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ACTIONS OF THE THYROID HORMONES AND ANALOGUES *IN VITRO* ON CATECHOL-O-METHYLTRANSFERASE*

A. D'IORIO and C. MAVRIDES

Department of Biochemistry, School of Medicine, University of Ottawa, Canada

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Abstract—3,5-Diiodothyronine, 3,3′,5-triiodothyronine and their formic, acetic, and propionic acid derivatives are shown to act as inhibitors *in vitro* of catechol-O-methyltransferase. Thyroxine is ineffective under the same conditions but its formic, acetic, and propionic acid derivatives are also inhibitors of the enzyme. The inhibition constants of all inhibitors, as well as the kinetic constants of the normal substrates used—namely, epinephrine and S-adenosylmethionine, are given. The physiological implications are discussed.

THE POTENTIATION of the catecholamine effects by thyroid hormones is a well-established phenomenon and has been observed both *in vivo* and *in vitro*.[1–5] The suggested biochemical interpretation of this synergism has been the inhibition of MAO† activity.[6,7] Recent work by Zile and Lardy[8] substantiates this hypothesis. Treatment of rats with thyroxine exerts a marked decrease of the MAO activity of the liver. However, addition of thyroxine or its derivatives to the enzyme *in vitro* was without effect.

D'Iorio and Leduc[9] in 1960 reported that in rats made thyrotoxic by injections of thyroxine, the level of catechol-O-methyltransferase (COMT) decreased by 45 per cent. It was also reported that, after injection of radioactive epinephrine, there was a marked decrease of urinary metanephrine in thyrotoxic rats. Again, addition of thyroxine to the enzyme *in vitro* was without effect.

Moreover, D'Iorio and Mavrides[10,11] have shown more recently that 3,5-diiodo-4-hydroxybenzoic acid and several iodophenols are good inhibitors of COMT *in vitro*. In view of these results it was felt that the lack of inhibition of thyroxine reported was due to its lack of solubility in the incubation medium. The present work is mainly concerned with a study of the effects of several analogues and derivatives of thyroxine on a semipurified preparation of COMT.

EXPERIMENTAL

A four- to fivefold purification of rat liver COMT was achieved with a slight modification of the method of Axelrod and Tomchick.[12] Instead of the phosphate gel adsorption step, the protein was dialysed against water overnight and subsequently lyophilized. The lyophilized preparation is stable for at least 2 months when kept at −20°.

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† Abbreviations used are: MAO, monoamine oxidase; COMT, catechol-O-methyltransferase; S-AME, S-adenosylmethionine; E, epinephrine.

The assay method used for the enzyme was that of Axelrod and Tomchick.[12] In this method the substrate is epinephrine bitartrate which is enzymatically methylated by the methyl donor (S-AME) to metanephrine. Metanephrine is then measured spectrofluorometrically in an Amico–Bowman spectrofluorometer and the activity expressed in fluorescence units. The incubation medium in a final volume of 2·0 ml was buffered with 100 μmoles phosphate buffer, pH 7·9, and the enzyme was fortified by the addition of 50 μmoles magnesium chloride. 10 mg of the enzyme preparation was invariably used, dissolved in water. The epinephrine concentration [S] for the kinetic experiments ranges from $0{\cdot}5 \times 10^{-4}$ M to 3×10^{-4} M. S-Adenosylmethionine concentrations [S-AME] ranged from 1×10^{-4} M to 1×10^{-3} M.[13] All compounds tested were first dissolved in 0·1 N NaOH, and 0·1 ml of the solution was added to the incubation mixture. An equal amount of 0·1 N NaOH was added to the control tubes. This addition raised the pH to 8·4 with no noticeable effect on the activity of the enzyme. Under our experimental conditions the reaction was usually linear with time up to 5 min, and the activity was consequently expressed in fluorescence units produced in the first 5 min of incubation. In one set of experiments, presented in Fig. 3, the concentration of S-AME had to be lowered, and under the circumstances the reaction was linear only up to 1 min and the velocity was accordingly expressed.

For the calculation of kinetic constants, the double reciprocal plot proposed by Lineweaver and Burk[14] was generally used. The results expressed in the various figures represent the mean values of one duplicate run. However, each duplicate experiment was repeated two to five times with comparable results.

All the compounds tested are commercially available.

RESULTS

Substrate Constants

Since our experiments involved the calculation of inhibition constants, it was felt that the kinetic constants for the normal substrates used in the assay method (epine-

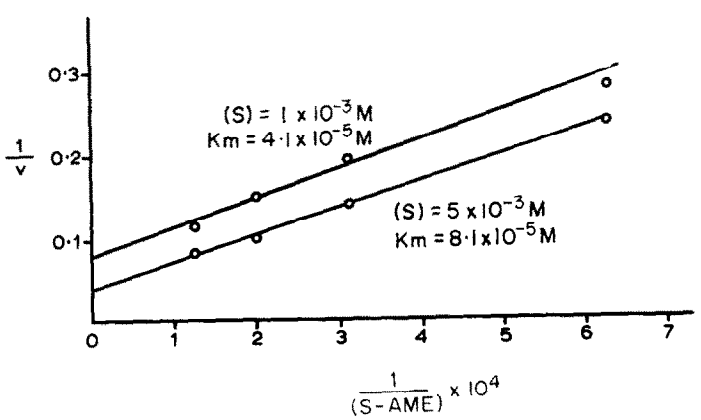

FIG. 1. Lineweaver and Burk plots for S-adenosylmethionine at two epinephrine concentrations [S] namely 1×10^{-3} M and 5×10^{-3} M. The respective apparent K_m values are found to be $4{\cdot}1 \times 10^{-5}$ M and $8{\cdot}1 \times 10^{-5}$ M. Velocity is expressed in fluorescence units produced in the first minute of incubation.

phrine and S-AME) should also be determined. A direct means of assessing the relative affinities of the inhibitors and the normal substrates would thus be available.

Axelrod and Tomchick[12] have reported a K_m value for E of $1{\cdot}2 \times 10^{-4}$ M, but the concentration of S-AME is not given and is probably very low. Crout[15] reports an approximate K_m value of 3×10^{-4} M for norepinephrine at optimal S-AME concentration. He reports a K_m of 4×10^{-5} M for S-AME, but the concentration of norepinephrine is not stated. Since, however, the reaction catalysed by COMT involves two substrates, the catechol and S-AME, it is to be expected that the apparent K_m value for one substrate would be a function of the concentration of the other.[16]

The reaction mechanism is unknown, but Axelrod and Tomchick[12] propose an enzyme–catechol complex followed by a nucleophilic attack of the hydroxyl group of the substrate on the methyl group of S-AME. Whether the methylating step involves an enzyme–S-AME complex, or whether it can merely be considered a bimolecular reaction between the enzyme–catechol complex and S-AME, remains to be answered, but straight lines can be obtained by making use of the Lineweaver and Burk plot and K_m values for S-AME calculated. Figure 1 illustrates the case. Two different K_m

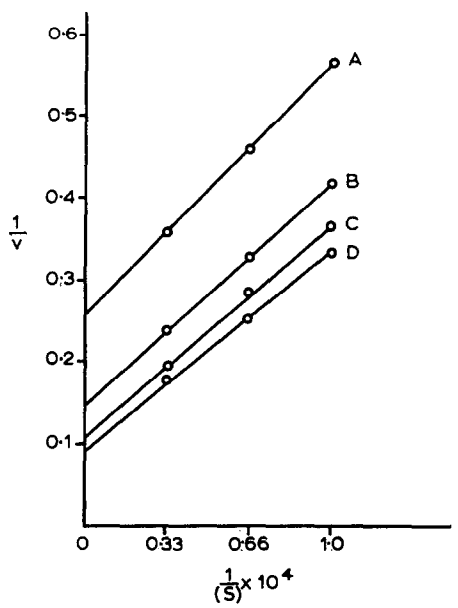

FIG. 2. Lineweaver and Burk plots for epinephrine at four S-adenosylmethionine concentrations. [S-AME] used: 1×10^{-4} M, 3×10^{-4} M, 6×10^{-4} M, and 1×10^{-3} M for lines A, B, C, and D respectively. The respective K_m values calculated from above plot are: $1{\cdot}25 \times 10^{-4}$ M, $2{\cdot}10 \times 10^{-4}$ M, $2{\cdot}47 \times 10^{-4}$ M, and $2{\cdot}70 \times 10^{-4}$ M. Velocity is expressed in fluorescence units produced in the first 5 min of incubation.

values for S-AME are obtained: $4{\cdot}1 \times 10^{-5}$ M and $8{\cdot}1 \times 10^{-5}$ M at 1×10^{-3} M and 5×10^{-3} M concentrations of E respectively. The first value is practically identical with that reported by Crout.[15]

Figure 2 illustrates the effect of increasing [S-AME] from 1×10^{-4} M to (optimal) 1×10^{-3} M on the apparent K_m value for E. More than a twofold increase in the

K_m value results from a tenfold increase in [S-AME]. In Fig. 3, K_m values for E calculated from Fig. 2 are plotted versus maximum velocity. A straight line is obtained[16] which, on extrapolation, gives the K_s for E, which is shown to be equal to $0{\cdot}5 \times 10^{-4}$ M.

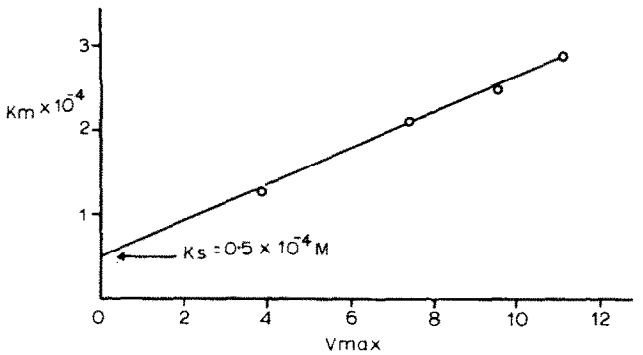

FIG. 3. K_m values from Fig. 2, plotted versus maximum velocity. The intersect at the vertical axis gives the substrate constant for epinephrine: $K_s = 0{\cdot}5 \times 10^{-4}$ M. Velocity is expressed as in Fig. 2.

TABLE 1. SUMMARY OF THE EFFECTS IN VITRO OF THYROID HORMONES AND ANALOGUES ON CATECHOL-O-METHYLTRANSFERASE

Compounds tested	Type of inhibition	K_i
3,5-Diiodothyronine	Mixed type	$2{\cdot}1 \times 10^{-3}$ M
3,5-Diiodothyroformic acid	Noncompetitive	$1{\cdot}3 \times 10^{-3}$ M
3,5-Diiodothyroacetic acid	Noncompetitive	$1{\cdot}3 \times 10^{-3}$ M
3,5-Diiodothyropropionic acid	Noncompetitive	$1{\cdot}3 \times 10^{-3}$ M
3,3′,5-Triiodothyronine	Mixed type	$2{\cdot}6 \times 10^{-3}$ M
3,3′,5-Triiodothyroformic acid	Mixed type	$7{\cdot}5 \times 10^{-4}$ M
3,3′,5-Triiodothyroacetic acid	Mixed type	11×10^{-4} M
3,3′,5- Triiodothyropropionic acid	Mixed type	$8{\cdot}5 \times 10^{-4}$ M
Thyroxine	No effect	
3,5,3′,5′-Tetraiodothyroformic acid	Mixed type	$1{\cdot}9 \times 10^{-4}$ M
3,5,3′,5′-Tetraiodothyroacetic acid	Mixed type	$2{\cdot}3 \times 10^{-4}$ M
3,5,3′,5′-Tetraiodothyropropionic acid	Mixed type	$3{\cdot}8 \times 10^{-4}$ M

Inhibition Experiments

Table 1 summarizes the action of a dozen compounds on the catechol-O-methyltransferase. The type of inhibition is indicated and the K_i values for each one of them are given.

It is noted that thyroxine itself has no effect. The problem of solubility might be responsible for this lack of activity. We observed that, to obtain a 3×10^{-4} M solution of thyroxine, the pH had to be raised to 10·4. At this pH the methylation reaction is completely quenched. As in the earlier experiments, no direct action of thyroxine on the enzyme could therefore be demonstrated. The other compounds were added in amounts sufficient to yield concentrations of the order of 10^{-3} M even though most of them, and especially the tetraiodo derivatives, would exhibit inhibition at lower concentrations.

The K_i values indicate that the affinity of the enzyme for the inhibitors increases with increasing numbers of iodine atoms. In the case of the tetraiodo derivatives, the inhibition constants suggest that the affinity of the enzyme for these compounds is of the same order of magnitude as that shown for E. They suggest, furthermore, that lengthening of the side chain in the tetraiodo derivatives results in poorer inhibition since the K_i for tetraiodothyropropionic acid is higher than that for tetraiodothyroformic acid by a factor or two, while the acetic acid derivative has a K_i value between the two. No such effect is evidenced for the diiodo and triiodo analogues. Another and more interesting effect is the one caused by the introduction of the alanine side chain. Diiodothyronine and triiodothyronine are poorer inhibitors than the diiodo and triiodo derivatives, respectively, bearing the formic, acetic, and propionic acid residues. Furthermore, whereas the triiodo derivatives seem to be generally slightly better inhibitors than the diiodo derivatives, this is not the case when the alanine side chain is present. Thus, triiodothyronine is not a better inhibitor than diiodothyronine. One is reminded of the striking effect of substituting the alanine side chain for the pyruvic acid chain in 3,5-diiodo-4-hydroxy-phenylpyruvic acid (DIHPA) which results in complete abolition of the inhibitory properties of DIHPA.[11] The observed patterns of inhibition fall into two categories—the strictly noncompetitive type and the mixed type.[16] The former is illustrated in Fig. 4 and is displayed by 3,5-diiodo derivatives

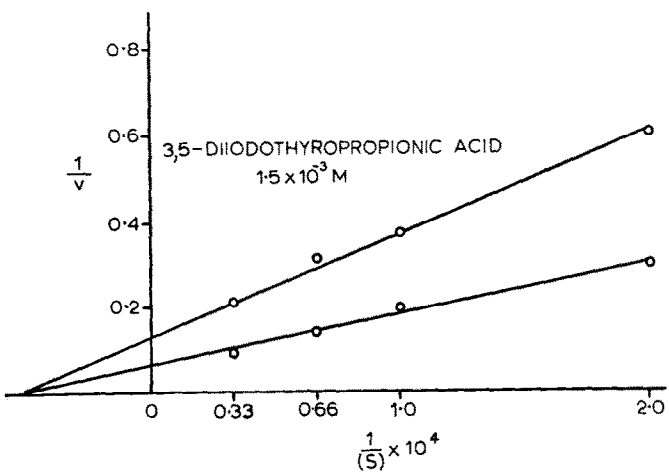

FIG. 4. Lineweaver and Burk plot showing inhibition of catechol-O-methyltransferase by 3,5-diiodothyropropionic acid. [S-AME] = 1×10^{-3} M. Velocity is expressed as in Fig. 2.

with the exception of diiodothyronine. The mixed type of inhibition is illustrated in Fig. 5 and is displayed by all other 3,3′,5-triiodo and 3,5,3′,5′-tetraiodo derivatives, and by 3,5-diiodo thyronine.

DISCUSSION

Triiodo- and tetraiodothyronine derivatives exert a mixed type of inhibition. The competitive portion of this inhibition is probably due to the proximity of the iodine and hydroxyl group. This would be in keeping with previous results[11] indicating that 3,5-diiodo-4-hydroxy-benzoic acid and diiodosalicylic acid act as competitive inhibitors of COMT.

This competitive component was also to be expected since Roche *et al.*[17] have found in the organism traces of O-methylthyroxine. More recently Tomita[18] and Tomita and Cha[19] have isolated an enzyme distinct from COMT which can O-methylate iodophenols. From the latter experiments one might be tempted to conclude that the observed inhibition is due to competition for the methyl donor S-AME. However, in

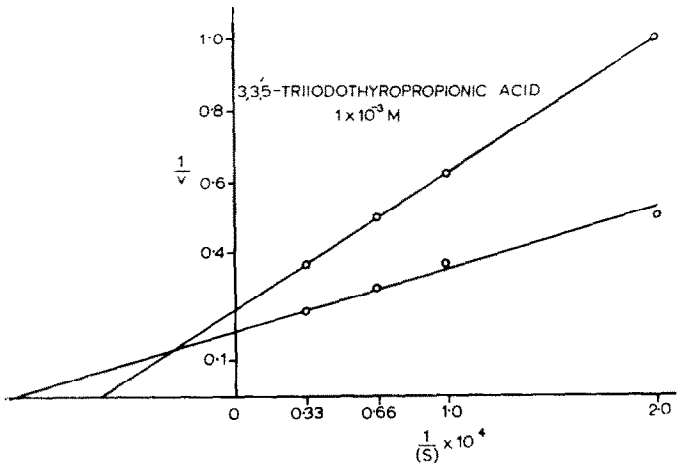

FIG. 5. Lineweaver and Burk plot showing inhibition of enzyme by 3,3',5-triiodothyropropionic acid. [S-AME] = $1{\cdot}1 \times 10^{-4}$ M. Velocity is expressed as in Fig. 2.

our experimental conditions,[10] it was impossible to show O-methylation of iodophenols. In the same series it was not possible to show reversal of the inhibition by adding excess amounts of S-AME. The same was true in the present experiments; a tenfold variation of S-AME from 10^{-4} M to 10^{-3} M had no effect on the inhibition pattern or K_i values. Furthermore, if the inhibition was due to removal of S-AME by a second enzymatic system, one might expect competitive inhibition. This is not the case since all the derivatives reported in Table 1 behave as noncompetitive inhibitors. Finally, the methylation of iodophenols occurs at a very slow rate, at an optimum pH of 6, so that very little interference should be expected under our present conditions.

On the other hand, 3,5-diiodothyroformic, thyroacetic, and thyropropionic acids act as strictly noncompetitive inhibitors. Thyronine, which is not included in the table, does not cause inhibition. It can then be postulated that the iodine substituents in positions 3,5 are essential for the inhibitory properties. It is possible that the oxygen of the ether bridge and the iodine in the ortho position might form a complex with COMT.

However, 3,5-diiodothyronine does not conform with this pattern and even though it has no *o*-iodophenolic function, its inhibition is of the mixed type.

We can offer no explanation for this anomaly except for the possible influence of the alanine side chain. It is to be noted that in all cases where the alanine side chain is present, the inhibition is lower than for acidic derivatives. This is also in conformity with previous experiments using one-ring systems.[11] It was found that 3,5-diiodotyrosine did not inhibit COMT, whereas its pyruvic acid analogue was a powerful titrating inhibitor.

Our present studies indicate that, except for thyroxine and thyronine, all the other derivatives tested exert a noncompetitive or a mixed type of inhibition. The K_i values suggest that these inhibitions take place at concentrations above the normal physiological range. But experiments *in vitro* do not always reproduce accurately the scale of biochemical events in the living organism. Furthermore, there is no way of knowing the real, active concentration of thyroid hormones or their derivatives in the various cell compartments.

The multiplicity of indirect effects induced by the thyroid hormones could serve as a basis for arguing against any specific effect on COMT resulting in potentiation of catecholamines. While this may be the case, our studies clearly indicate the possibility of a direct inhibition and this should be borne in mind as at least one of the contributing factors in the potentiating effects of the thyroid hormone on catecholamines.

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