THE EFFECTS OF TETRAZOLIUM SALTS ON MONOAMINE OXIDASE ACTIVITY

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1. Introduction

Tetrazolium salts are frequently used as histochemical reagents for the detection of monoamine oxidase (MAO; EC 1.4.3.4 monoamine: O_2 oxidoreductase deaminating) activity [1]. However, it was recently found that the enzymic reduction of INT (2-*p*-iodophenyl-3-*p*-nitrophenyl-5-phenyl-2*H*-tetrazolium chloride) and other tetrazoles by MAO substrates (amine-tetrazolium reductase activity) differs from MAO activity in several respects, e.g. in its substrate specificity [2] (see also [3–6]). It has also been reported that tetrazolium salts can act as inhibitors of MAO [2, 3, 7]. The present investigation was therefore initiated in order to determine, by a more sensitive radioassay of MAO [8]: (a) the extent to which the activity of partly purified preparations of soluble MAO from guinea-pig liver [2, 6] is affected by INT; and (b) the possible relationship of amine-INT reductase activity to the oxidative deamination reaction catalyzed by MAO, which is shown below,

$$R{-}CH_2NH_2 + O_2 + H_2O \longrightarrow R{-}CHO + NH_3 + H_2O_2.$$

2. Materials and methods

Partly purified soluble MAO preparations were derived from the high-speed supernatant fraction of guinea-pig liver homogenates and freed from aldehyde dehydrogenase activity as previously described [2, 6]. MAO activity was determined by measuring the aldehyde and/or acid formed from ^{14}C-labelled amine substrates (dopamine, tryptamine and tyramine) following the procedure of Robinson et al. [8], as adapted by Collins and Southgate [9]. Amine-INT reductase activity was assayed by the method of Lagnado and Dzodzoe [2]. In this assay, reaction mixtures contained the following ingredients, in 2.0 ml: 1.0 ml of 0.1 M-Na-phosphate buffer, pH 7.6; 20 μmole neutralized amine substrate; 1.2 μmole INT and enzyme (2–4 mg protein). The reaction was started by addition of INT and substrate after 10 min preincubation of the enzyme-buffer mixture at 37° to minimize endogeneous tetrazolium reduction (i.e., reduction occurring in the absence of added amine substrate), and stopped after 10–30 min incubation with 3.0 ml 50% cold TCA. Formazan was extracted with 10 ml ethylacetate and the colour was read at 520 nm (molar extinction of INT-formazan = 13,300).

3. Results

3.1. *The effects of INT on MAO activity*

It was previously reported that tetrazolium salts, including INT, inhibit MAO activity as measured by oxygen uptake [2, 3, 7]. The data shown in fig. 1 demonstrate that the soluble enzyme purified from guinea-pig liver was progressively inhibited by INT concentrations ranging between 3.33 and 66.6 X 10^{-5} M (final concentration). Similar results were obtained using ^{14}C-dopamine as substrate (not shown). The complete inhibition of product formation seen at the

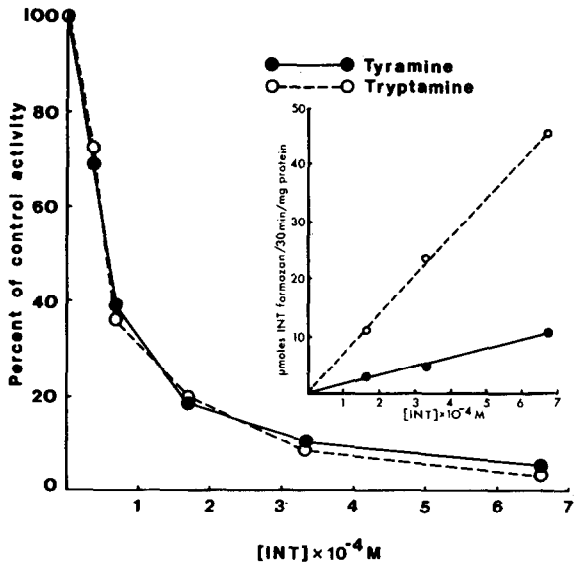

Fig. 1. The effect of INT on the activity of purified guinea-pig liver MAO. The enzyme was preincubated for 10 min at 37° with INT at the concentrations indicated, before the addition of substrate. Final volume for MAO assay [9]: 0.6 ml MAO activity expressed as a percentage of control values obtained in the absence of INT. INT-reductase activity was measured as indicated in sect. 2, using tryptamine (o- - - - o) or tyramine (•——•) as substrates (see inset).

highest concentration tested was confirmed, using tryptamine and dopamine as substrates, by thin-layer chromatography of ethyl acetate extracts of the complete reaction mixture on DEAE-cellulose or silica gel coated foils [10].

However, INT was reduced at all concentrations tested, and the amount of formazan produced was proportional to the amount of salt added (fig. 1). At a concentration of tetrazolium salt causing complete inhibition of product formation (66.6×10^{-5} M, final), formazan production, expressed as μmoles INT-formazan/30 min/mg protein, gave values of 46, 11 and 107 for tryptamine, tyramine and dopamine, in that order. This is in marked contrast to the substrate specificity pattern seen in the absence of added INT, where the activities, expressed as μmoles total deaminated product formed/30 min/mg protein gave values of 35.1 (tryptamine), 42.5 (tyramine) and 26.2 (dopamine).

Preincubation of the enzyme with INT (5.3×10^{-5} M, final) for zero to 30 min at 37° prior to addition of the substrate did not appear to alter the extent of the inhibition observed (about 50%, cf. fig. 1).

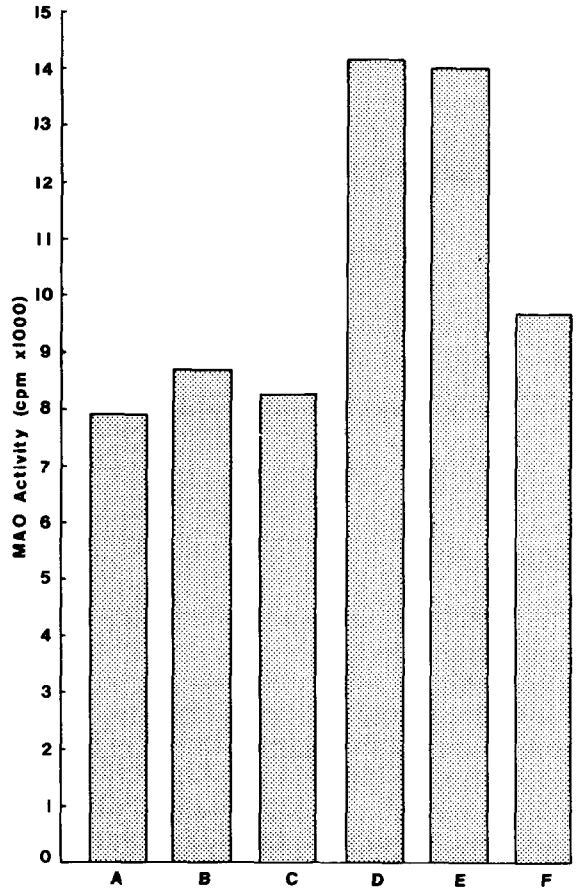

Fig. 2. The effects of INT and INT-formazan on the activity of guinea-pig liver MAO. Substrate: ^{14}C-tyramine. In (A)–(C), MAO activity was measured [9] after 15 min incubation; in (A), no additions; 200 μg INT-formazan or INT were added in (B) and (C), respectively, at the end of the 15 min incubation period. In (D)–(F), MAO activity was measured after 30 min incubation; 200 μg INT formazan or INT were added in (E) and (F), respectively, after the first 15 min incubation; in (D), no additions.

3.2. *The effects of INT-formazan on MAO activity*

The results shown in fig. 2 indicate that aqueous suspensions of the INT-formazan were without apparent effect on MAO activity under conditions where identical concentrations of INT caused an immediate arrest of enzyme activity. Control experiments in which INT or its formazan were added to the assay mixtures at the end of the incubation period showed that neither compound appears to interfere with the MAO assay procedure, since recovery of the radioactivity due to the products of deamination was not affected (fig. 2A–C).

Table 1
The effects of MAO inhibitors on amine-INT reductase (A) and MAO (B) activities of purified guinea-pig liver MAO preparations.

Inhibitor	Final concentration of inhibitor (mM)	% Inhibition of	
		A	B
1-isonicotinoyl-2-isopropylhydrazine	0.2	70	90
	2.0	96	98
Transphenylcyclopropyl amine	0.2	76	96
	2.0	92	97
α-methyltryptamine	0.2	76	97
	2.0	98	100
p-chloromercuribenzoate	0.2	44	25
	0.5	100	65
mercuric chloride	0.1	41	28
	0.2	85	58
o-phenanthroline	1.0	21	
	5.0	94	75

The enzyme was preincubated with inhibitors for 15 min at 37° and incubated a further 30 min after addition of substrate (tryptamine in all cases). Enzyme assays carried out as described in sect. 2.

3.3. *The effects of MAO inhibitors*

The effects of three typical MAO inhibitors on amine-tetrazolium reductase activity were compared to those seen measuring MAO product formation in parallel experiments. The results shown in table 1 indicate that the reaction catalyzed by soluble MAO of guinea-pig liver was strongly inhibited in both assay systems, although differences in the sensitivity to various inhibitors are apparent depending on the nature of the final electron acceptor. These results, taken together with earlier data [2–5], are consistent with the view that MAO is responsible for the enzymic reduction of tetrazolium salts by monoamines (see sect. 4).

4. Discussion

It was previously established that the reduction of INT by purified preparations of MAO from guinea-pig and rat liver in the presence of MAO substrates could not be attributed to the oxidation of aldehydes formed during amine oxidation [2]. In the case of the guinea-pig soluble MAO, only a single band of enzyme activity was seen after disc electrophoresis in 5% polyacrylamide gels [2], following the procedure of Youdim et al. [11]. Furthermore, it was shown that after electrophoresis of such preparations in polyacrylamide gels, sites of enzyme activity detected by the tetrazolium histochemical technique coincide in all cases with bands exhibiting MAO activity as measured by the radioassay method [9]; the intensity of tetrazolium reduction, however, does not necessarily reflect the level of MAO activity found in different forms of MAO [11, 12]. These observations, together with earlier data (see [3–5]) support the view that MAO is responsible for the enzymic reduction of INT and other tetrazoles in the presence of monoamine.

The present results, however, clearly show that amine-tetrazolium reductase activity is not coupled with the formation of the aldehyde and/or products which should be expected during amine oxidation by MAO. This suggests that INT (or its formazan) interacts directly with MAO causing its inactivation, rather than simply replacing oxygen as final electron acceptor. It was shown in control experiments that neither INT nor its formazan appear to interfere with the recovery of the products formed during amine oxidation (fig. 2). It seems also that formazan itself was not responsible for the inhibition of the enzyme. This is supported by the finding that inhibition was proportional to INT concentration (fig. 1) but was independent of the time of preincubation of the tetrazolium salt with the enzyme. On this basis, the inhibition observed appears to be a competitive nature (see also [7]).

On the basis of the present work, it does not appear necessary to implicate a different enzyme to account for tetrazolium reduction (cf. however [13]). Indeed, amine-tetrazolium reductase activity is sensitive to a wide variety of compounds that inhibit MAO (table 1; see also [2–5]). It is nevertheless difficult to understand why INT reduction occurs under conditions when MAO activity is completely inhibited (fig. 1). It is conceivable, however, that tetrazolium salts (or their formazan), may prevent the release of deamination products from the enzyme protein.

Further investigations are in progress to elucidate the nature of the reaction catalyzed by purified MAO

during tetrazolium reduction and the possible basis for INT reduction which occurs without the concomitant formation of the products of amine oxidation.

References

[1] G.C. Glenner, H. Burtner and G. Brown, J. Histochem. Cytochem. 5 (1957) 591.
[2] J.R. Lagnado and C.Y. Dzodzoe, submitted for publication.
[3] J.R. Lagnado and T.L. Sourkes, Can. J. Biochem. Physiol. 34 (1956) 1095.
[4] T.L. Sourkes and J.R. Lagnado, Experientia 13 (1957) 476.
[5] J.R. Lagnado and T.L. Sourkes, Can. J. Biochem. Physiol. 36 (1958) 587.
[6] H. Weissbach, B. Redfield and S. Udenfriend, J. Biol. Chem. 229 (1957) 953.
[7] C. Lallemant and C. Baron, Experientia 23 (1967) 953.
[8] D. Robinson, W. Lovenberg, H. Kreiser and A. Sjoerdsma, Biochem. Pharmacol. 17 (1968) 109.
[9] J. Southgate and G. Collins, Biochem. Pharmacol. 18 (1969) 2285.
[10] I. Smith, Chromatographic and Electrophoretic Techniques, Vol. 1 (Heinemann, Medical Books, London, 1969).
[11] M.B. Youdim, G. Collins and M. Sandler, in: Enzymes and Isoenzymes, Vol. 18 ed. D. Shugar (Academic Press, London, 1970) p. 281.
[12] M.B. Youdim and K. Tipton, unpublished observations.
[13] S.R. Guha and S.K. Ghosh, Biochem. Pharmacol. 19 (1970) 2929.