

THE USE OF PYROPHOSPHATE BUFFER FOR THE MANOMETRIC ASSAY OF XANTHINE OXIDASE

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Methods for the determination of xanthine oxidase (XO) activity include (a) study of the reduction of suitable hydrogen acceptors such as oxygen (1), methylene blue (2), and cytochrome *c* (3), and (b) study of the disappearance of substrate (4) or the appearance of the end-product, uric acid (5). The manometric procedure has, however, been the method of choice, but is limited in its application to the determination of liver XO activity owing to the high rate of oxygen consumption in the absence of substrate. In the method of Axelrod and Elvehjem (1) this endogenous respiration is largely suppressed by incubating the liver homogenate for 40 minutes prior to tipping in xanthine. However, in many experiments a large endogenous respiration is encountered which entails incubation for longer periods before linear oxidation of xanthine substrate is attained. Also, with protein-depleted animals, the method gives some anomalous results, a number of "zero" livers being found. This is attributed to the inhibitory effect of an excess of substrate on the small amount of enzyme already saturated with endogenous substrate. Removal of the endogenous substrate from the homogenate by dialysis gives positive XO activity in these cases.

It has been suggested that the respiration in the endogenous flask could be suppressed by addition of 6-pteridylaldehyde, which specifically inhibits XO, but in tissue homogenates with small XO activity this inhibitor interferes with other enzyme systems as well (6). Dixon and Elliott (7) have reported that pyrophosphate (PP) inhibits endogenous respiration and has no effect on the reduction of methylene blue by the XO system.

In the course of our studies on the elaboration *in vitro* of XO activity in liver slices (8), we had observed that PP buffer at pH 8.6 effectively inhibited endogenous respiration without any effect on xanthine oxidation, which was the same as at pH 7.4 when phosphate buffer was employed. The observations were extended and confirmed with a purified liver enzyme (9), with milk XO (10), and with rat livers of varying XO activity produced by dietary means. Activities were also compared by study of xanthine disappearance with both buffers.

EXPERIMENTAL

Methods—The liver was removed from a decapitated adult rat and chilled in cracked ice. A portion of the liver was weighed and immediately homogenized in a chilled glass homogenizer with 5 volumes of cold glass-distilled water. For the determination of xanthine oxidation 1 ml. of the homogenate containing 160 mg. of the tissue was added to the main compartment of a Warburg flask containing 1 ml. of either phosphate buffer (0.015 M), pH 7.4, or PP buffer (0.015 M), pH 8.6, unless otherwise specified. In the side arm was placed 1 ml. of sodium xanthate solution equivalent to 1 mg. of xanthine adjusted to the appropriate pH. The center well contained 0.2 ml. of a 10 per cent solution of KOH, making the total fluid volume 3.2 ml. Oxygen uptake was measured manometrically at 37° with air as the gas phase. The xanthine solution was tipped in either after an equilibration period of 10 minutes or after incubation of the flasks in the manometric bath for 40 minutes (1). Readings were taken every 20 minutes for 2 hours. In every experiment, correction for oxygen consumption in the absence of added xanthine was effected so as to obtain the true rate of xanthine oxidation. The values reported in this paper do not always represent XO activity, which is usually calculated only when the oxidation rate for added xanthine becomes linear (1).

Uricase activity was followed by the same procedure that was used for XO. The side arm contained lithium urate equivalent to 1 mg. of uric acid.

Residual xanthine was determined after precipitation of the proteins from the reaction mixture by addition of 1 ml. of a 10 per cent solution of sodium tungstate and 1 ml. of 0.66 N H₂SO₄ to every 3 ml. The resulting mixture was centrifuged and xanthine determined in the clear supernatant fluid by the method of Williams (11).

Results

Effect of PP Buffer on Endogenous Respiration—PP buffer markedly decreased the endogenous respiration of rat liver homogenate, which nearly reached completion within 20 to 30 minutes (Table I). With phosphate buffer this endogenous uptake was large and continued even up to 100 minutes. The rate during the first 20 minutes was the same in both cases.

When phosphate buffer of pH 8.6 instead of pH 7.4 was employed, the endogenous rate decreased slightly, but not as much as with PP; this suggests an effect by PP ions on endogenous respiration. PP in a final concentration of 0.005 to 0.0005 M had the same inhibitory effect. That this effect was due to PP and not merely to pH was also ascertained by studying the endogenous respiration at pH 8.6 with Sørensen borate buffer, Krebs-Ringer-bicarbonate buffer, and tris(hydroxymethyl)aminomethane buffer

(Sigma). PP at pH 7.4 also inhibited endogenous respiration, but was not suitable owing to its low buffering action.

Effect of PP on Xanthine Oxidation—The total oxygen consumption in the presence of substrate was the same with both phosphate and PP buffers (Table I). Tipping in xanthine after 40 minutes incubation of the homogenate also gave the same rate, though the endogenous respiration in the flask containing PP was nearly dissipated (Fig. 1). These observations show that PP buffer of pH 8.6 has no effect on xanthine oxidation. This was also ascertained with an XO preparation from liver precipitated according to Richert *et al.* (9) (Table I).

TABLE I

Effect of PP Buffer on Endogenous Respiration and Oxidation of Xanthine

The results are in microliters of O₂ per hour.

	Endogenous respiration		Total O ₂ uptake in presence of xanthine	
	Phosphate buffer, pH 7.4	PP buffer, pH 8.6*	Phosphate buffer, pH 7.4	PP buffer, pH 8.6
Liver homogenate, 1 ml. = 160 mg. tissue	70	46	72	72
	89	48	97	99
	82	43	116	116
	84	40	94	95
Precipitated enzyme† = 160 mg. tissue	0	0	51	51
	9	7	60	60

* Endogenous respiration dissipated after 20 to 30 minutes.

† Richert *et al.* (9).

Effect of PP on Xanthine Oxidation by Various Rat Organs—Besides liver, the spleen, kidney, lung, and intestine were studied with the two buffers and after an initial 10 minute equilibration (Table II). In the case of lung, spleen, and intestinal homogenates, endogenous uptake was comparatively small and did not therefore affect the oxidation of added xanthine, as with liver. PP decreased the endogenous uptake considerably with all tissues. With kidney homogenate, there was no oxidation of added xanthine in the absence of methylene blue. Addition of methylene blue resulted in a total oxygen uptake which was again essentially the same with the two buffers.

When the livers from rats which were protein-depleted by feeding a nitrogen-free ration (8) were employed for xanthine oxidation in the presence of phosphate buffer, they showed activities ranging from 0 to 260 μ l. of O₂ per gm. of dry liver in 2 hours. These "zero" livers when assayed by using PP buffer gave values for xanthine oxidation during 2 hours

ranging from 300 to 500 $\mu\text{l.}$ of O_2 . Typical results are included in Table II. The cause of "zero" livers is attributable to the inhibitory effect of the added substrate on the small amount of enzyme already saturated with endogenous substrate. Use of PP buffer removes the anomaly of "zero" livers.

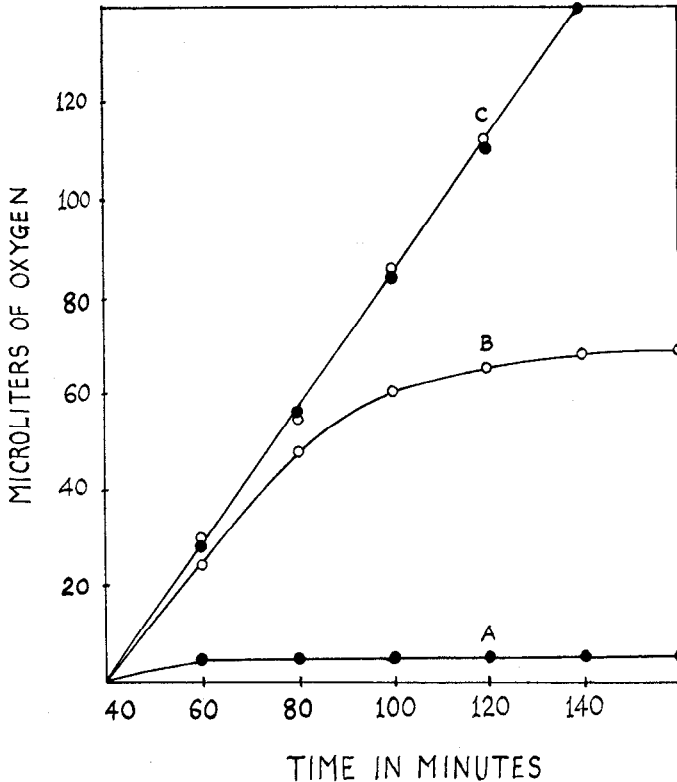


FIG. 1. Effect of incubation of liver homogenate for 40 minutes prior to tipping in xanthine on endogenous respiration and oxidation of xanthine. Curve A, endogenous with PP buffer of pH 8.6; Curve B, endogenous with phosphate buffer of pH 7.4; Curve C, oxygen uptake in the presence of xanthine with phosphate buffer, pH 7.4, or PP buffer, pH 8.6.

The inhibition of endogenous respiration by pteridine aldehyde, a specific inhibitor of XO (12), shows that much of the endogenous respiration is due to XO. This indicates that the values obtained by the method of Axelrod and Elvehjem (1) are minimal.

Effect of Tissue and Substrate Variations on Enzyme Activity—Oxidation of xanthine was followed with liver homogenate varying in amount from 50 to 300 mg. of tissue per flask, and it was observed that oxidation was

not a function of the quantity of enzyme used, provided the xanthine concentration was proportionally adjusted. Use of 100 to 300 mg. of tissue per flask caused no variation in oxidation with 1 mg. of xanthine as substrate. Substrate inhibition at high concentrations is known.

Study of Disappearance of Xanthine—From determinations of residual xanthine in the reaction mixtures with phosphate and PP buffers, it was

TABLE II

Endogenous Respiration and Xanthine Oxidation by Rat Organs

The results are in microliters of O₂ per gm. of dry tissue per 2 hours.

	Endogenous respiration		Xanthine oxidation*	
	Phosphate buffer, pH 7.4	PP buffer, pH 8.6	Phosphate buffer, pH 7.4	PP buffer, pH 8.6
Normal rat liver	2000	800	550	1800
	2360	950	700	2100
Protein-depleted rat liver	1200	600	0	460
	1050	520	0	500
	1600	780	260	800
Rat kidney†	1000	400	500	1115
	1040	415	460	1080
" intestine‡	200	160	1020	1030
	180	145	1230	1200
" lung	340	100	1680	1700
	280	85	1080	1100
" spleen	325	35	1300	1250
	440	48	1985	2062

* These figures represent the total oxygen uptake over the stated time interval and are not necessarily identical with figures which represent xanthine oxidase activity.

† In these determinations 0.2 ml. of 0.015 M methylene blue was added to each of the reaction vessels.

‡ Intestinal XO was determined for 30 minutes (13).

observed that disappearance of added xanthine was greater in the latter case. The rate of disappearance was, however, nearly the same at both pH values (Fig. 2). This meant that oxidizable purine substrates were being produced in the homogenates with phosphate buffer as fast as they were oxidized during the first 60 to 80 minutes. With PP buffer such endogenous substrates were produced to a lesser extent. There was fair agreement between the results for xanthine disappearance and oxygen uptake as measured manometrically.

The values obtained with the use of PP buffer of pH 8.6 are maximal and true, since (a) the endogenous respiration is minimal, and (b) inhibition

of the endogenous uptake is not due to the action of PP on XO activity, but to the formation of endogenous oxidizable substrates.

Effect of PP on Uricase Activity—A 20 to 25 per cent inhibition of the uricase activity of rat liver homogenate was observed with PP buffer. However, the oxidation of lithium urate was much faster than that of xanthine and in no way limited xanthine oxidation.

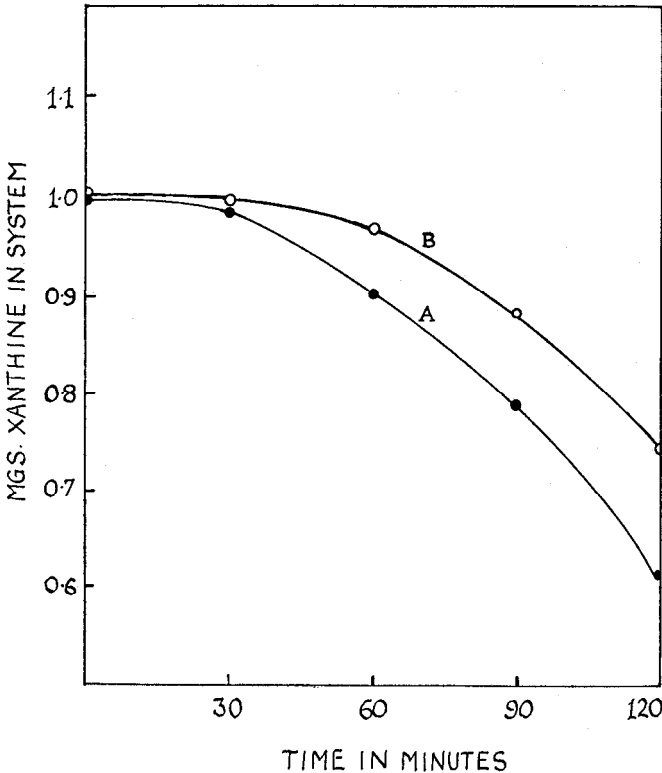


FIG. 2. Disappearance of added xanthine from liver homogenate. Curve A, liver homogenate with (0.015 M) PP buffer, pH 8.6; Curve B, liver homogenate with (0.015 M) phosphate buffer, pH 7.4.

DISCUSSION

Ordinarily, in calculating xanthine oxidase activity, the values for endogenous oxygen uptake are subtracted from the values for uptake in presence of added xanthine. The resulting differences are then plotted against time, and the activity is obtained from that portion of the curve which is nearest a straight line (1). It may be inferred from Fig. 1 that such a difference curve with PP buffer (Curve C minus Curve A) would

give a straight line after 60 minutes. With phosphate buffer, on the other hand, the difference curve (Curve C minus Curve B) would give a straight line only after 140 minutes. The xanthine oxidase activities thus obtained would be approximately the same with either buffer. Thus, the course of xanthine oxidation during 1 hour is a true picture of xanthine oxidase activity when PP buffer is employed, whereas with phosphate buffer the oxidation has to be continued for 2 hours or longer, when only the endogenous uptake levels off. With abnormal livers, as in protein-depleted animals, high endogenous uptakes may result in the loss of co-factors needed for xanthine oxidation and hence lead to "zero" livers.

SUMMARY

1. Pyrophosphate inhibits the endogenous respiration of rat liver homogenate. It has no effect on xanthine oxidase.
2. Oxidation of xanthine has been followed by a manometric method with pyrophosphate buffer of pH 8.6. Results in good agreement with the values for xanthine disappearance are obtained.

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