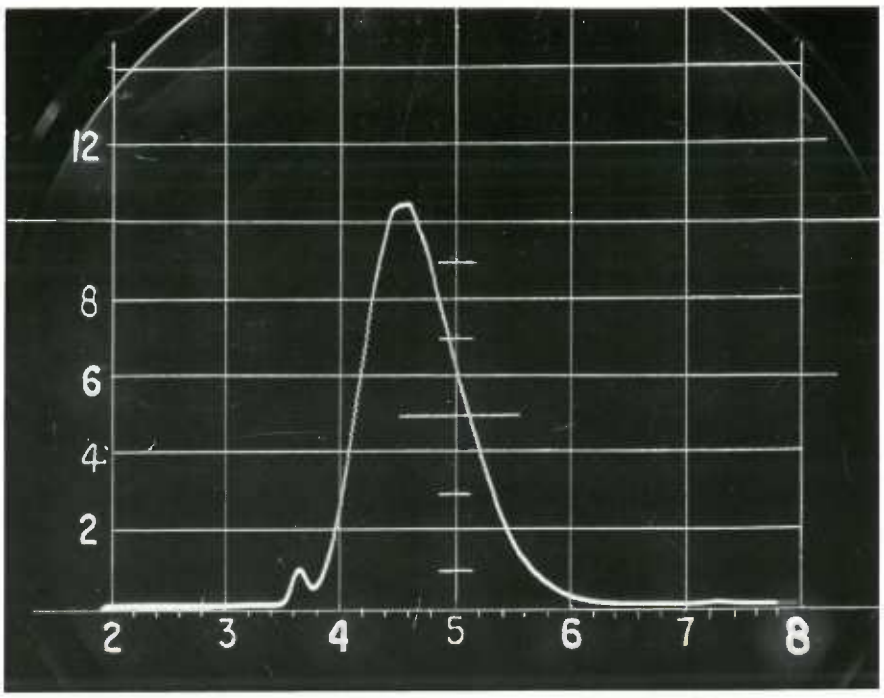
which has a major peak of absorption in 360 mu region, when added to the irradiation mixture consisting of LDH + psoralen, completely blocked the photosensitizing action of psoralen. One can conceive a competitive absorption phenomenon when two absorbing materials are absorbing in the same wavelength region. The effective wavelength of light would be certainly less accessible to psoralen molecules. This in turn would reduce the photosensitizing action. Flavin mononucleotide is a well-known photosensitizer. Addition of this mixture was found to be highly detrimental to the enzyme activity. FMN showed an absorption maxima in the region of 373 mu. Both psoralen and FMN therefore could act independently and induce the biological damage. Results with B_{12}, FMN and DPNH thus strengthened the belief that the effective wavelengths for psoralen action were near about 360 mu.

Although addition of hydroquinone seemed to protect the enzyme against psoralen photosensitization, this effect was however not real. Hydroquinone was found to reduce pyruvic acid in the reaction Pyruvic acid + DPNH $\rightleftharpoons$ lactic acid + DPN. This explained why linear curves with slopes identical to control specimens were obtained and indicated an apparent protective effect. Addition of substrate (pyruvate) also did not alter the action of psoralen. Pyruvate was thus incapable of preventing the photosensitized inhibition of LDH.

Action spectrum of psoralen : The biological studies reported in the preceding pages and observations reported by several workers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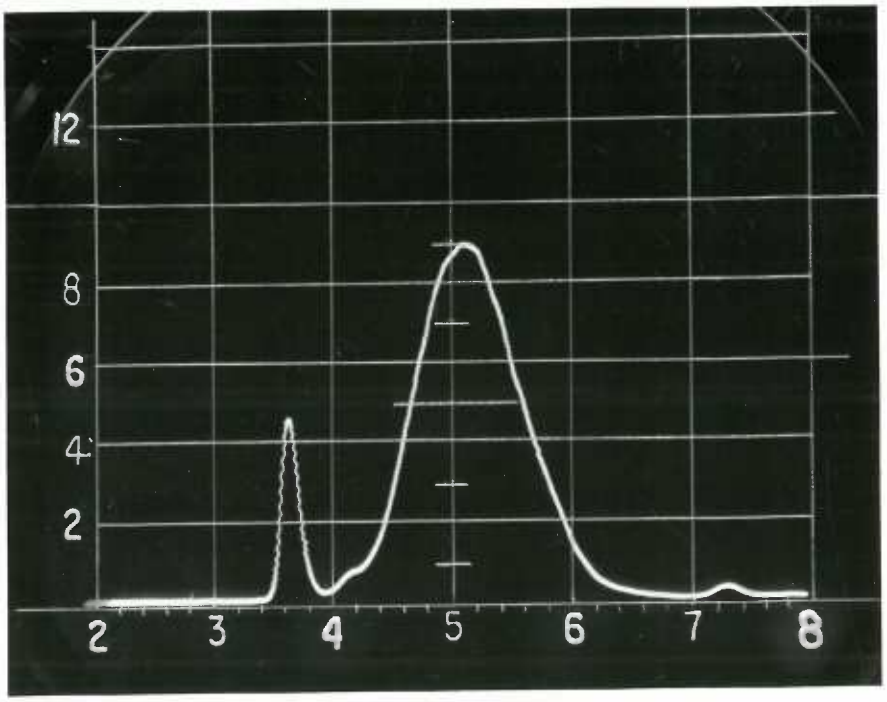
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Figure 11: Activating and Fluorescent Wavelengths of Psoralen and 5', 4-dimethyl-8-aminopsoralen.

The horizontal axis represents wavelength, each number when multiplied by 100 gives the wavelength in mu. The vertical axis represents the intensity of fluorescence, (an arbitrary scale).

Top figure shows the fluorescence peak of psoralen. The activating wavelength was 360 mu and the fluorescence peak was at 435 mu.

Bottom figure shows 380 mu as activating wavelength and 530 mu the fluorescent wavelength of 5', 4-dimethyl-8-amino psoralen. This compound was incapable of inducing photosensitized erythemal response.

(Fitzpatrick et al.[32], Fowlks et al.[34], Oginsky et al.[69], Musajo et al.[65]) has brought out one fact that long-wave ultraviolet light, by itself inocuous and ineffective in inducing any photochemical reaction in absence of psoralen or 8-MOP, was capable of inducing photosensitized reaction in presence of these two photosensitizers. So far it was not known which specific wavelengths of long-wave ultraviolet light was effective. Studies reported in relation to activating and fluorescence wavelengths of several furocoumarin derivatives and related compounds revealed the precise region of the wavelengths which were activating psoralen derivatives. The activating wavelengths for the photosensitizing action of furocoumarins were found to be in the region of 265-280 mu in the short ultraviolet range and between 340-380 mu in the long ultraviolet range (Fig. 11). The fluorescence peaks for these activating wavelengths were observed in the region of 420-460 mu. An interesting correlation between the photosensitizing activity and excitation wavelengths emerged. Only those compounds which were found to be biologically active, capable of inducing erythemal response as well as inhibition of enzymes showed this region of activating and fluorescent wavelengths. The inactive furocoumarins in general did not show these specific activating and fluorescent wavelengths. Data concerning the relationship between absorption spectrum of psoralen and the wavelengths of light-inducing photosensitized inhibition of lacticdehydrogenase revealed the nature of the light absorber in this photobiological process. The inhibition of LDH was observed by those wavelengths which corresponded with the absorption spectrum of

psoralen. If psoralen was the chromophore, the action spectrum of psoralen should superimpose on its absorption spectrum and should exhibit greatest effectiveness at maxima of absorption and least effectiveness at minima of absorption. This assumption turned out to be correct. The inhibition of LDH by psoralen was observed at 240, 290, 320 and 360 mu which corresponded very closely with the absorption peaks of 245, 295, 325 mu in the psoralen molecule. The 360 mu wavelength which was also found to be effective did not correspond to the absorption peak of psoralen. At 360 mu psoralen in very low concentration has very little absorption and yet it exhibited inhibition at this wavelength. Franck and Livingston[37] have proposed a theory in dye sensitized reactions and their view has now become more generally accepted. It emphasizes the importance of a triplet state. It is recognized that the high intensity absorption bands of photoactive dyes correspond to singlet-singlet transitions. The ordinary fluorescence corresponds to a transition-singlet excited state→ground state. A radiationless transition also occurs from the excited singlet state to a triplet state (chemically a diradical). It is the latter form which phosphoresces and has a lifetime sufficiently long to give rise to photobiological effects. It is the reactive, tautomer of the excited molecule. In the ground state the molecule will normally have no unpaired spins. On irradiation, however, a molecular bond can be disrupted and impaired electrons give rise to excitation to a triplet state. It is this state which is postulated to occur in psoralen photosensitization.

Whether psoralen forms a free radical or whether it excites the

protein to undergo a free radical state or whether the protein forms the free radical and psoralen stabilizes it or whether the activated state of psoralen at 360 mu induces the formation of a small steady state concentration of free radicals remains to be investigated. Answers to such basic questions can in part be obtained through studies utilizing electron spin resonance detection.

The mechanism of psoralen (P) photosensitization is postulated as follows: $P + h\nu \rightarrow P^*$ excitation by absorption

I $P^* \longrightarrow P + h\nu$, deactivation (fluorescence)

$P^* \longrightarrow P^{**}$ (metastable triplet state)

$P^{**} \longrightarrow P + h\nu_2$ reverting to the ground level

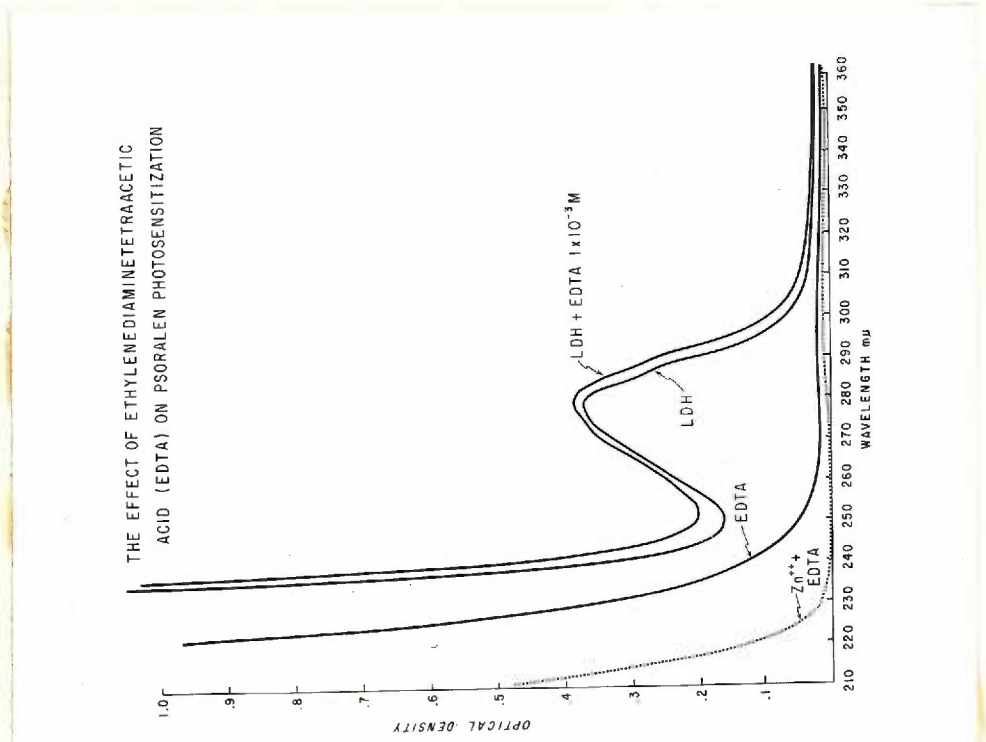
II $\left.\begin{array}{l} \text{Protein} + P \xrightarrow{+\,h\nu} \\ \text{Protein} \xrightarrow{+\,h\nu} \end{array}\right\}$ free radical formation.

In either case the high energy associated with P** or protein + P giving a free radical, will induce biological changes in the system.

The date presented for the activating and fluorescent wavelengths of all the photosensitizing furocoumarins supports this postulation. The decrease of activity in substituted psoralens may result from their inability to lose electrons or participate in free radical formation. Furthermore the detection of free radicals in several proteins following irradiation at 360 mu (Allen and Ingram[1]) and the fact that activation of reactive psoralen derivatives was found to be in the region of

340-380 mu strongly suggests such a mechanism.

Studies related to action spectrum of 8-MOP have been very recently reported by Oginsky et al.[69]. Bacterial cells of two E. coli strains were irradiated under long-wave ultraviolet lamp. Corning color filters differing in transmittance were used to select certain wavelength regions. Their results indicated that wavelengths longer than 390 mu were as effective as those shorter than 390 mu. These authors therefore commented, "It is reasonable to consider that 8-MOP is not the primary chromogen in this system." They plotted the surviving fraction of the bacterial colony under the given filter against the relative intensity of transmitted radiation by the same filter. Their observations and the concluding comment that action spectrum studies do not clearly implicate 8-MOP as the chromogen in thissystem were rather perplexing in the light of the action spectrum studies reported in this thesis. Upon examining the characteristics of UV transmittance of the Corning filters that were used by Oginsky et al.[69] it was evident (see Corning Glass Color Filters, a bulletin by Corning Glass Works, Corning, New York) that nine filters out of a total of twelve filters that were used by these investigators did show a significant transmittance (45-85%) in the region of 360 mu. Two other filters had about 10-15% transmittance in the region of 360 mu. Only one filter showed a cut-off at at 380 mu and did not transmit 360 mu. This was the filter which was least effective in inducing photosensitized bactericidal effect of 8-MOP. The nine filters that transmitted significant light in the region of 360 mu were the most effective in exhibiting the lethal effect. The two


THE EFFECT OF ETHYLENEDIAMINETETRAACETIC ACID (EDTA) ON PSORALEN PHOTOSENSITIZATION
OPTICAL DENSITY
WAVELENGTH mμ
LDH + EDTA 1x10⁻³M
LDH
EDTA
Zn⁺⁺ + EDTA

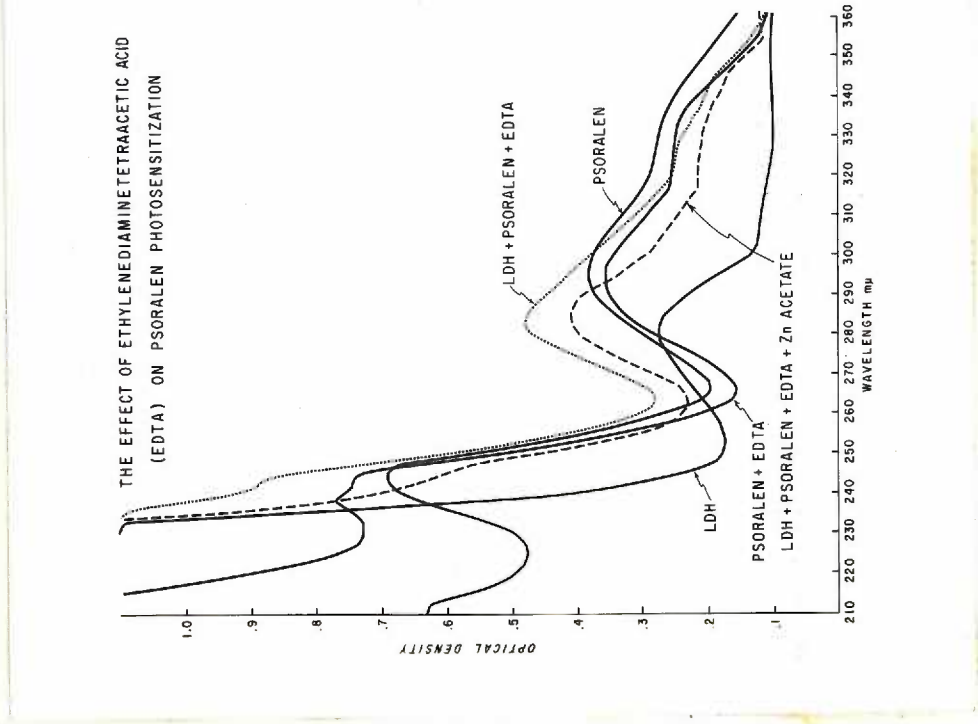

THE EFFECT OF ETHYLENEDIAMINETETRAACETIC ACID (EDTA) ON PSORALEN PHOTOSENSITIZATION
OPTICAL DENSITY
WAVELENGTH mμ
LDH + PSORALEN + EDTA
PSORALEN
LDH
PSORALEN + EDTA
LDH + PSORALEN + EDTA + Zn ACETATE

Figure 12a, b. The Effect of Ethylenediaminetetraacetic acid (EDTA) on psoralen photosensitization.

The absorption spectra of LDH + psoralen, LDH + psoralen + EDTA, and LDH + psoralen + EDTA + Zn^{++}. No shift of absorption peaks was observed. The absorption spectra were essentially identical to the sum of the spectra of the separate components.

other filters (transmittance 10-15% at 360 mu) showed the lethal effect but were less effective. In fact, their results certainly support the data presented in this thesis that the effective wavelength region in psoralen photosensitization is around 360 mu ± 20 mu. Proteins, as we know, show absorption peak in 280 mu region. The activating and fluorescence peaks of LDH as determined by spectrophotofluorimeter were 290 ± 5 mu and 345 mu respectively. In order to accept the quantum of energy of a specific wavelength, the recipient molecule must absorb in the same spectral region. Since LDH in low concentration does not show any absorption peak in this region of 340-380 mu, one can say that it was not the primary chromogen. It was considered that an LDH:psoralen complex with an absorption maximum in this region might have been formed in the mixture and was absorbing the quanta. However, upon examination of the absorption spectrum of LDH + psoralen mixture, it was observed that the spectrum of a mixture of LDH and psoralen did not show any shift of absorption peaks, it was essentially identical to the sum of the spectra of the separate components (see Fig. 12 a, b). Even the activation peaks and fluorescent peaks remained identical. (285/350 for LDH and 350/460 for psoralen.) It was therefore apparent that primary chromogen must be psoralen.

Studies related to the effects of structural alterations on the photosensitizing activity of furocoumarins were primarily carried out with an aim to relate the structure of the compound to its biological activ-

ity. What was hoped for ideally, was the emergence of a common denominator of structure or functional group relationship which would provide clues into the mechanism of biological activity or chemical reactivity. Surprisingly, however, no such definitive relationship was forthcoming in this study. An important varient which has not been controlled in these studies was that of solubility of the test material in the physiological milieu in which biological activity was tested. Differences in solubility and permeability within the cellular environment can modify and alter the response. However, not withstanding this limitation of approach, certain common factors did emerge from these experiments. For example, methylation at carbon atoms 4, 5' and 8 of the psoralen nucleus did not markedly modify the biological response, but methylation at 4' position reduced the activity. Likewise methylation at the 3 position also resulted in loss of activity.

Examination of the data revealed that molecules possessing linear tricyclic systems like psoralen were generally active but nonlinear structures such as isopsoralen were inactive. Substitution with groups which may be designated as electron enriching (such as methoxy, amino, acetamido, etc.,) as well as electron withdrawing (such as nitro and aceto) imparted a partial or complete loss in erythemal response. Hydrogenation of the 4', 5' double bond or substitution of a nitrogen atom for one of the carbon atoms in the furan ring eliminated activity. Indeed any substitution which markedly altered the resonance with-

in the psoralen system mitigated or destroyed the activity.

This was not unexpected from the other findings reported earlier, where it was shown that almost all compounds of this series which were biologically active and induced photosensitization possessed absorption and fluorescent peaks in the range of 320-360 mu/420-460 mu, respectively, while those which were inactive were outside these narrow limits. The significance of this observation is underlined by the fact that the long-wave ultraviolet (>3200 Å) light was employed to potentiate psoralen action (erythema and sun tanning). The augmentation of this response by the active psoralens is probably in large measure a result of the capture of radiation energy of this wavelength region (320-360 mu) and its stabilization to a metastable state. It is evident that alteration of the molecule with groups which alter the absorption and fluorescent spectra decrease the biological response. This response pivots primarily on the photoactivation of psoralen. The absorption of light at a specific wavelength and the emergence of light energy at another from a component in intimate contact with sensitive cellular structures thus appears to be crucial for photosensitizing activity. Evidently, any deviation from the character of this absorption will alter the capacity of the molecule to exhibit its photosensitized biological response. Loss of activity of psoralen derivatives may result from their inability to lose electrons or participate in free radical formation.

From the ultraviolet carcinogenesis experiments it can be concluded

that the findings of O'Neal and Griffin[70] indicating that 8-MOP when administered through diet provided protection against UV carcinogenesis are not confirmed. Dietary feeding of 8-MOP or psoralen (0.5g/kg. diet) did not reveal any protective effect. The basic difference between this study and that carried out by Griffin et al.[43, 81], was dose levels. Throughout their studies this group had used an arbitrarily chosen intraperitoneal dose of 0.4 mg/mouse/day. This dose was roughly 40 times the therapeutic dose usually recommended in clinical trials. Potentiation of carcinogenesis can follow in very high doses when one visualizes that these molecules can trap high energies of radiation. The primary purpose of psoralen administration was to build up an increase in corneum thickening, and increase in melanin pigmentation, thereby creating a barrier to reduce the penetration of harmful ultraviolet radiation. Such high doses as 0.40 mg/mouse/day would induce constant erythema and hyperplasia of the skin and would not let the damaged cells repair, nor permit the thickening of corneum. (An albino mice as we know cannot produce melanin pigment). In lower dose levels no such potentiation was observed. In fact, the rate of induction of tumor (tumor development time) was identical to the control groups. No evidence was there in lower dose levels to suggest that these two substances were carcinogenic and potentiated tumor induction and the rate of appearance of tumor. Moreover, psoralen being more reactive (it induced nearly three times as much erythema as 8-MOP

for the same dose level) did not show potentiation of carcinogenesis in lower dose levels. Only at higher dose levels 0.64 mg/mouse/day, both psoralen and 8-MOP showed potentiation of carcinogenesis and shortened the induction time, beginning at only 56 days. This acceleration of cancer induction is not surprising in view of the experimental damage to the animals on this high dose of 0.64 mg of 8-MOP or psoralen. It is of interest to note that the final tumor incidence did not increase in these animals.

As compared to albino mice, the pigmented mice showed strikingly very low incidence of skin cancer. The presence of melanin pigment in brown, black, grey and yellow pigmented mice appeared to protect these mice appreciably against UV carcinogenesis. It is also interesting to see that 8-MOP or psoralen did not potentiate carcinogenesis in these pigmented mice. Melanin granules being protein in nature can act as absorbers of carcinogenic wavelength and cut off the effectiveness of the radiation. It also seems likely that the increased attenuation of the radiation in presence of melanin is due to scattering by these particles. Daniels[23] has suggested that melanin being a stable free radical, is a repository of photoactively-produced electrons or radicals and may serve as depository ground for compounds produced by photochemical action which would be toxic or carcinogenic. Also Longuet-Higgins[107] believes that the melanin polymer can act as a one dimensional semiconductor with bound protons producing energy traps in the system.

The complex changes resulting from psoralen administration to humans as well as to experimental animals in fact represent a biological response to shield the living parts of the skin from solar as well as artificial UV light injury. Following exposure to light, the erythemal response potentiated by psoralens represents actinic injury to the cellular layers of epidermis and possibly even the dermis. It would seem reasonable to think that measures which would accelerate or augment these self preservative changes would be beneficial if they are specific and not inherently injurious to the other organs and more particularly to the skin and eyes. But recently free radicals have been postulated as taking part in carcinogenic activity by several workers (Allen & Ingram[1]). Psoralens can therefore be potentially dangerous if misused.

SUMMARY

1. The introductory part of the thesis deals with the theoretical background about the absorption of light by photoactive compounds and its effects on biological systems. The historical aspects of psoralen, their physiological properties and their distribution in nature have been outlined.

2. Destruction of succinicdehydrogenase activity by psoralen or 8-MOP photosensitization in vivo in the guinea pig skin and in vitro in isolated rat liver mitochondria has been demonstrated. Various treatments which rendered the rat liver mitochondria susceptable to the photosensitizing action of 8-MOP were studied. It was found that saline washing, suspension in distilled water and aging of the mitochondria rendered them more sensitive to photosensitization. It was observed that phosphate ions protected the SDH from photosensitized inhibition. Effect of preirradiating mitochondrial suspension, psoralen suspension and glutathione solution was investigated and it was concluded that no phototoxic agent was formed which was detrimental to enzyme activity. Furthermore, it has been shown that the photosensitizer and the UV illumination must be simultaneously present before any effects can be demonstrated in biological system. Effects of varying the concentration of 8-MOP and psoralen and also the dose of ultraviolet light were investigated. When the logarithm of enzyme activity was plotted against the dose of UV irradiation, the data indicated an exponential relation-

ship. Also with progressive increase in the concentration of these two photosensitizers and UV irradiation time, greater inhibition of the SDH and LDH activity was observed.

3. The degree of inhibition of a few enzyme systems that were studied was found to be low which indicated a low photochemical efficiency and also suggested that the mechanism of psoralen photosensitization was not a chain reaction.

4. The inactivation of enzymes such as cytochrome oxidase, lacticdehydrogenase and succinicdehydrogenase indicated that the effect of psoralens involved primarily a subcellular damage.

5. The effect of temperature on psoralen photosensitization was studied. The temperature coefficients for 8-MOP were less than 1.0. More enzyme activity was lost when illumination was carried out at reduced temperature.

6. The succinicdehydrogenase of rat liver mitochondria and the crystalline lacticdehydrogenase were not protected by addition of SH- reagents such as cysteine, BAL and Glutathione. The failure of these SH reagents to protect the enzyme activities led to the conclusion that the sulphydryl groups necessary for retaining enzyme activities were not primarily involved in photosensitization. Indeed, addition of GSH to rat liver mitochondria in progressively high concentrations was found to increase the degree of photosensitized inhibition of SDH.

7. Effect of addition of ethylenediaminetetraacetate was investigated. Incorporation of EDTA into biological systems (SDH+psoralen, LDH+psoralen, SDH+psoralen+GSH, LDH+psoralen+GSH) prior to irradiation revealed a significant protection. EDTA chelation with metal ions and prosthetic groups of these two enzymes as well, was investigated. However, no evidence was obtained which suggested that EDTA formed a complex with protein or its prosthetic group.

8. Effect of oxygen on photosensitizing action of succinicdehydrogenase and lacticdehydrogenase by psoralen and 8-MOP was investigated. In presence of oxygen of air as well as pure O_2, these two photosensitizers were found to inhibit these enzymes. Molecular O_2 was however found to retard SDH inhibition. When O_2 was replaced by nitrogen or helium, inhibition of these two enzymes could still be demonstrated. The mechanism of psoralen photosensitization did not meet the criteria of "photodynamic action" as defined by Blum[11]. It was found to be independent of oxygen and hence its biological action has been termed as "photosensitization."

9. The effect of metal ions such as Fe^{++}, Fe^{+++}, Zn^{++}, Cu^{++}, Mg^{++} was investigated with the hope that they would protect the structure and functional group of LDH. No protective effect was noticed.

10. The effect of incorporating various amino acids such as tyrosine, tryptophan, histidine, cysteine, methionine and glutathione in biological system was investigated with the assumption that aromatic groups

or SH groups of the enzymes were primary sites for photosensitization, and incorporation of these amino acids would block the psoralen photosensitized inactivation of LDH. No sparing effect of any kind by these amino acids was observed.

11. Substances which blocked the effective wavelengths of light which activated psoralen molecules and acted as optical filters were found to prevent psoralen action. Vitamin B_{12} and DPNH revealed such protective effect. Whereas flavine mononucleotide which is known to be photosensitizer potentiated psoralen action. Addition of bovine albumin and fibrinogen showed pronounced protective effect. These observations led to the hypothesis that a free radical or metastable triplet state of psoralen was involved in the mechanism of psoralen photosensitization.

12. Incorporation of quinones into LDH+psoralen system did not alter the photosensitization effect of psoralen. Hydroquinone effect appeared to be non-specific. Addition of substrate such as pyruvate did not reveal any protection against psoralen sensitization.

13. The activating and fluorescent wavelengthsstudies of several furocoumarins were carried out. The activating wavelengths for photosensitizing furocoumarins which were biologically active were found in two regions: (1) 265-280 mu; (2) 340-380 mu. The fluorescence peaks for these activating wavelengths were in the region of 420-460 mu. The inactive furocoumarins did not reveal such relationship. The in-

hibition of lacticdehydrogenase by a few selected furocoumarins also supported these observations. It is more likely that furocoumarins which show activation peaks in the region of 340-380 mu and concomitantly the fluorescent peaks in the region of 420-460 mu can cause photosensitization of skin and inhibit enzyme activities in this region of long-wave ultraviolet light. It is concluded that the action spectra for these photosensitizing compounds lies in the region of 340-380 mu.

14. The action spectrum determined by the photosensitized destruction of lacticdehydrogenase activity induced with psoralen was by 240, 290, 320 and 360 mu. This corresponded closely with the absorption spectrum of psoralen (245, 295 and 326 mu). The 360 mu wavelength which inhibited LDH in presence of psoralen corresponded with the activating peak of this molecule. It is postulated that the activating peak of 360 mu for maximum fluorescence of psoralen represents a metastable triplet state for this molecule, which being highly reactive induces photobiological changes.

15. The effect of structural alterations on the biological activity of furocoumarins with 36 furocoumarin and 42 coumarin derivatives was investigated. None of the compounds tested was more active than psoralen. Substitution with methyl groups at positions 4, 5^1 and 8 did not reduce the activity but methyl substitution at 4^1 or 3 positions significantly decreased the photosensitizing activity of psoralen. Simultaneous substitution at 3 and 4 positions also resulted in loss of activity. Substitution

of an alkyl group larger than methyl decreased the activity. Substitution with methoxy, amino, nitro, acetyl, acetamino, bromo, ethylcarbamyl at 5 or 8 positions resulted in either partial or complete loss of activity. Oxazolocoumarin derivatives, isopsoralen derivatives, were found to be inactive. Hydrogenation at 4' and 5' double bond practically eliminated the photosensitizing response. A benzo difuran derivative was found to be active, but hydrogenation resulted in loss of activity. Most of the coumarin derivatives were inactive. In short, any other substitution or modification of psoralen structure was found to either lower the response or mitigate the activity.

16. Based on these observations, it is visualized that a metastable triplet state of psoralen or more likely a free radical formation induced by psoralen or psoralen+protein mixture in presence of an effective wavelength in the region of 360 mu is inducing the biological changes both in vivo and in vitro systems.

17. Ultraviolet carcinogenesis studies in albino and pigmented mice receiving psoralen and 8-MOP, indicated that the findings of O'Neal and Griffin[70] suggesting that dietary administration of 8-MOP provided protection against UV carcinogenesis were not confirmed. No potentiation nor any protection was observed when 8-MOP or psoralen were administered orally in the dose levels ranging from 0.01 to 0.16 mg/mouse/day. Also the dietary feeding of 8-MOP or psoralen (0.5g/kg diet) did not indicate any protective effect. Higher dose levels of 0.64 mg/mouse/day accelerated cancer induction. The presence of melanin

pigment in several pigmented mice strains definitely protected the mice significantly against UV carcinogenesis. No potentiation or increased carcinogenesis by psoralen or 8-MOP feeding was observed in these pigmented mice.

Based on these observations it is concluded that photosensitization by psoralen reflects biological alterations initiated by light.

CONCLUSIONS

The inhibition or inactivation of enzymes such as succinicdehydrogenase, lacticdehydrogenase, cytochrome oxidase by psoralen or 8-methoxypsoralen in presence of ultraviolet irradiation, both in vivo and in vitro systems, indicated that the primary biological effects by these photoactive compounds involved subcellular molecular damage. The activation of psoralen by absorption of energy in the form of quanta and the dissipation of this energy in the biological systems is the underlying mechanism in psoralen photosensitization. Two hypotheses are advanced: (1) It is postulated that the mechanism of psoralen action involves a free radical formation in psoralen or psoralen plus protein mixture. (2) A metastable triplet state of psoralen has also been postulated which interacts and stabilizes free radical formed in proteins. It seems that the high energy and great reactivity associated with free radical or metastable triplet state is responsible for photosensitized biological reactions. This is supported by the observations that psoralen photosensitization was dependent on the concentration of psoralen, the dose of ultraviolet light and the wavelength of light. Low temperature increased the degree of photosensitization. The photosensitization by psoralen and 8-MOP was independent of oxygen. In fact, presence of molecular oxygen decreased the degree of photosensitizing action. Proteins such as albumin, fibrinogen, protected the enzyme LDH against psoralen photosensitization.

Failure of sulphydryl reagents such as BAL, cysteine and glutathione to protect the succinicdehydrogenase and lacticdehydrogenase from psoralen photosensitization make it tempting to conclude that SH groups necessary for activity of these enzymes were not primarily involved in this mechanism. Incorporation of EDTA into the biological systems completely blocked the action of these two furocoumarins. The photosensitization of psoralens can be blocked by substances which absorb the effective wavelengths which activate these molecules. Vitamin B_{12} and DPNH showed such an effect. The activating and fluorescent wavelength studies of several furocoumarins revealed the precise region of the wavelengths of light which enabled the biologically active furocoumarins to induce photosensitization. The activating wavelengths for photosensitizing action of furocoumarins were found in two regions: (1) 265-280 mu; (2) 340-380 mu. The fluorescence peaks for these activating wavelengths were in the region of 420-460 mu. The inactive furocoumarins did not exhibit these properties. Studies concerning the relationship between the absorption spectrum of psoralen and the wavelengths of light inducing photosensitized inhibition of LDH in presence of psoralen (the action spectrum) revealed that psoralen was the primary chromogen and also supported the existence of a metastable triplet state of psoralen. From the study of the effects of structural alterations on photosensitizing activity of furocourmarins and related compounds, one fact emerged and that was that

any substitution which markedly altered the resonance within the psoralen nucleus mitigated or destroyed the activity. A linearly annulated, unsubstituted condensed tricyclic system like psoralen was the most active furocoumarin. Ultraviolet carcinogenesis studies in albino and pigmented mice receiving psoralen and 8-MOP, indicated that the findings of O'Neal and Griffin[70], suggesting that 8-MOP administration through diet provided protection against UV carcinogenesis were not confirmed. Under the experimental conditions no potentiation, nor any protection, was demonstrated by 8-MOP or psoralen in dose levels ranging from 0.01 to 0.16 mg/mouse/day. Nor the dietary feeding of 8-MOP or psoralen indicated any protective effect. Higher dose levels of 0.64 mg/mouse/day accelerated cancer induction and shortened the tumor development time. The presence of melanin pigment in several pigmented mice strains certainly protected the mice significantly against UV carcinogenesis. No potentiation of carcinogenesis by psoralen or 8-MOP was observed in these pigmented mice. It is concluded that measures that would accelerate or augment psoralen photosensitized responses may be potentially dangerous to biological systems.

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