# CCXXXIV. URICASE PURIFICATION AND PROPERTIES

By C. G. HOLMBERG

*Molteno Institute, University of Cambridge*

(*Received 26 July 1939*)

SINCE the work of Schittenhelm [1905], Wiechowsky & Wiener [1909], Batelli & Stern [1909; 1912] and others, it is well known that cells of different organisms contain an enzyme catalysing the oxidation of uric acid to allantoin. Batelli & Stern, who described this enzyme under the name of uricase, were the first to determine its fundamental properties and the mechanism of the reaction it catalyses. It was shown by this work that during the oxidation of one molecule of uric acid to allantoin, one atom of oxygen and one molecule of water are taken up while one molecule of $CO_2$ is given off, the R.Q. of the reaction being equal to 2. Other workers [Felix *et al.* 1929; Schuler, 1932] have found, however, that under certain conditions the $O_2$ uptake and the $CO_2$-formation have different *p*H-optima. This result, according to Schuler, strongly supports the view that the reaction takes place in two steps: (1) oxidation of uric acid to an intermediate compound (oxy-acetylen-diurein-carboxylic acid) catalysed by uricase, and (2) decarboxylation of this compound which is either spontaneous or catalysed by another enzyme.

On the other hand, Keilin & Hartree [1936, 1] have been unable to find any departure from the theoretical R.Q. of 2 at different *p*H values.

As an oxidizing enzyme, uricase occupies an intermediate position between the typical dehydrogenases and the oxidases. Although it is reversibly poisoned by KCN it combines with the substrate and activates it, as shown by the inhibitory effect on the reaction produced by some derivatives of uric acid, which act as competitive inhibitors. On the other hand the substrate activated by this enzyme does not react with any known hydrogen acceptor except $O_2$ which is invariably reduced to $H_2O_2$ [Keilin & Hartree, 1936, 2].

All attempts to purify uricase have been without great success until quite recently when Davidson [1938, 1, 2] succeeded in obtaining a purified preparation with a $Q_{O_2}$ about 600 times higher than that of the acetone-dried pig's liver used as the starting material. As this purified preparation was practically free from copper and haematin but contained between 0·15 and 0·20 % of iron, Davidson suggested the possibility that iron is a constituent of the enzyme.

In this paper I propose (1) to describe an improved method of purification of uricase, and (2) to examine the properties of the purified preparation [*vide* also Holmberg, 1939].

## *Method of preparation*

(1) Fresh pig's liver (or liver stored for one day at +4°) is minced in the Latapie mincer and treated twice with 4 parts of acetone. The acetone is sucked off on a large Büchner funnel, the residue is left to dry for a few hours on filter papers and overnight in a desiccator. The dry preparation is ground in a porcelain mill and passed through a fine sieve. The resulting fine powder is used for the extraction.

(2) The extraction is carried out according to Davidson's method. 200 g. of powder are first washed with 2 l. of cool *M*/10 phosphate buffer *p*H 7·3. The residue is centrifuged off and extracted for 20 min. at 40° with 4 l. *M*/10 alkaline borate buffer solution *p*H 10.

(3) The crude extract is precipitated with 25–50 g. of barium acetate, centrifuged and the precipitate discarded. The excess of barium is removed with an equivalent amount of ammonium sulphate.

(4) The first two steps of purification are also identical with those used by Davidson. The cooled extract is precipitated with an equal volume of saturated ammonium sulphate, the precipitate centrifuged off and redissolved in distilled water so that the concentration of ammonium sulphate becomes about 1/10 saturation. This solution, while mechanically stirred, is heated to 55–60° for 5 min., rapidly cooled and the resulting precipitate centrifuged off and discarded.

(5) The turbid solution is treated twice with 40 g. barium acetate (dissolved in small amounts of distilled water); the precipitates are centrifuged off each time and discarded.

(6) The solution is brought to *p*H 7 and adsorbed with 50 ml. (about 2 g. dry weight) alumina C-$\gamma$. The adsorbate is centrifuged off and discarded. The solution is filtered through a folded filter, giving a clear, pale yellow solution.

(7) The solution is again precipitated with an equal volume of saturated ammonium sulphate, which has previously been neutralized with NaOH. The resulting small precipitate is centrifuged down, redissolved in about 200 ml. distilled water and rapidly filtered. After standing for 1–2 hr. a fine white precipitate is formed. It is centrifuged down, washed several times with distilled water and suspended in 25–30 ml. of distilled water. This precipitate, which contains the uricase in a state which is only sparingly soluble in alkaline buffer solutions, will be referred to as "purified uricase". The yield of this preparation is about 7 mg. per 200 g. of acetone powder and it contains 10–14 % of the uricase present in the original extract.

*Determination of activity*

The activity of the preparation is estimated from the velocity of $O_2$ uptake during the first 15 min. in presence of 2 mg. of lithium urate as measured in Barcroft differential manometers in air at *p*H 9 and 39°. The flask of the manometer receives the enzyme in 0·1 *M* borate buffer solution, while the substrate is introduced from a dangling cup. As the crude preparation of the enzyme always contains catalase, the activity of the purified enzyme can be compared with that of the crude enzyme only on addition to the former of a drop of pure catalase solution which decomposes the $H_2O_2$ formed during the reaction [Keilin & Hartree, 1936, 1]. Without catalase the activity of the purified enzyme seems to be about 30 % higher than in its presence (Fig. 1). That $H_2O_2$ accumulates during this catalytic oxidation is shown by a strong and instantaneous colour reaction obtained on addition of peroxidase and *p*-phenylenediamine to the reaction vessel even after the first 5 min. of uricase activity. If, on the other hand, peroxidase and *p*-phenylenediamine are added before the beginning

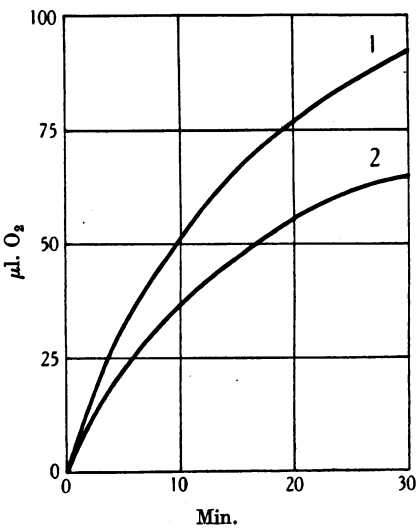


Fig. 1. Effect of catalase on $O_2$ uptake of 2 mg. uric acid and 30 $\mu$g. uricase. Curve 1: uricase + uric acid. Curve 2: uricase + uric acid + catalase.

of the reaction, the colour is only gradually developed [Keilin & Hartree, 1936, 1].

The accumulation of $H_2O_2$ can also be demonstrated by the addition of catalase from a dangling tube at the end of an experiment. The rapid decomposition of $H_2O_2$ which takes place immediately after mixing the solution with catalase produces a positive pressure which can be read on the manometer.

The enzyme concentration used in these experiments was so adjusted that the velocity of the reaction did not exceed 60$\mu$l. per 15 min. The activity of the purified uricase preparation estimated in this way and expressed as $Q_{O_2}$ is approximately 6000. This preparation is therefore 1200 times more active than the acetone-dried powder of liver, the $Q_{O_2}$ of which is approximately 5.

*Properties of purified uricase*

The purified uricase preparation is insoluble in water and forms a very fine precipitate which settles very easily. It is insoluble in neutral and slightly alkaline buffers, and only sparingly soluble at *p*H 10. In the dried state it has a pale brownish-green colour. The preparation gives a negative reaction for carbohydrate. The nitrogen content of a sample, which was not completely free from inorganic contamination with barium phosphate, was about 13·5%. The iron content of the same sample was 0·025%. The suspensions and alkaline solutions of such preparations containing about 5 mg. dry weight per ml. are colourless and show no absorption bands in the visible region of the spectrum. Only when treated with pyridine and reducer do they reveal the very faint $\alpha$-band of a protohaemochromogen.

The preparation can be kept as a suspension in distilled water in the cold for at least one week without an appreciable loss of activity.

*Effect of inhibitors*

Purified uricase is very sensitive to cyanide, the presence of $10^{-4}$ *M* KCN in the dangling cup being sufficient to abolish its activity. This inhibitory effect, as in the case of a crude enzyme preparation [Keilin & Hartree, 1936, 1] is perfectly reversible.

Sodium azide produces only a very slight inhibition at *p*H 7, but this effect lasts only for the first 5 min. of the reaction and then vanishes.

Sodium sulphide, $\alpha\alpha$-dipyridyl, dithizone and diethyldithiocarbamate have no inhibitory effects. Among metals copper exerts a very strong poisoning effect, $2\times10^{-5}$ *M* copper sulphate giving 75% inhibition using 60$\mu$g. of enzyme. Iron is much less effective, $10^{-3}$ *M* ferrous sulphate inhibits the same amount of enzyme by only 35%.

Manganese inhibits the enzyme in concentration as low as $10^{-4}$ *M* but this effect only develops gradually.

$H_2O_2$, as in the case of other enzymes, has a marked inhibitory effect which can, however, be completely abolished by the presence of 0·2% of gelatin. Very small amounts of diethyldithiocarbamate also abolish the inhibition by $H_2O_2$. It seems probable, therefore, that this inhibition is due to the presence of traces of copper (Fig. 2).

*Formation of $CO_2$ in the oxidation of uric acid by purified uricase*

It was suggested previously [Schuler, 1932] that the oxidation of uric acid to allantoin is a two-step reaction with the formation of an intermediate compound undergoing decarboxylation either spontaneously or through co-operation with another enzyme.

The study of the R.Q. of this reaction catalysed by a highly purified uricase does not, however, support the view as to the existence of a decarboxylating enzyme working in co-operation with uricase. In fact, the R.Q. of the reaction

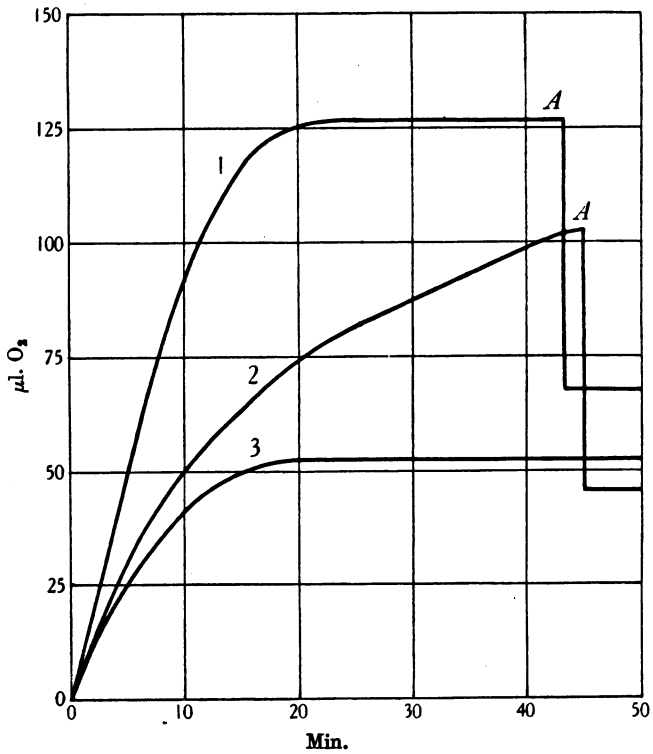


Fig. 2. Experiment showing poisoning action on uricase of accumulated $H_2O_2$, and protection by diethyldithiocarbamate. Uric acid 1 mg. + uricase 35 $\mu$g. The amount of $H_2O_2$ accumulated is shown by the increase in pressure after adding catalase. Curve 1: uricase + uric acid + 20 diethyldithiocarbamate. Curve 2: uricase + uric acid. Curve 3: uricase + uric acid + catalase. At *A* catalase added from dangling cup.

measured at *p*H 7·3 in presence and in absence of catalase are 1 and 2 respectively. On the other hand, it seems very unlikely that the complicated procedures of the purification could lead to a preparation containing two entirely distinct enzymes.

### *Coupled oxidations*

It is well established now that the $H_2O_2$ formed in the primary oxidation reaction can be utilized for secondary or coupled oxidation. In the coupled oxidation $H_2O_2$ is activated either by metals, haematin or peroxidase present in or added to the preparation [Thurlow, 1925; Harrison & Thurlow, 1926]. It was thus possible with $H_2O_2$, formed in the primary oxidation catalysed by purified uricase, to oxidize substances like sodium sulphide, hydroxylamine, *p*-aminophenol and *p*-phenylenediamine.

An entirely different type of secondary or coupled oxidation reaction is that of alcohol. It was shown by Keilin & Hartree [1936, 2] that alcohol added to the crude uricase and uric acid doubles the $O_2$ uptake by this system. They have also

shown that alcohol produces a similar effect on xanthine oxidase and hypoxanthine, only if catalase is added to the system. In both cases, however, alcohol undergoes oxidation to acetaldehyde. They have naturally concluded that in crude uricase preparation, the oxidation of alcohol by $H_2O_2$ formed in the primary reaction was also catalysed by catalase present in the preparation. That this is so can be clearly demonstrated now with the purified uricase preparation which is completely free from catalase. As is shown in Fig. 3 the addition of

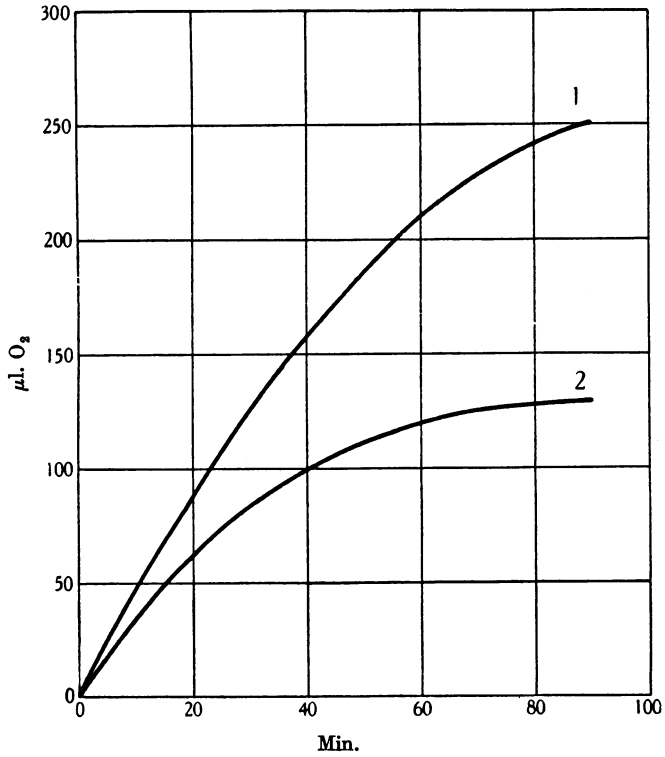


Fig. 3. Effect of 12 mg. ethyl alcohol on $O_2$ uptake by 2 mg. uric acid + 30 μg. uricase + one drop *strong* catalase. Curve 1: uricase + uric acid + catalase + alcohol. Curve 2: uricase + uric acid + catalase. The same amount of alcohol has no effect, on the $O_2$ uptake by uricase + uric acid without catalase.

alcohol to the purified uricase-uric acid system has no effect on this system while in presence of catalase it doubles the $O_2$ uptake and undergoes oxidation to aldehyde.

*Nature of uricase*

The fact that cyanide reversibly inhibits the catalytic action of uricase makes it very probable that this enzyme contains a heavy metal in its active group.

Although the amount of iron in these purified preparations is very small (0·02 %) the possibility that uricase contains iron cannot be definitely ruled out. In fact, as we have no indication yet as to the degree of purity of these preparations, and assuming that they are only 10–20 % pure, it is still conceivable that the iron or even the haematin which is just detectable, might be associated with the enzyme.

SUMMARY

A modification of the method described by Davidson is given whereby it has been possible to obtain uricase with a $Q_{O_2}$ of about 6000. The preparation has a very low iron content (about 0·025 %). The purified preparation has the same R.Q. as crude preparations and it is, therefore, considered improbable that the oxidation and decarboxylation of uric acid are catalysed by different enzymes.

An account is given of the action of different inhibitors on the purified uricase preparation, and of some coupled oxidations that can be produced by the $H_2O_2$ formed in the reaction are described.

I wish to express my deep gratitude to Prof. D. Keilin for giving me facilities to work in his laboratory and for all the help he has given me with this work, and also to Dr T. Mann for much help and advice.

REFERENCES

Batelli & Stern (1909). *Biochem. Z.* **19**, 219.
—— —— (1912). *Ergebn. Physiol.* **12**, 199.
Davidson (1938, 1). *Nature, Lond.*, **141**, 790.
—— (1938, 2). *Biochem. J.* **32**, 1386.
Felix, Scheel & Schuler (1929). *Hoppe-Seyl. Z.* **180**, 90.
Harrison & Thurlow (1926). *Biochem. J.* **20**, 217.
Holmberg (1939). *Nature, Lond.*, **143**, 604.
Keilin & Hartree (1936, 1). *Proc. roy. Soc.* B, **119**, 114.
—— —— (1936, 2). *Proc. roy. Soc.* B, **119**, 141.
Schittenhelm (1905). *Hoppe-Seyl. Z.* **46**, 354.
Schuler (1932). *Hoppe-Seyl. Z.* **208**, 237.
Thurlow (1925). *Biochem. J.* **19**, 175.
Wiechowski & Wiener (1909). *Arch. exp. Path. Pharmak.* **60**, 185.

ADDENDUM

*Note added 1 October 1939.* A purified uricase preparation analysed by the dithizone method for lead and zinc gave the following results:

A 6 mg. sample was found to be free from lead, while another 10 mg. sample was fonnd to contain 13 $\mu$g. zinc; i.e. 0·13 %. Considering that liver, the source of uricase preparations, is rich in zinc no definite conclusion can be drawn from this single estimation. It suggests nevertheless that further study of the relationship between uricase and zinc carried out on a larger scale will be of interest.