Brazilian Journal of Medical and Biological Research (2000) 33: 355-361
ISSN 0100-879X

Thyroid peroxidase activity is inhibited by amino acids

D.P. Carvalho, A.C.F. Ferreira, S.M. Coelho, J.M. Moraes, M.A.S. Camacho and D. Rosenthal

Laboratório de Fisiologia Endócrina, Instituto de Biofísica Carlos Chagas Filho, Universidade Federal do Rio de Janeiro, Rio de Janeiro, RJ, Brasil

Correspondence
D. Rosenthal
Laboratório de Fisiologia Endócrina
Instituto de Biofísica, UFRJ
21949-900 Rio de Janeiro, RJ
Brasil
Fax: +55-21-280-8193
E-mail: doris@biof.ufrj.br

Research supported by FINEP, CEPG/UFRJ, FAPERJ and CNPq.

Received June 17, 1999
Accepted January 21, 2000

Abstract

Normal *in vitro* thyroid peroxidase (TPO) iodide oxidation activity was completely inhibited by a hydrolyzed TPO preparation (0.15 mg/ml) or hydrolyzed bovine serum albumin (BSA, 0.2 mg/ml). A pancreatic hydrolysate of casein (trypticase peptone, 0.1 mg/ml) and some amino acids (cysteine, tryptophan and methionine, 50 µM each) also inhibited the TPO iodide oxidation reaction completely, whereas casamino acids (0.1 mg/ml), and tyrosine, phenylalanine and histidine (50 µM each) inhibited the TPO reaction by 54% or less. A pancreatic digest of gelatin (0.1 mg/ml) or any other amino acid (50 µM) tested did not significantly decrease TPO activity. The amino acids that impair iodide oxidation also inhibit the TPO albumin iodination activity. The inhibitory amino acids contain side chains with either sulfur atoms (cysteine and methionine) or aromatic rings (tyrosine, tryptophan, histidine and phenylalanine). Among the amino acids tested, only cysteine affected the TPO guaiacol oxidation reaction, producing a transient inhibition at 25 or 50 µM. The iodide oxidation inhibitory activity of cysteine, methionine and tryptophan was reversed by increasing iodide concentrations from 12 to 18 mM, while no such effect was observed when the cofactor (H_2O_2) concentration was increased. The inhibitory substances might interfere with the enzyme activity by competing with its normal substrates for their binding sites, binding to the free substrates or reducing their oxidized form.

Key words
- Thyroid
- Thyroperoxidase
- Amino acids
- Iodide oxidation
- Lactoperoxidase

Introduction

Thyroid peroxidase (TPO) is a heme-containing, membrane bound, glycoprotein enzyme that plays a key role in the biosynthesis of thyroid hormones (1,2). The oxidation of iodide, the iodination of thyroglobulin and the coupling of iodotyrosyl residues of thyroglobulin are catalyzed by TPO (2,3). TPO activity has been analyzed in various human thyroid glands, and in most studies a wide variability in TPO activity was found, even when the enzyme was extracted from different parts of the same gland (4). Hosoya et al. (5) have previously called attention to the fact that there seems to be no correlation between the TPO iodide oxidation activity and the TPO guaiacol oxidation activity in abnormal thyroids, as opposed to the good correlation found in normal human and por-

cine thyroid tissues. The presence of a poorly characterized TPO inhibitor has been previously described in some sporadic and dyshormonogenetic goiters due to absent or defective TPO (6-9). These findings are consistent with the fact that the iodide oxidation activity might be affected by factors that do not influence the guaiacol oxidation activity, as described by Hosoya et al. (5).

As reported here, the enzymatic hydrolysates of TPO preparations and of bovine serum albumin (BSA), as well as some amino acids and peptides can act as TPO iodide oxidation inhibitors, suggesting that a peptide or even some amino acids might interfere with the *in vivo* TPO iodide oxidation and iodination reactions. However, with the exception of cysteine, the inhibitory amino acids do not inhibit the TPO guaiacol oxidation reaction.

Material and Methods

Human thyroid tissue samples from diffuse toxic goiters (DTG) and from the paranodular tissue of a nodular goiter with normal TPO activity (NT) were obtained by thyroidectomy, immediately frozen and stored at -20°C until further processing. The thyroid tissue was cleaned of fibrous tissue and calcified or hemorrhagic areas on an ice-cooled glass plate. Thyroid peroxidase was extracted as previously described (4,9). Briefly, the thyroid tissue samples were cleaned, homogenized in 50 mM Tris-HCl buffer, pH 7.2, containing 1 mM KI, and centrifuged (100,000 *g*, 4°C, 60 min). The pellet was suspended in 2 ml digitonin (1%, w/v) and incubated at 4°C for 48 h. The digitonin-treated suspension was centrifuged (100,000 *g*, 4°C, 60 min), and the supernatant containing solubilized TPO was used in the activity and inhibitory assays. Protein concentration was measured by the method of Warburg and Christian (10).

DTG-TPO and NT-TPO iodide oxidation assays were performed using 12 mM KI in 50 mM phosphate buffer, pH 7.4, and glucose-glucose oxidase as the hydrogen peroxide (H_2O_2) generating system, as previously described (4,9). For the guaiacol oxidation assay a minor modification of the method described by Hosoya et al. (5,9) was used. Briefly, the reaction mixture contained 33 mM guaiacol (Aldrich Chemical Co., Milwaukee, WI, USA, a kind gift from Dr. Alvin Taurog, South-Western Medical School, UT, Dallas, TX, USA) in 50 mM phosphate buffer, pH 7.4, and glucose-glucose oxidase as the H_2O_2 generating system. The increase in absorbance at 353 nm for the iodide oxidation assay (ΔA_{353}) or at 470 nm for the guaiacol oxidation assay (ΔA_{470}) was monitored using a U-3300 (Hitachi) double beam spectrophotometer. TPO activity was estimated from the ΔA_{353}/min or ΔA_{470}/min rate determined from the linear portion of the reaction curve. One unit of iodide (or guaiacol) oxidation activity is defined as ΔA_{353}/min = 1.0 (or ΔA_{470}/min = 1.0). Specific activity was defined as units of iodide (or guaiacol) oxidation activity per gram of protein in the enzyme preparation.

The TPO iodination activity was determined as previously described, using BSA as iodine acceptor, and trichloroacetic acid (TCA) precipitation of the iodine bound to 660 µg BSA (11). The assay mixture contained TPO, 660 µg BSA, 30 nmol ^{125}I-iodide, and glucose-glucose oxidase as the H_2O_2 generating system and was made up to a final volume of 1.0 ml with 50 mM sodium phosphate buffer, pH 7.4. The reaction was carried out at 37°C for different periods of time, BSA was then precipitated with 10% TCA and the fraction of total iodine bound to protein was determined.

Protein preparations were hydrolyzed with 1 mg/ml pronase (Protease type XIV, Sigma Chemical Co., St. Louis, MO, USA) for 90 min, at 37°C. The hydrolysis was stopped by boiling for 30 min; non-hydrolyzed preparations incubated at 100°C for 30 min (denatured proteins) and a denatured

pronase solution (100°C for 30 min) were used as controls.

For the iodide oxidation inhibition assays, the same iodide oxidation activity (ΔA_{353}/min) of either active DTG-TPO or lactoperoxidase (Sigma) was assayed with and without the addition of a) different concentrations of propylthiouracyl (PTU) or methimazole (MMI); b) 0.4 mg of hydrolyzed BSA (Sigma) or its control; c) 0.3 mg of hydrolyzed NT-TPO preparation or its control; d) 0.2 mg of pancreatic digest of casein (trypticase peptone, BBL Microbiology Systems, Cockeysville, MD, USA); e) 0.2 mg of pancreatic digest of gelatin (gelysate peptone, BBL); f) 0.2 mg of casamino acids (Difco, Detroit, MI, USA), or g) the L-amino acids (different concentrations/assay) from the Aldrich Library of Chemical Standards (Aldrich), except alanine, aspartic acid, glycine and serine.

The possibility of inhibition caused by competition with the substrate (iodide) or with the enzyme cofactor (H_2O_2) was evaluated by increasing H_2O_2 generation (11 mM glucose + 20 µl 0.1% glucose oxidase; originally 5.5 mM glucose + 10 µl 0.1% glucose oxidase) or iodide concentration. Furthermore, to determine if the inhibitory amino acids were able to scavenge H_2O_2, 4.0 µM H_2O_2 (Merck S.A., Rio de Janeiro, RJ, Brazil) was incubated in the presence or absence of the amino acid concentrations necessary to inhibit TPO iodide oxidation activity by 50 and 100%. Aliquots of 100 µl were transferred to a tube and 1 ml of 0.2 M sodium phosphate buffer, pH 7.8, containing scopoletin (5 µM) and horseradish peroxidase (5 µg/ml) was added. Fluorescence was measured with a Hitachi (F4000) spectrofluorometer (excitation = 360 nm, emission = 460 nm), as previously described (12). The fluorescence measurements were plotted against H_2O_2 concentrations.

To determine the kinetic parameters of TPO iodide oxidation inhibition, the concentration necessary to inhibit 50% of the original TPO activity (IC_{50}) of the inhibitory amino acids was assayed in the presence of a given TPO activity and variable iodide concentrations. Results are the mean of at least three different experiments.

Since amino acids were less potent inhibitors of TPO iodination activity, for the iodination inhibition assays the active DTG-TPO was assayed with and without the addition of 50 µM of the iodide oxidation inhibitory amino acids or of some of the non-inhibitory ones. Results are the mean of at least three different experiments and are expressed as percentage of DTG-TPO activity at 5, 10, 20 or 30 min of incubation.

Results

The iodide oxidation specific activity of DTG-TPO (1169 U/g protein) and NT-TPO (431 U/g protein) was within our previously reported ranges for DTG and normal tissue TPO activities (4). The concentrations of PTU and MMI necessary to produce 50% inhibition of TPO-mediated thyroglobulin iodination have been reported to be 19.5 and 10 µM, respectively (1). Under our experimental conditions we found similar differences in the IC_{50} values for the inhibitory effects of PTU (10 µM) and MMI (4 µM) on the TPO iodide oxidation reaction. Thus, our TPO assay system can be compared with those reported by other authors, at least for the well-known antithyroid drugs PTU and MMI.

The DTG-TPO iodide oxidation activity was inhibited by hydrolyzed NT-TPO or hydrolyzed BSA, whereas no inhibitory activity was present before proteolysis. In fact, the addition of non-hydrolyzed NT-TPO before boiling produced an increase of 71% over the original DTG-TPO activity. Denatured (boiled) BSA or NT-TPO preparations did not affect the original DTG-TPO activity (Figure 1). TPO activity was also unaffected by the addition of denatured pronase.

The iodide oxidation activity of DTG-

TPO was determined in the presence of several peptides or amino acids, and the extent of inhibition of the original TPO activity is shown in Figure 2. The pancreatic digest of gelatin did not significantly change TPO activity, and inhibited the original DTG-TPO activity by no more than 10%. In contrast, the pancreatic digest of casein completely abolished DTG-TPO activity. Nevertheless, a mixture of amino acids derived from acid hydrolysis of casein (casamino acids) inhibited TPO activity only partially (54% inhibition). Among the tested amino acids, only cysteine (Figure 3A), methionine and tryptophan completely inhibited TPO iodide oxidation at the concentration of 50 µM. Tyrosine (50 µM) caused only partial inhibition (47%), and phenylalanine (50 µM) and histidine (50 µM) produced a maximal inhibition of 20-25% even at higher concentrations (100 µM). The concentrations of inhibitory amino acids necessary to inhibit 50% of the original TPO iodide oxidation activity (IC_{50}) were 12.5 µM cysteine, 13 µM methionine and 9.7 µM tryptophan. Other amino acids tested did not alter the enzyme iodide oxidation activity. The iodide oxidation activity of lactoperoxidase (LPO) was inhibited by the same amino acids that inhibited the TPO-catalyzed reaction, and to a similar extent (data not shown).

The guaiacol oxidation reaction was not significantly influenced by the amino acids, except for a very marked time lag produced by cysteine (Figure 3B). This time lag decreased when cysteine concentration was reduced (25 µM) or the concentration of the enzyme was doubled (data not shown). In contrast to cysteine, cystine did not significantly alter the iodide oxidation or the guaiacol oxidation TPO activity (Figure 3A and B).

The increase in H_2O_2 in the reaction mixture did not change the proportion of TPO iodide oxidation inhibition by amino acids. Furthermore, none of the inhibitory amino acids were able to scavenge H_2O_2 *in vitro*. However, when the amount of iodide was increased to 18 mM, the inhibition produced by casamino acids, tyrosine, phenylalanine or histidine was decreased, resulting in a 10-25% increment in the iodide oxidation activity. The same increment in iodide did not change the strong inhibition promoted by 50 µM cysteine, tryptophan and methionine. Nevertheless, this strong inhibition was dependent on the amino acid concentration in the assay. Kinetic iodide oxidation studies showed that the inhibition produced by cysteine (12.5 µM), methionine (13 µM) or tryptophan (9.7 µM) was reversible, since the original inhibition is reversed at higher iodide concentrations, and the enzyme $K_{0.5}$ for iodide was significantly increased in the

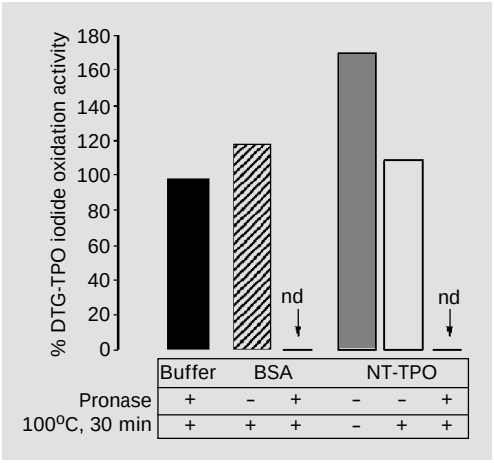

Figure 1 - Effect of hydrolyzed proteins on diffuse toxic goiter-thyroid peroxidase (DTG-TPO) iodide oxidation activity. The TPO iodide oxidation activity was measured in the presence of non-hydrolyzed (-) or pronase-hydrolyzed (+) bovine serum albumin (BSA, 0.4 mg) or normal TPO (NT, 0.3 mg) preparations. The amount of solubilized TPO producing a fixed iodide oxidation activity ($\Delta A_{353\ nm}$/min = 0.1) was assayed for each experimental condition. nd = Activity not detectable.

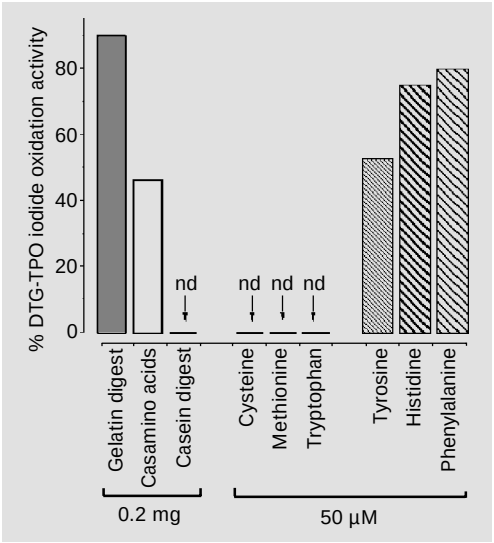

Figure 2 - Iodide oxidation inhibitory assays. The same iodide oxidation activity ($\Delta A_{353\ nm}$/min = 0.1) of active diffuse toxic goiter-thyroid peroxidase (DTG-TPO) was assayed with or without 0.2 mg of pancreatic gelatin digest, 0.2 mg of casamino acids, 0.2 mg of pancreatic casein digest, or 50 µM L-amino acids. nd = Activity not detectable.

presence of these amino acids, indicating a competitive inhibition of the reaction, at least at the concentrations tested (Table 1).

DTG-TPO iodination activity was also inhibited by all the amino acids that inhibited the TPO iodide oxidation reaction, but the inhibitory patterns were different. In the iodide oxidation reaction tryptophan and cysteine were the most potent inhibitors, with equivalent increases in $K_{0.5}$ (Table 1); however, cysteine was more potent than tryptophan in inhibiting the iodination reaction (Figure 4). Tyrosine also inhibited the iodination reaction, but to a lesser extent, while 50 µM phenylalanine, histidine, proline or cystine did not inhibit the iodination activity.

Discussion

The presence of endogenous dialyzable inhibitors of TPO iodination activity in the soluble fraction of bovine and ovine thyroid gland homogenates was first reported in studies carried out in the sixties (13,14), which suggested that endogenous sulfhydryl-containing compounds such as glutathione were responsible, at least in part, for iodination inhibition. It has also been shown that ascorbic acid, cysteine, epinephrine, norepinephrine, serotonin, NADH, and NADPH can inhibit tyrosine iodination (13). More recent studies have shown that some exogenous substances such as dietary flavonoids and sulfamethazine may impair TPO activity, at least *in vitro* (15-17).

As shown here, enzymatic proteolysis of BSA, and even of a TPO preparation, led to the release of TPO inhibitors. Furthermore, a pancreatic digest of casein (but not of gelatin) and a mixture of amino acids derived from acid hydrolysis of casein were able to significantly impair TPO activity *in vitro*, suggesting that some amino acids, the component(s) of these mixtures, could be responsible for TPO inhibition.

Since the pancreatic digest of gelatin was unable to alter the TPO iodide oxidation reaction, it probably contains only small amounts of specific TPO inhibitory amino acid residues, as expected from the primary structure of different types of collagen (18). We have found that the amino acids that impair TPO activity *in vitro* contain side chains with sulfur atoms (cysteine and methionine) or aromatic rings (tyrosine and

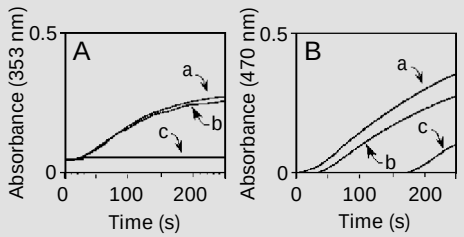

Figure 3 - Diffuse toxic goiter-thyroid peroxidase (DTG-TPO) inhibitory assays. Iodide oxidation (A) or guaiacol oxidation (B) activities of a) DTG-TPO, b) DTG-TPO + cystine, and c) DTG-TPO + cysteine. DTG-TPO iodide oxidation or guaiacol oxidation assays were performed in the absence or presence of cystine (50 µM) or cysteine (50 µM).

Table 1 - Kinetic parameters of thyroid peroxidase (TPO) iodide oxidation in the presence of some amino acids.

Results are reported as mean ± SEM. [a]n = 1. *P<0.05; **P<0.001 *vs* TPO (paired Student *t*-test).

TPO iodide oxidation activity ± amino acids	V_{max} ($\Delta A_{353\ nm}$/min)	$K_{0.5}$ (mM iodide)
TPO	0.120 ± 0.007	7.70 ± 0.55
TPO + 12.5 µM cysteine	0.140 ± 0.025	18.81 ± 1.12**
TPO + 9.7 µM tryptophan	0.144 ± 0.016	18.92 ± 2.82**
TPO + 13.0 µM methionine	0.105 ± 0.005	12.37 ± 0.77*
TPO + 12.5 µM cystine[a]	0.120	9.12
TPO + 12.5 µM phenylalanine[a]	0.121	8.80
TPO + 12.5 µM tyrosine[a]	0.085	7.71

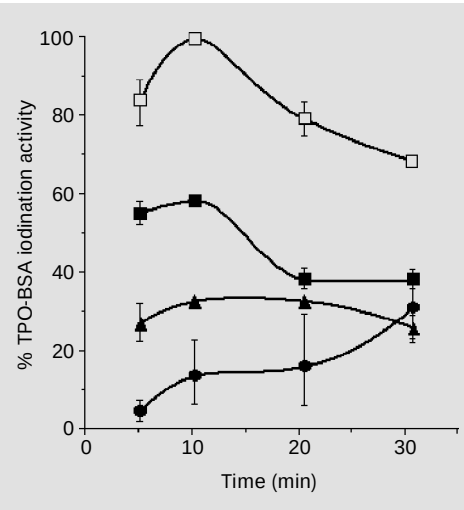

Figure 4 - Effect of some amino acids (50 µM) on diffuse toxic goiter-thyroid peroxidase (DTG-TPO) iodination activity. The iodination of bovine serum albumin (BSA) by DTG-TPO was measured in the absence or in the presence of tyrosine (open squares), tryptophan (filled squares), methionine (triangles) or cysteine (circles).

tryptophan). However, the extent of TPO inhibition is quite different among the inhibitory amino acids.

The present findings show that amino acids other than cysteine can inhibit the TPO iodide oxidation and iodination activities. However, cysteine was the most potent inhibitor of the iodination reaction and was the only amino acid that inhibited both iodide and guaiacol oxidation activities, although the guaiacol oxidation inhibition was self-limited, and the lag period was reduced when the cysteine concentration was halved. A transient inhibition of the TPO iodination reaction has also been described for thioureylene drugs, depending on their concentration (1). Furthermore, the degree of iodide oxidation inhibition produced by cysteine, methionine and tryptophan seems to be dependent on iodide concentration, as also shown by Taurog (1) for the inhibitory effects of thioureylene drugs. So, it would seem that the mechanism of TPO inhibition by these amino acids resembles that of thioureylene drugs as proposed by Taurog (1). As suggested by this author, since the oxidized form of iodide might act to oxidize the thioureylene drugs, as well as to iodinate tyrosine residues, the thioureylenes may compete with the tyrosyl residues for the oxidized iodide and so inhibit the iodination reaction. Some authors have also demonstrated that several mechanisms can be responsible for the inhibition of TPO- and LPO-catalyzed reactions, such as suicide inactivation of the enzyme, rapid equilibrium binding, and alternate substrate competition for the iodinating intermediate (15).

Thus, it is tempting to speculate that, at least under the conditions of the guaiacol oxidation assay, cysteine might be oxidized, just as proposed for the thioureylenes, into a non-inhibitory form such as cystine. Guaiacol oxidation was not inhibited by tryptophan and methionine, indicating that these amino acids do not interact with the oxidized form of guaiacol. However, tryptophan and methionine, like cysteine, seem to be competitive inhibitors of the iodide oxidation reaction, and therefore may react with the oxidized form of iodide, as we propose for cysteine, and/or compete reversibly with the iodide ion for its site on TPO.

In conclusion, our findings indicate that the inhibition of the *in vitro* peroxidase activity of some amino acids may be produced by their interaction with the oxidized form of iodide and/or with the iodide site on the TPO molecule. Further studies are needed to define a possible physiological role for amino acids in thyroid gland regulation.

Acknowledgments

We would like to thank Dr. Alvin Taurog for providing a generous sample of the “old Aldrich” guaiacol, and Dr. Luiz Francisco Macedo for critical advice.

References

1. Taurog A (1996). Hormone synthesis. In: Braverman LE & Utiger RD (Editors), *Werner and Ingbar's The Thyroid. A Fundamental and Clinical Text*. 7th edn. Lippincott-Raven, Philadelphia, PA, 47-84.
2. DeGroot LJ, Larsen PR & Hennemann G (Editors) (1996). Thyroid hormone synthesis and secretion. In: *The Thyroid and its Diseases*. 6th edn. Churchill Livingstone, New York, NY, 33-60.
3. Dème D, Pommier J & Nunez J (1976). Kinetics of thyroglobulin iodination and hormone synthesis catalysed by thyroid peroxidase. Role of iodine in the coupling reaction. *European Journal of Biochemistry*, 70: 435-440.
4. Moura EG, Rosenthal D & Carvalho-Guimarães DP (1989). Thyroid peroxidase activity in human nodular goiters. *Brazilian Journal of Medical and Biological Research*, 22: 31-39.
5. Hosoya T, Sato I, Hiyama Y, Yoshimura H, Niimi H & Taturani O (1985). An improved assay method for thyroid peroxidase applicable for a few milligrams of abnormal human thyroid tissues. *Journal of Biochemistry*, 98: 637-647.
6. Pommier J, Dominici R, Bougneres P, Rahmoun B & Nunez J (1977). A dialysable inhibitor bound to thyroglobulins from four simple goiters and from two goiters with iodine organification defect. *Journal of Molecular Medicine*, 2: 169-177.
7. Medeiros-Neto GA, Okamura K, Cavaliere H, Taurog A, Knobel M, Bisi H, Kallas WG & Mattar E (1982). Familial thyroid peroxi-

dase defect. *Clinical Endocrinology*, 17: 1-14.
8. Rosenthal D, Carvalho-Guimarães DP, Knobel M & Medeiros-Neto GA (1990). Dyshormonogenetic goiter: presence of an inhibitor of normal human thyroid peroxidase. *Journal of Endocrinological Investigation*, 13: 901-904.
9. Carvalho DP, Rego KGM & Rosenthal D (1994). Thyroid peroxidase in dyshormonogenetic goiters with organification and thyroglobulin defects. *Thyroid*, 4: 421-426.
10. Warburg O & Christian W (1941). Isolierung und Kristallisation des Gärungsferments Enolase. *Biochemische Zeitschrift*, 310: 384-421.
11. Carvalho-Guimarães DP, Ramos CF & Rosenthal D (1989). A technical improvement for the thyroid peroxidase iodination assay. *Brazilian Journal of Medical and Biological Research*, 22: 821-823.
12. Carvalho DP, Dupuy C, Gorin Y, Legue O, Pommier J, Haye B & Virion A (1996). The Ca^{2+}- and reduced nicotinamide adenine dinucleotide phosphate-dependent hydrogen peroxide generating system is induced by thyrotropin in porcine thyroid cells. *Endocrinology*, 137: 1007-1012.
13. Schussler GC, Ingbar SH & Eveleth P (1961). The role of intermediary carbohydrate metabolism in regulating organic iodinations in the thyroid gland. *Journal of Clinical Investigation*, 40: 1394-1412.
14. Klebanoff SJ, Yip C & Kessler D (1962). The iodination of tyrosine by beef thyroid preparations. *Biochimica et Biophysica Acta*, 58: 563-574.
15. Doerge DR & Decker CJ (1994). Inhibition of peroxidase-catalyzed reactions by arylamines: mechanism for the anti-thyroid action of sulfamethazine. *Chemical Research in Toxicology*, 7: 164-169.
16. Doerge DR & Divi RL (1995). Porphyrin π-cation and protein radicals in peroxidase catalysis and inhibition by anti-thyroid chemicals. *Xenobiotica*, 25: 761-767.
17. Divi RL & Doerge DR (1996). Inhibition of thyroid peroxidase by dietary flavonoids. *Chemical Research in Toxicology*, 9: 16-23.
18. Smith EL, Hill RL, Lehman IR, Lefkowitz RJ, Handler P & White A (Editors) (1983). Connective tissue. In: *Principles of Biochemistry*. 7th edn. McGraw-Hill Book Co., New York, NY, 211-242.