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STUDIES ON PEROXIDASE, 2,4-DICHLOROPHENOL STIMULATED AND L-THYROXINE STIMULATED DPNH OXIDASE ACTIVITIES IN RAT UTERUS

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STUDIES ON PEROXIDASE, 2,4-DICHLOROPHENOL STIMULATED AND L-THYROXINE STIMULATED DPNH OXIDASE ACTIVITIES IN RAT UTERUS

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LIST OF ABBREVIATIONS

Following are the explanations of the abbreviations used throughout this writing:

ADP	Adenosine diphosphate
AMP	Adenosine monophosphate
ATP	Adenosine triphosphate
DCP	2,4-dichlorophenol
DEAE	Diethylaminoethyl ether, cellulose
DPN	Diphosphopyridine nucleotide (oxidized)
DPNH	Diphosphopyridine nucleotide (reduced)
EDTA	Ethylenediaminetetraacetic acid
IAA	Indoleacetic acid
IOB	Iodosobenzoic acid
GSH	Glutathione (reduced)
GSSG	Glutathione (oxidized)
2-ME	2-mercaptoethanol
PCMB	p-chloromercuribenzoate
Pi	Inorganic Phosphate
TCA	Trichloroacetic acid
TPN	Triphosphopyridine nucleotide (oxidized)
TPNH	Triphosphopyridine nucleotide (reduced)
Tris	Tris (hydroxymethyl)aminomethane

LIST OF ABBREVIATIONS (continued)

Dichlorophenol Activity	Dichlorophenol stimulated DPNH oxidase Activity
Thyroxine Activity	Thyroxine stimulated DPNH oxidase activity

STUDIES ON PEROXIDASE, 2,4-DICHLOROPHENOL STIMULATED AND L-THYROXINE STIMULATED DPNH OXIDASE ACTIVITIES IN RAT UTERUS

CHAPTER I

INTRODUCTION

Since the demonstration by Lucas et al.(1), in 1955, of the existence of peroxidase in the rat uterus, many reports have appeared concerning the nature and possible function of peroxidase. Lucas reported that peroxidase appeared to be more prevalent in proliferating tissues. Uterine peroxidase activity varies considerably during the estrous cycle, presumably in response to changes in circulating estrogen.

Peroxidase has been considered more characteristic of plants than of animals (2). Demonstration of the conversion of peroxidase to a peroxidase-oxidase by manganese (2) has indicated new possibilities for identifying the physiological roles of this enzyme. When acting as an oxidase, peroxidase utilizes molecular oxygen rather than hydrogen peroxide (3).

The oxidation of reduced diphosphopyridine nucleotide by uterine peroxidase-oxidase requires the presence of a phenolic compound (4). This catalytic phenol requirement is relatively non-specific; apparently the main necessity is an aromatic hydroxyl group (5). Temple et al.(6) have

suggested that no difference exists in the mechanism mediating stimulation by different phenols. Bever et al. (7), however, have reported evidence that implies a distinction in mechanism between the two phenolic stimulators, dichlorophenol and thyroxine. In addition, Bever et al.(7) reported dichlorophenol stimulated reduced diphosphopyridine nucleotide oxidase, but not thyroxine stimulated reduced diphosphopyridine nucleotide oxidase activity to be further stimulated by inorganic phosphate (Pi).

Baker and Schultz (8) reported recently that peroxidase was inactivated by loss of inorganic phosphate by dialysis. Orthophosphate could be replaced by adenosine diphosphate and adenosine triphosphate but not by adenosine monophosphate.

Several mechanisms based on free radicals have been proposed to explain the participation of catalytic quantities of manganese and phenol, and the requirement of one-half mole of molecular oxygen per mole of reduced diphosphopyridine nucleotide oxidized. Direct evidence of the participation of free radicals produced from peroxidase substrates was provided by Yamazaki, Mason and Piette (9).

Inhibition of peroxidase-oxidase activity by compounds such as cysteine and glutathione have been reported (6) and substantiated several times (10,11,12). No attempt has been made to advance a reason for the observed inhibition of the peroxidase-oxidase activity other than suggesting the possibility of free radical trapping by these thiol compounds.

The purpose of the present study was to determine whether a distinction at the level of the enzyme molecule could be made between the mechanism of the two phenolic stimulators, dichlorophenol and

thyroxine. One of the first requirements of such a study would be to purify the enzyme as much as possible. Since all previous attempts to preserve enzyme activity have proved unsuccessful, it was hoped that techniques could be developed to aid in stabilization. A more complete examination of the phosphate stimulation observed by Bever and a survey of inhibitors and their kinetic implications should yield data clarifying the requirements of the enzyme with respect to the two phenols.

CHAPTER II

LITERATURE REVIEW

Distribution of Peroxidase

Although there are many exceptions to the rule, catalases are more abundant in animals and peroxidases in plants (2). The richest sources of peroxidase known at present appear to be the sap of the fig tree and the root of horseradish plant. Peroxidase reaction is rarely found in microorganisms but this may be due to unsuitable choices of acceptors for the reaction. Plant peroxidases in general have a relatively low specificity for hydrogen donors but certain animal peroxidases show considerable substrate specificity (13).

The most recent animal sources of peroxidase to be investigated are uterine tissue (1) and lumenal fluid (7). Most of the physiological acceptors are not known for the peroxidases found in mammalian tissues. Table 1 summarizes mammalian tissue peroxidase sources. Neufeld *et al.* (14) present data to substantiate the suggestion of Lucas *et al.* (1) that the presence of peroxidase might be characteristic of proliferating tissue. The organs with significant concentration of peroxidase and a high peroxidase-cytochrome oxidase ratio are among those which Leblond *et al.* (17) found to have a high rate of cell renewal. In contrast, the tissues without peroxidase are those with little or no cell renewal under normal conditions.

TABLE 1

Sources of Mammalian Tissue Peroxidase

High Activity	Low Activity
Uterus (1)	Heart (14)
Lumenal fluid (7)	Kidney (15)
Walker Carcinoscarcoma No. 256 (14)	Brain (14)
	Skeletal muscle (14)
Intestine (14)	Liver (15)
Stomach (14)	Milk (14)
Spleen (15)	Leucocytes (14)
Lung (15)	
Thyroid (16)	

The inverse relation of peroxidase and cytochrome oxidase in the tissues that were studied led to the hypothesis by Neufeld et al. (14) that peroxidase may partially substitute for the oxidase in the oxidative metabolism of the proliferating tissue.

Peroxidase Substrates

Since peroxidase has been shown to be widely distributed in mammalian tissue indicated in Table 1, considerable effort has been expended to find possible physiological substrates. Peroxidase has the capacity to oxidize a large number of substances and consequently exhibits a low substrate specificity for hydrogen donor compounds while it simultaneously maintains a high specificity for hydrogen peroxide. Direct physical evidence by electron spin resonance indicates that free radicals are formed from substrates in enzymic oxidation-reduction (9). Some of the more widely used substrates in peroxidatic oxidation are: Indoleacetic acid (18), epinephrine (19,20), glutathione (13), ergothioneine (21), luciferin (10), homogentisic acid (17), methionine (22), tryptophan (23,24), tyramine (23), tyrosine (23), ferrocytochrome c (20),

hydroquinone, ascorbic acid, dihydroxyfumaric acid (9), sulfite ions (19), resorcinol (12) and DPNH or TPNH (12). DPNH or TPNH have been found to replace each other successfully in reactions. Estradiol (5) has also been proved to act as a substrate with a low efficiency but its role as such is believed to be unrelated to its effectiveness as a physiological stimulator (6).

Estradiol

Lucas et al. (1) found that injections of estrogens into ovariectomized rats caused a pronounced activity of peroxidase in the uterus, whereas this activity is normally very low. The peroxidase activity was related to the amount of estrogen injected. Bever et al. (25) demonstrated that 17β-estradiol will elevate uterine DPNH oxidase activity in ovariectomized rats, but also observed that an elevated enzyme level could not be maintained by constant daily injections. Maximal activity occurred in 3 days and was followed by a minimal activity in 5-6 days, approximating the original activity of castrate non-treated rats. Figure 1 illustrates the effect of various levels of 17β-estradiol on uterine peroxidase.

The effect in vitro of estradiol on DPNH oxidation as discussed later is not related to its biological activity (6). According to a hypothesis by Mueller (26), the interaction of the estrogen with the cells appears to set in operation a process leading initially to the release or activation of a number of proenzymes. This is followed by a period of production of "specific" enzymes. According to this view the early increase in molecular oxygen uptake serves to support the energy requirements of "enzyme activating" period (27).

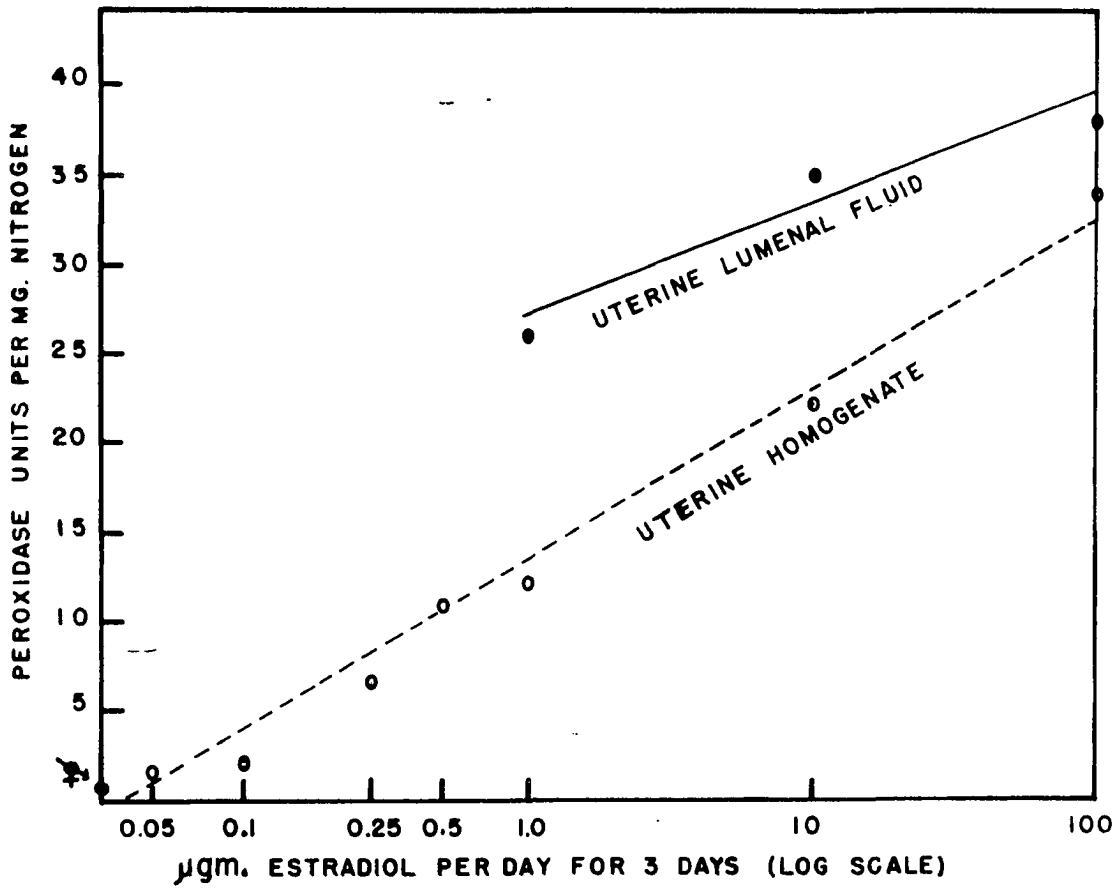

Figure 1. Effect of 17β-estradiol on uterine peroxidase of ovariectomized Rats.

Hollander and Stephens (28) found that a significant increase in enzyme activity occurred within two hours after a single intra-cardiac injection of estradiol was given to spayed rats. This increase occurred during the period of concomitant rapid water imbibition (29) and enhanced oxygen consumption (30).

The uterus of the ovariectomized rat has an 80% water content which rises to a maximum of 85% within six hours after the administration of estradiol and returns to 80% water content in 72 hours (31). A study using immature rats given intracardiac injections of estradiol indicates that the uterus may show an increase in weight as a result of estrogen stimulation without showing an accompanying gain in DPNH oxidase activity (28). In addition to increased peroxidase activity, Scott and Lisi (27) have noted changes in amount of hexokinase, transhydrogenase, glucose-6-phosphatase and phospho-gluconate dehydrogenase in the uterus following injections of estradiol.

The development of hormonally stimulated enzymatic activity after administration of estradiol is not due to an increase in some thermostable cofactor in the stimulated tissue (6). Temple et al. (6) found that active homogenates from stimulated animals were completely inactivated by heating at 100° C. for 5 minutes. Since fresh weight and dry weight remain at an elevated level throughout the period of stimulation (25), the activity does not appear to be related to the general protein response induced by estradiol administration.

Bever (32) and Bever and Campbell (33) made studies of the effect of (a) hypophysectomy, (b) adrenalectomy, and (c) combined hypophysectomy and adrenalectomy upon the enzymatic activity in ovariectomized rats.

They concluded that the enzyme response to daily estradiol injections in ovariectomized rats is not dependent primarily upon the pituitary or adrenal glands, although the adrenal glands may have a modifying influence. These findings strengthen the probability that the decline phase of the response is due to the effects of metabolic products of estrogen catabolism.

Bever et al. (7) found that the lumenal fluid in the uterus contains peroxidase activity which is increased by estradiol stimulation, as seen in Figure 1. Table 2, which illustrates the differences of activity between lumenal fluid and uterus homogenate, shows the varying values produced by different phenolic stimulators of DPNH oxidation by peroxidase after hormone injections.

TABLE 2

Effects of Phenolic Stimulators on DPNH Oxidation after Hormone Stimulation

Stage of Treatment	Source	P	DCP	Thyr	Est
Intact early estrus	Homog.	18	5.3	1.3	1.1
	Lum. Fluid	32	2.0	None	None
Castrated + 0.5 µg estradiol	Homog.	10	1.1	0.2	Trace
	Lum. Fluid	120	6.0	None	None
Castrated + 0.5 µg estradiol + 0.5 mg progesterone	Homog.	5	0.5	Trace	None
	Lum. Fluid	97	6.0	None	None

Peroxidase (P), Dichlorophenol (DCP), Thyroxine (Thyr) and Estradiol (Est) Activities based on Change in O. D./minute/mg N.

These data suggest that uterine and lumenal peroxidase and dichlorophenol stimulated peroxidase-oxidase activities respond similarly to hormonal treatment and that phenolic stimulation of peroxidase-oxidase by thyroxine

and estradiol tend to parallel each other. In addition, it can be seen that thyroxine and estradiol stimulated DPNH oxidase activities are absent in lumenal fluid, while peroxidase and dichlorophenol activity is quite high. The parallel responses between peroxidase and dichlorophenol and between estradiol and thyroxine, and the non-parallelism between the two groups, suggest that the mechanism mediating dichlorophenol response and that mediating thyroxine and estradiol, are not necessarily the same.

As previously stated, the response of uterine peroxidase increases in specific activity is not unique nor specific to injected estrogen. Injections of estrogen have proved to be a valuable tool in obtaining more active starting source of uterine peroxidase. Figure 1 demonstrates that the specific activity of uterine peroxidase increases linearly with the log of injected estrogen (7). Table 3 indicates the manner in which peroxidase responds to the normally fluctuating levels of circulating estrogens during the estrous cycle. Injected estrogen is assumed to magnify a normal physiological response and at the same time yield a predictable and reproducible source of uterine peroxidase.

TABLE 3

Peroxidase Response to Estrogen Levels During Estrous Cycle

Cycle Stage	P (U/mgN)	DCP (U/mgN)	Thyr (U/mgN)
Diestrus	0.53	0.30	0.17
Early proestrus	4.6	1.03	0.50
Proestrus	7.3	2.14	0.35
Early estrus (ballooned)	16.5	5.03	1.05
Full vaginal estrus	1.1	1.58	0.62
Early estrus/diestrus ratio	31	17	6

Peroxidase (P), Dichlorophenol (DCP) and Thyroxine (Thyr) Activities

Molecular Oxygen Requirement

Klebanoff (3) associated the oxidation of DPNH by a thyroxine stimulated horseradish peroxidase system in the absence of added hydrogen peroxide with uptake of oxygen. The reaction was followed manometrically. Approximately one-half mole of oxygen was taken up for every mole of DPNH oxidized.

For the oxidation of DPNH or TPNH by peroxidase the presence of Mn^{++} and oxygen may substitute for hydrogen peroxide (3). With the oxidation of DPNH or TPNH by the Mn^{++}-peroxidase-oxidase, the oxidation occurs in the absence of added hydrogen peroxide and is associated with an oxygen uptake. Peroxidase has been found to catalyze the oxidation of a number of hydrogen donors in the absence of added hydrogen peroxide if molecular oxygen and small amount of Mn^{++} are present in the reaction mixture (5).

Hollander and Stephens (4) also determined that one-half mole of oxygen was required for each mole of DPNH oxidized by a phenol-stimulated peroxidase reaction catalyzed by uterine tissue. Table 4 indicates the consumption of oxygen and production of DPN in the peroxidatic oxidation of DPNH.

TABLE 4

Oxygen Consumption Studies

	DPNH Added μ moles	O_2 Consumed μ moles	DPN Formed μ moles
Complete system-DPNH	6.0	0	0
Complete system-DCP	6.0	0	0
Complete system-enzyme	6.0	0	0
Complete system	2.0	1.08	1.5
Complete system	4.0	1.36	3.0
Complete system	6.0	1.88	4.5

Production of DPN in the oxidation of DPNH is indicated by Hollander and Stephens (4) in Figure 2. This figure illustrates the effect of additional DPNH after reaction has gone to completion and also shows the accumulation of DPN, which is reduced by the alcohol dehydrogenase.

Manganese

Oxidation by peroxidase of dihydroxyfumaric acid, phenylacetaldehyde, phenylpyruvic acid, dicarboxylic acid, indoleacetic acid, DPNH, TPNH, ferrocytochrome c, glutathione and methionine all require, or are stimulated by, manganese (34). Mudd and Burris (35) have shown that Ce^{+++} can replace Mn^{++} in peroxidase-catalyzed oxidations of DPNH, IAA, and 2-nitropropane. Mazelis (34) in the peroxidase oxidative decarboxylation of methionine, found that Mn^{++} could not be replaced by the following metals: Mg^{++}, Co^{++}, Zn^{++}, Cu^{++}, Al^{+++}, Fe^{+++}, Ni^{++}, Ca^{++} or Sn^{++}.

The requirement for Mn^{++} appears to be specific only in the aerobic oxidase activities of peroxidase. Yamazaki and Souzu (18) using horseradish peroxidase, demonstrated this as summarized in Table 5. Manganese appears to promote reaction between free radicals and O_2 to form hydrogen peroxide (18), however, hydrogen peroxide will not replace Mn^{++} requirement in aerobic reactions (34).

Different results occur with varying concentrations of Mn^{++}. Mudd and Burris (36) suggest that peroxidase-catalyzed oxidations may proceed by different mechanisms depending on the concentrations of manganese present in the reaction. Hollander and Stephens (4) indicate that in the phenol stimulated oxidation of DPNH by uterine

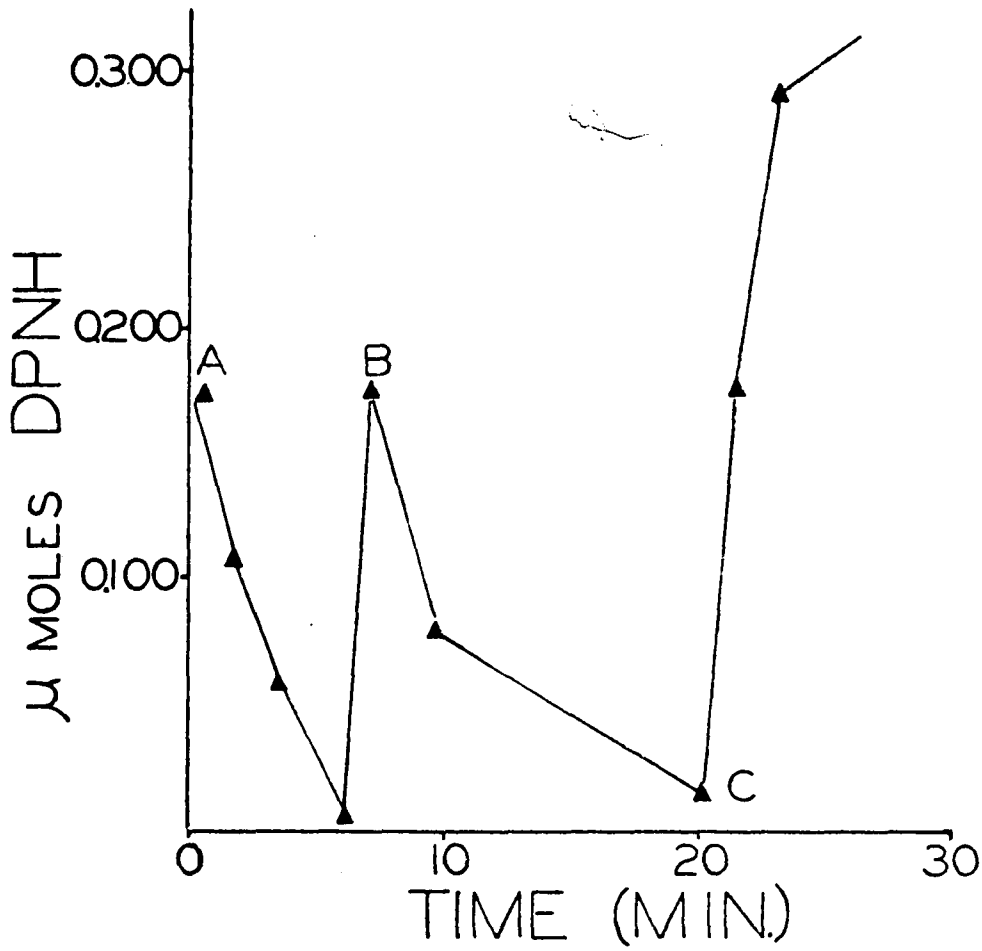

Figure 2. Production of DPN in the oxidation of DPNH. At A, 0.60 μmole of dichlorophenol was added to a system consisting of 0.016 M phosphate buffer, pH 7.0; 6 x 10^{-5} M DPNH; 5 x 10^{-5} M $MnCl_2$; 0.10 ml. of 5% uterine homogenate in a total volume of 3.0 ml. At B, 0.17 μmole of DPNH was added. At C, addition of alcohol dehydrogenase shows accumulation of DPN.

peroxidase the oxidase activity is trebled over control activity by 3×10^{-5} M Mn^{++} and completely inhibited by 10^{-3} M Mn^{++}. The change in activity with varying concentrations of Mn^{++} is shown in Figure 3. The reaction mixtures contain 0.05 M phosphate buffer, pH 7.0; DPNH, 7×10^{-5} M; 0.6 μM dichlorophenol and the concentration of Mn^{++} indicated.

TABLE 5

Manganese Effects on Oxidase Activities of Peroxidase

H-Acceptor	H-Donor	Effect of Mn^{++}
(Aerobic, without added H_2O_2)		
O_2	Triose reductone	+
O_2	Dihydroxyfumarate	+
O_2	Indoleacetic	+
Methylene blue	Triose reductone	+
Cytochrome c	Triose reductone	+
Fe^{+++}	Indoleacetic	+
(Anerobic, with added H_2O_2)		
H_2O_2	Triose reductone	-
Methylene blue	Triose reductone	-
Cytochrome c	Indoleacetic	-
Cytochrome c	Triose reductone	-
Cytochrome c	Hydroquinone	-
Fe^{+++}	Indoleacetic	-

(+) indicates stimulation (-) indicates no effect or inhibition

Akazawa and Conn (12) considered the mechanism of DPNH oxidation to be different from peroxidase-catalyzed oxidation of indoleacetic acid because a lower optimal concentration of manganese is required for the oxidation of DPNH. For indoleacetic acid oxidation the optimal concentration of manganese is of the order of 10^{-3} M to 10^{-2} M; for DPNH oxidation 10^{-5} M is the optimal concentration of manganese.

Citrate and pyrophosphate buffers inhibited the peroxidase-catalyzed oxidation of indoleacetic acid when either manganese or

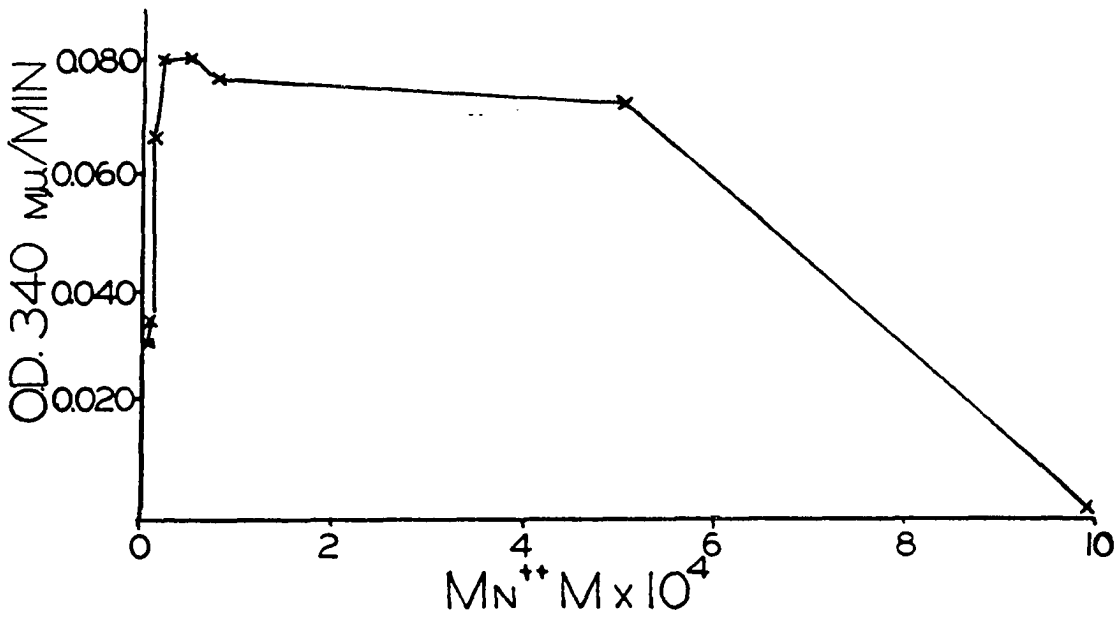

Figure 3. Effect of Mn^{++} on phenol-stimulated oxidase. Incubation conditions identical to Figure 2 except for variation in Mn^{++} concentration and initial volume of DPNH, which was 7×10^{-5} M.

cerium was used as metallic cofactors. The metals appear to give similar results.

Akazawa and Conn (12) have reported that horseradish peroxidase catalyzed oxidation of DPNH is not inhibited by citrate and pyrophosphate. They state that increase in manganese concentration increases inhibition by citrate.

Hollander and Stephens (4), using uterine peroxidase, demonstrated the failure of citrate or pyrophosphate to inhibit DPNH peroxidase catalyzed reactions. This observation seems to be inconsistent with a mechanism involving reversible oxidation of Mn^{++} to Mn^{+++}. This reversible oxidation of Mn^{++} during the oxidation of indoleacetic acid was shown in reactions catalyzed by horseradish peroxidase preparations by Kenten and Mann (37). Citrate and pyrophosphate formed stable complexes with Mn^{+++} and inhibited the aerobic oxidation of indoleacetic acid in their study.

The conversion by manganese of a peroxidase to a peroxidase-oxidase (2) which then does not require hydrogen peroxide, but requires a phenol and molecular oxygen in addition to manganese demonstrates two functions for manganese ion. The first is a redox function as with indoleacetic acid. The second function is concerned in the oxidation of DPNH and involves no change in valence state.

Phenols

Akazawa and Conn (12) first demonstrated that horseradish peroxidase can catalyze the aerobic oxidation of DPNH or TPNH. This aerobic oxidation requires Mn^{++} or Ce^{+++} and is stimulated by certain phenolic compounds (3,12,38), aromatic amines (39), and sulfite ions

(4,14). The phenolic compounds and aromatic amines are believed to act as oxidation-reduction catalysts, being alternately oxidized, probably to a free radical intermediate, by the peroxidase system and reduced by DPNH or TPNH (40).

Akazawa and Conn (12) indicate that in the oxidation of DPNH by peroxidase it seems reasonable to suggest that a ternary complex catalyzes the oxidation of the phenols by oxygen with hydrogen peroxide and an oxidized phenolic product. Though it is possible that DPNH might react with oxygen under similar conditions, several observations indicate that phenol is the reductant. There is no spectrophotometric evidence for oxidation of DPNH unless the mixture contains peroxidase, Mn^{++}, and a phenol. If the DPNH were the reductant, some indication of oxidation should be observed when no phenols were present in the reaction mixture. Several compounds other than DPNH and TPNH undergo similar aerobic oxidation in the presence of peroxidase, Mn^{++}, and certain phenols. This suggests that formation of an oxidized phenolic product is an initial step in the reaction. This product could, by the action of peroxidase, oxidize a variety of other compounds or could be further reduced by DPNH (12).

Beard and Hollander (41) indicate in their study of the oxidation of scopoletin by uterine peroxidase that a very reactive substance is generated from dichlorophenol when scopoletin is omitted from the system. This reactive substance is not a quinone since 2,4,6-trichlorophenol serves as a phenolic catalyst in the reaction and its structure precludes the formation of a quinone-hydroquinone electron transport system (4). The complete regeneration of the phenol from the enzymatic reaction

mixture is compatible with the role of a free-radical, such as (RO·) in the non-enzymatic reaction. Beard and Hollander (41) demonstrated that the phenol is not chemically altered in the presence of excess substrate in the phenol-stimulated enzymatic oxidation of DPNH.

Figure 4 (Akazawa and Conn, 12) illustrates the stimulatory effect of a particular phenol, resorcinol, on the oxidation of DPNH by horseradish peroxidase.

Akazawa and Conn (12) propose the following explanation of the action of phenols in the oxidation of DPNH by peroxidase.

$$2\ ROH + O_2 \longrightarrow 2\ RO\cdot + H_2O_2$$

$$2\ ROH + H_2O_2 \longrightarrow 2\ RO\cdot + H_2O$$

$$4\ RO\cdot + 2\ DPNH + 2\ H^+ \longrightarrow 4\ ROH + 2\ DPN^+$$

$$2\ DPNH + 2\ H^+ + O_2 \longrightarrow 2\ DPN^+ + 2\ H_2O$$

A variety of phenolic substances have been shown to catalyze the oxidation of DPNH by uterine peroxidase. Dichlorophenol was found by Temple et al. (6) to have the greatest catalytic effect of any of the phenolic cofactors studied in the oxidation of DPNH by uterine oxidase. Hollander and Stephens (4) indicate similar findings. In their studies they found the optimal pH for phenol catalyzed DPNH oxidation to be 5.6; however, to avoid acid destruction of DPNH they used pH 7.0 for their observations. Effects of varying concentrations of dichlorophenol in their system is shown in Figure 5. The reaction mixture contained 0.016 M phosphate buffer, pH 7.0; DPNH, 6×10^{-5} M; $MnCl_2$, 5×10^{-5} M; and uterine homogenate in addition to the concentration of dichlorophenol indicated.

Goldacre and Galston (42) note that the superiority of dichlorophenol in the peroxidase-oxidase system may be due to its capacity to

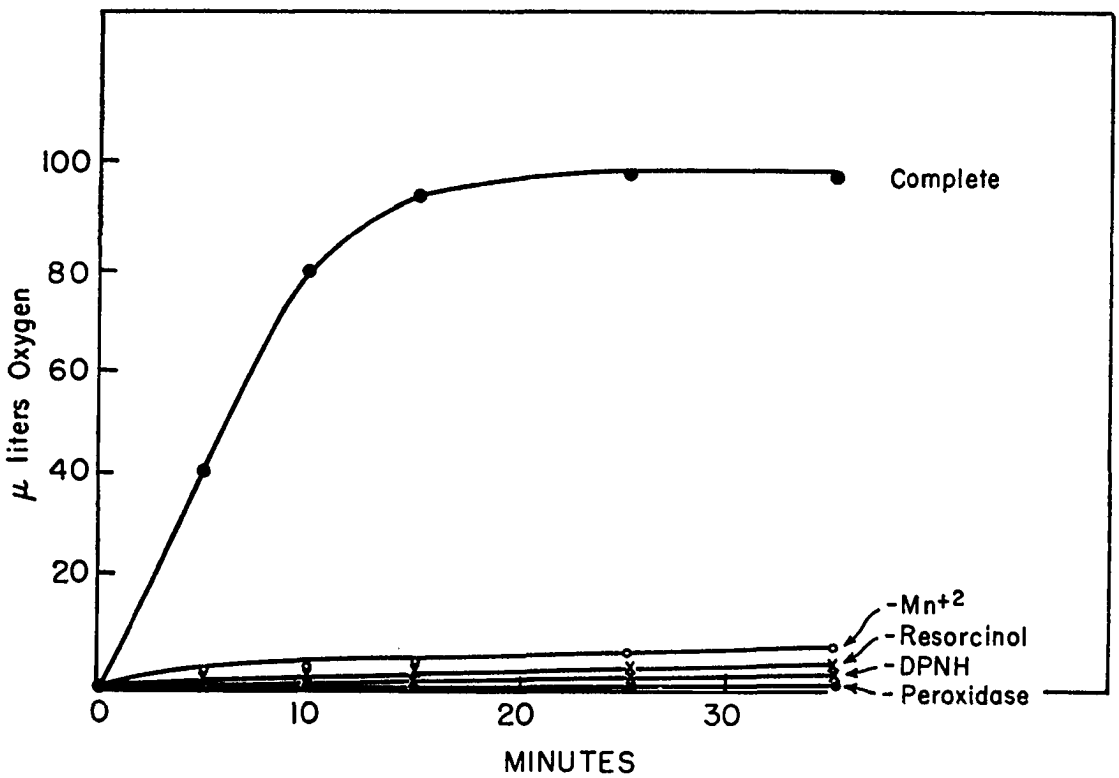

Figure 4. Effect of Resorcinal on DPNH oxidation. The reaction mixture contained 0.25 μM resorcinol; 0.3 μM $MnCl_2$; 7.6 μM DPNH and horseradish peroxidase in 0.8 M Tris buffer, pH 7.4.

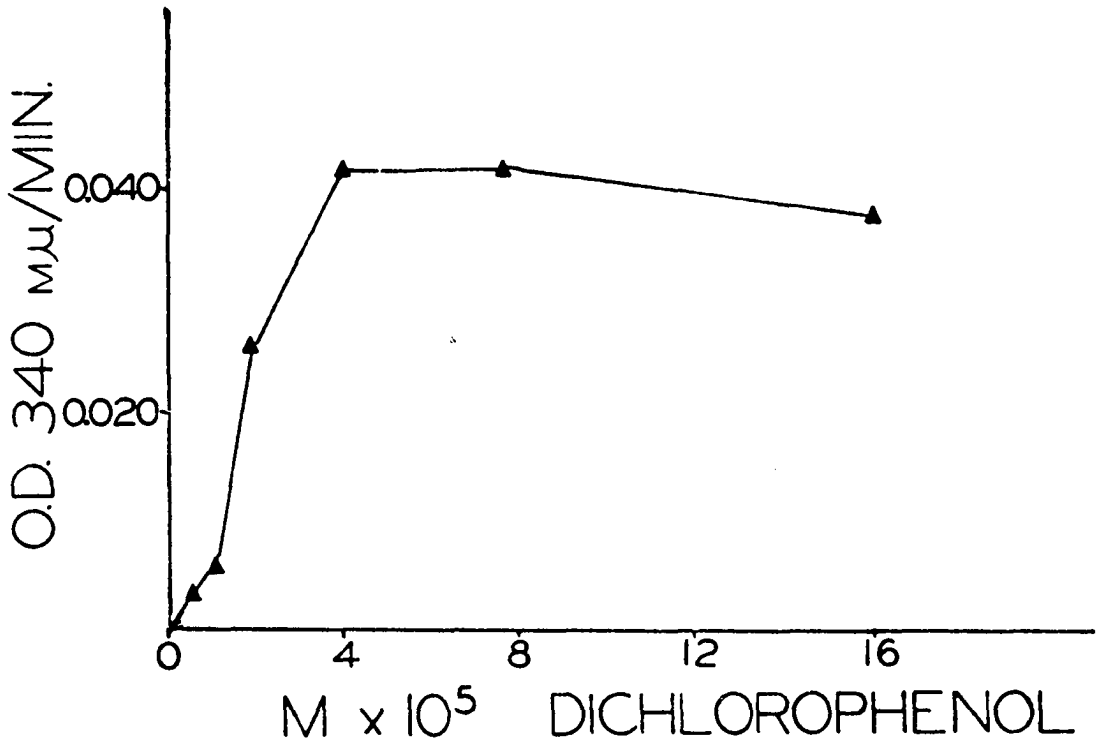

Figure 5. Effect of dichlorophenol on phenol-stimulated oxidase. Conditions identical with those of Figure 1 except variation in concentration of dichlorophenol and initial concentration of DPNH which was 7×10^{-5} M.

inhibit catalase. In this manner it prevents destruction of the hydrogen peroxide produced in the system.

Temple et al. (6) state that there is no reason to believe that the activation by phenolic estrogens affects a system different from that activated by dichlorophenol. The administration of large doses of estrogen increases the activity of phenol-activated oxidase so that the effect of the weak activator, estradiol, can be observed. Estradiol catalyzed DPNH oxidation does not go to completion, but increase in the amount of estradiol increases the final extent of the reaction. The catalytic phenol effect of estradiol is a general one and its activity in vitro bears no relationship to estrogenic activity in vivo (6).

Klebanoff and Segal (5) indicate the importance of an intact phenolic hydroxyl group in the inactivation of estradiol by the peroxidase system in their work with 17β-estradiol, estradio-3-acetate, and estradiol. They found the phenolic ester of estradiol to be inactive. They state that the protective effect of esterification in the 3-position suggests that the oxidation occurs through the phenolic hydroxyl group. In an earlier publication, Klebanoff (19) suggested that thyroxine, another phenolic compound which is capable, in physiological concentrations, of acting as a phenol catalyst of DPNH oxidation by horseradish and myeloperoxidase, may also act by means of a phenolic hydroxyl group. This is indicated by the observation that the methyl ether of a thyroxine analog is inactive in conditions where thyroxine is active.

As with dichlorophenol and estradiol stimulated DPNH oxidation by uterine peroxidase, variation in concentrations of thyroxine shows different results. Figure 6, which is from the work of Bever et al. (7),

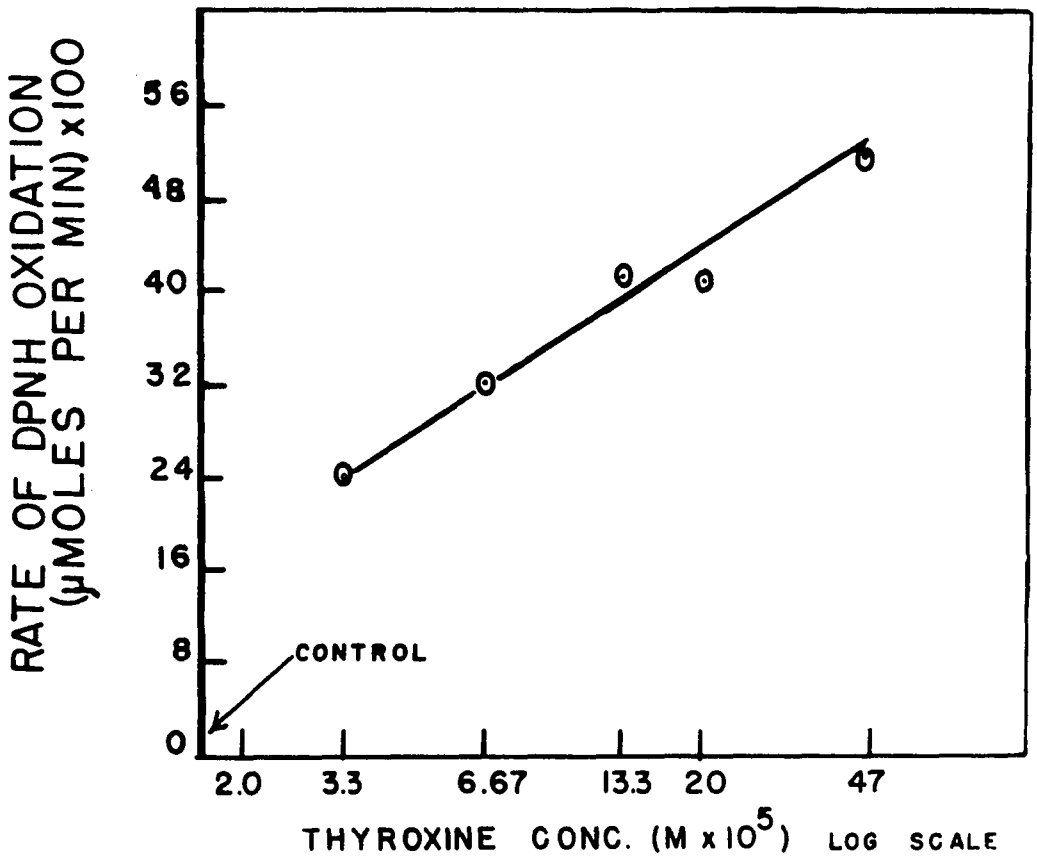

Figure 6. Effect of Thyroxine on DPNH oxidation. Reaction mixture contained 0.025 M phosphate buffer, pH 7.8; 1 x 10^{-4} M DPNH; 3.3 x 10^{-5} M Mn^{++}; and 0.1 ml.% uterine Homogenate.

demonstrates the effect of various concentrations of thyroxine in a reaction mixture containing 0.025 M phosphate, pH 7.8; DPNH, 1×10^{-4}; $MnCl_2$, 3.3×10^{-5}; 0.1 ml 5% uterus homogenate and the indicated concentration of thyroxine.

Both estradiol and thyroxine may themselves be oxidized by peroxidase in the presence of hydrogen peroxide. Under conditions in which a high accumulation of hydrogen peroxide would be expected in a manganese-DPNH system, estradiol and thyroxine may again be enzymically oxidized. The latter oxidations, i.e., without added hydrogen peroxide, require unusually favorable conditions which are not ordinarily encountered.

Bever et al.(7) state two lines of evidence which indicate that different mechanisms may be involved in dichlorophenol stimulated and thyroxine stimulated DPNH oxidation by uterine peroxidase. Their findings show optimal pH of 6.0 for uterine peroxidase, less than 6.0 for dichlorophenol stimulated DPNH oxidations, whereas for thyroxine stimulated DPNH an optimum pH of 7.4 was determined. They also found that uterine tissue from castrated rats treated with estrogen showed only peroxidase and dichlorophenol stimulated DPNH oxidation. No level of estrogen dosage was found which would restore thyroxine stimulated DPNH oxidation.

A comparison made by Bever et al. (7) of the in vitro effects of stimulation by dichlorophenol, estradiol and thyroxine on DPNH oxidation by uterine peroxidase is shown in Figure 7.

This investigation of three important phenolic stimulators indicates that the phenolic hydroxyl group is a principal requirement

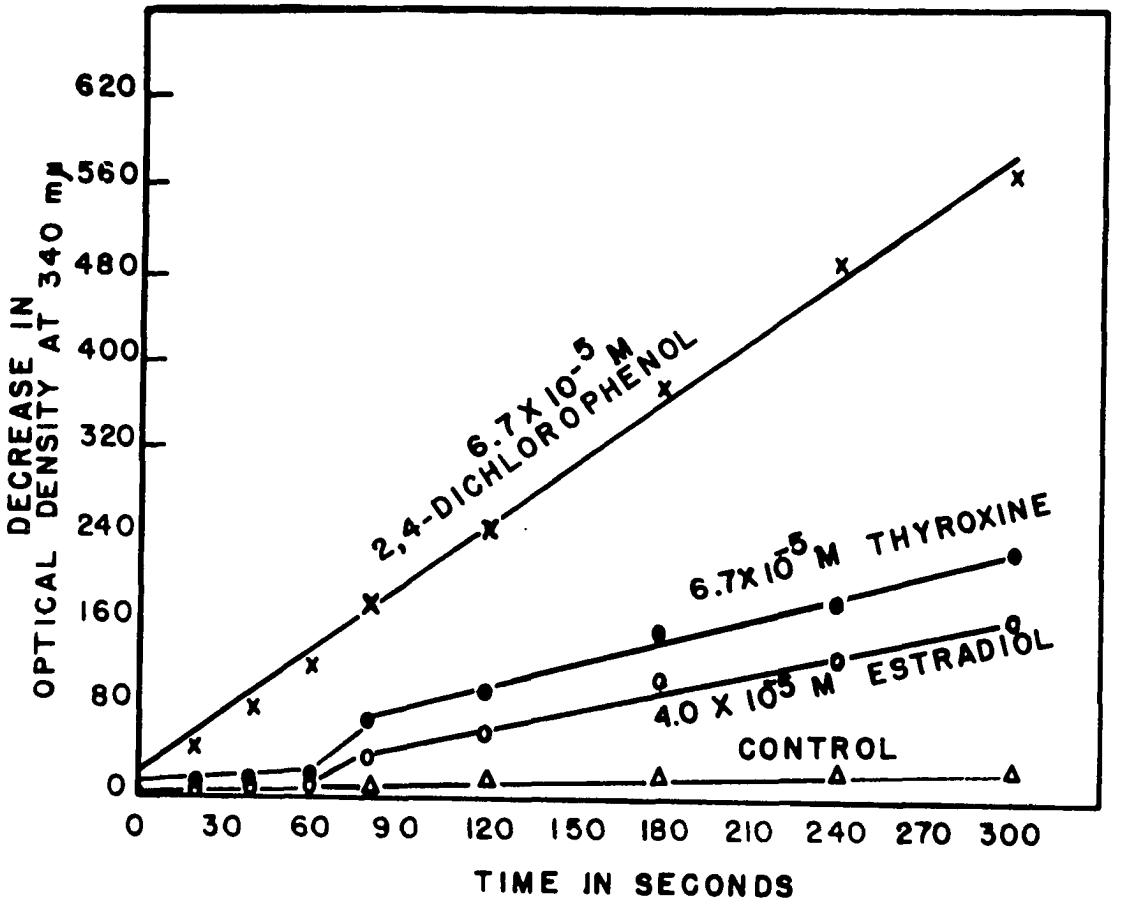

Figure 7. Stimulation of DPNH oxidation in Rat Uterus by three phenolic compounds.

for a phenolic stimulator of DPNH oxidation by peroxidase. In the following tables, compiled from Akazawa and Conn (12) and Klebanoff (3,4) are shown a number of phenolic compounds and their effects on DPNH oxidation by peroxidase.

TABLE 6

Phenolic Compounds Active in DPNH-Oxidizing Systems at Concentration of 1.7×10^{-5} M

Compound	Per cent Activity
2,4-dichlorophenol	100
2-chloro-4-phenylphenol	70
p-cresol	51
Resorcinol	44
p-chlorothiophenol	38
1-naphthol	32
3,4-dimethylphenol	27
m-cresol	17
o-cresol	15
Phenol	8
m-hydroxybenzoic acid	7
Orcinol	3

TABLE 7

Phenolic Compounds Active in DPNH-Oxidizing Systems at Concentration of 2×10^{-4}

Compound	Per cent Activity
2,4-dichlorophenol	100
o-chlorophenol	87
p-chlorophenol	82
2,4-dibromophenol	79
Phenol	58
p-benzoquinone	51
2,4,6-trichlorophenol	47
Hydroquinone	36
o-benzoquinone	33
Menadione sodium bisulfite	29
Resorcinol	23
2,6-dibromophenol	16
Tyrosine	15

TABLE 8

Phenolic Compounds Inactive in DPNH-Oxidizing System In Resorcinol Substrate	
Pyrogallol	p-toluquinol
Phloroglucinol	Naphthoresorcinol
2,6-dichlorobenzenone-indo-3-chlorophenol	Hydroxyhydroquinone
Thymol	Picric acid
	Salicylic acid

TABLE 9

Phenolic Compounds Inactive and Inhibitory in DPNH-Oxidizing System in Resorcinol Substrate	
Hydroquinone	Gallic acid
Catechol	3-hydroxycinnamic acid
o-aminophenol	3,4-dihydroxyphenylalanine
2,5-dihydroxybenzoic acid	Quercetin
2,4-dinitrophenol	2,4-dichloro-4-nitrophenol
4-methylcatechol	Chlorogenic acid
2,6-dichlorobenzenone	2,4-diaminophenol
Indophenol	2,4-dimethylphenol
3-hydroxyanthranilic acic	2-naphthol
Caffeic acid	Anthranilic acid
Dihydroxyfumaric acid*	17 β-estradiol*
Ascorbic acid*	p-nitrophenol*
Versene*	p-iodoaniline*

*Not in resorcinol substrate

Inhibitors

The use of inhibitors has been widely utilized as a tool to demonstrate the presence, characterization, and mechanism of action of a peroxidase. Peroxidase inhibition by cyanide, hydroxyl amine, and azide has been used as classical inhibitors of ferric-porphyrin enzyme (43); the light reversible carbon monoxide inhibition as characteristic of ferrous porphyrins (43) are typical examples. Table 10 is a list of compounds effective in low concentrations on the various sources and

TABLE 10

Inhibitors

Compound	Rat. Ut. Est. or Thyr. DPNH	Rat Ut. DCP DPNH	Rat Ut. Peroxidase	Luciferase Peroxidase
Cyanide	-(6)	-(4,49)	-(4)	-(10)
NH_2OH				
Azide		-(4)	-(4)	-(10)
Cu^{++}	-(4,6)	-(4)		
Catalase	-(6)	-(4)	-(4)	
Catalase (heat inactivated)				
Citrate		*(4)		
Pyrophosphate		*(4)		
Hydrogen peroxide	-(6)			
Resorcinol	-(6)			
Cysteine	-(6)			-(10)
Glutathione (Red.)	-(6)			-(10)
DPN (or TPN)	*(6)			*(10)
DPNH (or TPNH)				-(10)
PCMB				
Mn^{++}			-(2)	-(10)
Pyrogallol				-(10)
Catechol				-(10)
2-mercaptoethanol				-(10)
Indoleacetic acid				-(10)
Cystine				*(10)
Glutathione (Oxid.)				*(10)
Carbon monoxide				*(10)
Hydroquinone				
Diethyldithiocarbamate				
Ascorbic acid				*(10)
EDTA	-(4)			
Fluoride	*(4)			
Fe^{++}				
Ergothioneine				
Imidazole				
2-mercaptohistidine				
2-mercaptoimidazole				
1-methyl-2-mercapto-imidazole				
Histidine				
Sulfite	+(11)			

( ) indicates reference * = no effect + = stimulation - = inhibition

TABLE 10 (cont.)

Inhibitors					
HRP Thyroxine DPNH	HRP IAA	Ut. or HRP Sulfite DPNH, Thyr	Thyroid Peroxidase	Myelo Peroxidase	Peroxidase Sulfite Oxidation
-(12,19,5)			+(48)	-(19)	
-(12)					
-(5,19)	*(12)			-(19)	
-(3,12)		-(11)	-(50)		
-(3,12,36)	+(36)	*(11)	*(48)		-(11)
*(12)					*(11)
*(36)	-(36)	*(36)	*(50)		
*(36)	-(36)	*(36)			
-(12)				*(47)	
		-(11)			-(11)
			-(48,50)	*(47)	
-(12)					
	-(11)				-(11)
-(12)					
-(12)					
-(3)	-(11)			*(47)	-(11)
-(36)				*(47)	
				-(47)	
+(21)				+(21)	
-(21)				-(21)	
+(21)				+(21)	
+(21)				+(21)	
+(21)				+(21)	
*(21)					
-(4,11,14)					

functions of peroxidases. This compiled table is used as a basis for the following review of the mechanism difference and the site of inhibition of the peroxidase reactions.

Cyanide and azide are well known inhibitors of peroxidase because of their affinity for binding with the heme prosthetic groups of those enzymes. Carbon monoxide usually inhibits peroxidases when they function as peroxidase-oxidases, but does not inhibit most peroxidative reactions. This is because the iron in peroxidase, functioning strictly peroxidatively, is thought not to go into the ferrous state (2). Dure and Cormier (10) have demonstrated that cyanide and azide inhibition can be overcome with increasing concentration of hydrogen peroxide and therefore is indicative of the presence of a peroxidase.

Catechol, indoleacetic acid and pyrogallol are commonly known peroxidase substrates (2). If the peroxidase is capable of functioning as a DPNH oxidase, then these compounds would be expected to inhibit the reaction by competing with DPNH, or in the case of lucerifase, with luciferin. Table 10 indicates this to be the case. Other substrates having the same activity are ascorbic acid (3,10,11), resorcinol (6), hydroquinone (12) and ergothioneine (21).

Manganous ion is known to convert many peroxidases to peroxidase-oxidases (2). Inhibition by manganous ion of a peroxidase action and stimulation of its oxidase activity is also consistent with the proposal that uterine peroxidase and the phenol stimulated DPNH oxidase are identical proteins.

It has been demonstrated recently that free radicals are

produced during the enzymatic peroxidation of a variety of substrates (9). This observation is a confirmation of a previously proposed mechanism for peroxidation reactions in which free radicals were postulated as intermediates (18,44,45). Thus it is of interest that radical trappers such as 2-mercaptoethanol and cysteine also inhibit peroxidase reactions by competing with the substrate (10). The inhibition by free radical trappers has been used to identify peroxidases and as proof of their free radical mechanism (18,44).

Perhaps the most interesting and useful inhibitor of peroxidase-oxidase is catalase. Since heat-inactivated catalase does not inhibit, (3,4,6,11,12,36), the action of catalase is evidently associated with its catalytic ability (36). This inhibition by catalase has been used as evidence that hydrogen peroxide was an intermediate in the DPNH oxidation; however, attempts to detect as little as 0.05 μ molar concentration of hydrogen peroxide were completely unsuccessful (12).

Klebanoff (21) has reported that the stoichiometric oxidation of sulfite ions is initiated by peroxidase in the presence of Mn^{++} or certain phenolic compounds such as thyroxine or estradiol. This reaction is inhibited by a number of substances, i.e., GSH and hydroquinone, presumably as a result of their ability to react with free radical intermediates in the chain reaction (11). Klebanoff (11) has demonstrated the failure of sulfite oxidation to be insensitive to catalase. The mechanism of oxidation therefore must not proceed by production and subsequent utilization of hydrogen peroxide, and consequently sulfite oxidation cannot be considered a true peroxidase or peroxidase-oxidase mechanism.

The stimulatory effect of ergothioneine on DPNH oxidation by horseradish peroxidase is shared by 2-mercaptohistidine, 2-mercaptoimidazole and 1-methyl-2-mercaptoimidazole but not by histidine or imidazole (21). The sulfhydryl radical is the initial oxidation product of ergothioneine, and the operation of an ergothioneine-ergothioneine sulfhydryl radical oxidation-reduction system would be analogous to the systems proposed for the catalytic effect of certain monophenols (11, 12,38) and aromatic amines (46) on the aerobic oxidation of DPNH by peroxidase. Klebanoff (21) points out, however, that his data do not support, and, in fact, contraindicates an ergothioneine-ergothioneine disulfide oxidation-reduction system.

The chelation effect of EDTA (4,36) would be anticipated in view of the role of Mn^{++}. Hollander and Stephens (4) report that DPNH oxidation is not inhibited by citrate or pyrophosphate. The latter authors have utilized this chelation effect as evidence for a manganese valence change in the oxidation of indoleacetic acid, while DPNH oxidation depends only upon Mn^{++}. One further difference is with respect to catalase. DPNH oxidation is inhibited by catalase; indoleacetic acid oxidation is stimulated by catalase.

Very small quantities of hydrogen peroxide (10^{-9}M) stimulate the DPNH oxidase activity of peroxidase (6). This stimulation is limited to the elimination of an initial lag time reaction period. Temple *et al.* (6) postulates that this lag period is due to the time required for production of hydrogen peroxide by the Mn^{++}, phenol and O_2 system. At the end of this lag period further additions of hydrogen peroxide became increasingly inhibitory.

Schultz and Rosenthal (47) show that in the presence of oxygen ferrous ion inactivates purified preparation of myeloperoxidase. Data are presented which suggest that the mode of inactivation may be through the formation of oxygen reduction products. Horseradish peroxidase is not inhibited under similar conditions.

DPN (6,10) or TPN (10), respective products of DPNH or TPNH oxidation by peroxidase-oxidases, do not inhibit the former reaction.

Klebanoff (21) has suggested that imidazole is inhibitory to DPNH oxidation by horseradish peroxidase, and in the same report (21) indicated that histidine and imidazole are inactive as stimulants.

Thyroid peroxidase is insensitive to cyanide and catalase (48). Sulfite-potentiated DPNH oxidation by thyroxine stimulated uterine or horseradish peroxidase is catalase insensitive (11). Oxidation of indoleacetic acid by horseradish peroxidase is stimulated by catalase (36) and is azide insensitive (12). Myeloperoxidase is not inhibited by cysteine, ascorbic acid or EDTA (47). Luciferin peroxidase is carbon monoxide insensitive; it is inhibited by Mn^{++} and is therefore functioning simply as a peroxidase instead of a peroxidase-oxidase. Uterine peroxidase is inhibited by cyanide, azide, catalase, ascorbic acid, EDTA, and cysteine when functioning as a phenol activated DPNH oxidase (4,6).

This summary is intended to demonstrate that different sources of peroxidases respond dissimilarly to inhibitors and are not necessarily mediated by the same mechanism. It is to show that the inhibitory effects of cysteine, GSH, and 2-mercaptoethanol have been considered as inhibitory only to the free radical mechanisms. In addition, no

distinction could be made on the basis of the reported inhibitors to a difference between dichlorophenol and thyroxine or estradiol activated uterine peroxidase-oxidase mechanism or active site requirements.

Mechanism

Peroxidase

Peroxidase is an example of the mechanism shown in the following equation:

$$E + A \rightleftharpoons EA$$
$$EA + B \rightleftharpoons E + C + D$$

A Michaelis constant has been obtained for hydrogen peroxide, but the velocity is proportional to the concentration of hydrogen donor (52).

In a few cases, it is possible to determine the concentration of the complex ES directly, usually by spectrophotometric means. This field has been developed principally by Chance (52,53). Peroxidase has been shown by Keilin and Mann (54) to change its absorption spectrum when combined with hydrogen peroxide.

There are four distinct types of complexes that have been distinguished which bear relationship on the mechanism of peroxidase. Two of these complexes (III and IV) are red compounds formed with excess of hydrogen peroxide; these complexes are catalytically inactive and the reaction is inhibited when they are formed. The conversion of Complex I to Complex II proceeds more rapidly in the presence of a hydrogen donor and may therefore be assumed to contribute to the formation of the product of the reaction (52).

As to the chemical nature of the two complexes, there are two different hypotheses supported by two groups of investigators. Chance

(53) formulated Complex I as $Fe_p^{+++}(H_2O_2)$, i.e., an addition compound of the enzyme with hydrogen peroxide; the subscript p denotes that the iron forms a part of the haemoprotein enzyme. Complex II is considered to be $Fe_p^{+++}(OH\cdot)$, where the group $(OH\cdot)$ is merely written as a symbol for half reduced hydrogen peroxide. Electronic resonance absorption measurements show that Complex II does not contain a free radical, and in fact, Yamazaki et al. (9) have recently shown that AH_2 as the hydrogen donor does form a free radical. Thus the nature of Complex II is uncertain in Chance's formulation.

George (52,55), on the other hand, believes that the hydrogen peroxide oxidizes the iron in the enzyme to a valency state higher than that normally found, and suggests that Complex I contains quinquevalent and Complex II quadrivalent iron.

The following mechanism has been proposed by George (44) and by Yamazaki, Mason and Piette (9) for the peroxidatic oxidation:

$$Fe_p^{+++} + H_2O_2 \xrightarrow{k_1} \underset{\text{Complex I}}{(Fe_pO)^{+++}} + H_2O$$

$$\underset{\text{Complex I}}{(Fe_pO)^{+++}} + AH_2 \xrightarrow{k_2} \underset{\text{Complex II}}{(Fe_pO)^{++}} + \cdot AH$$

$$\underset{\text{Complex II}}{(Fe_pO)^{++}} + AH_2 \xrightarrow{k_3} \underset{\text{Peroxidase}}{Fe_p^{+++}} + \cdot AH$$

$$2\ \cdot AH \xrightarrow{k_d} A + AH_2 \quad (\text{or } HA\cdot AH\cdot)$$

where AH_2 is the substrate electron donor, $AH\cdot$ the free radical intermediate such as semiquinone, and A is the oxidized product.

In constrast to the mechanism of Chance, this process involves an oxidation and reduction of the enzyme. This is supported by George's observation that under certain conditions one molecule of hydrogen

peroxide gives rise to considerably more than one molecule of Complex II, which he explains by an oxidation of free enzyme by Complex I as follows (55):

$$\underset{\text{Peroxidase}}{Fe_p^{++}} + \underset{\text{Complex I}}{Fe_pO^{+++}} + H_2O \longrightarrow 2\,(Fe_pO)^{++} + 2H^+$$

Chance objects to George's mechanism on the ground that all component reactions are independent of pH and he considers that the existence of such higher oxidation states of iron is doubtful (52).

The general results of the direct studies of enzyme-substrate compounds of peroxidases prove that the rate of these reactions is governed by the concentration of active enzyme-substrate compounds.

The reaction mechanism differs, however, rather dramatically from that of invertase and other enzymes in which there occurs a rapid equilibrium of enzyme and substrate and an occasional spontaneous decomposition of the enzyme-substrate complex. In the reactions of the peroxidases, the combination of enzyme and substrate is very tight; the reversible reaction plays no significant part in the enzymatic activity. Furthermore, the enzyme-substrate complex, once formed, does not spontaneously decompose at a rapid rate; it is relatively stable until collision with the donor molecule occurs. The kinetics of peroxidases involve the consecutive reactions of substrate and donor molecule with the enzymes and with the enzyme-substrate complex. Since these two reactions may be equally effective in determining the activity, a specification of the activity of the peroxidases requires a measurement of both the velocity constant for the formation of the enzyme-substrate complex and for the reaction of the enzyme-substrate complex with the donor molecule. In addition, the classical Michaelis constant for the

enzyme-substrate complex becomes of little value in specifying the nature of the enzyme. For the peroxidases the Michaelis constant is a function of the donor concentration and, in fact, may be linearly related to it. The concept of optimum activity as applied to peroxidases has little meaning; theoretically the optimum activity would be found only at infinite substrate and donor concentrations--a satiety seldom achieved physiologically (52,53).

Peroxidase-Oxidase

As previously discussed in the section on manganese, manganese has the ability to convert peroxidases, which requires hydrogen peroxide, to peroxidase-oxidase (2). The latter then requires a phenol and molecular oxygen in addition to manganese for its oxidase function. In the oxidation of DPNH or TPNH, manganese undergoes no change in valence state (4,12). Divalent manganese can be replaced only by Ce^{+++} (35).

According to Yamazaki and Souzu (18) manganese appears to promote reaction between free radicals and oxygen to form hydrogen peroxide; however, hydrogen peroxide will not replace Mn^{++} in aerobic reactions (34). The stoichiometrics of Mn^{++} concentration (10^{-6} M) indicate a catalytic effect (4).

The structure of the final products of peroxidatic oxidation (2,56), the stoichiometrics and relative rate of reduction of peroxidase Compounds I and II (44,57), and the redox properties of substrate intermediates (45) make it likely that the intermediates are free radicals.

Recently Yamazaki et al. (9) have provided direct physical

evidence, with electron paramagnetic resonance spectroscopy, that free radicals are produced from substrates during the enzymatic peroxidation of a variety of substrates. This observation confirms a previously proposed mechanism for peroxidation reactions which postulated free radicals as intermediates (18,44,45). Yamazaki et al. (9,18) suggest that, in the main, the free radicals formed in this system are not combined with the enzyme, but exist free in solution.

Other additional supportive evidence for this free radical mechanism has been furnished by the fact that radical trapping compounds such as 2-mercaptoethanol (10), cysteine (6,10,12), and GSH (11) inhibit phenol stimulated DPNH peroxidase-oxidase. Presumably this inhibition is a result of their ability to react with free radical intermediates in the chain reaction produced from either the phenol or DPNH or both.

Oxidation-reduction systems have been proposed for the catalytic effect of certain monophenols (11,12,38) and aromatic amines (39) on the aerobic oxidation of DPNH by peroxidase. These compounds are believed to act as oxidation-reduction catalysts, being alternately oxidized and reduced by DPNH (or TPNH) probably to a free radical intermediate by the peroxidase system. It has been proposed (4) that DPNH is oxidized by highly reactive intermediates, and that this is a chain reaction initiated by free radicals generated by certain peroxidase catalyzed reactions (4,14,58).

Akazawa and Conn (12) suggested that a ternary complex of peroxidase, Mn^{++}, and H_2O_2 catalyzes the oxidation of the phenol (ROH) by oxygen to an oxidized form (RO·), (the latter presumably of a free radical nature) and H_2O_2. By the action of peroxidase, hydrogen

peroxide could then oxidize another molecule of the phenol to the oxidized (free radical) form. Reduced DPN could further reduce the oxidized phenol. According to Williams-Ashman, Cassman and Klavins (38), the process can be envisioned as:

$$2\ ROH + O_2 \longrightarrow 2\ RO\cdot + H_2O_2$$

$$2\ ROH + H_2O_2 \longrightarrow 2\ RO\cdot + 2\ H_2O$$

$$4\ RO\cdot + 2\ DPNH + H^+ \longrightarrow 4\ ROH + 2\ DPN^+$$

$$2\ DPNH + 2H^+ + O_2 \longrightarrow 2\ DPN^+ + 2\ H_2O$$

On the other hand, Yamazaki and Souzu (18) proposed a scheme for the promoting effect of Mn^{++} and substrate XH_2 on the oxidase reactions catalyzed by peroxidase (Figure 8). As illustrated in the figure, (H_2O_2) shows a trace amount of H_2O_2 which initiates the reaction. Mn^{++} may promote the reaction between the free radicals and O_2 to form H_2O_2. XH_2 promotes the oxidase reaction when YH radical is produced more rapidly by the reaction of YH_2 with XH radical than the direct peroxidase reaction of YH_2 (45). Although the ratio of one-half mole of O_2 per mole of YH_2 is correct, this mechanism could allow for accumulation of large amounts of H_2O_2 (18).

Molecular oxygen is consumed by the peroxidase oxidation of DPNH in a ratio of one-half mole of O_2 per mole DPNH (3,4). DPNH is not considered to be the direct reductant of O_2, but rather phenol (12). The initial step in the reaction is considered to be formation of the oxidized phenolic product (RO·)(12,41).

Studies with the three more commonly used phenols, dichlorophenol (41), thyroxine (19), and estradiol (5) have demonstrated the necessity of a free aromatic hydroxyl group, and that this phenol is not chemically

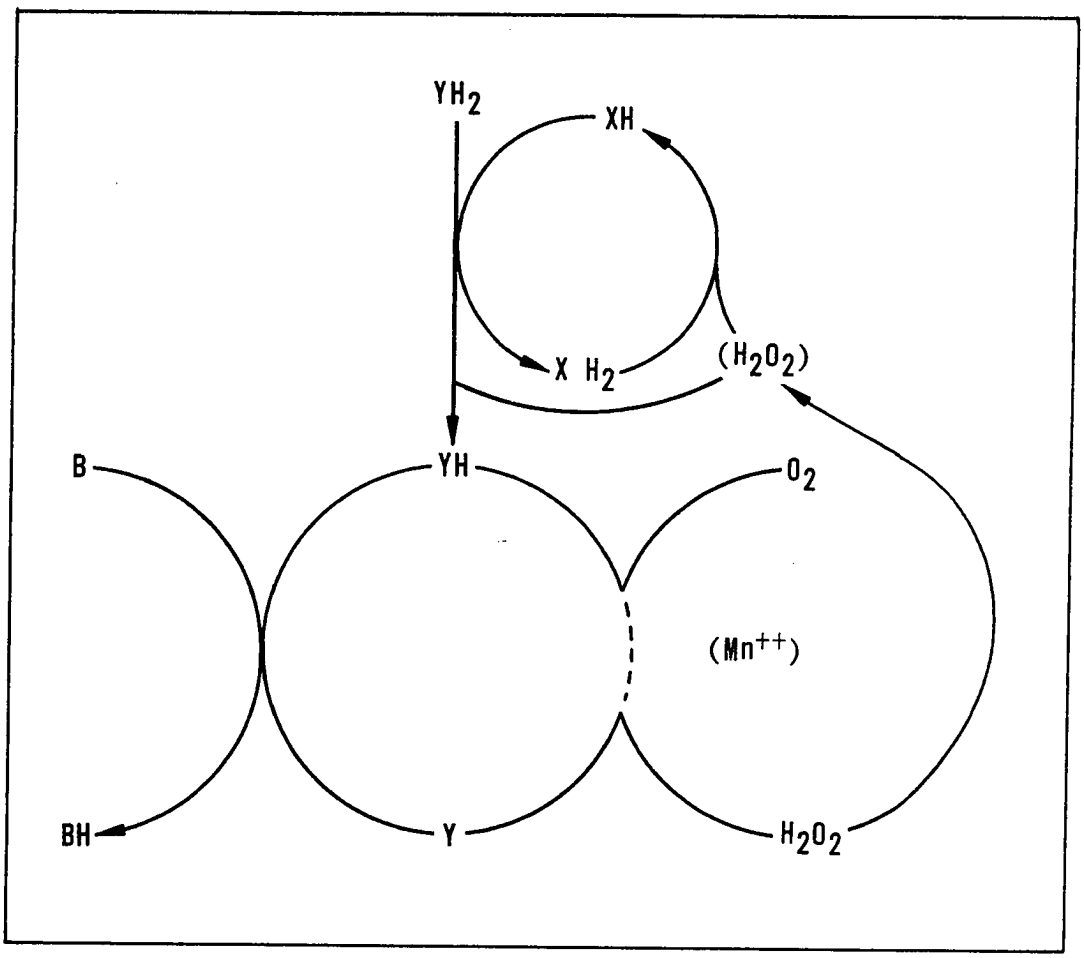

Figure 8. Effect of Mn^{++} and substrate XH_2 on oxidase reactions catalyzed by Peroxidase.

altered by the total reaction. These data were obtained by substitution for, esterification of, or deletion of the phenol hydroxyl group. In each case, the phenolic stimulation is lost.

In summary, peroxidase is converted to a peroxidase-oxidase by Mn^{++}. Mn^{++} can be replaced by Ce^{+++}. Mn^{++} does not undergo a redox reaction during DPNH oxidation. A rather non-specific phenol group is required. The phenol requirement seems to be only for a free aromatic hydroxyl group. The phenol is not chemically altered by the reaction but does temporarily contribute a free radical. A free radical mechanism is involved. Molecular oxygen (one-half mole/mole DPNH) is consumed, apparently utilized to form H_2O_2. DPNH (or TPNH) is readily oxidized to DPN (or TPN).

Preparation of Sample

Lucas et al. (1) prepared homogenates from mature, ovariectomized, estrogen-stimulated rats. The uteri were quickly removed, stripped of fat and chilled. A 20% homogenate in cold 0.1 M Na_2HPO_4-KH_2PO_4 buffer, pH 7.4, was prepared in a chilled, all-glass homogenizer. Hollander and Stephens (4), Temple et al.(6), and Hollander et al. (28) prepared 5% homogenates in 0.25 M sucrose using similar techniques. Lucas et al.(1) reported that homogenates prepared in distilled water undergo rapid loss of activity. Hollander et al.(4,28) filtered homogenate through nylon gauze before testing it.

Temple et al.(6) centrifuged homogenate for 10 minutes at 500 x g and found that the supernatant liquid retained its activity for several hours. Hollander and Stephens (4) fractionated the homogenate by centrifugation at 500, 5,000 and 100,000 x g and found no

great separation of activity in the particulate fractions. They observed that dialysis resulted in variable loss of activity.

Effective isolation and purification of peroxidase has thus far been relatively unsuccessful.

Beard and Hollander (41), who report purifications of 50-100 fold, prepared 20% homogenates in 0.005 M phosphate buffer, pH 7.0, containing 0.001 M Versene. The homogenates were centrifuged at 600 x g, frozen to solubilize activity, thawed, filtered through gauze and dialyzed against 0.005 M phosphate, pH 7.0, containing 0.001 M Versene and 0.15 M NaCl, then eluted with NaCl by gradient elution techniques. This procedure may be contrasted with reports that versene inhibits peroxidase activity by 77% in concentrations of 0.006 M and that freezing the homogenate causes a 50% loss of activity (4).

Lucas et al. (1) also noted that homogenates prepared in distilled water show rapid loss of activity and those prepared in 0.1 M Na_2HPO_4-KH_2PO_4 buffer, pH 7.4, remained essentially constant for several hours in the cold.

Hollander and Stephens (4) indicate that dialysis for 4 hours at 2° C. results in variable loss in activity. The loss of activity is considered to be due to removal of an endogenous amount of hydrogen peroxide, since addition of a catalytic amount of hydrogen peroxidase results in the oxidation of more DPNH than would be attributed to a stoichiometric reaction with peroxide.

Particulate fractions collected by centrifugation at 500, 5,000 and 100,000 x g contained phenol-activated DPNH oxidase and uterine peroxidase. These fractions were found to lose phenol-

activated DPNH oxidase activity more rapidly than uterine peroxidase activity was lost during storage at -20° C. (4).

pH

Variations in optimal pH values for different reactions have been observed by many. Temple et al. (6) indicated that the optimal pH of activation of phenol-stimulated DPNH oxidation depends on the nature of the cofactor.

Lucas et al. (1) found 6.0 to be the optimal pH for the oxidation of leuco dye by peroxidase. At this pH neither the rate of autooxidation nor the uncatalyzed oxidation of the dye by peroxide was significant.

Hollander and Stephens (4) determined that the optimal pH for phenol-catalyzed DPNH oxidase reaction was 5.6; however, to avoid acid destruction of DPNH most observations were carried out at pH 7.0.

Bever et al. (7) report that a pH study of thyroxine-stimulated DPNH oxidase showed a peak at pH 7.4 with an uncharacteristic shoulder in upper pH levels, as shown in Figure 9. This pH may be contrasted with the optima of uterine peroxidase at pH 6.0 and dichlorophenol stimulated DPNH oxidation at pH less than 6.0. Figure 10 illustrates the different pH optima for peroxidase and for peroxidase stimulated by estradiol and dichlorophenol. This figure is compiled after Lucas et al. (1), Hollander and Stephens (4), and Temple et al. (6).

Buffer Effects

The buffer most commonly used in assay mixtures for peroxidase oxidations is a phosphate buffer with pH ranging from 6.0-7.7. Klebanoff (21) found that glycylcine, tris, arsenate, and imidazole buffers were relatively ineffective and imidazole buffer had a strong inhibitory

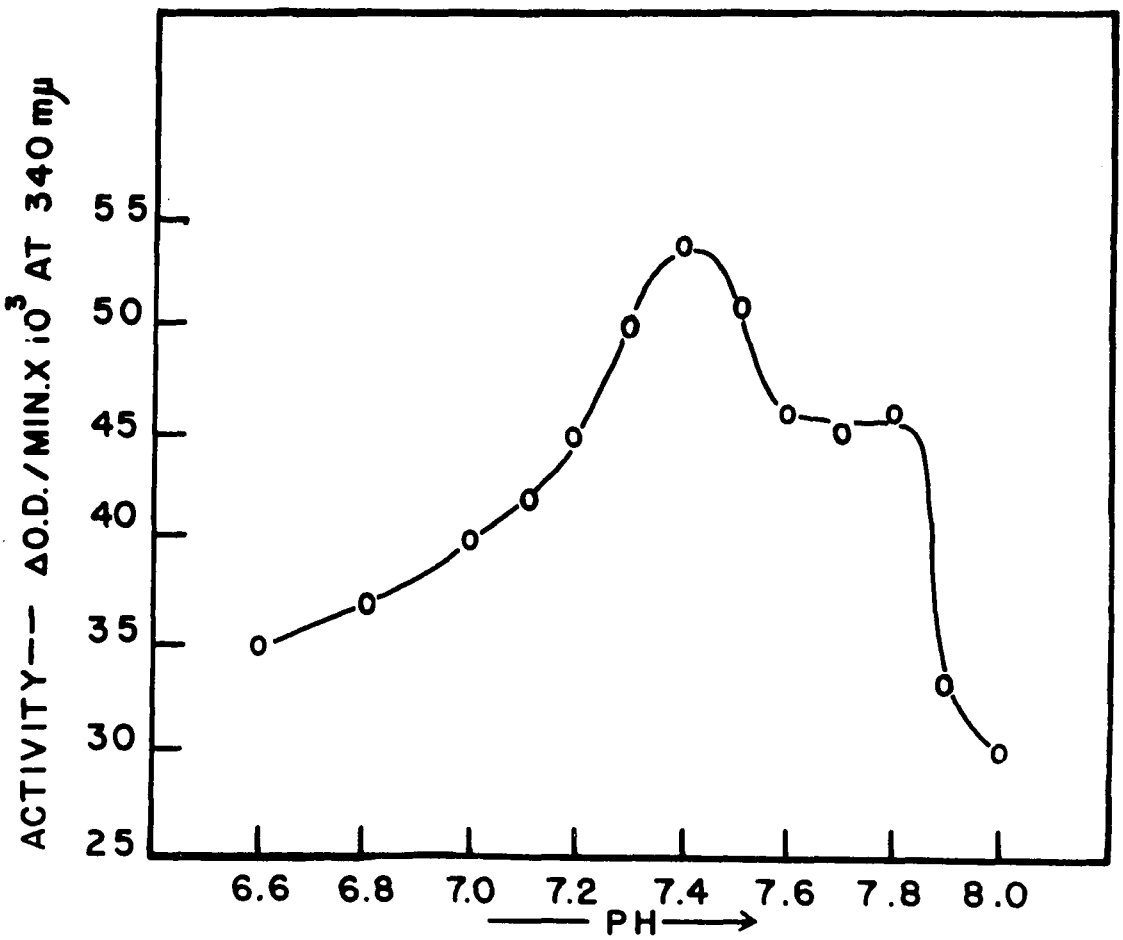

Figure 9. pH Effect on Activity of Thyroxine stimulated DPNH oxidase of Rat Uterus.

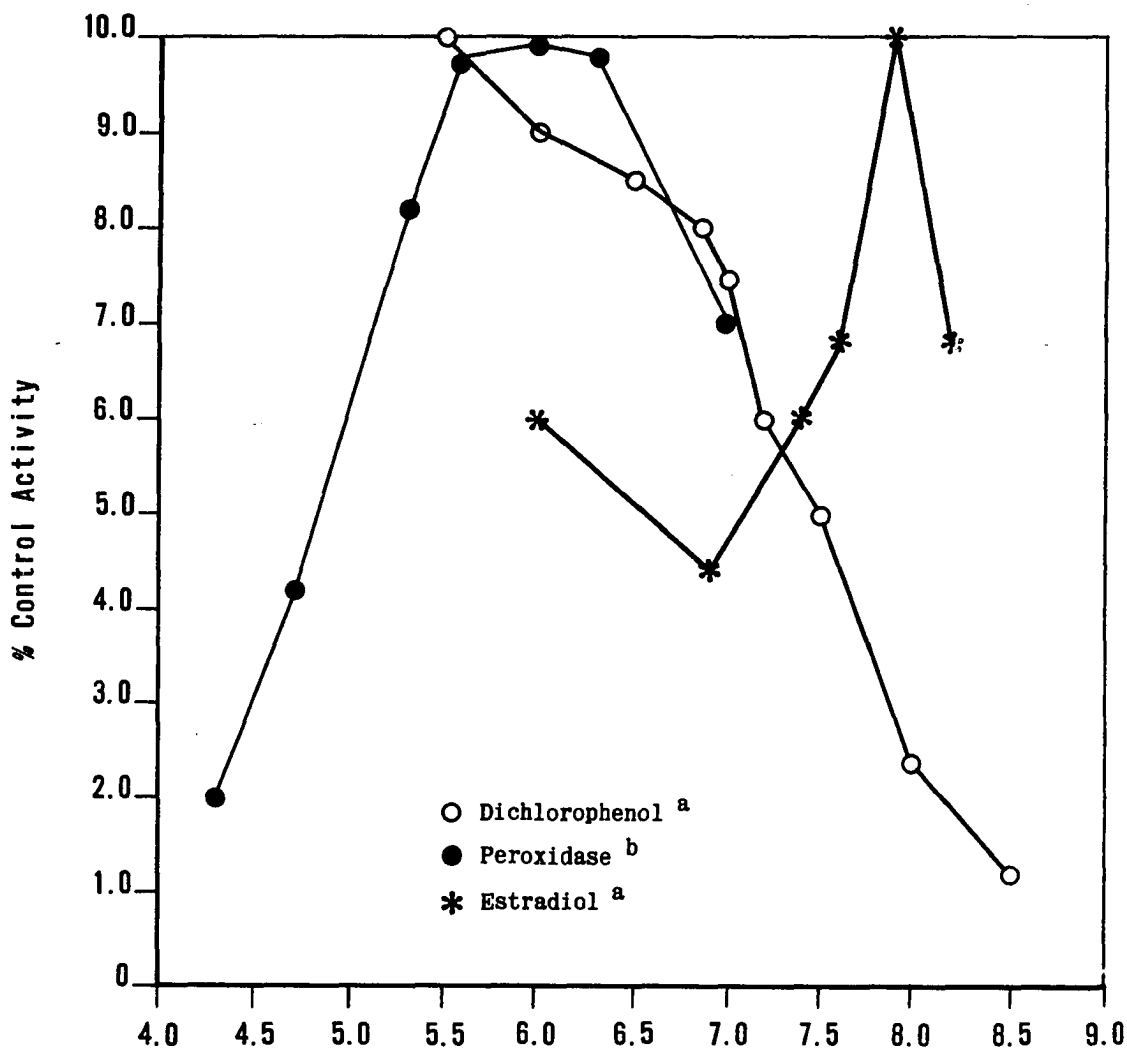

Figure 10. pH optima for Peroxidase and for Peroxidase stimulated by dichlorophenol and estradiol. This figure was compiled after Lucas et al (1), Hollander and Stephens (4), and Temple et al (6). a. Based on ΔO.D. 340 Mμ/min. b. Based on ΔO.D. 650 Mμ/min.

effect on ergothioneine oxidations when used with phosphate buffer. Lucas et al. (1) used 0.2 M phosphate-citrate buffer (McIlvaine's) at pH 6.0.

Klebanoff (21) observed that the peroxidase oxidation of ergothioneine indicated some dependence on certain buffers, and that phosphate was the most effective buffer.

Bever et al. (7) reported that borate buffer has little effect on peroxidase and thyroxine activities, but inhibits DPNH oxidase reactions stimulated by estradiol and dichlorophenol, as shown in Table 11.

TABLE 11

Borate Ion Effects

Stimulator	Buffer*	pH	Activity	% Inhibition
Dichlorophenol	Phosphate	7.0	180	---
Dichlorophenol	Borate	7.0	60	33
Thyroxine	Phosphate	7.8	40	---
Thyroxine	Borate	7.8	43	0
Estradiol	Phosphate	7.6	44	---
Estradiol	Borate	7.6	24	45

* Buffers 0.025 M

Upon investigating the cause of the borate ion effect, Bever et al. (7) determined that it was not the presence of borate but the absence of phosphate which produced the inhibition, since it was found that tris and glycylcine buffers in the absence of phosphate did not allow full activity. Table 12 and 13 illustrate the phosphate requirement of dichlorophenol and thyroxine stimulated oxidation.

Baker and Schultz (8) reports that under certain conditions the inactivation of peroxidase is Pi dependent. It was shown that a dialysable factor enhances the inactivation. Baker and Schultz

state that under the latter conditions there is a disappearance of Pi along with the inactivation process. ADP or ATP, but not AMP, can replace the Pi in the inactivation process.

TABLE 12

Effect of Phosphate on Dichlorophenol Stimulated DPNH Oxidase

Buffer System		Activity	
M Tris	M Phosphate	ΔO.D. x 10^{-3}	Per Cent Inhibition
0	2.5×10^{-2}	104	0
.025	0	31	70
.025	1.6×10^{-3}	57	45
.025	3.2×10^{-3}	77	26
.025	1.0×10^{-2}	105	0

TABLE 13

Effect of Phosphate on Thyroxine Stimulated DPNH Oxidase

Buffer System	Activity	% Inhibition
0.025 M Tris, No phosphate, pH 7.8	75	33
0.025 M Phosphate, No tris, pH 7.8	112	0

CHAPTER III

MATERIALS AND METHODS

Materials

Albino female rats weighing 140-160 grams were obtained from Holtzman Rat Company, Madison, Wisconsin.

The chemicals were obtained from the indicated sources: Oxidized and reduced di-and tri- phosphopyridine nucleotides, adenosine-5'-triphosphate, L-thyroxine, p-chloromercuribenzoate, o-iodosobenzoic acid, 17β-estradiol, and Sigma 7-9 Tris buffer--Sigma Chemical Company, St. Louis, Missouri; adenosine monophosphate, adenosine diphosphate--Pabst Brewing Company, Milwaukee, Wisconsin; o-dianisidine, 2-mercaptoethanol, thioglycolic acid--Eastman Kodak Company, Rochester, New York; thioglycerol--Evans Chematics Corp., Waterloo, New York; bovine albumin--Armour Labs., Kankankee, Ill.; imidazole and N-ethyl maleimide--California Corp. for Biochemical Research, Los Angeles, California; imidazole acetic acid--K & K Labs., Inc., Jamaica, New York; p-chloracetophenone--Matheson, Coleman and Bell, Norwood, Ohio; oxidized and reduced glutathione, L-cystine, cystamine, homocystine, D- and L- histidine, and iodoacetate--Mann Research Labs., Inc., New York; Sephadex G-25 and G-50--Pharmacia, Uppsala, Sweden.

Methods

Preparation of Sample

Albino rats of the Holtzman strain weighing 140-160 grams were utilized. Animals were estrogen "primed" by three daily subcutaneous injections of 10 µg of 17β-estradiol contained in 0.1 ml of sesame oil. The animals were sacrificed on the fourth day; the uteri were quickly removed and stripped of fat, connective tissue and blood vessels. All subsequent solutions and preparation steps were carried out at 5° C.

A 10% homogenate of fresh uteri (weight by volume) was made in 0.05 M Na,K-phosphate buffer, pH 6.0, in a Kontes all-glass homogenizer. The homogenate was centrifuged at 300 x g for 10 minutes, the supernatant discarded, and the sediment homogenized with 0.05 M phosphate buffer, then with 0.25 M sucrose and with 1.1% KCl. The supernatant is a very active source of peroxidase and dichlorophenol stimulatable DPNH oxidase activity.[1] The fraction insoluble in 5% KCl, when resuspended in 5% KCl or 0.5 M phosphate buffer, pH 6.0, is an active source of thyroxine stimulatable DPNH oxidase activity.

Demonstration of Ionic Strength Extraction Basis

The ionic strength of 0.5 M Na,K-phosphate buffer, pH 6.0, is 0.62 M. Equal ionic strength solutions were prepared of potassium chloride, sodium chloride and magnesium chloride. The sample was

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[1]Hereafter the following abbreviations are used: dichlorophenol or thyroxine activities refer to dichlorophenol or thyroxine stimulated DPNH oxidase activity.

prepared as described previously except that before centrifugation of the 1.1% KCl extraction preparation, the mixture was dividied into four equal amounts, each of which was then centrifuged. The supernatant was discarded. Equal volumes of the 0.5 M phosphate buffer, sodium chloride, potassium chloride and magnesium chloride were used individually to test their extraction effectiveness. Extraction was achieved by homogenizing the 1.1% KCl insoluble precipitate in a cold solution of each of the salts for 60 seconds, then centrifuging for 10 minutes at 500 x g. The supernatant was subsequently analyzed for peroxidase, dichlorophenol, thyroxine and protein. The experiment was repeated three times and each sample analyzed in triplicate.[2]

Dialysis

Samples were placed in dialysis tubing which had been pretreated by washing in 1 N HCl, 1 mM EDTA, and thoroughly rinsed in de-ionized water. The tubing was placed in 3 liters of distilled water or 1 mM EDTA, stirred slowly in the cold for 2 to 24 hours against three changes of water. The material inside the dialysis bag was centrifuged at 500 x g for 10 minutes to separate the insoluble material. This sediment was suspended by homogenation in 5% KCl.

Peroxidase Assay

The assay procedure used was developed by Worthington[3] and is based upon the use of o-dianisidine as the hydrogen donor. The assay

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[2]Unless otherwise indicated, all experiments reported in this present work were repeated three times and each analysis within the experiment was carried out in triplicate.

[3]Enzyme Descriptive Manual No. 11, Worthington Biochemical Corporation.

mixture contains 5.9 ml of 0.01 M H_2O_2 with 0.01 M phosphate buffer, pH 6.0; 0.05 ml 1% o-dianisidine in methanol and 0.1 ml of enzyme to be tested. For tests in which no phosphate was desired, the hydrogen peroxide was in 0.01 M tris buffer, pH 6.0. The rate of color development was measured spectrophotometrically at 460 mμ using a Bausch and Lomb Spectronic 340 instrument. The reaction was started with addition of sample and allowed to run for 2 minutes in most of the tests. A few selected reactions were followed for 5 or 10 minutes.

Specific activity of the samples was calculated in terms of change in optical density per minute at 460 mμ per gram of protein contained in the sample. Protein was measured by Lowry's method (59).

Inhibition studies were done in a manner similar to those with dichlorophenol except that the pH was adjusted and other changes were made which were necessary to adapt methods to conditions for peroxidase assay.

Dichlorophenol Assay

The normal assay mixture consisted of 3.0 ml of 0.05 M phosphate buffer, pH 7.0; 0.2 ml $MnCl_2$, 2 x 10^{-3} M; 0.2 ml dichlorophenol, 2 x 10^{-3}M; 0.6 ml DPNH, 1 mg/ml; sample and water to total 6.0 ml. In tests where no phosphate was desired 0.05 M tris buffer, pH 7.0, was used. In studies where given concentrations of phosphate were needed, micromolar amounts, usually 25 μM, of phosphate buffer replaced part of the tris buffer volume.

The reaction was started by the addition of DPNH and the disappearance of DPNH was followed spectrophotometrically for 2 minutes or longer with a Bausch and Lomb 340 at 340 mμ.

Various inhibition studies were carried out utilizing a 10 minute incubation period of enzyme, buffer, and inhibitor. In assays carried out in the presence of cofactors, the incubation included Mn^{++}, dichlorophenol and phosphate. All inhibitors were buffered to the appropriate pH for the reaction in which they were used. The change in optical density at 340 mμ per minute per gram of protein was the unit for the calculation of the specific activity of the samples.

Thyroxine

Assay of thyroxine stimulated DPNH reactions was done in the same manner as that of dichlorophenol. The pH for this assay was 7.8; 0.4 ml of DPNH (1 mg/ml) was used and 0.2 ml of L-thyroxine, 2×10^{-3} M, replaced the dichlorophenol.

Protein Analysis

A standard curve was prepared by using known quantities of crystalline bovine albumin. The color development, due to reaction of alkali copper-treated protein and the reduction of phosphomolybdic-phosphotungstic reagent (59), was read in a Bausch and Lomb 340 spectrophotometer at 500 mμ against a reagent blank.

Phosphorus Determination

Our method of determination is based upon the reduction of phosphomolybdic acid by N-phenyl-p-phenylenediamine as developed by Dryer, Tammes and Routh (60).

In determining inorganic phosphorus content, samples, prepared as described in the assay conditions section, were diluted to 10 ml with

1 N H_2SO_4. Of samples to which phosphate had been added, 0.1 ml of the 10 ml dilutions was further diluted to 3 ml with 1 N H_2SO_4. Three ml of the 10 ml dilution were required for determination of samples not containing added phosphate. To these 3 ml samples were added, in order, 1.0 ml of 0.025 M ammonium molybdate and 2.0 ml of p-semidine hydrochloride (50 mg of N-phenyl-p-phenyldiamine in 100 ml of 1% $NaHSO_3$). After a 10 minute waiting period, the color development was read against a blank of 1 N H_2SO_4 at 770 mμ with a Bausch and Lomb 340 spectrophotometer equipped with infra-red filter and tube.

The amount of the organic phosphate in samples was calculated by determining the total phosphorus content and subtracting from the total phosphorus the amount of inorganic phosphorus contained.

In the total analysis, 1.0 ml of 10% TCA was added to precipitate the protein in samples prepared by assay conditions. The samples were centrifuged and then diluted with 2 ml of 5 N H_2SO4 and digested. When dense white fumes appeared, a few drops of 30% H_2O_2 and then a few drops of water were added slowly to the samples after which they were treated as described above in the determination of inorganic phosphate content.

Relationship of Inorganic Phosphate Concentration to the Activity of Peroxidase, Dichlorophenol and Thyroxine Stimulated DPNH Oxidase

Three buffers were tested for their effect on each of the three activities at the appropriate concentration and pH of the standard assay. In the dichlorophenol assay, graded levels of Pi were added in the presence of acetate buffer, tris buffer or water.

Comparison of Na and K Prepared Extracts and Pi Source

All sodium salts (or all potassium salts) of chloride and phosphate were used to prepare the sample in the usual manner. The sample prepared in all sodium buffer was tested for a difference in response to equivalent sodium and potassium sources of Pi. The sample prepared in all potassium buffer was treated in the same manner, i.e., response to equal micromolar concentration of potassium or sodium salts of Pi.

Determination of Extent of DPNH Oxidation

Standard curves were prepared by the addition of known quantities of DPNH to a dichlorphenol system, incomplete with respect to enzyme. The density was determined at 340 mμ in a Bausch and Lomb 340 spectrophotometer.

Change in Pi Concentration as a Result of Dichlorophenol Activity

From this point on, unless otherwise specified, a concentration of 25 μM Pi was used in dichlorophenol assay. The usual assay conditions were used except reaction was allowed to proceed for 10 minutes. Pi was determined after a 10 minute incubation. The amount of enzyme and DPNH (3,6 and 9 μM) was varied. The quantity of DPNH oxidized in 10 minutes was determined by use of the standard DPNH curve.

Protein Phosphate Analysis Before and After Pi Treatment

The usual 10 minute incubation of dichlorophenol reaction conditions were used (25 μM Pi, 9 μM DPNH, 2×10^{-3} M dichlorophenol) unless otherwise indicated. Incubations for 10 minutes were stopped by protein precipitation by the addition of 10% TCA to the reaction

mixture. The precipitate was washed once with 10% TCA. The precipitate was collected in each case by centrifugation at 500 x g for 10 minutes. The sediment was digested with sulfuric acid and Pi analysis run, as described for organic phosphate analysis. The enzyme concentration was varied over a three fold range. Controls included 0 time, without DPNH, without dichlorophenol and without Pi. The possibility of replacement of Pi by equal molar concentrations of AMP, ADP, and ATP is included.

Dichlorophenol Enzyme Phosphorylation as Shown by 36,000 x g Centrifugation

Enzyme was assayed in the usual manner with and without 25 μM Pi and 9 μM DPNH. After 10 minutes incubation in which 6 μM DPNH had been consumed, the assay mixtures were centrifuged for 3 hours at 36,000 x g, the sediment washed once with 0.25 M sucrose and centrifuged as before. The enzyme was suspended either directly in tris buffer (0.05 M, pH 7.0) or first in 5% KCl, then placed in buffer and assay mixture.

Inhibition Studies

Dichlorophenol activity was determined in the absence or presence of inhibitors. DPNH was present in a 9 μM concentration; dichlorophenol, 2 x 10^{-3} M; 25 μM phosphate buffer, pH 7.0; enzyme, water and inhibitor to make a total volume of 6.0 ml. Incubation time was 10 minutes. Inhibitor concentration is the amount added to 6.0 ml. Inhibitor was incubated with enzyme and buffer for 10 minutes unless otherwise specified. Reversal of inhibition was done in the same manner, i.e., an additional 10 minutes incubation in the presence of reversal compound. Alpha-tocopherol (10 μg) when used, was added in

ethanol (0.02 ml) and rehomogenized with the fraction used. Solutions of inhibitors were corrected by dilute acid or base to the appropriate pH, i.e., pH 7.0 for dichlorophenol. Analogous procedures were followed for inhibition studies of peroxidase and thyroxine activities.

Sephadex G-25 Column

All attempts to obtain enzymatic activity after an active sample was placed on columns of DEAE cellulose, potato starch, Sephadex G-25 and G-50 have been without success. However, the "molecular sieve" action of the Sephadex G-25 column was used to separate free histidine from the protein. One ml of enzyme was passed through a Sephadex G-25 column, 1 x 4 cm., which had been equilibrated with 1.1% KCl. Elution was accomplished by a gradient elution with 10% KCl, with a 25 ml 1.1% KCl reservoir intraconnected. Ninhydrin was used to show separation of protein (3 ml fractions, #4-10) from histidine (fractions #12-20).

Trypsin Digestion

Digestion was followed at 280 mμ for the hydrolysis of peptide bonds, pH 8.0, 25° C., 0.001 mg trypsin added. The change in optical density at 280 mμ was followed at timed intervals.

CHAPTER IV

RESULTS

Activity Distribution during Sample Preparation

A 10% homogenate was prepared in 0.05 M phosphate buffer, pH 6.0. Separation of the insoluble material was accomplished by centrifugation at 500 x g for 10 minutes. Successive extractions with 0.25 M sucrose, 1.1% KCl and 5% KCl were made in the same manner. Results from a typical experiment are shown in Table 14.

The first extraction with 0.05 M phosphate buffer, pH 6.0, removed approximately 50% of the protein. A second wash with the same buffer removed an additional 15% of the protein now present. Next 0.25 M scurose removed 6-15% of the protein. Another 10-20% was removed by 1.1% KCl. All of these protein extractions were not enzymatically active, with the exception that a small amount of dichlorophenol activity was lost by 1.1% KCl. The 5% KCl soluble and insoluble fractions retain 75% of the original activities in 10% of the original protein. Peroxidase and dichlorophenol were present in the 5% KCl soluble while the insoluble fraction contains dichlorophenol and thyroxine but not peroxidase.

Ammonium Sulfate Fractionation

Each of the 5% KCl fractions was made 20% saturated with ammonium sulfate. The results on the basis of specific activity are

TABLE 14

Activity Distribution During Sample Preparation

Treatment	Total mg. Protein	Peroxidase		Dichlorophenol		Thyroxine	
		$\frac{\Delta O.D./min}{gm\ protein}$	Total Units	$\frac{\Delta O.D./min}{gm\ protein}$	Total Units	$\frac{\Delta O.D./min}{gm\ protein}$	Total Units
10% Homogenate	167	8	1,336	70	11,690	23	3,841
1st 0.05 M Phosphate Soluble	82	2	164	0	0	0	0
2nd 0.05 M Phosphate Soluble	12	0	0	0	0	0	0
0.25 M Sucrose Soluble	5	0	0	0	0	0	0
1.1% KCl Soluble	15	0	0	45	675	0	0
5% KCl Soluble	14	80	1,120	520	7,280	0	0
5% KCl Insoluble	15	0	0	201	3,015	224	3,360

presented in Table 15. Dichlorophenol specific activity was not changed by the precipitation of approximately 50% of the protein present by a 20% ammonium sulfate saturation. In the 5% KCl soluble fraction, peroxidase specific activity was doubled. The specific activity of thyroxine in the fraction insoluble in 5% KCl was also doubled by removal of approximately one-half of the protein present which is thyroxine inactive.

TABLE 15

Specific Activity and Ratios of DCP/T

Fraction	P	DCP	T	DCP/T
Original homogenate	8	70	23	3/1
5% KCl Soluble	80	520	0	
20% $(NH_4)_2SO_4$ supernatant of KCl soluble	185	490	0	
5% KCl insoluble	0	201	224	1/1
20% $(NH_4)_2SO_4$ supernatant of KCl insoluble	0	190	451	1/2.5

P, peroxidase activity; DCP, dichlorphenol activity; T, thyroxine activity

Basis for Demonstration of Ionic Strength Extractions

Previously it has been noted that the enzymatic activities extracted varied considerably with the strength of either potassium chloride or phosphate buffer. Table 16 represents data in which equal ionic strengths of 0.5 M phosphate buffer, pH 6.0, potassium chloride, sodium chloride and magnesium chloride were compared to test their effectiveness as extractors of enzymatic activity.

TABLE 16

Effect of Equivalent Ionic Strength

Treatment	mg Protein	Specific Activity		
		P	DCP	T
0.5 M Phosphate buffer, pH 6.0*	2.6	23	886	0 (141)
NaCl	2.5	23	1000	0 (135)
KCl	2.6	26	1050	0 (141)
$MgCl_2$	2.5	23	975	0 (129)

* Ionic strength = 0.62 M. () represents amount of T present in resuspended insoluble fractions.
P, Peroxidase activity; DCP, dichlorophenol activity; T, Thyroxine activity

Dialysis

All attempts to recover the activity after dialysis were unsuccessful. Both the resuspended precipitates and the supernatants were inactive. Combination of the precipitates and supernatants did not restore peroxidase, dichlorophenol or thyroxine activities.

Inorganic Phosphate Dependence of Dichlorophenol

Dichlorophenol shows a definite Pi dependence with a steep graded response between 1 and 35 μM Pi; above 35 μM a plateau seems to be reached. Figure 11 demonstrates the response of a 5% KCl soluble enzyme preparation to graded levels of inorganic phosphate (Pi).

Peroxidase or thyroxine shows neither a dependence nor stimulation by Pi, sodium or potassium. At equal molarities acetate buffer seems to be superior to phosphate or Tris buffers (Table 17) for peroxidase. Tris buffer (0.05 M, pH 7.0) seems to inhibit the dichlorophenol activity since the same activity elicited by Pi cannot be attained at the same Pi levels in the presence of Tris. The activity varies in relation to the concentration of Tris present.

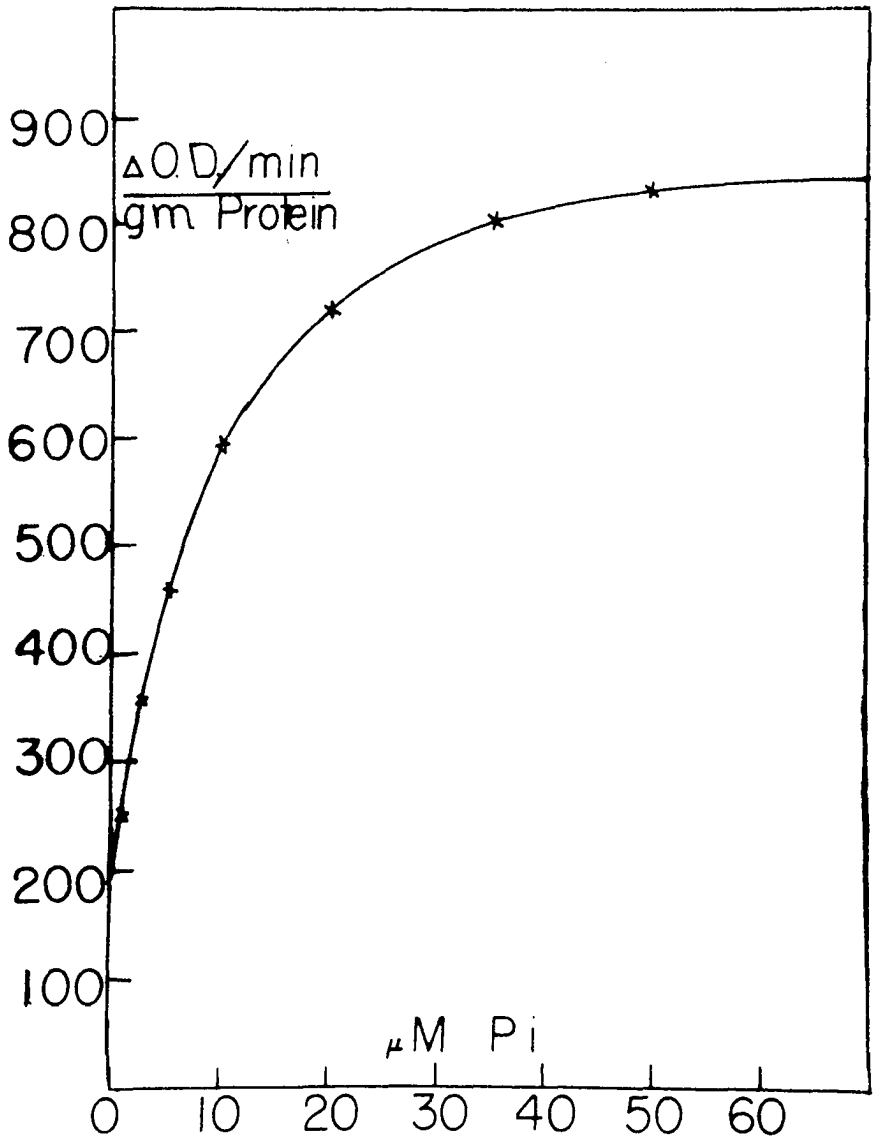

Figure 11. Phosphate Dependence of Dichlorophenol Stimulated DPNH Oxidase. Reaction mixture contained 0.05 Tris buffer, pH 7.0; 0.4 μM $MnCl_2$; 0.4 μM Dichlorophenol; 9 μM DPNH; enzyme, water, and the indicated amount of phosphate to a total volume of 6.0 ml.

TABLE 17

Activity in Different Buffers

Buffer	Peroxidase[a]		DCP[b]		Thyroxine[c]	
	K[1]	Na[2]	K[1]	Na[2]	K[1]	Na[2]
Tris	60	44	0	0	290	317
Acetate	66	50				
Sodium Phosphate	55	46	906	1,070	308	321
Potassium Phosphate	58	46	920	1,055	300	330
150 µM Pi			906			
150 µM Pi + 20 µM Tris			838			
150 µM Pi + 30 µM Tris			638			

a. Buffer 0.01 M, pH 6.0 b. Buffer 0.05 M, pH 7.0
c. Buffer 0.05 M, pH 7.8
1 Sample prepared in all potassium salts
2 Sample prepared in all sodium salts

Change in Pi Concentration as a Result of Dichlorophenol Activity

Table 18 shows that the consumption of Pi depends upon enzyme concentration and not DPNH concentration, although a certain minimum oxidation of DPNH is necessary to meet a 1/1 ratio of P/DPNH. When TPNH is substituted for DPNH, similar results are obtained, mole for mole.

TABLE 18

Pi Consumption vs Enzyme or DPNH Concentration

µM DPNH added	3		6		9		18	
ml enzyme	Pi	P/DPNH	Pi	P/DPNH	Pi	P/DPNH	Pi	P/DPNH
0.1	1	1/2	2	2/4	2	2/4		
0.2	2	2/2	4	4/4	4	4/6		
0.3	2	2/2	4	4/4	6	6/6	6	6/12

In this particular assay, each 0.1 ml enzyme requires a maximum of 2 µM Pi. This maximum consumption of Pi (i.e., conversion of inorganic phosphate to protein bound phosphate) will only be reached when sufficient

DPNH is oxidized (at a 1/1 rate). After this first requirement of Pi for DPNH oxidation, DPNH oxidation continues without respect to Pi.

Protein Phosphate Analysis Before and After Pi Treatment

Inorganic phosphate binding to the protein as a function of enzyme concentration is again demonstrated in Table 19. In addition, no uptake of Pi by protein is noted without the presence of DPNH (or TPNH) or dichlorophenol. ADP and ATP but not AMP could substitute for Pi in the DPNH oxidation. However, when ADP or ATP are used in lieu of Pi, no protein phosphorylation occurs. As in the previous experiment, TPNH can replace DPNH.

TABLE 19

Protein Phosphate Analysis

Condition	Enzyme Concentration			μM DPNH Consumed
	.25 ml	0.5 ml	0.75 ml	
	μM Pi Protein			
0 minutes	.02	.05	.05	0
10 minutes	3.2	6.1	6.0	6
No DPNH	.02	.06	.08	0
No DCP	.04	.07	.09	0
AMP (no Pi)	.02	.03	.04	0
AMP (with Pi)	3.1	5.9	6.15	6
ADP (no Pi)	.02	.05	.06	6
ADP (with Pi)	.07	.07	.10	6
ATP (no Pi)	.03	.03	.06	6
ATP (with Pi)	.08	.11	.07	6

Dichlorophenol Enzyme Phosphorylation as Shown By 36,000 x g Centrifugation

As previously shown, Pi disappears from the reaction medium, and reappears as phosphorylated protein. This experiment (Table 20) clarifies the effect of ionic strength on dichlorophenol activity with respect to Pi. This Pi is attached to the protein so that it is not liberated by

0.25 M sucrose and the enzyme is not Pi requiring. On the other hand, high ionic strength does liberate Pi so that added Pi is now required for activity. Since some Pi is apparently lost from the control, some Pi stimulation of the sucrose treated preparation is seen.

TABLE 20

Recovery of Pi Independent Enzyme

	Original		36,000 x g Residue			
			0.25 M sucrose		5% KCl	
μM Pi Added	0	25	0	25	0	25
μM Pi consumed per 10 minutes	0	5.7	3.9	5.1	0	5.0
μM DPNH consumed per 10 minutes	0	6.1	4.3	5.5	0	5.6

Concurrent Loss of Pi and Gain of Protein Phosphorus

Twenty-five μM of Pi and 9 μM of DPNH were added to the usual reaction conditions. Table 21 illustrates that the loss of Pi from the reaction medium is equal to that gained by the protein by the same sample at the same time during a 10 minute incubation.

TABLE 21

Concurrent Loss of Pi and Gain of Protein Phosphorus

ml Enzyme	Loss of Pi from Medium	Gain in Organic P
0.1	1.9	1.7
0.2	3.8	3.8
0.3	5.7	5.6

Replacement of Pi Requirement by Arsenate

Dichlorophenol activity in the presence of both Pi and arsenate was compared at equal molar concentration of the two ions (Table 22). As can be seen, arsenate is almost as effective, on a mole for mole ratio, as Pi, under normal assay conditions.

TABLE 22

Pi Replacement by Arsenate

μM Phosphate or Arsenate	Specific Activity	
	Pi	Arsenate
0	0	0
5	250	220
10	409	394
20	526	502
35	611	597

Thiol Inhibition

Table 23 shows the results of a group of inhibitory or stimulatory compounds on the activities of peroxidase, dichlorophenol, and thyroxine. The low concentrations which are effective (0.1 and 1 μM) indicate enzyme binding effects. Peroxidase, thyroxine, and dichlorophenol activities are inhibited by compounds such as cysteine, reduced glutathione (GSH), and 2-mercaptoethanol which can destroy -S-S- bond integrity and trap free radicals. Figure 12 demonstrates a mixed competitive and non-competitive inhibition by 2-mercaptoethanol. This "mixed" type of inhibition presumably can be ascribed to two separate inhibition sites (61). The implication is that some of its inhibition is competitive with DPNH. This inhibition is not reversible or cofactor protected. Compounds which are S-H bond inhibitors (i.e.,

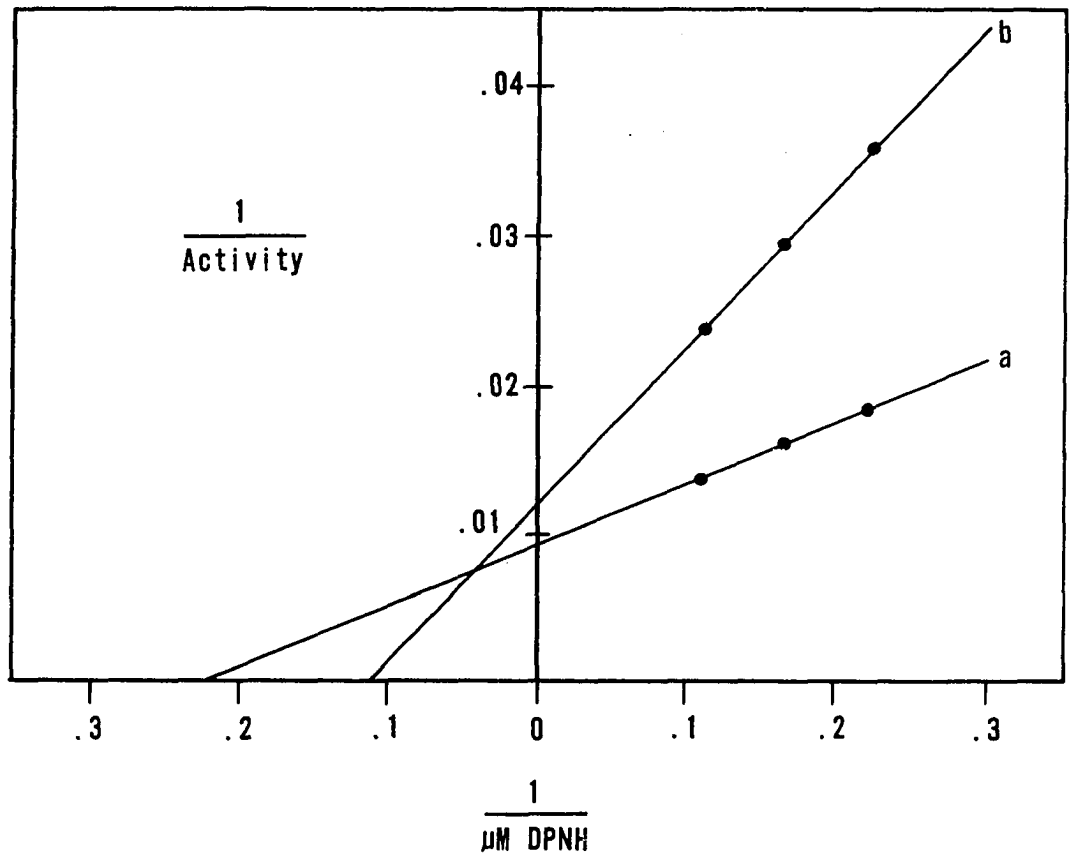

Figure 12. Mixed Inhibition by 2-Mercaptoethanol of Dichlorophenol Activity. a. Without inhibitor; b. With 1 μM of 2-Mercaptoethanol Added. Reaction mixture contained 0.05 Tris buffer, pH 7.0; 0.4 μM $MnCl_2$; 0.4 μM Dichlorophenol; 9 μM DPNH; 25 μM PO_4, enzyme and water to make total volume of 6.0 ml. Each sample had a 10 minute incubation of enzyme, buffer, and inhibitor, when used.

oxidized glutathione, PCMB, IOB, N-ethyl maleimide, and iodoacetate), are effective inhibitors of dichlorophenol activity but are ineffective against peroxidase and thyroxine.

TABLE 23

Thiol Inhibitors

μM Compound Added	0.1	1	0.1	1	0.1	1
Inhibitor	DCP		Thyroxine		Peroxidase	
2-mercaptoethanol	17	0	0	0	0	0
Thioglycerol	43	0	0	0	0	0
Thioglycolic acid	22	0	--	--	--	--
Cysteine	17	5	0	0	0	0
Glutathione (red.)	85	13	31	0	0	0
Glutathione (ox.)	108	37	100	100	100	100
Cystine	56	51	100	100	126	112
Homocystine	82	63	100	100	155	142
Cystamine	63	56	100	100	100	100
NaHS	43	62	--	--	--	--
$HgCl_2$	70	51	100	52	100	100
Iodoacetate	--	--	100	100	100	100
$Pb(NO_3)_2$	30	9	100	73	102	95
Chloroacetophenone	75	61	100	100	88	88
PCMB	56	43	100	95	100	100
N-ethyl maleimide	68	52	100	100	100	100
Iodosobenzoate	69	56	100	100	100	100
Arsenite	56	56	100	31	100	100

Data are expressed as per cent of control activity. Ten minute incubation in the absence of cofactors, DCP, Pi and Mn^{++}.

Cofactor Protection of S-H Groups of Dichlorophenol

Most S-H group inhibitor compounds inhibit dichlorophenol activity (but not peroxidase or thyroxine) only when incubated in the absence of cofactors (dichlorophenol, Pi and Mn^{++}). (See Table 24). If the enzyme is first exposed to the cofactors and then the S-H inhibitor is added, no inhibition is seen, and some stimulation occurs. Inhibition of -S-S- groups is not appreciably affected by the absence or presence of cofactors. Figure 13 is a Lineweaver-Burke (62) plot of

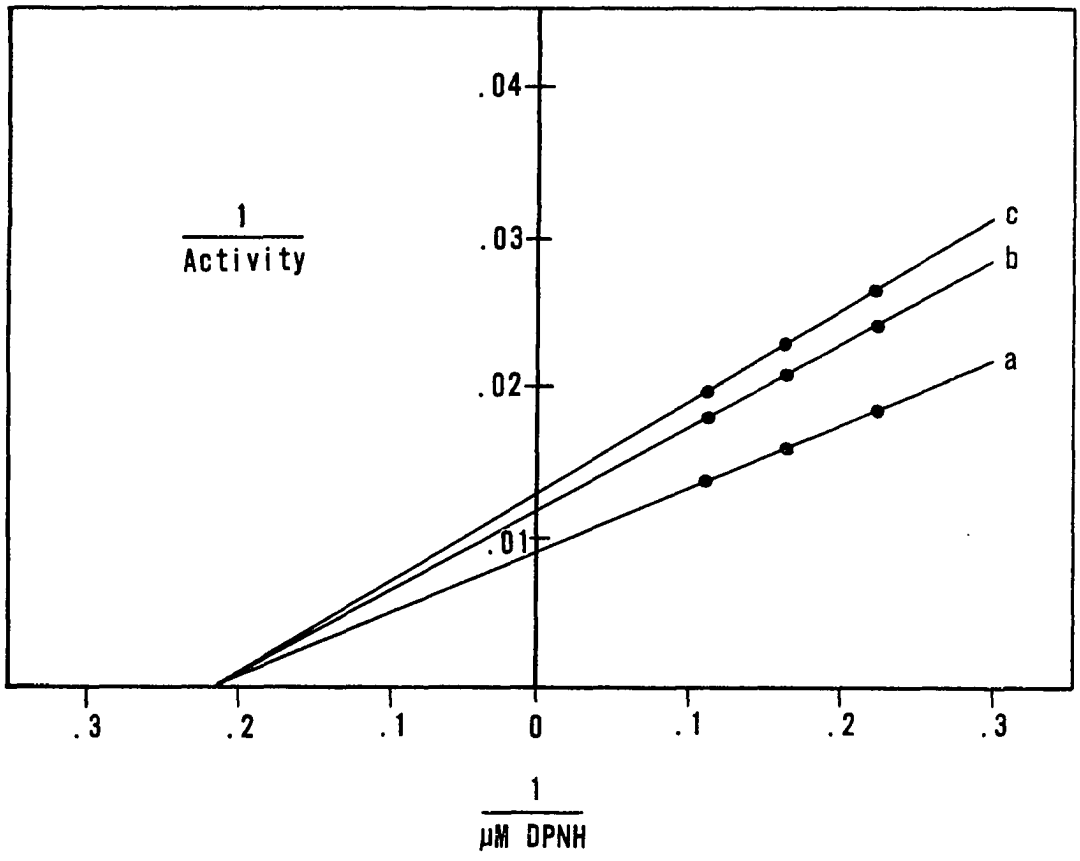

Figure 13. Non-competitive Inhibition of Dichlorophenol Activity. a. Without inhibitor; b. With 1 μM of Chloroacetophenone; c. With 1 μM of N-ethyl maleimide. Reaction mixture same as in Figure 12 except for inhibitors. The 10-minute incubation period was also used.

the effect of N-ethyl maleimide and chloroacetophenone (1/s vs 1/v). Non-competitive inhibition with respect to DPNH concentration is illustrated by the S-H bond inhibitors.

TABLE 24

Cofactor Protection of S-H Group

Compound	Enzyme	Enzyme + Cofactors
GSH	13	60
GSSG	37	118
Cysteine	5	17
Cystine	56	135
PCMB	56	102
IOB	56	94
2-mercaptoethanol	0	0
Chloroacetophenone	61	106
N-ethyl maleimide	52	98

Data are expressed as per cent of control activity. Ten minute incubation in the absence of cofactors, DCP, Pi, and Mn^{++}.

Reversal of S-H Inhibition of Dichlorophenol Activity

The inhibition of S-H bonds on the enzyme by -S-S- compounds can be reversed by the addition of S-H compounds, but only to the extent of the inhibition caused by the added compounds. Data in Table 25 indicate a stoichiometric relationship for reversal. Inhibition of the enzyme -S-S- groups was not found to be reversible; instead a cumulative inhibition was demonstrated. A ten minute incubation of indicated compound with buffer and enzyme was done. Reversal was accomplished by an additional 10 minute incubation in the presence of 0.1 μM of reversal compound. The usual dichlorophenol assay was used.

TABLE 25

Reversibility of S-H Inhibition of Dichlorophenol by S-H Compounds

Reversal Compound Inhibitor (μM)	1 μM	0.1 μM	1 μM + 0.1 μM GSH	0.1 μM + 0.1 μM GSH	0.1 μM + 0.1 μM GSSG
GSH	13	85	12	81	85
GSSG	37	108	50	87	90
Cysteine	5	17	5	16	17
Cystine	56	51	65	81	50
PCMB	43	56	48	78	50
IOB	56	69	62	81	64
2-mercaptoethanol	0	17	0	15	18

Activity reported as per cent control activity

Tocopherol Protection of S-H Groups

When 10 μg of α-tocopherol was pre-incubated with the enzyme before addition of 1 μM of PCMB, no significant inhibition was seen. (See Table 26). This concentration of PCMB (1 μM) was sufficient to cause a half-maximal inhibition of dichlorophenol activity. If tocopherol was added after PCMB, no appreciable protection was manifested.

TABLE 26

Tocopherol Protection from PCMB

Enzyme	Per cent of Control Activity
Enzyme	100
Enzyme + 1 μM PCMB	56
Enzyme + 10 μg Tocopherol	100
Enzyme + 10 μg Tocopherol after 10 minutes with 1 μM PCMB	94
Enzyme + 1 μM PCMB after 10 min. with 10 μg Tocopherol	58

Effect of DPN (or TPN)

DPN (or TPN) was not effective in altering the amount of DPNH (or TPNH) oxidized; neither does this oxidation product affect the inhibition caused by thiol compounds (Table 27).

TABLE 27

Effect of DPN or TPN

9 μM DPNH Present		9 μM TPNH Present	
Addition	μM DPNH consumed	Addition	μM TPNH consumed
0	6.1	0	6.1
4.5 μM DPN	6.0	4.5 μM TPN	6.4
9.0 μM DPN	6.1	9.0 μM TPN	6.3
9.0 μM DPN + 1 μM 2-ME	0.2	9.0 μM TPN + 1 μM 2-ME	0.5
9.0 μM DPN + 1 μM PCMB	2.7	9.0 μM TPN + 1 μM PCMB	3.1
1 μM 2-ME	0.3	1 μM 2-ME	0.4
1 μM PCMB	2.5	1 μM PCMB	3.0

DPNH or TPNH were added after 10 minute pre-incubation of the indicated compound in the standard dichlorophenol assay.

Effect of Histidine Analogs on Peroxidase, Dichlorophenol and Thyroxine

When histidine and the analogs histamine, imidazole and imidazole acetic acid were incubated for 10 minutes in the presence of cofactors, either stimulation, inhibition or no effect was observed (Table 28). No effect was elicited by the presence of 100 μM of glycine, alanine, phenylalanine, tyrosine, urocanic acid, tryptophan or lysine. With increasing concentration, the effect produced by the analog changed from no effect either to inhibition or to stimulation. The presence of the imidazole ring alone could yield stimulation. The presence of both the imidazole ring and the α-amino group caused inhibition. These effects

of histidine and its analogs were noted only on dichlorophenol activity. Neither stimulation nor inhibition of peroxidase or thyroxine activities could be demonstrated. Unfortunately, only 10-15% of the 5% KCl soluble fractions were sensitive to histidine inhibition; those samples sensitive to histidine inhibition lost this sensitivity in 24 hours. All attempts to produce fractions consistently sensitive to histidine inhibition were not successful. Those samples which were inhibited by histidine produced consistent and reproducible data.

TABLE 28

Effect of Histidine Analogs

µM added	0.01	0.1	1.0	10	100
L-Histidine	100	94	80	45	10
Histamine	100	100	81	62	16
Imidazole	100	85	94	88	145
Imidazole acetic	100	95	94	176	145
D-Histidine	100	100	100	98	85

Standard dichlorophenol assay with 10 minute incubation of enzyme, buffer and inhibitor. Activity reported in per cent control activity.

Non-Competitive Inhibition of Histidine With Respect to DPNH

A Lineweaver-Burke plot (62) of 1/v vs 1/s (Figure 14) at 5 µM concentrations of histidine demonstrates non-competitive inhibition of histidine where s is DPNH concentration in the presence of 25 µM Pi. The K_m apparent is 2.2×10^{-6} M/L. Four concentrations (1.33, 4.5, 6.0 and 9.0 µM) of DPNH were utilized.

Non-Competitive Inhibition of Histidine With Respect to Pi

Figure 15 shows a 1/v vs 1/s Lineweaver plot. Five levels of Pi were analyzed in the presence of six levels of histidine. This plot

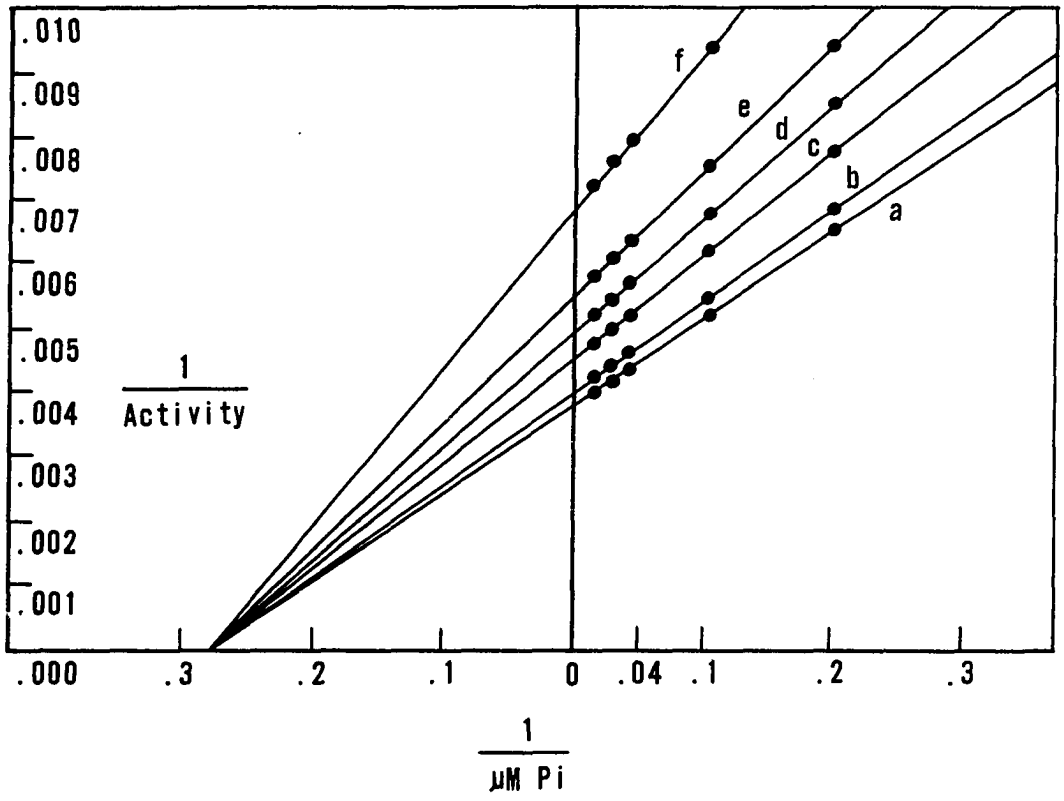

Figure 14. Non-competitive Inhibition of Histidine with respect to Pi. a. No histidine; b. 1 μM histidine; c. 5 μM histidine; d. 10 μM histidine; e. 20 μM histidine; f. 50 μM histidine. Reaction mixture contained 0.05 M Tris buffer. pH 7.0; 0.4 μM $MnCl_2$; 0.4 μM Dichlorophenol; enzyme, water and indicated amounts of Histidine and phosphate to make total of 6.0 ml. A 10 minute incubation period of enzyme, buffer, and inhibitor was used.

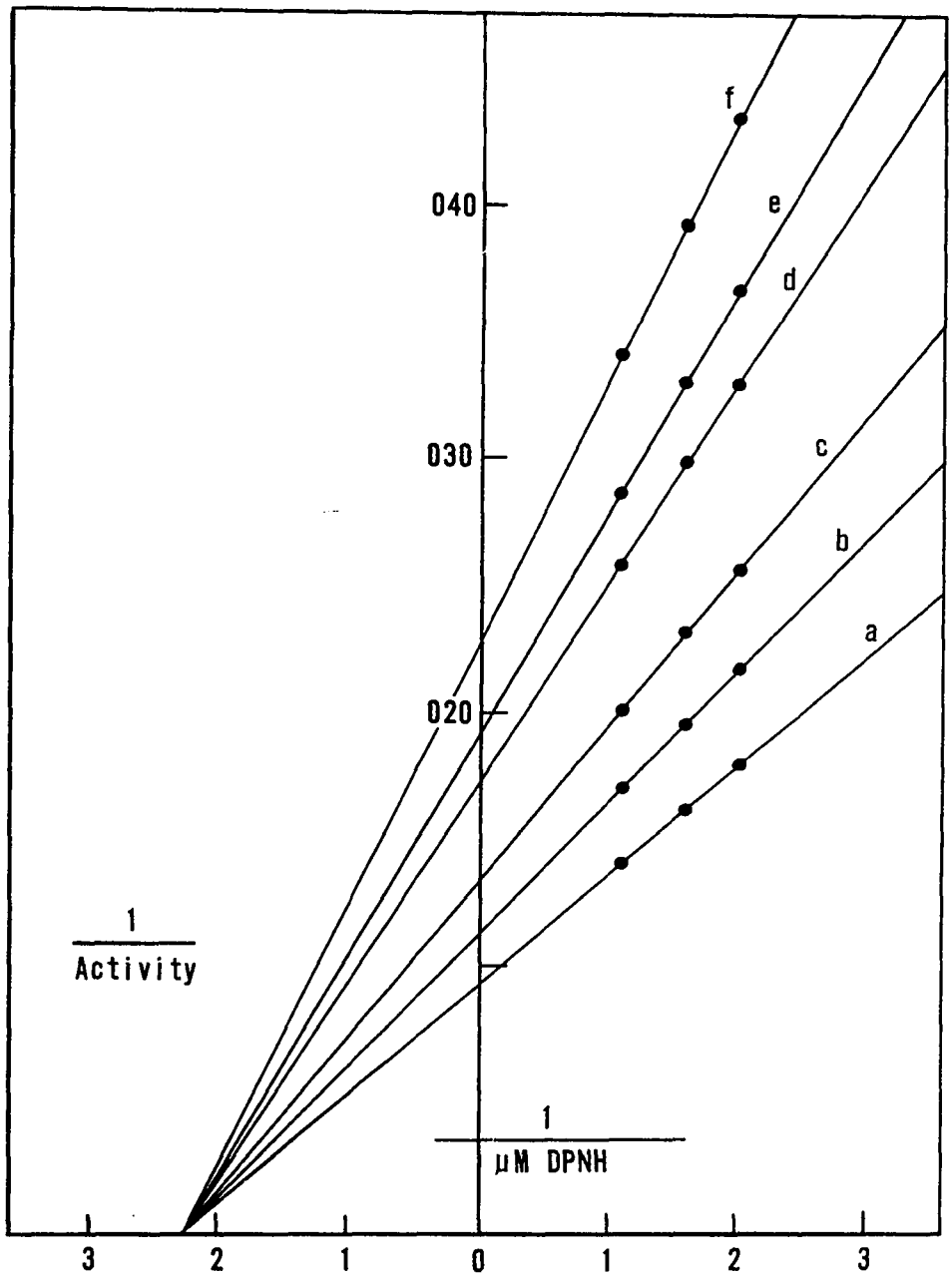

Figure 15. Non-competitive Inhibition of Histidine with respect to DPNH. a. No histidine; b. 1 μM histidine; c. 5 μM histidine; d. 10 μM histidine; e. 20 μM histidine; f. 50 μM histidine. Reaction mixture contained 0.05 M Tris buffer, pH 7.0; 0.4 μM $MnCl_2$; 0.4 μM Dichlorophenol; 25 μM phosphate, enzyme, water and indicated amounts of histidine and DPNH to make a total of 6.0 ml. A 10 minute incubation period of enzyme, buffer and inhibitor was used.

illustrates that histidine inhibition is non-competitive with respect to Pi. The apparent K_m was 2.8×10^{-6} M/L.

Inhibitor-Activation by Histidine

A dual nature of the histidine inhibition is demonstrated by a plot of S/v vs I (63) in Figure 16. The two widely different K_i values depend on concentration of histidine. These two different slopes were not apparent in 1/v vs 1/s plots. The K_i present at the higher concentrations is the result of inhibitor-activation by histidine.

Enzyme as Modified by Histidine Shown by Trypsin Digestion

The previous experiment suggested a dual nature of the histidine effect. Since a histidine sensitive sample would lose its sensitivity to histidine inhibition with time, the question presented itself as to whether histidine could still affect the enzyme at a point other than inhibiting the observed oxidation of DPNH. To test this possibility a histidine insensitive and a histidine sensitive sample were treated with 5 μM histidine for 10 minutes. The free histidine was separated by a Sephadex G-25 column. Control enzyme preparations (duplicate samples not treated with histidine) also were passed through the Sephadex column. All fractions were eluted with 10% KCl. No enzymatic activity was recovered. The histidine treated enzymes (both sensitive and insensitive to histidine inhibition) were twice as sensitive to trypsin digestion as non-treated (Figure 17). When the sensitivity to trypsin digestion was measured on aged enzyme preparations that had lost histidine sensitivity, the rates obtained were indistinguishable from those in Figure 17. Histidine alters the enzyme so that its trypsin digestion

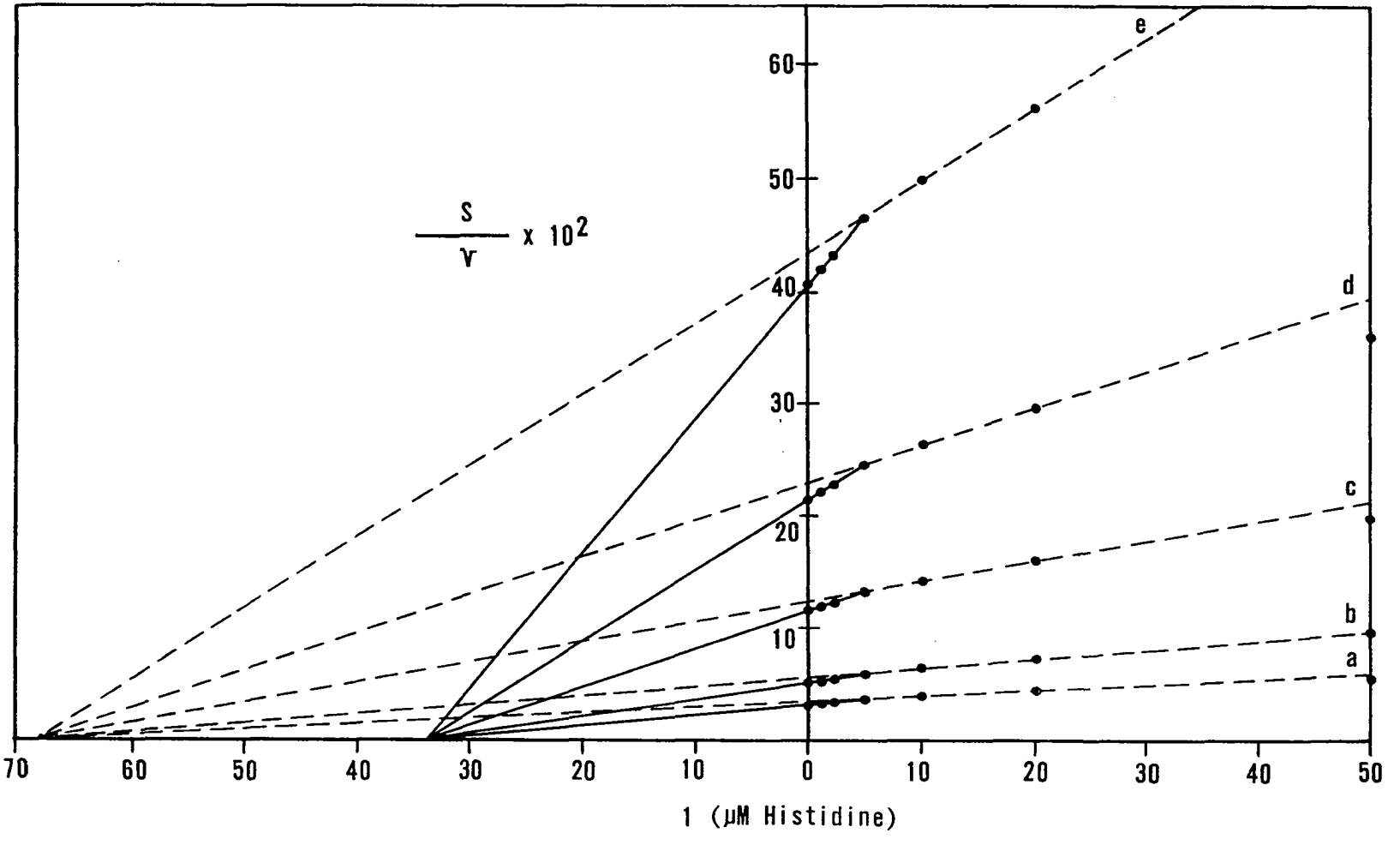

Figure 16. Dual nature of Histidine inhibition. ------ indicates slope above 5 μM. ——— indicates slope below 5 μM. Reaction mixture same as in Figure 14. a. 5 μM Pi; b. 10 μM Pi; c. 20 μM Pi; d. 50 μM Pi; e. 100 μM Pi.

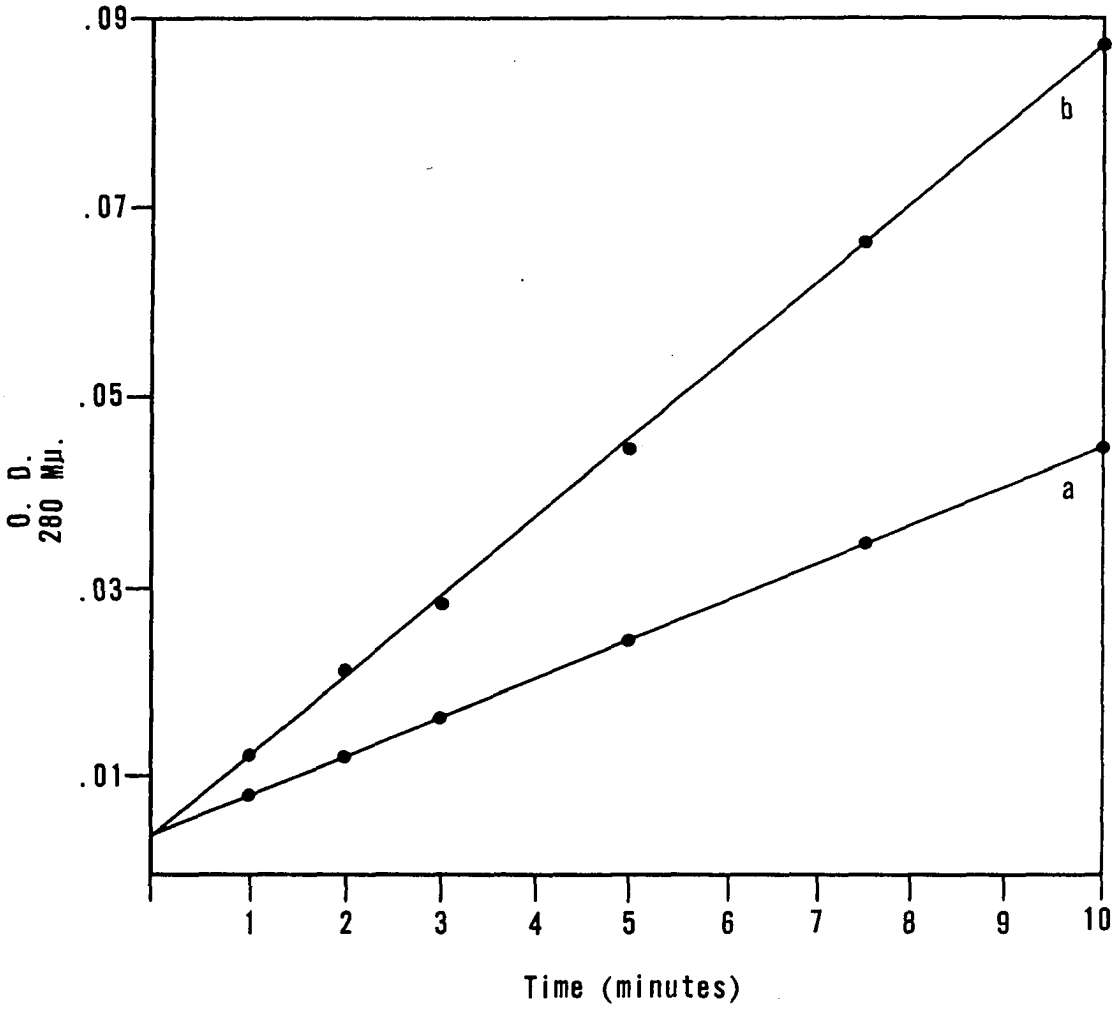

Figure 17. Trypsin Digestion of Histidine. a. Control without Histidine. b. Histidine treated enzyme. Enzyme incubated for 10 minutes with buffer and 5 μM of histidine. Free histidine separated from enzyme by Sephadex G-25 (see text for conditions of Sephadex column). Trypsin digestion was followed at 280 Mμ, pH 8.0; 25°C with 0.001 mg. Trypsin added. Fresh histidine sensitive, aged histidine sensitive and histidine insensitive preparations yield similar curves.

rate is increased whether the enzyme is sensitive or insensitive to inhibition by histidine. This observation supports the hypothesis that the binding of histidine by the enzyme does not by itself account for histidine inhibition.

CHAPTER V

DISCUSSION

The extraction procedure described in this work differs significantly from that of other workers on the enzyme in that a partial fractionation of peroxidase, dichlorophenol stimulated, and thyroxine stimulated activities is achieved. The basis of this fractionation is a difference in solubility in varying ionic strengths. Two main fractions are obtained. Peroxidase and dichlorophenol activities are present in a 5% KCl soluble fraction, while the fraction insoluble in 5% KCl contains dichlorophenol and thyroxine but not peroxidase activities. The absence of thyroxine activity from the highly active soluble fraction indicates that thyroxine activity does not necessarily have the same solubility characteristics as peroxidase and dichlorophenol. The absence of peroxidase from the insoluble fraction, which contains high thyroxine and moderate dichlorophenol activities is indicative that differences exist on the enzyme in response to these phenolic stimulators. This is further strengthened by the fact that the ratio of specific activity of dichlorophenol to thyroxine undergoes a six fold change during fractionation (Table 15). If the physical and chemical requirements for the enzymic expression of the effect of these two phenolic stimulators were the same, this ratio would be expected to have remained constant throughout fractionation.

Demonstration that ionic strength was the basis of the extraction resides in the fact that equal ionic strengths of phosphate buffer, potassium chloride, sodium chloride and magnesium chloride have the same effect. Low ionic strength solutions, i.e., 1.1% KCl are ineffective as extractors while higher strengths (Table 16) result in fractionation.

Three major difficulties were encountered which hindered further utilization of this technique. Ammonium sulfate at higher concentrations interfered with peroxidase, dichlorophenol and thyroxine activity assays. Ammonium sulfate could not be removed by dialysis since dialysis always resulted in complete loss of activity. In addition, protein precipitates by higher concentrations of ammonium sulfate were enzymatically inactive. To date, any technique which results in precipitation of enzymatic activity is concomitant with loss of activity.

The use of high ionic strength extractions yielded a preparation distinctly different from any previously described. Dichlorophenol stimulated DPNH oxidase activity can be obtained only in the presence of added phosphate. Peroxidase and thyroxine activities are not affected by presence or absence of phosphate. Bever et al. (7), using a sample prepared under milder ionic conditions, reported stimulation of dichlorophenol activity by added Pi, but not dependency on the Pi. Klebanoff (21) reported that maximal activity obtained in phosphate buffer could not be achieved with other buffers. A dependency of dichlorophenol activity upon Pi has not been previously reported.

The original homogenate and the sample washed in weak ionic strength are not dependent upon phosphate, although some stimulation by Pi can be demonstrated. Phosphate dependency occurs only after high

ionic strength extraction. This would suggest that Pi (or its equivalent) is liberated from the enzyme by the higher ionic strength. This hypothesis is supported by the data in Table 20. A Pi dependent sample is converted to a Pi independent preparation by dichlorophenol stimulated DPNH oxidation in the presence of Pi. This enzyme, after separation from the Pi containing medium by 36,000 x g centrifugation, does not require added Pi for activity. If this sample is treated with high ionic strength potassium chloride, it again becomes Pi dependent.

Another line of evidence which suggests an active phosphorylated enzyme is that the oxidation of DPNH, stimulated by dichlorophenol, shows a linear correlation to the uptake of Pi from the reaction medium. The maximum disappearance of Pi by a given amount of enzyme will be reached only when sufficient DPNH is oxidized. No uptake of Pi was noted without the presence of DPNH (or TPNH) or dichlorophenol. Specifically, the oxidation of reduced nucleotide was necessary for Pi uptake. The oxidation of 1 μM DPNH yields uptake of 1 μM Pi until the enzyme becomes completely phosphorylated. After this first requirement of Pi, DPNH oxidation continues without further consumption of Pi (Table 18).

Table 18 shows that the consumption of Pi depends upon enzyme concentration and not upon DPNH concentration, although a certain minimum oxidation of DPNH is necessary to meet a 1/1 ratio of P/DPNH. A definite quantitative correlation is established between the amount of Pi disappearance from the reaction medium and the amount of enzyme added.

Strong evidence of enzyme phosphorylation is established by the

correlation of Pi disappearance from the reaction medium with the appearance of an equivalent increase in protein phosphorus (Table 21). Evidence that arsenate is almost as effective on a mole for mole basis as Pi, under normal assay conditions is additionally suggestive of phosphorylation (See Table 22).

ADP and ATP, but not AMP, can substitute for the Pi requirement for DPNH oxidation. When ADP or ATP are used in lieu of Pi, no protein phosphorylation occurs.

Substantiating evidence, obtained from inactivation processes, has been reported recently by Baker and Schultz (8). They reported that under certain conditions the inactivation of peroxidase is Pi dependent. Baker and Schultz state that there is a disapperance of Pi along with the inactivation process. A three fold increase in the rate of inactivation takes place when ADP or ATP but not AMP replace the Pi.

Thiol inhibitors have proved to be a useful tool in demonstrating another area of difference between the two phenolic stimulators. Dichlorophenol activity can be inhibited by S-H, -S-S-, and free radical inhibitors. Peroxidase and thyroxine activities are inhibited by -S-S- inhibiton and free radical trapping compounds, but not by sulfhydryl inhibitors. The ability of 2-mercaptoethanol, cysteine, and reduced glutathione to act as -S-S- bond inhibitors (2) and as free radical trapping agents (10) is well established. If these compounds, i.e., 2-mercaptoethanol, were acting only by -S-S- bond inhibition, a Lineweaver-Burke plot of 1/v vs 1/s would demonstrate a non-competitive inhibition, provided the -S-S- bond involved was not at the active site. Free radical trapping would demonstrate competitive inhibition with respect to either DPNH concen-

tration or H_2O_2 production. The latter possibility is ruled out since small amounts of added H_2O_2 did not reverse or reduce the inhibition. A mixed competitive and non-competitive inhibition shown by 2-mercaptoethanol with respect to DPNH (Figure 12) is indicative that two separate inhibition sites are involved. Thus, the assignment of a sensitive -S-S- bond necessary for peroxidase, dichlorophenol and thyroxine activities is in agreement with the data. This labile group is not present at the active site for DPNH. This -S-S- bond could not be demonstrated to be cofactor protected or reversible. The latter result is suggestive of a conformation (structure or configuration) change of the enzyme molecule.

Compounds which are S-H bond inhibitors are effective inhibitors of dichlorophenol activity and ineffective inhibitors of peroxidase and thyroxine activities. Again a distinction between requirements of these phenolic stimulators is evidenced with respect to the enzyme as such.

Figure 13 demonstrated non-competitive inhibition of chloroacetophenone and N-ethyl maleimide with respect to DPNH. These compounds were selected from a list of many possible sulfhydryl group inhibitors because they were considered to be highly specific for the sulfhydryl group (64). Compounds such as iodoacetate and mercury are capable of reacting with other groups (64).

Most of the compounds effective as sulfhydryl group inhibitors inhibit dichlorophenol activity only when incubated in the absence of the cofactors, dichlorophenol, orthophosphate and Mn^{++}. In the presence of cofactors, compounds such as oxidized glutathione and cystine show some stimulation rather than inhibition. This stimulation would imply

that some of the inherent S-H groups exist as -S-S- bonds. The reduction of these -S-S- bonds to form S-H groups results in an increased activity.

The existence of S-H bonds which can undergo reversible oxidation-reduction is supported by the observation that reduced glutathione can partially reverse the inhibition caused by oxidized glutathione and cystine. This reversal of S-H bond inhibition caused by -S-S- compounds by the addition of sulfhydryl reagents, is limited to the extent of the inhibition caused by the added sulfhydryl reagent. The inhibition, due to the sulfhydryl, is the effect on the non-reversible -S-S- bonds. The point of emphasis is that the inherent S-H bonds can be reversibly oxidized and reduced with concomitant recovery of activity. These S-H bonds are also protected by the enzymatic cofactors. The -S-S- bonds can neither be reversibly oxidized and reduced with recovery of enzymatic activity nor cofactor protected.

Tocopherol protects the enzyme from sulfhydryl inhibitors. This protection is similar to the cofactor protection in that no protection is manifested if PCMB is added before tocopherol. Corwin and Schwarz (65) report similar results with α-keto glutarate oxidase *in vivo* and *in vitro*. Since the protection required tocopherol at the beginning of the incubation period, they suggest this indicated that protection is due to protecting the sensitive S-H groups from oxidation.

The postulated thiol inhibition-stimulation mechanism in Figure 18 illustrates the above mentioned interpretations.

The presence of labile S-H and -S-S- bonds necessary for dichloro-

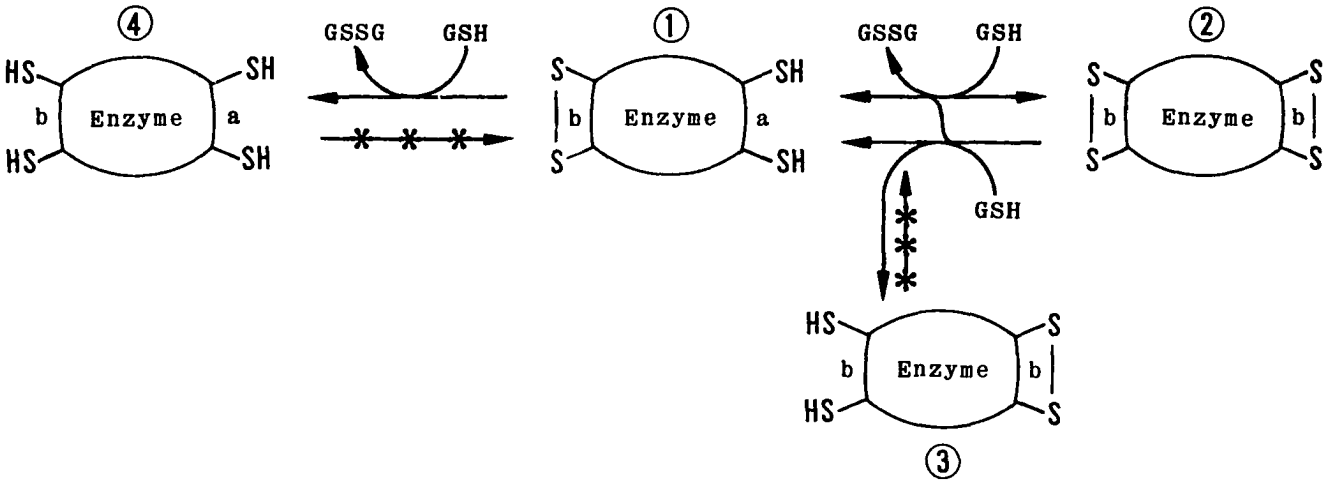

Figure 18. Postulated Thiol Inhibition-Stimulation Mechanism.

(a) SH - reversible group, also cofactor protected.

(b) $\begin{matrix} S \\ | \\ S \end{matrix}$ - non-reversible (for activity).

① Native enzyme - active.
② Inactive but reversible, maybe some native, would explain GSH stimulation.
③ Inactive, not reversible, explains inability for 100% reversibility since either compound could be formed although obviously ① predominates over ③
④ Inactive and not reversible.

phenol activity would partially account for the instability of the enzyme. The activity is quickly lost at room temperature, by freezing, storage at 5° C., upon application to chromatographic columns, and dialysis; neither can activity be protected by the presence of mild anti-oxidants or reductants (such as oxidized or reduced glutathione).

Histidine has proved to be an important tool in further demonstrating a distinction between the requirements on the enzyme molecule for the two phenolic stimulators of DPNH oxidation. Histidine and histamine yielded inhibition of dichlorophenol activity while imidazole and imidazole acetic acid resulted in stimulation. These effects of histidine and its analog were noted only on dichlorophenol activity. Neither stimulation nor inhibition of peroxidase or thyroxine activities could be demonstrated.

Apparently the presence of the imidazole ring could yield stimulation. The presence of both the imidazole ring and the α-amino group yields inhibition.

Non-competitive inhibition of histidine with respect to DPNH and orthophosphate was demonstrated by Lineweaver-Burke plots with apparent K_m values of 2.2×10^{-6} and 2.8×10^{-6} M/L respectively. An S/v vs I plot shows two widely different K_i values. The K_i value of histidine depends upon concentration. One K_i value is apparent at the lower concentration and another at higher concentrations. This would suggest a dual nature of the effect of histidine, i.e., two different inhibition affinities suggest two different sites.

According to classical inhibition kinetics, the number of inhibitor molecules interacting with an enzyme molecule can be

ascertained by plotting the ratio of control activity to activity in the presence of inhibitor at varying inhibitor concentrations (66). A linear relationship indicates that one inhibitor molecule interacts with one enzyme molecule. A curve that slopes away from an inhibitor concentration coordinate is interpreted as indicating an interaction of enzyme with two or more inhibitor molecules. When the data in Table 15 are so plotted, the curve slopes toward the inhibitor concentration axis, possibly suggesting that one histidine molecule interacts with more than one enzyme molecule (67). According to Martin (66), curvature of this type would be expected if a species of the enzyme that was insensitive to histidine were present, i.e., the curvature could be an artifact arising from the presence of some "histidine-insensitive" enzyme. The same type of curvature would result if two sites on one molecule were both subject to histidine attack, only one of which results in inhibition.

The fact that histidine sensitivity is lost rapidly with age, while the enzymatic activity remains high, and that many samples are obtained which were histidine-insensitive, would suggest that the inhibition of the enzyme by histidine is not necessarily the result of histidine interacting at the active site of the molecule. The finding that histidine inhibiton is non-competitive with orthophosphate and DPNH and that small quantities of H_2O_2 do not alter the inhibition corroborate this hypothesis.

Evidence is obtained from a study of trypsin digestion of the enzyme preparation that histidine can be bound to the enzyme whether or not it inhibits the enzyme. Figure 17 shows that the enzyme is

sensitive to trypsin digestion. Histidine-treated enzyme is digested at approximately two times the control rate. When the sensitivity to trypsin digestion is measured on histidine-insensitive enzyme, the curves obtained are indistinguishable from those obtained with histidine-sensitive enzyme. Histidine alters the enzyme so that its sensitivity to trypsin digestion is increased whether the enzyme is histidine-sensitive or histidine-insensitive. This observation supports the hypothesis that the binding of histidine by the enzyme does not in itself account for histidine inhibition. These experiments also suggest that histidine must alter the conformation (structure of configuration) of the enzyme molecule in some manner that renders the enzyme more sensitive to trypsin digestion.

Two lines of evidence suggest that the binding of histidine by the dichlorophenol stimulated DPNH oxidase is not sufficient to cause inhibition of enzyme activity. Histidine increases the rate of trypsin digestion of the enzyme. When an aged enzyme preparation which has become insensitive to histidine inhibition is tested for its sensitivity to trypsin, its rate of hydrolysis is identical with that of fresh enzyme. More significant, the rate of hydrolysis of histidine-insensitive enzyme is also increased by histidine treatment. One interpretation of these results is that histidine can bind to the enzyme and alter the trypsin sensitivity of the enzyme, but that the site of such binding is different from the site at which histidine binds when it inhibits the enzyme. An alternative explanation is that histidine binding at a single site is the only prerequisite to histidine inhibition and only interaction of the bound histidine with a second site results in enzyme inhibition.

A second type of experiment which suggests the presence of two sites of histidine binding came from the plot of S/v vs I. At low histidine concentration, one K_i is demonstrated, whereas at higher concentration another K_i is evidenced; i.e., histidine has two effects on the enzyme. As with the trypsin experiments, these data can be interpreted in terms of histidine interacting at one or more sites on the enzyme molecule.

The plot of S/v vs I demonstrates inhibition-activation.[4] That is, the velocity that would be projected on the basis of the initial slope is greater than would be expected with increasing inhibition concentration. Inhibition by histidine is not reversible. The two K_i values suggest the possibility of two enzyme sites, each having a different affinity for histidine. In some manner it would seem that attack by the first molecule of histidine results in inhibition; attack by the second molecule, at another site, results in decreasing the inhibition caused by the first histidine molecule.

The postulated scheme in Figure 19 depicts the effects of histidine on dichlorophenol activity. Two sites of histidine attachment to the enzyme molecule are represented as points (b) and (c). Site (b) is located close to, but not in, the active site at point (a). This is suggested since histidine inhibition is cofactor protected. Site (b) is represented as the only point of histidine inhibition. Since enzyme preparations are not always sensitive to histidine inhibition or lose their histidine sensitivity, this would imply that the necessary bonding requirements at site (b) are labile. When available, the affinity of

[4]The plot of S/v vs I and the scheme depicted in Figure 19 were the result of a personal communication with Dr. Paul W. Wigler.

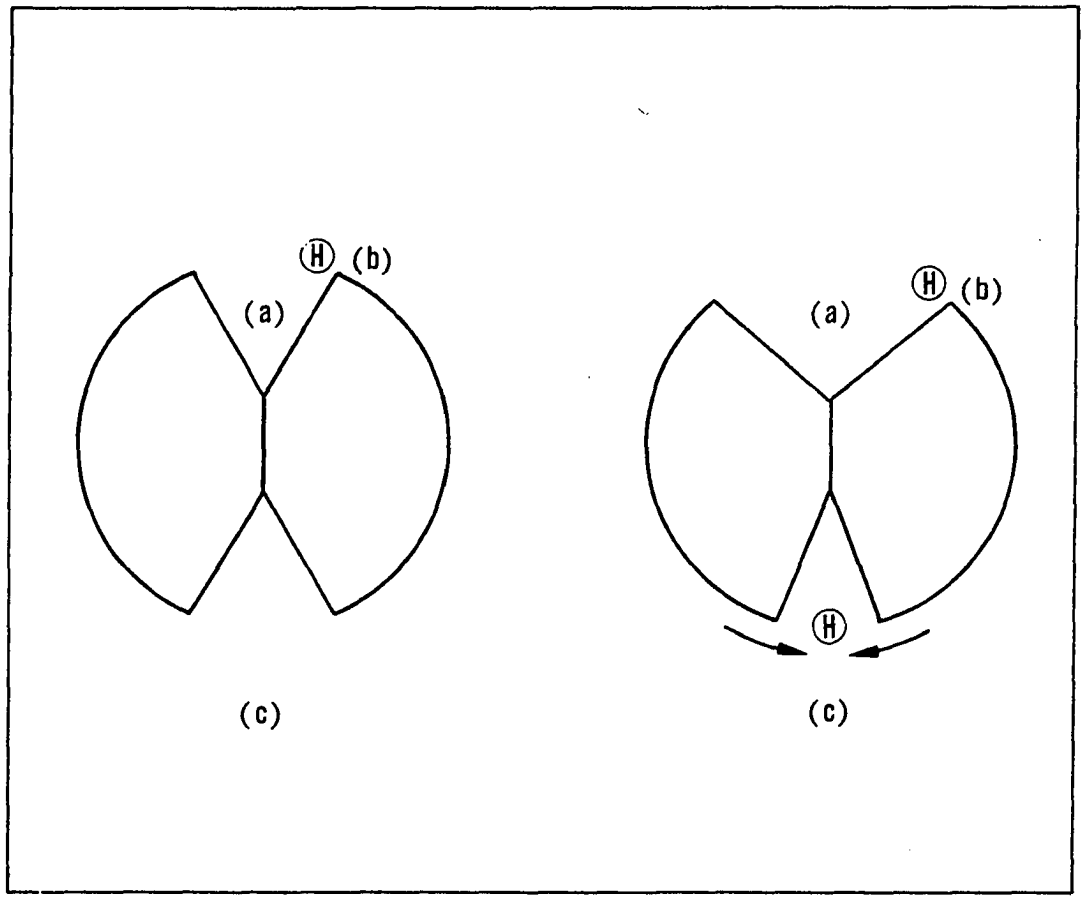

Figure 19. Postulated Scheme for non-reversible Inhibition Activation by Histidine on Dichlorophenol Activity. Histidine-inhibited enzyme, attack by first histidine Ⓗ molecule at point (b) close to active site (a). Effect of second histidine molecule at point (c), partially removes steric block by first histidine molecule.

site (b) for histidine is greater than that at site (c). Site (c) is represented as the point of attachment by the second histidine molecule. This attack results in a configuration change in such a manner to lessen the inhibition produced at site (b), i.e., inhibition activation. Site (c) is sensitive to histidine attack whether or not site (b) is available to histidine. This would explain why all histidine treated enzyme preparations, sensitive to histidine inhibition or not, undergo a digestion by trypsin at a rate twice that of untreated controls.

The data seem to suggest that histidine might act by causing a conformational change in the enzyme structure and by preventing cofactor participation rather than interfering with the binding of one of the substrates. This hypothesis is supported by the observation that histidine is a "non-competitive" inhibitor of the enzyme with respect to substrate, Pi and DPNH. The non-reversible inhibition-activation produced by the trypsin digestion study also demonstrates that some conformational alteration in the protein occurs in the presence of histidine. In view of the conclusion that histidine binding occurs in the absence of histidine inhibition, it does not appear that a conformational alteration of the protein is of importance in causing inhibition. The inhibition-activation study suggests that two sites of histidine sensitivity are involved. One site results in inhibition, the other site decreases the former inhibition, perhaps by conformational alterations.

CHAPTER VI

SUMMARY

A major difference in the chemical and physical requirements on the enzyme molecule has been shown to exist for eliciting the stimulation of DPNH oxidase in rat uterus by dichlorophenol and thyroxine. Four major lines of evidence resulting from this work substantiate the difference in enzymatic requirements of the enzyme molecule.

1. Fractions were prepared free of thyroxine stimulated DPNH oxidase, but which contained dichlorophenol stimulated DPNH oxidase. The preparation of a fraction containing a high level of thyroxine activity and a low level of dichlorophenol activity was demonstrated. A disproportionate ratio of dichlorophenol to thyroxine activity was evident during the fractionation process, i.e., a six fold overall change.

2. The use of high ionic strength for extraction of dichlorophenol activity resulted in a preparation which was dependent on phosphate. A phosphorylated protein was demonstrated as necessary for dichlorophenol activity, but not for peroxidase or thyroxine activities. ADP and ATP, but not AMP, substituted for orthophosphate.

3. Peroxidase, dichlorophenol and thyroxine activities were inhibited by free radical trappers and -S-S- bond inhibitors. A labile

S-H bond was demonstrated to be necessary for dichlorophenol activity but not for peroxidase and thyroxine. The sulfhydryl bond inhibition could be reversed and was protected by Pi, Mn^{++}, dichlorophenol and tocopherol. A thiol inhibition-stimulation mechanism was proposed.

4. Histidine and its analogs produced inhibition or stimulation of dichlorophenol activity. Histidine has been shown to be an effective non-competitive inhibitor of dichlorophenol activity with respect to phosphate and DPNH. Histidine could result in inhibitor-activation and had two K_i values. Peroxidase and thyroxine activities were not affected by histidine. It was proposed that histidine has two separate effects on the dichlorophenol activity. Histidine binding alone did not cause inhibition. Evidence has been presented that a conformational change is associated with histidine binding.

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