Biochemical Pharmacology, Vol. 41, No. 12, pp. 1871–1877, 1991
Printed in Great Britain.

0006-2952/91 $3.00 + 0.00

THE INHIBITION OF MONOAMINE OXIDASE BY BROFAROMINE

MARY C. ANDERSON, PETER C. WALDMEIER* and KEITH F. TIPTON†
Department of Biochemistry, Trinity College, Dublin 2, Ireland; and *Research Department, Pharmaceuticals Division, Ciba Geigy AG., CH-4002, Basle, Switzerland

(*Received* 12 *November* 1990; *accepted* 21 *January* 1991)

Abstract—The inhibition of rat liver mitochondrial monoamine oxidase-A (MAO-A) by brofaromine was time-dependent at low enzyme and inhibitor concentrations. The apparent sensitivity to inhibition decreased when the concentration of the mitochondrial preparation was increased. After preincubation of the enzyme with brofaromine repeated washing of the preparation, by sedimentation and resuspension, resulted in a gradual recovery of activity. This occurred more slowly than was the case when the reversible inhibitor amphetamine was used. After incubation with radioactively-labeled brofaromine the loss of radioactivity also occurred slowly. After incubation with radioactively-labeled pargyline polyacrylamide-gel electrophoresis in the presence of sodium dodecyl sulphage (SDS–PAGE) showed the radioactivity to be associated with a peptide of approximate M_r 50,000, corresponding to the subunit of MAO. Pretreatment with unlabeled pargyline depressed this labeling by pargyline, indicating the latter compound to bind to the active-site of the enzyme. Labeling experiments with radioactive brofaromine indicated that there was a high degree of non-specific binding but that no significant radioactivity remained associated with the enzyme on SDS–PAGE. Chromatographic techniques and determination of H_2O_2 liberation indicated that, in liver there was no appreciable metabolism of brofaromine under the conditions used in the inhibition experiments. These data indicate brofaromine to be a tight-binding, but reversible inhibitor of MAO.

Brofaromine is a selective inhibitor of the A-form of monoamine oxidase (monoamine: oxygen oxidoreductase (deaminating) (flavin-containing) EC 1.4.3.4; MAO) [1]. Studies on its interactions with the enzyme revealed unexpected differences between its *in vivo* and *in vitro* behaviour [1]. It was found to be a time-dependent inhibitor of the enzyme *in vitro* and the inhibition was not readily reversed by dilution or dialysis. *Ex vivo* studies on the inhibition of the enzyme in rat brain were also consistent with it acting as an irreversible inhibitor [1, 2]. In contrast the time-course of the effects of the compound and a number of other features of its behaviour *in vivo* were those that would be expected for a selective and reversible inhibitor of MAO-A.

Such an apparent discrepancy between the *in vivo* and *in vitro* behaviour of monoamine oxidase inhibitors has also been reported to occur with moclobemide [3] and SR 95191 [4]. The mechanisms involved in such behaviour are not only of intrinsic interest but also of practical importance since they suggest that the results of *in vitro* studies may not always reflect the reversibility or irreversibility of the *in vivo* actions of such drugs. In the present paper we present the results of a study on the mechanism of inhibition of MAO-A by brofaromine designed to investigate the causes of the apparent differences between the *in vivo* and *in vitro* behaviour.

MATERIALS AND METHODS

Rat liver mitochondria were prepared from livers of freshly killed rats [5]. The isolated mitochondria were stored in small aliquots at −20° until use. ^{14}C-Labeled 5-hydroxytryptamine (side-chain-2-^{14}C) creatine sulphate, (sp. radioact. 57 mCi/mmol) was obtained from Amersham International (Amersham, U.K.). ^{3}H-Labeled pargyline (phenyl-3-benzyl-^{3}H) hydrochloride (22 Ci/mmol) was obtained from New England Nuclear Products (Dreieich, F.R.G.). [^{3}H]Brofaromine (piperidine-3,4-^{3}H) (7.7 Ci/mmol) was synthesized in the Research Laboratories of Ciba Geigy U.K. Ltd, (Horsham, U.K.).

Reversibility studies. Mitochondrial preparations, containing 1 mg/mL protein, were incubated for 60 min at 37° with 1 μM brofaromine. Following this incubation, 100 μL aliquots were removed and placed in 900 μL of 100 mM phosphate buffer, pH 7.2, and centrifuged for 10 min using a Sorvall minifuge. The pellets obtained were resuspended in 900 μL of the buffer and centrifuged again. This washing and centrifugation procedure was performed between 1 and 5 times before assay. For determination of MAO activity the pellets obtained after centrifugation were resuspended in 100 μL of the phosphate buffer and assayed with 5-HT as substrate. Control samples of mitochondria incubated without brofaromine were taken through the same procedure and assayed in the same way for MAO activity. This washing procedure was performed at 4° and 37° in separate experiments.

The reversibility of inhibition was also investigated by dilution. Samples of mitochondria (500 μg) were incubated with 0.1 μM brofaromine for 60 min at 37° followed by dilution 10-fold with 0.1 M phosphate buffer, pH 7.2, at 37° and assayed after various time

† To whom correspondence should be addressed.

intervals. Control samples were incubated under the same conditions with either 0.1 μM or 0.01 μM brofaromine and subsequently assayed without prior dilution.

Binding studies. These were performed essentially as described previously [6]. Mitochondrial preparations, containing 50–100 μg protein, were incubated for 60 min at 37° with 10 μM [^{3}H]pargyline (sp. radioact.: 0.514 Ci/mmol) or 1 μM [^{3}H]brofaromine (sp. radioact.: 3.85 Ci/mmol) in 0.1 M phosphate buffer, pH 7.8. The effects of preincubation, for 60 min at 37°, with either 1 mM unlabeled pargyline or 10 μM unlabeled brofaromine on the binding of the radioactive inhibitors were also determined. After incubation, the mitochondria were washed extensively, by centrifugation (Sorvall minifuge) and resuspension in 0.1 M phosphate buffer, pH 7.8, and finally resuspended in 100 μL buffer and added to 10 mL 2:1 toluene/Triton (2:1, v/v) containing 0.4% PPO and the radioactivity was determined by liquid scintillation counting.

Binding of [^{3}H]brofaromine and [^{3}H]pargyline to mitochondrial preparations followed by polyacrylamide gel electrophoresis in the presence of sodium dodecylsulphate (SDS–PAGE). Binding of [^{3}H]brofaromine and [^{3}H]pargyline to the mitochondria was performed as described by Costa and Breakefield [7]. Binding mixtures contained 500 μg protein and 32.5 μCi [^{3}H]brofaromine, final concentration 1 μM, or 5 μCi labeled pargyline, final concentration 0.9 μM. The mixtures were incubated for 1 hr at 37° in 50 mM Tris–HCl buffer, pH 7.5. [^{3}H]Pargyline labeling was also measured after preincubation for 1 hr at 37° with 10 μM unlabeled brofaromine and [^{3}H]brofaromine labeling was determined in the same way following exposure of the mitochondrial preparations to 1 mM unlabeled pargyline for 1 hr at 37°. After extensive washing the pellets were resuspended in 200 μL sample buffer, which consisted of 5 mL 20% (w/v) SDS, 0.1 M dithiothreitol, 0.83 mL Tris–HCl buffer, pH 6.8, 5 mL glycerol, 0.3 mL 0.2% (w/v) bromophenol blue in ethanol and 39 mL distilled and deionized water, for application to SDS–polyacrylamide gels.

SDS–PAGE of samples. The gel was prepared using the procedure described by Ames [8]. Samples were applied in sample buffer the composition of which is described above. Standards of the molecular weight range 68–23 kDa were applied in addition to the four samples which were: [^{3}H]pargyline labeled; pretreated with unlabeled brofaromine prior to incubation with [^{3}H]pargyline; [^{3}H]brofaromine labeled, and pretreated with unlabeled pargyline prior to incubation with [^{3}H]brofaromine.

The gel was stained for protein using Coomassie brilliant blue and each track of the gel was sliced into 1 mm segments. These slices were placed in 250 μL of 30% (v/v) H_2O_2 and heated to 37° overnight to render the gel soluble. The samples were then mixed with to 10 μL of toluene/Triton (2:1, v/v) containing 0.4% (w/v) PPO and the radioactivity incorporated was measured by liquid scintillation counting.

Assay procedures. MAO-A activity towards 100 μM 5-hydroxytryptamine was determined radiochemically, in 0.1 M phosphate buffer, pH 7.2, at 37°, as described previously [9]. The liberation of H_2O_2 during the MAO-catalysed reaction was determined under the same conditions by a luminometric procedure involving the coupled oxidation of luminol in the presence of peroxidase (O'Brien and Tipton, manuscript in preparation). The assay mixture contained, in a total volume of 3.0 mL, 0.1 M phosphate buffer, pH 7.2, the mitochondrial preparation at final protein concentrations of up to 2.5 mg/mL, and brofaromine (1–100 μM). The reaction was allowed to proceed at 37° for periods up to 240 min. Under these assay conditions the procedure was capable of detection of the formation of 1.8 nmol H_2O_2/min. Protein concentrations were determined by the method of Markwell *et al.* [10].

High performance liquid chromatography (HPLC) was performed using a μ bondapak C_{18} column (Waters) eluting with 60:40 methanol/10 mM NaH_2PO_4 at a flow rate of 1.2 mL/min. Eluted material was detected spectrophotometrically at 254 nm. Under these conditions the retention time for brofaromine was 7 min and as little as 0.341 ng of applied material could be detected. Samples of 100 μM brofaromine were also preincubated at 37° in 100 mM phosphate buffer for periods of up to 240 min with the mitochondrial preparation (5 mg/mL) prior to precipitation of the protein using 0.2 M perchloric acid and application to the column.

The possible metabolism of brofaromine by MAO in tissue homogenates was also assessed by thin layer chromatography (TLC). Homogenates of brain (1 g/4 mL), heart (1 g/10 mL) and liver (1 g/10 mL) were prepared, from a 220 g male Tif:RAIF (SPF) rat, in 0.1 M potassium phosphate buffer, pH 7.9. Triplicate samples of each homogenate (0.1 mL) were mixed with an equal volume of the phosphate buffer containing 300 μM pargyline and incubated at 37° for 1 hr. Parallel samples, also in triplicate, were incubated in the same way but in the absence of pargyline. After this preincubation 10 μCi [^{3}H]brofaromine were added, in a volume of 0.1 mL phosphate buffer, to give a final concentration of 4.33 μM and incubation was continued for a further 23 hr. After that period 0.1 mL of 0.4 M HCl was added, the mixtures were shaken thoroughly and then centrifuged at 40,000 *g* for 5 min. The supernatants were evaporated to dryness in a vacuum centrifuge and the residues were dissolved in 10 μL ethanol and centrifuged in a Beckman microfuge for 2 min. One microlitre samples of each supernatant were spotted on to 10 × 20 cm HPTLC 60 F_{254}TLC plates (Merck, Darmstadt, F.R.G.) and the chromatograms were developed with ethyl acetate: ethanol:25% ammonia (60:30:10). They were subsequently scanned using a Berthold LB 2723 thin layer radioactivity scanner. The peaks were then cut out and weighed for quantitative evaluation.

RESULTS

Figure 1 shows the degree of inhibition of MAO activity as a function of brofaromine concentration, determined without preincubation between enzyme

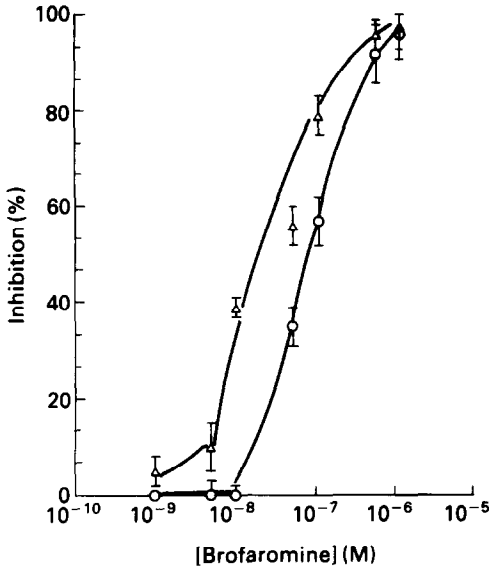

Fig. 1. The effect of brofaromine on the deamination of 5-HT by MAO in rat liver mitochondria. Brofaromine was incubated at the indicated concentrations with rat liver mitochondria (protein concentration 50 μg protein/assay) for 60 min at 37° prior to the addition of 5-HT to assay for activity (Δ). To determine the inhibition at zero time (○), the inhibitor was preincubated with 5-HT, prior to the addition of enzyme (50 μg) to initiate the reaction. In each case results are shown as the mean ± range of two separate determinations of percentage inhibition of MAO activity (with respect to samples preincubated for the same time with buffer).

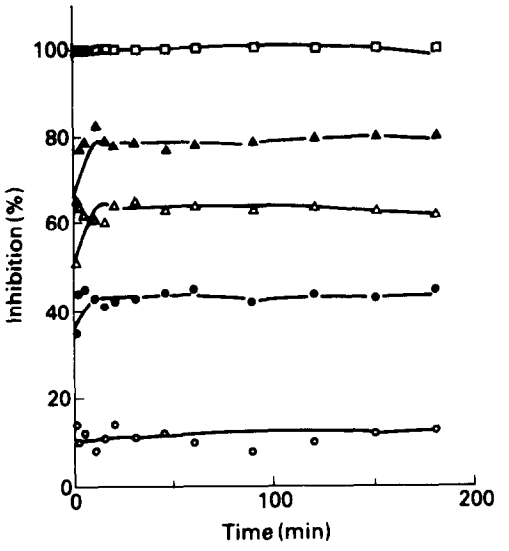

Fig. 2. Time-course of the inhibition of rat liver MAO by increasing concentrations of brofaromine. Rat liver mitochondria (50 μg protein) were incubated, for the time intervals shown, with the following concentrations of brofaromine: (□) 10^{-6} M, (▲) 10^{-7} M, (△) 5×10^{-8} M, (●) 10^{-8} M and (○) 5×10^{-9} M. At the times shown, the samples were assayed for MAO activity using 5-HT as substrate and compared to control samples which had been incubated for the same time in the absence of inhibitor.

and inhibitor and after a preincubation period of 60 min before the addition of substrate. The increase in inhibitory potency after preincubation is in agreement with earlier results [1] and might suggest a covalent interaction between enzyme and inhibitor. Despite the time-dependence shown in Fig. 1, it was not possible to determine the time-course of the inhibitory process with accuracy since inhibition developed rapidly under the conditions used here, being complete within 5 min of enzyme–inhibitor preincubation in a reaction mixture containing 50 μg of the mitochondrial preparation and 0.1 μM brofaromine. At the higher brofaromine concentration of 1 μM the inhibitory reaction was more rapid (see Fig. 2) and at mitochondrial concentrations of 500 μg, or greater, there was no detectable time-dependence in the inhibition by 1 μM brofaromine.

The apparent sensitivity to inhibition by brofaromine decreased as the protein concentration was increased (Fig. 3). The addition of 10 mg/mL bovine serum albumin had no significant effects on the inhibition curves (results not shown), suggesting that the effect of protein concentration was not a result of general and non-specific binding of brofaromine to protein. Such behaviour might be expected for an inhibitor that interacted stoichiometrically with the enzyme, either because of an irreversible reaction involving covalent bond formation (see Ref. 6) or high affinity, reversible binding in which the

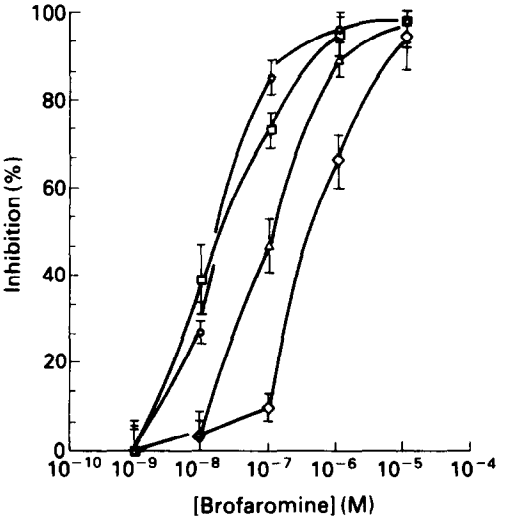

Fig. 3. The effects of increasing protein concentration on the inhibition of rat liver MAO by brofaromine. Rat liver mitochondria were incubated with brofaromine in the concentration range 10^{-9}–10^{-5} M for 30 min. After this time, the mitochondria were assayed for MAO activity using 5-HT as substrate as described in the methods section. The level of inhibition was determined by comparing the activities of these samples with mitochondria, of the same protein concentrations, which had not been exposed to inhibitor. The amounts of protein used were: (○) 250 μg, (□) 500 μg, (△) 1 mg and (◇) 2.5 μg. The results shown are the mean ± range of two separate experiments each performed in triplicate.

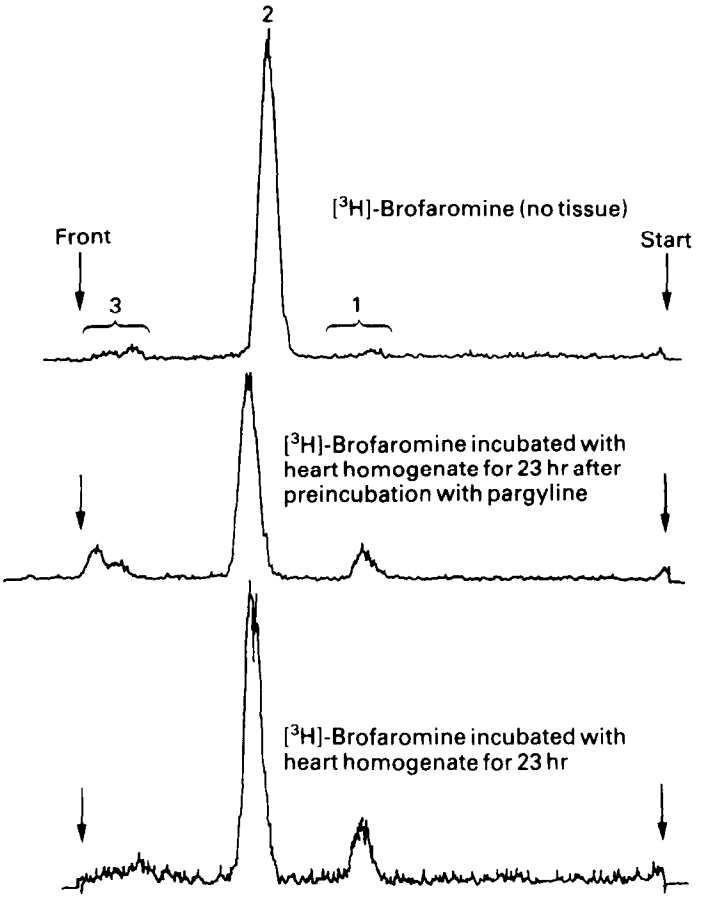

Fig. 4. Thin layer chromatography of ^{3}H-labeled brofaromine. Samples of brofaromine were incubated for 23 hr at 37° with a rat heart homogenate or with a similar homogenate which had been preincubated with 300 μM pargyline before analysis by TLC. The behaviour of a sample of the brofaromine which had not been treated with the tissue preparation is also shown.

amount of brofaromine required for inhibition was comparable with the enzyme concentration [11, 12].

After the initial increase the level of inhibition of the enzyme preparation by brofaromine remained constant for a period of up to 180 min at 37° (Fig. 2). These results suggest that metabolism of the inhibitor to produce a less, or more, potent species was not occurring under these conditions. Incubation of the preparation with brofaromine in the concentration range 1–100 μM for up to 240 min resulted in no detectable formation of H_2O_2. Neither was any breakdown of brofaromine or product formation detected by HPLC after up to 240 min incubation between brofaromine and the enzyme preparation.

Representative samples of scans of radioactivity on the thin layer chromatograms of the supernatants resulting from incubation of rat heart homogenates for 23 hr with [^{3}H]brofaromine are shown in Fig. 4. Radioactivity was found in three areas, giving peaks with approximate R_f values of 0.53 (peak 1) and 0.72 (peak 2) and in a broader area near the solvent front (designated area 3). There were no apparent differences between the chromatograms of samples that had been pretreated with pargyline and those that had not. Similar results were obtained when the brain and liver homogenates were analysed in this way. The distributions of radioactivity between the three areas, expressed as percentages of the total, are shown in Table 1. The results show that the percentage of radioactivity contained in peak 2, which corresponds to unchanged brofaromine was the same whether or not the samples had been pretreated with pargyline to inhibit the MAO activity. The same applies to peak 1 and area 3, which correspond at least partly to impurities in the starting material (see Fig. 4). Thus even over these long incubation periods brofaromine was not metabolized by MAO to any detectable extent.

Repeated centrifugation and resuspension of mitochondrial samples that had been preincubated for 60 min at 37° with 1 μM brofaromine were used to assess whether it was an irreversible or a reversible tight-binding inhibitor of MAO-A. The results, shown in Fig. 5, indicate the activity was regained only after extensive washing by this procedure. For comparison, the recovery of activity after pretreatment with the reversible inhibitor amphet-

Table 1. Distributions of radioactivity on chromatograms of supernatants of incubations of [^{3}H]brofaromine with homogenates of rat tissues

	− Pargyline			+ Pargyline		
	Area 3	Peak 2	Peak 1	Area 3	Peak 2	Peak 1
Brain	7.0	77.1	15.8	6.4	77.6	16.1
Heart	12.3	73.7	14.1	15.6	74.1	10.4
Liver	2.0	93.0	5.0	3.3	91.9	4.8
Starting material	6.0	92.1	1.9			

Data are means of triplicates of the percentage area of the radioactivity contained in peaks 1 and 2 and near the front (area 3), as indicated in the example given in Fig. 4.

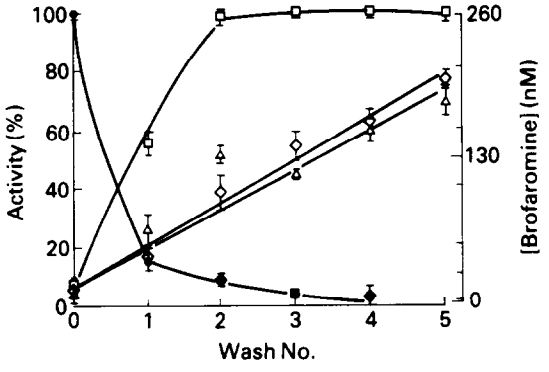

Fig. 5. The effects of repeated centrifugation and resuspension on the recovery of MAO activity and the loss of [^{3}H]brofaromine after treatment of rat liver mitochondria with brofaromine. Rat liver mitochondria (2 mg/mL) were incubated with 1 μM brofaromine or 80 μM amphetamine for 60 min. Samples were subjected to the spin/wash procedure, as described in the text, and assayed using 5-HT as substrate. (△) Washing performed at 4°, (○) washing performed at 37°, (□) amphetamine-treated mitochondria. Results are shown as percentage inhibition values, calculated with respect to control samples taken through the same procedure without inhibitor. (●) Removal of [^{3}H]brofaromine. Results shown are the mean ± range of two separate determinations, each performed in triplicate.

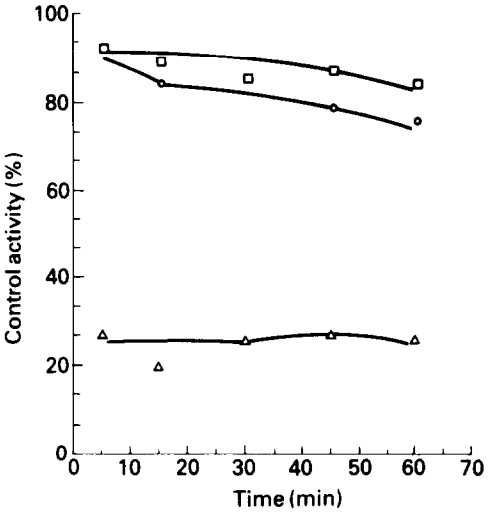

Fig. 6. Recovery of MAO activity following dilution of brofaromine in rat liver mitochondrial preparations. Rat liver mitochondria were incubated with 10^{-7} M brofaromine for 20 min. Following this the mitochondria and inhibitor mixture was diluted 1:10 into 100 mM phosphate buffer, pH 7.2 (final assay volume 500 μL) for the time intervals shown prior to assaying for MAO activity using 5-HT as substrate (○). In two separate experiments mitochondria were incubated with either 10^{-7} M (△) or 10^{-8} M (□) brofaromine for the same time intervals. The final protein concentration in all three cases was 50 μg. The MAO activities of the three test experiments were compared to mitochondria incubated for the same time intervals without inhibitor.

amine [13] is shown. Figure 5 also shows the loss of radioactivity from a sample of the enzymes that had been preincubated with the same concentration of ^{3}H-labeled brofaromine during the spin–wash procedure. The radioactive inhibitor can be seen to be removed as activity is regained. However, since rat liver mitochondria prepared in this way contain some 5.5 pmol MAO/mg protein [6] and about 260 nM brofaromine was initially associated with the preparation these results would indicate that there must be considerable binding of brofaromine sites other than the active site of MAO in a manner that was not readily removed by the washing procedure.

In the dilution experiments to assess reversibility the control and diluted samples were each at the same final protein concentration. As can be seen from Fig. 6 comparison between the diluted and control samples, each at the same final brofaromine concentration of 0.01 μM, showed the inhibition to be reversible under these conditions.

When ^{3}H-labeled pargyline was preincubated for 1 hr with the mitochondrial preparation and the peptides separated by polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulphate, a single radioactively-labeled band of approximate M_r 50,000 could be detected by liquid scintillation counting of the gel slices, as shown in Fig. 7. Such results are consistent with those reported by others [7, 14]. Similar experiments in which 10 μM [^{3}H]brofaromine was used in the same way showed no significant amount of label in this region (Fig. 7). Some residual binding of brofaromine to components other than MAO (<1 pmol total) was apparent. High concentrations of brofaromine (10 μM) prevented the incorporation of [^{3}H]pargyline into MAO.

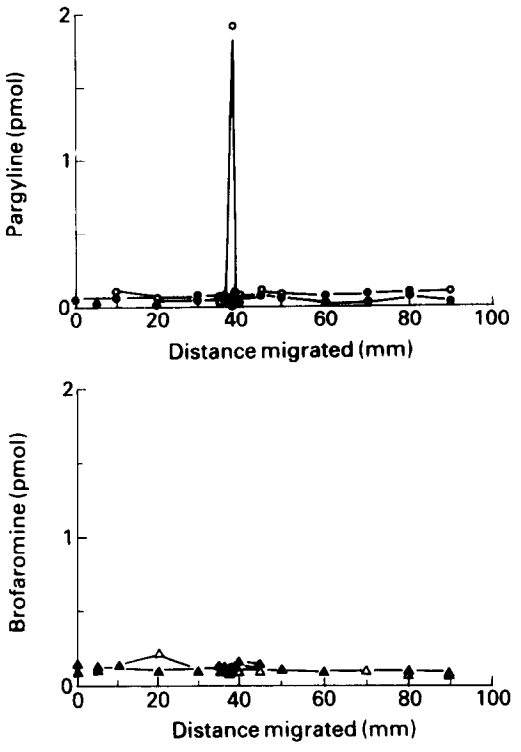

Fig. 7. Incorporation of [^{3}H]pargyline and [^{3}H]brofaromine into rat liver mitochondria preparations determined using SDS–PAGE. Rat liver mitochondrial preparations were treated with [^{3}H]pargyline or [^{3}H]brofaromine as described in the text. The samples were then subjected to SDS–PAGE. The protein bands were visualized using Coomassie brilliant blue stain. The gel was sliced into 1 mm sections and solubilized as previously described and incorporation of radioactive inhibitor was determined using liquid scintillation counting. (○) Total [^{3}H]pargyline incorporation, (●) [^{3}H]pargyline incorporation after pretreatment with 1 mM brofaromine, (△) total [^{3}H]brofaromine incorporation, (▲) [^{3}H]brofaromine incorporation after pretreatment with 1 mM pargyline.

In labeling experiments the enzyme was found to incorporate 5.5 pmol pargyline/mg protein [6]. This labeling was prevented by preincubation for 60 min at 37° with 1 μM brofaromine. Similar experiments with labeled brofaromine gave a very high apparent level of incorporation (3.5 nmol/mg) consistent with a large amount of non-specific binding occurring. Prior incubation of the samples, for 60 min at 37°, with 1 mM pargyline had no significant effect on the total binding, as might be expected from the small proportion of the total that represented MAO active sites.

DISCUSSION

The low IC_{50} value for the inhibition of MAO-A by brofaromine (see Fig. 1) places it into the category of 'tight-binding' inhibitors (see Ref. 11). In such cases the observed rate of enzyme inhibition can show time-dependence, not because of the chemical reaction between the enzyme and inhibitor but because at low enzyme and inhibitor concentrations the second-order rate of enzyme–inhibitor complex formation can be relatively slow [12]. In the present studies the observation of time-dependent inhibition at lower enzyme and inhibitor concentrations which becomes negligible as the concentrations were raised is fully in accord with such a mechanism of inhibition and with the time-dependent inhibition reported earlier [11].

An alternative explanation for such time-dependent inhibition would be that brofaromine is converted by the action of MAO into a more potent reversible inhibitor. Such a mechanism has been shown to explain the discrepancy between the time-dependent and apparently irreversible inhibition of MAO by moclobemide *in vitro* and its short duration of action *in vivo* [15]. In that case the inhibition was shown to be essentially irreversible on dialysis at 4° but to be readily reversible at 37°. Furthermore studies on the time-course at 37° showed inhibition to diminish at longer times as the metabolism of moclobemide proceeded to completion [15].

The results of the present study were not consistent with such a mechanism operating in the case of brofaromine since time-dependent inhibition was only observed at low enzyme and inhibitor concentrations and there was no diminution of the degree of inhibition over longer enzyme–inhibitor incubation times. Furthermore there was no evidence of brofaromine oxidation, as judged by the absence of detectable hydrogen peroxide formation. Neither could the appearance of any product, or disappearance of substrate, be detected by the HPLC or TLC procedures after incubation of MAO and brofaromine at 37° for up to 240 min or 23 hr, respectively. Although the TLC data might indicate some metabolism of brofaromine other than by MAO to occur in the heart and brain preparations during the extended incubation time used, no significant metabolism could be detected with the liver homogenate (see Table 1).

Studies with tight-binding enzyme inhibitors may yield misleading results unless care is taken to ensure that methods used to test for reversibility allow sufficient time for dissociation to occur [12] and also to remove the inhibitor from the system effectively. In the latter context, for example, a procedure that reduces the inhibitor concentration 100-fold will still result in 50% inhibition if the enzyme was initially incubated with 100 times the I_{50} inhibitor concentration. Furthermore dilution of an enzyme–inhibitor mixture might yield misleading results, suggesting that dissociation of inhibitor from the enzyme did not occur, if dilution resulted in an unchanged enzyme:inhibitor concentration ratio. Such results viewed together with apparent time-dependent inhibition may, all too easily, be interpreted as indicating irreversible inhibition. Such behaviour would account for the apparently irreversible inhibition observed with brofaromine in both *in vitro* and *ex vivo* experiments [1]. The present studies show that the inhibition by brofaromine is indeed reversible, both at 4° and at 37° providing the method used is sufficient to reduce

the free inhibitor concentration to a level significantly lower than the IC_{50} value.

The ability of brofaromine to prevent the binding of labeled pargyline to the enzyme preparation indicates that it binds to the active site of the enzyme. Brofaromine binding was essentially reversed by denaturation followed by electrophoresis in the presence of SDS, consistent with the inhibitory process not involving the formation of a covalent enzyme–inhibitor bond. The labeling data also show substantial binding of brofaromine to components other than the MAO active site in a manner that was only slowly reversed by repeated washing nor, indeed, completely removed by SDS–PAGE. Since brofaromine is a highly lipophilic compound (partition coefficient = 5.2 in *n*-octanol/water, pH 7.4, at 25°) this non-specific binding might involve hydrophobic interactions, which might be relatively resistant to the aqueous washing procedure used in the present work. Some dissociation from these sites and reassociation with the active site of MAO may also contribute to the slow recovery of activity during the repeated washing procedure. Such competition between the MAO active site and other high-affinity binding sites would significantly reduce the free concentration of brofaromine available for interaction with MAO and may thus account for the slower time-course of the inhibitory process seen when cruder tissue preparations were studied [1].

The results reported here indicate that brofaromine is a high-affinity inhibitor which binds to the active site of MAO-A but is not metabolised by the enzyme to any detectable extent. They also demonstrate that particular care is necessary in the evaluation of the behaviour of such 'tight-binding' inhibition in order to ensure that it is not confused with irreversible inhibition. However, with appropriately designed studies any apparent discrepancy between *in vitro* and *ex vivo* and *in vivo* behaviour can be readily resolved if it results from this cause.

REFERENCES

1. Waldmeier PC, Felner AE and Tipton KF, The monoamine oxidase inhibiting properties of CGP 11305A. *Eur J Pharmacol* **94**: 73–83, 1983.
2. Keller HH, Kettler R, Keller G and Da Prada M, Short acting novel MAO inhibitors *in vitro* evidence for the reversibility of MAO inhibition by moclobemide and Ro 16-6491. *Naunyn Schmeidebergs Arch Pharmacol* **335**: 12–20, 1987.
3. Da Prada M, Kettler R, Keller HH and Haefely WE, Neurochemical effects *in vitro* and *in vivo* of the antidepressant Ro 11-1163, a specific and short-acting MAO-A inhibitor. *Mod Probl Pharmacopsychiat* **19**: 231–245, 1983.
4. Kan J-P, Steinberg R, Leclerq J, Worms P and Biziere K, Monoamine oxidase-inhibiting properties of SR 95191, a new pyrazidine derivative, in the rat. Evidence for selective and reversible inhibition of monoamine oxidase type-A *in vivo* but not *in vitro*. *J Neurochem* **50**: 1137–1144, 1988.
5. Kearny EB, Salach JI, Walker WH, Seng RL, Kenny U, Zeszotek E and Singer TP, The covalently-bound flavin of hepatic monoamine oxidase: 1. The isolation of a flavin peptide and evidence for binding at the 8 α position. *Eur J Biochem* **24**: 321–327, 1971.
6. Gomez N, Unzeta M, Tipton KF, Anderson MC and O'Carroll A-M, Determination of monoamine oxidase concentrations in rat liver by inhibitor binding. *Biochem Pharmacol* **35**: 4467–4472, 1986.
7. Costa MRC and Breakefield XO, Electrophoretic analysis of [^{3}H]pargyline labeled monoamine oxidases A and B from human and rat cells. *Mol Pharmacol* **17**: 199–205, 1980.
8. Ames GFL, Resolution of bacterial proteins by polyacrylamide gel electrophoresis on slabs. Membrane, soluble and periplasmic fractions. *J Biol Chem* **249**: 634–644, 1974.
9. Tipton KF, Determination of monoamine oxidase. *Meth Find Exp Clin Pharmacol* **7**: 361–367, 1985.
10. Markwell MAK, Haas SM, Bieber LL and Tolbert NE, A modification of the Lowry procedure to simplify protein determination in membrane and lipoprotein samples. *Anal Biochem* **87**: 206–210, 1978.
11. Dixon M and Webb EC, *Enzymes*, 3rd Ed., pp. 361–368. Longman, London, 1979.
12. Tipton KF and Fowler CJ, The kinetics of monoamine oxidase inhibitors in relation to their clinical behaviour. In: *Monoamine Oxidase and Disease* (Eds. Tipton KF, Dostert P and Strolin Benedetti M), pp. 27–40. Academic Press, London, 1984.
13. Mantle TJ, Tipton KF and Garrett NJ, Inhibition of monoamine oxidase by amphetamine and related compounds. *Biochem Pharmacol* **25**: 2073–2077, 1976.
14. Parkinson D and Callingham BA, The binding of [^{3}H]-pargyline to rat liver mitochondrial monoamine oxidase. *J Pharm Pharmacol* **32**: 49–54, 1980.
15. Da Prada M, Kettler R, Cesura AM and Richards JG, Reversible, enzyme-activated monoamine oxidase inhibitors: new advances. *Pharm Res Commun* **20**(Suppl. 4): 21–23, 1988.