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Free Radical Generation by Thyroid Peroxidase and Its Effects on Cells in Vitro

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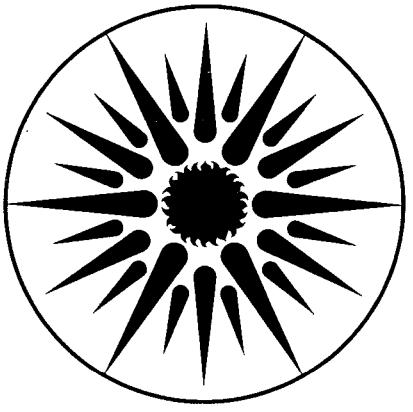
K.L. Moore
(Ph.D. Thesis)

December 1990



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FREE RADICAL GENERATION BY THYROID PEROXIDASE AND ITS EFFECTS ON CELLS *in vitro*

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Ph.D. Dissertation

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Free Radical Generation by Thyroid Peroxidase and its Effects on Cells *in vitro*

by

Katherine Louise Moore

Abstract

A new ESR method for quantifying micromolar quantities of hydrogen peroxide (H_2O_2) and peroxidase activity is described. The assay consists of measuring the one-electron oxidation of a reduced nitroxide by phenoxyl radicals produced by a peroxidase with H_2O_2. The method was developed to study the activity of small quantities of an impure peroxidase preparation coupled to a H_2O_2 generating system. The assay can be used for quantifying H_2O_2 or peroxidase enzyme activity in optically opaque biological samples that would not be suitable for work with conventional spectroscopic assays. The instantaneous readout availablility of this method is especially useful in the initial stages of enzyme characterization.

The ESR assay was applied to the study of free radical generation by membrane-bound thyroid peroxidase (TPO) with a variety of phenols. It was found that TPO can produce radicals besides those required for hormone synthesis. Endogenous and exogenous phenols, as well as iodide, can create these highly oxidizing species as a function of enzyme and H_2O_2 concentration.

The same conditions which generate radicals in the ESR assay were established in tissue culture experiments. Cells which had been transfected with and expressed the gene for human TPO (hTPO) provided peroxidase. H_2O_2 was generated continuously by glucose oxidase with glucose and dissolved oxygen. Phenols were present in the tissue culture media but were supplemented with other oxidation catalysts in some experiments. The same factors which promoted free radical generation in the ESR assay led to inhibition of the cells in tissue culture, suggesting that free radicals were responsible for

the cytotoxic effects. Partial prevention of the growth inhibition by a free radical trap provides evidence that radicals are involved in cell damage.

Tissue culture seems to be a good model for the study of the role of radicals in autoimmune thyroid diseases in which peroxidase activity and H_2O_2 generation are increased. Furthermore, tissue culture allows the effects of oxidizing conditions to be separated from those of the immune system. Preventative treatment of free radical effects may improve the prognosis for thyroiditis.

Free Radical Generation by Thyroid Peroxidase and its Effects on Cells *in vitro*

by

Katherine Louise Moore

Dedication

I dedicate this work to my family; Denis, Robert and Alan who supported me psychologically and physically throughout graduate school.

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Acknowledgements

I want to thank my advisor and dissertation co-chairman, Rolf Mehlhorn, for initiating my work with peroxidases and guiding me through electron spin resonance spectroscopy.

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Introduction

Peroxidases are capable of generating free radicals by oxidizing a variety of substrates. These substrates occur naturally, as phenolic amino acids, as thiol containing compounds, and as acetoacetate (Harrison et al) which is elevated in uncontrolled diabetes. Exogenous substrates, absorbed as drugs or environmental pollution, also form free radicals under oxidizing conditions.

Free radicals are known to damage tissue. Tissues with peroxidase, such as the breast (lactoperoxidase), have a high rate of cancer, as do tissues which have been exposed to inflammatory leukocytes (myeloperoxidase). Infiltrating phagocytes also provide a source of oxidants in infected tissue and are associated with the promotion of cancer (Muehlematter et al). Phenol, when coadministered with benzene, leads to myelotoxicity, presumably through free radical formation with myeloperoxidase (Eastmond, c).

The target of free radicals has been studied extensively in both whole animal studies by histological studies (Southorn, a,b) and on the cellular level by comprehensive biochemical characterization (Eastmond *et al*,a, Harrison *et al*, Mottley *et al*, O'Brien, b). The damage to the target may benefit the host as in the case of radiation therapy or defending against invading microorganisms. However, free radicals often contribute to human disease (Southern, b). They have been implicated in atherosclerosis (Harrison *et al*, b), ischemic-reperfusion injury (Southorn, b), and myelotoxicity (Eastmond *et al*, b). Free radicals attack many cellular components including membranes, DNA, proteins and polysaccharides (Southorn, a), fatty acids (Harrison, b), thiols, NADH, and arachidonate (Mottley, O'Brien, b, Nakamura M *et al*, c).

The thyroid gland both contains a peroxidase and generates an oxidant, hydrogen peroxide (H_2O_2) for the synthesis of thyroid hormone. An increase in peroxidase activity and H_2O_2 production occur under the direction of thyroid stimulating hormone (TSH) and long-acting thyroid stimulator (LATS) in Hashimoto's and Grave's

diseases, respectively (Weetman et al). As the diseases progress, the thyroid gland becomes enlarged, scarred and eventually destroyed. A low level of H_2O_2 is known to induce cellular proliferation (Burdon et al), while high levels of oxidants lead to growth inhibition and cell death (Cerutti). Thyroid tissue seems to be a perfect model for the damaging effects of free radicals. It normally produces H_2O_2 and radicals while synthesizing thyroid hormone. In circumstances of increased oxidant production, the results of increased H_2O_2 and radicals are seen, ie cellular proliferation and destruction.

Peroxidase activity and H_2O_2, the substrate of peroxidase, were assayed by electron spin resonance (ESR). It is specific for one-electron oxidation (radical production), very sensitive, and uses minute quantities of materials. The sensitivity of the ESR assay quickly showed that the ratio between H_2O_2 and peroxidase was critical for accurate measurements, a factor which is not mentioned in other methods of H_2O_2 and peroxidase assay. The small quantities of peroxidase necessary for ESR assay was especially advantageous when determining the most active fraction of thyroid homogenates, the best method of preservation, and the most suitable method of adding H_2O_2.

Membrane-bound and extracted thyroid peroxidase (TPO) was assayed by ESR with a variety of phenols to determine substrate specificity and peroxidase activity. Microsomal membranes from thyroid glands were used to mimic natural conditions. Deoxycholate was used to extract TPO from tissue culture cells.

The hypothesis that the radicals could be harmful to cells was tested in tissue culture. Chinese Hamster Ovary (CHO) cells, transfected with and expressing human thyroid peroxidase gene (hTPO), were utilized as a cellular model of TPO effects. The use of a nonthyroidal cells eliminates the possibility that other characteristics of thyroid cells are responsible for the effects being attributed to TPO.

The only available thyroidal cell line,FRTL-5, has no functional enzymatic activity (Derwahl et al).

Background

Thyroid Gland

The thyroid gland begins as an evagination of the pharyngeal floor and is composed of endothelial tissue. As it developes, it form a series of follicles which are surrounded by a rich capillary blood supply (Ingbar et al). The individual cells which form the follicles become extensively polarized. The basal sides becomes specialized in transporting iodide from the blood into the thyroid gland and the apical side concentrates the enzyme and mechanisms for forming thyroid hormone extracellularly; i.e. within the follicles. TPO is the primary enzyme involved in thyroid hormone synthesis (DeGroot et al) and is expressed on the apical surface of thyroid cells (Nilsson et al). Hydrogen peroxide (H_2O_2) generation by NAD(P)H-oxidase has been localized on the apical plasma membrane also (Mizukami et al).

Apparently all activities involved with thyroid hormone synthesis are regulated by thyroid stimulating hormone (TSH). Increased levels of TSH augment the rate of iodine uptake and oxidation, H_2O_2 generation, TPO activity, thyroglobulin synthesis and iodination, iodothyronine coupling, thyroglobulin processing and thyroid hormone release to the blood. TSH also regulates thyroid gland morphology. The stimulatory hormone is responsible for the shape and activity of the follicular cells and for the degree of vascularization of the gland. In conditions of high stimulation, as occur during low circulating thyroid hormone levels, the thyroid gland becomes enlarged. The hypertrophy, hyperplasia, and hypervascularity of the gland are termed goiter. High levels of thyroid gland stimulation and consequent goiter can be caused by the autoimmune diseases, Hashimoto's and Grave's.

Thyroid Hormone

Hormonogenesis in the thyroid glands consists of thyroglobulin synthesis, iodination of some of the tyrosine residues of the thyroglobulin, coupling of several of the iodotyrosyl residues, proteolysis of the thyroglobulin, secretion of iodothyronines into the blood, and deiodination of iodotyrosines within the thyroid and reutilization of the liberated iodide (Nunez et al and Taurog, a).

Iodide is carried across thyroid cell membranes against an electrical and concentration gradient as the first step in the biosynthesis of thyroid hormone. Thyroid cells are capable of concentrating iodide 40-500 times that of the blood depending on the concentration of TSH reaching them. The active transport of iodide is driven by the Na^+ gradient potentiated by a difference in affinity for I^- on the inside and outside of the membranes (Nakamura Y *et al*, d).

Iodide undergoes a two electron oxidation inside the follicular lumina just outside the apical membranes of the cells to form an intermediate compound which iodinates thyroglublin. Iodide oxidation occurs via thyroid peroxidase with H_2O_2 as the electron acceptor. Many intracellular sources of H_2O_2 have been suggested but a plasma membrane-associated NADPH-oxidase H_2O_2 generating system seems most likely (Nakamura Y *et al*, d; Bjorkman et al, Virion et al). The site of H_2O_2 generation is the apical surface of the follicle cell at the membrane-colloid interface (Labato et al).

Thyroglobulin, the precursor of thyroid hormone, is synthesized by follicular endothelial cells in the rough endoplasmic reticulum (RER), glycosylated and packaged in the Golgi, and delivered to the follicular lumina by fusion of the vesicle membranes with apical cell membranes. The glycoprotein has a molecular weight of 660,000 with about 140 tyrosine residues per mole of protein, between 10 and 50 of which become iodinated (Nunez et al).

It is believed that coupling is intramolecular and proceeds by a 1-electron transfer reaction involving thyroid peroxidase (Taurog) as

proposed by Johnson and Tewkesbury in 1942. The four step model for the coupling of the iodinated tyrosines includes: the oxidation of two iodotyrosyl residues to iodotyrosyl radicals; the formation of an unstable quinol ether by radical addition of these two iodotyrosyl radicals; the decomposition of the quinol intermediate to 1 mole of thyroid hormone and 1 mole of dehydroalanine; and the decompositon of the unstable dehydroalanine residue to pyruvic acid and ammonia during the hydrolysis of the protein (Nunez et al). The peroxidase reaction can be illustrated as:

Peroxidase + H_2O_2 -> Compound I

Compound I + AH -> Compound II + A·

Compound II + AH -> peroxidase + A·

2A· -> A-A

("A" in this scheme represents diiodotyrosine; "A·" its radical form; A-A dimerized diiodotyrosine; Compound I, the +5 form of thyroid peroxidase (native state is +3); and Compound II, the +4 form of the enzyme (Nakamura M et al, b).)

Iodination facilitates linkage. Spatial alignment of the interacting residues is also probably important for the condensation of the two diiodotyrosine residues. After iodination and coupling, a thyroglobulin molecule contains only about 2 molecules of hormone.

Luminal iodinated thyroglobulin undergoes proteolytic cleavage within the follicular cells of the thyroid gland before being secreted into the blood. Pseudopods form at the apical surface of the follicular cell, followed by endocytosis of colloid to yield droplets. Lysosomes fuse with the colloid droplets and the peptide bonds are hydrolyzed in the resulting structure. Free thyroxine (T_4) and triiodothyronine (T_3) are secreted into the capillaries. The other iodotyrosines liberated from thyroglobulin are dehalogenated. Most of the liberated iodide from monoiodinated tyrosine (MIT) and diiodinated tyrosine (DIT) is recycled for hormone synthesis but some of it is lost from the gland.

The major thyroid hormone in the blood is T_4 and is bound to thyroid hormone-binding proteins. Thyroxine-binding globulin (TBG)

has the highest affinity, *ie* avidity for thyroid hormone and therefore it binds most of the circulating thyroxine. Less hormone is bound to albumin, and a very small amount binds to a prealbumin called thyroxine-binding prealbumin (TBPA). The concentrations of binding proteins can influence the availability of hormone but usually do not. Levels of active, unbound hormone are generally maintained by a feed-back loop involving hormone binding and metabolism by tissue, TSH, and the thyroid gland.

Thyroid hormone binds to peripheral tissue, enters cells, and triiodothyronine (T_3) binds to receptors in the nuclei. Bound nuclear T_3 increases the synthesis of mRNA and ribosomal RNA. The mRNA that is formed dictates the formation of proteins which act as enzymes that modify cell function.

Most actions of thyroid hormone require several days to demonstrate. The variety of effects include the rate of oxygen consumption, metabolism, and growth and differention. Sufficient levels of thyroid hormone are critically important for the development of the nervous system during the early stages of life.

After circulating T_4 binds to peripheral tissues, it is enzymatically monodeiodinated to T_3 or reverse T_3. These hormones are further deiodinated and excreted in the urine. The deaminated and decarboxylated derivatives of thyroid hormones are conjugated in the liver to form sulfates and glucuronides, followed by biliary excretion.

Thyroid Peroxidase

Thyroid peroxidase (TPO) catalyzes the iodination of tyrosine or tyrosyl moieties in thyroglobulin and the formation of tetraiodothyronine (T_4). Iodination requires H_2O_2 to form an enzyme-associated iodinating intermediate (TPO-I_{oxid}), and an iodide acceptor, such as free or protein-bound tyrosine. The general reaction sequence for the oxidation of iodide and its addition to tyrosine is:

$$TPO + H_2O_2 \rightarrow TPO_{oxid}(\text{Cpd I}) + H_2O$$

TPO_{oxid}(Cpd I) +I^- -> TPO-I_{oxid}

TPO-I_{oxid} +acceptor -> I-acceptor + TPO

The coupling reaction is illustrated above in the section titled "Thyroid Hormone".

The rates of the reactions by TPO depend on its substrates and their concentrations. When H_2O_2 is added to TPO in small quantities, Compound I will be formed. Tyrosine is rapidly iodinated with a rate constant of $2.1X10^7$ $M^{-1}s^{-1}$ in the presence of iodide. Tyrosine and monoiodinated tyrosine (MIT) will be oxidized slowly ($4\text{-}7\ X10^4$ $M^{-1}s^{-1}$) by a two-electron reaction, while diiodotyrosine (DIT) will undergo a one-electron oxidation relatively quickly ($>2X10^5$ $M^{-1}s^{-1}$) (Ohtaki et al, b). In more recent experiments, Nakamura M et al, b concluded that TPO performs either a one- or a two-electron oxidation of phenols, depending on their substituents. They suggest that the mechanism of oxidation changes from a two-electron to a one-electron type when bulky or heavy atoms are substitued at the 2- and 6-positions of phenol. This may be a rare example in which biosynthesis is regulated by an electron-transfer mechanism. If Compound I is formed in the presence of iodide and no tyrosine is available, the enzyme-oxygen-iodine complex (TPO-I_{oxid}) can react with another iodide to form elemental iodine, I_2 + OH^- (Magnusson et al, b). If there is a slight excess of H_2O_2, the TPO-I_{oxid} will act as a catalase and degrade H_2O_2 to O_2 + H_2O + I^- . When H_2O_2 is added in a large excess (200 μM), TPO will be oxidized to a $^+6$ state, denoted Compound III. This form of the enzyme, Compound III, will slowly gain electrons and resume its native $^+3$ state, or will undergo irreversible inactivation (Kohler et al). Magnusson et al (a) suggest that methimazole and carbimazole, two drugs which inhibit hormone synthesis, compete with H_2O_2 for oxidized iodine.

TPO is an integral membrane-bound, heme-containing glycoprotein with a molecular weight of about 110,000 kD. The protein has a large extracellular (lumenal) enzymatically active portion with a disulfide

loop, a 24 amino acid hydrophobic membrane-spanning region and about a 60 amino acid intracytoplasmic carboxyl terminus (Yokoyama et al; Nakajima et al, Foti et al).

Thyroid stimulating hormone (TSH) stimulates TPO expression both *in vivo* and *in vitro*. TSH modulates the expression of TPO in FRTL-5 (a continuous line of rat epithelial cells derived from normal Fisher rat thyroids) cells (Chiovato et al) and is an absolute requirement for TPO activity in cultured dog thyroid cells (Magnusson et al (c)). TSH acts through cAMP in tissue culture systems. In the FRTL-5 cell system, it was shown that mRNA formation and subsequent protein synthesis is required for the expresson of TPO after TSH stimulation. Insulin and insulin-like growth factor-I (IGF-I) also positively regulate TPO mRNA levels in FRTL-5 cells (Zarrilli et al).

Many procedures have been developed to isolate TPO. They begin with fresh thyroid glands from a surgery or the slaughterhouse which are then trimmed and minced. The pieces of tissue are washed, centrifuged, and subjected to cholate and trypsin, and dialyzed (Morrison et al). Some methods include centrifugation on a density gradient (Yamashita et al). Hosoya et al (a) developed a procedure for the extraction and isolation of pig TPO from glands by gel filtration and chromatography. More recently TPO has been purified by immunoaffinity chromatography using a monoclonal antibody to TPO (Czarnocka et al). A great deal of work has been done to purify and obtain TPO because it has long been suspected to be the antigen in autoimmune thyroiditis (Hashimoto's). TPO has been expressed in E. coli in an effort to obtain an antigen to screen antibody production in Hashimoto's patients (Rapoport et al). It is now believed that the thyroid microsomal antigen found in autoimmune thyroid disease and TPO are identical (Portman et al, Seto et al). Work continues on the purification and characterization of human TPO (hTPO) because it appears that there are several epitopes (Taurog et al (b)). Large amounts of TPO can be generated by its expression in a nonthyroidal mammalian cell line. Wild type hTPO can be expressed on the cell

surface, as in thyroidal cells, (Kaufman et al) or a truncated, secreted form of the protein can be produced (Foti et al). Both the full-length and the truncated TPO are enzymatically and immunologically active.

Active Oxygen Species-hydrogen peroxide, superoxide, and hydroxyl radicals

Hydrogen peroxide (H_2O_2) is the oxidant essential for thyroid hormone synthesis. With the aid of thyroid peroxidase, H_2O_2 accepts two electrons from iodide to provide the form of iodine required for thyroglobulin iodination or one electron from iodotyrosine to create the iodotyrosyl radicals necessary for thyroid hormone formation. As was noted above, H_2O_2 is generated by thyroid follicular cells and its rate of formation is under the control of TSH (Perrild et al, Bjorkman et al).

Many origins of H_2O_2 in thyroid cells have been proposed and studied (Degroot et al). Candidates for the source include; NADPH-oxidase, NADH-oxidase (Labato et al), monoamine oxidase (Masini-Repiso et al), or xanthine oxidase. NADPH-oxidase is the best characterized H_2O_2 generating system in thyroid tissue (Dupuy et al, Nakamura Y et al,d). It is on the apical surface of the follicle cell at the membrane-colloid interface and donates electrons to O_2 to produce H_2O_2 . More recent work by Nakamura Y et al (f) suggests that the initial product in H_2O_2 formation is actually superoxide with its subsequent dismutation to H_2O_2. This mechanism is analogous to H_2O_2 formation by polymorphonuclear leukocytes, macrophages, and sea urchin eggs.

The fact that some cell types generate superoxide and/or H_2O_2 suggests that these oxidizing species have beneficial effects. Active oxygen species are naturally useful in phagocytosis and alcohol oxidation, and have been exploited for tumor destruction. Polymorphonuclear leukocytes and macrophages produce an oxidative

burst as the first step in killing and removing invading microorganisms. Oxidants from white cells are likely to stimulate tissue regeneration at the site of infection. Low levels of free radicals and/or H_2O_2 stimulate cellular proliferation by affecting the prostaglandin messenger system (Burdon et al) and by the induction of the protooncogenes, c-*fos* and c-*myc* (Crawford et al). Transitory enhancement of c-*fos* expression appears to be an immediate response to various forms of cellular stress. c-*myc* more likely plays a role in continuous cell cycling.

Cerutti suggests that promotable cells have higher levels of antioxidants and therefore, the oxidative stress experienced by them is in the low range which stimulates proliferation. Experiments have shown that promotable mouse epidermal cells have 2-3 fold higher catalase and Cu,Zn-superoxide dismutase (SOD) than in the non-promotable clone (Cerutti; Crawford et al; Larsson et al; Muehlematter et al). Another research group (Spitz et al) studied Chinese hamster fibroblasts after subjecting them to high levels of O_2. The increased exposure to O_2 presumably produces excessive activated oxygen species such as H_2O_2, superoxide (O_2^-), and hydroxyl radicals (HO·). Cells which had been adapted to stepwise increases in O_2 concentration were able to resist injury and death for 17-22 hours longer than the variants which had not been adapted and/or selected for growth in a highly oxidative environment. The H_2O_2-resistant cell line had 20-fold greater catalase activity, 2-fold greater CuZn SOD activity, and 1.5-fold greater glutathione peroxidase activity than the non-adapted cells. Similarly, there is transcriptional regulation of oxidative stress-inducible genes in bacteria (Storz et al). When bacterial cells are treated with low doses of H_2O_2, the synthesis of at least 30 proteins is induced and the cells become resistant to subsequent, otherwise lethal, doses of H_2O_2. The expression of nine H_2O_2 induced proteins, including catalase, is under the control of

one gene, *oxyR*.

The mechanisms of proliferation by radicals are believed to be through the induction of increases in intracellular free Ca^{+2} and of the protein kinase activity which phosphorylates ribosomal protein S6. S6 phosphorylation has been postulated to be required for the stimulation of protein synthesis in the acquisition of growth competence by quiescent cells. Any of three souces of active oxygen *i.e.* glucose/glucose oxidase (producing H_2O_2), xanthine oxidase (producing H_2O_2 and superoxide) or menadione (producing mostly superoxide) produce increased intracellular calcium and protein kinase activity when added to the promotable mouse epidermal cells in the experiments outlined above (Larsson et al).

Cellular proliferation, while beneficial in wound healing, can be undesirable. Although the thyroid gland is well protected against oxidants by high ascorbate levels, it is possible that supranormal levels of H_2O_2 contribute to the hypertrophy and hyperplasia of the epithelial cells lining the follicles in a goiterous thyroid gland. Cerutti has postulated a role for oxidants in tumor promotion in the clonal expansion of initiated cells (Cerutti). In this model, infiltrating phagocytes provide oxidants that select against non-promotable cells and stimulate the proliferation of initiated cells. If true, Cerutti's hypothesis explains the common occurence of tumors at sites of chronic inflammation.

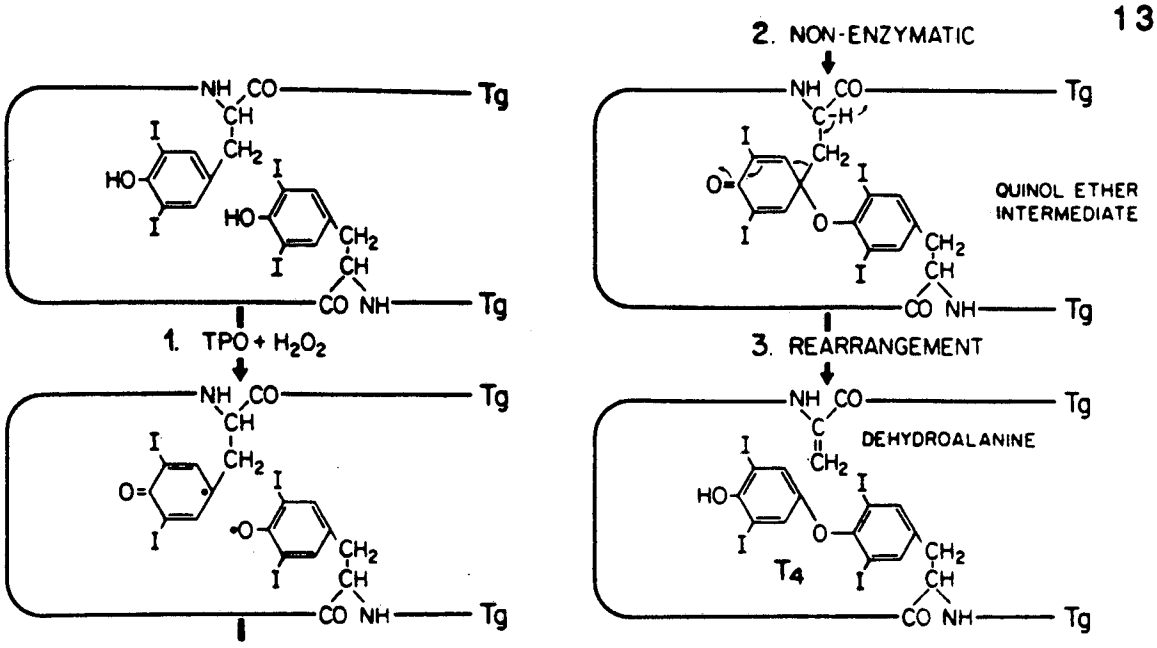
Exposure to excessive levels of H_2O_2 and/or free radicals leads to growth inhibition and cell death. Using xanthine/xanthine oxidase to mimic phagocytes, Muehlematter et al found that the active oxygen induced DNA strand breakage and poly ADP-ribosylation in mouse epidermal cells. It has been proposed that the DNA-breaks and poly ADP-ribosylation deplete NAD and ATP pools (Schraufstatter et al) and have cytostatic effects (Cerutti). High levels of H_2O_2 and/or free radicals also lead to decreased DNA, RNA, and macromolecule synthesis, lipid peroxidation, decreased levels of glutathione, and the

oxidation of SH-containing enzymes (O'Brien, b; Southorn (a)). Each of these effects damage cells and have been implicated in human diseases.

All aerobic cells possess mechanisms to mitigate the effects of H_2O_2 and/or free radicals. Low levels of H_2O_2 react with reduced glutathione (GSH) to form oxidized glutathione (GSSG) and water, a reaction catalyzed by glutathione peroxidase. GSH is regenerated from GSSG by NADPH. High concentrations of H_2O_2 are removed by the enzyme catalase which forms O_2 and H_2O. The mitochondrial cytochrome oxidase system consumes most of the oxygen which might form H_2O_2. Superoxide dismutase (SOD) catalyzes the dismutation of the superoxide anion radical to form H_2O_2, a much less oxidizing form of O_2. Cells also possess nonenzymatic antioxidants for the prevention of damage by free radicals. Vitamin E, a lipid phase antioxidant, protects membranes (Southern, a). Vitamin C is an aqueous phase antioxidant and is found in high concentrations in some tissues, especially the eye (Southern, a).

Free Radicals

Free radical generation occurs during intramolecular coupling of iodotyrosine residues for thyroid hormone formation. One electron is removed from the benzene ring at the junction with the alanine residue and the another electron is removed from the OH group of the iodinated tyrosine. The two iodotyrosine radicals then couple to form one hormone residue.



from Taurog in *Werner's The Thyroid*, p. 71.

However, the experiments reported here show that thyroid peroxidase is capable of generating free radicals besides those required for hormone synthesis.

Peroxidases catalyze the oxidation of a wide variety of xenobiotics to free radicals. Phenols and amines seem to be especially active substrates of radical formation. Low redox potential radicals can directly activate oxygen to form hydroxyl radicals or undergo scission to carbon radicals. The hydroxyl and carbon radicals readily oxidize biomolecules. Hydroxyl radicals can oxidizd sulfhydryl groups in proteins and cause strand breakage and deoxyribose degradation in DNA strands. If a radical has a high oxidation potential, it may oxidize NAD(P)H and GSH. Xenobiotics are believed to act as oxidation-reduction catalysts, being alternately oxidized to a free radical intermediate by the peroxidase system and reduced by GSH or NAD(P)H (O'Brien, b).

Free radicals formed by a peroxidase catalyzed one-electron oxidation either cause further oxidations or undergo an additional one electron oxidation to form an electrophilic two electron oxidation product. The two electron oxidation products and polymeric products of xenotiotic oxidation can bind to DNA, thereby interferring with its

normal function. GSH conjugate formation has also been attributed to the two electron oxidation product of peroxidase catalyzed metabolism of carcinogens and xenobiotics (O'Brien, b).

Cell injury associated with free radicals occurs either in disease states in which the protective antioxidant systems are impaired or, as is more likely in the thyroid gland, in situations in which there is excessive production of free radicals. Increased TSH stimulation due to hypothyroidism or long-acting thyroid stimulator (LATS) in Grave's disease increases H_2O_2 production and TPO activity. With increased substrate and enzyme activity, a greater production of radicals is to be expected. As noted above, these radicals would lead to cellular proliferation if present in low levels or if a population of oxidation resistant cells had been selected. If present in high levels, radicals would be cytotoxic. Two human thyroid diseases seem to illustrate both the proliferative and the cytotoxic effects of oxidative stress. In Grave's disease, the thyroid gland is enlarged and the follicular cells are hyperplastic. Eventually the thyroid gland becomes non-functional. Under high TSH stimulation due to the hypothyroidism of Hashimoto's disease, the thyroid gland also forms a goiter that is incapable of synthesizing thyroid hormone. In most Hashimoto's patients, there is destruction of epithelial cells and degeneration and fragmentation of the follicular basement membrane. In some cases, epithelial hyperplasia may be prominent. Fibrosis is generally present (Ingbar et al). These processes have been found in other diseases in which radicals are suspected to have a role. Oxidant resistant epithelial cells and a fibrotic basement membrane develop in pulmonary oxygen toxicity. It has been proposed that radicals released by leukocytes are related to endothelial damage and play a role in atherosclerotic disease (Southorn, b). The fact that agents that remove free radicals provide protection from the damage of some diseases supports the hypothesis that radicals are involved in their pathogenesis. Superoxide dismutase has been used to treat rheumatoid arthritis, Duchenne's muscular dystrophy (Southorn,b), and influenza (Oda et al).

It is reported to have beneficial effects, especially when conjugated with polymers to increase its circulating half-life (Oda et al) or is encapsulated in artificial liposomes to permit it to reach intracellular sites (Southorn, b).

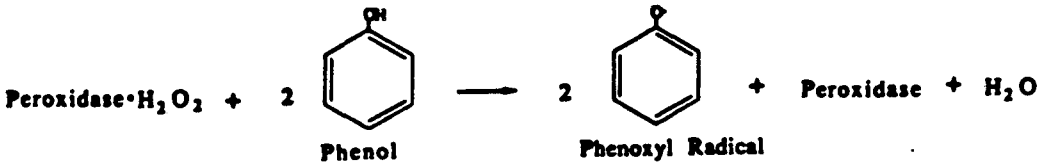
Peroxidases and ESR

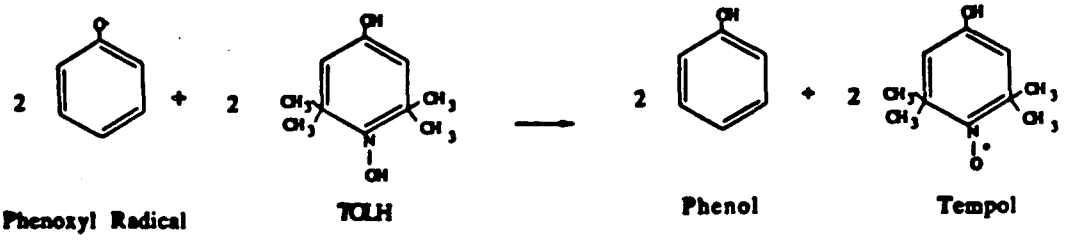
One of the first studies of radical intermediates in biological systems was the elucidation of the mechanism of reactions catalyzed by peroxidases (Yamazaki et al, a,b). These enzymes catalyze the two step reaction

$$H_2O_2 + 2e^- + 2H^+ \rightarrow 2\ H_2O$$

where the two electrons must be supplied by a substrate. Yamazaki et al used ascorbic acid as the electron donor and recorded its ESR signal directly. The development of spin probes has extended the technique of detecting free radicals to reactions which do not have substrates or products with unpaired electrons. Spin probes rapidly scavenge unstable free radicals and form relatively stable free radicals which are detectable by ESR spectroscopy. I have used the one-electron reductant, TOLH, to detect the radicals which develop during the oxidation catalyzed by peroxidase. TOLH is an ESR silent nitroxide which can be oxidized to the ESR detectable Tempol (TOL·). The reaction can be described as:

H_2O_2 + Peroxidase ⟶ Peroxidase·H_2O_2





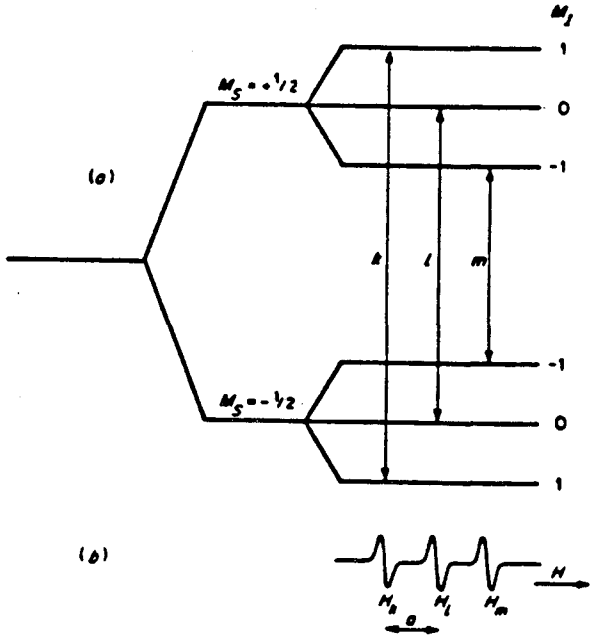
Spin Trapping

The success of a spin trapping experiment depends upon the rate of radical scavenging being faster than the rates of other reactions of the radical. The ideal spin probe would form a stable product which unreactive with other reagents in the environment. The spin probe must be soluble in the medium of interest with free diffusion of the spin probe to the location of the free radical event. TOLH freely reacts with phenoxyl radicals as both are water soluble. The rate of radical accumulation in my system reflects the rate of the reaction of peroxidase with H_2O_2. It is assumed that the reaction rates of the peroxidase-H_2O_2 complex with phenol and TOLH are non-limiting. It is also assumed that every radical produced is trapped and the magnitude (height) of the ESR signal is directly proportional to the number of radicals created (Janzen). The rate of radical formation can be recorded by locking on the peak of the first absorption curve. The signal height is continuously recorded and followed over time.

ESR Theory

ESR is based on the absorption of a quantum of energy by an unpaired electron as it moves from a low energy state to a higher one. Because there is always thermal energy in a sample, some of the electrons will be in a low and some in a high energy state. If the sample is put into a resonant magnetic field, a quantum of energy will be absorbed which excites spin flips between the two states and corresponds to the difference between energy levels. Net energy is absorbed from the radiating field because initially there are more electrons in the state which is parallel to the external magnetic field (a slightly lower energy state). This quantum of energy (E=hv) is in the giga hertz (GHz, 1GHz=10^9 cycles/sec) range. Energy absorption occurs at more than one field strength because the unpaired electron reacts to the magnetic field of the nearby nuclei as well as that of the externally applied magnetic field. The interaction of an unpaired electron with internal fields due to nuclear magnetic dipole moments

as well as with the applied field is termed 'the nuclear hyperfine interaction'. This results in the splitting of resonance lines into two or more components, so-called hyperfine splitting, and is expressed in units of gauss (g). The center of the signal of the resonant magnetic field is characteristic of the molecule under study. I have used a nitroxide, 2,2,6,6-tetramethyl-4-hydroxypiperdin-1-ol (TOLH), to trap phenoxyl radicals. The magnetic properties of this molecule arise from the unpaired electron localized primarily on the nitrogen atom. The nitrogen atom has a nuclear spin of one, which means that it has three possible nuclear spin states, +1, 0, and -1. The difference in energy of the unparied electron between a spin of +1/2 and -1/2 at the three different nuclear spin state of +1, 0 or -1, give three absorption values for a nitroxide. Usual ESR spectra are the first derivative of the microwave power absorbed plotted vs the applied magnetic field strength.

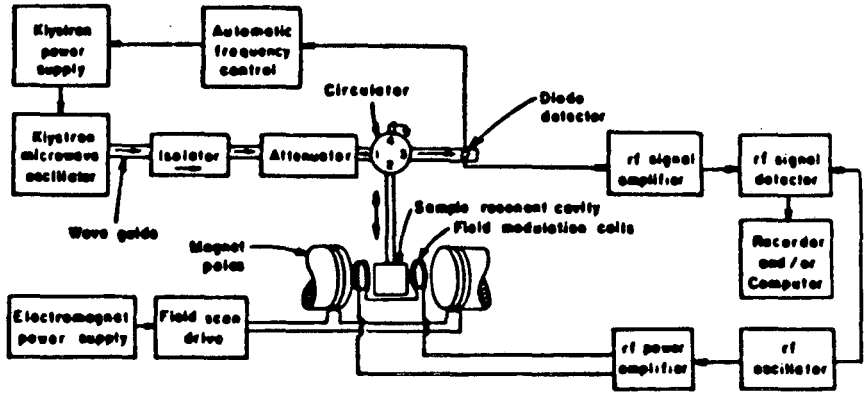


(a) nitroxide energy levels and allowed transistions, absorption spectra; (b) first derivative tracing-from Wertz and Bolton, p. 47.

ESR Machine

The major features of an ESR spectometer are as follows: (1) An

electromagnet provides a stable, linearly variable and homogeneous magnetic field that can be swept smoothly over a range of field intensities. (2) A klystron generates microwave irradiation and waveguides conduct the microwave beam into the sample cavity. The voltage applied to the vacuum tube (klystron) determines the frequency of the radiation. (3) The "cavity system" holds the sample and directs and controls the microwave beam to and from the sample. (4) A crystal or diode detects net microwave absorption by the sample when resonance occurs. Because of the small amount of energy absorbed, high-gain and noise-suppressing signal amplification are needed. Signal-to-noise enhancement is commonly achieved with phase-sensitive detection locked to a magnetic field modulation.



simplified block diagram of a typical ESR spectrometer-after Wertz and Bolton, p 24.

Other Methods of Quantifying Peroxidase Activity and H_2O_2

Peroxidase activity can be measured spectrophotometrically with a chromogenic donor such as ferrocyanide, cytochrome *c* and many dyes. In the case of thyroid peroxidase, guaiacol or iodide can be used with a moderately purified enzyme prepared from few mg of tissue (Hoyosa et al, b).

H_2O_2 can also be quantified spectrophotometrically by the

formation of cytochrome *c* peroxidase complex II, fluorometrically by the decrease of scopoletin fluorescence in the presence of HRP (Thurman et al) or in an oxygen polarograph by the release of O_2 (Clifford et al). These methods are sensitive to μM quantities of H_2O_2 but require larger samples than does ESR measurement. Chemiluminescence is an extremely sensitive method of O_2 metabolite detection but is prone to interferences which are difficult to control and does not discriminate among oxidizing species (Vilim et al). Chemiluminescence, especially when enhanced (Thorpe et al), is a multi-step process and each step can lead to artifacts. Some chemiluminescent systems require a high pH which is not compatible with enzyme activities.

ESR Assays of Hydrogen Peroxide and Peroxidase Activity

INTRODUCTION

An electron spin resonance (ESR) assay was developed to quantify peroxidase catalyzed free radical formation. The assay consists of measuring the oxidation of a reduced nitroxide by phenoxyl radicals generated by peroxidase with hydrogen peroxide. It can be used in one of two ways; to assay H_2O_2 concentration or peroxidase activity. The quantity of oxidized nitroxide reflects the amount of H_2O_2 in the sample in conditions of excess peroxidase activity. The rate of radical trap accumulation shows the rate of peroxidase activity with the proper (neither denaturing nor rate limiting) concentration of H_2O_2. A H_2O_2 generating system provides appropriate oxidant concentrations for assaying peroxidase activity.

This ESR method is very sensitive, permitting the assay of very small samples (5-50 µl) of thyroid peroxidase preparations. This allows the same preparation to be used for several assays. The minute quantities of tissue or cells required and the immediate readout available with this method are especially useful in the initial stages of enzyme characterization when concentration ranges of the components of the system, active cellular fraction, storage effects, etc. are being determined.

The ESR assay was used to assay optically opaque samples. As I thought it necessary to preserve the biological properties of the thyroid plasma membranes, the samples for assay were turbid. ESR spectroscopy is not affected by particles in solutions. In fact, high protein concentration increases the sensitivity of ESR spectroscopy. This is in contrast to light spectroscopy in which cloudy solutions are not suitable.

The instant read out of the ESR assay is convenient for the initial stages of enzyme characterization. Relative activities of various

fractions or substrate concentrations can be estimated without performing any mathematical calculations.

METHODS AND MATERIALS

ESR

All experiments were done in Teflon gas-permeable tubing (Zeus Industrial Products, Inc., Raritan, NJ), 0.032 +/- 0.003 in. I.D., 0.002 in. wall or 0.064 +/-0.003 in. I.D., 0.002+/- 0.001 in. wall. The tubing was folded so that four of the finer, or two of the heavier, widths were inserted into glass tubing and evaluated by electron spin resonance (ESR) spectroscopy. First derivative ESR spectra (100 kHz modulation) were recorded at ambient temperature (22°C) and atmospheric oxygen on a Bruker ER200 D-SCR spectrometer (X-band). Instrument settings: modulation amplitude 1.25 G, scan range 100 G, central field 3480 G, microwave power 10 mW. For kinetic studies, the low field peak of the nitroxide triplet was continuously recorded. Nitroxide concentration was estimated from the peak to peak height of the low field line of the first derivative spectrum.

All assays were performed in 0.14 M sodium phosphate buffer, pH 7.4, with a total volume of 50 µl/assay. If H_2O_2 was being used, a 30% stock solution of H_2O_2 was diluted immediately before the assays were performed. All reagents were purchased from Sigma, unless otherwise noted.

Buffer Preparation

Buffer salts (NaH_2PO_4 and Na_2HPO_4) were dissolved in commercially distilled water, incubated with insoluble, phosphate-glass, pH 7.4, until the phosphate-glass settled (about one week), and carefully removed from above the phosphate-glass. The buffer was then passed through a Chelex 100 (Bio-Rad, Richmond, Ca.) column which had been washed several times with buffer. Buffers were stored in polypropylene containers to avoid the possibility of

absorbing transistion metals from glass containers. Transition metal ions lead to reactions which oxidize spin traps and therefore need to be avoided or eliminated.

Some chelates of transition metal ions, e.g. Fe EDTA, as well as the ions themselves affect this assay. EDTA appears to chelate metal ions from glass containers and Hamilton syringes. 1 mM DTPA (diethylenetriaminepentaacetic acid) chelates of iron and copper exhibit very little TOLH oxidation activity (data not shown). The use of plastic tubes for mixing samples and plastic pipette tips avoids th eintroduction of metal ions into the TOLH assay mixtures. If a Hamilton syringe must be used to add reagents, it must be rinsed with that reagent immediately before use to remove metals arising from apparent corrosion of the needle.

Iodide (I^-) also must be minimized or eliminated. As little as 10^{-7}M free I^- will catalyze spin probe oxidation initiated by lactoperoxidase or thyroid peroxidase under some conditions.

Standardize Spin Probe

TOLH was prepared from Tempol (2,2,6.6-tetramethyl-1-piperidinoxy-4-ol) which was purchased from Aldrich Co. TOLH (2,2,6,6-tetramethyl-1-hydroxypiperidin-1-ol) was synthesized as previously described (Prolla et al). TOLH at two different concentrations was oxidized by 2 mM $K_3Fe(CN)_6$ to determine its concentration relative to known Tempol concentrations in 2mM $Fe(CN)_6$. After several minutes, low field line heights were compared with those of the Tempol standard.

Sheep Thyroid Peroxidase Preparation

Sheep thyroids were collected at a slaughterhouse, immediately packed in dry ice and transported to the laboratory where they were stored at -80° C. To prepare peroxidase-enriched tissue fractions, approximately 7 g of sheep thyroids were thawed, decapsulated, and the connective tissue removed. A volume 2X that of the trimmed

tissue 50 mM buffer, pH 7.4 buffer (10 ml buffer for 5 g tissue) was added to the thyroid. The tissue was then minced before being homogenized with a Polytron tissue grinder. All equipment and solutions were kept at 0-5°C during enzyme preparation. The homogenate was centrifuged for 10 min, at 2300 rpm in Sorval centrifuge with the SS34 rotor (approx. 400 g) to remove cell debris, mitochondria, and nuclei. The supernatant was stirred with a few grains of collagenase and DNAase on ice for 30 min. The treated supernatant was then centrifuged for 30 min at 108,000 g to collect the microsomes. The microsomal pellets were washed twice with 140 mM buffer. The high speed centrifugations were done in a tabletop ultracentrifuge in 1.5 ml Eppendorf tubes. The microsomes from each tube were suspended in 100 µl 140 mM buffer to yield a protein concentration of about 20 mg/ml. 5 g of trimmed tissue yields approximately 23 mg of protein. The microsomal TPO pellet remains active for at least 6 months when stored at -80°C or in liquid nitrogen.

Protein determinations for peroxidase preparations were done by the Lowry method using BSA as standard.

Rat Thyroid Peroxidase Preparation

Two rat whole thyroid glands were homogenized in 1 ml 50 mM buffer and spun 1 min in a microfuge. The pellet was resuspended in 1 ml 140 mM buffer and spun, twice. The pellet was resuspended in 500 µl 140 mM buffer for assay.

Rabbit Thyroid Peroxidase Preparation

The thyroid gland of one animal was trimmed of connective tissue and capsule, homogenized with 1 ml 50 mM buffer per gland and centrifuged for 2 min in microfuge. The supernantant was centrifuged at 108,000g for 15 min to obtain microsomes. The pellet was resuspended in 100µl 140 mM buffer and centrifuged at 108,000g for 15 min, twice. The pellet was resuspended in 100 µl 140 mM buffer for assay.

Tissue Culture TPO Preparation

TPO activity was assayed after extraction from cell microsomes. Cells from a 100-mm-diameter dish were prepared according to the rapid method described by Kaufman et al. Briefly, the cells were suspended in PBS with a rubber scraper, protein determination was done by the Bio Rad (Richmond, Ca) Protein Assay, and TPO was extracted in 0.1% deoxycholate. 3-100mm plates, plated 3 days previously at 10^6 cells/plate yielded approximately 10-12 mg protein for deoxycholate solubilization (0.2 ml deoxycholate/mg cellular protein). Supernatants were stored at -80°C.

Protein determinations on PBS suspensions of CHO-hTPO cells was determined for 5 µl aliquots of PBS suspension by the Bio-Rad and read on an Elisa machine. Bovine serum albumin was used as the protein standard.

RESULTS

Quantifying Hydrogen Peroxide by TOLH Oxidation

Conditions for Assay Illustrated with Horseradish Peroxidase

An excess of peroxidase will accurately measure H_2O_2 by oxidizing two moles of TOLH to ESR-detectable Tempol per mole of H_2O_2 (see reaction sequence in 'Background-Peroxides and ESR'). 5-25 µM aliquots of H_2O_2 added to 2 U/ml HRP and 40 µM phenol plateau rapidly and stoichiometrically oxidize TOLH (added at 3.5 mM) but large aliquots of peroxide lead to substoichiometric TOL· formation. An excess of H_2O_2 will inactivate peroxidase. The ESR tracing from more than 25 µM H_2O_2 in this assay shows a rapid initial oxidation followed by a progressively slower oxidation rate which does not reach the expected plateau. An aliquot of 100 µM H_2O_2 added to 2 U/ml HRP takes over 4 minutes to plateau and yields slightly less Tempol formation than expected. A bolus of 200 µM H_2O_2 added to the same HRP concentration begins to plateau after 3 min and reaches a

maximun of 270 μM Tempol, only 2/3 of the stoichiometric amount. The shorter time to reach maximum oxidation with 200 than 100 μM H_2O_2 reflects a quicker inactivation of the peroxidase.

Fig 1 shows a sample titration of 10-fold dilutions of HRP against a H_2O_2 generating system. One and 10 U/ml HRP quantitatively convert the H_2O_2 generated by 0.2 μg/ml glucose oxidase to Tempol. Both peroxidase concentrations catalyzed the oxidation of the H_2O_2 output of 0.2 μg/ml glucose oxidase to form 5 μM Tempol per minute . 10 U/ml HRP also accurately measured the H_2O_2 output from 2.0 μg/ml glucose oxidase. It catalyzed the oxidation of about 50 μM TOLH as would have been expected from a 10-fold increase in glucose oxidase. A quick indication of whether or not the peroxidase is able to convert the generated H_2O_2 to Tempol is the linearity of the tracing. If the tracing of Tempol production is linear over a period of several minutes, it indicates that there is no accumulation of H_2O_2 and that the peroxidase still has maximum activity. Later experiments show that TOLH oxidation in this experiment was approximately half that seen with glucose oxidase which had been freshly diluted from 10 mg/ml stock solutions.

Purified peroxidase can be added to a H_2O_2 assay to assure sufficient enzyme activity. However, if peroxidase concentration is limited, as in tissue homogenates, trials with several dilutions of the H_2O_2 generating system should be done to assure that the available peroxidase concentration is capable of converting all of the H_2O_2 to Tempol and H_2O.

Standard Curves with Horseradish Peroxidase and Lactoperoxidase

A calibration curve for the H_2O_2 assay done with HRP and H_2O_2 is shown in Fig 2. Points for this graph were obtained by sequentially adding 10 μM aliquots of H_2O_2 to two vials of 3.5 mM TOLH, 10 mM

glucose, 20 μM phenol, and 2 U/ml HRP, each. (The quantity of H_2O_2 must be decreased as 50 μl aliquots are removed from a vial.) The first mixture was used to assay 0-100 μM H_2O_2. The accuracy of the assay declines if more than about 10 samples have been removed from a vial. The second vial, which had had 10-1μl additions of 0.03% H_2O_2, was used to assay 110-200 μM H_2O_2.

The assay was repeated with lactoperoxidase (LPO) to determine the effect of iodide and tyrosine on TOLH oxidation. 10 μM H_2O_2 aliquots were added to 2 U/ml LPO, 100 μM tyrosine, 3.5 mM TOLH, 10 mM glucose, 1mM DPTA, +/- 100μM KI (Fig 3). Tyrosine with and without iodide gave nearly the same result (y=84 + 2.06x without I^- versus y=70 + 2.04x with I^-). The stoichiometric conversion of H_2O_2 to Tempol indicates that peroxidase was not forming iodinated tyrosine by performing a two-electron oxidation of iodide.

Assay of Hydrogen Peroxide Evolution from Glucose, Oxygen, and Glucose Oxidase with Horseradish Peroxidase, Sheep TPO

The glucose, glucose oxidase system is a convenient method of generating H_2O_2 to assess peroxidase activity in biological samples. The reactions involved in the glucose oxidase system are:

glucose + Enzyme-FAD --> E-$FADH_2$ + gluconolactone

E-$FADH_2$ + O_2 --> H_2O_2 + E-FAD.

A graphic representation of H_2O_2 generation and its coupling to peroxidase activity shows that for every H_2O_2 (O_2 consumed), 2 TOL· are formed (Fig 4). The O_2 was measured by an oxygen electrode.

The H_2O_2 output from a generating system must be measured under actual assay conditions. Oxygen polarograph experiments have shown that the substrates for the ESR assay, i.e. HRP, phenol and TOLH do not affect O_2 consumption. It is assumed that they also do

not effect H_2O_2 formation.

Glucose, one of the substrates for glucose oxidase in the evolution of H_2O_2, increases the rate of H_2O_2 evolution in concentrations of 2.5-25 mM. Concentrations between 25 and 100 mM increase the linear rate of H_2O_2 evolution very slightly. However, high concentrations of sugars have been shown to intercept radicals and therefore should be avoided. The high osmotic activity of concentrated glucose in parallel tissue culture experiments would also pose a problem. 10 mM glucose was used throughout the work reported to illustrate typical experimental conditions.

To confirm that the O_2 concentration is not limiting the H_2O_2 output from the glucose/glucose oxidase system, it is sufficient to demonstrate identical TOLH oxidation rates under different oxygen tensions. Experiments were done with a 60% oxygen, 40% nitrogen mixture flowing over the gas permeable tubing during ESR measurement. After correcting for line broadening by oxygen and comparing rates with an identical mixture assayed under 20% O_2 and 80% N_2, it was found that increased oxygen did not significantly increase the TOLH oxidation rate. This suggests that there is adequate O_2 availabilty in air through gas permeable tubing. (Data not shown.)

Fig 5 illustrates H_2O_2 quantification of the glucose, glucose oxidase system by microsomal sheep thyroid peroxidase. The graph shows that 0.2 μg/ml glucose oxidizes 4 μM TOLH/min, close to 5 μM TOL·/min recorded with HRP with the same glucose oxidase concentration. TOL· output is much less than would be expected for 1 and 2 μg/ml when compared with the output from 0.2 μg/ml glucose oxidase, indicating peroxidase inactivation, rather than catalase contamination. If catalase had been consuming a significant quantity of H_2O_2, the increased H_2O_2 generation capacity would have shown increased peroxidase activity.

Quantifying Peroxidase Activity by TOLH Oxidation

Conditions for Assay Illustrated with Horseradish Peroxidase

Two conditions must be demonstrated to accurately quantify peroxidase activity by TOLH oxidation. They are (a) TOLH oxidation rates must be directly proportion to peroxidase concentration and (b) TOLH oxidation rates must be linear with time. If these two conditions are fulfilled, it can be assumed that there is sufficient H_2O_2 to saturate the enzyme and that activity is not being inhibited by excessive H_2O_2. Non-limiting concentrations of the phenol catalyst and free radical trap are required to demonstrate the maximum velocity of the reaction. The peroxidase activity as measured by ESR will be the rate of TOLH oxidation (μM/min) divided by the enzyme concentration, if known, or by the protein concentration of crude enzyme preparations.

To ensure that the peroxidase is saturated several concentrations of H_2O_2 or H_2O_2 generating systems must be titrated against different concentrations of peroxidase. Fig 6 depicts the assay of steady-state HRP activity with the glucose, glucose oxidase H_2O_2 generating system. It can be seen that 0.2 μg/ml glucose oxidase supplies sufficient H_2O_2 to show maximum activity of 0.1 U/ml HRP and that 2.0 μg/ml glucose oxidase only of about 1 U/ml HRP. The measured activity of 1 U/ml HRP with 0.2 μg/ml glucose oxidase and 10 U/ml HRP with 2.0 glucose oxidase was limited by H_2O_2 availability. The highest linear rate of Tempol formation shows the true peroxidase activity. Maximum rates of Tempol formation are about twice as high in Fig 6 as in Fig 1, illustrating the importance of using fresh dilutions of glucose oxidase from concentrated stock solutions.

Excessive H_2O_2 will inactivate peroxidase. For example, 0.1 and 1.0 U/ml HRP plus 2 μg/ml glucose oxidase and 0-10 U/ml HRP plus 20 μg/ml glucose oxidase (Fig 1) show tracings which are non-linear; i.e., the rate of radical accumulation declines. If the TOLH oxidation

rate goes down during an assay, the peroxidase has been inactivated and cannot catalyze radical formation in proportion to its concentration. Further evidence that these rates are not valid can be inferred by the fact that the rates are not proportional to the glucose oxidase concentration.

Assay of Thyroid Peroxidase Activity with Sheep Thyroid Microsomes

The appropriate conditions for quantifying peroxidase activity can be established empirically by serial dilutions of a H_2O_2 source with peroxidase-containing tissue homogenates. Sheep thyroid microsomes were assayed with various concentrations of glucose oxidase to determine a suitable concentration of H_2O_2 for the assay of peroxidase activity (Fig 7). It can be seen that 0.2 µg/ml glucose oxidase generates H_2O_2 to form radicals at a constant rate for the first two min. To show that radical formation was peroxidase concentration dependent, 5, 10, or 25 µl sheep TPO was added to 0.2 µg/ml glucose oxidase (Fig 8). An activity of 7.5 µM TOL·/min for 25 µl, 0.3 µM TOL·/min/µl, or 0.015 µM TOL·/min/µg TPO suspension (20 mg/ml protein) can be calculated from the data. The same assay gave a rate of 12.4 µM TOL·/min/U/ml HRP which gives an equivalence of 0.0012 U/ml HRP activity/µg sheep TPO suspension.

Demonstration of Peroxidase Dependence of ESR Assay with Rat Thyroid Microsomes

To confirm that TOLH oxidation was dependent upon the peroxidase enzyme and not on transistion metal contamination, an ESR assay was done with or without microsomes (TPO source) and on the whole system with or without cyanide. Cyanide inhibits TPO by competing with H_2O_2 (Ohtaki et al, c). As can be seen in Table I, KCN greatly reduces the TOLH oxidation rate when compared with the complete assay system without KCN. The peroxidase was probably partially inactivated in these assays, due high H_2O_2 generation rates.

The results of this experiment should be considered as qualitative, rather than quantitative.

Table I

Rat Thyroid Microsomes	250 µM Tyrosine	2 µg/ml Glucose Oxidase	2mM KCN	µM TOL·/Min
-	+	-	-	0.48
-	+	+	-	1.93
+	-	-	-	1.59
+	-	+	-	4.05
+	+	+	-	14.32
+	+	+	+	3.66

Peroxidase Activity of Cultured hTPO-Transfected Cells

Peroxidase activity of 0.1 % deoxycholate solubilized membrane preparations of cultured CHO cells was assayed by ESR (Table II). It can be seen that the membrane preparation from the cells with the TPO gene (CHO-hTPO) have about 10X the peroxidase activity as the ones without (CHO-K1). Guaiacol is a much more efficient phenolic catalyst than is phenol for the cellular TPO extract in this assay. When the activity of 1 U/ml HRP with phenol is compared with that of 50 µl CHO-hTPO extract with guaiacol, it can be seen that 1 µl of the CHO-hTPO extract has an activity equal to 0.017 U/ml HRP.

Table II

Peroxidase	Phenol	µM TOL·/min
50 µl CHO-K1 extract	100 µM guaiacol	0.36
50 µl CHO-hTPO extract	100 µM guaiacol	2.65
50 µl CHO-hTPO extract	40 µM phenol	0.96
1 U HRP/ml	40 µM phenol	3.37

Conditions: 0.2 µg/ml glucose oxidase, 10 mM glucose, and 3 mM TOLH.

Other Substrate Concentration Effects: Phenols, Supporting Medium

Phenols

Minimal Detectable Phenol Concentration

As the oxidation of the nitroxide radical trap depends upon phenoxyl radicals, the measurement of Tempol formation can be used

to detect phenols. When various concentrations of phenol were added to 10 U/ml HRP and 0.2 μg.ml glucose oxidase with 10 mM glucose and 3.5 mM TOLH, 1 μM phenol was the mininum concentration detectable while 5 μM phenol gave the maximum rate of Tempol formation. Phenol concentrations cannot be measured by this assay.

Phenolic Activity of Media with or without Calf Serum, Hepes buffer, and Hank's Balanced Salt Solution (HBSS)

Because very little phenol is effective as catalyst for the production of radicals by peroxidase, it is possible that an assay system would have unknown phenolic activity. This was suspected in the tissue culture experiments when it was discovered that it was not necessary to add a phenol to damage to cells cultured with peroxidase. As Experiment 2 in Table III shows, 1 μl Ham's F-12 media with 5% calf serum in a 50 μl assay has phenolic activity comparable to phenol. Various solutions which would maintain cell viability were investigated as temporary media in which it could be unequivocally shown that cellular damage was phenol dependent. Both tissue culture media without serum and HBSS have sufficient phenolic catalytic activity to transfer free radicals to TOLH. It is assumed that the phenolic activity is high enough to also carry free radicals to vulnerable cellular structures. Hepes buffer was the only component of the tissue culture media which showed no phenolic activity in ESR assays.

Table III

Phenol Catalyst	µM TOL·/min
Expt 1	
-	5.3
40 µM phenol	59.8
10 µl media with 10% calf serum	14.0
25 µl media with 10% calf serum	25.1
10 µl HBSS	71.3

Conditions: 2 U/ml HRP; 1 µg/ml glucose oxidase; 10 mM glucose; 3.5 mM TOLH; 50 µl total assay volume.

Expt 2	
-	5.3
40 µM phenol	14.0
0.10 µl media	5.8
0.25 µl media	8.7
1.0 µl media	13.0
2.0 µl media	12.5
0.10 µl media with 5% calf serum	2.9
0.25 µl media with 5% calf serum	9.2
0.50 µl media with 5% calf serum	11.6
1.0 µl media with 5% calf serum	14.9
2.0 µl media with 5% calf serum	13.0
5 mM Hepes	3.4
10 mM Hepes	5.3
25 mM Hepes	1.9

Conditions: 10 U/ml HRP; 0.2 µg/ml glucose oxidase; 10 mM glucose; 3.5 mM TOLH; 50 µl total assay volume.

Comparison of Catalytic Competency of Various Phenols

Radical formation rate recorded by ESR can assess the activity of various phenols as substrates for a peroxidase in a particular environment. Each peroxidase has a different interaction with each phenol in each matrix. For example, peroxidase affinity for a phenol is influenced by whether or not the peroxidase is membrane bound. Table IV illustrates the varying efficiencies of several phenols with two thyroid peroxidases and lactoperoxidase (LPO). The rat thyroid microsomes (Expt 1) contain membrane bound TPO. The CHO-hTPO extract is a detergent solubilized membrane preparation and the LPO is a purified peroxidase preparation (Expt 2).

CHO-hTPO membrane extract best illustrates the activity of a peroxidase with a variety of phenols. The phenols were used in greatly

different concentrations in order to be equally effective as catalyst between TPO and TOLH (Table IV, Expt 2). For example, phenol concentrations in Expt 2 vary 500-fold (20 μM-10 mM) while the activity only changes 8-fold (0.24-1.93 μM TOL·/min). When 40 μM phenol is the catalyst, 1 μl hTPO extract has the same activity as 10 μU/ml HRP. However, if 10 mM guaiacol is the catalyst for CHO-hTPO, 1 μl hTPO extract is as active as 30 μU/ml HRP with 40 μM phenol.

An example of the same peroxidase in differing states of solubility can be seen by comparing thyroid peroxidase reactions with tyramine (Table IV). Although it is difficult to compare the rat TPO data with the CHO-hTPO data, due to different peroxidase and glucose oxidase concentrations, it appears that the tyramine concentration giving comparable activity to 40 μM phenol is much lower with CHO-hTPO deoxycholate extracts than with rat thyroid microsomes. Expt 1 (rat TPO) shows that 500 μM tyramine is about equivalent to 40 μM phenol when the baseline rate is subtracted while 40 μM tyramine and phenol showed the same rate of TOL· formation (Expt 2) with the CHO-hTPO extract. The differences in TPO affinity for tyramine may be due to species differences in the two preparations. However, it is more likely that the deoxycolate in the hTPO preparation gives better accessibilty between the peroxidase and tyramine.

The data from 100 μM arbutin and p-hydroxyphenyl pyruvic acid in Table IV, Expt 2 illustrate differing affinities of different peroxidases for the same phenol. If phenol is used as the standard of TOL· production, arbutin reacts more efficiently with CHO-hTPO extract than with the LPO. Similarly, OH-phenylpyruvate reacts more efficiently with the LPO than the TPO. Benzaldehyde and tyramine were equally as effective with either peroxidase.

The data on guaiacol in Expt 2, demonstrates the importance of optimizing phenol catalyst concentration in order to reflect the true capabilities of the peroxidase. (Phenol concentrations for the upper section of Expt 2 were optimal for LPO and were duplicated with CHO-hTPO extracts for comparison.) At the lower concentration of

guaiacol; i.e. 1 mM, it appears that 0.4 μg/ml glucose oxidase generates H_2O_2 at a rate which inactivates the TPO preparation as 0.4 μg/ml does not oxidize TOLH at a rate twice that of 0.2 μg/ml glucose oxidase. However, when the concentration of guaiacol was raised to 10 mM, closer to that in the light spectroscopy assay, the apparent rate of peroxidase activity reflected by TOLH oxidation is approximately proportional to the glucose oxidase concentration.

Table IV

Expt 1

40 μl Rat Thyroid Microsomes	Phenol	2μg/ml Gluc. Ox.	μM TOL per Min
-	250 μM tyrosine	+	1.98
+	-	+	1.59
+	250 μM tyrosine	+	7.42
+	40 μM phenol	+	5.11
+	2 mM tyramine	-	1.78
+	2 mM tyramine	+	16.87

Expt 2

	50 μl CHO-hTPO extract, 0.4 μg/ml gluc. oxidase		10 U/ml LPO, 1 μg/ml gluc. Ox.	
Phenol	μM TOL/min	% 40 μM Phenol	μM TOL/min	% 10μM Phenol
10 μM Phenol	-	-	35	100
40 μM Phenol	0.48	100	-	-
20 μM Arbutin	0.60	125	-	-
100 μM Arbutin	0.84	175	41	117
100 μM OH-phenylpyruvate	0.48	100	51	146
2 mM Benzaldehyde	0.24	50	23	67
200 μM ß-estradiol	0.36	75	-	-
40 μM tyramine	0.48	100	41	117
100 μM acetoacetic acid	0.36	75	-	-
	50 μl CHO-hTPO extract, 0.2 μg/ml gluc. oxidase			
100 μM ß-estradiol glucuronide	0.36	75		
1 mM ß-estradiol glucuronide	0.24	50		

Expt 2 (cont)

	50 µl CHO-hTPO extract, 0.4 µg/ml gluc. oxidase	10 U/ml LPO, 1 µg/ml gluc. Ox.
	µg/ml Gluc. Ox.	µM TOL·/min
		50 µl CHO-hTPO extract
1 mM guaiacol	0.2	0.48
1 mM guaiacol	0.4	1.57
10 mM guaiacol	0.2	1.08
10 mM guaiacol	0.4	1.93
		50 µl CHO-K1 extract
1 mM guaiacol	0.2	0.24
1 mM guaiacol	0.4	0.60
		0.1 U/ml HRP
40 µM phenol	0.4	120

Conditions: 50 µl CHO-hTPO or CHO-K1 extract; 10 mM glucose; 3 mM TOLH; 140 mM $NaPO_4$ buffer, pH 7.4; 50 µl total volume. 10 U/ml Lactoperoxidase (LPO); 10 mM glucose; 1 µg/ml glucose oxidase; 350 µM TOLH; 140 mM $NaPO_4$ buffer, pH 7.4; 50 µl total volume.

Complementary Visible Spectrophotometry

If parallel assays are being done with visible spectrophotometry and it is necessary to keep a tissue homogenate in suspension,a viscous material can be added to the buffer. However, the supporting medium may affect radical production rates. In a radical producing system with thyroid peroxidase, ESR assay showed that 1 M N-methyl-D-glucosamine had no effect or decreased TOLH oxidation, whereas 1M sucrose accelerated it. (Data not shown.)

ESR Discussion

This section describes the development and application of an electron spin resonance (ESR) assay for radical production by thyroid peroxidase (TPO) with a variety of substrates. The chemical reaction requires a phenol catalyst, H_2O_2, and a peroxidase. The phenol catalyst then oxidizes the reduced free radical trap, TOLH, to form Tempol, (TOL·), which can be detected by ESR. The ESR assay is a reliable and quantitative measure of peroxidase activity or hydrogen

peroxide (H_2O_2).

HRP, LPO prototypes

The ESR assay was developed with horseradish peroxidase (HRP) and lactoperoxidase (LPO) in an effort to optimize conditions for the assay of TPO activity. The former two peroxidases are commercially available in purified form. The extensive literature on HRP provided background, techniques and standards as the starting point for the development of the assay. LPO was used as a model for TPO after the basic parameters of the assay had been developed. LPO has many of the same characteristics as TPO (Magnusson et al,a; Kohler et al). Both LPO and TPO have five oxidation states, can iodinate proteins and can have catalase-like activity. Because of the similarities between the two peroxidases, all aspects of radical generation were assayed first with LPO and then with the relatively precious TPO.

Sample preparation

After developing the ESR assay with commercially purified reagents, the assay was applied to the study of TPO. Enzyme was prepared from whole thyroid glands in its natural membrane environment but free of its tyrosine substrates. The assay was especially helpful during the initial stages of purification. The high sensitivity of the assay, i.e. the equivalent of milli-units/ml HRP activity, minimizes sample size requirements. This is important for TPO experiments as thyroid tissue is scarce and TPO is a very small fraction of the tissue. The small quantities (10-50 μl) of enzyme necessary for this assay allow many assays to be run on the same preparation. The keen sensitivity of the assay was indispensable in finding a diluent which would release TPO from the cell while preserving enzyme activity. Various separation procedures were explored and the activity of each fraction could be quickly assessed. The assay was also useful for comparing methods and activity losses vs. length of storage.

Peroxidase quantification

Peroxidase activity was assayed in thyroid tissue preparations from rat, rabbit, and sheep. Rat thyroid microsomes had high TPO activity but there was insufficient tissue available to complete the study. Rabbit thyroid tissue was more readily available but the peroxidase preparations had low activity (data not shown). Sheep thyroids were plentiful and microsomal preparations contained sufficient activity to assay peroxidase activity and the effects of various H_2O_2 concentrations and phenol catalysts.

This ESR assay is useful for determining the pseudo-peroxidase activity of biological samples. Heme proteins, such as the cytochromes and oxidized hemoglobin, have peroxidase-like activity. The assay can be used to determine the potential of these other proteins for producing radicals. The different peroxidase activities in different membrane environments and states of oxidation or denaturation of a protein can be assayed. For example, highly oxidized peroxidase loses its activity as a peroxidase, as shown in results.

Peroxidase activity is directly proportional to the peroxidase concentration under appropriate conditions. Titrations of H_2O_2 against peroxidase will define windows of concentration which allow maximal enzyme activity without inactivating the enzyme. As was noted in the experimental results of H_2O_2 quantification, small but not large increases in H_2O_2 concentration yield proportional, rapid increases in spin probe oxidation. If H_2O_2 concentrations are excessive, Tempol formation will plateau, giving unexpectedly low increases in the ESR signal. Kohler et al suggest that a slight excess of H_2O_2 (6 μM) temporarily inactivates LPO or TPO (1.7 μM) while a large excess of H_2O_2 (200 μM) oxidizes the enzymes to a form which acts as a catalase. If an excess of H_2O_2 were to oxidize lacto- or thyroid-peroxidase to a form which can be slowly reduced to the active form in the ESR assay, there would be a slow increase in the amount of

TOL· after the initial plateau. If H_2O_2 converted lacto- or thyroid-peroxidase to a compound which act as catalase, some of the H_2O_2 would bypass the catalytic cycle which forms oxidized spin probe.

To determine whether excess H_2O_2 fails to stoichiometrically oxidize spin probe by incapacitating peroxidase or by forming H_2O and O_2, we added a large excess of H_2O_2 (200 μM) to 2 U/ml HRP. When TOLH oxidation plateaued at 82% of the expected value, another 100 μM H_2O_2 was added to the assay. This yielded a maximum of 51 μM Tempol (25% of expected oxidation), indicating that the peroxidase had some activity. Oxygen polarograph experiments confirm that under conditions of excess H_2O_2, the H_2O_2 which does not oxidize spin probe, does form O_2. It appears that under conditions of excess H_2O_2 concentration, HRP becomes both partially inactivated and has catalase-like activity. It was not possible to repeat this experiment with TPO as oxygen polarograph experiments require large amounts of enzyme.

H_2O_2 generation

To provide saturating H_2O_2 concentrations for sufficient incubation periods to obtain linear TOLH oxidation kinetics without inactivating peroxidase, it is most convenient to produce H_2O_2 continuously rather than to add it as a single aliquot. H_2O_2 can be generated by the glucose, glucose oxidase; xanthine, xanthine oxidase (McCord et al); D-amino oxidase (Krebs); and the flavin based photochemical (Prolla et al) systems. The glucose, glucose oxidase system was chosen for these experiments because the substrates are convenient, readily available, and compatible with tissue culture work. Actual oxidizing equivalents from each system must be defined under the assay conditions. H_2O_2 output from the glucose, glucose oxidase system under experimental

conditions reported here is 3-4 times lower than output under standard conditions. This can probably be explained by the lower temperature (20 vs 35°C) and higher pH (7.4 vs 5.1) in the experiments reported here.

H_2O_2 quantification

The ESR assay of H_2O_2 concentration is straightforward. In conditions of excess peroxidase and an effective phenol catalyst, each molecule of H_2O_2 is stoichiometrically converted to two molecules of Tempol (Fig 2).

The assay is suitable for measuring both accumulated H_2O_2 and rates of H_2O_2 production. It has been used in the experiments reported here to quantify the H_2O_2 output of the glucose, glucose oxidase system with purified peroxidases, TPO tissue preparations, and in tissue culture experiments. Others have checked the output of the photochemical system to be able to relate H_2O_2 levels to RBC damage. The dynamic quality of ESR spectroscopy could be used to determine the time course of H_2O_2 production by whole, live cells. Clifford and Repine have described the measurement of hydroxyl radical production by activated polymorphonuclear leukocytes using 5,5-dimehtyl-1-pyrroline N-oxide (DMPO) as a spin trap. A similar experiment to quantitate H_2O_2 production by white blood cells after phorbol ester stimulation could be done by ESR. A plasma membrane non-permeable spin probe with an impermeable phenol and HRP would produce a stable radical, continuously quantifying extracellular H_2O_2 production.

Phenol

The ESR assay of H_2O_2 or peroxidase is dependent upon micromolar concentrations of phenol. The assay is exquisitely sensitive for detecting compounds that can be oxidized to

one-electron oxidants (phenolic properties) in any biological or chemical sample. For example, the enigma of free radical damage to the cells in tissue culture without added phenol was solved by the finding that nutrient media contains a phenolic catalyst. Another unexpected finding was that iodide can act as a phenol in the ESR assay.

The phenol which is the most effective catalyst will vary with its environment. It can be confirmed that the assay is quantitative by the production of two Tempol per H_2O_2 in conditions of excess peroxidase. It is important to try a variety of phenols to show which phenol is the most suitable catalyst. The best phenol catalysts described in the peroxidase assays in this paper are phenol with HRP and guaiacol with detergent solubilized TPO. Other phenolic substances are the most effective catalyst with other peroxidases. In a system which produces tocopheroxyl radicals from tocopherol in membranes, arbutin proved to be much more efficient than phenol (Mehlhorn et al). This is presumably due to the hydrophilicity of arbutin. By remaining in the aqueous phase of the assay system, arbutin is accessible to the tocopherol on the surface of the membrane but, unlike phenol, does not penetrate the lipid bilayer, and avoids the complication of lipid peroxidation. A variety of phenols could be assayed with membrane-bound peroxidases and the results used to predict which phenols are likely to produce free radicals at physiological concentrations.

Light spectroscopy experiments in our lab with HRP, H_2O_2, and phenol showed that millimolar concentrations of phenols form phenol polymers via free radicals (data not shown). Although this was not repeated with TPO, it is assumed that phenol polymerization rather than radical formation led to the lower rate of radical formation with 1.0 than 0.1 mM ß-estradiol glucuronide, (0.24 vs 0.36 TOL·/min, respectively, Table IV, Expt 2). Phenol polymers are known to bind DNA and interfere with its function (O'Brien, a). Therefore, high levels of phenols in the presence of TPO and H_2O_2 could damage

thyroid DNA.

Iodide

Several groups (Magnusson et al ,b, Kohler et al and Nakamura et al, a) have shown the formation of either $I_2 + OH^-$ or $H_2O + O_2$ from lacto- or thyroid- peroxidase-H_2O_2-iodide complexes. Therefore, it would be predicted that a lacto- or thyroid-peroxidase-H_2O_2 complex would not form radicals with iodide in the absence of substrate for iodination. However, unbound iodide at concentrations of 10^{-7}M or more with LPO readily oxidizes TOLH rather than I^- or H_2O_2 (data not shown). The stoichiometric conversion of H_2O_2 to oxidized spin probe (TOL·) by LPO demonstrates radicals are formed efficiently in the presence of iodine even with an appropriate substrate for iodination (Fig 3). Extensive literature on the one- versus two-electron oxidations by TPO (Nakamura et al, b; Nakamura et al, c; Magnusson et al, b) indicate that TPO would act the same as LPO in the ESR assay. The relative ease of TOLH oxidation over tyrosine iodination suggests that free radical traps might be an effective therapy for thyrotoxicosis.

The quantity of H_2O_2 which oxidized Tempol with tyrosine and LPO was slightly less in the presence of iodide (Fig 3). This is presumably due to partial diversion of the enzyme-H_2O_2 complex to the two-electron oxidation of iodide, resulting in tyrosine iodination.

Conclusion

Thyroid peroxidase (TPO) from four tissue sources, i.e. cultured cells, and rat, rabbit, and sheep thyroids, formed free radicals with hydrogen peroxide and low concentrations of many endogenous and exogenous phenols. This finding gives credence to the hypothesis that TPO can form radicals other than its normal one, the diiodotyrosine radical for thyroid hormone synthesis. It is further hypothesized that the abnormal radicals would be produced, particularly from exogenous phenols, and cause tissue damage, rather than being quenched as

occurs during thyroid hormone production.

TPO quantitatively catalyzed free radical production in proportion to H_2O_2 (Fig 5) and peroxidase concentrations (Fig 8). Radical production in the thyroid gland is also likely to be commensurate with the level of available H_2O_2 and peroxidase. TSH increases both H_2O_2 production and TPO activity (Perrild et al). It is elevated in autoimmune thyroiditis and would be expected to increase radical creation in these diseases. Elevated H_2O_2 generation also would occur if the thyroid gland were infiltrated by white blood cells (WBCs), as happens during autoimmune thyroid disease. Very high levels of H_2O_2 would inactivate TPO, but it is unknown whether or not this happens *in vivo*.

A wide variety of substrates form free radicals with TPO. They range from naturally occurring phenols (tyrosine, estradiol) to ingested phenols (tyramine in ripe cheese) to compounds which accumulate during disease states (OH-phenylpyruvate in phenylketonuria, acetoacetic acid in uncontrolled diabetes) to pollutants (phenol, benzaldehyde) (Table IV, Expt 2). Even tissue culture media and Hank's Balanced Salt Solution, manufactured to simulate the environment of the cells in the body, have phenolic activity with HRP (Table III). (Rate constants for HRP are comparable with those of TPO except for thyroid hormone specific phenols such as tyrosine and iodinated tyrosines (Nakamura et al, b). It is probable that any phenol present in the blood would also be in the thyroid follicle, either as tissue filtrate or by being membrane permeable. It could be predicted that exposure to increased levels of peroxidase substrates would increase the chances of free radical formation and thyroid disease. In fact, severe diabetes (acetoacetate sometimes present) and thyroid disease often occur in the same patient.

In summary, ESR can be used to quantitatively assay free radical production by H_2O_2 and peroxidase. The assay can be used with either purified peroxidases or those in their natural membrane matrix.

The assay was applied to the study of thyroid peroxidase in this report.

Determination of Appropriate Horseradish Peroxidase Concentrations for the Quantification of H_2O_2

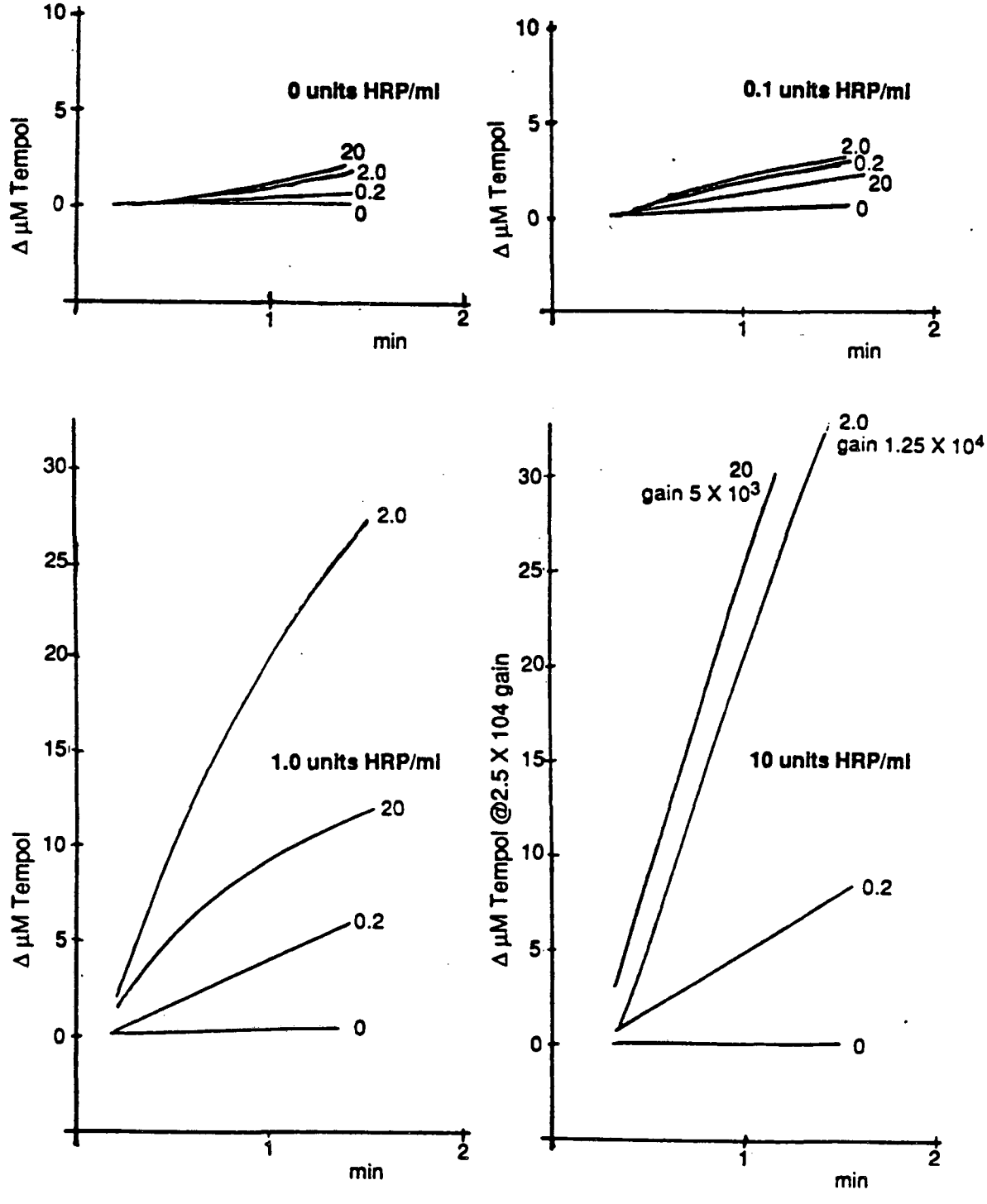


Fig 1. µg/ml glucose oxidase indicated by numbers on tracings. 40 µM phenol, 10 mM glucose, 3.5 mM TOLH 2.5 X 10^4 gain except where noted.

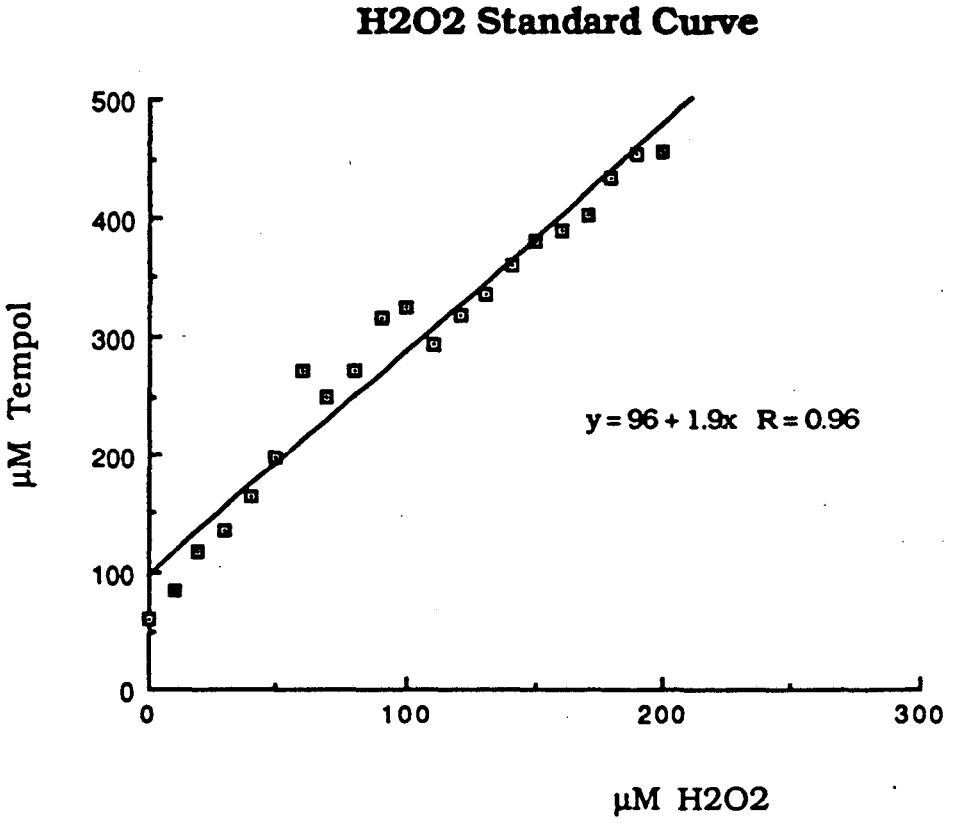


Fig 2. 2 U/ml horseradish peroxidase, 20 µM phenol, 10 mM glucose, 3.5 mM TOLH.

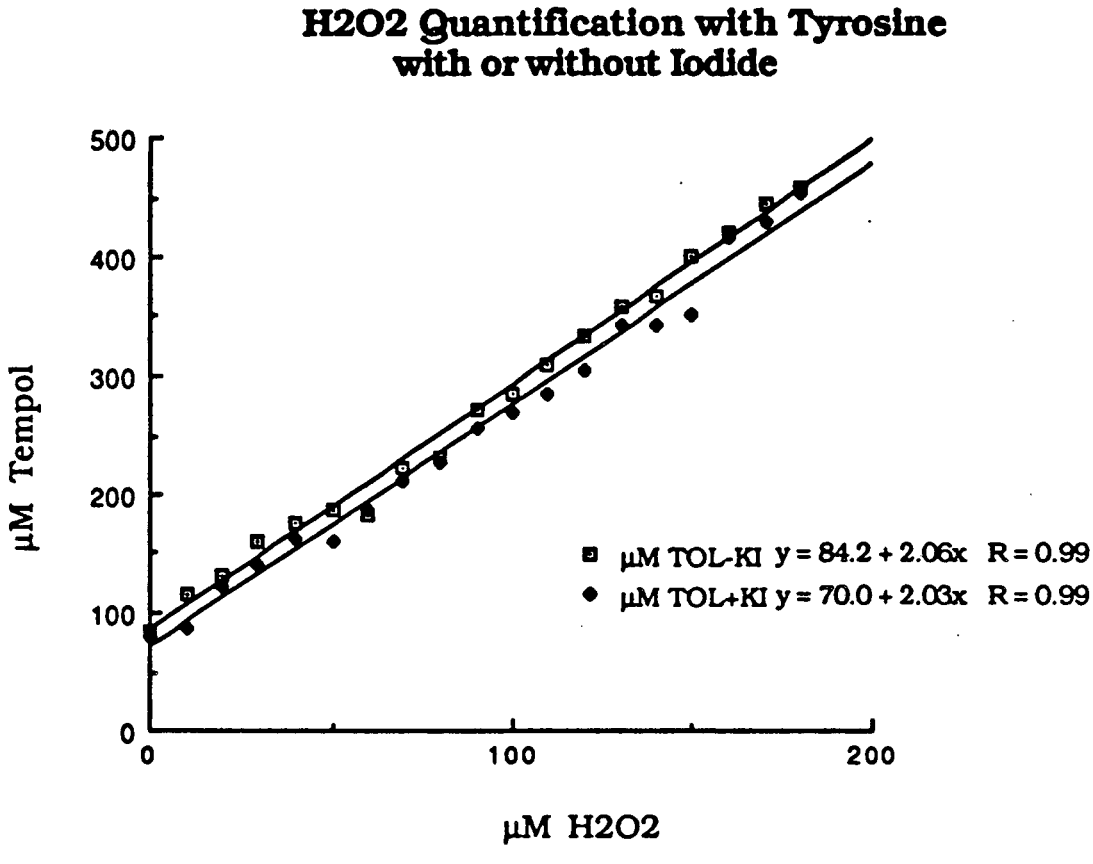


Fig 3. 2 U/ml lactoperoxidase, 100 µM tyrosine, 3.5 mM TOLH, +/- 100 µM KI.

Oxygen Consumption & Tempol Formation During H_2O_2 Generation

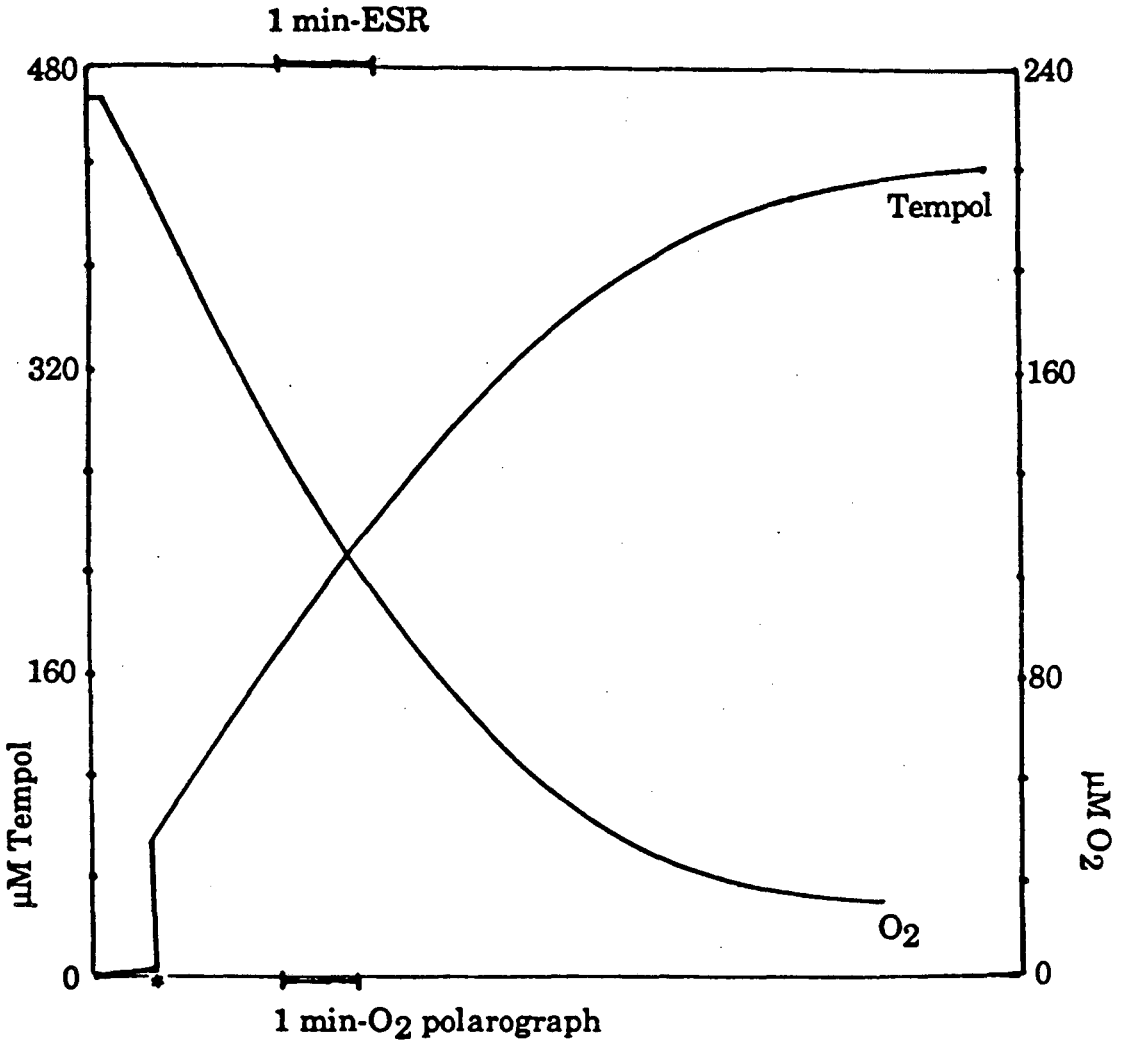


Fig 4. 25 U HRP/ml, 2 U/ml glucose oxidase, 10 mM glucose, 40 μM phenol, 3.5 mM TOLH. *on lower margin denotes glucose oxidase addition.

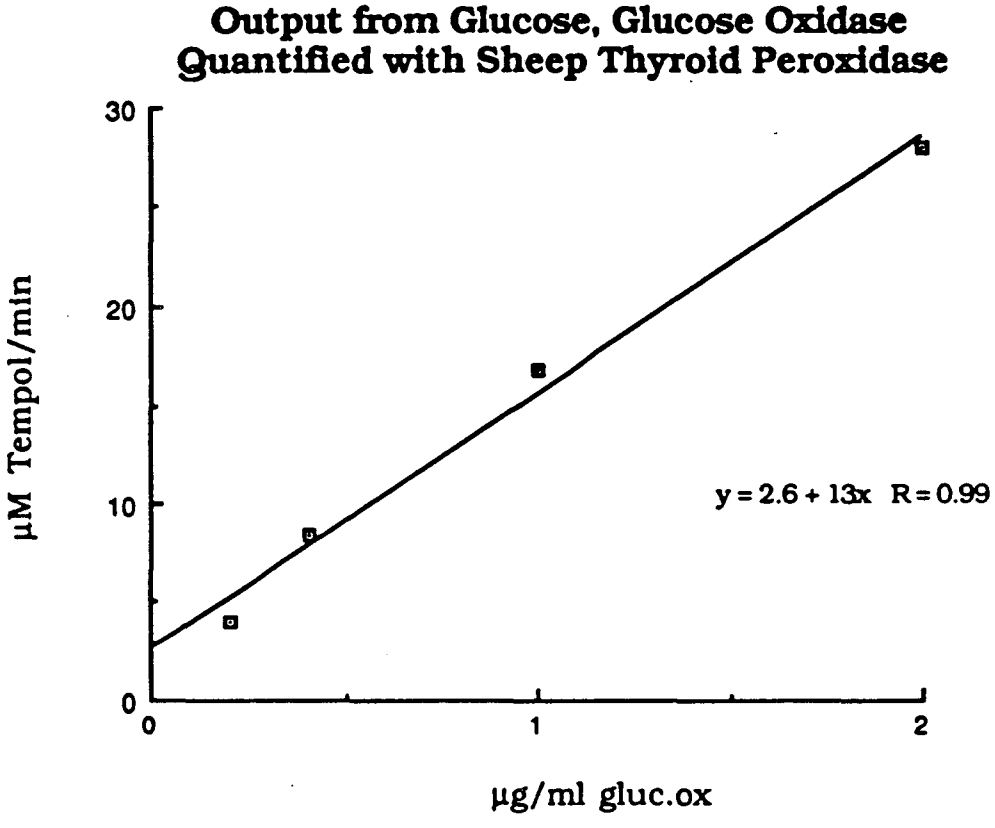


Fig 5. 25 µl sheep TPO, 40 µM phenol, 10 mM glucose, 3.5 mM TOLH.

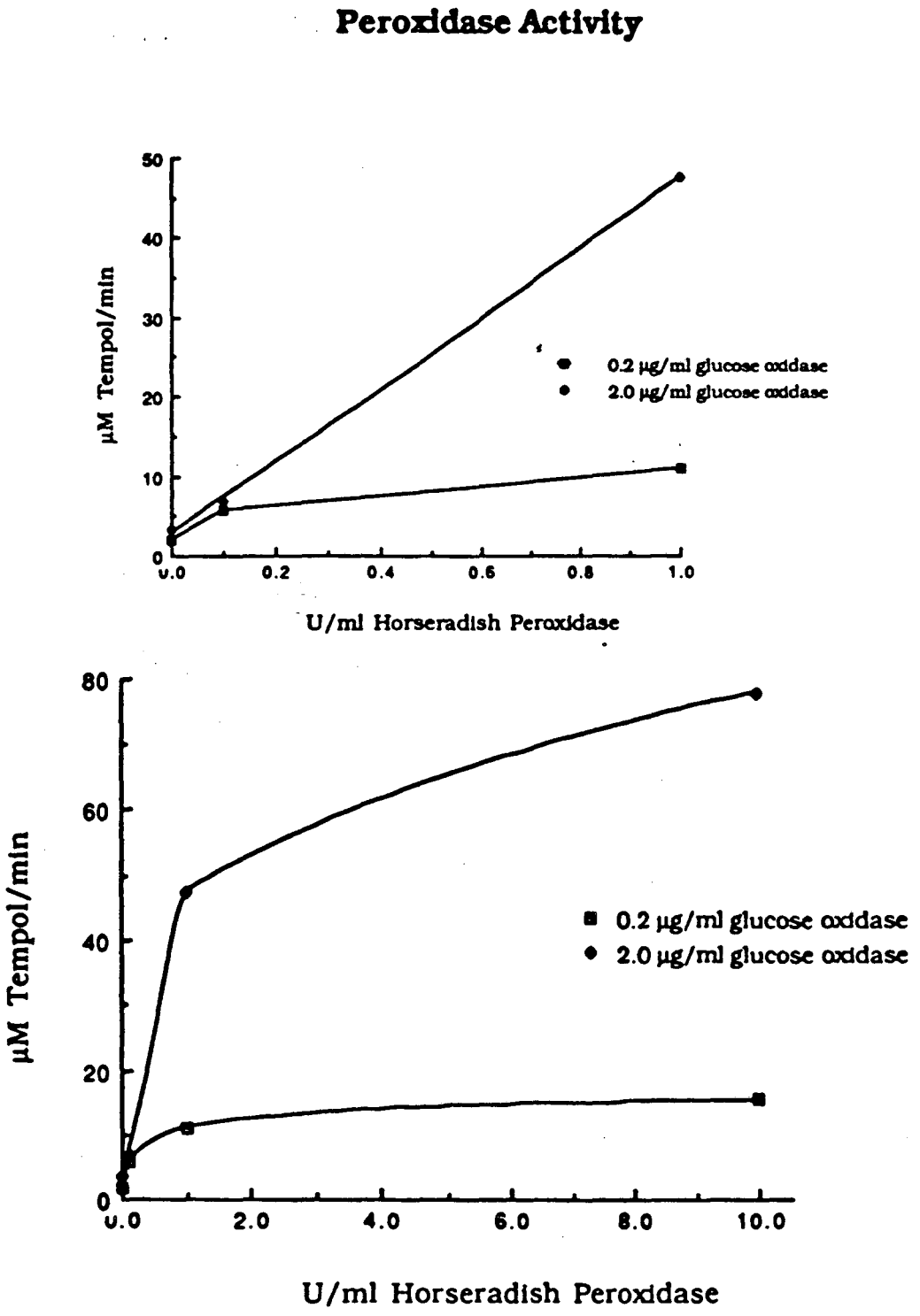


Fig 6. 40 µM phenol, 10 mM glucose, 3.5 mM TOLH.

Determination of Appropriate H_2O_2 Generation Rate for Assay of Peroxidase Activity in Sheep Thyroid Microsomes

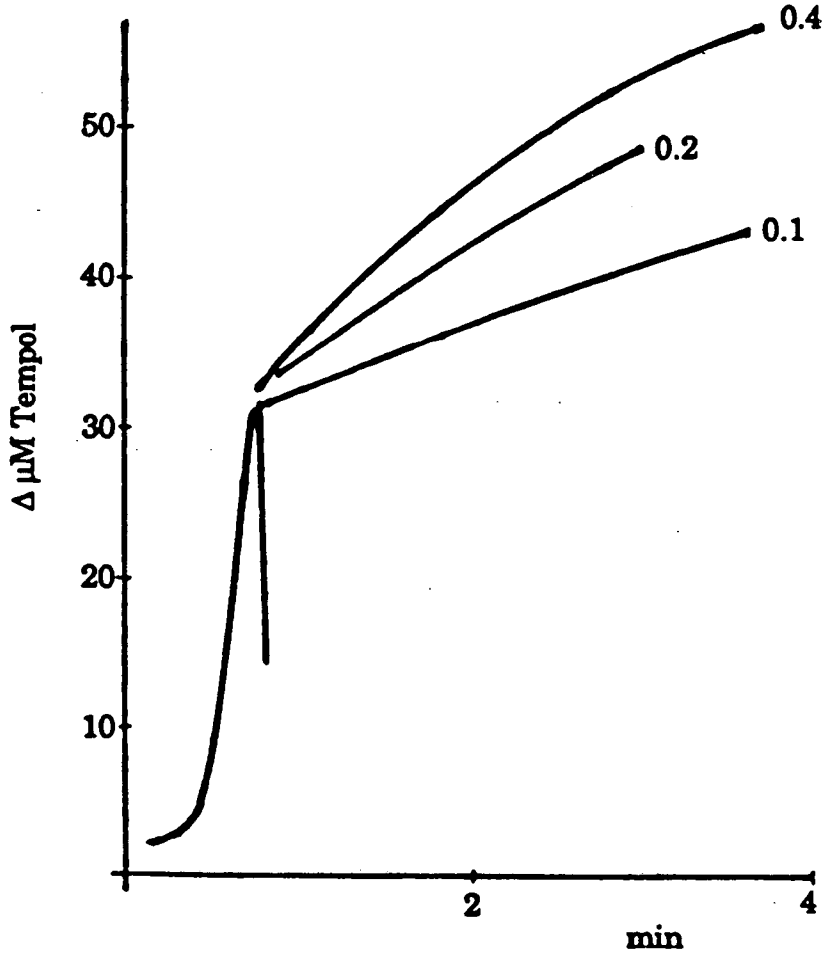


Fig 7. Numbers on fig denote μg/ml glucose oxidase. 25 μl sheep TPO, 40 μM phenol,10 mM glucose, 3.5 mM TOLH. 2.5 X 10^4 gain

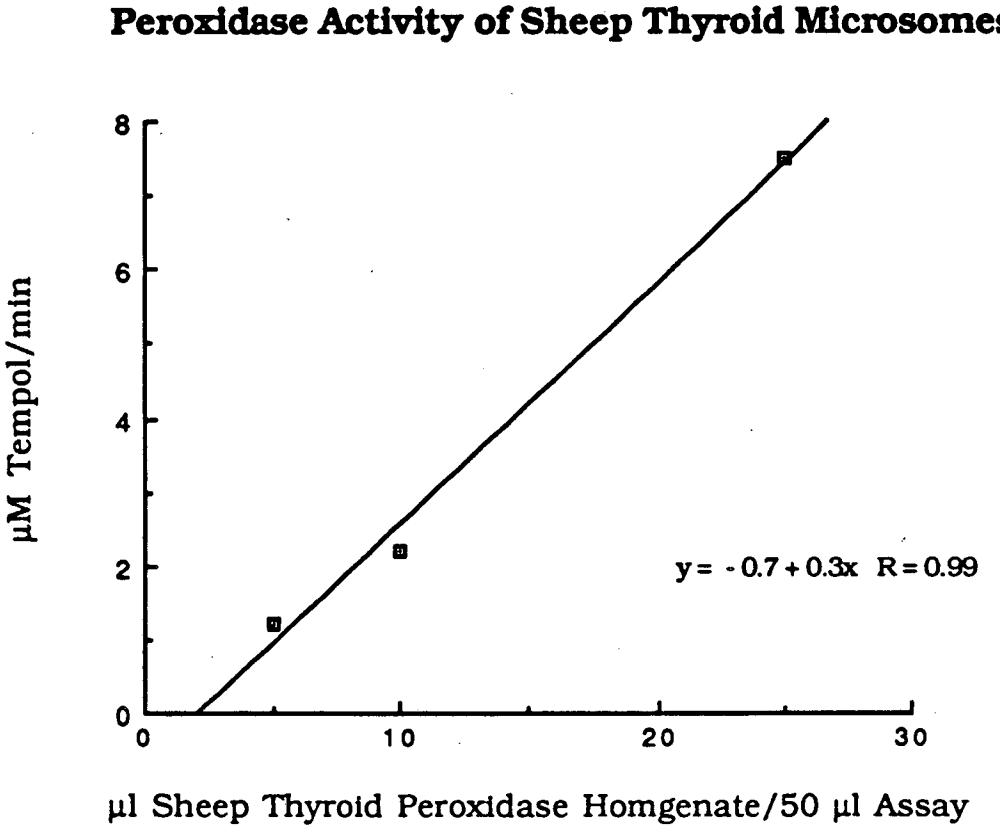


Fig 8. Thyroid peroxidase as microsomes with buffer added to total 50 µl, 0.2 µg/ml glucose oxidase, 40µM phenol, 10 mM glucose, 3.5 mM TOLH.

Tissue Culture

Introduction

A tissue culture system was chosen to test the hypothesis that the free radicals generated by thyroid peroxidase (TPO) are harmful to cells. The model incorporates conditions found both in the thyroid gland and ESR assay. Cells which synthesize TPO provided enzyme. Hydrogen peroxide (H_2O_2) was generated continuously at low levels, mimicking conditions in the thyroid gland. Phenol catalyst was present in culture media, as it would be in the plasma. It was expected that H_2O_2 would combine with TPO, oxidize phenol to phenoxyl radicals, and that the phenoxyl radicals would harm the cells. It was assumed that radical production in tissue culture would be proportional to the same substrates as in the ESR assay. Therefore, tissue culture experiments were done to correlate cell number with H_2O_2 and peroxidase concentrations and the presence of phenols. If cell viability were sensitive to radical production, then cell number would be negatively related to these substances. Other work has shown that radicals damage DNA, oxidize thiol groups within enzymes, bind to proteins and cause lipid peroxidation. It was expected that these effects would tax the metabolic activities of the cells and result in slower replication or possibly death. Spin trap was added to intercept free radicals as further evidence of radical involvement in cell viability.

Cells transfected with and expressing the gene for human thyroid peroxidase (hTPO) (Kaufman et al, Foti et al) were chosen as the simplest model of enzyme effects. Influences of thyroid cells, besides those caused by thyroid peroxidase, were eliminated by choosing a nonthyroidal cell line (Chinese hamster ovary (CHO)) to synthesize hTPO. CHO cells have a high transfection efficiency and these clones express either wild type, cell surface (CHO-hTPO) or truncated, secreted hTPO (CHO-M1K1). The enzyme from both cell types is active (Kaufman et al, Foti et al). Non-transfected cells (CHO-K1) or

those which had been transfected with the pSV_2-neo plasmid were used as controls (CHO-pSV_2-neo). The only well characterized thyroid cell line available, FRTL-5 rat thyroid cells, lacks TPO enzymatic activity (Derwahl et al).

Cell number, as determined by DNA content, was chosen as the measure of cell damage. Variation in replication rate or DNA content/cell among the various clones of transfected cells was normalized by expressing the results as a percentage of the same cell type without treatment. Early experiments were done by DABA (DNA) assay and trypan blue exclusion. Trypan blue permeability and diminished DNA were seen under the same conditions but could be observed earlier and measured more precisely by DNA assay. ^3H-thymidine uptake was also assayed as a sensitive measure of DNA activity. Changes in the rate of ^3H thymidine incorporation into DNA were expected to show parallel changes in cell number.

Methods and Materials

TIssue culture

CHO-K1, CHO-hTPO (Kaufman et al), CHO-M1K1 and CHO-pSV_2-neo (Foti et al) cells were the generous gift of Basil Rapoport of the Veterans' Administration Medical Center in San Francisco. They were maintained in Ham's F-12 medium supplemented with 10% fetal bovine serum and 5 μg gentamycin sulfate/ml media in air supplemented with 5% CO_2 at 37° C. Assays were done in 24 multi-well tissue culture plates in quadruplicate. Horseradish peroxidase, glucose, glucose oxidase, and phenols were obtained from Sigma, St. Louis, Missouri. The spin trap, TOLH, was obtained from Aldrich Co. and prepared according to Prolla et al.

Hydrogen Peroxide Generating System

A hydrogen peroxide (H_2O_2) generating system was added to the tissue cultures to provide long-term, low-level exposure to H_2O_2,

the enzyme substrate which enables thyroid peroxidase to perform oxidations. The glucose, glucose oxidase, oxygen system was chosen because all reagents are well tolerated by cultured cells. An additional 10 mM glucose was added to that already present in the culture medium to ensure adequate nutrition for the cells and substrate for glucose oxidase.

DABA Assay

The DABA (diaminobenzoic acid) assay (Hinegardner) is a fluorescent measure of cellular DNA. Cells were fixed to the bottom of the multi-well plate in which assays had been performed. After fixation, the purine nucleotides were hydrolyzed by DABA to a fluorescent compound proportional the DNA content. The assay is read in a Turner fluorimeter with samples of known cell number used as standards. Data is expressed here either as cell number, DABA absorbance, or percent of control (% DABA absorbance). % DABA absorbance was used to normalize results for differences in doubling time or DNA content.

3H thymidine

Thymidine incorporation is a measure of DNA synthesis and can be due to DNA repair or preparation for cell duplication. Pulses of 5.0 μCi ^{3}H-thymidine were added to 0.5 ml media/well in 24 multi-well plates to measure thymidine uptake into DNA. After incubation with ^{3}H-thymidine, the media was aspirated from the wells and the cells were fixed to the bottom of the plate (0.5 ml 100% methanol, 10 min). The cells were washed three times (2 ml cold 10% TCA) to remove any ^{3}H thymidine not incorporated into cellular DNA. After removing all TCA, an alkali solution (300 μl 0.3 N NaOH) was added to each well to lyse the cells and release the DNA. 100 μl aliquots were counted in 4 ml scintillation fluid on a scintillation counter. Counts were compared with cell number determined by DABA assay.

Conditioned media

Conditioned media for tissue culture peroxidase assays was collected of 100-mm plates with activity growing, non-confluent cultures with nearly the same density. It was spun at 3000 rpm for 10 min in a table top centrifuge to remove cells and cellular debris. If the conditioned media was concentrated, it was collected above a Centricon 30 filter (retains 30 KD and larger), spun at 6000 rpm in a Sorvall SA 600 rotor for 30 min. Both the wild type and the truncated hTPO are slightly larger than 100 KD. Centrifugation reduced the volume to about 14%, with slightly more CHO-M1K1 media being retained than CHO-pSV$_2$-neo. The concentrated conditioned media was then sterilized by Centrex filtration.

Results

Hydrogen peroxide generating system

Glucose and glucose oxidase were added to tissue cultures to generate low concentrations of H_2O_2 over extended periods. When media from test wells was assayed by ESR to determine the actual levels of H_2O_2 being generated during experiments, it was found that the glucose oxidase lost its activity with time (data not shown). Samples from tissue culture media which had been incubated with the H_2O_2 generating system for 24 hours had more glucose oxidase activity than those which had been incubated for 48 hours. This is probably due to dilution of the enzyme, as it loses its activity if not stored in concentrated solutions. Glucose oxidase activity was proportional to that added, indicating that H_2O_2 did not cause the inactivation. Glucose oxidase inactivation by radicals is unlikely as the addition of peroxidase to the culture media had no effect on its activity.

To determine whether or not adding glucose oxidase at shorter intervals would compensate for decreased H_2O_2 generation capacity, an experiment was done in which the media was replaced with media

with freshly diluted glucose oxidase every 12 or 24 hours for a total of 48 hours. The longer exposure to the same media caused more damage than shorter exposure (Fig 17). This suggests that H_2O_2 concentration builds over a 24 hour period. Thereafter, media was renewed at longer intervals to maximize H_2O_2 damage and peroxidase secretion by CHO-M1K1 cells.

Peroxidase Concentration Dependence

Various concentrations of horseradish peroxidase (HRP) were added to cultured CHO-pSV$_2$-neo cells with a hydrogen peroxide (H_2O_2) generating system to determine whether or not cell damage would correlate with peroxidase concentration (Fig 18). Cell number proved to be inversely proportional to peroxidase concentrations between 0-.02 U/ml HRP. CHO-M1K1 cells were cultured concurrently and their response to H_2O_2 was found to be equivalent to CHO-pSV$_2$-neo with 0.0048 U/ml HRP. The CHO-hTPO cells included in the experiment showed no effect from the H_2O_2 generated by 0.005 μg/ml glucose oxidase. 0.005 μg/ml glucose oxidase treatment for four days was chosen because previous experiments had shown that this regime led to discernable effects between peroxidase containing and control cultures.

Two approaches were used to confirm that the CHO-M1K1 cells secrete a factor (Foti et al) which amplifies the damage from H_2O_2 treatment. The first type of experiment was the co-culture of hTPO secreting cells with non-transfected cells (Fig 11). When cells expressing peroxidase (CHO-M1K1) were cultured with non-transfected (CHO-K1) cells in a ratio of 1:4, the proportion of surviving cells at the higher rates of H_2O_2 generation was the same as that of the transfected cells. The replication rate of non-transfected cells was affected much less than that of CHO-M1K1 cells by 0.015-0.025 μg/ml glucose oxidase. The number of cells in the

mixture would have been closer to that of the control cells if it had been determined by H_2O_2 alone. The experiment was repeated with 0, 20, 50 or 100% CHO-M1K1 cells with CHO-K1. Decrease in cell number proved to be proportional to the relative number of CHO-M1K1 with 0.01 or 0.02 μg/ml glucose oxidase (Data not shown).

The second type of experiment to show that CHO-M1K1 cells were secreting a factor which affects cells was to add conditioned media from them to cultures of control cells. Experiments done with unconcentrated conditioned media show a general toxic effect which is proportional to the amount of conditioned media. However, CHO-M1K1 media was more deleterious than CHO-pSV$_2$-neo media when it comprised 20-40% of the test media (Fig 12). A second experiment with less potent conditioned media was done to evaluate the effect of H_2O_2 on the secreted factor. CHO-M1K1 conditioned media potentiated the H_2O_2 effect (Fig 13).

0.1 or 0.2 ml of concentrated conditioned media from both cells types was stimulatory to tissue cultures with H_2O_2. 0.2 ml CHO-M1K1 concentrated media with glucose oxidase was able to partially overcome the stimulatory effect and decrease cell number by about 10% below that in wells with 0.1 ml CHO-M1K1 or 0.1 or 0.2 ml CHO-pSV$_2$-neo concentrated media (data not shown).

ESR experiments done to quantify hTPO activity in conditioned media revealed much more peroxidase activity than would be expected from the effect on the cells. Media (1ml media/well for 2 days) from CHO-M1K1 cells which had been plated three days previously at $1.6x10^4$ cells/well, had an ESR assayable peroxidase activity equal to approximately 0.15 U/ml HRP. Yet the cultures of CHO-M1K1 were affected as if they had 0.003 U/ml HRP. Media (1 ml media/well for 2 days) from CHO-M1K1 cells which had been plated five days previously at $1.2x10^4$ cells/well had an ESR assayable peroxidase activity equal to approximately 1 U/ml HRP. The CHO-M1K1 cultures showed no peroxidase potentiated effect from 0.005 μg/ml glucose oxidase while

control cultures with 0.003 U/ml HRP were decreased to 1/3. Media from CHO-pSV$_2$-neo cultures under the same conditions had negligible peroxidase activity. The difference in tissue culture effects and peroxidase concentrations as determined by ESR is probably due to the slow build-up of peroxidase enzyme in the media. Peroxidase activity in CHO-M1K1 media increased greatly by allowing the cells to remain in culture two more days. The HRP activity in tissue culture media is stable for the duration of an experiment according to ESR assay. (Data not shown.) It is assumed that hTPO activity secreted by the CHO-M1K1 cells is also stable.

Hydrogen peroxide concentration dependence

A time course was run to assess when H_2O_2 concentration dependent damage developes. Control (CHO-pSV$_2$-neo) and hTPO secreting (CHO-M1K1) cells were exposed to 0-0.020 μg/ml glucose oxidase for 12, 24, 36, or 48 hours (Fig 14). Neither group was affected after 12 hours treatment by the H_2O_2, except at the highest dose. Thereafter, both groups had a characteristic pattern of injury with its extent increasing at each time point. Loss plateaued in cells without TPO. This tendency became more pronounced as the length of treatment increased. CHO-M1K1 became more sensitive to glucose oxidase with time. The peroxidase secreting cells reacted in a dose-dependent manner after 48 hours. This experiment suggested that it would be necessary to treat cells with low levels of glucose oxidase for extended periods of time in order to distinguish between H_2O_2 with peroxidase and H_2O_2 only damage.

An experiment was done to optimize the difference between peroxidase and H_2O_2 damage (Fig 15). Four groups of cells were exposed to minimal (0-0.01 μg/ml) amounts of glucose oxidase for two, three or four days. The peroxidase containing cultures (CHO-pSV$_2$-neo plus 0.01 U/ml HRP and CHO-M1K1) showed the deleterious effect of 2 days of treatment, with the cells with added

peroxidase showing the greatest influence. The HRP treated cultures with 0.01 μg/ml glucose oxidase had only 48% of the cells not exposed to H_2O_2. The effect of H_2O_2 had developed dose-dependence in HRP treated cells (r=-.94) by the fourth day of treatment but was less severe than with shorter exposure (56% of 0 μg/ml glucose oxidase wells vs 42% and 48% on previous days). H_2O_2 treatment was deleterious to CHO-M1K1 after two or three days but showed the most serious effect after four days. By the fourth day of treatment, the decrease in cell number was dose dependent (r=-.94) with the highest glucose oxidase dose leading to 83% of the number of cells seen without H_2O_2. Control cells and cells with membrane-bound hTPO sustained minimal damage over the entire four days of treatment. The most significant effect to them was at an intermediate dose of glucose oxidase which led to about a 20% decrease in cell population in the CHO-pSV_2-neo cultures on the second day and in the CHO-hTPO culture on the third day of treatment. Thereafter, these cultures were unaffected by all administered H_2O_2.

Phenol effects

A variety of phenols was added to the four types of cell cultures to evaluate their potential for transferring free radicals to vulnerable cell structures. Fig 16,A demonstrates that the consequence of added phenols with a H_2O_2 generating system is greater than their additive effects when HRP is present in the media. Phenol and OH-phenylpyruvate were the most effective phenols while arbutin had the least effect in the HRP group. The addition of glucose oxidase to control cells with added 0.003 U/ml HRP and to CHO-M1K1 decreased both populations by 10% (Fig 16, B), indicating aproximately equal peroxidase activity. However, adding phenols to CHO-M1K1 did not decrease cell numbers below that of H_2O_2 treatment alone. In fact, the addition of phenols seemed to protect the cells slightly from H_2O_2. CHO-pSV_2-neo cells without peroxidase

showed very little effect of the addition of most of the phenols (Fig 16, D). OH-phenylpyruvate with H_2O_2 caused more distress to the control cells than to those with membrane bound hTPO. Tyramine plus H_2O_2 was slightly more detrimental to control cells than to those which secrete hTPO. Otherwise, none of the treatments, including H_2O_2, had any effects on CHO-hTPO (Fig 16, C).

Free Radical Trap Effect

TOLH, the reagent used to trap free radicals for ESR measurement, was added to tissue culture experiments to determine whether or not it would intercept radicals and prevent cell damage. Cell number in groups with added HRP and TOLH was dramatically increased over H_2O_2 or H_2O_2 plus phenol without oxidant probe (Fig 17). The group of cells with oxidant-sensitive probe, glucose oxidase, and HRP was twice as large as the one with glucose oxidase and HRP. TOLH added to cells with HRP, phenol and glucose oxidase prevented the near annilhation seen without it. The fact that a free radical trap could prevent the cytostatic effect of peroxidase and H_2O_2 in the HRP-treated group indicates that free radicals contributed to the damage.

TOLH was somewhat cytotoxic. Its poisonous effects were inversely proportional to the peroxidase content of the tissue culture. CHO-M1K1, CHO-hTPO, and CHO-pSV$_2$-neo were not affected by H_2O_2 or H_2O_2 with phenol. However, added TOLH decreased cell numbers to 85%, 70% or 60%, respectively.

Assay by DABA Absorbance vs 3H Thymidine Incorporation

Experiments were assayed by DABA absorbance and ^3H thymidine incorporation to evaluate the relative sensitivities of the tests. Total DNA, assumed to reflect cell number, was assayed by DABA absorbance. DNA synthesis and repair was monitored by ^3H thymidine uptake. DABA assay showed that CHO-hTPO cells replicated less well with

H_2O_2 than did CHO-K1 cells (Fig 18, A). ^3H thymidine uptake was higher at moderate doses of glucose oxidase in the CHO-hTPO cells than in control cells (Fig 18, B). At the highest rate of H_2O_2 generation, both cell types had the same amount of ^3H thymidine uptake/cell, probably reflecting impaired DNA processing in CHO-hTPO due to a toxic effect of H_2O_2 combined with cellular TPO. Dividing the counts/min (cpm) of ^3H thymidine incorporation into DNA by the DABA absorbance accentuates the disparity between CHO-hTPO and CHO-K1 (Fig 18, C). ^3H thymidine incorporation did not parallel cell number and was discontinued. As increased ^3H uptake did not result in increased cell number in CHO-hTPO, it is assumed that it was a measure of DNA repair.

Tissue Culture Discussion

The results of the tissue culture experiments described here present evidence that cells are damaged in proportion to the concentration of peroxidase and hydrogen peroxide in the presence of phenol, the three substrates required for phenoxyl radical generation. Cell injury, inferred by the lack of cell replication, correlates with free radical production in ESR assay. Partial prevention of the decrease in cell number by a free radical trap provides evidence that radicals are involved in cell damage.

Tissue culture experiments were done with eukaryotic cells transfected with and expressing the human thyroid peroxidase (hTPO) gene. The first available clone of these CHO (Chinese hamster Ovary) cells contained full-length hTPO expressed on the cell surface (CHO-hTPO) (Kaufman et al). Although CHO-hTPO cells responded to peroxidase substrates in tissue culture and ESR assays, their peroxidase activity was low. Furthermore, CHO-hTPO cultures drift rapidly to a population with few copies of the hTPO gene (personal communication). Experimental results were more dramatic with CHO-M1K1, the newly developed clone. CHO-M1K1 cells are

transfected with, express, and secret a truncated form of human thyroid peroxidase (Foti et al). Peroxidase expression by CHO-M1K1 is stable and at much higher levels than by CHO-hTPO. Both the wild type and truncated enzymes are active. CHO cells transfected with a plasmid containing a neomycin resistance gene only (CHO-pSV$_2$-neo) and CHO-pSV$_2$-neo cells with added horseradish peroxidase (HRP) were used as controls and prototypes, respectively.

Hydrogen Peroxide

Cells in tissue culture are killed by high, inhibited by moderate but stimulated by low levels of H_2O_2 (See Burdon et al review). The levels of H_2O_2 in the experiment illustrated in Fig 14 show that H_2O_2 was inhibitory within 12 hours and damage became more serious over the next 36 hours. Slower rates of H_2O_2 generation were also inhibitory at 48 hours (Fig 15). However, cells without peroxidase recovered from H_2O_2 treatment. In order to regain cell number, the cells increased their rate of replication. Doubling time was shorter with 0.01 μg/ml glucose oxidase treatment than in non-treated cells by 0.05- 0.2 days in CHO-hTPO, CHO-pSV$_2$-neo and CHO-pSV$_2$-neo + HRP after recovery from initial injury. This indicates that H_2O_2 was stimulatory after cells adapted to it.

Adaptation includes an increase in cellular oxidative defenses to lower effective oxidant concentrations and the stimulation of proliferation. OxyR, the product of a H_2O_2-inducible gene in bacteria, activates the transciption of catalase and an alkyl hydroperoxide reductase (Storz et al). Greater catalase, CuZn superoxide dismutase, and Se-dependent glutathione peroxidase activity have been found in a H_2O_2-resistant CHO cell line (Spitz et al). Similar increases in oxidative defenses have been found in mouse epidermal cells (Crawford et al). A promotable clone had higher levels of catalase and superoxide dismutase than a non-promotable clone. Growth rates of

both clones were the same without oxidative stress but were stimulated in the promotable clone by oxidative stress. Exposure to active oxygen generated extracellularly by xanthine oxidase (H_2O_2 and superoxide ($O_2\cdot^-$) retarded the growth of the non-promotable clone about 2-fold after 4 days. The same treatment decreased growth slightly in the promotable clone after 2 days but then stimulated proliferation, resulting in about 1.5x the number of cells without H_2O_2, $O_2\cdot^-$ treatment after 4 days (Muehlematter et al). The increase in cell number correlated with increased DNA synthesis as assayed by ^3H thymidine incorporation. More recent papers by the same group show that oxidative stress induces the proto-oncotgenes c-*fos* and c-*myc* and the phosphorylation of ribosomal protein S6 in the promotable clones (Crawford et al, Larsson et al). Crawford et al conclude that superior antioxidant defences in the form of higher levels of catalase and superoxide dismutase protect the promotable clone from excessive cytostatic effects of active oxygen and allow c-*fos* and c-*myc* to exert their functions. The induction of these early genes would be helpful in the recruitment of quiescent cells in wound healing and tissue regeneration, conditions with oxidative stress from invading leukocytes.

Cells with peroxidase react differently to H_2O_2 exposure than cells without peroxidase. H_2O_2-dependent damage develops more slowly in cells with peroxidase and becomes dose responsive with time. Whereas injury developed and plateaued within 24 hours without peroxidase (CHO-pSV$_2$-neo), it took 48 hours of H_2O_2 treatment to approach equivalent damage to CHO-M1K1 cells (Fig 14). Peroxidase seems to protect cells from H_2O_2 damage initially, perhaps by depleting H_2O_2. If peroxidase removed H_2O_2 to use it as a substrate, it would prevent initial H_2O_2 damage but cause insidious radical damage. Early, temporary effects of H_2O_2 and the gradual development of radical damage can also be seen in Fig 15. Symptons

appear more slowly than in the previously described experiment because glucose oxidase, and therefore H_2O_2, levels are lower. Cell number decreased to a plateau in CHO-pSV$_2$-neo, CHO-pSV$_2$-neo + HRP, and CHO-M1K1 cells after 48 hours, as it had in 24 hours of more intense H_2O_2 only treatment. The severe damage after two days in the cultures with added HRP is probably from the added effects of H_2O_2 and radicals. After treatment for four days, the CHO-M1K1 and HRP added cultures had developed dose-dependent H_2O_2 damage, typical of peroxidase mediated effects. It appears that the cells with added HRP recovered from H_2O_2 effects and developed more subtle lesions in four days. CHO-M1K1 took longer to display peroxidase-type injury than the HRP added group. The lag in damage to CHO-M1K1 is probably due to the sparcity of cells and therefore, very little secreted peroxidase in newly plated cultures.

Peroxidase

Peroxidase inhibits cell replication in proportion to its concentration in the presence of H_2O_2. This is most easily seen in cultures of control cells with added HRP (Fig 18). Parallel experiments with purified TPO could not be done because purified TPO is not available. However, two types of experiments were done with CHO-M1K1 to show that inhibition with a fixed amount of H_2O_2 is proportional to TPO. Co-culture experiments show that the potentiation of H_2O_2 damage to control cells is directly proportional to the percentage of peroxidase secreting cells (data not shown). Conditioned media experiments show that media from hTPO secreting cultures inhibit non-transfected cells more than media from non-hTPO transfected cultures (Fig 12).

Peroxidase potentiates H_2O_2 damage to cells, probably though free radical attack. ESR assay shows that peroxidase, H_2O_2, and phenol generate free radicals. Cells exposed to oxidative stress from

these compounds would be subjected to free radicals as well as from H_2O_2. The two effects are difficult to distinguish. Most reports in the literature generalize oxidant effects. Many studies use chemical rather than free radical systems to study oxidative regulation. Abate et al used sulfhydryl-modifying reagents to change the redox state and DNA-binding activity of Fos and Jun. Similarly, Storz et al controlled the state of OxyR oxidation with various antioxidants and reductants. oxyR is induced by low levels of H_2O_2. Its protein product, OxyR, is a transcriptional activator of genes and is activated directly by oxidation. Many papers do not distinguish between H_2O_2 and radical effects (Muehlematter et al, Halliwell). Experiments which do investigate the contribution of H_2O_2 vs radicals, usually study oxygen radicals. For example, Imlay and Linn found that hydroxyl radicals are responsible for a major portion of DNA damage from H_2O_2 in *E. coli.* There have been few studies of peroxidase mediated toxicity. Eastmond et al (b,c) have proven the involvement of myeloperoxidase in myelotoxicity from benzene exposure. Phenol and hydroquinone, the two principal metabolites of benzene, form benzoquinone in the presence of activated human leukocytes or HRP. The benzoquinone will bind added boiled rat liver protein or glutathione. They gave the benzene metabolites to mice and induced a significant loss in bone marrow cellularity. Free radical involvement in myelotoxocity would be implicated if free radicals could be trapped by ESR from their peroxidase, H_2O_2, benzoquinone system.

DNA is probably a major target of free radical damage. Experiments reported here reveal more ^3H thymidine uptake in peroxidase contaiing cells than in control cultures with identical H_2O_2 exposure (Fig 18). A greater incorporation of DNA percursor without a relative increase in DNA indicates either DNA repair or damage which inhibits further DNA synthesis. Imlay et al show that DNA nicks are proportional to radical generation. DNA breaks require repair. Muehlematter et al find a direct correlation between strand

breakage with consequent poly ADP-ribosylation and cytostatic effects after exposing cells to xanthine/xanthine oxidase (H_2O_2 and $O_2^{\cdot -}$). Excessive poly ADP-ribosylation could inhibit further DNA synthesis by depleting cells of NAD and ATP.

Phenol

Phenol is required for radical formation by the peroxidase-H_2O_2 complex. It would be expected that the addition of phenol to media would be needed to generate radicals and induce cellular damage. However, as was shown by ESR, media contains phenolic compounds which transfer free radicals from the oxidized enzyme. Adding phenols to media did increase the efficiency of radical damage to tissue cultures with added HRP (Fig 16, A). Phenols, except for OH-phenylpyruvate, had no effect and H_2O_2 addition caused only 9% inhibition of growth in the HRP group. Adding phenols with H_2O_2 to the cells with HRP caused an additional 5-80% decrease in cell number. Radicals transferred from peroxidase outside the cells in the HRP treated group either damage the exterior of the cells or transverse the plasma membrane to cause injury.

H_2O_2 generated by the glucose oxidase caused a 9% inhibition of growth in CHO-M1K1, just as it did in the HRP added group. Peroxidase was outside the cells in both groups. However, the addition of phenols with or without H_2O_2 did not affect CHO-M1K1 (Fig 16, B). In fact, phenols may have protected the peroxidase secreting cells from H_2O_2, as it does during initial H_2O_2 exposure. Phenols could increase the efficiency of radical production and divert H_2O_2 from the cells with peroxidase.

Arbutin and tyramine caused slightly more damage to CHO-hTPO than to CHO-M1K1 or controls (Fig 16, C). ESR experiments show that arbutin at tissue culture concentrations was the best catalyst with detergent extracted hTPO. Tyramine had about the same phenolic efficiency as phenol and OH-phenylpyruvate in the ESR assay.

Tyramine may have been more effective than other phenols in tissue culture as it is the most hydrophobic of the phenols tested. Membrane solubility might allow tyramine better access to the peroxidase. Radicals produced on a membrane would be expected to be more harmful than those created in the media.

The phenols which were added to tissue culture had been shown by ESR to generate free radicals with hTPO and HRP. Similar concentrations of phenols were used in ESR and tissue culture experiments, except for guaiacol. Guaiacol was used at lower doses in tissue cultures as it is toxic. The extent of cytotoxicity by individual phenols with H_2O_2 evidentally reflects enzyme specificity. Tyramine in CHO-hTPO cultures is the only case of hydrophobicity affecting toxicity.

Free Radical Scavenger

The partial preservation of cell number by the addition of a radical scavenger to a tissue culture with a radical generating system indicates that free radicals were involved in the destruction of the cells (Fig 17). TOLH dramatically increased cell number over that seen with H_2O_2 or H_2O_2 plus phenol in the HRP treated group. However, it did not allow growth at the rate seen with HRP only treatment. Either the TOLH concentration was not sufficient to intercept all the radicals or TOLH itself is somewhat toxic.

The addition of TOLH to otherwise harmless treatments, i.e. H_2O_2 alone or with phenol, was detrimental in the other three cells groups. If TOLH alone had been tested, it would have been possible to distinguish between TOLH and its products as the cytotoxic species. However, TOLH alone was not included as a control because it was expected to be benign as it had been with lower levels of H_2O_2 generation. It has been reported that Tempol, the oxidized product of TOLH, is mutagenic with xanthine/hypoxanthine (H_2O_2, $O_2\cdot^-$generator) when added to bacteria (Sies et al). The combination

of H_2O_2 and a Tempol producing system may cause both reversion in the bacteria and damage to the CHO cultures. The low levels of peroxidase in the CHO-M1K1 and CHO-hTPO appear to protect cells slightly from Tempol toxicity. Peroxidase may have diverted some of the H_2O_2 from the TOLH. In the short-term experiment in Fig 17, it appears that TOLH treatment would be beneficial only under conditions of high rates of phenoxyl radical generation. Treatment with TOLH or other free radical traps might be appropriate to alleviate damage from high concentrations of peroxidase and H_2O_2 such as would be expected in massive infections. Long-term tissue culture experiments could evaluate the relative harm or benefit from H_2O_2/peroxidase vs TOLH/H_2O_2/peroxidase vs H_2O_2/TOLH.

Many antioxidants have been used to counteract cell damage. Tissue culture experiments show that a variety of antioxidants partially and temporarily protect cells from oxygen radicals. Superoxide dismutase (SOD), catalase, glutathione peroxidase partially protect melanoma-, lymphoma- and leukemia-derived cells from the generation of toxic oxygen species by phenol oxidation (Passi et al). Butylated hydroxytoluene (BHT), vitamin E, and N-propylgallate guard somewhat against cell death and lipid peroxidation in H_2O_2-treated hepatocytes (Rubin et al). The antioxidants N,N'-diphenyl-p-phenylenediamine (DPPD) and promethazine protect the hepatocytes from an aliquot of H_2O_2 for at least three hours. However, if the H_2O_2 is supplied continuously by glucose/glucose oxidase treatment, DPPD and promethazine only prevents harm for one hour. The authors could not explain the inability of the hepatocytes to cope with continuously generated H_2O_2.

Antioxidant therapy has been tested in humans and animals to prevent tissue damage. Many antioxidant strategies have been tried to prevent oxidative injury during reperfusion of organs. SOD therapies have been used to alleviate the tissue damage in rheumatoid arthritis induced by radicals. SOD therapy protected mice from a potentially

lethal influenza virus infection, proving the involvement of oxygen radicals in the pathogenesis of influenza virus (Oda et al). Antioxidants delayed the onset of thyroid disease in Obese strain chickens (Bagchi et al). Butylated hydroxyanisole (BHA) and ethoxyquin were most effective. Vitamins C and E and ß-carotene had slight or negligible effects on the disease and its manifestations. Antioxidant benefits were still being provided as a dietary supplement when the disease developed, suggesting that two mechanisms are involved in thyroiditis in Obese chickens. The two phase damage parallels that of the tissue culture experiments depicted here. It is possible that the chickens were initially protected from H_2O_2-mediated damage by antioxidants but that thyroid peroxidase generated radicals which eventually induced disease.

Conclusion

Low levels of continuously generated H_2O_2 are cytotoxic to CHO cultures in a dose-dependent manner in the presence of thyroid peroxidase (TPO). Phenols intensify cellular inhibition by H_2O_2 and peroxidase in some cases. Strict phenol dependence could not be demonstrated because a phenol-activity-free tissue culture media could not be found. It is believed that peroxidase, H_2O_2, and phenol contribute to the lack of cell replication by generating phenoxyl radicals. Phenol dependent radical production in proportion to H_2O_2 and TPO concentrations had been previously shown in ESR experiments. The addition of the free radical trap, TOLH, prevented a large percentage of cell loss, indicating that radicals were responsible for some of the damage from the H_2O_2, peroxidase, phenol system in tissue culture.

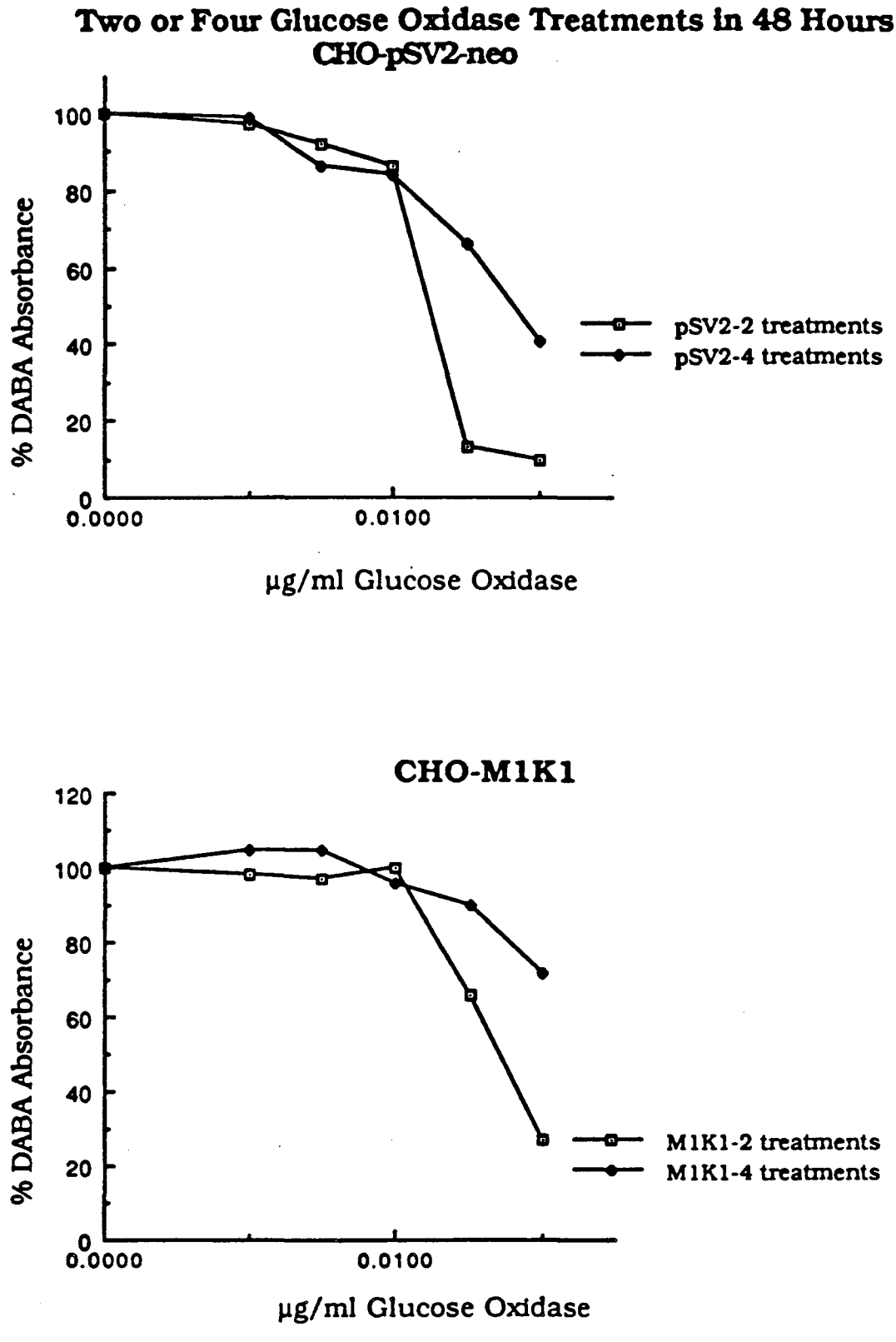


Fig 9. Plated at 1.6×10^4 cells/well. 24 hr rest. Treated at 12 or 24 hr intervals. All test wells fixed after 48 hr treatment.

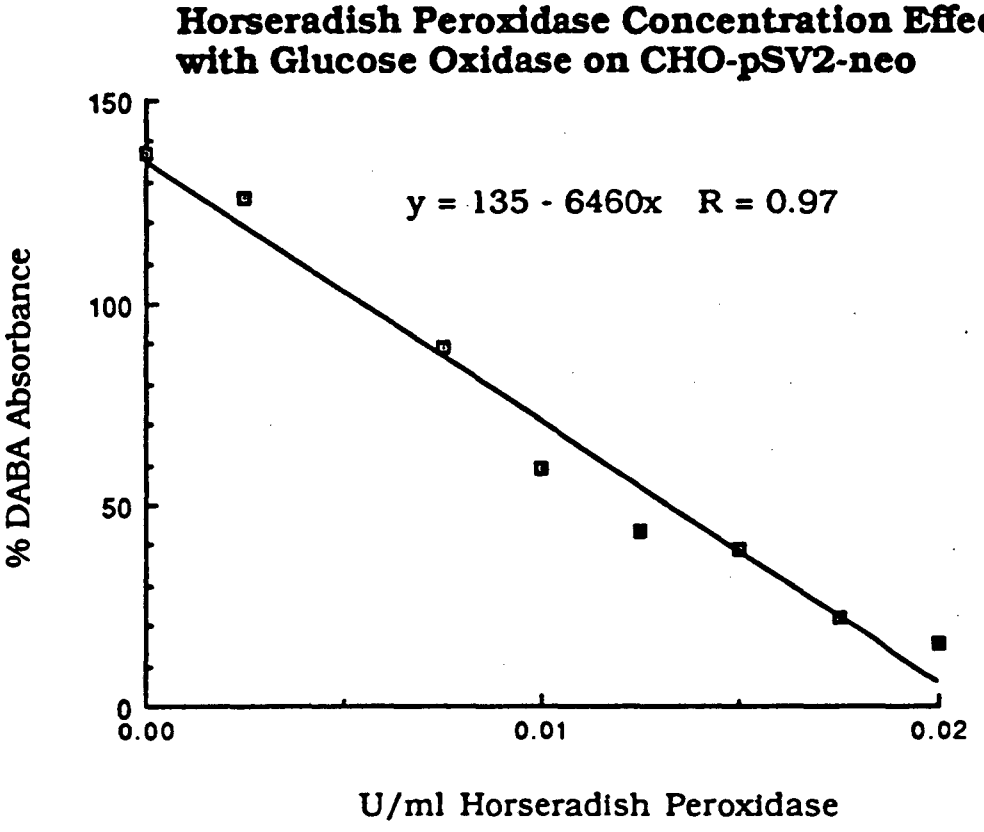


Fig 10. Treated with 0.005 μg/ml glucose oxidase, 10 mM glucose. Plated at $1.2x10^4$ cells/well. 24 hr rest. Treated on Days 0 & 2. Fixed on Day 4.

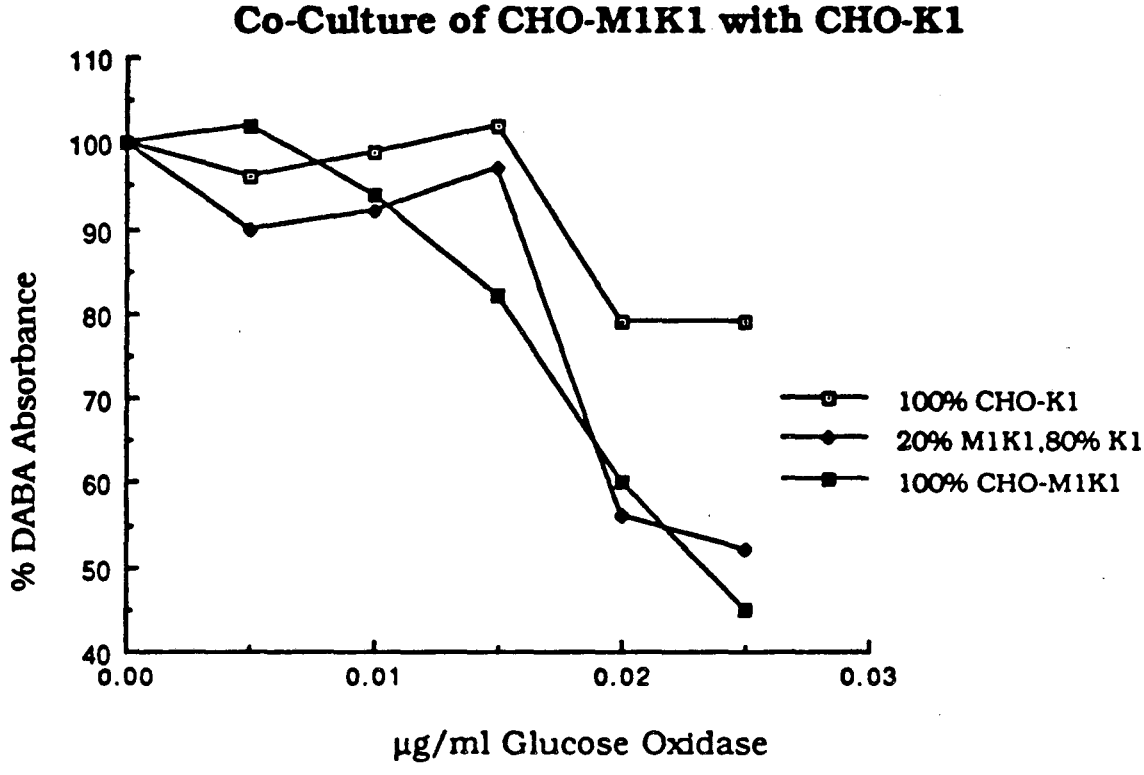


Fig 11. Plated at $1.6x10^4$ cells/well. 24 hr rest. Treated on Day 0. Fixed on Day 2.

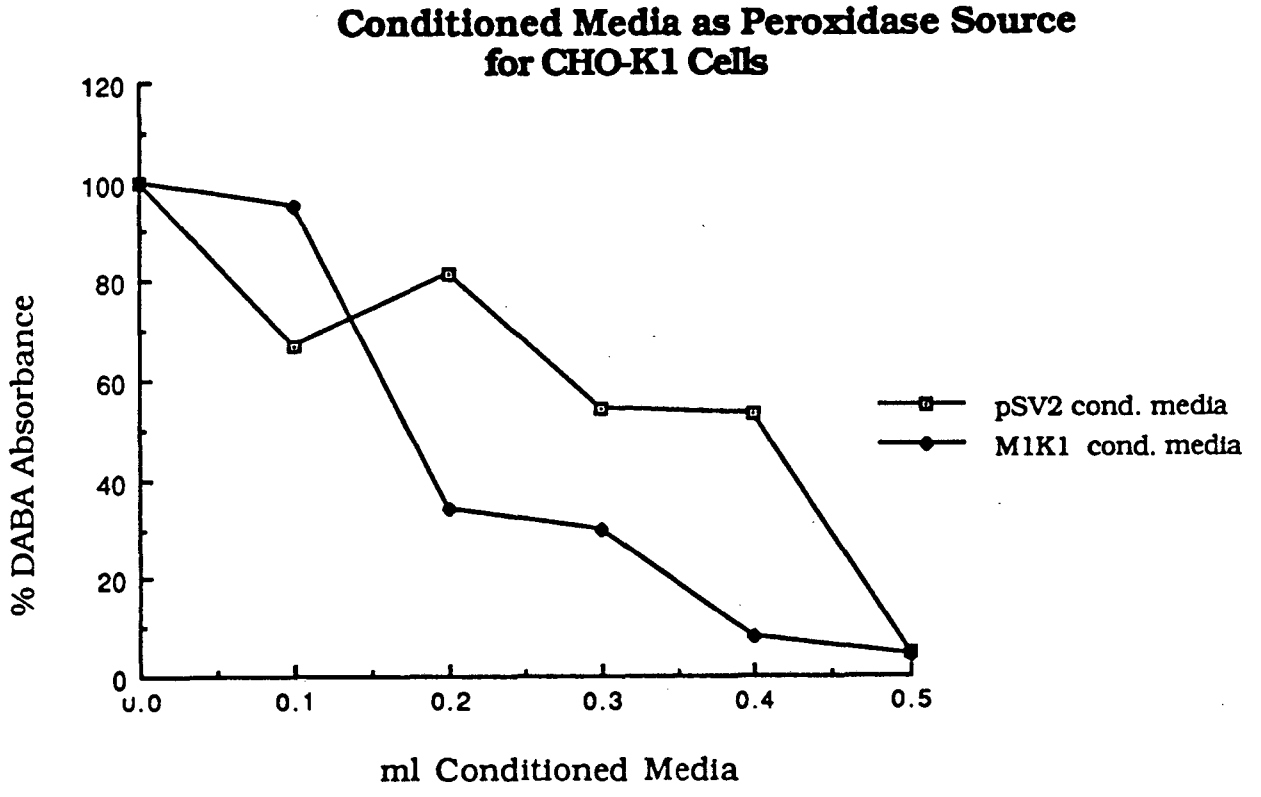


Fig 12. Treated with 0.02 μg/ml glucose oxidase and 10 mM glucose. Conditioned media from 10 ml media collected 3 days after $5x10^5$ cells plated in 100 mm dish. Test wells plated at $1.6x10^4$ cells/well. 24 hr rest. Treat on Day 0. Fixed on Day 2.

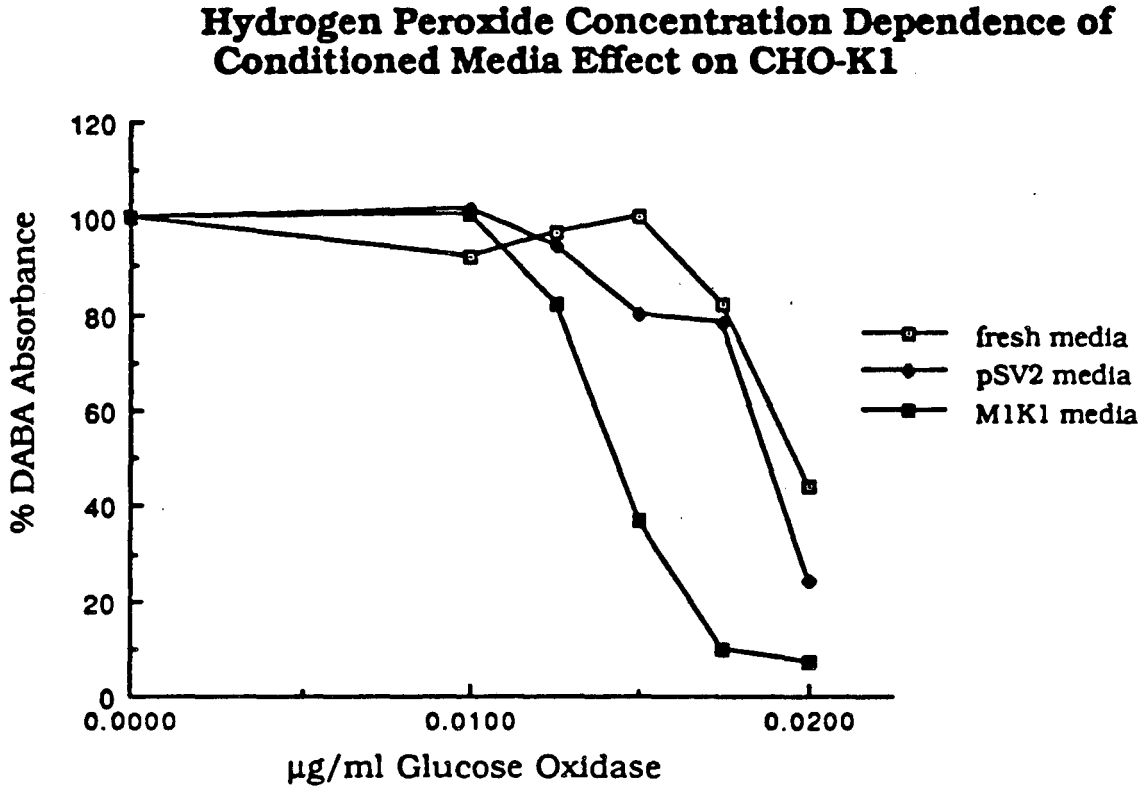


Fig 13. Plated at $1.6x10^4$ cells/well. 24 hr rest. Treated on Day 0. Fixed on Day 2.

Response to H2O2 After 12, 24, 36 or 48 Hours

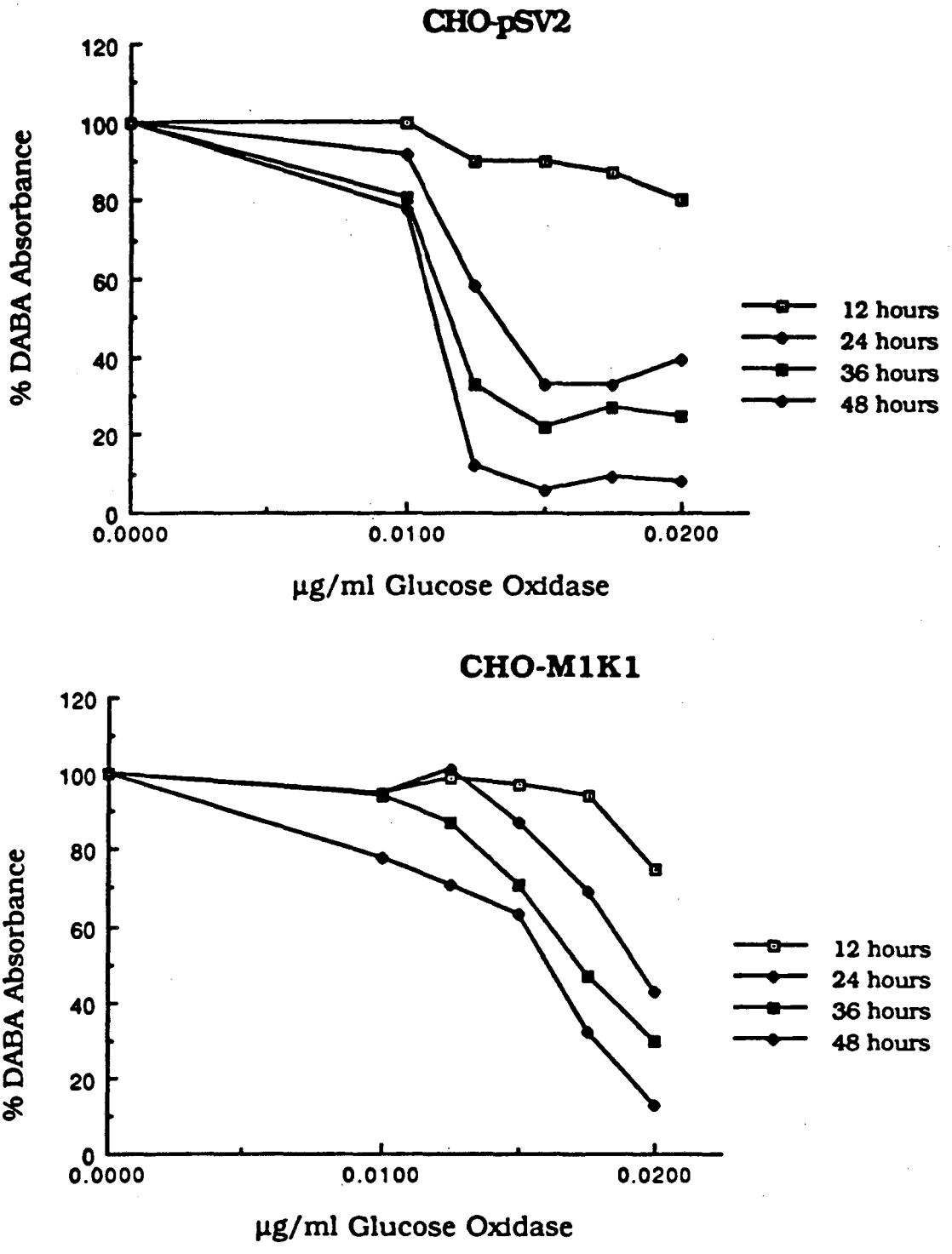


Fig 14. Plated at 1.6×10^4 cells/well. 24 hr rest. Treated once and fixed at 12 hr intervals.

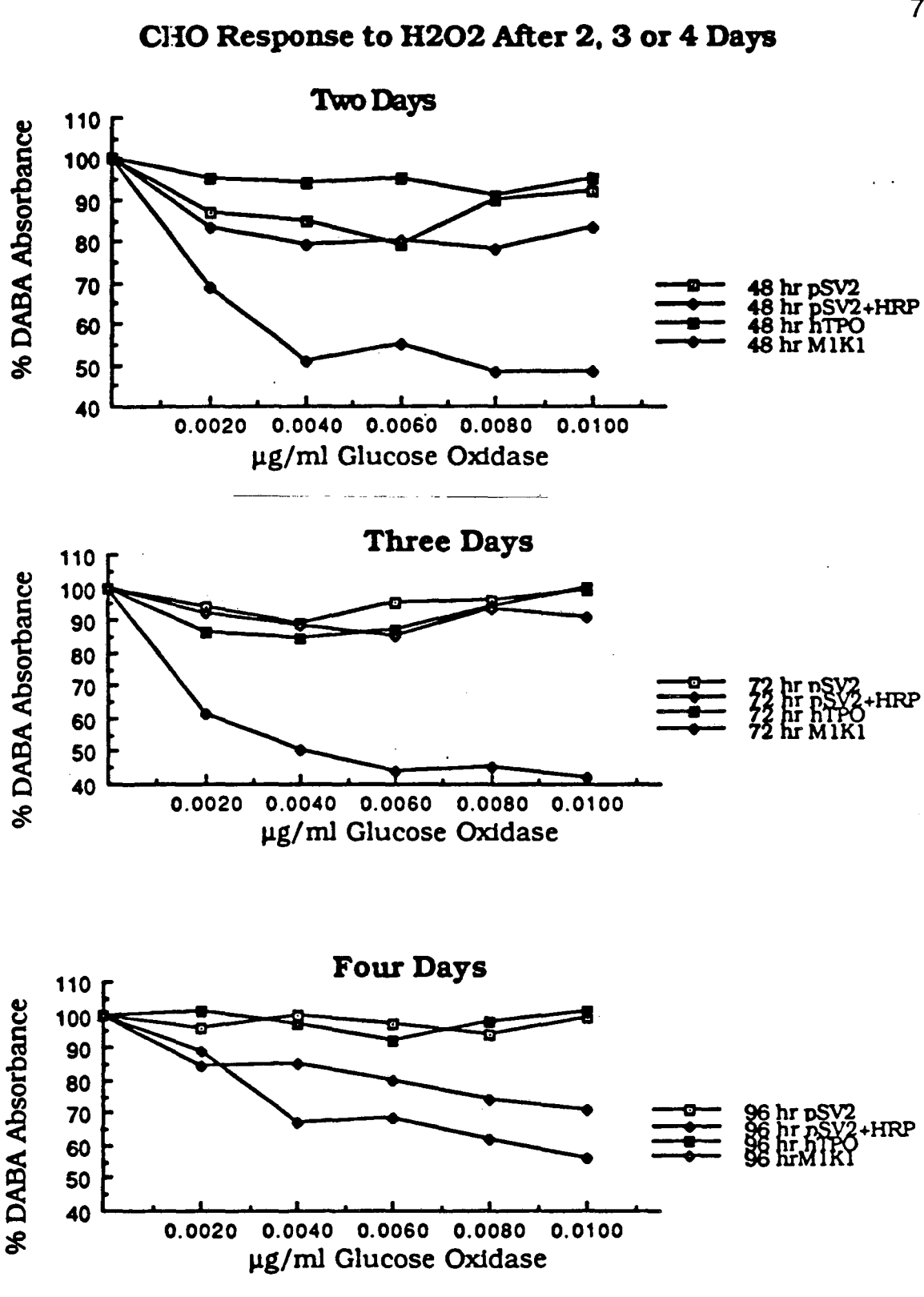


Fig 15. +/- 0.01 U/ml HRP. Plated at 1.2×10^4 cells/well. 24 hr rest. Treated on Days 0 & 2. Fixed on Day 4.

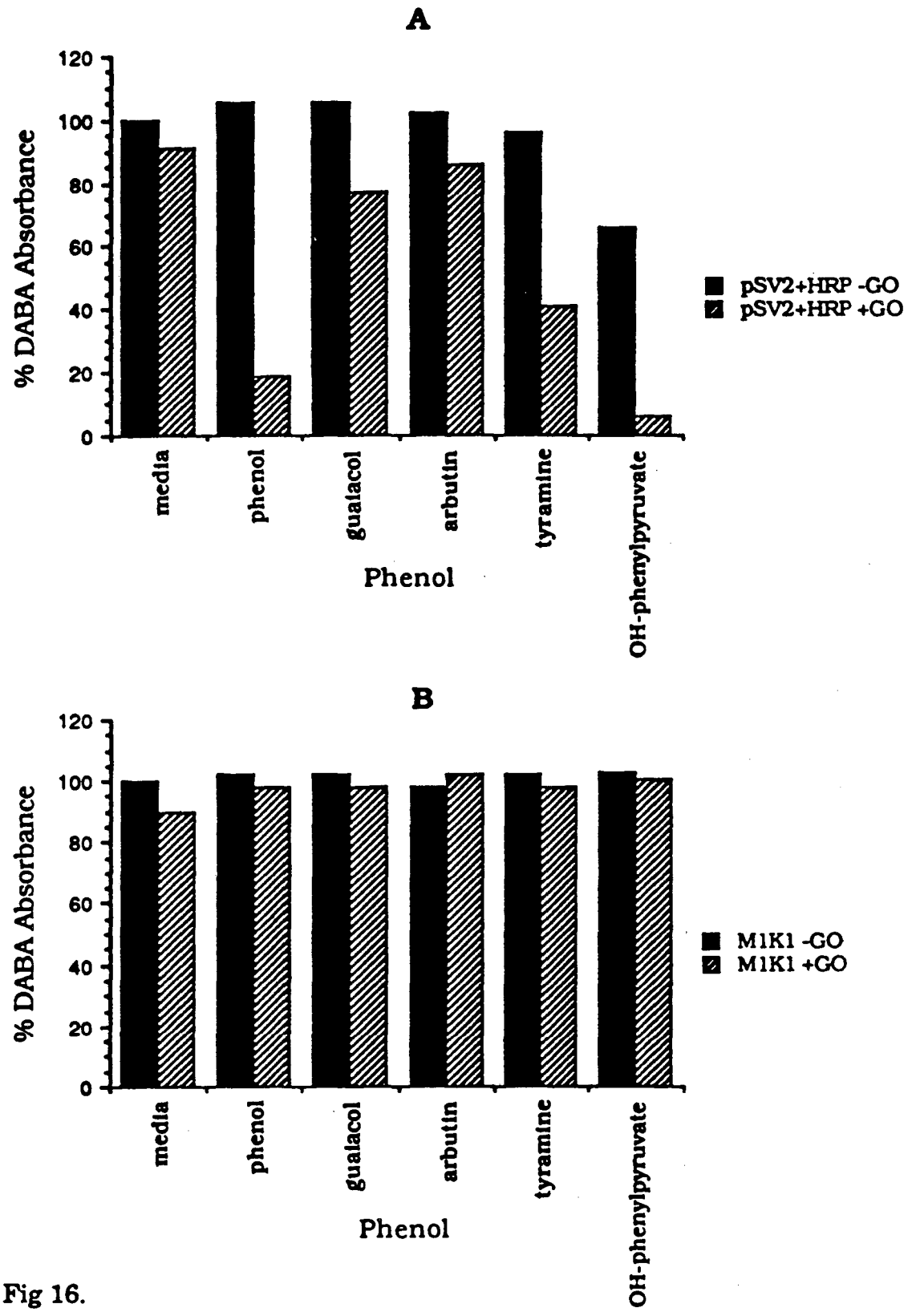

Phenol Effects
A
120
100
80
60
40
20
0
% DABA Absorbance
media
phenol
gualacol
arbutin
tyramine
OH-phenylpyruvate
Phenol
pSV2+HRP -GO
pSV2+HRP +GO
B
120
100
80
60
40
20
0
% DABA Absorbance
media
phenol
gualacol
arbutin
tyramine
OH-phenylpyruvate
Phenol
M1K1 -GO
M1K1 +GO

Fig 16.

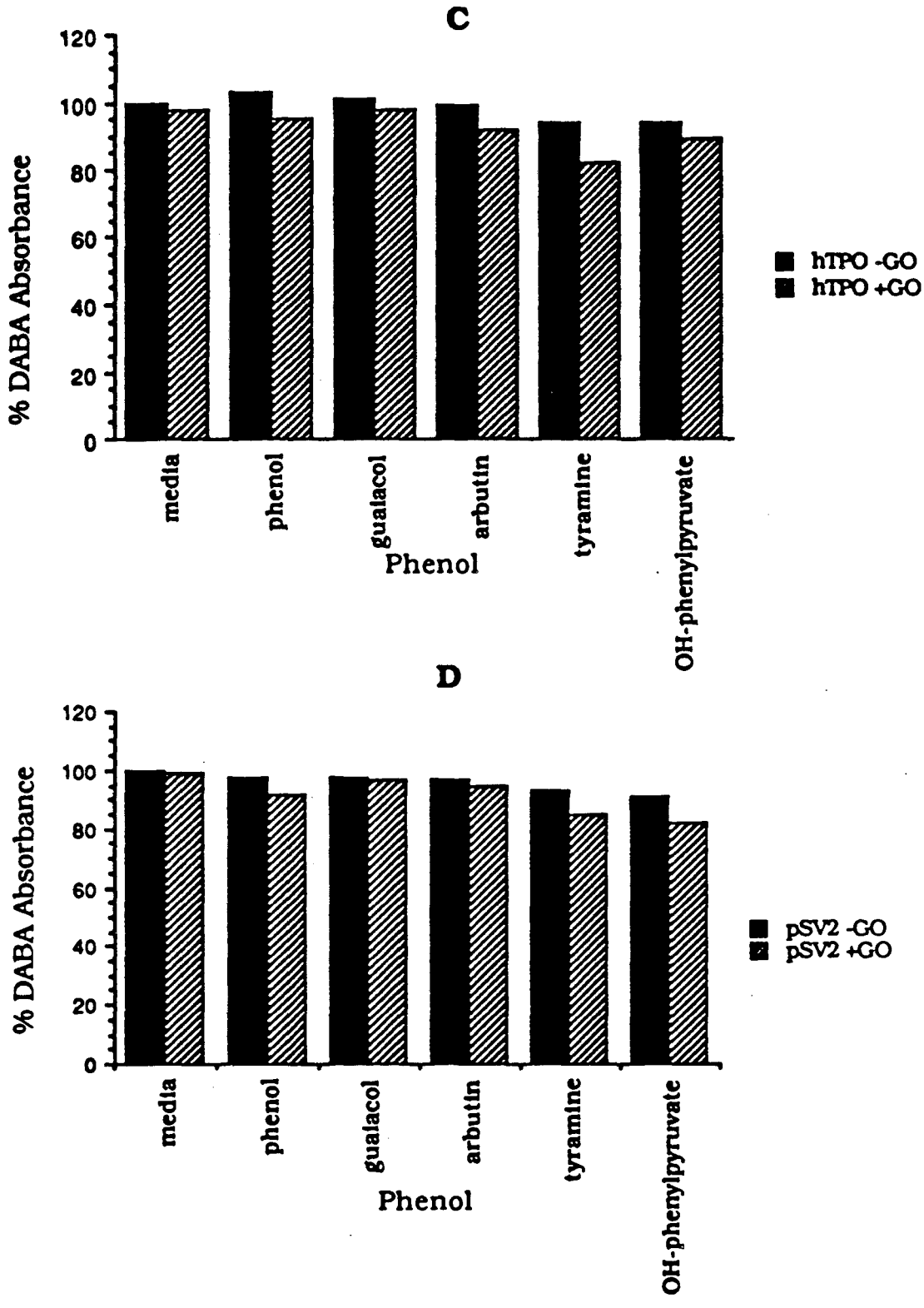


Fig 16. 0.003 U/ml HRP. 0.002 µg/ml glucose oxidase. 10 mM glucose. 20 µM phenol. 0.5 µM guaiacol. 50 µM arbutin. 40 µM tyramine. 25 µM OH-phenylpyruvate. Plated at $1.2x10^4$ cells/well. 24 hr rest. Treated on Days 0 & 2. Fixed on Day 4.

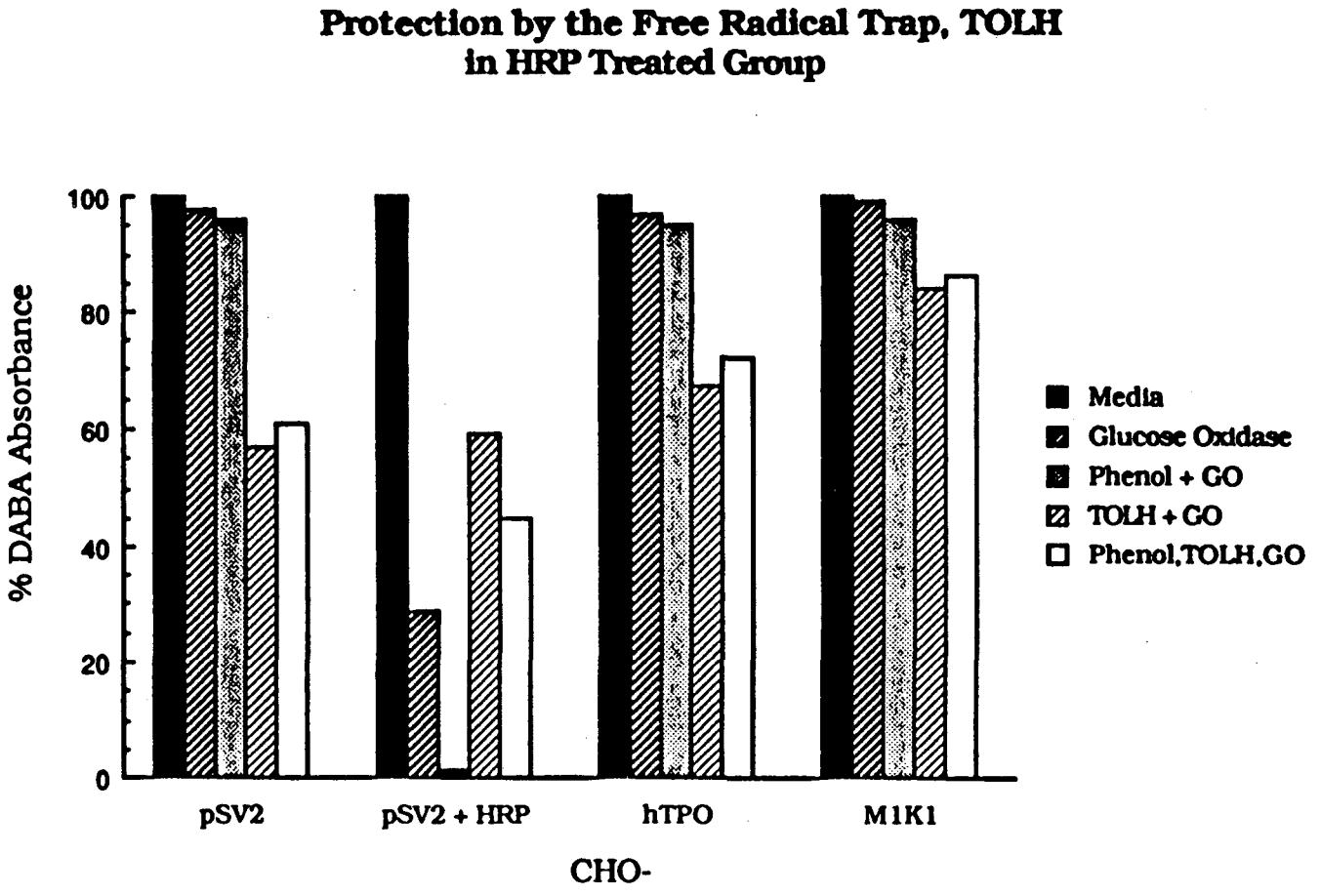


Fig 17. 0.003 U/ml HRP. 0.005 µg/ml glucose oxidase. 10 mM glucose. 40 µM phenol. 0.25 mM TOLH. Plated at 1.2×10^4 cells/well. 24 hr rest. Treated on Days 0 & 2. Fixed on Day 4.

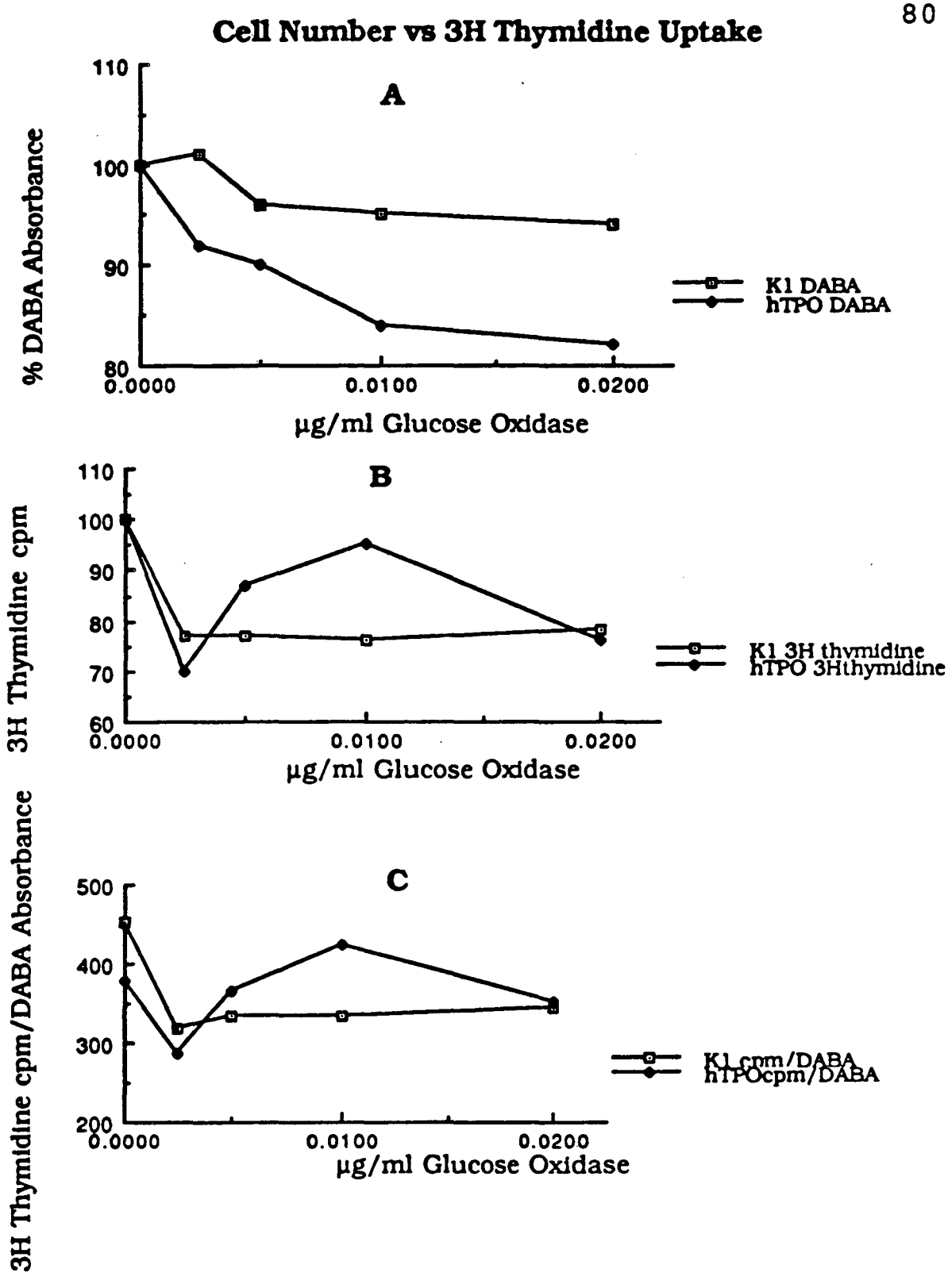


Fig 18. Plated at $1.6x10^4$ cells/well. 24 hr rest. Treated on Day 0. ^3H thymidine added on Day 1. All wells fixed on Day 2.

Conclusion

ESR experiments show that thyroid peroxidase forms free radicals in proportion to H_2O_2 concentrations and peroxidase activity and is phenol dependent. The natural phenol species in the thyroid gland is iodinated tyrosine which forms thyroid hormone. However, ESR experiments show that thyroid peroxidase can generate radicals with a variety of phenolic compounds and with iodide. It is suggested that highly oxidizing species are also made *in vivo* and might injure the thyroid gland.

Tissue culture experiments were done to test the effects of a radical generating system on cells. Cellular inhibition was H_2O_2 concentration dependent in the presence of peroxidase and vice versa. Phenol dependence could not be demonstrated in tissue culture because a phenol free media could not be found. However, the addition of specific phenols to media did enhance cellular destruction, suggesting that some phenols are more efficient catalysts than those found in media. It is predicted that thyroid gland exposure to phenols in the blood would increase the generation of spurious radicals.

The opportunity for free radical generation would be greater if H_2O_2 generation or peroxidase activity increase. These conditions occur in the autoimmune thyroid diseases, Hashimoto's and Grave's. Peroxidase activity and H_2O_2 generation are stimulated by thyroid stimulating hormone (TSH) in Hashimoto's and by long-acting thyroid stimulator (LATS) in Grave's. It seems likely that radical production is increased in autoimmune thyroiditis and is involved in the scarring and involution of the thyroid gland in these dieases.

Tissue culture provides the opportunity to evaluate the contribution of radicals to thyroiditis. If radicals are important in cell destruction, as tissue culture experiments indicate, perhaps antioxidant therapy would be beneficial for thyroiditis. Tissue culture experiments show that TOLH, a free radical probe, prevents some of the damage from a radical generating system. Although TOLH was somewhat toxic, its

analogues or a pure preparation might be valuable in the prevention or treatment of peroxidase mediated diseases.

Future Experiments

Electron Spin Resonance

The range of H_2O_2 concentration which will give nearly maximum peroxidase activity should be defined for the peroxidase assay. H_2O_2 concentration limits peroxidase activity if it does not saturate the enzyme or if it is excessive and oxidizes the enzyme to an inactive state. The tolerance of the enzyme delineates the dilutions of enzyme which can be accurately assayed with a given H_2O_2 concentration. For example, if peroxidase shows nearly maximal activity with 100% more or 50% less than optimal H_2O_2 concentration, then assays of peroxidase activity will only be meaningful within a four-fold range of H_2O_2 concentrations. The extremes of tolerated H_2O_2 concentration may be time dependent. There probably is a short time when a small excess of H_2O_2 is not reflected in decreased activity. The application of rapid-mix techniques to the ESR assay and computerized recording of spin probe oxidation would allow peroxidase activity to be measured before a significant percentage of the enzyme had been inactivated.

The H_2O_2 assay might be adapted to estimate catalase activity. The assay would be based on the successful competition of peroxidase with catalase for H_2O_2. It would be assumed that all added H_2O_2 would oxidize TOLH except that which had been consumed by catalase. Calculations of catalase activity can be simpified by changing the substrate for catalase from 2 H_2O_2 to 1 H_2O_2 and 1 ethanol per catalytic cycle by the addition of ethanol (Oshino et al). Values for catalase activity could be derived from parallel assays in buffer.

Tissue Culture

Peroxidase dependent H_2O_2 effects in tissue culture should be repeated with purified hTPO. Peroxidase concentration would be

known and constant with purified hTPO rather than dependent upon cell density and the time cells had been in the media, as was the case in the experiments reported here. Higher concentrations of hTPO might increase the rate of radical generation enough to test phenol specificity and TOLH protection. Purified hTPO could be obtained by filtration and electrophoresis of CHO-M1K1 media.

The effects of H_2O_2 could be distinguised from those of radicals in tissue culture experiments. The work reported here shows differences in timing, proliferative, and ^3H thymidine incorporation effects between H_2O_2 only and H_2O_2 with peroxidase exposure. A condition showing large changes in cellular activity could be explored for biochemical consequences such as; the induction of the proto-oncogenes c-*fos* and c-*myc*, phosphorylation of ribosomal protein S6, or the generation of DNA nicks.

It would be interesting to compare the cyctotoxicity of radicals generated by membrane-bound peroxidase with those from peroxidase in the media. Peroxidase mediated H_2O_2 effects could be compared between a clone of CHO-hTPO cells (membrane-bound hTPO) with a high copy number of the hTPO gene and CHO-M1K1 cells (secreted hTPO) with the same hTPO expression capacity.

Various types of antioxidant therapies for peroxidase mediated damage could be explored in tissue culture. Both tissue culture and animal treatments have shown that reactive oxygen species scavengers, such as catalase, superoxide dismutase, and glutathione peroxidase protect against some diseases. Antioxidants, such as vitamin E, BHA, and BHT protect membranes from radical damage. TOLH-like spin probes may protect cells and tissues from acute free radical attack. Long term experiments could differentiate between the delay or prevention of diseases with radical involvement and indicate possible side effects.

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