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MECHANISM OF ACTION OF PEROXIDASE AND ITS POSSIBLE PHYSIOLOGICAL FUNCTIONS*

JHARNA BHATTACHARYYA, RANAJIT K BANERJEE *and* ASOKE G DATTA

Department of Physiology, Indian Institute of Chemical Biology, 4, Raja S.C. Mullick Road, Calcutta 700 032, India

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The mechanism of action of peroxidase in the oxidation of an electron donor by H_2O_2 has been described. The possible physiological function of the submaxillary gland peroxidase in thyroid hormone formation in relation to its structure has been proposed. The role of gastric peroxidase in parietal cell function related to gastric acid secretion has been suggested. A new catalytic function of peroxidases in reducing iodine to iodide in presence of EDTA has been documented. Finally, the role of the submaxillary gland in controlling the growth of the uterus and induction of uterine peroxidase through ovary has been shown.

Key Words: Submaxillary Peroxidase; Gastric Peroxidase; Reaction Mechanism of Peroxidases

INTRODUCTION

PEROXIDASE is one of the oldest and well-studied enzyme. A large number of reports have come out suggesting its mechanism of action. However, very little is known about its physiological role except in few cases. In the present report, attempts will be made to discuss the role of this enzyme in explaining some of the physiological functions of some of the tissues. As this symposium is primarily directed towards the chemists, we shall pose an interesting chemical problem associated with peroxidase action and shall expect some suggestions which may help us in solving the problem.

MECHANISM OF ACTION

Before we discuss about the physiological role of this enzyme let me tell you briefly the mechanism of action of this enzyme. Most of these studies were carried with pure preparations of horse-radish peroxidase (HRP) which catalyzes the following reaction :

$$2\,AH + H_2O_2 \xrightarrow{HRP} 2A + 2\,H_2O \qquad ...(1)$$

During this catalytic process, the enzyme undergoes through two different types of complex formation as suggested by Chance[1] and the equation I may be rewritten as :

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$$HRP + ROOH \longrightarrow \text{Complex I}, \quad \ldots(2)$$

$$\text{Complex I} + AH \longrightarrow \text{Complex II} + A, \quad \ldots(3)$$

$$\text{Complex II} + AH \longrightarrow HRP + A \quad \ldots(4)$$

The existence of the above two complexes during peroxidase reaction was shown by the appearance of two distinct absorption maxima at 411 and 426mμ in the presence of H_2O_2 when the enzyme alone shows maxima at 390mμ.[1] Fig. 1 shows a sharp rise of O. D. at 411mμ (Complex I) and slow increase at 426mμ (Complex II) with sharp decrease of O. D. at 390mμ on addition of 1.3μM CH_3OOH. These two complexes can be titrated and the titration results are shown in Fig. 2. It can be seen that successive additions of peroxide take the complex I near to saturation. When corrections for complex II formation are made the value for complex I (Δ) formation tallys with one peroxide molecule per peroxidase molecule. It can be further seen from the figure that similar stoichiometry of I exists between disappearance of complex I with utilization of ferrocyanide. From these results Chance[1] modified the equation as :

$$HRP \xrightarrow{\text{Peroxide}} \text{Complex I} \xrightarrow{K_4Fe(CN)_6} \text{Complex II} \xrightarrow{K_4Fe(CN)_6} HRP \quad \ldots(5)$$

In a subsequent publication Theorell *et al.*[2] using chemical, kinetic and magnetomettic data postulated that, (*a*) complex I retains two oxidizing equivalents of the peroxide molecule and loses one during conversion to complex II, (*c*) complex II retains only one oxidizing equivalents which it loses during regeneration of the original enzyme and (*c*) complex I and complex II have three and two unpaired electrons respectively. The results may be depicted in the following reactions :

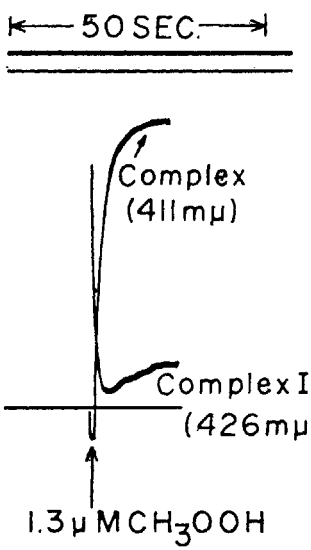

FIG 1 Effect of CH_3OOH on the kinetics of the formation of Complex I and Complex II.

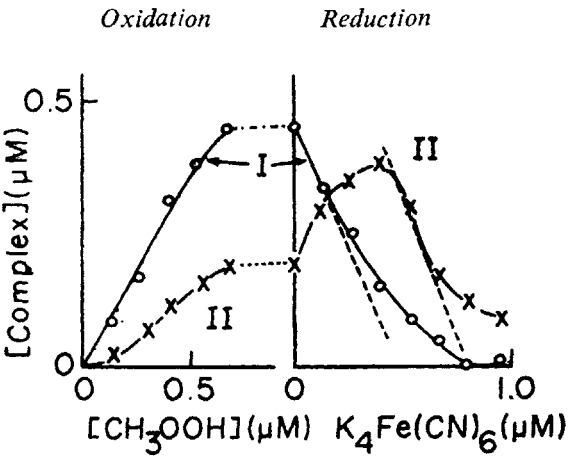

FIG 2 Titration of Complex I and Complex II formation with CH_3OOH and $K_4Fe(CN)$.

$$\text{HRP} \xrightarrow{H_2O_2} \text{Complex I} \xrightarrow{\text{2 Ox. Equi. donor}} \text{Complex II} \xrightarrow{\text{1 Ox. Equi. donor}} \text{HRP} \quad \ldots(6)$$

Physiological Functions

Although this enzyme has been discovered long time ago and shown to occur in most of the tissues, very little information is available about its function. The only function of this enzyme which has been well established is its role in thyroxine biosynthesis in thyroid gland. Thyroid peroxidase has been purified extensively and has been shown to catalyze the formation of monoiodotyrosine (MIT) and diiodotyrosine (DIT) from tyrosine.[3-5] The enzyme has also been suggested to catalyze coupling reaction between two DIT molecules to form one thyroxine molecule. Recent results from our as well as from other laboratories suggest that peroxidase may have some other functions also. These functions include formation of thyroxine in submaxillary gland, protection of multiple fertilization in ovum by ovoperoxidase, regulation of HCl secretion in stomach, oxidation of excess estradiol after estrogen surge in uterus etc.

Submaxillary Peroxidase

Existence of this enzyme was shown by Kirkwood and Faweet[6] in 1954 and was first purified in our laboratory.[7] The enzyme catalyzes the formation of MIT and DIT from tyrosine. The enzyme also uses 3,5–diiodothyronine (T_2) as the substrate for iodination reaction to produce thyroxide[8] (Fig. 3). These results clearly indicate that thyroxine is produced not only by coupling of two DIT molecules but also by iodination of preformed T_2 present in the submaxillary gland-catalyzed by submaxillary peroxidase.

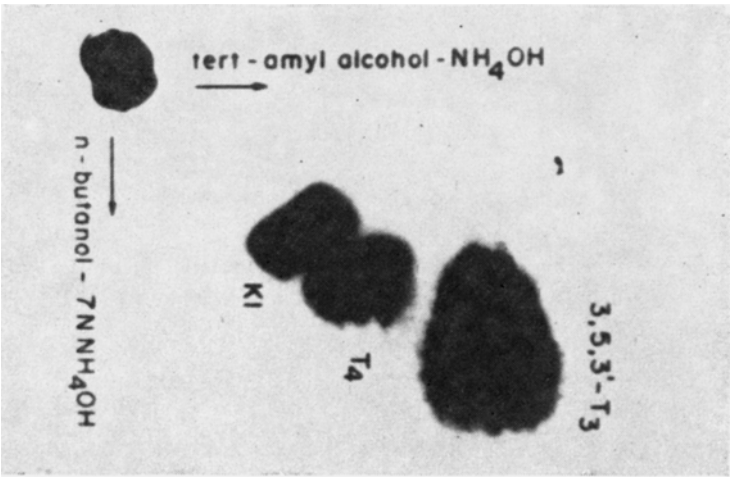

FIG 3 Formation of thyroxine by submaxillary peroxidase using 3,5-diiodothyronine.

Now a days, most of the people in thyroid field believe that iodination of free tyrosine to MIT and DIT does not lead to thyroxine formation, instead they believe that iodination of tyrosine residues in a protein molecule and subsequent coupling of two DIT molecules followed by proteolysis leads to thyroxine formation. This hypothesis is based on the fact that thyroid gland contains special protein, known as thyroglobulin and thyroxine formation occurs on this protein molecules catalyzed by thyroid peroxidase. Obviously, this finding poses a problem for us whether the sub-maxillary gland contains such type of protein or not. Fortunately, we could isolate a protein from submaxillary gland which on incubation with K^{131} I and H_2O_2 in presence of submaxillary peroxidase followed by proteolytic digestion produced significant amount of radiolabeled thyroxine.[9] The protein has been purified to homogeneity as judged by polyacrylamide gel electrophoresis (Fig. 4) and has a molecular weight of 120,000. The protein has two unequal subunits, one 800,000 (subunit I) and the other 42,000 (subunit II), and we have been able to separate these two subunits on urea sephadex column. The separated subunits (both I and II) can be iodinated by the action of submaxillary peroxidase and the products of iodination are MIT and DIT, but not thyroxine. It has already been mentioned above that the original native protein (120,000mw) gives significant amount of thyroxine after incubation in presence of the peroxidase, KI and H_2O_2. The two subunits can be reconstituted to original protein by combining together in equimolar concentration and dialysis under controlled condition. The reconstituted protein on incubation under the above condition produces thyroxine like the original protein

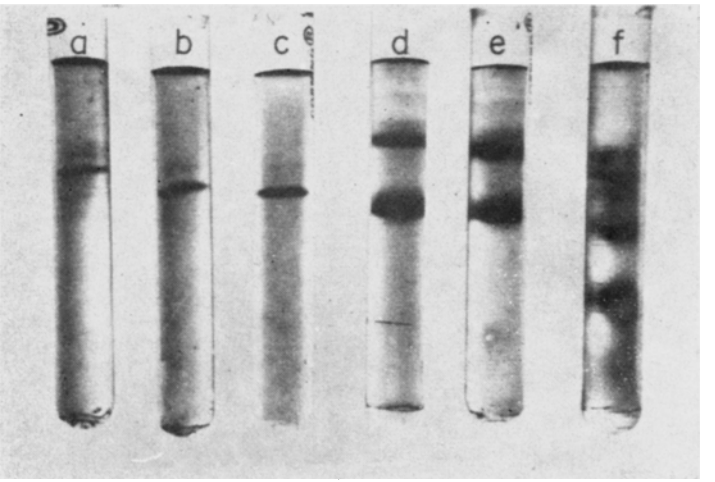

FIG 4 SDS-Polyacrylamide gel electrophoretic pattern of the iodinable protein from submaxillary gland. *a*. 40μg purified peroxidase without SDS. *b*. 40μg of purified protein. *c*. Reconstituted protein from subunit I and subunit II (without SDS). *d*. Same as *b* with SDS using 100μg protein. *e*. Iodinable protein purified from microsome. *f*. SDS gel electrophoresis of (1) lactoperoxidase, 78,000 (2) BSA, 67,000 (3) Ovalbumin 43,000 (4) Trypsin, 24,000 and (5) Cytochrome C, 12,000 as standard proteins.

(Table I). Hence, we postulate that thyroxine is formed by coupling of adjacent iodotyrosines residing in subunits I and II i.e. interchain coupling and not intrachain coupling as depicted in Fig. 5. For some time it was difficult for us to visualise, how two iodotyrosines couple together to form thyroxine with the elimination of an alanine residue from one of the iodotyrosines. Although we do not have any evidence but our imagination is presented in Fig. 6. From all these data we may conclude that submaxillary peroxidase, like thyroid peroxidase, can catalyze, thyroxine formation on a protein molecule also isolated from submaxillary gland.

Gastric Peroxidase

During the course of the above study another peroxidase has been isolated and purified in our laboratory. This peroxidase resides on the outer membrane of

TABLE I

Formation of different products on iodination of subunits one (I) and two (II), native and reconstituted proteins by submaxillary peroxidase

System	*n* moles of product formed/min/mg protein				
	Total	MIT region	DIT region	T_2 and T_3 region	T_4 region
Subunit I (80,000)	13.25	4.05	7.25	0.85	1.10
Subunit II (44,000)	11.29	3.56	6.23	0.62	0.88
Native (120,000)	11.62	3.54	3.82	1.42	2.64
Reconstituted (120,000)	11.30	3.67	3.68	1.30	2.45

The number in the parenthesis indicates the molecular weight. Preparation of subunit I and subunit II and also the reconstituted protein has been described in the text.

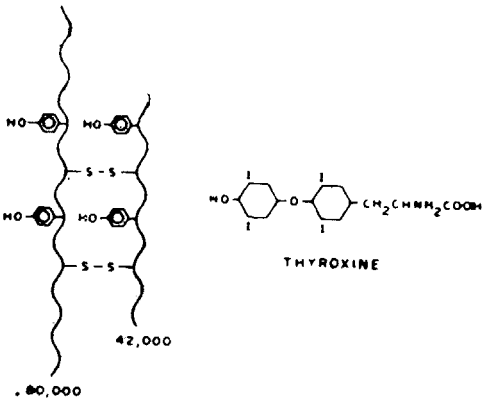

FIG 5 Interchain coupling of iodotyrosine residues of the protein to form thyroxine.

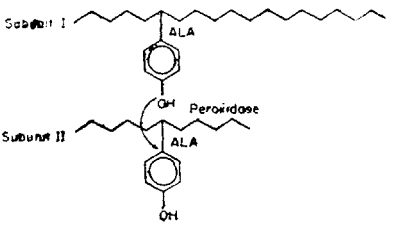

FIG 6 Diagramatic presentation of the mechanism of the formation of hyroxine on the protein backbone.

the mitochondria located in the parietal cells of gastric mucosa.[10,11] It may be mentioned that these parietal cells are responsible for the production of gastric HCl and preliminary results from our laboratory indicate that the gastric peroxidase may be involved in HCl production. This enzyme shows modulation with glucocorticoids, elevated on bilateral adrenalectomy and inhibited very strongly by dexamethasone.[12] The other unusual property of this enzyme is that it is completely inhibited by EDTA in *in vitro* experiments and the EDTA inhibition is completely reversed by equimolar concentration of Zn^{++}. This observation suggests that the enzyme contains Zn^{++} at or near the active site of the enzyme and that is why the enzyme is inhibited by EDTA and reversed by Zn^{++}. Unfortunately, the atomic absorption data show that the enzyme does not contain Zn^{++}. HRP also shows inhibition with EDTA and reversal with Zn^{++} and does not contain Zn^{++} in the enzyme molecule as shown by atomic absorption data. As highly pure HRP is available commercially some interesting experiments were carried out with HRP. The enzyme activity is normally measured using O-dianisidine, guiacol or KI as the electron donor. It is interesting to note that the enzyme activity is inhibited by EDTA only when KI is used as electron donor[11] and not O-dianisidine or guiacol. This clearly points out that EDTA does not really inhibit the enzyme but reacts either with KI or its product of oxidation i.e. I_3^-. The reaction between H_2O_2 and KI in presence of peroxidase may be depicted as follows :

$$2\,KI + H_2O_2 \longrightarrow I_2 + 2KOH \qquad ...(7)$$

$$I_2 + I^- \rightleftharpoons I_3^- \qquad ...(8)$$

I_3^- shows a maximum absorption band at 353mμ with a molar extinction value of 22,900 and this high value of I_3^- makes the assay very sensitive. It can be seen from Fig. 7 that EDTA completely inhibits the enzyme at 4mμ concentration if the

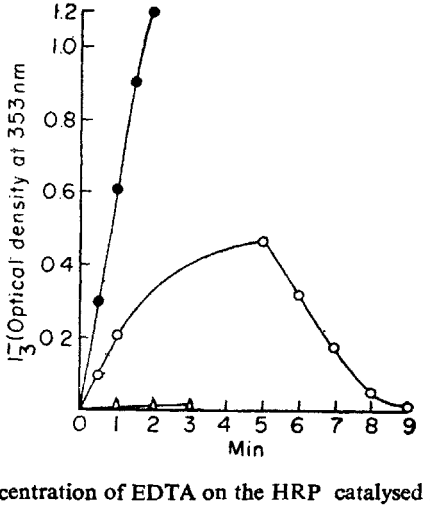

FIG 7 Effect of varying concentration of EDTA on the HRP catalysed I_3^- formation. —●— Control, —○— + 2mM EDTA, + 4mM EDTA. Peroxidase activity was assayed using iodide as electron donor.

inhibitor is present from the very beginning. However, if we use 2mM EDTA in the incubation mixture instead of 4mM, the O.D. increases to some extent and then goes down instead of remaining steady. This finding puzzles us and indiates that EDTA probably reacts with I_3^- and bleaches its colour. The bleaching of I_3^- colour due to EDTA is enzymatic and needs both H_2O_2 and peroxidase.

This led us to investigate the effect of addition of EDTA on the enzymatically generated I_3^-. Fig. 8 (open circle) shows that addition of three instalments of EDTA (1mM each time as shown by the upward arrow), the formation of I_3^- colour showed a tendency to decrease and addition of another 1mM EDTA (final concentration of 4mM), the bleaching of colour of I_3^- took place linearly and the extinction fell to zero within 2.5min when 2.3μg HRP was used. When 4.6μg of HRP was used (open triangle) instead of 2.3μg, both the initial rate of increase of O. D. due to I_3^- formation and subsequent decrease of O.D. on addition EDTA were almost double indicating that both these reactions were dependent on enzyme concentration. Addition of 3mM NaN_3 prior to the addition of last instalment of EDTA is indicated by downward arrows prevented the decrease of I_3^- colour. Fig. 9 shows that I_3^- generated non-enzymatically by incubating KI with H_2O_2, remained unaltered after the addition of four instalments (ImM each time) of EDTA (small change of O.D. was due to dilution with increasing amount of EDTA), but on addition of 2.3μg HRP, the extinction dropped to zero within two minutes. N_3^- inhibited the bleaching when added prior to the addition of peroxidase. Addition of 4mM Zn^{2+} to the completely bleached system (at about 7 min as shown in the figure), I_3^- formation restored to the original value indicating that HRP was still active and could catalyze the formation of I_3^- from the residual iodide and H_2O_2 present in the incubation as well as the iodide formed from the already bleached I_3^-.

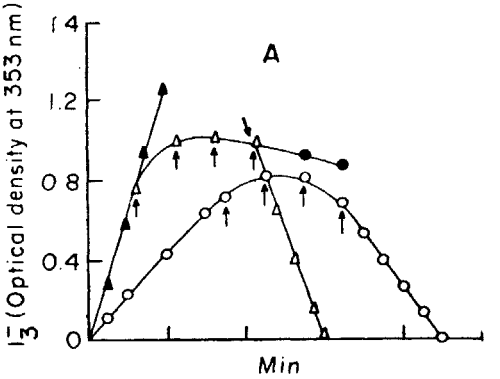

FIG 8 Effect of EDTA on the I_3^- generated by HRP catalyzed iodide peroxidation. Two different concentrations of HRP were used namely 2.3μg (—○—) and 4.6μg (—△—). First instalment of EDTA (1mM) was added when HRP catalyzed I_3^- formation reached a value of 0.7–0.75. The next instalments were added at 1 min interval as indicated by the upward arrows. The downward arrow indicates the addition of azide and —●— indicates the rate after azide addition. —▲—, I_3^- formation with 4.6μg HRP without EDTA.

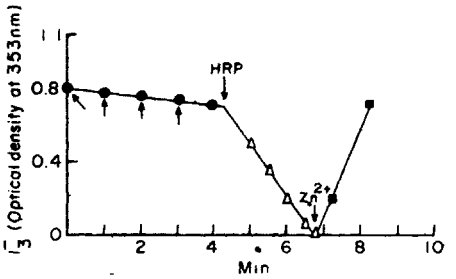

FIG 9 Role of peroxidase on the bleaching of I_3^- with EDTA. I_3^- was produced nonenzymatically for 1–2 hrs in a reaction mixture containing all the ingredients of peroxidase assay except the enzyme. When the extinction at 353nm reached around 0.8, 1mM EDTA was added at 1 min interval (total 4mM) as indicated by the upward arrows. After 1–1.5 min of the last EDTA addition, 2.3μg HRP (—△—) was added and the extinction of I_3^- was monitored. After the extinction dropped to zero at 6.75 min, 4mM $ZnSO_4$ was added (—□—).

It may be presumed that the loss of extinction of I_3^- may be due to an adduct of I_3^- with the EDTA in presence of the enzyme and H_2O_2. If it were so, the loss of I_3^- colour on addition of EDTA as well as reappearance of I_3^- colour after the addition of Zn^{2+} would have been instanteneous and not rate dependent. On the contrary, the results show that both the above-mentioned phenomena were time-dependent peroxidation reaction. The alternate possibility is the backward reaction resulting in the conversion of I_3^- to I^- with EDTA in presence of the peroxidase. However, require ment of H_2O_2 for the bleaching of I_3^- colour rules out a true back reaction. The disappearance of I_2 after the addition of EDTA could be further demonstrated by starch solution. Addition of starch solution to the incubation system before the addition of EDTA showed an absorbance of 2.0 at 660nm whereas addition of starch solution, a few minutes after the addition of EDTA, when I_3^- extinction went down nearly zero, absorbance at 660nm showed only 0.2. In order to check if the bleached product contained I^-, the experiment was carried out with the limiting amount of iodide. In Fig. 10, curve a 1μ mole of I^- (instead of 5μ moles) was allowed to be peroxidised to I_3^- with HRP till there was no further increase of extinction even after the addition of extra amounts of the enzyme and H_2O_2. Addition of 4mM EDTA at this point brought down the extinction to 0.02 and subsequent addition of equimolar amount of Zn^{2+} caused about 80 per cent recovery of I_3^- which could be completely prevented by the addition of N_3^- prior to the addition of Zn^{2+} (data not shown). This indicates that I_3^- was converted to I^- after the addition of EDTA and I^- could not be reconverted to I_3^- as long as EDTA was present in the system. I^- was peroxidised to I_3^- only after removal of EDTA by Zn^{2+}. The entire process of initial I_3^- formation, bleaching with EDTA and subsequent I_3^- formation on addition of Zn^{2+} were dependent on the enzyme concentration. When 14μg HRP (curve *b*) was instead of 7 μg as in curve a, all these three rates were found to be increased.

In order to check whether I^- was really produced as the reaction product after the addition of EDTA, the entire reaction as in curve a of Fig. 10 was allowed to

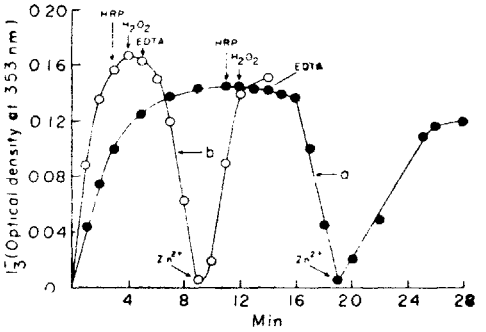

FIG 10 Effect of varying HRP concentration on the rate of I_3^- formation, I_3^- disappearance with EDTA and reappearance of I_3^- on addition of Zn^{2+} using limiting amount of iodide. In curve *a*, I_3^- was generated in the reaction mixture containing all the ingredients for peroxidase assay in the concentrations as mentioned in the text except that the concentration of KI was 1μ mole instead of 5μ moles. EDTA (4mM) was added when no further oxidation of iodide was observed on addition of extra amounts of HRP (3.5μg) and H_2O_2 (0.4μ mole) as indicated by the arrows. 4mM $Zn5O_4$ was added when EDTA induced loss of extinction reached around 0.025. In curve *b*, the same experiment was repeated on initial addition of 7.0μg of HRP.

proceed in presence of 131_I^-. The reaction mixture at zero time (before the addition of H_2O_2), at steady state (before the addition of EDTA) and when the extinction dropped almost to zero after the addition of EDTA, were extracted with benzene which dissolves iodine keeping the free iodide in the aquous layer. The benzene and aqueous layers were counted for radioactivity and the results are shown in Table II. It is clear from the table that at zero time almost all the counts of the reaction mixture remained as iodide since it could not be extracted by benzene. At the steady state before the addition of EDTA, the counts were almost evenly distributed in aqueous as well as in benzene layer suggesting 50 per cent conversion of iodide to

TABLE II

Distribution of iodine and iodide before and after the addition of EDTA to the assay system

	Organic phase I_2 CPM	Aqueous phase (I^-) CPM	Iodide spot CPM	Others spots
1. Reaction mixture at zero time	1,176	137,022	134,440	Nil
2. Before addition of EDTA	60,219	62,019	57,024	Nil
3. After addition of EDTA	5,121	133,836	132,480	Nil
4. After addition of Zn^{2+}	55,782	70,317	66,650	Nil

The experimental condition is exactly the same as in curve *a* of Fig 12 except that $Na^{131}I$ (150,000 CPM) was added alongwith KI. The entire reaction mixture at desired time was mixed with equal volume of benzene, vortexed and both the organic and aqueous phase were counted for radioactivity. An aliquot of the aqueous phase (0.3ml) was used for chromatography and auctoradiography. The solvent used was butanol : ethanol : 2N NH_3 (5 : 1 : 2).

iodide. After the addition of EDTA when the I_3^- extinction dropped to zero, almost all the counts could be recovered as iodide in the aqueous layer indicating quantitative reconversion to iodide from iodine. Finally, addition of Zn^{2+} (scavenger of EDTA) reestablished the distribution of radioactivity as was observed before addition of EDTA i.e. about 50 per cent iodide and 50 per cent iodine. Chromatography and autoradiography of the aqueous layer indicated the presence of only one spot having the same Rf value as iodide. No other radioactive spot could be detected on the autoradiogram when aliquots of aqueous layers were chromatographed on Whatmann 3MM paper using butanol-ethanol-2N NH_3 (5 : 1 : 2) as developer.

Results presented in this paper show clearly that HRP catalyzes two separate reaction (*i*) I^- to I_2 and (*ii*) I_2 to I^- and both the reactions required H_2O_2. The first reaction occurs in the absence of EDTA whereas the second reaction requires the presence of it. It may be possible that EDTA change the conformation of the enzyme and the altered enzyme catalyzes the reversal of the first reaction. But truly speaking, it is not a reverse reaction as H_2O_2 is not formed during the conversion of I_2 to I^-, rather H_2O_2 is required during this conversion.

Recently, Huwiler and Kohler[14] have shown a pseudo-catalytic degradation of H_2O_2 in lactoperoxidase—H_2O_2—iodide system. They have suggested that iodinium ion, I^+ being a highly reactive species, can react spantaneously in a redox reaction with H_2O_2 leading to the formation of iodide and O_2 according to the following reaction :

$$I^+ + H_2O_2 \longrightarrow O_2 + I^- + 2H^+ \qquad ...(9)$$

or

$$I_2 + H_2O_2 \longrightarrow O_2 + 2I^- + 2H^+ \qquad ...(10)$$

I_2 having zero polarization, exhibits a lower reactivity than I^+. The chemical oxidation of H_2O_2 by I_2 was, however, known for a long time.[15] Huwiler and Kohler[14] have depicted the following scheme for pseudo-catalytic degradation of H_2O_2 :

$$I^- \xrightarrow{H_2O_2} I^+ \xrightarrow{+I^-} I_2 \rightleftarrows I_3^- \qquad ...(11)$$

$$I^+ \xrightarrow{H_2O_2} O_2 + I^- + 2H^+ \qquad ...(12)$$

$$I_2 \xrightarrow{H_2O_2} O_2 + 2I^- + 2H^+ \qquad ...(13)$$

As early as in 1932, Leibhfsky[16] reported the formation of iodide from hypoiodite at the expense of H_2O_2 according to the following reaction :

$$IO^- + H_2O_2 \longrightarrow O_2 + H_2O + I^- \qquad ...(14)$$

Possibility of hypoiodite as an intermediate in iodide dependent catalytic degradation of hydrogen peroxide in thyroid peroxidase or lactoperoxidase catalyzed peroxidative reaction has recently been proposed.[17]

We are postulating that HRP besides catalyzing the oxidation of I^- to I_3^- may also catalyze any of the above two reactions (12) or (13) leading to the loss of extinction of I_3^- producing iodide. However, the role of EDTA in enhancing the rate of this reaction in our system is not clearly understood.

Uterine Peroxidase

Uterine peroxidase has been shown to act as a marker enzyme for estradiol action[18] and has been partially purified by calcium chloride extraction of 40,000xg pellet of uterine tissue.[18,19] It has also been reported that administration of estradiol in immature female rats induces uterine peroxidase.[20] Recently, we have made an observation of dramatic connection between submaxillary gland and uterus of immature female rats.[21] Results of these experiments are discussed in the following few pages.

Surgical removal of submaxillary glands produced a three- to four-fold increase in the size of the uterus as shown in Fig. 11. Histological examination of the transverse section of the uterine tissues from both normal and experimental animals shows that there was significant increase in the diameter of the uterine tissue of the experimental animals (Fig. 12A). It can be further seen that the inner luminar epithelial cells changed from cubical to columnar (Fig. 12B). Histological section of ovary from sub-maxillariectomized immature rats showed presence of matured graafian follicles (not shown here). All these are typical changes seen following estrogen administration in immature rats.[22,23] Assuming that these changes are due to increased production of estrogen and the ovary being the main organ for estrogen production, bilateral ovariectomy was performed in rats of the same age group before removal of submaxillary glands. Removal of submaxillary glands in ovariectomized rats abolished the changes in the size and histology of uterus following removal of the submaxillary glands, indicating involvement of the ovaries in the uterine changes.

Estrogen is an anabolic hormone and we, therefore, studied the incorporation of ^{14}C–phenylalanine into uterine protein of normal and submaxillariectomized animals. The results clearly indicated that removal of submaxillary gland increased incorporation of ^{14}C–phenylalanine into uterine protein (unpublished results). Involvement of increased estrogen production as a cause for the above-mentioned changes in submaxillariectomized animals was further evidenced by the following facts. It has been mentioned above that estrogen induces a membrane bound peroxidase in uterine tissue and this uterine peroxidase is considered to be the marker enzyme for estradiol action.[18] It is logical to accept that if the above mentioned changes are really due to increased formation of estrogen, then uterine peroxidase would be elevated on removal of submaxillary gland. Indeed, the uterine peroxidase was increased by 15-fold on removal of submaxillary gland over its normal counterpart (Table III). It could further be shown that on removal of submaxillary gland, the plasma estradiol level increased significantly (Table III). Estradiol values obtained by us are comparable to the values reported by Brown-Grant *et al.*[24]

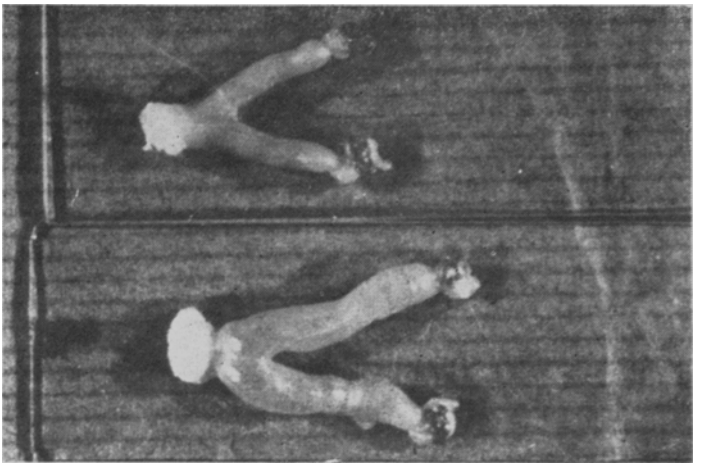

FIG 11 Surgical removal of submaxillary gland on the size of the uterus.

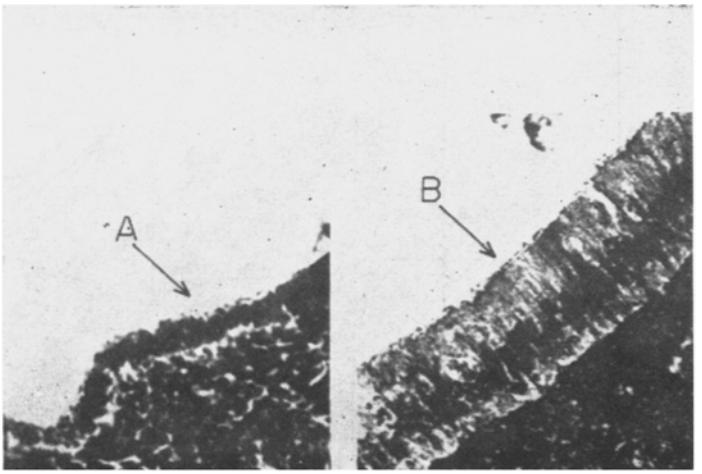

FIG 12 *A* and *B* histological examination of the transverse section of uterine tissue from normal and submaxillariectomized animal.

Thus, results from all the above experiments indicate that the blood estradiol level increased on submaxillariectomy and that the increased estradiol level caused increased uterine weight, histological changes in uterine tissues and induction of uterine peroxidase. The mechanism whereby these changes are associated with submaxillary gland, remains to be established. It may be assumed that the submaxillary gland produces a factor that holds estradiol formation in the ovary low levels in immature rats and that the removal of submaxillary glands reduces the circulating levels of this factor. This process is slow and possibly for this reason we do not observe any significant change in the uterus before twelve days after removal

TABLE III

Effect of submaxillariectomy on the uterine peroxidase and plasma estradiol level in immature rats

	Peroxidase O.D./min/ml	Plasma estradiol pg/ml
Normal	0.042 ± 0.002	1.3 ± 0.12
After submaxillariectomy	0.600 ± 0.012	5.2 ± 0.20
After submaxillariectomy + submaxillary extract	0.045 ± 0.005	1.1 ± 0.15

Values are the mean ± SE ($n = 4$).

Peroxidase activity was measured using guaiacol as electron donor and estradiol level was assayed by radioimmunoassay.

of the submaxillary gland. We have shown that intraperitoneal administration of extract of submaxillary glands to the submaxillariectomized rats, abolish the resulting changes and also the induction of uterine peroxidase. Administration of the extract to normal immature rats did not produce any significant change in the size and histology of the uterus or in the uterine peroxidase and blood estradiol levels.

Thus, from all the above evidences it may be concluded that the submaxillary gland contains a soluble factor which controls the formation of estradiol in ovary and thereby the estradiol associated changes in uterus as reported in this paper. The factor appears to be a new hormone and it is difficult to state whether the factor acts on the ovary or through pituitary.

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