Biochemical Pharmacology, Vol. 29, pp. 1185–1188
Pergamon Press Ltd. 1980. Printed in Great Britain.

THE EFFECT OF SONICATION UPON MONOAMINE OXIDASE-A AND -B IN THE RAT LIVER

CHRISTOPHER J. FOWLER,* BRIAN A. CALLINGHAM, MICHAEL D. L. O'CONNOR and E. KEITH MATTHEWS

Department of Pharmacology, University of Cambridge, Hills Road, Cambridge, CB2 2QD, U.K.

(*Received* 7 *September* 1979; *accepted* 24 *October* 1979)

Abstract—The effect of sonication upon the activity and properties of monoamine oxidase (MAO) was investigated. Short bursts of sonication produced a preparation of MAO that did not sediment upon centrifugation at 100,000 *g* for 60 min. The properties of the MAO in the high-speed supernatant fractions were essentially the same as those found for the enzyme in crude homogenates. However, the use of photon correlation spectroscopy to estimate particle size indicated that the MAO in the high-speed supernatant fractions was not soluble, but was localized in a particle of size in the range of 250–660 Å.

The most commonly used methods for the solubilization of monoamine oxidase (MAO, monoamine O_2: oxidoreductase, EC 1.4.3.4) involve treatment with the non-ionic detergent Triton X-100 [1], sonication [2] or a combination of both methods [3, 4]. However, solubilization by Triton X-100 treatment produces an alteration in the reaction pathway followed by MAO as well as changes in the substrate and inhibitor specificities of the enzyme [5–7]. Furthermore, Triton X-100 appears to inhibit MAO activity in a temperature-dependent manner [8].

In this study, the effect of sonication upon the multiple forms of MAO has been investigated. It has been reported that sonication of various membrane preparations produces a particulate, rather than a "soluble" fraction [9–12, for review see 13], and in consequence, the nature of the sonicated preparation has been investigated in the present study by use of a laser light scattering technique.

MATERIALS AND METHODS

Preparation of sonicated fractions. Mitochondrial membrane vesicles were made as described in the preceding paper [8] from rat liver crude homogenates in "sucrose buffer" (0.25 M sucrose, 10 mM potassium phosphate, pH 7.8). The vesicle fractions were set to a protein concentration of 8 mg.ml^{-1}, divided into 4 ml aliquots, and sonicated for periods of 30 sec separated by intervals of 15 sec. The times of sonication given in the text refer to the net time of sonication. For example, a sonication time of 4 min refers to 8 periods of sonication for 30 sec plus 8 intervals of 15 sec, i.e. a total time of 6 min. An MSE 150 Watt Ultrasonic Disintegrator operating at 20 kHz (nominal) at an amplitude of 8 μm (peak–peak), with an exponential head, was used. Local overheating was minimized by the immersion of the suspension in an ice–brine mixture. The sonicated fractions were centrifuged at 100,000 *g* for 60 min to give pellets ("membranes") and supernatant fractions ("high-speed supernatants"). The pellets were resuspended in sucrose buffer, and all fractions stored frozen until used for assay. Control membrane preparations were prepared in the same way, but with the sonication step omitted.

Enzyme assays. MAO was assayed radiochemically [14] with [^{3}H]-5-hydroxytryptamine (5-HT), [^{3}H]-tyramine, [^{14}C]-β-phenethylamine and [^{3}H]-benzylamine as substrates. When clorgyline was used to inhibit the activity of MAO, it was preincubated with the enzyme preparation for 20 min before the addition of substrate, to allow for the irreversible inhibition of MAO-A [15, 16].

Succinate dehydrogenase (SDH), fumarase, malate dehydrogenase (MDH) and acid phosphatase activities were assayed spectrophotometrically by standard techniques [17–20]. Protein content of the fractions was assayed by the method of Lowry *et al.* [21], with bovine serum albumin as standard.

Determination of particle size in the high-speed supernatants. Particle size in the high-speed supernatants was determined by the method of photon correlation spectroscopy [22]. Light from an 0.6328 μm, 5 watt helium–neon laser was scattered by a high-speed supernatant fraction within a cuvette placed in a water bath at 30°. The scattered light was observed at an angle of 90° by a photomultiplier (PM) tube. The PM signal was electronically shaped into discrete pulses and assayed by a Malvern K7023 Digital Autocorrelator. Analysis of the rate of decay of the measured correlation function gave the *z*-averaged diameter for monodispersed particles of spherical shape. The validity of this method was verified by the use of Dow latex particles of known size [see 22].

Materials. The radioactive substrates for MAO,

* Present address: Department of Pharmacology, University of Umeå, S-901 87 Umeå, Sweden.

5-hydroxytryptamine-[G-^{3}H]-creatinine sulphate and tyramine-[side chain-1,2-^{3}H]-hydrochloride were obtained from the Radiochemical Centre, Amersham, U.K. β-Phenethylamine-[ethyl-1-^{14}C]-hydrochloride was obtained from New England Nuclear GMBH, Dreieichenchain, Germany. Benzylamine-[ring-3-^{3}H]-hydrochloride was a custom synthesis by the Radiochemical Centre, Amersham, U.K. Clorgyline hydrochloride was a gift from May & Baker Ltd., Dagenham, U.K. All other reagents were of analytical grade wherever possible. Male Wistar rats were obtained from A. J. Tuck and Son, Rayleigh, U.K.

RESULTS

The yields of the total protein, the outer membrane marker enzyme MAO, the inner membrane marker SDH, and the mitochondrial membrane contaminants fumarase, MDH and acid phosphatase (marker enzymes for the mitochondrial matrix and lysosomes) after different periods of sonication are shown in Table 1. With each time of sonication, about 45 per cent of the MAO activity and the protein content was found in the high-speed supernatant fractions, the remainder being associated with the membrane fractions. There was a considerable inhibition of SDH activity, and little or no activity was recovered in the high-speed supernatant fractions. The contaminants fumarase, MDH and acid phosphatase were also released into the high-speed supernatants by the sonication process. Preliminary experiments indicated that sonication for periods of as little as 30 sec produced a considerable release of enzyme activities into the high-speed supernatant. The release was unchanged if the mitochondria were frozen and thawed prior to sonication for a net time of 4 min. In all subsequent experiments reported in this study, a net time of sonication of 4 min was used.

The substrate-specificity of MAO in the high-speed supernatant fractions after 4 min of sonication appeared to be the same as that found for the crude homogenates (Table 2). The sensitivity of the MAO to inhibition by Tris–HCl buffer, and the ratio of MAO activities assayed under an atmosphere of oxygen: atmosphere of air ("oxygen ratio") were not changed by the sonication procedure (Table 2), although the sensitivity to Tris–HCl and the oxygen ratio value depended upon the amine substrate used to assay for MAO activity, as reported previously [23, 24]. With two preparations, mean K_m values of the MAO (assayed under an atmosphere of oxygen) towards tyramine of 159 and 235 μM were found for the crude homogenate and high-speed supernatant fractions, respectively. The mean V_{max} values for these two fractions were 5.01 and 26.47 nmole (of tyramine metabolized). (mg protein)$^{-1}$.min^{-1}, respectively. The biphasic curves of inhibition of the deamination of tyramine by clorgyline in these two fractions were the same (Fig. 1).

Preliminary experiments indicated that the MAO in the high-speed supernatant fractions did not migrate when subjected to gel electrophoresis (5% acrylamide monomer, containing 5% w/w methylene-bis-acrylamide, chemically polymerized with tetraethyl methylene diamine and ammonium persulphate). When the material was loaded on to a Biogel A-50 column and eluted with sucrose buffer, the MAO activity was recovered in the void volume.

Table 1. The effect of various periods of sonication upon the yields of some marker enzymes of rat liver*

	Yield (%)					
	Protein	MAO	SDH	Fumarase	MDH	Acid phosphatase
No sonication						
High-speed S/N	14.2	7.7	1.8	30.5	3.4	15.5
Membranes	85.8	92.3	98.2	69.5	96.6	84.5
4 min sonication						
High-speed S/N	44.8	39.3	2.3	65.3	40.1	63.7
Membranes	56.6	65.7	37.0	9.8	60.2	39.0
10 min sonication						
High-speed S/N	50.0	40.6	7.0	71.6	35.3	49.1
Membranes	44.1	68.5	37.1	7.2	45.0	30.5
20 min sonication						
High-speed S/N	45.8	45.3	3.5	78.8	32.4	41.5
Membranes	52.3	71.9	53.3	4.3	56.1	41.4

* Mitochondrial membrane vesicles derived from a pool of livers from 6 rats (body weight 392 ± 18 g) were sonicated for 0, 4, 10 and 20 min (net) and then centrifuged at 100,000 g for 60 min to yield a high-speed supernatant fraction ("high-speed S/N") and a pellet, which was resuspended in sucrose buffer ("membranes"). The yields of the marker enzymes in the vesicle fractions were: protein, 9.6 per cent; MAO (tyramine), 36.6 per cent; SDH, 39.4 per cent; fumarase, 11.0 per cent; MDH, 8.8 per cent; acid phosphatase, 15.0 per cent, with respect to the total activity in the respective crude homogenate. All values shown in the table are yields, calculated as the total activity in each fraction as a percentage of the total activity in the combined high-speed S/N and membrane fractions of the unsonicated vesicle fraction. All assays were performed in triplicate. The substrate for MAO was tyramine (0.25 mM).

Table 2. Properties of MAO in crude homogenates and high-speed supernatant fractions (high-speed S/N) of rat liver*

	Substrate for MAO			
	5-HT (0.25 mM)	Tyramine (0.25 mM)	β-Phenethylamine (0.05 mM)	Benzylamine (0.25 mM)
Yield				
Crude homogenate	100	100	100	100
High-speed S/N	5.59 ± 1.27	5.83 ± 1.37	5.71 ± 1.33	5.63 ± 1.39
Oxygen ratio				
Crude homogenate	1.18 ± 0.06	1.47 ± 0.04	1.78 ± 0.05	1.21 ± 0.03
High-speed S/N	1.29 ± 0.07	1.51 ± 0.06	1.78 ± 0.12	1.21 ± 0.05
Tris sensitivity (%)				
Crude homogenate	39.9 ± 3.0	47.3 ± 1.6	54.8 ± 1.8	92.9 ± 7.2
High-speed S/N	38.5 ± 4.7	42.5 ± 2.7	48.2 ± 4.2	86.8 ± 4.4

* The net time of sonication was 4 min. Yields were calculated as the total activity in the high-speed S/N fractions as a percentage of the total activity in the respective crude homogenate fractions. "Oxygen ratios" were calculated as the activity of MAO assayed under an atmosphere of oxygen/activity in air. "Tris sensitivities" were calculated from the activity of MAO in the presence of 20 mM Tris–HCl, expressed as a percentage of the activity in the absence of Tris. All results are expressed in terms of means ± S.E.R. from triplicate determinations of activities in three groups of fractions, each derived from the livers of six rats (mean body weight 363 g).

On two preparations of high-speed supernatants, each derived from the livers of six rats, the particle sizes were determined. The results were:

1st experiment (high-speed supernatant in 0.25 M sucrose, no phosphate)—mean size of particles 660 ± 130 Å (half width of the gaussian distribution) calculated from 5 determinations of particle size;

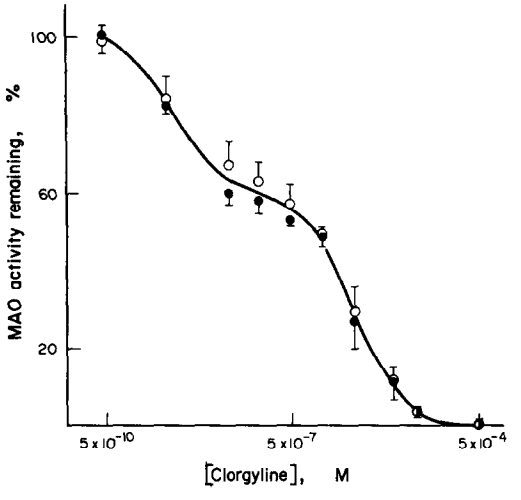

Fig. 1. The effect of clorgyline on the *in vitro* deamination of 0.25 mM tyramine by crude homogenates (●) and sonicated high-speed supernatant fractions (○) of rat liver. The high-speed supernatant fractions were obtained by sonication of mitochondrial membrane vesicle fractions for 4 min (net) followed by centrifugation at 100,000 *g* for 60 min. Each point represents the mean ± S.E.R. of triplicate determinations of activity in three groups of fractions, each derived from the livers of six rats (mean body weight 363 g), expressed as a percentage of the activity in the absence of clorgyline, plotted against the molar concentration of clorgyline.

2nd experiment (high-speed supernatant in 0.25 M sucrose, 10 mM phosphate, pH 7.8)—mean size of particles 277 ± 108 Å (half width of the gaussian distribution) calculated from 6 determinations of particle size.

DISCUSSION

Short bursts of sonication were found to be effective for the production of a preparation of MAO that did not sediment upon centrifugation at 100,000 *g* for 60 min. Both membrane integral proteins and contaminants were released to roughly the same extent by sonication, with the exception of SDH activity (Table 1), which was inhibited, in agreement with a previous study [25]. The MAO in the high-speed supernatant fractions had similar properties to the MAO in the corresponding crude homogenates, with respect to substrate specificity, sensitivity to inhibition by Tris–HCl and "oxygen ratio" (Table 2). The K_m of the MAO in the two fractions towards tyramine were similar, as were their responses to inhibition by clorgyline (Fig. 1). Thus it would seem that, unlike treatment with Triton X-100, sonication is without effect on the kinetic properties of MAO.

Although the MAO does not appear to sediment upon centrifugation at 100,000 *g* for 60 min, the MAO in the high-speed supernatant fractions appears to be particulate in nature, as it does not migrate upon gel electrophoresis under conditions where MAO solubilized by a combination of sonication and Triton X-100 does migrate [26], and is eluted in the void volume of a Biogel A-50 column, indicating that the MAO is attached to a particle of size at least 50 million daltons. Sonication has been reported to produce preparations that do not sediment upon centrifugation at 100,000 *g* for 60 min, but are still particulate in nature [9, 10]. These particles have been studied under the electron microscope, and their diameters estimated as 50–200 Å

for sonicated rat liver mitochondria [11], and 100–600 Å for sonicated erythrocytes [12]. In this investigation, the particles produced by sonication of rat liver vesicles are in the range of 250–660 Å, although this size probably represents the hydrated size of the particles, whereas the sizes estimated by electron microscopy are not hydrated sizes. These diameters are the same order of magnitude as found for phosphatidylcholine micelles, which had diameters of about 250 Å [27]. It is possible that the particle sizes determined in this study could be due to a single large disaggregated protein, or some sort of mixed "micelle" containing no MAO activity. Both possibilities are unlikely, as the MAO was eluted off the Biogel A-50 column with an estimated size greater than 50 million daltons (which would be detected by the laser scattering technique). As the molecular weight of purified rat liver MAO is in the range 150,000–380,000 daltons [28–31], thought to be composed of subunits of size approximately 60,000 daltons [32–34], it would seem that the laser light scattering technique is detecting a mixed "micelle", with MAO as one of its constituents. Thus it would seem reasonable to suggest that the effect of short bursts of sonication is to dissociate the membrane constituents, which then reaggregate to form mixed "micelles".

Acknowledgements—The authors are grateful to Dr. D. Parkinson for his advice and help. B.A.C. would like to thank the British Heart Foundation for a generous grant, and the Royal Society for the loan of an ultracentrifuge. At the time of these experiments, C.J.F. was a Medical Research Council Scholar. E.K.M. would like to thank the Royal Society and the British Diabetic Association for Grants. M.D.O'C. is an NIH Postdoctoral Research Fellow.

REFERENCES

1. K. T. Yasunobu, I. Igaue and B. Gomes, *Adv. Pharmac.* **6A**, 43 (1968).
2. M. Zile and H. A. Lardy, *Archs Biochem. Biophys.* **82**, 411 (1959).
3. M. B. H. Youdim and M. Sandler, *Biochem. J.* **105**, 43P (1967).
4. J. C. Shih and S. Eiduson, *J. Neurochem.* **21**, 41 (1973).
5. I. S. Severina and N. I. Zhivotova, *Biokhimiya* **38**, 202 (1973).
6. M. D. Houslay and K. F. Tipton, *Biochem. J.* **145**, 311 (1975).
7. K. F. Tipton, M. D. Houslay and T. J. Mantle, in *Monoamine Oxidase and its Inhibition.* Ciba Foundation Symposium 39 (Eds. G. E. W. Wolstenholme and J. Knight), pp. 5–16. Elsevier, Exerpta Medica, North Holland, Amsterdam (1976).
8. C. J. Fowler, B. A. Callingham, T. J. Mantle and K. F. Tipton, *Biochem Pharmac.* **29**, 1177 (1980).
9. M. R. J. Salton and A. Netschey, *Biochim. biophys. Acta* **107**, 539 (1965).
10. C. J. Brakenridge and H. S. Bachelard, *Int. J. Protein Res.* **1**, 157 (1969).
11. I. V. Verevkina, V. Z. Gorkin, V. M. Mityushin and I. Ye. El'piner, *Biofizika* **9**, 503 (1964).
12. S. A. Rosenberg and J. R. McIntosh, *Biochim. biophys. Acta* **163**, 285 (1968).
13. I. Kahane and S. Razin, *Biochim. biophys. Acta* **249**, 159 (1971).
14. B. A. Callingham and R. Laverty, *J. Pharm. Pharmac.* **25**, 940 (1973).
15. T. Egashira, B. Ekstedt and L. Oreland, *Biochem. Pharmac.* **25**, 2583 (1976).
16. C. J. Fowler and B. A. Callingham, *J. Pharm. Pharmac.* **30**, 304 (1978).
17. D. E. Green, S. Mii and P. M. Kohout, *J. biol. Chem,* **217**, 551 (1955).
18. E. Racker, *Biochim. biophys. Acta* **4**, 211 (1950).
19. L. Siegel and S. Englard, *Biochim. biophys. Acta* **54**, 67 (1961).
20. K. Linhardt and K. Walter, in *Methods of Enzymatic Analysis* (Ed. H. U. Bergmeyer), pp. 779–787. Academic Press, New York (1963).
21. O. H. Lowry, N. J. Rosebrough, A. L. Farr and R. J. Randall, *J. biol. Chem.* **193**, 265 (1951).
22. E. K. Matthews and M. D. L. O'Connor, *J. Physiol., Lond.* **278**, 1P (1978).
23. C. J. Fowler, B. A. Callingham and M. D. Houslay, *J. Pharm. Pharmac.* **29**, 411 (1977).
24. C. J. Fowler and B. A. Callingham, *Biochem. Pharmac.* **27**, 1995 (1978).
25. G. H. Hogeboom and W. C. Schneider, *Nature, Lond.* **166**, 302 (1950).
26. D. Parkinson, unpublished.
27. C.-H. Huang, *Biochemistry* **8**, 344 (1969).
28. M. B. H. Youdim and T. L. Sourkes, *Can. J. Biochem.* **44**, 1397 (1966).
29. T. L. Sourkes, *Adv. Pharmac.* **6A**, 61 (1968).
30. M. B. H. Youdim and G. G. S. Collins, *Eur. J. Biochem.* **18**, 73 (1971).
31. M. D. Houslay and K. F. Tipton, *Biochem. J.* **135**, 173 (1973).
32. R. McCauley, *Biochem. Pharmac.* **25**, 2214 (1976).
33. B. A. Callingham and D. Parkinson, in *Monoamine Oxidase; Structure, Function and Altered Functions* (Eds. T. P. Singer, R. W. Von Korff and D. L. Murphy). Academic Press, New York, in press.
34. B. Y. Yoo and L. Oreland, unpublished.