LXXIII. THE PHOTO-OXIDATION OF CERTAIN ORGANIC SUBSTANCES IN THE PRESENCE OF FLUORESCENT DYES.

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INTRODUCTION.

THE present work was undertaken to extend the recent investigations of Harris, Gaffron, and others on the photo-dynamic oxidation of certain organic substances and to establish new data on the dynamics of this type of reaction. It was hoped that the information thus obtained might provide a basis for the explanation of the skin lesions and other manifestations exhibited by individuals sensitised to light by the occurrence in their tissues of certain fluorescent porphyrins. Fischer [1924], Garrod [1922] and Gray [1924] have reported in detail cases of haematoporphyrinuria congenita in which uro- and copro-porphyrin can be identified in the organs and excreta. Hausmann [1926] has described the symptoms produced in man and in small animals by injections of small doses of haematoporphyrin on exposure to light. Raab [1903], Tappeiner and Jodlbauer [1905], Dreyer [1904], Ledous Lebard [1902] and others have produced analogous effects on protozoa, bacteria, enzymes, toxins and immune bodies by the action of various fluorescent synthetic dyes in the presence of light. Harris [1926] has shown that proteins such as egg-albumin, haemoglobin and serum-albumin, and amino-acids like tyrosine and tryptophan undergo photo-oxidation when exposed to ultra-violet light and that this oxidation is greatly accelerated by the presence of minute traces of haematoporphyrin and other fluorescent dyes. Gaffron [1926] has investigated the photo-oxidation of tyrosine, phenol and uric acid using haematoporphyrin as sensitiser in visible light.

Two main facts emerge from the work of these and other authors. Firstly, fluorescence appears to be an essential physical property of those dyes which exhibit photo-dynamic activity. Thus Tappeiner [1909], in studying a long series of dyes, found that only those possessing the property of fluorescence were toxic to *Paramoecium* in light. Similarly Gaffron showed that while haemin, and the copper and silver salts of haematoporphyrin, which are non-fluorescent, are inactive as sensitisers for egg-albumin, the fluorescent zinc salt is active. The writer has shown that turacin, the non-fluorescent copper salt of uroporphyrin,

is inactive as a sensitiser for *Glaucoma* and tyrosine, whereas uroporphyrin, itself powerfully fluorescent, is an efficient sensitiser. At the same time there is reason for believing that the degree of fluorescence bears no direct relation to that of photo-dynamic activity. Raab [1903] showed that the fluorescent dye aesculin in concentration 1 : 1000 was without action on *Paramoecium*. The blue fluorescent substances described by Peters, Kinnersley and Squires [1925] in animal tissues are unable to bring about the photo-oxidation of tyrosine, and do not protect *Glaucoma* against the lethal action of haematoporphyrin in light. Quinine sulphate in aqueous solution gives a brilliant blue fluorescence in ultra-violet light but shows no absorption bands or fluorescence in visible light and is unable to bring about the photo-oxidation of tyrosine.

There is, secondly, the relationship of oxygen to the photo-dynamic process to be considered. Tappeiner came to the conclusion that in this respect ultra-violet and visible light differed in their photo-chemical action. Ultra-violet light possessing a high energy value is able to bring about a molecular rearrangement in living cells, enzymes and simple chemical compounds without the aid of oxygen. In the presence of oxygen these decomposition products are then oxidised, enabling the reaction to proceed to completion. In all probability this is effected by the agency of active oxygen formed by ultra-violet light. Stedman and Mendel [1926] have reported the presence of hydrogen peroxide in water and in protein solutions exposed to the rays of a mercury arc lamp. In the case of visible light on the other hand no photo-chemical action occurs in the absence of oxygen even when fluorescent dyes are present. In the presence of oxygen irradiation by visible light brings about a ready oxidation as Gaffron has shown. The selective action of ultra-violet light on tyrosine and tryptophan leads to the hypothesis that the action of visible light on living cells is essentially a photo-oxidation of certain specific constituents of the cell.

METHOD.

In determining the ability of organic compounds to undergo photo-oxidation the Barcroft technique for measuring oxygen uptake was employed. The flasks of the Barcroft apparatus were contained in a large water-bath at constant temperature, 20°. The source of light employed was a 300-watt 105-volt Osram lamp contained in a cylindrical glass vessel through which water continually circulated. The vessel was itself immersed in the water-bath at a distance of approximately 12 cm. from the flasks of the Barcroft apparatus. Under the conditions specified no light of shorter wave length than 320 $\mu\mu$ actually reached the contents of the flasks. Into each flask 2 cc. of the substrate were introduced. To one was added an appropriate volume of the sensitiser, to the other an equal volume of distilled water or buffer solution. Carbon dioxide evolved during the oxidation was absorbed by strong sodium hydroxide placed in a small container inside each flask. At the commencement of an experiment the flasks were shaken in the dark with taps open to

the air until temperature equilibrium was established. The taps were then closed and shaking continued in the dark. So long as light was rigorously excluded no oxygen uptake occurred, but on switching on the light measurable oxidation occurred within half a minute. The p_H of the solution was adjusted to 7.5.

The substances investigated were in the first instance purified by re-crystallisation or distillation.

CHEMICAL NATURE OF SUBSTRATE.

The problem of the relation of chemical structure to photo-dynamic activity which is raised by the work of Harris on amino-acids has been investigated by comparing the ability of a wide variety of organic compounds to undergo photo-dynamic oxidation. Differences of solubility and of the nature of solvent employed (water or alcohol) in different cases make quantitative comparison difficult and this has not been attempted. The aim of the present work has been to discover what types of compound undergo oxidation and what types are inactive. It is obviously outside the scope of the present work to attempt any comprehensive catalogue and certain types have been selected which throw light on the possibility of any particular chemical grouping having definite relation to photo-oxidation.

It may be stated in the first place that no exception has been discovered to the generalisation that the aliphatic compounds are inactive. Table I summarises the substances investigated in this series.

Table I.

Glucose	Sodium acetate	Acetone	Ethyl alcohol	Ethylamine
Fructose	Sodium lactate	Acetaldehyde	Aspartic acid	
Lactose	Sodium propionate			
Hexosediphosphate	Sodium oleate			
	Sodium linoleate			

It is evident that the presence of easily oxidisable groupings such as those present in oleic acid or acetaldehyde are not in themselves sufficient to ensure photo-oxidation. Nor does this inactivity depend on their ability or otherwise to absorb ultra-violet light. It is not until a complexity of structure represented by the aromatic compounds is reached that photo-oxidation is observed. Among this class of compound a wide variety of response is shown, and Table II summarises the results obtained.

Of the benzene derivatives studied in the above series it will be seen that the introduction of a hydroxyl or amino-group into the ring renders the compound susceptible to photo-oxidation. All the other groups studied—carboxyl, nitro-, cyano-, the halogens and sulphuric acid—are without effect. This conclusion is in harmony with the well-known fact that oxidation by methods other than that involving the influence of light is rendered easy by the introduction of amino- or hydroxyl-groups into the benzene ring as compared with

the refractory behaviour of the other groups mentioned. It will be seen that *p*-hydroxybenzoic acid gave a far greater oxygen uptake than any of the other substances investigated.

Table II.

<i>Active.</i>			
Substance	O ₂ mm. ³ /hour	Substance	O ₂ mm. ³ /hour
<i>o</i> -Cresol	76.7	Tyrosine	425.0
<i>m</i> -Cresol	255.0	Benzaldehyde	360.0
<i>p</i> -Cresol	333.2	Phenylhydrazine	1025.0
Phenol	148.7	Cinnamic acid	55.0
<i>p</i> -Hydroxybenzoic acid	4500.0	Hypoxanthine	950.0
Aminobenzoic acid	525.0	Xanthine	2025.0
Salicylic acid	77.5	Uric acid	1000.0
Aniline	221.7	Guanine	900.0
α -Naphthol	320.0	Caffeine (p_H 7)	45.0
Tryptophan	1417.0	Caffeine (p_H 10)	170.0
Histidine	139.0		
Thiophen	300.0		
Quinoline	282.5		
<i>Inactive.</i>			
Substrate	Substrate	Substrate	
Benzene	Toluene	Anthracene	
Benzoic acid	Benzyl alcohol	Quinine	
Phthalic acid	Acetophenone		
Nitrobenzene	Hippuric acid		
Dimethylaniline	Nitrobenzaldehyde		
Cyanobenzene			
Iodobenzene			
Chlorobenzene			
Benzenesulphonic acid			

Turning now to the effect of introducing side-chains into the ring it will be seen that the presence of amino-groups such as those in tyrosine or phenylhydrazine or the occurrence of an unsaturated carbon as in cinnamic acid enables photo-oxidation to take place. It is of interest to compare phenylhydrazine and cinnamic acid on the one hand with hydrazine and oleic acid on the other. Although readily oxidisable groups are present in the latter pair photo-oxidation occurs only after introduction of the phenyl group.

The other side-chains investigated, as in benzyl alcohol, acetophenone, hippuric acid and toluene, are not oxidised. The only exception, benzaldehyde, is so unstable that oxidation is not surprising; and it was found that the introduction of a stabilising nitro-group (nitrobenzaldehyde) prevented oxidation.

The purine group, hypoxanthine, xanthine, uric acid, guanine and caffeine all showed a very large photo-oxidation. Owing to their insolubility they were investigated in alkaline solution at p_H 10. Caffeine in neutral solution was found to be nearly inactive. It is possible that the oxidation of nucleoproteins in the cell nucleus may be an important factor in the photo-oxidation of the living cell.

DYNAMICS OF PHOTO-OXIDATION.

The method adopted involved an examination of the rate of photo-oxidation of tyrosine in the manner already described under varying concentrations of substrate and sensitiser. In general the curve showing the rate of oxygen uptake closely resembled that obtained by Gaffron. The velocity of

the reaction is at first rapid, and in the case of higher concentrations of tyrosine nearly linear; but subsequently it becomes very slow and is almost at a standstill at the end of 9 hours. Fig. 1 shows the rates of oxidation of solutions of tyrosine at p_H 9 in a concentration of 200 mg. %, 100 mg. % and 50 mg. % respectively using 1 cc. of $M/2500$ solution of uroporphyrin as sensitiser.

At concentrations higher than 100 mg. % the velocity during the first hour is nearly linear and is independent of the initial concentration. The logarithm of the oxygen consumption when plotted against the duration of the reaction is in accordance with the view that the photo-oxidation of tyrosine is a monomolecular reaction following the law of mass action. The values

Fig. 1.

1. Tyrosine 200 mg. %.	2. Tyrosine 100 mg. %.
3. Tyrosine+glass powder.	4. Tyrosine 50 mg. %.
5. Tyrosine+glass powder.	6. Tyrosine 25 mg. %.

of k calculated at different stages of the reaction showed a gradual falling off. This is due in all probability to the gradual destruction of the catalyst used, namely porphyrin. The gradual change of colour of the reacting mixture during the course of the reaction from a pink to a reddish-brown, associated with a loss of fluorescence, is indicative of this change. The same effect is observed when methylene blue, eosin and haematoporphyrin are used as sensitisers. The nature of this slow destruction of the sensitiser is not clear but it appears to be an oxidative change. The porphyrins in the presence of oxygen and certain unknown impurities are slowly decomposed by daylight, especially in alkaline reaction, as shown by Squires, whereas eosin and methylene blue appear to be quite stable in the absence of a substance like tyrosine.

The interaction of substrate and sensitiser may possibly have some bearing on the problem of the relation of chemical structure of substrate to photo-

dynamic action. Harris found that the addition of small quantities of potassium cyanide had no influence on the velocity of the photo-chemical reaction in ultra-violet light, and this was confirmed in the case of visible light. It was found further that addition of small traces of ferrous or ferric iron to the system had no accelerating action, nor did the presence of these ions in the absence of a fluorescent dye bring about any photo-oxidation of tyrosine. This is hardly surprising since dilute solutions of these salts show only faint general absorption of light in the visible spectrum. It may be noted that Harris found that iron in ionisable form—particularly in the ferrous state—was able to act as an efficient light sensitiser, though less powerful in this respect than haematoporphyrin in ultra-violet light.

When varying concentrations of uroporphyrin were tried, the concentration of substrate remaining constant, it was found that the increased rate of oxidation at the higher level was not proportional to the increase in concentration of sensitiser. This is probably due to the fact that the surface layers absorb a large proportion of the incident light so that little oxidation occurs in the interior of the fluid.

The fact that the photo-chemical oxidation of tyrosine is of the monomolecular type suggested that the reaction might be a surface phenomenon analogous to that found for an enzyme action. It was thought of interest to see whether the rate of the reaction could be modified by increasing the available surface of the glass vessel by the addition of finely powdered glass. In order to remove grease and other impurities the powdered glass was boiled with nitric acid, washed in distilled water, alcohol and ether and finally dried. It was found that the addition of the powder to the flask containing tyrosine solution together with uroporphyrin or methylene blue produced no acceleration of oxidation. The curve of oxygen uptake could be superimposed on a curve obtained in the absence of glass powder. It was found, however, that above p_H 8.5 the autoxidation of uroporphyrin was very greatly accelerated, whereas this effect was negligible in the case of methylene blue.

In view of the importance of oxygen in the photo-dynamic effect of visible light in the presence of fluorescent dyes, experiments were made to test for the possible production of hydrogen peroxide during the course of the reaction.

It was found that whereas a preparation of catalase protected a culture of *Glaucoma* from the toxic effect of small concentrations of added hydrogen peroxide, no protective action was exhibited by catalase to the sensitisation to light of *Glaucoma* in the presence of haematoporphyrin. Tables III and IV show the results in the case of solutions of hydrogen peroxide and haematoporphyrin respectively.

On a number of occasions fluid from tubes containing *Glaucoma* culture and haematoporphyrin which had been exposed to light was tested by means of the guaiacum reaction, using freshly prepared alcoholic solution of guaiacum and an extract of horse-radish peroxidase. All these tests proved negative.

Table III.

Exp.	H ₂ O ₂ 0.077 %	H ₂ O ₂ 0.077 % catalase 1 drop	H ₂ O ₂ 0.077 % catalase 2 drops	H ₂ O ₂ 0.077% boiled catalase 2 drops	H ₂ O ₂ 0.73 % catalase 1 drop
O ₉	19'	Motility reduced, 12 hours	Motility reduced, 12 hours	—	—
O ₁₀	—	Not dead, 103'	—	25'	5' O ₂ evolved visibly
O ₁₁	19'	Some dead. Re- mainder slug- gish. 118'	—	—	5'

Table IV.

Exp.	<i>Glaucoma</i> 2 cc. Haematopor. 3 drops	<i>Glaucoma</i> 2 cc. Haematopor. 3 drops. Catalase 3 drops	<i>Glaucoma</i> 2 cc. Haematopor. 3 drops. Boiled catalase 3 drops	<i>Glaucoma</i> 2 cc. Haematopor. 3 drops Catalase 2 drops
YD	46'	—	—	74'
YE	66'	76'	76'	66'

It must be concluded that no hydrogen peroxide is formed during the course of a photo-chemical reaction in visible light. Some experiments were undertaken to see whether photo-dynamic action occurs in visible light when oxygen is replaced by a hydrogen acceptor. Methylene blue was chosen for this purpose since it can act both as hydrogen acceptor and as fluorescent sensitiser. Solutions of tyrosine together with a small amount of methylene blue were placed in Thunberg tubes which were then evacuated and exposed to visible light for periods up to 24 hours. Decoloration of methylene blue took place slowly and was nearly complete at the end of this time. Control tubes containing methylene blue and tyrosine kept in the dark after evacuation showed no trace of decoloration. It was found possible to replace tyrosine by other substances such as phenol or guanine which undergo photo-oxidation in the presence of oxygen. Sodium nitrite, which gives no oxygen uptake under aerobic conditions, failed to decolorise methylene blue anaerobically when exposed to light. Replacement of methylene blue by a sensitiser like haematoporphyrin, which does not act as a hydrogen acceptor, does not bring about the photo-oxidation of tyrosine under anaerobic conditions.

SUMMARY.

1. The relation of chemical structure to the phenomenon of photo-dynamic action has been investigated. None of the aliphatic compounds employed was found to undergo photo-oxidation. Of the benzene derivatives only those in which an amino- or hydroxyl group has been introduced into the ring were oxidised. Side chains in which the groups —NH_2 and —CH=CH— occur are oxidised. The other side chain compounds investigated are inactive. Certain other ring compounds, α -naphthol, tryptophan, thiophen and, to a marked degree, the purine derivatives, are oxidised.

2. The significance of this selective action in relation to the photo-oxidation in the living cell is pointed out.

3. The photo-oxidation of tyrosine corresponds to a monomolecular reaction following the law of mass action.

4. During the course of the reaction the photo-catalyst is gradually destroyed. This is true of uro- and haemato-porphyrin and of eosin and methylene blue. The last two do not undergo autoxidation as in the case of the porphyrins.

5. The presence of ionised iron salts and of glass powder has no accelerating influence on the reaction. Potassium cyanide does not inhibit the reaction. The behaviour of iron salts in visible light contrasts with their activity as sensitisers in ultra-violet light.

6. Hydrogen peroxide is not formed during the course of the reaction.

7. Methylene blue brings about the oxidation of tyrosine and other substances under anaerobic conditions in the presence of visible light. It appears to act in this respect both as photo-catalyst and hydrogen acceptor.

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