The control of peroxidase-catalysed iodination and de-iodination

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Summary

It has been demonstrated that the H_2O_2/I^- ratio is a critical factor in the control of iodination and de-iodination of covalently bound tyrosyl residues in proteins and free iodotyrosines by peroxidase enzymes. This has been shown for myeloperoxidase (MPO) isolated from normal human polymorphonuclear lymphocytes in particular, and also for peroxidases of animal origin such as thyroid peroxidase (TPO) and lactoperoxidase (LPO). It has also been shown that the H_2O_2/I ratio exerts a controlling influence on MPO-catalysed reactions of fully iodinated tyrosines, e.g. di-iodotyrosine, and of partially and completely iodinated thyronines such as thyroxine and tri-iodothyronine.

Using an *in vivo* model system it has been shown that MPO catalyses the sequential events of iodination, iodine exchange and de-iodination of tyrosines and, furthermore, that all three reactions are influenced by the rate of H_2O_2 generation and the iodide concentration of the reaction medium. The action of MPO on iodothyronine substrates only affects de-iodination irrespective of whether the iodothyronine is partially iodinated, as in tri-iodothyronine, or completely iodinated, as in thyroxine. This MPO-catalysed de-iodination of thyroxine and tri-iodothyronine can also be regulated by the H_2O_2/I^- ratio. Moreover, the results show that MPO-catalysed iodine exchange can only occur in completely iodinated tyrosines such as di-iodotyrosine (DIT). Iodine exchange in partially iodinated tyrosines such as mono-iodotyrosine (MIT) or in iodothyronines (T_3 and T_4) cannot be catalysed by MPO irrespective of the H_2O_2/I^- ratio.

These results introduce a new concept which may be important in understanding the control of thyroid activity in thyroid disease and the control of MPO activity in biological defence mechanisms in man.

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Peroxidase enzymes are remarkably versatile in being able to perform divergent multifunctional activities.[1-3] We are interested mainly in these functions and in the control of thyroid peroxidase (TPO), which is the main enzyme concerned with thyroid hormone formation, and myeloperoxidase (MPO),[4] obtained from polymorphonuclear lymphocytes, which plays a key role in the biological defence mechanism of man by virtue of its antimicrobial activity against micro-organisms.[4] For the activities of these enzymes in the thyroid as well as in the white blood cell, both iodination as well as de-iodination reactions need to be catalysed by their respective peroxidases, which are both H_2O_2-dependent.

Despite molecular dissimilarities and differences in haem content,[5,6] the peroxidases of animal origin (TPO, MPO and lactoperoxidase (LPO)), as well as plant peroxidases such as chloroperoxidase (CPO) and horseradish peroxidase (HRPO), are all capable of performing similar functions *in vitro*. These include the oxidation of iodide with subsequent iodination of tyrosyl residues,[7,8] the coupling of iodotyrosyl (DIT) residues to form thyroxine (T_4) and tri-iodothyronine (T_3),[9,10] the de-iodination of iodotyrosines and iodothyronines[11,12] and the exchange of inorganic iodine for organic iodine in DIT.[11,13,14] Peroxidases have also been shown to bring about post-translational effects on proteins such as oxidation of protein sulphydryls[15] and the cleavage of tryptophanyl peptide bonds.[16] In fact, with electron microscopic studies it has been observed that peroxidases can cause post-translational changes in the shape of the thyroglobulin molecule[17] and even cross-linking of the fertilization membrane of the ovum immediately after fertilization to prevent polyspermy.[18] It has recently been observed in our laboratory that TPO, CPO and LPO can bring about changes in the subunit composition of thyroglobulin (TG) and that they also have proteolytic effects as observed by SDS polyacrylamide gel electrophoresis of peroxidase-treated TG (unpublished data).

With the possible exception of peroxidase-catalysed structural changes in proteins, proteolytic effects and changes in subunit composition of TG, all the other functional activities, including di-tyrosine formation[19] in proteins, are H_2O_2-dependent. Thus drugs such as antithyroid substances and some non-steroidal anti-inflammatory agents can affect these functions either by having a direct effect on the peroxidase or influencing H_2O_2 generation.[3]

Since both iodination and de-iodination reactions are catalysed by H_2O_2-dependent peroxidases and since these reactions occur in the thyroid and in the neurophil for their respective functions of thyroid hormone formation and antibacterial action, a control mechanism is required in these systems whereby either iodination or de-iodination is triggered, depending on the need for either the one or the other process. If, on the other hand, both iodination and de-iodination reactions are catalysed simultaneously by peroxidase enzymes in the thyroid and the neutrophil, a control mechanism is required to determine whether the net effect will favour an iodination or a de-iodination process.

In the literature several clues exist to indicate that the inorganic iodine concentration is important for such a regulatory process. Thus, Wolff and Chaikoff[20] observed that the administration of excess iodide to normal rats inhibits thyroidal organic iodine formation although no satisfactory explanation exists for this effect. Nevertheless, this inhibitory effect has also been demonstrated by Taurog *et al.*[21] on TPO-catalysed iodination of bovine serum albumin (BSA) and TG *in vitro* using a system where H_2O_2 was added to the reaction medium and confirmed with stable iodine analyses.[3] Similarly, an iodide dependence of the TPO-catalysed iodine exchange reaction

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between ^{131}I-DIT and ^{125}I-I^- in a H_2O_2-generating system has also been demonstrated, as well as a less iodide-dependent peroxidase-catalysed de-iodination of T_4.[3]

The purpose of this study was to determine whether the degree of H_2O_2 generation and the inorganic iodine concentration of the medium have a controlling influence in switching on and directing the course of iodination and de-iodination reactions of peroxidases.

The concept is proposed that in the control of peroxidase-catalysed reactions for both thyroid hormone formation as well as for the biological defence mechanism of neutrophils in man and animals the H_2O_2/I^- ratio forms a crucial modulator of iodination and de-iodination reactions.

Materials and methods

Radioactive substances

Carrier-free ^{125}I and ^{131}I were obtained from the Radiochemical Centre, Amersham, England, and contained no reducing agents. ^{125}I-T_4 and ^{125}I-T_3 were also from Amersham and were supplied in 50% aqueous propylene glycol (sp. act. 40-60 μCi/μg). When the ^{125}I-labelled iodothyronines showed the presence of more than 15% free ^{125}I resulting from radiochemical breakdown, the compounds were repurified by chromatography in butanol : dioxane : 2M ammonia (BDA, 4:1:5). The ^{125}I-T_4 and ^{125}I-T_3 were mixed with the corresponding stable iodothyronine dissolved in 1mM KOH to give a final iodine concentration of 10 μg/ml in the iodothyronine for the enzyme experiments.

^{131}I-MIT and ^{131}I-DIT were prepared by injecting adult Wistar rats (200 g) with 300 μCi ^{131}I per rat. After 18 hours the thyroid glands were removed, homogenized in 0,2M phosphate buffer, pH 8,3, containing 0,2M KCl and centrifuged at 25 000 *g* for 30 minutes. The supernatant was mixed with an equal volume of water and digested with pronase for 5 hours at 37°C under vacuum. The digest was chromatographed in butanol : acetic-acid : water (BAW, 12:3:5 v/v/v) and the ^{131}I-MIT and ^{131}I-DIT bands were cut out, extracted separately with methanol : 15M ammonia (3 : 1 v/v) and evaporated to dryness at 37°C in a Buchler Evapo-Mix. The residues were dissolved in 1 mM KOH containing sufficient stable MIT or DIT to give a final iodine concentration of 10 μg/ml MIT or 10 μg/ml DIT for the enzyme experiments. These MIT and DIT solutions were again chromatographed in BAW, extracted from the paper with methanol : ammonia and, if necessary, filtered through a Millipore filter to exclude paper articles. The extract was dried and dissolved in 1 mM KOH just before use. The ^{131}I-MIT and ^{131}I-DIT samples so prepared were at least 90% pure as judged by chromatography in BAW and had specific activities of approximately 40 000 cpm/μg. Specific activities were determined from radioactive counts and stable iodine analyses as previously described.[26]

Enzymes

LPO was obtained from Miles Laboratories and had a haem/protein absorption ratio (A_{410}/A_{280}) of 0,74. Hog TPO was a gift from Dr Alvin Taurog (Department of Pharmacology, Southwestern Medical School, Dallas, Texas, USA) and was prepared according to the method of Taurog *et al.*[22] Its A_{410}/A_{280} ratio was 0,38. The enzyme had been stored at a protein concentration of 1,95 mg/ml in the frozen state in 0,02M PO_4, pH 6,8, for approximately 10 years, during which time it retained practically all its activity. The MPO was pepared from fresh normal human leucocytes according to the method of Theron *et al.*[23] and had an A_{410}/A_{280} ratio of 0,48. Glucose oxidase (GO) was purchased from Hopkins & Williams and had a specific activity of 750 Sorett units/ml.

Incubation system

A reaction mixture was used which permitted the simultaneous measurement of delabelling and labelling of ^{131}I-labelled iodotyrosines or ^{125}I-labelled iodothyronines. The components were as follows: ^{131}I-iodothyronine (20-40 nmol, approx. 4 x 10^5 cpm) or ^{125}I-iodothyronine (10-15 nmol, approx. 4 x 10^5 cpm), MPO (15-30 μg), ^{131}I or ^{125}I (0-10 mM, approx. 1,5 x 10^6 cpm), glucose (0,5 mg), glucose oxidase (0,001-0,1 units) and 0,1M phosphate buffer, pH 7,2. The total volume was 0,5 ml and the reaction was started at 37°C with the addition of glucose oxidase and stopped after incubation by rapid addition of propylthio-uracil to give a final concentration of 1-2 mM. Methylmercapto-imidazole was not used with MPO since it had been shown that it stimulates certain MPO-catalysed reactions.[24,25] In the LPO experiments in which H_2O_2 was added directly to the reaction medium at 37°C the reaction was stopped after 1 minute's incubation by rapid addition of methyl-mercapto-imidazole to a final concentration of 1mM.

Aliquots (50 μl) of the reaction mixture were applied to paper for chromatography either in BAW (^{131}I-MIT and ^{131}I-DIT experiments) or BDA and BEA (^{125}I-T_4 or ^{125}I-T_3 experiments). Chromatography and the location and counting of radioactive compounds on the chromatograms were performed as previously described.[3] In the double isotope experiments the ^{131}I spill-over counts into the ^{125}I channel were subtracted from the ^{125}I counts. Iodide levels (in nmol/ml) in the ^{131}I-MIT, ^{131}I-DIT, ^{125}I-T_4 or ^{125}I-T_3 used in the reaction mixture were calculated by determining the stable iodine content. The number of nanomoles of these compounds delabelled or converted to other iodo compounds were determined by multiplying that fraction of the total radioactive counts found in each chromatographic band by the nanomoles of iodide/ml present in the original compound. Similarly, nanomoles of ^{127}I incorporated were obtained by multiplying nmol/ml iodide in the reaction by the fraction of ^{125}I- or ^{131}I-iodide incorporated.

Results

Influence of iodide and H_2O_2 on peroxidase-catalysed iodination of un-iodinated tyrosyl residues in proteins

Inhibitory effects by excess iodide on peroxidase-catalysed iodination of tyrosyl residues in different proteins by several peroxidases are well known[21,27] and have been confirmed by stable iodine analyses.[11] Fig. 1 illustrates the influence of increasing concentrations of H_2O_2 on protein iodination by using LPO as the peroxidase and BSA as the un-iodinated protein.

The results show that iodination, expressed as μmmol iodide incorporated/mg protein/min, proceeds linearly with increased H_2O_2 concentrations, followed by inhibition at high levels of H_2O_2. Since these results were obtained at an I^- concentration of 10^{-4}M in the reaction medium, it is clear that when the H_2O_2/I^- ratio, expressed as mM H_2O_2/mM I^- in the reaction medium, exceeded the value of 20, inhibition of iodination commenced under the present experimental conditions. Whether the H_2O_2/I^- ratio for maximum iodination differs among different peroxidases or whether it changes with the degree of purity of a peroxidase or whether it is influenced by the number of tyrosyl residues of the acceptor protein, remains to be established.

Since the H_2O_2 was added and not generated, its influence is considered to be directly exerted on the peroxidase. Thus, at relatively low levels the H_2O_2 acts as an electron acceptor during the peroxidase-catalysed oxidation of iodide[1] but whether inhibition of peroxidase-catalysed iodination of tyrosine residues in proteins in the presence of excess H_2O_2 is due to partial destruction of the enzyme is still unknown. Nevertheless, the results show that LPO-catalysed iodination of covalently bound tyrosyl residues in BSA is influenced by the level of H_2O_2

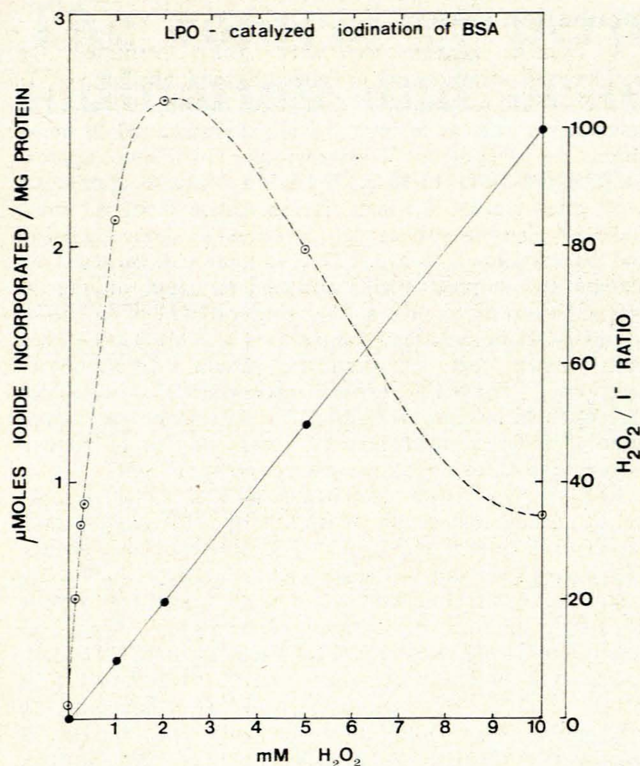

Fig. 1. Inhibition of LPO-catalysed iodination of BSA by excess H_2O_2. The incubation system contained BSA 500 μg/ml, $^{125}I^-$ 100 nmol/ml, LPO 220 pmol/ml in 0,1M PO_4 buffer, pH 7,2. After incubation for 1 min at 37°C the reaction was stopped by rapid addition of MMI to a final concentration of 5 mM. Short-term chromatography (1 h) in BAW was performed and the percentage radioactivity in the 'origin' determined. ^{127}I incorporated in protein (μmol/mg) was determined by multiplying μmol/ml iodide by the fraction of ^{125}I incorporated into BSA which was then calculated for 1 mg protein (○——○ iodination of BSA; ●——● H_2O_2/I^- ratio).

in the medium and thus may be influenced by the level of H_2O_2 generation. These values are indicative of a controlling influence of the H_2O_2/I^- ratio on peroxidase-catalysed iodination of proteins.

TABLE I. MPO-CATALYSED IODINATION AND DE-IODINATION REACTIONS WITH (A) ^{131}I-MIT, (B) ^{131}I-DIT, AND (C) ^{125}I-T_4 AS SUBSTRATES AT HIGH (10^{-3}M) AND LOW (10^{-7}M) STABLE IODIDE CONCENTRATIONS

(A)

IODINE CONCENTRATION	GO	ENZYMES	^{131}I DISTRIBUTION: MIT CPM	MIT %	DIT CPM	DIT %	I CPM	I %	TOTAL CPM	TOTAL %
10^{-3}M IODIDE	·08	NONE	32974	94	420	1	1729	5	35123	100
		- MPO + GO	29290	94	633	2	1173	4	31096	100
		- GO + MPO	27585	95	465	2	1075	3	29125	100
		+ GO + MPO	15681	55	8188	29	4447	16	28316	100
10^{-7}M IODIDE	·08	NONE	28109	95	405	1	1128	4	29642	100
		- MPO + GO	27649	95	338	1	1211	4	29198	100
		- GO + MPO	29688	95	344	1	1056	4	31087	100
		+ GO + MPO	23864	82	304	1	4808	17	28976	100

(B)

IODINE CONCENTRATION	GO	ENZYMES	^{131}I DISTRIBUTION: MIT CPM	MIT %	DIT CPM	DIT %	I CPM	I %	TOTAL CPM	TOTAL %
10^{-3}M I	·08	NONE	1272	3	39199	81	7901	16	48372	100
		- MPO + GO	1190	2	41146	86	5614	12	47950	100
		+ MPO - GO	695	1	40419	79	10189	20	51303	100
		+ GO + MPO	576	2	12110	50	11535	48	24221	100
10^{-7}M I	·08	NONE	1442	2	40745	72	14654	26	56841	100
		- MPO + GO	1873	4	39853	77	9767	19	51493	100
		+ MPO - GO	1593	3	39821	75	8379	22	53095	100
		+ GO + MPO	1667	5	19723	55	14365	40	35755	100

(C)

IODIDE CONCENTRATION	ENZYMES	^{125}I DISTRIBUTION: T_4 CPM	T_4 %	I CPM	I %	TOTAL CPM	TOTAL %
10^{-3}M IODIDE	NONE	12888	70	5600	30	18488	100
	- GO	11863	69	5221	31	17084	100
	- MPO	11053	67	5411	33	16464	100
	+ MPO + GO	3625	24	11391	76	15016	100
10^{-7}M IODIDE	NONE	13518	82	2860	18	16378	100
	- GO	15156	73	5617	27	20803	100
	- MPO	11653	71	4665	29	16319	100
	+ MPO + GO	11800	74	4063	26	15863	100

RESULTS REPRESENT MEAN OF TRIPLICATE ANALYSES

Influence of iodide concentration and H_2O_2 generation on peroxidase-catalysed reactions with partially iodinated (MIT) and completely iodinated (DIT) free iodotyrosines

In order to investigate the effects of iodide and H_2O_2 concentrations on peroxidase-catalysed reactions, the choice of partially iodinated tyrosine (MIT) and completely iodinated tyrosine (DIT) seemed to be important. They can serve as indicators for three peroxidase-catalysed reactions, namely iodination, exchange of inorganic iodine for organic iodine and de-iodination.

Since the study undertaken was mainly on MPO it was important to establish at the onset (*a*) whether the effects were indeed due to H_2O_2-dependent peroxidase activities, and (*b*) whether the H_2O_2 generated under our experimental conditions has the ability of oxidizing I^- non-enzymatically. This was considered important by virtue of the observation that in LPO exeriments non-enzymatic oxidation of I^- can occur at certain pH values.[13]

Results in Table I show that MPO alone without glucose-glucose oxidase (GO) (the H_2O_2-generating system) had no obvious influence on MIT, DIT, or T_4 at high (10^{-3}M) or low (10^{-7}M) iodide concentrations. They also show that the H_2O_2 generated in the absence of MPO did not oxidize iodide under the experimental conditions. When both enzymes (MPO and GO) were present, however, iodination of MIT was catalysed and reactions occurred with DIT and T_4 as substrates. These effects were all more pronounced at high iodide concentrations, but less so with a low iodide concentration in the reaction medium.

In the experiments summarized in Table I, samples of the incubated media were directly applied to chromatography paper at the end of the incubation time. A loss of total radioactivity counts in chromatograms obtained from the reaction medium of the complete systems (i.e. where both MPO and GO were present) was observed, particularly in the presence of high iodide. This shows that in experiments of this nature the reaction should be stopped by the addition of peroxidase inhibitors such as methylmercapto-imidazole (MMI), propylthio-uracil or sodium thiosulphate, which are all strong reducing agents. In view of the observation that MMI inhibits MPO-catalysed iodination reactions but has a stimulatory effect on the MPO-catalysed de-iodination of T_4,[24,25] it cannot be used to stop MPO-catalysed reactions.

Fig. 2 illustrates the effects of MPO- and TPO-catalysed iodination of ^{131}I-MIT during increased H_2O_2 generation. With the MPO-catalysed reactions it is observed that until there is a critical concentration of GO at which the reaction can be altered into one or other direction. Thus, up to about 0,02 units of GO/ml the ^{131}I-MIT disappears very rapidly with increasing GO concentrations, owing mainly to its iodination to ^{131}I-DIT.

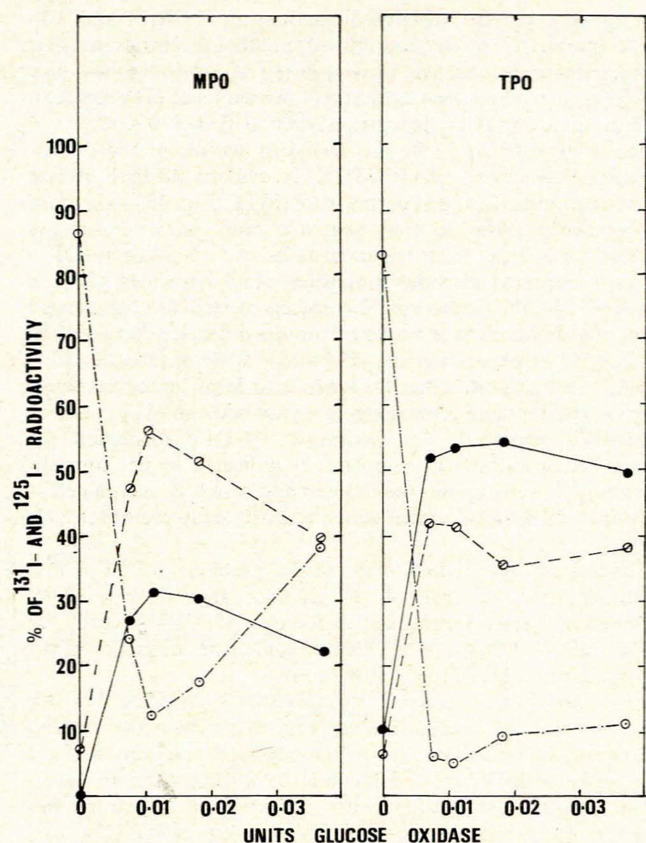

Fig. 2. Comparison of the influence of the rate of H_2O_2 generation on MPO- and TPO-catalysed effects on ^{131}I-MIT after incubation for 20 min at 37° C in the presence of 10^{-4}M I^-. Other procedures were as indicated in the legend of Fig. 3. At the end of the incubation, samples were directly applied to the chromatography paper and chromatographed in BAW. (O-.-O ^{131}I-MIT; Ø---Ø ^{131}I-DIT; ●——● ^{131}I-iodide).

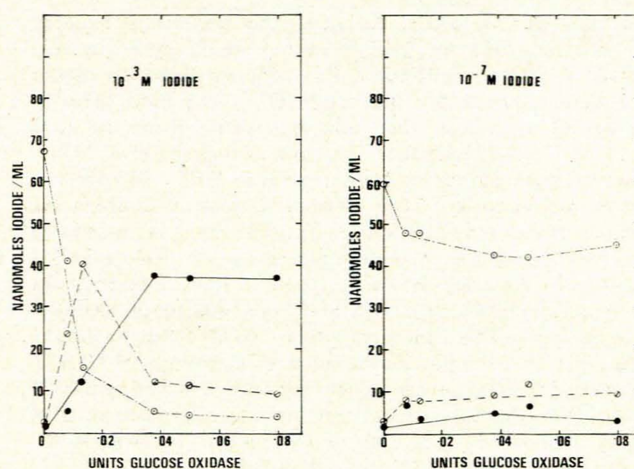

Fig. 3. Effect of H_2O_2 generation on MPO-catalysed reactions with ^{131}I-MIT after a 15-min incubation in the presence of either 10^{-3}M or 10^{-7} iodide. The reaction mixture (0,5 ml) contained ^{131}I-MIT (162 μM), MPO (25 μg), glucose (0,5 mg), glucose oxidase (0-0,8 U) and 0,1 M phosphate buffer, pH 7,2. After a 15-min incubation at 37° C, the reaction was stopped by the addition of 1mM PTU and 50 μl portions of the incubation mixture were chromatographed in BAW. The nmol of ^{131}I-MIT delabelled or converted to DIT were calculated as described in 'Materials and Methods' (O-.O ^{131}I-MIT; Ø---Ø ^{131}I-DIT; ●——● ^{131}I-iodide).

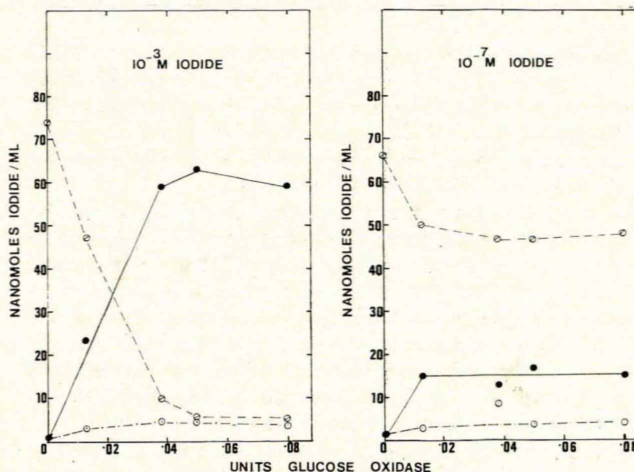

Fig 4. Effect of H_2O_2 generation on MPO-catalysed reactions with ^{131}I-DIT after a 15-min incubation in the presence of either 10^{-3}M or 10^{-7}M iodide. The procedure and method of calculation was as indicated in Fig. 3, except that the reaction mixture contained 120 μM ^{131}I-DIT (O---O ^{131}I-DIT; Ø-.-Ø ^{131}I-MIT; ●——● ^{131}I-iodide).

The increase in ^{131}I-iodide can be due to ^{131}I-MIT de-iodination or to the exchange of stable iodide in the reaction medium for organic iodine in DIT, which is coincident with DIT formation.

Judging from the ^{131}I distribution curves, MPO-catalysed reactions were more sensitive to changes in GO concentration than TPO. This may indicate that the leucocyte should be highly sensitive to peroxidase inhibitors and substances inhibiting H_2O_2 generation such as antithyroid and anti-inflammatory drugs.[3,23]

Since it is evident from the results in Fig. 2 that the degree of H_2O_2 generation influenced MPO-catalysed reactions on MIT in a medium containing 10^{-4}M iodide, the influence of inorganic iodide was subsequently investigated on MPO with ^{131}I-MIT and ^{131}I-DIT as substrates. This was done by looking at the effects in a high (10^{-3}M) and low (10^{-7}M) iodide concentration.

At the same time the GO concentrations were increased stepwise to 0,08 units/500 μl reaction medium while the incubation period was cut from 20 to 15 minutes. A comparison of the MPO-catalysed reactions on ^{131}I-MIT at 10^{-4}M iodide (Fig. 2) with those at 10^{-3}M iodide (Fig. 3) shows that some inhibition had been affected by the 10-fold increase in iodide concentration at equivalent GO concentrations, since the changes in ^{131}I-MIT in Fig. 3 were not so sharp as in Fig. 2 although the trends of the curves are comparable. This difference is most likely due to the Wolff-Chaikoff effect which starts to exhibit itself at 10^{-3}M concentrations of iodide.[31] Nevertheless, a comparison between the effects of two extreme iodide concentrations of 10^{-3}M *v.* 10^{-7}M is clearly illustrated in Fig. 3 (on ^{131}I-MIT) and Fig. 4 (on ^{131}I-DIT) at the same levels of H_2O_2 generation.

At the high iodide concentration in the medium the process of iodination of MIT to DIT is the first phenomenon observed at low GO concentrations. This is followed by a disappearance of ^{131}I-DIT at higher GO concentration, which could be partially explained by exchange of ^{125}I-iodide with ^{131}I in DIT and partially by de-iodination, as is indicated by the free ^{131}I-iodide released.

At the low iodide concentration in the medium, an increased GO concentration shows an initial disappearance of MIT with very little DIT formation and some small degree of de-iodination. These effects were so small, however, that the H_2O_2 dependence of the reactions was by and large lost in the low-iodide medium.

From the results shown in Fig. 3 it is therefore concluded that with ^{131}I-MIT as substrate all the MPO-catalysed reactions are grossly inhibited at all stages of H_2O_2 generation when the iodide concentration is 10^{-7}M.

With ^{131}I-DIT as substrate (Fig. 4) the drop in ^{131}I-DIT proceeded less rapidly with increased GO, as was the case with ^{131}I-MIT when both reactions took place in a medium

containing 10^{-3}M iodide. Although the maximal ^{131}I-iodide release from MIT or DIT occurred at an approximately equivalent GO concentration, this concentration was higher than that required for maximum DIT formation. This is interpreted to indicate that iodination seems to be the most sensitive H_2O_2-dependent reaction catalysed by MPO. However, since iodination cannot occur in DIT, ^{131}I release is due to the secondary H_2O_2-dependent reactions, which are exchange of iodine and de-iodination occurring at increased GO concentrations. If the ^{131}I-iodide appearance with increased GO was due to a de-iodination reaction, it is evident that de-iodination should occur at both the 3,5 positions simultaneously, since no appreciable concentration of ^{131}I-MIT was formed.

Again at 10^{-7}M iodide some degree of delabelling of ^{131}I-DIT occurred at low GO concentrations, which is reflected by the ^{131}I appearing in the iodide band and the insignificant amount of ^{131}I-MIT formed. The inhibitory nature of low iodide concentration in the H_2O_2-dependent MPO reactions on ^{131}I-DIT was again evident.

The experimental results in Figs 3 and 4 show that the environmental inorganic iodine concentration exerts a modulating influence upon the H_2O_2-dependent MPO-catalysed reactions on iodotyrosines and that the control is mediated by the level of H_2O_2 generation. For optimum efficiency a certain concentration of inorganic iodine is required. At low iodine concentrations the iodotyrosines are inclined to be reserved owing to a loss of H_2O_2 dependence on the peroxidase actions.

This effect of iodide concentration on H_2O_2-dependent MPO reactions with ^{131}I-DIT was studied in more detail at an incubation period of 15 minutes at 37°C, by varying the iodide concentration from 10^{-7}M to 10^{-2}M. The iodide effect was investigated at both a low GO level (0,01 U/500 μl) and a relatively high level (0,08 U/500 μl) (Fig. 5).

As is evident from the distribution of the iodine on the chromatograms, appreciable delabelling of ^{131}I-DIT started to occur with concentrations greater than 10^{-4}M iodide when the H_2O_2 generation was low. When more H_2O_2 was generated by increasing the GO concentration to 0,08 U a sudden spurt of ^{131}I-DIT delabelling occurred at an iodide concentration which was an order of magnitude lower than with 0,01 U GO. At this stage the mechanism of ^{131}I delabelling could only be ascribed either to iodine exchange or to de-iodination, or both.

Since it had been noticed that the incubation period is important to obtain comparable results and that sequential processes took place during incubation, kinetic studies were undertaken on MPO-catalysed reactions on ^{131}I-MIT and ^{131}I-DIT at high (10^{-3}M) and low (10^{-7}M) iodide concentrations (Fig. 6). At the same time the incorporation of ^{125}I-iodide was also monitored in an attempt to interpret the sequence of events at an effective rate of H_2O_2 generation (0,08 U GO/500 μl).

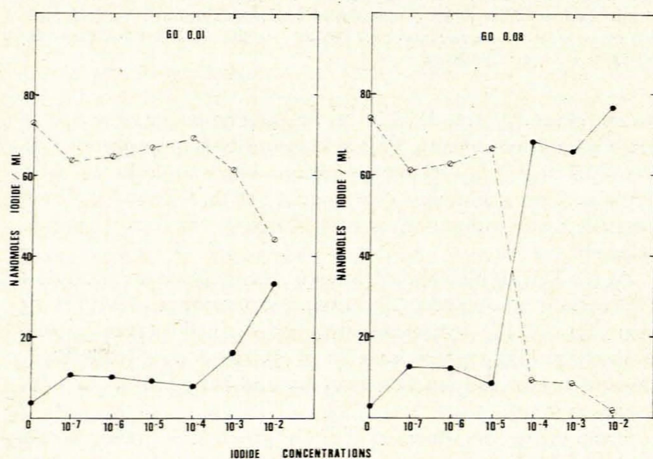

Fig. 5. Effect of iodide on MPO-catalysed reactions with ^{131}I-DIT after a 15-min incubation at low (0,01 U glucose oxidase) and high (0,08 U glucose oxidase) levels of H_2O_2 generation. The procedure and method of calculation was as indicated in Fig. 3, except that the reaction mixture contained 128 μM ^{131}I-DIT and the various concentrations of stable iodide as indicated (Ø---Ø ^{131}I-DIT; ●——● ^{131}I-iodide).

In Figs 6(A) and (B) the transient nature of the MPO-catalysed reactions on ^{131}I-DIT is evident at high iodide concentrations. In the experiment on MIT (Fig. 6B) iodination proceeded rapidly so that near-maximum incorporation of iodine (as judged from the nanomoles of ^{125}I-iodide in DIT) already occurred at about 5 minutes which coincides with the peak of ^{131}I-DIT formation. The process of exchange lags behind that of iodination, as is evident from the difference between the rate of ^{125}I incorporation into DIT and ^{131}I release into the iodide band. This indicates that DIT needs to form before exchange occurs and that iodine exchange does not occur in MIT, since no ^{125}I-MIT appeared. At maximum ^{125}I-DIT formation the exchange of iodine was complete, as indicated by the constant level of ^{131}I release into the iodide band which is maintained at about the same level as that which was originally present in ^{131}I-MIT.

This exchange of iodide for organic iodine in DIT is also indicated by the drop of ^{131}I-DIT at about 5-9 minutes' incubation. After 9 minutes the decrease of ^{125}I-DIT and ^{131}I-DIT indicated that a third process, namely de-iodination of the newly formed DIT, had commenced.

A comparison of the same experiment at high-iodide (Fig. 6B) and low iodide concentrations (Fig. 6C) shows the striking influence of iodide on the MPO-catalysed reactions and the conservative influence of a low iodide concentration on MIT.

A similar time study with ^{131}I-DIT as substrate was performed in high-iodide (Fig. 6A) and low-iodide (Fig. 6D) concentrations. With 10^{-3} M iodide in the medium, the rate of ^{131}I release compares favourably with the rate of ^{125}I incorporated into DIT up to about 5 minutes incubation, although the former was a little slower. The level of ^{131}I-iodide released was lower than the total ^{131}I put into the system, due to incorporation into tyrosyl residues of the enzyme protein as judged from an increase in radioactivity of origin material on the chromatograms. The de-iodination process of DIT, as judged from a decline of ^{125}I-DIT and an increase in ^{131}I-iodide, now appeared to start earlier and was even more pronounced than in the MIT experiment. Nevertheless, it is obvious that if an experiment is performed by analysing the samples only at the end of a 15-minute incubation, the de-iodination process would be the overriding effect and many of the iodide-dependent MPO-catalysed reactions would not be observed. Similarly, at the low iodide concentration (10^{-7}M) these iodide-dependent phenomena on DIT were not observed (Fig. 6D).

Since it was observed that the MPO-catalysed reactions on iodotyrosines were highly time-dependent and only took place in a relatively high-iodide environment, the effect of increasing H_2O_2 generation on the MPO-catalysed events were re-investigated at 5 minutes' incubation and with a 10^{-3}M iodide concentration. The results obtained are illustrated in Fig. 7 and can be summarized as follows: with ^{131}I-MIT as substrate for the MPO-catalysed reactions, the H_2O_2 generated at a level of 0,01 GO U/500 μl is not enough to initiate any appreciable event within 5 minutes of incubation. At higher levels of glucose oxidase ^{131}I-MIT iodination is almost linear to the GO increase. It is also evident that the ^{131}I of MIT appears in the iodide band only after ^{125}I-DIT formation, confirming that MPO-catalysed iodine exchange can only occur in DIT and not in MIT. Furthermore, no de-iodination occurred in DIT at 5 minutes even at the highest rate of H_2O_2 generation. This observation confirms that there is a specific sequence of events in MIT, de-iodination occurring only late in the sequence.

With ^{131}I-DIT as substrate iodine exchange was observed immediately as H_2O_2 was generated, and an excellent correlation exists, over a wide range of GO concentrations, between the rate

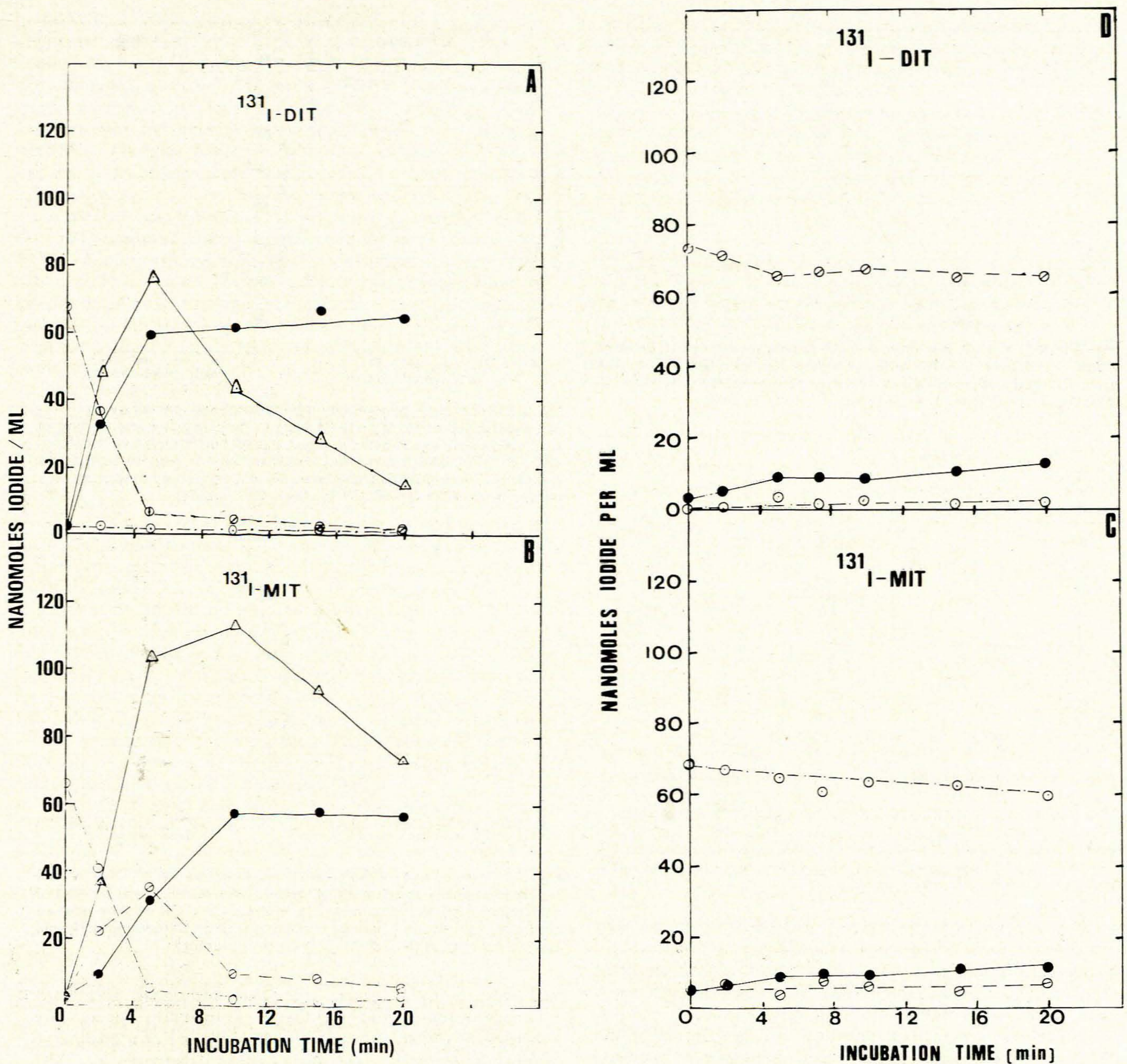

Fig. 6. Time course of MPO-catalysed reaction between either ^{131}I-MIT or ^{131}I-DIT and ^{125}I-iodide in the presence of either 10^{-3}M iodide (A and B) or 10^{-7}M iodide (C and D). The procedure and method of calculation were as indicated in Fig. 3, except that the reaction mixture contained either 153 μM ^{131}I-MIT or 123 μM ^{131}I-DIT, MPO (20 μg) and ^{125}I-iodide (10^{-3}M or 10^{-7}M). The reaction was stopped at the times indicated. The nanomoles ^{127}I incorporated into DIT or MIT were obtained by multiplying nmol/ml of iodide by the fraction of ^{125}I incorporated into DIT or MIT (Ø---Ø ^{131}I-DIT; ʘ---ʘ ^{131}I-MIT; ●——● ^{131}I-iodide; △——△ ^{125}I-DIT).

of ^{131}I-iodide released from DIT and that of ^{125}I-iodide incorporated into DIT. It appears that with this short incubation time exchange is the only reaction occurring in DIT, except at very high rates of H_2O_2 generation (0,3 U GO/500 μl) when a small amount of de-iodination of DIT may occur.

Influence of iodide and H_2O_2 generation on peroxidase-catalysed reactions with iodothyronines (T_4 and T_3)

The influence of iodide concentration, H_2O_2 generation and incubation times in MPO-catalysed reactions on thyroid hormones were investigated. Fig. 8 portrays the MPO-catalysed effects on ^{125}I-T_4 incubated for up to 60 minutes in high (10^{-3}M) and low (10^{-7}M) iodide concentrations containing ^{131}I and 0,1 U GO/500 μl. At both iodide concentrations the reactions were completed after 30 minutes' incubation at 37°C. No incorporation of ^{131}I into the ^{125}I-T_4 was observed at any stage of the incubation period, indicating that MPO-catalysed iodine exchange can occur only in DIT as substrate but not in T_4. The main phenomenon was de-iodination of T_4, which was less significant in an iodine-poor medium (about 95% at 10^{-3}M iodide compared with <40% at 10^{-7}M iodide).

The sensitivity of the MPO-catalysed de-iodination of T_4 to different levels of H_2O_2 generation at 10^{-3}M iodide and 10^{-7}M iodide is illustrated in Fig. 9. It can be seen that T_4 is very efficiently de-iodinated at extremely low GO concentrations although the extent of de-iodination is much less at 10^{-7}M iodide.

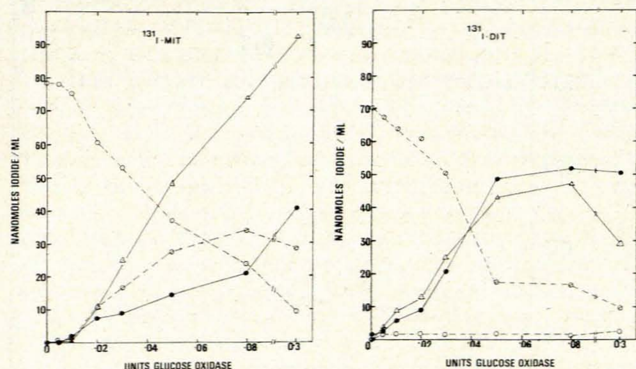

Fig. 7. Effect of H_2O_2 generation on MPO-catalysed reaction between either ^{131}I-MIT or ^{131}I-DIT and ^{125}I-iodide (10^{-3}M) after 5 min. The procedure and method of calculation were as indicated in Fig. 6 (O--O ^{131}I-MIT; Ø---Ø ^{131}I-DIT; ●——● ^{131}I-iodide; △——△ ^{125}I-DIT).

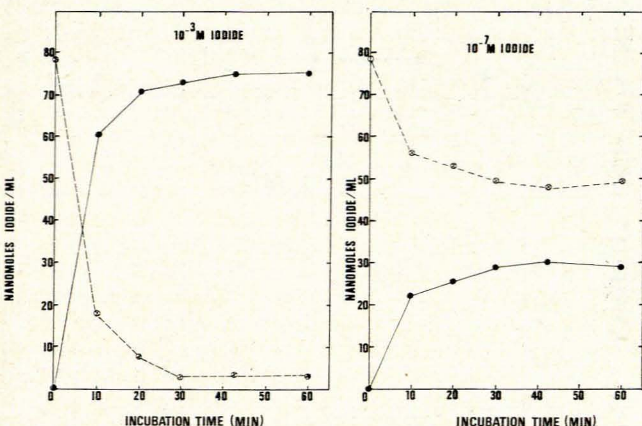

Fig 8. Time course of MPO-catalysed reactions with ^{125}I-T_4 in the presence of either 10^{-3}M or 10^{-7}M iodide. The procedure and method of calculation were as indicated in Fig. 3, except that the reaction mixture contained 119 μM ^{125}I-T_4 and 0,1 U glucose oxidase. Chromatography was carried out in BDA (⊗---⊗ ^{125}I-T_4; ●——● ^{125}I-iodide).

Chromatographic analyses of the incubated samples indicated that de-iodination of T_4 is not a simple sequential process, since no appreciable amount of ^{125}I-T_3 was formed at any stage of T_4 de-iodination, although the ^{125}I-T_4 was labelled in both the 3′ and 5′ positions of the thyronine molecule.

Similar results were obtained with ^{125}I-T_3 as substrate for the MPO-catalysed effects, namely that de-iodination occurred which was both iodide- and H_2O_2-dependent and that no ^{131}I-iodide was incorporated into ^{125}I-T_3 (i.e. no iodine exchange or T_4 synthesis occurred). These findings were surprising since it was expected that T_3 would be iodinated to T_4 rather than de-iodinated. In view of previous findings of iodine exchange reactions in iodothyronines with TPO[11] and LPO,[13] time studies were undertaken to see whether iodine exchange may perhaps appear as a transient process during incubation.

Fig. 10 shows the process of ^{125}I-T_3 de-iodination at 10^{-3}M iodide and 10^{-7}M iodide with time. It was observed that no ^{131}I incorporation into T_3 occurred. Furthermore, the extent of de-iodination of T_3 was much less at 10^{-7}M iodide, as was observed with T_4. It is concluded that MPO catalyses de-iodination of iodothyronines and that this phenomenon is very sensitive to the concentrations of H_2O_2 and iodide in the reaction medium.

Discussion

As yet no qualitative difference has been demonstrated among the multifunctional potential of different peroxidases of animal and plant origin except for the additional ability of chloroperoxidase and MPO to chlorinate appropriate substrates.[28] Other differences in function are of a qualitative nature. Nevertheless, the main catalytic activities of TPO concern thyroid hormone formation, whereas those of MPO in leucocytes and macrophages may be directed to thyroid hormone utilization for the antimicrobial activity affected by MPO.[24] It is however known that the neutrophil, like the thyroid cell, possesses an active iodide concentration mechanism, can iodinate cell membranes of ingested bacteria and, in fact, is capable of extrathyroidal iodotyrosine and iodothyronine syntheses.[24] In view of these common functional activities which are centred in their respective peroxidase enzymes, it is not surprising that some non-steroidal anti-inflammatory drugs as well as antithyroid substances will inhibit both MPO prepared from leucocytes and TPO obtained from thyroid cells.[3,23,25] Furthermore, these enzymes are both H_2O_2- and halide-dependent and during active thyroid hormone synthesis as well as during active phagocytosis of neutrophils and macrophages H_2O_2 generation is intensely increased.[29] A general concept for the regulation of these and other peroxidases therefore seems feasible.

Analysis of the results presented here shows that a peroxidase such as MPO catalyses a sequence of events starting with the iodination of tyrosyl residues and of partially iodinated tyrosine

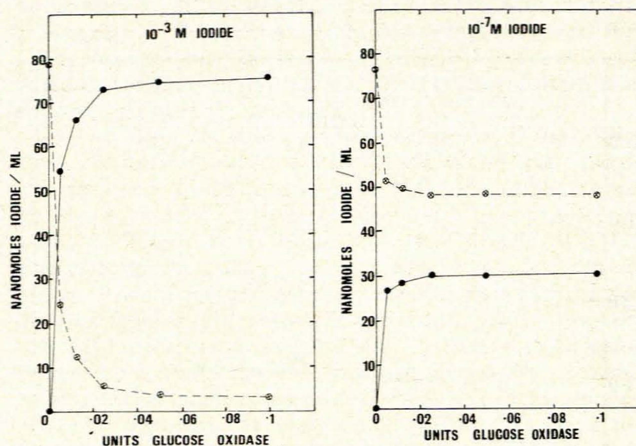

Fig. 9. Effect of H_2O_2 generation on MPO-catalysed reactions with ^{125}I-T_4 in the presence of 10^{-3}M or 10^{-7}M iodide. The procedure and method of calculation were as indicated in Fig. 3, except that the mixture contained 120 μM ^{125}I-T_4 and the various concentrations of glucose oxidase as indicated. The incubation time was 60 min and chromatography was carried out in BDA (⊗---⊗ ^{125}I-T_4; ●——● ^{125}I-iodide).

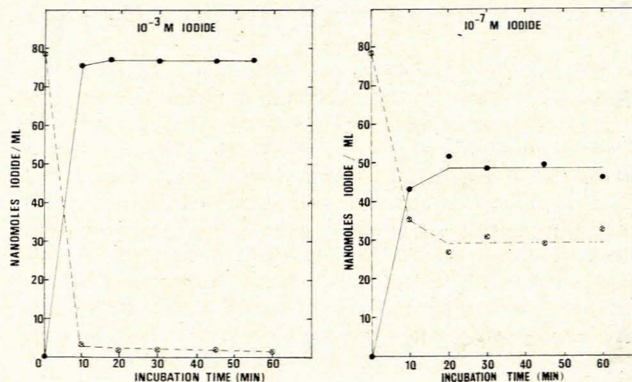

Fig. 10. Time course of MPO-catalysed reactions with ^{125}I-T_3 in the presence of either 10^{-3}M or 10^{-7}M iodide. The procedure and method of calculation were as indicated in Fig. 3, except that the reaction mixture contained 133 μM ^{125}I-T_3 and 0,1 U glucose oxidase. Chromatography was carried out in BEA (⊗---⊗ ^{125}I-T_3; ●——● ^{125}I-iodide).

to DIT. This is followed by a process of iodine exchange between inorganic iodine and DIT and eventually proceeds to the de-iodination of DIT. The results also indicate that, for every step in the chronological order of events, the rate of H_2O_2 generation plays a regulatory role and that the inorganic iodine concentration can modulate these H_2O_2-dependent peroxidase activities. The data further indicate that MPO *in vitro* is not capable of de-iodinating partially iodinated tyrosines and cannot effect exchange between inorganic iodide and MIT. It is clear from the literature that once DIT is formed, a time lag exists for the TPO-catalysed intramolecular coupling of iodotyrosines[10] (TPO coupling of free iodotyrosines cannot be achieved),[14] so that a higly iodinated TG with poor thyroxine content can be prepared. Iodotyrosine coupling therefore also falls into the sequence of kinetic events catalysed by both TPO and MPO.[30] The rate of the coupling function of peroxidases also seems to be influenced by the iodide[10] and H_2O_2[31] concentrations and therefore by the H_2O_2/I^- ratio.

It is noteworthy that the peroxidase-catalysed events on free iodotyrosines reported in this paper are graded by the rate of H_2O_2 generation, so that the most sensitive of these reactions to an increase in H_2O_2 concentration is iodination, followed by that of exchange. The final event of DIT de-iodination is comparatively less sensitive to H_2O generation and requires a high H_2O_2 concentration. All processes proceed poorly in an iodine-poor medium. This is strikingly different from the extreme degree of sensitivity of the MPO-catalysed de-iodination of iodothyronines to H_2O_2 generation. MPO-catalysed de-iodination of iodothyronines is also inhibited in an iodine-poor medium. The data show that 3,5,3'-T_3 is not iodinated by MPO to T_4 but is actively de-iodinated. However, the partially iodinated tyrosine (MIT) is only iodinated while DIT is de-iodinated by the simultaneous release of both its iodine atoms without the formation of MIT. These reactions of MPO all seem rational for the utilization of thyroid hormones by neutrophils for bacterial iodination.[32]

The H_2O_2/I^- ratio appears to play a functional role, since MPO-catalysed processes of iodination and de-iodination reactions on free iodotyrosines and iodothyronines show a differential sensitivity to the H_2O_2 concentration and since very specific and abrupt changes in these events can be effected by a very specific concentration of I^- in the medium (Fig. 5). It is postulated that the H_2O_2/I^- ratio is crucial in the thyroid and neutrophil for the control of TPO- and MPO-catalysed iodination, exchange and de-iodination reactions. It may be possible that the inorganic iodine concentration determines the degree of H_2O_2 generation whereby an H_2O_2/I^- ratio is established for the required effect and hence contributing to the autoregulatory processes in the thyroid cell and the neutrophil.

It remains to be established whether organic iodine, like iodide, has a modulatory influence on these peroxidase-catalysed reactions as is indicative of the opposing effects of free DIT on TG iodination versus T_4 formation.[14]

Because of the finding that some drugs inhibit TPO and MPO directly whereas others inhibit peroxidase activities by an additional influence on H_2O_2 generation, exogenous control of thyroid activity as well as neutrophil and macrophage activities during the inflammatory response seem possible.

The free gift of hog thyroid peroxidase presented to the Unit by Dr Alvin Taurog of the Pharmacology Department of the Southwestern Medical School, Dallas, is greatly appreciated.

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