8. The minimum value for the turnover number of xanthine oxidase is 313 min.^{-1} at 19° .

9. Xanthine oxidase has virtually no fluorescence when irradiated with blue light, compared to free FAD.

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The Reduction of Cytochrome *c* by Hypoxanthine and Xanthine Oxidase

By D. B. MORELL*

Molteno Institute, University of Cambridge

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The work by Bigwood, Thomas & Wolfers (1935) and Horecker & Heppel (1949) showing that xanthine oxidase, in the presence of its substrate, is able to reduce oxidized cytochrome *c* is of interest since it suggests the possibility that the reduced flavin prosthetic group of the enzyme may be re-oxidized via a cytochrome system rather than directly by oxygen. A puzzling finding by Horecker & Heppel was that the reduction of cytochrome *c* was very slow anaerobically and that the rate of the reduction increased markedly with increasing oxygen tension.

The present paper deals with a further study of the reduction of cytochrome *c* by hypoxanthine in the presence of xanthine oxidase. The reduction of cytochrome *c* under both anaerobic and aerobic conditions has been confirmed, but no evidence that oxygen increases the rate of this reduction has been found.

METHODS

The reduction of cytochrome *c* was followed at $550 \text{ m}\mu$ in the Beckman spectrophotometer using a slit width of 0.1 mm . For anaerobic experiments a Thunberg tube fused to a 5 mm . optical glass cell contained the reaction mixture (see

below). The reaction was started by adding the hypoxanthine from the hollow stopper. The readings were made against air. Anaerobic conditions were achieved by exhausting the tube at the pump and by flushing twice with N_2 freed of O_2 .

Aerobic experiments were made in open 1 cm . cells. The readings were made against a reference cell containing water. In plotting the results of anaerobic experiments the cell 'blank' (determined separately) was subtracted from the optical density reading and the resultant figure multiplied by two. This facilitated comparison between data for aerobic and anaerobic experiments.

Unless otherwise stated, the reaction mixture, in both aerobic and anaerobic experiments, contained $1.45 \times 10^{-5} \text{ M}$ -cytochrome *c*, $3.2 \times 10^{-4} \text{ M}$ -hypoxanthine, 0.38 mg./ml. albumin, 0.05 ml. of catalase and 0.1 ml. xanthine oxidase preparation in a final volume of 3.2 ml.

Cytochrome *c*. Preparations containing 0.34% Fe, prepared by the method of Keilin & Hartree (1945), were kindly supplied by Dr C. L. Tsou. The preparations were thoroughly dialysed against distilled water.

Albumin. A highly purified horse serum albumin was kindly supplied by Dr E. F. Hartree.

Catalase. This preparation was made according to the method of Keilin & Hartree (1937), omitting the ultrafiltration step; it contained some ferritin. It was thoroughly dialysed against distilled water.

Hypoxanthine solutions. ($7.35 \times 10^{-4} \text{ M}$). These solutions were prepared in distilled water every 3 days from the pure Roche product and kept at 5° when not in use.

* Present address: Institute of Medical Research, Royal North Shore Hospital, St Leonards, N.S.W., Australia.

Xanthine oxidase. The enzyme was prepared from milk as described in a previous publication (Morell, 1952). The preparations were dialysed against distilled water and diluted at 0° before use so that an optical density change at 550 m μ . of approximately 0.02/min. was obtained in the measurement with oxidized cytochrome *c*.

The concentrations of cytochrome *c*, albumin and hypoxanthine were the same as those used by Horecker & Heppel (1949). A very much higher concentration of catalase was used in the present study to obtain consistent results under the present conditions. In the aerobic experiments it was desired completely to break down the H₂O₂ formed by the oxidation of hypoxanthine. Dixon (1925) has shown that relatively large concentrations of catalase are necessary for this purpose. Horecker & Heppel (1949) used phosphate buffer, pH 7.4 (final concentration not stated), in their reaction mixture. In the present study, unless stated otherwise, the reaction mixtures did not contain added buffers because it was found that even low concentrations of buffer solution inhibited the reduction (see p. 668). The pH of the above reaction mixture was found to be 6.8 when measured with the glass electrode.

EXPERIMENTS

Comparison of the anaerobic and aerobic rates of reduction

In Fig. 1, curves A and B represent the changes in optical density at 550 m μ . with time, for the anaerobic and aerobic reactions respectively. Even anaerobically the cytochrome *c* was not fully reduced by hypoxanthine. This can be seen in Fig. 1 by comparing the extent of reduction caused by hypoxanthine (curve A) with that caused by the subsequent addition of dithionite (curve A, +Na₂S₂O₄). In fact only about 84% reduction was achieved anaerobically and 62% aerobically. These results were reproducible with the same enzyme preparation although, with different preparations, differences in the degree of reduction under aerobic conditions were found. The degree of reduction achieved could not be correlated with the purity of the enzyme preparations.

It can also be seen in Fig. 1 that, whereas the anaerobic reduction rate is almost linear for most of the reaction, the reduction under aerobic conditions is approximately logarithmic. Many enzyme and cytochrome *c* preparations have been examined in these studies and in all cases the anaerobic reduction rate was found to be greater than the aerobic reduction rate. Even the use of very high concentrations of catalase (0.2 ml. strong solution in 3.2 ml. mixture) did not affect the shape of the aerobic reduction. The shape of this curve, in the presence of a high concentration of catalase, is probably largely due to the fact that oxygen is competing with cytochrome *c* as the hydrogen acceptor.

The admission of air in the anaerobic experiment when the reduction was at a maximum (Fig. 1;

+ air) caused a rapid re-oxidation of the reduced cytochrome. Other experiments showed that partial re-oxidation occurs even in the presence of catalase. The cytochrome was almost completely re-oxidized in the absence of catalase. The complete reduction of the cytochrome *c* by dithionite after it had been oxidized in the absence of catalase (see Fig. 1) showed that the hydrogen peroxide was, in fact, oxidizing the reduced cytochrome and not destroying it.

Fig. 1. The reduction of oxidized cytochrome *c* (purified) by hypoxanthine and xanthine oxidase; curve A, anaerobic conditions; curve B, aerobic conditions. The reaction mixture was as described under Methods except that catalase was omitted for the anaerobic experiment. Separate experiments showed that catalase did not affect the anaerobic reduction.

The rate of reduction of cytochrome *c* by xanthine oxidase and hypoxanthine was found to be proportional to the concentration of the enzyme. The reduction did not occur in the absence of either the enzyme or its substrate. These results confirm those of Horecker & Heppel (1949).

With either xanthopterin or salicylaldehyde as substrate of the enzyme, the cytochrome *c* was reduced under anaerobic conditions. With xanthopterin as substrate, however, the reduction was very slow. Aerobically the reduction did not proceed at a measurable rate with salicylaldehyde as substrate. Aldehydes are known to inactivate xanthine oxidase under aerobic conditions (Horecker & Heppel, 1949; Corran, Dewan, Gordon & Green, 1939).

In Fig. 2 the inhibitory effect of various concentrations of phosphate buffer on the rate of anaerobic reduction of cytochrome *c* by hypoxanthine and xanthine oxidase is shown. Even the addition of phosphate buffer, pH 7.3, to a final concentration of 3.2×10^{-3} M caused a 44% inhibition. Glyoxaline

and glycine buffers of the same pH and concentration similarly inhibited the reduction. Sodium and potassium chloride also effectively inhibited the reduction. This inhibition by buffers and salts seems to be due to a general salt effect and not to the toxicity of a particular ion.

Fig. 2. The effect of phosphate buffer, pH 7.3, in inhibiting the anaerobic reduction of oxidized cytochrome *c* by hypoxanthine and xanthine oxidase.

The reduction of 'endogenous' cytochrome c of heart-muscle preparation

Keilin & Hartree (1949) believe that the cytochrome *c* present in a colloidal heart-muscle preparation is organized with respect to cytochrome oxidase and other components of the succinoxidase system in a manner similar to that existing in the cell. It was of interest in the present experiments, therefore, to compare the ability of the xanthine oxidase system to reduce this 'endogenous' cytochrome *c* with its ability to reduce the purified, extracted pigment. For this experiment heart-muscle preparation, prepared according to the method of Keilin & Hartree (1947) by Dr C. L. Tsou, was washed several times in distilled water, after precipitation at pH 5.5, to remove salts which inhibit the reduction, and finally suspended in distilled water.

When xanthine oxidase was added to the heart-muscle preparation plus hypoxanthine under anaerobic conditions and viewed under a low dispersion microspectroscope the *a*- and *c*-bands of cytochrome at 604 mμ. and 550 mμ. appeared slowly. For the comparison of the rates of reduction of 'endogenous' and soluble cytochrome *c*, an additional Thunberg tube was used with the same reagents as described above, except that the heart-muscle preparation was replaced by an approximately equal concentration of soluble cytochrome *c* (determined by comparing the intensities of fully reduced 'endogenous' and soluble cytochrome *c*).

On adding the enzyme in the stoppers simultaneously to the cytochrome-hypoxanthine mixtures

and on comparing the increase in the intensities of the *α*-band of cytochrome *c*, it was found that the rate of reduction of 'endogenous' cytochrome *c* was only about one-quarter that of soluble cytochrome *c*. Although it must be admitted that the method of comparing these rates is not very accurate it is certain that, under these conditions, the 'endogenous' cytochrome *c* is reduced at a slower rate than the soluble form.

DISCUSSION

The present study does not confirm Horecker & Heppel's (1949) finding that oxygen stimulates the reduction of cytochrome *c* by xanthine oxidase and hypoxanthine. Fig. 1 shows that oxygen has the inhibitory action expected from its ability to compete with cytochrome *c* as the hydrogen acceptor. In agreement with this finding Singer & Kearney (1950) have shown that the rate of reduction of cytochrome *c* by leucoriboflavin is decreased by oxygen.

Since milk xanthine oxidase reacts rapidly with oxygen, it is usually considered that this enzyme belongs to a small group of dehydrogenases (the aerobic dehydrogenases) which react directly with oxygen without the intervention of intermediary hydrogen carriers. The finding by Bigwood *et al.* (1935) and Horecker & Heppel (1949), confirmed in the present paper, that xanthine oxidase reacts with cytochrome *c* raises the question whether oxygen is the immediate hydrogen acceptor *in vivo*. The fact that the initial rate of reduction of cytochrome *c* is the same in the presence as in the absence of oxygen (Fig. 1) shows that the rate of reaction of the enzyme with cytochrome *c* is comparable with the rate with 20% oxygen. The concentration of cytochrome *c in vivo* is not known, but the oxygen tension is considerably less than 20%, and Horecker & Heppel (1949) have shown that the rate of the aerobic reaction decreases markedly with decreasing oxygen tension. It was found in the present study that xanthine oxidase reacted considerably more slowly with the 'endogenous' cytochrome *c* of a heart-muscle preparation than with soluble cytochrome *c*, but this does not exclude the possibility that in the tissues which contain this enzyme it is so situated spatially that the reaction with cytochrome *c* is accelerated. Richert, Vanderlinde & Westerfeld (1950) have recently reported experiments on the effect of Antabuse (tetraethylthiuram disulphide) on liver xanthine oxidase, which suggest that, in crude preparations, the enzyme is bound to some cell constituent and does not react readily with oxygen.

SUMMARY

1. The ability of oxidized cytochrome *c* to act as a hydrogen acceptor in the hypoxanthine-xanthine oxidase system has been confirmed.

2. In all the preparations studied the anaerobic rate of reduction of cytochrome *c* was greater than that under aerobic conditions. It is considered that oxygen and oxidized cytochrome *c* compete with one another as hydrogen acceptors in the xanthine oxidase system. This is contrary to the findings of Horecker & Heppel (1949).

3. The cytochrome *c* of heart-muscle preparation was found to be reduced by hypoxanthine in the presence of purified milk xanthine oxidase more slowly than the purified, extracted pigment.

4. Phosphate, glyoxaline and glycine buffers,

pH 7.3, and sodium and potassium chloride inhibited the rate of reduction of cytochrome *c* by hypoxanthine and xanthine oxidase considerably. Phosphate buffer, pH 7.3, 3×10^{-3} M, inhibited the anaerobic reduction by 44 %.

5. The possibility that reduced xanthine oxidase is not oxidized directly by oxygen in animal tissues is discussed.

I wish to thank Prof. D. Keilin, F.R.S., for his interest in this work and Dr E. C. Slater for helpful discussions. I also wish to thank the Australian National University for a scholarship.

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Studies in Rhodopsin

5. CHEMICAL ANALYSIS OF RETINAL MATERIAL

By F. D. COLLINS, R. M. LOVE AND R. A. MORTON

Department of Biochemistry, The University of Liverpool

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The chemical composition of the specialized tissues involved in vision is not well established. In respect of low-intensity or scotopic vision, the need is for analysis of the rod outer segments and of rhodopsin, that is of the receptor end-organ and the receptor substance. Retinas themselves have been analysed for various constituents, but the picture is very incomplete and much more work is needed. Rod outer segments (cf. Lythgoe, 1937; Saito, 1938) have not until recently been separable in amounts adequate for analytical work, but appreciable quantities can now be obtained (Collins, Love & Morton, 1952).

Krause (1937) showed that rhodopsin solutions contained phosphorus and choline, suggesting the presence of lecithin. Broda, Goodeve & Lythgoe (1940) reported that, in rhodopsin solutions, 20 % of the weight of solute could be ascribed to phospholipin. Wald & Ishimoto (1946) extracted rod preparations with light petroleum until no further phosphorus-containing material went into solution. Nevertheless, on exposing the extracted rod preparation to light, phospholipin in appreciable quantity was set free, presumably as a result of the

decomposition of rhodopsin, and could then be extracted.

Collins & Morton (1950*b*) suggested that nucleotides, probably as nucleic acid, were present in rhodopsin solutions. Ehrlich & Dische (1950) analysed whole retinas from several species of animals for nucleic acid and found a considerable difference from other tissues. They also found that the amounts and proportions of deoxypentose- and pentose-nucleic acids (DNA and PNA) did not vary as between dark and light adapted eyes.

It was decided, therefore, to study the distribution of phospholipin and of nucleic acid in retinas and isolated rod outer segments. For this, the fractionation procedure of Schmidt & Thannhauser (1945) has been followed.

EXPERIMENTAL

Materials. Cattle retinas were obtained from the local abattoir, and the rod outer segments were prepared as described by Collins *et al.* (1952). Some experiments were done using *Rana esculenta* imported from Holland.