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The effect of DSP-4 (*N*-[2-chloroethyl]-*N*-ethyl-2-bromobenzylamine) on monoamine oxidase activities in tissues of the rat

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The in vitro inhibition of monoamine oxidase (MAO) in rat liver, and of the clorgyline-resistant amine oxidase (CRAO) in rat heart and aorta, by DSP-4 (*N*-[2-chloroethyl]-*N*-ethyl-2-bromobenzylamine) has been studied. Inhibition of each enzyme activity was independent of prolonged preincubation, was reversed by dialysis, and also Ackermann-Potter plots were consistent with reversible inhibition. Simple linear competitive inhibition of MAO-A and MAO-B was observed, with K_i values of 6×10^{-6} and 8×10^{-5} M, respectively. CRAO was inhibited in a mixed, non-competitive manner (K_i of $3{\cdot}2 \times 10^{-5}$ and $7{\cdot}8 \times 10^{-6}$ M in heart and aorta, respectively) which conformed to a kinetic model in which the binding of DSP-4 to CRAO increased the affinity of substrate binding, but prevented product formation. The possible significance of these results for the in vivo actions of the drug is discussed.

Several recent reports have described the properties of DSP-4 (*N*-[2-chloroethyl]-*N*-ethyl-2-bromobenzylamine) as an inhibitor of neuronal noradrenaline uptake in central and peripheral tissues of rats and mice. This compound, which is a tertiary amine, in solution at physiological temperature and pH undergoes spontaneous cyclization to form a positively charged aziridinium derivative. It has been proposed that this aziridinium ion leads to alkylation and irreversible damage of a constituent of the adrenergic neuronal membrane, which may be the carrier for noradrenaline uptake. In addition, DSP-4 appears to induce the long-term degeneration of some noradrenergic neurons, particularly in the brain, although it is not clear whether or not this occurs solely as a result of actions on the neuronal membrane. Also, accumulation of the drug inside the neurons may play a secondary role (Ross 1976; Ross & Renyi 1976; Zieher & Jaim-Etcheverry 1980).

If DSP-4 does indeed accumulate at intracellular sites, it is possible that an additional property of this drug might be inhibition of mitochondrial monoamine oxidase (MAO). The aziridinium form of DSP-4 is extremely similar, structurally, to the adrenergic neuron blocking drug, bretylium, a quaternary amine which does not possess alkylating groups, but which nevertheless has been reported to inhibit MAO activity both in vitro and in vivo (Kuntzman & Jacobson 1963; Clarke 1970). MAO is now believed to exist in animal tissues in at least two forms, called MAO-A and -B, which are distinguished primarily by their relative sensitivity towards acetylenic inhibitors such as clorgyline and deprenyl (see Fowler et al 1978, for review). These two activities also show different substrate specificities. In the rat liver for example, 5-hydroxytryptamine (5-HT) and benzylamine (BZ) are substrates almost exclusively for MAO-A and -B, respectively (Hall et al 1969; Parkinson et al 1980). In addition, a clorgyline-resistant amine oxidase (CRAO) which is distinct from MAO-A and -B, and which also metabolizes BZ has been described in cardiovascular tissues of the rat (Lyles & Callingham 1975; Clarke et al 1980; Lyles & Callingham 1980). Since DSP-4 is a potential alkylating agent with a structure based on that of benzylamine, we have examined whether it had any inhibitory actions on these enzyme activities. We report the results of studies using MAO-A and MAO-B from rat liver, and CRAO from rat heart and aorta homogenates, and present a kinetic evaluation of those inhibitory effects of DSP-4 found.

MATERIALS AND METHODS

Materials

Radioactive substrates for MAO were [^{3}H]5-hydroxytryptamine creatinine sulphate and [^{14}C]benzylamine hydrochloride from the Radiochemical Centre, Amersham, U.K.

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DSP-4 hydrochloride was kindly provided by Dr S. B. Ross, Astra Läkemedel AB, Södertälje, Sweden.

Male Sprague-Dawley rats, about 200 g, were supplied by A. J. Tuck & Sons, Rayleigh, U.K.

Methods

Livers, hearts and aortae from four rats were removed, and stored deep-frozen until ready for use. When required, samples of tissue were thawed, pooled and homogenized in 1 mM potassium phosphate buffer, pH 7·8, with a ground glass hand homogenizer. Tissue:buffer ratios of homogenates (g ml^{-1}) were 1:10 (heart), 1:50 (liver) and 1:320 (aorta). Homogenates were centrifuged at 600 *g* for 10 min to remove cell debris, and the resultant supernatants were decanted and used for the subsequent inhibition experiments.

Enzymatic activities of amine oxidases in these homogenates were assayed by the method of Callingham & Laverty (1973). Assay mixtures normally contained 25 µl homogenate and 25 µl water or DSP-4 solution (in distilled water). However, for construction of Ackermann-Potter plots (Ackermann & Potter 1949), homogenate volumes of 5–25 µl were added to appropriate volumes of water (or DSP-4 solutions) to keep a total preincubation volume of 50 µl. These mixtures were preincubated at 37 °C for periods depending upon the experimental design (see Results). After preincubation, assay tubes were returned to an ice-bath and then remaining enzyme activity was assayed by the addition of 50 µl radioactive substrate solutions, made to appropriate concentrations in 0·2 M potassium phosphate buffer, pH 7·8. Specific radioactivities of substrates were 2 µCi μmol^{-1} for 5-HT (for assay of MAO-A), and 0·5 µCi or 10 µCi μmol^{-1} for BZ (for assay of MAO-B and CRAO, respectively). Assay tubes were flushed with O_2, capped with rubber stoppers and incubated for 5 min at 37 °C, during which time product formation was linear. The enzymatic reactions were then terminated by plunging assay tubes into an ice-bath, followed by rapid addition of 10 µl 3 M HCl to each tube. Radioactive deaminated metabolites were extracted into 0·6 ml ethyl acetate–toluene (1:1 v/v) and 0·4 ml of each sample was counted for radioactivity by liquid scintillation spectrometry with correction for quenching by means of automatic external standardization. In control experiments, DSP-4 (at 4×10^{-4} M) had no effects on extraction of metabolites of either 5-HT or BZ, produced in assays containing each of the three enzyme activities described above.

RESULTS

In preliminary experiments designed to investigate whether or not DSP-4 was capable of inhibiting MAO and CRAO, samples of rat liver and heart homogenates were preincubated for 20 min with various concentrations of DSP-4 (10^{-9} to 10^{-3} M during preincubation) before the addition of 5-HT or BZ at final concentrations of 100 µM to assay MAO-A or MAO-B activity respectively in rat liver, or BZ (1 µM) to assay CRAO activity in rat heart homogenates. Addition of substrate in these experiments results in a reduction of DSP-4 concentration in the assay to one-half of that present during preincubation. Under these initial conditions, all three enzyme activities were inhibited by increasing concentrations of DSP-4, with approximate IC50 concentrations (during preincubation) of 5×10^{-5} M (MAO-A), 4×10^{-4} M (MAO-B) and 3×10^{-5} M (CRAO).

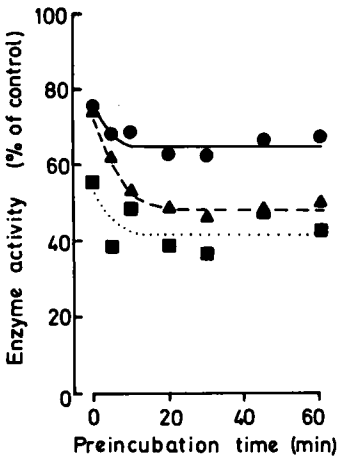

FIG. 1. Effect of preincubation time upon inhibition of amine oxidase activities. ●—● rat liver MAO-A (5-HT at 100 µM as substrate); ▲– –▲ rat liver MAO-B (BZ at 100 µM); ■ · · · · ■ rat heart CRAO (BZ at 1 µM). DSP-4 preincubation concentrations were 5×10^{-5} M (for MAO-A and CRAO) and 5×10^{-4} M (for MAO-B). Activities are expressed as percentages of corresponding controls containing no DSP-4. Each point is the mean of triplicate determinations.

The reversibility of the interaction between DSP-4 and these enzymes was examined in the following ways. First, experiments were performed to investigate whether or not the inhibition of each enzyme by DSP-4, used at or near these IC50 concentrations, was dependent upon the period of preincubation. Fig. 1 shows that that some inhibition of each enzyme occurred without preincubation. An additional small increase in the percentage inhibition was seen during

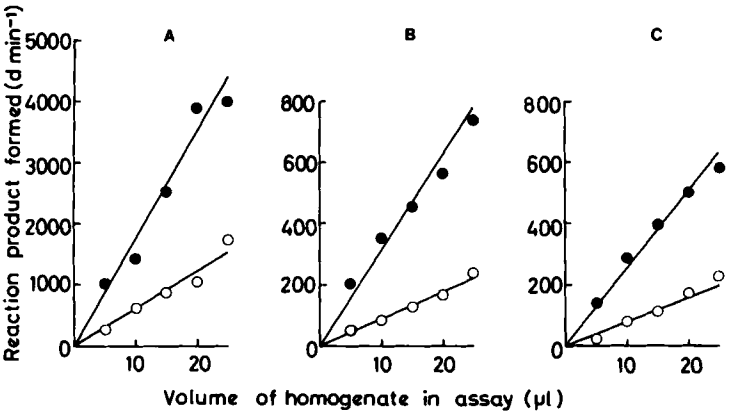

FIG. 2. Ackermann-Potter plots showing inhibition of rat liver MAO-A (panel A), MAO-B (panel B) and rat heart CRAO (panel C) by DSP-4. The reaction product formed by using different volumes of rat liver or heart homogenate in the assay was measured after preincubation for 10 min of samples in the absence of (●) or presence (○) of DSP-4. Concentrations of DSP-4 and substrates corresponded to those shown in Fig. 1. Each point is the mean of duplicate determinations.

the initial 10 min of preincubation, especially with MAO-B, but there was no evidence for a prolonged and continuous time-dependence up to 60 min. The lack of a prolonged time-dependence of inhibition by DSP-4 may indicate a predominantly reversible mode of inhibition, but this possibility was also confirmed more directly. Fig. 2 shows the influence of DSP-4 upon Ackermann-Potter plots for each enzyme, in which the reaction product formed is plotted as a function of the amount of enzyme (homogenate) in the assay (Ackermann & Potter 1949). Both the control plots, and those obtained in the presence of suitable preincubation concentrations of DSP-4 intersect at the origin, a result indicative of reversible inhibition. Although not shown, similar results were obtained with samples that had been preincubated for 60 min with DSP-4, showing that the mode of inhibition was not altered with prolonged preincubation. In addition, after preincubation for 10 min of comparable enzyme + inhibitor mixtures containing DSP-4 at concentrations of 5×10^{-5} M (MAO-A or CRAO) and 5×10^{-4} M (MAO-B), followed by dialysis of these mixtures at 4 °C for 40 h against 1 mM potassium

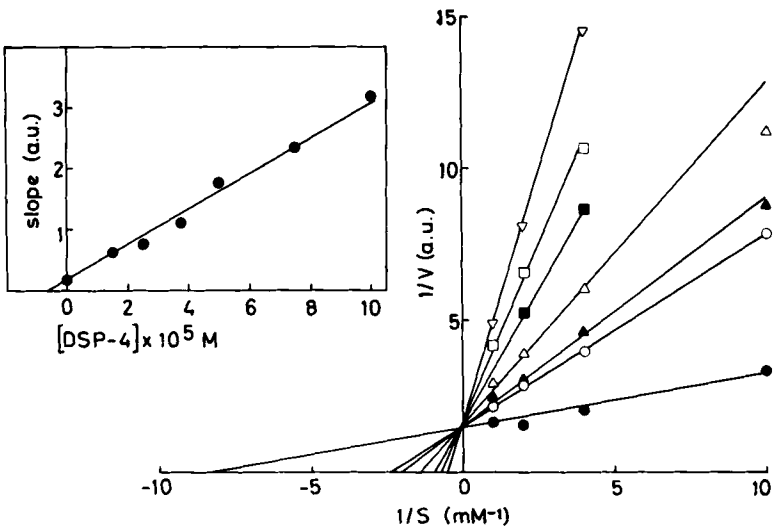

FIG. 3. Lineweaver-Burk plot showing inhibition of rat liver MAO-A by DSP-4. 5-HT used at substrate (S) concentrations of 0·05, 0·10, 0·25 and 0·5 mM. Reaction velocities (V) in arbitrary units (a.u.) DSP-4 concentrations ($\times 10^5$ M) were zero (●), 1·5 (○), 2·5 (▲), 3·75 (△), 5·0 (■), 7·5 (□), 10·0 (▽). Each point is the mean of duplicate determinations. Inset: Replot of slopes (●) against DSP-4 concentrations, estimated from the primary reciprocal plot.

phosphate buffer, pH 7·8, it was found that DSP-4 caused no detectable inhibition of enzyme activity, thus providing further evidence for the reversible nature of the inhibition.

As a result of these initial findings, a more detailed kinetic analysis of the inhibitory properties of DSP-4 was made by investigating the effects of different concentrations of DSP-4 upon initial reaction velocities of MAO-A, MAO-B and CRAO, measured over a range of concentrations of their appropriate substrates. In these studies, samples were preincubated for 10 min with inhibitor, and all DSP-4 concentrations given are those *after* the addition of substrate. These results were plotted by the double-reciprocal Lineweaver-Burk method in order to determine the type of inhibition involved.

Fig. 3 shows the results obtained with MAO-A. Deamination of 5-HT by rat liver homogenates in the absence of DSP-4 had a K_m of approximately 120 μM. DSP-4, at final assay concentrations from $1{\cdot}5 \times 10^{-5}$ to $1{\cdot}0 \times 10^{-4}$ M was a competitive inhibitor of MAO-A. The slope replot of these data was linear, and indicated a K_i value of about 6×10^{-6} M for DSP-4.

The deamination of BZ by MAO-B in rat liver homogenates had a K_m of about 180 μM. The inhibition of MAO-B by DSP-4 at final assay concentrations from $1{\cdot}5 \times 10^{-4}$ to 5×10^{-4} M was also competitive, with a linear slope replot indicating a K_i value of about 8×10^{-5} M for DSP-4 (Fig. 4).

The inhibition by DSP-4 of CRAO in rat heart and aorta homogenates was investigated by using BZ as substrate at concentrations from 1–8 μM (Figs 5,6). K_m values of BZ metabolism by CRAO in these tissues (see discussion) were 5·7 μM (heart) and 2·4 μM (aorta). In both tissues, CRAO was inhibited in a mixed fashion. These results are similar to those predicted by the intersecting, linear non-competitive inhibition model described by Segel (1975) shown below:

$$\begin{array}{ccccc} E + S & \overset{K_s}{\rightleftharpoons} & ES & \xrightarrow{k_p} & E + \text{product} \\ + & & + & & \\ I & & I & & \\ K_i \Vert & & \alpha K_s \Vert & & \alpha K_i \\ EI + S & \rightleftharpoons & ESI & & \end{array}$$

In this model, the dissociation constant (K_s) for substrate (S) binding to the EI complex differs from that to the enzyme (E) alone by a factor of α, and the resulting ESI complex is catalytically inactive. If $\alpha < 1$ the plots will intersect below the 1/S axis of primary reciprocal plots, as was found with the present data. The intercept on the [I]-axis of a replot of slopes (from the primary reciprocal plot) versus [I], gives the value of $-K_i$. A replot of the 1/V-axis intercepts versus [I], on the other hand gives an [I]-axis intercept of $-\alpha K_i$. From the analysis of the present results in this way (Figs 5,6), values for K_i were estimated to be $3{\cdot}2 \times 10^{-5}$ M (heart) and $7{\cdot}8 \times 10^{-6}$ M (aorta), and for α were 0·19 (heart) and 0·36 (aorta).

DISCUSSION

The present results show that DSP-4 is a competitive inhibitor of MAO-A and MAO-B in rat liver homogenates, and a mixed type inhibitor of CRAO activity in rat heart and aorta. There was little dependence upon preincubation, suggesting that the mode of inhibition is probably reversible and this

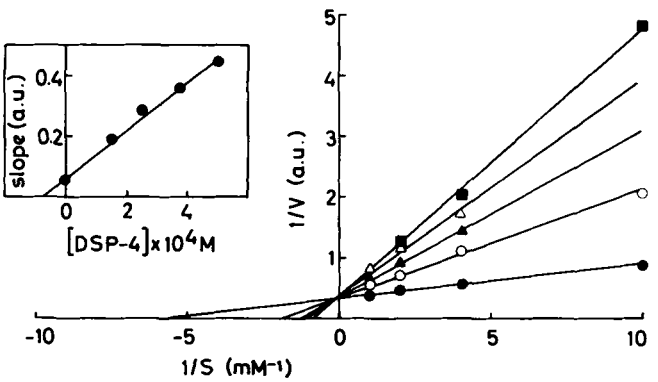

FIG. 4. Lineweaver-Burk plot showing inhibition of rat liver MAO-A by DSP-4. BZ used at substrate (S) concentrations of 0·05, 0·10, 0·25 and 0·5 mM. Reaction velocities (V) in arbitrary units (a.u.) DSP-4 concentrations (× 10^4 M) were zero (●), 1·5 (○), 2·5 (▲), 3·75 (△), 5·0 (■). Each point is the mean of duplicate determinations. Inset: Replot of slopes against DSP-4 concentrations, estimated from the primary reciprocal plot.

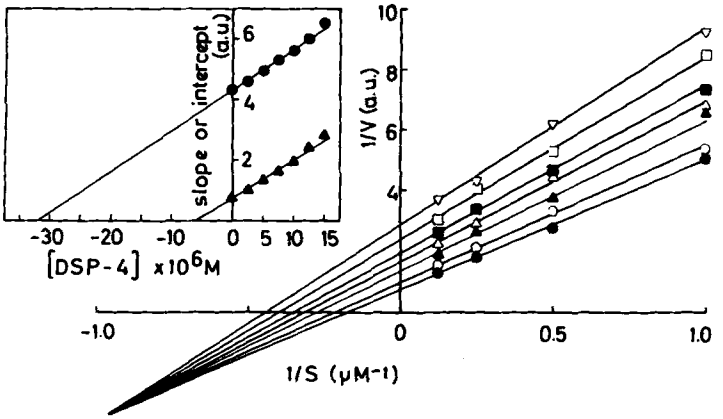

FIG. 5. Lineweaver-Burk plot showing inhibition of rat heart CRAO by DSP-4. BZ used at substrate (S) concentrations of 1, 2, 4, 8 μM. Reaction velocities in arbitrary units (a.u.) DSP-4 concentrations (× 10^6 M) were zero (●), 2·5 (○), 5·0 (▲), 7·5 (△), 10·0 (■), 12·5 (□), 15·0 (▽). Each point is the mean of duplicate determinations. Inset: Replots of slopes (●) and intercepts (▲) against DSP-4 concentrations, estimated from the primary reciprocal plots.

conclusion was confirmed by the method Ackermann & Potter (1949) and by reversibility of the inhibition upon dialysis.

DSP-4 has been reported to cyclize spontaneously, with a half-life of 7 min, to an aziridinium derivative upon incubation in aqueous media at physiological pH (Ross et al 1973; Zieher & Jaim-Etcheverry 1980). The completion of this transformation under our experimental conditions may be responsible for the slight time-dependence of inhibition, particularly of MAO-B, during the initial 10 min of preincubation at 37 °C. Alternatively, other factors such as diffusion or lipid solubility may regulate the access of the inhibitor to the active site of the enzyme, and thus influence the attainment of equilibrium conditions. Whether DSP-4 or its aziridinium derivative is the active agent, it is clear that the inhibition becomes unchanged and is also reversible after that time. Thus, unlike the effects on neuronal noradrenaline uptake (Ross 1976; Ross & Renyi 1976; Zieher & Jaim-Etcheverry 1980), there is little evidence to suggest that alkylation plays a role in the inhibition of amine oxidases by DSP-4.

From the present analysis, DSP-4 was a slightly more potent inhibitor of MAO-A (K_i of 6 × 10^{-6} M) than of MAO-B (K_i of 8 × 10^{-5} M). The inhibition of both MAO-A and MAO-B conformed to a simple linear competitive scheme. The oxidation of amines by MAO is believed to occur by means of a Ping-Pong mechanism, with the second substrate, oxygen, increasing amine metabolism in an uncompetitive manner as the oxygen concentration is raised

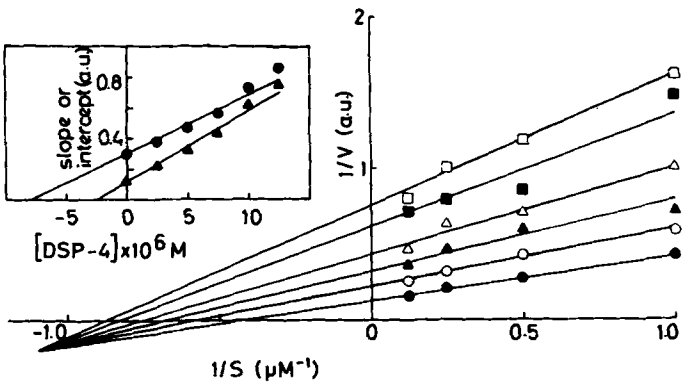

FIG. 6. Lineweaver-Burk plot showing inhibition of rat aorta CRAO by DSP-4. BZ used at substrate (S) concentrations of 1, 2, 4, 8 μM. Reaction velocities in arbitrary units (a.u.) DSP-4 concentrations (× 10^6 M) were zero (●), 2·5 (○), 5·0 (▲), 7·5 (△), 10·0 (■), 12·5 (□). Each point is the mean of duplicate determinations. Inset: Replot of slopes (●) and intercepts (▲) against DSP-4 concentrations, estimated from the primary reciprocal plot.

(Houslay & Tipton 1973). The present kinetic analysis of the actions of DSP-4 has been made only at a fixed oxygen concentration (obtained by flushing the assay tubes with oxygen before incubation). Since factors such as these can influence the kinetics of inhibition of Ping-Pong systems (Segel 1975; Roth 1979), effects of DSP-4 upon the binding of oxygen to the enzyme cannot be ruled out at the present time, and therefore the exact molecular interactions of DSP-4 with MAO-A and MAO-B remain unclear.

In the current experiments, the K_i values for inhibition by DSP-4 of CRAO ($3{\cdot}2 \times 10^{-5}$ M, heart; $7{\cdot}8 \times 10^{-6}$ M, aorta) were intermediate between those for inhibition of MAO-A and MAO-B. Although the metabolism of BZ in the rat heart is brought about by all three enzymic activities, CRAO has a very much lower K_m (here 5·7 μM) than either MAO-A or MAO-B for this substrate, and thus kinetic studies on CRAO alone in this tissue can be performed by the use of suitably low BZ concentrations, where the contribution of MAO-A and MAO-B is negligible (Lyles & Callingham 1975; Clarke et al 1980; Lyles & Callingham 1980). In the rat aorta CRAO alone is responsible for BZ metabolism (Clarke et al 1980) with a similarly low K_m (2·4 μM in the current studies). Inhibition by DSP-4 of CRAO from both sources could be described by a non-competitive model, in which the substrate (S) can bind to the EI complex with a higher affinity, than to E alone, although the resulting ESI moiety is catalytically inactive. The kinetic constants obtained were not identical for CRAO in each tissue, and it is not yet clear whether or not this indicates differences in CRAO contained within them. Although relatively little is known, to date, about the properties, function and catalytic mechanism of CRAO in rat cardiovascular tissues, other preliminary evidence has suggested that CRAO from rat heart and aorta may not be identical (Clarke et al 1980).

In conclusion, it appears that DSP-4 is capable of inhibiting MAO-A, MAO-B and CRAO activity in vitro. The possibility cannot be discounted that these inhibitions arise from DSP-4 acting as a substrate for some or all of these enzyme activities. In this regard, some tertiary (but not quaternary) amines have been reported to be oxidized by MAO, although generally very poorly (Blaschko 1952). The substrate specificity of CRAO has not yet been investigated in detail. It is also not clear if these inhibitory effects would have any significance for the drug's pharmacological profile in vivo. In order to inhibit MAO activity in vivo, which is believed to be located predominantly on the outer mitochondrial membrane (Schnaitman et al 1967), DSP-4 would need to be accumulated at appropriate concentrations within neurons and other cells. Information about whether this occurs is lacking, although this mechanism has been proposed for the structurally similar drug bretylium, which shows MAO inhibitory actions in vitro and in vivo (Kuntzman & Jacobson 1963; Clarke 1970). However, it is difficult to compare the in vitro potency of DSP-4 found here with that for bretylium since a proper kinetic analysis was not undertaken in those previous studies (Kuntzman & Jacobson 1963).

The location of rat cardiovascular CRAO activity is a subject of increasing interest, and has been found in subcellular fractions containing mitochondria, microsomes or cell cytosolic constituents (Coquil et al 1973; Lyles & Callingham 1975; Fowler & Callingham 1977; Parkinson et al 1980). Of particular relevance is a recent abstract which has proposed that this activity is found on the plasma cell membrane, at least in the rat aorta (Duong et al 1981) although the cell type involved is not clear. In addition, preliminary work in this laboratory by Dr M. A. Barrand shows that there may be a similar distribution of CRAO in the brown adipose tissue of the rat. This location of CRAO in the plasmalemma would indicate that intracellular accumulation of DSP-4 might not be needed for inhibition of CRAO in vivo. However, until more is known about the function and physiological substrate(s) of this enzyme, the possible significance of its inhibition by DSP-4, and other agents, will remain unanswered.

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