CXX. MICRODETERMINATION OF HYPOXANTHINE AND XANTHINE

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In the course of experiments on the formation of hypoxanthine in pigeon liver [see Edson *et al.* 1936] it was found desirable to have a micromethod for the determination of hypoxanthine. In this paper we describe a method based on the use of xanthine oxidase which has proved satisfactory in work on surviving tissues and on enzymes. Hypoxanthine and xanthine are quantitatively oxidized to uric acid, the former requiring 2 atoms of O, the latter 1 atom. If one purine base only is present and if the nature of this is known, its concentration can be calculated from the determination of the O₂ uptake. If both xanthine and hypoxanthine are present their concentrations can be calculated if the uric acid formation is measured in addition to the O₂ uptake.

Preparation of xanthine oxidase

The method requires a highly active preparation of xanthine oxidase. This is prepared in the following way [Dixon & Kodama, 1926]: fresh milk (0.5–1 l.) is clotted with a rennin preparation (Crosse and Blackwell's rennet tablets) and the clot is broken up with a glass rod in order to facilitate the separation of the whey from the curd; the whey is filtered off through muslin, one volume of milk yielding about 0.8 vol. of whey. In each 880 ml. of whey are dissolved 215 g. of solid ammonium sulphate. The solution is allowed to stand for about 30–60 min. until the globulins containing the enzyme have risen to the surface. The underlying almost clear solution is removed by suction and the surface layer amounting to less than one-fifth of the total volume is centrifuged for 10–15 min. The globulins now form a solid floating cake which is transferred to a filter paper and dried *in vacuo*. After extraction with ether to remove the fat, the ether-insoluble residue is dried again. The yield is 3–5 g. per l. of milk. The enzyme keeps *in vacuo* for several weeks. It is used in the form of a 10% solution in 0.1 *M* phosphate buffer of pH 7.4.

15 mg. of the enzyme (0.15 ml. 10% solution) should oxidize 0.5 mg. hypoxanthine in less than 1 hr. (40°, pH 7.4). We discarded less active preparations as the O₂ uptake in the presence of such preparations tended to be (up to 15%) in excess of the theoretical value. It appears that enzyme preparations of low activity are obtained from milk contaminated with inhibitors [see Philpot, 1938].

The activity of the enzyme is tested manometrically. The following solutions are required:

(1) Hypoxanthine stock solution, 12.5 mg. in 50 ml. 0.025 *M* phosphate buffer of pH 7.4.

(2) Aqueous pigeon liver extract: 1 g. pigeon liver is thoroughly ground with sand and 10 parts of water and centrifuged. The supernatant liquid is used. It keeps for several weeks if stored in the refrigerator and preserved with octyl alcohol.

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(3) 10% xanthine oxidase in 0.1 *M* phosphate buffer, pH 7.4.

(4) Phosphate buffer, 0.1 *M*, pH 7.4.

In addition to the thermobarometer two conical manometric flasks with a centre chamber and a side arm are required. The first flask, serving as "blank" contains 0.5 ml. phosphate buffer, 3.5 ml. water and 0.5 ml. liver extract in the main compartment, 0.2 ml. 2*N* sodium hydroxide in the centre chamber and 0.15 ml. xanthine oxidase solution in the side arm. The second flask contains 2 ml. hypoxanthine solution, 1.5 ml. water, 0.5 ml. phosphate buffer and 0.5 ml. liver extract in the main compartment. The centre chamber and side arm are filled as in the control. The bath temperature is 40°. The side arms are mixed when thermal equilibrium is reached and readings are taken at 10 min. intervals. Suitable enzyme preparations yield figures of the following order:

O ₂ uptake after 10 min.	20 μl.
"	20 min. 48 μl.
"	30 min. 81 μl.
"	40 min. 82.5 μl.
"	60 min. 82.5 μl.

(Calculated: 82.4 μl.)

The O₂ uptake should be complete in less than 60 min. and should agree with the theoretical value within $\pm 2\%$.

Determination of uric acid

We used two methods for the determination of uric acid formed in the presence of xanthine oxidase: the method of Edson & Krebs [1936] based on the conversion of uric acid into urea, and Kern & Stransky's [1937] modification of the colorimetric method of Folin. The latter method, though less specific, is more convenient and is therefore preferable when substances interfering with the colour formation are absent. This was the case in our experiments and we have therefore used the colorimetric method in most determinations.

The method of Edson & Krebs can be employed without modification. If the colorimetric method is used the concentration of uric acid in the sample must be of the same order as in blood filtrates for which the method was originally designed. It is therefore essential to dilute the solutions if the concentration of uric acid is relatively high. The determination of the O₂ uptake in the presence of xanthine oxidase indicates the order of magnitude of the uric acid present and we have used this guide in the following way.

If the O₂ uptake is below 25 μl., the solution is rinsed with water into a 50 ml. measuring flask and water is added to fill about three-quarters of the flask. Then are added 1/100th volume of acetone (0.5 ml.), 1/25th vol. of 10% Na₂WO₄ (2 ml.) and 1/25th vol. of 0.66*N* H₂SO₄. The flask is filled up to the mark and the contents filtered after 20 min. or more.

If the O₂ uptake is above 25 μl. the dilution is increased according to the following scale:

If the O ₂ uptake is 25–50 μl.	dilute to 100 ml.
"	50–100 μl. dilute to 200 ml.
"	100–200 μl. dilute to 400 ml., etc.

The relative proportions of acetone and deproteinizing reagents remain the same.

20 ml. filtrate are mixed with 5 ml. Na silicate solution (22 g. Na silicate cryst. Schering-Kahlbaum and 15 g. glycerol in 100 ml.) and 1 ml. Folin's phosphotungstic acid [see Kern & Stransky, 1937]. The colorimetric reading is taken after 5 min.

The standard uric acid solution is treated at the same time and in the same way as the unknown solutions: a known quantity of uric acid (about 0.03 mg.) is transferred into a 100 ml. flask and mixed with water and the reagents used in the xanthine oxidase experiments (xanthine oxidase, liver extract, buffer, acetone, tungstate, H_2SO_4) and filtered. 20 ml. of the filtrate are used.

Quantitative oxidation of purine bodies

Effect of liver extract. Hypoxanthine, xanthine and purine (see later) react with O_2 in the presence of xanthine oxidase as follows [Thurlow, 1925]:

The H_2O_2 formed inactivates and destroys the enzyme and unless it is removed reaction (1) does not go to completion. We found in accordance with Dixon [1925] and Dixon & Keilin [1936] that quantitative yields in respect to O_2 uptake and also to uric acid formation are obtained if pigeon liver extract, containing catalase, is added to xanthine oxidase. Mammalian liver is not suitable because it may contain uricase. Of the pigeon liver extract 0.5 ml. is added to 3–5 ml. solution; with smaller quantities we sometimes found incomplete uric acid yields though the O_2 uptake was correct.

Data on the O_2 uptake and uric acid formation in the presence of liver extract are given in Table I. The manometric arrangement was as described for the determination of xanthine oxidase activity.

Table I. *Oxidation of purine bodies in the presence of xanthine oxidase and liver extracts*

Substances added	O ₂ consumption and uric acid formation				Method used for uric acid determination
	Quantity	In equivalents of $\mu\text{l. O}_2^*$	O ₂ uptake ($\mu\text{l.}$)	Uric acid formation ($\mu\text{l.}$)	
	mg.				
Hypoxanthine	0.918	151.2	150	150.5	Edson & Krebs [1936]
"	0.612	100.8	101	99.5	"
"	0.306	50.4	49.3	52	"
"	0.153	25.2	25.7	25.8	"
"	1.028	169.0	174.5	172.5	Colorimetric
"	0.514	84.5	87.0	83.5	"
"	0.257	42.3	42.5	43.0	"
"	0.129	21.1	18.0	21.7	"
Xanthine	0.206	15.2	17.4	28.9	"
"	0.412	30.4	29.7	59.7	"
"	0.440	32.4	31.6	64.4	"
Purine	0.476	132.5	133.5	86.2	"

* Calculated on the assumption that hypoxanthine requires $10\frac{1}{2}$, xanthine $\frac{1}{2}\text{O}_2$ per mol.

Preparation of the solution for the determination of hypoxanthine and xanthine

The sample of purine base to be determined should be contained in a small volume of liquid (5–10 ml.) suitable for the manometric determination of the O_2 uptake. The reaction of the solution has to be adjusted with phosphate to

c. pH 7.4, but since the activity of xanthine oxidase does not vary much between pH 6.8 and 7.8, the adjustment need not be very accurate. The balanced salt solution of Krebs & Henseleit [1932] containing 0.025 *M* NaHCO₃ is adjusted to the correct pH by addition of 0.2 ml. 0.5 *M* KH₂PO₄ to 3 ml. saline.

If the purine bases are present in a volume too large for the manometric experiments, or if interfering substances (enzyme inhibitors) are present, it is advisable to precipitate the purines by means of the copper-bisulphite method and to redissolve them in a smaller volume. [See Krüger & Schmid, 1905; Thannhauser & Czoniczer, 1920; Kerr & Blish, 1932.]

Calculations

Let x_{O_2} = mol. O₂ absorbed in the presence of xanthine oxidase.
 $x_{\text{Uric acid}}$ = mol. uric acid formed in the presence of xanthine oxidase.
 $x_{\text{Hypoxanthine and xanthine}}$ = mol. hypoxanthine and xanthine present.

If hypoxanthine requires 1 mol. and xanthine 0.5 mol. O₂ for the conversion into uric acid (as is the case under suitable conditions):

$$x_{\text{Hypoxanthine}} = 2x_{O_2} - x_{\text{Uric acid}},$$

$$x_{\text{Xanthine}} = 2(x_{\text{Uric acid}} - x_{O_2}).$$

If there are other substances in addition to xanthine and hypoxanthine which absorb O₂ or produce uric acid in the presence of xanthine oxidase the formulae are not valid and the method is not applicable to the individual determination of hypoxanthine and xanthine. In this case, however, the method allows the calculation of the total purine bodies:

$$x_{\text{Total purine bodies}} = x_{\text{Uric acid}}.$$

Specificity of the method

Substances absorbing O₂ in the presence of xanthine oxidase. The specificity of xanthine oxidase, on which the method depends has recently been reviewed and examined by Booth [1938]. The enzyme absorbs O₂ in the presence of aldehydes and of a number of purine bodies. Among the purines Coombs [1927] found hypoxanthine, xanthine, 6:8-dihydroxypurine, 2-thioxanthine and adenine to react with the enzyme. Booth [1938] added 8-hydroxypurine and 2:8-dihydroxypurine to the list. Through the courtesy of Dr H. O. L. Fischer, Toronto, we were able to test another substance, purine, prepared by E. Fischer [1898]. We found that it reacts about as rapidly as hypoxanthine, yielding the theoretical amount of uric acid (Table II).

Table II. *Oxidation of purine in the presence of xanthine oxidase*

(Experimental conditions as described for testing the activity of xanthine oxidase; figures corrected for blanks.)

Substrate	0.476 mg. purine	0.514 mg. hypoxanthine	0.440 mg. xanthine
μl. O ₂ absorbed after 20 min.	29.2	31.2	18
" 40 min.	60.6	66.4	30
" 60 min.	89.2	86.5	31.6
" 120 min.	132.5	86.5	31.6
μl. O ₂ calculated for oxidation to uric acid	133.5	85	32.4
mg. uric acid formed	0.646	0.615	0.483
mg. uric acid calc.	0.666	0.636	0.486
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It follows that an O_2 uptake observed in the presence of xanthine oxidase does not finally prove the presence of xanthine or hypoxanthine. But of all the substances which may absorb O_2 in the presence of xanthine oxidase, only hypoxanthine, xanthine, adenine and a few aldehydes are known to occur in biological material. Uric acid determinations show whether aldehydes or purines are present: the presence of aldehydes is indicated if an absorption of O_2 but no formation of uric acid occurs. We have met with no such case in experiments on tissue extracts and body liquids, and we think it unlikely that aldehydes are found in animal tissues in quantities sufficient to interfere with the determination of purine bodies.

Adenine, if present, will be determined as hypoxanthine; its presence is suggested if the rate of oxidation is comparatively slow. Hypoxanthine reacts about 20 times faster than adenine [Booth, 1938].

Substances liberating hypoxanthine or xanthine. Nucleotides, nucleosides or guanine gradually liberate hypoxanthine or xanthine under the conditions of the determination. Among these potential precursors of purine bases muscle adenylic acid, free or as dinucleotide, is quantitatively the most important one in animal tissues. The method as described in this paper, does not distinguish between free and "bound" purine bodies (Table III). To differentiate between these, xanthine oxidase and catalase preparations free from nucleotidase, nucleosidase and guanase must be used. In the material, however, for which the method was designed, viz. pigeon liver, the concentration of bound purine is negligible as compared with the amount of free hypoxanthine produced and we have therefore made no attempts to prepare pure xanthine oxidase and catalase.

Table III. O_2 uptake in the presence of adenylic acid and of guanine

40 mg. "xanthine oxidase"; 0.5 ml. liver extract 1 : 10; 4 ml. $M/40$ phosphate buffer.

Substrate added:	4.25 mg. muscle adenylic acid	4.85 mg. yeast adenylic acid	2.1 mg. guanine	0.464 mg. hypoxanthine
$\mu l.$ O_2 absorbed after 20 min.	6	2	6	20
" 40 min.	16	5	11	72
" 140 min.	63	27	26	77
" 250 min.	91	42	38	77

Interfering substances

A number of substances interfere with the quantitative determination of the O_2 uptake because they undergo a "coupled oxidation" under the conditions of the determination. Such substances are nitrites [Thurlow, 1925] *p*-phenylenediamine [Harrison & Thurlow, 1926], methaemoglobin [Harrison & Thurlow, 1926; Dixon & Keilin, 1936], methyl, ethyl and propyl alcohols [Keilin & Hartree, 1936] and haemoglobin [Krebs, 1936; Bernheim *et al.* 1936]. Among these, haemoglobin and its derivatives are the only substances likely to be present in animal tissues. We find, however, that the traces of haemoglobin present in tissue extracts or in solutions in which slices have been kept do not affect the O_2 consumption if liver extract is present. Liver extract inhibits the coupled oxidation of haemoglobin and methaemoglobin (Table IV) although it promotes the coupled oxidation of alcohol [Keilin & Hartree, 1936]. If the concentration of haemoglobin or methaemoglobin is very high the inhibition of the coupled oxidation is not always complete. In this case, or in the presence

of alcohol, it is not possible to calculate the concentrations of hypoxanthine and xanthine from the O_2 uptake and only the concentration of the total purine bodies can be measured under these conditions.

Table IV. *Effect of liver extract on the "coupled oxidation" of haemoglobin*

Each flask contained 0.44 mg. hypoxanthine in 3 ml. 0.05 *M* phosphate buffer, pH 7.4; 15 mg. xanthine oxidase in 0.15 ml. The figures have been corrected for blanks. The haemoglobin solution was prepared by cytolysing washed pigeon red cells with 10 vol. of water. The solution was cleared by centrifuging. 1 ml. was equivalent to 108 μ l. O_2 .

Added to solution:		0.1 ml. haemo- globin solution	0.1 ml. haemo- globin solution; 0.5 ml. pigeon liver extract	0.5 ml. pigeon liver extract
μ l. O_2 absorbed after 10 min.	28	48	22	24
„ 20 min.	46	98.5	66	66
„ 30 min.	56	128	70	71
„ 40 min.	64	130.5	71	74
„ 50 min.	66	132	72	74
Calc. for 1 mol. O_2	72.6	72.6	72.6	72.6

SUMMARY

1. Conditions have been ascertained under which xanthine oxidase brings about the quantitative conversion of hypoxanthine and xanthine into uric acid.

2. A method based on the use of xanthine oxidase is described which permits the determination of hypoxanthine and xanthine in quantities from 0.1 mg. upwards. If both O_2 uptake and uric acid formation are measured it is possible to distinguish between hypoxanthine and xanthine, since the former requires 2 atoms, the latter 1 atom of oxygen.

3. In testing the specificity of the method it was found that purine reacts with xanthine oxidase as rapidly as hypoxanthine, but since purine has never been found in biological material it is improbable that it will interfere.

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