XVII. STUDIES ON XANTHINE OXIDASE. X. THE ACTION OF LIGHT.

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THE action of light on various enzymes has been studied by many investigators. In the great majority of cases the light has been found to produce a destruction of the enzyme, particularly in the presence of certain dyes. The enzymes which have been chiefly studied are invertase, diastase, pepsin, trypsin, zymase, urease, peroxidase, and catalase. In all these cases destruction has been found to occur. The presence of oxygen appears however to be necessary for the action of light. Tappeiner and Jodlbauer [1905] found that invertase and diastase underwent no destruction in the absence of oxygen, and Jamada and Jodlbauer [1908] and Zeller and Jodlbauer [1908] showed that this was also true of peroxidase and catalase respectively. These authors have found, however, that although oxygen is necessary in all these cases for the action of visible light, the destruction by ultra-violet light is independent of the presence of oxygen.

The action of light on the reducing enzymes of the dehydrase type has not been systematically investigated. In fact the only reference in the literature appears to be a statement in a paper by Harden and Norris [1915] on the reduction of methylene blue by the enzymes of dried yeast. They stated that the reduction was greatly accelerated in certain cases by exposure to light, but that this acceleration only occurred if the solutions contained dissolved oxygen.

We have investigated the effect of visible light on the reduction of methylene blue by the xanthine oxidase system. As the source of the enzyme we used either fresh milk, the caseinogen preparation of Dixon and Thurlow [1924], or the whey preparation of Dixon and Kodama [1926]. The reaction was carried out in all cases in glass Thunberg tubes in the usual manner.

THE ACTION OF LIGHT.

On comparing the rates of reduction of methylene blue by the xanthine oxidase system in the dark and in sunlight, effects were observed precisely similar to those obtained by Harden and Norris with the yeast enzymes. If the tubes were carefully freed from oxygen by thorough evacuation, shaking to remove all dissolved gases, refilling with nitrogen, and once more thoroughly evacuating, no acceleration was produced by the sunlight. If, however, the tubes were simply exhausted to a pressure of 5 cm. of Hg, so as to leave a

small amount of oxygen, the light produced a large acceleration. This is illustrated by the following experiment.

Exp. 1. Each tube contained 3 cc. of a 2 % solution of the whey preparation + 2 cc. phosphate buffer (p_H 7.6) + 1 cc. 1/5000 methylene blue + 0.1 cc. of a 0.2 % solution of hypoxanthine.

Oxygen-free Air at 5 cm. Hg.	Reduction times at 15°	
	In sunlight	In dark
	10' 15'	10' 45'

The apparent rate of reduction is naturally slower in the presence of the air owing to the reoxidation of the leuco-methylene blue by the oxygen, but it will be seen that in the presence of the air the reaction is accelerated threefold by the light. The experiment shows clearly that the presence of oxygen is necessary for the action of light.

Various sources of illumination were tried; sunlight, a mercury arc lamp, a carbon arc, and even an ordinary 100 watt electric bulb at a distance of 1 ft.; all gave large accelerations.

A very slight rise of temperature during the irradiation could not always be avoided, but it was always too insignificant to affect the reduction time appreciably, and the irradiation was never found to produce any effect in the absence of oxygen. In experiments where care was taken to keep the temperature absolutely constant during the irradiation similar results were obtained.

The next stage in the investigation of the action of light on the reaction was to determine the effect of previous irradiation of the components of the system individually in the presence of oxygen, the actual reaction being subsequently carried out in the absence of light and oxygen.

It was found that previous irradiation of the buffer solution or the hypoxanthine produced no effect on the reaction velocity. A brief preliminary irradiation of the enzyme (in the presence of oxygen) alone or, better, in the presence of methylene blue greatly increases its activity as the following experiment shows.

Exp. 2. The same quantities were used as in *Exp. 1* but with a different preparation of the oxidase. In tube A none of the constituents was irradiated. In tube B the enzyme and buffer were previously exposed to sunlight in a tube open to the air for half an hour, and the methylene blue and hypoxanthine subsequently added. In tube C the methylene blue was added to the enzyme and buffer and then exposed similarly to sunlight for half an hour, the hypoxanthine being added afterwards. In this and in all subsequent experiments all the tubes were cooled to the same temperature after irradiation, and immediately after adding the hypoxanthine all traces of oxygen were very carefully removed from the tubes by the methods used in *Exp. 1*. Errors due to temperature differences and reoxidation of the leuco-methylene blue were therefore eliminated.

Tube	Reduction time in dark at 15°
A	42'
B	35'
C	20'

It will be seen that the previous irradiation of the oxidase, particularly in the presence of the dye, has produced a large increase in its activity. The experiment is typical of many.

It was found that while short exposures produced an acceleration, longer exposures produced a deceleration, presumably owing to a destruction of the enzyme. This effect, as well as the acceleration, is dependent on the presence of oxygen, as the following experiment shows.

Exp. 3. The same quantities were used as in *Exp. 1*, except that only 0.5 cc. of methylene blue was taken, and that fresh milk was used instead of the solution of the oxidase preparation. (Precisely similar results have been obtained with the purified oxidase.) In tube A the components were not irradiated. In tubes B and C the milk, buffer and methylene blue were mixed and exposed to sunlight for 6 hours. Tube B was carefully freed from oxygen before the irradiation, while tube C was left open to the air and occasionally shaken during the irradiation. The day was cold and the temperature did not rise appreciably.

Tube	Reduction time in dark at 37°
A	3'
B	3.5'
C	Not reduced in 60'

Eosin also was found to be capable of acting as a sensitising dye instead of methylene blue.

Exp. 3 a. The procedure of the preceding experiment was repeated exactly, except that 0.5 cc. 1/500 eosin was added before the irradiation instead of the methylene blue. The methylene blue was added after the irradiation and it was found that the determination of its reduction-time was a perfectly easy matter in spite of the presence of the eosin, which is not reduced by the system.

Tube	Reduction time in dark at 37°
A	3'
B	5'
C	Not reduced in 60'

It is clear that the nature of the effect (acceleration or deceleration) produced by the irradiation depends on the length of the period of exposure. The following experiment shows the relation more completely.

Exp. 4. A series of open tubes each containing 2 cc. of a 2 % whey preparation + 2 cc. buffer (p_H 7.6) + 1 cc. methylene blue was irradiated by a carbon arc at a distance of 1 ft. At intervals tubes were removed and cooled (the temperature rose a few degrees during the irradiation), and the reduction times with hypoxanthine then determined at 15° after thorough evacuation. A second series with enzyme and buffer but without methylene blue was simultaneously irradiated, the methylene blue and hypoxanthine being added afterwards. The results are given graphically in Fig. 1. Fig. 2 gives a similar

curve obtained by exposing 0.5 cc. of fresh milk + 1 cc. methylene blue + 3 cc. buffer to strong sunlight for varying periods. In both cases the acceleration produced by short exposures and the destruction with long exposures is well marked.

Fig. 1.

Fig. 2.

Fig. 1. Effect of previous irradiation of oxidase with carbon arc.

A. Enzyme irradiated with methylene blue in presence of air.

B. Enzyme irradiated without methylene blue in presence of air.

C. Enzyme irradiated with methylene blue in absence of air.

"Activity" denotes reciprocal of reduction-time (in minutes) $\times 100$.

Fig. 2. Effect of previous irradiation of oxidase with sunlight.

A. Enzyme irradiated with methylene blue in presence of air.

B. Enzyme not irradiated.

Most of those who have studied the action of light on other enzymes have obtained only a destruction of the enzyme. In general it appears however that the exposures given were fairly long. In two cases the effect of short exposures has been studied, and in both cases results similar to those given above were obtained. Green [1897] found that exposing ptyalin to light at first markedly increased its activity, and then with longer exposures destroyed it. Bering and Meyer [1912] showed that exactly the same effects were produced in the case of peroxidase. It seems probable that the results obtained by Harden and Norris with the reducing enzymes of yeast may be due to a similar action. The fact that similar effects have been obtained with enzymes of such different origin and nature suggests that the phenomenon may be a fairly general one, and is a point of some interest in connection with the problem of enzyme constitution.

The fact that oxygen is necessary, as well as light, for both the phase of acceleration and the phase of destruction indicates that the two effects are due to the same cause—a photo-oxidation of the enzyme, the first stage of the oxidation resulting in an increase in its activity, possibly by the production of fresh active centres, the later stage causing its destruction.

It appeared possible from certain other considerations that the oxygen might form some active oxidising agent under the influence of the light in the presence of the dye, and that this agent might act on the enzyme to produce the effects observed.

The effect of previous irradiation of the methylene blue alone was therefore next tested in the presence and absence of oxygen.

Exp. 5. In each tube were placed 1 cc. methylene blue + 3 cc. buffer. Tube A was not irradiated. Tube B was left open to the air and was irradiated for 2 hours by the mercury vapour lamp. Tube C was thoroughly evacuated and then similarly irradiated. After the irradiation the tubes were cooled to the same temperature, 0.5 cc. milk (diluted 1 : 2) added, and the tubes allowed to stand for an hour. The reduction-times with 0.1 cc. hypoxanthine were then determined in the usual way.

Tube	Reduction time in dark at 15°
A	135'
B	165'
C	115'

It will be seen that the previous irradiation of the dye (in the presence of oxygen) has produced a distinct deceleration of the reaction so that the above hypothesis of the formation of the active agent from oxygen and the dye by the irradiation seems to be supported. If, however, the irradiated dye is not allowed to stand for some time with the enzyme before testing, no effect whatever is produced and the reduction-times with irradiated and non-irradiated dye are identical. The period of standing is necessary in order to allow the active oxidising agent to act upon the enzyme, as is shown in experiments given later.

The irradiation of the dye *in vacuo* resulted in a small acceleration (tube C). This acceleration is probably to be explained by the presence of a small trace of oxygen in the tube. This may have been sufficient for the formation of an amount of the oxidising substance too small to produce any destruction of the enzyme but enough to give a slight acceleration.

If the view expressed above is correct we should expect that a given amount of the active substance will have a destructive effect on a small amount of enzyme but an accelerating effect on larger amounts, since an amount sufficient to oxidise a small amount of enzyme as far as the phase of destruction will be insufficient to oxidise a larger amount beyond the acceleration phase. The following experiment, which is typical of several, shows that this is actually the case.

Exp. 6. Two tubes each containing 1 cc. methylene blue + 3 cc. buffer were irradiated $2\frac{1}{2}$ hours with the mercury vapour lamp, being left open to the air during the irradiation. To one tube was then added 0.2 cc. milk and to the other 1 cc. milk. Two control tubes were also put up with similar quantities, but with non-irradiated dye. All four tubes were then allowed to stand for an hour at room temperature. 0.1 cc. hypoxanthine was then added, the tubes were carefully evacuated, and the reduction-times determined as before.

	Reduction time in dark		
	Dye irradiated	Dye not irradiated	
0.2 cc. milk	26'	23.5'	(at 37°)
1.0 cc. „	14'	19.5'	(at 15°)

With 0.2 cc. milk the previously irradiated dye has produced some destruction of the enzyme, but with five times as much milk it has produced a marked acceleration; thus on changing the enzyme concentration an actual reversal of the effect of the previous irradiation occurs.

The effect of varying the enzyme concentration is also evident in the following experiment in which the enzyme and the methylene blue were previously irradiated together.

Exp. 7. In this experiment open tubes containing 1 cc. methylene blue + 2 cc. buffer + 2 cc. of a solution of the whey preparation of the strength given in the first column of the table were irradiated for 16 minutes with a carbon arc, and the activities compared as before with similar non-irradiated control solutions.

	Reduction times in dark at 15°	
	Previously irradiated	Not irradiated
2 % whey preparation	15'	16'
1 % " "	63'	35'
0.5 % " "	No reduction in 2 hours	45'

THE FORMATION AND ACTION OF H_2O_2 .

Certain facts in connection with the behaviour of the irradiated dyes had led us to consider the possibility that the active agent, which was formed during the irradiation in the presence of oxygen and which then acted on the enzyme to produce the effects observed, might be hydrogen peroxide.

In order to test whether hydrogen peroxide is actually formed when a methylene blue solution is irradiated in the presence of oxygen we endeavoured to see whether nitrites could be oxidised by such a solution in the presence of milk peroxidase, using the technique of Thurlow [1925]. Hydrogen peroxide oxidises nitrite rapidly in the presence of such a peroxidase, but not at an appreciable rate in its absence.

Exp. 8. The peroxidase was prepared by the method given by Thurlow. The Griess-Ilosvay reagent was used to detect and estimate roughly the nitrite remaining at the end of the experiment. By comparing the resulting colour with the colour given under the same conditions by an amount of nitrite equal to that originally added it was possible to determine how much had been oxidised during the experiment. A careful series of preliminary experiments showed that this was perfectly feasible in spite of the presence of the methylene blue. The concentration of the latter was the same in all tubes, and the blue colour in no way interfered with the detection of the red colour produced by the Griess-Ilosvay reagent. Six tubes were taken. Tubes A, B, C and F contained 1 cc. of a 5 % solution of milk peroxidase + 2 cc. buffer + 0.5 cc. methylene blue + 1 cc. $NaNO_2$ (= 0.01 mg.). In tube D the peroxidase was previously inactivated by boiling, the contents being otherwise the same, while tube E contained peroxidase, buffer, and nitrite but no dye. Tube A was a control and was tested immediately after the mixing. It served

as a standard to indicate the colour given by the amount of nitrite originally present. Tubes C, D and E were irradiated open to the air for $1\frac{1}{2}$ hours with the mercury vapour lamp. Tube F was similarly irradiated but with all traces of oxygen carefully removed from the tube. Tube B was not irradiated but stood meanwhile in the dark open to the air. At the end of the irradiation 0.5 cc. methylene blue was added to tube E and the Griess-Ilosvay reagent added to all tubes. The results were as follows:

Tube	Nitrite remaining unoxidised
A	+++
B	+++
C	--
D	+++
E	++
F	+++

In tube C which was irradiated and in which oxygen, dye and peroxidase were all present, the oxidation of the nitrite was complete. The presence of all factors is, however, necessary. No oxidation took place in the absence of light (tube B) or of oxygen (tube F) or with boiled peroxidase (tube D). Some oxidation indeed occurred in tube E where all the factors were present except the methylene blue. This is probably to be explained by the formation of a small amount of peroxide by the irradiated peroxidase preparation itself (compare the similar effect in Exp. 4).

We consider that these results constitute good evidence of peroxide formation during irradiation in the presence of oxygen.

It now remained to determine whether the effects of the irradiation on the xanthine oxidase could be duplicated by the action of hydrogen peroxide, as was to be expected if our view were correct that this was the agent responsible for the light effects.

It had already been shown [Dixon, 1925] that the xanthine oxidase, in common with most enzymes, was destroyed by small amounts of hydrogen peroxide. Still smaller amounts should, on our view, produce an increase in the activity of the enzyme. The action on the enzyme of hydrogen peroxide in varying amounts was therefore tested.

Exp. 9. A series of tubes was prepared containing 5 cc. buffer + 1 cc. methylene blue and in addition hydrogen peroxide of a final concentration varying from 0 to $M/1000$. 1 cc. of a 5 % solution of a caseinogen preparation of the oxidase was added to each tube, and the tubes were allowed to stand for 2 hours at room temperature, to allow time for the peroxide to act on the enzyme. 0.1 cc. hypoxanthine was then added to each tube, all the tubes were evacuated, and the reduction times determined as before.

The results are shown graphically in Fig. 3. It will be seen that the hydrogen peroxide in a concentration of one-millionth molar has practically doubled the activity of the enzyme, and that quite a marked effect is produced by a concentration of one-hundredmillionth molar. With concentrations of peroxide greater than $10^{-5} M$ the retarding effect of the peroxide is

partly due to its reoxidation of the reduced methylene blue diminishing the apparent velocity of reduction. Since the initial concentration of the methylene blue was about $10^{-4} M$ this effect can introduce no appreciable error into the results with peroxide concentrations less than $10^{-5} M$, but must be allowed for with concentrations greater than this. The falling part of the curve in Fig. 3 does not therefore necessarily represent an actual destruction of the enzyme in this experiment. With a weaker solution of enzyme, however, the destruction shows up clearly, even with H_2O_2 concentrations of the order of $10^{-6} M$ where the reoxidation effect is entirely negligible (*e.g.* in Exp. 11 below).

The optimum peroxide concentration for acceleration with enzyme concentrations of the order used in Exp. 9 was found in a number of experiments to be about one-millionth molar, and if the conditions are correctly adjusted, the increase of activity may become very large, as in the following experiment.

Fig. 3. Effect of varying concentrations of H_2O_2 . The horizontal straight line represents the activity without H_2O_2 .

Exp. 10. Two tubes were made up, each containing 0.5 cc. 5 % caseinogen preparation + 5 cc. buffer + 1 cc. methylene blue and the amount of peroxide given. The tubes were allowed to stand for $1\frac{1}{2}$ hours and the reduction-times with hypoxanthine then determined.

Amount of H_2O_2	Reduction time at 15°
0	61'
$10^{-6} M$	24'

The reversal of the effect by changing the enzyme concentration which was found in Exps. 6 and 7 was also obtained when using hydrogen peroxide instead of the light, as follows.

Exp. 11. Four tubes were prepared, each containing 5 cc. buffer + 1 cc. methylene blue. To one pair of the tubes 0.2 cc. 5 % caseinogen preparation was added, and to the other pair 0.5 cc. of the same solution. One tube of each pair contained $10^{-6} M$ hydrogen peroxide. The tubes were allowed to stand for $1\frac{1}{2}$ hours before the activities with hypoxanthine were tested.

Amount of oxidase	Reduction time at 15°	
	Without H_2O_2	With H_2O_2
0.2 cc.	170'	195'
0.5 cc.	84'	70'

The necessity for allowing sufficient time for the peroxide to act upon the enzyme is shown by the following experiment, in which the peroxide was allowed to act for varying periods of time before the activity was tested.

Exp. 12. Each tube contained 0.5 cc. 5 % caseinogen preparation + 5 cc. buffer + 1 cc. methylene blue + hydrogen peroxide in a concentration of $10^{-6} M$. The tubes were allowed to stand for varying times before the hypoxanthine was added and the reduction time determined. The results are given in Fig. 4.

Fig. 4. Time curve of action of $10^{-6} M$ H_2O_2 on oxidase. The horizontal straight line represents the activity without H_2O_2 .

In certain cases when using milk as the source of oxidase we have been unable to obtain effects with either hydrogen peroxide or irradiation. This we ascribe to the presence of catalase in these milks in amounts sufficient to prevent oxidation by the peroxide. Using the enzyme preparation which is free from catalase, on the other hand, we have never failed to obtain the effects, and we consider that the difference affords further support to the view that the irradiation effects are due to the formation of peroxide. It should be mentioned that curves exactly similar to that of Fig. 3 have been obtained with certain milks, which were presumably deficient in catalase.

Aldehydes are capable of replacing hypoxanthine in the oxidase system as the hydrogen donator, and nitrates can be used instead of methylene blue as the oxidiser in the reaction. The activity of the enzyme was therefore compared before and after the action of peroxide, using these substances as the reactants.

Exp. 13. The tubes contained 0.5 cc. 5 % caseinogen solution + 5 cc. buffer + 1 cc. methylene blue or 5 % $NaNO_3$, with in addition $10^{-6} M$ H_2O_2 in two of the tubes. They were allowed to stand 1 hour. Then 0.5 cc. 1 % acetaldehyde was added to each tube, instead of the usual hypoxanthine, and all the tubes immediately evacuated. In the case of the tubes containing methylene blue the reduction times were determined in the usual way. The tubes containing nitrate were kept at 15° for 2 hours; and they were then opened and the Griess-Ilosvay reagent was added to determine the amount of nitrate which had been reduced to nitrite.

Amount of H_2O_2	Reduction time methylene blue at 15°	Amount of nitrite formed
0	105'	++
$10^{-6} M$	85'	+++

The acceleration by small amounts of peroxide still occurs when aldehyde is substituted for hypoxanthine and nitrate for methylene blue, and this affords further support to the view that its action is on the enzyme itself and not on the reactants.

The photo-oxidation of the enzyme is unaffected by the addition of cyanide, so that it is presumably not dependent on the presence of traces of catalytic metals.

Exp. 14. In each of four tubes was placed 5 cc. 2% solution of a whey preparation of the oxidase + 1 cc. buffer + 1 cc. methylene blue. One pair of the tubes contained in addition potassium cyanide in a concentration of $M/400$. 0.1 cc. hypoxanthine was added to each tube and the tubes were exhausted to 10 cm. pressure (cf. Exp. 1). One tube of each pair was placed in the sunlight, the others in the dark.

	Reduction time at 15°	
	In light	In dark
Without KCN	10'	> 50'
With KCN	10'	> 50'

To sum up then, we consider that the foregoing experiments show that the mechanism of the action of light on the system is as follows. An active oxidising agent, very probably hydrogen peroxide, is formed from molecular oxygen under the influence of the light in the presence of the dye, or, to a lesser extent, in the presence of the enzyme preparation¹. This agent then acts upon the enzyme, producing first an increase and then a decrease of activity. We are at present unable to state how the increase of activity is brought about. It is possible that fresh active centres are formed from inactive precursor groups by the oxidation. It is also not impossible that the peroxide might destroy by oxidation some inhibitor substance associated with the enzyme, and so liberate fresh active centres. It would clearly be a difficult matter to decide experimentally between these hypotheses.

A number of facts in the literature are in harmony with the view given above. Straub [1904] showed that if eosin were irradiated in the presence of oxygen it would liberate iodine from potassium iodide, but that this only happened if oxygen were present. Downes and Blunt [1877] and Tappeiner and Jodlbauer [1905] showed that light was fatal to certain bacteria in the presence of oxygen, but not in its absence. Ledoux-Lebard [1902] found that eosin previously irradiated in the presence of oxygen killed paramoecia but that eosin irradiated in the absence of oxygen was harmless. All these effects might be expected if H_2O_2 were formed as we have supposed.

It should be pointed out that the formation of H_2O_2 by irradiation has no connection with the H_2O_2 formation shown by Thurlow [1925] to occur

¹ Which presumably contains some substance which acts in the same manner as the dye.

in the aerobic oxidation of hypoxanthine by the oxidase. Oppenheimer [1926] has suggested that the formation of H_2O_2 observed by Thurlow might in fact be due to the action of light on the enzyme preparation. Three facts show that this is not so: (a) Thurlow's results occur equally well in absolute darkness, as we have confirmed on several occasions; (b) Thurlow's peroxide formation occurs only in the presence of hypoxanthine or its equivalent, whereas the light effect occurs in the absence of hypoxanthine; (c) the amounts of H_2O_2 formed by light are infinitesimal in comparison with those formed in Thurlow's experiments.

We may contrast with the behaviour of the oxidase system that of the sulphhydryl system. Dixon and Tunnicliffe [1923] showed that the reduction of methylene blue by cysteine and glutathione was accelerated considerably by light. But this system differs fundamentally from the oxidase, in that the effect of the light is not dependent on the presence of oxygen and takes place equally well no matter how carefully the tubes are freed from oxygen.

Exp. 16. Great care was taken to ensure that all tubes were oxygen-free. Each tube contained 1 cc. cysteine solution (containing 5 mg. of the hydrochloride, neutralised) + 3 cc. buffer + 0.5 cc. methylene blue.

Amount of KCN present	Conditions of experiment	Reduction time
0	In sunlight	14'
0	In diffuse light	8'
M/100	In weak sunlight	5'
M/100	In dark	140'
M/500	In dim light (indoors)	43'
M/500	In dark	180'

The reduction times were determined at room temperature, but the three sets were not carried out at precisely the same temperature, and therefore the tubes in different pairs cannot be strictly compared. It will be seen that the effect of the light occurs also in the presence of cyanide, which prevents that part of the reaction which is due to metal catalysis [Harrison, 1927], so that the reduction is that due to the —SH group itself. The last pair of tubes shows that the system is extremely sensitive to light. Harrison found that it was difficult to obtain strictly reproducible results with metal-free sulphhydryl compounds, and suggested that this might be due to varying intensity of light on different days; and these figures seem to support the view.

By a somewhat curious coincidence Kendall and Nord [1926] found that the reduction of indigo carmine by cysteine was greatly accelerated by the addition of hydrogen peroxide. The apparent analogy of this system with the xanthine oxidase in this respect is, however, a false one, since the peroxide acceleration with the cysteine does not occur with methylene blue but only with indigo carmine, and has been traced by Dixon and Tunnicliffe [1927] to the action of the peroxide on this dye producing a catalytically active product.

SUMMARY.

1. The reduction of methylene blue by the xanthine oxidase system is markedly accelerated by light, but only if traces of oxygen are present.
2. Previous exposure of the oxidase solution or the methylene blue to light (in the presence of oxygen) for short periods produces an acceleration of the reaction. Exposure for longer periods produces a destruction of the enzyme. Neither of these effects occurs if oxygen is absent during the irradiation.
3. Both these effects are due to the formation of an active oxidising agent, probably hydrogen peroxide, from the oxygen during the irradiation. This oxidises the oxidase; the first stage of the oxidation produces a marked increase in the activity of the enzyme, and the later stages destroy the enzyme.
4. Both these effects are produced by the addition of hydrogen peroxide in small quantities to the oxidase. The presence of one-millionth molar peroxide in general more than doubles the activity of the enzyme, while a very definite acceleration is produced by one-hundredmillionth molar peroxide. In higher concentrations the peroxide destroys the enzyme.
5. The nature of the effect produced by a peroxide solution of given strength depends on the amount of oxidase present. One and the same solution of peroxide may produce an acceleration with one solution of enzyme, and a retardation with another (weaker) solution. The same is true of solutions of methylene blue which have been exposed to light and oxygen.
6. The accelerating action of added hydrogen peroxide on the enzyme can also be seen when aldehyde is substituted for hypoxanthine and nitrate for methylene blue.
7. In presence of peroxidase, the peroxide formed during the aerobic irradiation of methylene blue solutions is able to carry out secondary oxidations (*e.g.* nitrites). This oxidation does not occur in the absence of either peroxidase, or oxygen, or light.

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