URICASE AND BRITISH ANTI-LEWISITE

IS URICASE A ZINC-PROTEID?

by

E. PRÆTORIUS

Institute of Medical Physiology, University of Copenhagen (Denmark)

The nature of the enzyme uricase has been investigated by a number of authors. The studies of PRZYLECKI AND TRUSZKOWSKI[1], FOSSE *et al.*,[2] and KEILIN AND HARTREE[3] on the inhibitory effect of cyanide on the action of this enzyme led to the belief that uricase is a metalloproteid, either a copper-proteid[1,4] or an iron-proteid[5]. Later investigators[6,7], however, have suggested the presence of zinc as an integral part of the enzyme.

WEBB AND VAN HEYNINGEN[8] showed that 2 : 3-dimercaptopropanol (British Anti-Lewisite, BAL), produces inhibition of some enzyme systems and suggested that the inhibition might be due to combination of the dithiol with the metal of the prosthetic group. The findings of these authors have been confirmed by BARRON *et al*[9].

The question as to whether uricase is a metalloproteid or not, can hardly be expected to be definitely decided, until it has been possible to crystallize the enzyme or at least to get information as to the degree of purity of the preparations and of the size of the enzyme molecule. Nevertheless we have found it of interest to study the effect of BAL on highly fractionated uricase preparations.

In a paper by BARRON and co-workers[10] the following details, which are of peculiar interest to the present work, are found:

> In aqueous solutions BAL combines with a number of heavy metals, forming complex compounds, which in the case of iron, copper and nickel are coloured and almost or quite insoluble. With zinc, cadmium and mercury the dithiol forms white precipitates, and the magnesium compounds are colourless and soluble.
>
> BAL is non-autoxidizable, but oxidation into the insoluble disulphide complex is taking place in the presence of air, the reaction being catalysed by copper, but not by manganese, cobalt or nickel. With iron the oxidation is considerably slower than with copper, and magnesium decreases the velocity of the oxidation catalysed by copper.

The present paper deals with the effect of BAL on the action of uricase. Conditions under which BAL acts as an inhibitor of uricase are described, and the effect of zinc on this inhibition has been studied in detail. Finally it will be discussed whether the findings tend to support or to invalidate the assumption that zinc is a constituent of the uricase system.

Before entering into a description of the experimental results it was thought advisable to give an account of the technique and the methods used.

METHODS

The principles given by KALCKAR[11] of enzymatic determination of uric acid by

References p. 601.

differential spectrophotometry in the BECKMAN instrument with ultraviolet attachment, form the basis of the method elaborated for the present kinetic uricase studies. Strictly speaking, the BECKMAN spectrophotometer is not particularly suited to serve such purpose, since neither the temperature nor the oxygen tension can be regulated in the reaction mixtures, which are kept in open quartz cells. Even with these restrictions, however, the method offers much advantage beyond manometric determinations in WARBURG-BARCROFT apparatus. Nevertheless a few investigations of this kind were performed in order to make possible a comparison between the activity of the uricase here employed and the activities obtained by other investigators.

Optical methods

In all experiments the quartz cells contained samples of 3 ml volume and had a light path of 1 cm. The slit-width never exceeded 0.3 mm.

In the case of *simple spectrophotometry, i.e.*, when the spectrum of a compound is determined, the contents of the measuring cell differ from those of the reference cell only by the presence in the former of the compound whose spectrum is to be obtained. The extinction difference between the two cells is read directly on the transmission scale of the instrument, and is a measure of the concentration of the investigated compound.*

The *enzymatic differential spectrophotometry* is the determination of the extinction change taking place in the reaction mixture of a quartz cell as a result of the enzymatic conversion of one compound into another having a distinctly different absorption spectrum. Since the difference measured is not dependent on different contents of two cells, as is the case by simple spectrophotometry, but is an extinction change taking place in the same cell, the contents of the reference cell may be chosen arbitrarily.

Reagents

Uricase. The fine white precipitate, about 3 mg, which according to the directions of HOLMBERG[7] is obtained from 100 g of acetone-treated liver powder, is suspended in 2 ml of M/15 glycine buffer, pH 9.4.

Glycine buffer. $^2/_3$ M, pH 9.4. 25 g of crystalline glycine is dissolved in about 200 ml of boiled quartz-distilled water; 110 ml of N NaOH and 3 ml pure chloroform are added, and the volume, with stirring, is made up to 500 ml with quartz-distilled, CO_2-free water.

Stock solution of uric acid. A solution of lithium urate is prepared according to the directions of FOLIN[12]. The stability of this solution, however, cannot be ensured by the use of formaldehyde which even in the presence of a large excess of glycine produces a considerable inhibition of uricase. Therefore the urate solution was preserved by saturation with chloroform which at the concentrations of buffer and uric acid used in the spectrophotometric experiments has no measurable effect on the enzyme activity. In the manometric experiments only non-chloroformed, freshly prepared solutions were employed.

Diluted solutions of uric acid. 50 µl of the stock solution are diluted to 10 ml in a volumetric flask with M/15 glycine buffer. The concentration of uric acid in such dilutions is 5 µg per ml.

Solution of BAL. 500 µl BAL** is shaken with 3 ml of freshly boiled quartz-distilled water in a tightly stoppered tube. An unstable emulsion is formed; the water, which is saturated with the compound, contains 6.2 % BAL[13]. The non-dissolved part very soon settles as a coherent clear drop. Samples of a few µl are pipetted off just above the interface between the non-dissolved BAL and the solution.

* The extinction, E_λ, of an arbitrarily chosen concentration of a substance is $\log_{10} I^\circ_\lambda / I_\lambda$, where I° and I are the intensities of light before and after absorption by the investigated substance. λ is the wave-length of the light.

The concentration C of the compound is proportional to E according to BEER's law. When a light path of 1 cm is used $E_\lambda = K_\lambda \cdot C$. K_λ, the specific extinction, is accordingly defined as the extinction when the concentration is one. In this paper the unit of concentration is 1 µg per ml.

** Kindly furnished by Prof. R. H. S. THOMPSON and Dr L. A. STOCKEN, *Dep. of Biochemistry*, University Museum, Oxford.

References p. 601.

Determination of uric acid

The following experiment is cited in order to illustrate the agreement between the extinction value obtained by simple spectrophotometry and the extinction change produced by the enzymatic conversion of the uric acid. In this experiment was used the same concentration of uric acid 4.93 μg per ml, as in those described later.

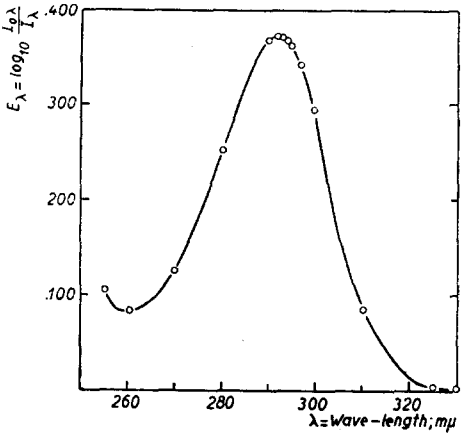

Fig. 1. The absorption spectrum of uric acid at pH 9.4. Abscissae: wave-length in mμ. Ordinates: extinction, $\log_{10} I_{\lambda}^{\circ}/I_\lambda$. Uric acid concentration 4.93 μg per ml. Light path 1 cm.

Absorption spectrum of uric acid (simple spectrophotometry). With M/15 glycine buffer as a reference the extinction of the uric acid dissolved in the same buffer is measured by light of various wave-lengths in the ultraviolet region. Fig. 1 shows the resulting spectrum. Abscissae: wave-length in mμ. Ordinates: the directly read extinction, E_λ, ($\log_{10} I_{\lambda}^{\circ}/I_\lambda$).

The wave-length of maximum absorption is 293 mμ. From the value of $E_{293\ m\mu}$, which is 0.370, and from the known concentration of uric acid, 4.93 μg per ml, the value of $K_{293\ m\mu}$ is calculated to be 0.075.

The enzymatic uric acid determination (differential spectrophotometry). Uricase suspension (9 μl) is added at zero time to the buffered uric acid solution, the spectrum of which is seen in Fig. 1. $E_{293\ m\mu}$ is read several times, in the beginning at short intervals; the exact time of each reading is written down.

Fig. 2 shows the variation of the extinction at 293 mμ after addition of the enzyme. Abscissae: reaction time in minutes. Ordinates: $E_{293\ m\mu}$. In the upper corner to the right the initial part of the curve is drawn on coordinates similar to those of the main curve, the only exception being the unit of abscissae, which has been made considerably longer to ensure accuracy of the extrapolation to zero time.

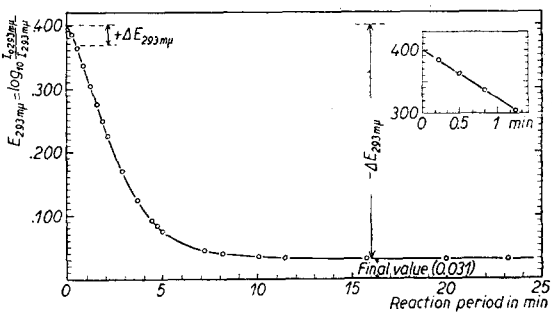

Fig. 2. The extinction change produced by uricase added at zero time to a solution of uric acid containing 4.93 μg per ml. pH 9.4. Tp. 23°. Wave-length 293 mμ. Abscissae: reaction period in minutes. Ordinates: extinction at 293 mμ. Before addition of uricase $E_{293\ m\mu}$ is 0.370. In the upper corner to the right: the initial part of the curve (the unit of abscissae is longer).

The difference between the value of extrapolation, 0.401, and the final value, 0.031, is 0.370. This difference, which is designated $-\Delta E_{293\ m\mu}$, was persistently found to be the same value of extinction as that observed by simple spectrophotometry.

This agreement is a confirmation of the statement of KALCKAR[11], that the absorption of uric acid at about 290 mμ completely disappears, when this compound is enzymatically oxidized. The absorption which persists when the enzymatic reaction has completed, corresponds exactly to the increase of $E_{293\ m\mu}$, ($+\Delta E_{293\ m\mu}$), produced by the addition of uricase to the buffered uric acid solution, *i.e.*, the value

References p. 601.

of extrapolation minus the extinction of the uric acid equals the final value of $E_{293\,m\mu}$.

Parallel to the readings of $E_{293\,m\mu}$, $E_{340\,m\mu}$ was read a number of times. The value of this was invariable (0.017), throughout the observation period, — an increased safeguard as regards uniform registration and against irrelevant extinction changes.

Activity determinations on uricase suspensions

The velocity of the fall of the extinction which is caused by the addition of a certain amount of uricase to the mentioned glycine buffered uric acid solution, furnishes a measure of the uricase activity. The period, $t_{1/2}$, in which half of the decrease is reached, may be read on coordinates or determined by interpolation. The initial velocity, V_i, of the extinction decrease is then defined and calculated by the equation: $V_i = -\Delta E_{293\,m\mu} : 2\ t_{1/2}$.

A suspension of uricase is said to have the *standard activity* of unity, when V_i is 0.100 per minute under following conditions: 10 μl of the enzyme suspension is added to 3 ml of a mixture of uric acid and glycine buffer of p_H 9.4 at 22°. The concentration of uric acid should be 5 μg per ml and the oxygen tension at equilibrium with air*.

Upon standing, most of the solid uricase, which is suspended in glycine buffer, will settle in 24 hours. Subsequently the supernatant shows only slight activity. If the sediment is stirred up, the standard activity will rise; during $\frac{1}{2}$–$\frac{3}{4}$ hour after the stirring the increase will amount to 25–50 % of the activity immediately after the resuspension of the sediment. The maximum activity will keep for one, often for two hours, whereupon the sedimentation causes a slow activity decrease. It is necessary, therefore, by kinetic experiments to make sure that the standard activity of the uricase suspension used is persistently the same throughout the experimental period.

Determination of dry-matter of uricase

500 μl uricase suspension is centrifuged at high speed for 5 min. The sediment is washed twice at 2° with quartz distilled water, dried over calcium chloride and weighed in the tared centrifuge tube.

EXPERIMENTAL RESULTS

The absorption spectrum of BAL

7 μl of saturated aqueous solution of BAL were added to each of three quartz cells, which contained 3 ml, respectively, of freshly boiled water, oxygen-saturated water, and glycine buffer (p_H 9.4). A series of extinction readings were made at various wave-lengths on the contents of the three cells, both immediately and following the addition of BAL. In the reference cell was water. All of the measuring cells contained 145 μg BAL per ml (1.2 μM per ml).

Fig. 3 represents the spectrum of BAL in freshly boiled water (•——•), in oxygen-saturated water after standing for one hour (o——o), and the spectrum of the glycine buffer (x——x). The immediate determination in oxygen-saturated water showed almost the same curve as in boiled water; to avoid confusion this result is not included in the figure. After standing for one hour in freshly boiled water BAL had a spectrum, the line of which should be drawn between the graphs •——• and o——o.

A number of spectra taken at various times after addition of BAL to water showed that a spectral change is taking place rather slowly, the velocity being dependent on the oxygen tension. Even in oxygen-saturated water the change was far from being completed in one hour.

Quite different are the changes, which occur, when BAL is added to the glycine

* The unit of standard activity and the mentioned standard conditions are chosen solely for practical reasons and for present purposes only. In a later communication[14] it will be demonstrated that the curve on Fig. 2 is not a true expression of uric acid disappearance. A transitory accumulation of an unknown intermediate oxidation product with a rather high absorption at 293 mμ offers interference. (Compare KLEMPERER[15]).

References p. 601.

buffer of p_H 9.4. The buffer itself (x——x) and BAL in water (•——•) produce only slight absorption at wave-lengths higher than 260 mμ. When BAL is added to the buffer solution, however, a considerable extinction increase will suddenly occur at all wave-lengths in the ultraviolet (◆——◆).

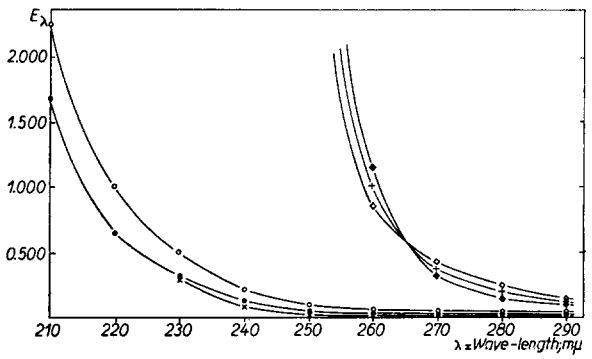

Fig. 3. The spectrum of a solution of BAL (145 μg per ml) in freshly boiled water •——•, in oxygen-saturated water after standing for one hour o——o, and the spectrum of glycine buffer (p_H 9.4) x——x. The spectrum of BAL in oxygen-poor glycine buffer determined immediately after the addition of BAL to the buffer ◆——◆, the spectrum of the same mixture 5 minutes later +——+, and 20 minutes later ◆——◆. Abscissa: wave-length in mμ. Ordinates: extinction.

In addition to this abrupt change, which is most pronounced in the short-waved region, further changes are taking place when BAL, in the presence of air, is kept in the alkaline buffer. The spectra +——+ and ◇——◇, which are taken respectively 5 and 20 minutes later than ◆——◆, indicate that the extinction at 270 mμ and higher wave-lengths is increasing, while the extinction at 260 mμ and under is decreasing.

The abrupt spectral change, which occurs when the reaction is made alkaline, may be produced by sodium hydroxide instead of buffer. If an equivalent amount of hydrochloric acid is added promptly, the spectrum will suddenly return to the shape before the addition of alkali to the neutral BAL solution, (the curve •——•). The instantaneous spectral change is reversible, therefore, and must be due to a change of p_H.

The gradual change of the BAL spectrum, however, must be the result of the unavoidable oxidation of BAL, since the velocity of the change is dependent on the oxygen tension and increases with increasing p_H. Furthermore, BARRON *et al.*[10], demonstrated that the oxidation of BAL is accelerated by copper ions and retarded by cyanide. This is also true for the gradual change of the BAL spectrum.

The wave-lengths 260 mμ and 293 mμ are the wave-lengths at which the uric acid spectrum has a minimum and a maximum, respectively. In the following section it will be shown how the extinction of these wave-lengths is gradually changed as the oxidation of BAL proceeds in an alkaline buffer.

The oxidation of BAL at p_H 9.4

In Fig. 4 it is shown $E_{260\,m\mu}$ decreases when BAL is kept in oxygen-poor (•——•) and oxygen-rich (o——o) glycine buffer, respectively. Abscissae: standing time of the BAL solution in minutes. Ordinates: $E_{260\,m\mu}$. As before 7 μl of saturated aqueous solution of BAL were added to 3 ml of the buffer (p_H 9.4), at zero time.

Fig. 5 shows the increase of the $E_{293\,m\mu}$ after addition of 7 μl of the saturated solution of BAL to oxygen-poor (•——•), oxygen-rich (o——o), and oxygen-containing glycine buffer (+——+), respectively. In the last case the oxygen is at equilibrium with air. Abscissae: standing time of the BAL solution in minutes. BAL is added to the buffer at zero time.

Even in the original oxygen-saturated buffer the spectral change owing to the

References p. 601.

oxidation of BAL is incomplete. In the oxygen-poor buffer the oxidation takes several hours. From a comparison with the experiments illustrated in Fig. 3, it may be concluded, moreover, that the velocity of the oxidation in a neutral solution is only a small fraction of that in an alkaline medium. These results are utilized in experiments described later (*"Dialysis of uricase against BAL"*).

From Fig. 4 appears that $-\Delta E_{260\,m\mu}$ by complete oxidation is 0.800–0.900 when the concentration of BAL is 1.2 μM per ml. In Fig. 5 is apparent that the corresponding value of $+\Delta E_{293\,m\mu}$ is 0.110–0.130.

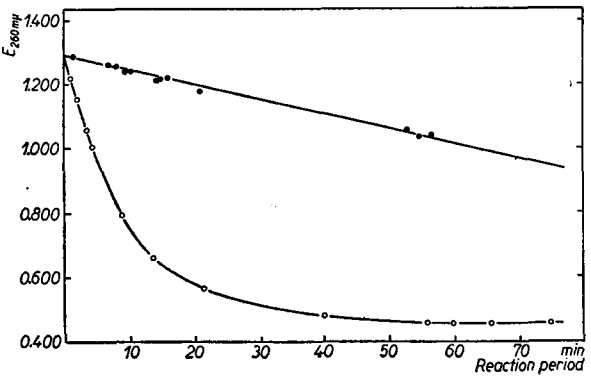

Fig. 4. Change in $E_{260\,m\mu}$ during standing of BAL in glycine buffer of pH 9.4. The concentration of BAL is 145 μg per ml. •——• originally oxygen-free, o——o originally oxygen-saturated buffer. BAL is added to the buffer at zero time. Abscissae: standing time of the BAL in buffer. Ordinates: $E_{260\,m\mu}$.

Inhibition of the enzymatic reaction by BAL

Each of six cells with 3 ml of the so-called *standard mixture* (compare *"Activity determinations"*), contains uric acid at a concentration of 5 μg per ml in M/15 glycine buffer. p_H is 9.4. In five of the cells the oxygen tension is at equilibrium with air. The last one is oxygenpoor. (The reference cell contains water).

At certain intervals 7 μl saturated aqueous solution of BAL is added to the first two cells, to the third 7 μl of ¼-saturated and to the fourth 7 μl $^1/_{12}$-saturated BAL solution; to the remaining two cells 7 μl water is added. The BAL concentrations of the four cells are accordingly 1.2, 1.2, 0.3 and 0.1 μM per ml, respectively. The cell poor in oxygen is not supplied with BAL.

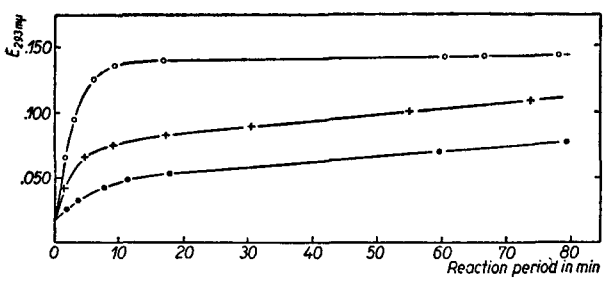

Fig. 5. $E_{293\,m\mu}$ as a function of standing time of BAL in glycine buffer of pH 9.4. The concentration of BAL is 145 μg per ml. •——• originally oxygen-free, o——o originally oxygen-saturated, and +——+ oxygen containing buffer, originally at equilibrium with air. BAL is added to the buffer at zero time. Abscissae: standing time of BAL in buffer. Ordinates: $E_{293\,m\mu}$.

Exactly half an hour after the addition of BAL (or water), 9 μl of uricase suspension is added to each of five of the cells. To one of those containing 1.2 μM BAL per ml, 9 μl glycine buffer is added instead of the enzyme suspension. Nitrogen is bubbled through the BAL-free, oxygen-poor cell for two minutes immediately before the addition of uricase; (the cell chamber is also strongly ventilated with nitrogen during the enzymatic reaction).

After addition of the enzyme, $E_{293\,m\mu}$ is read several times over a period of 30 minutes, at first at short intervals. Fig. 6 gives the extinction change at 293 mμ as a function of the reaction time. The curves are drawn from the same origin on the ordinate (0.375), the extinction value which corresponds to the concentration of uric acid in the cells.

References p. 601.

In Fig. 6 it is seen that an increase of the concentration of BAL causes a decrease of the steepness of the curve, the slope of which is mainly determined by the velocity of uric acid disappearance. In the case of the BAL-free, nitrogen-bubbled cell, however, the slope of the curve is almost equal to that of the cell with 1.2 μM of BAL. It appears therefore that addition of 1.2 μM BAL per ml half an hour before the start of the enzymatic reaction, will produce nearly the same effect on the slope as does nitrogen in the BAL-free standard mixture; (compare the increase of $E_{293\,m\mu}$ corresponding to the continued BAL oxidation in the cell without uricase).

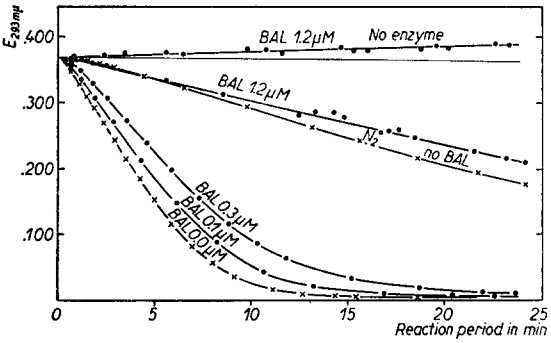

Fig. 6. The inhibitory effect of BAL added half an hour before zero time compared with the inhibitory effect of oxygen deficiency produced by bubbling with nitrogen 2 minutes before the addition of uricase at zero time to a glycine buffered solution of uric acid, the concentration of which is 5 μg per ml. pH 9.4. Abscissae: reaction period in minutes. Ordinates: $E_{293\,m\mu}$. The uppermost curve shows the slight extinction increase which occurred half an hour after the addition of BAL to a control system containing no uricase.

So far there is no reason to believe that the inhibition of uricase caused by BAL in the experiments illustrated is a specific one.

The effect of zinc on the inhibition produced by BAL

To five cells, which all of them contain 3 ml of the standard mixture of uric acid and buffer, is added BAL 15 minutes before the addition of uricase. The concentration of the dithiol is 1.2 μM per ml. Cell number six, which only contains uric acid, buffer and enzyme, represents the standard system (compare "*Activity determinations*").

Exactly 2 minutes after the start of the enzymatic reaction a small volume (10 μl) of salt solution is added to three of the BAL-containing cells: zinc sulphate, magnesium sulphate, and barium acetate, respectively. All of the salt solutions are strong enough to make the concentration of metal ions 1.2 μM per ml of the mixture.

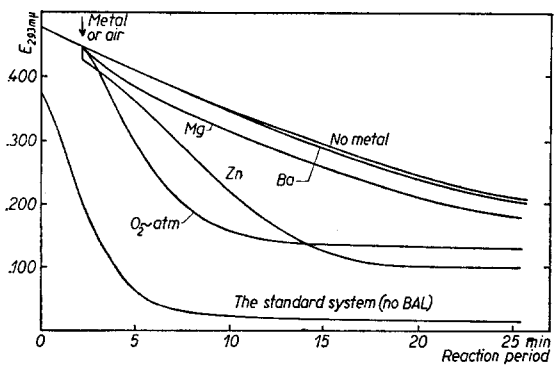

Fig. 7. The effect of bubbling with air (¾ of a minute) and of the addition of metal ions (1.2 μM per ml) 2 minutes after the start of the enzymatic reaction on which inhibition is produced by BAL (1.2 μM per ml) which is added 15 minutes before zero time. pH 9.4. The lowest curve represents the standard system.

The remaining two cells with BAL are otherwise treated. 10 μl water is added to one 2 minutes after the addition of uricase. At the same time the other is bubbled with air.

In Fig. 7 it is shown that addition of zinc considerably reduces the inhibition caused by the presence of BAL. Barium has no effect at all and the effect of magnesium is very small.

A priori one might be inclined to explain this as an indication of the importance of zinc to the activity of uricase. Since, however, a much stronger effect than that of zinc is obtained by bubbling with air (Fig. 7), it remains to be demonstrated

References p. 601.

if the effect of zinc might be ascribed to an interference on the oxidation of BAL.

If the coefficient of oxygen absorption in the contents of the cells is not essentially different from that of the oxygen absorption in pure water, the original concentration of oxygen in the cells is about 1 μM per ml. From the BAL oxidation curve (+——+ Fig. 5), it is evident that the oxygen concentration must be considerably reduced in the quarter of an hour after the addition of BAL to the buffered solution, when the concentration of BAL is 1.2 μM per ml of the mixture.

The slope of the curve (Fig. 7) after the addition of zinc is steeper the earlier this procedure takes place after the addition of BAL to the standard mixture of uric acid and buffer. If zinc is added before BAL, or promptly thereafter, the curve will be identical with that of the BAL-free standard system.

The assumption that the inhibition which is produced by BAL on the enzymatic conversion of uric acid, is simply due to the reduction of the oxygen concentration of the system, not only agrees with the data given by DAVIDSON[6] of the uricase activity at various oxygen tensions, but is furthermore supported by the following experiments.

The effect of zinc on the oxidation of BAL

Fig. 8 represents the changes of $E_{293\,m\mu}$ corresponding to the oxidation of BAL in the glycine buffer. The addition of barium is without any effect, magnesium produces a slight inhibition, (this is in agreement with BARRON *et al.*[10]), but zinc causes an instantaneous decrease of $E_{293\,m\mu}$, after which the extinction is quite constant.

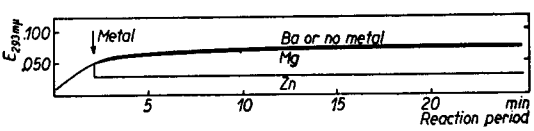

Fig. 8. The effect of metal ions on the BAL oxidation curve. BAL is added to glycine buffer (pH 9.4) at zero time. 2 minutes later metal salt at equimolar concentration is added (1.2 μM per ml).

It has been checked, that the presence of stock solution of uric acid (see "*Reagents*") has no influence on the shape of the curves.

It is evident accordingly, that the reduction produced by zinc on the inhibition of uricase by BAL, is simply due to the fact that a continued oxygen consumption for the oxidation of BAL is prevented by the presence of zinc.

Incubation of uricase with BAL

In order to illustrate the method of examination of uricase after incubation with BAL, the following example is described in detail.

18 μl suspension of uricase in glycine buffer is mixed in a micro vessel (volume 300 μl), with 14 μl of saturated aqueous solution of BAL and kept for two hours at 2°. A sample of 18 μl of uricase suspension diluted by 14 μl of water serves as a control.

At the end of the incubation period the contents of each of the vessels are stirred automatically for 20 minutes at room temperature, (22–24°), by means of a CARLSBERG micro titration arrangement[16], in which the burette is replaced by a fine tube, from which the micro vessels are ventilated with oxygen to ensure that practically all of the BAL is oxidized; (compare the curve o——o, Fig. 5).

To each of two cells, which contain 3 ml of the standard mixture of uric acid and the buffer (pH 9.4), is then added 16 μl of the enzyme-BAL mixture, and 16 μl of the enzyme dilution, respectively; to a third cell is added 9 μl of undiluted uricase suspension. The curves, which represent $E_{293\,m\mu}$ as a function of the reaction time, are all of the same shape as that in Fig. 2, and the $-\Delta E_{293\,m\mu}$ per minute, (the slope of the initial part of the curve), does not differ from the mean value by more then 5%. (See "*Activity determinations*").

The same result was seen in a number of similar experiments with shorter incubation periods, in which the oxidation of BAL was not completed.

Accordingly, uricase is not affected by incubation with BAL in high concentration (3%).

References p. 601.

Dialysis of uricase against BAL

To demonstrate the experimental conditions by dialysis of uricase the following is cited in detail.

The sediment after centrifugation of 3 ml uricase suspension, which is designated A, is washed twice with quartz distilled water and then resuspended in 12 ml of water. 8 ml of this suspension are dialysed through cellophane for 24 hours at 2° against 2 liters of a 0.6% solutions of BAL, and the remaining part of the suspension (4 ml) against 2 liters of water (without BAL) for 48 hours. Vigorous shaking of the cellophane bags ensure that the suspended particles will not settle. Upon centrifuging the BAL-dialysed uricase the sediment is washed twice and again resuspended in water (8 ml) and finally dialysed against pure water for 24 hours.

When the period of dialysis is over, half of the contents of the large bag and all of those of the small one are centrifuged separately, and each of the two deposits is suspended in 1 ml glycine buffer.

The specimen dialysed against BAL is designated uricase B, and that exclusively dialysed against water, uricase C.

(The non-centrifuged moiety of the contents of the large dialysing bag, the uricase of which has been dialysed against BAL, is used for zinc determination in a quartz spectrograph).

The results of dry-matter determinations and determinations of standard activity on uricase A, B and C are entered in Table I.

TABLE I

Enzyme preparation	Dry-matter mg/ml	Standard activity $10 \cdot (-\Delta E_{293\,m\mu})$/min	Standard activity per mg/ml
A	6.04	1.60	0.264
B	2.44	0.60	0.246
C	2.54	0.70	0.276

Enzyme activity and concentration of dry-matter of uricase in three suspensions, A, B and C (all of them from the same batch) in glycine buffer. The uricase in A has not been dialysed, that in B has been dialysed against BAL for 24 hours and then against water for 24 hours. The uricase in C has been dialysed against water for 48 hours.

It appears from the table that a considerable loss of dry-matter occurs by the procedures (some uricase is dissolved in the washing water, some of it adheres to the wall of the centrifuge tube). On the other hand no significant change in activity per mg of dry matter is seen.

This and other experiments with only 2–7 hours of dialysis indicate that uricase does not lose in activity when dialysed against BAL.

That considerable amounts of non-oxidized BAL persist at the end of the dialysing period, was not only to be expected from the data (Fig. 3) of the rate of oxidation in freshly boiled water, (compare "*The oxidation of BAL*") but was also shown experimentally by examination of the dialysis medium at the end of the dialysis period.

70 μl of the dialysis fluid (corresponding to 7 μl saturated aqueous solution of BAL) was mixed with 3 ml oxygen-saturated glycine buffer of pH 9.4 in a quartz cell at zero time and $E_{293\,m\mu}$ was read several times. An increase of $E_{293 m\mu}$ giving a curve similar to that of Fig. 5 (o——o) was the result; in one hour $+\Delta E_{293 m\mu}$ was more than 0.050. Another sample gave a decrease of $E_{260\,m\mu}$ as does the curve o——o in Fig. 4. In less than one hour $-\Delta E_{260\,m\mu}$ was about 0.400.

From these values of the extinction changes it is evident that only half of the amount of BAL undergone oxidation.

Manometric activity determinations

In addition to the activity determinations given in Table I the activity of uricase A and B was measured in a WARBURG-BARCROFT apparatus in order to compare these specimens with those of DAVIDSON[6] and HOLMBERG[7].

References p. 601.

The reaction mixture consisted of 2.24 mg of uric acid in M/15 glycine buffer at p_H 9.4, the total volume being 3 ml. Uricase A amounted to 57 μg and uricase B to 40 μg. The atmosphere was oxygen. Carbon dioxide was absorbed by 50 μl of 10% potassium hydroxide.

The initial velocity of the oxygen uptake calculated from the first 6 minutes of reaction was, respectively, 97 and 107 μl O_2 per minute per mg, which corresponds to a Q_{O_2} of 6000,—in agreement with the results of the authors mentioned.

Zinc analysis in quartz spectrograph

Part of the uricase, which had been dialysed against BAL was washed with quartz distilled water in a centrifuge tube, which together with some pipettes and a stirrer had been previously boiled in hydrochloric acid for one hour and subsequently 5 times in quartz distilled water.

After spontaneous sedimentation of the washed enzyme, most of the slightly active supernatant was drawn off and discarded. The main part of the concentrated suspension was then transferred to a weighing vessel.

Each of the following weighings was carried out on a micro balance after 3 days of drying in a desiccator ($CaCl_2$ and vacuum)

Vessel + uricase . . .	2516.63 mg
Vessel	2515.83 mg
Uricase	0.80 mg

As much as possible of the dried uricase was transferred to the carbon electrode of a quartz spectrograph, (Zeiss Qu 24). After renewed drying the weight of the weighing vessel with the remainder of the enzyme was 2516.01 g. To the electrode, therefore, 0.62 mg had been transferred.

A spark spectrum taken immediately showed *no zinc lines**. With diluted zinc salt solutions, however, it was possible by simultaneous exposures to point out distinctly amounts of zinc as small as 0.25 μg. (The plates used were designated "Silbercorin, Perutz", light sensitivy $\frac{12^\circ}{10}$ DIN").

0.50 μg zinc gave the following distinct lines: 2138.5, 3302.6 and 3345.0 Ångström. The first mentioned is the strongest one and was clearly observed even at 0.25 μg zinc. (4722.2 and 4810.5 are also zinc lines of relatively high energy but cannot be utilized in experiments with carbon electrodes, which always contain iron).

In 0.62 mg of uricase, the zinc content was accordingly less than 0.2 5μg *i.e.*, the content of zinc in this specimen was less than 0.4‰. The activity, however, was just as high as that of the most active preparations recorded by DAVIDSON and HOLMBERG.

It has been possible therefore—by dialysis against BAL—to prepare a uricase with a zinc content per unit of active enzyme, less than half of the value of the lowest one hitherto reported, (0.9‰, DAVIDSON[5]).

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References p. 601.

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SUMMARY

1. The activity of uricase is estimated from the velocity of the spectral change, which is caused by the enzymatic conversion of uric acid.

2. A method for quantitative determination of BAL is outlined on the basis of the spectral changes which occur when the compound is oxidized by air in an alkaline medium.

3. Inhibition of the action of uricase may occur in the presence of BAL, which, being oxidized itself, reduces the oxygen tension of the system.

4. The activity of uricase is unaffected by dialysis of the enzyme against BAL, and is also unaffected by incubation of the enzyme with this compound.

5. BAL combines instantaneously with zinc ions with the formation of a stable compound. The oxidation of BAL ceases suddenly when equivalent amounts of zinc are added.

6. By dialysis of uricase against BAL a sample of the enzyme has been prepared, the specific activity of which is as high as that of the best preparations hitherto reported, although the zinc content is less than 0.04 %. In a later experiment with larger amount of uricase the zinc content was less than 0.008 %.

7. The findings do not lend support to the assumption that uricase may be a zinc-proteid.

RÉSUMÉ

1. L'activité de l'uricase est mesurée par la vitesse à laquelle le spectre d'absorption de l'acide urique change, sous l'influence de l'enzyme.

2. Une méthode de détermination quantitative de BAL est décrite. Elle est basée sur les changements du spectre, produits par l'oxydation du produit par l'air, en milieu alcalin.

3. Une inhibition de l'action de l'uricase peut se produire en présence de BAL. BAL étant lui même oxydé, il réduit la tension d'oxygène du système.

4. L'activité de l'uricase n'est pas influencée par la dialyse contre BAL; elle n'est pas non plus influencée par incubation de l'enzyme en présence de BAL.

5. BAL se combine instantanément avec des ions de zinc en formant un composé stable. L'oxydation de BAL s'arrête immédiatement lorsque des quantités équivalentes de zinc sont ajoutées.

6. En dialysant de l'uricase contre du BAL, une préparation d'enzyme a été obtenue, dont l'activité spécifique atteint celle des meilleures préparations connues, bien que le taux de zinc soit inférieur à 0.04 %. Dans une expérience plus récente, conduite avec une plus grande quantité d'uricase, le taux de zinc était inférieur à 0.008 %.

7. Les résultats ne supportent pas l'hypothèse, que l'uricase serait une protéine de zinc.

ZUSAMMENFASSUNG

1. Die Uricaseaktivität wird bestimmt aus der Geschwindigkeit der spektralen Veränderung, die durch die enzymatische Umsetzung von Harnsäure hervorgerufen wird.

2. Eine Methode zur quantitativen Bestimmung von BAL wird angegeben, die auf den spektralen Veränderungen beruht, die bei der Oxydation dieser Verbindung durch Luft in alkalischem Milieu auftreten.

3. Eine Hemmung der Uricasewirkung kann bei Anwesenheit von BAL auftreten, das die Sauerstoffspannung des Systems verringert, indem es selbst oxydiert wird.

4. Die Uricaseaktivität wird durch Dialyse des Enzyms gegen BAL nicht beeinflusst, und ebensowenig durch Inkubation des Enzyms mit dieser Verbindung.

References p. 601.

5. BAL verbindet sich momentan mit Zinkionen unter Bildung einer stabilen Verbindung. Die Oxydation von BAL hört sogleich auf, wenn aequivalente Zinkmengen zugefügt werden.

6. Durch Dialyse von Uricase gegen BAL wurde ein Enzympräparat bereitet, dessen spezifische Aktivität ebenso hoch ist wie die der besten bisherigen Präparate, obwohl der Zinkgehalt geringer ist als 0.04%. In einem späteren Experiment mit grösserer Uricasemenge betrug der Zinkgehalt weniger als 0.008%.

7. Die Befunde geben keine Stütze für die Annahme, dass Uricase ein Zinkproteïd sei.

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