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Antithyroid Effect of 2,3-Dihydroxypyridine *in Vivo* and *in Vitro*[1] (41454)

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Abstract. The antithyroid effect of 2,3-dihydroxypyridine (2,3-DHP) was investigated *in vivo* and *in vitro*. Thyroid studies were carried out on two groups of rats; one group of 14 rats were fed a low-iodine diet containing 1% 2,3-DHP for 25 days, and a control group of 12 rats received low-iodine diet alone. 2,3-DHP-treated rats had reduced thyroid function with a significant rise in serum thyrotropin (TSH) levels and decreased serum triiodothyronine (T3) levels; thyroxine (T4) and T3 contents of the thyroid gland were 20 and 10%, respectively, lower than those of controls. *In vitro*, 2,3-DHP inhibited peroxidase activity, and its antiperoxidase potency was similar to the commonly used thioureylene antithyroid drug, propylthiouracil. In addition, 2,3-DHP was a much weaker inhibitor of the thyroid conversion of T4 to T3 *in vitro* than propylthiouracil. 2,3-DHP represents a new class of antithyroid drug and its main action appears to be inhibition of peroxidase-catalyzed thyroid hormone formation in the thyroid gland.

Hyperplastic goiter has been reported in cattle grazing on the tropical legume, *Leucaena leucocephala* (1, 2). Hegerty *et al.* found that 3,4-dihydroxypyridine (3,4-DHP) isolated from the seed of leucaena was responsible for development of goiter (3). Later, this compound was shown to inhibit thyroid peroxidase and to induce hypothyroidism in mice (4). Recently a closely related compound, 2,3-dihydroxypyridine (2,3-DHP), has been reported to possess antithyroid activity by inhibiting thyroid peroxidase *in vitro* (5). Since 2,3-DHP is more readily available than 3,4-DHP, we investigated the anti-thyroid effect of 2,3-DHP in rats by analyzing serum thyroid hormone levels, serum TSH levels, and thyroid hormone content in the thyroid gland. Also, the relative antithyroid potency of 2,3-DHP was compared to commonly used thioureylene drugs, propylthiouracil and methimazole *in vitro*.

Materials and Methods. Fourteen male Sprague–Dawley rats weighing 250–300 g were fed a low-iodine diet (Teklad, Madison, Wisc.) containing 1% 2,3-DHP for 25 days. Twelve control rats received only low-iodine diet. Water and food were given *ad libitum*. On the 25th day, rats were killed and serum was obtained for radioimmunoassay of T_3, T_4, and thyrotropin (TSH) (6–8).

The thyroid gland of each animal was weighed and homogenized in 1 ml of 0.05 *M* sodium phosphate buffer, pH 7.0, for 60 sec by use of Polytron homogenizer equipped with a PT-7 generator. This small generator was necessary to homogenize the small thyroid glands. Digestion of 500 μl of the thyroid homogenate was carried out with Pronase (3 mg/ml, Calbiochem, La Jolla, Calif.) for 24 hr according to the method of Inoue *et al.* (9). The completeness of digestion of thyroglobulin by this method was about 85% in our hands. Extraction of thyroid hormone was carried out by adding 400 μl ethanol to 100 μl of thyroid digest. After a small aliquot of ethanol extract was dried under N_2, 100 μl of T_3- and T_4-free serum was added for T_4 assay and further dilution was made for T_3 assay. Results are expressed as nanograms of T_3 or T_4 per milligram of thyroid.

The potency of 2,3-DHP, propylthiouracil, and methimazole was tested *in vitro* using two different systems of peroxidase-catalyzed reaction: iodination of thyro-

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globulin (10) and guaiacol oxidation (11). Since lactoperoxidase catalyzes the iodination of protein in a manner similar to thyroid peroxidase (12), lactoperoxidase was employed in this experiment. For iodination of thyroglobulin, the standard incubation medium contained 1 mg bovine thyroglobulin (Type 1, Sigma Chemical Co., St. Louis, Mo.), 1 mg glucose, 1.5 μg glucose oxidase (Type IV, Sigma Chemical Co.), 1 μg lactoperoxidase (L-8257, Sigma Chemical Co.), 50 μM KI, 80 nM [^{125}I]iodide, and 100 μl of diluted antithyroid drugs in a total volume of 1.0 ml of 0.05 M sodium phosphate buffer, pH 7.0. Incubation was carried out for 20 min at 37°C and the reaction was terminated by adding 50 μl of 10 mM methimazole. A small aliquot was then applied to Whatman 3 MM paper and chromatographed to measure the degree of thyroglobulin iodination (10). For guaiacol assay, the incubation medium contained 1 mM guaiacol, 1 μg lactoperoxidase, and 100 μl of diluted antithyroid drug in a total volume of 1 ml in 0.05 M sodium phosphate buffer, pH 7.0. The reaction was started by the addition of 100 μl of 88 mM H_2O_2 at room temperature, and the rate of guaiacol oxidation was followed spectrophotometrically on a Gilford spectrophotometer-240 (Gilford Instrument Laboratory, Inc., Oberlin, Ohio) at 470 nm.

An *in vitro* conversion of T_4 to T_3 was carried out according to the method of Ishii *et al.* (13). An enzyme for the conversion of T_4 to T_3 was prepared from the thyroid of a patient with Graves' disease who was treated with methimazole before surgery. Three grams of the thyroid gland was minced and homogenized for 60 sec in 12 ml of cold 0.05 M Tris–0.25 M sucrose buffer, pH 7.0, containing 5 mM dithiothreitol (DTT). The homogenate was centrifuged at 100,000g for 60 min. The 100,000g pellet was suspended in 4 ml of Tris–sucrose buffer containing 5 mM DTT, followed by homogenization for 10 sec, and used as a T_4 monodeiodinating enzyme. The concentration of protein was measured by Lowry's method (14). The incubation system was 1 ml total volume containing 2 μg T_4 (Sigma Chemical Co.), 5 mM DTT, 0.8 mg enzyme, 1 μM–1 mM 2,3-DHP or propylthiouracil, and 0.05 M Tris–0.25 M sucrose buffer, pH 7.0. Incubation was carried out at 37° for 60 min in a polypropylene test tube (12 × 75 mm). At the end of incubation 2 ml of ice-cold 95% ethanol was added to the incubation tube, which was then kept at −10° for 12 hr. Ethanol-insoluble material was removed by centrifugation at 1000g for 20 min. Twenty microliters of ethanol extract was then added to 300 μl of T_3- and T_4-free serum, and T_3 was measured by radioimmunoassay. The blank tubes which did not contain either DTT or enzyme in the incubation medium did not produce T_3.

Statistical analysis was performed by unpaired t test.

Results and Discussion. Table I shows the results of the thyroid studies in rats. The thyroid function tests showed that administration of 2,3-DHP reduced thyroid function in rats, as evidenced by a 3.5-fold increased serum TSH level compared to that of controls. Serum T_4 levels in the two groups of rats were similar, but serum T_3 levels were significantly lower in 2,3-DHP-treated rats than in controls ($P < 0.05$). Measurement of the T_3 and T_4 contents of the thyroid gland provided convincing evidence of the inhibitory action of 2,3-DHP on the process of thyroid hormone formation; the T_3 content of the thyroid glands from 2,3-DHP-treated rats was only 1/10th that of the controls and the T_4 content was about 2/10th that of the controls. Since the weights of thyroid glands in the two groups of rats were not significantly different, ingestion of 1% 2,3-DHP for 25 days was not sufficient to induce a significant goiter in rats. In addition, 2,3-DHP-treated rats gained less weight than the controls. It should be noted, however, that the size of goiter and the amount of weight gained may not be an accurate index to assess hypothyroidism, since variable size of goiter and body weight have been reported after treatment with antithyroid drugs (15). Lee *et al.* reported that the diet containing 1% 2,3-DHP did not produce a significant goiter in mice after 5 weeks of treatment, but goiter was found after 11 weeks of treatment (5). In contrast, 0.001% propylthiouracil in drinking water has been shown

TABLE I. THYROID STUDIES IN CONTROL RATS AND 2,3-DHP-TREATED RATS

	No.	Body weight (g)	Thyroid weight (mg)	Serum T3 (ng/dl)	Serum T4 (μg/dl)	Serum TSH (μU/ml)	Thyroid hormone content (ng/mg thyroid) T3	Thyroid hormone content (ng/mg thyroid) T4
Control rats	12	375 ± 10.9[a]	27.8 ± 1.7	43.4 ± 3.6	3.3 ± 0.2	77.2 ± 9.0	26.2 ± 4.1	72.1 ± 14.3
2,3-DHP-treated rats	14	325 ± 7.3	34.5 ± 2.5	31.1 ± 3.3	3.1 ± 0.2	274.8 ± 62.2	2.6 ± 0.5	16.3 ± 2.5
P		<0.001	NS	<0.05	NS	<0.001	<0.001	<0.001

[a] Values presented here are the mean ± SEM.

to produce a goiter as early as the fourth day of treatment (18). Therefore, 2,3-DHP appears to be a weak goitrogen, even though it is as potent an inhibitor of peroxidase as propylthiouracil *in vitro* (vida infra).

The iodination of thyroglobulin *in vitro* showed that concentrations of 2,3-DHP, propylthiouracil, and methimazole producing 50% inhibition of peroxidase activity were 3.7×10^{-5} *M*, 2.5×10^{-5} *M*, and 7×10^{-6} *M*, respectively, (Fig. 1A). Thus, 2,3-DHP was an almost equally potent peroxidase inhibitor as PTU in the iodination of thyroglobulin. In the guaiacol assay, 8.8×10^{-4} *M* 2,3-DHP, 4.4×10^{-4} *M* propylthiouracil, and 1.3×10^{-5} *M* methimazole caused 50% inhibition of peroxidase activity (Fig. 1B). The relative potency of methimazole and propylthiouracil determined by our guaiacol assay is slightly different from the result reported by Taurog (16). His guaiacol assay with thyroid peroxidase showed that methimazole has a 13-fold greater potency than that of propylthiouracil; in our guaiacol assay with lactoperoxidase, methimazole is 34-fold as potent as propylthiouracil. Although this discrepancy is not fully explained, the different source of peroxidase and its activity, and the different assay conditions could in part account for it. Nevertheless, the inhibitory effect of 2,3-DHP on lactoperoxidase-mediated guaiacol oxidation was similar to propylthiouracil. It is possible that the relative potency of 2,3-DHP and propylthiouracil may be different if highly purified thyroid peroxidase were used for the assay.

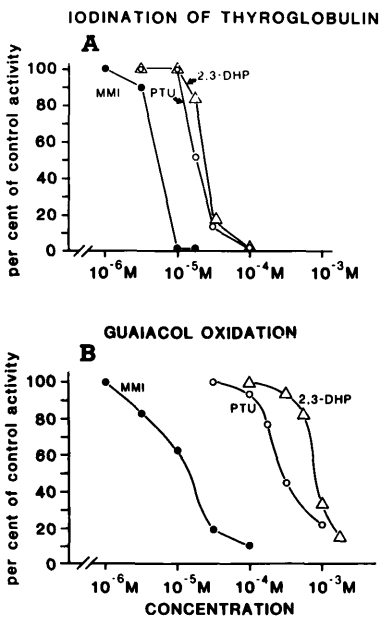

FIG. 1. Dose-inhibition curves for inhibition by 2,3-DHP and thioureylene drugs of iodination of thyroglobulin (A) and guaiacol oxidation (B). Control activity is defined as the rate of iodination of thyroglobulin or guaiacol oxidation without inhibitors. Values shown here are the mean of duplicate determinations. MMI, methimazole; PTU, propylthiouracil.

2,3-DHP at the concentration of 1 m*M* caused a 20% reduction of conversion of T_4 to T_3 *in vitro*. Propylthiouracil, a well-known inhibitor of peripheral conversion of T_4 to T_3 (17), caused about 40% inhibition of thyroid T_4 to T_3 conversion at the concentration of 10 μ*M*. The production of T_3 from T_4 by the thyroid enzyme preparation was completely inhibited by 1 m*M* propylthiouracil (Table II). Thus, the potency of 2,3-DHP to inhibit the thyroid conversion of T_4 to T_3 was less than 1/100th that of propylthiouracil.

The antithyroid activity of 2,3-DHP was confirmed in this study, since rats receiving

TABLE II. EFFECT OF 2,3-DHP AND PROPYLTHIOURACIL ON THE CONVERSION OF T4 TO T3 *IN VITRO*

Molar concentration of antithyroid agent	Production of T3 (ng/mg enzyme/60 min)	
	2,3-DHP	Propyl-thiouracil
0	54.7 ± 1.7	57.8 ± 4.6
10^{-6}	53.7 ± 3.1	56.9 ± 3.3
10^{-5}	53.0 ± 2.8	33.3 ± 0.7
10^{-4}	52.1 ± 2.6	7.4 ± 0.6
10^{-3}	44.0 ± 3.2	ND

Note. Thyroid tissue from a patient with Graves' disease was used as a T4 to T3 converting enzyme. Values presented here are the mean ± SEM of triplicate samples. ND, not detectable.

2,3-DHP showed reduced thyroid function with a significant rise in serum TSH. Although ingestion of 2,3-DHP decreased only serum T_3 levels without significant change in serum T_4 levels, the thyroid content of T_3 and T_4 was greatly reduced in 2,3-DHP-treated rats. It is conceivable that the release of thyroid hormone from the thyroid glands in 2,3-DHP-treated rats was greatly stimulated by TSH, despite their low hormone content, thus resulting in almost the same level of serum T_4 as in controls.

Lower serum T_3 levels in the 2,3-DHP-treated rats than in controls raised the possibility that 2,3-DHP could be an inhibitor of the conversion of T_4 to T_3. Our *in vitro* finding indicates that the concentration of 2,3-DHP necessary to inhibit the conversion of T_4 to T_3 was high compared to that of propylthiouracil. Therefore, the inhibitory effect of 2,3-DHP on the conversion of T_4 to T_3 may not be significant *in vivo*, unless a large dose of 2,3-DHP is employed.

Depletion of thyroid hormone content of the thyroid gland found in the 2,3-DHP-treated rats could arise from impairment of iodide trapping by the thyroid gland, or inhibition of intrathyroidal hormone synthesis, or both. Lee *et al.* reported that 2,3-DHP did not inhibit iodide trapping (5). Since 2,3-DHP inhibited peroxidase activity *in vitro*, the mechanism of antithyroid effect of 2,3-DHP appears to be inhibition of the thyroid hormone catalytic enzyme, thyroid peroxidase. It is of interest to note that 2,3-DHP, which is chemically distinct from the thioureylene antithyroid drugs, possesses properties similar to propylthiouracil.

1. Holmes JHG. Growth of Brahman cross heifers grazing leucaena. Proc Soc Anim Prod **11**:453–456, 1976.
2. Jones RJ, Blunt CG, Holmes JHG. Enlarged thyroid glands in cattle grazing leucaena pastures. Trop Grassl **10**:113–116, 1976.
3. Hegerty MP, Lee CP, Christie GS, Court RD, Haydock KP. The goitrogen 3-hydroxy-4 (1H)-pyridone, a ruminal metabolite from leucaena leucocephala: Effects in mice and rats. Aust J Biol Sci **32**:27–40, 1979.
4. Christie GS, Lee CP, Hegarty MP. Antithyroid properties of 3-hydroxy-4 (1H)-pyridone: Antiperoxidase activity and effect on thyroid function. Endocrinology **105**:342–347, 1979.
5. Lee CP, Christie GS, Hegarty MP. Antithyroid and antiperoxidase activity of some isometric dihydroxypyridines. In: Stockigt JR, Nagataki S, eds. Thyroid Research VIII. Canberra, Aust Acad Sci, pp137–140, 1980.
6. Chopra IJ, Ho RS, Lam R. An improved radioimmunoassay of triiodothyronine in serum: Its application to clinical and physiological studies. J Lab Clin Med **80**:729–739, 1972.
7. Chopra IJ. A radioimmunoassay for measurement of thyroxine in unextracted serum. J Clin Endocrinol Metab **34**:938–947, 1972.
8. Pekary AE, Hershman JM, Parlow AF. A sensitive and precise radioimmunoassay for human thyroid stimulating hormone. J Clin Endocrinol Metab **41**:676–684, 1975.
9. Inoue K, Taurog A. Digestion of ^{131}I-labeled thyroid tissue with maximum recovery of ^{131}I-iodothyronines. Endocrinology **81**:319–332, 1967.
10. Nagataki S, Uchimura H, Kuzuya N, Masuyama Y, Kumagai L, Ito K. Iodinating activity of thyroid tissue in toxic diffuse goiter. J Clin Invest **59**:723–729, 1977.
11. Hosoya T, Kondo Y, Ui N. Peroxidase activity in thyroid gland and partial purification of enzyme. J Biochem (Tokyo) **52**:180–189, 1962.
12. Taurog A, Dorris MA, Lamas L. Comparison of lactoperoxidase and thyroid peroxidase-catalyzed iodination and coupling. Endocrinology **94**:1286–1294, 1974.
13. Ishii H, Inada M, Tanaka K, Mashio Y, Naito K, Nishikawa M, Imura H. Triiodothyronine generation from thyroxine in human thyroid. Enhanced conversion in Graves' thyroid tissue. J Clin Endocrinol Metab **52**:1211–1217, 1981.

14. Lowry OH, Rosebrough NJ, Farr AL, Randall RJ. Protein measurement with the Folin phenol reagent. J Biol Chem 193:265–275, 1951.
15. Alexander WD, Wollf J. Thyroidal iodide transport. VIII. Relation between transport, goitrogenic and anti-goitrogenic properties of certain anions. Endocrinology 78:581–590, 1966.
16. Taurog A. The mechanism of action of the thioureylene antithyroid drugs. Endocrinology 98:1031–1046, 1976.
17. Geffner DL, Azukizawa M, Hershman JM. Propylthiouracil blocks extrathyroidal conversion of thyroxine to triiodothyronine and augments thyrotropin secretion in man. J Clin Invest 55:224–229, 1975.
18. Männistö PT, Ranta T, Leppaluoto J. Effects of methylmercaptoimmidazole (MMI), propylthiouracil (PTU) and potassium iodide (KI) on the serum concentrations of thyrotropin (TSH) and thyroid hormones in the rat. Acta Endocrinol 9:271–281, 1979.

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