REDUCED PYRIDINE NUCLEOTIDES AS ACTIVATORS OF CERTAIN REACTIONS CATALYZED BY PEROXIDASE .

S. J. KLEBANOFF
The Rockefeller Institute, New York, N.Y. (U.S.A.)

(Received March 28th, 1960)

SUMMARY

Conditions are described in which the aerobic oxidation of epinephrine and ferrocytochrome *c* by peroxidase is dependent on the presence of DPNH or TPNH in the reaction mixture and is stimulated by thyroxine, certain thyroxine analogues and estradiol. In the case of epinephrine oxidation, DPNH and thyroxine abolished a lag period which is associated with the aerobic oxidation of epinephrine in the absence of these substances. The oxidation of epinephrine and ferrocytochrome *c* by peroxidase in the presence of Mn^{++}, DPNH, thyroxine and oxygen is inhibited by catalase and by certain thyroxine analogues. The latter inhibition is overcome by an increase in DPNH concentration.

Thyroxine is inactivated by the H_2O_2–peroxidase system and this inactivation is inhibited by the presence of a suitable hydrogen donor (*e.g.* DPNH, epinephrine) in the reaction mixture. A DPNH dependent inactivation of thyroxine by the Mn^{++}-peroxidase–oxygen system also is described.

It is suggested that the Mn^{++} dependent aerobic oxidation of reduced pyridine nucleotides by peroxidase facilitates certain secondary oxidations as a result of the generation of hydrogen peroxide in the course of the reaction.

INTRODUCTION

Thyroxine has been found to stimulate greatly the oxidation of a number of hydrogen donors by the H_2O_2–peroxidase system[1,2]. The suggestion has been made[1,2] that thyroxine facilitates peroxidatic reactions by acting as an electron carrier, being alternately oxidized by the H_2O_2–peroxidase system and reduced by the hydrogen donor. The phenolic hydroxyl group of thyroxine is essential for this effect. Certain hydrogen donors are oxidized by peroxidase in the absence of added H_2O_2, if Mn^{++} and oxygen are present in the reaction mixture (see ref. 3 for early bibliography). DPNH and TPNH are among the hydrogen donors which are oxidized by peroxidase in this fashion[3]. Thyroxine[2] and estradiol[2,4] as well as other phenolic compounds[3] stimulate the Mn^{++} dependent aerobic oxidation of DPNH and TPNH by peroxidase.

In the present study thyroxine and estradiol sensitive oxidations are described which require peroxidase, manganese and oxygen but which differ from the classical

Abbreviations: TPNH, reduced triphosphopyridine nucleotide; DPNH, reduced diphosphopyridine nucleotide.

Mn^{++} dependent aerobic oxidations by peroxidase in that the addition of DPNH or TPNH also is required.

METHODS AND MATERIALS

Ferricytochrome *c* (Sigma Chemical Co.) was reduced with sodium borohydride and the latter was removed by dialysis[5]. The horseradish peroxidase (RZ 1.1) was obtained from Worthington Biochemical Corp. and the myeloperoxidase was prepared as previously described[1]. The myeloperoxidase solution employed had a ratio 430 mμ/390 mμ of 1.62 and an O.D. at 430 mμ of 0.090. All other materials were obtained as previously described[1,2]. The thyroxine analogues were kindly supplied by Drs. R. C. KROC AND A. W. RUDDY of the Warner-Lambert Research Institute. The spectrophotometric measurements were made at 25° with a Cary M 14 recording spectrophotometer. The blank was air unless otherwise indicated.

RESULTS

Epinephrine oxidation

The oxidation of epinephrine by H_2O_2 and peroxidase is stimulated by thyroxine[1]. Estradiol also stimulates this reaction and, as in the case of thyroxine, the stimulation is a function of the phenolic hydroxyl group. Fig. 1 demonstrates that for the immediate oxidation of epinephrine by peroxidase in the absence of added H_2O_2, DPNH was required in addition to Mn^{++} and thyroxine or estradiol. DPNH could be replaced by equimolar concentrations of TPNH. The concentration of DPNH required for the oxidation of epinephrine under the conditions employed in Fig. 1 was higher in the estradiol stimulated system than in the thyroxine stimulated system. At a DPNH

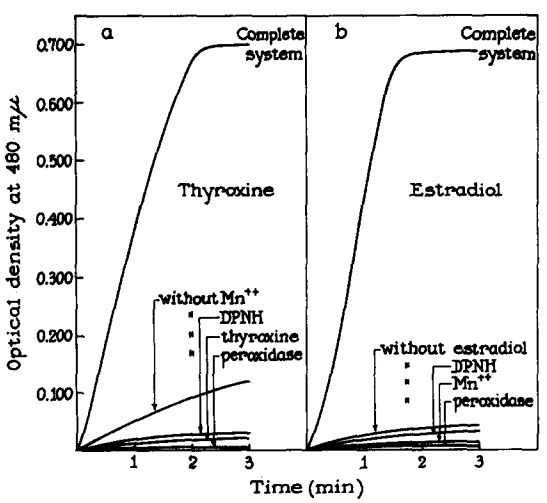

Fig. 1. Stimulation by thyroxine or estradiol of the oxidation of epinephrine by peroxidase in the presence of Mn^{++}, DPNH and oxygen. The complete system contained 120 μmoles of phosphate buffer pH 7.0, 0.5 μmole of epinephrine, 0.5 μmole of manganous chloride, 50 μg of horseradish peroxidase and water to a final volume of 3.0 ml. In addition *a* contained 0.3 μmole of DPNH and 0.05 μmole of thyroxine, whereas *b* contained 1.2 μmole of DPNH, 0.05 μmole of estradiol and ethanol to a final concentration of 1.7 %. The reaction was begun by the addition of peroxidase. The blank contained all components except peroxidase.

concentration of 0.3 μmole/3.0 ml of reaction mixture, epinephrine was rapidly and completely oxidized by the peroxidase system in the presence of thyroxine whereas only a slight oxidation was observed in the presence of estradiol. Approximately 1.2 μmoles of DPNH were required to produce a complete oxidation of epinephrine by the estradiol stimulated system.

The oxidation of epinephrine by peroxidase, Mn^{++}, DPNH and thyroxine was associated with an oxygen uptake and could be followed manometrically. Under the conditions employed, epinephrine was oxidized in the absence of DPNH and thyroxine, at a rate equal to that observed in the presence of DPNH and thyroxine following a lag period of approx. 30 min. The lag period was completely abolished on the addition of DPNH and thyroxine but not by either alone. In the absence of DPNH, 5 μmoles of oxygen were taken up during the oxidation of 2.0 μmoles of epinephrine following the lag period indicating that several oxygen consuming steps occur during the oxidation of epinephrine. If thyroxine (0.1 μmole) and DPNH (2.0 μmoles) also were added to the reaction mixture, the oxygen uptake was increased to 6.5 μmoles.

Catalase inhibited the oxidation of epinephrine by peroxidase in the presence of Mn^{++}, DPNH, thyroxine and oxygen. In a typical experiment the rate of oxidation of epinephrine was inhibited 90 % by 800 μg of catalase, 17 % by 800 μg of heated catalase (10 min at 100°) and 12 % by 800 μg of crystalline bovine albumin.

Of a number of thyroxine analogues tested at a final concentration of $1.7 \cdot 10^{-5} M$, 3,5-diiodo-L-tyrosine, *n*-butyl 3,5-diiodo-4-hydroxybenzoate, DL-thyronine and 3,5-diiodo-L-thyronine had very little effect on epinephrine oxidation under the conditions employed in Fig. 2, whereas 3,3′,5-triiodo-L-thyronine and 3,3′,5′-triiodo-DL-thyronine were approximately half as active as thyroxine. An increase in the concentration of thyronine or diiodothyronine to 20 times that employed in Fig. 2 had little effect on the oxidation of epinephrine whereas a stimulation was produced

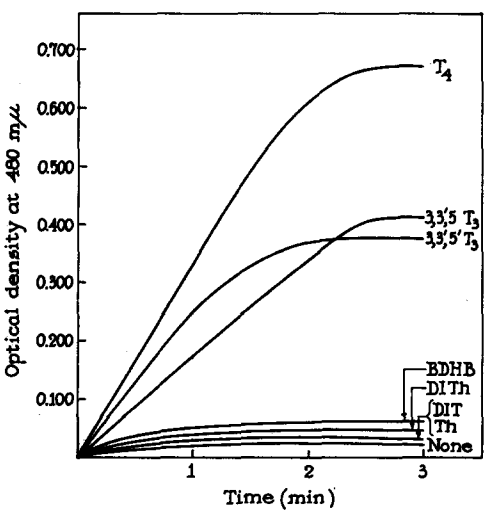

Fig. 2. Effect of thyroxine analogues. As in Fig. 1a except that 0.05 μmole of thyroxine (T_4), 3,3,5′-triiodo-L-thyronine (3,3,5′ T_3), 3,3′5′-triiodo-DL-thyronine (3,3′5′ T_3), 3,5-diiodothyronine (DITh), thyronine (Th), 3,5-diiodotyrosine (DIT) and *n*-butyl-3,5-diiodo-4-hydroxybenzoate (BDHB) were added as indicated.

by high concentrations of diiodotyrosine and *n*-butyl 3,5-diiodo-4-hydroxybenzoate. These latter analogues were less than 1 % as active as thyroxine.

Thyronine, at concentrations equimolar to that of thyroxine, largely prevented the stimulation of epinephrine oxidation by thyroxine under the conditions employed in Table I. An inhibition also was observed with 3,5-diiodothyronine and 3,3′5′-triiodothyronine although higher concentrations of these analogues were required. The antithyroxine effect of these analogues was abolished by an increase in the DPNH concentration.

Ferrocytochrome c *oxidation*

ALTSCHUL, ABRAMS AND HOGNESS[6] described the presence in yeast of a peroxidase with a very high affinity for ferrocytochrome *c*. This was confirmed by CHANCE[7], who also observed that the oxidation of ferrocytochrome *c* was a general property of peroxidase whether isolated from horseradish, milk, white blood cells or yeast. Fig. 3 demonstrates the stimulatory effect of thyroxine on the oxidation of ferrocytochrome *c* by horseradish peroxidase and H_2O_2. The product of oxidation was ferricytochrome *c* as indicated by its absorption spectrum and by its reduction to ferrocytochrome *c* on the addition of ascorbic acid (Fig. 3).

Added hydrogen peroxide could be replaced by the glucose–glucose oxidase system, horseradish peroxidase by myeloperoxidase and thyroxine by a number of thyroxine analogues or by estradiol. Thyronine was approximately 10 times and estradiol 4 times as active as thyroxine on a molar basis when horseradish peroxidase was employed, whereas thyroxine was approx. 1.5 times as active as thyronine, and thyroxine and estradiol were equally active when myeloperoxidase was employed.

TABLE I

ANTITHYROXINE EFFECT OF CERTAIN THYROXINE ANALOGUES

As in Fig. 1a except that thyroxine, thyronine, 3,5-diiodothyronine (DITh) and 3,3′5′-triiodo-DL-thyronine (3,3′5′ T_3) were added in the amounts indicated. The reaction was begun by the addition of peroxidase. The results are expressed as the increase in O.D. at 480 mμ during the first minute of the experiment.

Additions	*Increase in O.D. at 480 mμ in 1 min*
None	0.010
DPNH	0.010
DPNH + thyroxine (0.05 μmole)	0.330
DPNH + thyroxine (0.05 μmole) + thyronine (0.01 μmole)	0.245
DPNH + thyroxine (0.05 μmole) + thyronine (0.02 μmole)	0.125
DPNH + thyroxine (0.05 μmole) + thyronine (0.03 μmole)	0.090
DPNH + thyroxine (0.05 μmole) + thyronine (0.05 μmole)	0.060
DPNH + thyroxine (0.05 μmole) + thyronine (0.1 μmole)	0.045
DPNH + thyroxine (0.05 μmole) + DITh (0.05 μmole)	0.420
DPNH + thyroxine (0.05 μmole) + DITh (0.2 μmole)	0.290
DPNH + thyroxine (0.05 μmole) + DITh (0.5 μmole)	0.145
DPNH + thyroxine (0.05 μmole) + DITh (1.5 μmoles)	0.95
DPNH + thyroxine (0.05 μmole) + 3,3′5′T_3 (0.05 μmole)	0.495
DPNH + thyroxine (0.05 μmole) + 3,3′5′T_3 (0.2 μmole)	0.290
DPNH + thyroxine (0.05 μmole) + 3,3′5′T_3 (0.5 μmole)	0.250

The decrease in the rate of oxidation of ferrocytochrome *c* with time was much more pronounced with the myeloperoxidase preparation than with the horseradish peroxidase preparation, as was found for the oxidation of epinephrine[1].

AKAZAWA AND CONN have reported that ferrocytochrome *c* is aerobically oxidized in the presence of peroxidase, Mn^{++} and certain phenols[3]. Ferrocytochrome *c*, however, was not oxidized by peroxidase in the presence of Mn^{++}, oxygen and thyroxine or estradiol under the conditions employed in Table I. The rate of oxidation of ferrocytochrome *c* was followed for 5 min only and therefore an oxidation following a lag period of greater length, as was observed with epinephrine cannot be excluded. If

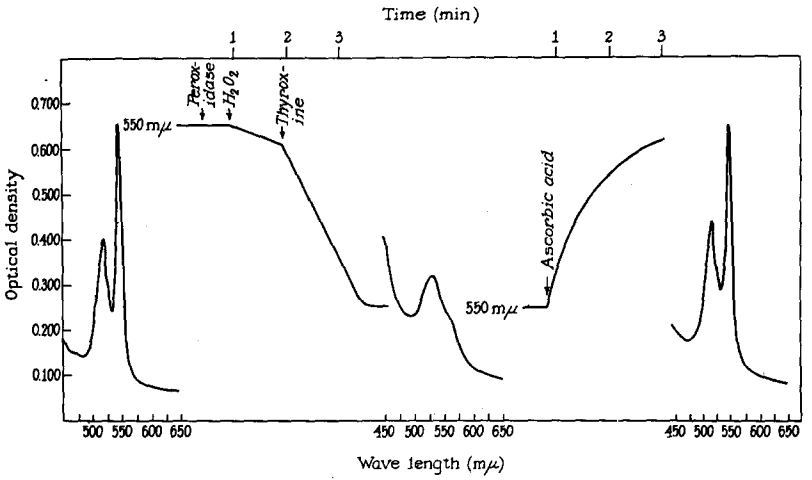

Fig. 3. Effect of thyroxine on the oxidation of ferrocytochrome *c* by the H_2O_2–peroxidase system. The reaction mixture contained initially 120 μmoles of phosphate buffer pH 7.0, 0.07 μmole of ferrocytochrome *c* and water to a final volume of 3.0 ml. Horseradish peroxidase (5 μg), H_2O_2 (1.0 μmole), sodium-L-thyroxine (0.05 μmole) and ascorbic acid (1.0 μmole) were added as indicated. The absorption spectrum of the solution was determined where indicated, by scanning from 450 mμ to 650 mμ at a rate of 2.5 mμ/sec. The wavelength was maintained at 550 mμ between spectra. The blank was air.

TABLE II

OXIDATION OF FERROCYTOCHROME *c* BY PEROXIDASE IN THE ABSENCE OF ADDED HYDROGEN PEROXIDE

The reaction mixture contained 120 μmoles of phosphate buffer pH 7.0, 0.07 μmole of ferrocytochrome *c* and water to a final volume of 3.0 ml. $MnCl_2$ (0.5 μmole), horseradish peroxidase 50 μg, DPNH (0.6 μmole), TPNH (0.6 μmole) and thyroxine (0.05 μmole) were added as indicated.

Supplements	*O.D. change/min at 550 mμ*
Mn^{++} + peroxidase	0.000
Mn^{++} + peroxidase + thyroxine	0.000
DPNH	0.000
Mn^{++} + peroxidase + DPNH	0.040
Mn^{++} + peroxidase + DPNH + thyroxine	0.256
TPNH	0.000
Mn^{++} + peroxidase + TPNH	0.040
Mn^{++} + peroxidase + TPNH + thyroxine	0.312

DPNH or TPNH also was added to the reaction mixture, the immediate oxidation of ferrocytochrome *c* was observed and this oxidation was greatly stimulated by the addition of thyroxine (Table I). Estradiol could replace thyroxine in this system but, as in the case of epinephrine oxidation, a considerably higher concentration of DPNH or TPNH was required. Horseradish peroxidase could be replaced by myeloperoxidase. Catalase inhibited the aerobic oxidation of ferrocytochrome *c* as was observed for the oxidation of epinephrine.

Inactivation of thyroxine

The pre-incubation of thyroxine with peroxidase and hydrogen peroxide for 15 sec prior to the addition of DPNH resulted in a fall in the rate of oxidation of the DPNH to the level observed in the absence of thyroxine (Fig. 4, o—o). Pre-incubation of thyroxine with H_2O_2 or with peroxidase alone for 3 min had no influence on the rate of oxidation of DPNH. Of the components of the reaction mixture, only the addition of a second aliquot of thyroxine increased the rate of oxidation of DPNH to that observed in the absence of a pre-incubation period.

The rapid inactivation of the H_2O_2–peroxidase–thyroxine system on pre-incubation in the absence of DPNH was not observed when DPNH was present in the reaction mixture during the pre-incubation period (Fig. 4, ●—●). The rate of oxidation of DPNH remained high for a period which corresponded to the time required for the complete oxidation of the initial aliquot of DPNH (Fig. 4, – – – –). Pre-incubation beyond this period resulted in a rapid inactivation of the peroxidase system. When the pre-incubation was less than 1 min, the concentration of DPNH in the reaction mixture following the addition of the second aliquot was higher than the concentration present when the pre-incubation period was greater than 1 min.

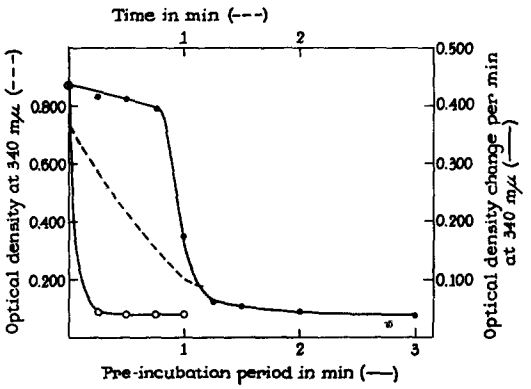

Fig. 4. The inactivation of thyroxine by peroxidase and hydrogen peroxide. o—o, the reaction mixture which contained 120 μmoles of phosphate buffer pH 7.0, 0.05 μmole of thyroxine, 100 μg of horseradish peroxidase, 1.0 μmole of H_2O_2 and water to a final volume of 3.0 ml was pre-incubated at room temperature (28°) for the periods indicated. DPNH (0.3 μmole) was added at the end of the pre-incubation period except in the flask represented by zero pre-incubation in which case the DPNH was added prior to H_2O_2. The rate of oxidation of the DPNH was expressed as O.D. change per min at 340 mμ. ●—●, as above except that 0.3 μmole of DPNH was present in the reaction mixture at the beginning of the pre-incubation period. – – – –, the oxidation of the initial aliquot of DPNH is indicated by the fall in absorption at 340 mμ. A second aliquot of DPNH (0.3 μmole) was added following the pre-incubation of the reaction mixture for the periods indicated and the rate of oxidation of the DPNH was determined.

However, variation in the initial concentration of DPNH over wide limits did not affect its rate of oxidation[2].

These data indicate that the pre-incubation of thyroxine with H_2O_2 and peroxidase results in the oxidation of thyroxine to a form which is inactive as a stimulant of reactions catalyzed by peroxidase and that the presence of a suitable hydrogen donor in the reaction mixture protects thyroxine from this inactivation. DPNH was employed in Fig. 4 as the hydrogen donor. However, similar results were obtained with epinephrine.

In contrast to the results observed with the H_2O_2–peroxidase system (Fig. 4), the pre-incubation of thyroxine with Mn^{++}, peroxidase and oxygen for 4 min had little effect on the rate of oxidation of DPNH added at the end of the pre-incubation period (Fig. 5, o—o). However, if DPNH was present in the reaction mixture during the pre-incubation period, a rapid inactivation of the peroxidase system occurred, following a lag period which corresponded to the time required for the complete oxidation of the initial aliquot of DPNH (Fig. 5, ●—●). Again, only the addition of thyroxine restored the peroxidase activity to the initial high levels. This

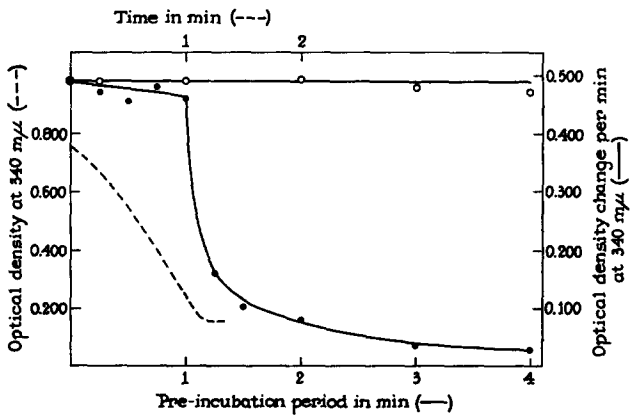

Fig. 5. The inactivation of thyroxine by peroxidase, Mn^{++} and oxygen. As in Fig. 4 except that Mn^{++} (0.5 μmole) was added instead of H_2O_2.

would suggest that the oxidation of DPNH by peroxidase in the presence of Mn^{++}, oxygen and thyroxine results in the accumulation of an oxidant, possibly H_2O_2, in sufficient quantity to inactivate the thyroxine when the protective effect of DPNH is gone. AKAZAWA AND CONN could not demonstrate an accumulation of H_2O_2 during the aerobic oxidation of DPNH by horseradish peroxidase[3] while H_2O_2 does appear to accumulate during the aerobic oxidation of triose reductone by turnip peroxidase[8]. The oxidation of thyroxine and certain thyroxine analogues by H_2O_2 and peroxidase has been reported previously by MAYRARQUE-KODJA, BOUCHILLOUX AND LISSITZKY[9].

DISCUSSION

In the oxidation of DPNH by peroxidase in the presence of Mn^{++}, oxygen and certain phenolic compounds, 2 moles of DPNH are oxidized for every mole of oxygen taken up[2–4]. Catalase inhibits the oxidation of DPNH by this system[2–4] which suggests

that H_2O_2 is formed and utilized during the course of the reaction. AKAZAWA AND CONN were unable to demonstrate the accumulation of H_2O_2 during the oxidation of DPNH by horseradish peroxidase in the presence of Mn^{++}, resorcinol and oxygen[3] which suggests that under these conditions the steady state concentration of H_2O_2 is very low. The process can be envisaged as follows:

$$\text{DPNH} + \text{H}^+ + \text{O}_2 \longrightarrow \text{DPN}^+ + \text{H}_2\text{O}_2 \quad (1)$$

$$\text{DPNH} + \text{H}^+ + \text{H}_2\text{O}_2 \longrightarrow \text{DPN}^+ + 2\text{H}_2\text{O} \quad (2)$$

$$2\text{DPNH} + 2\text{H}^+ + \text{O}_2 \longrightarrow 2\text{DPN}^+ + 2\text{H}_2\text{O} \quad (3)$$

Eqns. 1 and 2 have been simplified for the purpose of this discussion and represent overall reactions which also involve Mn^{++} and a phenolic compound. The inhibitory effect of catalase on the DPNH dependent oxidation of epinephrine and ferrocytochrome *c* by peroxidase suggests that H_2O_2 is an intermediate in these reactions. It is suggested therefore that the initial oxidation of DPNH by the peroxidase system generates an oxidant which is either H_2O_2 itself or is dependent on H_2O_2 for its formation and that this oxidant can be utilized in a secondary oxidation if the second substance being oxidized can successfully compete with DPNH for the H_2O_2 formed.

Estradiol on a molar basis had a greater stimulatory effect on the oxidation of ferrocytochrome by H_2O_2 and horseradish peroxidase than did thyroxine. However, the Mn^{++} dependent aerobic oxidation of ferrocytochrome *c* in the presence of DPNH occurred more efficiently (*i.e.* at a lower DPNH concentration) with thyroxine than with estradiol. Similarly the relative effect of the thyroxine analogues on the oxidation of epinephrine by the Mn^{++}–peroxidase–DPNH–O_2 system (Fig. 2) was very different from the effect of these substances on the oxidation of epinephrine by peroxidase and H_2O_2 (see ref. 1). These data suggest that the rate limiting step in the coupled oxidation of epinephrine and ferrocytochrome *c* is the oxidation of DPNH and that the oxidation of DPNH in the presence of thyroxine can be more effectively coupled to secondary oxidations (possibly as a result of the greater availability of H_2O_2) than the oxidation of DPNH in the presence of estradiol or the thyroxine analogues tested.

In certain instances (*e.g.* ferrocytochrome *c* and epinephrine oxidation) the secondary oxidation in the coupled system is sensitive to thyroxine and estradiol. However, this need not be the case for the overall reaction to be phenol sensitive since the rate limiting step in the coupled oxidation is the oxidation of DPNH and the latter reaction is stimulated by thyroxine and estradiol as well as by other phenols. In a previous study[10], estradiol was found to be inactivated by peroxidase, manganese and oxygen if DPNH (or TPNH) was present in the reaction mixture and a stimulation by thyroxine was evident when suboptimal concentrations of DPNH (or TPNH) were employed. The DPNH or TPNH dependent inactivation of estradiol by peroxidase is an instance in which the secondary oxidation, *i.e.* the inactivation of estradiol by H_2O_2 and peroxidase, does not appear to be thyroxine sensitive whereas the overall reaction is thyroxine sensitive[10]. Indeed according to this formulation, the secondary oxidation need not involve peroxidase at all but may involve catalase, or be nonenzymic and still be sensitive to thyroxine or estradiol. In this way the number of substances whose oxidation by peroxidase is associated with an oxygen uptake and is stimulated by thyroxine and estradiol as well as by certain other phenolic compounds would be increased considerably.

ACKNOWLEDGEMENTS

This work was supported in part by research grant A-2772 from the National Institutes of Health. I am grateful to Dr. R. M. ARCHIBALD and Dr. H. JAFFE for the use of their facilities and for their helpful discussions of this work.

REFERENCES

1 S. J. KLEBANOFF, *J. Biol. Chem.*, 234 (1959) 2437.
2 S. J. KLEBANOFF, *J. Biol. Chem.*, 234 (1959) 2480.
3 T. AKAZAWA AND E. E. CONN, *J. Biol. Chem.*, 232 (1958) 403.
4 H. G. WILLIAMS-ASHMAN, M. CASSMAN AND M. KLAVINS, *Nature*, 184 (1959) 427.
5 A. P. MARTIN, H. A. NEUFELD, F. V. LUCAS AND E. J. STOTZ, *J. Biol. Chem.*, 233 (1958) 206.
6 A. M. ALTSCHUL, R. ABRAMS AND T. R. HOGNESS, *J. Biol. Chem.*, 136 (1940) 777.
7 B. CHANCE, in J. T. EDSALL, *Enzymes and Enzyme systems*, Harvard Univ. Press, Cambridge, 1951, p. 93.
8 I. YAMAZAKI, K. FUJINAGA, I. TAKEHARA AND H. TAKAHASHI, *J. Biochem. (Tokyo)*, 43 (1956) 377.
9 A. MAYRARQUE-KODJA, S. BOUCHILLOUX AND S. LISSITZKY, *Bull. soc. chim. biol.*, 40 (1958) 815.
10 S. J. KLEBANOFF AND S. J. SEGAL, *J. Biol. Chem.*, 235 (1960) 52.