BIOCHEMISTRY OF FUROCOUMARINS

by

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A THESIS

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INTRODUCTION

Furocoumarins.

The fusion of pyrone ring with benzene nucleus gives rise to a class of heterocyclic compounds known as benzo pyrones, of which two distinct types are recognized: (A) benzo pyrones, commonly called coumarins, and (B) benzo pyrones called chromones, the latter differing from the former only in position of the carboxy group in heterocyclic ring.

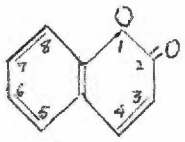


Fig. A. Benzo pyrone (coumarin)

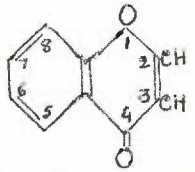


Fig. B. Benzo pyrone (chromone)

If the furan ring is fused on suitably substituted coumarin and chromone derivatives, it leads to the formation of two classes of compounds generally known as furano coumarins or furocoumarins and furano chromones or furochromones (Figs. C and D).

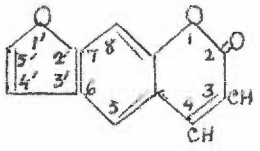


Fig. C.(Furan + coumarin) furocoumarin

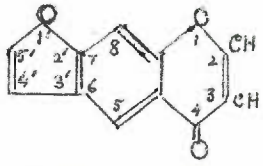


Fig. D.(Furan + chromone) furochromone

Depending upon the fusion of the furan ring with coumarin ring, several isomers are possible, two of which are psoralen and isopsoralen. Shown in Fig. E and F.

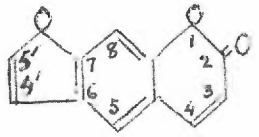


Fig. E. Psoralen

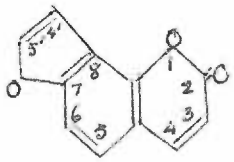


Fig. F. Isopsoralen

The following furocoumarins have been isolated so far from natural sources:

1. Psoralen (ficusin)
2. 5-Methoxypsoralen (bergapten)
3. 8-Methoxypsoralen (xanthotoxin)
4. 5, 8-Dimethoxypsoralen (isopimpinellin)
5. Bergaptol (5-hydroxypsoralen)
6. Bergamotin (5-geranyloxy)
7. Isoimperatorin (5-isopentenyloxy)
8. Ostruthol
9. Oxypeucedanin (5-epoxyisopentenyloxy)
10. Imperatorin (8-isopentenyloxy)
11. Xanthotoxol (8-hydroxy)
12. Byak angelicol (5-methoxy-8-epoxyisopentenyloxy)
13. Byak angelicin (5-methoxy-8-(2, 3-dihydroxyisopentyloxy))
14. Phellopterin (5-methoxy-8-isopentenyloxy)
15. Nodakenin (2', 3'-dihydro-2'-(1-glycosoxy isopropyl))
16. Marmesin (optical isomer of aglucone of nodakenin)
17. Angelicin (isopsoralen)
18. Isobergapten (5-methoxy isopsoralen)
19. Sphondin (6-methoxy isopsoralen)
20. Pimpinellin (5, 6-dimethoxy isopsoralen)
21. Athamantin (2', 3'-dihydro-2'-(1-hydroxyisopropyl)-3'-hydroxy diisovaleryl ester)
22. Prangenin (8-butyloxy)
23. Psoralidin (5-hydroxy-8-isoprene)
24. Thamnosmin
25. Isopimpinellin
26. Peucedanin
27. Oreoselone
28. Oroselone

The structural formulae and the distribution of these furocoumarin compounds in the plant kingdom has been summarized and presented in Table I.

Furochromone compounds are represented by visnagin (2-methyl-5-methoxy furochromone), chellol glucoside (2-glucosoxymethyl-5-methoxy furochromone) and khellin (2-methyl-5, 8-dimethoxy furochromone). Only a few of these furochromones are known, mostly they have been isolated from umbelliferous plants and particularly from Ammi visnaga (Geissman, 1).

The important genera of plants possessing furocoumarins are psoralea (Leguminosae), Umbelliferae, Rutaceae, Moraceae. Since psoralen, xanthotoxin (8-methoxypsoralen), bergapten, (5-methoxypsoralen)), isopimpinellin have been studied more recently in medicine, their distribution in different species has been specificially summarized in Table II. The distribution of other naturally isolated furocoumarins has been presented in Table III. It is evident that furocoumarins in general are confined to 4 or 5 plant natural orders, mainly Leguminosae, Umbelliferae, Rutaceae and Moraceae. More often, they have been isolated from Rutaceae and Umbelliferae plant species.

Psoralea corylifolia is the characteristic species of genus Psoralea. The genus Psoralea belongs to the family Leguminosae, comprising of 100 - 115 species (60).

Table I. Naturally Occurring Furocoumarins

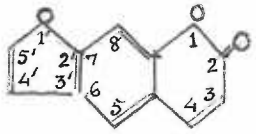
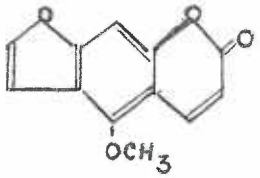
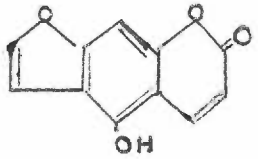
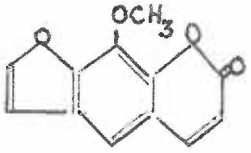
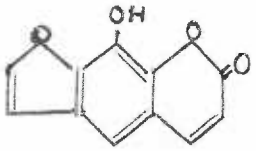
Common name	Chemical name	Structure	Sources
1. Psoralen	Furo(2', 3':6, 7)coumarin		Psoralea corylifolia, ficus carica, coronilla glauca, phebalium argenteum, xanthoxylum flavum
2. Bergapten	5-methoxypsoralen		Ammi majus, pimpinella saxifraga, heracleum-sphondylium, ficus carica, citrus bergamia, fagara xanthoxyloides, skimmia laureola, seseli indicum, ligusticum acutifolium, fagara schinifolia, West Indian lime (lime oil), pastinaca sativa, ruta graveolens, citrus acida, heracleum giganticum, heracleum neplaense, angelica archengilica, pimpinella magna, petrosalinum sativum, apium graveloiens
3. Bergaptol	5-hydroxypsoralen		citrus bergamia (bergamot oil)
4. Xanthotoxin	8-methoxypsoralen		Ammi majus, fagara xanthoxyloides, luvunga scandens, agelica archengelica, ruta chalepensis, ruta montana, ruta graveolens, aegele marmelos, pastinaca sativa
5. Xanthotoxol	8-hydroxypsoralen		Angelica archengelica

Table I. Naturally Occurring Furocoumarins (continued)

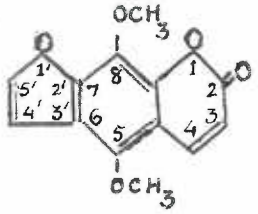
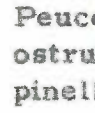
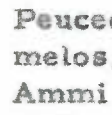
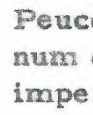
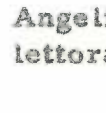
Common name	Chemical name	Structure	Sources
6. Isopimpinellin	5, 8-dimethoxypsoralen		Pimpinella saxifraga, heracelum sphondylium, seseli indicum, West Indian lime, luvunga scandems, fagara oil anthoides, skimmia laureola, thamnosma montana, citrus acida, ferula alliaces
7. Prangenin	8-butyloxypsoralen	R-8-($OCH_2 \cdot CH_2-CH_2-CH_3$)	Prangas pabularia
8. Isoimperatorin	5-isoamylenoxypsoralen	R-5-($OCH_2 \cdot CH{:}C$ )	Peucedanium ostruthium, imperatoria ostruthium, pastinaca sativa, pimpinella magna
9. Imperatorin	8-isoamylenoxypsoralen	R-8-($OCH_2 \cdot CH{:}C$ )	Peucedanum ostruthium, aegle marmelos, imperatoria ostruthium, Ammi majus
10. Oxypeucedenin	5-(2'', 3'' epoxyisoamylenoxy-furocoumarin	R-5-($O-CH_2-CH(O)-C$ )	Peucedanum ostruthium, peucedanum officinale, prangos pabularia, imperatoria, ostruthium
11. Byakangelicon	8-(2'', 3'' epoxyisoamylenoxy-5-methoxy furocoumarin	R_1-8 " R_2-5 $-OCH_3$	Agelica glabra, thamnosma montana, ferula allicaes
12. Phellopterin	8-isoamylenoxy-5-methoxypsoralen	R_1 8-$OCH_2 \cdot CH{:}C$  R_2 5-OCH_3	Angelica glabra, chellapferin lettorales

Table I. Naturally Occurring Furocoumarins (continued)

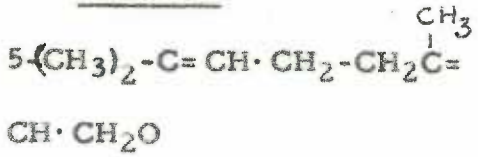
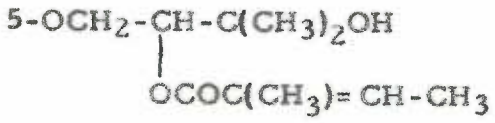
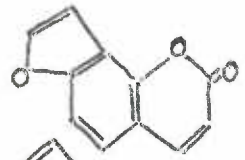
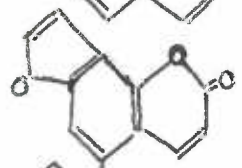
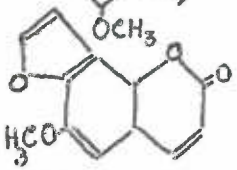
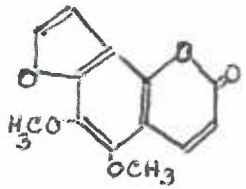
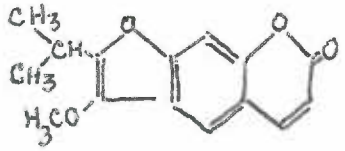
Common name	Chemical name	Structure	Sources
13. Bergamotin	5-geranyloxy furocoumarin		Citrus bergamia (lime)
14. Ostruthol			Peucedanum ostruthium, imperatorium ostruthium
15. Angelicin	Furo(2', 3':7, 8)coumarin (isopsoralen)		Angelica archengelica, psoralea corylifolia
16. Isobergapten	5-methoxyisopsoralen		Pimpinella saxifraga, pimpinella magna, heracleum sphondylium, thamnosa montana
17. Sphondin	6-methoxyisopsoralen		Pimpinella saxifraga, heracleum sphondylium, pimpinella manga, thamnosa montana
18. Pimpinellin	5, 6-dimethoxyisopsoralen		Pimpinella saxifraga, heracleum sphondylium, pimpinella magna
19. Nodakenin	2', 3'-dihydro-2'-(l glucosoxyisopropyl)		Peucedanum decursivum
20. Peucedanin	2'-isopropyl-3'-methoxy furocoumarin		Peucedanum offincinale, prangos pabularia, aegle marmelos, ruta chalepensis

Table I. Naturally Occurring Furocoumarins (continued)

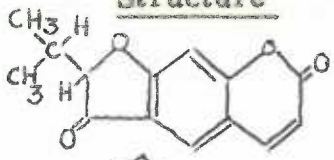
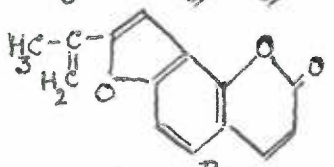
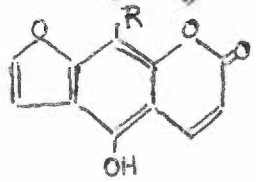
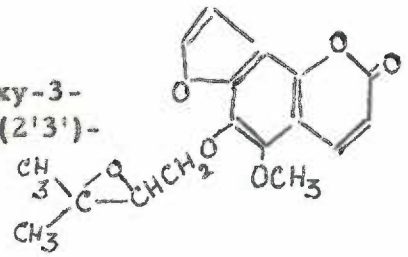
Common name	Chemical name	Structure	Sources
21. Oreoselone			Aegle marmelos, peucedanum officinale
22. Oroselone			Peucedanum oreoselinam
23. Psoralidin	R = CH_2-CH=C.$(CH_3)_2$		Psoralea corylifolia
24. Marmesin	Optical isomer of the aglucone of nodakenin		Aegle marmelos
25. Athamantin	2'3'-dihydro-2'-(1 hydroxy) isopropyl-3'-hydroxy di-iso-valeryl ester of angelcin		Peucedanum oreoselinum
26. Byak angelicol	5-methoxy-8-epoxyiso-pentenyloxy psoralen		Constituent of Japanese drug (Byakusi) angelica glabra, thamnosa montana
27. Thamnosmin	5-methoxy-6-(2, 3-epoxy-3-methyl butoxy-2H-furo(2'3')-1 benzopyran 2 one		Thamnosma montana

Table II. Naturally Occurring Furocoumarins (Psoralen, Xanthotoxin, Bergapten and Isopimpinellin)

Psoralen plants	Natural order	References
1. Ficus carica	Moraceae	2, 3
2. Psoralea corylifolia	Leguminosae	4, 5
3. Coronilla glauca	"	19
4. Phebalium argenteum	Umbelliferae	19
5. Xanthoxylum flavum	Rutaceae	19
Xanthotoxin (8-methoxypsoralen)		
1. Angelica archengelica	Umbelliferae	8
2. Ammi majus	"	9, 10, 11, 12, 13
3. Pastinaca sativa	"	22
4. Fagara xanthoxyloides	Ructaceae	5, 6
5. Luvanga scandems	"	7
6. Ruta chalepensis	"	14, 15
7. Ruta montana	"	19
8. Ruta graveolens	"	15, 19
9. Aegle marmelos	"	16, 19
Bergapten		
1. Ficus carica	Moraceae	17, 19
2. Fagara xanthoxyloides	Ructace	18, 19

Table II. Naturally Occurring Furocoumarins (Psoralen, Xanthotoxin, Bergapten and Isopimpinellin) (continued)

Bergapten (continued)	Natural order	References
3. Skimmia laureola	Ructace	1, 19
4. Citrus bergamia	"	1, 19, 20
5. Ruta graveolens	"	15, 19
6. Ligusticum acutifolium	"	1, 19
7. Heracleum sphondilium	"	1, 19
8. Heracleum giganteum	"	19
9. Heracleum nepalense	"	19
10. West Indian lime	Rutaceae	1, 19
11. Citrus acida	"	1, 19
12. Fagara schinofolia	"	1, 19
13. Seseli indicium	Umbelliferae	1, 19
14. Pastinaca sativa	"	1, 22
15. Angelica archengelica	"	14, 21,
16. Ammi majus	"	10, 12, 13
17. Pimpinella magna	"	19
18. Pimpinella saxifraga	"	1, 19
19. Petroselinum sativum	"	19
20. Apium graveiolens	"	19

Table II. Naturally Occurring Furocoumarins (Psoralen, Xanthotoxin, Bergapten and Isopimpinellin) (continued)

Isopimpinellin (5,8-dimethoxy-psoralen)	Natural order	References
1. Pimpinella saxifraga	Umbelliferae	1
2. Heracleum sphondylium	"	1
3. Seseli indicum	"	1
4. West Indian lime	Rutaceae	1
5. Luvunga scandems	"	1
6. Thamnosma montana	"	23
7. Fagara oilanthoides	"	1

Table III. Reported Distribution of Furocoumarins in Nature

Plants	Natural order	Compounds reported	References
1. Psoralea corylifolia	Leguminosae	Psoralen Isopsoralen Psoralidin	4, 24
2. Coronilla glauca	"	Psoralen	19
3. Acanthus mollis	Acanthaceae	Psoralen	
4. Ficus carica	Moraceae	Ficusin (psoralen) Bergapten	2, 3
5. Aegle marmelos	Rutaceae	Xanthotoxin Imperatorin Marmesin Bergapten	16, 1
6. Citrus acida	"	Bergapten	1
7. Citrus bergamia	"	Bergaptol Bergapten Bergamotin	19
8. Fagara oilanthoides	"	Isopimpinellin	1
9. Fagara schinofolia	"	Bergapten	1
10. Fagara xanthoxyloides	"	Xanthotoxin	1, 5, 19, 25
11. Luvangus scandems	"	Xanthotoxin Isopimpinellin	1, 7, 19
12. Ruta chalepensis	"	Peucedanin Imperatorin Xanthotoxin	14, 26
13. Ruta graveolens	"	Bergapten	15, 19
14. Skimmia laureola	"	Bergapten	1, 19
15. Ruta montana	"	Xanthotoxin	19

Table III. Reported Distribution of Furocoumarins in Nature (continued)

Plants	Natural order	Compounds reported	References
16. Thamnosma montana	Rutaceae	Thamnosmin Isopimpinellin Byakangelicin Isobergapten	23
17. Zanthoxylum flavum	"	Psoralen	19
18. Ammi majus	Umbelliferae	Xanthotoxin Bergapten 8-isoamylenoxypsoralen	9, 10, 11, 12, 13
19. Angelica archengilica	"	Xanthotoxin Bergapten Imperatorin Angelecin (Isopsoralen) Xanthotoxol	14, 26, 1, 19
20. Angelica glabra	"	Imperatorin Byakangelicol Byakangelicin Phellopterin	14, 26, 21
21. Archangelica offincinale	"	Angelecin	21
22. Heracleum-sphondylium	"	Bergapten Isopimpinellin Isobergapten Pimpinellin Sphondin	1, 19
23. Ferula allicaes	"	Isopimpinellin Byakangelicon	1
24. Imperatoria-ostruthium	"	Imperatorin Isoimperatorin	1, 16
25. Ligusticum acutifolium	"	Bergapten	1, 19
26. Peucedenum decursivum	"	Nodakenin	1

Table III. Reported Distribution of Furocoumarins in Nature (continued)

Plants	Natural order	Compounds reported	References
27. Peucedenum officinale	Umbelliferae	Peucedanin imperatorin Oxypeucedanin Oreoselone	14, 26
28. Peucedenum oreoselium	"	Athamantin Oreoselone	1
29. Peucedenum ostruthium	"	Ostruthol Oxypeucedanin Isoimperatorin Imperatorin	1
30. Pimpinella magna	"	Bergapten Isoimperatorin Isobergapten Pimpinellin Sphondin	19, 1
31. Pimpinella saxifraga	"	Isopimpinellin Isobergapten Pimpinellin	1
32. Phellopterum littoralis	"	Phellopterin	1
33. Prangae pabularia	"	Peucedanin Oxypeucedanin	1
34. Seseli indicum	"	Bergapten Isopimpinellin	19
35. Phebalium argenteum	"	Psoralen	19, 29
36. Pastinaca sativa	"	Bergapten Isoimperatorin	28
37. Petroselium sativum	"	Bergapten	19
38. Heracleum giganticum	"	Bergapten	19
39. Heracleum nepalense	"	Bergapten	19

These species include strongly scented herbs, shrubs and under shrubs distributed very widely in tropical and subtropical regions. Psoralea corylifolia Linn is indigenous to India and is common herbaceous weed growing throughout the country. It has several local names in India, such as Babchi (in Hindi, Marathi, Punjabi), Latak kasturi (Bengali), Sugandhakantak (Sanskrit) Karop-Karishi (Tamil), Kalagiuja (Telugu). Psoralea esculenta which grows in the United States and Canada, which was synonimously called Indian bread root, has been widely used as an article of food by American Indians and by early settlers (60). Psoralea grandulosa Linn and P. pentaphylla Linn are reported to have been used medicinally in West Indies and in Mexico. Psoralea butiminosa, (Arabia) p. physodes (California), p. melilotoides (Virginia) and many other varieties have been used in medicinal practice as aromatic bitter tonic and emmenagogue. Other species of psoralea (p. polystica and p. pinneta) are widely used medicinally in South Africa as aromatic tonics.

Historical background of psoralens.

In most of the countries of the world, perhaps among 80% of the world's people, crude drugs derived from plants constitute the basis of prevailing medical practice. It is not surprising, therefore, that plant derivatives have been used for centuries in treatment of leucoderma (vitiligo). Vitiligo is a disfiguring disorder of the skin pigmentation, especially in pigmented peoples (Asiatic, Indian, Negroid, etc.).

The ancient civilization of India, China, Egypt and many of the oriental countries had greatly advanced in natural cures and supplemented their medical beliefs by use of crude plant constituents and plant extracts. The primitive experiences and beliefs about cure of leucoderma, and the art of healing the depigmented areas can be traced back in Indian, Egyptian and Chinese civilizations. The oldest and most sacred books of Hindus (India) which are collectively called "Vedas", a word literally signifying knowledge, has one of the four earliest documents. "Atharveda" (approximately 1400 B. C., even earlier 3001 B. C. ?) in which there maybe found allusions to cure of many diseases. Bloomfield (61) and Whitney (62),who have translated the hymns of "Atharveda", mention the earliest use of black seeds by ancient Hindu physicians for the treatment of leprosy and white skin spots (vitiligo). Though not identified and confirmed, these seeds are presumed to be similar to psoralea corylifolia (Vide Laufer, 63). These seeds were recommended for white leprosy and were given orally as well as applied topically in the form of paste or ointment. The drug was considered so efficaceous in white leprosy and inducing even color of the skin, that it was known in Sanscrit as "Kusthanashini", meaning leprosy destroyer. These seeds were described by Sayana as "Svetakushtha panodanya" and as "Svetakushtha bhaishagyani" by Kesava. From this earliest documents of India the following translated version has been reproduced below to indicate that though white leprosy and white skin spots were not known

as separate entities, yet the use of this plant along with other plant materials was specially directed to induce even color of the skin.

"Leprosy cured by this dark plant, born by night art thou, O plant, dark black, sable; Do thou, that art rich in color, stain this leprosy and the light grey spots."

"The leprosy and the grey spots drive away from here,
May thy native color settle upon thee
The white spots of the skin cause to fly away.

"Sable is thy hiding place, sable art thou, O plant,
Drive away from here these speckled spots.

"Leprosy which has originated in the bones,
And that which has originated in the body,
And upon the skin, and the white
Marks begotten of corruption,
Destroy them with thy charm."

In the same book further mention of this plant together with other plant constitutents is found to cure leprosy and white skin disease.

"The eagle (Suparna) that was born at first,
his gall thou wast, O plant!
The Asuri having conquered this (gall), gave
it to the trees of their color.

"Asuri was the first to construct this remedy
for leprosy, this destroyer of leprosy, she has
destroyed the leprosy and has made the
white skin of even color.

"Even color is the name of thy mother,
Even color is the name of thy father,
Thou, O plant, producest even color
Render this spot to even color.

"The black plant that produces even color
has been fetched out of the earth,
Do thou now, pray, perfect this,
render anew the colors."

The mode of application of this plant mixture has been also described in the same book. The priest rubs the drug (paste) of these even color seeds until the area is red. He smears upon the people the substances mentioned in the verses (Mantras). Kesava and Sayana, also mention Bhringarga (Ecclipta prostata), haridra (yellow sandal or yellow turmeric curcuma longa) and Indra varuni (Colocynth). These substances having been applied, the patient performs the rites to Maruts (Air) in the sun. According to Kaus, this was intended to put the patient outside under the sun until he sweated (open air sweating, effect of ultraviolet radiation).

It is hard to identify this plant by such poetical expressions, but in Bower manuscript (64), translated by A. F. R. Hoernle (1893-1912) which deals with the manuscript remains of Buddhist literature found in Eastern Turkestan (Turkey) and also studies in medicine of ancient India, (about 200 A. D.) mention of white skin diseases (leucoderma) and its cure by a plant "Vasuchika", which is said to be an old form of Bavache (Psoralea corylifolia) has been distinctly mentioned.

In further support of this historical evidence, one finds in "Sino-Iranica", written by Laufer (63), a drug by the name pu-ku-čí (bu-ku-tsi) distinctly identified with psoralea corylifolia and first mentioned by Mačí and collaborator in the Kai pao pen tscao (A. D. 968-976) of the Sung period, as growing in all districts of Lin-nan

(Kwan-tun), Kwan-si and in the country of Po-se in China. In Nan Čou Ki (ancient Chinese book of the 5th century) written by Sci-Piao, the same plant is mentioned as Hu-Kui tse, the Allium odorum of Hu. However, Li ši scen annotes that the Hu name of this plant is po-ku-ci (bwa-ku-či, Ba-ku-či) popularly, but erroneonsly written as po-ku-ci. Laufer adds comments to the evidence presented by the Chinese sources, that the word Ba-ku-či, not an original word of Chinese language, is of Indian origin. It answers the sanskrit "Va-ku-ci" or Va su chi ka, which has been now identified as the plant Psoralea corylifolia. The close similarity of the name for this plant in different Indian languages further reveals the fact that this plant has been used for centuries in Indian "Ayur Vedic" system of medicine. (The body of literature that gradually grew up on the subject of medicine is called "Ayur Veda", literally meaning "the knowledge of life, 400 B.C. - 350 A.D. approximately).

Today we know that psoralea corylifolia contains the active furocoumarin "psoralen", which can induce skin pigmentation in depigmented areas. How this plant attracted the attention of our ancient people remains a guess, but it is fascinating to see this early history which has showed us the track which remained untrodden for centuries, but now which has attracted our attention to pursue the secrets of nature in the mechanism of cutaneous responses of increased erythema and pigmentation by the active ingredients of these plants.

In further support of earlier use of plants containing furocoumarins (groups) in cure of vitiligo, an ancient history of another very important plant deserves a special mention. Egyptian civilization has contributed immensely in understanding many secrets of natural cures. The dried fruit or extracts of plant Ammi Majus Linn has been known to Egyptians for their value in the treatment of depigmented area of skin for many centuries. Ibn El Bitar (65) gave a description of Ammi Majus in his famous book Mofradat El Adwiya (13th century) and stated that it was commonly employed as a remedy for leucoderma by "Ben-Shoeib," a Berberian tribe of north western African Desert. In the same book, the author quotes a statement of a famous physician El sherif for the therapeutic uses, dose and necessity of exposing the person to sun-light for inducing pigmentation after the powder of Ammi Majus was administered to the patient. In 1898 Dragendorff (66) described this plant and later on Dawood El Antaki (67) in 1923 confirmed the earlier use of Ammi Majus in the treatment of leucoderma.

The credit of revealing the therapeutic usefulness of Ammi Majus in leucoderma goes to Fahmy and collaborators at the University of Cairo. Fahmy (68) in 1931 observed that some Egyptian herb doctors were using a grey green powder called "Atrillal" (Gazar El Shitan or Regl El Ghoral) for the treatment of vitiligo. It was distributed through only one or two dealers who would not reveal its nature. Fahmy later on ascertained that "Atrillal" powder was obtained from

the fruits of a weed that grew all along the river Nile; this weed was identified as Ammi Majus Linn. Earlier Fahmy and Elkeiya (68) had analysed the fruits of Ammi Majus and isolated an amorphous glucosidal principal along with oleoresin and many other constituents. Later on Fahmy and Hameed (69) reported the isolation of a crystalline principle from this plant, which they named ammoidin. In his further studies, Fahmy (70) in collaboration with Abushady isolated three crystalline compounds which they believed were the active ingredients of the crude powder. These were found to be furocoumarins and were named after the plant from which they were isolated as ammoidin ($C_{12}H_8O_4$), ammidin ($C_{16}O_{14}O_4$) and majdin ($C_{12}H_8O_4$). This was an unfortunate selection of names, for the two compounds had already been known. Ammoidin or 8-methoxypsoralen had been isolated from different plant source Fagra Xanthoxyloides to the extent of 2% (Thom, 71; Hans Priess, 72, 1911). 8-Methoxypsoralen or xanthotoxin had already been synthesized by Spaeth, 1933 (73). Later on Alexander Shonberg and Aly Sina (12, 13) showed that ammoidin was similar to xanthotoxin. Xanthotoxin was also reported earlier by Bose and Mookergie to be present in the berries of Luvanga Scandens (7). The chemical study and synthesis of this drug had been carried out by several investigators (Thoms, 71; Spaeth and his associates, 6, 8, 73, 74, 75, 76, 77). Majudin or 5-methoxypsoralen was a well known constituent of oil of bergamot which had long been used in perfume

industry and known to be a causative factor in causing a light sensitized dermatitis (Kuske, 1938, 35; Jensen and Hansen, 1939, 38).

Recent history.

Modern period of psoralen research began when Kuske, 1938 (35) investigated the "phytophotodermatitis," the term suggested by Klaber (36) to include all eruptions appearing on the skin and parts of the body which have been in contact with certain plants after subsequent exposure to sunlight. An additional aspect inherent in this photosensitizing action is characteristic residual pigmentation. Kuske obtained compounds from oil of bergamot (bergapten, 5-methoxypsoralen), oxypeucedenin from master worts (Peucedanum ostruthium) and ficusin (psoralen) from figs, and demonstrated bullous erruptions and residual pigmentation following the application of these compounds in presence of sunlight. Later on Jensen and Hansen (38) elucidated the same effect with wild parsnip. He also investigated the biophysical aspect of ultraviolet wavelength effective in producing this dermatitis and pigmentation. Clinical trials with 8-methoxypsoralen by El-Mofty (78) from Egypt, Sidi and Bourgeois (79) from France, and Fitzpatrick et al. (80) from Oregon, revealed promising results in the treatment of vitiligo and increasing the solar tolerance of skin. Since then in England, France, Africa, U.S.A. and India psoralens have stimulated great interest in the field of pigmentation.

Furocoumarins and phytophotodermatitis.

Before going further in the other developments of furocoumarin research, it will be interesting to recall some relationship of plants which are believed to cause phytophotodermatitis and residual pigmentation and the compounds present in these plants which could cause these cutaneous responses. Recently a body of evidence is coming forward to suggest that phytophotodermatitis caused due to external contact with many of the plants is due to furocoumarin group of compounds present in these plants.

As early as 1926, Oppenheim (31) first described meadow dermatitis due to eau de cologne and fig leaves in presence of light. The cow parsnip and wild growing parsnips were perhaps the first plants reported subsequently to produce the characteristic features of this dermatitis. Later on Klaber (36) suggested the term phytophotodermatitis for this type of photosensitization. It has long been known that contact with figs may result in inflammation of the skin followed by persistant pigmentation (33, 8). Employees engaged in canning industries processing fruits and vegetables such as carrots, celery, parsley, etc. have been reported to develop dermatitis and pigmentation. Reviews have been published by Klaber (36), Bellringer (40), Goldsmith and Hellier (82) and Klauder and Kimmich (39) pointing to the relationship of phytophotodermatitis and botanical species that cause this photosensitization. A brief summary has been prepared in Table IV. The common names of plants along with their botanical

Table IV. List of plants reported to give phytophotodermatitis.

Common name	Botanical name	Natural order	References
1. Fig	Ficus carica	Moraceae	30
2. Parsnip	Pastinaca sativa	Umbelliferae	31, 32, 33, 34,
a)cowparsnip	Heracleum sphondylium	"	35, 36, 37, 38,
b)hogwood	" "	"	52
c)garden parsnip	Heracleum giganteum	"	
d)wild parsnip	Heracleum mantegazzianum	"	
3. Fennel	Foeniculum vulgarae	"	36, 39
4. Dill	Anethum graveolens	"	36, 39, 40
5. Parsley	Peucedenum oreoselium	"	36, 39, 40
6. a)wild carrot b)garden carrot	Daucus carrot	"	36, 39, 40, 41, 42
7. Master worts	Peucedenum ostruthium	"	35
8. Celery	Apium graveolens	"	45, 46, 47
9. Atrillal	Ammi majus	"	48
10. Angelica	Angelica species	"	50, 51, 56
11. Common rue	Ruta graveolens	Rutaceae	43
12. Gas plant	Dictamus albus	"	53
13. Lime bergamot	Citrus bergamia	"	36, 39, 40
14.	Dictamus fraxinella	"	56
15. Lime	Citrus aurantifolia	"	59
16. Butter cup	Renunculus species	Reninculaceae	39, 55

Table IV. List of plants reported to give phytophotodermatitis (continued).

Common name	Botanical name	Natural order	References
17. Mustard	Brassica species	Cruciferae	39, 40
18.	Sinapis arevensis	"	55
19. Bind weed	Convolvulus arevensis	Convolvulaceae	36, 39, 40
20. Agrimony	Agrimony eupatoria	Rosaceae	39, 54
21. Yarrow (milfoil)	Achilleae millefolium	Compositae	55, 57, 58
22. Goose foot	Chenopodium species	Chenopodiaceae	39, 40
23. Bavachi	Psoralea corylifolia	Leguminosae	49
24. St. John's wort	Hypericum perforatum Hypericum angustifolium	Hypercaceae	39

names, natural order of their family has been presented with a view to indicate the botanical relationship, the distribution of these plants in a few families or plant genera and to bring forth an evidence that many of these species contain active furocoumarins (compare Tables I, II, III). Contact with these species in presence of sunlight (ultra-violet) can bring a photodynamic effect resulting in phytophotodermatitis.

It is evident from the data presented in Table IV that species of Umbelliferae are more predominent in causing dermatitis and exerting photodynamic action. The Rutacea are the next most frequent in capacity to photosensitize. This includes the common rue gas plant, and many citrus fruits. Several varieties of lime and bergamot are known to cause these biological changes. Moreaceae family is represented by fig, whose photogenic action has long been known. Other botanical families included to a less extent are Convoloulaceae, Compositae, Cruciferae, Rosaceae and Renunculaceae. The etiology of this condition does not seem clearly established. One thing is clear that light plays an important part. Chlorophyll has been suspected as the photosensitizer (Rogin and Sheard, 83) but the action spectrum does not seem to support this. If Table IV and Tables I, II, and III are compared with respect to plants and the distribution of furocoumarin compounds, it will be seen that plants containing

furocoumarin compounds have been generally implicated in phytophotodermatitis. Without going into detailed aspect of this photodynamic action, it will suffice to point out that members of furocoumarin group could be the causative factors for this type of dermatitis. Plants like fig, parsnip, Ammi Majus, psoralea corylifolia, master worts, parsley, goose foot, bergamot (Citrus bergamia), etc., are known to contain some of the active furocoumarins like psoralen, 8-methoxypsoralen (xanthotoxin), bergapten (5-methoxypsoralen), etc. These compounds are photodynamically active (Fowlks, et al., 84), and in presence of sunlight or long wave ultraviolet light can bring about these skin changes. Very little evidence has been presented by dermatologists in this connection to suggest that furocoumarins could be the chief cause, but the occurrence of these furocoumarins in many of the plants which are reported to cause photosensitization in human skin, sheep and cattle suggests that these furocoumarins may be one of the chief causative agents in presence of active light wavelength.

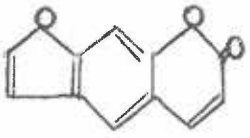
Chemical history of furocoumarins.

Spaeth et al. (2, 6, 8, 74-77), Jois and co workers (4, 5), Thoms (5, 18, 71), Schonberg and associates (12, 13), Fahmy and his colleagues (68, 69, 70) were some of the early pioneering workers who have contributed to the understanding of the structures, methods of synthesis and chemistry of furocoumarins group of compounds. Since the main

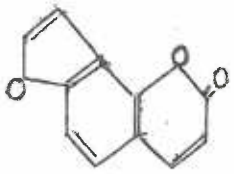
interest in furocoumarins is confined to a few compounds, namely, psoralen, 8-methoxypsoralen (xanthotoxin), bergapten (5-methoxypsoralen) and few other naturally occurring substances like isopsoralen, isopimpinellin, etc., reference to these compounds will be made and more often 8-methoxypsoralen will be abbrivated as 8-MOP.

The chemical study and synthesis of xanthotoxin (8-methoxypsoralen) was carried out by Thoms (5, 18), Spaeth and coworkers (73), Spaeth in collaboration with several of his associates in an extensive series of papers (2, 6, 8, 73, 74, 75, 76, 77) reported the synthesis, chemistry and isolation of 8-methoxypsoralen, isopsoralen (Angelicin), bergapten, psoralen, etc., from various plants sources such as Ficus carica, Fagara Xanthoxyloids, Angelica archengelica, Aegle marmelois, Rutagraveolens, etc.

Jois et al. (4) reported the isolation of psoralen from the petroleum ether extract of seeds of psoralea corylifolia. They obtained a dark reddish brown oil and a crystalline solid $C_{11}H_6O_3$ m.p. 162° which they named as psoralen. On further investigation, Jois and Manjunath (5) observed that the two furocoumarins, psoralen and isopsoralen were present in this plant. Structure I and II were assigned to psoralen and isopsoralen molecules respectively.



I
Psoralen



II
Isopsoralen

The synthesis of psoralen was reported by Spaeth et al. (87) and Horning and Reisner (27). Presently there is no full agreement between the melting point of synthetic and natural products of psoralen material (Mukerji, 49). It is possible that in presence of light and particularly ultraviolet light, these molecules get dimerised, and this dimerisation could alter the melting points (Fowlks, 86). As stated earlier, Egyptian workers (Fahmy and his associates, 68, 69, 70) had isolated 3 crystalline compounds from Ammi Majus ammoidin ($C_{12}H_8O_4$ up to 0.5%), ammidin ($C_6H_{14}O_4$ up to 0.3%) and majudin ($C_{12}H_8O_4$ up to 0.04%). However, Alexander S chonberg and Sina (12, 13) later on showed that ammoidin was identical with xanthotoxin, ammidin with imperatorin or 8-iso-amylenoxypsoralen, and majudin was similar to bergapten (5-methoxypsoralen).

Spaeth et al., reported the isolation from the roots of Anglica archengelica, a furocoumarin, Angelecin or isopsoralen, and established its structure by synthesis (85). Okahara (2, 88) studying the leaves of Ficus carica (fig) isolated a furocoumarin "ficusin" m.p. 161.5° - 162°C. which was shown by Jois and Manjunath (89) to be identical with psoralen. Subsequently 5-methoxypsoralen (bergapten) was isolated and identified from ficus carica by Spaeth and Hiller (17, 60). Since there are a number of the plants studied for the chemistry and isolation of the active compounds, it was thought easier to prepare a summarized chart to give an overall picture of furocoumarins at

a glance. Very little attempt has been made by workers in the field of furocoumarins to gather the widely scattered information about their natural occurrence. In Table III a general list of furocoumarins and plants reported to have these different compounds, has been therefore presented.

Chromatography of furocoumarin compounds.

These compounds occur together in nature along with many coumarins and flavonoid compounds. This makes the identification and separation of this group of compounds more difficult. Owing to the insolubility of these compounds in water, the paper chromatography of this class of compounds also presents considerable difficulty. However, a large number of naturally occurring coumarins, furocoumarins and flavonoid substances exhibit characteristic fluorescent colors on the developed chromatograms when examined under ultraviolet light which helps therefore in identification of these compounds under different solvent systems. A chromatographic detailed study of the furocoumarins and coumarin compounds has been reported by Swain (111) and also by Chakraborty et al. (114). Swain recommended Whatman No. 1 filter paper, plain or buffered with 0.1 M Na_2HPO_4 or 0.1 M $NaBO_3$. A large variety of solvent systems such as phenol water, m. cresol:acetic acid: water (50:2:48), amylacohol:acetic acid: water (4:1:5), butanol water, ethyl acetate:water, isopropenol:water (1:4), acetic acid water (10:90, and plain water, etc., were employed.

This study and also observations of Chakraborty et al. indicated that the degree of separation of furocoumarins in commonly used solvent systems was slight. The use of single phase systems containing large amounts of water or the aqueous phase of two phase systems was found to be very useful in achieving good separation. Fowlks (112) has studied this problem very carefully particularly with a variety of furocoumarin compounds. His recommendation for achieving separation of these compounds and identification based on Rf values tend to favor 15% acetic as the most favorable solvent system. The fluorescence behavior of developed chromatograms by subjecting it to ammonia vapors or by spraying it with 2 N. NaOH followed by exposing to HCl vapors was also suggested to be studied for identification of compounds. Using a large size all glass tank (30 x 30 x 60 cm.) with a fitting glass lid and Whatman paper strips No. 2x22½, 3 mm. thick, a good separation of many furocoumarins was observed. Running the column at room temperature (21° - 25° C.) for approximately four hours till the solvent system reached exactly 25 cm. hieght, the following Rf values were found.

	Solvent system 15% acetic acid
Psoralen	0.61
5-Methoxypsoralen	0.55
8-Methoxypsoralen	0.57
5,8-Dimethoxypsoralen	0.60

Well defined round spots are obtained, there is no tailing. This solvent system was usually employed in this study for establishing the purity of compounds and other identification studies.

Biogenesis of furocoumarins.

The intriguing question of chemical and biochemical mechanisms by means of which plants synthesize these furocoumarins has been a subject of speculation. Just as a number of theories on the biosynthesis of flavones and anthocyanins have been proposed, an attempt has been made to bring together a biogenetic approach to this problem starting with apparently simple but most complicated process of photosynthesis. There are several possible synthetic routes, but a more probable from a less probably biosynthetic process has been postulated. As earlier stated, coumarins, furocoumarins, chromones and furochromones compounds occur very commonly together in nature (Umbelliferae, Rutaceae, Leguminosae, etc.). It is evident, therefore, that a close biogenetic relationship exists between these compounds, and it is axiomatic that metabolic processes of these plants are intimately bound up with the synthesis of these compounds through a common pathway of building up a basic structural unit. Geismann (1) has extensively reviewed the subject of biogenesis of flavonoid compounds represented by flavones, flavanones, chalcones, anthocyanins, etc., together with other compounds such as coumarins, furocoumarins, furochromones, lignanes, etc. In this review, he suggested that there exists a common

anatomical structural relationship between these different compounds. He postulated that the aromatic rings and aliphatic side chains of numerous groups of these naturally occurring substances are related. In broader terms he showed that all these compounds have $C_6 - C_3$ carbon skeleton which becomes a part of common synthetic pool of carbon compounds which are then utilized in the synthesis of complex constituents. (C_6 represents benzene nucleus, and C_3 moiety represents different oxidation levels of 3 carbon aliphatic side chain such as CHOH-CHOH-CHO, CHOH-CHOH-CHOH, etc.) This structural carbon skeletal relationship is briefly suggested as follows:

1. Flavonoid $C_6 - C_3 - C_6$
   a. flavonols
   b. flavones
   c. benzal coumaranones
   d. flavanols
   e. flavanones
   f. chalcones, dihydrochalcones
   g anthocyanidins
   h. catechins
   i. isoflavones

2. Coumarins $C_6 - C_3$

3. Lignanes $(C_6 - C_3)_2$

4. Furocoumarins $C_6 - C_3$ + furan ring

5. Furochromones $C_6 - C_3$ + chromone ring

In general the biogenesis of furocoumarins could be postulated as follows:

1. formation of hexose precursors of the carbocyclic C_6 fragment

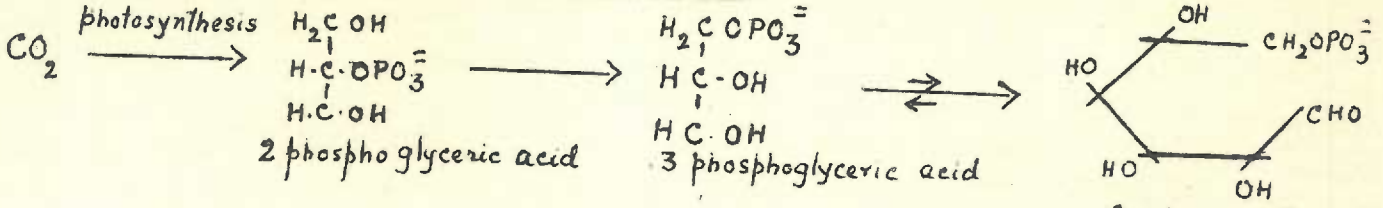
2. the derivation of the C_3 side chain and its attachment to C_6 fragment

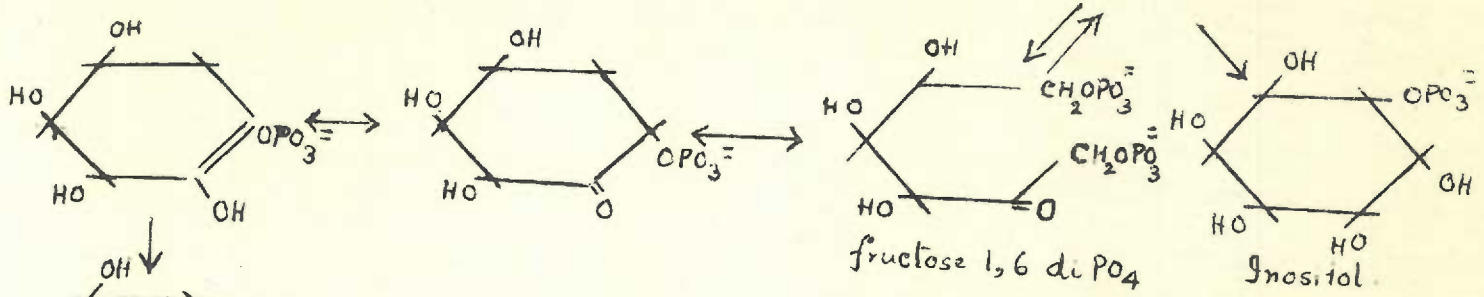
3. formation of furan ring from two carbon keto alcohol or from isopentenyl group

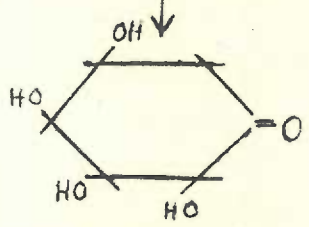
Compounds which may serve as immediate progenitors of six carbon moieties of C_6 - C_3 compounds are: first hexoses (Geissman, 1), second cyclitols (inositol and other related compounds) (1), third shikimic acid and quinnic acid (Davis, 90). Mechanisms by which plants accomplish the feat of synthesizing various benzenoid compounds from non-aromatic source materials are many; only Geissman's (1) hypothesis, together with recent work of Davis (90), Sprinson (91) and Sreenivasan et al. (92) is included in the flow sheet of biogenesis. The close relationship of glucose and fructose to trioses such as glyceraldehyde and dihydroxy acetone makes one to favor the hypothesis of direct relationship between 6 carbon sugar unit and C_6 unit (aromatic). Geissman proposes that hexose sugar could be immediate precursor for the origin of C_6 (benzenoid) fragment. Since there is wide spread occurrence of quinnic acid, shikimmic and cyclitols (inositols) in plants, it appears that the synthesis of aromatic nucli proceeds through these derivatives which are known to be formed from hexoses. The work of Davis, Sprinson and Sreenivasan adds additional support to this hypothesis, and it is now generally believed, and more or less accepted that shikimic acid is a precursor of aromatic ring phenylalanine, tyrosine, and tryptophane (Davis, 93). Based on Geissman, Davis,

Biogenesis of Furocoumarins (Scheme I)

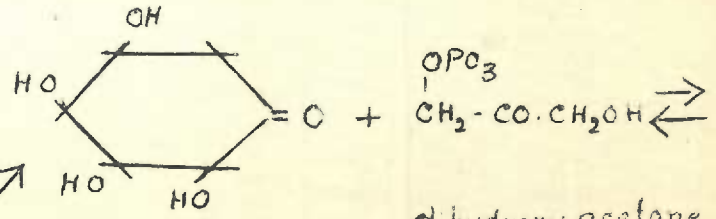
A Formation of Carbocyclic C_6 fragment (Geissman[1])







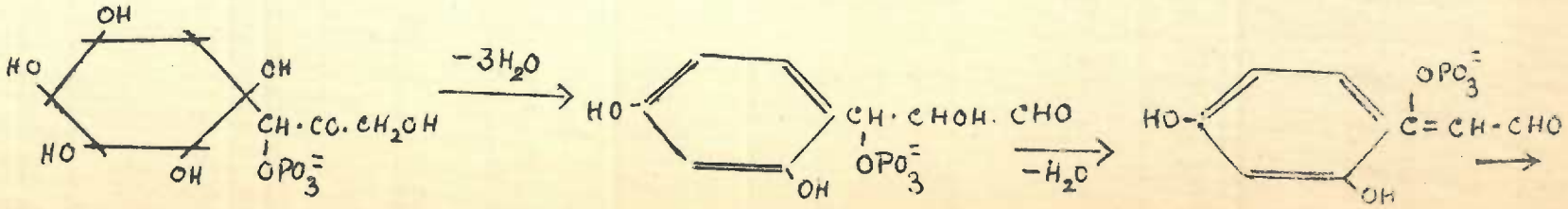
C_6 UNIT (hypothetical)



C_6 UNIT

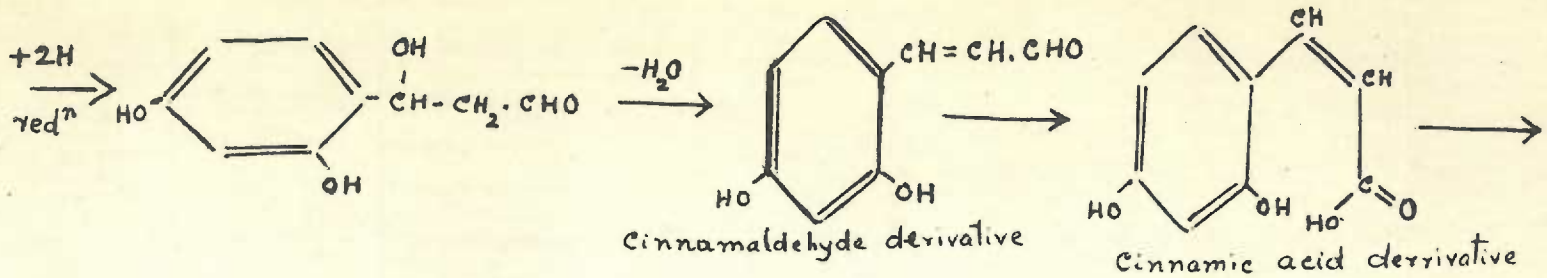
dihydroxy acetone (photosynthesis)

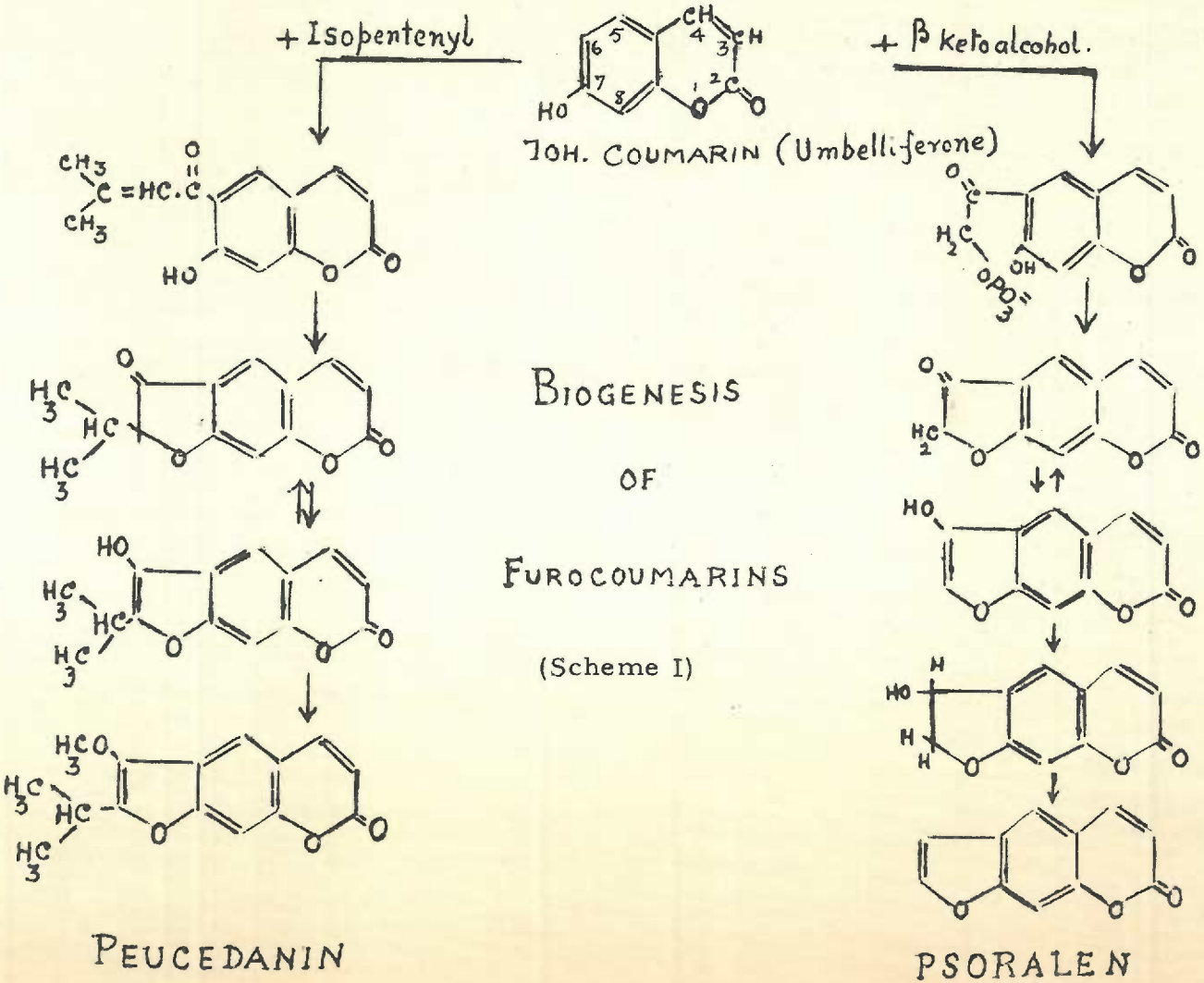
B Biogenesis of Coumarin (7OH, Umbelliferone)



(continued)

(B)





BIOGENESIS OF FUROCOUMARINS

(Scheme I)

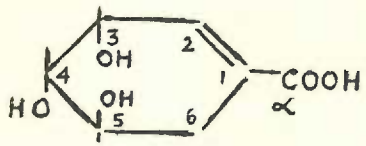
Enzymic Synthesis of Shikimic acid (C_6 Unit) (Sprinson & Sreenivasan) 91,92

Biogenesis of Furocoumarins (Scheme II)

(A)

(a) Fructose 6 PO_4 + Glyceraldehyde 3 PO_4 $\rightleftharpoons$ Erythrose 4 PO_4 + Pentose sugar

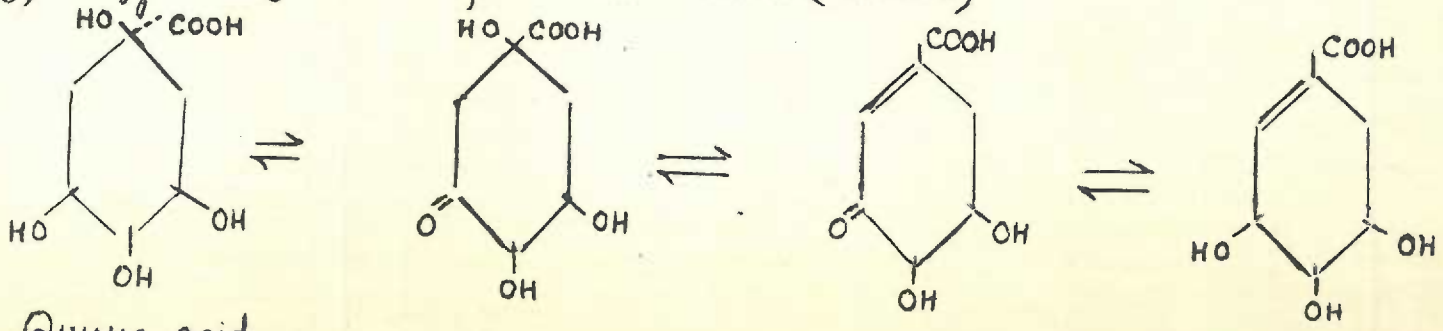
(b) Fructose 6 PO_4 + Erythrose 4 PO_4 $\rightleftharpoons$ Sedoheptulose PO_4 $\rightarrow$ Shikimic acid.



Shikimic acid

Carbons 1,2 & α are derived from 3 carbon intermediate of glycolysis, carbons 3,4,5,6 are derived from pentose - sedoheptulose pathway

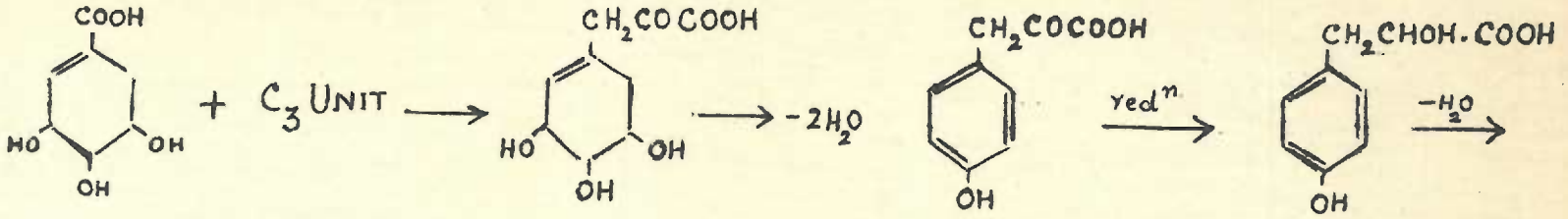
(B) Enzymic Synthesis of Shikimic acid. (Davis)[90]



Quinic acid

Shikimic acid.

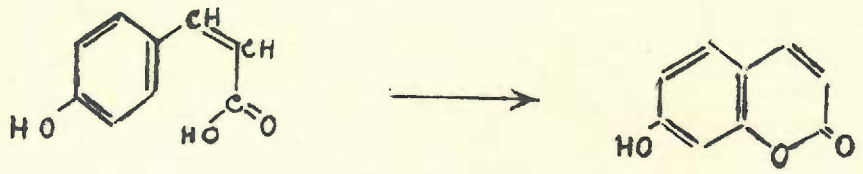
Biosynthesis of Umbelliferone (7hydroxy coumarin) (Scheme II, continued)



Shikimic acid

3,4,5 tri hydroxy phenyl pyruvate

4 hydroxy phenyl lactate



4 OH. Cinnamic acid.

7hydroxy Coumarin (Umbelliferone)

Rest of the steps leading to the synthesis furocoumarins would be the same as shown earlier on page A, B, C, D

Sprinson, and other published work, on the origin of benzenoid and other C_6 - C_3 structural configuration compounds from non-aromatic compounds, a postulated hypothesis on the biogenesis of furocoumarins in nature has been schematized.

The biogenesis of furocoumarins would be preceded by the formation of coumarins. Except for coumarin itself, most of the naturally occurring coumarins have a hydroxyl function in 7 position (para to C_3 side chain), therefore, the biogenesis of 7 hydroxycoumarin (Umbelliferone) has been described. In order to limit the space, furocoumarin biosynthesis has been postulated schematically and shown step wise in shceme I and II.

Biological function of furocoumarins.

It becomes pertinent to know the biological function of these substances in nature. They appear to have specific biochemical properties which may contribute to the survival of certain plant species. Specifically, these compounds belong to that group of substances which can inhibit certain plant growth without otherwise harming the plant. When one reads the observations of Crosby (23), Rodighiro (94), about the inhibitory effects of these compounds on germinating seeds, particularly when furocoumarin containing seeds and other normal seeds (like legume, tomatoes, etc.) are allowed to germinate together one observes that the furocoumarin containing seeds tend to inhibit the normal growth of the other seeds. Even when one sees the plants growing in the

vicinity of certain weeds containing furocoumarins, one can notice their inhibitory effect on normal plant growth. One naturally concludes that furocoumarins are plant growth inhibitors. However, the functional role of furocoumarins in the plant life remains to be answered. It appears that they are eventually regulating the ultimate plant growth. If one germinates the seeds containing furocoumarins like psoralen, xanthotoxin, bergapten, etc. (seeds of psoralea corylifolia, Ammi Majus, etc.), one notices that they do not germinate so rapidly as seeds of legume plants (beans, etc.). The obvious reason is the concentration of furocoumarins, which is high initially, to retard sprouting, but eventually gets low due to the diffusion process in the surrounding soil in presence of water. When this change of lowering concentration or alteration of furocoumarin due to sun light influence occurs, these seeds begin to germinate. It is known that furocoumarins of plant Ammi Majus, namely, 8-methoxypsoralen (xanthotoxin), 5-methoxypsoralen (bergapten) and 8-isoamylenoxypsoralen (imperatorin) are concentrated in the pericarp of the seeds. Likewise, the psoralen and angelicin of psoralea corylifolia are also found in the pericarp of the seeds. Thus the germ (embryo) of the seed is surrounded by a tissue containing a germinating inhibitor which could regulate the time when sprouting will occur, by the rate at which it diffuses into the surrounding soil. It appears that this growth inhibitory quality in fact, is a protective means deviced by nature.

The growing plant (like Ammi Majus) contains very little furocoumarins (personal observations in the course of investigations), only when the plant produces inflorescence, one observes increased synthesis of these compounds. When they reach the seed forming stage the concentration reaches its maximum. Likewise, Bose and Mookerjee (7) found no furocoumarins in unripe berries of luvunga scandems. In other words, they serve the function of inhibiting the further plant growth, their increased synthesis regulating the subsequent growth, and probably giving a signal for the plant to orient its metabolic and physiologic activities towards preserving its future progeny in the form of fertilized seeds. This growing seed which is still moist and which has not very thick cuticle, is subject to damaging effect of ultraviolet radiation. These substances which are usually present in pericarp can therefore act as a filter by absorbing these harmful radiation, and thus preventing their penetration towards embroynic plant present in the fertilized seed.

Studies carried out by Fowlks et al., (84) on photodynamic effect of furocoumarins on bacteria resulting in their inactivation and bactericidal effect, suggests another functional role of these substances in nature. Since bacteria and fungi can easily find a host site in various small plants in nature for their propogation, it appears that these plants containing furocoumarins devised a self defensive mechanism through the formation of these furocoumarins. These substances can bring about inactivation and death of bacteria by virtue of their photo-

dynamic properties. The presence of these compounds in moist seeds, and preponderance of sun rays (ultraviolet rays) can certainly favor the activation of these molecules, bringing about a photodynamic effect on invading organisms.

These compounds are reported to be fish poisons (Spaeth, 73). They seem to affect the respiratory gills of the fish. They are also known to have anthelmintic properties. It will be improper to comment without more supportive evidence to give credit to this insecticidal property, however, it will not be inappropriate to suggest that these substances might have this kind of a role to play in nature to a certain extent.

These compounds might have influenced human skin pigmentation. In today's world, we see different colored races. The tropical race is dark in color. Even among these tropical races some have very dark skin like Negroes of Africa, Ceylonese of Ceylon, and Indians of South India. Some people are more brown, others are simply tanned. Then there are people of Caucasian skin color. There are white skin people in temperate and cold climate countries of Europe, U.S.A. and other continents. They all have functional melanocytes producing melanin pigments, but still the skin color is different in different races. One of the primary causes for this difference in color is the effect of ultraviolet radiation which is well known to alter the skin pigmentation. The people of these different regions have been exposed to solar radiation. The relative amount and proportion of different wavelengths of solar

ultraviolet spectrum varies and depends on density of atmosphere, sea level and proximity to the equator. This could result in different degree of skin pigmentation. There is a possibility of another factor which could be postulated to be one of the causes to influence the relative variation of skin pigmentation. The eating habits of these people. In tropics particularly many people are vegetarian and eat fair amounts of green vegetables as one of their constituents of food. The green vegetables like carrots, celery, parsley, and many green legumes, are known to have various furocoumarin substances. In India, luvunga scandens containing 8-methoxypsoralen & isopimpinellin is used for chewing as a habit after every meal. Ficus fruits (common figs) are eaten in large number in tropical countries. Since no detailed study has been reported about the proportion of different furocoumarins present in these food materials, it is hard to draw valid hypothesis, but a preliminary survey of many of these edible foods which were screened for the presence of furocoumarin substances (personal observations) reveals that there are number of these compounds which are being consumed every day in our daily food. Imagine the role these substances might have played in altering the skin pigmentation. Constant intake of these furocoumarins and a continued exposure of skin to hot tropical sun rich in ultraviolet light (the two primary and most essential things for inducing pigmentation), bringing about deeply melanized skin. Melanin pigment is known to act as a filter for protecting the

skin against the damaging effects of ultraviolet radiation (sunburn particularly). It is likely, therefore, that these substances played a beneficial role in altering the skin pigmentation of these people. People in the desert areas, particularly in Arabia, Egypt, etc., where the sun is very hot, but where there is very little intake of green vegetables in daily food, are usually more tanned (not brown or black). It is likely that the relative absence of furocoumarins in their diet keeps their skin less melanized (light complexion).

Psoralens and suntanning.

Psoralen, 8-methoxypsoralen and other active furocoumarins have attracted the attention of dermatologists, biologists, and biochemists because of their several useful properties. Today, psoralens afford one of the only hopeful treatments for repigmentation of vitiligo (leucoderma) skin. In pigmented people (Asiatic, Indians, Negroes) vitiligo, a variety of leucoderma, which refers to an acquired idopathic or functional deficiency of normal melanin pigmentation, is a great social problem. Not only for cosmetic reasons it creates a great inferiority complex in individuals suffering from this disease, but also it brings a great tragedy in their life. They are hated by their own people, people avoid their company because of a fear that vitiligo might be contagious, they lose the opportunity for work, do not have the privileges to marry and lead a normal life. They are, in fact, at the mercy of the kindness of people. To these people, furocoumarin

compounds like 8-methoxypsoralen, psoralen have given a ray of hope in restoring their skin pigment. Biochemically speaking, vitiligo appears to be a disorder of melanogenesis characterized by depigmented areas of otherwise normal skin. There may be absolute functional loss in which histologically no Dopa (dihydroxyphenylalanine) positive melanocytes (converting Dopa to melanin) can be demonstrated, or there may be cells which are very slightly Dopa positive which have lost their function of melanin formation due to some inhibitory process (inhibitors like glutathione containing sulphydryl groups). The treatment with psoralens might produce repigmentation in vitiligenous areas in one or more of the following ways: 1) It might destroy the inhibitors and restore the tyrosinase activity. 2) It might induce the migration of melanocytes to the affected areas from the surrounding normal skin or hair bulbs. 3) It might increase the number of melanocytes which would subsequently induce pigmentation.

It is known that short wave and mid-wave ultraviolet radiation (2000-3200 A°) is not beneficial to human skin. It has the property to cause sunburn; it can cause carcinogenesis in exposed skin areas (95). The energy of long wave ultraviolet which is known to be least harmful, can be used as a beneficial tool to induce least harmful and more beneficial effects on human skin. Furocoumarins like psoralen, 8-methoxypsoralen have been found to be photodynamically activated in presence of long wave ultraviolet light (19, 80, 84). This activation

phenomenon (to be discussed later) of furocoumarins brings about biological changes of skin erythema and augmented pigmentation. It is this pigment inducing property which has opened a vast field for psoralen research. Not only it has revealed the properties of restoring the pigment in depigmented skin, but also it has shown augmented skin tanning responses which increases the solar tolerance in individuals susceptible to sunburn. To these people, psoralens have given hopes of going out in the sun for doing outdoor jobs (farmers and other outdoor working groups). They can now enjoy sunshine, share the pleasure of participating in sports activities during hot day time. It has opened a vast field to study the possibility of a protective effect it can augment by way of increased pigmentation and increased corneum thickening against ultraviolet induced skin cancer (studies of O'Neal and Griffin, 95).

It is well known that negro skin and brown skin of Asiatic people which have relative high melanin pigment, are well protected against sunburn. This decrease in sensitivity to ultraviolet radiation is believed to be due to increased melanin pigmentation and thickening of corneum. Psoralens increase melanization and also the thickness of corneum. Thus by increasing the melanin content in people easily susceptible to sunburn, psoralen have revealed a promising means to reduce the amount of harmful ultraviolet radiation reaching the malpigian layer. It has offered a new tool to study the photodynamic action which still

remains unsolved (Fowlks, 84, 99). The photodynamic property of these molecules can serve a useful means to inactivate bacteria and fungi in large scale industries like slaughter houses, canning factories and food processing houses.

Sunburn and psoralen action:

Psoralen action on the skin represents a photodynamic action brought about in presence of long wave ultraviolet light (19, 80, 84). The photodynamic action manifests itself in the biological changes of increased erythema response followed by brown or black skin pigmentation resulting from redistribution and increase of melanin pigment of the epidermis. The effects generally observed are probably consequences of cell damage initiated by photodynamic action. To understand these effects, the process of sunburn requires to be elicited briefly.

Sunburn: An exposure of human skin to solar radiation (one hour or so) is followed by reddening of the exposed area which is described as "erythema". This erythema of sunburn is the gross manifestation of dilatation of the minute vessels of dermis. The red color results from the increased amount of blood flow in these vessels. It is suggested that photochemical changes in the epidermis lead directly or indirectly to the elaboration of some mediating substance in the corneum or substances in the corneum and malpigian layer. There is a latent period for the appearance of erythema, which is presumably required in the

elaboration of these dilator substances and their penetration into the dermis. The biological changes induced by ultraviolet light are detectable in the dermis, but this is not the site of photochemical reaction, since only a very small fraction of the incident u. v. radiation, virtually restricted to longer wavelengths (>3400 A°),ever reach the most superficial vessels of the dermis. Yet the shorter wavelengths which are absorbed in the epidermis only, do elicit erythema. This therefore leads to the hypothesis of a mediating substance which is formed in the epidermis and which moves down to the dermis to cause dilation.

The erythema of sunburn fades almost imperceptably into suntan. This tanning represents the change in position and increase in quantity of melanin pigment in the epidermis. That this brown or black color is principally due to melanin granules has been shown by studies of the spectral distribution of the radiation reflected from the tanned and untanned skin. The ultraviolet radiation exhibits melani - zation in two ways. Wavelengths shorter than 3200 A° induce formation of new melanin granules. There also occurs darkening of preformed melanin which is brought about by 3200 to 4200 A° wavelength range. This darkening represents the oxidation of melanin granules., already present in the skin in the reduced leuco state (see Fig. 1).

Figure 1a. Showing absorption spectra of psoralen, 8-methoxypsoralen, 5-methoxypsoralen in relation to solar erythema and melanogenesis. The action spectrum range for solar erythema lies in the mid ultraviolet zone between 2900 A to 3200 A. Psoralens are photodynamically active in long wave ultraviolet range $>$ 3200 A.

Figure 1b. Absorption spectra of:

psoralen	absorption peaks	212, 245, 295 mu
5-methoxypsoralen	" "	221, 267, 313 mu
8-methoxypsoralen	" "	217, 248, 303 mu
5,8-dimethoxypsoralen	" "	223, 269, 316 mu

4×10^{-5} M solutions of each of the furocoumarins in 25% ethanol were used for recording absorption spectra.

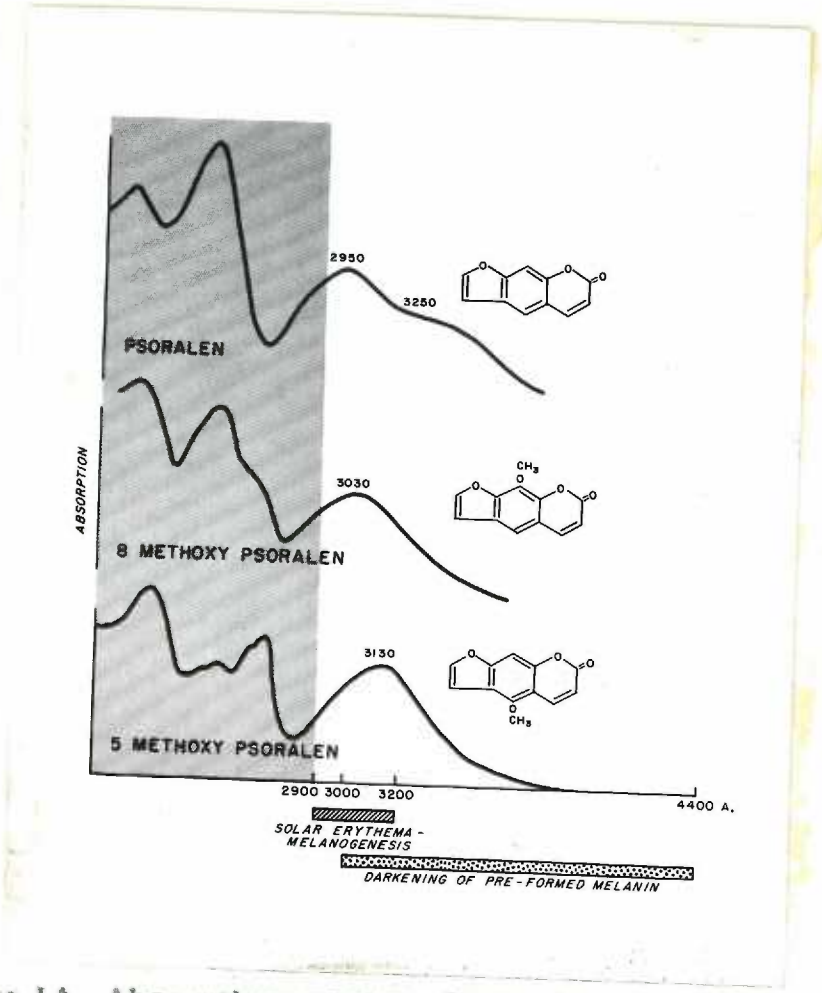
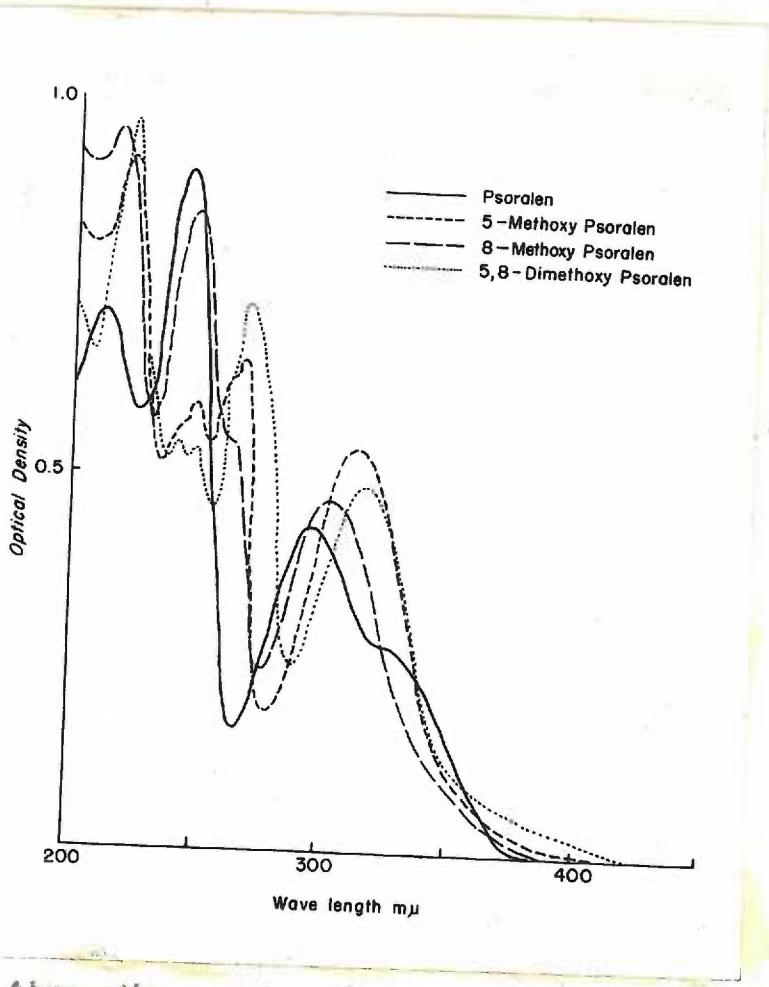


Figure 1A. Absorption spectra of psoralen, 8-methoxy-psoralen and 5-methoxypsoralen. Also the wave length range of solar erythema and melanogenesis



1B. Absorption spectra of :

a) psoralen.....absorption peaks 212, 245, 295 mu.
b) 5methoxy psoralen " 221, 267, 313 mu.
c) 8-methoxypsoralen " 217, 248, 303mu.
d) 5, 8-dimethoxypsoralen " 223, 269, 316 mu.

Wavelength requirements: The action spectrum for the production of erythema is well known. It shows a maximum at 2500 A°, a minimum at 2800 A° and very sharp maximum at 2970 A°. It falls sharply from 2970 A°, nearly reaching zero at 3200 A°. The strong absorption by the corneum at 2800 A° probably accounts for the minimum effectiveness of this wavelength in producing erythema.

Wavelengths longer than 3300 A° (u. v. 3300 - 4200 A°, visible and infrared) seem to have very little specific effect on the skin of normal individuals

Psoralen action shows some similarity to the process of sun burn. Initially there is a long latent period which intervenes between the exposure to sunlight or u. v. light and appearance of erythema. Within 8 to 12 hours one observes erythema similar to sunburn. This reddish color changes gradually to brown or tan color. The action spectrum, however, does not resemble the sunburn spectrum. Studies of Daniels, and Fitzpatrick (96) indicate that longwave ultraviolet light of greater than 3400 A° is more effective in causing the photodynamic action whereas the action spectrum of sunburn is limited to radiation shorter than 3200 A°. The absorption spectra of psoralen, 8-methoxypsoralen and bergapten, which are photodynamically active compounds, has been shown in Fig. 1. These compounds, though absorb strongly below 3000A, do show some absorption characteristic in the region of 3200 A°. It appears that

action spectra of active furocoumarins like psoralen, 8-methoxypsoralen and other derivatives which otherwise show more absorption in short and mid ultraviolet range gets altered in a biological system through variation in physical, chemical and biochemical conditions.

Theories of mechanism of photodynamic action.

Earlier it was believed that histamine or histamine like H substance was elaborated as a result of u.v action on living cells. It was thought that the molecule which initiates the response in a biological system was histidine and that the mechanism was simple photochemical production of histamine. However, it has been shown that histamine action does not resemble the erythema and pigmentation response of sun burn.

Mitchell (1938) (97) proposed that the light absorber in ultraviolet radiation is protein. From the published work it appears highly probable that proteins are oxidizable substrates in most of the photodynamic action and that tyrosine, tryptophane and histidine are amino acids affected within the protein molecule. Blum (98) presents evidence that the locus of action is nucleoprotein and the light absorber in erythema response is nucleic acid. Fowlks (99) has recently reviewed this whole literature of photodynamic action. From the studies of the effects of ultraviolet light on the living cell in presence of photodynamically active substances (reported by several investigators) he presented a clearer picture of this mechanism of photosensitized

biological responses to irradiation. In a system photosensitized by methylene blue he summarized the following biochemical changes in photodynamic action.

1. A formation of protein methylene blue complex which usually causes a shift in the absorption spectrum of the dye (about 20 mμ towards red).

2. Histidine is the most readily altered of all the amino acids (opening of imidazole ring).

3. Tryptophan is the most easily damaged amino acid.

4 Tyrosine is oxidized possibly to dopa (dihydroxyphenylalanine). Dopa is probably melanized.

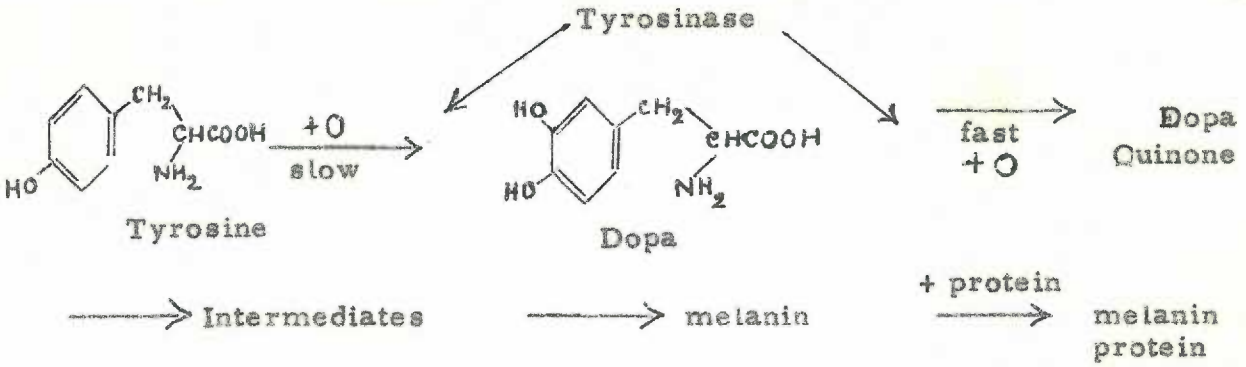
5. Cystine has the -ss- bond broken.

6. Methionine sulfoxide is formed from methionine.

7. Peptide bonds are not broken as a primary result of photodynamic action.

Biochemical aspect.

So far, theoretical, physical and physiological factors were briefly described in the process of erythema and pigmentation brought about by sunburn and photodynamic action. It is believed that melanin formation associated with ultraviolet induced suntan, or psoralen tan, follows the same chemical steps as *in vitro* reaction leading to the formation of melanin from tyrosine. This process of melanization, as proposed by Lerner and Fitzpatrick (100), can be briefly schematized:



The process of melanin pigment formation includes the oxidation of colorless amino acid tyrosine to 'dopa' (3, 4-dihydroxyphenylalanine) by the enzyme tyrosinase, a copper protein complex, eventually leading to the formation insoluble, brown black polymer melanin, through a series of intermediatary steps.

Fitzpatrick and Lerner (101) have proposed that ultraviolet radiation acts in vivo to bring about the oxidation of tyrosine to dopa by direct photochemical effect, in a manner parallel to the in vitro reactions shown by Arnow (102) (oxidation of tyrosine to dopa in presence of u. v.). Formation of new dopa brings about further acceleration of tyrosine to dopa reactinn.

Primary effect of photosensitized psoralen action is the injury to the cells of corneum and malpighian layer. Melanization in a common response of epidermis to this injury. Theoretically psoralens might produce pigmentation in one of the following ways.

1. Long wave ultraviolet induced photodynamic effect of psoralen may bring the oxidation of tyrosine to dopa, which would further accelerate the conversion of tyrosine to melanin.

2. It is known that tyrosine-tyrosinase reaction in mammalian tissue is regulated by many physical and chemical factors. One of the major factors regulating this mechanism are compounds containing SH groups such as glutathione, perhaps by binding the copper present in the enzyme complex tyrosinase. Physical agents like u. v., x-rays, radium, infrared, etc., can oxidize the inhibitory SH groups thereby releasing the normally inhibited enzyme. Psoralen action potentiates this release process and brings about activation of tyrosinase.

3. The melanocytes might migrate to the affected areas, from the surrounding skin and increase melanogenesis.

4. There can be increase in number of melanocytes due to this treatment which might induce more pigmentation.

5. It may induce the migration of pigment granules already formed. One may assume that the migration of pigment from the undamaged basal cells into the injured cells of epidermis is due some kind of "tropic" action which is augmented by psoralens.

6. Increased erythema caused by psoralen action causes more dilatation of capillaries which results in increased blood flow. This increased blood flow may remove the tyrosinase inhibitory factors (SH group compounds) and induce formation of new melanin. Increased temperature of skin during this process could accelerate melanogenesis.

Furocoumarin studies (abstract)

The studies reported here were carried out from several aspects. These compounds are known to be photosensitizers (Fitzpatrick 80; Musajo, 19; Fowlks, 84), and their action has been referred to as "photodynamic action." Blum (103) has defined photodynamic action as photochemical reaction in a biological system brought about by a chemical agent which serves as a light absorber. The main features of photodynamic action are: 1) that it requires light of wavelength longer than 3200 A°, which has no erythema or sunburn producing action, 2) it is an oxygen obligate photosentization of living systems in other words, photodynamic action requires the presence of molecular oxygen. 3) it requires a long latent period for producing biological effects after exposure to ultraviolet radiation. The mechanism of action of furocoumarin compounds like psoralen and 8-methoxypsoralen was investigated with a view to know whether they exhibit all these above mentioned features characteristic of photodynamic action. Using red blood cells and albino rabbit ears, in vitro and in vivo photodynamic action was studied.

More recently great interest has been aroused on the possible pigment stimulating properties of some of these compounds like 8-methoxypsoralen, imperatorin, psoralen, etc., particularly in repigmentating the vitiligenous skin and increasing the skin tolerance to solar radiation.

Presently very few compounds are commercially available, but in the future, many new products are destined to come up for therapeutic trials. There are many known furocoumarin compounds (listed in Table I). Not all of these compounds exhibit photodynamic action. With the increased availability of synthetically prepared derivatives, a need for bioassay method for screening active and inactive compounds becomes apparent. So far only Musajo (19) has reported relative activity of coumarins and furocoumarins by applying various substances topically on human skin. Since all these compounds do not exhibit the same activity topically as well as orally (some of them are inactive when given orally), the investigations reported here were designed with the following purposes: 1) To set up a screening method for determining the active and inactive compounds when given orally to guinea pigs or applied topically on guinea pig and human skin. 2) To study the rate of excretion of some of the active compounds with a view to standardize bioassay methods for determining the latent period and total time of ultraviolet radiation essential for optimum response. 3) To determine their minimum effective concentration for erythema response, on guinea pig skin and correlate this data with the results obtained with human skin. 4) To compare their relative activity in terms of structural configuration, so that a relationship between molecular configuration and the photodynamic activity of the compound could be ascertained.

Toxicity of these compounds has not been investigated in detail. Hakim (28) had carried out in vitro effects of 8-methoxypsoralen and other compounds on isolated mammalian organs. Fitzpatrick et al (104) earlier had reported preliminary observations on acute toxicity studies with 8-methoxypsoralen. Recently Fitzpatrick et al. (105) carried out liver function test studies after oral feeding of 8-methoxypsoralen to a group of medical students for a period of 3 months. Acute toxicity study was undertaken with a view to know M. L. D. and LD50 for psoralen and 8-methoxypsoralen. Since these compounds are being administered for along period, it was essential to study the chronic toxicity of 8-methoxypsoralen after ingesting it daily for a length of time in varying doses to mice. Its effect on intraoccular pigmentary changes in presence of long wave ultraviolet light in the course of prolonged oral feeding was also studied. Finally, a preliminary psoralen action hypothesis in terms of pigmentation response has been also investigated.

The present studies with 8-methoxypsoralen and psoralen reveal all the features characteristic of photodynamic action. In vitro studies of photodynamic action in terms of red cell hemolysis in presence of 8-methoxypsoralen and psoralen, and in vivo studies on erythema production in albino rabbits in presence of 8-methoxypsoralen show that the biological action is oxygen dependent and is fecilited in presence of oxygen. It requires a latent period and is brought about by wavelength in the region of 3600 A°.

Using albino guinea pigs, activity of different furocoumarins, coumarins and related compounds can be assayed conveniently. Both topically and orally, the relative activity of different compounds was determined and compared against the responses observed on human skin. The lag period (latent period) essential for observing for maximum response, and the total ultraviolet radiation time required for reproducable response has been determined. In topical and oral studies 45 minutes u. v. radiation produces optimum response. A lag period of 30 to 45 minutes in topical studies and 1 1/2 hours in oral studies if observed before radiation, will elicit the desired response for evaluation. Orally fed compounds in one dose reach their optimum concentration in blood at the end of 1 1/2 hours and are excreted by the end of 6 hours almost completely. No response can be observed at the end of 8 hours.

The parent compound furocoumarin psoralen exhibits maximum activity The order of activity of other active compounds apppars to be(1) psoralen,(2) 4, 5', 8-trimethyl psoralen, (3) 5'-methyl psoralen, (4) 5'8-dimethyl psoralen, (5) 8-methoxypsoralen, (6) 5-methoxypsoralen, (7) 4'5'-dihydro xanthotoxin (topically only), (8) psoralen glucoside, (9) 8-isoamyloxypsoralen. Compounds like isobergapten and 4,5'-dimethyl isopsoralen showed weak activity. The furan ring and coumarin ring configuration compounds do not show any photoactivation. The compound to be active photodynamically in inducing erythema and

pigmentation should have a linear angular fused ring structure of furan and coumarin ring as present in psoralen molecule. Non-linear angular structure like isopsoralen molecules does not exhibit any attivity. Valence bonds between carbon 3-4 and 4'5' and attachment of furan ring at carbon 6 and 7 are the active centers in the molecule. Intact lactone ring is essential for the compound to be active. Methyl substitutions at carbon atoms 4, 5', 8 inpsoralen molecule do not increase the relative activity of the basic molecule but retain its photodynamic activity. Methoxy substitution in 8 or 5 position, however, decreases the activity. Hydroxy, nitro, amino group substitution render the compound inactive. It appears that substituting radicals which increase the electron density of reactive centers retain the activity of the molecule, whereas radicals which decrease the electron density lower the photodynamic activity.

Acute toxicity and chronic toxicity studies with mice reveal that psoralen and 8-methoxypsoralen, the two most important compounds, are least toxic. LD50 range for both of these compounds is very high (psoralen 1000 mg/kg, 8-methoxypsoralen 600-700 mg/kg); they do not disturb the normal rate of growth and can be tolerated even in high concentration. Histologic studies also show no pathological changes. There is no discernable intraoccular damage in guinea pig eyes even after ingesting high doses of 8-methoxypsoralen and subjecting the animal daily for 15 minutes to long wave ultraviolet radiation for a period of 7 months.

In vitro and in vivo psoralen action shows no conclusive observation as yet to suggest the action underlying this process. It seems, however clear that psoralen activates the melanocytes Histologically they are more dopa (dihydroxyphenylalanine) positive melanocytes, suggesting that tyrosinase is activated in the system.

MATERIALS AND METHODS

Studies on photodynamic action of psoralen and 8-methoxypsoralen.

In vitro studies: (Red cell hemolysis) Human erythrocytes were used throughout this study. It is known that photodynamically active compounds induce red cell hemolysis in presence of oxygen and light at wavelength greater than 3200 A° (Blum, 103). The photodynamic effect of furocoumarins was studied by the method proposed by Blum and his coworkers (106). Blood specimens were obtained from healthy persons and defibrinated by slow swirling motion in presence of glass beads. Red cells were separated by centrifuging at 2000 r.p.m. for 15 minutes and were washed 3 times with buffered normal saline solution pH 7.0 (0.85% saline buffered with sodium phosphate salts at pH 7.0, 0.001 M). Care was taken to avoid any trace of hemolysis as a result of washing cells, or prolonged centrifugation. A 50% cell suspension (v/v) was prepared in buffered saline, and 0.5 ml. of this suspension added to 4.5 ml. of buffered saline solution, with or without furocoumarin compounds (mainly psoralen or 8-methoxypsoralen). Pyrex petri dishes with covers, which transmit light of wavelength 3200 A° and above, were used for irradiation of red cell suspension. Uniformly surface leveled petri dishes were selected,

otherwise erroneous results of hemolysis could be observed because of surface drying of cell suspension due to uneven fluid level. Necessary controls were kept simultaneously in the dark without radiation. Several such determinations were carried out. Initially, irradiation was carried out for one hour at room temperature under Wood's ultraviolet light (to be described in the following pages). After centrifuging the specimens at the end of radiation, the degree of hemolysis was determined immediately by estimating the hemoglobin present in the supernatent fluid. Hemoglobin was determined by the micro method (Bing and Baker, 107), (as modified by Crosby and Furth[113]), which is highly quantitative and reproducible. Duplicate hemoglobin determinations were carried out for each specimen. The degree of hemolysis shown in various tables represents the difference between the cell free hemoglobin, estimated in the supernatent in the irradiated and non-irradiated systems. 0.5 Ml to 1 ml. of the supernatent fluid (obtained after centrifugation) was used to determine the hemoglobin content. The purple color obtained in presence of benzidine hydrochloride and other reagents, was suitably diluted to 25 ml. or 50 ml. with 10% acetic acid solution (depending upon the degree of hemolysis) and the intensity measured in Bausch and Lomb Model No. Spectronic 20 at 515 mu.

Temperature effects due to radiation heat were minimized by slow blast of air; there was practically no change in total volume, still at the end of radiation, each specimen was finally diluted to 5 ml. in a graduated centrifuge tube with buffered saline.

The insolubility of psoralen and 8-methoxypsoralen in water or physiological saline presents a problem in obtaining higher concentration of these compounds in solution. Boiling the saline solution with a known quantity of psoralen or 8-methoxypsoralen increased their solubility (5 mg/100 ml.). However, 7% ethanolic solution in buffered saline was found to retain approximately 10 mg/100 ml. of these compounds. In earlier experiments, ethanolic buffered saline solution was used. This low concentration of ethanol did not induce any hemolysis by itself even after keeping the cells in contact for 24 hours (108). In the later experiments, solutions of psoralen or 8-methoxypsoralen dissolved in normal saline were only used, particularly when the role of oxygen in photodynamic hemolysis was being investigated.

Oxygen free systems: Experiments were designed in the following two ways: (1) to test the hypothesis that photodynamic action is oxygen dependent and (2) that no photosensitized hemolysis is observed if oxygen is removed from the system.

I. Conical flasks (100 ml.) fitted with surface aerating glass tubes were designed with fitting rubber stoppers and connected in series by rubber tubings. Hydrogen was used to replace the oxygen of the system and was bubbled on the surface of red cell suspension for at least 45 minutes before irradiation. Control flasks receiving no ultraviolet light were placed in the above series with thick black paper, uniformally covering the flasks. Hydrogen was passed in

continuous flow through this system consisting of 5 flasks in the following order: one flask covered with black paper (control dark) without any psoralen or 8-MOP, 2 flasks without any psoralen or 8-MOP, 2 flasks with either psoralen or 8-MOP. The flasks were radiated for one hour at 8 inches distance with long wave ultraviolet light (see next page). The degree of hemolysis was determined as stated above and compared against the degree of hemolysis observed when the similar run was repeated with the same stock cell suspension except that no hydrogen was bubbled, but only the oxygen of the air served as oxygen dependent test system.

In the second type of experimental design, small pyrex petri dishes with fitting lids were used. They had a side arm through which any gas could be made to flow over the surface of cell suspension. A stock 5% suspension of red cells in normal saline, with and without psoralen was prepared (psoralen 25 µg/ml.). In this system nitrogen gas was used instead of hydrogen gas. The run was as follows:

1. 5 ml. red cell suspension without psoralen was radiated with the same long wave ultraviolet light source in different petri dishes, for 1 minute, 2 minutes, 4 minutes and ten minutes periods. The gas phase was ordinary air.

2. Five ml. red cell suspension with psoralen (25 µg. ml.) was radiated similarly in 4 different petri dishes.

3. Five ml. red cell suspension with psoralen (25 µg/ml.) was radiated similarly as in 1 and 2, but this time air was replaced by pure oxygen. Oxygen was bubbled for at least 10 minutes before the petri dishes were radiated. It was kept bubbling during the interval when cell suspension was subjected to 1 minute 2 minutes, 4 minutes and 10 minutes radiation.

4. Five ml. red cell suspension with 25 µg/ml. psoralen radiated similarly as in 1, 2, and 3, but this time oxygen was completely displaced from the system by bubbling pure nitrogen. N_2 bubbling was carried out similar to O_2 bubbling. Radiation was carried out under identical conditions.

5. Suitable control samples for each run without radiation were pipetted out before the radiation run was carried out. Effect of just bubbling oxygen and nitrogen for period of 10 minutes without any ultraviolet radiation was also investigated in cell suspension in presence or absence of psoralen. Degree of hemolysis was determined as stated earlier.

Ultraviolet light source.

All investigations reported in the present study were carried out with ultraviolet lamp emitting long wave u. v. radiation. A Glo Craft black light unit Model A45 G. E. company (Switzer Brothers, Inc., Ohio) was used. This 250 watt mercury lamp operated from 110 volts line current emits major portion of its light in the region of 3650 A°.

It has a "Roundel" pressed glass filter No. 41, which cuts off all the wavelength below 3200 A° and above 4000 A°. With this filter there is 52% light transmission at 3650 A°, 25% transmission at 3400 A° and 35% transmission at 3800 A°. There is less than 1% transmission at 3200 A° and 4000 A°. Thus, the photodynamic action studies have been carried out with long wave ultraviolet light only. The distance of irradiation was kept at 12 cm. unless stated otherwise.

In vivo studies.

Albino rabbit ears were used to study the in vivo characteristics for photodynamic action. The terminal half portion of the rabbit ear can be easily depleted of oxygen supply by means of specially devised clamping unit. The ears were gently shaved of hair. One ml. of 1% ethanolic solution of 8-methoxypsoralen was applied uniformly to each ear. After a lag period of 30 minutes, the terminal half portions of both the ears were depleted of oxygen supply with the aid of screw clamps. The clamping device was as follows: Each ear was placed in between the two square bakelite plates (2 square inch area). The top bakelite plate had a 2.5 cm. diameter circular hole in the center, so that a circular 2.5 cm. diameter skin area when depleted of oxygen could be easily radiated at a fixed angle. Six small size clamps were placed at different angles and screwed gradually and firmly. Within 15 minutes, the terminal portions of clamped ears showed change of color (pink to

purple, an index of oxygen depletion). At the end of 20 minutes, the ears were subjected to ultraviolet radiation in a horizontal position for one hour at a distance of 12 cm. Throughout this period the terminal portion of ears remained in oxygen depleted cyanosed condition. The lower half portion of each ear which was not depleted of oxygen supply, served as a control area for each ear. At the end of radiation, the clamps were removed and animals kept in light protected cages. Observations on photodynamically induced biological change of erythema were recorded at the end of 24, 48 and 72 hours. Five rabbits were used; three of them gave satisfactory results.

Bioassay method for determining relative activity of photodynamically active compounds:

Albino guinea pigs weighing 400-600 grams were used for assaying compounds. By topical application and oral feeding of various compounds relative activity was evaluated in terms of cutaneous responses of erythema. Photodynamically active compounds are known to induce erythema and residual pigmentation of skin following its exposure to solar light or ultraviolet light. Pigment response cannot be ascertained in an albino guinea pig skin, but if the concentration of the active compound is in the range which does not induce cell damage in the form of functional loss ordeath due to blistering, edema, etc., the degree of erythema produced in presence of a known amount of a photodynamically active substance can reflect the relative activity

of the compound. The minimum effective concentration for inducing erythema and the degree of erythema produced at this concentration will indicate the photodynamic potency of the compound.

The hair on the back of guinea pig was clipped, the whole back skin area was made smooth and free from projecting hair by applying a mild depilatory. An adhesive tape 6" long and 3" wide having 1" x 1" square windows was prepared each time for assaying different compounds and fixed on the back of guinea pig. The animal was kept in a flat position on a wooden board, its back facing the ultraviolet light (see figure IIA). Movement of the animal during radiation was completely checked by keeping it immobile through fastening strings fixed with thumb tacks. Varying concentration of compound dissolved in 95% ethanol was applied topically in each square inch window area. In each square area, 0.1 to 0.2 ml. solution was applied slowly and uniformly with the aid of small graduated micro pipette. Not less than 3 different concentrations of each compound were applied initially to ascertain the activity of the compound. Following this, serially diluted solutions were prepared for active compounds (concentration ranging from 5 µg. to 400 µg./0.2 ml.) and minimum effective concentration for inducing erythema was determined. In control area 0.2 ml. of 95% ethanol was applied. In topical studies a lag period (the interval after applying the compound and before radiating the

Figure 2a. Bioassay of furocoumarins and other photodynamically active compounds.

After smooth shaving, the albino guinea pig is laid flat on a wooden board and made immobile as shown in figure 2a. An adhesive tape with eight 1" x 1" square windows is fixed on the back. Topically different compounds in varying concentrations can be applied quantitatively by micropipette, and after a lag period of 30 to 45 minutes, the animal can be radiated under ultraviolet light. Each compound can be administered orally, and at the end of 1 1/2, 2 and 2 1/2 hours interval, photodynamic response can be similarly tested.

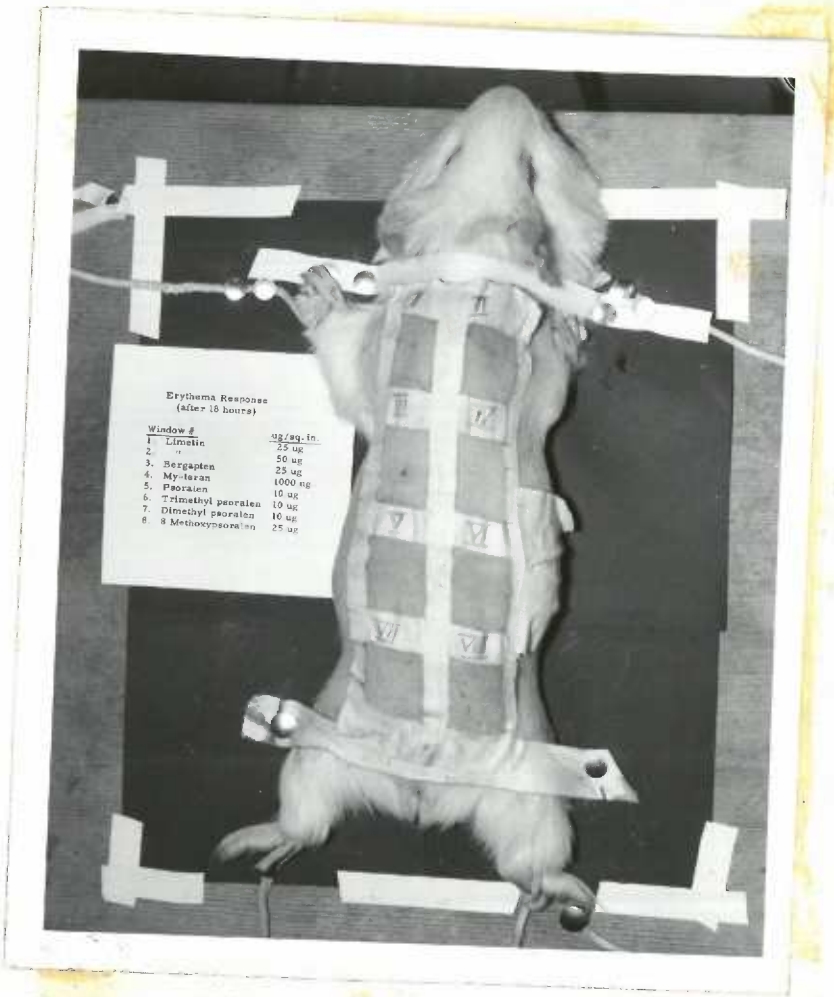


Figure 2a. Bioassay of furocoumarins -- to test photodynamic activity of various compounds, the experimental animal (guinea pig) is laid flat after applying various compounds topically or feeding orally. Each square inch window area can be radiated under ultraviolet light either simultaneously or separately at different time intervals. The eyes of the animal are covered with adhesive tape while being radiated.

animal) of 30 minutes, lasting up to 45 minutes (when series of compounds were applied topically) has been kept constant.

The guinea pig was radiated for 45 minutes under ultraviolet light at a distance of 12 cms.

In topical as well as oral studies, the total time of ultraviolet radiation and the lag period essential to be observed for eliciting maximum response was initially determined. These observations have been presented separately. For subsequent assay purposes, 45 minutes total radiation time has been observed in topical as well as oral studies. In oral studies, after feeding the compound to guinea pig, a minimum of 1 1/2 hours lag period has been observed, since this is the interval essentially required for the compound to reach its peak concentration in animal blood (see the studies on the rate of excretion of furocoumarins).

Temperature effects were minimized beneath the lamp area as stated earlier.

In oral studies, the compound was weighed in small capsules and administered orally on weight basis (mg/kg) taking care to feed the capsule quantitatively. After a lapse of 45 minutes, about 2 ml. water was given slowly through a dropper to the animal to allow the compound under test to be drained quantitatively into the stomach.

The degree of erythema developed at the end of 18 and 36 hours after radiation was employed as a measure of relative activity of the

compound. The erythema was read on photoelectric reflection meter (Photovolt Model 610 T, Photovolt Corporation, New York) with standard red and green filters after adjusting 100% reflection on a standard white enamelled disc with red filter. Reflection readings with standard green filter were evaluated as % difference in transmission between control area and area under test in presence of compound. Visible erythema has been also recorded in terms of conventional + or - signs as follows: - sign represents no erythema, ± just barely visible erythema, + clearly visible erythema of low intensity, ++ and +++ are relative stages of increasing intensity of erythema usually interpretably by little experience, ++++refers to maximum erythema at the end of 18, 36 and 72 hours after radiation.

Relative activity of furocoumarins on human skin.

The relative activity of different compounds as observed on guinea pig skin was compared on human skin also. Various compounds were applied topically on innerside of forearms of medical students. Twelve medical students (Caucasian) were used as subjects. The inside forearms of these students were shaved, taped with adhesive tape windows as described in guinea pig bioassay procedure. Initially at least three different concentrations of each compound under assay were applied quantitatively on each subject. Finally, the minimum effective concentration was determined by serially applying graded concentration of

active compound. A lag period of 30 to 45 minutes was used for each compound. Each arm was radiated in a flat position. Radiation time, however, was kept to 15 minutes only, since longer period exhibited undesirable effects. Distance of radiation was 12 cm. in all these cases. Observations of erythema and pigmentation response were recorded at the end of 18, 36, 72 and 96 hours. All these subjects were again observed for pigmentation response at the end of 15 to 20 days after radiation. Erythema and pigmentation response was evaluated on reflection meter readings (using red and green filters) and visual gradings as described earlier.

In topical and oral studies care was taken to avoid exposure of the skin directly to fluorescent light or sunlight, before and after radiation.

Rate of excretion of furocoumarins.

Using albino guinea pigs, the rate of excretion of psoralen, 8-methoxypsoralen, 5-methoxypsoralen and trimethylpsoralen was investigated with a view to know the time when maximum effect in terms of erythema response is observed, and to know their rate of excretion, when one single heavy dose of the compound has been given orally. These compounds were administered orally to albino guinea pigs (25 mg/kg. approximately). Guinea pigs weighing approximately 500 grams were fed these compounds separately in the form of a capsule as described earlier. At the end of 1/2, 1, 1 1/2, 2, 2 1/2, 3, 4, 5, 6, 7, 8 and 9 hours period after ingestion of the compound, respective

windows were exposed to ultraviolet radiation for 45 minutes. After radiation, black paper was fixed with adhesive tape on the exposed window so as to avoid its further exposure to ultraviolet in the process when remaining windows were being radiated at different time intervals. Appearance of erythema served as an index for determining the optimum lag period and the rate of excretion. During this interval, little water and some cabbage was given to the animal to withstand the 9 to 10 hours experimental stress. Degree of erythema was read on reflection meter. The results for each compound have been separately presented in a graphic way.

Toxicity studies.

Acute toxicity of 8-methoxypsoralen and psoralen was tested on healthy 8 week old albino Swiss mice. All these mice were grouped on their weight basis.

There were 5 mice in each group and each mouse of the group had nearly the same weight (±- 0.5 to 1 gram). 8-Methoxypsoralen toxicity study included 11 groups as follows: Group I - control group, receiving no 8-MOP. Group 2 to 11 received 100, 200, 300, 400, 500, 600, 700, 800, 900, and 1000 mg/kg body weight of 8-methoxypsoralen respectively. Psoralen toxicity study included 14 groups as follows: Group I - control group without psoralen. Group 2 to 14 received 100, 200, 300, 400, 500, 500, 600, 600, 700, 800, 900, 1000, 1200 mg/kg. body weight of psoralen respectively. Varying concentrations

of 8-MOP or psoralen (calculated in terms of body weight and shown in the respective tables) were very finely suspended in 0.5% sterlized gum acacia solution and administered intraperitoneally. The volume of injected fluid in each mouse was 0.5 ml. The control group received plain gum acacia solution. The number of deaths in each group were recorded at the end of 24 and 48 hours. The minimum lethal dose (MLD) and LD50 was determined by the method described in the book of biological standardization by Burn (115).

Various organs of these mice which had died in 48 hours period were examined microscopically for pathological changes.

Chronic toxicity of 8-methoxypsoralen.

Chronic toxicity effect of 8-methoxypsoralen administered intraperitoneally every day to albino Swiss mice for period of 8 weeks was investigated. Young 7 to 8 weeks old mice (Jackson Memorial Laboratory, Bar Harbor, Maine) were divided in 6 groups. Each group consisted of 10 mice, which weighed approximately equally ($\pm$ 1 to 1.5 grams).

Group I was a control group, which received 0.2 ml. of 0.5% sterile gum acacia solution intraperitoneally. Groups II, III, IV, V and VI received respectively 2, 4, 6, 8 and 10 mg. per kg. body weight of 8-methoxypsoralen intraperitoneally suspended in 0.2 ml. sterilized gum acacia solution (8-MOP was given on average weight basis to each group). On every alternate day they were weighed, and

mean gain in weight for each group for every week was recorded. Accordingly, the concentration of 8-MOP present in 0.2 ml. of injected solution was increased due to the gain in weight by the group. During this 8 week period, all the mice were fed laboratory Rockland mouse pellets and water ad libitum. The effect of 8-MOP in terms of growth (gain in weight) has been analyzed statistically. At the end of 2 months period, all the mice were sacrificed and different organs (liver, spleen, kidney, etc.) examined microscopically for pathological changes.

Intraoccular effects with psoralen.

Since these compounds are photodynamically active, and people move out in the sun after their oral intake, the question arises whether these substances will bring harmful effects to the eyes (in the form of pigmentary disturbances resulting in rise of intraoccular pressure which would harm the normal function of the eye). A study was undertaken to feed 8-methoxypsoralen orally to guinea pigs (albino as well as colored) for a period of 7 months and its effects investigated after radiating the animal eyes daily to long wave ultraviolet light. Four guinea pigs (1 albino and 3 colored) were selected and examined initially for normal anatomy of eye. One colored guinea pig served as a control animal receiving no 8-methoxypsoralen. Three other guinea pigs (1 albino and 2 colored) received 8-methoxypsoralen daily for a period of 7 months as follows:

1. 1st 15 days -- 0.71 mg/kg = 50 mg/70 kg
2. up to 2 months - 2.15 mg/kg = 150 mg/body weight/70 kg.
3. from 3 to 7 months -- 4.4 mg/kg = 308 mg/70 kg.

After feeding the 8-MOP suspension, guinea pig eyes were radiated under long wave ultraviolet lamp (Wood's light) for 15 minutes daily (6 days a week). One eye of each animal served as its own control. It was covered with thick adhesive tape every day during the course of radiation.

Dr. John Harris, Associate Professor of Ophthamology, University of Oregon Medical School, very kindly consented to examine these animals for pathological changes in the eye.

Also another investigation was carried out to see whether heavy oral doses of 8-methoxypsoralen result in the diffusion of this compound into the aqueous fluid of the eye. Four animals were fed 10 mg. capsules of 8-methoxypsoralen (equivalent to approximately 20 mg/kg or equivalent to about 1.4 grams /70 kg. body weight). At the end of 2 hours when 8-MOP concentration is known to reach its peak value in the blood, aqueous fluid from both the eyes of each animal was withdrawn and examined for 8-methoxypsoralen fluorescence under ultraviolet light. Each of these specimens was spotted on chromatography paper and examined for the presence of 8-MOP or 8-MOP protein complex, if any, after running the spotted paper in 15% acetic acid solvent system.

Mechanism of psoralen action.

It is known that energy in the form of radiation is absorbed by a photodynamically active substance, which is thereby transformed to an excited or activated state. This activated molecule can transfer its energy of activation to another molecule by collision. The secondarily activated molecule is capable of combining with a molecule of oxygen resulting in the oxidation products. Similarly, whether activated psoralen molecule can, photodynamically, oxidize tyrosine to Dopa (dihydroxyphenylalanine) was investigated. A stock solution of tyrosine (50 mg/100 ml.) was prepared after boiling in distilled water. Two other stock solutions were prepared as follows:

Solution B -- 50/mg. tyrosine and 5 mg. psoralen /100 ml.

Solution C -- 50/mg. tyrosine and 5 mg. 8-methoxypsoralen/100 ml.

Five ml. of each of the solutions A, B and C were pipetted out in pyrex glass petri dishes (at least 3 dishes for each solution for each run). They were radiated under long wave ultraviolet light (as described earlier) for 1 hour, with and without glass covers. Another set of these three solutions in 10 petri dishes were radiated for 1 hour without glass covers under another ultraviolet lamp which emitted mostly short wave and mid-ultraviolet light. The distance of radiation was 50 cm. Since these dishes were without any cover, evaporation loses could not be avoided. All solutions were finally quantitatively diluted back to 5 ml. volume with distilled water. Similarly u. v. irradiation for 2 hours was

carried out under identical conditions, using both kinds of long wave and short wave ultraviolet radiations.

Evidence of Dopa formation was ascertained by chromatographic separation under isobutanol-acetic acid-water (4:1:5) solvent system and also under butanol-methyl ethyl ketone-water-diethyl amine solvent system. Aliquots (50, 100 and 250 ul) of each radiated specimen were spotted along with standard Dopa and tyrosine solution. Chromatograms were developed with 0.25% ninhydrin solution in butanol. Dopa formation in each of the radiated solutions was ascertained by estimating Dopa colorimetrically by the method proposed by Arnow (102).

In vivo action of psoralen.

A C57 black mice strain, obtained from Jackson Memorial Laboratory, Bar Harbor, Maine, was used. About 1" long area at the base portion of the mice tail (near rectum) was gently shaved. Four groups of mice were treated in the following manner:

Group A (control A) consisted of 3 mice which received neither ultraviolet nor any 8-methoxypsoralen. (Non-radiated control)

Group B (control B) -- 4 mice which received long wave ultraviolet radiation for 30 minutes without any 8-MOP (Radiated control).

Group C (topical 8-MOP) -- 4 mice, 0.2 ml. of ethanolic 8-MOP solution (500 ug) was applied topically on the upper portion of the tail and radiated under Wood's light at the end of 30 minutes, for a period of 30 minutes.

Group D (oral 8-MOP) -- 5 mice, 20 mg/kg (0.64 mg. per mouse) of 8-MOP was given orally.

By the end of 1 hour, mice were radiated for 30 minutes. After radiation, at the end of 72 hours, skin biopsy specimens (at least 3 specimens from each mouse) were obtained from the treated areas. Dermis was separated from epidermis at 0° C. through enzymic digestion with trypsin (buffered solution at pH 7.4, as described by Szabo, 109). Epidermis sections were tested for dopa oxidase reaction as described by Laidlaw and Blackberg (110). The degree of darkening of melanocytes was evaluated after studying the mounted tissue sections under microscope.

RESULTS

Photodynamic effects of psoralen and 8-methoxypsoralen.

In vitro studies: Human erythrocytes suspended in buffered saline or plain saline solution containing a photosensitizing substance like psoralen and 8-methoxypsoralen, show a definite indication of red cell hemolysis (see Table V, fig. 11, Table VI, fig. 3, and also Tables VII, VIII, and IX). This hemolysis, though of a low magnitude, has been observed in several determinations. It appears that photodynamically activated psoralen or 8-MOP molecules induce hemolysis of a very low degree, unlike other photodynamic hemolysis observed in presence of eosin or methylene blue type photosensitizing dyes, where the degree of hemolysis is very high. It should be also seen that ultra-

violet light >3400 A°, does not cause any detectable hemolysis by itself, only in presence of these active molecules, hemolysis is observed. The degree of hemolysis was found to increase when the radiated cell suspension containing psoralen or 8-MOP was kept at 5° C. for 24 hours in the dark (Table VII, fig. 4 and 5). After varying the concentration of 8-MOP (Fig. VIII), it was observed that the degree of hemolysis increased slightly, but not significantly. This hemolysis was not proportional to the concentration of 8-MOP. The 8-MOP concentration curve shows initial tendency of increased hemolysis, followed by very little rise. By increasing the time of ultraviolet radiation, there was slight increase in the process of hemolysis (Table IX, fig. 7), but there was never a complete hemolysis of all cells. In fact, later experiments reveal that one hour radiation time is not needed (fig. 6). This study, therefore, indicates that there is definite evidence of small, but consistent degree of hemolysis in presence of a photodynamically active substances and long wave ultraviolet radiation.

Role of oxygen in photodynamic hemolysis.

Cell suspension containing psoralen or 8-methoxypsoralen, radiated under ultraviolet light (> 3400 A°) in an atmosphere of ordinary air shows hemolysis and cell damage (fig. 4, 5 and 6). In absence of oxygen this hemolysis was significantly retarded (also see Tables VII and VIII). There was marked degree of hemolysis after 24 hours in the

Table V. Photodynamic effect of 8-MOP* on human erythrocytes.

		Hemoglobin cc. in supernatent (µg)	Hemolysis per cc. in 1 hour (µg Hb)	Total hemolysis in 5 cc. supernatent (µg Hb)	Total degree of hemolysis per 5 cc. supernatent (µg Hb)
4.5 ml. buffered saline pH 7.0 + 0.5 ml. r.b.c. suspension (control)	A. Dark	50		250	
	U.V.	80	30	400	150
	B. Dark	50		250	
	U.V.	80	30	400	150
	C. Dark	35		175	
	U.V.	65	30	325	150
	D. Dark	35		175	
	U.V.	75	40	375	200
4.5 ml. buffered saline pH 7.0 containing 450 µg. 8-MOP + 0.5 ml. r.b.c. suspension	A. Dark	50		250	
	U.V.	207	157	1035	785
	B. Dark	50		250	
	U.V.	230	180	1150	900
	C. Dark	45		225	
	U.V.	175	130	875	650
	D. Dark	45		225	
	U.V.	185	140	925	700

Note: See figure 2.

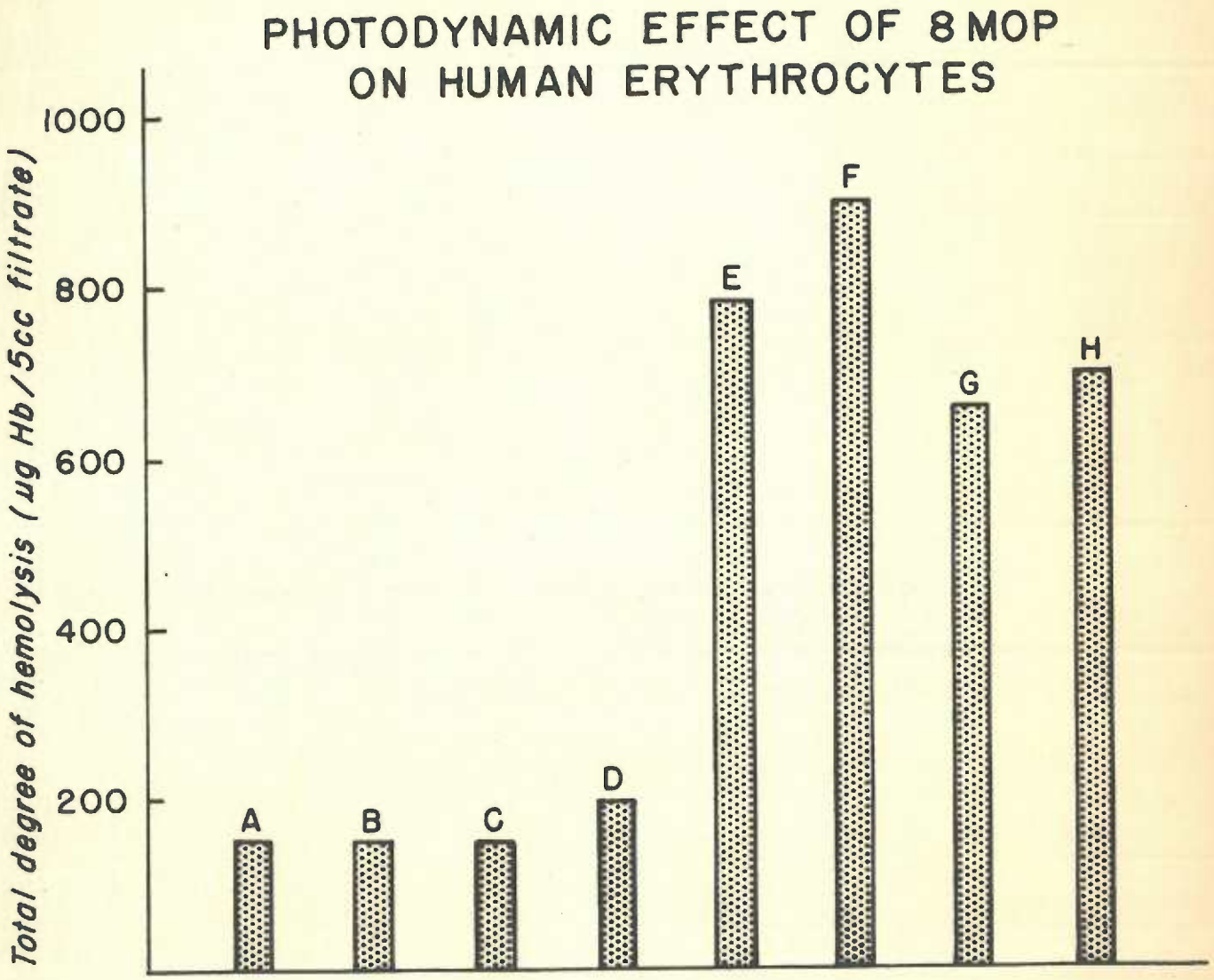
1. U.V. irradiation -- 1 hour
2. Distance between petri dishes and U.V. lamp -- 11 cm.
3. Light source -- Wood's lamp

* 8-Methoxypsoralen

Figure 2. Photodynamic effect of 8-methoxypsoralen on human erythrocytes.

Note the high degree of hemolysis in presence of photodynamically active compound 8-methoxypsoralen.

Figure 2



A, B, C, D - 4.5cc buffered saline pH 7.0 + 0.5cc r.b.c. – irradiated 1 hr.

E, F, G, H - 4.5cc buffered saline pH 7.0 containing 450 µg 8 MOP + 0.5 cc r.b.c. irradiated 1 hr.

Distance of irradiation 11 cm. µV source Woods light c filter (big size)

Table VI. Photodynamic effect of psoralen on human erythrocytes.

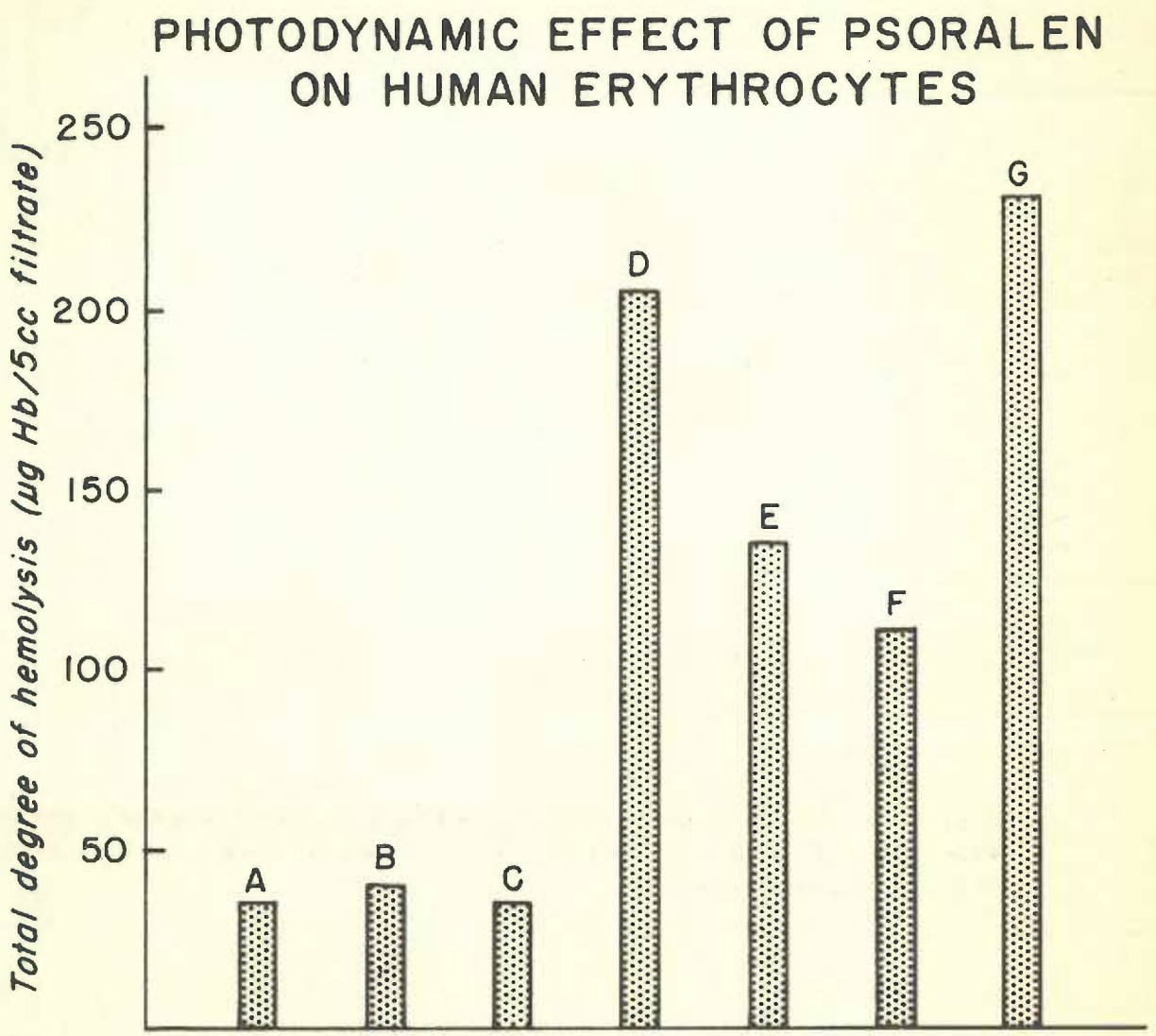
		Hemoglobin cc. in supernatent (µg)	Hemolysis per cc. in 1 hour (µg Hb)	Total hemolysis in 5 cc. supernatent (µg Hb)	Total degree of hemolysis per 5 cc. supernatent (µg Hb)
4.5 ml. buffered saline pH 7.0 + 0.5 ml. r.b.c. suspension (control)	A. Dark	42		210	
	U.V.	49	7	245	35
	B. Dark	54		270	
	U.V.	62	8	310	40
	C. Dark	36		180	
	U.V.	43	7	215	35
4.5 ml. buffered saline pH 7.0 containing 400 µg psoralen + 0.5 cc r.b.c. suspension	A. Dark	84		420	
	U.V.	125	41	625	205
	B. Dark	74		370	
	U.V.	101	27	505	135
	C. Dark	48		240	
	U.V.	70	22	350	110
	D. Dark	74		370	
	U.V.	120	46	600	230

Note: See Figure 3

1. U.V. irradiation -- 1 hour
2. Distance between petridishes and U.V. lamp -- 12 inches.
3. Light source: small Wood's lamp.

Figure 3. Photodynamic effect of psoralen on human erythrocytes. Note the high degree of hemolysis in presence of photodynamically active compound psoralen.

Figure 3



A, B, C - 4.5 cc buffered saline pH 7.0+0.5cc r.b.c. pH 7.0

D, E, F, G - 4.5 cc buffered saline pH 7.0 containing 400 µg. psoralen + 0.5 cc r.b.c.

Distance of irradiation - 12" U.V. source - Small Wood's lamp

Time of irradiation - 1 hour

Table VII. Photodynamic action of Psoralen and 8-MOP on human erythrocytes.* (radiation 1 hour, at 12 cm.)

Psoralen		1 Hemoglobin/ cc. supernatent (ug)	2 Hemoglobin/ 5 cc. supernatent (ug)	3 Degree of hemolysis after 1 hr. (ug)	4 Hemoglobin/ 5 cc. supernatent after 24 hrs.	5 Degree of hemolysis after 24 hrs.
With Oxygen						
4.5 ml. buffered saline pH 7.0 + 0.5 cc. r.b.c. suspension	Dark	61	305		370	
	U.V.(a)	67	335	30	445	75
	U.V.(b)	64	320	15	430	60
4.5 ml. buffered saline pH 7.0 containing 435 ug psoralen + 0.5 ml. r.b.c. suspension	Dark	96	480		720	
	U.V.	112	560	80	2500	1780
	Dark	99	495		765	
	U.V.	118	590	105	2550	1785
Without Oxygen						
4.5 ml. buffered saline pH 7.0 + 0.5 cc. r.b.c. suspension	Dark	134	670		735	
	U.V.(a)	99	495	Nil	605	Nil
	U.V.(b)	99	495	Nil	610	Nil
4.5 ml. buffered saline pH 7.0 containing 435 ug psoralen + 0.5 ml. r.b.c. suspension	Dark	134	670		765	
	U.V.	112	560	Nil	925	160
	Dark	125	625		700	
	U.V.	105	525	Nil	840	140

* See figures 4 and 5.

Figure 4. Photodynamic action of psoralen on human erythrocytes (effect of oxygen)

In presence of oxygen, psoralen and long wave ultraviolet radiation, there is evidence of more hemolysis, in absence of oxygen this photosensitized hemolysis is absent. After a lapse of 24 hours, the degree of hemolysis was significantly higher in those specimens which were radiated in presence of oxygen whereas there was practically no hemolysis in specimens radiated in absence of oxygen. Also note that long wave ultraviolet light by itself did not induce any hemolysis, only in presence of psoralen this hemolysis was observed.

PHOTODYNAMIC ACTION OF PSORALEN ON HUMAN ERYTHROCYTES

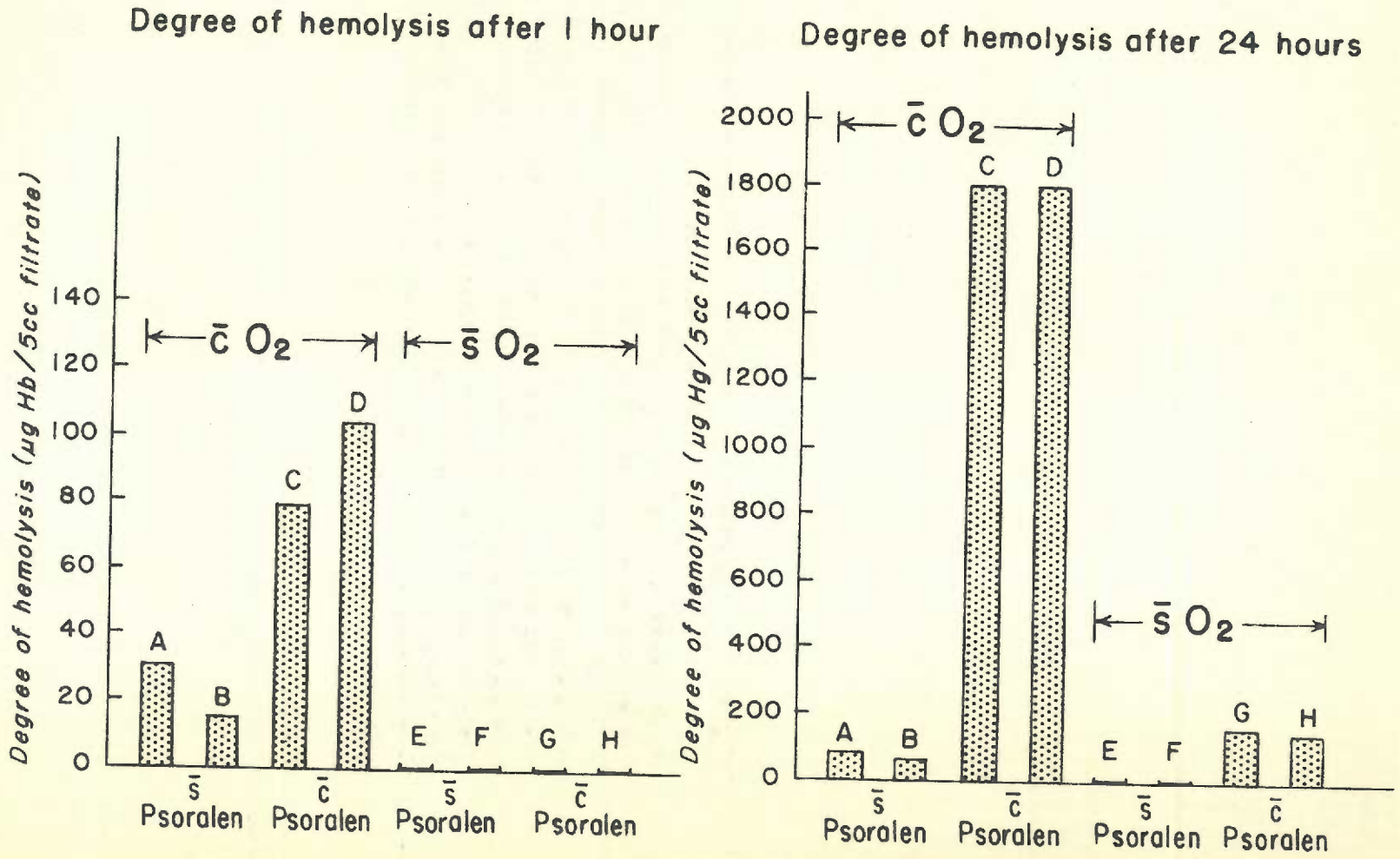


Figure 4

Table VII. (continued)

8-Methoxypsoralen		1 Hemglobin/ cc. supernatent (ug)	2 Hemoglobin/ 5 cc. supernatent (ug)	3 Degree of hemolysis after 1 hr. (ug)	4 Hemoglobin/ 5 cc. supernatent after 24 hrs.	5 Degree of hemolysis after 24 hrs.
			With Oxygen			
4.5 ml. buffered saline pH 7.0 + 0.5 cc. r.b.c.	A. Dark	119	595		600	
	U.V.	125	625	30	720	120
	B. Dark	36	180		300	
	U.V.	55	275	95	440	140
4.5 ml. buffered saline pH 7.0 + containing 300 ug 8-MOP + 0.5 ml. r.b.c.	A. Dark	106	530			
	U.V.	246	1230	700		
	B. Dark	123	615		720	
	U.V.	242	1210	595	2080	1360
	C. Dark	55	275		280	
	U.V.	390	1950	1685	2200	1920
			Without Oxygen			
4.5 ml. buffered saline pH 7.0 + 0.5 cc. r.b.c.	A. Dark	96	480		500	
	U.V.	60	300	Nil	320	Nil
	B. Dark	96	480		480	
	U.V.	60	300	Nil	300	Nil
4.5 ml. buffered saline pH 7.0 + containing 300 ug 8-MOP + 0.5 ml. r.b.c.	A. Dark	92	460		500	
	U.V.	74	370	Nil	490	Nil
	B. Dark	98	490		500	
	U.V.	70	350	Nil	580	80
	C. Dark	91	455		700	
	U.V.	85	425	Nil	425	Nil

Figure 5. Photodynamic action of 8-methoxypsoralen on human erythrocytes (effect of oxygen)

In presence of oxygen, 8-MOP and long wave ultraviolet radiation, there is evidence of more hemolysis, in absence of oxygen this photosensitized hemolysis is absent. After a lapse of 24 hours, the degree of hemolysis was significantly higher in those specimens which were radiated in presence of oxygen, where as there was practically no hemolysis in specimens radiated in absence of oxygen. Also note that long wave ultraviolet radiation by itself did not induce any hemolysis, only in presence of 8-methoxypsoralen this hemolysis was observed.

PHOTODYNAMIC ACTION OF 8 METHOXY PSORALEN ON HUMAN ERYTHROCYTES

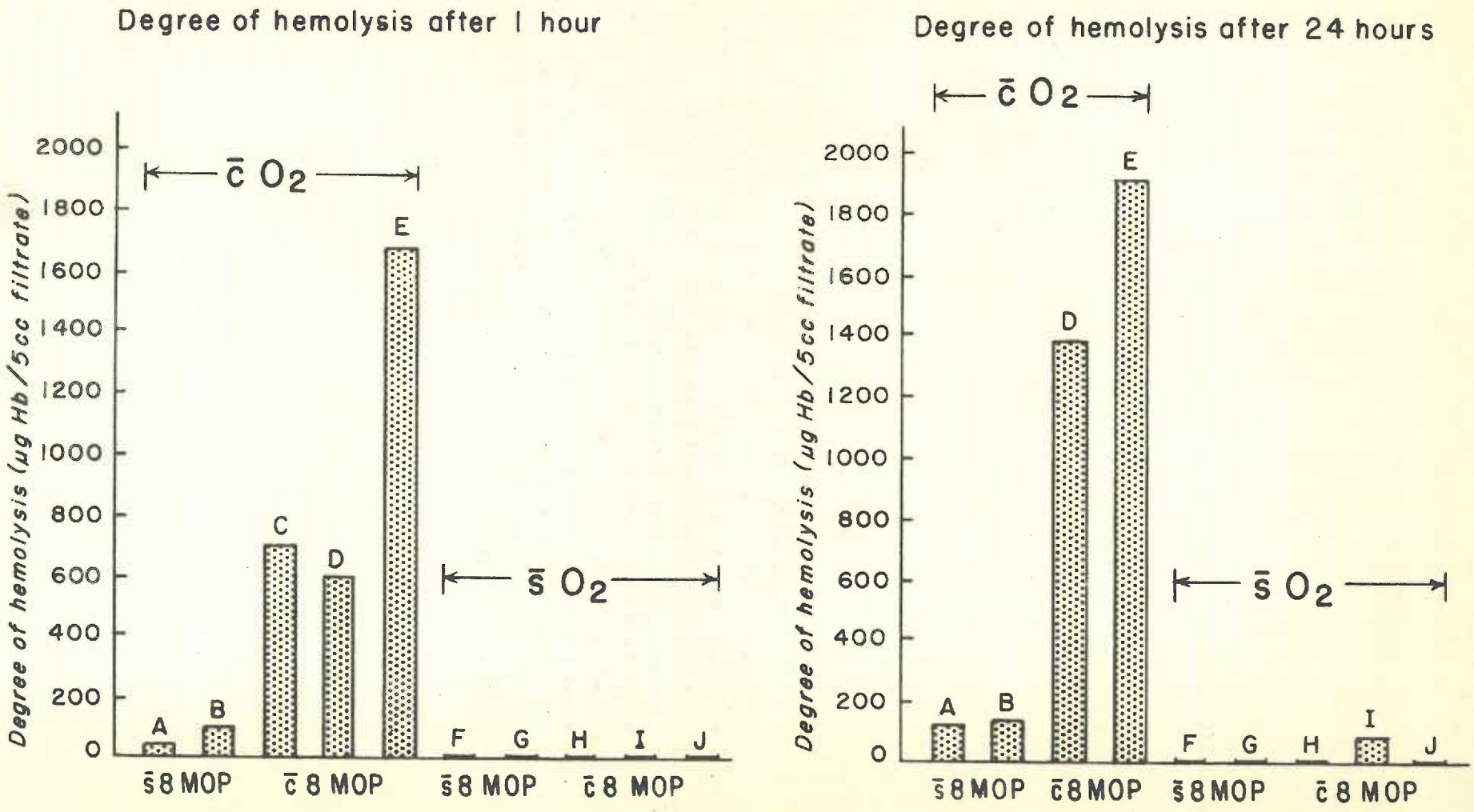


Figure 5

Table VIII. Photodynamic action of psoralen in erythrocytes.*

	U. V. radiation	Degree of hemolysis (hemoglobin µg/5 cc. supernaten				
		0	1	2	4	10
(A) Control 5 ml. of 5% red cell suspension in saline	In air	115.0	97.5	162.5	115.0	113.7
(B) 5 ml. of 5% red cell suspension in saline + 25 µg psoralen/ml saline	In air	113.75	130.0	341.0	357.5	292.5
(C) "	Under 0_2	114.5	162.5	341.0	162.5	162.5
(D) "	Under N_2	113.75	113.7	130.0	130.0	130.0
A1. 5 cc. of 5% red cell suspension in saline	No radiation specimen in test tube for 2 hours	113.75				
B1. 5 cc. of 5% red cell suspension in saline + 25 µg. psoralen/ml. saline	"	114.5				
C1. "	No radiation specimen under 0_2 for 10 minutes	97.5				
D1. "	No radiation specimen under N_2 for 10 minutes	113.75				

*See Figure 6.

Figure 6. Photodynamic effect of psoralen (role of oxygen). In presence of oxygen of the air or pure O_2 and photodynamically active compound psoralen, there is distinct hemolysis of red cells. In absence of oxygen (under nitrogen) otherwise, this hemolysis is almost completely absent. Also note that long wave ultraviolet light (3200 A°) by itself did not induce any red cell hemolysis. Only in presence of psoralen this hemolysis was observed.

Figure 6

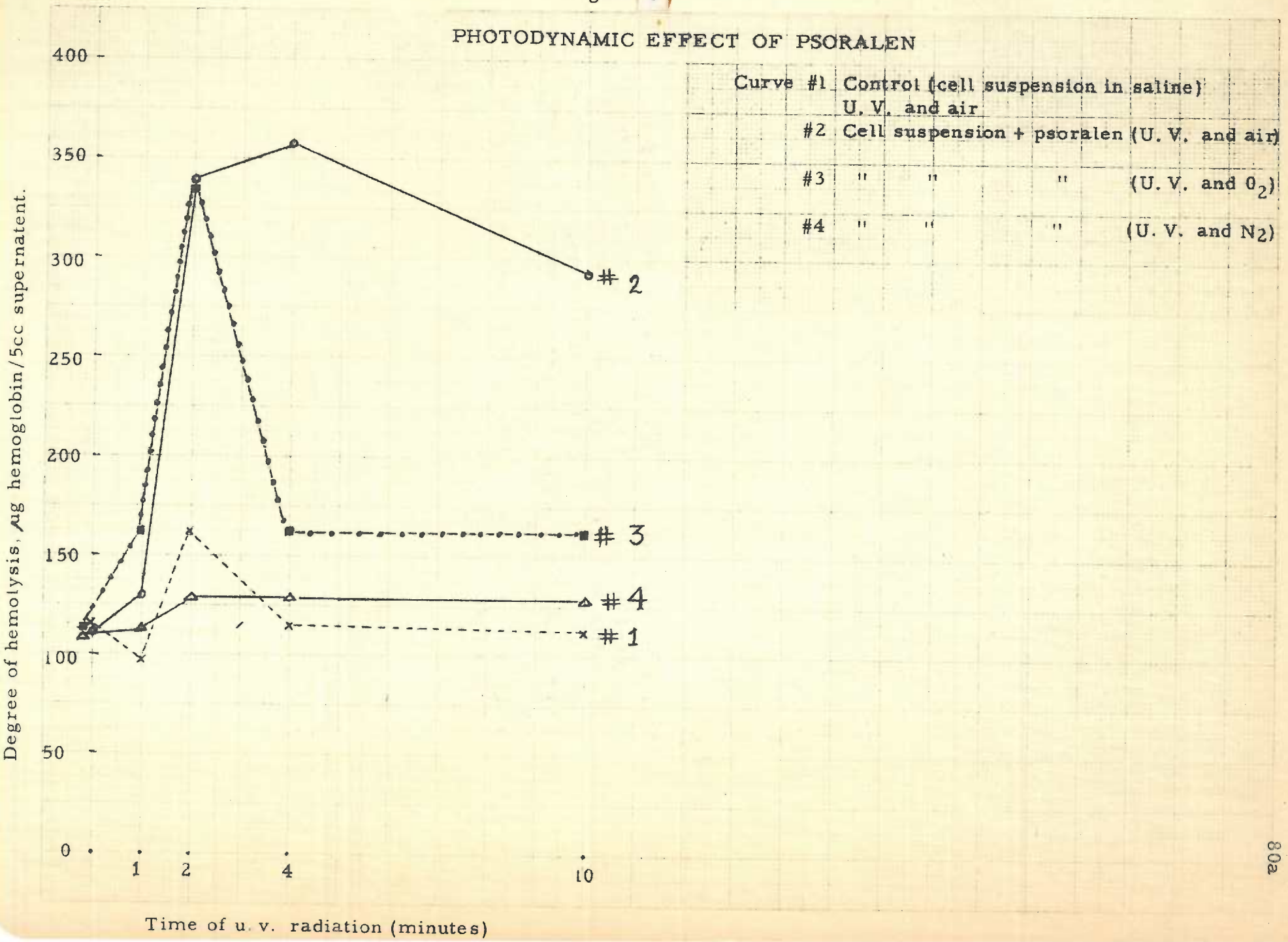

PHOTODYNAMIC EFFECT OF PSORALEN
Curve #1 Control (cell suspension in saline) U. V. and air
#2 Cell suspension + psoralen (U. V. and air)
#3 " " " (U. V. and 0_2)
#4 " " " (U. V. and N_2)
Degree of hemolysis, ug hemoglobin/5cc supernatent.
400
350
300
250
200
150
100
50
0
1
2
4
10
2
3
4
1
Time of u. v. radiation (minutes)

Table IX. Photodynamic effect of 8-MOP after varying the concentration and time of irradiation. *

	8-MOP concentration / 5 ml. (µg.)		U. V. irradiation 1 hour: Hemoglobin / cc. supernatent (µg.)	U. V. irradiation 1 hour: Degree of hemolysis per cc. (µg)	U. V. irradiation 1 hour: Total degree of hemolysis per 5 cc. supernatent	U. V. irradiation 2 hours: Hemoglobin / cc. supernatent (µg.)	U. V. irradiation 2 hours: Degree of hemolysis per cc. (µg)	U. V. irradiation 2 hours: Total degree of hemolysis per 5 cc. supernatent
4. 5 ml. buffered saline pH 7. 0 + 0. 5 ml. r. b. c. suspension	Nil	Dark	210			200		
		U. V.	230	20	100	210	10	50
	112	Dark	70			70		
		U. V.	144	74	370	110	40	200
	225	Dark	54			54		
		U. V.	132	78	390	183	129	645
	337	Dark	62			62		
		U. V.	156	94	470	164	102	510
	450	Dark	74			74		
	a)	U. V.	148	74				545
	b)	U. V.	156	82	390	183	109	

* See figure 7.

Figure 7. Photodynamic effect of 8-methoxypsoralen after varying the concentration and time of ultraviolet radiation. Note that without 8-MOP, even at the end of one hour or 2 hours ultraviolet radiation, there was practically no hemolysis. Only in presence of 8-MOP increased degree of hemolysis was observed.

Figure 7

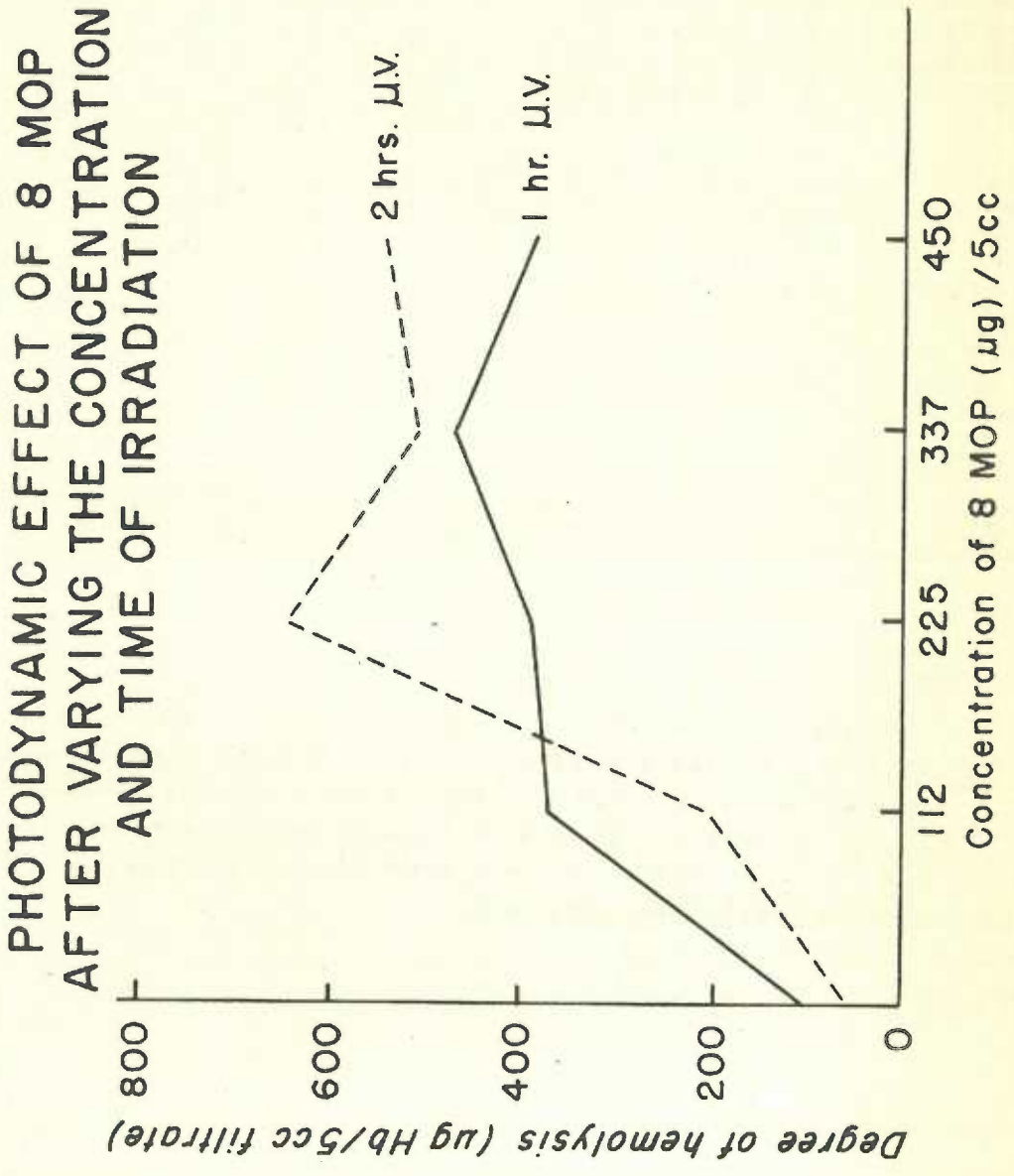
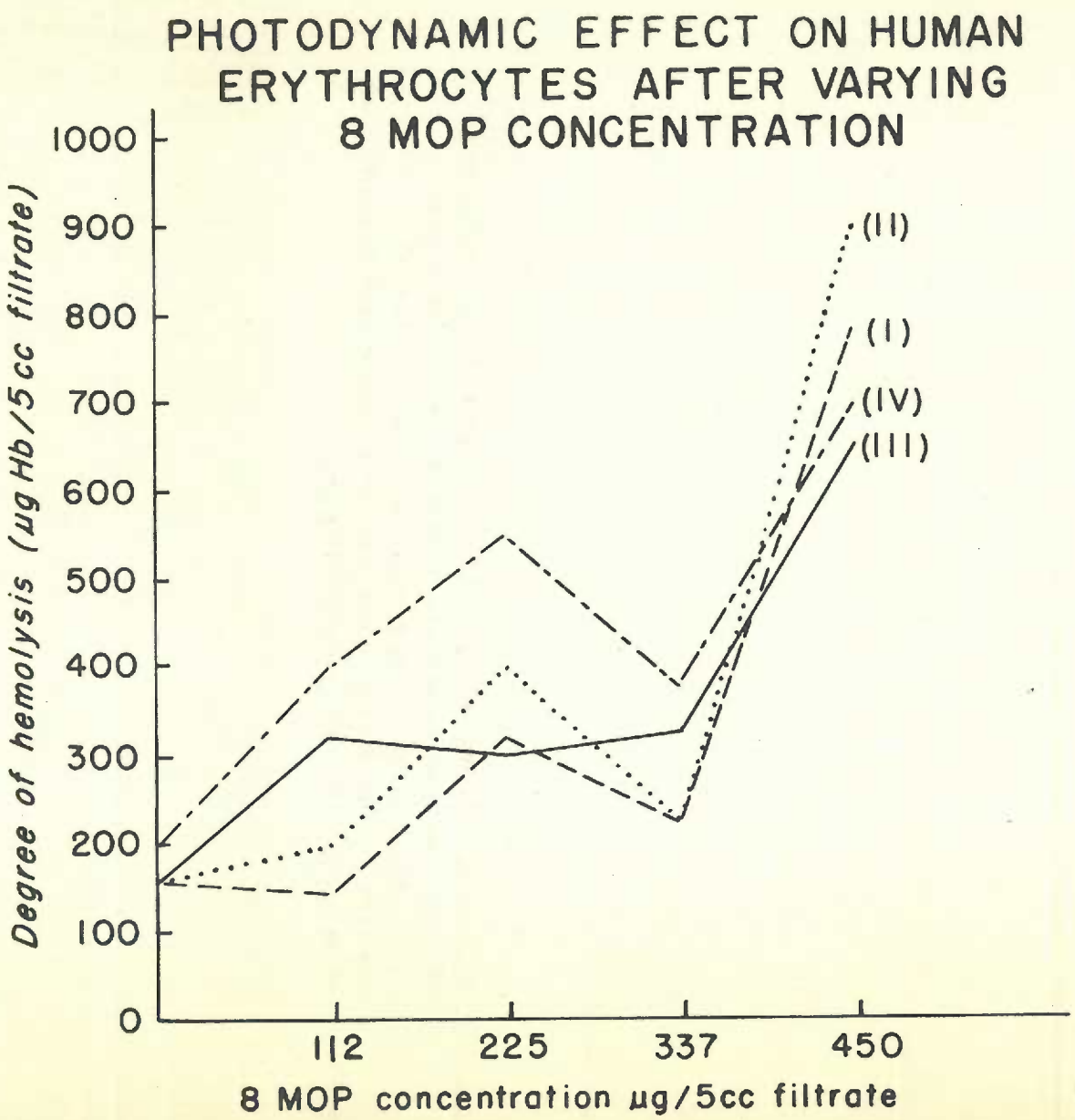

PHOTODYNAMIC EFFECT OF 8 MOP
AFTER VARYING THE CONCENTRATION
AND TIME OF IRRADIATION
2 hrs. U.V.
1 hr. U.V.
800
600
400
200
0
112
225
337
450
Concentration of 8 MOP (µg) / 5cc
Degree of hemolysis (µg Hb/5cc filtrate)

Figure 8. Photodynamic effect on human erythrocytes after varying 8-methoxypsoralen concentration. The degree of hemolysis was not proportional to concentration of 8-methoxypsoralen, even though there was evidence of increased degree of hemolysis at high concentrations.

Figure 8


PHOTODYNAMIC EFFECT ON HUMAN ERYTHROCYTES AFTER VARYING 8 MOP CONCENTRATION
Degree of hemolysis (µg Hb/5cc filtrate)
1000
900
800
700
600
500
400
300
200
100
0
112
225
337
450
8 MOP concentration µg/5cc filtrate
(II)
(I)
(IV)
(III)

specimens which were radiated in presence of oxygen, where as there was negligible hemolysis in absence of O_2. In fig. 6, it can be seen again that in presence of ordinary oxygen of the air and ultraviolet light, the hemolytic cell damage is in operation only in those specimens which had psoralen (curve #2 and 3), but no such hemolytic damage was observed in absence of psoralen. In a pure oxygen atmosphere, there was evidence of hemolysis up to 2 minutes radiation, but subsequently it was less (but still more than the control specimens). Probably the pure O_2 inhibits this process after some time (either cell diffusion or other process like high O_2 tension which causes the cells to be refactory to hemolysis).

In presence of inert gas (curve # 4, fig. 6) this hemolytic process is absent, the activated psoralen molecules are unable to bring about this biological change.

It appears that in this photodynamically induced hemolytic cell damage, oxygen enhances the photosensitizing action. In absence of oxygen, the photodynamically active molecules of psoralen or 8-methoxy-psoralen, do not cause hemolysis.

In vivo studies on photodynamic action of 8-methoxypsoralen.

In vivo experiments as performed on rabbit ears again[5] show that in presence of oxygen, 8-methoxypsoralen induces biological changes of increased erythema. In absence of oxygen these changes are not observed. In control areas painted with 8-MOP solution and exposed

Figure 9. In vivo photodynamic effect of 8-methoxypsoralen. When oxygen supply at the terminal portions of the rabbit ears was depleted and ears radiated under long wave ultraviolet light in presence of 8-methoxypsoralen, no erythema response could be observed. In the control areas (base portions of both ears) where oxygen supply was not depleted, marked erythema response was observed. Photodynamic response is definitely fecilitated in presence of oxygen.

A B

Figure 9. In vivo. Photodynamic effect of 8-methoxypsoralen. The terminal portions on both ears of each rabbit show no erythema in absence of oxygen. The base area of both ears show erythema in presence of oxygen.

under ultraviolet (> 3200 A°) without depleting the O_2 supply, there was visible erythema after 24 hours, and by the end of 48 hours, the erythema in these areas reached its peak. In O_2 depleted areas of the ears (terminal portion), there was no erythema observed after 24 hours, and even at the end of 72 hours period, the skin areas did not appear erythematous (see fig. 9). Out of five rabbits, three showed definite absence of erythema in oxygen depleted areas.

Thus, in these in vitro and in vivo studies, one sees one of the characteristic features of a photodynamic action described by Blum (103). Studies on relative activity of different furocoumarins reported elsewhere in this thesis, both on human skin and guinea pig skin, conclusively show that these compounds induce marked erythema which does not appear immediately after radiation, but manifests itself after a long latent period of 8 to 10 hours. This latency is therefore another characteristic feature of a photodynamic action. Furthermore, these changes have been induced in presence of long wave ultraviolet light of wavelengths greater than 3400 A°. All these characteristics follow the hypothesis proposed by Blum for a photodynamic action.

Bioassay method for determining the relative activity of furocoumarins.

Lag period and ultraviolet radiation time for an optimum response was initially determined to standardize the bioassay procedure.

Lag period.

From Tables X and XI it can be seen that after applying the compound topically, a 30 minute lag period is needed for an optimum erythema response. Increasing the lag period does not alter the response. A lag period of only 5 or 15 minutes, however shows less erythema response when compared with 30 minutes and 45 minutes lag time.

Time of ultraviolet radiation.

For a guinea pig skin which is quite thick, a total 45 minutes long wave ultraviolet radiation gave maximum response (Table X and XI). Radiation for 15 or 30 minutes, although showed characteristic erythema, but the degree of erythema particularly in presence of low concentrations of active compounds was not optimum. When relative activity of compounds are required to be determined, an optimum response is always desired, therefore, 45 minutes ultraviolet radiation time is recommended. For testing the activity of compounds topically for bioassay purposes, this study indicates that a lag period of 30 to 45 minutes and long wave ultraviolet radiatinn for 45 minutes is essential to be observed. Since the intensity of the light emitted by long wave ultraviolet lamp is usually low, it is desirable to keep the animals close to u. v. lamp.

Oral response.

Table XII shows that after feeding the compound orally, a lag period of minimum 1 hour is needed before a response can be observed. Optimum response was observed after a lag period of 1 1/2 hours. In

Table X.

DETERMINATION OF LAG PERIOD AND U. V. RADIATION TIME FOR MAXIMUM RESPONSE OF FUROCOUMARINS

Topical studies with 8-MOP on guinea pigs

Light source: Woods light emitting >3200 A°
Distance: 12 cm.
Concentration of 8-MOP: 15 µg. per square inch in each area
Erythema: Response expressed as + or - after 18 and 36 hours

	Ultraviolet Radiation Time											
	15 minutes			30 minutes			45 minutes			60 minutes		
Lag period	con-trol	18 hrs.	36 hrs.	con-trol	18 hrs.	36 hrs.	con-trol	18 hrs.	36 hrs.	con-trol	18 hrs.	36 hrs.
5 min.	-	-	-	-	-	±	-	-	++	-	-	+
15 min	-	-	-	-	-	+	-	+	++	-	+	+
30 min	-	-	±	-	-	++	-	+	++	-	+	++
45 min	-	-	+	-	+	++	-	++	+++	-	+	+++
60 min	-	-	±	-	-	++	-	+	+++	-	+	+++

Conclusions:

I. Minimum lag period. 30 Minutes is needed for erythema response.

II. After topically applying the drug, if 30 to 45 minutes lag period is observed, maximum response can be obtained.

III. Minimum u. v. radiation time required is 30 minutes, however, 45 minutes total u. v. radiation gives the maximum response.

Table XI.

Determination of U. V. Radiation Time for Maximum Erythema Response After Topically Applying Varying Concentration of 8-MOP

Topical studies

Light source: Woods light > 3200 A°
Distance: 12 cm.
Lag period: 45 minutes
Erythema response expressed as + or - after 18 and 36 hours.

Concentration of 8-MOP	Ultraviolet Radiation Time					
	15 minutes		30 minutes		45 minutes	
µg/sq. inch	18 hrs.	36 hrs.	18 hrs.	36 hrs.	18 hrs.	36 hrs.
10 µg.	-	±	±	±	±	±
15 "	-	+	+	++	+	++
25 "	-	+	+	++	++	+++
50 "	-	+	++	++	++	+++
100 "	-	+	++	++	++	+++
Control	-	-	-	-	-	-

Conclusions: Minimum radiation time 30 minutes for minimum response, 45 minutes u. v radiation time with a 30 to 45 minute lag period will produce maximum response.

Table XII.

Determination of Lag Period and U.V. Radiation Time for Maximum Response of Furocoumarin When Given Orally

Light source: Woods light emiting >3200 A°
Distance: 12 cm.
Amount of 8-MOP administered : 25 mg/kg
Erythema response expressed as + or - after 18 and 36 hours.

	Ultraviolet Radiation Time					
	15 minutes		30 minutes		45 minutes	
Lag period	18 hrs	36 hrs	18 hrs.	36 hrs	18 hrs.	36 hrs.
15 minutes	-	-	-	+	+	+
30 minutes	±	-	+	+	++	+++
1 hour	+	+	+	++	++	+++
1 1/2 hour	+	++	+	+++	++	+++
2 hours	+	++	++	+++	+++	++++
2 1/2 hours	+	++	++	+++	+++	++++
3 hours	+	+	++	++	+++	+++
4 hours	+	+	+	++	++	++
Control	-	-	-	-	-	-

Conclusions: I. Lag period of minimum of 1 hour needed for a response

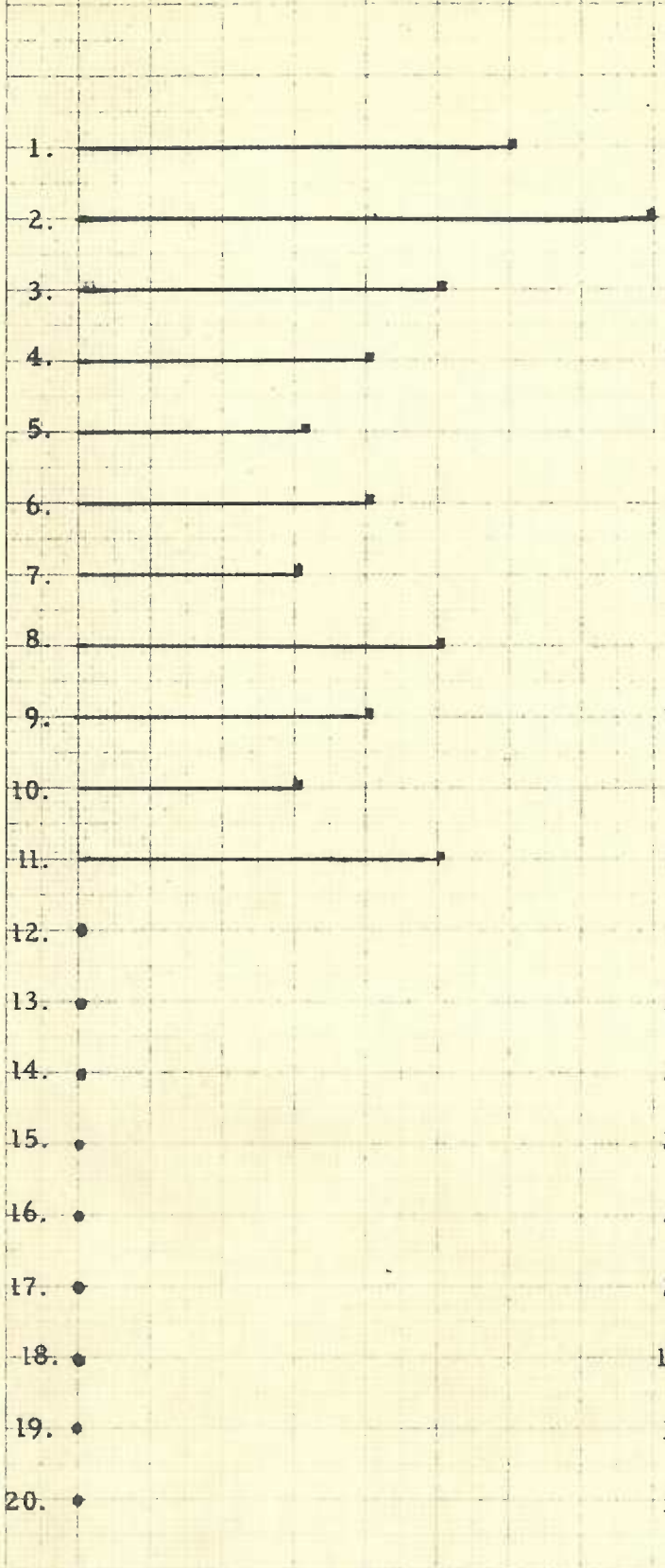
II. Maximum response observed after 1 1/2 hour lag period and lasts up to 3 hours, peak of response lies between 1 1/2 to 2 1/2 hours.

III. Maximum radiation time for optimum response appears to be 45 minutes.

Figure 10. Relative activity of furocoumarins (topical studies - guinea pigs)

Note the minimum effective concentration and the degree of erythema observed in presence of different furocoumarin compounds. Psoralen and trimethyl psoralen showed highest response.

Figure 10. Relative Activity of Furocoumarins (Topical Studies, Guinea Pigs)



	M.E.C.* ug/Sq"	Visible erythema	Compound
1.	10	++	Psoralen
2.	10	+++	Trimethyl psoralen
3.	10	++	5', 8-Dimethyl psoralen
4.	10	++	5'-Methyl psoralen
5.	125	+	Psoralen glucoside
6.	15	++	8-Methoxypsoralen
7.	50	+	5-Methoxypsoralen
8.	75	+	4',5' Dihydro-8-methoxypsoralen
9.	100	+	8-Isoamylenoxy-psoralen
10.	250	+	Isobergapten
11.	500	+	4,5' Dimethyl isopsoralen
12.	2000	-	Psoralen quinone
13.	2000	-	Thiofurocoumarin
14.	2000	-	5, 8 Dimethoxypsoralen
15.	2000	-	5 Amino-8-methoxy-psoralen
16.	2000	-	5 Nitro-8-methoxy-psoralen
17.	2000	-	8-Hydroxypsoralen
18.	1000	-	Khellin (furochromone)
19.	1000	-	Chellol glucoside (furochromone)
20.	1000	-	Visnagin (furochromone)

0 1 2 3 4 5 6 7 8

Erythema Response at M.E.C.
(Reflection meter % difference, control-test area)
*M.E.C. -- Minimum effective concentration

testing the activity, the animal should be radiated at the end of 1 1/2 hours after feeding the compound orally. Since the erythema response declined by the end of 3 hours, radiation with ultraviolet should be carried out before the end of 3 hours. This lag period interval, agrees very well with the observations of Daniels and Fitzpatrick (96), carried out on human subjects.

Relative activity of furocoumarins.

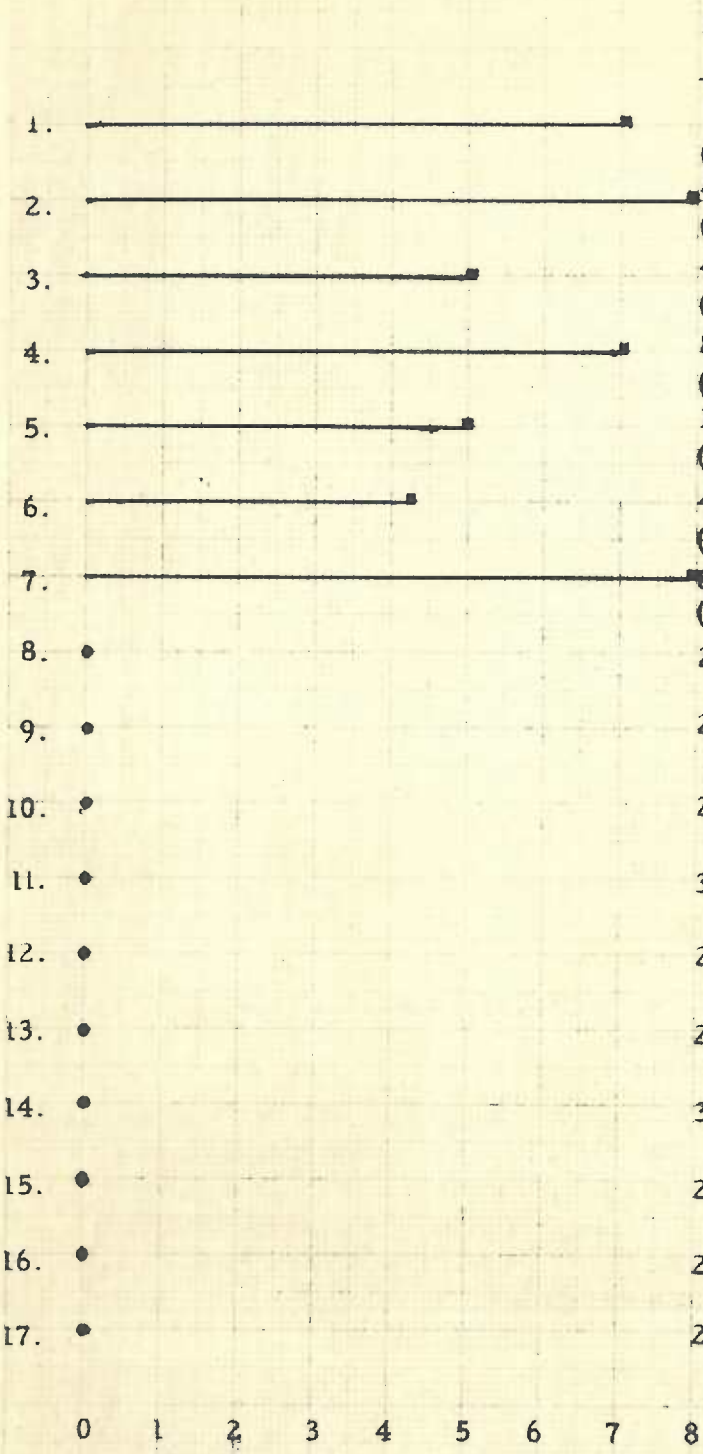
Topical studies (guinea pig skin). Seventeen furocoumarin compounds were tested topically for relative activity in varying concentrations ranging from 2.5 µg up to 1000 µg per square inch area of the skin. Since all these observations would require a number of tables for presentation, and therefore, to conserve space, only minimum effective concentration (M.E.C.) for erythema response has been graphically presented in figure 10. (M.E.C. is that minimum effective concentration of the compound which is required to produce clearly visible erythema.)

The reflection meter readings (% difference between control area and the test area, with green standard filter) has been shown for each compound along with the degree of erythema observed by visual grading system. It can be seen, that out of 17 furocoumarins, 11 showed varying activity. By comparing the erythema response at minimum

Figure 11. Relative activity of furocoumarins (oral studies - guinea pigs)

Note the minimum effective concentration and the degree of erythema observed in presence of different furocoumarin compounds. Figures in parenthesis in the column of minimum effective concentration represent concentrations in terms of millimoles. Psoralen and trimethyl psoralen showed highest response.

Figure 11
Relative Activity of Furocoumarin (Oral Studies, Guinea Pigs)



	M.E.C.* ug/kg.	Visible erythema	Compound
1.	3.72 (0.02 M)	++	Psoralen
2.	4.36 (0.02 M)	++	Trimethyl psoralen
3.	4.3 (0.02 M)	+	Dimethyl psoralen
4.	8.0 (0.04 M)	++	5' Methyl psoralen
5.	14.7 (0.04 M)	+	Psoralen glucoside
6.	4.3 (0.02 M)	+	8-Methoxypsoralen
7.	8.6 (0.04 M)	++	5-Methoxypsoralen
8.	25	-	5, 8-Dimethoxypsoralen
9.	20	-	4', 5'-Dihydro 8-methoxypsoralen
10.	25	-	8-Isoamylenoxy-psoralen
11.	30	-	Isobergapten
12.	25	-	Psoralen quinone
13.	20	-	Thiocoumarin
14.	32	-	5 Amino-8-methoxy-psoralen
15.	25	-	5 Nitro-8-Methoxy-psoralen
16.	28.5	-	8-Hydroxypsoralen
17.	20	-	4, 5'-Dimethyl isp-psoralen

0 1 2 3 4 5 6 7 8

Erythema Response at M.E.C.
(Reflection meter % difference, control-test area)

*M.E.C. -- Minimum effective concentration
Figures in parenthesis represent millimols.

effective concentration, the order of activity of various compounds was as follows: trimethyl psoralen, psoralen, dimethyl psoralen, 5' methyl psoralen, 8-methoxypsoralen, 5-methoxypsoralen, 4', 5'-dihydroxy-8-methoxypsoralen, 8-isoamylenoxypsoralen, psoralen glucoside, isobergapten, 4, 5'-dimethylisopsoralen. Other compounds like thiocoumarin, psoralen quinone, 5, 8-dimethoxypsoralen, 5 amino-8-methoxypsoralen, 5-nitro-8-methoxypsoralen, 8-hydroxypsoralen, were inactive; even 1000 µg concentration of these compounds did not exhibit any erythema effect.

Oral studies (guinea pigs): When fed orally some of the compounds, however, do not exhibit the response as observed in topical studies. As shown in figure 11, 4', 5'-dihydro-8-methoxypsoralen, 8-isoamylenoxy psoralen, 4, 5'-dimethyl isopsoralen and isobergapten (5-methoxypsoralen) were found to be inactive. Even in very large doses (25-30 mg/kg) they showed no activity. Otherwise, the order of activity was similar to topical data as presented. Trimethyl psoralen and psoralen appear to be most potent compounds.

It is interesting to see that on molar basis, minimum effective concentration for most of the active compounds was nearly 0.02 millimoles, but the response elicited by different compounds was varying, which further reveals their relative activity.

In this topical and oral bioassay studies, it is evident that one can screan these compounds very effectively and determine their relative activity. Not only a photodynamic response can be demonstrated but also quantitatively, the relative activity of the photosensitizing compound can be evaluated.

Activity of furochromone compounds.

Three compounds, mainly khellin, chellol glucoside and visnagin possessing a furochromone ring configuration, were tested topically for their activity. As shown in Table 13, none of these compounds showed any response even in very high concentration.

Coumarin compounds. (Topical and oral studies).

In all 28 compounds representing a spectrum of substituted coumarin compounds (substitution at various carbon atoms, with different groups like methyl, ethyl, isopropyl, hydroxyl, etc.) were tested with a view to investigate whether coumarin ring configuration present in active furocoumarin compounds is the active basic ring system for photodynamic action. Except limetin (5,7-dimethoxy coumarin) and 2-thiocoumarin, none of these other compounds shown in Table 14 exhibited any erythema inducing action. Even the methyl substituted derivatives (mono, di and tri methyl derivatives) were inactive. It is evident from Table 14 that coumarin and its other derivatives do not possess the active molecular configuration for photodynamic action.

Table XIII.

Relative activity of furochromones and other compounds.

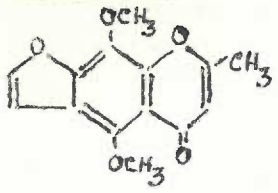
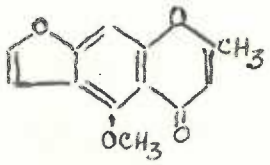
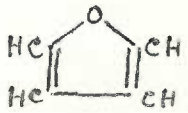
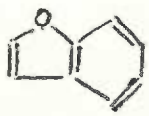
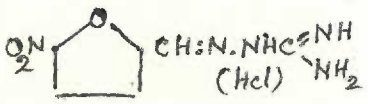
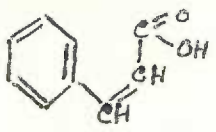
Compound	Structure	Topical erythema response (guinea pig skin)
1. Khellin		Inactive 1000 µg/sq. in.
2. Chellol glucoside (2 glucosoxy methyl-5-methoxy furochromone)		"
3. Visnagin		"
Other Compounds		
1. Furan		Inactive 1000 µg/sq. in.
2. Benzofuran		"
3. Furacin (5-nitro-2-furaldehyde amino guanidine Hcl)		"
4. Cinnamic acid		"

Table XIV.

Relative activity of coumarin derivatives.

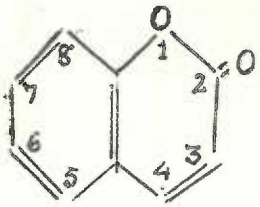
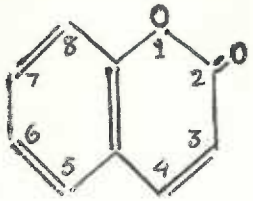
Compound		Activity (Erythemal response) Topical effect 1000 ug/sq. in.	Oral effect
1. Coumarin		Inactive	No response 30 mg/kg
2. 7-Hydroxy coumarin (umbelliferone)		"	"
3. 4-Hydroxy coumarin		"	"
4. 7-Hydroxy 4 methyl coumarin (4 methyl umbelliferone)		"	"
5. 3-Methyl coumarin		"	"
6. 4-Methyl coumarin		"	No response 25 mg/kg
7. 6-Methyl coumarin		"	"
8. 7-Methyl coumarin		"	"
9. 5, 7-Dimethyl coumarin		"	"
10. 6, 7-Dimethyl coumarin		"	"
11. 4, 5, 7-Trimethyl coumarin		"	No response 33 mg/kg
12. 3-Ethyl coumarin		"	No response 26.1 mg/kg
13. 3, 4-Dihydro 5 methyl coumarin		"	
14. 3, 4-Dihydro 6 methyl coumarin		"	No response 28.6 mg/kg
15. 3, 4-Dihydro 7 methyl coumarin		"	
16. 3, 4-Dihydro 6, 7 dimethyl coumarin		"	

Table XIV.

Relative activity of coumarin derivatives. (continued)



Compound	Activity (Erythemal response) Topical effect 1000 ug/sq. in.	Oral effect
17. 5, 7-Dihydroxy 4 methyl coumarin	Inactive	
18. 8-Isopropyl 5 methyl coumarin	"	No response 25. 3 mg/kg
19. 4-Methyl 7 ethoxy coumarin	"	
20. 2-Thio coumarin	Active + 1000 ug/sq. in.	No response 25 mg/kg
21. 3-Chloro coumarin	Inactive	No response 20 mg/kg
22. 4-Methyl benzo coumarin	"	
23. 4-β methyl benzo coumarin	"	
24. Aesculine 6, 7 dihydroxy 6 glucoside	"	
25. 7-Acetoxy 4 methyl coumarin 7-(O-C-CH_3)	"	
26. 7-Allyloxy coumarin 7-(O-CH_2-CH_2 = CH_2)	"	
27. β Methyl napthyl coumarin	"	
28. 5, 7-Dimethoxy coumarin (Limetin)	Active + 200 ug/sq. in.	No response 20 mg/kg

Other compounds.

Absence of photodynamic activity in coumarin ring configuration compounds prompted to investigate whether furan ring present in furocoumarin molecule exhibits any photodynamic response. Musajo (19) had reported furan and benzofuran to be inactive compounds. Furacin another furan derivative showed no erythema inducing properties. Cinnamic acid (transform) an open lactone ring compound also showed no activity.

Thus it appears that furan ring configuration alone does not possess any photodynamic action, nor coumarin ring configuration compounds exhibit similar action. Only when these two rings are fused together (as in psoralen molecule) one observes photodynamic activity.

Relative activity of furocoumarins on human skin. (Topical studies)

In this study there were 12 subjects, a tabulation of each subject's erythema and pigmentation response would occupy unduely large space, and therefore, observations for various cancentrations of different compounds have been expressed interms of mean response only. This mean erythema response, as measured by reflection meter readings, has been summarized in Table XV for different concentrations to indicate the relative activity of these compounds on human skin. A typical graded response after topically applying psoralen, 8-MOP, 5-MOP and trimethyl psoralen has been shown in figure 12. It can be seen from Table XV

Table XV. Relative activity of furocoumarins (topical studies - human skin).

Compounds	Concentration µg/sq. in. area	Mean erythema response: Reflection meter % diff.(control-test area) with green filter	Mean erythema response: Degree of visible erythema	Mean pigmentation response: Reflection meter % diff.(control-test area) with green filter	Mean pigmentation response: Degree of visible pigmentation	Remarks
1. Psoralen	10	5	+	6	++	
	25	7	++	9	+++	blistering
	50	8	++	13	+++	"
	100	9	+++	14	++++	"
2. Trimethyl psoralen	10	4	+	3	±	
	25	5	+	5	++	
	50	5	+	6	++	blistering
	100	6	++	8	++	"
3. 5'[illegible]methyl psoralen	50	7	++	6	++	blistering
	100	8	+++	10	+++	
4. 5'8 Dimethyl psoralen	50	8	++	5	++	blistering
	100	9	+++	18	+++	"
5. 8 Methoxy-psoralen	50	4	+	4	+	--
	100	6	+	6	++	--
	200	6	++	9	+++	--
6. 5 Methoxy-psoralen	100	3	+	3	+	--
	200	3	+	5	++	--
	300	5	++	8	++	--
7. Psoralen glucoside	100	3	+	3	+	--
	200	4	+	5	++	--

Table XV. Relative activity of furocoumarins (topical studies - human skin) (continued).

Compounds	Concentration ug/sq. in. area	Mean erythema response		Mean pigmentation response		Remarks
		Reflection meter % diff.(control-test area) with green filter	Degree of visible erythema	Reflection meter % diff.(control-test area) with green filter	Degree of visible pigmentation	
8. 4'5' Dihydro xanthotoxin	50	-	-	2	±	--
	100	-	-	2	±	--
	200	-	-	5	+	--
9. 4, 5' Dimethyl isopsoralen	50	-	±	2	+	--
	100	-	±	3	+	--
	200	3	±	3	+	--
10. Isobergapten	100	2	±	2	±	--
	200	2	±	3	±	--
11. 8 Isoamylenoxy psoralen	100	2	-	2	±	--
	200	2	+	2	±	--
12. 8 Hydroxy psoralen	500	-	-	-	-	--
13. Psoralen quinone	500	-	-	-	-	--
14. Isopimpinellin	500	-	-	-	-	--
15. 5 Nitroxantho toxin	500	-	-	-	-	--
16. 5 Amino xanthotoxin	500	-	-	-	-	--

Figure 12. Relative activity of furocoumarins (topical studies - human skin)

#1 Psoralen and 8-methoxypsoralen

#2 5-Methoxypsoralen and trimethyl psoralen

It can be seen that psoralen is the most active compound, followed by trimethyl psoralen, 8-methoxypsoralen and 5-methoxypsoralen.

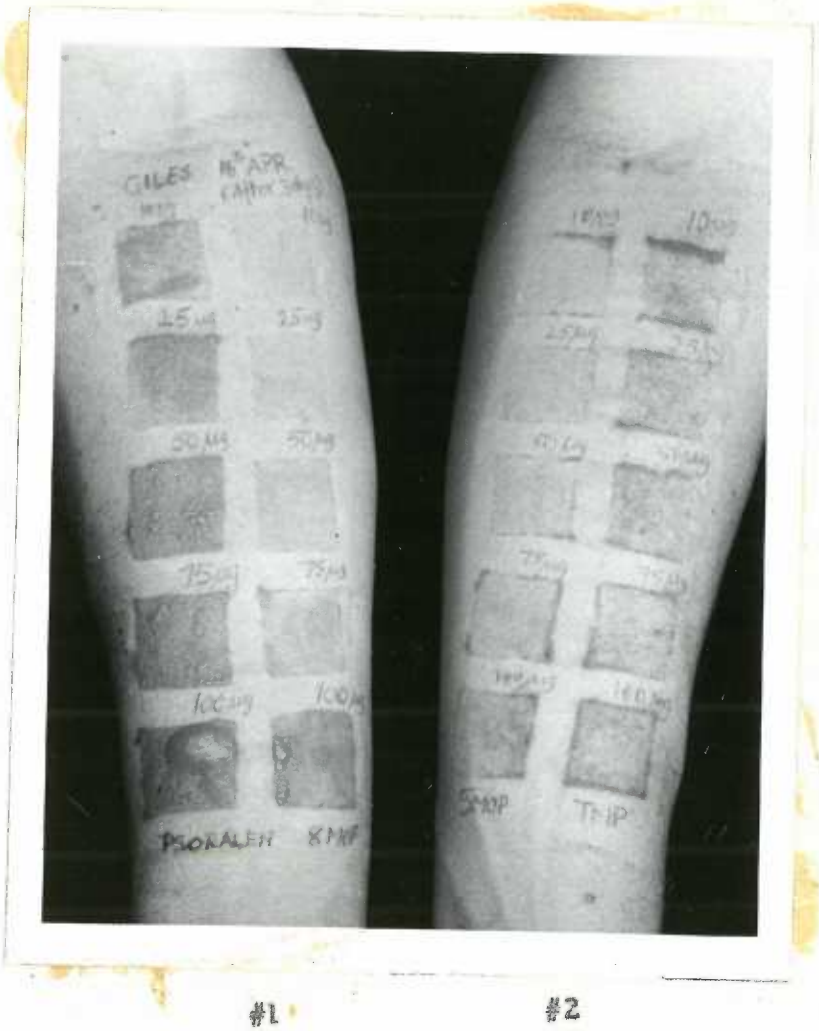


Figure 12. Relative activity of furocoumarins (topical studies - human skin)

#1 Psoralen and 8-MOP (8-methoxypsoralen)

#2 5-MOP (5-methoxypsoralen) and TMP (4, 5'8-trimethyl psoralen)

(It can be seen that psoralen is the most active compound, followed by trimethylpsoralen, 8-MOP (8-methoxypsoralen) and 5-methoxypsoralen)

and figure 12, that psoralen is the most potent compound, followed by trimethyl psoralen. The other 2 methyl derivatives were also quite active. All these 3 methyl substituted psoralens were second best compounds. Although trimethyl psoralen showed highest activity on guinea pig skin, but the difference in activity between psoralen and trimethyl psoralen was not very significant. On human skin psoralen always showed the highest erythema and pigmentation response.

8-Methoxypsoralen possesses intermediate activity. The degree of erythema and pigmentation with this compound was certainly less than psoralen or other methyl substituted psoralens. It did not even produce severe burns (blisters) as psoralen, or 5'-methyl psoralen or trimethyl psoralen had produced. 5-Methoxypsoralen was the 6th compound in order of activity.

5-Methoxypsoralen response in general was always less than 8-methoxypsoralen. Psoralen glucoside was less active than 8-MOP or 5-MOP. Even on molar basis, the erythema response was less than these two compounds. 4'5'-Dihydro-8-methoxypsoralen, 8-isoamylenoxy psoralen, 4,5'-dimethyl isopsoralen, and isobergapten were similarly less active. Other compounds like psoralen quinone, thiofurocoumarin, 5,8-dimethoxypsoralen, 5-amino and 5-nitro derivatives of 8-methoxypsoralen and 8-hydroxypsoralen were completely inactive. All these compounds, in general, showed activity in the same order as observed

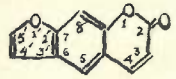
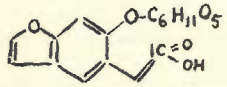
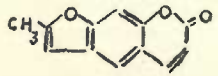
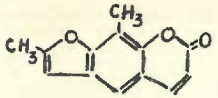
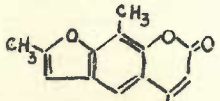
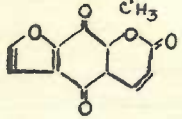
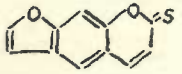
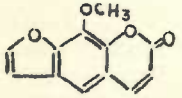
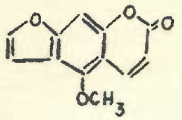
on guinea pig skin. The pigmentation response was very similar to erythema response. Psoralen treated areas showed highest pigmentation, followed by trimethyl psoralen. 5'-Methyl psoralen and 5',8-dimethyl psoralen were equally effective as trimethyl derivative. 8-Methoxypsoralen was the fifth best compound in this order of activity. The pigment response with 5-methoxypsoralen was always less than 8-methoxypsoralen. Thus, it appears that unsubstituted linearly angular structure of psoralen exhibits highest pigmentation inducing property.

The relative activity of these substances based on guinea pig and human skin responses has been summarized and presented in Table XVI, along with their structural formulae. The relationship between molecular configuration and the activity of the compound will be discussed under discussion section.

Rate of excretion of orally ingested furocoumarins.

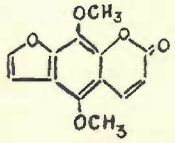
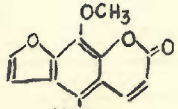
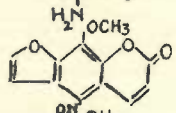
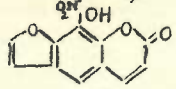
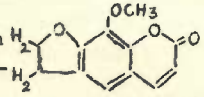
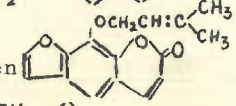
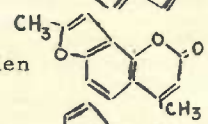
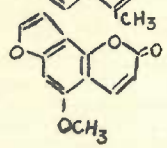
The rate of excretion of 4 compounds, namely, psoralen, 8-methoxypsoralen, 5-methoxypsoralen and trimethyl psoralen has been determined by guinea pig bioassay method. After orally feeding a large dose (25 mg. kg approximately) of each of these compounds separately to guinea pigs (as shown in figure 13), it was observed that the ingested compound reached its maximum concentration in blood at the end of 1 1/2 hours and exhibited optimum response between 1 1/2 to 3 hours. By the end of three hours, the effective concentration of each furocoumarin started

Table XVI. Relative activity of furocoumarins

Furocoumarin	Structure	Relative Activity on Albino Guinea Pig Skin					Relative Activity on Human Skin			
		Topical	Oral	M. E. C.* for erythemal response			Erythemal response		Pigmentation response at	
				Topical ug/sq. in.	Oral mg/kg	Degree of erythema	Topical	M. E. C.* ug/sq. in.	M. E. C.*	50 ug/sq. in.
Psoralen		++++	++++	5 ug.	3.72 mg (0.02 mM)	++	++++	10 ug.	++	+++
Psoralen glucoside		+	+	125 ug.	7.32 mg. 14.7 mg. (0.04 mM)	± + +	+	100 ug	+	-
5'Methyl psoralen		+++	+++	10 ug.	8 mg. (0.04 mM)	++	+++	10 ug.	+	++
5', 8 Dimethyl psoralen		+++	+++	5 ug.	4.3 mg. (0.02 mM)	+	+++	10 ug.	+	++
4,5', 8 Trimethyl psoralen		++++	++++	2 5 ug.	4.6 mg. (0.02 mM)	++	+++	10 ug.	+	++
Psoralen quinone		inactive	inactive	inactive up to 2000 ug.	inactive 25 mg.	inactive	inactive	inactive	inactive	inactive
Thio furocoumarin		"	"	"	inactive 20 mg.	"	"	"	"	"
8 Methoxypsoralen (8-MOP) xanthotoxin		+++	+++	15 ug.	4.32 mg. (0.02 mM)	+	++	10 ug ± 25 ug ±	±	+
5 Methoxypsoralen (5-MOP) bergapten		++	++	50 ug.	8.6 mg. (0.04 mM)	++	+	25 ug ± 50 ug +	±	±

*Minimum effective concentration

Table XVI. Relative activity of furocoumarins (continued)

Furocoumarin	Structure	Relative Activity on Albino Guinea Pig Skin					Relative Activity on Human Skin			
		Topical	Oral	M.E.C.* for erythemal response			Erythemal response		Pigmentation response at	
				Topical ug/sq. in.	Oral mg/kg	Degree of erythema	Topical	M.E.C.* ug/sq. in.	M.E.C.*	50 ug/sq. in.
5, 8 Dimethoxypsoralen (isopimpinellin)		inactive	inactive	inactive No erythema up to 2000 ug.	inactive 25 mg.	inactive	inactive	inactive	no pigmentation	no pigmentation
5 Amino 8 methoxy psoralen (5 NH_2 8-MOP)		"	"	"	inactive 32 mg.	"	"	"	"	"
5 Nitro 8 methoxy psoralen (5 NO_2 8-MOP)		"	"	"	inactive 25 mg.	"	"	"	"	"
8 Hydroxy psoralen (xanthotoxal)		"	"	"	inactive 28.5 mg.	"	"	"	"	"
4'5' Dihydro xanthotoxin (4'5' dihydro 8 methoxy-psoralen)		+	"	75 ug.	inactive 25 mg.	"	+	150 ug.	±	"
8 Isoamyleneloxy psoralen		+	"	100 ug.	inactive 25 mg.	"	+	200 ug.	±	"
4, 5' Dimethyl isopsoralen		±	"	500 ug.	inactive 20 mg.	"	±	150 ug.	+	"
Isobergapten (5 methoxy isopsoralen)		+	"	250 ug.	inactive 30 mg.	"	±	200 ug.	±	"

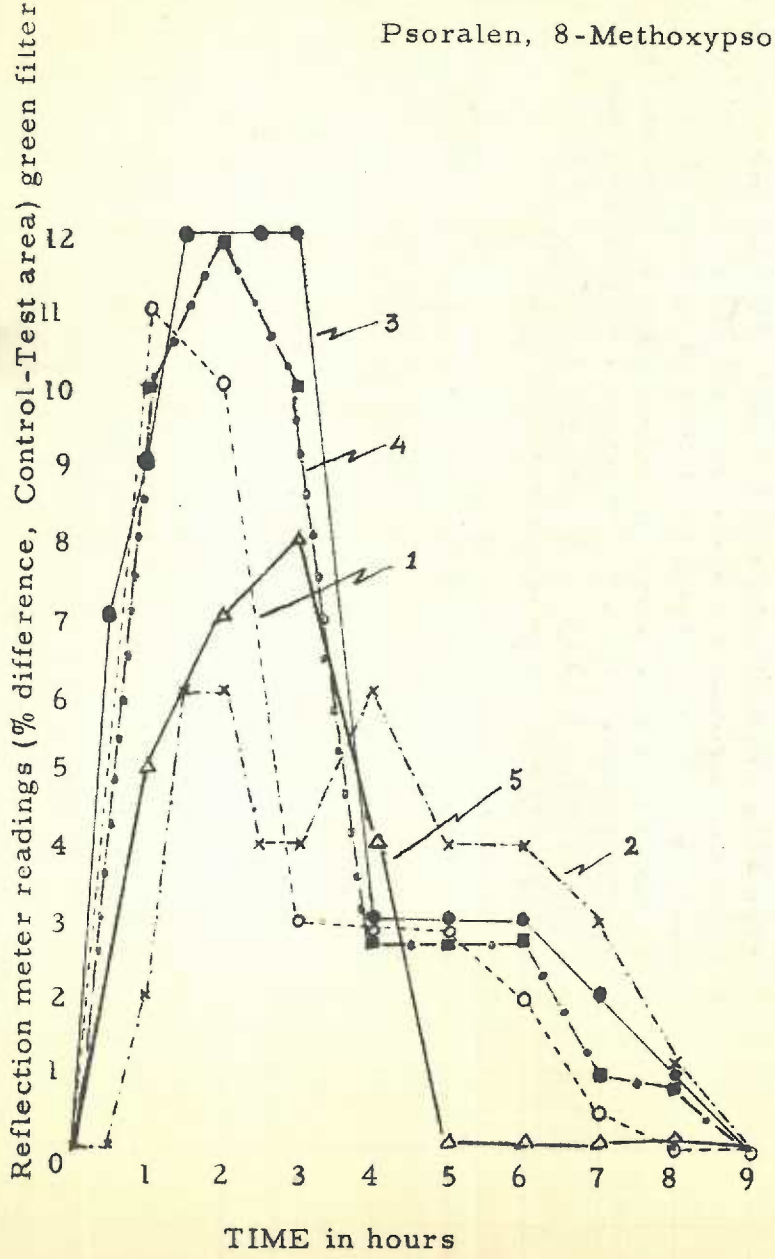
*Minimum effective concentration

Figure 13. Rate of excretion of active furocoumarins. Optimum response is observed by the end of 1 1/2 hours after feeding the compounds, and lasts up to 2 1/2 hours. By the end of three hours, the active concentration starts declining and by the end of 6 hours very little response is observed. It appears that at the end of 8 hours, most of the ingested compound is excreted out.

Figure 1-3

RATE OF EXCRETION OF ACTIVE FUROCOUMARINS

Psoralen, 8-Methoxypsoralen, 5-Methoxypsoralen, Trimethoxypsoralen



1. Psoralen. . . . 25 mg/kg. . . . o-------o
2. 8-MOP. . . . . .22 mg/kg. . . . x-.-.-.-x
3. " . . . . . 25 mg/kg. . . . •——•
4. 5-MOP. . . . . 25 mg/kg. . . . ■-.-.-.-■
5. TMP . . . . . 25 mg/kg. . . . △——△

Erythema reading after 24 hours.

declining and dropped to a very low level by the end of 5 hours. The ingested compounds were almost completely excreted at the end of 8 hours. This study also shows close agreement with the earlier studies in relation to the lag period reported in Table XII.

Acute toxicity of 8-methoxypsoralen and psoralen.

Whether psoralen and 8-methoxypsoralen, when administered in large doses, are highly toxic and produce lethal effects, was ascertained by acute toxicity studies. The number of deaths in each group for both these compounds have been tabluated along with other pertinent data in Table XVII and XVIII. It is evident that psoralen which had LD50 of 1000-1030 mg/kg, is the least toxic drug. 8-MOP showed similarly a wide range of LD50 (685-700 mg/kg). Human subjects are usually given 20 to 30 mg/70 kg. of 8-methoxypsoralen, considering this low therapeutic dose, and wide range of LD50 in mice, it can be said that these compounds will not be harmful even if a slightly large dose is given by chance at any time. There is a wide safety limit in therapeutic and toxic doses.

The organs of these mice, after their death were examined macroscopically and microscopically. The liver in these mice, particularly those receiving very large doses (500 mg/kg) showed white discoloration and cloudiness. The lungs appeared hemorrhagic and also there was evidence of intestinal hemorrhage. Other viscera, however, appeared normal.

Table XVII.

TOXICITY STUDIES — MLD and LD_{50} for 8-Methoxypsoralen

Groups	1	2	3	4	5	6	7	8	9	10	11
8-MOP(mg/kg)	control	100	200	300	400	500	600	700	800	900	1000
No. of mice	5	5	5	5	5	5	5	5	5	5	5
Average wt. (g) (± 0.5 g)	15.06	16.6	12.5	14.5	13.0	12.1	16.2	11.5	16.3	13.5	17.0
mg. 8-MOP/ mouse I. P.	0	1.66	2.5	4.35	6.5	6.05	9.12	8.05	13.04	12.15	17.0
Deaths after 24 hours	none	none	none	none	1	1	1	4	2	3	3
Deaths after 48 hours	none	none	none	none	none	2	none	none	none	1	2
Total deaths	none	none	none	none	1	3	1	4	2	4	5

Results:

MLD 400 mg/kg

LD50 (1) 685 mg/kg
(2) 700 mg/kg

LD 100% 1000 mg/kg

Table XVIII.

TOXICITY STUDIES MLD and LD_{50} for Psoralen

Groups	1	2	3	4	5	6	7a	7b	8a	8b	9	10	11	12	13	14
Psoralen(mg/kg)	con-trol	50	100	200	300	400	500	500	600	600	700	800	900	1000	1100	1200
No. of mice	10	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5
Average weight (G) (± 0.5 G)	14.8	17.2	19.5	21.0	20.9	21.1	21.3	12.8	24.8	14.5	12.7	15.0	12.8	14.5	14.5	15.2
mg. psoralen per mouse	0	0.86	1.95	4.2	6.27	8.44	10.65	6.4	14.9	8.7	8.9	12.0	11.52	14.5	15.95	18.24
Deaths after 24 hours	none	none	none	none	none	none	none	none	none	none	none	2	2	none	none	3
Deaths after 48 hours	"	"	"	"	"	"	"	"	"	"	"	none	2	1	1	2
Total deaths	none	none	none	none	none	none	none	none	none	none	none	2	4	1	1	5

Results:

MLD = 800 mg/kg

LD50 (1) 1000 - 1100 mg/kg
(2) 1030 " "

LD 100% 1200 mg/kg

Microscopic findings:

Liver cells showed fatty degeneration near the peripheral portion. The fatty globules tended to be smaller and partially filling the hepatic cells. The cells appeared congested. There was inflammation in the blood vessels. It appeared that there was lipoid depletion of liver. Glomeruli and nephrons appeared congested. In spleen, there was evidence of congestion Lymphocytes and erythrocytes were seen in large number. The spleen presented a picture of relative enlargement probably due to the portal congestion.

Chronic toxicity effect:

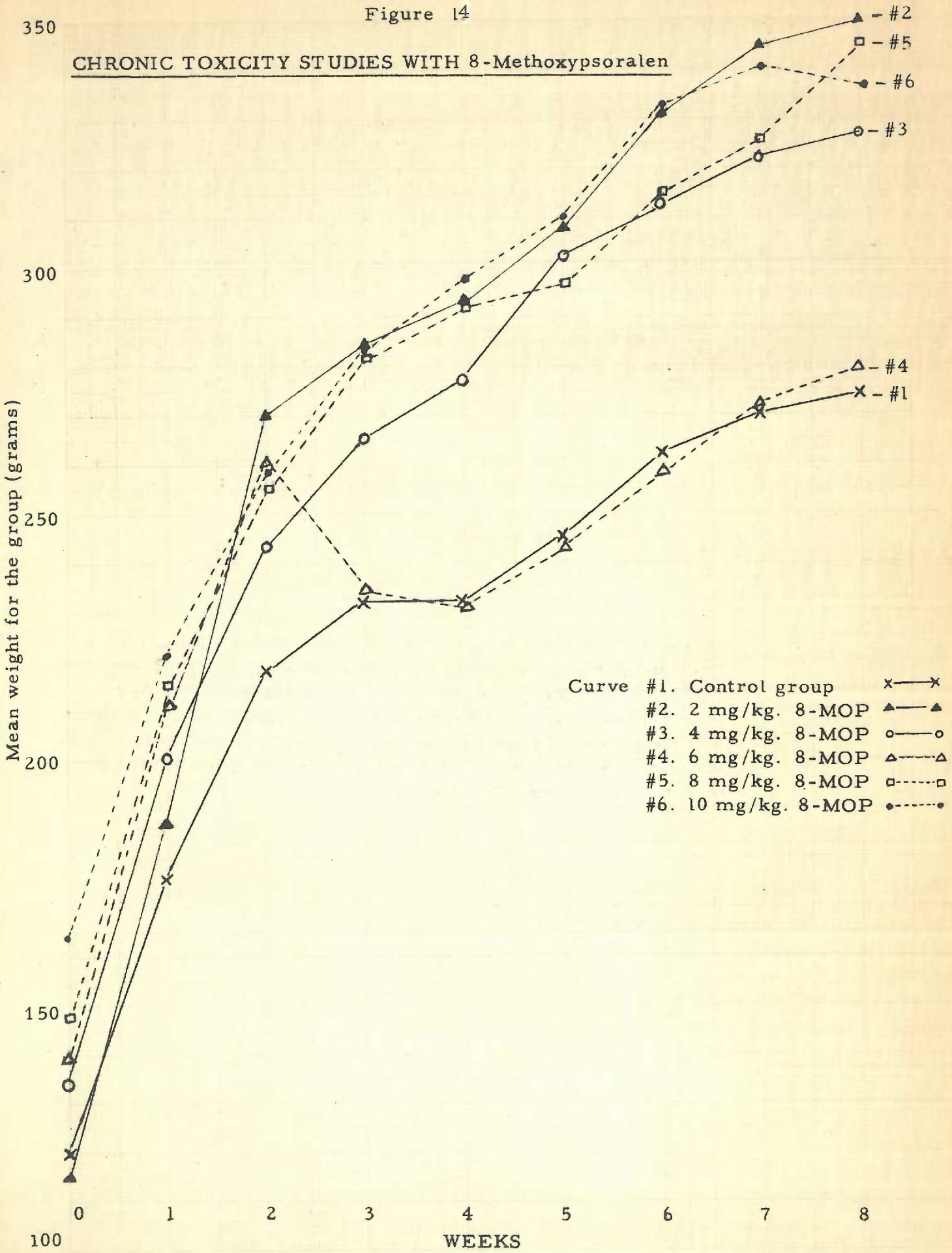
The growth rate for a period of 8 weeks has been plotted for all the 5 groups receiving varying concentration of 8-methoxypsoralen and along with the control group, this data has been presented in figure 14. The effect of daily injecting 8-methoxypsoralen intraperitoneally in these groups has been statistically investigated with a view to know whether the increase or decrease in weights at the end of 4 weeks and 8 weeks period was in any way of statistical significance.

Figure 14 shows the average gain in weight for all the groups for a period of 8 weeks. It can be seen that 4 of the groups receiving 8-methoxypsoralen showed invariably higher gain in weight as compared to control group. Only one group receiving 6 mg/kg. 8-methoxypsoralen showed some indication of weight loss. But the other 2 groups receiving 8-MOP in higher dose (in the order of 8 and 10 mg/kg. respectively)

Figure 14. Chronic Toxicity Studies with 8-Methoxypsoralen. The mean gain in weight for different weeks for each group receiving varying concentration of 8-methoxypsoralen has been plotted along with the weight of the control group. Note that all groups showed gain in weight. 8-Methoxypsoralen appears to be non-toxic substance. This data has been also analysed statistically (Table XIX).

Figure 14

CHRONIC TOXICITY STUDIES WITH 8-Methoxypsoralen



did not reveal any loss in weight. Hence "t" test was carried out for group mean gain in weight. As shown in Table XIXB, "t" values for control versus different 8-methoxypsoralen groups shows only one statistically significant value (for group receiving 2 mg/kg 8-MOP - t value was found to be 3.45 and 3.6 for 4th week and 8th week respectively, which is statistically significant at 0.05 level). This indicates that 8-methoxypsoralen in lower doses stimulated the growth. Otherwise "t" values for rest of the groups were not statistically significant.

Since the effect of 8-methoxypsoralen was to be evaluated in terms of growth as represented by gain in weight, it was essential to find out that the differences in weight gains or losses for all the groups receiving varying doses of 8-methoxypsoralen were largely due to 8-methoxypsoralen effect and not due to some influence of initial weights of mice in each group. Therefore, the weight gains were analysed by analysis of covariance procedure which adjusted the group means for the difference in the initial weights. The resultant analysis (summarized in Table XIXA) gave a variance ratio of 0.46 and 0.37 at four weeks and 8 weeks period. From the small variance ratio (F) it is concluded that no difference in weight gains among the group is statistically demonstrable.

It is thus clear that continuous administration of 8-methoxypsoralen does not lead to toxic symptoms. These dose levels of 8-methoxypsoralens (from 2 to 10 mg/kg) are in fact, much higher than needed

Table XIX.

A. Chronic toxicity studies with 8-methoxypsoralen.

Analysis of covariance	Degree of freedom-df	Initial weight x^2	xy		Gain in weight- y^2		Mean square (weight)		Variance ratio 'F'	
			4th week	8th week	4th week	8th week	4th week	8th week	4th week	8th week
Among group	5	150.36	-51.86	-84.35	437.23	613.04	87.5	122.6	.46	0.37
Within group	54	15.0	-34.86	-5.97	937.04	1658.47	17.35	30.7		
Total	59	165.36	-86.72	-90.32	1374.27	2371.51				

"t" values within groups: 4th week = 0.59, 8th week = 0.73; mean standard error: 4th week $\pm$ 0.389, 8th week $\pm$ 0.54

B. "t" Test -- Comparison of group means.

Comparison of group means	Control vs. 2 mg/kg 8-MOP 't' for difference	Control vs. 4 mg/kg 8-MOP 't' for difference	Control vs. 6 mg/kg 8-MOP 't' for difference	Control vs. 8 mg/kg 8-MOP 't' for difference	Control vs. 10 mg/kg 8-MOP 't' for difference
4th week	+3.45*	+1.62	-0.43	+1.71	+0.83
8th week	+3.6*	+1.78	-0.38	+1.82	+1.2

* Significance at 0.05 level.

for human use. The low toxicity is also evident from the very low death incidence. The total number of deaths for this period was as follows: control -- 2; 8-MOP groups: 2 mg/kg -- none, 4 mg/kg -- 1, 6 mg/kg -- 4, 8 mg/kg -- none, 10 mg/kg -- none.

The microscopic findings in all these 50 mice under 8-methoxypsoralen trial did not reveal any gross pathological changes.

The liver cells appeared normal, there was no evidence of necrosis, and rarely a degerative type of nucleus was seen. The characteristic findings in few mice was the evidence of inflammation process around the blood vessels, round cells resembling polymorphonuclear cells were seen infiltrated round this area. Sinusoids appeared normal. The kidney showed no gross pathological change. Glomeruli and nephrons appeared normal. The tubules showed no change. Spleen, however, showed hyperplasia of lymph follicles, which appeared to be proliferating outside. Sinusoids appeared congested. Lymphocytes and erythrocytes were seen in proportionately increased number. The heart, lungs, and intestines showed no detectable pathological changes.

Intraoccular effects of 8-methoxypsoralen (chronic effect).

The cumulative effect of oral administration of 8-methoxypsoralen on eye (such as the changes in the intraoccular tension, retintis and retinal pigmentary changes, odema of cornea or changes in the opacity of lens, etc.) were examined. Since one eye of each guinea pig (left eye) served as its own control (receiving no ultraviolet radiation) pathological

changes in the intraoccular region in the ultraviolet radiated eyes were easy to be detected. In this long duration experiment lasting for 7 months, the animals were examined four times. At no time, any definite or even any suggestive pathological change in any of the animals' eyes was observed (Table XX). The radiated eyes appeared normal. There was neither any visible intraoccular tension or pigmentary changes detected (by Dr. John Harris). This study indicates reasonably, that there is no harmful effects on the eye after oral administration of 8-methoxypsoralen.

Intraoccular effect of 8-methoxypsoralen (acute effect).

Table XXB. The aspirated aqueous specimens after feeding massive dose of 20 mg/kg (= 1.4 gram/70 kg) did not reveal any presence of either free 8-methoxypsoralen or 8-methoxypsoralen bound with any protein fraction. 8-Methoxypsoralen is highly fluorescent material (as low as 2 to 4 ug can be detected by fluorescence test), even in combined form with any protein fraction (as in serum) it exhibits a characteristic fluorescence.

If 8-methoxypsoralen (atomic weight 216) or protein bound fraction of this molecule were permeable into the aqueous humor through the vascular capillary bed of cilliary bodies of the eye, it would certainly be detected in aqueous humor. Absence of any 8-methoxypsoralen in the aqueous fluid favors the hypothesis that this molecule does not diffuse into the aqueous humor.

Table XX. Intraoccular effect of 8-methoxypsoralen.

A. Chronic effect

Animals (guinea pigs)	U. V. radiation >3200 A°	Dose of 8-MOP mg/kg (oral)	No. of days radiated	Left eye -- $\bar{s}$ u. v. radiation Control eyes	Right eye -- $\bar{c}$ u. v. radiation Radiated eyes
With 8-MOP:					
2 brown	15 min/day 6 days/week	50	15 days	No change	No change
1 albino	" "	150	2 months	No detectable pathological change	No detectable pathological change
Without 8-MOP:					
1 control brown	" "	308	5 months	No detectable pathological change	No intraoccular tension, retinal or pigmentary changes, no detectable change in lens, cornea or fundus

B. Acute effect

Animals	Dose	Total aqueous humor specimens	Observations
3 guinea pigs	20 mg/kg 8-MOP	6	Clear fluid 1) No visible fluorescence under ultraviolet. 2)Paper chromatography: showed no spot and fluorescence of 8-MOP, or 8-MOP-protein complex (not even in micro quantities).
2 guinea pigs	without 8-MOP	4	Clear fluid, no fluorescence

Thus it is evident from both these studies (acute and chronic effect), that no harmful effects to the guinea pig eyes are associated with the intake of this compound.

Mechanism of 8-methoxypsoralen action.

Tyrosine solutions can be oxidized to dihydroxyphenylalanine after a long time u. v. radiation (Arnow, 102; Fitzpatrick, et al., 101). Whether photodynamically active psoralen or 8-MOP can potentiate this oxidation and thus indicate a link in the in vivo mechanism of psoralen action in the process of skin pigmentation, was examined by radiating solutions of tyrosine with and without a photodynamic agent. It can be seen from Table XXIA that chromatographically as well as colorimetrically no dopa formation in detectable quantities was observed in presence of long wave as well as short wave ultraviolet radiation. It appears that this oxidative mechanism requires a long time ultraviolet radiation probably of a high intensity. The photodynamically active molecules do not accelerate the oxidative process.

In vivo studies.

The skin section (only epidermis) obtained at the end of 72 hours after radiation reveal interesting observations. The incubated specimens in presence of 3,4-dihydroxyphenylalanine showed that melanin producing cells (melanocytes) from 8-methoxypsoralen treated group (topical as well as oral) were more "dopa" positive, they were deeply pigmented, suggesting more dopa oxidase activity and increased con-

Table XXI. A. Mechanism of 8-methoxypsoralen action.

Solutions	U. V. radiation	No. of petridishes radiated For 1 hr.	No. of petridishes radiated For 2 hrs.	Observations Colorimetric	Observations Paper chromatography
1. 5 ml. tyrosine	1) 3200 A°	3	3	No DOPA* detected in any specimen	No DOPA* spot observed
	2) 2200 - 4000 A°	4	2	" "	" "
2. 5 ml. tyrosine + 50 ug/ml. 8-MOP	1) 3200 A°	3	3	" "	" "
	2) 2200 - 4000 A°	3	3		
3. 5 ml. tyrosine + 50 ug/ml. psoralen	1) 3200 A°	4	4	" "	" "
	2) 2200 - 4000 A°	3	4		

B. Histologic studies.

Animals (C57 black mice) tail region	No. of animals	Dose of 8-MOP	U. V. 3200 A°	Observations (after 72 hours) Relative DOPA* + ve melanocytes
a. control	3	none	none	± to +
b control	4	none	30 minutes	+
c. 8-MOP topically	4	500 ug	30 "	++ to +++
d. 8-MOP orally	5	20 mg/kg	30 "	++ to +++

* 3, 4-dihydroxyphenylalanine

version of dopa to melanin. The melanocytes of the control group receiving no 8-MOP (with and without u.v. radiation) were light in color (weak "dopa" positive reaction) they appeared less pigmented (see Table XXIB).

It appears that long wave ultraviolet radiation which is otherwise not activating the process of melanogenesis, is activating these pigment producing cells in presence of a photodynamically active molecule like 8-methexypsoralen. The enzyme tyrosinase is probably released from the inactive state and activated to form more melanin pigment.

DISCUSSION

A photochemical reaction observed in a photodynamic action involves at least 3 steps: 1) activation of the sensitizing molecule (a photodynamic agent), 2) collision of this activated molecule with the substrate and 3) collision of the substrate with the oxygen. All of these factors are known to influence or determine the overall photodynamic reaction rate. Blum (103) has also presented evidence to indicate that the effectiveness of photodynamic process (as represented by red cell hemolysis) largely depends on the extent to which photosensitizing substances are taken up by erythrocytes The manner of uptake is also important. substances that penetrate the cells (such as neutral red, methylene blue, etc.) are less effective than those that are held at the surface of the cell (fluorescein dyes).

The degree of hemolysis observed in presence of psoralen and 8-methoxypsoralen in the present study is undoubtedly low, and hence presents a difficulty in revealing an identical hemolytic picture as observed in presence of eosin or other dyes, where the degree of hemolysis is significantly high. But the fact that there is definite hemolysis in presence of oxygen is more important. The low degree of hemolysis observed in these experiments may be due to several causes. It is possible that the outer layers of the red cell suspension containing psoralens, absorb so much of incident energy that the intensity reaching the inner layers of cell is much reduced and hence ultimate photodynamic hemolysis is very less.

There may be another possibility to suggest that 8-MOP or psoralen which are permeable to cell wall, are not held at the surface of the cell in enough concentration, so as to induce a high degree of photosensitized hemolysis. It is also possible that under u. v. radiation first phase of photodynamic effect, namely, "photochemical process" is taking place, but the subsequent lytic process resulting in the rupture of the cell wall and hemolysis is of low magnitude and only confined to particularly susceptible cells (of different age, size and structure).

But in general there was hemolytic cell damage in presence of oxygen, light and photosensitizing compounds. In absence of oxygen this hemolytic process was not observed. The <u>in vivo</u> photodynamic action

on rabbit ears manifested in the form of erythema also shows that the presence of oxygen enhances this biological action. In absence of oxygen this photodynamic action could not be demonostrated.

Fowlks (99) has investigated the photosensitization of bacteria by psoralen and related compounds. His findings, however, do not support Blum's hypothesis and observations regarding the role of oxygen in a photodynamic process. He believes that simultaneous presence of bacteria and sensitizing compound is only required to bring about long wave ultraviolet induced photodynamic effect. He, however, comments that oxygen may enhance the photosensitizing action of many compounds but that photodynamic process is not necessarily oxygen dependent.

It is difficult to comment more precisely about the role of oxygen, whether or not it is definitely required in a photodynamic action brought about by psoralen and its derivatives, but the fact that presence of oxygen enhances this action both in vitro and in vivo tends to suggest that this process of photodynamic action, if not entirely dependent on oxygen, is definitely ficilitated by its presence. There still remains to be investigated the role oxygen has to play in this complex photosensitized reaction.

The effects generally observed in sun burn action and psoralen action are similar, but the mechanism for the two are fundamentally different. Psoralen action is not just a sun burn action, it is a photo-

dynamic action. Photodynamic process, as stated earlier, requires three characteristic features, namely, 1) photodynamic action spectra are of longer wavelength, generally greater than 3300 A°, 2) it requires a long latent period, 3) it occurs in presence of oxygen only. Sun burn can occur in absence of oxygen. The action spectrum for sun burn is limited to radiation shorter than 3200 A°, where as most of the photodynamic processes are exhibited in longer wavelength.

In vitro studies of red cell hemolysis also confirms the wavelength and oxygen requirement for a photodynamic process. Hemolysis by wavelengths shorter than 3300 A° takes place independently of O_2, whereas photodynamic hemolysis requires presence of oxygen and wavelength greater than 3300 A°. Longer wavelengths do not induce cell hemolysis without the presence of a photodynamic agent.

The present study with psoralen and its derivatives reveals all these features described by Blum (103). One can say that psoralen action is a photodynamic action.

As reported earlier in introductory chapter, photosensitization of the skin following contact with various species of plants is a very common observation. The causative factors have been usually investigated in a qualitative way, generally by rubbing the extracts on human skin. A laboratory method for determining photodynamic activity was a necessity. Using various bacterial cultures, Fowlks, et al (84) have standardized an excellent method for determining photodynamic activity

for various compounds. However, many of the compounds which were reported by them to be photodynamically active on bacteria did not reveal the same photosensitization response on human skin and guinea pig skin in this study. It was therefore necessary to standardize a bioassay method for screening active and inactive compounds which could exhibit photodynamic action on mammalian skin. By topical and oral feeding, it has been possible to demonstrate photodynamic activity of any photosensitizing compound. Besides screening the compounds for their activity, this bioassay method has an advantage of determining the relative activity of various compounds.

Keeping the distance of radiation and source of light constant, albino guinea pig skin exhibits an easy means to evaluate comparative photodynamic response. By observing the standard conditions for lag period, and total ultraviolet radiation time, any compound can be tested and assayed quantitatively for its photosensitizing action. Topically as well as orally photodynamic response can be elicited. No such quantitative method has been so far proposed or standardized. The relative response on guinea pig skin for all the compounds which were tested, was very similar to the one observed on human skin.

By topical application and oral feeding of different furocoumarins, coumarins, furochromones, a relationship of the molecular configuration to the activity of the various compounds has been possible to be evaluated both on guinea pig skin and human skin. As stated earlier, the components

of furocoumarin, psoralen molecule, are furan ring and coumarin ring of lactone configuration. This basic molecule and some of its derivatives are active in long wave ultraviolet light and bring about biological changes of augmented erythema and pigmentation.

The furan ring does not possess any erythema inducing action. The coumarin ring configuration compounds totalling 27 do not show any photoactivation on guinea pig skin. Even when tested on human skin they were inactive,(orally as well as topically.) Even the different monomethyl, dimethyl and trimethyl derivatives of coumarin which presumably increase the electron density, do not render the compound to become active. But the fused ring structure of furocoumarin molecule, psoralen shows the highest activity. The question therefore arises as to what are the structural specificity and active centers which influence the activity. Firstly, the absorption spectra of psoralen molecule reveals three peaks in the region of 2450 A°, 2950 A° and 3200 A°. Though the action spectra and absorption spectra for photodynamic activity of this molecule have not been confirmed to suggest the active wavelength which brings about photodynamic action, but the absorption in long wave ultraviolet region can be said presumably to endow a property in this molecule to be photodynamically active (Daniels and Fitzpatrick, 96). Long wave ultraviolet light as such does not cause increased erythema and pigmentation, but in presence of this molecule these biological changes are observed.

There are active sites in this molecule which are favorable for photon induced activation. The active regions in psoralen molecule are (a) valence bonds between carbon 3 and 4, (b) carbon atoms 5 and 8, (c) intact lactone ring, (d) furan and coumarin ring fusion at carbon 6 and 7 to give linearly angular structure of furocoumarin molecule, (e) unsaturated linkage between carbon 4' and 5'.

Furochromone molecule as studied in three of its derivatives, khellin, visnagin and chellol glucoside, has an altered 3-4 valence bond. These compounds are inactive. Fowlks (86) has shown that open lactone ring compound furocoumaric acid without any substitution at other carbon atoms, is an inactive molecule. 2-Thiofurocoumarin molecule, in which oxygen of the keto group has been replaced by sulfur, in completely inactive molecule both topically as well as orally.

Methyl radicals which are known to increase the electron density and carcinogenic activity in carcinogenic compounds, however, do not increase the erythema inducing property of psoralen in human skin. But trimethyl psoralen exhibits highest response in guinea pig skin as compared to other active compounds. Methyl substitution does not affect the active centers in psoralen molecule by virtue of its electron donating property. It can be said that methyl derivatives exhibit equally high activity and that methyl substitution does not alter the activity of psoralen molecule.

8-Methoxypsoralen, 5-methoxypsoralen are less active compounds. 5-Methoxypsoralen shows less activity than 8-methoxypsoralen. Substitution with methoxy groups at carbon 5 or 8 results in decreased activity. 5, 8-Dimethoxypsoralen is completely inactive. Substitution with amino or nitro group at carbon 5 or hydroxyl group at carbon 8 reveals the same effect of inactivation. It appears that substitution with methoxy, nitro, amino or hydroxy group results in decreased electron density at carbon atoms 5 and 8 which therefore render the compound inactive. Unsubstituted molecule of psoralen (without methoxy group at carbon 5 or 8) shows highest activity.

4', 5'-Dihydro-8-methoxypsoralen possesses very low activity when applied topically and when given orally does not show any photoreactivation. Methyl substitution at carbon 5' does not alter the activity. The unsaturated linkage is needed at these carbon atoms. Hydrogenation of furan ring completely alters the activity of furocoumarin molecule.

The two components of furocoumarin ring, furan and coumarin, do not exhibit photodynamic activity. Only when they are fused together, the resultant molecule shows photodynamic response. Non-linear angular structure present in isopsoralen or isobergapten molecules where the furan ring is not attached at carbon atoms 6 and 7 as in psoralen molecule but is attached at carbon atoms 7 and 8 instead, results in the loss of photodynamic activity of the molecule.

The linkage of furan ring with coumarin ring at carbon 6 and 7 is essential. Furan ring attached to carbon 7 and 8 as shown in isobergapten and dimethyl isopsoralen structures inactivates these compounds. It is apparent that photodynamic activity of the furocoumarin molecule is directly related to its molecular configuration. Substitution with methoxy, hydroxyl, nitro or amino groups at carbon atoms 5 and 8 positions alter or reduce the activity of unsubstituted psoralen molecule. If there is substitution at carbon atoms 5 and 8 simultaneously, the resultant molecule loses its photodynamic action. Hydrogenation of furan ring at carbon 4' and 5' results in virtual loss of photodynamic activity. Substitution of oxygen with sulfur at 2 keto group similarly results in complete loss of photosensitizing activity of the furocoumarin molecule. Angular and non-linear structure of isopsoralen molecule (isobergapten, dimethyl isopsoralen) exhibits practically very little photodynamic response topically and none orally. It is thus evident that linearly angular unsubstituted structure of furocoumarin molecule like psoralen possesses the maximum activity. Non-linear structure loses its activity.

The toxicity effect of psoralen and 8-methoxypsoralen has been investigated from various angles. Not only it has been possible to determine the acute toxicity and chronic toxicity of the two important compounds, but also its rate of excretion and the effect it might produce on mammalian eye which is so vital for life. That psoralen and 8-methoxypsoralen can be easily tolerated without toxic effects, is evident from the

LD50 range. Psoralen, which showed LD50 of 1000-1030 mg/kg and 8-methoxypsoralen, which had a range of 670-700 mg, if converted to body weight of a human subject, would be equivalent to 70 grams and 49 grams per 70 kg body weight respectively. The human requirements for these compounds are only 20-30 mg/70 kg. It would not be inappropriate to say, therefore, that these compounds are least toxic. The chronic toxicity studies further supports this observation. Not only the average weight data, but also the statistical data, conclusively show that these compounds do not induce toxic effects. The experimental animals receiving 8-MOP gained more weight than the control group. There was no such indication in any of the groups which suggested that 8-MOP acted as a toxic drug. The microscopic findings particularly of liver, kidney, and spleen further adds support to this statement that 8-MOP is relatively non-toxic drug. The excretion rate study further reveals that they are quickly eliminated from the system. Undoubtedly this excretion rate data does not rule out the possibility of the retention of these compound in liver cells, but certainly it can be said that these compounds are fairly rapidly eliminated from the system. The concentration of psoralen, 8-MOP and other compounds left in the system at the end of 8 hours is way below the minimum effective concentration for producing erythema (by oral feeding). Observations made on mice liver after feeding heavy doses of 8-MOP reveal that there is no deposition of this compounds. Presence of this substance would have been detected by its characteristic

fluorescence. In reference to the toxicity effect of these compounds, recent studies carried out by Fitzpatrick, et al., on 24 normal human subjects, before, during and after oral administration of 30 mg. 8-methoxypsoralen deserves special mention. In this study, a double-blind experimental design was used. A group of 12 subjects received 8-MOP and another 12 received placebos for a period of 3 months. There was no significant difference in the battery of liver function tests they carried out in these two groups. No subject had a change in liver function tests that would indicate liver damage.

Effect of feeding 8-MOP orally to guinea pig for a period of 7 months in concentrations approximately 8 to 10 times more than the human requirements, further reveals that there are no toxic reactions associated with the daily intake of this compound. The animals were in excellent healthy condition. The absence of any intraoccular toxic effect on eyes removes the fear that psoralen or 8-MOP, which are photodynamically active agents, might induce harmful effects to the eyes, particularly effects such as changes in intraoccular fluid tension, odema, retinitis or sypmptoms similar to pigmentary glaucoma. These guinea pigs were, in fact, exposed to large doses of ultraviolet radiation every day, unlikely to be experienced at such high levels at any single occasion by a person. The absence of any intraoccular pathological changes after such prolonged treatment certainly favors to comment that 8-MOP has no harmful effects. The anatomy of the human eye, its location in a

well protected area of the face where direct solar radiation do not approach, further removes the fear that this molecule might induce harmful effects. People in Egypt, India, Europe and even in the U.S. have been taking psoralen for the past several years. So far no harmful ocuular effects have been reported from any quarters. It can be said that psoralen and 8-methoxypsoralen do not induce any structural, functional or biological toxic and harmful effects if given in a well controlled manner.

The in vitro and in vivo studies in the mechanism of psoralen or 8-MOP action towards promoting skin pigmentation does not conclusively reveal the exact mechanism of action of these photodynamically active compounds. Psoralen or 8-MOP did not promote "dopa" (3,4-dihydroxyphenylalanine) formation from tyrosine in presence of long wave ultraviolet radiation (>3200 A°), as well as ultraviolet radiation from high pressure mercury arc lamp emitting radiation in the range of 2200 to 4000 A° (more of the short and middle ultraviolet rays). Dopa is well known to accelerate the tyrosine to melanin reaction (Fitzpatrick)[100,101]. Although formation of small amounts of "dopa", which may then catalyze the formation of larger amounts, has attractive aspects, but the in vitro absence of dopa formation suggests very little possibility of its in vivo formation. Ultraviolet radiation is no doubt, known to promote dopa formation from tyrosine, but this requires very large doses of carbon arc radiation. It is possible that dopa might have been formed in micro

quantities in the *in vitro* system, but the colorimeteric and chromatographic methods were not sensitive enough to detect such micro quantities.

In vivo studies by topical application of 8-methoxypsoralen to mice skin and its subsequent exposure to ultraviolet rays, however, brings about alteration in the activity of the melanocytes. These melanocytes were more darker in color before incubation with 'dopa' solution as compared to control mice with and without ultraviolet radiation. After incubation in 'dopa' solution they showed a strong positive 'dopa' reaction. They were deeply pigmented, suggesting high 'dopa' oxidase activity.

The dentric cells in the control groups were much lighter in color before incubation in 'dopa' solution, after incubation, the degree of darkening was still quite less. The degree of darkening reveals the relative activity of melanocytes which ultimately reflects the activity of the enzyme tyrosinase. The strong dopa positive reaction favors the hypothesis that psoralen activates these melanocytes and promotes melanogenesis by releasing the tyrosinase from its inhibitors. It appears that primary effect of psoralen action in presence of ultraviolet radiation is the injury to the cells and this injury initiates the process of melanization. This injury may lead to the removal of inhibiting substances and activate tyrosinase.

Besides activation of melanocytes, it is possible that psoralen action may promote the increase in number of melanocytes due to epidermal injury and thereby promote more pigmentation,but apparently in this study there was no evidence of increased number of melanocytes. After a continuous irradiation, however one does find increased number of melanocytes. It appears, therefore, that psoralen action promotes melanogenesis through both of these processes.

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