BBA 65565

MONOAMINE OXIDASE IN *TRIBOLIUM CONFUSUM* DUVAL (COLEOPTERA)

K. D. CHAUDHARY, U. SRIVASTAVA* AND A. LEMONDE**
Department of Biochemistry, Faculty of Medicine, Laval University, Quebec (Canada)

(Received October 25th, 1966)

SUMMARY

1. Certain biochemical characteristics of monoamine oxidase (monoamine:O_2 oxidoreductase (deaminating), EC 1.4.3.4) in *Tribolium confusum* homogenate have been determined by using a radioisotope method.

2. Like the mammalian enzyme, the insect monoamine oxidase shows maximum activity at pH 7.4. Besides, this enzyme possesses a very high affinity for tryptamine used as substrate ($K_m = 8.7 \cdot 10^{-9}$ M) and has an activation energy (ΔE) of the order of 10 440 cal/mole within the limits of optimum temperature (37°).

3. At a final concentration of 0.5 M, EDTA causes about 70% loss in the activity. Addition of Zn^{2+} and Co^{2+} (final concentration 10^{-3} M of each) in the presence of EDTA not only recovers the lost activity but also activates the enzyme considerably.

4. Both parnate sulfate and harmine inhibit monoamine oxidase reversibly, their respective inhibition constants (K_i) being $0.26 \cdot 10^{-3}$ M and $1.4 \cdot 10^{-3}$ M.

5. Monoamine oxidase activity is lodged in the particulate fraction of which the mitochondria, in terms of specific activity, contains about 5.7 times more activity than nuclei. The enzyme is tightly bound to mitochondria.

6. During the life cycle of the insect, monoamine oxidase titer follows a more or less inverse trend to that of acetylcholinesterase (acetylcholine acetyl-hydrolase, EC 3.1.1.7).

INTRODUCTION

Recent work has stimulated renewed interest not only in the physiological role of monoamine oxidase (monoamine:O_2 oxidoreductase (deaminating), EC 1.4.3.4), but also in the fundamental nature of the enzyme[1]. The enzyme activity is not restricted to vertebrate tissues alone but is also known to occur in some invertebrate phylla: molluscs, annelids and echinoderms[2]. Of particular interest is the finding of monoamine oxidase in molluscs where serotonin has been reported to act as a neuro-

* Holder of a postdoctoral fellowship from the Muscular Dystrophy Association of Canada.
** Holder of a Medical Research Associateship. Medical Research Council, Ottawa, Canada.

transmitter substance. In insects, even though the presence of several biogenic amines which constitute the natural substrates of monoamine oxidase has been demonstrated[3–5], no attempt has so far been made to study this enzyme. The only evidence suggesting the occurrence of this enzyme in *Periplaneta americana* L. is from the work of BLASCHKO, COLHOUN AND FRONTALI[6]. In the present investigation, we have undertaken the biochemical characterization of monoamine oxidase in the crude homogenate of *T. confusum*. We have also measured relative titers of this enzyme throughout various developmental stages of this insect*.

MATERIALS AND METHODS

The insects used were from our permanent cultures maintained under constant condition of temperature (28±1°) and relative humidity (70±5%). The methods of rearing *T. confusum* have been described elsewhere[7].

For the determination of monoamine oxidase activity, appropriate quantities of whole insects at various ages were homogenized in 0.9% cold KCl to give a 10% homogenate. Monoamine oxidase activity was determined by the method of WURTMAN AND AXELROD[8]. In a typical assay, 100 µl of the enzyme preparation, 100 µl of [^{14}C]tryptamine (30 000 counts/min; specific activity 2.73 mC/mmole) and 200 µl of 0.3 M phosphate buffer (pH 7.4) were mixed in a 15 ml glass stoppered centrifuge tube and incubated at 37° for 20 min. The reaction was stopped by the addition of 400 µl of 6 M HCl, and the deaminated radioactive material was extracted with 6 ml of toluene by shaking. After centrifugation, a 3 ml aliquot of the organic layer was transferred to a vial containing 2 ml of distilled toluene and 10 ml of diluted liquifluor solution (Nuclear Chicago Corp., Des Plaines, Ill.) and counted for 10 min in a liquid scintillation spectrophotometer. A small amount of [^{14}C]tryptamine is extracted by this procedure. A correction was made for this blank value (42 counts/min by incubating [^{14}C]tryptamine with boiled enzyme. Each assay was done in triplicate.

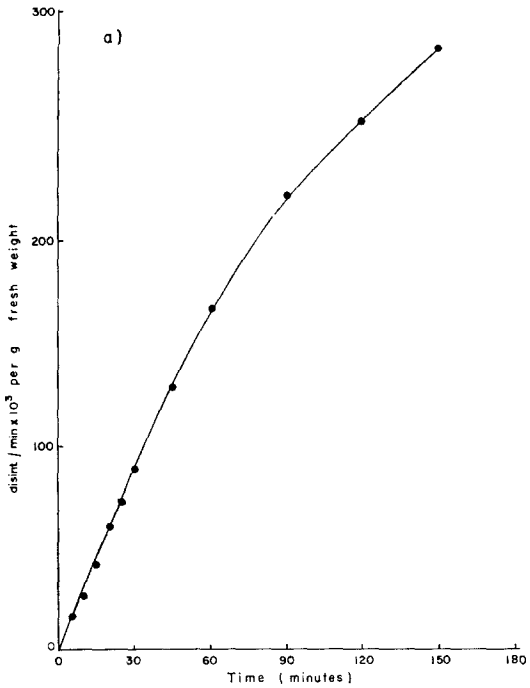
Since pupae contained the highest enzymatic activity, this stage was selected for studying the intracellular localization of monoamine oxidase. Total homogenate was subjected to differential centrifugation to obtain nuclei (600 × *g*) mitochondria (10 000 × *g*), microsomes and high-speed supernatant (105 000 × *g*). Details of the procedure have been published earlier[9]. To determine whether monoamine oxidase occurred as a free or bound enzyme, mitochondria were subjected separately to freezing and thawing, deoxycholate treatment, Triton treatment and change of pH. Protein determination was carried out according to the method of GOA[10].

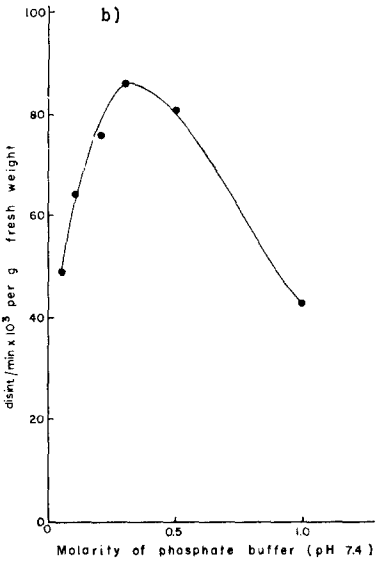
RESULTS

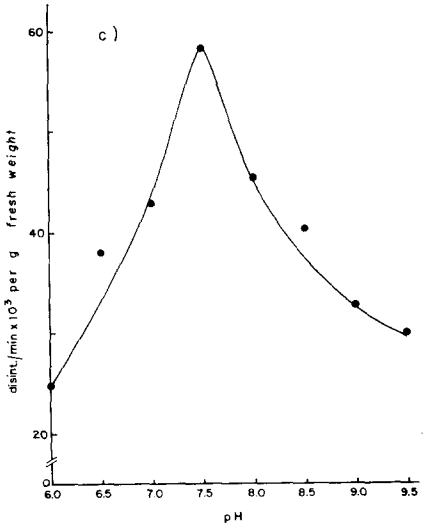
Effect of time

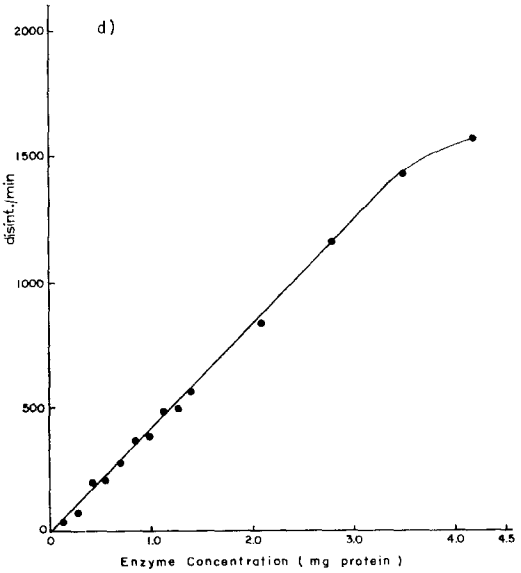
Results showing the effect of varying the incubation periods are illustrated in Fig. 1a. The reaction was linear during the first 40 min. Thus a standard assay time of 20 min was adopted for the measurement of other properties of monoamine oxidase.

* The life cycle of *T. confusum* can be divided into 5 well-defined stages: (i) embryonic (6 days), (ii) larval (14 days), (iii) prepupal (3 days), (iv) pupal (6 days), and (v) adult (up to 2 years).


a)
disint./min x 10³ per g fresh weight
Time (minutes)


b)
disint./min x 10³ per g fresh weight
Molarity of phosphate buffer (pH 7.4)


c)
disint./min x 10³ per g fresh weight
pH


d)
disint./min
Enzyme Concentration (mg protein)

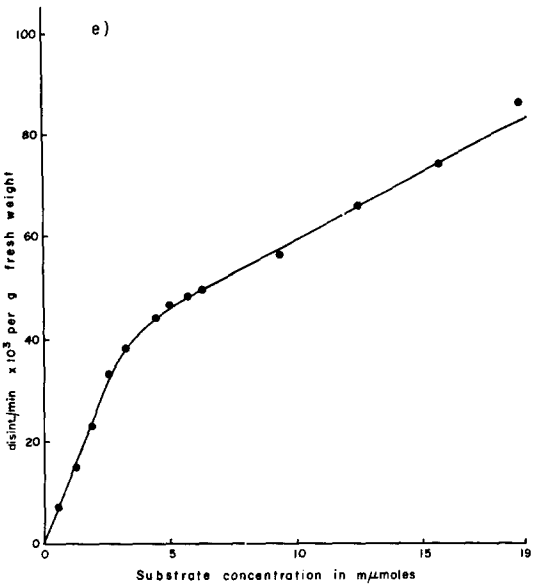


Fig. 1. Hydrolysis of [^{14}C]tryptamine by *T. confusum* monoamine oxidase as function of (a) time, (b) molarity of the buffer, (c) pH, (d) enzyme and (e) substrate concentration. The reaction mixture comprising 200 μl of 0.3 M phosphate buffer (pH 7.4), 100 μl of [^{14}C]tryptamine (30 000 counts/min, specific activity 2.73 mC/mmole), and 100 μl of enzyme preparation (10% homogenate) was incubated at 37° for 20 min. The resulting radioactive [^{14}C]indoleacetic acid was determined by the method of WURTMAN AND AXELROD[8] as described in the text.

Effect of pH

Maximum activity with an effective buffering action was obtained by using phosphate buffer at a final molar concentration of 0.3 M as shown in Fig. 1b. Under the optimal conditions, maximum activity was obtained at pH 7.4 as shown in Fig. 1c. The optimum pH at 0.3 M concentration was taken for the standard assay technique.

Effect of enzyme concentration

Solutions of enzyme at various concentrations and containing 0.14–4.2 mg of protein were used (Fig. 1d). A proportional increase in the activity resulted from a corresponding increase in the enzyme concentration, provided the latter was not too high. Protein determination gave a value of 14 mg of protein in 1 ml of 10% homogenate. This enzyme preparation had a mean specific activity of 396 disint./min per mg of protein.

Effect of varying substrate concentration (Michaelis constant)

A zero-order reaction was maintained provided the substrate concentration was not too high (Fig. 1e). Fig. 2 is a double-reciprocal graph showing the dependence of reaction velocity on substrate concentration. The Michaelis constant (K_m) calculated from these data by the method of Lineweaver and Burk is $8.7 \cdot 10^{-9}$ M (tryptamine) for the stated conditions.

Effect of temperature

Maximum monoamine oxidase activity was shown at 37°, being followed by a

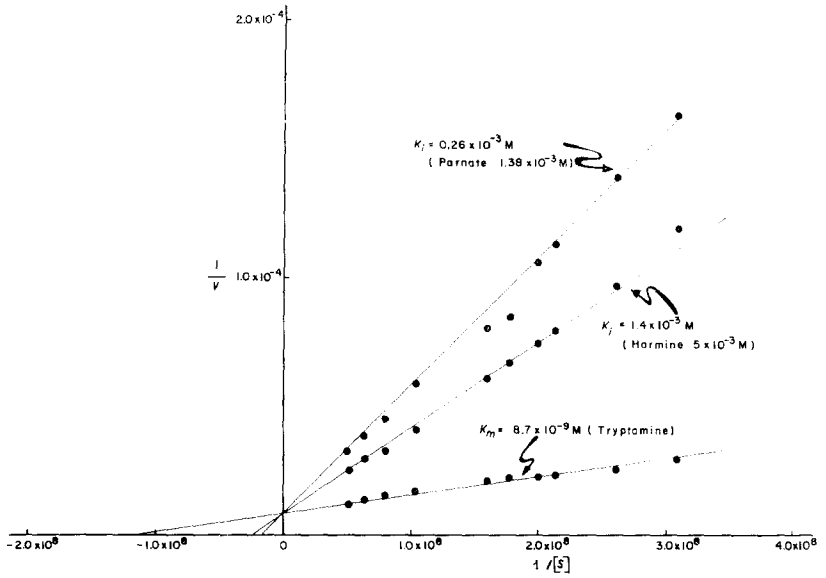


Fig. 2. Lineweaver–Burk plot of $1/v$ *vs.* $1/[S]$ for Michaelis constant (K_m) and the respective inhibition constants (K_i) for enzyme inhibition by parnate sulfate and harmine. Assay conditions are the same as in Fig. 1 except for the addition of inhibitors, where necessary.

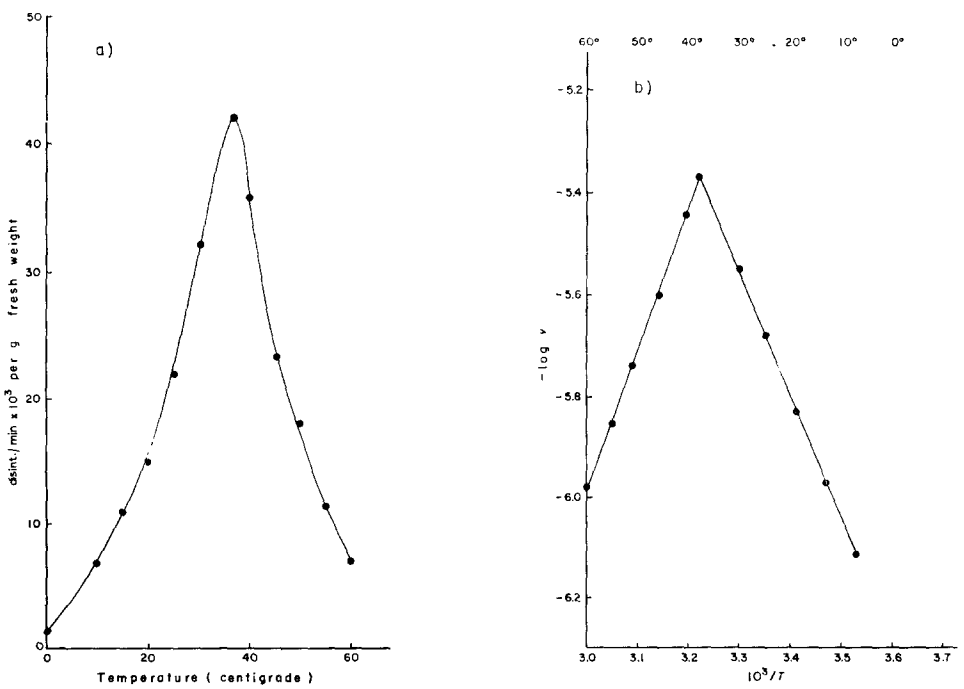


Fig. 3. (a) Effect of temperature on monoamine oxidase activity and (b) Arrhenius plot of temperature effects on the enzyme . The following mean values were obtained for Q_{10} and activation energy, respectively: 1.8 and 10 440 calories. Assay conditions are the same as in Fig. 1.

sharp decline in the activity which was probably due to enzyme denaturation. These data are presented in Fig. 3a. The mean activation energy of 10 440 calories and the Q_{10} value of 1.8 were calculated from the Arrhenius plot (Fig. 3b).

Effect of metallic ions

Effect of metallic ions was shown after chelating endogenous metal ions present in whole homogenate by the addition of EDTA at a final concentration of 0.5 M (Table I). Dialysis of the total homogenate resulted in 70% loss in activity which was not reversed by the addition of various metallic ions. EDTA (0.5 M final concentra-

TABLE I

EFFECT OF METALLIC IONS

Addition	*Concentrations (M)*	*Specific activity (disint./min per mg protein)*	*% total activity**
None		484	100
None (dialysed homogenate)		144	29
EDTA	0.5	148	30
Cu^{2+}	10^{-5}	232	47
	10^{-3}	256	52
	10^{-1}	38	7.5
Ni^{2+}	10^{-5}	402	83
	10^{-3}	370	76
	10^{-1}	230	47
Zn^{2+}	10^{-5}	550	113
	10^{-3}	714	147
	10^{-1}	300	62
Co^{2+}	10^{-5}	484	100
	10^{-3}	556	114
	10^{-1}	472	97
Mn^{2+}	10^{-5}	126	26
	10^{-3}	456	94
	10^{-1}	26	5.0
Fe^{2+}	10^{-5}	156	32
	10^{-3}	206	42
	10^{-1}	200	41
Mg^{2+}	10^{-5}	162	33
	10^{-3}	212	43
	10^{-1}	156	32

* Expressed on the basis of the activity of the non-dialysed homogenate as 100. Assay conditions are the same as in Fig. 1.

tion) also inhibited monoamine oxidase activity by approx. 69%. Addition of Zn^{2+} and Co^{2+} at a final concentration of 10^{-3} M in the presence of EDTA not only recovered the lost activity but also activated the enzyme considerably. Co^{2+} has less stimulatory effect than Zn^{2+}. Mn^{2+}, Ni^{2+} and Cu^{2+} at 10^{-3} M final concentration restored 94%, 76% and 52% of the total activity, respectively.

TABLE II

INTRACELLULAR LOCALIZATION OF MONOAMINE OXIDASE IN *T. confusum* PUPAE

Assay conditions are the same as in Fig. 1.

Fraction	*Concentration of protein (mg/ml)*	*Monoamine oxidase activity*	
		disint./min per ml	*disint./min per mg protein (specific activity)*
Nuclei	8.5	2190	257
Mitochondria	2.93	4350	1484
Microsomes	0.62	—	—
Supernatant	1.25	—	—

Effect of inhibitors

(1) Parnate sulfate (Smith, Kline and French; cis-trans-phenylcyclopropylamine sulfate). Parnate sulfate inhibited strongly the monoamine oxidase activity, the inhibition being of a competitive nature, as shown in Fig. 2. The K_i value calculated from the figure at a final concentration of $1.38 \cdot 10^{-3}$ M of this inhibitor was $0.26 \cdot 10^{-3}$ M.

(2) Harmine (Penick; 7-methoxy-1-methyl-9-pyrid(3,4-b)indole). Harmine also inhibited the monoamine oxidase activity, but to a lesser extent than parnate sulfate as shown in Fig. 2. Here also the inhibition was of a competitive nature. The K_i value calculated from the figure at a final concentration of $5 \cdot 10^{-3}$ M of harmine was $1.4 \cdot 10^{-3}$ M.

Intracellular localization of monoamine oxidase

The maximum specific activity of monoamine oxidase is located in the mitochondrial fraction of *T. confusum* homogenate. The nuclear fraction contained approximately half the activity possessed by the mitochondrial fraction (Table II) when

TABLE III

EFFECT OF VARIOUS TREATMENTS ON MONOAMINE OXIDASE FROM ISOLATED MITOCHONDRIA IN *T. confusum* PUPAE

The supernatant was obtained by centrifuging the mitochondrial fraction at 105 000 × *g* after each treatment. Assay conditions are the same as in Fig. 1.

Treatment	*Specific activity (disint./min per mg protein)*	
	Supernatant	*Residue*
None (intact mitochondria)	—	1484
Freezing and thawing (5 times)	—	1780
Deoxycholate treatment	—	1840
Triton (X-100) treatment	—	1740
Change of pH (pH was changed to 9 slowly and then quickly brought back to 7.5)	—	1790

the results were expressed in disint./min per ml of homogenate. However, when the same activity was expressed in terms of per mg protein in the fraction (specific activity), mitochondria had 5.7 times more activity than nuclei. No monoamine oxidase activity could be detected in microsomal and supernatant fraction. The mitochondrial fraction, when subjected to various treatments, showed no monoamine oxidase activity in the supernatant of disrupted mitochondria. All the activity was found to be in the residue obtained after these treatments (Table III).

Monoamine oxidase during growth and metamorphosis

As shown in Fig. 4, the monoamine oxidase activity is lowest in the eggs followed by an increase in the larval and pupal stages. It reaches a plateau in the pupal stage followed by a sharp decline in adults.

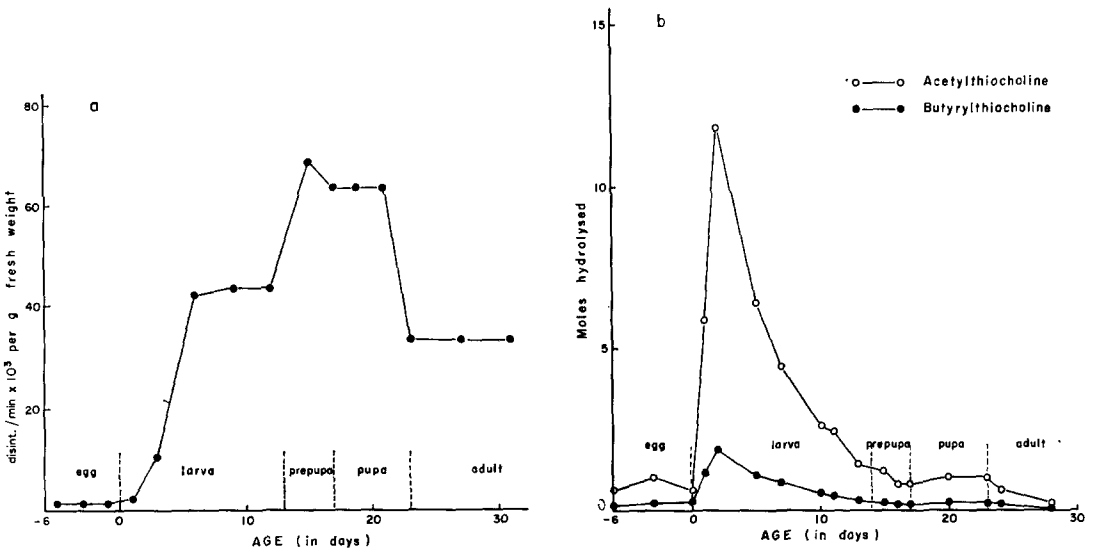


Fig. 4. Activities of (a) monoamine oxidase and (b) acetylcholinesterase during the growth and metamorphosis of *T. confusum*. Assay conditions are the same as in Fig. 1. Part (b) of the figure is reproduced from our earlier publication[18] to compare the activities of 2 enzymes. Acetylcholinesterase activity is expressed as rate of hydrolysis of the substrates in moles/min $\times 10^{-6}$/g fresh weight.

DISCUSSION

The data concerning the hydrolysis of [^{14}C]tryptamine by *T. confusum* monoamine oxidase offer the following points of interest.

The insect monoamine oxidase shows an optimal pH of 7.4 (Fig. 1) which is very close to the pH optimum of 7.3 reported for tyramine oxidation by the corresponding mammalian enzyme[1]. The enzyme is inactivated below pH 6. *T. confusum* monoamine oxidase possesses a relatively very high affinity for tryptamine as shown by a very low K_m value of $8.7 \cdot 10^{-9}$ M (Fig. 2). The K_m values reported for the solubilized beef liver mitochondrial enzyme are, however, of the order of $5 \cdot 10^{-3}$ M and $2.6 \cdot 10^{-3}$ M for tyramine and phenylbutylamine, respectively[11]. As in mammalian systems, ap-

proximately half the activity is destroyed at 50°, probably due to denaturation of the enzyme protein[1].

It is interesting to notice that Zn^{2+} activates the enzyme about 5 times more than Cu^{2+} (Table I) especially in view of the fact that the latter is an essential co-factor for the mammalian monoamine oxidase. In fact, plasma monoamine oxidase has been demonstrated to be a copper-bound protein with pyridoxal phosphate as the prosthetic group[12,13]. BUFFONI AND BLASCHKO[14] have also shown that monoamine oxidase of pig plasma is a copper-containing enzyme. BLASCHKO *et al.*[15] reported that no monoamine oxidase activity was detectable in pig plasma with a low Cu^{2+} content. The studies of MILLS, DALGARNO AND WILLIAMS[16], however, suggest that either other metallic ions may substitute for Cu^{2+} to a limited extent to maintain amine oxidase activity at a low level in plasma of low Cu^{2+} content or that traces of a monoamine oxidase without a metallic component exist in ovine plasma.

Like the mammalian enzyme, insect monoamine oxidase is inhibited by parnate sulfate (long duration of action) and harmine (short duration of action). Parnate sulfate acts essentially irreversibly on monoamine oxidase. However, ZELLER[11] has demonstrated that by replacing the usual substrate, tyramine, by the closely related phenylbutylamine, the inhibition becomes reversible. This receives support from our findings on insect monoamine oxidase where parnate sulfate inhibits the enzyme reversibly when the substrate used is tryptamine (Fig. 2). In fact a competitive type of effect may sometimes be observed with irreversible inhibition also, in that the substrate will protect the enzyme from the inhibitor by reducing the velocity constant of the inhibition[17].

Intracellular localization studies show that the insect enzyme, like mammalian monoamine oxidase, is particulate bound *i.e.* most of the activity is tightly bound to mitochondria (Table III).

During the course of growth and metamorphosis, monoamine oxidase titers follow certain interesting variations especially in the light of our earlier findings on acetylcholinesterase (acetylcholine acetyl-hydrolase, EC 3.1.1.7)[18] (Fig. 3). Acetylcholinesterase records its highest activity on the 2nd or 3rd day of the larval period when monoamine oxidase activity is relatively very low. On the whole these 2 enzymes follow a more or less inverse trend. In fact monoamine oxidase is widely distributed in the body and is usually located where acetylcholinesterase levels are low[1]. Just as cholinesterases are responsible for the degradation of acetylcholine, monoamine oxidase must inactivate excess of biogenic amines, some of which have been reported in insects. In molluscs, serotonin has been reported to act as a neurotransmitter substance[2]. In insects acetylcholine has so far been shown to have a function in the nervous system but not in the synaptic transmission at neuro-muscular junctions[4]. Whether biogenic amines play any role in neuro-transmission in insects has not been demonstrated, although claims that they do so have been made[19]. It is perhaps not surprising that catecholamines have been found in view of the part that they play in sclerotization. Where catecholamines serve as neurohumoral function it is usual to find amine oxidase in association with nervous system, but BLASCHKO *et al.*[15] found no significant amount of this enzyme in the cockroach. Our results on *T. confusum* seem to confirm these findings, since the relative titer of monoamine oxidase in this insect is approx. 700 and 200 times lower than that in rat liver and brain, respectively, under the same experimental conditions (A. HO-VAN-HAP, unpublished data).

ACKNOWLEDGEMENTS

This work was supported by a grant from Medical Research Council, Ottawa, Canada. The authors deeply appreciate the technical assistance of Miss DANIELLE LANGLAIS and some invaluable suggestions of Miss ALICE HO-VAN-HAP of the Department of Biochemistry, School of Medicine. Parnate sulfate and harmine were very kindly provided by Professor L. POIRIER of the Department of Physiology, School of Medicine, Laval University.

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