THE DEMONSTRATION, CHARACTERIZATION AND PARTIAL PURIFICATION OF A PEROXIDASE AND NADH OXIDASE IN UTERINE TISSUE

by

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B. S., Oregon State University, 1961

A MASTER'S THESIS

submitted in partial fulfillment of the

requirements for the degree

MASTER OF SCIENCE

Department of Biochemistry

KANSAS STATE UNIVERSITY
Manhattan, Kansas

1966

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INTRODUCTION

A peroxidase and a NADH oxidase system have previously been demonstrated in rat uterine tissue by several workers[5,19,47,48,77,81]. The purpose of this investigation is to characterize these enzymes further, to extend such studies to other species, and to uncover their physiological role, if any.

It has been suggested[5] that peroxidase and oxidase are actually one enzyme, however, that work was done only on rat tissue. An attempt will be made here to show that the two enzymes can indeed be separated. Since there should be a similar pattern of intracellular enzyme distribution in higher animals, it will also be of interest to isolate these enzymes from the uteri of several species. These studies will include tissue from human, monkey, sheep, cow, pig, rabbit, cat, guinea pig and rat. Evidence will be given to show that the components of the system are separate entities and that the intracellular distribution of the enzymes is similar in all animals tested except the rat.

The peroxidase component of uterine tissue was further studied by comparing it with model porphyrin enzymes. This was done in an attempt to classify the activity of the uterine peroxidase in comparison with known peroxidase models. The absorption spectrum of this enzyme also was determined. From this and other experimental evidence an attempt will be made to show that this enzyme is a peroxidase distinct from previously isolated peroxidases.

A possible physiological role of the oxidase system in the uterus was studied. Since the NADH oxidase activity is dependent on a monohydric phenol, it was hypothesized that such a phenol could, in vivo, actually be a phenolic hormone such as estrogen or progesterone. A series of phenols and

hormones were therefore studied with this system, and evidence will be presented to show that hormones might well control the NADH oxidase reaction by synergistic and antagonistic mechanisms.

LITERATURE REVIEW

History

Uterine peroxidase was first demonstrated by Lucas et al.[77] in 1955 and since that time many reports have appeared concerning the nature and possible function of the peroxidase. The in vivo concentration of peroxidase was found to be highly estrogen-dependent and the amount of enzyme activity isolated was directly related to the amount of 17β-estradiol or diethyl stilbestrol injected into the animal. Lucas compared the rates of oxidation of three hydrogen donors by the uterine peroxidase as well as by peroxidases from other sources, and found the uterine enzyme to have similar donor oxidizing abilities as lactoperoxidase, but different from verdo-, yeast cytochrome c, horseradish and carrot peroxidases[77]. Neufeld et al.[91] suggested that the peroxidase may be characteristic of proliferating tissues, that organs with high peroxidase/cytochrome oxidase ratios in general had a high rate of cell renewal, and that peroxidase may partially substitute for cytochrome oxidase in proliferating tissues.

In 1963 Coleman[19] fractionated NADH oxidase and peroxidase enzyme systems from rat uterine tissue after extracting with solutions of high salt concentration. He found 2,4-dichlorophenol (DCP) and thyroxine-stimulated NADH oxidase activities, as well as peroxidase activity. He is the only worker up to this time who has simultaneously studied peroxidase and NADH oxidase activities in uterine tissue.

The oxidation of reduced pyridine nucleotides by horseradish peroxidase and oxygen was first clearly demonstrated by Akazawa and Conn[2] in 1958. Both reduced nicotinamide adenine dinucleotide (NADH) and reduced nicotinamide adenine dinucleotide phosphate (NADPH) were rapidly oxidized in the presence of horseradish peroxidase, molecular oxygen, catalytic amounts of Mn^{++} and certain phenols. The oxidase function of peroxidases had previously been observed with other donors. For example, Theorell and Swedin[112,116] in 1939 demonstrated that in the presence of Mn^{++} and oxygen, peroxidase could catalyze the oxidation of dihydroxyfumarate. Chance[11] in 1951 found that NADH could serve as hydrogen donor for peroxidases by reacting directly with horseradish peroxidase complex II. Conn et al.[20] found NADH to be oxidized by an enzyme complex of the wheat germ which was resolved into two components, one of which was a peroxidase. In 1953 Dolin[30] isolated a peroxidase from Streptococcus faecilis which oxidized NADH. This unusual enzyme was found to be a flavoprotein and contained no hematin prosthetic groups. Hydrogen peroxidase was the specific oxidant and oxygen could not be used. Stanbury[108,109] reported a NADPH oxidizing enzyme in sheep thyroid and rat liver. Therefore, the direct oxidation of reduced pyridine nucleotides is not unique to uterine tissues, but is found throughout the animal and plant kingdom.

Klebanoff[61,62,64] reported that thyroxine and other phenolic compounds stimulated NADH or NADPH oxidation by horseradish peroxidase (acting as an oxidase) in the presence of Mn^{++} and molecular oxygen. In this case the presence of Mn^{++} and oxygen replaced hydrogen peroxide. He also found that catalase and ascorbic acid inhibited the oxidation of NADH. In addition to NADH, substrates such as epinephrine, dihydroxyphenylalanine, ascorbic acid and ferrocytochrome c were oxidized at increased rates after the addition of

thyroxineor other phenol activators.

Hollander and coworkers[47,48,115] continued with work of Klebanoff by using phenolic estrogens as activators in a similar system. In 1960 they also studied the in vitro effect of added estradiol on the NADH oxidase system of rat uterus. Williams-Ashman[127] verified the finding that phenolic estrogens stimulated the NADH oxidation by the Mn^{++}- horseradish peroxidase - oxidase system, and found that estrogens can act as intermediary hydrogen carriers in such reactions.

Aromatic amines were also found to stimulate NADH oxidation by a horseradish peroxidase system. This finding by Williams-Ashman[128] is in agreement with that of Kenton[59] who showed that aniline accelerated the oxidation of indoyl-3-acetic acid in the presence of Mn^{++} and peroxidase. Yamazaki[132] also showed that m-phenylenediamine and aniline enhanced the oxidation of triose reductone by a turnip peroxidase.

The oxidation of NADH and NADPH by a peroxidase was found by Klebanoff[66,67] to be further enhanced by the addition of bisulfite ions. Apparently, the bisulfite is first oxidized and initiates the oxidative activity of the peroxidase possibly by a free radical mechanism. Hemoglobin, myoglobin, myeloperoxidase, uterine peroxidase, and a high concentration of inorganic ferrous ion could replace the horseradish peroxidase catalyst.

Klebanoff[69], in 1962, found NADH oxidizing capacity in a Mn^{++} and phenol supplemented peroxidase system from beef thyroid particles. The iodination of tyrosine and oxidation of NADH activities were both located in the particulate fraction and both activities were stimulated by hydrogen peroxide supplementation using either glucose-glucose oxidase or flavin mononucleotide. Yip[138] recently separated these two activities in a solubilized preparation by employing column chromatography on CM-sephadex C-50. The coupling of the

peroxidase-iodinase system was envisioned by Klebanoff to have the following mechanism:

1. NADH (or NADPH) + H^+ + O_2 $\xrightarrow{\text{peroxidase, } Mn^{++}}$ NAD^+ (or $NADP^+$) + H_2O_2
2. H_2O_2 + I^- $\xrightarrow{\text{peroxidase}}$ "oxidized iodide" + H_2O
3. "oxidized iodide" + tyrosine $\xrightarrow{\text{iodinase}}$ iodotyrosine

Klebanoff[68] also in 1962 found that ergothioneine (see structure in Appendix I) stimulated the oxidation of NADH and NADPH by horseradish peroxidase, Mn^{++} and oxygen. Ergothioneine was oxidized to ergothioneine disulfide in the course of the reaction. Certain sulfhydryls may therefore stimulate in a manner much like phenols and aromatic amines.

The first experimental work on the NADH oxidase in uterine tissue was done by Beard and Hollander[5] in 1959. Preliminary results indicated that the peroxidase and the NADH oxidase may be separable since their stabilities were so different. However, in later work[47] they failed to separate the two activities after considerable purification.

Coleman[19] accomplished a partial fractionation of rat uterine peroxidase, DCP stimulated and thyroxine stimulated NADH oxidase activities. A first fraction contained the peroxidase and some DCP activity, while a second fraction had DCP- and thyroxine-stimulated NADH oxidase activities. This separation took advantage of solubility differences in solutions of varying ionic strength. Thus, although some fractionation has been achieved, it has not been possible to clearly separate peroxidase activity from all oxidase activity in rat uterus up to this time.

Peroxidase Mechanism

Peroxidases are enzymes which catalyze the oxidation of various donors by hydrogen peroxide. They catalyze the transfer of 2 electrons from substrates to hydrogen peroxide, forming water and oxidized donors. There are

four major types of electron donors for peroxidatic oxidations as outlined by Mason[83]: (1) aromatic amines, (2) phenols, (3) aromatic acids, and (4) miscellaneous substances, such as cytochrome c, NADH, iodide, and leucomalachite green.

It was initially thought that peroxidase and catalase were the same, but basic differences were revealed as early as 1901 by Raudnitz. The mechanism of peroxidatic action was not studied until much later. Major reviews in this field have been given by Chance[11], Chance and Fergusson[14], Mason[83], Theorell[117,118], Wyman[130], Paul[96], Stern[110] and Saunders et al.[103].

Chance[11] proposed a mechanism differentiating peroxidase from catalase as follows:

$$\begin{array}{l} \text{Enzyme} + H_2O_2 \longleftrightarrow \text{Enzyme}\cdot H_2O_2 \\ \text{Enzyme}\cdot H_2O_2 + AH_2 \longleftrightarrow \text{Enzyme} + A + 2H_2O \end{array}$$

In the catalatic reaction AH_2 is also hydrogen peroxide and A becomes O_2. In the peroxidatic reaction AH_2 is the oxidizable substrate and A is the oxidized product. Catalase may thus be looked upon as a specialized peroxidase which requires H_2O_2 for both donor and oxidant. Chance obtained direct kinetic data on the rates of formation of enzyme-substrate complexes by means of rapid flow spectroscopy. Although he studied horseradish peroxidase, the mechanism may well be pertinent to peroxidases in general. The peroxidase reaction mechanism can be summarized by the 3 following steps[14]:

Step I. Formation of the enzyme-substrate complex:
$$\text{Peroxidase} + H_2O_2 \longleftrightarrow \text{Peroxidase-}H_2O_2 \quad \text{(Complex I)}$$

Step II. Transition from primary to secondary complex:
$$\text{Peroxidase-}H_2O_2 + AH \longleftrightarrow \text{Peroxidase Complex II} + A$$

Step III. Liberation of the enzyme from Complex II:
$$\text{Peroxidase Complex II} + AH \longleftrightarrow \text{Peroxidase} + A + 2H_2O$$

Steps I and II are the rate-limiting steps for Step III proceeds very rapidly. Reaction kinetics of Complex II with NADH has been further studied by Yamazaki[135].

The existence of a half-oxidized donor molecule, i.e., a free radical, was also proposed by Chance[14] in the following steps:

$$\text{Step I:} \quad \text{Enz-Fe}^{+++}(H_2O) + H_2O_2 \rightleftharpoons \text{Enz-Fe}^{+++}(H_2O_2) + H_2O$$
$$\text{Step II:} \quad \text{Enz-Fe}^{+++}(H_2O_2) + AH_2 \rightleftharpoons \text{Enz-Fe}^{+++}(OH\cdot) + AH\cdot + H_2O$$
$$\text{Step III:} \quad \text{Enz-Fe}^{+++}(OH\cdot) + AH\cdot \rightleftharpoons \text{Enz-Fe}^{+++}(H_2O) + A$$

The importance of the free radical will be discussed later.

Oxidase Mechanism

The oxidation of a substrate by peroxidase in the presence of manganous ions and oxygen was studied first by Lemberg and Legge[74], and later by Chance[13] using a system in which dihydroxyfumaric acid was oxidized to diketosuccinic acid with horseradish peroxide. The stoichiometry of the reaction was very difficult to determine, however. Chance suggested a ternary Mn^{++}-H_2O_2-peroxidase system and put forth the following mechanism:

$$O_2 + \text{dihydroxyfumarate} \rightleftharpoons H_2O_2 + \text{diketosuccinate}$$
$$H_2O_2 + \text{dihydroxyfumarate} \rightleftharpoons 2H_2O + \text{diketosuccinate}$$

This mechanism differs from a more complicated one suggested by Lemberg and Legge[74]. The above system is inhibited by catalase and cyanide, indicating the involvement of both H_2O_2 and a heme protein. Mason[82] has used ^{18}O to study this reaction. His results indicate that the ferriperoxidase combines with molecular oxygen and activates it toward aromatic substrates.

A somewhat similar mechanism was suggested by Tanaka and Knox[113] for the oxidation of tryptophan in liver. However, their system was not inhibited by catalase or cyanide after the reaction began, showing involvement of hydrogen peroxide only in the initiation step.

A mechanism accounting for the oxidation of NADH by a peroxidase-Mn^{++}-oxygen system was first postulated by Akazawa and Conn[2]. In the first reaction a phenol is half-oxidized and it can in turn oxidize other compounds.

The second reaction may proceed enzymatically or non-enzymatically. The hydrogen peroxide produced in step 1 could then oxidize more phenol in a typical peroxidase reaction:

1. $2\ ROH + O_2 \xrightarrow{\text{peroxidase} + Mn^{++}} 2\ RO\cdot + H_2O_2$
2. $2\ RO\cdot + NADH + H^+ \longrightarrow 2\ ROH + NAD^+$
3. $2\ ROH + H_2O_2 \longrightarrow 2\ RO\cdot + 2\ H_2O$

The half-oxidized phenol probably exists as a phenoxy radical, associated with the enzyme in a complex and could be reduced by NADH as shown in step 2. In this case, the subsequent oxidation of NADH is analogous to the oxidation of various donor molecules by the peroxidase-OH· complex proposed by Chance[14].

A mechanism for oxidation of NADH by uterine peroxidase-Mn^{++}-oxygen was suggested by Beard and Hollander[5]:

1. $NADH + O_2 + H^+ \longrightarrow NAD^+ + H_2O_2$
2. $H_2O_2 + 2\ ROH \longrightarrow 2\ RO\cdot + 2\ H_2O$
3. $2\ RO\cdot + Mn^{++} + 2\ H^+ \longrightarrow 2\ ROH + Mn^{++++}$
4. $2\ RO\cdot + NADH + H^+ \longrightarrow NAD^+ + 2\ ROH$

They claimed that H_2O_2 in the first step was detected with starch iodide, and the formation of NAD^+ was observed by an increase in absorbance at 340 mμ after addition of alcohol and alcohol dehydrogenase. This corresponds to the finding of Andreae[3] that H_2O_2 is produced during the oxidation of indoleacetic acid. In reaction 2 the H_2O_2 formed in step 1 is shown to oxidize the phenol to a phenoxy radical. This step had also been suggested by Akazawa[2] and Williams-Ashman[127]. They suggested that the phenoxy radical may then take one of two possible steps. Step 3 shows the radical being reduced by the Mn^{++} and accounts for the formation of Mn^{++++} observed during the oxidase reaction by Beard and Hollander[5]. Kenton and Mann[55,56] also observed that Mn^{++} was oxidized to Mn^{++++} in the presence of horseradish peroxidase, phenols and H_2O_2. It has been shown by Maclachlan[78] that Mn^{++++} will oxidize indoleacetic acid nonenzymatically. The phenoxy radical could also be reduced by NADH as

shown in step 4. Although not shown above, it seems likely that any Mn^{++++} generated would oxidize NADH to NAD^+ also.

The actual transitory existence of free radical intermediates has been shown by several workers. Mason and coworkers[133], for example, in 1960 identified free radicals enzymatically generated from the substrates hydroquinone, ascorbic acid and dihydroxyfumarate, by the action of horseradish peroxidase. Yamazaki[134] also identified free radical intermediates in the indoleacetic oxidase reaction catalyzed by horseradish peroxidase. This system is similar to the uterine system in that Mn^{++} is a necessary cofactor. The existence of transient free radical forms of estrogens was demonstrated only recently by Borg[9]. Not only could diethylstilbestrol and estradiol free radicals be resolved, but free radical intermediates were also found for the iodothyronines, insulin and indoles.

Role of Manganese

The oxidation of phenylacetaldehyde[58], phenylpyruvic acid[21], dicarboxylic acids[57], indoleacetic acid[123], ferrocytochrome c[88], glutathione[88] and 2-nitropropane[76], as well as NADH and dihydroxyfumarate discussed previously, all require or are stimulated by manganese-supplemented peroxidase. It would simplify interpretation if the role of Mn^{++} was the same in each case, but apparently this is not so. The reaction mechanisms differ with the concentration of Mn^{++} and certain chelators. For example, NADH oxidation catalyzed by horseradish peroxidase-Mn^{++} is inhibited by catalase and EDTA, but not by citrate or pyrophosphate. However, indoleacetic acid oxidation[89] catalyzed by horseradish peroxidase-Mn^{++} is not inhibited by catalase or EDTA, but is inhibited by citrate and pyrophosphate. It is therefore apparent that the reaction mechanisms are different. Several workers[2,5,19,88,89] have found

that Ce^{+++} may be substituted for Mn^{++} in peroxidase-oxidase systems. Mudd and Burris[88] found this substitution possible for the horseradish peroxidase-catalyzed oxidation of NADH, indoleacetic acid and 2-nitropropane. These workers found that the optimum concentration of Mn^{++} increased as the concentration of the phenolic compound increased.

Manganous ion has been found by Chappell et al.[17] to greatly stimulate the respiration of rat liver mitochondria at concentrations up to 1 mM. They found that these mitochondria actually accumulate Mn^{++}. Saris[102] found Mn^{++} and Sr^{++} to cause swelling and a slow pH change in the mitochondria, as well as to initiate active transport of adenosine triphosphate (ATP). These ion effects on the mitochondria may profoundly affect the activity of oxidative enzymes contained in the mitochondria.

Since Mn^{++} is a necessary cofactor in the peroxidase-oxidase system, it is of interest to find if Mn^{++} is concentrated in tissues where such systems might be present. Ray[29] found the uptake of Mn^{++} to be especially great in the thyroid gland. The thyroid has 0.1 to 0.3 mg per cent dry weight Mn^{++} and this increases 4 times in goiterous condition[39]. The thyroid is therefore noteworthy in that it has a great capacity for storing Mn^{++}, as well as possessing an iodinating and NADH oxidizing ability. Another significant finding was that of Mikhailov[86] who showed that administration of Mn^{++} caused a marked proliferation in glands of internal secretion, especially the thyroid gland. Little experimental work has been done on the manganese content of the uterus, however. de Azcona[27], using spectrographic methods, found manganese in the uterus to be in the range of 0.01 to 0.1%, similar to that found in the ovaries, stomach and thyroid[100]. Uterine washings were found by Heap[46] to contain no detectable manganese. Although this wide range prevents specific comparison with the other organs, uterine manganese content is approximately the same as

other organs. It is notably higher than the average abundance in the human body which is 0.001%. It cannot be said, however, that the uterus is outstanding in its manganese content. A summary of the manganese content of various tissues and glands found in the literature is shown in Table I.

Table I. Concentration of manganese in tissues.

Tissue	% w/w	Ref.	Tissue	% w/w	Ref.
Liver	0.21	38	Kidney	0.06	38
Brain	0.03	28	Uterus	0.01-0.1	42
Heart	0.04	71	Ovary	0.01-0.1	100
Muscle	0.05	38	Stomach	0.01-0.1	100
Lung	0.02	38	Thyroid	0.01-0.1	100

Model Peroxidases

The study of model peroxidases has been of great interest since 1940 when Theorell cleaved hemin from horseradish peroxidase and inactivated it. Paul[94] also split horseradish peroxidase, then combined it with various hemins, and tested the activities of the new compounds. Maehly[80] prepared hemin derivatives in which either the iron or carboxyl groups were missing, and studied the combination of these with apo-horseradish peroxidase. Falk[35] described a reconstitution of horseradish peroxidase apoenzyme with meso- and hematohemes which produced a greater activity than when protoheme, the natural prosthetic group, was used. These studies showed that the peroxidatic activity depended to a great extent on the type of heme compound as well as the heme-protein binding. Recent work on model enzyme systems employing simple ligands with iron porphyrins has been done by Nakamura[90,120]. They have demonstrated oxidase and peroxidase activity with simple heme derivatives using leucomalachite green as a hydrogen donor.

Hormone Effect on Enzymic Activity

According to Williams-Ashman[129], there are two major reasons why hormonal effects recently have generated so much interest among enzymologists. First, hormones exert their actions on in vitro enzyme systems at extremely low concentrations, and second, in some of these reactions the hormones appear to participate in hydrogen or electron transfers by undergoing rapid alternate oxidation and reduction. When hormones function in this manner, they exhibit co-enzyme like characteristics. It is of great interest whether any of the observed in vitro effects is related to the biochemical action by which these hormones regulate the structure and function of the cell.

There is a great abundance of literature on the effects of specific hormones on specific enzyme systems, as well as on overall biological activity. It has been found that estrogens will stimulate uterine respiration and glycolysis[99,104], nucleic acid formation[114], amino acid incorporation[43,75], inorganic phosphate incorporation[45], glycogenolysis[104], RNA synthesis[42] and oxidation of Kreb cycle intermediates[6]. The activities of enzymes such as malic dehydrogenase, succinic dehydrogenase, cytochrome oxidase and ATPase have also been found to parallel hormonal activity. Strangely enough, some enzyme systems are inhibited by the same hormones which stimulate other systems. Scott and Lisi[105] found the effect of estradiol on the in vitro activity of glucose-6-phosphate dehydrogenase, phosphogluconate dehydrogenase and hexokinase activities in rat uterus to depend on the concentration. At low concentrations estrogen stimulated the activities, which were then inhibited at increasing hormone concentration. This inhibitory effect of steroid hormones has also been demonstrated in oxidizing systems, such as succinoxidase[10,125] and mitochondrial NADH oxidase[8,115]. These steroidal inhibitions are generally found only at unphysiologically high concentrations, and are quite

unspecific as to their mode of inhibition[129].

An enzyme, 17β -hydroxysteroid dehydrogenase, has been isolated from human placenta and shown to be hormone mediated[121]. This soluble enzyme catalyzes the reduction of NAD^+ and $NADP^+$ by 17β-estradiol and the oxidation of NADH and NADPH by estrone. It is thought that the dehydrogenase catalyses the transfer of hydrogen from NADPH to NAD according to the reaction:

$$\begin{array}{l} \text{NADPH} + \text{estrone} + H^+ \rightleftharpoons \text{NADP}^+ + \text{estradiol} \\ \text{NADP}^+ + \text{estradiol} \rightleftharpoons \text{NADH} + H^+ + \text{estrone} \\ \hline \text{NADPH} + \text{NAD}^+ \rightleftharpoons \text{NADP}^+ + \text{NADH} \end{array}$$

There are several propositions by workers in this field as to how hormones affect enzyme activity. Lehninger[73] suggested that hormones may exert their characteristic control of enzymes by affecting the mitochondria where the enzyme is located. He proposed 3 sites of hormone interaction with mitochondria: (1) By controlling the concentration of substrates outside the mitochondrion, and thus indirectly affecting the enzyme, (2) by rearranging the multienzyme systems within the mitochondrion, and (3) by affecting the semi-permeable membrane surrounding the mitochondrion.

Mueller[85] envisioned metabolic alterations induced by hormones at 3 possible sites of protein anabolism. The first may be the "unmasking" of the template, initiating the synthesis. Second, the hormone may regulate the combination of the newly-forming protein with the template by inducers and suppressors. The third site, Mueller suggests, may be the final dissociation of new protein from the template.

Yielding[137] also proposed 3 approaches to hormonal regulation of enzyme activity: (1) A hormone may regulate an enzyme by altering its structure, (2) control of the enzyme structure and activity may be a multivalent process requiring the simultaneous or antagonistic action of more than one compound, and (3) the control exerted on an enzyme may involve a location

other than the active site of the enzyme.

Biochemistry of the Uterus

The biochemical composition of the uterus is of importance in understanding the relation of enzyme and hormone balance. The respiration of the uterus of many animals has been shown to vary with hormone concentration and the ovarian cycle. Aerobic metabolism and glycolysis in the uterus are both under hormone control. This is not strictly characteristic of the uterus, however, since the respiration of rat liver, ovaries and anterior pituitary varies cyclically also[98]. Uterine glycogen deposition, which is also under hormonal control, is not uniform but is found in highest concentration in the endometrium, and this region is also richer in lipase, amylase and trypsin than any other portion of the organ.

The water content[22] of the uterus is about 80%, depending on the reproductive state. A single injection of estrogen was found to increase the weight of uteri in rats by 60% in a six-hour period[98], indicating the sensitivity of water balance to hormonal control. The rate of water uptake in the endometrium is much greater than in the myometrium.

Uterine tissue varies in its inorganic composition[23] from the other types of muscular organs in that it contains much more sodium and calcium and less potassium, magnesium and phosphorus. Uterus is most sensitive to calcium activation which can completely reverse the inhibitory effect of progesterone on uterine muscle. Strontium has a similar, but less effective action on the uterus. These two ions apparently modify the ATP activation of actomyosin and thus modify the contraction mechanism.

Buffering capacity of the uterus varies in different phases of the ovarian cycle and this property of the organ is rapidly lost in the absence of hormones.

The period of highest uterine acidity during the estrous cycle in rats coincides with the period of maximum oxygen consumption. The tissue becomes increasingly alkaline so that at metestrus, the acidity is less than at any other time. Hydrogen ion concentration increases rapidly again to approximately 6.7 which the normal nongravid uterus then maintains. The buffering mechanism in uterine tissue is thought, by Reynolds[98], to be necessary for the dynamic equilibrium between actomyosin and ATP.

The oxidation-reduction potential[98] of the uterus is effected by a balance of ascorbic acid and glutathione. An increase in ascorbic acid in the endometrium coincides with an increase in active proliferation of the endometrium as well as the myometrium. Glutathione appears to be an essential constituent of the uterus, but acts as an antagonist of ascorbic acid. In fact, it has been shown[98] that the concentration of glutathione decreases as the concentration of ascorbic acid increases, and vice versa.

Gautheron et al.[37] found that pig uterine muscle contained only about one-seventh as many mitochondria as pig cardiac muscle, and the uterine mitochondria seemed much more fragile than heart mitochondria. The number and size of uterine mitochondria was found[38] to increase greatly by the in vivo administration of progesterone, indicating hormone control even of the intact mitochondria.

Soma[107] reported that a solvent-extractable hormone-like substance was produced in the uterus. This hormone was thought to cooperate with the follicle stimulating hormone (FSH) in controlling uterine growth. The uterus, therefore, appears to have an endocrine function of its own.

MATERIALS AND METHODS

Tissue Sources

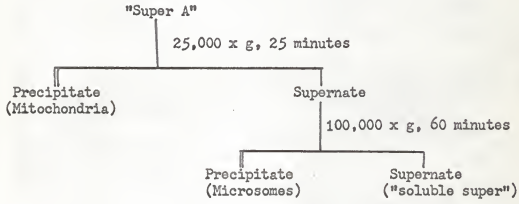
Uterine tissue from nine different animals was used in this study. Tissues were obtained fresh from bled animals when possible and chilled immediately. Tissues which were still reddish appearing were frozen, sliced and rinsed in cold deionized water to remove blood. Some materials not available locally were obtained frozen on dry ice from Pel-freez Biologicals Company (see location of distributors in Appendix II). Immature or gravid tissues were not used. All tissues, whether obtained fresh or as dry ice-packed commercial samples, were stored frozen at -20° before enzyme extraction.

Sheep and beef uteri were obtained from Kansas State University Animal Industries slaughters. Uteri were taken from the killed and bled animals and immediately put on ice. Another group of sheep uteri was obtained from the Animal Husbandry department during hysterectomy of experimental ewes. Guinea pigs were obtained from the Bacteriology Department. The uteri were taken from animals which were ether anesthetized, killed and bled for three minutes. The uteri were removed, rinsed in ice cold water and frozen. Cat uteri, excised and treated in the same manner, were obtained through the Veterinary Clinic. A sample of normal human uterine tissue was obtained through the cooperation of Dr. G. Mowry and the St. Mary's Hospital surgical staff. The tissue was chilled as soon as it was released from surgery, frozen, sliced and rinsed to remove blood.

Monkey, dog, rabbit and rat uteri were obtained from Pel-freez Biologicals Company. The monkey and rat tissues were relatively free from blood. The dog and rabbit uteri contained a moderate amount of blood and were therefore frozen, sliced and rinsed as previously described.

Handling of Tissue and Extraction of Enzyme

The frozen, blood-free tissue was blotted dry, minced as finely as possible with a stainless steel knife and weighed. A 15% homogenate of the tissue (w/w) in 0.25 M sucrose was prepared by homogenizing three times for one minute each in a Waring blendor. The chamber was cooled on ice between each blending. The homogenate was strained through cheesecloth and centrifuged at 1000 x g for 20 minutes in a Lourdes refrigerated Model A Beta-fuge. The precipitate was discarded, and the supernate labelled "Super A". The following centrifugation steps were done to "Super A":



The supernate remaining after the 100,000 x g centrifugation was called "Soluble Super". The precipitated subcellular components were made up in a convenient volume of 20 mM phosphate buffer, pH 6. For the 25,000 x g centrifugation, a Lourdes centrifuge with a 9RA head was used and for the 100,000 x g spin, a Spinco Model L Preparative Ultracentrifuge with a number 40 rotor was employed. Each fraction was then assayed for peroxidase and for NADH oxidase activity.

Enzyme Assay Methods

Leuco TIP Peroxidase Assay. The peroxidase assay used was a modification

of the method of Smith, Robinson and Stotz[106], which was improved by Lucas and coworkers[77]. This method is based on the peroxidatic reoxidation of reduced (leuco) 2,3',6-trichloroindophenol (TIP) dye (see structure on page 25) obtained from Eastman Distillation Products and used without further purification. The modification worked out in this study employed the convenient and reliable reducing action of thiosulfate to produce a leuco dye substrate. The approach had many advantagesover previous methods which used hydrogen gas and a palladium catalyst for reduction. When optimally reduced, the dye turned from bright blue to a pale yellow color. It was found necessary to grind the TIP to a fine powder before complete solution and subsequent reduction could be obtained. The dye solution was then made up in a volumetric flask to which buffer and reducing agent had previously been added. In the presence of hydrogen peroxide and a uterine extract, the rate of blue color formation due to reoxidation of leuco TIP was proportional to enzyme concentration.

The oxidation rate of leuco TIP by the peroxidase was measured at 10 second intervals on a Model 6A Coleman Junior Spectrophotometer by plotting the absorbance change at 675 mμ against time. The slope was usually linear for the first 45 seconds, but fell off rapidly after that. A unit of enzyme is that amount causing a change of 1 absorbance unit per minute in the linear region. The tubes were incubated in a 30° water bath, and the spectrophotometer zeroed with all components except peroxide. Each tube contained 1.0 ml of 0.2 M phosphate buffer, pH 6.0, 2.0 ml of 1 mM leuco TIP, enzyme and water to 6.0 ml total volume. The reaction was started with 1.0 ml of 8.4 mM H_2O_2. The hydrogen peroxide solution was made fresh daily from a refrigerated stock solution and the concentration determined colorimetrically with a titanium sulfate reagent[34].

NADH Oxidase Assay. The NADH oxidase assay used was that of Klebanoff[56]. The rate of oxidation of NADH is determined by following the decrease in absorbance at 340 mμ on a Beckman DB spectrophotometer equipped with a recorder. The initial rate of change is proportional to the enzyme concentration.

Components of the 1 cm cuvette are as follows: 1.0 ml of 0.2 M phosphate buffer, pH 6.0, 0.1 ml of 5 mM $MnCl_2$, 0.1 ml of 10 mM $NaHSO_3$, 0.1 ml of 20 mM 2,4-dichlorophenol (DCP), enzyme and water to 2.9 ml, and 0.1 ml of 3 mM NADH to start the reaction. Identical components were added to the reference cell, except 0.1 ml of water was added in place of the 0.1 ml NADH. The cells were balanced in spectrophotometer at zero and infinite absorbances before addition of the NADH. There was an initial lag period of about 30 seconds, a linear portion for about 3 minutes, and then a falling off due to depletion of NADH. The rate was measured only over the linear region. A unit of enzyme is that amount producing a change of 1 absorbance unit per minute under these conditions. The NADH and NADPH which were prepared fresh daily were obtained from the Sigma Chemical Company and were more than 95% pure. All other chemicals were Baker and Adamson reagent grade.

Starch-Iodide Peroxidase Assay. This method, taken from Cunningham et al.[24], takes advantage of the blue color formed by iodine with starch. After prior incubation at 30°, 5.0 ml of starch-iodide assay medium (0.28 M acetate, pH 4.6, 0.01% starch and 0.56 mM KI) and 1.0 ml of diluted enzyme are added to a colorimeter tube and the instrument is zeroed. Then 1.0 ml of 1.4 mM H_2O_2 is added to start the reaction. After a lag period a linear rate of blue color formation is measured at 600 mμ. A unit of activity in this study was defined as on page 18 rather than the reciprocal time definition originally employed.

Pyrogallol Peroxidase Assay. This method was taken from Chance and Maehly[15] and modified by D. Louie of this laboratory. To 4.0 ml of 0.1 M pH 5.5 acetate buffer is added 1.0 ml of 5.6 mM pyrogallol and 1.0 ml of diluted enzyme. After zeroing the instrument, 1.0 ml of 7 mM H_2O_2 is added and the rate of color formation read at 560 mμ. A unit of activity was defined as on page 18.

Guaiacol Peroxidase Assay. This method was also taken from Chance and Maehly[15] and modified by L. Nicholson of this laboratory. To 4.0 ml of 0.1 M Tris pH 7.4 buffer was added 1.0 ml of 0.1 M guaiacol and 1.0 ml of diluted enzyme. The reaction was started by the addition of 1.0 ml of 1.4 mM H_2O_2 and was read at 470 mμ. A unit of activity was defined as on page 18.

Dianisidine Peroxidase Assay. This method was taken from Coleman[19]. To 5.9 ml of 10 mM H_2O_2 in 0.01 M phosphate buffer, pH 6.0 was added 0.05 ml of 1% dianisidine in methanol. Then 0.1 ml enzyme was added to start the reaction which was read at 460 mμ. A unit of activity was defined as on page 18.

4-Methoxy-α-Naphthol Peroxidase Assay. This method was taken from Guilbault and Kramer[44] and was slightly modified. To 0.5 ml of 0.1 mM 4-methoxy-α-naphthol was added enzyme and water to 6.0 ml total volume. The reaction was started by the addition of 1.0 ml of 12 mM H_2O_2 and the rate of color formation was measured at 620 mμ. A unit of activity was defined as on page 18. Considerable non-enzymatic oxidation of the reagent was found and this was subtracted.

Protein and Iron Determinations

Protein Assay. Protein was determined by a modification of Yonetani's

version[139] of the Gornall method[41]. To a protein sample of less than 8 mg is added 0.5 ml of 3% hydrogen peroxide, 2.0 ml of biuret reagent (1.5 gm of $CuSO_4 \cdot 5H_2O$, 6.0 gm of sodium potassium tartrate, and 40 ml of carbon dioxide-free 75% sodium hydroxide, all diluted to 500 ml) and water to make a final volume of 5.0 ml. The tubes were well mixed, allowed to stand for 30 minutes at room temperature, and the absorbance at 540 mμ measured against a reagent blank. This method is especially useful in peroxidase and cytochrome work for peroxide bleaches out the alkaline hemochromagen of heme proteins which would otherwise interfere with the biuret color. A final molarity of 0.2 M ammonium sulfate was found to have a negligible effect on the absorbance at 540 mμ. Crystalline bovine plasma albumin from Armour Pharmaceutical Company was used as a protein standard.

Iron Determination. Iron determinations were done by the method of King and Nickel[60], from a modification of the method of Yonetani[138]. The samples to be assayed were dried at 105° overnight in micro Kjeldahl digestion flasks which had been preboiled in concentrated HCl to remove any iron contamination. The dried samples were digested in about 2 ml of concentrated sulfuric acid plus 1 ml of 30% hydrogen peroxide until the color was gone. Five ml of deionized water was then added to each tube and the samples boiled until white fumes evolved. This was repeated two times to remove traces of hydrogen peroxide which would interfere with the colorimetric assay. The digested sample was then made up to 10 ml with deionized water.

o-Phenanthroline obtained from Fisher Scientific Company was used for the colorimetric iron determinations. A standard iron solution of 50 ug per ml was prepared from Baker and Adamson primary standard iron wire. An aliquot of the digested iron sample (2.5 to 25 ug Fe) was diluted to 3 ml and to this solution 1.0 ml of 0.25% o-phenanthroline, 0.5 ml of 1% hydroquinone (prepared

fresh daily) and 2.5 ml of 25% sodium citrate was added. The pH then was carefully adjusted to approximately 4 with sulfuric acid or ammonium hydroxide by checking with pHydrion vivid 3-9 pH paper. The tubes were made up to 10 ml, allowed to stand at room temperature for 60 minutes, the absorbance was read at 509 mμ and unknowns were determined from a standard curve.

Miscellaneous Determinations

Absorption Spectra. Absorption spectra, run on either Beckman DB or Cary Model 11 recording spectrophotometers, were scanned from 700 to 370 mμ. Sodium dithionite was used to reduce the samples and potassium ferricyanide to oxidize them. Both were added in minimal amount to prevent artifacts. To the reduced sample also was added separately: NaN_3, KCN, H_2O_2 and KOH to form derivatives.

The pyridine hemochromagens were prepared according to the method of Keilin and Hartree[54]. A suitable volume of preparation (not greater than 4 mg protein) was pipetted into a 10 ml volumetric flask containing 0.4 ml of 1 M NaOH to which 2 ml pyridine was then added, followed by about 50 mg $Na_2S_2O_4$. The flask was taken to volume with distilled water, stoppered, mixed and read after 30 minutes.

Hydrogen Peroxide Standardization. Standard hydrogen peroxide was prepared from a 30% solution, by titrating with standard $Ce(SO_4)_2$ with ferrous-o-phenanthroline (Ferroin) as indicator[50]. Dilutions of this refrigerated stock were prepared daily and were routinely analyzed by the colorimetric titanium sulfate method[34].

Solubilization Procedures. Several attempts were made to rupture the mitochondrial fraction. A freeze-thaw experiment was done using an Aminco refrigerated bath set at -16°. A large volume (120 ml) of diluted mitochondria

was slowly frozen and thawed 9 times. After each freeze-thaw an 11 ml aliquot was taken, homogenized and centrifuged 45 minutes at 100,000 x g. The precipitates after the centrifugation were made up in a convenient volume of 20 mM phosphate buffer, pH 6.0. The soluble and the particulate fractions were then assayed for NADH oxidase activity.

Trypsin and digitonin (obtained from Nutritional Biochemical Corporation) were used in the proteolytic digestion-digitonin method described by Klebanoff[69] for solubilizing the NADH oxidase activity from the particulate fraction of beef thyroid preparations. The mitochondria were suspended in a 1% digitonin solution and 2 mg trypsin per 10 mg protein added. The solution was stirred at 0-4° for 1.5 hours, centrifuged at 100,000 x g for 45 minutes, and both particulate and soluble fractions assayed for NADH oxidase activity.

A synthetic non-ionic detergent 1012-6[a] from the Continental Oil Company was used in another solubilization study. A final concentration of 1% detergent was added to the mitochondria, and the solution stirred for 40 minutes at0-4°. Then the solution was centrifuged at 100,000 x g for 45 minutes, and both soluble and particulate fractions assayed for NADH oxidase activity.

Model Enzyme Experiments. Heme compounds used in this study included hematoporphyrin hydrochloride, crystalline hemin, bovine hemoglobin, horse heart cytochrome c and beef liver catalase (all from Sigma Chemical Company). Horseradish peroxidase (Nutritional Biochemical Company) was also used.

Hormone Experiments. Hormones tested included 17α-estradiol, 17β-estradiol, estriol, estrone, 17α-ethynyl-estradiol, Δ^5-pregnen-3-ol-20-one, androsterone, dehydroisoandrosterone, testosterone, 19-nortestosterone,

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[a] a polyoxyethylene (60%) derivative of straight chain alcohols, probably C 12 to C 16.

17α-testosterone and diethyl stilbestrol (all from the Sigma Chemical Company). Progesterone, thyroxine and monoiodotyrosine (from Mann Research Laboratory) and norepinephrine and DOPAamine (3,4-dihydroxyphenylethylamine) (both from Nutritional Biochemical Company) were also used.

Electrophoresis. Disc elcctrophoresis[94] was carried out according to the procedure of Davis[26]. Polyacrylamide gel columns were prepared in carefully cleaned glass tubes. The gel was composed of three layers, (1) A large-pore gel containing the protein, (2) a large-pore spacer gel in which electrophoretic concentration of the sample was completed, and (3) a small-pore gel in which separation takes place. The small-pore solution was prepared from 7% acrylamide, N,N'-methylenebisacrylamide (BIS), N,N,N',N'-tetramethylethylenediamine (TEMED), and Tris buffer, pH 8.9. Approximately 250 ug of protein from various uterine enzyme preparations was used in each tube. After about 45 minutes at 240 volts the tubes were withdrawn from the apparatus, the gels removed, and the proteins fixed in sulfosalicylic acid. The protein bands were stained in Coomassie Brilliant Blue R 250 (I. C. I. Organics, Inc.) for about an hour and destained by soaking in several changes of water. The blue-stained protein bands were indefinitely stable in 7% acetic acid solution.

RESULTS

Peroxidase Assay Method

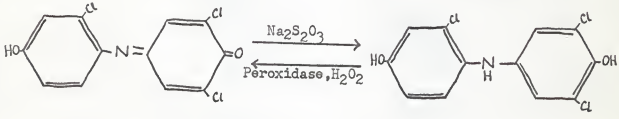
It was desirable to find a convenient assay method for the uterine peroxidase before undertaking further work on the enzyme. A number of hydrogen donors therefore were tested with a uterine homogenate. These included 2,3',6-trichloroindophenol (TIP), starch-iodide[24], pyrogallol[15], guaiacol[15], dianisidine[19] and 4-methoxy-α-naphthol[44]. Only leuco TIP was found to be rapidly oxidized by the sheep enzyme so it was chosen for routine

analyses. The rat enzyme however, showed a different pattern, as shown in Table II.

Table II. Comparison of hydrogen donors in the assay for sheep and rat uterine peroxidases. Activity of each is expressed as absorbance change per minute per ml enzyme and related to the leuco TIP activity.

Hydrogen Donor	Sheep -5[a]	Rat -2[a]
leuco TIP	100	100
starch-iodide	1.9	648
pyrogallol	2.7	6.1
guaiacol	2.3	--
dianisidine	0	--
4-methoxy-α-naphthol	8.0	--

The structural changes TIP undergoes during oxidation and reduction is as shown:



TIP (blue)

Leuco TIP (colorless)

A major drawback in using leuco TIP as hydrogen donor is its susceptibility to oxidation by hemoglobin and other heme-protein components of tissue. This non-enzymatic oxidation was subtracted after measuring the oxidation of leuco TIP by boiled enzyme each time of assay. Also, control of pH was very important in this assay, because at alkaline pH there was increasing non-enzymatic autooxidation which must also be subtracted.

[a]Animal numbers refer to experimental number in laboratory records.

Leuco TIP was first used as a hydrogen donor for measuring peroxidatic activity by Smith et al.[106], and was modified and used for uterine peroxidase systems by Lucas et al.[77]. These workers reduced the TIP with hydrogen gas and a palladium catalyst. The rate of reoxidation of the dye by hydrogen peroxide was found proportional to the enzyme concentration. Reduction of TIP with hydrogen gas and palladium was found to be quite laborious and indefinite, so an alternate method was sought using chemical reducing agents in place of hydrogen. Ascorbic acid, sodium dithionite, glutathione, sodium thiosulfate, sodium sulfite, sodium bisulfite and sodium borohydride were tested in the following manner. To identical concentrations of TIP solution was added a minimum of carefully weighed reducing agent. A minimum is that amount necessary to cause decolorization (reduction) of the dye after 15 minutes. Each of the reduced solutions was then tested with identical volumes of enzyme and hydrogen peroxide to see if reoxidation could be obtained. Results are seen in Figure 1, page 27. Glutathione (GSH) reduced the TIP to a purple-brown color which soon precipitated and had little activity with the enzyme. Hydrogen reduced dye had limited activity with the enzyme compared with others and had no linear region in the reoxidation curve. The uterine peroxidase was incapable of oxidizing leuco TIP when the dye was reduced minimally with Na_2SO_3 and $NaHSO_3$. Sodium dithionite ($Na_2S_2O_4$) was very effective in reducing TIP at very low concentrations, but the resulting mixture was also resistant to reoxidation by the peroxidase. The two best reducing agents tested were sodium thiosulfate and sodium borohydride. Thiosulfate was chosen over borohydride because of its stability, ease of preparation as a standard solution and enhanced activity for the peroxidase. Optimum reduction was obtained when the thiosulfate was buffered at pH 6.

Experiments run to determine the optimum concentration for reduction of

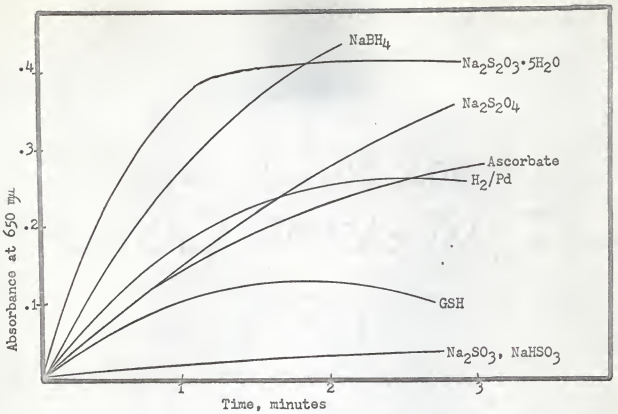


Fig. 1. Effect of different reducing agents on leuco TIP activity with uterine peroxidase. Two mM TIP was reduced with a minimum amount of reducing agent. The rate of reoxidation of the leuco TIP by identical aliquots of pig uterine homogenate was measured in a system containing 28 mM phosphate buffer, pH 6.0 and 1.2 mM H_2O_2 in a final volume of 7 ml.

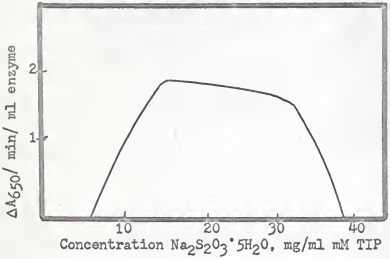


Fig. 2. Effect of $Na_2S_2O_3$ concentration in TIP reduction mixture on enzyme activity. Conditions for reoxidation same as Figure 1.

the dye to a colorless solution showed that if excess reducing agent was added, a definite lag period occurred before peroxidase could reoxidize the leuco TIP. If too little reducing agent was added the dye either remained oxidized or was soon air-oxidized. A standard TIP solution was prepared by suspending 64 mg of solid dye in 20 ml of 20 mM phosphate buffer, pH 6. It was necessary to grind the solid with a small mortar and pestle in order to obtain complete solution. The mixture was brought up to a final volume of 100 ml which gave a TIP concentration of 2 mM. One ml of this stock solution, reduced with varying amounts of standard thiosulfate, was diluted to 2 ml final volume and assayed with constant volumes of pig uterine homogenate. As shown in Figure 2, page 27, a threshold was found at low reducing agent concentration below which there was no activity. A plateau region occured at optimum reduction, and at concentrations of reducing agent greater than this, there was a definite fall-off due to the excess reducing agent. The dye concentration was constant for each assay (it was carefully weighed to 0.1 mg). It is also easy to control the concentration of reducing agent, and this was done to insure reproducible results from day to day.

An optimum relation was found between the weight of reducing agent in the dye mixture and the time necessary to reduce TIP to its leuco form. This is shown in Table III, page 29. At thiosulfate concentrations less than 10 mg per ml of mM TIP, considerable time was required for reduction. Since at the optimal reductant concentration for enzyme activity the time of reduction was only 15 minutes, that level was chosen rather than a higher concentration. Routinely, 32 mg (100 umole) TIP was macerated in 20 ml of 0.2 M pH 6 phosphate buffer. The mixture was dissolved in about 60 ml of water, standard thiosulfate (15 mg $Na_2S_2O_3 \cdot 5H_2O$ per umole TIP) added and the solution diluted to 100 ml. After 15 minutes at room temperature optimally reduced 1 mM leuco

TIP substrate was obtained and stored on ice until use.

Table III. Effect of reducing agent concentration on reduction time.

Weight, mg, of thiosulfate per ml of 1 mM TIP	Time, minutes, to reduce TIP at room temperature
5	More than 40
10	20 - 25
15	15
20	10
25	2 - 3

An absorption spectrum run on the enzymatically oxidized TIP showed a peak at 675 mμ. The reduced dye, however, had little absorbance at this wavelength, as shown in Figure 3, page 30. Therefore, 675 mμ was used as the wavelength at which to record enzymatic oxidation of leuco TIP.

The effect of temperature on TIP reduction was tested by incubating aliquots of the dye at 22°, 30° and 37°. It was found that the rate of reduction was slightly greater at elevated temperatures for equal concentration of reducing agent. The increased rate of reduction, however, was not great enough to warrant using higher than room temperature.

Optimum leuco TIP concentration for enzyme activity was next determined. A range of leuco TIP concentrations from 0.06 to 0.9 mM was run with constant volumes of pig uterine homogenate in order to determine the optimum substrate concentration. The results are shown in Figure 4, page 30. The apparent optimum leuco TIP concentration is 0.29 mM which is the final concentration achieved when 2.0 ml of 1 mM leuco TIP is diluted to a final volume of 7.0 ml in the assay tube.

Stability of the reduced dye was studied in order to determine how long a solution could remain reduced during the course of an experiment before there

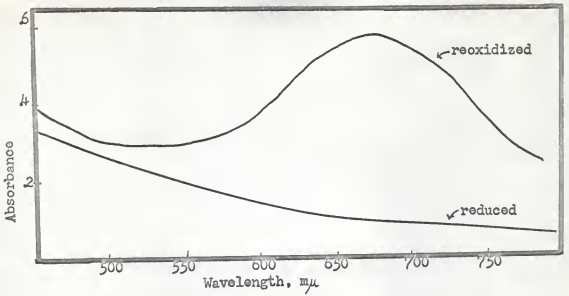


Fig. 3. Absorption spectra of TIP which was thiosulfate reduced and enzymatically reoxidized. Conditions same as Figure 1, page 27.

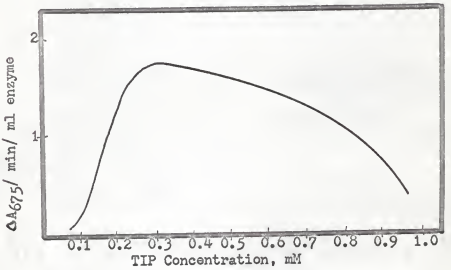


Fig. 4. Effect of leuco TIP concentration on peroxidase activity. Conditions for reoxidation same as Figure 1, page 27.

would be an apparent loss of activity for the enzyme. One mM TIP was optimally reduced and incubated at 30° for a period of 48 hours. Aliquots were taken at time intervals over 48 hours and assayed with a constant amount of freshly prepared enzyme. In five hours, the TIP had lost 8% of its original activity. After 24 hours, 32% was lost, and after 48 hours, 56% was lost. This is shown in Figure 5, page 32. This experiment showed that approximately 1.6% of the enzymatic redox capability of the leuco TIP system was lost per hour when stored at 30°c. In contrast to the results at 30°, it was found that optimally reduced dye lost none of its capability of being oxidized by uterine peroxidase after storage for 12 hours on ice and after 24 hours, only 8% was lost. Frozen leuco TIP lost 9% of its activity in 12 hours. It is not known why the frozen material appeared to lose activity at a slightly greater rate than that stored at 0°.

The pH optimum for the enzyme assay with leuco TIP was tested. The rate of oxidation of the reduced dye by uterine peroxidase was measured over a pH range of 6 to 11. At each pH a non-enzymatic control was run by substituting an equal volume of boiled enzyme for the active sample. It was found that there is a definite pH optimum, but at this optimum there is a 40 to 50% non-enzymatic oxidation which must be subtracted. This is shown in Figures 6 and 7, page 32. Because of the high non-enzymatic activity at the optimum pH of 9, an assay pH of 6 was chosen so that the non-enzymatic activity would be less than 10%.

The effect of buffer salt concentration on the TIP assay was examined. Phosphate buffers at pH 6.0 were used at final concentration of 7 to 140 mM. Decreasing activity was found at increasing buffer salt concentrations, as shown in Figure 8, page 34. A compromise was therefore made between the need for buffering and the inhibiting effect of buffer salt. It was decided

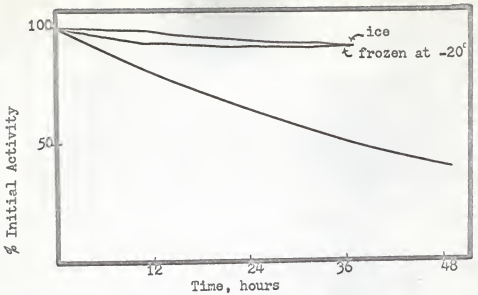


Fig. 5. Stability of reduced TIP at 30°, at ice temperature, and at -20°. Assay conditions same as Figure 1, page 27.

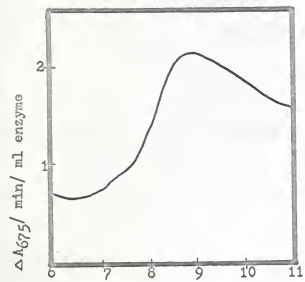


Fig. 6. Effect of pH on peroxidase activity. Phosphate, Tris and bicarbonate buffers at varying pH, but all 28 mM, were tested. Other assay conditions as in Figure 1, page 27. Activities corrected for non-enzymatic activity in Fig. 7.

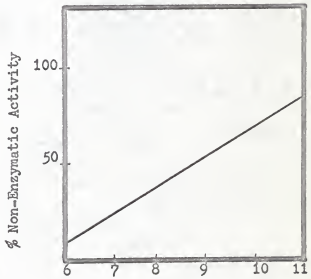


Fig. 7. Effect of pH on non-enzymatic activity. Assay same as in Figure 6, except the enzyme was first inactivated by boiling.

that a final concentration of 41 mM phosphate buffer would routinely be used.

Another important parameter studied was optimal hydrogen peroxide concentration for the enzme assay. All previously determined optimal conditions were used, except the reaction was run with varying concentrations of hydrogen peroxide and activity measured at final peroxide concentrations up to 10 mM. At concentrations greater than 4 mM there was definite inhibition. A final concentration of 1.2 mM peroxide was chosen for routine assays. Standard 8.4 mM hydrogen peroxide was prepared fresh daily from a refrigerated stock solution of 60 mM peroxide by diluting with deionized water. One ml was used to start the reaction in a final total volume of 7 ml, giving a final concentration of 1.2 mM hydrogen peroxide. The effect of hydrogen peroxide concentration on the enzyme activity is shown in Figure 9, page 34.

Finally, using optimum conditions for the assay as experimentally determined, an essentially linear relationship was found between the rate of oxidation of leuco TIP and volume of enzyme assayed. This is shown in Figure 10, page 35.

A summary of the components of the optimal leuco TIP assay for uterine peroxidase is as follows:

1.0 ml of 0.2 M phosphate buffer, pH 6.0,

2.0 ml of 1 mM reduced[a] TIP in 40 mM phosphate buffer, pH 6.0,

enzyme (usually 0.3 ml or less) in 20 mM phosphate buffer, pH 6.0,

deionized water to 6.0 ml,

1.0 ml 8.4 mM H_2O_2 to start the reaction.

The procedure is carried out as described on page 18. Final concentrations in this system are:

41 mM phosphate buffer,

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[a] Reduction procedure on page 28.

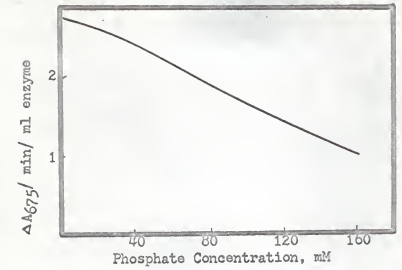

Fig. 8. Effect of buffer salt concentration on peroxidase activity. The assay system contained phosphate buffer pH 6.0, at concentrations from 0 to 130 mM. Other conditions as in Figure 1, page 27.

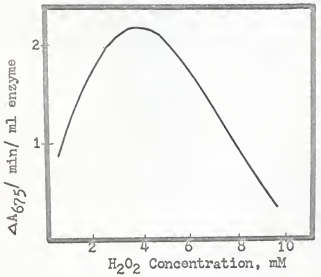

Fig. 9. Effect of H_2O_2 concentration on peroxidase activity. The assay system contained peroxide concentrations from 0.5 to 10 mM. Other conditions as in Figure 1, page 27.

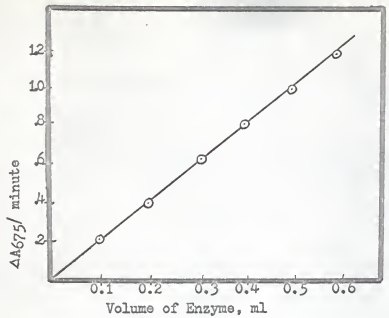

Fig. 10. Effect of enzyme concentration on the oxidation rate of leuco TIP. Assay conditions were 41 mM phosphate buffer, pH 6.0, 0.28 mM reduced TIP, 1.2 mM H_2O_2 and a pig uterine homogenate.

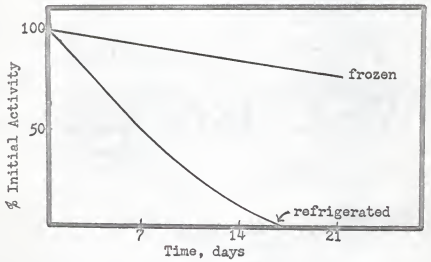

Fig. 11. Stability of uterine peroxidase when refrigerated or frozen for 21 days. Pig uterine homogenate was used. Assay conditions as in Figure 10.

0.28 mM leuco TIP,

1.2 mM H_2O_2.

The above optimum assay conditions were used for testing the activity of other peroxidases in oxidizing leuco TIP. Horseradish, lacto-, thyroid, lacrimal and salivary peroxidases were all capable of oxidizing leuco TIP.

Characteristics of Uterine Peroxidase

Distribution. Uterine peroxidase was located principally in the non-particulate, soluble portion of the cell. Routinely about 80% of the initial peroxidase activity in the supernate after 1000 x g centrifugation was recovered in the supernate after 100,000 x g centrifugation except for the rat tissues. Examples are given in Table IV, page 37. The particulate fractions were almost devoid of peroxidatic activity but were extremely high in NADH oxidase activity (see page 53).

Stability. The stability of freshly prepared uterine peroxidase was tested after storage at 0 to 4° and at -20°. Small aliquots were put into a series of tubes which were frozen and withdrawn just prior to testing in order to prevent thawing and refreezing of the samples. The enzyme was tested over a period of three weeks. It was found that frozen samples lost 15% of their original activity in the first week and 25% in three weeks. The refrigerated samples lost 40% of their activity in the first week and essentially all of their activity in three weeks. This is shown in Figure 11, page 35.

The stability of uterine peroxidase was studied at various temperatures. An enzyme sample was put into a bath initially at ice temperature. The temperature of the bath was gradually raised and with constant stirring, enzyme aliquots taken at intervals, assayed and the temperature noted. At temperatures greater than zero and up to 10°, there was a 10% decrease in activity,

Table IV. Distribution of peroxidase activity in uterine subcellular fractions. "Super A" is the supernate after 1000 x g centrifugation of the homogenate, "Mito" is the mitochondrial precipitate brought down at 25,000 x g, "Micro" is the microsomal precipitate sedimented at 100,000 x g and "Sol Super" is the supernate after 1000,000 x g.

Animal	Fraction	Peroxidase Activity, Total Units	Per Cent Recovery
Monkey	Super A	10.2	83
	Mito	0.7	
	Micro	0.2	
	Sol Super	8.5	
Suman	Super A	9.0	81
	Mito	0.8	
	Micro	0.6	
	Sol Super	7.3	
Cat -2	Super A	40.4	82
	Mito	4.8	
	Micro	3.5	
	Sol Super	33.0	
Sheep -2	Super A	580.0	69
	Mito	23.8	
	Micro	6.9	
	Sol Super	396.0	
Dog	Super A	29.5	98
	Mito	0.4	
	Micro	0.3	
	Sol Super	28.4	
Rat -2	Super A	101.8	42
	Mito	34.8	
	Micro	14.2	
	Sol Super	49.7	
Guinea Pig	Super A	48.6	75
	Mito	4.0	
	Micro	4.2	
	Sol Super	36.4	

but after this initial loss, the enzyme was stable up to 40°. The temperature of the enzyme incubation mixture was then maintained at 37 to 39° for 11 hours. There was no significant loss of activity during the first 200 minutes and the enzyme solution after this time was still clear and slightly red colored. After 4 hours a slight haze was noted, but there was only a 6% additional loss in activity. This experiment shows that enzyme solutions can be equilibrated to the reaction temperature with no danger of activity loss udring the assay procedure.

The uterine peroxidase was also found to be stable toward dialysis. There was no change in activity upon dialysis for 18 hours in 20 mM phosphate buffer, pH 7 in the cold.

Specificity. A characteristic of the uterine enzyme is its high specificity for leuco TIP as artificial hydrogen donor. As previously described and shown in Table II, page 25, uterine peroxidase in tissues other than rat, has at least 50 times the activity toward TIP as with starch-iodide, pyrogallol, guaiacol or dianisidine. 4-Methoxy-α-naphthol was found to be second most active but it still had only 8% of the enzyme's activity with TIP.

Partial purification. A partial purification of the uterine peroxidase was accomplished by ammonium sulfate fractionation of the supernate after 100,000 x g centrifugation. Ammonium sulfate (to give a calculated per cent saturation) was added to the supernate, dissolved with stirring, the solution allowed to stand with occasional stirring on ice for 20 minutes and then centrifuged at 25,000 x g for 25 minutes at 4°. After each precipitation the protein was made up to a convenient volume in 20 mM pH 6 phosphate buffer and the peroxidase activity determined. A sixfold purification was obtained. The results of a typical purification such as done on rabbit uterine extract are given in Table V, page 39.

Table V. Results of ammonium sulfate fractionation of rabbit uterine peroxidase and its activity toward leuco TIP.

Fract. No.	$(NH_4)_2SO_4$ % sat'n	Units ml	Protein mg/ml	Volume ml	Units mg	Total Units	%Activity Recovery	Fold Purif.
1	0	0.623	4.90	81.0	0.127	50.5	----	1.0
2	0--28	0.065	4.38	9.0	0.015	0.6	1	0.1
3	28--45	0.092	10.40	11.5	0.009	1.1	2	0.1
4	45--73	0.993	12.00	11.5	0.083	11.4	23	0.7
5	73--100	5.010	6.36	8.0	0.788	40.1	80	6.2

The ammonium sulfate fraction from 73 to 100% saturation contained most of the peroxidase activity. The enzyme was concentrated and purified 6.2 times. Similar ammonium sulfate fractionations were done on other species: monkey, 3.5, human, 4.3, guinea pig, 3.1, and sheep, 4.0 fold purifications at 64-85, 64-85, 64-85 and 70-100% ammonium sulfate saturations, respectively. Absorption spectra were run on the various ammonium sulfate fractions. The initial material (Super A) had small peaks at 574 and 538 mμ and a reduced Soret band at 413 mμ. Fraction 5, however, had a much enhanced spectra while fractions 2 to 4 had negligible absorbance. This shows that the catalytic activity is associated with a heme protein-like material. The specific absorbance of the Soret band per mg of uterine peroxidase protein increased from 0.276 in Fraction 1 to 0.685 in Fraction 5, a 2.5 fold increase while the specific activity increased 6.2 fold.

Control spectra of reduced hemoglobin and horseradish peroxidase were run at the same time. Horseradish peroxidase had a Soret peak at 440 m and minor peaks at 590 and 560 mμ, while hemoglobin had a Soret band at about 430 and one minor peak at 550 mμ. The absorption spectra findings are compared in Table VI, page 40. The presence of α and β peaks at 538 and 574 mμ in the uterine peroxidase, and Soret peak at 413 mμ indicated that it is a heme

protein different from hemoglobin or horseradish peroxidase.

Table VI. Comparison of spectral findings of uterine peroxidase, horseradish peroxidase and hemoglobin. Samples were minimally reduced with $Na_2S_2O_4$ and scanned from 700 to 370 mμ.

Sample	α	β		Soret (γ)	
Uterine peroxidase	574	538		413	
Horseradish peroxidase	590	560	(556)[a]	440	(437)[a]
Hemoglobin	---	550	(553)[b]	430	

Similar ammonium sulfate purification results were obtained for all species tissues tested, except rat. For this species and unlike others tested, ammonium sulfate fractions of 0 to 30% and 30 to 70% precipitated significant peroxidase activity. At best, a two-fold purification was obtained in the protein precipitated in the 70 to 100% saturation range compared with 4 to 6 fold in other species. Each fraction was tested for TIP activity, starch-iodide activity, and its absorption spectrum determined. The results of the enzyme assays are given in Table VII. It can be seen that considerable TIP activity is located in both fractions, while the starch-iodide activity is located principally in the 0 to 70% range.

Table VII. Comparison of TIP and starch-iodide activities in the ammonium sulfate fractions of rat -2b. Units of both activities, ΔA/ min.

Fract. No.	$(NH_4)_2SO_4$ % Sat'n	Leuco TIP Activity		Starch-iodide Act.	
		Total Units	Units/ml	Total Units	Units/ml
1	0	29.4	0.200	67.2	0.456
2	0--70	15.9	0.121	74.4	0.563
3	70--100	9.7	0.428	2.0	0.084
4	Super of #3	0	0	0	0

[a] Data in brackets from Archer et al., Biochim. et Biophys. Acta 99:96. 1965.
[b] Data in brackets from Sidwell et al., J. Bio. Chem. 123:33. 1938.

Absorption spectra. The absorption spectra of semi-purified uterine peroxidases from rabbit (6 fold) and sheep (4 fold) were investigated. An appropriate dilution of purified and dialyzed material was treated with dithionite or ferricyanide, for reduced or oxidized spectra respectively, and with NaN_3, NaF, NaCN, KOH, H_2O_2 or pyridine for characteristic derivatives. The spectra for all reduced non-rat enzymes showed peaks at 574, 538 and 413 mμ. The KOH and H_2O_2 derivatives differed slightly showing a Soret shift to 409 and 410 mμ respectively. The oxidized spectra showed peaks at 625, 574, 497 and 404 mμ. The pyridine hemochromagen derivative showed peaks at 620 (shoulder), 590, 572 and 395 mμ. The rat enzyme showed peaks at 625, 574, 537 and 407 for oxidized; 625, 574, 530 and 406 mμ for reduced material. Other derivatives could not readily be demonstrated with rat material. The major difference between uterine peroxidase spectra thus appears to be the decidedly lower Soret value of 406 mμ for rat compared with 413 mμ for other species. In addition, the rat preparation had much lower heme extinctions per mg of purified protein. A typical spectra is shown in Figure 12, page 42.

Iron content. Iron and protein determinations were done on purified monkey, rabbit, human and guinea pig peroxidases and on untreated sheep, cat and rat enzymes. The procedure followed is described on page 21. All protein-iron samples were carefully controlled by running a reagent blank through the entire drying, digestion and assay procedure. The results obtained are given in Table VIII, page 43.

Disc electrophoresis. The purified sheep and rat peroxidases were electrophoresed as described on page 24. In both samples there was one major band with a narrow light band preceding it and a light diffuse band (or bands) trailing it. These samples are shown in Figure 13, page 42. This experiment shows that further purification of the preparation will be

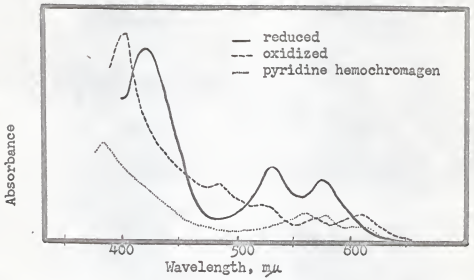

Fig. 12. Absorption spectra of uterine peroxidase. Samples were either reduced with dithionite or oxidized with ferricyanide. The pyridine hemochromagen derivative was formed as described on page 22.

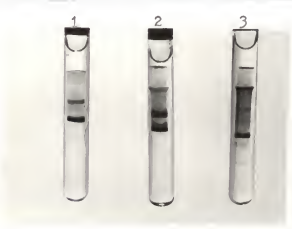

Fig. 13. Photograph of electrophoretic gels. (1) is unpurified sheep uterine peroxidase. (2) is sheep uterine peroxidase purified 4 times, and (3) is rat uterine peroxidase purified 2 times. Gels (2) and (3) were run at the same time.

required before conclusions can be drawn about the physical properties of the peroxidase enzyme.

Table VIII. Iron composition of uterine peroxidases. The samples were dried, digested and assayed by the o-phenanthroline method as described on page 21. Per cent iron is given as milligram Fe per milligram enzyme protein.

Species	Fold Purified	% Fe w/w
Monkey	3.5	0.149
Rabbit	6.2	0.119
Human	4.3	0.113
Guinea Pig	3.1	0.087
Sheep	1	0.120
Cat	1	0.103
Rat	1	0.450

Model Enzyme Study

A series of iron porphyrin compounds was tested for their activity in catalyzing the oxidation of reduced TIP. It was hoped that by using various hematoporphyrin-related constituents to replace enzyme, the catalytic properties of uterine peroxidase might be approximated.

The oxidation of the leuco TIP catalyzed by Fe^{++} was studied first. It was found that the non-enzymatic oxidation of leuco dye was only 2% of the enzymatic rate at iron concentrations as high as 0.01 M. This indicated that iron contamination will not be a problem in the TIP assay. It also points out, however, that ferrous ion alone can catalyze the oxidation of leuco TIP at appreciable rates between 0.1 and 1.0 M. The results of this study are given in Table IX, page 44.

A series of porphyrins were studied next. The concentration of iron was kept constant in all cases, except for hematoporphyrin itself which contains

no iron. For this compound a solution was made to the same molar concentration as hemin. In another solution of hematoporphyrin, an amount of iron was added equivalent to that of natural hemin. All these solutions were then tested for "enzymatic" activity for the leuco dye and hydrogen peroxide.

Table IX. Effect of iron concentration of the oxidation rate of leuco TIP. Varying concentration of Fe were added to the optimum assay conditions with a boiled pig uterine homogenate. The rate of TIP reoxidation was measured as ΔA_{675}/ min/ assay tube.

mg Fe/ml	Final Fe^{++} concentration, Moles/liter	Activity, Units/tube
0.00056	0.00001	0.012
0.0056	0.0001	0.017
0.056	0.001	0.024
0.56	0.01	0.043
5.59	0.1	0.131
55.85	1.0	12.0

Since the iron content of uterine peroxidase preparations had been determined, it was possible to compare the enzymes with various models as well as with known peroxidases. The results of this study are given in Table X, page 45. The activity of untreated and boiled model enzymes was compared. The iron concentration in each case was 5 ug per sample. As can be seen, there are several categories of results obtained. Some uterine peroxidases could be distinguished from hemoglobin and cytochrome c, but some could not. These data, along with the absorption spectra, strongly indicate that the uterine peroxidase is a heme (porphyrin) enzyme similar to some previously characterized peroxidases. It is apparent, however, that horseradish is much more reactive toward the leuco TIP than uterine peroxidase.

An interesting event was uncovered in the course of the model enzyme study. A hematoporphyrin-Fe solution was tested for leuco TIP peroxidase

Table X. Comparison of uterine peroxidase with other peroxidases and peroxidase models. Boiled enzyme activity was done to show non-enzymatic activity. Fe^{++} alone was used as reference, and all the other compounds were compared with it.

Compound*	Untreated: Units Activity 5 ug Fe	Boiled: Units Activity 5 ug Fe	Fold Activity over Fe^{++}
Fe^{++}	0.05	0.020	1
Hematoporphyrin	0.004	0.010	1
Hematoporphyrin+Fe^{++}	0.029	0.038	1
Hemin-HCl	0.110	0.159	2-3
Hemoglobin	0.302	0.025	6
Cytochrome c	0.266	----	5
Horserad. Peroxidase	1.44×10^6	3.38×10^3	2.8×10^7
Catalase	----	0.7--1.84	14-36
Sheep-2 Uterine Peroxidase	1.30**	---**	26
Monkey	1.51	---	30
Guinea Pig	5.35	---	107
Cat-2	0.44	---	9
Rat	0.16	---	3
Rabbit	3.32	---	66
Human	0.47	---	9

* Structures are given in Appendix I

** Non-enzymatic activities were less than 1% and were subtracted from the untreated activities before entry into the table.

activity the day of its preparation, and found to be about four times less than the natural hemin. This solution was tested again after standing 10 days sealed at room temperature. It was found that not only had the synthetically-prepared hemin changed color, but that it then had an activity which equallyed the natural hemin. Apparently, complexing had occurred between the porphyrin and the iron, generating hemin. The results of this experiment are shown in Table XI, page 46.

Table XI. Comparison of porphyrin activities toward leuco TIP before and after storage at room temperature for 10 days. All four systems had equivalent Fe concentration, i.e., 5 ugm/ml. Their non-enzymatic activity in oxidizing reduced TIP was measured using optimum conditions as described in Figure 10, page 35.

Compound	Initial Activity Units 5 ug Fe	Activity after 10 days Units 5 ug Fe
Fe^{++} alone	0.050	0.040
Hematoporphyrin alone	0.004	0.002
Hematoporphyrin + Fe^{++}	0.029	0.119
Hemin=HCl	0.111	----

NADH Oxidase Assay Method

In order to facilitate the study of NADH oxidase in uterine tissue, it was thought desirable to confirm optimal concentrations for the assay method. Two published methods were available, but the recommended concentrations and number of components differed somewhat. Hollander et al.[48] and Klebanoff et al.[69] both used an assay system with phosphate buffer, $MnCl_2$, 2,4-dichlorophenol (DCP) and NADH, but Klebanoff also found sodium bisulfite[67] to be an activator. The oxidation of NADH is measured as a decrease in absorbance at 340 mμ, and the rate of this change is directly proportional to enzyme concentration. DCP, in addition to fulfilling the requirement of phenol activator for the reaction, might also act as a catalase inhibitor[40]. Since these workers found different optimum concentrations for assay medium components, it was decided that they should be checked.

Tris buffer was found to inhibit the NADH oxidase assay, while phosphate seemed to stimulate. Identical volumes of enzyme were tested in systems of 0.167 mM $MnCl_2$, 0.667 mM DCP, 0.33 mM $NaHSO_3$, and 0.10 mM NADH

(after Klebanoff[69]) containing equivalent final concentrations of either phosphate or Tris buffers. Five times more activity was found with phosphate buffer than with Tris buffer so phosphate was consistently used after this experiment.

The optimum pH for NADH oxidase assay was then determined for phosphate buffer. As can be seen in Figure 14, page 48, a plateau is reached between pH 6 and 7 with a decrease in activity on either side of these values. A pH of 6 was chosen because the peroxidase assay is also at pH 6 and enzyme fractions would already be made up in pH 6 buffer.

Optimum buffer salt concentration was also determined. In contrast to the uterine peroxidase which was inhibited by increasing salt concentration (see Figure 8, page 34), the oxidase was initially stimulated with increasing salt concentration. This is another important difference in the two enzymes which had not been previously described. As can be seen in Figure 15, page 48, the activity quickly rose until a plateau was reached at 30 mM phosphate and then fell off only gradually. At final concentrations greater than 120 mM a definite fall-off was noted. A final concentration of 67 mM was chosen for convenience.

The optimum concentration of Mn^{++} was determined next, although this study was complicated by appreciable non-enzymatic oxidation of NADH at high Mn^{++} concentrations. It was found, however, that there was virtually no activity in the absence of Mn^{++}. With increasing Mn^{++} there was increasing enzymic activity up to 0.167 mM as shown in Figure 16, page 50. At concentrations greater than this, the non-enzymatic oxidation of NADH became greater than 50% of the total enzymatic activity. The concentration of Mn^{++} chosen was 0.167 mM final. It was found that an equimolar amount of ethylenediaminotetraacetic acid (EDTA) caused a 90% activity loss probably by chelating the

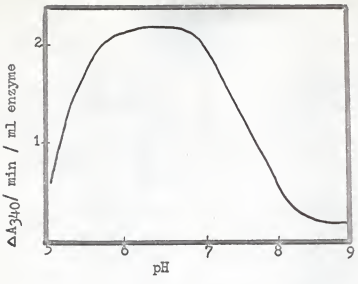

Fig. 14. Effect of pH on NADH oxidase activity. Pig uterine homogenate tested in 67 mM phosphate buffer from pH 5 to 9. Other assay conditions were 0.167 mM $MnCl_2$, 0.333 mM $NaHSO_3$, 0.667 mM DCP and 0.100 mM NADH to start the reaction.

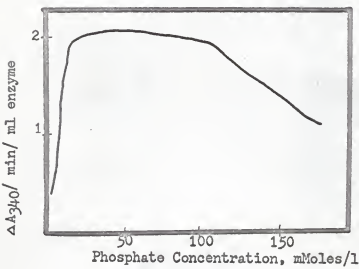

Fig. 15. Effect of phosphate buffer salt concentration on NADH oxidase activity. Assay conditions same as in Figure 14, except the final molarity of pH 6 phosphate buffer was varied.

Mn^{++}. When EDTA was added to the assay system in which no Mn^{++} had also been added, there was less activity than Mn^{++} plus EDTA. This suggests the presence of a small amount of endogenous Mn^{++}. When 10 mM to 10 uM hydrogen peroxide was added in place of the Mn^{++}, there was no activity.

Optimum DCP concentration was somewhat difficult to evaluate because at higher Mn^{++} concentrations, higher DCP concentrations were necessary for optimal activity. However, when the Mn^{++} concentration was held at 0.167 mM, an optimum of 0.667 mM DCP was found, as shown in Figure 17, page 50.

Sodium bisulfite was found by Klebanoff[67] to greatly stimulate the oxidation of NADH by a peroxidase-Mn^{++} system from beef thyroid[69]. A range of concentrations of $NaHSO_3$ was therefore added to the assay cuvettes and an optimum found between 0.33 and 0.66 mM, as shown in Figure 18, page 50. The addition of optimal bisulfite (o.33 mM) to the assay medium caused a nearly 3-fold increase in activity.

It was found that there was a sharp optimum concentration of NADH for the oxidase assay, as illustrated in Figure 19, page 51. Although there was an initial linear dependence on substrate concentration, a rather marked inhibition occured above 0.1 mM NADH. Therefore, 0.1 mM NADH was chosen for the optimal condition.

Various enzyme dilutions were next assayed using all the optimum concentrations described so far. A linear relationship between the rate of NADH oxidation and volume of enzyme was observed as shown in Figure 20, page 51. The optimum range for accurate readings is from 0.05 to 0.20 ΔA/min. At activities greater than 0.2 ΔA/min, errors would be introduced by the curvilinear response. A unit of activity is defined as that amount of enzyme which causes an absorbance decrease at 340 mμ of 1.0 per minute.

In summary, the optimum NADH oxidase conditions found were:

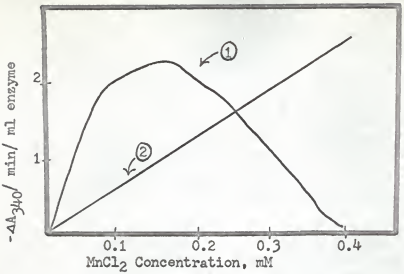

Fig. 16. Effect of Mn concentration on NADH oxidase activity. Conditions, except for Mn^{++}, as in Figure 14, page 48. Curve ①, enzymatic (pig uterine homogenate), curve ②, non-enzymatic.

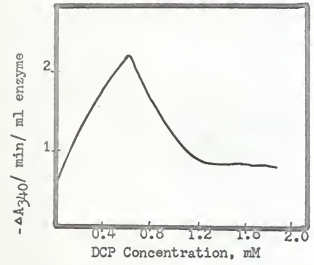

Fig. 17. Effect of DCP concentration on NADH oxidase activity. Assay conditions except for DCP as in Figure 14, page 48.

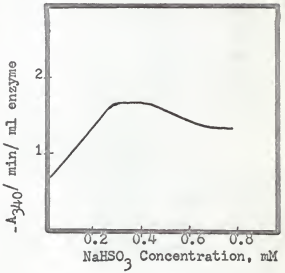

Fig. 18. Effect of $NaHSO_3$ concentration on NADH oxidase activity. Assay conditions except for $NaHSO_3$ as in Figure 14, page 48.

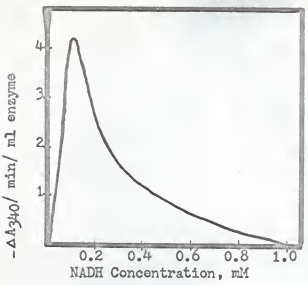

Fig. 19. Effect of NADH concentration on NADH oxidase activity. Cat-2 mitochondrial preparation was used. Conditions other than NADH concentration, same as in Figure 14, page 48.

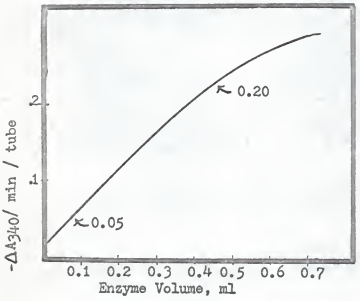

Fig. 20. Effect of enzyme concentration on rate of NADH oxidation. Increasing concentrations of pig-6 uterine mitochondria were added. Assay conditions include 67 mM phosphate buffer, pH 6, 0.167 mM $MnCl_2$, 0.333 mM $NaHSO_3$, 0.667 mM DCP and 0.100 mM NADH.

1.0 ml of 0.2 M phosphate buffer, pH 6, final concentration 67 mM,

0.1 ml of 5 mM $MnCl_2$, final concentration 0.167 mM,

0.1 ml of 10 mM $NaHSO_3$, final concentration 0.333 mM,

0.1 ml of 20 mM DCP, final concentration 0.667 mM,

enzyme in 0.02 M phosphate buffer, pH 6, usually 0.1 ml,

deionized water to 2.9 ml final volume,

0.1 ml 3 mM NADH, final concentration 0.100 mM, to start reaction.

All components except NADH were added to 1 cm cuvettes. To the reference cuvette was added 0.1 ml of deionized water in place of NADH and the instrument chart adjusted to zero and infinite absorbance with cuvettes in the light path. The NADH was quickly added to the recording cuvette, mixed and its net absorbance recorded continuously.

The optimal concentrations found for the components are identical with those found by Klebanoff[69]. The effect of omission of any of these components resulted in a loss of activity as shown in Figure 21, page 55. No activity was found without NADH or enzyme, and more moderate decreases were found with the omission of Mn^{++}, DCP or $NaHSO_3$. This finding substantiates findings of Klebanoff[56].

Characteristics of NADH Oxidase

Distribution. Essentially all the NADH oxidase was found in subcellular particles, i.e., the mitochondria and microsomes of uterine cells, and this was true of all species tested except the rat. The specific activity and total activities of NADH oxidase in several species is given in Table XII, page 53. Two major characteristics of the enzyme are evident. First, the enzyme is located in the particulate fractions (mitochondria and microsomes). Only in rat is the distribution of oxidase not significantly concentrated in

the particulate. Secondly, a recovery calculation cannot be made of the total activity because an inhibitor is present in the supernate after 1000 x g centrifugation. This inhibitor was not eliminated by dialysis or storage of the protein.

Table XII. Distribution of NADH oxidase in some uterine subcellular fractions. "Sup A" is the supernate after 1000 x g, "Mito" is the precipitate after 25,000 x g, "Micro" is the sediment after 100,000 x g and "Sol Sup" is the supernate after 100,000 x g.

Animal	Fraction	Specific Activity Units/mg	Total Activity Units
Sheep -2	Sup A	0.02	37
	Mito	4.54	1350
	Micro	9.16	1105
	Sol Sup	0.12	88
Dog	Sup A	0.29	76
	Mito	0.96	25
	Micro	0.62	10
	Sol Sup	0.11	21
Monkey	Sup A	1.36	116
	Mito	1.99	31
	Micro	3.64	32
	Sol Sup	1.27	89
Rat -1	Sup A	163	16700
	Mito	103	1980
	Micro	227	1700
	Sol Sup	171	8120

Stability. It was evident from the start of this study that this enzyme was much less stable than the peroxidase located in the cytoplasm. Considerable oxidase activity was lost if uteri were not chilled immediately after removal from slaughtered animals. For example, uteri taken during hysterectomy of experimental ewes (sheep -3 and -5) and chilled immediately had at least 3 times the NADH oxidase activity of those sheep uteri obtained during routine

slaughtering (sheep -2). Optimal NADH oxidase activity was obtained from those small animals which were killed in the laboratory and whose uteri were excised within a few minutes. These activity variations, however, had no effect on the subcellular distribution of the enzyme. The oxidase activity was always concentrated in the mitochondria and microsomes, except in rat.

The stability of the mitochondrial enzyme toward dialysis was studied. It was found that mitochondria lost half their NADH oxidase activity after dialysis overnight in the cold against pH 6.0 0.02 M phosphate buffer. However, the mitochondria and microsomes did not lose significant activity when frozen in the same buffer over a period of several months.

Solubilization. The mitochondria were treated in several ways in attempts to release a soluble NADH oxidase. Further purification of the oxidase would be feasible only after release from the particulate fraction. First, the classical freeze-thaw method was attempted. Cat -2 mitochondria was used because of its high activity and concentration. A fraction was taken before treatment, the total volume slowly frozen (15-20 minutes) in a -16° bath, thawed in cold water and an 11 ml aliquot taken. The aliquot was homogenized and centrifuged at 100,000 x g for 45 minutes. This was repeated for a total of nine freeze-thawings and after each time the supernates and particulates (made up in 20 mM pH 6 phosphate buffer) were assayed for NADH oxidase activity. No release of oxidase from the mitochondria could be obtained with subsequent freeze-thawings, as shown in Figure 22, page 55.

The method of solubilizing mitochondrial enzymes described by Klebanoff[69] was then attempted. In this method mitochondria are incubated at 0 to 4° with stirring for 1.5 to 2 hours in final concentration of 1% digitonin and 2% trypsin, centrifuged as before, and assayed. This procedure released less than 10% of the NADH oxidase from mitochondria.

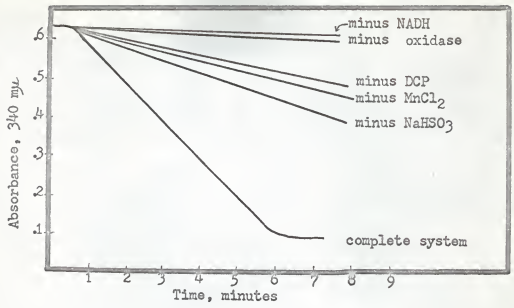

Fig. 21. Effect of omission of assay components on apparent activity. Assay conditions same as in Figure 20, page 51.

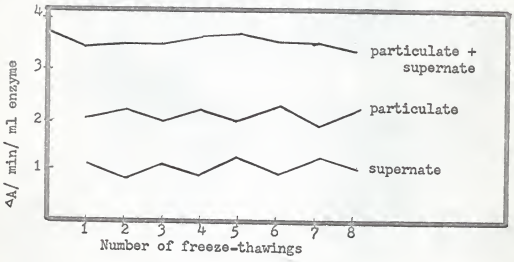

Fig. 22. Effect of freeze-thawings on release of NADH oxidase from mitochondria. Assay conditions as in Figure 20, page 51.

The method of Nicholson[92] using Continental Oil Company 1012-6 non-ionic detergent was also tried. A suspension of mitochondria in 1% detergent was prepared, stirred for 40 minutes at 5°, centrifuged at 100,000 x g for 45 minutes and assayed as before. This method released about 25% of the NADH oxidase from mitochondria. Further attempts at solubilizing the NADH oxidase from mitochondria are planned in the future in hopes of obtaining improved yields.

Iron content. An iron assay was done on the uterine mitochondria of guinea pig, cat, rat, human and monkey. The method[60] was that used on uterine peroxidase. The results obtained are given in Table XIII, page 57. The average umole iron per gram protein is 52.8. The literature does not appear to reveal information concerning iron content of uterine mitochondria. However, it is known that rat liver mitochondria contain 22 umoles iron per gram[16] and that beef heart mitochondria[60] contain about 8.8 umoles iron per gram protein. One can therefore expect to observe considerable variations of iron content between types of mitochondria. The average per cent iron in uterine mitochondria from several species varies little from 0.3%, however, the NADH oxidase activity per ug iron varies considerably. The especially high value for rat mitochondria may be a further reflection of the consistent deviation of this species from others in regard to its uterine oxidase and peroxidase pattern.

Substrate specificity. It was found that NADH oxidase is capable of oxidizing NADPH at the same rate as NADH when correction was made for the extinction of NADPH being only 60% that of NADH at 340 mμ.

Metal ion requirement. The substitution of other metals for Mn^{++} in the NADH oxidase assay was studied. Several workers have found Ce^{+++} to replace Mn^{++} 2,5,19,88,89 and Akazawa and Conn[2] also found that Co^{++} had a

Table XIII. Iron content of uterine particulates. Samples were dried, digested and assayed as described on page 21. Activity data was taken from the day of isolation of the mitochondria.

Tissue	% Fe w/w	umole Fe / gm protein	Total Units Activity / 5 ug Fe
Cat microsomes	0.25	45.2	16.25
Rat mitochondria	0.31	55.2	166.50
Human mitochondria	0.34	61.4	2.80
Monkey mitochondria	0.28	49.4	3.80

stimulatory effect on NADH oxidase. The metal ions tested were in their lower oxidation state and were therefore theoretically capable of being oxidized during the NADH oxidase reaction. All the metals were prepared at a concentration of 5 umole per ml and added in place of Mn^{++}. Only Co^{++}, Ce^{+++} and Fe^{++} gave appreciable activity although Sr^{++} and Mg^{+} appeared to stimulate to a small extent.

Table XIV. Substitution of other metals for Mn^{++} in NADH oxidase reaction. Assay conditions as in Figure 20, page 51. Identical volumes of Cat -2 mitochondria and assay components were used so variations in activity are due entirely to the metal ion effect. All metals were present in a final concentration of 0.167 mM.

Metal	Activity Units/ml enz	% Mn^{++} Activity	Metal	Activity Units/ml enz	% Mn^{++} Activity
Mn^{++}	2.15	100	Ni^{++}	0	0
Mg^{++}	0.10	5	Co^{++}	0.60	28
Pb^{++}	0	0	Sr^{++}	0.15	7
Ba^{++}	0.10	5	Sn^{++}	0	0
Ca^{++}	0	0	Fe^{++}	0.35	16
Cu^{+}	0	0	Ce^{+++}	0.30	14
Hg^{+}	0	0			

Phenol requirement. The specificity of the NADH oxidase for certain phenolic compounds was also examined. Akazawa and Conn[2] tested a series of phenols with a horseradish-Mn^{++} system and found the most active to be in

this order: 2,4-DCP > 2-chloro-4-phenylphenol > p-cresol > resorcinol > p-chlorothiophenol. It is of interest to find which substituted phenols are most active with the present NADH oxidase and thereby discover possible in vivo phenolic stimulators of the enzyme. If hormones are natural stimulators, the 3,4 and 4 substituted phenols would probably be most active. The results of this study are given in Table XV, page 59. In each series the activity of DCP was considered 100%. In most cases the phenols were added at several levels from 3.3 mM to 3.3 uM, and the most active concentration used. The most active phenols, in general, were those with 2,4 substituents although phenols with substitutions in the 3 or 3,5 positions were also quite active. These substitutions on the ring appear to alter the reactivity of the phenolic hydroxyl group. A lag period was found in the present study, similar to that reported by previous workers[97,124] who implicated a phenol in the lag effect. Waygood[124], for example, suggested that the phenolic compound might react directly with Mn^{++} and tie it up. Ray[97] proposed that phenols inhibit manganous oxidation by virtue of their property as free radical inhibitors.

Recent work by Lucas et al.[122] suggested the presence of a phenol oxidase in rat uterus which was not Mn^{++} dependent so each of the above phenols was also tested in the absence of Mn^{++}. No activity was found in the Mn^{++}-free systems from the three species tested. Perhaps the presence of a phenol oxidase only in rat uterine tissue makes it behave differently from the other animals.

Inhibitors. A series of compounds was tested for inhibition of NADH oxidase. All components of the assay system were added at their optimal concentration. Inhibitors were added as shown in Table XVI, page 59. Inhibition of the enzyme by cyanide, azide and hydroxylamine indicated the possible involvement of heme. Ascorbic acid probably blocks the oxidation-reduction

reaction, while o-phenanthroline inhibition indicates the presence of a ferrous compound.

Table. XV. Relative activities obtained by substitutions for DCP in the NADH oxidase system. The phenols were tested in concentrations close to that of the DCP (0.667 mM). Activity was calculated as units per mole of phenol and related to the DCP activity. Mitochondria from three tissue sources were tested.

Phenol*	Sheep-2	Monkey	Dog
DCP	100	100	100
Phenol	3	6	--
Catechol	0.1	--	--
p-Nitrophenol	50	56	--
2,4-Dinitrophenol	46	50	--
p-Hydroxybenzoic acid	4	--	--
Pyrogallol	--	3	--
Resorcinol	--	64	58
Hydroquinone	--	--	11
Orcinol	--	--	45
p-Phenylphenol	--	--	44

*Structures given in Appendix I

Table XVI. Effect of inhibitors on NADH oxidase activity.

Compound	Concentration, mM	Inhibition, %
Cysteine	0.167	100
KCN	0.167	76
NaN_3	0.167	79
Ascorbic Acid	0.167	100
$NH_2OH \cdot HCl$	0.167	100
o-Phenanthroline	12	100

Effect of Hormones on NADH Oxidase Activity

The effect of hormones on NADH oxidase activity was studied in an attempt to find a phenol which might have an in vivo role in the reaction. Hormones may conceivably be involved in the oxidase for three reasons. First,

many of the steroid hormones have phenolic rings. Second, many of the hormones have been shown to exist briefly as free radicals after oxidation by a rat uterine homogenate (for example, Borg[9] showed this to be true of estradiols, estrone and diethylstilbestrol). Thirdly, uterine physiology is known to be markedly influenced by steroid hormones and involvement with oxidase reactions might be one type of mechanism.

The activity of NADH oxidase was found very sensitive to low concentrations of various hormones added to replace DCP. The concentration of added hormone had to be carefully controlled because of its variable effect at increasing concentration. A series of hormones was studied for the stimulation of the NADH oxidase system at concentrations from 0.33 pM to 3.3 mM. It was generally found, as shown in Figure 23, page 61, that concentrations less than 1 nM had no effect on the enzyme, while at concentrations greater than this, the hormones inhibited or stimulated the oxidase activity. It was found that in general the hormones tested had three different effects on the enzyme. One group actually stimulated NADH oxidase activity with increasing concentration, as androsterone and dehydroisoandrosterone. Another group strongly inhibited enzyme activity with increasing concentration. Members of this group included norepinephrine, 17β-estradiol and diethylstilbestrol. The third group had little effect on the activity until unphysiologically high concentrations were reached. Since the hormones were prepared in absolute ethanol, all activities approached the same level, which is that of the ethanol acting as a DCP replacement. The concentration of ethanol in all tests was 0.559M.

It is significant that these three hormone groups are structurally similar. The first group (the major inhibitors) all have a phenolic group. These hormones cause 60 to 100% inhibition of NADH oxidase activity at increasing hormone concentration from 33 nM to 33 uM as indicated in Table XVII, page 62. Hormones

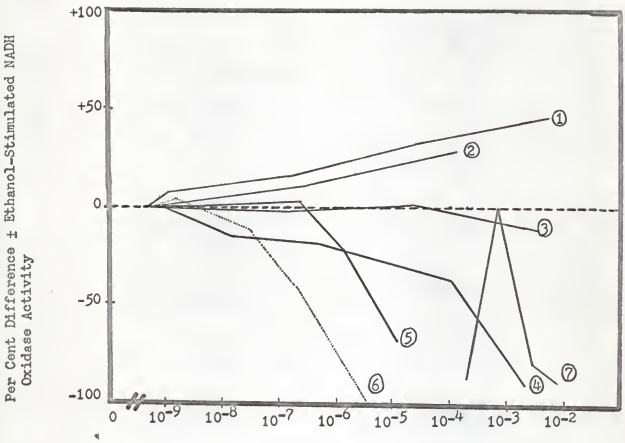

Fig. 23. Effect of increasing hormone or DCP concentration on NADH oxidase activity. Curves shown are: ① androsterone, ② dehydroisoandrosterone, ③ progesterone, ④ diethyl stilbestrol, ⑤ 17β-estradiol, ⑥ norepinephrine and ⑦ DCP. All hormones except norepinephrine were prepared in absolute ethanol. Theactivity of NADH oxidase with 0.559 M ethanol was set as basis for comparing the effect of increasing hormone. Dotted line represents extrapolation to activity in 0.559 M ethanol for sample made up in water. DCP at optimum concentration of 0.667 mM produced the same activity as ethanol at 0.559 M.

Table XVII. Representative hormonal effects on NADH oxidase activity. Activity conditions as in Figure 20, page 51. Each concentration was added in 0.1 ml absolute ethanol whose activity was approached by that of the most dilute samples.

Hormone	Concentration, Moles/liter	Activity* Units/tube
Diethyl Stilbestrol	3.3 pM	3.15
	330 pM	3.20
	33 nM	3.20
	3.3 nM	2.70
	330 uM	1.20
	3.3 mM	0
Progesterone	3.3 nM	0.75
	33 nM	0.90
	330 nM	1.15
	3.3 uM	1.00
	33 uM	0.70
Androsterone	0.33 pM	2.90
	33 pM	2.85
	3.3 nM	3.00
	330 nM	3.30
	33 uM	3.55
	3.3 mM	3.70

having this characteristic effect on the NADH oxidase system were found to be: 17α-estradiol, 17β-estradiol, 17ethynyl-estradiol, estriol, estrone, norepinephrine, DOPAamine and diethylstilbestrol. These structures are all given in Appendix I. The stimulators of NADH oxidase at increasing hormone concentration were characterized by a completely saturated ring 1 with a hydroxyl at position 3. The three examples of this group were found to be androsterone, dehydroisoandrosterone and pregnenalone. The third group of hormones tested had little effect on the NADH oxidase system until high concentration was reached. Examples of this group are progesterone, testosterone, 17 methyl-testosterone, and nortestosterone. The only exception to

* Absolute enzyme activities are not comparable among groups.

the above finding was thyroxine and monoiodotyrosine which are phenolic, yet were found to have little inhibitory effect on the NADH oxidase.

Table XVIII. Effect of hormones at three levels on the activity of NADH oxidase. Hormone-stimulated activity is given as log units per mole and this is compared with the same activity with DCP per mole at 3 concentrations. Each hormone was corrected for the activity of 0.1 ml of ethanol in the absence of hormone, except for DCP, norepinephrine and DOPAamine which were water soluble.

Hormone or Phenol	33 uM Hormone		0.33 uM Hormone		3.3 nM Hormone	
	Log Units mole	Fraction DCP Act.	Log Units mole	Fraction DCP Act.	Log Units mole	Fraction DCP Act.
DCP	6.538	1				
	5.053*	1				
17α-Estradiol	6.133	0.3	8.326	19	10.436	7900
17β-Estradiol*	3.663	0.1	6.412	25	8.502	3030
17α Ethynyl Estradiol	6.137	0.3	8.410	22	10.549	10025
Estriol	0	0	7.478	3	10.385	7040
Estrone*	3.477	0.1	6.436	26	8.542	3320
Norepinephrine	6.079	0.4	7.929	25	10.346	6430
DOPAamine	6.079	0.4	8.038	32	10.394	7180
Diethyl Stilbestrol	0	0	8.086	35	10.228	4900
Progesterone	5.955	0.3	8.105	37	10.094	3590
Testosterone	5.623	0.1	8.093	36	10.061	3330
17α methyl-Testosterone	5.881	0.2	8.038	32	10.083	3500
Nortestosterone	5.959	0.3	8.061	33	10.143	4040
Thyroxine*	3.881	0	6.672	37	8.690	4300
Monoiodotyrosine*	0	0	5.929	7	8.415	2300
Androsterone*	4.764	0.5	6.520	30	8.592	3490
Dehydroisoandrosterone*	4.881	0.6	6.643	39	8.463	2550
Pregnenalone*	4.362	0.2	6.079	12	---	---

The effect of hormones was compared with the effect of DCP by calculating the activity produced per mole of phenol (or hormone). The activity per mole of DCP was set at unity and the hormone effect was expressed relative to DCP. As can be seen in Table XVIII, the stimulation obtained per mole from 3.3 nM hormone was at least 3000 times the stimulation per mole using the same concentration of DCP. However, when both DCP and hormone were 0.33 uM, the hormone

*These data are applicable to the indicated DCP value.

was about 10 times less effective per mole than DCP. Perhaps due to the steric hindrance of the large steroid molecule, equal molar concentrations of hormone and DCP would not necessarily be equally reactive at high concentrations.

Survey of Species Distribution of the Peroxidase and Oxidase System

Nine different animal uteri were examined for the presence of peroxidase and NADH oxidase systems. It was found that all species had these enzymes, and that the subcellular distribution pattern was similar for all animals except rat. The results of this study are shown in Table XIX, pages 65-66. Reviewing designations, "Mito" is the mitochondrial particle brought down at 25,000 x g, "Micro" is the microsomal particles brought down at 100,000 x g and "Sol Super" is the supernate after 100,000 x g. A unit of activity is defined as a change of one absorbance unit per minute. Units per mg is the activity per milligram enzyme protein. Units per gram is the total activity per gram of starting material.

As can be seen in Table XIX, the units of peroxidase activity recovered per gram is fairly constant, while the units of oxidase activity varies widely. It can also be readily seen that although the rat uterine peroxidase is fairly similar within the other species, its NADH oxidase and enzyme distribution is very much different.

Sheep -2 was obtained during ewe slaughters and remained in the dead animals about 20 minutes before removal was possible. Sheep -3 and -5 uteri were obtained during hysterectomies and were put at -20° within one minute after removal from the animals. Cow, dog, rabbit, monkey and rat uteri were obtained frozen on dry ice from a biological supply house. Guinea pig and cat uteri were chilled within three minutes after death of the animal.

Table XIX. Species distribution of the peroxidase and oxidase system.

Animal	Fraction	Peroxidase Activity		NADH Oxidase Activity	
		Units mg protein	Units gm tissue	Units mg protein	Units gm tissue
Sheep -2	Mito	0.109	0.50	4.54	30.5
	Micro	0.107	0.01	9.16	24.9
	Sol Sup	0.526	8.9	0.12	2.0
Sheep -3	Mito	0.005	1.3	1.46	160.5
	Micro	0.262	10.8	0.13	0.2
	Sol Sup	0.038	16.0	0.02	0.3
Sheep -5	Mito	0.068	0.22	0.73	2.3
	Micro	0.076	0.13	0.88	1.6
	Sol Sup	0.302	6.06	0.03	0.5
Cow	Mito	0.010	0.02	0.19	0.3
	Micro	0.033	0.03	0.15	0.2
	Sol Sup	0.056	0.80	0.01	0.1
Dog	Mito	0.014	0.1	0.96	0.9
	Micro	0.025	0.1	0.62	0.4
	Sol Sup	0.146	1.0	0.11	0.8
Rabbit	Mito	0.084	0.2	0.12	0.3
	Micro	0.074	0.1	0.11	0.2
	Sol Sup	0.147	2.6	0.04	0.7
Monkey	Mito	0.044	0.2	1.99	8.8
	Micro	0.025	0.1	3.64	9.0
	Sol Sup	0.120	2.4	1.27	25.5
Human	Mito	0.077	0.2	1.90	5.6
	Micro	0.106	0.2	2.52	4.1
	Sol Sup	0.174	2.2	0.39	4.9
Rat -1	Mito	0.326	1.1	103	330
	Micro	0.231	0.3	227	284
	Sol Sup	0.125	0.9	171	1352
Rat -2	Mito	0.169	1.7	7.48	73.2
	Micro	0.207	0.7	12.44	40.4
	Sol Sup	0.156	2.4	0.92	13.9
Guinea Pig	Mito	0.102	0.6	0.67	4.2
	Micro	0.106	0.7	0.55	3.6
	Sol Sup	0.296	5.9	0.17	3.4

Table XIX. continued

Animal	Fraction	Peroxidase Activity		NADH Oxidase Activity	
		Units mg protein	Units gm tissue	Units mg protein	Units gm tissue
Cat -1	Mito	0.148	0.5	3.13	10.5
	Micro	0.190	0.6	3.41	10.7
	Sol Sup	0.086	2.0	2.16	44.7
Cat -2	Mito	0.074	0.3	11.05	41.3
	Micro	0.053	0.2	8.22	31.1
	Sol Sup	0.105	1.9	0.11	2.1

DISCUSSION

Nine different animal uteri were examined for the presence of a peroxidase and a NADH oxidase system. It is evident that a leuco TIP peroxidase system is located in the soluble part of the cell, while a NADH oxidase is located in the mitochondria and microsomes. An interesting exception is rat uterine tissue where both enzymes were found in the soluble and particulate fractions of the cell. The rat peroxidase was found in usual magnitude, while the NADH oxidase activity greatly exceeded that of any other species tested. This different system is not characteristic of rodents, however, because the guinea pig preparation approximated the enzyme distribution found in seven other animals.

About 2 units of peroxidase activity were recovered rather consistently per gram of uterine tissue except from sheep and guinea pig. The sheep peroxidases in three different preparations yielded higher peroxidase activity than the other animals, including rat. Sheep preparation 2 was taken from uteri which had been excised from slaughtered ewes, while preparations 3 and 5 were made from uteri taken during hysterectomies and immediately put on ice.

Although sheep consistently gave the highest yields of peroxidase, guinea pig was almost as high and was about 3 times greater than the other 7 species. Since guinea pig uteri were excised in the same manner as cat, the higher level of guinea pig peroxidase cannot be attributed to tissue handling.

The yield of NADH oxidase varied from 0.1 to 161 units per gram of tissue, except in rat where the yield was as great as 1352 units per gram. The oxidase is quite labile and higher activity was found in those tissues which were chilled immediately after death of the animal. Some of the lower specific activities of the oxidase could therefore have resulted from denaturation.

Certain characteristics of the partially purified peroxidase and the crude oxidase were determined. The soluble peroxidase was very stable at physiological as well as ice temperatures, and was stable toward dialysis. The enzyme was precipitated by ammonium sulfate at 70 to 100 per cent saturation with a resulting 4 to 6 fold purification. The absorption spectra of all eight partially purified animal peroxidases showed minor peaks at 574 and 538 $m\mu$, and a strong Soret band at 413 $m\mu$. This spectra was compared with that of peroxidases and heme proteins previously described and found to be different from all well-known compounds, although it exhibits typical heme absorption bands. An average iron content of 0.115% was found in 7 preparations of the peroxidase enzyme.

The NADH oxidase which was located in the particulate portions of the cell, was found to be unstable toward freezing and dialysis. The mitochondria and microsomes were completely resistant to release of the enzyme by freezing and thawing. About 10% of the activity was released by a digitonin-trypsin method, and 25% by a non-ionic detergent. The NADH oxidase was found to be equally active with NADPH as substrate. Perhaps this enzyme would be more logically named "reduced pyridine nucleotide oxidase". Manganous ion was found to be

indispensable for NADH oxidase activity, although certain other metals such as Co^{++}, Ce^{+++} and Fe^{++} were partially active. At equal concentrations, Co^{++} was 30% as effective, while the other metals tested were much less. The NADH oxidase system was most activated by phenols substituted in the 2,4 or 3,5 positions. This finding is significant since the phenolic steroid hormones are substituted in the 3,4 positions relative to the hydroxyl group. The enzyme system was found to be inhibited by cyanide, azide, cysteine, ascorbic acid and o-phenanthroline.

A model enzyme study was undertaken in order to further study the uterine peroxidase. A series of porphyrin compounds was tested for ability to oxidize leuco TIP. The oxidation rate of the dye by ferrous ion alone and a series of heme compounds was compared at constant iron content using purified uterine peroxidase. Hemin, hemoglobin and boiled catalase had, respectively, 2,6 and about 25 times the activity of ferrous ion alone. On equivalent iron basis, the uterine peroxidases had an average activity 35 times that of ferrous iron, and was significantly above that of boiled catalase. This would indicate a similarity of the catalase prosthetic group (protoferriheme IX)[110] with that of uterine peroxidase.

The absorption spectrum of uterine peroxidase revealed it to be different from other common heme proteins, although it had a spectral shift when reduced or oxidized which was generally characteristic of heme proteins. The spectrum of the pyridine hemochromagen did not closely approximate any common porphyrins, therefore, its heme group may not simply be protoferriheme IX.

A study of the activating effect of hormones on the NADH oxidase was undertaken in an attampt to find a possible in vitro activator. The hormones studied could be divided into 3 groups depending on their structure and their effect on the NADH oxidase. Those steroid hormones with a phenolic ring were found to be

inhibitory at increasing concentrations, while those with a hydroxyl at position 3 and a saturated ring 1 had the opposite or stimulatory effect. Steroids with a hydroxyl at position 3 and with a partially saturated ring 1 had little or no effect. Substitution of groups at positions other than 3 had no effect on the activation by the hormone. It was absolutely necessary to have a free hydroxyl at position 3, however, for the hormone to have any effect. Williams-Ashman[126,127] suggests that this is because it is no longer possible for the hormone to undergo formation of an oxidized intermediate. If the hormones did undergo such an oxidation, it would be reasonable to hypothesize their role in the NADH oxidase reaction as one of mediating hydrogen transport.

An important feature of hormone control via the NADH oxidase system might be the inhibition of activity observed at increasing hormone concentration. Yielding[137] found inhibition of enzyme activity (glutamic dehydrogenase) at increasing estrogen concentration. Klebanoff[64] found 92% inhibition of NADH oxidase by increasing the in vitro epinephrine concentration one hundred-fold.

The present results suggest that a balance of hormone inhibitors and stimulators might well control a NADH oxidase system in the uterus. Since 2 moles of NADH are probably consumed for every mole of oxidant reduced, this reaction would involve the sacrifice of 6 moles of ATP because the reaction occurs outside the cytochrome chain. The role of such an alternate pathway might be to influence basal metabolic rate. The effect of low concentrations of hormones in controlling the enzyme system was illustrated by the fact that in our system 33 nM hormone concentration would inhibit or stimulate the NADH oxidase. This observation is consistent with results of others[126].

Klebanoff[70] recently suggested that the uterine peroxidase was actually eosinophil peroxidase. Such a suggestion was also made in a footnote by Paul[96] and in a discussion by Rytomaa and Teir[101]. They based their reasoning on the

fact that the number of eosinophil granules in rat uterus has been shown to increase under estrogen stimulation. It also increased in cervical carcinoma. These granules are rich in peroxidase and increased enzyme is recovered under such conditions. Therefore, these workers suggested that the higher peroxidase content of estrogen-stimulated rat uterus might be due to the increased number of eosinophilic granules. McNary[84] found also that eosinophil granules were high in manganese, a necessary cofactor in the oxidase. Thus the eosinophil level might conceivably alter NADH oxidase activity also. Archer[4], however, had recently isolated eosinophil peroxidase from the blood of rats and its physical characteristics and absorption spectra vary greatly from the peroxidase isolated from the uterus during this study. Our evidence is that the uterine peroxidase from the eight species tested other than rat is different from eosinophil peroxidase and any other peroxidases yet found.

Another significant finding of this investigation is that rat uterine leuco TIP peroxidase and NADH oxidase systems differ significantly from similar enzymes in 8 other species tested. No study has been reported on these systems in tissues other than rat. The subcellular distribution of the rat enzymes was wide with only the NADH oxidase showing any localization. An ammonium sulfate fractionation of the soluble rat uterine peroxidase yielded 2 activities. The first precipitated at 0 to 70% saturation and showed strong activity in oxidizing iodide to iodine, while it had little ability to oxidize leuco TIP. The second, which showed the reverse activities, precipitated at 70 to 100% saturation. The rat peroxidase had an absorption spectrum similar to the others but with a much reduced extinction per unit activity. An iron determination of rat uterine peroxidase showed that it contained 4 times as much iron as the average from the other species analyzed. Gel electrophoresis of the purified rat enzyme gave an electrophoretic pattern comparable to a purified sheep

enzyme which was run at the same time. It is therefore evident that more work will have to be done to completely rationalize the species differences and differences in reactivity reported here.

SUMMARY

A peroxidase and a NADH oxidase system have been demonstrated in the uterine tissue of the following species: man, monkey, cow, sheep, dog, rat, cat, and guinea pig. Similar intracellular distribution was found for both enzymes in all species tested except the rat.

The peroxidase, found in the soluble fraction of the cell, was measured by the rate at which it reoxidized reduced trichloroindophenol (TIP). Uterine peroxidase was found to be very stable at physiological as well as ice temperatures, and was stable toward dialysis. The enzyme was precipitated from the soluble fraction of the cell by increasing the ammonium sulfate concentration from 70 to 100% saturation, with a resulting 4 to 6 fold purification. The partially purified uterine peroxidase, when reduced, had characteristic absorption maxima at 574, 538 and 413 mμ and when oxidized, at 628, 535, 497 and 404 mμ. The purified rat enzyme had less intense peaks per mg protein at the same wavelengths. The pyridine hemochromagen had peaks at 628, 535, 497 and 404 mμ. These peaks do not closely match any of the common porphyrin spectra, thus more work will be done before a complete analysis of the prosthetic group can be given. An iron assay of the preparations, other than rat, showed an average of 0.115% Fe, while the purified rat enzyme had 0.45% Fe.

A model enzyme study tested a series of porphyrin compounds for their activity in catalyzing peroxidation of reduced TIP. The relative activity of the porphyrin-containing compounds was compared with the uterine peroxidases on an equivalent iron basis. It was found that only denatured catalase with a

protoporphyrin IX prosthetic group approached the activity of the uterine enzyme. Horseradish peroxidase also with a protoporphyrin IX prosthetic group had activity toward leuco TIP greatly exceeding that of the uterine enzyme.

The phenol-activated NADH oxidase, found in the particulate fractions of the cell, was much less stable than the peroxidase and yields varied according to the manner in which the uteri were handled. Optimum activity was found in those uteri which were taken immediately after death of the animal, and chilled or frozen. The NADH oxidase was dependent on a phenol activator and on manganous ion. NADPH could substitute for NADH as a substrate for the enzyme. The enzyme reaction was stimulated by bisulfite and phosphate buffer, and inhibited by cysteine, cyanide, azide, o-phenanthroline, ascorbic acid and hydroxylamine. The phenols most active in the NADH oxidase assay were those with 2,4 and 3,5 substitutions.

Hormones might well be in vivo activators of NADH oxidase. Three general effects were obtained by the in vitro addition of hormones to the oxidase system. Phenolic hormones, such as estrogens and norepinephrine, were inhibitory at increasing concentration. The steroid hormones with a saturated ring 2, such as androsterone and pregnenalone, were stimulatory. Those steroid hormones with a partially saturated ring 1, such as progesterone and testosterone, had little effect on the activation of the oxidase. The possibility of physiological control of the NADH oxidase system by hormones is discussed.

ACKNOWLEDGMENTS

The author wishes to express her sincere appreciation to her major professor, Dr. B. A. Cunningham, Department of Biochemistry, for his advice, guidance, and assistance throughout this investigation, and to Dr. T. E. King, Oregon State University, for early enzyme training.

The animal contributions from Dr. J. E. Mosier of the Veterinary Clinic, Dr. B. Eleftheriou of the Zoology Department, Dr. A. Eisenstark of the Bacteriology Department and Dr. G. Mowry of the Manhattan Medical Center are much appreciated.

The author is also grateful for the use of specialized equipment borrowed from Drs. R. E. Clegg, G. D. Johnson and W. S. Ruliffson.

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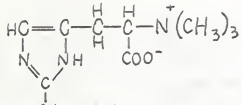
APPENDIX I



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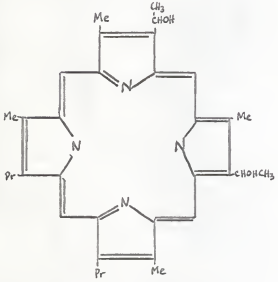

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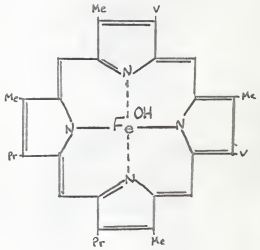

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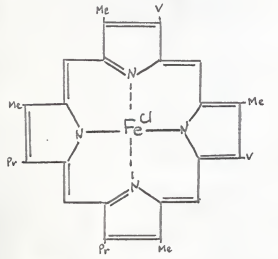

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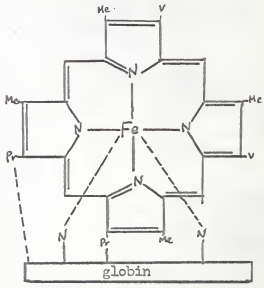

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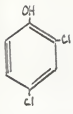

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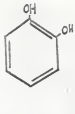

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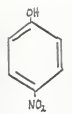

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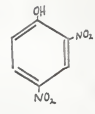

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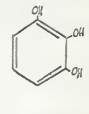

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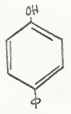

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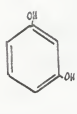
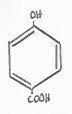


Resorcinol



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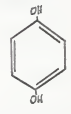
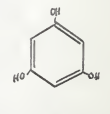
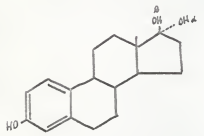


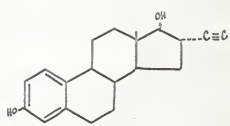
Hydroquinone



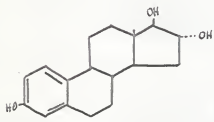
Orcinol



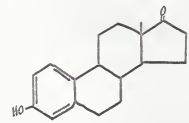
Estradiol



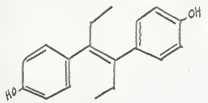
17α Ethynyl Estradiol



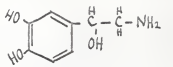
Estriol



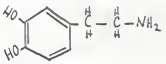
Estrone



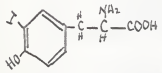
Diethyl Stilbestrol



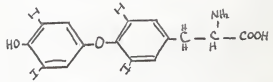
Norepinephrine



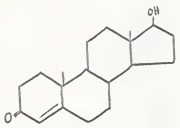
3,4-Dihydroxyphenylethylamine
(DOPAamine)



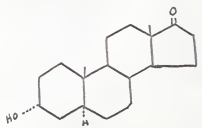
Monoiodotyrosine



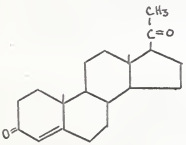
Thyroxine



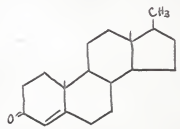
Testosterone



Androsterone



Progesterone



17α Methyl Testosterone

APPENDIX II

Locations of chemicals and equipment supply houses utilized:

Pel-freez Biologicals Company, Rogers, Arkansas

Distillation Products Industries, Rochester, New York

Sigma Chemical Company, St. Louis, Missouri

MicroEssential Laboratories, Brooklyn 10, New York

Nutritional Biochemical Corporation, Cleveland, Ohio

California Corporation for Biochemical Research, Los Angeles 63, Cal.

Mann Research Laboratory, New York 6, New York

I. C. I. Organics, Inc., Providence, Rhode Island

THE DEMONSTRATION, CHARACTERIZATION AND PARTIAL PURIFICATION OF A PEROXIDASE AND NADH OXIDASE IN UTERINE TISSUE

by

KAREN LOUISE NICKEL

B. S., Oregon State University, 1961

AN ABSTRACT OF A THESIS

Submitted in partial fulfillment of the

requirements for the degree

MASTER OF SCIENCE

Department of Biochemistry

KANSAS STATE UNIVERSITY
Manhattan, Kansas

1966

ABSTRACT

A peroxidase and a NADH oxidase system have been demonstrated in uterine tissue from nine species of animals. These enzymes were separated by simple centrifugation techniques in all tissues except rat. Peroxidases were found in the cytoplasm, and NADH oxidase in the mitochondrial and microsomal fractions.

The peroxidase was found to be quite stable to thermal inactivation and could be purified by precipitation between 70 and 100 per cent saturation of ammonium sulfate. The absorption spectrum of the peroxidase from all nine species was similar and had maxima at 574, 538 and 413 mμ for reduced material and at 628, 535, 497 and 404 for oxidized sample. The pyridine hemochromagen indicated that the enzyme may contain an analogue of protoporphyrin IX as its prosthetic group. A model enzyme study with porphyrin compounds also suggested this, in that only those porphyrins with protoporphyrin IX approached the activity of the uterine peroxidase toward leuco TIP. An iron assay of the peroxidase indicated a 0.115% Fe content by weight. Electrophoresis of the purified peroxidase showed one major band with several diffuse trailing bands.

The NADH oxidase was found to be rather unstable toward dialysis and storage. The enzyme is dependent on a phenol and manganous ions for activity. NADPH could be substituted for NADH in the reaction. The enzymic reaction was stimulated by sodium bisulfite and phosphate buffer, but was inhibited by cysteine, cyanide, azide, o-phenanthroline, ascorbic acid and hydroxylamine. Release of NADH oxidase from the mitochondria was negligible after freeze-thawing, however, some activity was released by digitonin-trypsin or non-ionic detergent treatments. A study of the phenol specificity of the NADH oxidase indicated those phenols with 2,4 or 3,5 substitutions were most stimulatory. The manganous requirement was found to be only partially satisfied with cobaltous or cerous ion substitution.

Rat uterine tissue was found to vary from the other tissues with regard to its peroxidase and NADH oxidase distribution, concentration and reactivity. This is a significant discovery since no work on uterine peroxidase up to this time has been done on other than rat tissue. Neither the peroxidase nor the NADH oxidase of rat uterus was located in one portion of the cell, but seemed to coexist in both soluble and particulate fractions. Rat tissue was also found to be extremely active in NADH oxidase activity, although its peroxidase activity was of the same order of magnitude as the other animal tissues. The cytoplasmic fraction of the cell was found to contain two peroxidatic activities which could be separated by ammonium sulfate fractionation. One fraction was highly active in oxidizing iodide to iodine but had little ability to oxidize the reduced TIP. The other fraction had predominantly TIP activity. This ability to oxidize iodide to iodine was not found in any of the other tissue preparations. Rat peroxidase iron content of 0.45% Fe was four times that found for the other tissues. Rat NADH oxidase was found to be Mn^{++} dependent and stimulated by 2,4-dichlorophenol or hormones, as was the NADH oxidase from the other species.

The manner in which hormones affected NADH oxidase activity was investigated and three general effects noted. These included: (a) inhibition by phenolic steroid hormones, (b) stimulation by the cyclic alcohol steroid hormones, and (c) little effect by the hormones with a partially saturated ring 1. The possibility of physiological control mediated by hormone effects on the NADH oxidase system in the uterus is discussed.