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THE TURNOVER OF THE A- AND B-FORMS OF MONOAMINE OXIDASE IN RAT LIVER

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Abstract—The inhibition of rat liver monoamine oxidase was determined following the intraperitoneal injection of the inhibitors clorgyline and pargyline in order to establish the concentration ranges in which substrate-selective inhibition occurred. The rate of recovery of the activity of the A-form of the enzyme after inhibition by clorgyline was determined using tyramine and serotonin as substrates, and the rate of recovery of the activity of the B-form after inhibition by pargyline was determined using tyramine and 2-phenylethylamine as substrates. No significant differences could be detected between the rates of recoveries of the two forms which corresponded to a rate constant for degradation of the enzyme of about 0.27 day^{-1}.

Studies with the irreversible monoamine oxidase inhibitor clorgyline led Johnston [1] to conclude that there were two species of the enzyme in rat brain which differed in their sensitivities to inhibition and substrate specificities. The species that was more sensitive to inhibition was active towards serotonin, whereas the activity towards benzylamine was less sensitive to inhibition. Johnston termed these the A- and B-forms of the enzyme, respectively, and showed that they were both active towards tyramine. Subsequent work has shown the behaviour of the enzyme from rat liver to be similar to that of the brain enzyme [2, 3] and 2-phenethylamine has been shown to resemble benzylamine in being a specific substrate for the B-form [3, 4], although the specificities of the two forms are not the same in all other organs and animal species (see ref. 5 for review). In addition the irreversible inhibitors Lilly 51641 [6] and PCO [7] have been shown to resemble clorgyline in being more potent inhibitors of the A-form of the enzyme, whereas deprenyl [8] and pargyline [9] are more potent inhibitors of the B-form.

The nature of the two forms of the enzyme has been the subject of considerable speculation (see refs. 10 and 11 for reviews). It has been suggested that they may represent two different enzymes, but attempts to separate them in a pure state, so that their protein structures could be compared, have been unsuccessful. A close similarity between the two forms from rat liver is implied by the observation that they are immunologically identical [12]. The possibility that the two forms of the enzyme could result from the presence of two different active sites on the same enzyme molecule has also been considered [13, 14].

An alternative suggestion is that the two forms could result from modifications of the properties of a single enzyme by its membrane-bound environment [15]. A considerable amount of evidence has been educed to show that the properties of the enzyme are indeed affected by its lipid environment (see refs. 10 and 16 for reviews) and the observation that in human liver the two forms are located on different faces of the mitochondrial outer membrane [17] might imply that they exist in different environments. In addition, it has been shown that the selective effects of clorgyline and deprenil may be, at least in part, mediated by their lipid solubilities [18], but there has been no report of successful interconversion of the two forms by lipid substitution.

Several groups have determined the rate of degradation of the enzyme by measuring the rate of reappearance of enzyme activity following inhibition with pargyline [19–22], but these have not attempted to distinguish between the recoveries of the two different forms. In this paper we wish to report studies on the turnover of rat liver monoamine oxidase designed to investigate possible differences between the rates of degradation of the two forms.

MATERIALS AND METHODS

[1-^{14}C]-labelled serotonin creatinine sulphate and tyramine hydrochloride were obtained from the Radiochemical Centre, Amersham, Bucks, U.K. and [1-^{14}C]-labelled 2-phenethylamine hydrochloride was obtained from New England Nuclear, Boston, MA, U.S.A. Clorgyline (M & B 9302) and pargyline hydrochloride were kind gifts from May and Baker, Dagenham, Essex, U.K. and Abbott Laboratories, Queenborough, Kent, U.K., respectively. Other chemicals were of the highest purity commercially available.

Assay methods. The activity of monoamine oxidase was determined by a modification of the method of Otsuka and Kobayashi [22]. The reaction mixtures contained in a total volume of 0.5 ml, 80 mM sodium phosphate buffer, pH 7.2, 0.05 μCi labelled substrate and the enzyme sample. When tyramine and serotonin were used as substrates, their final concentrations in the assay were 1.0 mM, whereas a concentration of 0.1 mM was used with phenethylamine, these values representing between 5 and 10

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times the K_m value in each case [3]. The buffer–enzyme mixtures were incubated at 30° in a shaking water bath and the reaction was then initiated by the addition of the substrate.

After 20 min the reaction was stopped by the addition of 0.5 ml of 1.0 M citric acid. Blanks were prepared by adding the citrate to the reaction mixture before the substrate. The products of the reaction were extracted into 10 ml of toluene–ethylacetate (1 : 1 v : v) containing 0.6% 2,5 diphenyloxazole (P.P.O.) and the organic phase was separated, after freezing the aqueous layer, and counted in a Packard liquid scintillation counter. Time-courses of the reaction were used to ensure that product formation proceeded linearly for at least 30 min in each case. Proportionality between initial velocity and protein concentration was also established within the concentration ranges represented by the enzyme samples.

Protein concentration was determined by the micro-biuret method of Goa [23] using bovine serum albumin as the standard.

Inhibition studies. Male Wistar rats in the weight range 140–160 g were injected intraperitoneally with clorgyline or pargyline in 0.9% NaCl and in a total volume of 0.5 ml/150 g body weight. Controls were injected with a similar volume of the saline solution. The rats were decapitated and the livers and brains were removed and homogenized in 5 vol of 10 mM sodium phosphate buffer, pH 7.2. The samples were frozen and stored at −20° until required for assay.

The presence of free inhibitors in the samples was determined by assaying control and inhibitor-treated homogenates separately and as a mixture of the same volumes of the two. In all cases the assay mixtures were incubated for 30 min at 30° in the absence of substrate before the assay was commenced. A difference between the sum of the activities obtained when the two samples were assayed alone and that obtained with the mixture served as an indication that the treated sample contained a significant amount of free inhibitor.

Kinetic studies. Equations describing the reappearance of monoamine oxidase activity following its irreversible inhibition have been presented by Neff and Goridis [20] and by Della Corte and Callingham [21]. After inhibition, the net rate of reappearance of activity will depend upon its rates of synthesis and degradation according to the equation:

$$\frac{\mathrm{dMAO}}{\mathrm{d}t} = k_s - k_d \cdot \mathrm{MAO}, \qquad (1)$$

where MAO represents the enzyme concentration, t represents time, k_s is zero-order rate constant for the enzyme synthesis and k_d is the first-order rate constant for enzyme degradation. After a sufficient time the activity of the enzyme will have risen to a constant steady-state level ($\mathrm{dMAO}/\mathrm{d}t = 0$), when the rate of synthesis will be balanced by the rate of degradation:

$$k_s = k_d\,\mathrm{MAO}_{ss}, \qquad (2)$$

where the subscript *ss* denotes the constant steady-state enzyme concentration.

Combining equations (1) and (2) gives:

$$\frac{\mathrm{dMAO}}{\mathrm{d}t} = k_d\,(\mathrm{MAO}_{ss} - \mathrm{MAO}). \qquad (3)$$

Thus the rate of reappearance of monoamine oxidase activity should follow first-order kinetics depending only upon the rate constant for enzyme breakdown. Since the inhibitor concentrations used were not sufficient completely to inhibit enzyme activity, the activity at the beginning of the time-course was not zero. If the activity at zero-time is defined as MAO_o and that at time t is defined as MAO_t, the equation may be integrated to give:

$$(\mathrm{MAO}_t - \mathrm{MAO}_o) = (\mathrm{MAO}_{ss} - \mathrm{MAO}_o)\,(1 - e^{-k_d t}), \qquad (4)$$

which can be expressed in linear form as:

$$\ln[(\mathrm{MAO}_{ss} - \mathrm{MAO}_t)/(\mathrm{MAO}_{ss} - \mathrm{MAO}_o)] = k_d t. \qquad (5)$$

The value of k_d may be evaluated from a semilogarithmic plot of the rate of reappearance of activity against time. However, the accuracy of this method depends on accurate assessments of the final steady-state concentration of the enzyme and would also be affected by the presence of any residual free inhibitor during the time in which reappearance of activity was being measured. Because of this, the method described above was used only to obtain an initial estimate of the value of k_d which was then used to fit the exponential form, equation (4), by an iterative procedure that was independent of these factors [21]. The half-life of the enzyme ($t^{1/2}$) was calculated from the relationship:

$$\frac{0.693}{k_d} t_{1/2} =$$

RESULTS AND DISCUSSION

Inhibition of monoamine oxidase in vivo. The dose–response curves shown in Figs. 1 and 2 indicate, in agreement with the results of others [25–28], that the selective properties of clorgyline and pargyline can be observed after *in vivo* treatment. At the higher concentration of clorgyline used in these studies (30 mg/kg), inhibition of the activity towards 2-phenethylamine was still relatively small, but incubation of samples of the rat liver homogenate at 30° for 15 min with 10 μM semicarbazide did not result in any significant degree of further inhibition, suggesting that no carbonyl-reagent sensitive activity towards this substrate was present (see, e.g. ref. 24). Inhibition occurred rapidly at that clorgyline concentration, having reached its maximum value when rats were killed 15 min after injection.

The presence of free clorgyline and pargyline in the livers was determined by measuring its ability to inhibit added monoamine oxidase as described earlier. When rats were killed 1 hr after injection of 0.8 mg/kg pargyline, no free inhibitor could be detected in the liver homogenate when 2-phenethylamine was used as the substrate. In contrast, suf-

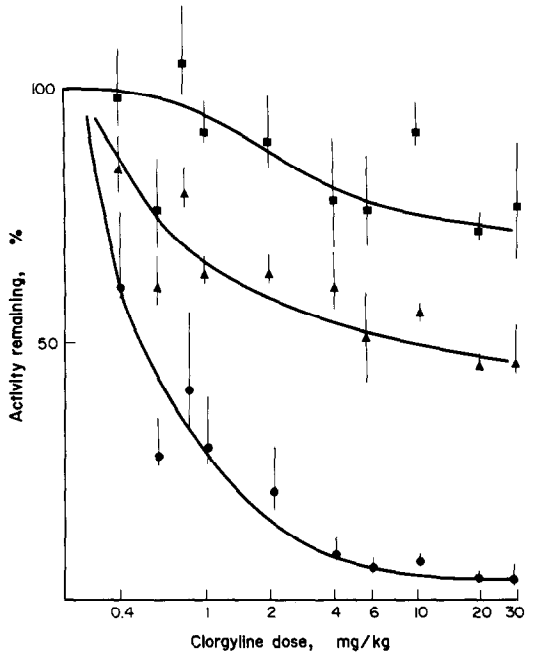

Fig. 1. Inhibition of rat liver monoamine oxidase by *in vivo* treatment with clorgyline. Rats were injected intraperitoneally with the indicated doses of clorgyline in 0.9% saline, and were killed 60 min later. The activities of monoamine oxidase towards serotonin (●), tyramine (▲) and 2-phenethylamine (■) were determined as described in the text. Three rats were used at each concentration, assays were done in duplicate. The error bars indicate the S.E. for each point, which were determined using Fieller's Theorem (see ref. 35) for the standard errors of ratios.

ficient free clorgyline to inhibit 50 per cent of the added monoamine oxidase, assayed with serotonin, could be detected 1 hr after its injection at a concentration of 2 mg/kg. Studies at a higher concentration (30 mg/kg) of intraperitoneally injected clorgyline indicated that the amount of free inhibitor declined rapidly and was not significant after about 3 hr. Nevertheless, this could cause appreciable errors in estimating the true rates of recovery of the enzyme at relatively short times after clorgyline treatment if zero-time was assumed to be the time of injection. The use of an analytical method that does not require a knowledge of the zero-time such as that of Guggenheim [29] or the non-linear iterative method used here [21] should, however, obviate any inaccuracies of this sort.

Studies of the activities of the enzyme in brain homogenates prepared after intraperitoneal injection of clorgyline or pargyline indicated that selective inhibition could also be demonstrated with the enzyme in that organ (see e.g. Fig. 3). Thus the methods used here could also be used for studying the turnover of the brain enzyme.

Recovery of monoamine oxidase activity after inhibition. Doses of 2 mg/kg clorgyline and 0.8 mg/kg pargyline were used to achieve substantial inhibition of the one form of the enzyme without too great inhibition of the other. The time-courses of recovery are shown in Figs. 4 and 5. The zero-time values were taken as those obtained 2 and 3 hr after injection of pargyline and clorgyline, respectively. Estimation of the best fits by the iterative procedure of Della Corte and Callingham [21] gave the results shown in Table 1. The results obtained for the

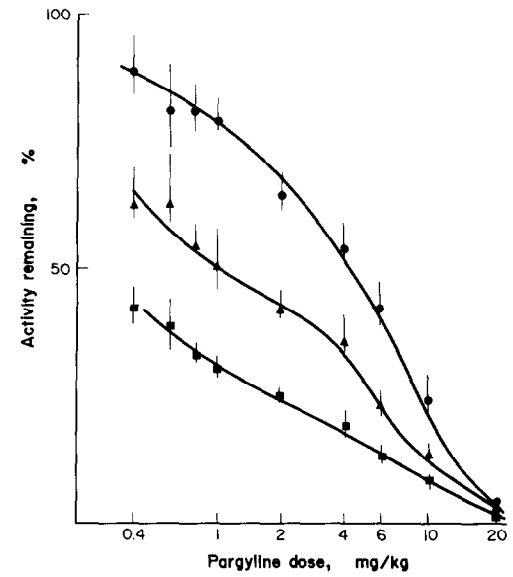

Fig. 2. Inhibition of rat liver monoamine oxidase by *in vivo* treatment with pargyline. The methods used were as described in Fig. 1. Each experimental point represents the mean value (± S.E.) obtained from three rats. (●) Activity towards serotonin, (▲) activity towards tyramine, (■) activity towards 2-phenethylamine.

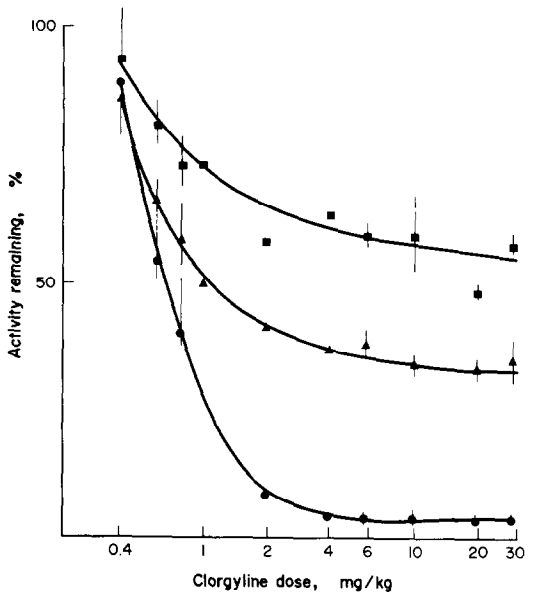

Fig. 3. Inhibition of rat brain monoamine oxidase by *in vivo* treatment with clorgyline. The methods used were as described in Fig. 1. Each point represents the mean value (± S.E.) obtained from three rats, when standard error bars are not shown the value represents the mean of values from two rats. (●) Activity towards serotonin, (▲) activity towards tyramine, (■) activity towards 2-phenethylamine.

Table 1. Kinetic parameters for the degradation of rat liver monoamine oxidase. The data were analysed as described in the text from groups of five rats that were killed at increasing time intervals up to 16 days after injection of the inhibitor; each experimental point was given a weighting factor inversely proportional to its relative standard error in the non-linear regression analysis

Inhibitor	Substrate	K_d (day $^{-1}$) Value ± S.E.	95% confidence limits	Half-life (days) Value (S.E. interval)	95% confidence limits
Clorgyline	Serotonin	0.227 ± 0.035	0.146 0.308	3.05 (2.64–3.61)	2.25 4.75
	Tyramine	0.264 ± 0.022	0.213 0.315	2.62 (2.42–2.86)	2.20 3.25
	2-Phenethylamine	0.245 ± 0.071	0.081 0.409	2.83 (2.19–3.98)	1.69 8.55
Pargyline	Tyramine	0.285 ± 0.067	0.130 0.440	2.43 (1.97–3.18)	1.57 4.91
	2-Phenethylamine	0.284 ± 0.022	0.233 0.335	2.44 (2.26–2.64)	2.07 2.83

recovery of activity towards 2-phenethylamine following injection of clorgyline were relatively inaccurate because of the low level of inhibition achieved initially (see Fig 1). There was no significant difference between any of the calculated rate constants for enzyme degradation which corresponded to a half-life of about 2.7 days for the enzyme. This value is similar to that calculated for the rat liver enzyme by other workers [30, 31] which ranged from 1.8 to 4 days.

The half-life of the enzyme appears to vary between different organs and values between 9 and 11 days have been reported for rat brain monoamine oxidase [19, 20, 31] and Neff and Goridis [20] have reported difference between the half-lives in different brain regions. The situation in rat heart is more complicated, since the half-life increases with age from about 6 days in young rats (40–45 days old) up to 17 days for older rats [21].

In studies of the fall in incorporation of ^{14}C-labelled pargyline into rat liver monoamine oxidase, Erwin and Deitrich [30] calculated the half-life of the enzyme associated with the microsomal fraction to be about 1 day, whereas the value they obtained for the mitochondrial enzyme was 3.5 days. Such a difference might be expected to lead to the recovery curves departing from a single exponential form when liver homogenates are used for the studies. However, the relative similarities of the two rate constants would make this difficult to observe experimentally. In addition, the small proportion of the enzyme activity that is actually associated with the microsomal fraction, rather than arising from mitochondrial damage (see e.g. ref. 32), suggests that any contribution from the enzyme activity in this fraction would have a rather small effect on the recovery rates measured.

The results reported here indicate that, within

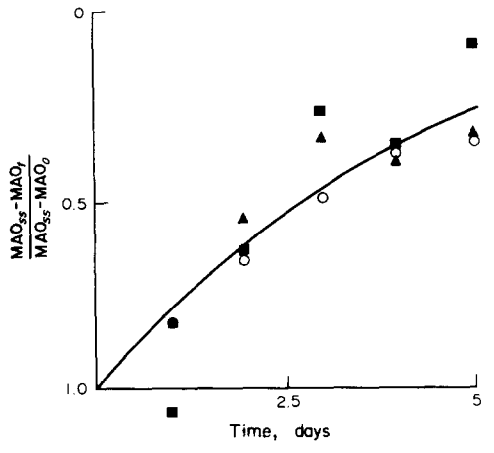

Fig. 4. The recovery of monoamine oxidase activity *in vivo* after inhibition by clorgyline. Details of the assay methods and precedures used are given in the text. The rats were injected intraperitoneally with 2.0 mg/kg clorgyline and were killed at the times indicated. The experimental points each represent the mean values of five rats. (○) Activity towards serotonin, (▲) activity towards tyramine, (■) activity towards 2-phenethylamine.

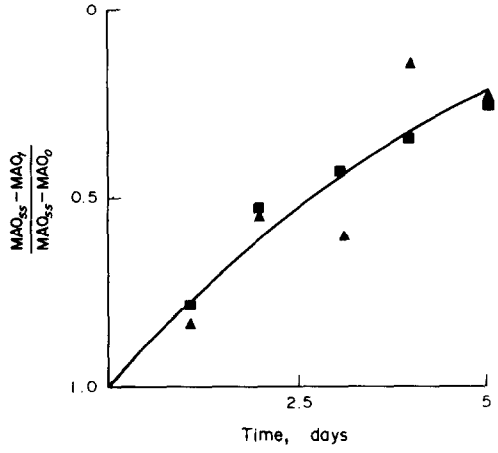

Fig. 5. The recovery of monoamine oxidase activity *in vivo* after inhibition by pargyline. Details of the assay methods and procedures used are given in the text. The rats were injected intraperitoneally with 0.8 mg/kg pargyline and were killed at the times indicated. The experimental points represent the mean values of five rats. (▲) Activity towards tyramine, (■) activity towards 2-phenethylamine.

experimental error, there is no significant difference between the rates of degradation of the two forms of the enzyme. The relatively short half-life of the enzyme and the experimental errors attendant upon this type of *in vivo* approach would, however, obscure any small differences that there might be in the rates of degradation of the two forms and, in this context, it might be of interest to use this approach to study turnover in organs such as rat brain and mature rat heart where the enzyme has a longer half-life. Recently, however, Felner and Waldemeier [36] have shown there to be little difference between the half-lives of the two forms in rat brain. Little is known about the mechanism of degradation of monoamine oxidation *in vivo* (see ref. 21 for review) and the nature of the rate-limiting step in the process is unknown. The lipid environment of the membrane-bound enzyme has been shown to protect it from the action of trypsin [33], but there appears to be little difference between the susceptibilities of the different forms to proteolytic digestion [34]. The relative rates of degradation of the two forms would not be easy to predict if removal of the FAD co-factor, removal from the membrane, or an unfolding process were limiting.

Since the completion of this paper, Campbell, Murphy, Sears and Pinkett (manuscript in preparation) have measured the reappearance of activity following chronic treatment with phenelzine and confirmed that there is no significant difference between the half-lives of the two forms of the enzyme in the rat liver. The half-life determined by these workers was somewhat longer than that observed here, but this may reflect the different strain of rat (Sprague–Dawley) used in that work. In rat brain, however, the results of this group suggest that the B-form of the enzyme may have a shorter half-life than the A-form.

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