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The Reaction between Peroxides and Leucomalachite Green Catalyzed by Heme in the Presence of Organic Solvents

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In a previous paper[1] it was reported that peroxides of fats and fatty acids, in the presence of heme, react with easily oxidizable substances. The reaction was found to be very much accelerated when carried out in an organic solvent. In the quantitative study of the reaction, and for comparison with the corresponding reaction involving hydrogen peroxide instead of organic peroxides, leucomalachite green was used as the easily oxidizable compound. This substance, as well as the malachite green formed in the reaction, are fairly stable compounds, and the colour of malachite green is not much influenced by the pH.

EXPERIMENTS

The reaction between hydrogen peroxide and leucomalachite green has been studied before by other investigators, for instance, Willstätter and Weber[2]. The leucomalachite green used was purified by repeated recrystallisations, as described by these authors. The substance is easily soluble in organic solvents. Aqueous solutions were prepared by saturating 0.05 *N* acetic acid with the substance. With impure products, the solutions were unstable, and the yield of malachite green was not quantitative.

Solutions of hydrogen peroxide were tested quantitatively by titration with potassium permanganate, and fresh dilutions, with redistilled water, were made up before each experiment. Various oils and fatty acids were used as organic peroxides, the peroxide values of which were determined by the method of King *et al.*[3], and by the authors' method[4].

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In most of the experiments, a solution prepared by dissolving 20 mg hemin * in a mixture of 5 ml pyridine and 10 ml glacial acetic acid was used as a source of heme. All solvents should be rigorously pure, as reported in a previous paper [1].

Most of the experiments were carried out by adding 0.8 ml of a dilution of peroxide to a mixture of 4 ml of the leucomalachite green solution and 0.2 ml heme solution in a test tube. The intensity of the colour was read in the Beckman photometer at 620 mμ or in the Klett-Summerson colorimeter using filter 610. Extinction curves were constructed for malachite green. The results were calculated in terms of milliequivalents of peroxide per kg. One equivalent of malachite green corresponds to 2 moles of peroxide. One milliequivalent per liter of the reaction mixture corresponds to an extinction of 25 in a 1 cm layer.

With hydrogen peroxide in an aqueous medium at room temperature the colour reached its maximum in the course of 30 to 50 minutes. Under these circumstances the yield was quantitative. With 0.1 ml of a 1 : 100 000 dilution of 30 per cent hydrogen peroxide a strong colour was obtained, and even 0.1 ml of a 1 : 1 000 000 dilution could be determined since it gave an extinction of about 0.05 in 1 cm layer in 5 ml total volume. Thus it is possible by this method to determine very small amounts of hydrogen peroxide.

The influence of variations in certain of the experimental conditions on the yield and velocity of the reaction was studied. As mentioned above this problem has been studied by other investigators, and their results could be confirmed. The optimum value for the pH was found to be about 4.1. An increase in the temperature also increased the velocity of the reaction. High concentrations of leucomalachite green favours the reaction velocity. A concentration of heme as given above is suitable. Too high concentrations can result in a decrease in the yield, probably due to a catalatic action of heme. With heme alone, without the presence of pyridine, the velocity of the reaction is very low.

When a peroxidized fat is tested under the same circumstances instead of hydrogen peroxide, no quantitative reaction will take place. On shaking some drops of a highly peroxidized oil with 4 ml of an aqueous leucomalachite green solution plus 0.2 ml heme solution for half an hour, the fluid will assume a green colour which will, however, be very faint as compared with the result of a stoichiometric reaction. It was found necessary, therefore, to cause the reaction to take place in a one phase system by means of a fat solvent. In order to study the influence of a fat solvent on the reaction, the influence of

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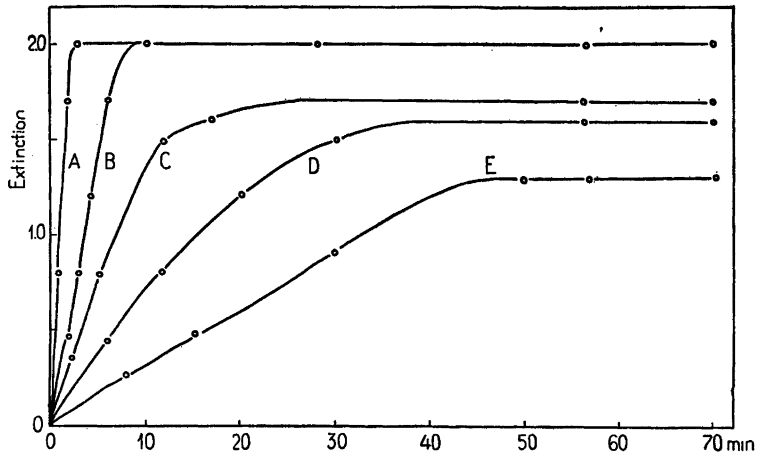

Fig. 1. Heme-catalyzed reactions between hydrogen peroxide and leucomalachite green carried out in mixtures of acetone and water.

Four ml substrate + 0.2 ml hydrogen peroxide solution + 0.2 ml heme solution.
Substrate: Mixtures, in the proportions given below, of 0.4 per cent leucomalachite green solutions, in 0.4 N. aqueous acetic acid and in acetone, respectively.
Hydrogen peroxide solution: A dilution of 5 : 100 000 of a 30 per cent stock solution.
Heme solution: 20 mg heme + 100 ml pyridine + 100 ml glacial acetic acid.
Readings at 615 mμ in the Beckman spectrophotometer.

Curve A: 100 per cent acetone solution,
or 80 » » » + 20 per cent aqueous solution,
or 50 » » » + 50 » » » »
Curve B: 25 » » » + 75 » » » »
» C: 12 » » » + 88 » » » »
» D: 5 » » » + 95 » » » »
» E: 100 » » aqueous solution.

substituting part or all of the water in the hydrogen peroxide reaction by different fat solvents was investigated.

The influence of acetone (or ethyl alcohol) on the reaction between hydrogen peroxide and leucomalachite green can be seen from Fig. 1. The experiments were made in the same manner as described above, and with the same concentrations of active substances, but instead of water, acetone or acetone-water mixtures in various concentrations were used as a solvent for the leucomalachite green. In order to facilitate quick readings of the colours, the experiments were carried out in test tubes which could be put directly into a Klett-Summerson colorimeter where the colour development could be followed continuously. Fig. 1 shows an example of the influence of different concentrations of acetone, which are indicated in the curve.

The curves show that acetone accelerates the reaction greatly. However, the fact that the end-point of the reaction is situated at a much higher colour intensity indicates that the reaction is different from that which occurs when water is absent. When water is present the reaction stops when the hydrogen peroxide has been used up, whereas in the presence of large amounts of acetone the reaction is not stoichiometric.

When the reaction is carried out in acetone there seems to be an induction period of a few seconds during which no formation of colour can be observed. After this period the development of colour accelerates. In water no such induction period can be observed. Therefore, a graphic representation of the resulting colour of the reaction in relation to time will give an S-shaped curve when acetone is used as a solvent, but not when water is used. While the amount of hydrogen peroxide present in the acetone or alcohol reactions does not greatly influence the amount of malachite green formed, it does greatly influence the velocity of the reaction, and especially the length of the induction period. Finally, the reaction in acetone shows a much greater tendency to take place without the presence of peroxides, that is to say, that a solution of heme-pyridine and leucomalachite green is much less stable in acetone, or alcohol, than in water.

As mentioned above, peroxidized fats and fatty acids give a similar reaction with leucomalachite green and pyridine-hemochromogen. Also in this case, the amount of peroxide influences the velocity of the reaction. It is a proof of the identity of the two reactions that the same amount of peroxide, either in the form of hydrogen peroxide or of organic peroxide, gives the same acceleration of the reaction. This can be seen from the experiment reported in Fig. 2 which at the same time examplifies the special characteristics mentioned above of the reaction in an organic solvent.

The experiment was carried out in the following way: In test tubes which could be put directly into a Klett-Summerson colorimeter were placed 4 ml of a 0.06 per cent solution of leucomalachite green in absolute alcohol, and 1 ml of a solution of hydrogen peroxide or peroxidized linseed oil in absolute alcohol having a peroxide content in milliequivalents per liter as indicated in the curves of the graph. Then 0.1 ml pyridine-hemochromogen solution was added, and at the same time the stop-watch was started. The tube was then inserted in the colorimeter and the colour development was followed.

The curves are most easily explained by a chain-reaction which as soon as inhibiting substances are used up, proceeds very rapidly until one of the reacting compounds is also used up. These inhibiting substances can be oxidized by peroxides.

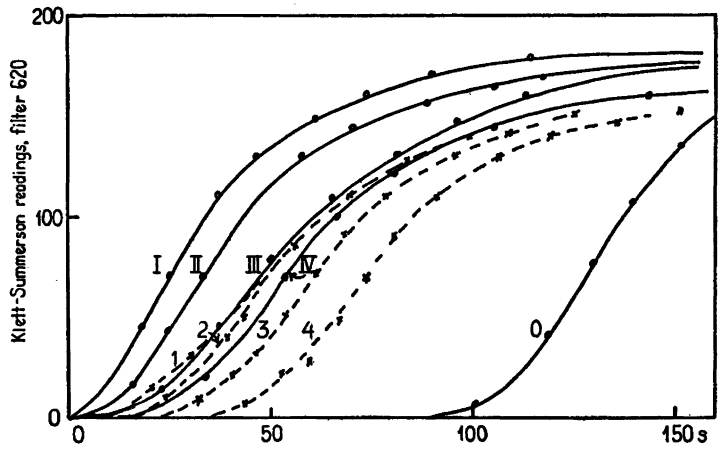

Fig. 2. Heme-catalyzed oxidation of leucomalachite green in alcohol in the presence of inorganic or organic peroxides.

Four ml substrate + 0.9 ml peroxide + 0.1 ml heme solution.
Substrate: 0.06 per cent leucomalachite green in 96 per cent alcohol.
Peroxide: Solutions, of the concentrations given below, of hydrogen peroxide or peroxidized linseed oil in alcohol.
Heme solution: 20 mg hemin + 5 ml pyridine + 10 ml glacial acetic acid.
Milliequivalents of peroxide added:

Curve	*0*	*No peroxide.*		
»	*1*	*0.0012*	*milliequivalents*	*hydrogen peroxide.*
»	*2*	*0.0006*	»	» »
»	*3*	*0.0003*	»	» »
»	*4*	*0.00015*	»	» »
»	*I*	*0.0032*	»	*organic* »
»	*II*	*0.0016*	»	» »
»	*III*	*0.0008*	»	» »
»	*IV*	*0.0004*	»	» »

Atmospheric oxygen is involved in the reaction. This can be seen from some experiments carried out in modified Thunberg-tubes, in which the experiments could be carried out *in vacuo*. They consisted of tubes of such a diameter that they could be put directly in a Klett-Summerson colorimeter for reading. In the bottom of the Thunberg-tube was put leucomalachite green in alcohol plus peroxide, and pyridine-hemochromogen in the small side-bulb. Then the carefully greased glass-stopper was inserted, and the tube was evacuated through a small tube beneath the stopper and provided with a tap which was then closed. The contents of the tube were then mixed, and the tube inserted in the colorimeter. When the reaction in alcohol or acetone

was allowed to take place under such conditions, and the results depicted graphically, no S-shaped curve could be constructed, but a curve similar to that for a reaction in aqueous medium was obtained.

The reaction as a rule does not proceed so that all the leuco-compound is oxidized but stops before, probably because the hemin has been broken down, which can be seen in different ways by the disappearing of the hemochromogen colour. However, by adding more hemochromogen to a test tube wherein a reaction has been carried out until maximal colour, only a small increase in the colour intensity is seen, and the addition of more peroxide also gives rise to only a small increase in the colour intensity. It seems, therefore, as if breakdown products, which inhibit the reaction, have been formed. These may have been formed from the hemin or from the dye, since the dye when the reaction is violent is also destroyed by the formation of a purple pigment.

The presence of chromic acid also accelerates the reaction, whereas ferric chloride or cupric sulfate does not. This or similar reactions can be observed with different sources of peroxides, with different kinds of easily oxidizable substances (leucomalachite green, leucodichlorophenolindophenol, guajac resin), and with heme prepared by Anson and Mirsky's [5] method, or a commercial preparation of hemin. However, the presence of organic solvents such as ethyl or methyl alcohol, acetone, dioxan, *etc.*, is necessary. The nature of the influence of the organic solvent remains to be elucidated.

SUMMARY

Hemin in the presence of organic solvents strongly catalyzes the oxidation of a number of easily oxidizable compounds by oxygen. A similar strong catalysis is not seen in an aqueous solution, but a certain quantity of an organic solvent must be present. The reaction is accelerated by inorganic or organic peroxides.

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