Brit. J. Pharmacol. (1955), 10, 1.

THE MODE OF ACTION OF ANTITHYROID DRUGS: FURTHER OBSERVATIONS ON *IN VITRO* INHIBITION OF OXIDATIVE PROTEIN IODINATION

BY

RUSSELL FRASER, M. G. W. HANNO, AND W. R. PITNEY

From the Department of Medicine, Postgraduate Medical School of London, Ducane Road, W.12

(RECEIVED FEBRUARY 18, 1954)

A preliminary report by Pitney and Fraser (1953) showed that the potency of antithyroid drugs may be assessed by two *in vitro* tests, and that such drugs may differ in their modes of action. In this communication we report further observations with these tests.

The enzyme test—the main test—measures the inhibition by a drug of an enzyme system which iodinates protein. The mechanism of such inhibition may be analogous to that which occurs in the thyroid. The peroxide test measures the ability of the drug to interfere with a non-enzymic but chemically similar iodination of protein. In the tests, two standardized systems are made; one consists of a milk enzyme powder with xanthine, casein and radioiodide, and the other of peroxide, casein and radioiodide. After a standard incubation of the tubes with and without added drugs, the proteins are precipitated and the residual radioiodide in the supernatant fluid is counted to ascertain the degree of protein iodination. These tests are based on the original observation of Keston (1944) that unpasteurized milk with added xanthine will bind radioiodide to its protein, transforming a large proportion to thyroxine, and that this binding is inhibited by thiourea. Keston (1944) postulated that the xanthine oxidase of the milk produces peroxide which releases free iodine and so iodinates the protein. The milk peroxidases are doubtless also important factors in the enzyme reaction of this test. In confirmation of this hypothesis, Pitney and Fraser found that approximately equivalent iodination could be produced in a mixture of iodide and protein by adding either the milk enzymes and xanthine, or peroxide.

The organic binding of iodide by the thyroid gland is generally believed to involve an oxidative mechanism, which may be activated by a peroxidase (Raben and Astwood, 1949; Dempsey, 1944; deRobertis and Grasso, 1946). Without more precise knowledge of the mechanism in the thyroid, it remains uncertain whether this *in vitro* enzyme test is sufficiently analogous; it is, however, encouraging that the test arranges the antithyroid drugs investigated in approximately the order of their biological potency. The test should therefore be useful for a preliminary screening of new antithyroid drugs.

The test also gives some indication of the mode of action of antithyroid drugs. Three main hypotheses have been suggested to account for the inhibition of the organic binding of iodide by antithyroid drugs: (1) that they prevent the liberation of any free iodine, either by their ability to combine with iodine, by their reducing power, or by both (Pitt-Rivers, 1950); (2) that they inhibit (*a*) the thyroid cell peroxidase (Westerfeld and Lowe, 1942; Dempsey, 1944), or (*b*) other oxidative enzyme systems such as cytochrome-oxidase (Schachner, Franklin, and Chaikoff, 1943). The last two hypotheses might be bracketed together as the enzyme-inhibiting theory, since the enzymes concerned in the thyroid are not yet known. Results with the test suggest that the most potent antithyroid drugs may act mainly by inhibiting an enzyme, while many of the weaker drugs may act mainly as substrate competitors or chemical removers of iodine.

METHOD

The Enzyme Test

The test procedure and the method of preparing the enzyme powder have been fully described by Pitney and Fraser (1953). The dry enzyme powder keeps satisfactorily in the refrigerator. No decrease in potency was noticed during the six months' period of the tests reported here. For these tests 50 mg. of enzyme powder was used in each tube, and, in the absence of drugs, this gave 80% (75% to 85%) protein-binding of iodide. The drugs were compared in terms of molar concentrations. Usually they were tested up to a strength giving 100% inhibition, but sometimes difficulties of solubility prevented the testing of the

full range of concentrations desired—e.g., with 2-thiouracil and *n*-butyl-4-hydroxy-3:5-di-iodobenzoate.

To each of a series of centrifuge tubes are added: 3 ml. phosphate buffer *p*H 7.4, 1 ml. xanthine solution (0.45 mg./ml. just brought into solution in hot alkali), 5 ml. casein suspension (3 g./100 ml. H_2O), 1 ml. enzyme solution (50 mg./ml.), 1 ml. iodide solution (iodide 10^{-7} M and ^{131}I 1 μc./ml.), and 1 ml. of drug solution. After incubation for half an hour at 37° C., 3 ml. of 25% trichloracetic acid is added; then, after centrifugation, the supernatant fluid is removed and its radioactivity counted. Two pairs of control tubes are run with each test; control A contains neither drug nor enzyme and the supernatant-fluid count gives the figure for 100% inhibition of binding; control B contains enzyme but no drug, and the supernatant-fluid count gives the figure for no inhibition or maximal binding. With the aid of these control values the counts obtained in the other tubes are then each converted to an index of antithyroid activity as follows:

$$\%\ \text{Inhibition of binding by drug in tube X} = \frac{(\text{Count X} - \text{count B}) \times 100}{\text{count A} - \text{count B}}$$

The Peroxide Test

The peroxide test is performed similarly except that 1 ml. of 0.1 M-peroxide is added instead of the enzyme and the xanthine. This test need only be used when it is desired to study the mode of action of the antithyroid drug. It is then only necessary to include a pair of tubes containing the lowest concentration of drug found to give 100% inhibition with the enzyme test.

Results

Fig. 1 shows the results of tests made on a series of known antithyroid and allied drugs; the main findings are summarized in Table I. In the figure, the drugs are compared over a range of concentrations by the percentage inhibition of protein-binding which they induced. These concentrations are expressed as molar equivalents relative to the iodide present in the testing system (molar $\times$ $10^{-8.08}$). The figure shows the results both with the enzyme test and with the peroxide or enzyme-free test. Figures 2a, 2b, and 2c show the curves obtained with each test from three sample drugs. Table I shows various indices derived from these curves for each of the drugs: (1) their potencies with each test (the reciprocal of the molar equivalents for 30% inhibition, comparisons being made at this level, since 50% inhibition was not reached on the peroxide test with some of the drugs); (2) an index of the non-enzymic action in the enzyme test (the percentage inhibition found in the peroxide test with the lowest drug concentration causing 100% inhibition in the enzyme test); and (3) an index of the speed of transition in the enzyme test from minimal to maximal effect with

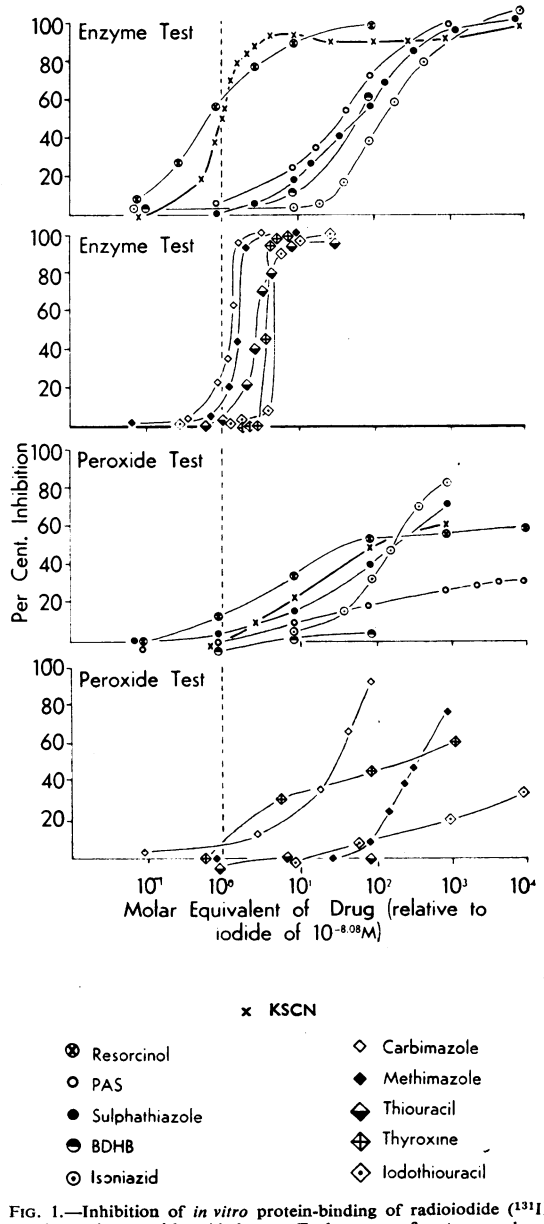

FIG. 1.—Inhibition of *in vitro* protein-binding of radioiodide (^{131}I) by various antithyroid drugs. Each curve refers to one drug. The drug concentration is shown in molar equivalents, relative to the iodide present during the test ($10^{-8.08}$M). NOTE: (a) *The enzyme test:* (i) the drug concentration for any chosen level of inhibition arrays the drugs in order of potency, and (ii) the slope of the inhibition curve from minimal to maximal effect varies and indicates different types of action—some, e.g. carbimazole, with steep slopes suggesting enzymic inhibition, and others, e.g. sulphathiazole, with gentle slopes suggesting substrate competition. (b) *The peroxide test:* the degree of inhibition is less and the order of the drugs by potency is different, tending to confirm the indications of enzyme-inhibition by some of the drugs on the former test.

TABLE I

CHARACTERISTICS OF THE INHIBITION BY VARIOUS DRUGS OF THE *IN VITRO* OXIDATIVE PROTEIN IODINATION

Drug (Mol. Wt. in Parentheses)	Potency (At 30% Inhibition)			Non-Enzymic Action in First 100% Enzyme Effect (See Footnote)	Speed of Transition from Minimal to Maximal Inhibition (With Rising Drug Concn.)		Summary	
	Enzyme Test	Peroxide Test	Ratio Enzyme: Peroxide		Enzyme Test (10–90%)	Peroxide Test (10–30%)	Potency	Type of Action
Carbimazole (186)	0·84	0·07	12·0	10%	0·35	0·11	Strong	E
Methimazole (114)	0·63	0·005	126·0	0	0·56	0·56		E
Resorcinol (110)	2·82	0·145	19·4	55%	0·011	0·10		C
Thiocyanate (K salt) (97)	1·12	0·056	20·0	60%	0·10	0·22		I
Thiouracil (128·2)	0·35	—	—‡	0	0·25	—	Medium	E
Iodothiouracil (276)	0·18	0·0003	600·0	2%	0·55	0·035		E
L-Thyroxine (800)	0·28	0·159	1·8	25%	0·66	0·35		I
p-Aminosalicylate (211·2)	0·067	0·0004	168·0	28%	0·005	0·003	Weak	I
BDHB* (an antithyroxine) (446)	0·035	—	—‡	Over 5%	Over 0·09‡	—		I
Sulphathiazole (Na salt) (367·4)	0·050	0·022	2·3	80%	0·009	0·15		C
Isoniazid (173)	0·014	0·0126	1·1	85%	0·047	0·40		C
Perchlorate (K salt) (138·5)	0	0		—			Nil	
Quinine HCl (400)	0	0		—				

* BDHB = *n*-Butyl-4-hydroxy-3:5-di-iodobenzoate (Sheahan, Wilkinson, and Maclagan, 1951).

‡ Too insoluble for adequate testing (2-thiouracil and BDHB): to derive the figures shown in columns 5 and 6 for BDHB the 50% inhibition values have been used.

E = Enzymic; I = intermediate; C = chemical.

Potency for 30% inhibition = reciprocal of drug concn. for this effect (in molar equivalents relative to the iodide present).

Non-enzymic action in first 100% enzyme effect = % inhibition found on peroxide test with the drug concn. just causing 100% inhibition on enzyme test.

Speed of transition from minimal to maximal inhibition = concn. for lower inhibition (10%) divided by that for higher inhibition (30% or 90%).

rising drug concentration (the ratio of the drug concentration for 10% inhibition to that for a greater inhibition—90% for the enzyme test and 30% for the peroxide test, since with the latter 90% was not always reached).

The drugs have been arranged in Table I in four main groups according to their potency on the enzyme test—high, medium, low and nil. The other characteristics shown in the table have helped to divide the drugs into three main types —enzymic inhibitors (E), chemical inhibitors (C) and drugs with intermediate action (I). In each potency range, the type E drugs have been entered first, since enzymic inhibition may be the optimal type of antithyroid action for clinical use.

Potency

It will be noted that in either test there is little inhibitory action with drug concentrations of less than molar equivalence with iodide ion. Most of the strong inhibitors achieve 100% inhibition in the enzyme test at concentrations between 3 and 10 times molar equivalence. These figures in the enzyme test may suggest the range of concentrations of the drugs which are likely to be required in the thyroid for an antithyroid effect. However, it must be remembered in using these figures that many potent antithyroid drugs are concentrated in the thyroid, while other drugs may not enter the thyroid cell.

It will be noted that all drugs appear more potent by the enzyme test—though the enzyme/peroxide potency ratios for 30% inhibition are far from constant (from 1.1 to 600). Some of this variation might be expected, since the high peroxide concentrations involved in the peroxide test might degrade a number of the drugs (McQuillan, Morton, Stanley, and Trikojus, 1954). The peroxide concentration was chosen to give approximately equivalent protein-binding in the absence of drugs. This is one reason why the peroxide test is not advocated as a reliable index of potency. However, some of the differences between the enzyme/peroxide ratios of different drugs may be due to the different modes of action, and this is suggested by the other analyses shown in Table I and discussed below.

Type of Action

Irrespective of potency, there are striking differences in the inhibition achieved in the peroxide test at the concentration which causes 100% enzymic inhibition (" non-enzymic contribution "). This " non-enzymic contribution " is roughly inversely correlated with the " speed of transition " from minimal to maximal inhibition in the enzyme test. The " speed of transition " in the enzyme test is apparently a good inverse index of the " non-enzymic contribution " to the inhibition in the enzyme test, thus confirming the validity of

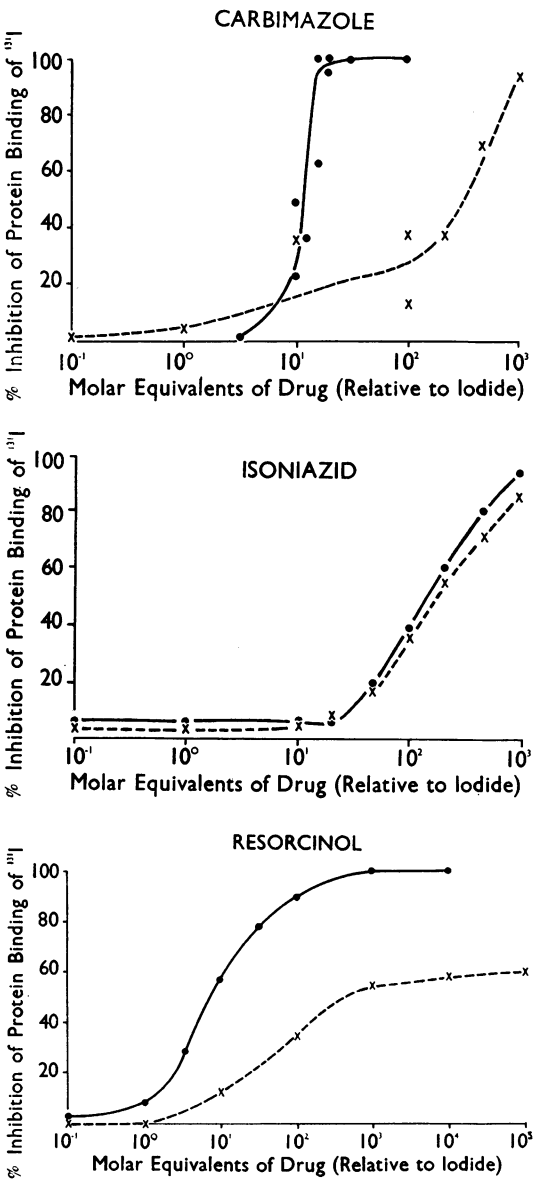

FIG. 2a, b, c.—Individual enzyme and peroxide test results with 3 drugs illustrating different types of result. ●——● enzyme test; ×- - - -×, peroxide test.

this subdivision into types. With thiouracil, *p*-aminosalicylate and resorcinol, the "non-enzymic contribution" is less than would be expected from the "speed of transition." Possibly these drugs are partly destroyed by the high peroxide concentration in the peroxide test. The "speed of transition" is therefore probably the more reliable index of the type of action. By this criterion the drugs have been classed in Table I into three types: *type E*, apparently mainly enzyme-inhibitors with a small "non-enzymic contribution" and rapid "speed of transition" on the enzyme test; *type C*, showing the opposite features and so apparently active mainly by iodine-removal or substrate-competition; and *type I*, showing intermediate characteristics, but resembling more closely type C.

Type E: carbimazole (2-carbethoxythio-1-methyl-iminazole), methimazole (2 - mercapto - 1 - methyl-iminazole), 2 - thiouracil and iodothiouracil (4 - hydroxy-5-iodo-2-mercaptopyrimidine).

Type I: Thiocyanate, L-thyroxine and *p*-amino-salicylate.

Type C: resorcinol, sulphathiazole and isoniazid.

It will be noted that, in general, type E drugs have strong, and type C drugs weak, antithyroid action, both by this enzyme test and by biological tests, with type I drugs more allied to type C. The two main exceptions are thiocyanate and resorcinol, both strong inhibitors on the enzyme test though otherwise resembling type C drugs.

DISCUSSION

The enzyme test assesses the inhibition of peroxidase-activated iodination of protein. Such peroxidase activity has already been shown to be inhibited by some antithyroid drugs (Westerfeld and Lowe, 1942; Dempsey, 1944). Apart from the identity of the enzyme concerned, conditions in the enzyme test may be comparable to those in the thyroid—the hydrogen ion concentration, the presence of a large excess of protein, and a low iodide concentration. Confirmation of its validity as a test of antithyroid activity can only be final when the enzyme that is active in the organic binding of iodide by the thyroid cells has been isolated. A peroxidase is as likely to be the enzyme involved as any other enzyme suggested (Raben and Astwood, 1949). Direct biological comparison would not be valid, since this also involves the ability of the drugs to enter the thyroid gland and is complicated by differences in sensitivity between various species. It is, however, suggestive that the enzyme test arranges the tested drugs approximately in order of known biological activity, and that no activity has been found with two drugs not known to inhibit the organic binding of iodide by the thyroid (quinine and perchlorate). It is now clear that, in the thyroid, inorganic iodide ion is first concentrated, and then subsequently incorporated into thyroxine (Astwood, 1944–5).

The enzyme test can only be expected to assess activities affecting the second stage. By *in vivo* tests, both perchlorate (Stanbury and Wyngaarden, 1952) and thiocyanate (Vanderlaan and Vanderlaan, 1947) have been found to inhibit the first stage, i.e. concentration of iodide by the thyroid gland, without apparently affecting the second stage. However, this is not discordant with the *in vitro* finding that thiocyanate is an inhibitor, since this drug is known to remain mostly, if not entirely, extracellular in the body. This does, however, emphasize the importance of supplementing this *in vitro* screening test with subsequent biological testing, before drawing final conclusions; for whatever the *in vitro* potency, antithyroid drugs are useless unless they penetrate the thyroid cell.

A theoretically important finding is that antithyroid drugs vary in their mode of inhibiting peroxidase-activated iodination of protein, one group being largely enzyme inhibitors, one group largely substrate competitors or iodine removers, and a third intermediate group showing both types of action. It is probable that drugs similarly vary in their action on the thyroid. Most potent antithyroid drugs rapidly pass from minimal to maximal effect in the enzyme test and have a relatively slight inhibitory action in the non-enzymic peroxide test. Both these features suggest that enzymic inhibition is the major factor in their action in the enzyme test and so presumably in the thyroid. Practically all the antithyroid drugs also inhibit at higher concentrations in the non-enzymic peroxide test. However, the concentrations involved suggest that this may not be their action *in vivo*. The results of the peroxide test must be interpreted with caution, since a much higher concentration of peroxide was used than is likely to be found in the body; some of the drugs tested may not be stable in such a concentration. The results of this test have been presented mainly for the support they give to the interpretation of the enzyme tests. They tend to confirm that the steep curves of the enzyme test indicate enzyme inhibition.

The drugs differ not only in potency but also in their relative inhibitory effect on the enzymic and the non-enzymic tests. Only one group, consisting mostly of the weaker drugs, shows an approximately equivalent effect in both tests and is thus likely to have a mainly non-enzymic action. It appears that antithyroid action may be partly affected by the drug's chemical activity as an iodine-remover or substrate competitor. However, the potent antithyroid drugs also have a more important enzyme-inhibiting action (probably peroxidase-inhibiting). This is contrary to the view expressed by Pitt-Rivers (1950) that the antithyroid action of drugs depends on either an ability to bind iodine or reducing power or both. However, Miller, Roblin, and Astwood (1945) did not find that iodine-removal activity correlated adequately with biological antithyroid potency; nor did Arnott and Doniach (1952) find that the comparative biological antithyroid activity of a series of compounds chemically allied to resorcinol supported this hypothesis.

It is likely that, potency apart, the type E drugs —the enzyme inhibitors—would be the better drugs for clinical use, because their action should involve concentration in the thyroid by attachment to the enzyme. Thus, with an effective antithyroid dose, there should be a lower concentration elsewhere in the body and so a lesser general toxic risk. This test should therefore help in screening new potential antithyroid drugs by picking out from the most potent those which are also enzyme-inhibitors. The two most potent of the type E drugs which we have tested were carbimazole and methimazole—the two drugs reported to give the least toxic effects in a clinically adequate antithyroid dosage (Fraser, Garrod, Hanno, and Jadresic, 1954). Conversely, resorcinol is potent but mainly a type C drug and known to be clinically toxic; and iodothiouracil, found clinically to be merely a weak antithyroid drug, needs about double the molar concentration of 2-thiouracil for equivalent *in vitro* action.

The test should also help to detect undesirable antithyroid action in drugs intended for long-term use for other purposes. This is unlikely to matter with drugs to be used only for short periods, since unless an antithyroid drug is used for longer than three months it will not exhaust the normal thyroid's store of preformed hormone. It will be noted that we found that *p*-aminosalicylate gives 50% inhibition of the enzyme test at about ten times the molar concentration required for thiouracil, or at about thirty times molar equivalence with iodide. Total inhibition does not occur until about thirty times this concentration. If for a rough preliminary assessment we consider an average dose of the drug (5 g.) distributed in 50 litres of body water, its molar ratio to iodide would be about 474:1 (assuming body fluid to contain approximately 1×10^{-6} mols. iodide/l.). Thus, ignoring excretion and the unknown possibilities of concentration in the thyroid, an antithyroid effect is possible with this dose given three times daily and might be important clinically after

three months' administration. This has been observed (Balint, Fraser, and Hanno, 1954). Similar rough calculations for other drugs may help to indicate the possibilities of such undesirable antithyroid effects and the need to look out for them during clinical trials.

Summary

1. Further experience with two *in vitro* tests confirms their value for determining the potency and the mode of action of antithyroid drugs.

2. The antithyroid action of some drugs may be attributed to their chemical ability to remove or compete with iodine, but that of the most potent ones probably involves enzymic inhibition of a peroxidase.

3. For screening possible new antithyroid drugs the enzyme test is advocated, using various drug concentrations so as to give from 0–100% inhibition. Positive results should be confirmed *in vivo*.

4. To be suitable for clinical trial, antithyroid drugs should show enzyme inhibition in this test, and be more potent than currently available drugs.

5. If any drug which is likely to be used for prolonged periods is found to give over 50% inhibition in the enzyme test at concentrations likely to be achieved in the body, a watch should be kept for antithyroid effects during clinical trials.

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