9·0 at 5 min, 16·9 at 15 min, and 20·0 at 30 min. Amantadine at 1×10^{-4} M inhibited this uptake by 13, 19 and 23 per cent respectively.

The emphasis of this study was on the comparison of values obtained from different systems with similar experimental procedures. The actual numbers can be manipulated by varying certain parameters such as order of addition, preincubation, incubation time, etc. In fact, the extent of inhibition can be increased by preincubating the synaptosomes with amantadine for various times. But since this is true for all preparations, the comparative picture remains the same.

The complete lack of effect of 1×10^{-4} M amantadine on dopamine uptake by striatal synaptosomes argues strongly against this being involved in the mechanism of the anti-Parkinson activity of this drug. The inhibition of dopamine uptake by hypothalamic synaptosomes probably reflects an effect on exchange of dopamine with endogenous norepinephrine, since both the uptake and the inhibition are drastically reduced by reserpine pretreatment.

Amantadine does inhibit the uptake of norepinephrine, particularly into hypothalamic synaptosomes, and the lack of effect of reserpine pretreatment suggests that this is a direct effect on the membrane process itself. Whether or not this effect has any physiological significance will most likely depend on the dose of amantadine and may assert itself at high levels. Its effects in the normal amantadine dose range of 2–3 mg/kg are almost certainly minimal unless Parkinsonian patients exhibit a greatly increased sensitivity.

Recently, effects of amantadine on the synthesis and/or release of dopamine have been proposed with some supporting evidence.[11,15] The validity of these proposals will have to be determined by further experimental work, but the hypothesis that uptake inhibition is involved seems very unlikely in view of the results reported here.

*Central Research Department** W. F. HERBLIN
E.I. du Pont de Nemours & Company,
Wilmington, Del. 19898, *U.S.A.*

* Contribution No. 1865.

REFERENCES

1. R. S. SCHWAB, A. C. ENGLAND, JR., D. C. POSKANZER and R. R. YOUNG, *J. Am. med. Ass.* **208,** 1168 (1969).
2. J. D. PARKES, K. J. ZILKHA, D. M. CALVER and R. P. KNILL-JONES, *Lancet* **1,** 259 (1970).
3. G. C. COTZIAS, P. S. PAPAVASILIOU and R. GELLENE, *New Engl. J. Med.* **280,** 337 (1969).
4. D. B. CALNE and M. SANDLER, *Nature, Lond.* **226,** 21 (1970).
5. J. T. COYLE and S. H. SNYDER, *Science, N.Y.* **166,** 899 (1969).
6. V. FLORIO and V. G. LONGO, *Physiol. Behav.* **6,** 465 (1971).
7. J. E. THORNBURG and K. E. MOORE, *Pharmacologist* **13,** 202 (1971).
8. E. A. FLETCHER and P. H. REDFERN, *J. Pharm. Pharmac.* **22,** 957 (1970).
9. S. SYMCHOWICZ, C. A. KORDUBA and J. VEALS, *Life Sci.* **10,** 35 (1971).
10. U. STRÖMBERG, T. H. SVENSSON and B. WALDECK, *J. Pharm. Pharmac.* **22,** 960 (1970).
11. B. SCATTON, A. CHERAMY, M. J. BESSON and J. GLOWINSKI, *Eur. J. Pharmac.* **13,** 131 (1970).
12. J. GLOWINSKI and L. L. IVERSEN, *J. Neurochem.* **13,** 655 (1966).
13. J. T. COYLE and S. H. SNYDER, *J. Pharmac. exp. Ther.* **170,** 221 (1969).
14. A. S. HORN, J. T. COYLE and S. H. SNYDER, *Molec. Pharmac.* **7,** 66 (1971).
15. R. P. GRELAK, R. CLARK, J. M. STUMP and V. G. VERNIER, *Science, N.Y.* **169,** 203 (1970).

Biochemical Pharmacology, Vol. 21, pp. 1995–1998. Pergamon Press, 1972. Printed in Great Britain.

Multiple forms of monoamine oxidase. Comparison of *in vitro* and *in vivo* inhibition patterns

(*Received* 21 *January* 1972; *accepted* 7 *March* 1972)

MITOCHONDRIAL monoamine oxidase (MAO; EC 1.4.3.4) from various animal species and tissues has recently been purified and shown to occur in several molecular forms.[1–6] Although these *in vitro*

findings appear to be consistent, the extent to which they represent the *in vivo* status of the enzyme is at present unknown. Whilst certain evidence points to the multiple forms being genuine constituents of the mitochondrion,[7] direct proof of this supposition is difficult to obtain. This paper describes an indirect approach to the problem, based on the different sensitivities of the isoenzymes to MAO inhibitors (MAOI). After MAOI pretreatment of the rat prior to sacrifice, differential inhibition of subsequently isolated multiple forms was observed. This result is a further indication that the multiple forms are unlikely to be artifactual.

Materials and Methods

Mitochondrial MAO from rat and human liver was prepared and purified as described previously.[3] Briefly, mitochondria were isolated,[8] washed in 0·3 M sucrose and suspended in ice-cold phosphate buffer (0·05 M, pH 7·4). After sonication and the addition of 1·5% v/v Triton X-100, the enzyme was fractionated by ammonium sulphate precipitation and purified by filtration through Sephadex G-200. As previously reported,[3] polyacrylamide gel electrophoresis of the purified enzyme preparation resulted in five bands of activity, designated MAO.[1–5] This separation procedure is consistent and reproducible.[7] The protein content of the gel in the area of each band was extracted[3] and stored at 4° until required.

To measure the inhibition of MAO in the intact rat, groups of five animals were injected intraperitoneally with either tranylcypromine (0·5 mg/kg), pargyline (2·0 mg/kg) or clorgyline (2·0 mg/kg). After 2 hr, they were killed by cervical dislocation and MAO isoenzymes were prepared from pooled liver samples as described above. Enzyme activities were estimated spectrophotofluorimetrically using kynuramine as substrate[9] and radiochemically with ^{14}C-tyramine as substrate.[10] Protein nitrogen was estimated[11] using bovine serum albumin as standard. The inhibitors used did not interfere with these assay systems.

Kynuramine was obtained from Sigma Chemicals Co., ^{14}C-tyramine (50 mc/mM) from the Radiochemical Centre, Amersham; clorgyline (M & B 9302) was a gift from Dr. D. R. Maxwell, May & Baker, Ltd., Dagenham. All chemicals used were of the highest purity obtainable.

Results

As enzymes in the presence of irreversible inhibitors do not obey Michaelis–Menten kinetics, it was only possible to measure apparent K_i values. Enzyme samples were incubated with different concentrations of inhibitor at 37° for 30 min prior to the addition of substrate. Using at least two substrate concentrations, the results were expressed graphically,[12] from which an estimate of apparent K_i value was made.

TABLE 1. THE EFFECT OF VARIOUS INHIBITORS *in vitro* ON RAT AND HUMAN LIVER MITOCHONDRIAL MONOAMINE OXIDASE ISOENZYMES

Inhibitor	Kynuramine				^{14}C-Tyramine			
	MAO_1	MAO_2	MAO_3	MAO_4	MAO_1	MAO_2	MAO_3	MAO_4
				Rat				
Iproniazid	9·7	1·2	1·4	3·9	3·4	3·5	2·1	1·2
Pheniprazine	6·5	7·5	2·9	6·1	0·18	0·06	0·08	0·65
Pargyline	0·08	0·04	0·035	0·02	0·02	0·05	0·02	0·09
Harmaline	240	160	130	650	140	170	520	270
Clorgyline	0·44	0·61	0·14	5·0				
				Human				
Iproniazid	5·5	6·4	4·1	0·35	1·5	0·3	1·0	68
Pheniprazine	0·34	0·38	0·95	0·12	0·05	0·05	0·13	0·59
Pargyline	0·06	0·055	0·08	0·11	0·02	0·01	0·01	0·47
Harmaline	350	220	140	830	180	180	310	780

Enzyme samples were incubated for 30 min prior to the addition of kynuramine or ^{14}C-tyramine as substrates. Apparent inhibitor constants (K_i values, μM) were calculated from Dixon plots[12] using at least two substrate concentrations. MAO_5 was not examined. Each value is the mean of at least two estimations.

Table 1 shows the inhibitory effect of iproniazid, pheniprazine, pargyline, harmaline and clorgyline on rat and human liver MAO,[1-4] using kynuramine and tyramine as substrates. The sensitivity of the isoenzymes to a single inhibitor varied widely; for example, using kynuramine as substrate and iproniazid as inhibitor, the apparent K_i for human MAO_2 is almost twenty times that of MAO_4. The sensitivity of the isoenzymes to inhibitors appeared to vary with the substrate employed; thus the K_i of rat liver MAO_1 towards pheniprazine was more than thirty times higher with kynuramine than with tyramine. Although human and rat liver enzymes each exist in five different forms and present a similar gross electrophoretic pattern, their respective sensitivities to inhibitors are not identical; using, for example, pheniprazine as inhibitor and kynuramine as substrate, the K_i of human liver MAO_4 is at least fifty times lower than that of the same isoenzyme from rat liver. It was not possible to harvest sufficient MAO_5 (the isoenzyme moving from anode to cathode) for reliable measurements to be made.

TABLE 2. THE *in vivo* EFFECT OF INHIBITORS ON RAT LIVER MITOCHONDRIAL MONOAMINE OXIDASE ISOENZYMES

	Kynuramine				^{14}C-Tyramine			
Inhibitor	MAO_1	MAO_2	MAO_3	MAO_4	MAO_1	MAO_2	MAO_3	MAO_4
Clorgyline	69	30	63	59	42	75	62	0
Tranylcypromine	47	96	52	44	69	99	18	4
Pargyline	61	95	31	40	81	79	10	0

Rats were injected intraperitoneally with either pargyline, tranylcypromine or clorgyline (2·0, 0·5 and 2·0 mg/kg respectively) and sacrificed 2 hr later. The results are expressed as per cent inhibition compared with saline-injected controls, using two different substrates and are mean values of three separate determinations. MAO_5 was not examined.

After injection of rats with pargyline, tranylcypromine or clorgyline, specific activities of the subsequently prepared isoenzymes were measured and expressed as percentages of control values (see Table 2). Pronounced differences were observed. Using tyramine as substrate, pretreatment with all three drugs resulted in a lesser degree of inhibition of MAO_4 than of the other isoenzymes investigated. This effect was not observed when kynuramine was used as substrate. There was little correlation between extent of *in vivo* inhibition and corresponding K_i values estimated *in vitro*.

Discussion

The wide variation in *in vitro* inhibition characteristics of rat and human liver MAO are in line with previous observations both on these and other tissues.[13] The apparent K_i values reported have little meaning in terms of kinetic constants; however, because all experiments were carried out under identical conditions, it is possible to make comparisons between the enzyme forms and inhibitors used. As to the relevance of these findings to the *in vivo* situation, it is at least clear that variations in degree of inhibition of the different forms do occur in response to drug pretreatment (Table 2). These data point strongly to the existence of MAO isoenzymes in the intact mitochondrion, for if the enzyme multiplicity were a result of the preparative procedure, it might be expected that all forms would be inhibited to a similar extent.

However, the sensitivity of the enzyme forms to MAO inhibitors *in vitro* does not indicate the effectiveness of the inhibitors when administered to the whole animal. It seems possible that the presence of compartmental barriers in living tissues might account for the differences observed compared with the action of these drugs on isolated solubilized enzyme protein. Moreover, MAO inhibitors in the intact animal may be metabolized to form secondary compounds, also active inhibitors of MAO but perhaps with a different pattern of inhibition; iproniazid, for example, is thought to be transformed *in vivo* to another active compound, isopropylhydrazine.[14] In addition, the affinity of MAO for both substrates and inhibitors may be altered by the solubilization procedure.

A further point arises of potential clinical importance. The variable hypertensive effect of tyramine in patients treated with different MAO inhibitors is well described, with tranylcypromine being potentially one of the most dangerous.[15] If a particular isoenzyme were responsible for a major proportion of the *in vivo* inactivation of a substrate such as tyramine, its selective inactivation by a relatively specific inhibitor might have deleterious consequences in the presence of an excess of that

substrate. The intense inhibitory effect of tranylcypromine on rat MAO_2 using tyramine as substrate (Table 2) points to an urgent need for comparable observations in man.

Acknowledgements—G. G. S. Collins and M. B. H. Youdim were respectively supported by the Medical Research Council and the Wellcome Trust.

Bernhard Baron Memorial Research Laboratories,
and Institute of Obstetrics and Gynaecology,
Queen Charlotte's Maternity Hospital,
London

G. G. S. COLLINS*
M. B. H. YOUDIM
M. SANDLER

* Present address: Department of Pharmacology, School of Pharmacy, Brunswick Square, London WC1N 1AX.

REFERENCES

1. M. B. H. YOUDIM and M. SANDLER, *Biochem. J.* **105,** 43 (1967).
2. G. G. S. COLLINS, M. B. H. YOUDIM and M. SANDLER, *FEBS Letters* **1,** 215 (1968).
3. M. B. H. YOUDIM, G. G. S. COLLINS and M. SANDLER, in *Enzymes and Isoenzymes. Structure, Properties and Function*, Vol. 18 (Ed. D. SHUGAR), p. 281. Academic Press, London (1970).
4. H. C. KIM and A. D'IORIO, *Can. J. Biochem.* **46,** 295 (1968).
5. V. Z. GORKIN, *Experientia* **25,** 1142 (1969).
6. B. GOMES, I. IGAUE, H. G. KLOEPFER and K. T. YASUNOBU, *Archs. Biochem. Biophys.* **132,** 16 (1969).
7. M. B. H. YOUDIM and G. G. S. COLLINS, *Eur. J. Biochem.* **18,** 73 (1971).
8. J. HAWKINS, *Biochem. J.* **50,** 577 (1952).
9. M. KRAML, *Biochem. Pharmac.* **14,** 1684 (1965).
10. D. S. ROBINSON, W. LOVENBERG, H. KEISER and A. SJOERDSMA, *Biochem. Pharmac.* **17,** 109 (1968).
11. O. H. LOWRY, N. J. ROSEBROUGH, A. L. FARR and R. J. RANDALL, *J. biol. Chem.* **193,** 265 (1951).
12. M. DIXON and E. C. WEBB, *Enzymes*, 2nd Ed. Longmans, London (1963).
13. M. B. H. YOUDIM, G. G. S. COLLINS and M. SANDLER, *Nature, Lond.* **223,** 629 (1969).
14. A. N. DAVISON, *Biochem. J.* **67,** 316 (1957).
15. B. BLACKWELL, *Lancet* **2,** 849 (1963).