25. The Purification of Uricase

2. Some Properties of Purified Uricase

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(Received 10 February 1942)

In a previous communication [Davidson, 1938], a method was described for the purification of the enzyme uricase which catalyses the oxidation of uric acid. The purified preparation contained 0.15–0.20 % of Fe. Recently, Holmberg [1939], using a modification of this method, obtained a preparation containing only 0.025 % Fe. In a note added at the end of his paper Holmberg reports that one sample of purified enzyme contained 0.13 % Zn. Another sample was free from Pb.

Wachtel *et al.* [1941] verified the presence of Zn in preparations of pig-liver uricase in the early stages of purification only and showed that a 200-fold increase in activity resulted in a fourfold increase in Zn content. Rats on a Zn-deficient diet showed a high plasma uric acid content, but liver uricase was not decreased.

The presence of Zn in at least one enzyme is already known. Keilin & Mann [1940] have shown that purified preparations of carbonic anhydrase contain about 0.33 % Zn, and these observations have been confirmed by Hove *et al.* [1940, 2].

Moreover, Zn ions have been shown to increase the activity of yeast carboxylase [Lohmann & Kossel, 1939], of yeast peptidase [Johnson, 1940], and of crude intestinal phosphatase preparations [Hove *et al.* 1940, 1].

In this paper some properties of purified uricase are discussed with particular reference to the presence of Zn.

Enzyme preparations. The starting material used was again pig liver. The specific activity of the crude acetone liver powder was 0.12–0.15 $\mu\text{l.}/\text{mg.}/\text{min.}$ as determined in the manometric test previously described [Davidson, 1938]. A few preparations of purified uricase were made by the original method of Davidson [1938]. These had a specific activity of 85–90 $\mu\text{l.}/\text{mg.}/\text{min.}$ Several preparations were also made by the modification of Holmberg [1939] which is in some ways more convenient, and avoids the dialysis at low temperatures of considerable volumes of fluid. These preparations had a specific activity of 80–90 $\mu\text{l.}/\text{mg.}/\text{min.}$, i.e. both methods result in a product of about the same specific activity.

Heavy metal content. Zn was determined by the dithizone method of Schwaibold *et al.* [1938] as described by Keilin & Mann [1940] and Fe was determined by a modification of the method of Kennedy [1927].

Table 1. *Fe and Zn contents of uricase at various stages of purification*

Specific activity $\mu\text{l.}/\text{mg.}/\text{min.}$	Fe %	Zn %
0.12–0.15	0.04–0.08	0.010–0.014
0.33–0.43	0.20–0.30	0.02
0.5	0.35	0.03
1.1–1.8	0.10–0.18	0.03–0.06
6.6–13.8	0.21–0.30	0.06–0.07
60–90	0.10–0.20	0.06–0.09

The Fe and Zn contents of uricase preparations at different stages in the purification process are shown in Table 1. It will be seen that the Fe content of the preparation is

high at all stages. Although it varies considerably from stage to stage there appears to be no correlation between the enzyme activity and the Fe content. In none of the purified preparations was the Fe content as low as that obtained by Holmberg (0.025%). It was always greater than the Zn content, and in the purest preparation was not lower than 0.2%.

The Zn content, on the other hand, increases as the purification of the enzyme proceeds. The figures obtained for preparations in the early stages of purification agree fairly well with those quoted by Wachtel *et al.* [1941] who, however, did not use uricase preparations with a greater specific activity than about 10 μ l./mg./min. In no preparation was a Zn content as high as 0.13% obtained. The purest preparation contained 0.09% Zn.

In the purified preparations of uricase Zn was identified both as Zn-Hg thiocyanate and as Rinnmann's green [cf. Keilin & Mann, 1940].

In order to determine the nature of the other metals, if any, present in the purified enzyme spectrographic methods were employed. Such methods have occasionally been found convenient for other enzymes, e.g. by Richards & Hellermann [1940] in the case of arginase. The method employed was the cathode layer arc procedure described by Mitchell [1940] for soils, using a Hilger Large Quartz Spectrograph. The dialysed enzyme preparation was dried, powdered on an agate mortar and introduced into the hollow cathode. Owing to the volatility of Zn two exposures were made, one during the first minute and the second during the remainder of the life of the cathode (about 4 min.). The sensitivity of the method towards Zn is not very great, nevertheless the presence of appreciable quantities of Zn and Fe was demonstrated. Traces of other heavy metals, including Cu, Pb, Mn, Mg, Al and Sn, were also detected. No attempt was made to carry out a quantitative estimation of the metals by the spectroscopic method but the relative amounts of the various metals could be approximately assessed.

The spectroscopic observations were carried out by Dr R. L. Mitchell of the Macaulay Institute, Craigiebuckler, to whom grateful thanks are expressed.

Effect of varying oxygen tension

It has been shown that the velocity of the oxidation of uric acid by crude liver powder is dependent on the O_2 tension, being twice as great in pure O_2 as in air [Battelli & Stern, 1909]. With pure uricase this point was further investigated, using mixtures of O_2 and A prepared in the apparatus described by Warburg *et al.* [1931]. The results, shown in Fig. 1, reveal that over a wide range the velocity of the reaction is proportional to the partial pressure of O_2 .

Effect of carbon monoxide

The previous experiment was repeated, using CO in place of the inert gas, the manometric measurements being made in the dark room lest any effect of CO might be light-sensitive. At every tension of O_2 tested the velocity of the reaction was the same in the presence of CO as in the presence of inert gas. No inhibition of uricase by CO occurred even when the ratio CO/ O_2 was 49/1. Keilin & Hartree [1936] have reported that at a ratio CO/ O_2 of 8/1 no inhibition of uricase in crude liver powder occurred.

Fig. 1. Effect of varying the partial pressure of oxygen on the rate of oxidation of uric acid in the presence of uricase. Temperature 38°, pH 9.0, substrate 2.24 mg. uric acid as lithium urate, gas space— O_2 -A mixture, total volume 3.0 ml., time 20 min.

Kubowitz [1938] showed that the 'copper-proteid' polyphenoloxidase takes up no CO in the presence of 100 % CO. When substrate is added the Cu is reduced and CO is immediately taken up, one molecule being absorbed for every two atoms of Cu in the enzyme.

A similar experiment was carried out with CO and uricase (Table 2). The manometers were shaken in a thermostat at 20° in the dark room for 14 hr., but no appreciable uptake

Table 2. *Effect of CO on uricase after addition of substrate*

		Temperature 20°. pH 10.		
		Manometers		
		1	2	3
Main chamber		30 mg. uricase in borate buffer	30 mg. uricase in borate buffer	Borate buffer without enzyme
Side bulb		2.24 mg. uric acid as lithium urate	2.24 mg. uric acid as lithium urate	2.24 mg. uric acid as lithium urate
Gas space		100 % CO	100 % A	100 % CO
Time after addition of uric acid (min.)		μ l. CO	μ l. CO	μ l. CO
10		0.0	+0.5	0.0
25		-1.0	0.0	0.0
50		-0.5	+0.5	0.0
170		-2.0	+0.5	0.0
260		-3.0	+0.5	-1.0
830		-8.0	+2.0	-6.5

of CO occurred either before or after addition of substrate despite the very large amounts of uricase present. CO does not therefore react with the metal present in uricase in stoichiometric proportions as it does with Cu in Kubowitz's enzyme.

Inhibitors

Cyanides. The inhibition of purified uricase by cyanide has already been discussed [Davidson, 1938; Holmberg, 1939; Wachtel *et al.* 1941]. The effect is completely reversible and full activity is restored after the cyanide has been removed by dialysis.

Potassium thiocyanate. Hove *et al.* [1940, 2] have shown that KCNS has a marked inhibitory action on carbonic anhydrase, 93 % inhibition being produced by a concentration of 10 mM. On the other hand, KCNS was reported by Wachtel *et al.* [1941] to have no effect even in a concentration of 22 mM on uricase. In the present series of experiments the effect of KCNS on purified uricase was investigated, and was found to be rather curious (Fig. 2). During the first 5 min. after the addition of substrate no effect, or only a very slight effect, was obtained but after this initial latent period inhibition was pronounced but was never complete. At a concentration of 5 mM a very slight effect was produced but at concentrations of 10 mM and 20 mM the effect was marked. Even at a concentration of 100 mM the inhibition was never complete (Fig. 2). This effect was repeatedly obtained with different preparations of uricase and with all samples of KCNS tested.

Potassium ferrocyanide. $K_4Fe(CN)_6$ was found to cause slight inhibition (10 %) in a concentration of 15 mM.

Sulphanilamide. The powerful inhibitory action of this substance on carbonic anhydrase [Mann & Keilin, 1940] was not seen with purified uricase, on which it had no effect.

Heavy metals. Many heavy metals inhibit purified uricase, Cu being effective at much lower concentrations than any of the other metals tested. Holmberg's [1939] observations on inhibition by Cu (75 % inhibition at 0.02 mM), Fe (35 % at 1 mM) and Mn were confirmed, as were those of Wachtel *et al.* [1941] on Zn (80 % inhibition at 2.0 mM). Inhibition was also caused by Co (60 % at 1 mM) and Ni (40 % at 1 mM).

Dithizone. Hove *et al.* [1940, 2] have reported that carbonic anhydrase is partially inhibited by saturated aqueous dithizone. In the case of uricase Holmberg [1939] reported that dithizone was inactive. In the present work dithizone was found to increase slightly the activity of purified uricase preparations.

A number of other substances were found to accelerate the uptake of O_2 in the oxidation of uric acid by uricase. These included pyrophosphate (30 mM), phenanthroline, $\alpha\alpha'$ -dipyridyl, thiolacetic and dithioacetic acids, thiourea (80 % acceleration at 80 mM), quinaldinic acid (30 % at 60 mM), sodium diethyl dithiocarbamate [cf. Holmberg, 1939], histamine, thiamine and peptone. The activity of carbonic anhydrase is known to be increased by aneurin and histamine [Main & Locke, 1941] and by peptone [Scott & Mendive, 1941, 1].

Fig. 2.

Fig. 3.

Fig. 2. Effect of different concentrations of KCNS on uricase. Temperature 38° , pH 9.0, substrate 2.24 mg. uric acid, gas space 100 % O_2 , total volume 3.0 ml.

Fig. 3. Course of the reaction. Temperature 38° , pH 9.0, substrate 1.12 mg. uric acid as lithium urate, gas space 100 % O_2 , total volume 3.0 ml. Curve A, purified uricase alone. Curve B, purified uricase plus catalase. Curve C, impure uricase. Curve D, crude extract of acetone liver powder. Theoretical (1), O_2 uptake with H_2O_2 formation, 149 μ l. Theoretical (2), O_2 uptake without H_2O_2 formation, 74.5 μ l.

The nature of the reaction catalysed by uricase

In the course of the oxidation of uric acid H_2O_2 is produced [Holmberg, 1939; Keilin & Hartree, 1936] and the reaction is probably therefore:

Using 1.12 mg. uric acid the theoretical O_2 uptake, if this reaction were allowed to go to completion, would be 149 μ l. The actual O_2 uptake found with purified uricase was rather less, probably owing to poisoning of the enzyme by H_2O_2 . In most cases it was found to be about 120 μ l. (Fig. 3). When this point was reached a slight positive pressure, due presumably to a decomposition of some of the H_2O_2 , developed before a steady state was reached.

In the presence of traces of catalase the H_2O_2 is decomposed and the reaction becomes:

and the theoretical O_2 uptake with 1.12 mg. uric acid would be $74.5\mu l$. This amount was actually found with purified uricase to which a small amount of catalase had been added (Fig. 3). Purified uricase is itself catalase-free.

With crude uricase, in which catalase is present as an impurity, the second reaction occurs and the theoretical O_2 uptake of $74.5\mu l$. was obtained.

In the second reaction the R.Q. is 1 and in the first it is 2. These values for the R.Q. were actually found by Holmberg [1939] in the presence and absence respectively of catalase.

End-product of uric acid oxidation

It is usually considered that uricase catalyses the oxidation of uric acid to allantoin through the possible intermediary stage of hydroxy-acetylene-diurein carboxylic acid [Schuler, 1932], and Kleinmann & Bork [1933] using very crude uricase, have reported the isolation of allantoin from among the reaction products, together with some urea and oxalic acid. In the case of xanthine oxidase, however, Ball [1939] found that although the impure enzyme catalysed the oxidation of xanthine or hypoxanthine to a product which was oxidized by uricase and which was therefore presumably uric acid, with purified xanthine oxidase the product of the reaction was a substance which did not act as a substrate for uricase, and which appeared to differ from ordinary uric acid. It was therefore thought desirable to determine whether allantoin was in fact produced in the reaction with purified uricase.

500 mg. of lithium urate in borate buffer pH 9 were treated with successive additions of uricase till tests for uric acid were very faint. The reaction mixture was kept at 37° with O_2 bubbling through it. The mixture was then neutralized with HCl, taken to dryness and extracted in a Soxhlet apparatus with absolute alcohol (in which allantoin is slightly soluble) for 15 hr. The alcohol was evaporated off and the residue dissolved in a small amount of water. Chlorides were removed with Ag acetate, and excess Ag with H_2S . The filtrate was aerated to remove H_2S and was then treated with an equal volume of Wiechowski's [1908] reagent. The heavy precipitate of the allantoin compound was allowed to settle overnight and was then centrifuged off and decomposed with H_2S . The filtrate from the sulphide precipitate was taken to dryness and dissolved in hot alcohol. On cooling, crystals separated. They were recrystallized from hot alcohol.

Yield 30 % of theoretical. M.P. 235° (decomp.). Allantoin melts at 238° . Found: C, 30.4; H, 3.8; N, 35.8 %. $C_4H_6N_4O_3$ requires C, 30.4; H, 3.8; N, 35.5 %.

DISCUSSION

The reversible inhibition of uricase by cyanide suggests that the enzyme is almost certainly a metallo-protein compound but whether it is in the nomenclature of Warburg [1938] a 'zinc-proteid' or an 'iron-proteid' or neither, is still in doubt. The presence of Zn in purified or partially purified uricase preparations is significant since although Zn is fairly widely distributed throughout animal tissues, apart from those enzymes which are activated by Zn ions the only enzyme known to be a compound of Zn and protein is carbonic anhydrase. The results presented here demonstrate that the Zn content of the uricase preparations rises as the specific activity increases although a 600-fold increase in activity is accompanied by only a ninefold increase in Zn content. At the same time it would be unwise to conclude for this reason alone that Zn is of necessity the active group. The possibility of Zn being an impurity in uricase, as Cu is in impure carbonic anhydrase, cannot at this stage be completely ruled out. In the case of carbonic anhydrase, a true 'zinc-proteid', Keilin & Mann [1940] have shown that in the early stages of purification the Cu content rises with increasing activity to a concentration of almost 0.1 % and then suddenly falls.

Although purified uricase contains Zn, other proteins also containing Zn but without uricase activity occur in impure uricase preparations.

If, for example, the mother liquor from which uricase is precipitated in Holmberg's method of preparation is dialysed against distilled water and the precipitated proteins are rubbed up with ice-cold phosphate buffer (the phosphate extract being discarded) and then with borate buffer at room temperature, the residual uricase dissolves in the borate leaving a pure white protein, quite insoluble in water and borate buffer, containing about 0.04 % of Zn. It is possible of course that this substance may consist in part of inactivated uricase.

The Fe content of uricase remains high at all stages and the purest preparations contain the amount of Fe previously reported (0.2 %). In no case was the low figure (0.025 %) of Holmberg obtained. The high Fe content at all stages in the purification procedure suggests that much, and perhaps even all, of it is present in the form of impurities. Even purified catalase, an 'iron-proteid', contains only 0.09 % of Fe [Sumner *et al.* 1940].

The other heavy metals which were detected spectroscopically are probably all impurities.

In Holmberg's method of purification the final stage consists in the precipitation of uricase from a mother liquor consisting of a solution of an ammonium sulphate precipitate in water. The specific activity of the enzyme increases in this stage some 40–60 times and it is highly probable that the precipitated uricase might carry with it heavy metals present in the mother liquor as impurities either derived from the liver powder or introduced in the reagents.

The action of inhibitors gives few clues to the nature of the heavy metal in uricase. Inhibition by KCNS, which inhibits carbonic anhydrase, might support the suggestion that Zn is the active group but this inhibition is incomplete. Dithizone does not inhibit uricase as it does carbonic anhydrase; in fact it activates it. Most of the substances which activate uricase are those which might be supposed to act by removing traces of inhibiting heavy metals.

If uricase is in fact a 'zinc-proteid', on the assumption that the Zn content would be similar to that of Keilin & Mann's carbonic anhydrase (i.e. 0.33 %), then the present preparation would be about 30 % pure.

However, the percentage of Zn in Zn protein complexes is variable. Scott & Mendive [1941, 2] report the presence of only 0.15 % of zinc in purified carbonic anhydrase while Hove *et al.* [1940, 2] report that one of their specimens of carbonic anhydrase contained as much as 0.51 % of Zn. In the case of insulin Scott & Fisher [1935] found that crystalline insulin contained 0.52 % of Zn. Later, however, Cohn *et al.* [1939] reduced the Zn content of crystalline insulin to 0.36 % while Sahyun [1941] has recently reported the presence of only 0.15 % of Zn in crystalline insulin. Schorn [1929] reported the formation of compounds of Zn and albumin containing 0.63 % of Zn.

It is therefore impossible at the present time to assess the degree of purity of the uricase preparations. It may be significant that purified uricase obtained by two different methods, the original one of Davidson and Holmberg's modification, which differ considerably in their later stages, has about the same specific activity. All further attempts at purification have been hindered by the great insolubility of the enzyme in water or buffer solutions. Even in alkaline buffers of pH 9 or 10 its solubility is not great, and the insoluble nature of the enzyme appears to become more and more pronounced as purification proceeds.

SUMMARY

The presence of Zn in purified preparations of uricase has been confirmed. Such preparations also contain Fe and the purest samples obtained contained 0.09 % Zn and 0.2 % Fe.

Uricase is partially inhibited after a latent period by KCNS but not by CO even in high concentrations. The effects of other inhibitors and activators are reported and the results are discussed in relation to the heavy metal content of the enzyme.

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