Effect of Haematin on the Oxidation of Succinic Acid by Tissue Preparations

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While investigating the possibility that free haematin might act as a carrier analogous to cytochrome *c* in the oxidation of succinate by heart muscle preparations, we found on the contrary that it had a marked inhibitory effect on the oxidation system. In order to analyze the mechanism of this inhibition we attempted to reverse it by addition of such substances as organic bases and denatured proteins which co-ordinate with haematin Fe.

MATERIALS AND METHODS

Heart-muscle preparation was obtained as described in the preceding paper (Keilin & Hartree, 1947).

Cytochrome c used in these experiments contained 0.34% Fe and was prepared as previously described (Keilin & Hartree, 1945).

Pigeon breast-muscle preparation. The breast muscles of 2 pigeons were passed through a Latapie mincer and stirred for 10 min. with 1 l. distilled water. The mixture was squeezed out through muslin and the cloudy fluid centrifuged hard. The resulting clear red fluid was rejected and the brown residue shaken gently with 100 ml. water to form a suspension of fine particles without disturbing the mat of fibrous material at the bottom of the tube. The suspension was diluted to 250 ml. with 0.2 M- Na_2HPO_4 and centrifuged for 5 min. The cloudy fluid, carefully separated from the solid material, was mixed with 100 g. crushed ice and the pH was adjusted to 5.5 by cautious addition with stirring of *N*-acetic acid. The precipitate was immediately centrifuged down and resuspended in 20 ml. 0.1 M-phosphate buffer pH 7.3.

Both the heart and the pigeon breast-muscle preparations can be preserved in a refrigerator for 4–5 days. Throughout this paper they will be referred to as 'oxidase preparations'.

Haematin solution. 2×10^{-3} M-crystalline haemin was made up in 0.1 N-NaOH and preserved in a well stoppered flask. When necessary, fresh dilutions were made with 0.1 N-NaOH.

Denatured globin. The procedure is based upon the method of Anson & Mirsky (1930) for the preparation of native globin. An amount of 30 ml. corpuscles, obtained from defibrinated blood and washed 3 times with 0.9% saline solution, was lysed with 60 ml. distilled water. 50 ml. 0.1 N-HCl was added and the mixture centrifuged in an angle centrifuge to remove stroma. The 100 ml. clear red fluid so obtained was run as a thin stream into a well-stirred mixture of 1 l. acetone and 20 ml. conc. HCl. The precipitate of denatured globin was filtered on a Buchner funnel and rapidly washed at first with acetone containing 1% conc. HCl and finally with pure acetone. The white

powder was dried in air and preserved in a stoppered bottle. It is soluble in water but is completely precipitated on addition of alkali sufficient to bring the solution to pH 6–8.

The activity of the succinic system in the oxidase preparations was estimated aerobically in Barcroft differential manometers at 39°. The manometer flasks contained 0.1 ml. heart muscle or 0.04 ml. breast muscle preparation in 3.3 ml. fluid comprising 0.07 M-phosphate pH 7.3, 0.04 M-succinate and 4×10^{-6} M-cytochrome *c*. The addition of sodium succinate after temperature equilibration and closing of the manometer taps was effected by means of dangling tubes suspended by platinum hooks from the central tubes of the flasks.

Fig. 1. Modified Thunberg tube (B) with two hollow stoppers (A and C).

Estimations of dehydrogenase activity by the rate of reduction of methylene blue were carried out either in Thunberg tubes with hollow stoppers in the usual way or in a special type of vacuum tube with an extra hollow stopper (Fig. 1).

RESULTS

The effect of haematin on the aerobic oxidation of succinic acid

Our experiments with the heart muscle preparation are summarized in Table 1. They show that 2×10^{-4} M-haematin strongly inhibits the oxidation of succinic acid by the oxidase preparation + cytochrome *c*. However, denatured globin and glyoxalin,

haematin, namely proto-, deuto- and haemato-haematin produce approximately the same inhibitory effect on the succinic system while Fe^{+++} , porphyrin and globin parahaematin (cathemo-globin) have no effect on it.

Similar experiments with the pigeon breast-muscle preparation are summarized in Table 2 and show that although denatured globin does not activate the succinic system in this material it protects it from the inhibition by haematin.

Table 1. *The oxidation of succinic acid by heart muscle preparation (oxid.); its inhibition by haematin and protection against this inhibition by glyoxalin and denatured globin*

(Estimations carried out manometrically at 39° : 0.1 ml. oxid.; 0.3 ml. 1% cytochrome *c*; 0.04M-sodium succinate; 2×10^{-4} M-haematin; 0.3 ml. 0.1M-neutralized glyoxalin; 0.3 ml. 1.4% denatured globin. Volumes made up to 3.3 ml. with 0.1M-phosphate buffer pH 7.3.)

Content of manometric flasks	O ₂ uptake in 1st 15 min. (μ l.)	Added at 15 min.	O ₂ uptake in 2nd 15 min. (μ l.)
Oxid. + cyt. <i>c</i> + succin.	110	—	—
Oxid. + cyt. <i>c</i> + succin. + glyoxalin	110	—	—
Oxid. + cyt. <i>c</i> + succin. + glyoxalin* + haematin	125	—	—
Oxid. + cyt. <i>c</i> + succin. + globin	233	—	—
Oxid. + cyt. <i>c</i> + succin. + globin* + haematin	215	—	—
Oxid. + cyt. <i>c</i> + succin. + haematin	4	Glyoxalin	24
Oxid. + cyt. <i>c</i> + succin. + haematin	4	Globin	20

*Added before haematin to oxid. + cyt. *c*.

provided that they are added before the haematin, protect the oxidizing system from this inhibitory effect. Whereas glyoxalin acts only as a protective substance, denatured globin does more than prevent the inhibition; it brings about a considerable acceleration of the reaction.* The addition of other denatured proteins or even of boiled oxidase preparation also protects succinic dehydrogenase from inhibition by haematin.

The inhibitory effects of two concentrations of haematin (10^{-4} M and 4×10^{-5} M) tested under standard conditions with a wide range of succinate concentrations were found to be approximately constant. This shows that there is no appreciable competition between haematin and succinate; in other words, they do not compete for the same point of attachment on the succinic dehydrogenase protein.

Table 2. *The oxidation of succinic acid by a pigeon breast-muscle preparation (oxid.); its inhibition by haematin and protection against this inhibition by denatured globin*

(Estimations carried out manometrically at 39° : 0.04 ml. muscle extract; 0.3 ml. 1% cytochrome *c*; 0.04M-sodium succinate; 2×10^{-4} M-haematin; 0.3 ml. 1.4% denatured globin; 0.1M-phosphate buffer pH 7.3.)

Contents of manometric flasks	O ₂ uptake/20 min. (μ l.)
Oxid. + cyt. <i>c</i> + succin.	209
Oxid. + cyt. <i>c</i> + succin. + haematin	18
Oxid. + cyt. <i>c</i> + succin. + globin	188
Oxid. + cyt. <i>c</i> + succin. + globin + haematin	176

If succinate be replaced by *p*-phenylenediamine in order to measure cytochrome oxidase activity, the same concentration of haematin produces less than 25% inhibition. It is important to note that three

* This phenomenon, which may also be observed with other proteins, will be considered in a separate paper.

The effect of haematin on the anaerobic oxidation of succinic acid

In order to determine whether the inhibitory effect of haematin was due to its reaction with succinic dehydrogenase and not with some other component of the system, experiments were carried out in vacuum tubes with methylene blue as hydrogen acceptor. Four tubes (i, ii, iii, iv) were filled with reagents as shown in Table 3. Tubes i and iii were normal Thunberg tubes with one hollow stopper, whereas tubes ii and iv were provided with two hollow stoppers (Fig. 1, A, C). The results of these experiments clearly show that a marked inhibition of methylene blue reduction is obtained under anaerobic conditions and this inhibition is partly reversed by cyanide which has a high affinity for haematin. The efficiency of cyanide in these experiments is less marked than that of glyoxalin or denatured globin in aerobic experiments. This may be due to the fact that we are dealing here with a genuine reversibility of inhibition and not merely

Table 3. *The effect of haematin and cyanide on the anaerobic oxidation of succinic acid by heart muscle preparation (oxid.) in presence of methylene blue (MB)*

(Experiments carried out in Thunberg tubes (*B*) with two hollow stoppers (*A* and *C*); $T=39^{\circ}$. 0.5 ml. oxid.; 0.04M-sodium succinate; 0.5 ml. 10^{-3} M-MB; 0.3 ml. 2×10^{-3} M-haematin in 0.1N-NaOH or 0.3 ml. 0.1N-NaOH. When KCN in solid form was added (5 mg.) in *C* an equivalent amount of H_2SO_4 (0.7 ml. 0.1N) was added from *A*. Volumes made up to 4 ml. with 0.1M-phosphate buffer pH 7.3 in *B*. After evacuation and warming to 39° , *C* was added to *B*, then *A* added at zero time.)

Tube no.	<i>A</i>	<i>B</i>	<i>C</i>	Reduction time (min.)
i	Succinate	Oxid. + MB + NaOH	—	2.25
ii	Succinate + H_2SO_4	Oxid. + MB + NaOH	5 mg. KCN	3
iii	Succinate	Oxid. + MB + haematin	—	25
iv	Succinate + H_2SO_4	Oxid. + MB + haematin	5 mg. KCN	8.5

with a protection of the enzyme against the inhibitory effect of haematin. The use of cyanide in aerobic experiments is precluded owing to its powerful inhibitory effect on cytochrome oxidase.

By placing the haematin in one stopper and methylene blue with succinate in the other stopper of a special vacuum tube it was possible to add haematin to the oxidase preparation after the complete removal of air. In such experiments a marked inhibition by haematin was still obtained, the reduction time being of the order of 25 min. This shows that O_2 is not necessary for the inhibition of the enzyme by haematin.

The aerobic experiments summarized in Table 1 show that although glyoxalin will protect the dehydrogenase from the effect of haematin it cannot reverse the effect once the haematin has been added to the oxidase preparation. It has been possible, however, to reverse the effect to a considerable degree if the heart-muscle preparation is incubated *in vacuo* with haematin and succinate and then treated with glyoxalin. If the mixture is now placed in a manometer a rapid O_2 uptake ensues, but if the succinate is omitted during the incubation and only added in the manometer flask, the preparation is found to be inactive. For this experiment four double-stoppered vacuum tubes (v, vi, vii and viii) are filled as shown in the left half of Table 4, evacuated and warmed to 39° . The haematin and glyoxalin are then added at intervals as indicated in

the table and the tubes cooled and opened. Definite volumes containing equal quantities of oxidase are then withdrawn from the tubes and tested manometrically with excess of cytochrome *c* and succinate. The results of these experiments which are given in the right half of Table 4 show that incubation of the heart preparation with haematin in absence of succinate produces the usual strong inhibition (vii and viii) but when succinate is present (v and vi) the inhibition is largely abolished.

Haematin and other enzymes

The effect of haematin was also tested on lactic dehydrogenase and the oxidation of glucose by crushed baker's yeast, *D*-amino-acid oxidase of pig kidney, lactic dehydrogenase of heart muscle and glucose oxidase (notatin) of *Penicillium notatum*. In no case did haematin exert an appreciable inhibitory effect. In the case of the cruder enzyme preparations this may be due to the presence of excess of inactive proteins which, especially if denatured, would compete for the haematin. Thus the conclusion of Yamafuji, Fujii & Imagawa (1943), that the activity of preparations oxidizing succinate is unaffected by haematin, may arise from the presence of denatured protein in their enzyme preparations. On the other hand, a more comprehensive investigation with purer materials may show that certain other enzymes are as sensitive to haematin as is succinic dehydrogenase.

Table 4. *Protection of succinic dehydrogenase against the inhibitory effect of haematin by incubation with succinate and glyoxalin*

(4 ml. 1:50 dilution heart preparation (oxid.) in phosphate buffer (0.1M, pH 7.3), 0.6 ml. 0.4M-sodium succinate, 0.2 ml. 0.004M-haematin in 0.1N-NaOH or 0.2 ml. 0.1N-NaOH. Thunberg tubes (*B*) with two hollow stoppers (*A* and *C*) evacuated and warmed to 39° . *A* added to *B* and incubated 25 min. at 39° ; *C* then added. Tubes cooled and opened and contents used for manometric experiments.)

Tube no.	Filling of Thunberg tubes			Filling of manometer flasks				
	<i>A</i>	<i>B</i>	<i>C</i>	Volume taken from Thunberg tube	0.4M-succin. (ml.)	1% cyt. <i>c</i> (ml.)	pH 7.3 buffer (ml.)	O_2 uptake in manometers (μ l. O_2 /10 min.)
v	Haematin	Oxid. + succin.	Glyoxalin	2.7	0.3	0.3	—	74
vi	NaOH	Oxid. + succin.	Glyoxalin	2.7	0.3	0.3	—	107
vii	Haematin	Oxid.	Glyoxalin	2.4	0.3	0.3	0.3	0
viii	NaOH	Oxid.	Glyoxalin	2.4	0.3	0.3	0.3	100

DISCUSSION

The inhibition by haematin of succinic dehydrogenase is of special interest on account of its apparent specificity and the fact that the activity of this enzyme *in vivo* is intimately bound up with that of the cytochrome system which is composed of haematin compounds.

There are three possible explanations of the inhibition: (1) Haematin, being a good oxidation catalyst, may irreversibly inhibit the dehydrogenase by oxidizing certain groups essential for its activity. (2) The two propionic acid side chains of haematin, being in close proximity, may enable it to behave as a competitive inhibitor in the same way as malonate. (3) A parahaematin may be formed by reaction between haematin and some Fe-binding group essential for the catalytic activity of the dehydrogenase.

The evidence against the oxidation hypothesis is that the inhibition is equally marked in both aerobic and anaerobic experiments and also that globin parahaematin (cathae moglobin) and especially glyoxalin parahaematin, which are much more efficient oxidizing catalysts than free haematin, have no inhibitory effect. Opposing the second suggestion are the facts that there is no competition between haematin and succinate and that, although two propionic groups are present in mesoporphyrin, the latter produces no inhibition. On the other hand, the third suggestion, namely the formation of a co-ordination compound between the haematin Fe and some important functional groups of succinic dehydrogenase, appears to be supported by the following three considerations: (a) Whereas iron and porphyrin alone are inactive their compound, haematin, is a strong inhibitor of the dehydrogenase, (b) proto-, haemato-

and deuterohaematin are equally effective, and (c) glyoxalin and denatured globin, which both form very stable parahaematin compounds, show marked protection against inhibition by free haematin. The marked sensitivity of succinic dehydrogenase to free haematin suggests that not only the cytochrome components, catalase and peroxidase, but all other forms of intracellular haematin exist as compounds with proteins.

SUMMARY

1. Free haematin in a concentration of $2 \times 10^{-4} \text{M}$ inhibits almost completely the oxidation of succinic acid by highly active muscle preparations.

2. In this respect proto-, haemato- and deuterohaematin are equally effective though neither iron nor porphyrins alone produce a comparable effect.

3. When haematin is bound to nitrogenous substances such as glyoxalin or denatured globin, forming the corresponding parahaematin compounds, it loses its inhibitory property. Hence the prior addition of glyoxalin or denatured protein will protect the enzyme preparation from inhibition by haematin.

4. Since the oxidation of succinic acid is also inhibited under purely anaerobic conditions in presence of methylene blue as hydrogen acceptor and since the oxidation of *p*-phenylenediamine is very little affected, the inhibitory effect is due to a reaction between haematin and succinic dehydrogenase and not to any influence upon the cytochrome system.

5. The inhibition is probably due to the formation of a co-ordination compound between haematin iron and some groups in succinic dehydrogenase important for its activity.

REFERENCES

- Anson, M. L. & Mirsky, A. E. (1930). *J. gen. Physiol.* **13**, 469.
Keilin, D. & Hartree, E. F. (1945). *Biochem. J.* **39**, 289.
Keilin, D. & Hartree, E. F. (1947). *Biochem. J.* **41**, 500.
Yamafuji, K., Fujii, M. & Imagawa, H. (1943). *Biochem. Z.* **316**, 70.