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The effect of tris buffers on rat liver mitochondrial monoamine oxidase

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The effect of tris buffers upon the monoamine oxidase (MAO) activity in rat liver mitochondria has been investigated. Tris buffer was shown to inhibit MAO in a non-competitive manner with a Ki of 15–25 mM. Tyramine, 5-hydroxytryptamine and β-phenethylamine but not benzylamine oxidations were all inhibited by tris buffer. All inhibitions, except that of 5-HT, were completely reversible. It is suggested that these effects are produced by conformational changes in the structure of the MAO. The significance of these results is discussed with respect to the use of tris buffers in the extraction and estimation of the activity of MAO.

Previous work in this laboratory (Browne, Laverty & Callingham, 1973) has shown that rat liver and heart monoamine oxidase (E.C. 1.4.3.4., MAO) is inhibited by tris-HCl buffer, when tyramine but not benzylamine is used as substrate. Yasunobu & Oi (1972) have stated that, in bovine liver, tris buffer is an uncompetitive inhibitor of MAO, with respect to benzylamine and a competitive inhibitor with respect to oxygen. As many investigators use tris buffers in experiments involving MAO, a more complete study of possible interactions between MAO and tris buffers seemed appropriate.

MATERIALS AND METHODS

Materials

The radioactive substrates for MAO [^{3}H]tyramine and [^{14}C]β-phenethylamine were obtained from New England Nuclear GMBH, Dreieichenhain, Germany; [^{3}H]5-hydroxytryptamine from The Radiochemical Centre, Amersham, U.K.; and [^{14}C]benzylamine from ICN Pharmaceuticals, Hersham, U.K. Tris [tris-(hydroxymethyl)-methylamine] was of analar grade and obtained from Fisons Scientific Apparatus, Loughborough, Leicestershire, U.K. All other reagents were standard laboratory reagents of analytical grade wherever possible. Male Wistar rats were obtained from A. J. Tuck & Son, Rayleigh, Essex.

Methods

Rats, after weighing, were killed by a blow to the head. The livers of the animals were rapidly removed, weighed and homogenized 1:10 in ice cold isotonic potassium phosphate buffer at either pH 7·4 or 7·8. Both conical glass homogenizers and an MSE Atomix blender were used; no effect on the subsequent results could be detected due to differences in the method of homogenization, other than the absolute activity recovered, which was always lower following the use of the blender. The homogenates were centrifuged twice at 600 *g* for 10 min to remove nuclei and cell debris. The resulting supernatants were then centrifuged at 15 000 *g* for 10 min to yield crude mitochondrial pellets. The pellets were divided and half resuspended in isotonic phosphate. The other half was resuspended in isotonic tris-HCl buffer of the same pH as the phosphate. These fractions were centrifuged twice at 15 000 *g* for 10 min, with the pellets being resuspended each time in their respective buffers. The final fractions are referred to as 'phosphate fraction' (P-fraction) and 'tris fraction' (T-fraction) respectively. All experiments were done using these fractions.

The radiochemical assay for MAO was that of McCaman, McCaman & others (1965) as modified by Callingham & Laverty (1973). In those experiments where the MAO activity was determined in the presence of various concentrations of tris buffer, the tris was added to the P-fraction immediately before the addition of the substrates. [^{3}H]tyramine, [^{3}H]5-hydroxytryptamine (5-HT), [^{14}C]β-phenethylamine and [^{14}C]benzylamine were used as substrates. MAO values are expressed as specific activities, i.e., in nmol (of substrate consumed) (mg protein)$^{-1}$ h^{-1}, calculated as means ± standard error of the mean.

Protein contents of the fractions were estimated by the micro-biuret method of Goa (1953).

Statistical significance between fractions was determined by Student's *t*-test.

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RESULTS

The MAO activities of the P- and T-fractions from three groups of rats were determined and the results are shown in Table 1. The three groups were: Group 1:4 homogenates, each derived from the livers of two rats (body wt 200 g), at pH 7·4. Group 2:4 homogenates, each derived from the livers of four rats (body wt 100 g), at pH 7·8. Group 3:8 homogenates, each derived from the livers of 3 rats (body wt 150 g), at pH 7·8.

Table 1. *The effect of tris buffer upon rat liver mitochondrial fractions.* For details of each experimental group see text. All assays were performed in duplicate on all the fractions from each group. MAO activities from Group 1 were obtained from initial velocities calculated by computer program from incubation periods of 5, 10 and 20 min at 37°. Groups 2 and 3 values were calculated from 5 min incubation periods. 25 μl of each fraction was used in 100 μl of assay mix to produce a final concentration of 38·5 mM of tris.

Group	Substrate (mM) 5-HT	P-fract.	T-fract.	Relative Act. $\left(\frac{T}{P} \times 100\right)$	
1	1	278·2 ± 13·0	81·9 ± 11·0	29·4	< 0·001
2	0·2	150·7 ± 10·7	33·2 ± 8·8	22·1	< 0·001
3	0·2	268·1 ± 17·3	55·8 ± 3·8	20·8	< 0·001
	Tyramine				
1	1	821·4 ± 78·4	288·3 ± 23·7	35·1	< 0·01
2	0·2	216·7 ± 3·6	52·6 ± 11·6	24·3	< 0·001
	1	466·4 ± 20·2	197·0 ± 30·8	42·2	< 0·001
3	0·2	285·5 ± 11·2	85·0 ± 5·8	29·8	< 0·001
	1	508·4 ± 22·6	164·6 ± 8·8	32·4	< 0·001
	β-Phenethylamine				
1	1	673·0 ± 68·1	238·1 ± 22·3	35·4	< 0·001
2	0·05	159·5 ± 5·2	39·3 ± 7·2	24·6	< 0·001
3	0·05	306·0 ± 10·7	83·6 ± 4·9	27·3	< 0·001
	Benzylamine				
1	1	347·5 ± 21·9	290·0 ± 26·0	83·5	NS
2	0·25	176·8 ± 6·6	204·1 ± 33·0	115·4	NS
3	0·25	216·1 ± 19·7	212·3 ± 22·3	98·2	NS

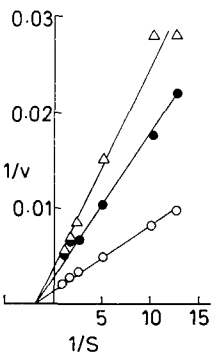

FIG. 1

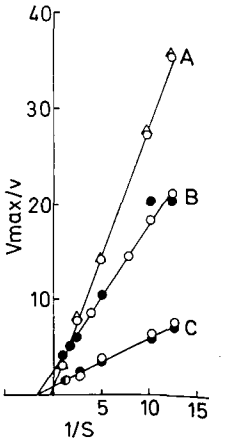

FIG. 2

FIG. 1. Inhibition of liver mitochondrial MAO by tris-HCl using tyramine as substrate. Double reciprocal plot; abscissa, 1/(substrate concentration in mM); ordinate, 1/(initial velocity). Initial velocities were calculated by computer program from progress curves of MAO activity at 5, 10, 15 and 20 min, and expressed as nmol (of substrate consumed) (mg protein)$^{-1}$ h^{-1}. All assays were performed in duplicate and results expressed as means. ○— P-fraction from group 3; ●— P-fraction in presence of 19·25 mM tris; △— T-fraction from group 3 (i.e., 38·5 mM tris).

FIG. 2. Comparison between theoretically derived double reciprocal plots and experimental data from Fig. 1. Abscissa, 1/(substrate concentration in mM); ordinate, Vmax/v (i.e., the ratio of the maximal velocity of the P-fraction from group 3 to the initial velocity). A: (theoretical) ○— Ko = 0·1 mM^{-1}, Ksi = 0; △— Ko = 3 μM^{-1}, Ksi = 10 mM^{-1}, assuming a tris concentration of 38·5 mM. B: ○— theoretical values for non-competitive inhibition, assuming a tris concentration of 38·5 mM and a Ki for tris of 22 mM; ●— experimentally determined values for T-fraction of group 3 (in presence of 38·5 mM tris). C: ○— theoretical values assuming that there is no inhibition of MAO and a Km of 0·56 mM; ●— experimental values of uninhibited P-fraction from group 3.

Substantial inhibition of MAO activity was caused by 38·5 mM tris in the T-fraction with all substrates except benzylamine which was unaffected under these conditions. Inhibition was seen whether substrates were at 1 mM or at concentrations near the Km values determined by Houslay & Tipton (1974).

Kinetic studies were performed on Group 3 P- and T-fractions and on the Group 3 P-fractions to which tris had been added to give a final assay concentration of 19·25 mM. Four time intervals (5, 10, 15 and 20 min) were used with 6 concentrations of tyramine as substrate in order to calculate the initial velocities of the reaction of each substrate concentration. These results are summarized in Fig. 1.

Tris buffer was found to reduce the Vmax value without effect on the Km of tyramine for the MAO. Similar results were also obtained using Group 1 fractions when tyramine, 5-HT and β-phenethylamine were used as substrates. Replotting the data from Fig. 1 by the method of Dixon (1953) gave straight lines intersecting at a single point on the abscissa, which is consistent with pure non-competitive inhibition. A Ki for tris of 16 mM was found.

Control experiments showed that the addition of tris did not influence the extraction of the deaminated metabolites into the organic layer in the assay procedure (1:1 benzene–ethyl acetate saturated with water). The inhibition was not due to the high con-

centration of chloride ions found in the tris-HCl buffer since isotonic KCl caused no inhibition at all. The degree of inhibition of the P-fraction in the presence of 19·25 mM tris, was the same regardless of the anion used with the exception of ascorbate which afforded a significant amount of protection (Table 2).

Table 2. *Effect of different tris salts upon the inhibition of tyramine oxidation by MAO.* The Group 3 P-fractions were used and incubated with either 19·25 mM phosphate or tris buffers, pH 7·8. The substrate concentration was 0·2 mM. Assays were performed in duplicate on each of the 8 fractions of this group. The results are expressed as the means and standard errors of the ratios (× 100) of the tris: phosphate activities. 95% confidence limits were calculated (Goldstein, 1967) from the absolute values of the enzyme activities compared with tris-HCl values.

Tris salt (19·25 mM)	% Activity	95% Confidence limits
Tris-HCl	44·1 ± 3·5	—
Tris-phosphate	43·9 ± 3·7	0·71–1·46
Tris-acetate	49·6 ± 1·9	0·93–1·41
Tris-ascorbate	57·2 ± 1·3	1·11–1·58*
Tris-citrate	50·2 ± 3·0	0·92–1·43
Tris-oxalate	48·9 ± 2·7	0·96–1·31

* Significantly different from tris-HCl.

The possibility that the inhibition produced by tris buffer could be due to the formation of complexes between the buffer and the substrates for the enzyme was examined by comparing the results shown in Fig. 1 with theoretical plots for pure non-competitive inhibition and complex formation. The comparison is shown in Fig. 2. The experimentally obtained results were in close agreement with the theoretical plot for non-competitive inhibition, but were widely different from the theoretical plots for complex formation, obtained by the method described by Segel (1975) (see Discussion).

The effect of tris buffer was the same whether the assay mixture had been exposed to oxygen during the reaction or to air, suggesting that the tris does not affect the binding of oxygen to the enzyme.

The reversibility of the inhibition produced by tris was tested with homogenates from Group 3 P-fraction. The P-fraction was exposed to increasing concentrations of tris up to that found in the T-fraction. Diluting this final fraction reduced both enzyme and inhibitor concentrations. The reduction in enzyme concentration was compensated by expressing its activity in terms of specific activity, thus essentially changing only the inhibitor concentration (see Webb, 1963). If the inhibitor is reversible, the recovery in specific activity of the enzyme should be superimposable upon the curve of the increasing inhibition with addition of tris. This was found to be so with tyramine and β-phenethylamine as substrates, but the inhibition of 5-HT oxidation was only partially reversible (Fig. 3).

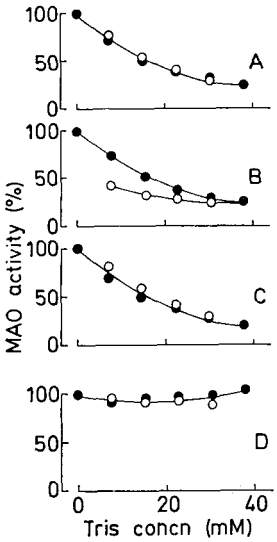

FIG. 3. Reversibility of the inhibition of group 3 P-fraction MAO by tris-HCl. Abscissae, tris concentration in mM; ordinates, MAO activity as percent of control. The values are the means of duplicate determinations from the 8 P-fractions of group 3. The s.e. of the ratios are within the circles in all cases. ●— increasing tris concentrations; ○— reduction of tris concentrations by serial dilution, with compensation for the decrease in protein concentration. Substrates: A; tyramine (0·2 mM); B, 5-HT (0·2 mM); C, β-phenethylamine (0·05 mM); D, benzylamine (0·25 mM), used to check that, in the absence of inhibition by tris, this method was reproducible throughout the serial additions and dilutions.

DISCUSSION

By the use of a radiochemical method for the estimation of MAO activity, it has been possible to confirm and extend our observation that tris inhibits the oxidative deamination of tyramine but not of benzylamine (Browne & others, 1973). Moreover, it has been found that tris inhibits the oxidation of 5-HT and of β-phenethylamine in mitochondrial fractions.

Several mechanisms could, at first sight, be put forward to account for these results. First, a substantial amount of the substrate complexes with the tris to yield a complex that is not oxidized by the

enzyme or extracted into the organic solvent used in the assay procedure. Secondly, a small amount of complex formation occurs, the resulting complex being the real inhibitor of the enzyme. In both these cases, there would be no complex formation with benzylamine. Thirdly, the inhibition is due to the chloride ions present in the tris-HCl buffer. Fourthly, the tris buffer affects the uptake of the deaminated metabolites into the organic layer. Finally, the tris inhibits the MAO enzyme system itself.

In order to reveal the presence of any tris-substrate complex, the kinetics of the inhibition have been examined. If a complex had been formed the reaction could be described by:

$$\begin{array}{ccccc} & & \overset{Ks}{} & \overset{kp}{} & \\ E + S & \rightleftharpoons & ES & \longrightarrow & E + P \\ & + & & & \\ & I & & & \\ {\scriptstyle Ko}\downarrow\uparrow & & {\scriptstyle Ksi} & & \\ E + SI & \rightleftharpoons & ESI & & \end{array}$$

(see Segel, 1975)

In the first case above, Ko is large, Ksi = 0. In the second case, Ko is small and Ksi is large. With these assumptions, calculations similar to those described by Segel (1975) can be made, and theoretical Lineweaver-Burk plots made for different values of Ko and Ksi assuming further that the Km of the enzyme for tyramine is 0·56 mM. This value has been obtained by the method of Wilkinson (1961) from the data in Fig. 1 for the phosphate fractions. Two of these theoretical plots are shown in Fig. 2 where they have the appearance of competitive inhibition. Furthermore, it can be shown in the general case that the velocity of the reaction would approach the uninhibited Vmax as the substrate concentration tended to infinity. The best fit to the actual experimental results shown in Fig. 2 is found when the interaction is non-competitive with a Ki in the range of 15 to 25 mM. From the data shown in Fig. 3 it would seem that the Ki values for all the substrates affected are in this range, and a Ki of 16 mM using tyramine as substrate is found upon Dixon analysis.

The mechanism of MAO action in the rat liver has been shown to be a 'Ping-Pong' or double displacement reaction (Houslay & Tipton, 1973; 1975). Carrying out the reaction in an atmosphere of oxygen rather than air increases the MAO activity in an uncompetitive manner (Tipton, 1972). Therefore, reactions done in oxygen will yield values for the apparent Km higher than those done in air. If the inhibition by tris were due to either form of complexing described above, the two-fold change in Km due to the use of either air or oxygen (Houslay & Tipton, 1974; 1975) would change the effective concentration of substrate (Segel, 1975), and thus the observed inhibition by tris. In non-competitive inhibition, the percentage activity remaining at a single inhibitor concentration is not dependent on Km but is given by: $100 \times (1 + i/Ki)$, and so change in Km would have no effect unless tris directly affected the binding of oxygen to MAO, which is improbable, as benzylamine oxidation is unaffected by addition of tris. As there is no change in the % inhibition with change in oxygen tension, it is unlikely that complex formation plays any part in the inhibition produced by tris.

Van Woert & Cotzias (1966) have suggested that anion inhibition of MAO can occur in the rat liver, and bovine thyroid gland microsomal MAO is sensitive to halide ions (Mushawar, Oliner & Schulz, 1972). It is thus possible that the inhibition of MAO in these experiments could have been due to the presence of chloride ions in the buffer. The P-fractions of Group 3 were tested in the presence of 38·5 mM KCl and no significant inhibition found. No other anion tested changed the inhibition by tris, except ascorbate, which reduced it, possibly due to its anti-oxidant property (Table 2).

No evidence could be found to support the suggestion that tris altered the uptake of deaminated metabolites into the organic layer used in the assay.

All the present experiments would suggest that tris interacts directly with the enzyme in a pure non-competitive manner. This differs somewhat from that found in bovine liver, where the inhibition of benzylamine oxidation is uncompetitive with respect to tris (Yasunobu & Oi, 1972). In the present experiments, benzylamine, of all substrates tested, was the only one whose oxidation was not influenced by tris.

There is now a considerable amount of evidence to indicate that MAO in many tissues can exist in at least two forms resolvable by their different substrate and inhibitor specificities (see Neff & Yang, 1974). But tris would appear to show no selectivity for either of the two forms of MAO in the rat liver, since it inhibits the oxidation of 5-HT (MAO-A), β-phenethylamine (MAO-B) and tyramine (MAO-A and -B). Linear Dixon plots with tyramine as substrate indicate that tris inhibits both the MAO-A and -B fractions with very similar Ki values. Moreover, it inhibits both forms in a pure non-competitive fashion. Any change in the mechanisms of the inhibition by tris, with respect to the two forms, would appear as non-linear plots in both Dixon and double reciprocal analysis as discussed by Houslay & Tipton (1974), as would change in Ki values (Houslay, Garrett & Tipton, 1974). However, benzylamine

is also oxidized by MAO-B (Hall, Logan & Parsons, 1969; Houslay & Tipton, 1974) and its oxidation is not inhibited by tris.

These observations are compatible with the theory of substrate binding to membrane-bound MAO-A and -B by Houslay & Tipton (1974), who suggested that a primary locating point of substrates in the active site was through their amino nitrogen. Thus the aromatic ring of benzylamine derivatives and β-phenethylamine (and 5-HT) derivatives would occupy different positions in the enzyme active site, a crucial point in determining the substrate specificity of MAO-A and -B in rat liver mitochondria.

We therefore suggest that tris causes some conformational change in the enzyme that impairs the access of the substrates with two methylene groups in their side chains but not the shorter benzylamine.

The inhibition of tyramine and β-phenethylamine oxidation was fully reversible, while that of 5-HT was not. Again, this would suggest that tris has no selective effect on either of the two forms of MAO in the rat liver. It is possible that the effect of tris on the enzyme is not completely reversible and some residual conformational change remains to impede the access of the bulky 5-HT molecule.

The model for amine oxidation by rat liver mitochondria outlined above, predicts that 5-HT should be a substrate for both MAO-A and -B as recently demonstrated in bovine heart (Mantle, Houslay & others, 1976). However, a site of steric hindrance had to be invoked, which would prevent 5-HT oxidation by MAO-B, but still allow it to bind to MAO-B with a similar Ki to the Km it exhibits as a substrate for MAO-A (Houslay & Tipton, 1974).

We think it reasonable that tris could act selectively, with respect to 5-HT oxidation, merely because of the position that the indole nucleus must take up on the enzyme surface in order to be oxidized. Recently, Gillespie & McKnight (1976) have shown that tris may influence the responses of a variety of smooth muscle preparations to adrenergic motor nerve stimulation and to noradrenaline. For instance, tris reduced the contractions of rat anococcygeus muscle and vas deferens following nerve stimulation but potentiated the responses of the perfused rabbit ear artery to noradrenaline. We are tempted to suggest that this latter action of tris could be due to the partial inhibition of MAO.

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