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Some Limitations in the Use of the I^- Method for Measuring the Peroxidase Activity from Bovine Thyroid Gland *

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Studies for measuring the peroxidase activity from thyroid gland have usually been achieved on the basis of the H_2O_2 oxidation of I^- to I_3^- catalized by peroxidase. The activity assay has been found to depend on several factors such as the relative order of reagent addition, protein content of the enzyme preparation, presence of detergent and the pH of the reaction medium. At below 7.0 pH, the contribution of the non-enzymic transformation of I^- to total activity became quite significant, to the extent that at below 6.5 pH, the chemical reaction predominates over the enzymic one. At values above 7.0 pH, a very rapid decomposition of the product was observed.

Guaiacol oxidation has been considered to be a more reliable method than the iodide one, especially when the substrate concentration and temperature vary, and when the activity of relatively rich in protein samples, as well as of some other substances that might interfere with the I_3^- formation, are going to be measured.

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Abbreviations used: TPO, thyroid peroxidase. BSA, bovine serum albumin. T_3, triiodothyronine. T_4, thyroxine.

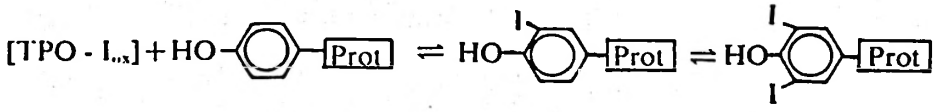
The peroxidases a group of heme-containing enzymes, catalyze the oxidation by H_2O_2 of a wide variety of organic substrates, mainly phenols and aromatic amines, to yield as end products, polymerized compounds whose formation can be recorded colorimetrically. One of the most used substrates has been guaiacol (5). However, another method of measuring enzymatic activity for thyroid peroxidase (TPO) has been the monitoring of the oxidation I^- to I_3^- by the increase

of absorbance at 353 nm (1). Thyroid gland peroxidase plays a very important role in the biosynthesis of thyroid hormones, catalyzing three different reactions:

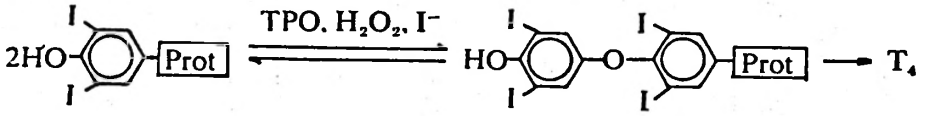
1) Iodide peroxidation to yield oxidized iodine bound to the enzyme (6).

$$I^- + H_2O_2 + TPO \rightleftharpoons [TPO - I_{ox}] + H_2O$$

2) Thyroglobulin iodination with the formation of mono- and di-iodinated derivatives of tyrosine residues.



3) Condensation of the iodinated residues to produce thyroid hormones T_3 and T_4 (9).



Althoug I^- oxidation method has been widely used, several authors have raised objections (2, 4, 10, 12, 14-16) such as: 1) A fast loss of linearity in the time course of I_3^- formation. 2) A loss of linearity between the rate of formation of product and the concentration of enzyme. 3) A dependence of I_3^- absorbance on the I^- concentration.

Better linearity has been achieved when bovine serum albumin (BSA) was included in the reaction medium, but in some cases the existence of a lag period has also been reported (2, 4, 10, 16). In view of these dificulties, a re-investigation of the iodide method has been done in order to achieve a better understanding of the anomalies previously commented upon.

Materials and Methods

Chemicals. BSA and Triton X-100 were purchased from Sigma. General chemicals were obtained from Merck. All were of analytical reagent grade and were used without further purification.

Reagents. Substrate solutions, H_2O_2, I^- and guaiacol were prepared daily. The H_2O_2 solution was protected from sunlight to avoid decomposition.

Solubilization of Thyroid Peroxidase. Thyroid glands from freshly killed beef were obtained from a slaughter-house (Mercomurcia), cleaned of connective tissue, blood and fat, and immediately wrapped in plastic bags and stored at —30° C. The frozen tissue was stored in plastic bags for up to a year without any loss in enzyme activity. The glands were cut into small pieces and were homogenized in 100 mM carbonate buffer pH 10.5 containing 0.1 mM KI (7). The ratio of tissue/buffer was 1:2 (w/v). The homogenate was spun at $700 \times g$ for 10 min. The pellet was discarded and the supernatant centrifuged at $10{,}000 \times g$, for 20 min. The mitochondrial pellet was discarded and the second supernatant was spun at $105{,}000 \times g$ for 1 h in a Beckman L-3-50 ultracentrifuge. The resulting pellet was considered to contain the microsomal fraction in accordance with NEARY *et al.* (11). This pellet was resuspended in 50 mM carbonate buffer pH 10.5, containing 0.1 mM KI, in a Potter-Elvehjem homogenizer. After determination of the protein content the level was adjusted to 2 mg $\times$ ml^{-1} with buffer. Triton X-100 (10 % w/v) was added to this fraction to reach a final detergent concentration of 0.6 %. This Triton containing frac-

tions was incubated for 5 h at 4° C before re-centrifugation at 105,000 × *g* for 1 h. The resulting supernatant was considered to be «solubilized enzyme». This preparation was shown to be fairly stable for several weeks at 4° C.

Protein estimation. The method of WANG and SMITH (17) was used to prevent interference due to the Triton X-100.

Methods of assay of enzyme activity. The assays were performed in a Varian-Techtron UV-Vis 635 spectrophotometer using glass cuvettes of 1 cm pathlength, thermostated at 25° C, with iodide or guaiacol as substrates.

Iodide oxidation method. The total reaction volume was 2.5 ml and contained 0.6 ml of 40 mM KI, 0.1 M phosphate buffer, 10 mM H_2O_2 solution and 50 µl of enzyme preparation. One unit of activity was defined as the increase of one unit of absorbance at 353 nm per minute, and corresponded to 2.18×10^{-7} mol of I^- oxidized per minute ($\epsilon^{353}{}_{I_3^-} = 22.9 \times 10^3\,M^{-1}\,cm^{-1}$).

Guaiacol oxidation. The assay with guaiacol was performed by monitoring product formation at 470 nm. The reaction mixture contained 1 ml of 50 mM guaiacol dissolved in 50 mM Tris-HCl buffer pH 8.2; 1.4 ml of buffer; 50 µl of 10 mM H_2O_2 solution and 50 µl of enzyme preparation. Control assays were performed under the same conditions except that the enzyme was replaced by distilled water. A unit of activity was defined as $\Delta A_{470} = 1.0$ per minute and correspond to $4{,}5 \times 10^{-7}$ mol of guaiacol oxidized per minute ($\epsilon^{470}{}_{tetraguaiacol} = 5.57 \times 10^3\,M^{-1}\,cm^{-1}$).

Results and Discussion

Relative order of addition of reagents. The results obtained in the peroxidase activity assays by the guaiacol method were shown to be completely independent of the relative order of addition of reagents. However, the iodide method was somewhat anomalous in this respect. When the KI and H_2O_2 were mixed, a small amount of I_3^- was produced non enzymatically as evidenced by UV absorbance measurements. Therefore, the enzyme was added immediately after the two substrates were incubated for one minute prior to the enzyme addition. Incubation times longer than 1 min had no effect on the absorbance values.

When a small amount of I_3^- was added (5 µM final concentration in measurement cell) to the reaction medium to get 0.01 units of initial absorbance, there was no need to incubate prior to the addition of enzyme to obtain the maximum activity. On the other hand if the KI and the enzyme extract were mixed and the H_2O_2 was added after incubating, the observed activity decreased when the incubation period increased. Table I shows that the increase of the lag period was dependent on the incubation time.

Influence of proteins and detergent on the measured activity. Some workers (16) in routine assays use BSA in the reaction mixture to improve linearity of product formation *versus* time. In table II are contained the results of measurements

Table I. *Relationship between lag period and incubation time for peroxidase activity by iodide method.*

Samples containing 1.8 ml 0.1 M phosphate buffer pH 7.3; 0.6 ml of 40 mM KI and 50 µl of enzyme preparation were incubated at 25° C. The reaction was initiated with 50 µl of 10 mM H_2O_2.

Incubation time (min)	Activity (ΔA/min)	Recording time interval (s)	Lag period (s)
0.75	0.216	50-110	28
4.0	0.150	80-140	48
8.0	0.116	110-170	95

Table II. *Effect of added protein on the peroxidase activity by iodide method.*
The assays were performed by mixing in the cuvette (1.8 — x) ml of 0.1 M phosphate buffer pH 7.3; 0.6 ml of 40 mM KI; x ml of BSA (2 mg/ml); 50 μl of 10 mM H_2O_2. After a preincubation period of 1 min, the reaction was initiated by the addition of 50 μl of enzymic extract.

Volume of added BSA (μl)	Activity (ΔA/min)	Lag period (s)
0	0.110	—
20	0.112	—
50	0.116	10
100	0.132	28
150	0.148	44
200	0.152	52
400	0.144	150

performed in the presence of different amounts of BSA. It was noted that the lag period increased as the protein concentration does. However after the lag period, the rate of formation of I_3^- slowly increased with the protein concentration until a final value of 0.4 mg of BSA.

A similar effect has been observed by TAUROG *et al.* (16) but so far a clear interpretation of this phenomenon has not been proposed. The existence of such lag period has been shown in several papers (10) and although several authors have suggested that this was due to the presence of either membrane-bound enzyme or subcellular organelles in the extracts (2), an explanation has not been offered yet. This lag period could be result of some kind of competition between I_3^- formation and iodination of the protein tyrosine residues present in the incubation medium. That explanation assumes that iodination of the tyrosine residues would preferentially occur before I_3^- formation, in accordance with the K_m values for I^- and peptides containing tyrosine (8, 13).

In agreement with that hypothesis, it has been observed that the lag period does appear, even without BSA in the incubation mixture, using enzymatic preparations richer in protein than the standard ones. Furthermore, substances able to undergo iodination such as phenol, tyrosine and 2,4-dichlorophenol, in the absence of BSA, also induced the existence of such a lag period. These results are in clear contrast with those obtained by POMMIER *et al.* (13). The reason for these different findings are unknown but it is interesting to note that POMMIER'S results were obtained from proteolytically solubilized enzyme and in this case the enzyme was solubilized with detergent.

In the reaction mixture without BSA, the presence of Triton X-100 up to a final concentration of 0.1 % had no effect on the activity assay. However in the presence of BSA, the detergent decreased the extent of the lag period and it could even be abolished without any effect on the reaction rate. It is possible that detergent can prevent the iodination of the tyrosine residues of the BSA.

The guaiacol method was unaffected by the presence of Triton X-100 or BSA in the incubation medium.

Influence of pH. The chemical reaction for the I^- oxidation by H_2O_2 is pH dependent (fig. 1). The plot of peroxidase activity against pH showed an optimum value at pH 7.3 and this is in fair agreement with several other workers (1, 4, 9), who found a pH of maximum activity between 7 and 7.4.

At pH lower than 7.0 measurement of activity is difficult due to the fast rate of the blank reaction. At pH 6.5 the extent of the non-enzymic reaction was even larger thant that of the enzymic one, and therefore the I^- method is not suitable for measuring the activity in an acid pH range.

At pH higher than 7.4, a sudden decrease of the activity was observed. At pH 8.5 there was practically no increase in the absorbance, whilst under identical

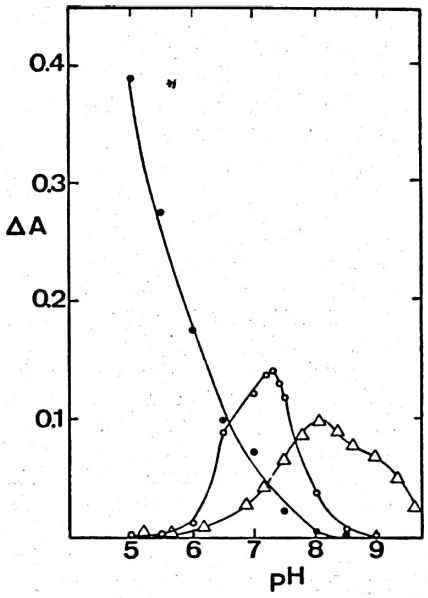


Fig. 1. *Effect of the pH on peroxidase activity and I_3^- formation.*
(●) Chemical oxidation of I^- by H_2O_2. (○) Enzymic oxidation of I^- by H_2O_2 catalyzed by TPO. I_3^- was measured by absorbance increase at 353 nm as described in Materials and Methods. (△) Guaiacol oxidation. Tetraguaiacol was measured by absorbance increase at 470 nm. The buffers used were: 0.1 M citrate-phosphate buffer (pH 5-7); 0.1 M phosphate buffer (pH 8-9) and 0.1 M Tris-chloride buffer (pH 8-9).

conditions the guaiacol method leads to a very high value of activity.

The existence of a decomposition of the I_3^- formed at basic pH was confirmed by the following experiment. Aliquots of known I_3^- concentration in 0.1 M phosphate buffer pH 7.3 were incubated in the presence of KI and H_2O_2 in a similar way as an enzymatic assay. A rather fast decrease in the absorbance was measured as a function of the initial I_3^- concentration (table III, assay condition A). The absorbance decrease was more drastic at high value of pH and was linear respect to the incubation time. It was also higher when the H_2O_2 concentration was increased (table III, assay condition B). A parallel release of O_2 could be observed with the disappearance of I_3^-.

The results can be explained from the well known existence of dismutation reaction at basic pH:

$$I_3^- + H_2O \rightleftharpoons 2I^- + IO^- + 2H^+ \quad (I)$$

The anion IO^- is responsible for the non-enzymic iodination of the tyrosine residues at basic pH (1, 4, 9). Once the dismutation has taken place, the IO^- reacts with H_2O_2:

$$IO^- + H_2O_2 \rightleftharpoons I^- + O_2 + H_2O \quad (II)$$

$$E_o = +0.64 \text{ v.}$$

Accordingly reactions (I) and (II) it is possible to explain some anomalies observed by different workers. TAUROG (15) showed that I_3^- formation was dependent on the I^- concentration when it was

Table III. *Decomposition of I_3^- in the I^- oxidation at pH 7.3 under maximum peroxidase activity conditions, by iodide method.*
Assay condition, A: The reaction mixture contained (2.5 — x) ml of 0.1 M phosphate buffer pH 7.3; 50 μl of 5 mM H_2O_2 and x ml of 400 μM I_3^-. Assay condition, B: The reaction mixture contained (2.5 — y) ml of 0.1 M phosphate buffer pH 7.3; 180 μl of 400 μM I_3^- and y ml of 5 mM H_2O_2. The I_3^- was prepared by dissolving I_2 in an excess of KI. Decomposition rate is measured by absorbance decrease at 353 nm.

Assay condition	Volume of I_3^- (μl)	Volume H_2O_2 (μl)	Decomposition rate (ΔA/min)
A	80	50	0.016
	100	50	0.019
	140	50	0.028
	180	50	0.040
B	180	50	0.039
	180	100	0.067
	180	150	0.110
	180	200	0.150

smaller than 5 mM. Because the dissociation constant for the reaction,

$$I_3^- \rightleftharpoons I_2 + I^-$$

is quite low (K_d = 1.4 mM at 25° C), this phenomenon cannot be explained by dissociation of I_3^-. But taking into account reaction (I), it can be deduced that if the I^- concentration is low the equilibrium could shift toward the decomposition of I_3^-, and therefore the measured absorbance is lower than should be.

Alternatively the known catalytic effect of I^- on the H_2O_2 decomposition (3) which diminishes the activation energy from 18 to 13.5 Kcal/mol, could correspond to the sequence of reactions:

$$3I^- + H_2O_2 \rightleftharpoons I_3^- + 2OH^-$$

$$I_3^- + 2OH^- \rightleftharpoons 2I^- + IO^- + H_2O$$

$$IO^- + H_2O_2 \rightleftharpoons I^- + O_2 + H_2O$$

and adding,

$$2H_2O_2 \rightleftharpoons 2H_2O + O_2$$

By the above argument, the iodide method, although widely used in the literature for measuring thyroid peroxidase activity, presents several disadvantages with respect to the guaiacol one. It can be utilized in a well buffered medium in a very narrow interval of pH, keeping the substrate concentrations fixed and preferably using purified enzyme. However, for kinetic and thermodynamic studies, where conditions of pH, temperature and reagents concentration have to be modified, it is not profitable to use the iodide method due to the above commented equilibrium shift and the presence of branch reactions.

On the other hand, the iodide method is non-specific for thyroid peroxidase, because other peroxidases, such as lactoperoxidase and horseradish peroxidase catalyze also iodide oxidation (8). Finally the I_3^- accumulation has no place in physiological conditions, because *in vivo* the species containing oxidized iodine bound to the enzyme are used for direct iodination of tyrosine residues.

Resumen

Los estudios para medir la actividad peroxidasa de tiroides son normalmente realizados en base a la oxidación del I^- a I_3^- por el H_2O_2, y dependen de diferentes factores, tales como orden de adición de reactivos, contenido de proteína de la preparación enzimática, presencia de detergente y sobre todo del pH del medio. Así, a pH inferior a 7,0, se hace significativa la contribución de la transformación no-enzimática del I^- a la actividad total, de manera que si el pH es menor que 6,5, la reacción química predomina sobre la enzimática. A pH superior a 7,0 se observa una descomposición rápida del producto.

Debido a estos problemas, se considera que el ensayo de actividad peroxidasa basado en la oxidación del guayacol es más adecuado que el del ioduro, en particular cuando se varía la concentración de substrato y la temperatura, y cuando se mide la actividad de muestras que contienen una cantidad de proteína relativamente alta, así como compuestos que interfieren con la formación del I_3^-.

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