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Some Observations on the Ability of Heme to Catalyze the Oxidation of Easily Oxidizable Substances by Peroxides of Fats and Fatty Acids *

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It is well known that hemin and related compounds show a peroxidase-like action by catalyzing the oxidation of easily oxidizable substances, for instance, benzidine, by hydrogen peroxide. This fact is the basis of the benzidine and similar reactions for the detection of blood. The peroxidase action of heme has been studied quantitatively by several workers, among them Kuhn and Brann [1] and Reuter *et al.* [2]

As far as the author has been able to determine an analogous reaction in which, instead of hydrogen peroxide, peroxides of fats and fatty acids took part, has been described only by Frehden [3], who used the conversion, by peroxides, of 2,7-diaminofluorene in the presence of hemine to blue meriquinoid oxidation products as a spot reaction for the detection of rancidity in fats. On the other hand, the catalytic influence of hemin on the oxidation of fatty acids by atmospheric oxygen to peroxides, has been studied by several workers, for instance, Robinson [4], Kuhn and Meyer [5] and Franke [6].

Since none of the investigators, who have studied other catalytic functions of hemin, have studied the reactions between peroxides of fats and fatty acids and easily oxidizable substances, this paper will deal with the study of such reactions.

EXPERIMENTAL

The heme used in most of the experiments was prepared by Anson and Mirsky's method [7]. In other experiments a commercial preparation of hemin was used ***.

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Various peroxidized fats and fatty acids were tried, such as oleic acid, lard, cod liver oil, *etc.* The peroxide values were determined by the method of King *et al.*[8], and by the authors' method [9], and calculated as milliequivalents per 1000 g of fat.

Especially for quantitative studies, it was found necessary to use rigorously purified solvents. The acetone, pyridine, xylene, *etc.*, must not contain traces of either reducing or oxidizing substances. They can be tested by adding a few drops of a solution of leuco-dichlorophenolindophenol (containing a little dichlorophenolindophenol) dissolved in ethyl alcohol and acidified with acetic acid, to 20 ml of the solvent in a test tube. After heating this mixture in a water-bath to 70° C, or to the boiling-point of the solvent, the intensity of the red color of the indophenol should show neither a decrease nor an increase.

HEME-CATALYZED OXIDATION OF DIFFERENT SUBSTANCES BY PEROXIDES OF FATTY ACIDS

When benzidine is used as the oxidizable substance in the reaction, the following experiments can be made. In a test tube containing 1 ml of a 2 per cent solution of benzidine in 96 per cent ethyl alcohol, 0.1 ml of a 0.008 per cent solution of heme in distilled water and a few drops of an oil having a peroxide value of about 25 are added. After shaking, only a very faint bluish color can be observed. However, if instead of a peroxidized oil an equivalent amount of hydrogen peroxide is added, a strong blue color will appear.

The blue color produced by a peroxidized oil can be better observed when the benzidine and the heme solutions are mixed and placed on a porcelain dish, and the oil allowed to float on the dish. On the contact surface between the oil and the alcohol-water mixture, a faint but distinctive bluish-green color appears which reaches its maximum in the course ofa few minutes, and then fades out.

The color reaction is still more clearly seen on a filter paper. When the filter paper is impregnated with the benzidine-heme solution and a drop of the oil is brought onto it, a bluish-green ring appears around the oil in the course of a few minutes. It is possible by means of such a paper to make a rough estimation of peroxide values. Oils having a peroxide value of about 20 give a pronounced blue color. At a peroxide value of about 10, the color is fainter, and, due to the color of the oil and the heme, more greenish. A peroxide value of about 3 only makes the color of the paper darker brown, while still lower peroxide values will give no distinct color at all.

The results obtained with benzidine show that this substance reacts with fatty peroxides in the presence of heme, but that the reaction is much weaker than that with hydrogen peroxide. This is probably due to the fact that the reaction does not take place in one phase but in a non-homogenous system, and, consequently, is much slower. Therefore, the unstable colored products obtained by the oxidation of benzidine will not be formed in sufficiently great

amounts to be observed. In order to obtain a stronger color reaction an attempt was made to use substrates which on oxidation would produce more stable colors, and to carry out the reaction in one phase.

When the oxidation of benzidine is carried out in the presence of α-naphthol, a coupled reaction, with the formation of a stable compound — an indophenol — takes place. An intensive red color is developed when 1 drop of a peroxidized oil and 0.1 ml of the heme solution mentioned above are added to 1 ml of a solution containing 1 per cent of benzidine and 1 per cent of α-naphthol in 96 per cent ethyl alcohol.

Similar strong colors can be obtained by other coupled oxidations in the presence of peroxidized fat and heme. Dimethyl-*p*-phenylenediamine + α-naphthol, and *p*-phenylenediamine + α-naphthol will give blue and red colors, respectively.

The colors will appear faster when the mixtures are heated for a few minutes at 70° C on a water bath. Furthermore, faster reactions and more intensive colors are obtained when heme is used in the forms of imidazol-, pyridine-, or other hemochromogens. A solution of 20 mg hemin in a mixture of 120 ml pyridine and 40 ml distilled water, or in a mixture of 5 ml pyridine and 10 ml glacial acetic acid can be used. When 0.1 ml of such a solution is added to a solution of benzidine in alcohol and shaken with a drop of a peroxidized oil, a comparatively strong color is developed. Cyanide inhibits the reactions.

The colors are stronger if the peroxidized oil does not form a suspension in alcohol but is brought into a true solution, for instance, by the addition of a little ether or by carrying out the reaction in acetone. In xylene or other hydrocarbons in which the oil is easily soluble a strong reaction is also seen, but after a short time the hemin precipitates and the reaction stops. If a few drops of a peroxidized oil is shaken with an aqueous pyridine-hemochromogen solution, a weak reaction may sometimes be observed.

Certain other easily oxidizable substances give very strong colors in the reaction, especially when pyridine-hemochromogen is used. This is the case with leucomalachite green, and the colorless compound 3,5-dichloro-4,4'-hydroxyphenylenediamine which is obtained by reduction of the well known redox-indicator 2,6-dichlorophenolindophenol, for instance, with ascorbic acid. Guajac resin can also give a very strong color, whereas pyrogallol gives only a weak color, and tyrosin is not oxidized.

DISCUSSION

The experiments show that peroxides of fats and fatty acids react in the presence of heme, especially when it is in the form of a suitable hemochromogen, with a great variety of easily oxidizable substances. The reaction is

strong, especially if it is carried out in one phase, but the speed of the reaction decreases for more heterogenous mixtures.

The question arises as to whether or not the reaction has a biological significance or function, that is, if enzymes or other heme-containing compounds may function in the plant or animal organism in a manner analogous to peroxidase, but with fat peroxides as oxygen sources. Much evidence of the biological significance of peroxides of unsaturated fatty acids has accumulated in the past years [10], and an enzyme catalyzing the peroxidation has been isolated [11], but no enzyme catalyzing the transfer of peroxidic oxygen from such peroxides has been observed. It is well known that peroxides from horse-radish can react with certain organic peroxides, for instance, ethyl ether peroxide. We have been unable to observe any reaction between a potent extract of horse-radish, peroxidized fats, and easily oxidizable substances; not even when the fats were brought into a very fine suspension by means of 'Tween 80'.

A study of the reaction between peroxides and leucomalachite green, catalyzed by heme, in the presence of organic solvents will be reported in a following paper. A histochemical method for the demonstration of peroxidized fat, based on the reactions described here, has already been published [12].

SUMMARY

Heme, especially in the form of pyridine-hemochromogen, catalyzes the oxidation of benzidine, guajac resin, leucomalachite green and other easily oxidizable substances by peroxides of fats and fatty acids. The reaction is strong when carried out in acetone, ether-alcohol, or other suitable solvents.

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