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THE BINDING OF [^{14}C]PHENETHYLHYDRAZINE TO RAT LIVER MONOAMINE OXIDASE

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Abstract– A study has been made of the binding of the active site-directed inhibitor [^{14}C]phenethylhydrazine to native rat liver monoamine oxidase (MAO), its multiple forms and sub-units. Inhibition of enzyme activity was time-dependent and was accompanied by irreversible binding of the drug to the enzyme protein. When fully inhibited, the ratio of moles inhibitor bound per 150,000 g of enzyme was in all cases approximately 1:1. It is concluded that each molecule of native enzyme and its multiple forms consists of two sub-units, only one of which possesses an active site. Other evidence presented suggests that the preparation contains two types of MAO.

Certain hydrazines have been used for many years in the treatment of depression (for references, see Biel *et al.* [1]) and their efficacy has been assumed to be the result of inhibition of the enzyme monoamine oxidase [monoamine:oxygen oxidoreductase (deaminating), EC 1.4.3.4]. Studies of the *in vitro* kinetics of inhibition of monoamine oxidase (MAO) by such irreversible inhibitors not only provide information about their mechanism of action, but also about the active centre of monoamine oxidase itself [2–5]. When the inhibitors are radioactively labelled, their binding to the enzyme protein may be directly monitored [2, 6].

Recent experiments have shown that MAO from both rat [7] and pig liver [8] consists of at least two sub-units of similar molecular weight. From the published values of the flavin content of rat liver MAO [9], it would appear that only one of the sub-units possesses a catalytic site (see Youdim and Collins [7]). In the present paper the binding of [^{14}C]phenethylhydrazine to purified rat liver MAO and its constituent sub-units and multiple forms has been studied in order to determine the ratio of bound drug to enzyme protein.

METHODS AND MATERIALS

Preparation of enzymes. Soluble MAO was prepared from rat liver mitochondria using the method of Youdim and Sourkes [10] as modified according to Youdim and Collins [7]. The method involved sonication and the use of a non-ionic detergent (Triton X-100) to achieve solubilization of the enzyme followed by ammonium sulphate fractionation and chromatography using Sephadex G-200 and DEAE Cellulose to purify the enzyme protein.

Disc electrophoresis. Polyacrylamide gel electrophoresis of the purified enzyme was carried out as previously described [11] using a Shandon disc electrophoresis apparatus. The electrode buffers were Tris–HCl (0·05 M, pH 9·1) and after passing a current of 4 mA per tube at room temperature for 2–3 hr, five enzyme forms were separated. The enzyme remaining at the origin (form-1) may result from polymerization of the enzyme when in contact with Sephadex G-200 [7] and may therefore be artifact of the loading procedure [12]. A second (form-5) migrated towards the anode and the rest towards the cathode (forms-2, 3 and 4). The proteins were extracted from the gels and stored at 4° until required [11]. The protein contents of the native enzyme and multiple forms were estimated by the method described by Lowry *et al.* [13] using bovine serum albumin as standard.

Estimation of MAO activity. Enzyme activity was measured using kynuramine as substrate [14]; the incubation mixture consisted of MAO (0·1 ml containing 0·05 mg of enzyme protein), 0·5 ml of phosphate buffer (0·1 M, pH 7·4), kynuramine (to give a final concentration of 0·1 mM) and water to give a final volume of 1·0 ml. Incubation was carried out at 37° for 10 min, after which the 4-hydroxyquinoline formed was measured spectrophotofluormetrically [14].

Determination of the binding of phenethylhydrazine to MAO. For these experiments [^{14}C]phenethylhydrazine (1·66 and 12·2 m-Ci/m-mole, from Warner-Chillcot Laboratories, New Jersey, U.S.A.) was used. The inhibitor was diluted with phosphate buffer (0·05 M, pH 7·4) and 1·0 ml added to 4·0 ml of the enzyme preparation (containing 5·0 mg protein/ml) to give a final concentration of 5 μM of inhibitor. After incubation at 37° for 40 min, a 0·1-ml aliquot was assayed for enzyme activity [14] in order to ascertain that the enzyme was inhibited. The remainder of the mixture was diluted 10-fold with the phosphate buffer and dialysed at 4° against buffer containing 100 μM of non-radioactive phenethylhydrazine until the counts associated with the enzyme reached a constant low value. The binding of [^{14}C]phenethylhydrazine to the multiple forms of rat liver monoamine oxidase was also measured; the multiple enzyme forms were separated by polyacrylamide gel electrophoresis, extracted from the gels and concentrated by re-precipitation using ammonium sulphate (55% saturation). The residue was isolated by

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centrifugation (12,500 *g* for 20 min at 0°) and diluted with the phosphate buffer to give a solution containing 5·0 mg MAO protein/ml. Radioactive phenethylhydrazine was added to each enzyme preparation to give a final concentration of 5 μM. Incubation was carried out at 37° for 40 min, after which the mixtures were diluted and dialysed as described above. The protein contents of the dialysis residues were measured together with the bound radioactivity, the latter being the difference between the counts in the dialysis sacs and those in the surrounding buffer. Assuming the molecular weight of rat liver MAO to be 150,000 [7] the number of moles of inhibitor bound to each mole of monoamine oxidase protein was calculated. Similar studies were carried out in the presence of benzylamine (0·5–10 mM).

The binding of [^{14}C]phenethylhydrazine to the subunits of MAO. In these experiments the radioactive inhibitor was incubated for 60 min with the whole enzyme under the conditions described above. The excess drug was removed by gel filtration through a 2·5 × 30-cm bed of Sephadex G-25 equilibrated with 0·05 M phosphate buffer, pH 7·4. Sodium dodecyl sulphate (SDS) was added to the samples to give a final concentration of 1% w/v, and in some samples 0·1 M 2-mercaptoethanol was also present. After incubation at 37° for 2 hr, polyacrylamide gel electrophoresis was carried out in gels containing 1° w/v SDS [7]. The molecular weights of the bands of protein so separated were estimated using the method devised by Weber and Osborn [15]. The proteins were extracted from the gels [11], passed through 2·5 × 10-cm columns of Sephadex G-25 and the radioactivity and the protein contents of the eluates determined.

Materials. [^{14}C]phenethylhydrazine was a gift from the Warner–Chilcott Laboratories, New Jersey, U.S.A. Kynuramine dihydrobromide and 4-hydroxyquinoline were from the Sigma Chemical Co., St. Louis, U.S.A. Sodium dodecyl sulphate and crystalline bovine serum albumin were from the Sigma Chemical Co., London, U.K. All chemicals used were of the highest obtainable purity.

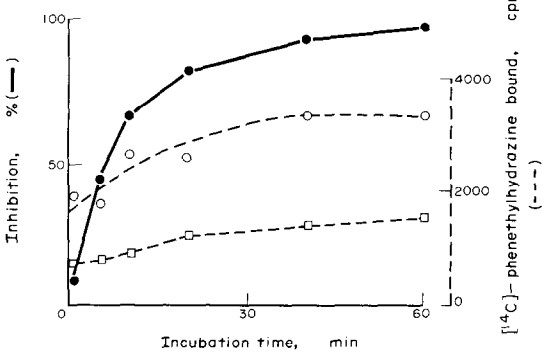

Fig. 1. The binding of [^{14}C]phenethylhydrazine to rat liver monoamine oxidase. Purified rat liver monoamine oxidase (MAO) was incubated with [^{14}C]phenethylhydrazine (5 μM) and the residual activity (●——●) estimated using kynuramine as substrate (see Methods). The binding of the radioactive inhibitor was estimated after dialysis of samples against phosphate buffer alone (○------○) or against buffer containing 100 μM phenethylhydrazine (□-----□) until the counts associated with the enzyme protein reached a constant low value (see Methods). Each point is the mean of three observations

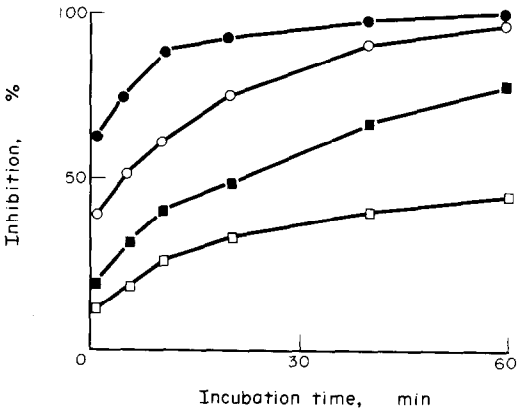

Fig. 2. The effect of various concentrations of benzylamine on the inhibition of rat liver MAO by phenethylhydrazine. The incubation, dialysis and enzyme assay procedures are described in the Methods and Materials section. Each point is the mean of three experiments: ●——●, 0; ○——○, 0·5 mM; ■——■, 1 mM and □——□, 10 mM benzylamine.

RESULTS

Phenethylhydrazine is an active site-directed irreversible inhibitor of MAO. The inhibition was time-dependent, approximately 40 min incubation at 37° being necessary for 95 per cent inhibition of activity at a drug concentration of 5 μM (Fig. 1). Furthermore, the inhibition was a function of the amount of inhibitor bound to the enzyme protein (Fig. 1). The difference in the amount of bound labelled phenethylhydrazine on dialysis of the enzyme preparation in buffer alone and in buffer containing 100 μM non-radioactive inhibitor is of interest and suggests that non-specific binding of [^{14}C]phenethylhydrazine may be occurring; the addition of a large molar excess of the inhibitor presumably displaces non-covalently bound radioactive drug from the sites of attachment. However, the amount of inhibitor bound is approximately the same whether the excess is removed by dialysis (see below) or by gel filtration through Sephadex G-25 (see Table 1). When the substrate benzylamine was in the incubation mixture, the inhibitory action of the phenethylhydrazine on enzyme activity was blocked (Fig. 2), presumably by competition for the active site, thereby reducing the binding of the drug to the enzyme (Fig. 3).

After incubation of [^{14}C]phenethylhydrazine (5 μM) for 40 min with the multiple forms of MAO, with the exception of form-5, inhibition was complete. This lack of inhibition of form-5 may have been due to an insufficient incubation time. Unfortunately, incubation of this form for more than 50 min led to significant losses in activity presumably due to enzyme denaturation and so longer incubation times could not be studied. However, following prolonged dialysis against buffer containing 100 μM of non-radioactive phenethylhydrazine, 1·03 moles of the drug were bound to each 150,000 g of native monoamine oxidase (mean of three experiments) whereas 0·89, 0·99, 0·90, 0·92 and 0·06 moles (mean of two experiments) were bound to each 150,000 g of enzyme forms-1 to 5 respectively. When the concentration of [^{14}C]phenethylhydrazine was increased to 100 μM, not only was the activity of

Table 1. The binding of [^{14}C]phenethylhydrazine to native MAO and its sub-units

Preparation	Protein (μg)	[^{14}C]phenethylhydrazine (nmoles)	Protein (g/mole phenethylhydrazine)
Enzyme before electrophoresis	1675	11·3	148,500
SDS-treated enzyme after electrophoresis (2-mercaptoethanol)	147	1·0	147,000
SDS-treated enzyme after electrophoresis (NO 2-mercaptoethanol)			
Band A	105	0·73	138,500
Band B	83	0·53	154,000

For explanation and experimental details, see text.

form-5 completely inhibited but 0·91 moles of labelled inhibitor were bound to each 150,000 g of enzyme.

When [^{14}C]phenethylhydrazine-treated MAO was submitted to SDS-polyacrylamide gel electrophoresis in gels containing 0·1 M 2-mercaptoethanol, a single protein band migrating from cathode to anode was visible. Calculation of the molecular weight of this band gave a value of 60,500 (mean of 3 experiments), similar to that previously described for the untreated enzyme [7]. In the absence of 2-mercaptoethanol, 2 bands of protein were visible; the molecular weight of the faster travelling band (B) was 75,000 and that of the more slowly migrating band (A) approximately 72,500 (both values the mean of three experiments). Table 1 shows the binding of [^{14}C]phenethylhydrazine to the whole enzyme and its sub-units. The number of grammes of protein associated with each mole of inhibitor is similar for the whole enzyme and for the protein bands separated by SDS-polyacrylamide gel electrophoresis in the presence and absence of 2-mercaptoethanol.

DISCUSSION

The present experiments have demonstrated that not only does [^{14}C]phenethylhydrazine bind irreversibly to MAO but that the extent of inhibition of enzyme activity is a function of the amount of drug bound. Presumably, binding occurs at the active site of the enzyme for the presence of the substrate benzylamine effectively blocks both the inhibitory action of phenethylhydrazine [3] but also its binding (see Results). If one assumes that the molecular weight of rat liver is 150,000 [7, 10] then each mole of enzyme protein binds approximately 1 mole of inhibitor. Previous work [7] has shown that when the purified enzyme is treated with SDS and then subjected to SDS-polyacrylamide gel electrophoresis in the presence of 2-mercaptoethanol, the enzyme dissociates to give a single band of protein having a molecular weight of approximately 78,000. This suggests that each molecule of MAO consists of at least two sub-units. On the basis of these results and of the reported flavin content [9] and the phenethylhydrazine binding capacity of the enzyme, we originally postulated that only one of the sub-units should possess an active site [7]. Recently, Oreland and co-workers [8] showed that if the 2-mercaptoethanol is omitted from the incubation mixture and gels then two bands of protein are separated during electrophoresis each, perhaps, representing one of the sub-units. However, both protein bands bind the inhibitor to equal extents (see Table 1) and must both possess active sites. Moreover, the amount of drug bound is similar to that bound to the intact enzyme. These results with phenethylhydrazine are in close agreement with those of Oreland *et al.* [8] who measured the binding of the non-hydrazine MAO inhibitor pargyline to the pig liver enzyme. It is unlikely that the two protein bands separated are artefacts [8] so that one can only conclude that each represents a different monoamine oxidase.

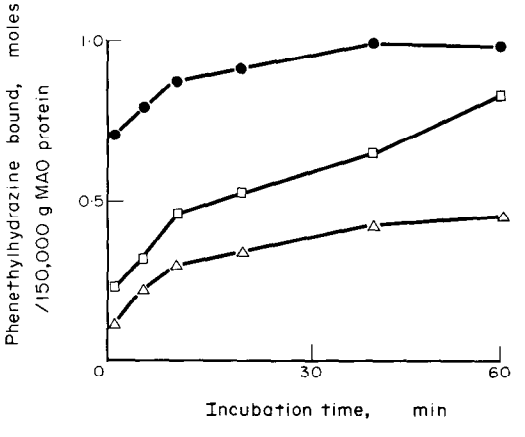

Fig. 3. The effect of various concentrations of benzylamine on the binding of [^{14}C]phenethylhydrazine to rat liver MAO. The details of incubation and dialysis procedures are described in the Methods and Materials section. Each point is the mean of three experiments. ●—●, 0; □—□, 1 mM and △—△, 10 mM benzylamine.

In addition to estimating the binding of [^{14}C]phenethylhydrazine to the whole enzyme we also measured to binding to the five multiple forms of rat liver MAO. Under the experimental conditions employed, with the exception of form-5, approximately 1 mole of inhibitor was irreversibly bound to each 150,000 g of enzyme protein. A similar binding was achieved with form-5 when the concentration of inhibitor was increased (see Results). Assuming that the molecular weights of the multiple forms are similar [7], the results indicate that each enzyme form possesses the same number of active sites. The results do nothing, however, to clarify the question as to whether

the enzyme forms exist *in vivo* or are an artefact of the preparation procedure.

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