THE ACTION OF ANTITHYROID SUBSTANCES ON THE FORMATION *IN VITRO* OF ACETYLTHYROXINE FROM ACETYLDIIODOTYROSINE

by

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In 1943, MACKENZIE AND MACKENZIE[1] showed that the administration of sulphonamides, thiourea and certain substituted thioureas to rats, mice and dogs produced hyperplasia of the thyroid gland and a lowering of the B.M.R. This effect could not be abolished by adding inorganic iodide to the animals' diet but the administration of thyroxine afforded complete protection from the goitrogenic action of these drugs. The authors therefore concluded that the mechanism of this action was not the same as that of previously described goitrogenic compounds, that is, the drugs did not owe their activity to their power of inhibiting the iodine concentrating mechanism of the thyroid. ASTWOOD, SULLIVAN, BISSELL AND TYSLOWITZ[2] showed in the same year that sulphonamides and thiourea produced the same histological changes in the thyroid as those described by MACKENZIE AND MACKENZIE, with loss of colloid. Again, iodide was shown to be ineffective and thyroid powder completely effective in abolishing the action of the drugs tested, from which the authors concluded that they acted primarily by inhibiting hormone synthesis. This theory received much experimental support from other workers (FRANKLIN, LERNER AND CHAIKOFF[3]; LARSON, KEATING, PEACOCK AND RAWSON[4]; REINEKE, MIXNER AND TURNER[5]; VANDERLAAN AND BISSELL[6]), but the mechanism by which such an inhibition is effected was not fully explained. CAMPBELL, LANDGREBE, AND MORGAN[7] suggested that in the case of thiourea the activity was due to its reaction with iodine to give formamidine disulphide, thereby keeping the iodine in the thyroid in the reduced form.

The *in vitro* reaction of goitrogenic compounds with iodine was examined by MILLER, ROBLIN AND ASTWOOD[8] who isolated the oxidation product of 2-thiouracil as the sodium salt of thiouracil disulphide; further, they demonstrated that, in general, highly active antithyroid substances reacted rapidly in bicarbonate buffer with iodine (more than one molecular equivalent), while those compounds with low activity such as the sulphonamides and *p*-aminobenzoic acid reacted only very slowly, and then with only a fraction of an equivalent of iodine. These authors also showed that iodination of tyrosine and casein could be inhibited by thiouracil. It was therefore concluded that these drugs owed their activity to their power of combining with iodine, although the biological inactivity of 6-amino-2-thiouracil and 5-carbethoxy-2-thiouracil could not be explained since they reacted *in vitro* with iodine in the same way as the biologically active 2-thiouracil.

It has recently been shown (PITT-RIVERS[9]) that if acetyldiiodotyrosine is incubated at 37° at p_H 7.4, acetylthyroxine is formed. In the present communication experiments

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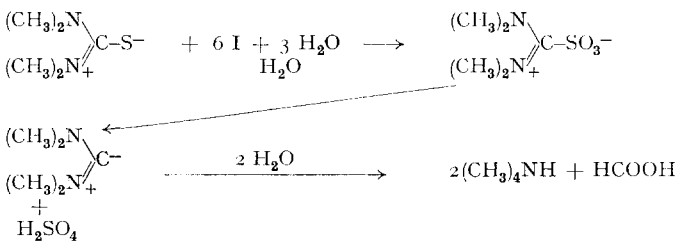
are described which show that this reaction can be inhibited by the presence of goitrogenic substances, and the products of iodine oxidation of one of them have been isolated. Tetramethylthiourea (1 mol) is oxidized in bicarbonate buffer by 6 atoms of I with the formation of sulphuric acid (1 mol) and dimethylamine (2 mol). It is suggested that the oxidation takes place as follows: firstly the unstable tetramethylformamidine sulphonic acid is formed and this is rapidly hydrolysed to tetramethylformamidine and sulphuric acid; further hydrolysis yields dimethylamine and formic acid:

Fig. 1



Formic acid has not been isolated, but the reaction mixture has been shown to contain a substance which reduces ammoniacal silver salts.

During the incubation of tetramethylthiourea with acetyldiiodotyrosine, sulphate is formed and quantitative estimation of sulphate and iodide after such an incubation reveals that these are present in the ratio of 5.5 : 1; this suggests that the inhibition reaction follows the same course as that outlined in Fig. 1, and demonstrates that the slow decomposition of acetyldiiodotyrosine results in the liberation of iodine as such, that is, as a potential oxidising agent.

The iodination of casein has been carried out in the presence of thiourea in such a way that (*a*) iodination has been completely inhibited, thus confirming MILLER, ROBLIN AND ASTWOOD's results with thiouracil, and (*b*) iodination of the protein has been controlled so that substitution only has resulted, with inhibition of the secondary reaction, the oxidation of diiodotyrosine to thyroxine.

The mechanism of these inhibitions is discussed.

EXPERIMENTAL

INHIBITION EXPERIMENTS

The following substances were tested as inhibitors of acetylthyroxine formation: 2-thiouracil, 6-amino-2-thiouracil, thiourea, tetramethylthiourea, 2-amino-thiazole, 2-mercapto-4-methylimidazole, ergothioneine, *p*-aminobenzoic acid, sulphanilamide and sulphaguanidine. Tetramethylthiourea was prepared from tetramethylthiuramsulphide by the method of DELÉPINE[10].

Method. Acetyldiiodotyrosine ½ H_2O (MYERS[11]) was dissolved in water with the aid of 1.78 equivalents of *N*-NaOH, the initial p_H of all solutions being 7.4. In experiments with the more soluble inhibitors such as thiourea and tetramethylthiourea, stock solutions of these were made, adjusting the p_H where necessary to 7.4, and varying amounts were added to the acetyldiiodotyrosine solutions. In the case of the insoluble inhibitors such as thiouracil and sulphaguanidine, the inhibitor was dissolved in the

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whole amount of water required for the experiment, acetyldiiodotyrosine was suspended in the solution and was dissolved by addition of the requisite amount of alkali. With each new set of experiments, a control experiment was done to determine the amount of acetylthyroxine formed in the absence of inhibitor. The final concentration of acetyldiiodotyrosine in all experiments was 0.085 M. The solutions were incubated at 37° for 11 days and then chilled at 4° for 12 hours; the insoluble sodium salt of acetylthyroxine which had been precipitated during the incubation period was separated at the centrifuge, thoroughly drained from the mother-liquor, dissolved in 0.1 N NaOH and precipitated at the boiling point with dilute HCl. In many experiments the crude precipitates of acetylthyroxine were crystalline and had melting points between 210–220° in which case the products were not further purified. In those experiments where the melting points of the crude acetylthyroxine was below 210°, the products were crystallised by the method of ASHLEY AND HARINGTON[12].

In the experiments with thiourea considerable amounts of sulphur were liberated, and with 2-mercapto-4-methylimidazole uncharacterised insoluble oxidation products were formed during the incubations; the sodium salt of acetylthyroxine was separated from these by filtration through hard filter paper (WHATMAN No. 42) of its solution in hot dilute NaOH. The gross yields were determined of acetylthyroxine only, since recovery of unchanged acetyldiiodotyrosine was complicated in some of the experiments. For instance, with thiourea, unchanged acetyldiiodotyrosine could only be recovered pure after several wasteful crystallisations, as it was mixed with an amorphous product which, after several reprecipitations from alkaline solution with acid and extraction with organic solvents, could not be crystallised. This product contained iodine, nitrogen and sulphur; repeated extraction with CS_2 did not remove this sulphur, whence it was concluded that thiourea had reacted with acetyldiiodotyrosine with the introduction of sulphur into the molecule.

The results are given in Table I.

Since the variation in the yields of acetylthyroxine obtained in the control experiments is about 7% (between 104 and 112 mg), no inhibition which is less than 10% is considered significant.

The oxidation of tetramethylthiourea with iodine. Tetramethylthiourea (0.264 g, 2 mmol) was dissolved in water (10 ml) containing sodium bicarbonate (1.5 g); 1.00 *N*-iodine solution was added slowly to the warmed solution (40°) until there was no further uptake after waiting 15 min for the iodine to disappear; 5.9 atoms of iodine were used. The solution was then acidified with dilute HCl and treated with excess of barium chloride and the barium sulphate was collected; it weighed 0.4191 g (1.8 mmol). In a duplicate experiment the barium sulphate obtained weighed 0.4408 g (1.9 mmol).

Identification of the base. Tetramethylthiourea (0.132 g) was dissolved in water (10 ml) containing sodium bicarbonate (1.0 g). 1.00 *N* iodine (5.9 ml) was added slowly to the warmed solution. When all the iodine had reacted, the solution was made strongly alkaline by addition of 10 *N* NaOH and was extracted several times with ether. The combined ether extracts were dried over KOH pellets and treated with excess of ethereal picric acid solution. The salt which separated was collected, washed with ether and dried; it had m.p. 158–9°, and the mixed m.p. with authentic dimethylamine picrate was not depressed. The yield was 0.33 g (60% of the theoretical). Attempts to isolate dimethylamine quantitatively as the picrate were unsuccessful.

Tetramethylthiourea 0.0660 g (0.5 mmol) was oxidised with 5.9 atoms of iodine

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TABLE I

THE ACTION OF ANTITHYROID SUBSTANCES ON THE IN VITRO FORMATION OF ACETYLTHYROXINE FROM ACETYLDIIODOTYROSINE

Inhibitor (molecular equivalents)	Acetylthyroxine formed (mg per c.mol of acetyldiiodotyrosine)	Inhibition %
2-Thiouracil		
0	108	
0.04	59	45
0.1	28	74
0.2	0	100
6-Amino-2-thiouracil		
0	112	
0.04	106	5
0.1	69	38
Thiourea		
0	110	
0.1	103	6
0.2	40	64
0.5	2	98
Tetramethylthiourea		
0	112	
0.005	104	7
0.01	94	16
0.02	85	24
0.1	31	73
0.2	0	100
2-Aminothiazole		
0	105	
0.05	61	42
0.1	40	62
0.2	11	90
2-Mercapto-4-methylimidazole		
0	104	
0.05	57	45
0.1	36	65
0.2	trace	nearly 100
1.0	0	100
Ergothioneine		
0	112	
0.04	74	34
p-Aminobenzoic acid		
0	110	
0.1	73	34
0.5	50	55
1.0	36	67
Sulphanilamide		
0	108	
0.1	92	15
1.0	31	71
Sulphaguanidine		
0	108	
0.1	95	12

in 5 ml water containing 0.5 g sodium bicarbonate in a micro-Kjeldahl flask. At the end of the oxidation, 10 ml of 10 *N*-NaOH were added and the volatile base formed was steam-distilled into standard acid. The amount of base found was 0.964 mmol.

A control experiment was done by passing steam through an alkaline solution of tetramethylthiourea which had not been oxidised with iodine. No volatile base was distilled into the standard acid.

The one-carbon atom residue left unaccounted for was not isolated, but a solution of oxidised tetramethylthiourea was shown to reduce ammoniacal silver sulphate, indicating that this residue was probably formic acid or formaldehyde.

Oxidation of tetramethylthiourea during aerobic incubation with acetyldiiodotyrosine at p_H *7.4.* Acetyldiiodotyrosine (4.84 g) (1 cmol) was incubated for 11 days at 37° with 0.2 molecular equivalents of tetramethylthiourea. No acetylthyroxine was formed. The solution was then halved and after removal of unchanged acetyldiiodotyrosine with (*a*) dilute sulphuric acid and (*b*) dilute hydrochloric acid, iodide and sulphate were determined. Iodide found, 5.3 mmol; sulphate found, 0.97 mmol whence the ratio was 5.5 : 1.

Effect of thiourea on the iodination of casein. A. Casein (Kahlbaum) containing 6.6%

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of tyrosine by determination (27.5 g) was iodinated with 4.3 atoms of iodine per molecule of tyrosine as described by LUDWIG AND VON MUTZENBECHER[13], using 4.3 *N* iodine in KI solution instead of powdered iodine. At the end of the reaction, inorganic iodine was removed by prolonged dialysis and the product was precipitated, collected and dried *in vacuo*. The iodine content of the product was determined by a modification of HARINGTON AND RANDALL'S[14] method*. Found: Total I, 7.4%, acid-insoluble I, 0.9%.

B. Casein (27.5 g) was iodinated in the presence of thiourea (4 equivalents/mol tyrosine). The product contained no iodine.

C. Casein (27.5 g) was iodinated in the presence of thiourea (0.5 equivalents/mol tyrosine). The product contained 3.8% total iodine, all of which was acid-soluble; as the product had been freed from iodide by dialysis, this was diiodotyrosine iodine.

DISCUSSION

It will be seen from Table I, that antithyroid substances can inhibit the *in vitro* formation of acetylthyroxine from acetyldiiodotyrosine, that the degree of inhibition is proportional to the amount of inhibitor present, and that there is a marked variation between the activities of the different compounds tested.

Comparison of this *in vitro* effect with the *in vivo* action of these substances shows that there is a definite parallelism between the two. Firstly, the *in vitro* proportionality between dose and effect is in agreement with the findings of ASTWOOD AND BISSELL[16], who showed that, in rats, graded doses of thiouracil produced graded effects both in the amount of thyroid enlargement and in the iodine concentration in the glands. Secondly, the difference between the activities of the inhibitors *in vitro* is comparable with the differences between their antithyroid activity *in vivo*: 2-thiouracil and 2-aminothiazole are considerably more active than thiourea, while the sulphonamides and *p*-aminobenzoic possess only low activity. However, when a quantitative comparison is attempted, it is complicated by the variation found in the activities of individual antithyroid compounds towards different animals. For instance, WILLIAMS[17] found that when tetramethylthiourea was administered to thyrotoxic patients it had an antithyroid action similar to that of 2-thiouracil, whereas ASTWOOD, BISSELL AND HUGHES[18] found that in rats the estimated activity of tetramethylthiourea was only 30% of that of 2-thiouracil. Similarly MACKENZIE AND MACKENZIE[1] found that the changes produced by sulphaguanidine in the thyroids of rats, mice and dogs were absent when the drugs were administered to chicks and guinea-pigs; ASTWOOD, BISSELL AND HUGHES[19] have shown that *p*-aminobenzoic acid and sulphadiazine are also without effect on the chick's thyroid. STANLEY AND ASTWOOD[20] demonstrated the differences in antithyroid potency of 32 substances as assayed in man (by the authors) and in the rat. But although this species variation makes quantitative comparison between the *in vivo* and *in vitro* effects difficult, it is nevertheless apparent that the highly active antithyroid drugs can inhibit acetylthyroxine formation in low concentration, while the compounds with low antithyroid activity are correspondingly less active *in vitro*.

* The method of chemical assay of the thyroid gland of HARINGTON AND RANDALL[14] has been criticised by LELAND AND FOSTER[15] on the grounds that time of hydrolysis of the protein is too short and that incomplete hydrolysis leads to iodine other than thyroxine iodine appearing as 'acid-insoluble'. The following modification was therefore used: the iodinated protein (100 mg) was boiled under reflux for 6 h with 10 ml of 2*N*-NaOH, after which the procedure of HARINGTON AND RANDALL[14] was followed.

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The results obtained with 2-mercapto-4-methylimidazole, ergothioneine and 6-amino-2-thiouracil call for some comment. The high *in vitro* activity of the mercapto-methylimidazole was not unexpected in view of the known high goitrogenic potency of many of the mercaptomethylimidazoles; the activity of ergothioneine was more surprising, and could unfortunately only be tested at a single low dose level because of the small amount of material available, but the test shows that its *in vitro* activity is of the same order as that of 2-mercapto-4-methylimidazole. This finding is at variance with the antithyroid activity of ergothioneine in animals: although LAWSON AND RIMINGTON[21] found that it had considerable activity in rats, their results were not confirmed by ASTWOOD AND STANLEY[22], who found that ergothioneine was completely inactive in rats and in man; this inactivity in man has been confirmed by LAWSON AND RIMINGTON[23]. The divergence between the *in vivo* and *in vitro* activities of ergothioneine may be due to the fact that *in vitro* it cannot be lost from the reaction mixture except as a result of the reaction itself, while in the body it may, either by rapid metabolism or rapid excretion (LAWSON AND RIMINGTON[23]), be prevented from accumulating in an effective concentration in the thyroid gland.

The *in vivo* inactivity of 6-amino-2-thiouracil was explained in a similar way by MILLER, ROBLIN, AND ASTWOOD[8], who showed that this compound is equally reactive towards iodine as is 2-thiouracil; they therefore suggested that aminothiouracil might owe its inactivity to oxidation in the body, although other factors, such as solubility and distribution between the tissues and body fluids, were also considered. However, none of these factors accounts for the low *in vitro* activity of aminothiouracil, and this remains at present unexplained.

In considering whether the factors governing the *in vitro* and the *in vivo* inhibition of thyroxine synthesis are the same, one must first consider what are the factors governing thyroxine synthesis in the body. ASTWOOD[24] first emphasized that two separate processes are at work in the thyroid gland which control iodine absorption: a concentrating mechanism which collects iodine from the circulation and a synthesising mechanism which controls the incorporation of the collected iodine into the gland tissue in organic combination. In a recent paper, VANDERLAAN AND VANDERLAAN[25] have examined the former mechanism quantitatively, and have shown that in the rat normal glands can concentrate iodide from the circulation so that the ratio of thyroid iodide to serum iodide is 25 : 1. Administration of thiocyanate to the animals not only inhibited this concentration but caused the discharge of pre-existing iodide from the glands within a short tome of administration; propylthiouracil caused no such immediate impairment of the iodine-concentrating mechanism; it was in fact without effect on it. On the other hand, the hyperplasia of the gland which is a secondary result of prolonged administration of antithyroid substances was accompanied by an enhancement of the iodine-concentrating mechanism, as shown by an increase in the ratio between the iodide of the thyroid and of the serum. Nevertheless the total iodine in these hyperplastic glands was far lower than in normal glands, where the organically bound iodine forms about 80% of the total, because of the complete prevention of iodine incorporation and hormone synthesis.

The action of many goitrogenic compounds in preventing the organic incorporation of ingested iodide in the thyroid has been used recently by STANLEY AND ASTWOOD[20] as a measure of the activity of these compounds in man. Radioactive iodide was given by mouth and the radioactive iodine content of the thyroid was measured after ingestion

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at given intervals of time; in normal humans this was found to rise in a constant slope up to 24 hours; administration of the goitrogenic substances caused a lowering or even a complete flattening of the slope, this change being used to determine the activity of the compound tested. The rise in inorganic iodide demonstrated by VANDERLAAN AND VANDERLAAN[25] would not interfere with this determination for the reason given above: that organic iodine forms the greater part of the total thyroid iodine, and the failure to synthesise the former produces an overall lowering of the total iodine which could not be significantly affected by changes in iodide concentration.

The action therefore of such antithyroid substances as the thioureas and thiouracils, thiazoles, mercapto-imidazoles and sulphonamides controls only the effectiveness of the second function of the thyroid discussed above, its ability to incorporate iodine in organic combination. It is now suggested that the quantitative similarity between the activities of these different groups of compounds *in vitro* and *in vivo* indicates a common mechanism: the preferential reaction of the antithyroid compound with iodine keeps the latter in the reduced form, and thus prevents not only substitution of tyrosine to diiodotyrosine in the thyroid protein, but also subsequent oxidation of diiodotyrosine to thyroxine in both cases. The antithyroid activity of any compound in the body will naturally be controlled by another factor: its effective concentration in the thyroid gland; if its destruction, distribution or excretion prevent this concentration being reached, it will not exhibit the antithyroid properties which it might do *in vitro*, where an effective concentration can be assured.

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SUMMARY

1. The inhibitory action of several antithyroid compounds towards the formation of acetylthyroxine from acetyldiiodotyrosine during aerobic incubation has been tested.

2. The *in vitro* activity of these compounds has been found to be roughly proportional to their *in vivo* activity; the mechanism of these inhibitions is discussed.

RÉSUMÉ

1. L'action inhibitrice de diverses substances antithyroïdiennes envers la synthèse de l'acétylthyroxine à partir de l'acétyldiiodotyrosine *in vitro* a été étudiée.

2. Une comparaison est faite entre le degré d'inhibition produit par ces substances *in vitro* et leur activité antithyroïdienne *in vivo*; le mécanisme de ces réactions est discuté.

ZUSAMMENFASSUNG

1. Die hemmende Wirkung mehrerer Antithyroidverbindungen auf die Bildung von Acetylthyroxin aus Acetyldijodtyrosin bei aerober Inkubation wurde geprüft.

2. Die Aktivität dieser Verbindung *in vitro* ist, wie gefunden wurde, ihrer Aktivität *in vivo* grossen Zügen proportional; der Mechanismus dieser Hemmungen wird diskutiert.

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