Studies on Milk-xanthineoxidase

By

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Introduction

HORBACZEWSKI,[1] SPITZER[2] and SCHITTENHELM[3] (1891-1905) recognized the ability of different tissues to oxidize hypoxanthine and xanthine enzymatically to uric acid in the presence of oxygen. The name of xanthine oxidase was given to this enzyme by BURIAN[4] (1905). In 1902 SCHARDINGER observed that if formaldehyde and methyleneblue were added to milk, the dye was rapidly decolorized, and MORGAN, STWART, and HOPKINS discovered that milk was rich in xanthine oxidase activity. DIXON, THURLOW,[5] and BOOTH[6] (1924-1938) recognized that Schardinger's enzyme was the same enzyme as xanthine oxidase.

It has been recognized that the xanthin oxidase oxidizes at least ten kinds of different purines and thirty one kinds of aldehydes. Oxygen, dyes, and nitrate can act as the hydrogen acceptor for the xanthine oxidase. HORECKER and HEPPEL[7] (1949) have demonstrated the reduction of cytochrome c by this enzyme system.

The xanthine oxidase activity was determined by such several methods as described below: AXELROD and ELVEHJEM[8] (1942) have described the manometric method for the oxidation of xanthine in the Warburg's respirometer at 37°, FIGGE and STRONG[9] (1941) have employed methylene blue as a hydrogen acceptor in the Thunberg method, which is a modification of the assay used by GREEN and DIXON[10] (1938). Van Meter and OLESON[11] (1950) have used 2-amino-4-hydroxy-6-pteridine carboxyaldehyde to inhibit the activity of the xanthine oxidase in the endogeneous respiraton. KALKAR[12] (1947) has employed the spectrophotometric determination of the enzymatic oxidation of hypoxanthine or xanthine to uric acid which results in a marked increase in the absorption at 290mμ. Recently, LITWACK[13] (1953-1954) has represented the xanthine oxidase activity by the colorimetric determination of the xanthine remaining after enzyme reaction.

For many years (1936-1952), BALL,[14] SZENT-GYÖRGYI,[15] DIXON, KEILIN,[16] KALKER,[17] KREBS, NORRIS,[18] LOWRY,[19] KNOX,[20] PHILLPOT,[21] BERNHEIM,[22] GAY, FELSHER,[23] WESTERFELD,[24] BEILER, MARTIN[25] and FEIGELSON et al. have recognized that the various substances such as cyanide, copper, oxidized-p-aminophenol, pyrogallol, 6-pteridinealdehyde, antabuse, L-ascorbic acid, and numerous flavonoids can act as the inhibitors of xanthine oxidase in vitro.

Recently, MILLER,[27] WILLIAM,[28] ELVEHJEM, WESTERFELD,[29] RICHERT,[30] LITWACK,[31] FEGELSON, WAINO[32] and BASS[33] (1948-1953) have studied the influence of dietary protein on the activity of rat liver xanthine oxidase and recognized the relationship between the availability of amino acid and the activity of liver xanthine oxidase.

Molybdenum is known to be required by some plants and microorganisms. The first demonstration of a rôle for molybdenum in animal nutrition, and the first association of molybdenum with on enzyme, have been reported by RICHERT, WESTERFELD, GREEN, BEINERT,[34] and FRIEDMAN[35] (1953). They have observed that purified milk xanthine oxidase contained 0.03 per cent of molybdenum. RICHERT and WESTERFELD have indicated that purified milk xanthine oxidase contained iron, riboflavin and molybdenum in a ratio of 8 : 2 : 1 respectively, and had a minimal molecular weight of approximately 32,000.

I have investigated the inhibitory action of

a number of inhibitors on the milk xanthine oxidase and the physico-chemical properties of this enzyme.

Experimental and Discussion

(1) Isolation of milk xanthine oxidase

The milk xanthine oxidase was isolated from the raw cream by the procedure of BALL (1939) described below. The fresh whole milk was centrifuged and the cream layer was suched off and recentrifuged. To 350gm of the cream (contained 40 per cent of fat) 350ml of 0.2M Na_2HPO_4 were added and the mixture was warmed to 38° and shaken gently at this temperature in a shaking machine for 2 hours. The mixture was then centrifuged, at 0° to solidify the fat and the aqueous portion was drawn off from under the fat layer. (The fat was discarded, as a second extraction is not worth while). The last traces of the fat were removed from the enzymecontaining extract by recentrifuging. The extract so obtained (600ml.) had a milky appearance. To 600ml. of the above extract, 700mg. of a commercial preparation of pancreatic lipase was added and the mixture incubated at 32° for 4 hours. The mixture was cooled to 20°. The precipitate of calcium phosphate produced by the addition of 0.5M $CaCl_2$ (640ml) to this solution, was centrifuged off and the yellowish clear solution of the enzyme was obtained. The above solution was saturated with ammonium sulfate up to 60 per cent and allowed to stand at 0° overnight. The precipitate (50gm in the wet weight) was centrifuged off and dissolved in a water (500ml). This solution was saturated with ammonium sulfate up to 30 per cent and the precipitate centrifuged off and discarded, and the supernatant brought to 50 per cent saturation with ammonium sulfate. The resulting precipitate was centrifuged (wet weight 10gm) and dissolved in water (100ml.). The enzyme solution so obtained was clear golden brown color, its $QO_2=230$. Such a preparation has been used for experiments described below.

(2) Determination of milk xanthine oxidase activity

The determination of the activity per unit of dry weight of the enzyme preparation during the process of the purification was manometrically carried out by determining the oxygen consumption with xanthine as the substrate at 37°. The manometric test was arranged as follows: the main chamber of a conical manometric flask contained 1.0ml of 0.1M phosphate buffer of pH 7.5, 0.1ml to 0.5ml of the enzyme solution, and enough water to make a total volume of 3.0 ml respectively. The center well contained 0.2ml of 5 per cent KOH, therefore, no CO_2 was developed during the oxidation. The side bulb contained 0.2ml of 0.05M solution of xanthine in 0.05M NaOH. After the incubation was carried out for 30 minutes, xanthine solution was mixed and the oxygen consumption was observed in each interval of 10 minutes. Dry weights of the preparation used were obtained either by the calculation from the results of the protein nitrogen determination or by the direct estimation on a dialysed preparation. The activity was expressed in terms of QO_2, i. e., cmm of O_2 absorbed per hour per mg of the dry weight. The amount of the oxygen-consumption was observed during the first 30 minutes of the reaction being employed.

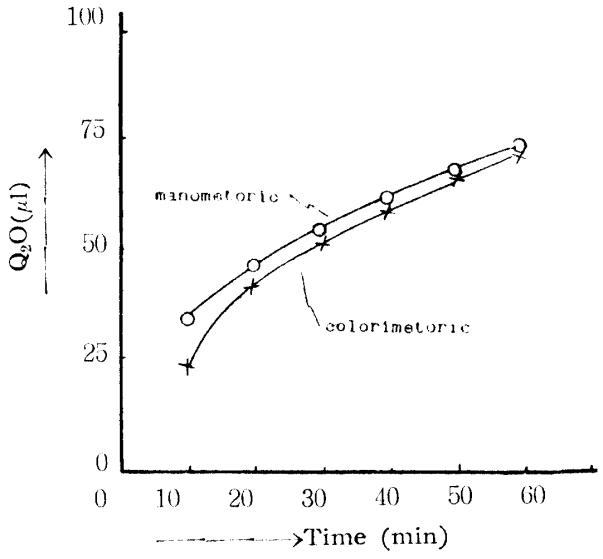
It has been recognized by above manometric oxygen consumptionmethod that the optimum pH, temperature and the Michaelis constant of isolated milk xanthine oxidase were 7.5, 37° and 0.6×10^{-5}M respectively and that this enzyme was stable against the various pH values (pH 2.5-pH 9.0) and the temperature (20°-45°).

The same flask which was used in the manometric determination placed in the bath and shaken for 30 minutes. At this time xanthine was mixed. 1ml. of the aliquots were removed immediately after mixing and the other 1 ml. of the aliquots were removed from the other manometric flask every 10 minutes. 1 ml. aliquots were pipetted into flasks containing 1 ml. of 40 per cent sodium tungstate, 9 ml. of water and 1 ml. of 2N sulfuric acid. The flasks were brought to the 12 ml volume, and the proteins centrifuged. After centrifugation, to 0.5 ml of supernatant, 2.5 ml of water was added, followed

by 1 ml of the diluted Folin-Ciocalteu reagent, and the color was developed by the addition of 5 ml of saturated sodium carbonate solution. A reagent-blank was used which consisted of the following : 1 ml of 10 per cent sodium tungstate, 5 ml of water, 1 ml of 2 N sulfuric acid, and 5 ml of water, 1 ml, of Folin-Ciocalteu reagent, and 5 ml of saturated sodium carbonate. The color intensity was then determined with a 660 mμ filter in the Hitachi colorimeter. The standard curve was set up over a range from 10γ to 60γ of xanthine. The enzyme activity was calculated from the quantity of xanthine which was consumed by 1 mg of enzyme for 1 hour, because according to the result that for the oxidation of 1 mol. xanthine 1 mol. of oxygen was needed.

A comparison of the xanthine oxidase activity determined by the colorimetric method with that obtained by the manometric method was shown in Fig. 1.

Fig-1



(3) Effect of inhibitors on milk xanthine oxidase

The xanthine oxidase activity was determined manometrically by the method described above. The main chamber of a conical manometric flask contained 1ml of 0.1M of the phosphate buffer of pH 7.5, 0.1 ml of the enzyme solution, x ml of a solution of the compound to be tested, and enough water to make a total volume fo 3.0 ml. The center wellcontained 0.2 ml of 5 per cent KOH and a small piece of rolled up filterpaper, while the side bulb contained 0.2 ml of a 0.05M xanthine in 0.05M NaOH. The flasks were attached to the manometers and allowed to come to temperature-equlibrium for 30 minutes at 37°. Then the contents of the side bulb were introduced tipped into the center compartment and readings were taken at 10 minutes intervals for 60 minutes. Control were also run without added inhibitors.

The results of the effects of the several inhibitors for milk xanthine oxidase were summarized in Table-1.

(a) Effects of inhibitors on SH-enzymes.

The effects of the inhibitors on the SH-enzymes were shown in the A group of Table-1. From these results, it has been observed that the milk xanthine oxidase was inhibited completely by $CuSO_4$, $HgCl_2$, $AgNO_3$ and parachloromercuribenzoate (PCMB) in the concentration of 10^{-3}M, and that by CH_2ICOOH and cystins in the same concentration the enzyme-activity was inhibited to 50 per cent. But $ZnSO_4$ could not act as an inhibitor for milk xanthine oxidase, because the reactivity for the SH-radical by Zn^{++} was lower than those of Cu^{++}, Hg^{++} Ag^{+}. The inactivated milk xanthine oxidase which was inhibited by $HgCl_2$, $CuSO_4$, $AgNO_3$, PCMB, CH_2ICOOH and cystine has recovered its activity by the addition of cysteine. From the results of these experiments, it has been concluded that milk xanthine oxidase is a thiol enzyme.

(b) Effects of inhibitors on heavymetal-enzymes.

The effects of inhibitors on heavymetal-enzymes were shown in the B group in Table-1. The milk xanthine oxidase was inhibited by KCN completely, but not by NaN_3, KCNS and KF. From these results, on the mode of the action of KCN for the xanthine oxidase, it may be considered that KCN did not react with Mo or Fe which are the constituents of this enzyme, but reacted with the protein-moiety of this enzyme.

(c) Effects of cysteine, $Na_2S_2O_3$, $Na_2S_2O_4$, L-

Table 1 The Effects of Several Inhibitors for Milk Xanthine Oxidase

	Inhibitor	Concentration of inhibitor	Activity (Qo_2)	Degree of inhibition (%)
A	Control	—	118.4	—
	$CuSO_4$	2×10^{-3}M	4.3	96.4
	$HgSO_4$	〃	8.6	92.7
	$ZnSO_4$	〃	83.9	29.1
	$CdSO_4$	1×10^{-3}M	102.0	13.9
	$MnSO_4$	〃	129.3	0.0
	$MgSO_4$	〃	98.4	16.9
	$AgNO_3$	2×10^{-3}M	12.9	89.1
	Sr $(No_3)_2$	1×10^{-3}M	11.6	0.0
	H_2O_2	1.5×10^{-3}M	98.4	16.9
	Cystine	1×10^{-3}M	51.0	56.9
	CH_2 ICOOH	5×10^{-3}M	73.2	38.2
	Cl-Hg-C_6H_6-COOH	1×10^{-3}M	6.3	94.7
	Indophenol	〃	72.9	38.4
	K_3Fe $(CN)_6$	〃	132.9	7.0
	$FeSO_4$ $(NH4)_2$ SO_4	〃	72.9	38.4
	H_3AsO_3	〃	54.6	53.0
	$Na_2B_4O_7$	〃	118.4	0.0
B	KCN	2×10^{-3}M	27.9	76.4
	KCNS	〃	118.4	0.0
	NaN_3	〃	116.6	0.0
	KF	〃	114.1	3.6
C	$Na_2S_2O_3$	1×10^{-3}M	136.6	0.0
	$Na_2S_2O_4$	〃	136.6	0.0
	Cysteine	〃	136.6	0.0
	Ascorbic acid	4.6×10^{-7}M	49.2	58.5
	F.M.N.	1×10^{-3}M	134.8	0.0
D	Phenol	2×10^{-3}M	114.8	0.0
	Hydroquinone	〃	49.5	58.2
	Catechol	〃	114.1	3.6
	Resorcine	〃	118.4	0.0
	Phloroglucine	〃	103.3	12.7
	Pyrogallol	〃	43.0	63.7
	Protocatechuic acid	1×10^{-3}M	134.8	0.0
	Gallic acid	〃	85.6	27.7

ascorbic acid and flavin-mononucleotide on milk xanthine oxidase.

The effects of cysteine, $Na_2S_2O_3$, $Na_2S_2O_4$, L-ascorbic acid and flavinmononuleotide on milk xanthine oxidase were shown in the C group in Table-1. It was recognized that cysteine, $Na_2S_2O_3$ and $Na_2S_2O_4$ could not act as the inhibitors, but L-ascorbic acid act a strong inhibitor for milk xanthine oxidase, and in the trace amount of it such as 4.6×10^{-7}M, it inhibited the xanthine oxidase activity up to 50 per cent. The inhibition of L-ascorbic acid has been discussed by FEIGELSON (1952) and he has attributed its inhibition to the combination of the enzyme and it.

(d) Effects of phenols on milk xanthine ox-

idase.

The effects of several phenols on milk xanthine oxidase were shown in the D group of Table-1. From these results, it was recognized that the milk xanthine oxidase was inhibited by hydroquinone, pyrogallol and gallic acid, but was not inhibited by resorcine, protocatechuic acid, catechole and phloroglucine.

(4) Recovery effect of milk xanthine oxidase activity by addition of cysteine

It has been investigated whether the inactivated milk xanthine oxidase which was inhibited by mercaptide forming agents, can be recoverable its activity in the presence of reversing agent such as cysteine.

From these results in Table-2, it has been recognized that when cysteine previously existed in the enzyme-solution, even if any inhibitor such as $HgCl_2$, $AgNO_3$, PCMB, CH_2ICOOH and cystine, were added to the solution, these inhibitors could not affect the activity of the enzyme. This may be attributed to the property that the affinity of cysteine to these inhibitors was stronger than that of the SH-radical in the enzyme to these inhibitors. From the fact that the substances forming mercaptide could cause instantaneously the inhibitory action for the enzyme activity, it may be considersd that the SH-radical in the milk xanthine oxidase was a sluggish type.

As shown in Table-3, if $HgCl_2$ and $CuSO_4$ were previously added before cysteine was introduced into the solution of the enzyme, the enzyme-activity was recovered to 50 per cent by the addition of cysteine. But when PCMB, CH_2IPOOH and cystine were previously added before cysteine was introduced into this enzyme-solution, the total activity of the enzyme was recovered by the addition of cysteine. On the other hand, if $AgNO_3$ already existed in the enzyme-solution, the effect of cysteine added was not observed.

From these results, it has been considered that the inhibition of the enzyme-activity caused

Table 2 Effects of Inhibitors in pre-existence of cysteine (cysteine conc. : 2×10^{-3}M)

Inhibitor	Inhibifor addedto enzyme+cysteine		
	Concentration	Activity	Inhibition %
Control	——	118.4	——
$HgCl_2$	2×10^{-4}M	120.2	0
$CuSO_4$	〃	81.9	31
$AgNO_3$	〃	118.4	0
Cl-Hg-C_6H_6-COOH	1×10^{-4}M	118.4	0
CH_2ICOOH	5×10^{-3}M	114.8	0
Cytine	2×10^{-3}M	115.7	0

Table 3 Recovery effect of cysteine for inhibitors (cysteine conc. 2×10^{-3}M)

Inhibitor		Cysteine added toenzyme+inhibitor	
	Concentration	Activity	Recovery %
Control	——	118.4	——
$HgCl_2$	2×10^{-4}M	45.5	38
$CuSO_4$	〃	81.9	69
$AgNO_3$	〃	3.6	3
Cl-Hg-C_6H_6-COOH	1×10^{-4}M	116.6	100
CH_2ICOOH	5×10^{-3}M	120.2	100
Cystine	2×10^{-3}M	117.0	100

by $HgCl_2$, $CuSO_4$, and $AgNO_3$ may be attributed to the formation of the irreversible inhibitor-enzyme complex.

Summary

It has been demonstrated that the manometric oxygen consumptionmethod and the colorimetric method were very suitable for the determination of the xanthine oxidase in milk, and that the optimum pH-value, the temperature and the Michaelis constant of this oxidase were 7.5, 35° and 0.6×10^{-5}M respectively, and that this oxidase was stable against the various pH-values and the temperatures.

It has been investigated that the various substances, such as (A) $CuSO_4$, $HgSO_4$, $ZnSO_4$, $CdSO_4$, $AgNO_3$, cystine, CH_2ICOOH, PCMB, indophenole and $K_3Fe(CN)_6$, (B) KCN, KSCN NaN_3 and KF, (C) cysteine, $Na_2S_2O_3$ $Na_2S_2O_4$, L-ascorbic acid and flavinmononucleotide, (D) phenol, hydroquinone, catechol, resorcine, phloroglucine, pyrogallol, protocatechuic acid, gallic acid, etc., could act as the inhibitors and the activators of the milk xanthine oxidase in vitro.

The activity of the milk xanthine oxidase, which was inactivated by the inhibitors such as $HgCl_2$, $CuSO_4$, $AgNO_3$, PCMB, CH_2ICOOH and cysteine, has recovered its activity by the addition of cysteine and the mechanism of the activation has been discussed.

It has been concluded that the milk xanthine oxidase was a thiolenzyme which contained the sluggish SH-radical.

Literature

1) J. Horbaczewski ; *Monatsh* **12** 221 (1891).
2) W. Spitzer ; *Arch, ges, Physiol.* **76** 192 (1899).
3) A. Schittenhelm ; *Z. physiol. Chem.* **42** 251 (1904) ; **45** 121 (1905).
4) R. Burian ; *Z. physiol. Chem.* **43** 497 (1905).
5) M. Dixon & S. Thurlow ; *Biochem. J.* **18** 976 (1924).
6) V. H. Booth ; *Biochem. J.* **29** 1732 (1935) ; **32** 494 (1938).
7) B. L. Horecker & C. A. Heppel ; *J. Biol. Chem.* **178** 683 (1949).
8) A. E. Axelrod & C. A. Elvehjem ; *J. Biol. Chem.* **140** 725 (1941).
9) F. H. Figge & L. C. Strong ; *Cancer Res.* **1** 779 (1941).
10) D. E. Dixon & M. Green ; *Biochem. J.* **28** 237 (1938).
11) J. C. Van Meter & J. J. Oleson ; *J. Biol. Chem.* **187** 91 (1950).
12) H. M. Kalkar ; *J. Biol. Chem.* **167** 429 (1947).
13) G. Litwack. et al ; *J. Biol. Chem.* **201** 261 (1953) ; *J. Nutrition* **47** 299 (1953) ; **49** 579 (1951).
14) E. G. Ball ; *J. Biol. Chem.* **128** 51 (1939).
15) A. Szent-Györgyi ; *Biochem. Z.* **173** 275 (1926).
16) M. Dixon & D. Kelin ; *Proc. Roy. Soc. London* **119** 159 (1936).
17) H. Kalkar & H. Klenow ; *J. Biol. Chem.* **172** 349 (1948).
18) E. G. Krebs & E. R. Norris ; *Federation Proc.* **8** 216 (1949).
19) O. G. Lowry et al ; *J. Biol. Chem.* **174** 771 (1948).
20) W. E. Knox ; *J. Biol. Chem.* **163** 699 (1946).
21) F. J. Phillpot ; *Biochem. J.* **32** 2013 (1938).
22) F. Bernheim & M. L. C. Bernheim ; *Am. J. Physiol.* **121** 55 (1938).
23) S. T. Gray & R. Z. Felsher ; *Proc. Soc. Exp. Biol. and Med.* **59** 287 (1945).
24) D. A. Richert, R. Vanderlinde & W. W. Westerfeld ; *J. Biol. Chem.* **186** 261 (1950).
25) J. M. Beiler & G. J. Martin ; *J. Biol. Chem.* **192** 831 (1951).
26) P. Feigelson ; *J. Biol. Chem.* **197** 843 (1955).
27) L. L. Mieler ; *J. Biol. Chem.* **172** 113 (1948).
28) J. N. William et al ; *J. Biol. Chem.* **181** 559 (1949) ; **183** 519 (1950) ; **200** 303 (1953) ; **201** 261 (1953) ; *Proc. Soc. Exp. Biol. Med.* **72** 386 (1949) ; *J. Nutrition* **47** 299 (1953) ; **49** 579 (1951).
29) W. W. Westerfeld et al. *Proc. Soc. Exp. Biol. Med.* **71** 181 (1949) ; *J. Biol. Chem.* **184** 163 (1950).

30) D. A. RICHERT & W. W. WESTERFELD et al; *J. Biol. Chem.* **192** 35, 49, 451 (1951); **203** 915 (1953).
31) G. LITWACK et al; *J. Biol. Chem.* **181** 605 (1950).
32) W. W. WAINO et al; *J. Nutrition* **49** 107 (1955).
33 A. D. BASO et al; *Proc. Soc. Exp. Biol. Med.* **73** 687 (1950).
34) D. D. E. GREEN & H. BEINERT; *Biochimica et Biophysica Acta* **11** 599 (1953).
35) H. C. FRIEDMANN; *Biochimica et Biophysica Acta* **11** 585 (1953).

要　　約

牛乳キサンチン酸化酵素の活力測定には検圧法，比色法何れも好都合であつた．検圧法によつて該酵素の至適温度，至適 pH，及びミカエリス恒数を測定して，それぞれ35°C, pH7.5, 0.6×10^{-5}M の値を得た．同時にこの酵素は各種の pH，温度に対して可成り安定であることを認めた．更に (A). $CuSO_4$, $HgSo_4$, $CdSO_4$, $ZnSO_4$, $AgNO_3$, cystine, CH_2ICOOH, PCMB, indophenol, $K_3Fe(CN)_6$; (B) KCN, KSCN, NaN_3, KF,; (C) cysteine, $Na_2S_2O_3$, $Na_2S_2O_4$, ascorbic acid, FMN; (D) phenol, hydroquinone, catechole, resorcinol, phloroglucinol, pyrogallol, protocatechuic acid, gallic acid etc の各種の物質がそれぞれ invitro で牛乳キサンチン酸化酵素の阻害剤及至賦活剤となることを認め，その阻害度，賦活度を表示した．$CuSO_4$, $AgNO_3$, PCMB, CH_2ICOOH, cystine などによつて阻害された酵素は cysteine によつて恢復されるのであつて，この activation の機作について考察した．以上の各種実験から牛乳キサンチン酸化酵素は sluggish-SH 基をもつ thiol enzyme と推定出来た．