XANTHINE OXIDASE OF THE CLOTHES MOTH, *TINEOLA BISSELLIELLA*, AND SOME OTHER INSECTS

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Summary

Xanthine oxidase activity in *Tineola* larvae averages 200 μmoles of uric acid per g whole larva (wet weight) per hr and in *Tenebrio, Lucilia, Anthrenocerus, Ephestia,* and *Anthrenus* larvae activity ranges between 13·4 and 1·3. The optimum pH for *Tineola* xanthine oxidase lies between pH 7·7 and 8·0, and the optimum concentration of xanthine is at or below $1{\cdot}3 \times 10^{-3}$M. Methylene blue in concentrations up to $5{\cdot}3 \times 10^{-3}$M has no toxic effect on this enzyme, and the lower concentrations of methylene blue have a limiting effect. Cyanide and 6-pteridyl aldehyde inhibit *Tineola* xanthine oxidase. The insect xanthine oxidases are demonstrated to be dehydrogenases. DPN, and pyruvate and DPN together, stimulate uric acid production by *Tineola* xanthine oxidase in the absence of methylene blue. In *Tenebrio* larvae there is a higher concentration of xanthine oxidase in the midgut and fat-body than in the remaining tissues.

I. INTRODUCTION

A very wide distribution of xanthine oxidase in the animal kingdom was demonstrated by Morgan (1926) and Florkin and Duchateau (1941). Larvae of *Tenebrio molitor* L. were shown to have outstandingly high activity, but no investigations on the properties of insect xanthine oxidase have been carried out.

Day (1951) reported the presence of xanthine oxidase in the gut of *Tineola bisselliella* (Humm.) larvae and suggested that the xanthine-uric acid system might be responsible for the maintenance of the very low oxidation-reduction potential found in the gut of this wool-digesting insect. It was apparent that more information was required on this enzyme, its properties, and distribution in insect tissues, before its role in the maintenance of the low oxidation-reduction potential could be evaluated.

Since the Thunberg methylene blue technique, as employed by Florkin and Duchateau (1941), and methods using tetrazolium salts (Anderson and Patton 1954) are liable to be affected by many different dehydrogenases in a crude enzyme solution, and since the hydrogen acceptor in the above mentioned methods is restricted to these dyes, a more specific method of measuring xanthine oxidase activity was adopted. This was based on the estimation of uric acid produced in the reaction mixture and by this method it has been shown that insect xanthine oxidases are true dehydrogenases and not oxidases.

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II. Materials and Methods

(*a*) *Reagents*

The following reagents were used:

$6{\cdot}6 \times 10^{-3}$M xanthine solution (monosodium xanthine, Schwarz Laboratories),

0·1M tris(hydroxymethyl)aminomethane, buffer pH 7·9 ("Tris," Sigma Chemical Co.),

0·027M methylene blue solution (B.D.H.),

0·005M sodium cyanide (B.D.H.),

2-amino-4-hydroxy-6-formylpteridine,*

0·025M sodium pyruvate† (98 per cent. purity),

Diphosphopyridine nucleotide (DPN) (Sigma Chemical Co.).

(*b*) *Insect Tissue Homogenates*

Preparations were obtained from larvae of *Tineola bisselliella, Tenebrio molitor, Lucilia cuprina* (Wied.), *Anthrenocerus australis* (Hope), *Ephestia kühniella* Zell., and *Anthrenus flavipes* Le Conte by homogenizing tissues or whole insects in water or in saline solution in glass-"Teflon" tissue grinders at 0°C. With *Tenebrio* homogenates, very light centrifugation was applied to remove the larger fragments of hard cuticle. For the study of the distribution of xanthine oxidase, the midgut of *Tineola* and midgut and fat-body of *Tenebrio* were homogenized separately. To retain optimal conditions for uric acid estimation, a concentration of 25 mg tissue per ml of *Tineola* homogenate, 80 mg per ml of *Tenebrio*, and 100 mg per ml of other insects was required.

Dialyses were carried out against saline at 0°C with continuous mechanical stirring, renewing the saline every 20 min. After 2 hr 20 min, most of the endogenous uric acid had been removed, and after 3 hr the concentration of uric acid had fallen below the level at which it could be detected by the method employed for estimation. No loss of xanthine oxidase activity was caused by dialysis for 3 hr.

(*c*) *Estimation of Xanthine Oxidase Activity*

Activity of xanthine oxidase was measured by estimation of uric acid production from xanthine at 37°C. The reaction mixture contained 1 ml of insect homogenate, 1 ml of $6{\cdot}6 \times 10^{-3}$M xanthine solution, 1 ml of 0·1M "Tris" buffer solution pH 7·9, 1 ml of 0·027M methylene blue solution, and 1 ml of water. When only molecular oxygen was provided as the hydrogen acceptor, water was substituted for the methylene blue solution. All solutions, with the exception of the homogenate, were pre-warmed to 37°C. After the addition of the homogenate, the tube was shaken thoroughly. Two 1 ml aliquots were immediately removed, and the tube was placed in a shaker apparatus in a water-bath at 37°C. The incubation period was governed by xanthine oxidase activity and varied from 30 min for *Tineola* to 2 hr for other insects. After the required incubation time, two 1 ml aliquots were again removed and pipetted

* Kindly supplied by Dr. D. B. Morell and † Dr. C. C. Kratzing.

directly into wide test tubes containing 1 ml of 10 per cent. sodium tungstate, 7 ml of water, and 1 ml of 2/3 N sulphuric acid, under which conditions protein was precipitated (Folin and Wu 1919). This treatment also caused precipitation of methylene blue. However, care was necessary to ensure that the filtrate

TABLE 1

XANTHINE OXIDASE ACTIVITY OF INSECT TISSUE HOMOGENATES AND URIC ACID CONTENT IN EXCRETA

Insect	Without Methylene Blue	With Methylene Blue		Uric Acid Content in Excreta (%)
	Aerobic		Anaerobic	
	μmoles Uric Acid per g Larva per hr			
T. bisselliella	6·5	200	200	31·7
E. kühniella	0·4	1·8	2·5	—*
T. molitor	0·5	13·4	13·4	—*
L. cuprina	0	2·2	6·5	—*
A. australis	0	2·9	4·0	8·7
A. flavipes	0	1·0	1·3	1·1

* No analyses carried out.

was completely clear and colourless since the methylene blue precipitate was very fine and mobile and had a tendency to creep around the filter paper when only small amounts of protein were present in solution. Uric acid was estimated by Newton's (1937) method, in 1 ml aliquots of the filtrate, using a Coleman

TABLE 2

EFFECT OF STORAGE ON *TINEOLA* XANTHINE OXIDASE ACTIVITY

Treatment	Loss of Activity (%)
Homogenate kept at 18°C for 24 hr	91·5
Homogenate kept at 0°C for 24 hr	13·0
Homogenate kept at 0°C for 120 hr	58·0
Homogenate kept at −22°C for 120 hr	0

Junior spectrophotometer for optical density readings at a wavelength of 680 mμ. Xanthine oxidase activity was expressed in μmoles of uric acid produced per g of wet insect tissue per hr.

For activity estimation under anaerobic conditions, Thunberg tubes were used. After aliquots at zero time were taken, the tubes were quickly evacuated, and twice flushed with oxygen-free nitrogen. After a further evacuation they were placed in a water-bath for incubation. The uric acid estimation was then carried out in the same way as for aerobic conditions.

Controls in which the xanthine solution was replaced by water were treated the same way as test estimations.

III. Results

The activity of xanthine oxidase in *Tineola* larvae varied in different homogenates, but in all instances was very high, averaging 200 μmoles uric acid per g whole larvae (wet weight) per hr. Activity in other insects (Table 1) was of a much lower order and it ranged between 1·0 μmoles for *Anthrenus* and 13·4 μmoles for *Tenebrio*. In controls (omitting xanthine) for *Tineola* and *Lucilia* preparations no increase of uric acid was measurable, but for other insects uric acid produced in μmoles per g per hr ranged between 0·4 for *Tenebrio* and 2·5 for *Anthrenocerus*. The uric acid content in excreta of *Tineola*, *Anthrenocerus*, and *Anthrenus* (Table 1) is directly related to the xanthine oxidase activity of these insects.

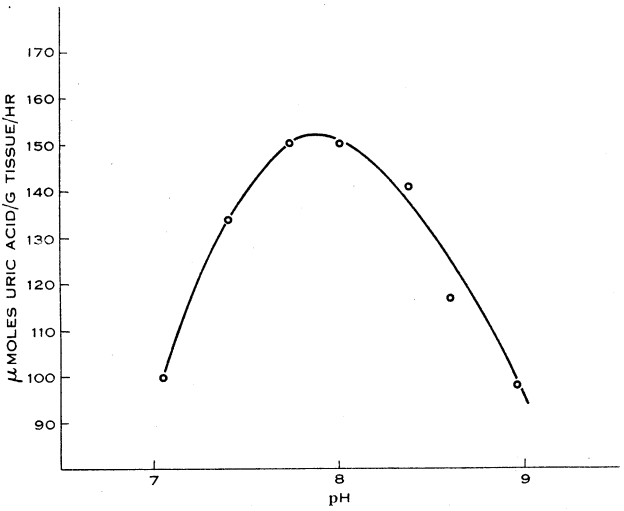

Fig. 1.—Effect of pH on xanthine oxidase activity of *Tineola* homogenate. Different pH values were established by addition of 1 ml of 0·1M "Tris" buffer of 7-9 pH range to the reaction mixture of final volume 5 ml. Incubated for 30 min at 37°C and uric acid measured.

Tineola homogenate lost its activity quite rapidly when kept at room temperature (about 18°C) (Table 2) but when stored in a refrigerator (about 0°C) for 5 days it retained 42 per cent. of the original activity. Homogenates which were deep-frozen (about − 22°C) showed no decrease of activity after

5 days. The pH optimum for xanthine oxidase activity in *Tineola* preparations lies in a narrow range between pH 7·7 and 8·0 (Fig. 1).

High concentrations of xanthine have an inhibitory effect on *Tineola* xanthine oxidase and 27 per cent. decrease of xanthine oxidase activity was caused by the increase of xanthine concentration from $0{\cdot}66 \times 10^{-3}$M to $5{\cdot}28 \times 10^{-3}$M (Fig. 2, homogenate *A*) and 45 per cent. decrease was caused by increase of xanthine concentration from $1{\cdot}3 \times 10^{-3}$M to $15{\cdot}8 \times 10^{-3}$M (Fig. 2, homogenate *B*). The highest activity was obtained with a xanthine concentration of $1{\cdot}3 \times 10^{-3}$M and this concentration was chosen for further work.

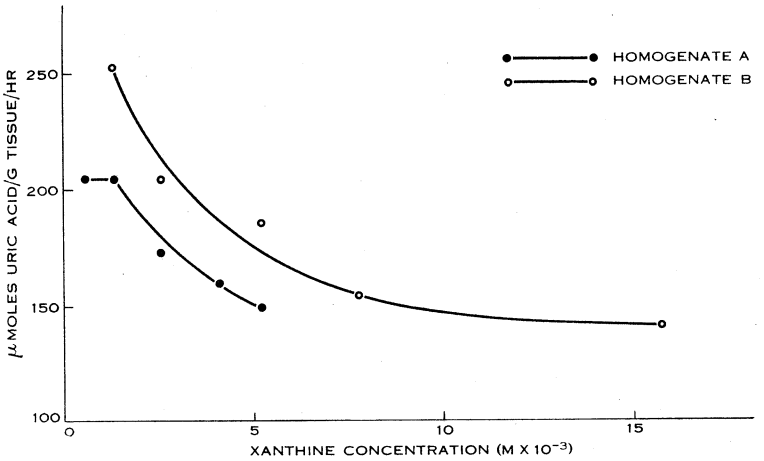

Fig. 2.—Effect of substrate concentration on xanthine oxidase activity of *Tineola* homogenates. Varying amounts of $13{\cdot}2 \times 10^{-3}$M or $39{\cdot}6 \times 10^{-3}$M xanthine solution and water were added to the reaction mixture to ensure final concentrations of xanthine of $0{\cdot}66 \times 10^{-3}$M to $15{\cdot}84 \times 10^{-3}$M and retain a final volume of 5 ml. Incubated for 30 min at 37°C and uric acid measured.

A very pronounced effect of methylene blue concentration on *Tineola* xanthine oxidase is demonstrated in Figure 3. The highest activity was measured when the methylene blue concentration was equal to or higher than $13{\cdot}5 \times 10^{-4}$M and activity dropped 48 per cent. when the methylene blue concentration was decreased to $8{\cdot}4 \times 10^{-5}$M. A final concentration of $5{\cdot}4 \times 10^{-3}$M of methylene blue was used throughout this work. *Tineola* xanthine oxidase activity at this concentration of methylene blue lay on the plateau of the curve (Fig. 3) so that the dye produced no toxic effect. With this concentration of methylene blue, activities of the same order were obtained when estimations were carried out under either aerobic or anaerobic conditions.

Xanthine oxidase of *Tineola* homogenates was completely inhibited when these were pre-treated with sodium cyanide (Table 3). Addition of 2-amino-4-hydroxy-6-formylpteridine in a final concentration of 2 and 0·2 μg per ml to

the normal reaction mixture caused an inhibition of activity of 69 and 23 per cent. respectively.

TABLE 3

EFFECT OF CYANIDE AND 2-AMINO-4-HYDROXY-6-FORMYLPTERIDINE ON *TINEOLA* XANTHINE OXIDASE ACTIVITY

Compound Added to Reaction Mixture	Final Concentration of Compound Added	Inhibition (%)
Sodium cyanide	0·01 M*	100
Sodium cyanide	0·001 M*	100
2-amino-4-hydroxy-6-formylpteridine	2 μg/ml	69
2-amino-4-hydroxy-6-formylpteridine	0·2 μg/ml	23
2-amino-4-hydroxy-6-formylpteridine	0·02 μg/ml	0

* Homogenate was pre-treated with sodium cyanide for 30 min at 37°C. In test without cyanide, homogenate was pre-treated the same way with water and the activity was 148 μmoles uric acid per g wet tissue per hr.

Sodium nitrate at 0·02M final concentration did not function as a hydrogen acceptor in the place of methylene blue under aerobic or anaerobic conditions. Similarly, a suspension of cystine in the presence or absence of DPN had no stimulating effect on uric acid production by *Tineola* homogenates. Replacing methylene blue solution with the same amount of 0·025M sodium pyruvate solution (final concentration 0·005M) did not cause any increase in activity of the *Tineola* homogenate. However, the addition of DPN at a final concentration of 8 μg per ml produced some increase, and a still greater increase was stimulated by the addition of pyruvate together with DPN. A typical example of this effect is presented in Table 4. In controls with DPN plus pyruvate but without xanthine no increase in uric acid was found.

TABLE 4

EFFECT OF SODIUM PYRUVATE AND DPN ON URIC ACID PRODUCTION BY *TINEOLA* HOMOGENATE

Compound Added to Reaction Mixture	Final Concentration of Added Compound	Per Cent. of Activity*	
		Non Dialysed Homogenate	Dialysed Homogenate
Water		5·3	0
Sodium pyruvate	0·005M	5·3	0
DPN	8 μg/ml	11·4	2·7
Sodium pyruvate +DPN	0·005M 8 μg/ml	22·3	17·1

* As per cent. of activity of the same homogenate in presence of methylene blue at a final concentration of $5{\cdot}4 \times 10^{-3}$M.

In the study of xanthine oxidase distribution in *Tineola* and *Tenebrio* larvae, homogenates of different tissues were prepared and the activity estimated. All calculations for distribution were made on the assumption that the midgut of *Tineola* constitutes about 10 per cent. of the wet weight of the intact larva; for *Tenebrio* the midgut forms 2·5 per cent. and the separated fat-body 10 per cent. of a whole larva. About 10·5 per cent. of total xanthine oxidase activity in *Tineola* larvae was recovered from the midgut and 84·5 per cent. was recovered from the rest of the body (Table 5). The loss of about 5 per cent. of activity is attributed to loss of tissue and some inactivation during the process of dissection. In *Tenebrio* larvae 8·6 per cent. of xanthine oxidase activity was found in the midgut, 33·6 per cent. in the fat-body, and 57·8 per cent. in the rest of the body.

TABLE 5

DISTRIBUTION OF XANTHINE OXIDASE IN TISSUES OF *TINEOLA* AND *TENEBRIO* LARVAE

Insect	Insect Tissues	Per cent. of Total Body Weight	Specific Activity (μmoles uric acid per g tissue per hr)	Per cent. of Total Activity in Larva
T. bisselliella	Midgut	10	198	10·5
	The remainder of the body	90	176	84·5
	Whole larva	100	188	100
T. molitor	Midgut	2·5	49·4	8·6
	Fat-body	10	48·2	33·6
	The remainder of the body	87·5	9·5	57·8

IV. DISCUSSION

The activity of xanthine oxidase in *Tineola* larvae is several times higher than that in rat and chicken liver. Williams and Elvehjem (1949) reported 190 μl 0_2 per g rat liver (wet weight) per hr, which corresponds to 8·5 μmoles of uric acid per g per hr, and van Pilsum (1953) 7·7 μmoles uric acid per g per hr. Morell (personal communication) has found for chicken liver that up to 50 μmoles uric acid per g per hr is produced.

The pH optimum for *Tineola* xanthine oxidase lies in a narrow range between pH 7·7-8·0 (Fig. 1), whereas that for crude milk xanthine oxidase was found to lie in a wide range pH 6 to 9 (Dixon and Thurlow 1924) and rat liver xanthine oxidase pH 8 to 9 (van Pilsum 1953).

High concentrations of xanthine have an inhibitory effect on *Tineola* xanthine oxidase and it appears that optimum xanthine concentration is not greater than $1{\cdot}3 \times 10^{-3}$M (Fig. 2). For milk xanthine oxidase the optimal xanthine concentration lies below $0{\cdot}9 \times 10^{-3}$M (Dixon and Thurlow 1924). Rat liver homogenate tolerated a much higher xanthine concentration, up to

4×10^{-2}M, without any inhibitory effect on its activity (van Pilsum 1953). According to this author, however, a purified preparation of rat liver xanthine oxidase was quite rapidly inhibited when the concentration of xanthine was increased from 4×10^{-3}M to $3{\cdot}6 \times 10^{-2}$M (about 90 per cent. inhibition). Xanthine oxidase of *Tineola* homogenates appears to be similar to milk and purified rat liver xanthine oxidase in its reaction to increased concentrations of xanthine, although the inhibitory effect was not as marked with the crude insect enzyme.

Methylene blue in higher concentrations (up to $5{\cdot}4 \times 10^{-3}$M) did not have any toxic effect on *Tineola* xanthine oxidase and the relation of methylene blue concentration to xanthine oxidase activity resembles the relation of a substrate concentration to enzyme activity (Fig. 3).

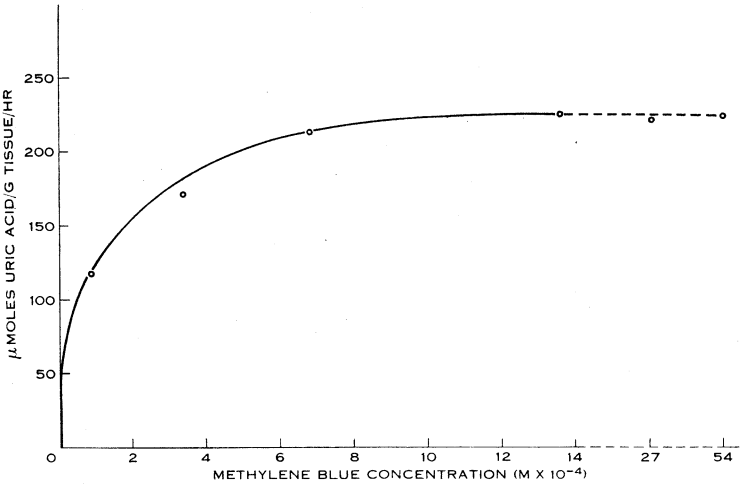

Fig. 3.—Effect of methylene blue concentration on xanthine oxidase activity of *Tineola* homogenate. Methylene blue solutions of varied concentrations were added to the reaction mixture to obtain final concentrations of methylene blue of $0{\cdot}8 \times 10^{-4}$M to $5{\cdot}4 \times 10^{-3}$M. Incubated for 30 min at 37°C and uric acid measured.

The inhibition of *Tineola* xanthine oxidase by pre-treatment with cyanide (Table 3) is of the same character as the corresponding inhibition of milk xanthine oxidase (Dixon and Keilin 1936). Kalckar, Kjeldgaard, and Klenow (1948) have demonstrated a very potent inhibitory effect of 2-amino-4-hydroxy-6-pteridyl aldehyde on xanthine oxidase. This inhibitor had a similar, but less pronounced, effect on *Tineola* xanthine oxidase.

When methylene blue was omitted from the incubation mixtures, the activity of xanthine oxidase in insect homogenates dropped to a very small fraction of the previous value or disappeared completely (Table 1). Furthermore, after 3 hr dialysis of *Tineola* homogenate, no activity could be found in the absence of methylene blue (Table 4). This suggests that insect xanthine oxidases are dehydrogenases. Richert and Westerfeld (1951) demonstrated that pigeon kidney and chicken and turkey liver homogenates oxidize xanthine to uric acid

at a very low rate when molecular oxygen only was offered as the hydrogen acceptor, and that the addition of methylene blue increased several fold the rate of this oxidation. Morell (1955) has shown that methylene blue increased uric acid production by chicken liver xanthine oxidase about 12- to 16-fold. The same author has also demonstrated stimulation of chicken liver xanthine oxidase activity by DPN, pyruvate, D-ketoglutarate, and fumarate. DPN alone and pyruvate plus DPN increased the production of uric acid by *Tineola* homogenates (Table 4), whereas pyruvate without DPN had no effect at all. This suggests that the level of DPN concentration in *Tineola* larvae is very low or that destruction of DPN occurred in the preparation of the homogenates. The stimulating effect of DPN and pyruvate on *Tineola* xanthine oxidase is interesting in view of the suggestion (Morell 1955) that xanthine oxidase in chicken liver is linked to lactic dehydrogenase through DPN. On the other hand, if DPN is directly reduced by xanthine oxidase in the presence of xanthine and if the concentration of DPN of 8 μg per ml ($1{\cdot}2 \times 10^{-5}$M) is sub-optimal for this reaction, the addition of pyruvate in the presence of lactic dehydrogenase would increase DPN and thus increase uric acid production.

Only a small fraction of the xanthine oxidase activity is located in the gut of *Tineola* (10·5 per cent.) and *Tenebrio* larvae (8·6 per cent.), although the gut is as active as any other tissue examined. In *Tenebrio* larvae 33·6 per cent. of xanthine oxidase was found in the fat-body, but undoubtedly this figure is too low, as some of the fat-body could not be separated from the rest of the body. Specific activities of midgut and fat-body tissue of *Tenebrio* are of much higher order than the specific activity of the remaining tissues (Table 5) and this is in agreement with the findings of Leifert (1935) and Anderson and Patton (1954).

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