

## ***In Vitro* Interaction of 5-Hydroxytryptamine with Cytosolic Molybdenum Hydroxylases as a Potential Inhibitor for Initial Rates Activities**

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**Abstract: Problem statement:** The role of 5-HT has been investigated in many behavioral activities. Thus, studies using raphe lesion showed that 5-HT is involved in sleep, general activity levels, habituation, aggression, pain sensitivity and morphine analgesia, avoidance behavior, self-stimulation and water consumption. **Approach:** The metabolic interaction between serotonin (5-hydroxytryptamine) and indole-3-aldehyde and xanthine via aldehyde oxidase (EC 1.2.3.1) and xanthine oxidase (EC 1.1.3.22), respectively, were studied in liver tissue homogenate of Dunkin-Hartley guinea pigs by following the decrease in substrate concentration using spectrophotometer. Homogenates of liver were incubated with indole-3-aldehyde in the presence and absence of serotonin or (chlorpromazine and allopurinol a potent and selective inhibitors for aldehyde oxidase and xanthine oxidase, respectively). Oxidation of indole-3-aldehyde to indole-3-acetic acid was reduced up to 63.2% in the presence of serotonin (100  $\mu$ M), while oxidation of xanthine to uric acid was reduced up to 51.6% under the same conditions. **Results:** In comparison, incubation of the substrates with their specific inhibitors (100  $\mu$ M of chlorpromazine and 100  $\mu$ M allopurinol) give almost complete inhibition. These results demonstrate that in the guinea pig liver a metabolic interaction between serotonin and indole-3-aldehyde or xanthine via molybdenum hydroxylases system may take place in liver, which is the main tissue for xenobiotics detoxification. **Conclusion:** The overall conclusion from this research is that serotonin could be a protector for neurons and other tissue from the insult of oxidation of aldehydes and xanthines by molybdenum hydroxylases.

**Key words:** Indole-3-aldehyde, 5-hydroxytryptamine,  $\beta$ -estradiol, aldehyde oxidase, xanthine oxidase

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### **INTRODUCTION**

Molybdenum-enzymes are needed for key reactions in the metabolism of carbon, sulfur and nitrogen containing compounds and up to now more than 50 different mononuclear molybdenum-enzymes have been found in nature. However, only two classes are present in human and other mammals, namely, xanthine oxidase family which represented by xanthine dehydrogenase, aldehyde oxidase, pyridoxal oxidase and nicotinate hydroxylase, the second class represented by sulfite oxidase and nitrate reductase. In general, reactions catalyzed by molybdenum-enzymes are characterized by the transfer of an oxygen atom, ultimately derived from or incorporated into water, to or from a substrate in a two-electron redox reaction. Aldehyde oxidase proteins are cytoplasmic enzymes that catalyze the oxidation of a variety of aromatic and non-aromatic heterocycles and aldehydes (indole-3-

aldehyde), thereby converting them to the respective carboxylic acid (indole-3-acetic acid). As a result of this reaction, aldehyde oxidase is capable of producing superoxide as well as hydrogen peroxide (Al-Fayez *et al.*, 2007; Beedham, 1987; Beedham *et al.*, 1995).

The central or midline (raphe) location of the large neurons of the brain stem has attracted the attention of anatomists since the time of Ramon y Cajal, who described these cells as large multipolar neurons with uncertain projections. No one suspected that they contained the same chemical substance distributed throughout the body. For many years, investigators had known of a blood-brone chemical that produced vasoconstrictions (a 'Serum' factor that affected blood vessel 'tonus' hence the same Serotonin) and of a substance present in the gut that increased intestinal motility (enteramine). In the mid-twentieth century, serotonin or 5-Hydroxy Tryptamine (5-HT), the single compound producing both these effects, was isolated

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and synthesized and its molecular structure was elucidated (Ruddell *et al.*, 2008).

Shortly thereafter, 5-HT was found to be present in the mammalian CNS in significant quantities and to be concentrated in varying amount in different regions of the brain. This led to the proposal of 5-HT as a CNS neurotransmitter. Localization of the cell bodies and axon terminals was initially visualized in the 1960's with the Falck-Hillarp method of formaldehyde-induced histofluorescence (Osborne, 1973). However, the instability of the fluorophore produced meant that this method could not provide information on the serotonergic innervation of the brain at the same level of sensitivity as it did for the catecholaminergic innervation. Alternatively methods were subsequently used to localize 5-HT in the brain: Autoradiography either at the light microscope or at the electron microscope level (Al-Balool, 2007; Reader, 1982); lesion studied combined with biochemistry (Khler and Steinbusch, 1982; Steinbusch, 1981) or histochemistry (Johansson *et al.*, 1981). More recently, the preferred method of immunohistochemistry with antibodies against 5-HT has demonstrated the full extent of serotonergic innervation (Lidov and Molliver, 1982a; 1982b; 1982c). The serotonergic cell bodies are restricted to clusters in the brain stem, but their fibers using most of the known longitudinal pathways, innervate nearly every area of the brain (Lidov and Molliver, 1982b; 1982c) the serotonergic cell bodies are restricted to clusters in the brain stem, but their fibers, using most of the known longitudinal pathways, innervate nearly every area of the brain (Costa *et al.*, 1982; Mulligan and Trk, 1987). The neurons in the entire mammalian CNS number in billions, whereas serotonergic cells number in the thousands and they constitute ~1/1,000,000 of all CNS neurons. However, their influence on their target sites appears to go far beyond these numbers. In the rat brain it is estimated that there are  $\sim 6 \times 10^6$  serotonergic varicosities/mm cortical tissue. By extrapolation, this means that each serotonergic neurons projecting to the cortex may be responsible for  $5 \times 10^5$  serotonergic varicosities, that each of their cortical target neurons receives  $\sim 200$  varicosities and that serotonergic terminals may account for as many as 1/500 of all axon terminals in rat cortex (Daubert and Condron, 2010).

The 5-HT cell bodies in the brain are located in the brain stem (Chojnacka-Wojcik *et al.*, 1995; Verhofstad *et al.*, 1981), in raphe nuclei groups which extend from the midbrain to the medulla oblongata. These areas are classified into nine regions (B1-B9) (Verhofstad *et al.*, 1981) rostral 5-HT neurons have ascending projections that innervate virtually all areas

of the brain, whereas the caudal cell groups project to the spinal cord (Geyer and Vollenweider, 2008).

Serotonin receptors were originally classified as D and M types in the 1950's and later as 5-HT1, 5-HT2, 5-HT3 and more recently, 5-HT4. The advent for molecular cloning techniques brought about an explosive increase in the number of distinct 5-HT receptors subtype that can be identified reaching the current number of 14. 5-HT3 is a ligand-gated ion channel, while the rest belong to G-protein coupled types (Pearl *et al.*, 1995). Serotonergic cell bodies appear very early (E12) in the embryonic development of the rat, when they form a cluster on either side of the floor plate of the rhombencephalon, accompanied by short, non-varicose fibers (Soleimanzadeh, *et al.*, 2010). This cluster gives rise to the ascending fibers, while a second one, appearing caudally at E14, gives rise to the descending ones. The dorsal and median raphe nuclei, which will be examined in this thesis, arise from the first cluster.

The role of 5-HT has been investigated in many behavioral activities. Thus, studies using raphe lesion showed that 5-HT is involved in sleep (Petitjean *et al.*, 1972), general activity levels (Vergnes *et al.*, 1972), habituation (Hamid *et al.*, 2007), aggression (Vergnes *et al.*, 1974), pain sensitivity and morphine analgesia, avoidance behavior, self-stimulation and water consumption (Hole and Lorens, 1975; Lorens and Yunger, 1974; Lorens *et al.*, 1976).

## MATERIALS AND METHODS

**Chemicals and reagents:** All chemicals were purchased from Sigma Chemical Company Ltd. All reagents and solvents,  $\text{KH}_2\text{PO}_4$  and  $\text{K}_2\text{HPO}_4$  (for buffer preparation), were of analytical grade.

**Preparation of enzyme fraction:** Partially purified molybdenum hydroxylases have been prepared and manipulated according to the reported method by (Al-Fayez *et al.*, 2007; Beedham, 1987).

**Determination of kinetic constant:** Oxidation rates were determined spectrophotometrically using a Shimadzu 2101 UV/VIS spectrophotometer, which was linked to pye-Unicam cell temperature control unit. With the exception of enzyme, which was kept in ice until mixing with other components, all solutions were pre-warmed to 37°C. The spectrophotometer was computer-controlled by Shimadzu UV-210 spectroscopy software package with additional kinetics software.

Molybdenum hydroxylases, aldehyde oxidase and xanthine oxidase, activity was monitored, at 37°C,

using 100  $\mu\text{M}$  indole-3-aldehyde (enzyme fraction was diluted 1:40 dilutions) and 50  $\mu\text{M}$  xanthine as substrates, respectively, in 67 mM Sorenson's phosphate buffer, pH 7.0, containing 100  $\mu\text{M}$  EDTA.

**Calculation of inhibitor kinetic constants:** Dixon plots have been used to determine  $K_i$ , by plotting inhibitor concentrations, serotonin, against the reciprocal of initial rates,  $1/V$ , which measured in the presence of inhibitor.

**Statistical analysis of data:** Metabolic formation and substrate breakdown is expressed  $\pm$  Standard Deviation (SD). Statistical significance, p-values, between control and inhibited incubations were evaluated using a one-tailed or two-tailed, paired student's t-test. Linear relationship between variables was determined by least-squares method using linear correlation coefficient (r). Mean, standard deviation, p-values and linear correlation coefficient were all determined using Excel 2007 for Windows.

## RESULTS

Serotonergic neurons synthesize 5-HT from dietary tryptophan, which is converted to 5-hydroxy-tryptophan, in turn, converted to 5-HT by aromatic-L-amino acid decarboxylase. The production rate is believed to be adjusted by impulse-coupled regulation of tryptophan hydroxylases activity (Yulug, 2009; Anden *et al.*, 1971). Serotonin itself is metabolized initially into 5-hydroxy-indole-acetaldehyde by Monoamine Oxidase (MAO), the enzyme that oxidatively deaminates all monoamines to their corresponding aldehydes; further oxidation by aldehyde dehydrogenase leads to the formation of 5-hydroxy-indole acetic acid (Tecott, 2007; Kooy *et al.*, 1981).

Serotonin is released by stimulation of the serotonergic cell bodies in the raphe nuclei. These cells possess 5-HT autoreceptors, suggesting that 5-HT regulates its own release. However, other neurotransmitters and modulators (dopamine, noradrenaline, acetylcholine) have also been implicated in this regulation. Drugs and experimental compounds like reserpine,  $\pi$ -chloroamphetamine (PCA) and Methylenedioxymethamphetamine (MDMA/ 'Ecstasy') can also cause release of 5-HT (for review, see ref. 29, 30). The action of 5-HT upon postsynaptic receptors, as studied by iontophoretic application, is chiefly inhibitory (Tecott, 2007). This action is terminated by reuptake into the presynaptic terminal; the uptake system has a high specificity and does not

interact with other monoamines. It has been known from long time that serotonin is synthesized and functioning outside the CNS and it had been found in almost all tissue in the human body (Kooy *et al.*, 1981).

In this study, the oxidation of 50  $\mu\text{M}$  xanthine and 100  $\mu\text{M}$  indole-3-aldehyde by partially purified molybdenum hydroxylases fractions was followed by monitoring the decrease in absorbance at 295 and 300 nm, due to the formation of uric acid and indole-3-acetic acid, respectively, using oxygen as an electron acceptor. In addition, the oxidation of the two substrates was measured in the presence of artificial electron acceptor, potassium ferricyanide ( $\text{K}_3\text{Fe}(\text{CN})_6$ ). These reactions were monitored by following the decrease in absorbance at 420 nm resulting from the reduction of potassium ferricyanide to potassium ferrocyanide. Using either oxygen or potassium ferricyanide gave equivalent results. In the presence of 100  $\mu\text{M}$  serotonin the oxidation of 50  $\mu\text{M}$  xanthine as well as 100  $\mu\text{M}$  indole-3-aldehyde was reduced by 63.2 and 51.6% using oxygen as an electron acceptor. Inhibition of indole-3-aldehyde oxidation by varying serotonin concentration, using potassium ferricyanide as electron acceptor, indicates that the molybdenum centre could be the site of interaction between the inhibitor and substrates (Fig. 1). Although serotonin is less effective than equimolar concentrations of chlorpromazine (100  $\mu\text{M}$ ), which inhibited indole-3-aldehyde oxidation by  $\approx 98\%$ , it has a significant inhibitory effect on substrate oxidation catalyzed by guinea pig liver enzyme.

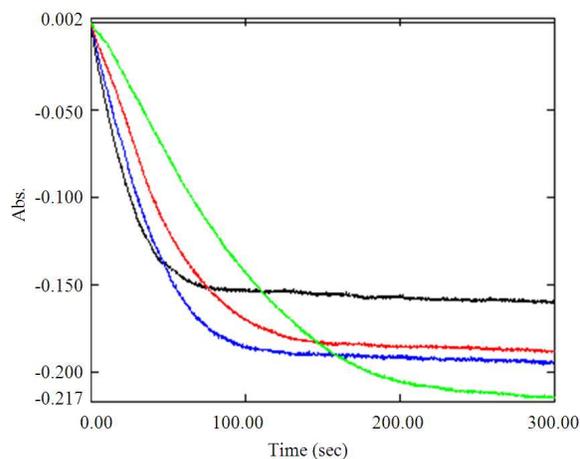


Fig. 1: The effect of varying serotonin concentrations on the reduction of 76  $\mu\text{M}$  potassium ferricyanide by electrons generated during the oxidation of 100  $\mu\text{M}$  indole-3-aldehyde by guinea pig liver aldehyde oxidase

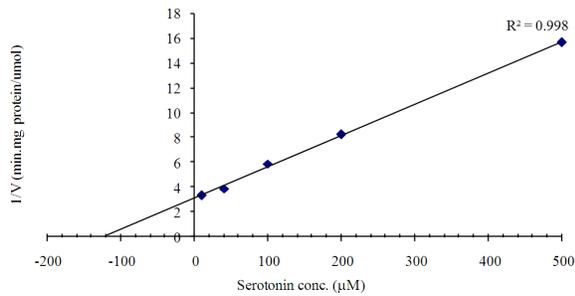


Fig. 2: Dixon plot of indole-3-acetic acid formation by guinea pig liver aldehyde oxidase in the presence of 10, 40, 100, 200 and 500 μM serotonin ( $r = 0.9986$ )

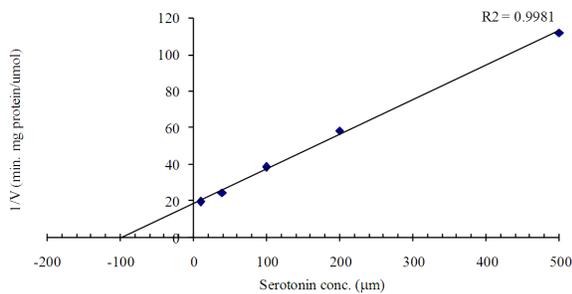


Fig. 3: Dixon plot of uric acid formation by guinea pig liver xanthine oxidase in the presence of 10, 40, 100, 200 and 500 μM serotonin ( $r = 0.9981$ )

However, serotonin could act as a competitive substrate, thus 100 μM serotonin was incubated with partially purified guinea pig fraction in Sorenson's phosphate buffer, pH 7.0, using potassium ferricyanide as electron acceptor. No change in absorbance at 420 nm was detected. The neurotransmitter was also incubated with the enzyme preparation using oxygen as electron acceptor and the incubation was scanned between 200-700 nm several time for up to 10 min. Again, there were no changes observed in the spectrum of serotonin. It was therefore concluded that serotonin is not a substrate for our enzyme preparation.

The effect of serotonin on the initial rate of potassium ferricyanide reduction and that measured at 80 sec during the oxidation of indole-3-aldehyde are shown in Fig. 1. Figure 1 indicates that serotonin caused a marked inhibition of initial rates of substrate oxidation but may increase overall production electron by aldehyde oxidase. The inhibitory effect is investigated further in Fig. 4.

Inhibitor constant ( $K_i$ ) for serotonin with aldehyde oxidase was estimated to be  $120 \pm 13$  μM ( $n = 4$ ), using the initial rates over 5 minutes for a Dixon plot of serotonin concentration versus  $1/V$  (Fig. 2).

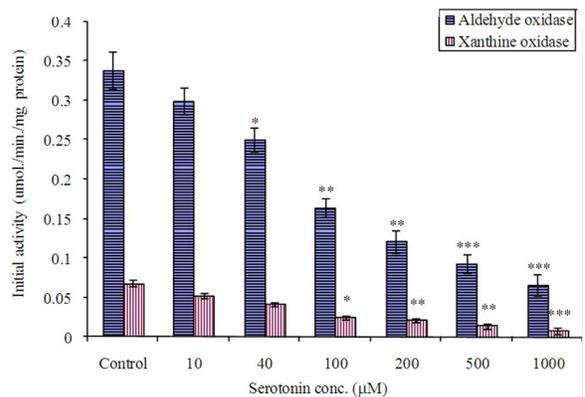


Fig. 4: Initial rates of indole-3-acetic acid and uric acid formation by guinea pig liver molybdenum hydroxylases using different concentrations of substrates (2.5-100 μM) in Sorenson's phosphate buffer, (67 nM), pH 7.0, containing 100 μM EDTA at 37°C, oxygen as electron acceptor (Mean  $\pm$  SD,  $n = 4$  male guinea pigs, \* $p < 0.1$ , \*\* $p < 0.05$ , \*\*\* $p < 0.02$ )

This indicates the serotonin is a moderate inhibitor of guinea pig liver aldehyde oxidase. Similarly, the  $K_i$  for serotonin with xanthine oxidation by xanthine oxidase was found to be  $94 \pm 9$  μM ( $n = 4$ ). This indicates that serotonin is more potent inhibitor for xanthine oxidation than indole-3-aldehyde (Fig. 3). Further investigation of mode of inhibition exhibited by serotonin was carried out in our laboratory using spectrophotometric assay.

## DISCUSSION

Interestingly, serotonin was found to act as a competitive inhibitor for aldehyde oxidase and non-competitive inhibitor of guinea pig liver xanthine oxidase. This manuscript was not designed to explain this controversy. Further studies will focus on this discrepancy.

Interaction of serotonin with both enzyme was studied for the first time in this work. With the exception of 10 μM serotonin, all other concentrations had a significant inhibitory effect ( $p < 0.1-0.02$ ) on the oxidation of indole-3-aldehyde and xanthine oxidase by guinea pig liver molybdenum hydroxylases (Fig. 4).

Noteworthy, the effect of potent aldehyde oxidase inhibitor, chlorpromazine (100 μM) and traditional xanthine oxidase inhibitor, allopurinol (100 μM), on indole-3-aldehyde and xanthine oxidation by the enzyme preparation, respectively, indicates that these substrates are specific and excellent.

Although the results presented in this study have not been obtained with human liver molybdenum hydroxylases, previous studies have shown that guinea pig liver molybdenum hydroxylases are a good model for the human liver enzyme (Al-Fayez *et al.*, 2007; Beedham *et al.*, 1995).

### CONCLUSION

The overall conclusion from this research is that serotonin could be a protector for neurons and other tissue from the insult of oxidation of aldehydes and xanthenes by molybdenum hydroxylases. More details on the mechanism of reactive oxygen species production by molybdenum hydroxylases in the presence of serotonin are needed and will be studied in near future by the author.

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