XXXVI. HAEMOGLOBIN AND METHAEMOGLOBIN AS OXIDATIVE CATALYSTS.

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That iron plays a part as a catalyst in the living cell is inherently probable. The work of Warburg and Meyerhof, upon sea urchin eggs, has provided experimental evidence to show that it catalyses biological oxidations, and Warburg [1914] has claimed for it a predominant influence in respiratory processes. For the most part, experimental studies bearing on this matter have dealt with the effect of adding inorganic ionisable iron salts to preparations from living tissues, or to tissue constituents such as lecithin [Thunberg, 1910]. The tissues, however, contain organic compounds of iron, and among these are haemoglobin and, probably, some of its derivatives.

So far back as 1865 Kühne showed definitely that haemoglobin is present in blood-free muscle fibres. He then suggested that the pigment played some part in the oxidation processes of the contractile substance, his view being supported by the familiar fact that red muscles, which contain most of the pigments are those most capable of sustained activity.

No attempt seems to have been made to test this suggestion directly. It is well known, of course, that haemoglobin and those of its derivatives which contain iron can act as peroxidases or pseudoperoxidases [Buckmaster, 1907] in the sense that they promote the blueing of guaiacum tincture by peroxides, but this fact brings no proof that they play a part in oxidations which have metabolic importance.

At Prof. Hopkins' suggestion the following experiments were therefore undertaken. They deal especially with haemoglobin, methaemoglobin and haemin as catalysts in the oxidation of unsaturated fats.

MacMunn [1884, 1887], as is well known, claimed that in some cases at least, the pigments present in the tissues are special derivatives of blood pigments which, in the case of muscle, he called myohaematins. Whether his view be correct would seem still to be an open question. No attempt has yet been made to separate these substances in a form in which their catalytic activity could be tested.

The substrate actually employed in my observations was linseed oil. This is, of course, a drying oil, and clearly the results obtained might be supposed

to come into close relation with the whole subject of "driers," which are known to act as catalysts of oxidation of oils. This subject has been studied on scientific lines by E. Rideal and others. Rideal and Taylor [1919] point out that efficient driers are usually oxides or salts of metals which can exist in two states of oxidation; they act most effectively when added in some form which is soluble in the oil, cobalt, for instance, in the form of a linoleate, manganese as rosinate, etc.

The curves of oxygen uptake given by Rideal indicate that the uncatalysed autoxidation shows the typical sinuous curve of autocatalysis, while in the catalysed action the period of induction still exists, though it is reduced. It is therefore postulated that the catalytic action depends on an increased production of an autocatalytic agent, normally present in small quantities, which is probably of peroxide form, analogous to that formed by turpentine. The autocatalyst originally present is said to be destroyed by boiling.

The subject has also been studied by Coffey [1922] by the method of measuring the increase of weight of oil spread in very thin films on a glass plate, and by a manometric method, when pieces of filter paper were soaked in a solution of oil in light petroleum and the gaseous exchanges were studied. In the latter case, evidence of CO_2 formation among other volatile decomposition products was obtained. A period of induction of about 15 minutes was a feature of these results.

The apparatus used in the present research was the Barcroft differential manometer, with a bottle of suitably modified form which enables oxidation to be effected more rapidly than is the case for the original pyriform shape, owing to larger surfaces exposed to the air or other gas filling the bottle, which is calibrated for 3 cc. of material. A cup within the bottle may contain 40 % potash so that any carbon dioxide evolved may be absorbed and approximately estimated.

The linseed oil was used in the form of an emulsion, in water or buffer solution, 50 mg. of oil being shaken up with 10 cc. of water or buffer, and 1 or 2 cc. of the ensuing uniform suspension being used for each experiment.

As the oxidation of the oil in my experiments was carried out in a system physically different from that existing in the investigations of the authors just quoted, it seemed well to study the effects of known "driers" when added to an emulsion and shaken in the Barcroft apparatus. The experiments throughout were carried out at 37°. Experiments with cobalt oxide gave a curve for oxygen uptake showing the typical sinuous form obtained by Rideal and others, with a well marked preliminary induction period of about an hour. In control experiments, that is in absence of the catalyst, the oxygen uptake was exceedingly small and never attained appreciable dimensions in nine hours (Fig. 1).

Before describing in detail my observations on blood pigments, it may be stated generally that haemoglobin, methaemoglobin and haemin catalyse with efficiency the autoxidation of linseed oil, in which linolenic acid is doubtless

the constituent chiefly responsible for oxygen uptake. Haematoporphyrin, on the other hand, is without effect. While it would seem, therefore, that the presence of iron in the molecule is essential to the catalytic activity, it is much more difficult to decide whether the mechanism of catalysis is identical with that of inorganic driers in general.

The samples of haemoglobin used were supplied by Mr T. R. Parsons and were prepared from sheep's blood by the freezing centrifuge method. The samples were therefore free from reagents, except possible traces of sodium chloride.

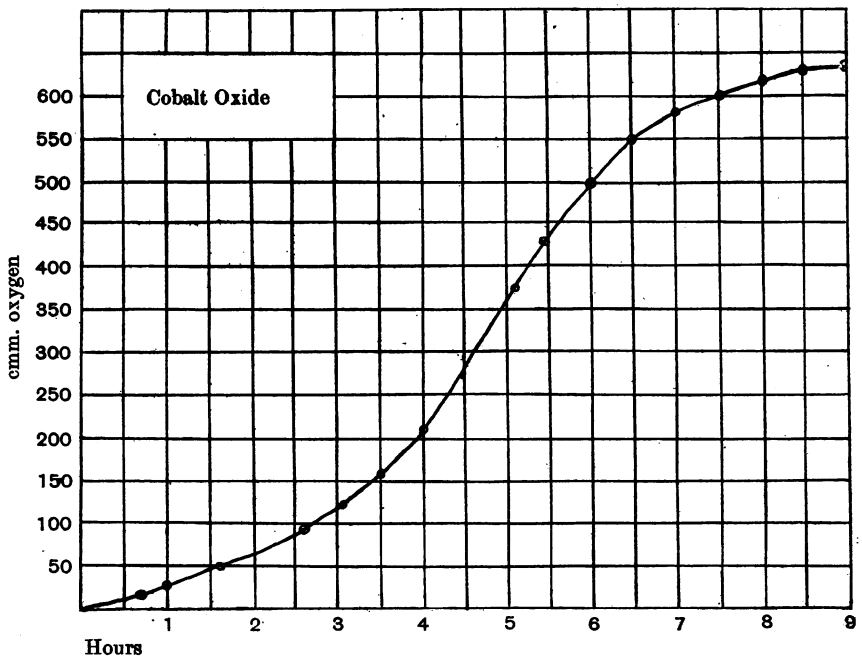

Fig. 1. The catalytic effect of cobalt oxide. 5 mg. linseed oil. 50 mg. cobalt oxide.

Using 1 cc. of a haemoglobin solution, containing 1 mg. haemoglobin per cc. a marked catalysis of oxidation is found to take place, differing from that of the so-called driers in that the preliminary induction period is absent. The acceleration of oxygen uptake, while more or less proportionate to the amount of haemoglobin present when the latter is at low concentrations, failed to increase proportionately for higher concentrations (Fig. 2). Corresponding methaemoglobin solutions, made from crystalline methaemoglobin prepared in a similar manner to the haemoglobin used, were found to exhibit similar catalytic activity, as shown in Fig. 3, where the effects of solutions of haemoglobin and methaemoglobin of equal concentrations are compared. The slight differences in the shapes of the curves for haemoglobin obtained from day to day are due to slightly altered rates of shaking caused by unavoidable variations of current.

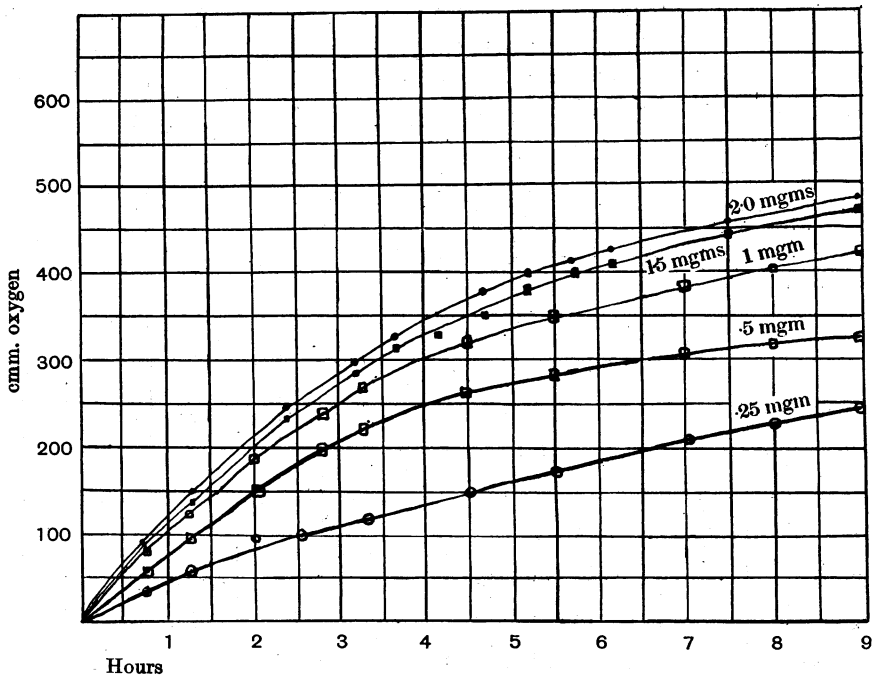

Fig. 2. The effect of haemoglobin, in different concentrations. 5 mg. linseed oil with 2·0, 1·5, 1, ·5 and ·25 mg. of haemoglobin. Control curve (oil without catalyst) as in Fig. 3.

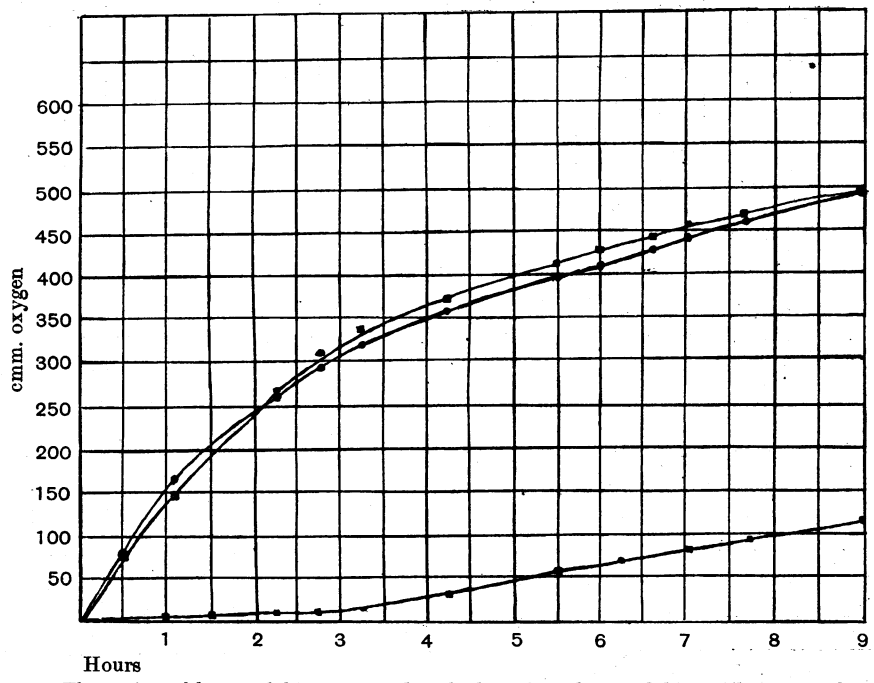

Fig. 3. The action of haemoglobin compared with that of methaemoglobin, with control curve 5 mg. linseed oil with 5 mg. haemoglobin, and the same with 5 mg. methaemoglobin.

There would seem to be little doubt that the oxygen in oxyhaemoglobin is in some special relation with the iron. Apart from general probabilities the work of Laidlaw [1904], showing that the entry of oxygen into the haemoglobin molecule stabilises the iron, offers significant evidence for this. If the pigment be an oxidative catalyst as well as (in so pre-eminent a degree) an oxygen carrier, and both in virtue of its iron content, its position in the former category might be expected to be exceptional. It would not seem, however, that the quite different association with oxygen, found in methaemoglobin, affects to any important degree, the catalytic effect of the molecule.

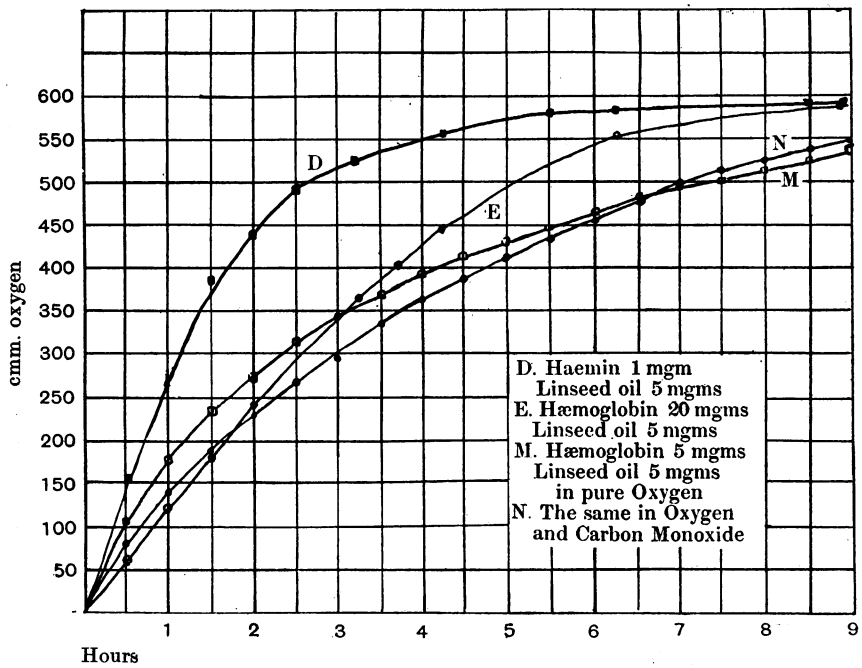

Fig. 4. *Curves N and M.* The action of haemoglobin in an atmosphere of one part of CO and five parts O_2 compared with that in pure oxygen. 5 mg. linseed oil. 5 mg. haemoglobin. *Curves D and E.* The action of haemin compared with that of a strong haemoglobin solution containing an approximately equivalent amount of iron. 5 mg. linseed oil with 1 mg. of haemin, and with 20 mg. of haemoglobin.

An attempt was then made to see if the seemingly catalytic activity of haemoglobin were removed by the action of carbon monoxide. The experiments were carried out in two ways:

(1) Coal gas, or pure carbon monoxide (prepared from formic acid) was bubbled for half an hour into the bottle of the Barcroft apparatus containing the haemoglobin to be used for the experiment. The linseed oil emulsion and buffer or water were then added, and the experiment carried out as usual.

(2) The oil emulsion and haemoglobin solution having been placed in the bottle, and the apparatus arranged for the experiment, the air in both bottles was replaced by a known gas mixture containing one part of carbon monoxide

and five parts of oxygen. In a control experiment carried out simultaneously, the bottles were filled with pure oxygen. No inhibition of catalysis, but rather a slight increase of activity was observed (Fig. 4, Curves *N* and *M*).

Haemin gave curves for catalysis of oxygen uptake of a type rather different from that observed for haemoglobin and methaemoglobin, the initial stages of oxidation being much more rapid (Fig. 4, Curves *D* and *E*). The concentration of iron in haemin is approximately twenty times greater than in haemoglobin. When very weak solutions of haemin were used, in which the iron content approximated to that in a haemoglobin solution containing 2 mg. haemoglobin per cc. the haemin curves tended to resemble those

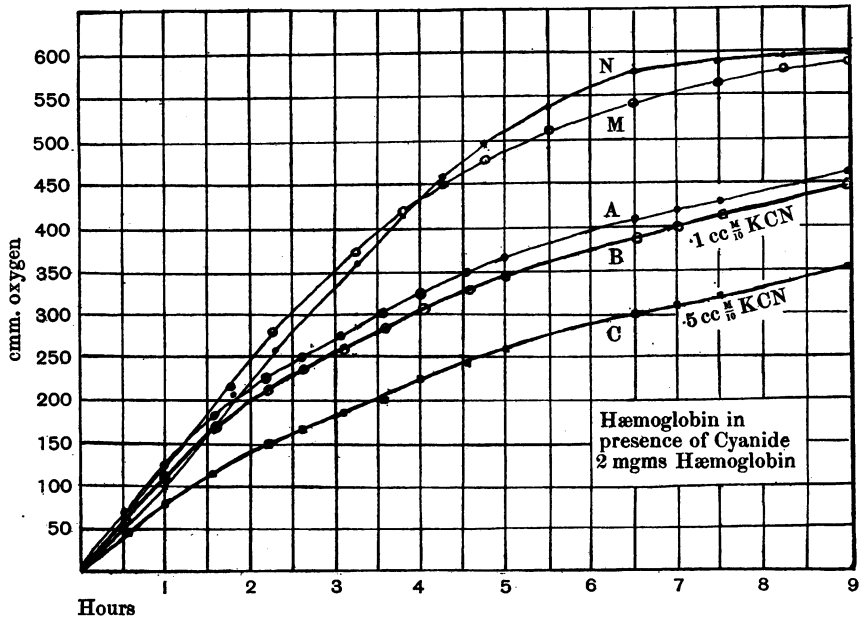

Fig. 5. *Curves M and N.* The action of a very weak haemin solution compared with that of a haemoglobin solution containing an approximately equivalent amount of iron. 5 mg. linseed oil with 0·2 mg. haemin, and with 4 mg. haemoglobin.
Curves A, B, C. The effect of cyanide in different concentrations. 5 mg. linseed oil and 2 mg. haemoglobin with 1 cc. and 0·5 cc. *M*/10 KCN.

obtained by the use of haemoglobin. The converse however, did not appear to be true, that is, increased concentrations of haemoglobin, such as solutions containing 20 mg. per cc. never gave rise to curves resembling the typical haemin curve in its initial stages (Fig. 5, Curves *M*, *N* and *A*; Fig. 4, Curves *D* and *E*). The difference may be in the colloidal character of haemoglobin.

As the results obtained seem to indicate action on the part of iron, particularly on account of the inactivity of haematoporphyrin, attempts were made to observe if the catalysis could be inhibited by the action of cyanide. Warburg has stated that, for the sea urchin's egg, the amount of cyanide necessary to cause total inhibition of respiration is that required to transform the iron present into the inactive ferrocyan-ion.

With haemoglobin and methaemoglobin, the addition of such a strength of cyanide was found to cause no change in the oxygen uptake; even the presence of $M/600$ potassium cyanide was practically without effect, this amount of cyanide being equivalent to five times the concentration of iron present. If, however, the strength of cyanide used were above $M/300$ a distinct but incomplete inhibition was observed (Fig. 5, Curves *A*, *B*, *C*). This effect however was found to be entirely absent in the case of haemin. These results are difficult to interpret; it is assumed that the cyanide exerts an effect on the colloidal catalysts owing perhaps to some change in the physical system, particularly as the inhibition is much more marked if a solution of blood be used instead of one of crystalline haemoglobin. The addition of cyanide to

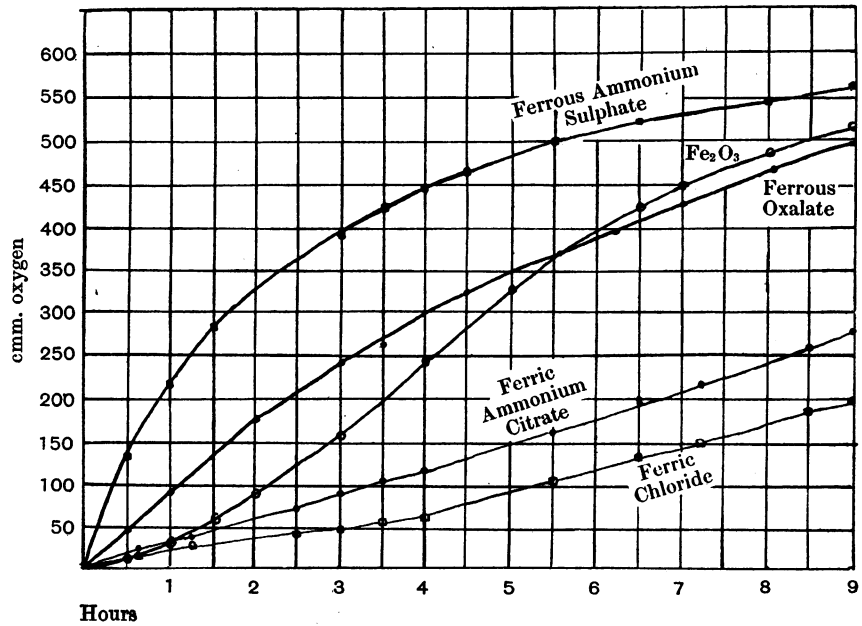

Fig. 6. The action of ferrous ammonium sulphate, and of ferrous oxalate, compared with that of iron sesquioxide and of ferric salts. 5 mg. linseed oil with 1 cc. ferrous ammonium sulphate (0·00112 g. iron); with 1 cc. saturated ferrous oxalate; with 50 mg. iron sesquioxide; and with solutions of ferric chloride and ferric ammonium citrate containing 0·00112 g. Fe in 1 cc.

haemoglobin and methaemoglobin brings about the formation of cyanohaemoglobin, as can be seen by the change in colour and in the spectrum. Apparently this pigment can also exert a catalytic effect.

It is noteworthy that the curves of oxygen uptake of linseed oil, when the blood pigments are employed as catalysts, show no signs of the period of induction which is so characteristic of those obtained in the presence of the driers referred to above. It would not seem, however, that this is universally present when the action of inorganic catalysts is concerned, indeed, to judge by my experiments, it is not seen in the case of such inorganic salts of iron as ferrous ammonium sulphate, ferrous oxalate and ferric ammonium citrate

(Fig. 6). Other ferrous salts have been difficult to obtain, except the lactate, which is very slightly soluble in cold water.

The ferric salts which were tried seemed to exhibit less catalytic activity. In this connection the work of Buckmaster may again be cited (Fig. 6). If an equivalent of cyanide be added to ferrous ammonium sulphate the resulting ferrocyanide exerts no catalytic effect.

It is noteworthy that if the concentration of the ferrous salt solution be such that the iron is present in similar strengths to those existing in the solutions of blood pigments, such as in haemoglobin solutions containing 1 mg. haemoglobin per cc. no catalysis is evident; the concentration of the solutions of ferrous salts used was such that 1 cc. contained 0·00112 g. iron, but the minimal amount that would exert catalytic activity was not determined.

Discussion.

The experiments described show conclusively that haemoglobin, methaemoglobin and haemin act in very low concentrations as efficient catalysts in the oxidation of linolenic acid. It is probable therefore that when blood pigments are found within the structural elements of a tissue they take a definite place among the agents which promote oxidation in that tissue. Since haematoporphyrin has no similar activity it seems clear (as might indeed be expected) that the above mentioned compounds function in virtue of their content of iron. In the case, at least, of the particular oxidation studied, haemoglobin and its derivatives are—if comparison be based upon the actual amount of iron present—more efficient as catalysts than inorganic salts of the metal. None of the accepted explanations applied to the influence of ionised iron as a catalyst seems however to fit the facts in the case of these organic compounds.

Since there is no doubt that the atom of iron in the haemoglobin molecule plays a primary part in oxygen carriage, it would appear likely that when haemoglobin acts as a catalyst of oxidations the same affinities of the iron atom would be concerned with the oxygen transfer there involved. It is remarkable therefore that methaemoglobin, in which the relation of oxygen to the iron atom is certainly different, and carbon monoxide haemoglobin, in which the locus of oxygen uptake is (presumably) occupied, should both, when in equal concentrations, act with a catalytic effect equal to that of haemoglobin itself. Cyanides, when added in concentrations at all commensurate with the amount of iron concerned, have no effect upon catalysis by these substances. If the accepted explanation of the effect of cyanides in inhibiting oxidations catalysed by inorganic iron is correct—namely, that it is due to the formation of an inactive complex ion—the absence of any effect upon non-ionised iron in the organic molecule might be expected. The fact, however, makes it even less easy to decide precisely how the iron exercises its influence in the phenomena described.

In the slow autoxidation of unsaturated fatty acids it is commonly assumed that the first occurrence is peroxide formation at the unsaturated linkages. This circumstance has been supposed to explain the fact that the curves of oxygen uptake show the characteristic of an autocatalysed reaction; oxidation increasing in velocity as the concentration of active oxygen rises.

Haemoglobin and its iron-containing derivatives are known to act as peroxidases or pseudoperoxidases, and it is possible that, in the case of the unsaturated fatty acids, actual oxidation of the molecule, by the active oxygen which first appears at the unsaturated linkage, is promoted by the presence of the organic iron compounds. Under their influence oxidation may proceed with a much lower concentration of peroxide than is the case for the uncatalysed reaction. On this view it is interesting to observe that the period of induction, notable in the spontaneous reaction, and representing the gradual attainment of an efficient concentration of active oxygen, disappears when haemoglobin or its derivatives are present. The system then pictured would be analogous to plant oxidase systems, as described by M. W. Onslow [1920]. In these, peroxide formation occurs in relation with adjacent hydroxy-groups of catechol derivatives. Actual oxidation of the catechol molecule follows upon such preliminary formation of peroxide, this process being greatly accelerated by a peroxidase.

Unfortunately, the precise lines on which peroxidases exert their action are unknown, and it would seem that the presence of a metal in their constitution is not essential to their activity. In any case it is of course possible that haemoglobin, in promoting the oxidation of fats, acts along other lines. The main purpose of the experiments described in this paper is to show that, apart from its function in the blood, haemoglobin, which was shown long ago by Kühne to be present in tissues, may play its own part in promoting oxidations.

Summary.

1. The blood pigments haemoglobin, methaemoglobin and haemin, are shown to act as efficient catalysts of the autoxidation of linseed oil; haematoporphyrin, however, shows no similar activity. The catalysis is attributed to the iron content of the molecule.

2. The concentration of iron, present as blood pigment, necessary to produce the same catalytic effect, is very much smaller than that required if the iron be in the form of an inorganic salt.

3. Addition of neutralised potassium cyanide in amounts equivalent to the iron present is without effect on the catalytic action. It is suggested that the slightly inhibitory effect of high concentrations of cyanide may be due to some physical change in the colloidal system.

I wish to thank Prof. Hopkins for suggesting this work and for the help he has given throughout its course. I am indebted to Mr T. R. Parsons for supplies of crystalline haemoglobin, and for assistance in the preparation of methaemoglobin.

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