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IN VITRO AND *IN VIVO* INHIBITION BY BENSERAZIDE OF CLORGYLINE-RESISTANT AMINE OXIDASES IN RAT CARDIOVASCULAR TISSUES

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Abstract—Benserazide (D,L-serine 2-[2,3,4-trihydroxybenzyl]-hydrazide) has been shown to inhibit the clorgyline-resistant amine oxidase (CRAO) activities which metabolize benzylamine in homogenates of rat aorta, heart and brown adipose tissue. *In vitro* studies showed a concentration- and time-dependent inhibition of CRAO in heart and aorta which was reversed by dialysis for 18hr. At high concentrations (10^{-4}–10^{-3}M) benserazide appeared to increase enzyme activity towards and occasionally above control value. These increases became more prominent after long periods of preincubation (especially in the presence of saturating benzylamine concentrations) and remained after dialysis of those homogenates preincubated with benserazide. The administration of benserazide for one or seven days in daily doses of 5–150 mg/kg also inhibited CRAO activity *in vivo* in a dose-dependent manner, with greater inhibition after seven days treatment. Reversal of inhibition, by dialysis of tissue homogenates from benserazide-treated rats, was much slower than was found with homogenates incubated *in vitro* with the drug. After benserazide administration to rats, MAO-A activity towards 5-hydroxytryptamine was generally not inhibited, and in fact was significantly increased in some cases. The administration of L-DOPA (250 mg/kg) together with benserazide (40 mg/kg) resulted in a similar degree of CRAO inhibition in aorta and heart to that seen after benserazide alone. These findings are discussed with regard to the use of these drugs in the therapy of Parkinson's Disease, although the paucity of information about the physiological function of CRAO makes the significance of its inhibition by benserazide unclear.

Two forms of the outer mitochondrial membrane enzyme monoamine oxidase (MAO), called MAO-A and MAO-B, can be distinguished by their different sensitivities to inhibition by acetylenic inhibitors such as clorgyline and deprenyl (see [1] for review). In recent years, much attention has been directed towards the properties of these MAO forms in a variety of animal tissues, and several reports describing their distribution and substrate specificities in rat cardiovascular tissues have appeared [2–11].

It is now clear that MAO-A and MAO-B are not the only enzymes able to catalyse the oxidative deamination of particular amines in rat cardiovascular tissues. Coquil *et al.* [2] found that part of the tyramine deamination by homogenates of rat mesenteric and femoral arteries was resistant to inhibition by clorgyline at *in vitro* concentrations sufficient to inhibit MAO-A and MAO-B activities completely. Our studies with rat heart homogenates also revealed a clorgyline-resistant component of benzylamine metabolism and showed that this enzyme, like that of rat arteries, was sensitive to inhibition by carbonyl reagents such as semicarbazide [3]. Recently, we have adopted the term clorgyline-resistant amine oxidase (CRAO) to describe this enzyme, in order to distinguish it from MAO-A and MAO-B which also contribute to benzylamine metabolism by rat heart homogenates [3]. Other amine substrates such as 2-phenylethylamine [4, 9, 12], kynuramine [5] and dopamine [12] appear to be metabolized by CRAO in certain cardiovascular tissues of the rat.

At present, relatively little is known about the biochemical properties and physiological importance of CRAO. In rat heart homogenates, the K_m for deamination of benzylamine by CRAO (around 5 μM) is much lower than the corresponding values for MAO-A and MAO-B [3]. Thus, CRAO activity can be assayed selectively in this tissue without any significant interference from MAO activities if a sufficiently low concentration of benzylamine, for example 1 μM, is used [13]. Similarly, in the rat aorta and brown adipose tissue, CRAO alone is responsible for metabolism of benzylamine at low micromolar concentrations [13, 14]. Thus, the use of these tissues, along with suitable assay conditions, can provide a useful means for studying the properties of CRAO in the rat.

In an earlier paper, we reported that the administration to the rat of benserazide, a drug used clinically as an inhibitor of L-aromatic amino acid decarboxylase in patients suffering from Parkinson's Disease, resulted in a reduction of benzylamine metabolism by homogenates of hearts prepared from the animals. It was suggested that this observation may be due to *in vivo* inhibition of cardiac CRAO activity by benserazide [15]. Further studies have been undertaken to investigate this possibility, and in the present paper we describe the inhibitory properties of benserazide upon CRAO, both *in vitro* and *in vivo*, in the rat heart, aorta, and in some

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instances brown adipose tissue. Some of these results have been reported in preliminary form [16].

MATERIALS AND METHODS

Male albino Sprague–Dawley rats usually weighing about 140–160 g were supplied by A. J. Tuck and Son (Rayleigh, U.K.). [G-^{3}H]-5-Hydroxytryptamine creatinine sulphate and [methylene-^{14}C]benzylamine hydrochloride from the Radiochemical Centre (Amersham, U.K.) were used as radioactive substrates. L-Dihydroxyphenylalanine (L-DOPA), pyrogallol and D,L-serine were purchased from Sigma (London) Chemical Co. (Poole, U.K.). Benserazide (D,L-serine 2-[2,3,4-trihydroxybenzyl]-hydrazide, Ro 4-4602) hydrochloride was kindly supplied by Roche Products Ltd. (Welwyn Garden City, U.K.).

Animals were weighed and then killed by cervical dislocation. The heart, abdominal aorta, and in some cases interscapular brown adipose tissue were dissected, and adhering blood removed by blotting with absorbent paper. The tissues were then weighed and stored at −20° until required for enzyme assays. Tissue homogenates were prepared in 1 mM potassium phosphate buffer pH 7.8 by the use of a hand-held ground-glass homogenizer with a tissue (g):buffer (ml) ratio of 1:10 (heart and brown adipose tissue) and 1:40 (aorta). These crude homogenates were centrifuged at 600 *g* for 10 min to remove cell debris, and the supernatants were decanted and used for assay of amine oxidases. For *in vitro* studies of drugs, pooled homogenates of tissues from 3 or 4 rats were prepared, and then subdivided into several suitable portions which were stored frozen, and then used as required for the experiments described. In contrast, tissues from animals treated with drugs *in vivo* were homogenized individually and enzyme activities of each tissue were assayed immediately after preparation of the homogenate.

Amine oxidase assays were based upon the method described by Callingham and Laverty [17]. Assay mixtures contained 25 μl of tissue homogenate, 25 μl of distilled water and 50 μl of 0.2 M potassium phosphate buffer pH 7.8 containing appropriate concentrations of radioactive substrate. For assay of MAO-A activity, the homogenates were prepared for use as described in the previous paragraph, and after inclusion in the assay mixture, they were incubated at 37° for 5 min (heart), 20 min (brown adipose tissue) or 60 min (aorta) with 5-HT (sp. act. 2 μCi/μmole) at a final concentration of 1 mM. Under these conditions, 5-HT is metabolized by MAO-A alone in these tissues [3, 14] and metabolite formation is linear with both time and protein concentration in the assay. CRAO activity was assayed with 1 μM benzylamine (sp. act. 10 μCi/μmole), for either 5 min (heart, aorta) or 10 min (brown adipose tissue). Although heart homogenates could be used without further dilution for CRAO assays, it was necessary to dilute samples of the aorta and brown adipose tissue homogenates described previously by an additional 8-fold and 16-fold, respectively, with 1 mM potassium phosphate buffer pH 7.8, in order to ensure linear metabolite production with time, and to avoid greater than about 10% total depletion of substrate during the course of the assay.

During studies to investigate the *in vitro* effects of drugs upon CRAO activity, 25 μl of the appropriate drug solution (prepared in 0.2 M potassium phosphate buffer pH 7.8 to prevent any pH change due to benserazide being the hydrochloride) was included instead of distilled water in the assay described above. Control assays contained the corresponding volume of buffer without inhibitor. The resulting 50 μl assay mixture containing homogenate and drug solution was preincubated at 37° for periods which depended upon the experiment (see Results), before addition of the radioactive benzylamine. Enzyme assays were terminated by plunging reaction tubes into ice, followed by the addition of 10 μl 3 N HCl. Blank assays consisted of mixtures to which HCl was added before incubation with substrate. Deaminated metabolites were extracted into 0.6 ml ethyl acetate/toluene (1:1 v/v), and 0.4 ml of the organic phase was counted for radioactive metabolites by liquid scintillation spectrometry with automatic external standardization to correct for quenching.

Protein concentrations of homogenates were assayed by the microbiuret method of Goa [18], modified in the case of samples of brown adipose tissue to contain sodium dodecyl sulphate (0.06% final concentration by volume) in the assay to remove turbidity due to fat droplets.

For *in vivo* studies, rats were treated by i.p. injection of L-DOPA and/or benserazide, prepared in 0.9% saline (NaCl, w/v in distilled water) as previously described [15]. Control injections of saline were given where appropriate. Tissues were removed 24 hr after final drug injections.

Statistical significance of differences between mean values of control and drug-treatment groups was tested by the non-parametric rank-sum method of Wilcoxon (two-tailed analysis).

RESULTS

In vitro *inhibition of CRAO by increasing benserazide concentrations*. In preliminary experiments to investigate whether or not benserazide was capable of inhibiting CRAO activity, samples of rat heart and aorta homogenates were preincubated for 20 min at 37° with benserazide at concentrations from 10^{-7} to 10^{-2}M, before addition of benzylamine to assay for remaining enzyme activity. Figure 1 shows that CRAO activity in both tissues was inhibited in a concentration-dependent manner, with approximately 50% inhibition being obtained at benserazide concentrations (IC_{50}) of 8×10^{-7}M (aorta) and 4×10^{-6}M (heart). A plateau, or slight reduction in the percentage of inhibition occurred at around 10^{-4}–10^{-3}M, before virtually complete inhibition was seen at 10^{-2}M. Further studies on this 'plateau' effect are described later.

Pyrogallol (1,2,3-trihydroxybenzene) and D,L-serine, which correspond to constituent parts of the benserazide molecule were compared with benserazide as potential inhibitors of CRAO. These experiments used drug concentrations between 10^{-7}M and 10^{-2}M, with identical homogenates and experimental

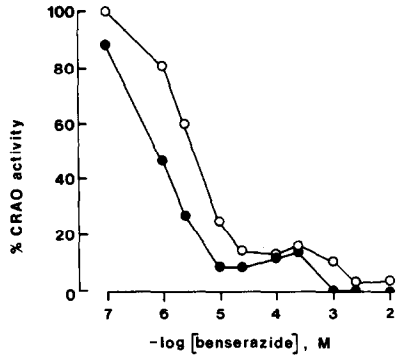

Fig. 1. *In vitro* inhibition of CRAO activity by benserazide. Homogenates of rat aorta (●) or heart (○) were preincubated for 20 min at 37° with various concentrations of benserazide, before addition of benzylamine (1 μM) to assay for remaining activities which were expressed as percentages of control samples preincubated without benserazide. Each point is the mean of triplicate determinations with S. E. R. (not shown) of less than ± 6%.

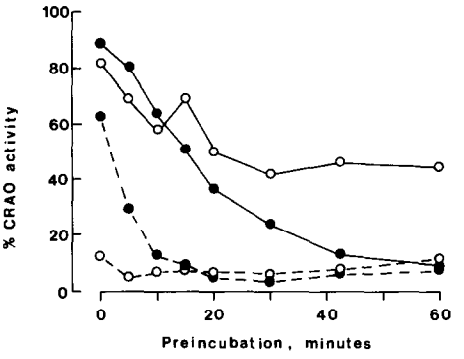

Fig. 2. Effect of preincubation time upon inhibition of CRAO activity by benserazide. Homogenates of rat aorta (●) or heart (○) were preincubated for various periods at 37° with either 1×10^{-6}M (—) or 1×10^{-5}M (– –) benserazide before addition of benzylamine (1 μM) to assay for remaining activities which were expressed as percentages of control samples preincubated without benserazide. Each point is the mean of duplicate determinations.

conditions to those used with benserazide. Pyrogallol was a much less potent inhibitor (IC_{50} of approximately 10^{-3}M in heart and 10^{-2}M in aorta) than benserazide, while D,L-serine was completely inactive.

Time-dependent inhibition. The possibility of time-dependent inhibition by benserazide was investigated by preincubating benserazide (10^{-6}M and 10^{-5}M) for varying periods (0–60 min) with rat aorta and heart homogenates, before assay of CRAO activity. With both concentrations of benserazide, some inhibition was observed even in the absence of preincubation (Fig. 2). In the rat aorta, further inhibition became apparent with increasing preincubation, although with 10^{-5}M benserazide, CRAO was almost totally inhibited after about 20 min. Rat heart CRAO was also inhibited in a partially time-dependent manner by 10^{-6}M benserazide, the percentage inhibition reaching a limiting value of about 55% after 30 min preincubation. Little time-dependence was seen with 10^{-5}M, since this concentration caused almost complete inhibition even without preincubation.

Ackermann–Potter plots. Effects of benserazide upon CRAO activity were also studied by the use of different amounts of aorta or heart homogenate in the assay, in order to analyse the results by the method of Ackermann and Potter [19]. Figure 3 shows that without preincubation, the slight inhibition of CRAO by 1×10^{-6}M and 2.5×10^{-6}M benserazide produced plots of reduced slopes compared with controls, passing through the origin. After preincubation for 20 min, these plots showed even further reduced slopes, but also now with an intercept on the enzyme concentration axis.

Potentiation of CRAO activity by benserazide. Further studies were undertaken to investigate the effects of benserazide upon CRAO activity which might be responsible for the plateau in the inhibition curve of Fig. 1 at around 10^{-4}M benserazide. Preliminary experiments (not shown) suggested that an 'activating' effect upon CRAO may be superimposed

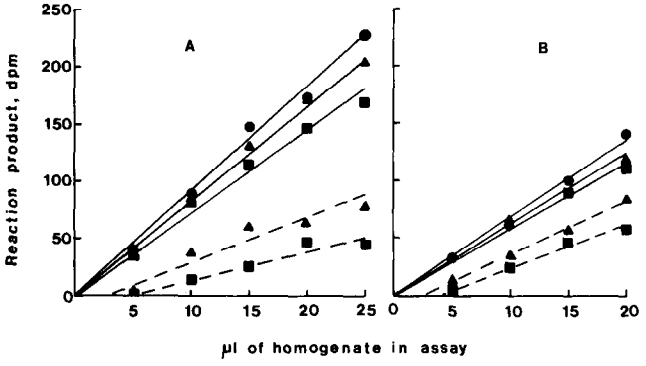

Fig. 3. Ackermann–Potter plots of inhibition of rat aorta (panel A) and heart (panel B) CRAO activity by benserazide. Samples were preincubated for zero (—) or 20 min (– –) at 37° with benserazide at concentrations of 1×10^{-6}M (▲) or 2.5×10^{-6}M (■). Control samples (●) contained no benserazide (preincubation for 20 min had no effect upon control activities). Each point is the mean of duplicate determinations.

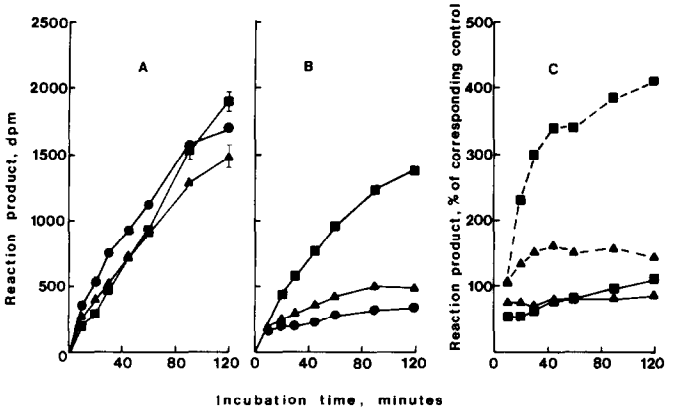

Fig. 4. Influence of benserazide upon CRAO activity assayed by the use of various incubation periods. Homogenates of rat aorta (panel A) and heart (panel B) were incubated with benzylamine (1 mM), in the absence (●) and presence of benserazide (▲, 2.5×10^{-4}M, ■, 2.5×10^{-3}M). Heart homogenates had been preincubated with 7.5×10^{-4}M clorgyline to inhibit MAO activities (see text). Each point represents the mean of triplicate determinations ± S.E.R. when exceeding symbol size. Panel C shows these activities of aorta (—) and heart (– –) homogenates containing benserazide expressed as percentages of the corresponding control samples at each assay time.

upon the inhibitory effects of benserazide at these concentrations, and it appeared that this activation was favoured by prolonged incubation of homogenates with benserazide, particularly in the presence of high concentrations of benzylamine to assay for enzyme activity.

These conditions were studied in greater detail in the following manner. Assay mixtures containing rat aorta homogenate, benserazide (2.5×10^{-4}M or 2.5×10^{-3}M) and 1 mM benzylamine (sp. act. 0.5 μCi/μmole) were incubated at 37° for varying periods between 10 and 120 min. Radioactive metabolites of benzylamine were extracted and quantified from these assays in the usual manner (see Materials and Methods). Figure 4A shows that at short assay times, metabolite production was lower in those samples containing benserazide than in control assays without benserazide. However, after 120 min incubation, samples containing benserazide (2.5×10^{-3}M) now produced greater amounts of metabolite than their corresponding control samples.

In order to carry out similar experiments with rat heart homogenates it was necessary first to preincubate homogenate samples (without benserazide or benzylamine) for 20 min at 37° with 7.5×10^{-4}M clorgyline in order to inhibit completely the MAO-A and MAO-B activities which would normally contribute to benzylamine deamination at 1 mM [3]. After this preincubation period, benserazide and benzylamine were added to the mixture, and the rest of the experiment carried out in an identical manner to that with the aorta. These results are shown in

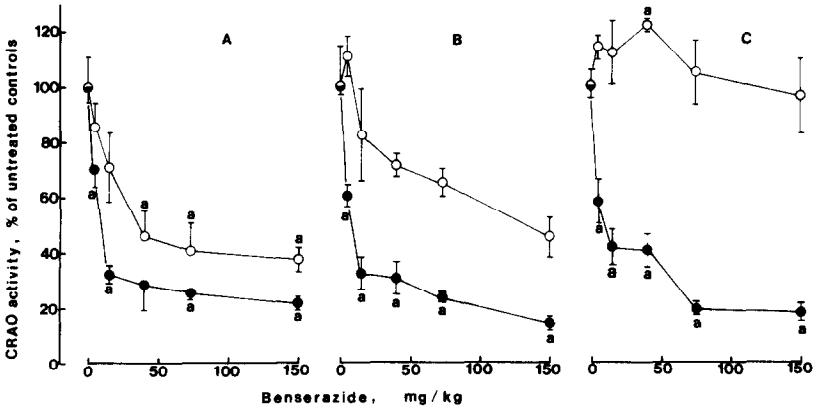

Fig. 5. *In vivo* inhibition of CRAO activity by benserazide. Rats were treated with daily i.p. injections of various doses of benserazide for either one (○) or seven (●) consecutive days. CRAO activities were assayed with 1 μM benzylamine in homogenates of aorta (A), heart (B) and brown fat (C) from individual rats. Mean activities (±S.E.R.) of each group (containing 3–5 rats) are expressed as percentages of the corresponding control group which received saline injections. Control specific activities (nmoles/hr/mg protein) were 22.4 ± 2.3 (aorta), 0.75 ± 0.11 (heart) and 4.5 ± 0.3 (fat) after one day; 14.0 ± 0.8 (aorta), 0.91 ± 0.03 (heart) and 6.5 ± 0.9 (fat) after seven days. Symbol 'a' indicates significantly different ($P < 0.05$ or less) from corresponding controls.

Fig. 4B. Under these conditions, incubation for 10 min with either 2.5×10^{-4}M or 2.5×10^{-3}M benserazide resulted in measured activities similar to controls. However, with increasing incubation times, metabolite production became much greater in samples containing benserazide than in those samples incubated without the drug. These results for the heart and aorta are summarized in Fig. 4C, in which the activities of samples containing benserazide are expressed as percentages of the corresponding controls at each incubation time. Expression of the results in this way shows even more clearly the increasing CRAO activity, particularly in the rat heart, which occurs under these conditions of prolonged incubation with benserazide.

Dialysis of homogenates after in vitro *benserazide treatment.* Samples (0.5 ml) of homogenates were preincubated for 2 hr at 37° with 0.5 ml solutions of benserazide (in 0.2M potassium phosphate buffer, pH 7.8) to give preincubation concentrations between 2×10^{-6}M and 2×10^{-4}M. Control samples were preincubated with 0.5 ml buffer containing no benserazide. These preincubation mixtures were then dialysed for 20 hr at 4° against several 1 litre changes of 1 mM potassium phosphate buffer, pH 7.8. CRAO activity was then assayed in dialysed samples as well as in corresponding undialysed preincubation mixtures. Percentage activities in nondialysed and dialysed preincubation mixtures containing heart samples, respectively, compared with appropriate controls were 32 and 99% (2×10^{-6}M benserazide); 24 and 116% (2×10^{-5}M benserazide); and 37 and 153% (2×10^{-4}M benserazide). For aorta homogenates, the corresponding values were 14 and 104% (2×10^{-6}M); 39 and 112% (2×10^{-5}M); and 40 and 132% (2×10^{-4}M). Each value is the mean of duplicate samples within each category, assayed in triplicate. It is apparent from these results that the inhibition found after preincubation with benserazide was completely reversed by dialysis, and in fact those samples treated with the higher benserazide concentrations provided evidence for increased benzylamine deamination compared with controls after dialysis.

In vivo *inhibition of CRAO activity by different doses of benserazide.* Rats weighing approximately 200 g were divided into several groups and treated daily for one or seven days with benserazide in doses of up to 150 mg/kg. They were then killed 24 hr after their final injections.

Figure 5 shows the mean CRAO activities in the three tissues studied from each group of rats. CRAO was inhibited in a dose-dependent manner in the rat aorta, with significant decreases in activity at most doses of benserazide. Similar dose-dependent inhibition of rat heart CRAO activity was obtained, although the enzyme appeared to be rather less sensitive to benserazide than the rat aorta CRAO after one day of treatment, with no changes reaching statistical significance. With both tissues, seven days treatment with a given dose of benserazide produced a greater degree of inhibition than treatment for one day.

CRAO activity of brown adipose tissue was not inhibited after one day by any dose of benserazide, and in fact a slight but significant increase was obtained with 40 mg/kg. In contrast, after seven days all doses of benserazide now produced a significant inhibition of enzyme activity in a dose-dependent manner.

MAO-A activities were also assayed in these tissue homogenates with 5-HT as substrate. These results are shown in Fig. 6. No significant changes in MAO-A activity of rat aorta and heart were found after one day of benserazide treatment. However, in brown adipose tissue, MAO-A activity was significantly increased above controls after this time with 40 and 150 mg/kg. After seven days treatment with benserazide, MAO-A activity was also increased significantly in rat aorta (40 and 150 mg/kg) and in the rat heart (75 mg/kg). In contrast, no significant change in MAO-A activity of brown adipose tissue was found after seven days.

A separate experiment was performed to investigate whether or not the inhibition of CRAO in tissue homogenates prepared from rats treated with benserazide could be reversed by dialysis. Three rats (approx. 200 g) received two i.p. injections of

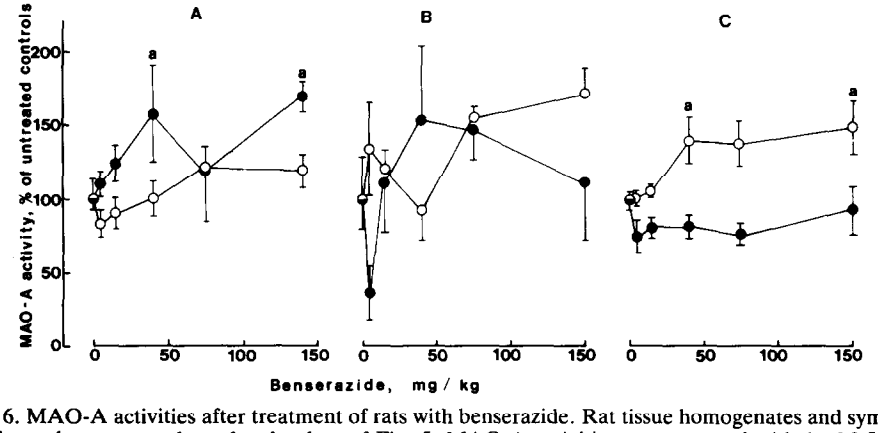

Fig. 6. MAO-A activities after treatment of rats with benserazide. Rat tissue homogenates and symbols used are the same as those for the data of Fig. 5. MAO-A activities were assayed with 1 mM 5-HT. Control activities (nmoles/hr/mg protein) were 31.3 ± 4.3 (aorta), 63.0 ± 18.4 (heart) and 17.3 ± 0.9 (fat) for one day; 17.0 ± 1.2 (aorta), 72.4 ± 14.3 (heart) and 19.2 ± 1.4 (fat) for seven days.

40 mg/kg benserazide 4 hr apart; three control rats received corresponding saline injections. They were killed 18 hr later and homogenates of heart (1:10 w/v) and aorta (1:320) prepared as previously described. One ml samples of each homogenate were dialysed for 18 hr or 68 hr against several 1 litre changes of 1 mM potassium phosphate buffer, pH 7.8. CRAO activity was then assayed in these and corresponding non-dialysed samples of the same homogenates. Mean specific activities ± S.E. (nmoles/hr/mg protein) of non-dialysed homogenates from control and benserazide-treated rats, respectively were (aorta) 25.9 ± 2.9 and 7.6 ± 0.4; (heart) 0.91 ± 0.02 and 0.44 ± 0.02. After 18 hr dialysis the corresponding values were (aorta) 27.0 ± 2.5 and 15.0 ± 1.1; and (heart) 0.82 ± 0.01 and 0.59 ± 0.02. After 68 hr dialysis activities were (aorta) 16.2 ± 1.8 and 14.1 ± 0.4; and (heart) 0.39 ± 0.02 and 0.34 ± 0.01. These results indicated a slow reversal of inhibition upon dialysis, consistent by semi-logarithmic analysis (not shown) with a first-order recovery process with a half life of about 27 hr and 33 hr for aorta and heart, respectively. It should be noted, however, that although CRAO activity in the control homogenates was stable to dialysis for 18 hr, a considerable loss in activity occurred after 68 hr.

Effects of benserazide and L-*DOPA treatment upon CRAO.* Two experiments were performed to investigate the effects of combined administration of benserazide and L-DOPA upon CRAO activity in rat aorta and heart. In the first experiment, groups of rats weighing 120–140 g were treated daily with benserazide (40 mg/kg) or L-DOPA (250 mg/kg) alone, or with both drugs as previously described [15], and rats were killed from these groups 24 hr after 1, 2, 3 or 4 days treatment. In the second experiment, rats weighing 160–180 g were treated with benserazide and L-DOPA for seven consecutive days, before being killed 24 hr later.

CRAO activities in these groups of animals are shown in Table 1. Treatment with L-DOPA alone had no significant effect upon rat aorta CRAO, but did produce a trend towards higher CRAO activity in the rat heart compared with corresponding animals, although this difference only reached statistical significance after four days. Benserazide treatment resulted in the expected inhibition of CRAO activity in both the rat aorta and heart. In addition, administration of both benserazide and L-DOPA resulted in CRAO activity being reduced below control values, generally to an extent which was similar to that produced by benserazide alone.

DISCUSSION

These results show that benserazide is an inhibitor, both *in vitro* and *in vivo* of CRAO activity in rat cardiovascular tissues. Under the *in vitro* conditions used here, IC_{50} concentrations of around 10^{-6}M were obtained with CRAO in rat aorta and heart homogenates, although these values would be dependent upon factors such as preincubation time and amount of enzyme used. Ackermann–Potter plots [19] involving no preincubation of homogenates with benserazide showed reduced slopes compared with controls, which still passed through the origin, a result consistent with reversible inhibition. After 20 min preincubation, the plots showed even greater reductions in slope together with a small intercept on the enzyme concentration axis, suggesting the development not only of a greater degree of reversible inhibition, but also the possibility that some 'irreversible' inhibition may take place.

However, Ackermann–Potter plots do not allow one to distinguish between covalent irreversible

Table 1. Effects of L-DOPA (250 mg/kg) and benserazide (40 mg/kg) upon CRAO activity in rat heart and aorta

Treatment	CRAO activity nmoles/hr/mg protein 1 day	2 days	3 days	4 days	7 days
Heart					
Control	0.98 ± 0.04 (100)	0.64 ± 0.03 (100)	0.79 ± 0.07 (100)	0.57 ± 0.03 (100)	0.90 ± 0.03 (100)
L-DOPA	1.01 ± 0.06 (103)	0.73 ± 0.05 (114)	0.90 ± 0.05 (114)	0.72 ± 0.02† (126)	0.98 ± 0.03 (109)
Benserazide	0.73 ± 0.01† (74)	0.39 ± 0.01† (61)	0.51 ± 0.01† (65)	0.44 ± 0.01† (77)	0.43 ± 0.03‡ (48)
DOPA + benserazide	0.76 ± 0.04* (78)	0.50 ± 0.07 (78)	0.69 ± 0.06 (87)	0.46 ± 0.03 (81)	0.47 ± 0.02‡ (52)
Aorta					
Control	20.3 ± 2.2 (100)	19.5 ± 3.0 (100)	15.4 ± 1.4 (100)	14.3 ± 1.7 (100)	22.7 ± 3.2 (100)
L-DOPA	16.2 ± 2.7 (80)	20.5 ± 3.4 (105)	16.3 ± 1.7 (106)	14.5 ± 1.2 (101)	26.4 ± 1.4 (116)
Benserazide	11.5 ± 3.0 (57)	8.6 ± 0.9† (44)	6.1 ± 0.6† (40)	5.1 ± 0.5† (36)	5.6 ± 0.7‡ (25)
DOPA + benserazide	12.8 ± 3.5 (63)	9.5 ± 1.9 (49)	2.1 ± 0.5† (15)	3.9 ± 0.4† (27)	4.2 ± 0.4‡ (19)

Specific activities of CRAO in each group given as mean ± S.E.M. (n = 5–7 rats in each group).
* $P < 0.05$, † $P < 0.01$, ‡ $P < 0.005$ vs corresponding control group.
Values in parentheses represent activities expressed as percentage of corresponding controls.

binding and pseudo-irreversible inhibition involving tight reversible interactions. The fact that dialysis was able to reverse inhibition of CRAO produced either *in vitro* or *in vivo* suggests that irreversible covalent interactions are unlikely to play a significant role.

It was of interest that the reversal of CRAO inhibition by dialysis appeared to be much slower in homogenates from tissues inhibited *in vivo* than those from *in vitro* experiments. In the latter case, no inhibition could be detected after 18 hr dialysis of samples preincubated with benserazide at concentrations which produced marked inhibition before dialysis. A considerable degree of inhibition was found to survive after *in vivo* treatment, bearing in mind that 18 hr elapsed after drug treatment before the removal of tissues and their subsequent homogenization in buffer, conditions which might be expected to favour a reduction in reversible enzyme inhibition. In this context, metabolic studies by others have revealed an extremely rapid excretion of benserazide from the rat with a half-life of 26 min after intraarterial administration [20]. However, the subsequent reversal of CRAO inhibition by dialysis of homogenates from benserazide-treated rats required almost three days to become complete.

A possible interpretation of these results may be that the *in vivo* interaction between benserazide and CRAO is more stable than that produced by preincubation with tissue homogenates, perhaps representing a pseudo-irreversible type of inhibition. It is known that the D,L-serine residue can be split from benserazide, at least *in vivo*, to generate free trihydroxy-benzylhydrazine [20] and it is therefore possible that this compound may be responsible for part or all the effects observed. In support of this, our *in vitro* studies revealed that D,L-serine and pyrogallol, representing constituent parts of the benserazide molecule were inactive or much less active, respectively, as inhibitors of CRAO. Since a number of other compounds with free or substituted hydrazine groupings (e.g. phenelzine, iproniazid, isoniazid) inhibit the various amine oxidases with different degrees of selectivity [21], the hydrazine moiety may well be the most important feature of benserazide for inhibition of CRAO.

In a separate study, we reported that the *in vivo* recovery of CRAO activity after benserazide treatment was consistent with a first-order process with a half-life of about six days [22]. From the present results, it is unclear whether this recovery was due to a very slow removal *in vivo* of reversibly bound benserazide, or alternatively if enzyme turnover plays a significant role. The use of a covalently-binding irreversible inhibitor of CRAO is required to answer this problem.

The *in vitro* inhibitory effects of benserazide upon CRAO activity were countered somewhat by an apparent 'activating' action of drug concentrations around 2×10^{-4}M. This became more prominent after incubating benserazide with homogenates for prolonged periods in the presence of high substrate concentrations, with the rationale that benzylamine might protect the enzyme's active-site from the inhibitory effects of benserazide, and at the same time the 'activating' effect would result in increased benzylamine metabolism. The results obtained appear to be consistent with this idea, and the 'activation' was also evident after dialysis of homogenates preincubated with 2×10^{-4}M benserazide. Benserazide has been reported to undergo gradual oxidation in solutions around neutral pH, to produce a mixture of complex polyphenol compounds [20] which presumably are responsible for the yellow–brown colour which developed in our preincubation mixtures containing benserazide at concentrations of 2×10^{-4}M and above. This colour was not removed by dialysis of homogenates, and we are tempted to speculate that potentiation of enzyme activity arises from this strong association with unidentified homogenate constituents (membranes?) although the exact nature of this phenomenon remains to be identified.

The *in vivo* inhibition of CRAO by benserazide was found to be dependent upon the dose used and the period of treatment. In addition, some evidence for tissue-selective inhibition was obtained, particularly after a single injection of various doses, with the enzyme in rat aorta being inhibited rather more than the heart enzyme, while that in brown adipose tissue was not significantly inhibited at this time. Possibly these results may reflect differences in the initial distribution of benserazide to these tissues since tissue-selective inhibition was much less evident when the cumulative effects of treatment for seven days were examined.

MAO-A activity in the same tissues was, in general, not inhibited by these doses of benserazide, except for a slight effect in brown adipose tissue. These results are in agreement with those of Burkard *et al.* [23], who showed that MAO activity in rat brain and liver was only weakly inhibited by a single injection of benserazide at doses greater than about 100 mg/kg. At present we have no obvious explanation for the observations that some doses of benserazide produced significant increases in MAO-A activity of the tissues, and these potentially interesting findings require confirmation by additional studies. Furthermore, it should be noted that, as with our previous experience, the activity of MAO-A showed quite considerable variation between individual animals of similar weight, particularly in the cardiovascular tissues, and thus with the small groups of animals used in the present studies, some relatively large changes in activity after benserazide failed to reach the level of statistical significance.

The possibility that L-DOPA administration might modify the inhibitory effects of benserazide upon CRAO activity was also studied. Doses of L-DOPA (250 mg/kg) and benserazide (40 mg/kg) were those used in some of our earlier studies on the effects of these drugs upon MAO activities in various tissues [15, 24, 25]. In addition, 40 mg/kg benserazide produces a selective inhibition of L-DOPA decarboxylation in peripheral tissues of the rat, with little penetration to the brain [23].

L-DOPA, administered by itself, had no significant effect upon CRAO activity in the rat aorta, although evidence for a slight increase in cardiac CRAO activity was obtained, particularly after four days. These findings were similar to those reported earlier

[25]. In contrast significant increases in MAO-A activity have been found under similar circumstances [25]. The administration of both L-DOPA and benserazide resulted in inhibition of CRAO activity which was not markedly different in degree as a whole from that produced by benserazide alone. These results are of interest in the context that the administration of both L-DOPA and peripheral decarboxylase inhibitors such as benserazide, to patients suffering from Parkinson's Disease has been a prominent feature of the therapy of this condition over the past few years [26].

Several recent reports have shown that in addition to inhibition of aromatic amino acid decarboxylase, benserazide also inhibits some of the enzymes of the tryptophan catabolic pathway [27–29], as well as catechol-*O*-methyl transferase [30], by acting as a substrate of the latter enzyme [31]. The present paper shows that inhibition of CRAO activity should be added to the recognised spectrum of biochemical actions shown by this compound. However, the possible significance of this finding remains elusive until the nature and physiological function of CRAO in animal tissues is more clearly defined. In this latter respect, valuable clues may exist in evidence first that this enzyme may have a subcellular localization on the cell plasmalemma in the rat aorta and brown adipose tissue [14, 32], and second that it may have a selective localization in smooth muscle, as indicated by histochemical studies with human uterus [33]. Although administration of benserazide to rats over long periods can result in skeletal deformities [34], there appears to be little evidence for any obvious adverse side-effects after administration to Parkinsonian patients [35, 36]. The recommended daily clinical dosage of benserazide to produce peripheral decarboxylase inhibition lies between 50 and 250 mg [37] which (assuming a 70 kg patient) would correspond closest to the lowest (5 mg/kg) of our doses used here in the rat. On the other hand, our studies include doses (up to 40 mg/kg) reported to be selective for peripheral decarboxylase inhibition in the rat, as well as some higher doses likely to cause cerebral decarboxylase inhibition [23].

Further investigations into the physiological role of CRAO in human and animal tissues may shed more light on these questions, and benserazide, as an inhibitor of this enzyme, may be a useful tool for such studies.

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