PHOTOREACTIONS BETWEEN FLAVIN COENZYMES AND SKIN-PHOTOSENSITIZING AGENTS

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It is known that a furocoumarin, xanthotoxin or 8-methoxy-psoralen, is routinely used in the treatment of vitiligo, a skin disease which is defined as "an acquired cutaneous achromia characterized by variously sized and shaped single or multiple patches of milch white color[1]", lacking the pigment normally present in man's skin.

This disease affects all human races, so to speak, particularly those with a more heavily pigmented skin, although the incidence differs greatly from country to country. According to recent statistics, the incidence in some regions of the United States of America would be at least one per cent of the population; in India, this figure is even higher and, according to some authors, may even reach three per cent[2].

Vitiligo was treated in very ancient times by popular medicine in Egypt and India, through the ingestion of drugs, such as the fruit of *Ammi majus* (in Egypt) or grains of *Psoralea corylifolia* (in India), and successive exposure to sunlight.

Nowadays, in practice, matters have not changed because xanthotoxin is merely one of the substances present in *Ammi majus*. Moreover, *Psoralea corylifolia* contains psoralen and angelicin (isopsoralen)—two other furocoumarins.

These three substances and another, still of the same family, bergapten (5-methoxy-psoralen), form the group of so-called photodynamic natural furocoumarins, as they have the property, when applied to the skin, of provoking erythema followed by pigmentation on successive exposure to sunlight or ultra-violet light.

These substances are responsible for some types of photodermatitis which appear through contact of the skin with some plants, such as fig leaves, *Ruta graveolens*, *Pastinaca sativa*, *etc.*, and also with essence of bergamot, which is used in the preparation of eau-de-cologne. In fact, this essence contains bergapten. Exposure to sunlight is always necessary.

Present treatment of vitiligo with xanthotoxin is carried out by administering the substance orally or else painting it on the leukodermic patches and then exposing them to sunlight or ultra-violet light. In many cases, a slow re-pigmentation of the leukodermic patches is obtained. The most effective radiations for this purpose are those falling within the long ultra-violet waves.

The effects obtained with xanthotoxin treatment are a clear example of

photosensitizing effect and are to be classified within the wider field of photodynamism of furocoumarins.

My colleagues and I have for many years devoted our efforts to these studies, which have passed through various stages, by means of which we have recently arrived at the point of revealing a photoreaction between flavin coenzymes and skin photosensitizing substances. This is the subject of the present paper.

When we began to study this subject little was known about it; the only certain data were those of Kuske[3] obtained with two furocoumarins, bergapten and oxypeucedanin, which he found to be responsible for some types of photodermatitis due to vegetables. As I have already mentioned, xanthotoxin was later introduced into the treatment of vitiligo[4].

We first dealt with the problem of the relationships between the chemical structure and the photosensitizing activity; by extraction from vegetable products or by synthesis, we have succeeded in obtaining about sixty furocoumarin derivatives, besides many other coumarins. On testing these by means of tests which we ourselves have perfected, we have been able to obtain a quantitative, although rough, estimation of the activity of all these substances[5–12]. Photosensitizing furocoumarins and their activities are given in *Table 1*. The quantitative results which have been obtained on testing these substances on human skin, are given in *Table 2*. We have taken the activity of psoralen, which is the most active of all the substances, as 100.

The main relationships between chemical structure and photosensitizing activity which appear clearly from our results and which were confirmed some years later by analogous researches carried out by American investigators[13–15], are summarized in *Table 3*.

(*i*) The most active substance is psoralen, the parent furocoumarin; the activity is undoubtedly linked to the furocoumarinic nucleus: hydrogenation of the double bond of either the furan or the α-pyron ring destroys the activity.

(*ii*) In the furocoumarinic system the linear structure is more active than the angular one; in fact, psoralen and bergapten are much more active than angelicin, isobergapten and allobergapten.

(*iii*) The introduction of a hydroxyl group in position 5 or 8 of the psoralen leads to inactive compounds (bergaptol, xanthotoxol). Methylation of these hydroxyls re-establishes a large portion of the lost activity; in fact, xanthotoxin and bergapten are, along with psoralen, the most active natural furocoumarins.

Lengthening of the alkyl chain reduces the activity gradually to zero (5-ethoxy-psoralen, 5-isopropyloxy-psoralen, 5-n-propyloxy-psoralen, 5-n-butyloxy-psoralen, *etc.*).

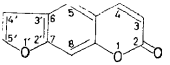
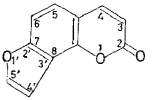
Introduction of two methoxy-groups in the 5 and 8 positions causes the compound to be inactive (isopimpinellin).

(*iv*) Introduction of methyl groups in various positions of the molecules of psoralen, xanthotoxin and bergapten lead, in general, to a lessening of activity.

(*v*) Introduction of nitro-, amino-, acetylamino-groups cancels the activity of the parent compound.

The activity of these substances, *i.e.* mainly of psoralen, xanthotoxin and

Table 1. Qualitative test performed on human skin[5, 7]. 25 γ of substance/cm² Philips HPW 125 lamp (3655 Å) at 15 cm from the skin; irradiation for 30 minutes: ++++ maximum activity; — inactive substances

No.	*Natural furocoumarins*		*Activity*
1	Psoralen		++++
2	Xanthotoxin	(8-methoxy-psoralen)	+++
3	Bergapten	(5-methoxy-psoralen)	+++
4	Angelicin		+
5	Isobergapten	(5-methoxy-angelicin)	+
6	Oxypeucedanin	[5-(β,γ-oxido-isoamyloxy)-psoralen]	+ (sunlight only)
7	Xanthotoxol	(8-hydroxy-psoralen)	—
8	Imperatorin	(8-isoamylenoxy-psoralen)	—
9	Bergaptol	(5-hydroxy-psoralen)	—
10	Isopimpinellin	(5,8-dimethoxy-psoralen)	—
11	Ostruthol	[5-(β,γ-dihydroxy-isoamyloxy)-psoralen angelic acid monoester]	—

Synthetic furocoumarins

Related to psoralen

12	4′,5′-Dihydro-psoralen	—
13	3,4-Dihydro-psoralen	—
14	4′-Methyl-psoralen	+++
15	4,4′-Dimethyl-psoralen	+++
16	4′-Phenyl-4-methyl-psoralen	—
17	Dimer of psoralen	—
18	Thyopsoralen	—

Related to xanthotoxin

19	3,4-Dihydro-xanthotoxin	—
20	3-Methyl-xanthotoxin	+
21	4-Methyl-xanthotoxin	+++
22	4′-Methyl-xanthotoxin	++
23	4′,3-Dimethyl-xanthotoxin	+
24	4′,4-Dimethyl-xanthotoxin	+
25	5′,4-Dimethyl-xanthotoxin	+++
26	5′-Phenyl-4-methyl-xanthotoxin	—
27	5-Chloro-xanthotoxin	+
28	5-Nitro-xanthotoxin	—
29	5-Amino-xanthotoxin	—
30	5-Acetylamino-xanthotoxin	—
31	8-Benzyloxy-psoralen	+
32	Thyoxanthotoxin	—

Related to bergapten

33	4′,5′-Dihydro-bergapten	—
34	4-Methyl-bergapten	+
35	4-Methyl-4′,5′-dihydro-bergapten	—
36	Allobergapten	+
37	4′,5′-Dihydro-allobergapten	—
38	4′,5′,3,4-Tetrahydro-allobergapten	—
39	4-Methyl-allobergapten	+
40	4-Methyl-4′,5′-dihydro-allobergapten	—
41	5-Ethoxy-psoralen	++
42	5-Isopropyloxy-psoralen	+
43	5-n-Propyloxy-psoralen	— (sunlight +)
44	5-n-Butyloxy-psoralen	— (sunlight +)
45	5-Isoamyloxy-psoralen	— (sunlight +)
46	Psoralen-5-oxyacetic acid ethyl ester	—
47	5-Benzyloxy-psoralen	—
48	8-Nitro-bergapten	—
49	8-Amino-bergapten	—
50	8-Acetylamino-bergapten	—
51	Bergapten-8-carboxylic acid methyl ester	+
52	4′,5′-Dihydro-bergapten-8-carboxylic acid methyl ester	—
53	Bergapten-8-carboxylic acid	—
54	4′,5′-Dihydro-bergapten-8-carboxylic acid	—
55	Dimer of bergapten	—

Related to angelicin

56	4-Methylangelicin	+
57	Dimer of angelicin	—

Related to isopimpinellin

58	5,8-Dihydroxy-psoralen[8]	—
59	Psoralenquinone	—

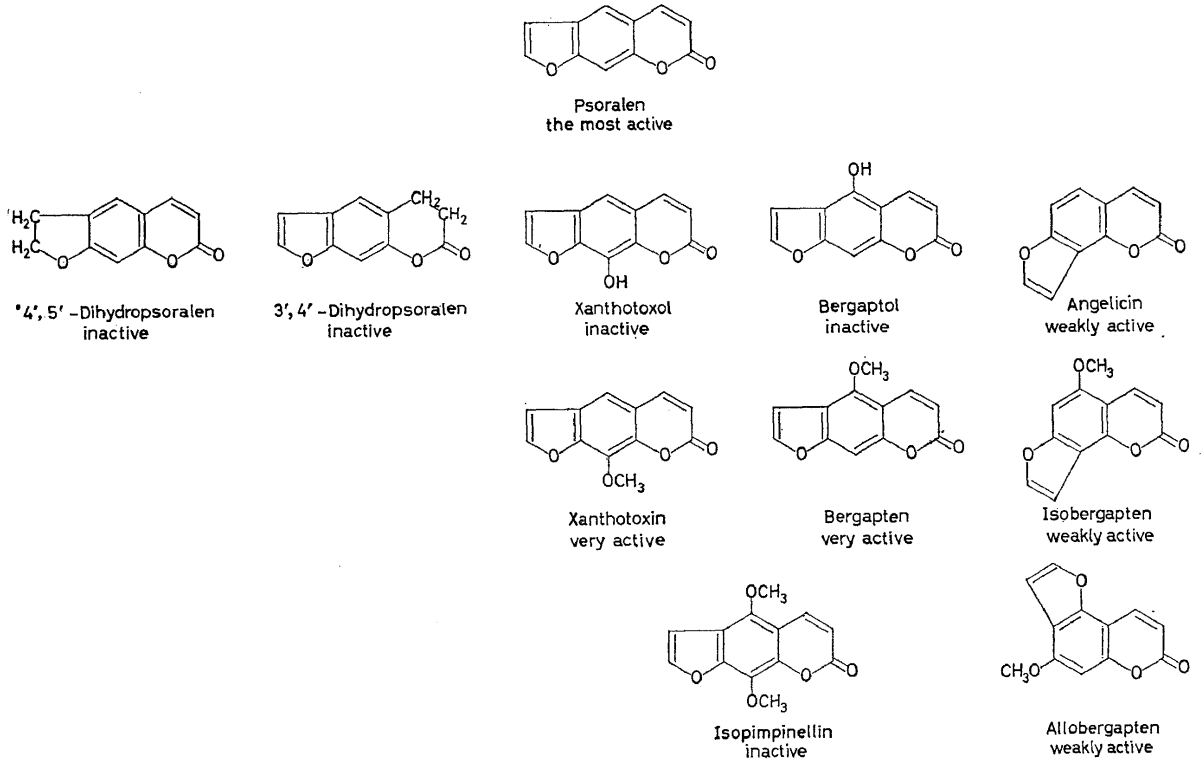
Table 2. Quantitative test[5, 7]: 5 γ of substance/cm^2 of human skin; Philips HPW 125 lamp (3655 Å) at 15 cm from the skin; variable time of irradiation

Compounds	*Minimum length of irradiation requested for outcome of erythema* (min)	*Relative activity*
Psoralen	6	100
4′-Methyl-psoralen	10	60
Xanthotoxin	16	37·5
4,5′-Dimethyl-xanthotoxin	18	33·3
4,4′-Dimethyl-psoralen	20	30
4-Methyl-xanthotoxin	20	30
Bergapten	22	27·5
5-Ethoxy-psoralen	25	24
4′-Methyl-xanthotoxin	25	24
5-Isopropyloxy-psoralen	35	17·1
4-Methyl-bergapten	40	15
5-Chloro-xanthotoxin	40	15
Angelicin	50	12
4′,4-Dimethyl-xanthotoxin	50	12
Allobergapten	50	12
4-Methyl-allobergapten	50	12
Bergapten-8-carboxylic acid methyl ester	50	12
3,4′-Dimethyl-xanthotoxin	55	11
3-Methyl-xanthotoxin	60	10
Isobergapten	60	10
4-Methyl-angelicin	60	10
8-Benzyloxy-psoralen	60	10

bergapten, is really remarkable; in fact, just a few γ/cm^2 of skin and a few minutes of irradiation are sufficient to cause, after a latent period lasting a few hours, signs of erythema and even the appearance of bullae. In view of their diffusion in nature, an extensive research has been carried out to see if they are present in vegetables used for human consumption; it has, in fact, been found that bergapten is present in celery and parsley[16, 17]. This fact, besides indicating the possible cause of dermatitis due to celery which has been described several times in medical literature, also reveals that small amounts of active substances are ingested, and thus poses the problem of their possible biological function at the level of the skin.

However, the most obscure part of the whole question has always been the mechanism of action, in spite of intensive research on our part and on the part of other investigators[18]. First of all, we wished to ascertain the position of the photodynamism of furocoumarins with respect to the photodynamism shown by other well-known substances such as haematoporphyrin, methylene blue, hypericin, phagopyrin, fluorescein dyes, benzpyrene, *etc.* We chose comparative tests by means of which we tested these last-named substances and the furocoumarins. Our tests comprised: photo-oxidation of terpinen to ascaridol, haemolysis of red cells, photo-oxidation of blood serum proteins followed by means of polarography, and the response of guinea-pig skin after epicutaneous application or intradermal injection[19, 20]. This extensive research clearly led to the conclusion that furocoumarins form a group of substances possessing special properties; more precisely, we have classified the photodynamic substances tested, into three groups:

Table 3. Relationships between structure of some furocoumarins and their photosensitizing properties on the skin



(I) The first group can comprise haematoporphyrin, hypericin, phagopyrin, fluorescein dyes, methylene blue, *etc.*, which provoke an immediate, but short-lasting, photoreaction in guinea-pigs. Its mechanism of action can be related to photo-oxidation processes involving protein substrates, as has been demonstrated by various investigators, among whom I should mention Santamaria[21, 22] who has dealt extensively with this subject.

(II) The second group consists of the furocoumarins which have no, or hardly any, influence on protein photo-oxidation and which cause dermatitis of the skin, even that of guinea-pigs, with a latent period lasting several hours and which is shown by erythema slowly followed by pigmentation lasting several months.

(III) The third group comprises benzpyrene and some other polycyclic aromatic hydrocarbons showing properties similar to both group I and group II substances, that is, they are capable of photo-oxidizing protein substances and on the skin give a response similar to that of the furocoumarins.

But although the limits of the problem have thus been defined, the mechanism of action of the furocoumarins has remained substantially unexplained. Nor has there been any marked progress since the studies, so interesting from several aspects, of Pathack *et al.*[23] on the fluorescence of furocoumarins and on the relationships between the wavelength of the exciting light and the wavelength of the radiations emitted, and the more recent research by the same investigators on the formation of free radicals following irradiation of furocoumarins[24].

However, we have persisted in our work and have recently succeeded in revealing an *in vitro* photoreaction between FMN (flavin mononucleotide) and photosensitizing furocoumarins[25]. The first indications were obtained spectrophotometrically; we then observed that on irradiating water–ethyl alcohol and water–methyl alcohol solutions of FMN and furocoumarins with ultra-violet light at 3655 Å various new compounds are formed, as was shown by paper chromatography of the irradiated solutions; spots were observed which did not appear on irradiating FMN or the furocoumarins alone, under the same conditions. The reactions involved are highly complex and lead to the formation of numerous new products.

These photoreactions are now of great interest to us since we have become aware of the fact that they occur only in the case of furocoumarins active on the skin; no formation of any new compounds is seen with inactive furocoumarins (*Table 4*). Moreover, we have observed that a certain protection can be obtained in guinea-pigs with FMN, although at very high doses. On administering this compound by subcutaneous injection after irradiation of guinea-pigs painted with bergapten, the erythema either does not appear, or else is more attenuated, than in the case of the irradiated control animals, *i.e.* treated in exactly the same way but without FMN. We have, therefore, extended the study of these photoreactions from both the chemical and biological points of view. We have considered the flavin coenzymes FMN and FAD (flavin adenine dinucleotide) on the one hand, and on the other the three most active furocoumarins namely, psoralen, xanthotoxin and bergapten. We also considered benzpyrene which, as I have already mentioned, sometimes behaves analogously to the furocoumarins. Few of the results

Table 4. Chromatographic studies on the photoreaction between FMN and some furocoumarin derivatives

Furocoumarin derivatives with skin-photosensitizing properties in sun and u.v. light	*Spots of new compounds*	
	R_F 0·13–0·24	R_F 0·85–0·90
Psoralen	+	+
4′-Methyl-psoralen	+	+
4′,4-Dimethyl-psoralen	+	+
Xanthotoxin	+	—
3-Methyl-xanthotoxin	+	—
3′,4-Dimethyl-xanthotoxin	+	—
4′-Methyl-xanthotoxin	+	+
4′,4-Dimethyl-xanthotoxin	+	—
5′,4-Dimethyl-xanthotoxin	+	—
5-Chloro-xanthotoxin	+	—
8-Benzyloxy-psoralen	+	—
Bergapten	+	+
4-Methyl-bergapten	+	+
5-Isopropyloxy-psoralen	+	+
Isobergapten	+	+
Allobergapten	—	+
4-Methyl-allobergapten	+	—
Angelicin	—	+
Bergapten-8-carboxylic acid methyl esther	+	—
5-n-Propyloxy-psoralen	—	+
5-n-Butyloxy-psoralen	—	+
5-Isoamyloxy-psoralen	—	+

No new spots have been noted with the following *photodynamically inactive* derivatives: xanthotoxol, bergaptol, imperatorin, isopimpinellin, 5,8-dihydroxy-psoralen, psoralenquinone, 4-methyl-4′-phenyl-psoralen, 4-methyl-5′-phenyl-xanthotoxin, 3,4-dihydro-psoralen, 4′,5′-dihydro-psoralen, 4′,5′-dihydro-bergapten, psoralen-5-oxyacetic acid ethyl ester, ostruthol, 5-nitro-xanthotoxin, 5-acetamino-xanthotoxin, 8-nitro-bergapten, dimer of psoralen, thyopsoralen, thyoxanthotoxin, umbelliferon, erniarin, citropten, seselin.

obtained in the last two years have been published previously. Neglecting the experimental details, I will say that the results of chemical nature can be summarized as follows:

(*i*) Psoralen, xanthotoxin and bergapten photoreact *in vitro* only with FMN and not with FAD.

(*ii*) Benzpyrene photoreacts only with FAD and not with FMN.

As regards the furocoumarins, the new compounds formed, isolated by extractions and chromatography on columns of various types, can be divided into two types:

(*a*) colourless substances with varied and strong fluorescence in ultra-violet light (violet, blue, yellow-green) almost insoluble in water and soluble in ether, which have R_F 0·85–0·90 with Partridge's solvent;

(*b*) yellow substances showing also yellow fluorescence in ultra-violet light, very soluble in water and insoluble in ether, having R_F 0·13–0·24 with Partridge's solvent.

These two groups of substances can be clearly separated by thoroughly extracting the photoreaction liquid with ethyl ether; type (*b*) substances remain in the aqueous layer, while type (*a*) substances pass into the ether and are then fractionated and isolated on silica gel and alumina columns.

All substances of type (*a*) are derived from the furocoumarins and have undergone transformation of the furan ring.

In the bergapten photoreaction three new coumarin derivatives have been isolated: one is 5-methoxy-7-hydroxy-coumarin-6-acetic acid and another is the corresponding methyl or ethyl ester according as to whether methyl or ethyl alcohol was present in the irradiated solution. The formation of ester following irradiation in aqueous alcoholic vehicles is not new; it has been described, for instance, by Japanese researchers[26] who, on irradiating riboflavin in water–alcohol solutions obtained the methyl, ethyl or propyl esters of flavin-acetic acid according to the alcohol present. When heated, 5-methoxy-7-hydroxy-coumarin-6-acetic acid gives the corresponding lactone, and treated with diazomethane forms the methyl ester of 5,7-dimethoxy-, coumarin-6-acetic acid, identical with that recently obtained by French investigators through chromic oxidation of a natural coumarin extracted from *Toddalia aculeata*[27]. The third substance, isolated in small quantities, is probably 4′,5′-dihydro-4′-oxo-5-methoxyfurocoumarin, the acetyl derivative of which can be prepared as for the other coumaranones. The above reactions of bergapten are summarized in *Table 5.*

Two substances have been obtained from the photoreaction between FMN and psoralen in water–methyl alcohol solution: one, in very small amounts, and with a violet fluorescence, is considered to be the methyl ester of 7-hydroxy-coumarin-6-acetic acid; the second, isolated in greater amounts, is 7-hydroxy-6-formylcoumarin, the acetyl derivative and the phenylhydrazone of which have been prepared. This aldehyde was already known and we too have obtained it from psoralen by ozonolysis; the two substances were found to be identical (*Table 6*).

No new compounds of type (*a*) are formed in the photoreaction between FMN and xanthotoxin, but only yellow substances of type (*b*).

The yellow substances, which we have called type (*b*), remain in the aqueous phase when the irradiated solutions are extracted with ether. They have been separated from the unaltered FMN and from the other flavin products forming during FMN photolysis (riboflavin, lumiflavin, lumichrome) by concentration through lyophilization and successive chromatography on columns of powdered cellulose, using butyl alcohol saturated with water as the solvent. All are very unstable substances, so that their isolation has been accomplished only with great difficulty, in all cases avoiding temperatures above 20°. We will call these new yellow substances *flavin photo-compounds* (FPC).

Two substances of this type have been isolated from the photoreaction between bergapten and FMN: flavin photo-compound I (FPC-I), with an R_F 0·21 (Partridge's solvent) and flavin photo-compound II (FPC-II), in very small amounts, with an R_F 0·24. The photoreaction between xanthotoxin and FMN has also led to the isolation of two yellow substances: flavin photo-compound III (FPC-III), with an R_F 0·13 (always with Partridge's solvent) and flavin photo-compound IV, also found only in small quantities, with an R_F 0·16. In the photoreaction between psoralen and FMN, paper chromatography reveals the formation of small quantities of new yellow substances, especially if the irradiation is performed at low temperature. However, probably due to the instability of these products, it has so far been impossible to isolate them. The greater part of the research up-to-now has dealt with two main substances, FPC-I and FPC-III.

Table 5. FMN + bergapten photoreaction

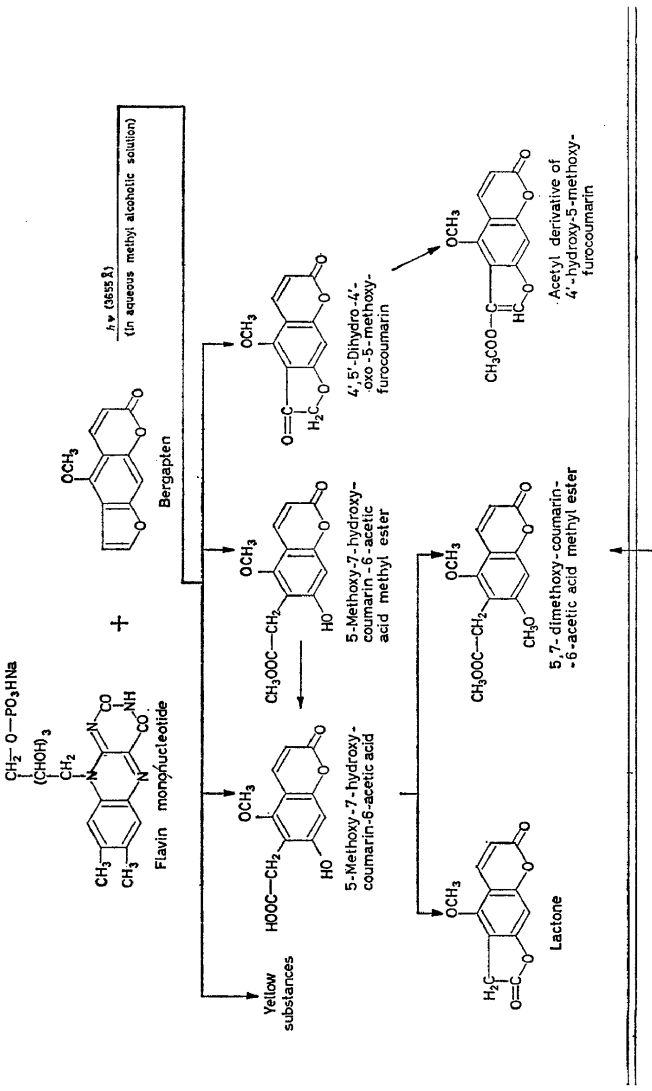
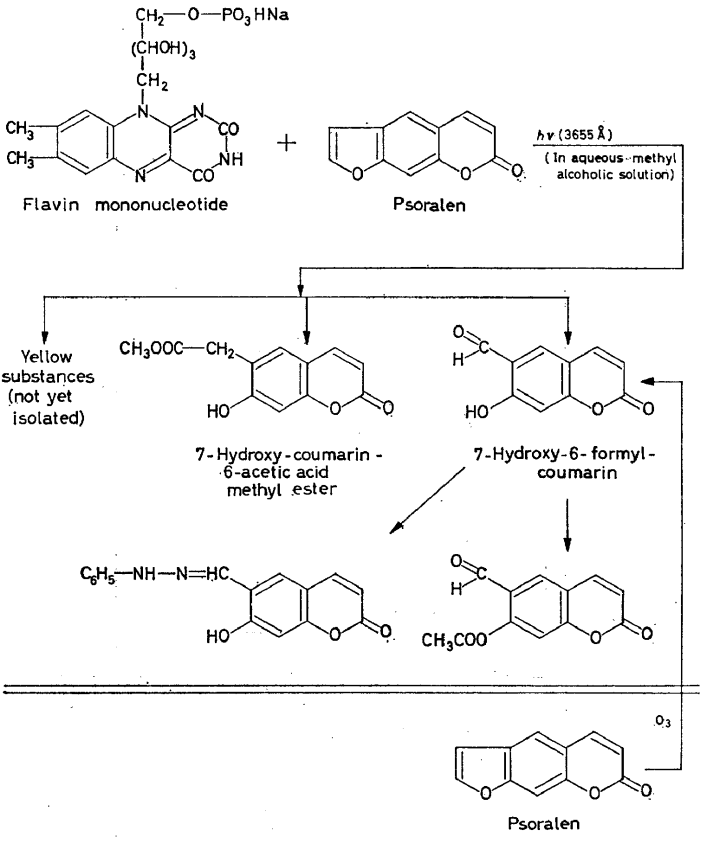


Table 6. FMN + psoralen photoreaction



I must state immediately that these substances, although obtained pure on paper chromatography, have not yet been obtained in the analytically pure state, as they are insoluble in all solvents except water, in which they are too soluble. The lyophilized products so far obtained have given analytical data revealing an excessive mineral residue. However, these are undoubtedly flavin derivatives; their ultra-violet absorption spectra have a general course similar to that of FMN (*Figure 1*).

On paper chromatography they always show a single spot, even when developed with a series of different solvents, and generally have R_F values intermediate between FMN and riboflavin. Also in the case of paper electrophoresis under various conditions (at pH 5 and pH 8), they show a behaviour midway between FMN and riboflavin.

They are easily decomposed on heating with water or, better, with dilute hydrochloric acid, giving mainly FMN, lumichrome, and a coumarin compound with a blue or blue-violet fluorescence, besides traces of riboflavin and lumiflavin.

It has to be especially noted that when FPC-I is heated with diluted hydrochlonic acid, a substance is obtained showing sky-blue fluorescence in

ultra-violet light and an R_F 0·70 with Partridge's solvent. The substance, at present under investigation, can be obtained more easily through ozonolysis of bergapten and it could be 5-methoxy-7-hydroxy-6-formyl-coumarin, already known as apoxanthoxyletin, or one of its derivatives.

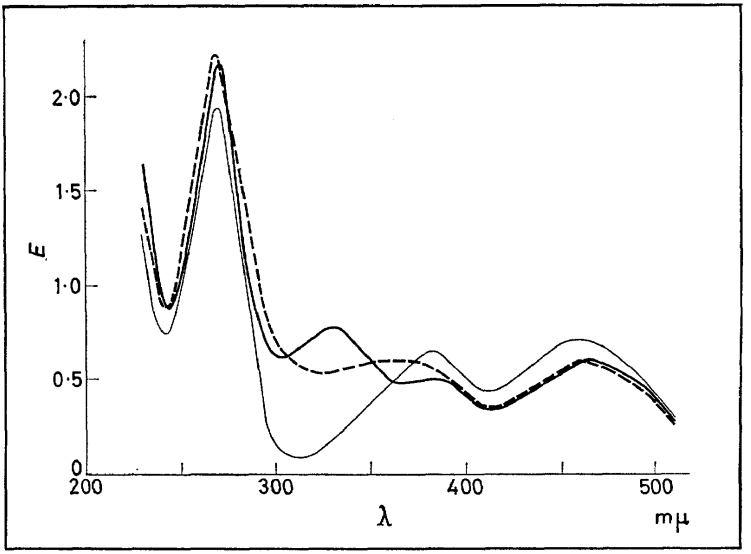


Figure 1. u.v. Absorption spectra of aqueous solutions of FMN (——), flavin photo-compound I obtained from bergapten (- - - - -) and flavin photo-compound III obtained from xanthotoxin (——)

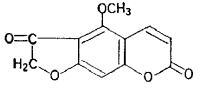
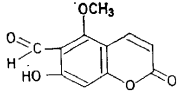
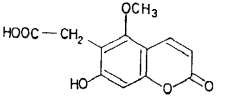
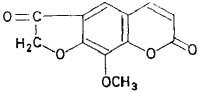
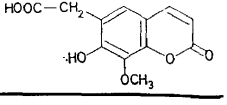
Table 7 gives the products formed on decomposition due to heating the flavin photo-compounds with water or dilute hydrochloric acid. In this table the composition of the coumarin derivatives is indicated as probable since the identification has been made chromatographically on paper and by means of spectrophotometry, and must be confirmed. It has not been possible to isolate the substances in view of the great difficulty in preparing a sufficient quantity of flavin photo-compounds from which the decomposition products can be obtained.

The study of the flavin photo-compounds is extremely difficult and it is, therefore, as yet impossible to speak of their composition. Nevertheless, it may be stated that they definitely contain a flavin portion always holding FMN and a part deriving from the furocoumarin used in the reaction. In this connection, I should like to point out that it has been stated that riboflavin, FMN and FAD may form "complexes" with other organic molecules in solution, for example, with tryptophan, serotonin, other indole compounds[28], chlorpromazine[29], chlortetracycline[30], atebrin[31], but these complexes have so far only been demonstrated by spectrophotometry in solutions, and have never been isolated.

At this point, I must return to FAD* and benzpyrene. As already mentioned, furocoumarins do not react with FAD, while benzpyrene, by contrast,

* I am indebted to Takeda Pharmaceutical Industries, Osaka, Japan, for the generous supply of FAD used in this research.

Table 7. Decomposition products of flavin photo-compounds obtained from FMN and bergapten or xantothoxin

Flavin photo-compounds	*Flavin derivatives*	*Probable coumarin derivatives*	
Flavin photo-compounds I from bergapten R_F 0·21 (Partridge's solvent)	FMN-lumichrome riboflavin (traces) lumiflavin (traces)	4′,5′-Dihydro-4′-oxo-5-methoxy-furocoumarin	
		Apoxanthoxyletin or 7-hydroxy-5-methoxy-6-formyl-coumarin (only in HCl decomposition)	
Flavin photo-compound II from bergapten R_F 0·24 (as above)	FMN-lumichrome riboflavin (traces) lumiflavin (traces)	7-Hydroxy-5-methoxy-coumarin-6-acetic acid	
Flavin photo-compound III from xantho-toxin R_F 0·13 (as above)	FMN-lumichrome riboflavin (traces) lumiflavin (traces	4′,5′-Dihydro-4′-oxo-8-methoxy-furocoumarin	
Flavin photo-compound IV from xantho-toxin R_F 0·16 (as above)	FMN-lumichrome riboflavin (traces) lumiflavin (traces)	7-Hydroxy-8-methoxy-coumarin-6-acetic acid	

does not photoreact with FMN, but only with FAD. Irradiation of water–alcohol solutions of equimolecular mixtures of benzpyrene and FAD has been performed using the normal lamps emitting light at 3655 Å. This photoreaction also gives a complicated series of products; among these, two yellow substances have been isolated in the chromatographically pure state, although in extremely small amounts: flavin photo-compound V (FPC-V) and flavin photo-compound VI (FPC-VI); they are extremely soluble in water, and in paper chromatography with Partridge's solvent have an R_F 0·19 and 0·33. Their flavin nature is clearly demonstrated by their ultraviolet absorption spectra; moreover, on heating with hydrochloric acid they also decompose giving numerous compounds, partly identified and partly under investigation.

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* *

I must point out that none of the substances isolated is active on the skin and that the transformations of the furan ring from furocoumarin, by irradiation in the presence of a flavin derivative such as FMN, do not cause any special surprise; it is known from studies by Schenck *et al.*[32] that other furan derivatives also become oxidized on irradiation in the presence of photodynamic substances, such as eosin and Bengal rose. It is likewise known that riboflavin is an active photodynamic substance capable, for instance, of oxidizing methionine to the corresponding sulphoxide[33].

The most interesting aspect of these photoreactions is undoubtedly that of the new flavin photo-compounds which, as already mentioned, decompose to reveal a flavin portion and a portion deriving from the skin-photosensitizing agent. These complex substances are probably the first products of the photoreaction, while the oxidized coumarin derivatives would be formed from their decomposition. In confirmation of this we have observed that if the irradiation is carried out at low temperatures (around 0°) mainly yellow substances are formed, while the coumarin derivatives are either absent or present in much smaller amounts.

The different stability of these flavin photo-compounds may govern the course of the photoreactions; this would explain the difference in behaviour of psoralen, where the formation of coumarin derivatives predominates in the photoreaction, compared with that of xanthotoxin, where these deriatives are practically absent, and that of bergapten, which is halfway between the two.

The interest in the flavin photo-compounds is all the more obvious if we consider them from the coenzymatic aspect. Now, whether derived from FMN and bergapten or from FMN and xanthotoxin, they are incapable of restoring the activity of the apoenzyme of the Warbrug's old yellow enzyme, while, by contrast, this occurs regularly with FMN[34]. This restoration of the activity of the apoenzyme may also be obtained with the FPC-I and FPC-III after they have been decomposed with hydrochloric acid, which again demonstrates that FMN is reformed when they decompose (*Figure 2*).

The two yellow substances FPC-V and FPC-VI isolated in the photoreaction between FAD and benzpyrene, are of complex flavin nature as I have already stated, and are incapable of functioning as coenzyme with the apoenzyme of D-amino-acid oxidase, as, instead, FAD does naturally[35].

In other words, with regard to these enzymatic systems, Warburg's yellow enzyme and D-amino-acid oxidase, the flavin photo-compounds have lost the coenzymatic properties peculiar to FMN and FAD from which they are derived.

It is also known that in animal tissues the most important coenzyme is flavin adenine dinucleotide (FAD), rather than flavin mononucleotide (FMN), and that FAD is synthesized enzymatically, starting from FMN. We have now observed that the enzyme system which can be isolated from yeast and which controls the biosynthesis of FAD from FMN[36], cannot transform FPC-I or FPC-III, obtained from FMN and furocoumarins into FAD (*Figure 3*). These facts indicate that, although sufficiently unstable to make their chemical study difficult, these flavin photo-compounds, nevertheless, have reasonable biological stability, so much so that the flavin portion, FMN or FAD, linked in them is blocked coenzymatically.

Two questions arise at this point: are these flavin photo-compounds formed *in vivo*? Are they involved in the skin photo-sensitization produced by furocoumarins and by benzpyrene? An answer may be given to the first question from experimental results.

It has been possible to ascertain that photoreactions progressing, at least partly, analogously to those studied *in vitro* may occur also in the skin of guinea-pigs (which behave like man with regard to the furocoumarins). In fact, if the skin of guinea-pigs is irradiated after being painted with a bergapten solution, and is then immediately removed, homogenized and lyophilized, a large portion of the bergapten which had been deposited on the skin can be recovered from the same skin by extraction with ether. If the residue from the extraction is then treated with warm dilute hydrochloric acid and again extracted with ether, it is possible to obtain a substance with a brilliant sky-blue fluorescence, which is identical with the substance obtained on hydrochloric acid decomposition of the FPC-I, isolated from the *in vitro* photoreaction between FMN and bergapten. The same substance may also be isolated, in better yield, from guinea-pig and even pig liver homogenates irradiated in the presence of bergapten. The chromatographic and spectrophotometric behaviour of these products is the same. The fluorescence spectra also coincide.

It must be noted that the first ether extraction of the homogenized and lyophilized tissue does not yield these substances; it is, therefore, logical to suppose that it is at first combined in such a way as to be insoluble in ether, and that it is freed only after treatment with dilute hydrochloric acid. An explanation of this fact may be given by admitting the formation between the FMN of the skin and the furocoumarins of flavin photo-compounds, which are then decomposed by the hydrochloric acid, thus freeing the coumarin compound which is extracted with the ether.

After these experiments, we may reasonably conclude that photoreactions occur *in vivo* which are analogous to those taking place *in vitro* between skin-photosensitizing agents and flavin coenzymes.

I shall reply to the second question raised before with the hypothesis, which is moreover based on many experimental results, that the flavin photo-compounds could also be, *in vivo*, the first products formed by the flavin coenzymes and photosensitizing agents, through the action of light.

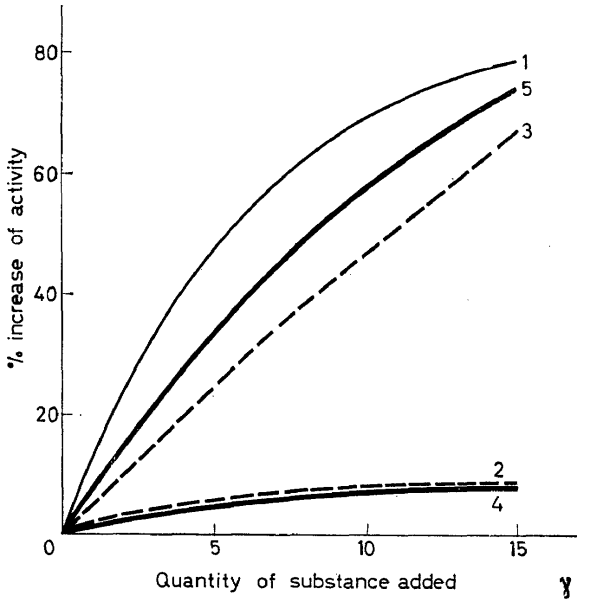

Figure 2. Percentage increasing of the residual activity of the apoenzyme of the Warburg's old yellow enzyme when FMN or the flavin photo-compounds were added (the activity was measured by the dichlorphenol-indophenol spectrophotometric method);
1: FMN; 2: FPC-I, obtained from bergapten (not decomposed); 3: the same, after decomposition with hydrochloric acid; 4: FPC-III, obtained from xanthotoxin (not decomposed); 5: the same, after decomposition with hydrochloric acid

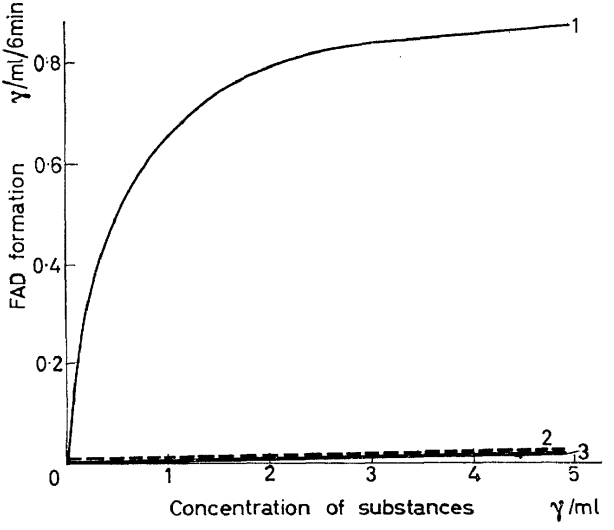

Figure 3. Amount of FAD enzymically formed from FMN (1), from the flavin photo-compound I obtained from bergapten (2) and from the flavin photo-compound III obtained from xanthotoxin (3)[36]. (FAD was estimated by means of the apoenzyme of the D-amino-acid oxidase)[35]

The consequent disappearance of the coenzymatic properties of the flavin compounds could be responsible for the skin lesions observed. Inactivation of this type could also occur with sun burns which, from many aspects, resemble to a minor degree the effects of the furocoumarins.

This is a hypothesis, and more precisely a working hypothesis that, we think, can give rise to further research.

I wish to thank, warmly, Professor Giovanni Rodighiero, with whom I developed these experiments, my other co-workers Professor G. Caporale and Drs C. Antonello, F. Baccichetti, U. Fornasiero, F. Dall'Acqua, G. Malesani, C. Giacomelli and M. Razzi, and also Professor G. Azzone of the Institute of General Pathology of the Padua University, for his assistance in the enzymologic work.

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