CI. STUDIES ON XANTHINE OXIDASE. VI. A CELL OXIDATION SYSTEM INDEPENDENT OF IRON.

BY MALCOLM DIXON AND SYLVA THURLOW.

From the Biochemical Laboratory, Cambridge.

(Received June 27th, 1925.)

WARBURG [1921; 1923, 1, 2] has developed a theory of oxidations in the cell which states that molecular oxygen must always be activated by iron before it can oxidise organic substances. He considers iron to be the only substance in the cell with which molecular oxygen reacts: "Das Oxydationsmittel der Atmung ist der molekulare Sauerstoff, die Primärreaktion der Atmung die Reaktion zwischen molekularem Sauerstoff und Eisen, und *nur* in dieser Reaktion, nicht mit den organischen Molekülen, vermag der molekulare Sauerstoff in der Zelle zu reagieren."

His theory is based, partly on the study of the respiration of the sea urchin's eggs, partly on the consideration of certain chemical systems, namely, the oxidations occurring on the surface of iron-containing charcoal, the oxidation of sulphhydryl compounds, and the oxidation of fructose in the presence of phosphate. In all these systems the addition of cyanide inhibits the oxidation, and the addition of minute quantities of iron markedly catalyses the reaction.

Although Warburg extends his theory to include all oxidations occurring in the cell, the only evidence with regard to enzyme systems is afforded by the work of Fleisch [1924] and Szent-Györgyi [1924] on the succinic oxidase of animal tissues. These workers found that the addition of cyanide inhibited the oxygen uptake by succinate in the presence of the enzyme, but had no effect upon the reduction of methylene blue by the same system. They concluded that in this and similar systems oxygen activation was necessary, and that the cyanide inhibited the activator. These experiments would seem to show that Warburg's view, of oxygen activation by iron applies also to enzyme systems.

Experimental evidence will, however, be given in this paper to show that this is not the case with the xanthine oxidase system. Moreover, we have recently obtained results, which will be published later, showing that in the succinic oxidase system it is very doubtful whether oxygen activation by iron occurs.

A detailed study of the xanthine oxidase of animal tissues has been made by the authors [1924, 1, 2, 3; 1925] and, as this enzyme can easily be prepared free from other enzymes, it was thought to be of interest to see whether

oxygen activation by iron was necessary in this system. The experiments were carried out by means of the Barcroft respirometer, the technique of which has been previously described. In all cases identical solutions were placed in both flasks of the apparatus, but hypoxanthine, the oxidisable substance, was placed only in one flask. This ensures that the experiments are properly controlled. All experiments were carried out at 40° and at p_H 7.6.

We have already shown [1924, 2] that the reduction of methylene blue by the xanthine oxidase system is practically unaffected by cyanide, except in concentrations exceeding $M/100$, when a slight inhibition is produced. This is due to a slight destruction of the enzyme itself. We have found the oxygen uptake of the system to be affected by cyanide in exactly the same way as was the reduction of methylene blue. Cyanide in concentrations as high as $M/100$ produced no inhibition in the rate of oxygen uptake, whereas, in the systems previously mentioned, other workers have found concentrations of the order of $M/1000$ to produce complete or almost complete inhibition.

The following typical experiment shows that the xanthine oxidase system is not inhibited by cyanide.

The contents of the flasks were:

- A. 1 cc. xanthine oxidase solution + 0.5 cc. (1 mg.) hypoxanthine + 1.5 cc. p_H 7.6 phosphate buffer.
- B. 1 cc. xanthine oxidase solution + 0.5 cc. (1 mg.) hypoxanthine + 0.1 cc. $M/10$ KCN + 1.4 cc. p_H 7.6 phosphate buffer.
- C. 1 cc. xanthine oxidase solution + 0.5 cc. (1 mg.) hypoxanthine + 0.2 cc. $M/10$ KCN + 1.3 cc. p_H 7.6 phosphate buffer.
- D. 1 cc. xanthine oxidase solution + 0.5 cc. (1 mg.) hypoxanthine + 0.5 cc. $M/10$ KCN + 1.0 cc. p_H 7.6 phosphate buffer.

All solutions were adjusted to p_H 7.6 before addition. The xanthine oxidase was prepared from milk by the method described by us [1924, 1].

Since the effect of cyanide is negative, the curves are all of the same form and hence only isolated points are quoted. The figures denote oxygen absorbed in cubic millimetres corrected to normal temperature and pressure and to dryness.

Time in minutes	A (no KCN)	B (KCN = $M/300$)	C (KCN = $M/150$)	D (KCN = $M/60$)
20	60	72	63	55
55	124	138	110	100
80	143	145	126	103

It can be seen that even relatively high concentrations of cyanide have practically no effect on the oxygen uptake. The uptake in *D* is, however, somewhat smaller than in the others. This was shown to be due to destruction of the enzyme by the cyanide, by testing the solutions at the end of the experiment with methylene blue and an added trace of hypoxanthine. Practically no enzyme was left in either flask of *D*, whereas in *A*, *B* and *C* there still remained a considerable quantity of active enzyme. This destruction in *D* must not be confused with the destruction of the enzyme by the hydrogen peroxide normally formed in the reaction, which occurs in the experimental flask only

[Dixon, 1925]. No peroxide is formed in the control flask, so that the destruction observed here must be due to the cyanide.

In order to eliminate any possibility that the xanthine oxidase in milk might behave differently from that in the tissues, an experiment was carried out on a liver suspension which contained much xanthine oxidase, and it was found that in this case also cyanide produced absolutely no inhibition of the oxygen uptake by this oxidase system, although in the same preparation a considerably smaller concentration of cyanide completely inhibited the oxygen uptake of the succinic oxidase system. It is clear that unless a specific oxygen activator is required for each enzyme, an assumption highly improbable, the xanthine oxidase system does not require active oxygen. The inhibition of the succinic system is due, according to Warburg's theory, to the prevention of oxygen activation, but, although no active oxygen is present, the xanthine oxidation still occurs. It is therefore clear that the xanthine oxidase system, since it is not affected by cyanide, forms an exception to the general statement made by Warburg that cyanide inhibits all tissue respiration systems.

Warburg and Sakuma [1923], Harrison [1924] and Meyerhof and Matsuoka [1924] have shown that pyrophosphates can replace cyanide in the inhibition of iron catalysis. We have repeated the preceding experiment on the xanthine oxidase, using pyrophosphate instead of cyanide, and again have obtained no inhibition. The maximum concentration of pyrophosphate used by us was $M/20$, whereas Harrison found that the oxidation of glutathione was markedly inhibited by $M/50$ pyrophosphate.

These two experiments afford strong evidence that iron plays no part in this system, since the concentration of inhibiting substance was far larger than that required to produce inhibition of iron catalysis.

Further evidence is afforded by observations made on the effect of the addition of iron to the xanthine oxidase system. Warburg, Harrison and Meyerhof and Matsuoka have found that, in all the systems which they have investigated, the addition of very small amounts of iron salts produces a marked acceleration of the oxygen uptake. We therefore added iron in varying concentrations to the xanthine oxidase system. The following experiment shows that no acceleration was produced even by relatively high concentrations of iron.

The contents of the flasks were:

- A. 1 cc. xanthine oxidase solution + 0.5 cc. (1 mg.) hypoxanthine + 1.5 cc. p_H 7.6 buffer.
- B. 1 cc. xanthine oxidase solution + 0.5 cc. (1 mg.) hypoxanthine + 0.1 cc. $FeCl_3$ (0.1 mg. Fe) + 1.4 cc. p_H 7.6 buffer.
- C. 1 cc. xanthine oxidase solution + 0.5 cc. (1 mg.) hypoxanthine + 0.5 cc. $FeCl_3$ (0.5 mg. Fe) + 1.0 cc. p_H 7.6 buffer.

The figures again represent cubic millimetres of oxygen absorbed.

Time in minutes	A (no Fe)	B (0.1 mg. Fe)	C (0.5 mg. Fe)
10	40	36	37
20	69	65	66
55	115	104	112

The ferric chloride solution was not neutralised, but it was found that the addition of the amount in question produced no appreciable change in the p_H of the solution. It is not possible to add an amount of iron greater than that in *C* when using phosphate buffer, as the limit of solubility of ferric phosphate is here reached.

The figures show that no acceleration of the oxygen uptake of the xanthine oxidase system is produced by the addition of an iron salt.

We have, then, in the xanthine oxidase a system occurring in the cell in which molecular oxygen brings about oxidation without having been activated by iron. Warburg's statement quoted above that molecular oxygen can react only with iron, and not with organic molecules, is thus shown to be incorrect. Molecular oxygen seems to act as a direct acceptor of hydrogen in this system, as it has been shown [Thurlow, 1925] that hydrogen peroxide is formed as an intermediary product of the reaction. The system therefore forms an exact illustration of Wieland's [1922] theory of oxidation.

SUMMARY.

1. The oxidation of hypoxanthine by molecular oxygen in presence of the xanthine oxidase is not inhibited by cyanide or pyrophosphate.
2. The addition of iron to the system produces no acceleration.
3. Oxygen activation by iron does not occur in this system, which thus forms an exception to Warburg's statement that molecular oxygen can react only with iron and not with organic molecules.

We wish to express our thanks to Sir F. G. Hopkins for his interest in this work. One of us (M. D.) is also indebted to the Royal Commissioners for the Exhibition of 1851 for a senior Studentship.

REFERENCES.

- Dixon (1925). *Biochem. J.* **19**, 507.
Dixon and Thurlow (1924, 1). *Biochem. J.* **18**, 971.
—— — (1924, 2). *Biochem. J.* **18**, 976.
—— — (1924, 3). *Biochem. J.* **18**, 989.
Fleisch (1924). *Biochem. J.* **18**, 294.
Harrison (1924). *Biochem. J.* **18**, 1009.
Meyerhof and Matsuoka (1924). *Biochem. Z.* **150**, 1.
Szent-Györgyi (1924). *Biochem. Z.* **150**, 195.
Thurlow (1925). *Biochem. J.* **19**, 175.
Warburg (1921). *Biochem. Z.* **119**, 134.
—— (1923, 1). *Biochem. Z.* **136**, 266.
—— (1923, 2). *Biochem. Z.* **142**, 518.
Warburg and Sakuma (1923). *Pflüger's Arch.* **200**, 203.
Wieland (1922). *Ergeb. Physiol.* **20**, 477.