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CHARACTERIZATION OF RAT SKELETAL MUSCLE MONOAMINE OXIDASE*

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Abstract—Optimal conditions for deamination of 5-hydroxytryptamine in rat skeletal muscle were determined. The presence of monoamine oxidase (MAO) A and MAO B isozymes was demonstrated by the use of tyramine (a substrate of both forms), specific substrates (serotonin and benzylamine), and specific inhibitors (clorgyline and deprenyl) of MAO A and B respectively. A 6.5:3.5 ratio of MAO A to B was found using a whole muscle homogenate, while a 7.5:2.5 ratio was found with isolated mitochondria. Thermal inactivation studies demonstrated that skeletal muscle MAO A is more susceptible to heat inactivation than MAO B. The approximate proportion of muscle homogenate MAO which is present in sympathetic nerves was found to be 18 per cent, as determined by treating rats with 6-hydroxydopamine and quantifying the decrease in activity. Significant inhibition of MAO activity was observed after administration *in vivo* of the MAO inhibitors pargyline, tranylcypromine and harmaline.

There has been considerable recent interest in the role of bioamines in the pathogenesis of Duchenne-type muscular dystrophy (DMD). Specifically, the possibility that serotonin (5-HT) or monoamine oxidase (MAO, EC 1.4.3.4) is relevant to the etiology of DMD has been proposed on the basis of myopathies which develop in rats after the administration of 5-HT to aortic-ligated or imipramine-pretreated rats [1, 2] or after the administration of the MAO inhibitor, pargyline [3]. The latter treatment produced much greater pathology in the soleus, a distal muscle, than in the vastus lateralis, a proximal muscle. 5-HT may be directly toxic to skeletal muscle, as originally proposed by O'Steen *et al.*, [4] or via its effects on the vasculature [1, 2]; inhibition of MAO or a genetically determined deficiency of MAO could potentiate the toxic effects or vascular effects of 5-HT and thereby promote muscle damage.

There has been very little biochemical study of skeletal muscle MAO. In several studies, the deamination of tryptamine by muscle homogenates has been used as a marker for mitochondrial MAO [5–7]. However, there are no studies which differentiate the enzyme present in mitochondria from muscle fibers from that in other components of muscle homogenates, e.g. sympathetic nerve endings, connective tissue, blood vessels, etc. Since it has not yet been possible to demonstrate muscle fiber MAO histochemically by techniques which demonstrate MAO in other tissues (Ref. 8 and H. Y. Meltzer, unpublished data), it is conceivable that MAO activity in muscle fibers is low. MAO activity has been studied in homogenates of muscle from DMD patients and controls; no apparent difference in total activity was noted but there was no examination of possible difference in the isozymes of MAO, kinetic constants, inhibitor sensitivity or other parameters [9].

We, therefore, decided to study MAO in rat skeletal muscle homogenates in order to further characterize it and to establish its localization in skeletal muscle fibers.

METHODS AND MATERIALS

Animals. The rats used in this study were Sprague–Dawley males, obtained from Sprague–Dawley, Inc., Madison, Wis. They weighed 150–200 g at the time of study. Rats were housed in a room at 24° with a 12 hr light–dark cycle.

Reagents. Tyramine HCl, tryptamine HCl, benzylamine HCl and serotonin creatinine sulfate were purchased from Sigma Chemical Co. The radioactive substrates, [2-^{14}C]5-HT binoxalate (sp. act. 27 mCi/m-mole), and [1-^{14}C]tyramine hydrochloride (sp. act. 55 mCi/m-mole) were purchased from Amersham/Searle Corp.; [2-^{14}C]benzylamine HCl (sp. act. 4.0 mCi/m-mole) was purchased from ICN Isotope and Nuclear Division, Cleveland, Ohio and [2-^{14}C]-tryptamine bisuccinate (sp. act. 47.0 mCi/m-mole) was purchased from New England Nuclear, Boston, Mass. Tranylcypromine and pargyline were gifts of Smith, Kline & French, Inc., Philadelphia, Pa., and Abbott Laboratories, Chicago, Ill. respectively. Harmaline HCl and 6-hydroxydopamine (6-OH-DM) were purchased from Sigma Chemical Co. All the other chemicals used were of analytical grade quality.

Enzyme assay. Adult rats were killed by decapitation and quadriceps muscle was taken and immersed in relaxing media (0.1 M KCl, 2 mM $MgCl_2$, 2 mM EGTA, 10 mM Tris, and 1 mM Na-pyrophosphate, adjusted to pH 7.4) [10]. After 5 min, fatty material was removed and the muscle was homogenized in the same media (5%, w/v) with a glass homogenizer.

Monoamine oxidase activity was determined by the modified ratioisotopic method of McCaman *et al.* [11] using [^{14}C]5-HT, tryptamine, tyramine and benzylamine as substrates. In brief, the reaction mixture, containing enzyme activity (0.1 to 0.6 mg pro-

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tein), sodium phosphate buffer (25 mM, pH 7.4) and 0.65 mM [^{14}C]5-HT, [^{14}C]tryptamine, [^{14}C]tyramine or [^{14}C]benzylamine in a total volume of 0.2 ml, was incubated at 37° for 20 min with shaking. The reaction was terminated by the addition of 50 μl of 3 N HCl. The ^{14}C-deaminated products formed were extracted by shaking with 5 ml toluene for 10 min. A 1-ml aliquot of the extract was added to a scintillation vial containing 10 ml of scintillation fluid [toluene, containing 2.5 g of 2,5 diphenyloxazole (PPO) and 1,4-bis-2(4-methyl-5-phenyloxazolyl)-benzene, (dimethyl POPOP) 43.6 mg/liter] and the radioactivity was determined in a Nuclear Chicago scintillation counter. The blank experiments were done with buffer and amine, without the addition of homogenate.

Isolation of mitochondria. Mitochondria were isolated following the procedure of Zak *et al.* [10] and resuspended in the relaxing buffer. MAO activity was determined as described above.

Determination of pH optimum for MAO activity. The pH optimum for deamination of serotonin was determined by the radioisotopic method described above. The reaction mixture was as previously described and included [2-^{14}C]5-HT binoxalate (0.65 mM) as the substrate. Sodium phosphate was used in the range pH 6.4 to 7.8, while sodium pyrophosphate was used to obtain pH 7.4 to 8.4. All subsequent assays of MAO activity were done at pH 7.4 using sodium phosphate buffer.

Thermal inactivation of MAO activity. The heat stability of muscle MAO activity were determined by heating the tissue homogenate (7 mg/ml) in buffer at 50°. Aliquots were removed at various times for determination of enzyme activity as described above.

Inhibition studies. Studies *in vitro* were performed by incubating the homogenate with inhibitor at 37° for 15 min prior to the addition of substrate. The mixture was then incubated for an additional 30 min. The degree of inhibition was calculated from the difference in the number of counts/min in the sample with and without inhibitor. Studies were also performed *in vivo*. Pargyline (75 mg/kg), tranylcypromine (10 mg/kg), harmaline (50 mg/kg), or saline was injected intraperitoneally into groups of three rats daily for 7 days. The rats were killed 2 hr after the last dose; quadriceps muscle was taken and MAO activity with 5-HT as the substrate was determined.

Skeletal muscle fiber MAO vs sympathetic nerve MAO. Skeletal muscle homogenates were made from four muscles which differ in the proportion of type I fibers: abductor longus > soleus > lateral gastrocnemius > tensor fascia [12]. Type I fibers have more mitochondria than type II fibers [13]. MAO activity with 5-HT as substrate was determined in all four muscles.

Further, six 80 to 100-g male rats were injected intravenously with 6-hydroxydopamine (6-OH-DM), 100 mg/kg, dissolved in 0.1 N HCl containing 100 mg/ml of ascobic acid on days 1 and 6. This treatment destroys sympathetic nerve endings [14]. Control rats received vehicle injections. Both groups were sacrificed 2 days after the last injection. Lateral gastrocnemius and soleus muscle specimens were taken and the deamination of serotonin was studied in the homogenates.

Protein determination. Protein concentration was determined following the method of Lowry *et al.* [15] using bovine serum albumin as standard.

RESULTS

Comparison of substrates. After incubation of the muscle homogenate with radioactive amines, ^{14}C-deaminated metabolites were extracted with toluene and the results are shown in Table 1. As evident from the table, 5-HT was found to be the best substrate followed by tyramine, benzylamine and tryptamine. Extraction of the product was also done by ether, but no significant difference in the enzymatic value and the blank value, which was much higher than that with 5-HT, was observed (data not presented). The radioactivity extracted into toluene from the blanks was less than 0.2 per cent of the amount of 5-HT, tryptamine, tyramine or benzylamine. The blank value with 5-HT as substrate was equivalent to 0.02 per cent of the amount of 5-HT in the buffer-substrate mixture. The radioactivity extracted in the enzymatic reaction was 75–80 per cent of the metabolite formed. Hence, in all experiments, toluene was used for the extraction of the metabolite.

Relation between incubation time and enzyme activity. The reaction mixture was incubated for different time intervals with 5-HT as a substrate. Results were plotted as enzyme activity vs time of incubation. The MAO activity was found to be linear up to 40 min. After 75 min, no increase in activity was observed. Similarly, deamination of benzylamine was found to be linear up to 40 min, while deamination of tyramine was found to be linear up to 60 min. Hence, in all experiments, the incubation was performed for 30 min.

Effect of substrate concentration. Muscle homogenate MAO activity was determined using 5-HT as a substrate at different concentrations (0.13 to 1.3 mM). The results were plotted as reciprocal of substrate concentrations vs reciprocal of velocity (Lineweaver–Burk plot). The intercept at the abscissa yielded a K_m value of 9×10^{-5} M. When MAO activity was determined with isolated mitochondria, the K_m was found to be 4×10^{-5} M.

Effect of enzyme concentration. For the study of the effect of enzyme concentration, MAO activity was determined as a function of concentration of protein in the homogenate. It was found that the reaction

Table 1. Comparison of muscle homogenate MAO activity with various substrates*

Substrate	MAO activity (nmoles/mg protein)
Serotonin	3.8 ± 0.6
Tyramine	1.8 ± 0.1
Benzylamine	1.4 ± 0.2
Tryptamine	1.1 ± 0.1

* Homogenate (0.32 mg protein) was incubated with 0.65 mM (sp. act. 1.12 mCi/m-mole) of [^{14}C]serotonin, [^{14}C]tryptamine, [^{14}C]tyramine or [^{14}C]benzylamine. The metabolite was extracted and counted as described in the text. Each experiment was done in duplicate and the values represented are mean ± S. D. and expressed as nmoles of products formed/mg of protein/hr.

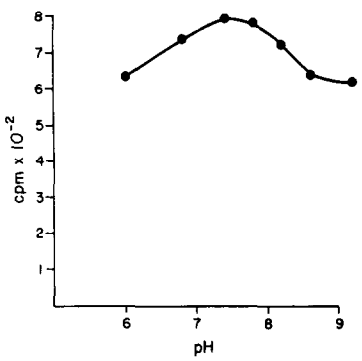

Fig. 1. Effect of pH on enzyme activity. The pH optimum for muscle homogenate was determined for 5-HT as the substrate. The reaction mixture contained 0.65 mM 5-HT (sp. act. 1.12 mCi/m-mole) and 0.32 mg enzyme in 0.1 mM phosphate or pyrophosphate buffer. Enzyme activity was determined as described in the text.

was linear up to 0.5 mg homogenate protein with 5-HT as a substrate for a 30-min incubation period. However, with benzylamine and tyramine as the substrates, reaction was found to be linear up to 0.8 mg of the protein.

pH Requirement. The optimum pH for the deamination of 5-HT was found to be 7.4 (Fig. 1). No significant difference in enzyme activity was observed on increasing the pH to 7.8; however, enzyme activity decreases with pH greater than 7.8. Similarly, no difference in enzyme activity was found with sodium phosphate or sodium pyrophosphate as buffers. No significant difference in enzyme activity was observed from changing the pH 7.4–9.4 for benzylamine deamination studies, while pH 7.4 was found to be optimum for tyramine deamination studies. Hence, in all experiments sodium phosphate buffer (pH 7.4) was used. Similarly, when MAO activity was determined with mitochondria, a pH of 7.4 was found to be optimum.

Heat stability of MAO activity. The effect of heat denaturation on MAO activity was determined using serotonin and benzylamine as substrates. 5-HT is considered to be a specific substrate for MAO A isoenzyme [16, 17], while benzylamine is specific for MAO B isoenzyme [18]. As evident in Fig. 2, MAO A was more heat sensitive than MAO B. After 10 min of heating, MAO A loses 67 per cent of its original activity, while MAO B loses 43 per cent. MAO A activity is entirely lost whereas MAO B still retains 24 per cent of the original activity after 120 min of heating.

Inhibition studies in vitro. With tyramine as substrate and clorgyline as inhibitor, a plot of the per cent of inhibition vs the log of inhibitor concentration revealed increasing inhibition, a plateau, and then further increase in inhibition (Fig. 3), rather than a simple sigmoid curve. This is indicative of the presence of more than one isozyme or form of MAO. Following the view of Johnston [19], there is a 6.5:3.5 ratio of MAO A to B in rat skeletal muscle. Similar results were obtained when inhibition studies were performed with mitochondria. However, the ratio of MAO A to B was 7.5:2.5. The presence of MAO A and B in muscle homogenate was also studied using their specific inhibitors, i.e. clorgyline for MAO A and deprenyl for MAO B. Inhibition of the deamination of 5-HT by clorgyline could be detected at a very low concentration, i.e. 26 per cent at 10^{-10} M (Fig. 4). Complete inhibition occurred at a concentration of 10^{-7} M. Similarly the presence of MAO B was confirmed by studying the effect of deprenyl on the deamination of benzylamine. Deprenyl inhibits deamination of benzylamine at a low concentration, e.g. 25 per cent at 10^{-9} M; a maximum inhibition of 59 per cent was obtained at 10^{-3} M (Fig. 5). The presence of MAO A was further confirmed by studying the effect of deprenyl on 5-HT metabolism. As expected, deprenyl does not inhibit the oxidation of 5-HT at a low concentration but it does inhibit at a higher concentration, i.e. 42 per cent at 10^{-5} M (Fig. 4). At a higher concentration, deprenyl has been

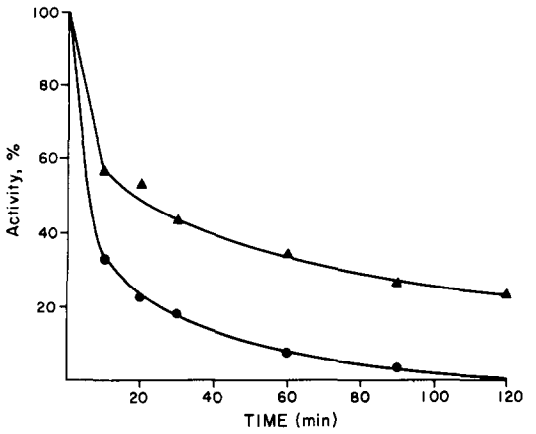

Fig. 2. Effect of heat treatment on MAO activity. Muscle homogenate (7 mg/ml) in buffer was incubated at 50°. Samples were taken at time intervals indicated and assayed for MAO activity. The reaction mixture contained 0.65 mM of serotonin or benzylamine and homogenate (0.35 mg). Results are plotted as activity remaining vs time of incubation. The substrates used were benzylamine (▲) and 5-HT (●).

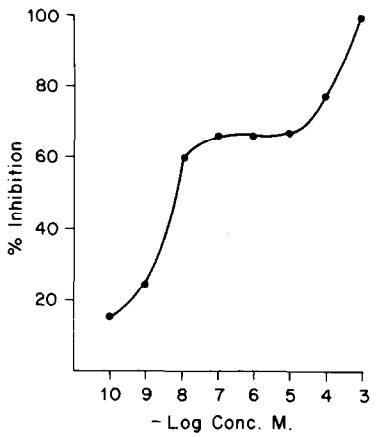

Fig. 3. Inhibition of the deamination of tyramine by increasing the concentration of clorgyline. Muscle homogenates (0.35 mg) were preincubated with clorgyline at 37° for 15 min and then tyramine was added (0.65 mM). The incubation was continued for an additional 30 min. Data are presented as a mean (duplicate) of percentage inhibition of MAO activity.

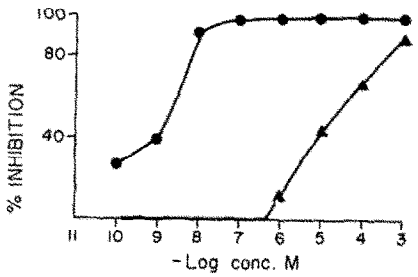

Fig. 4. Inhibition of 5-HT deamination by increasing the concentration of clorgyline and deprenyl. Muscle homogenate (0.35 mg) was preincubated for 15 min at 37° with the drugs after which serotonin (0.65 mM) was added and the incubations were continued for an additional 30 min. Data are reported as mean (duplicate) percentage inhibition of MAO activity; MAO activity without inhibitors was 3.6 nmoles 5-HT deaminated/hr/mg of protein. Key: clorgyline (●), and deprenyl (▲).

reported to inhibit both forms of MAO [20]. Similar results were reported earlier for rat brain [21] and bovine brain homogenate [22].

Inhibition in vivo. Complete inhibition of 5-HT deamination was found after pargyline treatment, while tranylcypromine and harmaline inhibited the deamination of 5-HT by 85 and 50 per cent, respectively (data not presented).

MAO activity in skeletal muscles vs sympathetic nerve. MAO activity of homogenates of different muscles toward 5-HT as a substrate is shown in Table 2. As evident from the table, the specific activity of MAO in these four muscles parallels the proportion of type I (mitochondrial-rich) muscles. These results suggest that MAO is localized in skeletal muscle fibers rather than in blood vessels, connective tissue or nerves, since the red and white muscles differ from each other mainly in the number of mitochondria and the activity of glycolytic enzymes rather than the presence of other tissues. The observation that administration of 6-OH-DM to produce sympathetic nerve denervation caused only a 10–20 per cent loss of

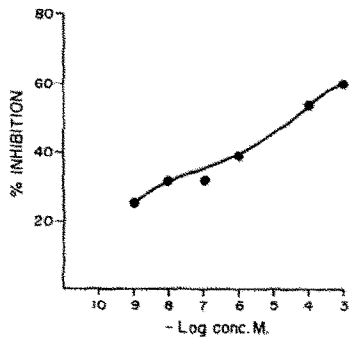

Fig. 5. Inhibition of benzylamine deamination by increasing the concentration of deprenyl. Muscle homogenate (0.35 mg) was preincubated with deprenyl for 15 min at 37° before the addition of benzylamine (0.65 mM). Enzyme activity was determined as described in the text. Data are presented as mean (duplicate) percentage inhibition of MAO activity. MAO activity without the inhibitor deprenyl was 1.5 nmoles benzylamine deaminated/hr/mg of protein.

Table 2. Comparison of MAO activity in different muscle homogenates*

Enzyme source	% Type I/ muscle fibers†	MAO activity (nmoles/mg protein)
Abductor longus	88	4.5 ± 0.1
Soleus	84	3.4 ± 0.2
Lateral gastrocnemius	5	2.8 ± 0.1
Tensor fasiae latae	0	2.4 ± 0.3

* Each muscle homogenate (0.4 to 0.5 mg protein) was incubated with [^{14}C]serotonin (0.65 mM) as described in the text. Each experiment was done in duplicate and the values are mean ± S. D. and expressed as nmoles metabolized/mg of protein/hr.

† See Ref. 12.

MAO activity in the lateral gastrocnemius and soleus muscle also indicates that MAO in skeletal muscle homogenates is mainly muscle fiber MAO (Table 3).

DISCUSSION

The studies reported here identified the optimum pH, duration of incubation and extraction conditions for determination of rat skeletal muscle MAO activity. It was demonstrated that rat skeletal muscle has at least two isozymes of MAO which have many features, such as pH optimum, heat stability and inhibitor sensitivity, in common with the isozymes of MAO previously reported in brain [18] and liver [23]. In accordance with current nomenclature, this would indicate the presence in rat skeletal muscle of an MAO A which is active toward 5-HT as substrate, is inhibited by clorgyline and is relatively heat sensitive [16, 24] and an MAO B which is active toward benzylamine as substrate, is sensitive to inhibition by deprenyl and is relatively heat stable [18, 25]. The apparent K_m (4×10^{-5} M) found for serotonin deamination by skeletal muscle mitochondria was identical to that reported earlier for rat liver mitochondria (4×10^{-5} M) [26] and purified human brain MAO (4×10^{-5} M) [27].

Studies *in vitro* with clorgyline inhibition of tyramine oxidation suggest that there is roughly twice

Table 3. Effect of chemical sympathectomy on MAO activity*

Enzyme source	MAO activity (cpm/mg protein) Control	6-OH-DM	% Inhibition
Soleus	3068.0	2677.0	12.7
	3440.0	2581.0	25.0
Lateral gastrocnemius	2721.0	2527.0	7.1
	2725.0	2528.0	7.9

* Groups of six male rats were injected intravenously with 6-OH-DM, 100 mg/kg, dissolved in 0.1 N HCl containing 100 mg/ml ascorbic acid on days 1 and 6. Control rats received vehicle injections. Both groups were sacrificed 2 hr after the last injection. Each muscle was homogenized separately and MAO activity with [^{14}C]serotonin as substrate was determined as described in the text. Each experiment was done in duplicate and the values are the average.

as much MAO A as MAO B in rat skeletal muscle. In light of the small decrease in MAO activity produced by 6-OH-DM treatment, it appears that most of the MAO A in skeletal muscle is muscle fiber MAO rather than sympathetic nerve MAO, unless the 6-OH-DM did not entirely destroy sympathetic nerves. It seems unlikely that the 6-OH-DM would affect skeletal muscle fibers without destroying the sympathetic nerve endings first. Lowe *et al.* [28] found no significant loss of MAO activity in rat heart after 6-OH-DM treatment, indicating the presence of extraneuronal MAO in cardiac muscle. The correlation of muscle homogenate MAO activity with the relative amount of mitochondrial-rich type I fibers in each muscle also suggests the extraneuronal location of MAO in skeletal muscle, since it is unlikely that the various fiber types differ markedly in sympathetic nerve supply. Thus, the difficulty in demonstrating MAO activity with histochemical methods in frozen sections of skeletal muscle is not the result of lack of MAO activity, particularly toward MAO A substrates such as tyramine and serotonin.

The reported toxicity of pargyline for soleus muscle [3] could be based on MAO inhibition as we observed complete inhibition of MAO under the conditions employed. However, since pargyline produced complete inhibition of MAO of vastus lateralis muscle as well as soleus, it is curious that no morphologic pathology developed in the vastus lateralis [3]. Conceivably, MAO inhibition leads to an increase in the levels of bioamines in the circulation which are directly or indirectly (perhaps via vascular effects) more toxic to the soleus muscle than the vastus lateralis muscle.

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