AN INTERRELATIONSHIP BETWEEN ERGOTHIONEINE, CERTAIN PHENOLIC HORMONES AND PEROXIDASE

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SUMMARY

1. The oxidation of ergothioneine by peroxidase and H_2O_2 is stimulated by thyroxine or estradiol. The main product of oxidation appears to be ergothioneine disulfide.

2. An aerobic oxidation of ergothioneine by peroxidase was observed in a system supplemented by Mn^{2+}, DPNH and thyroxine. A partial recovery of ergothioneine was obtained on the addition of glutathione. The aerobic oxidation of ergothioneine by peroxidase was inhibited by catalase.

3. Ergothioneine stimulates the oxidation of DPNH (or TPNH) by peroxidase, Mn^{2+} and oxygen. The effect of ergothioneine is inhibited by H_2O_2 and stimulated by catalase. Ergothioneine is effective in catalytic concentrations.

4. The stimulatory effect of a combination of ergothioneine and thyroxine or ergothioneine and estradiol on the Mn^{2+}-dependent aerobic oxidation of DPNH by peroxidase is considerably greater than additive.

5. It is proposed that ergothioneine acts as an oxidation–reduction catalyst for the aerobic oxidation of reduced pyridine nucleotides by peroxidase, and the possibility of a relationship between the actions of peroxidase, ergothioneine and thyroxine or estradiol *in vivo* is considered.

INTRODUCTION

Ergothioneine is widely distributed in mammalian tissues (see ref. 1 for an extensive review of the literature). Its presence appears to be dependent on dietary intake since blood and tissue levels can be greatly reduced by dietary restriction, and all efforts to demonstrate synthesis by mammalian tissues have failed. Efforts to demonstrate a role for ergothioneine in the metabolism of the cell have been largely unsuccessful. Thus no striking effect on bodily functions has been produced by the depletion of body ergothioneine or by the administration of ergothioneine to the intact animal. A few effects of ergothioneine on isolated enzyme systems have been described and are reviewed by MELVILLE[1]. The most promising of the *in vitro* effects of ergothioneine is the sensitization of certain ribosidases to inhibition by nicotinamide which prompted the designation of ergothioneine as a coenzyme of red blood cell nicotinamide riboside phosphorylase[2].

AKAZAWA AND CONN[3] first demonstrated that peroxidase can catalyze the aerobic oxidation of reduced pyridine nucleotides (DPNH and TPNH). The aerobic oxidation

of DPNH (or TPNH) by peroxidase requires Mn^{2+} (or Ce^{3+}, ref. 4) and is stimulated by phenolic compounds[3,5,6], aromatic amines[7] or sulfite ions[8,9]. Among the phenolic compounds which are active in this way are thyroxine and certain of its analogues and phenolic estrogens[6,5]. In the course of the study of the influence of thyroxine and estradiol on the aerobic oxidation of DPNH (or TPNH) by peroxidase, it was observed that ergothioneine has a stimulatory effect on that reaction. This prompted a study (1) of the oxidation of ergothioneine by peroxidase and either H_2O_2 or oxygen, (2) of the stimulation by ergothioneine and certain related substances of the oxidation of DPNH (or TPNH) by peroxidase, and (3) of the influence of thyroxine and estradiol on these reactions.

METHODS AND MATERIALS

Oxygen uptake was determined by standard Warburg manometric techniques. The oxidation of ergothioneine was determined by the fall in absorption at 258 mμ[10] and the oxidation of DPNH (or TPNH) by the fall in absorption at 340 mμ. The spectrophotometric measurements were made at 25° in a Cary MII recording spectrophotometer using fused quartz cells 1 cm in length. The blank contained all components except the substance the oxidation of which was being measured.

Horseradish peroxidase (R.Z. 1.1) and crystalline beef-liver catalase (150000 units/ml) were obtained from Worthington Biochemical Corporation. A preparation of lactoperoxidase with a 412 mμ/280 mμ ratio of 0.5 was kindly supplied by Dr. M. MORRISON and myeloperoxidase was prepared from canine leucocytes as previously described[11]. L-Ergothioneine and 2 mercapto-L-histidine were obtained from California Foundation for Biochemical Research, 2-mercaptoimidazole was obtained from K & K Laboratories and 1-methyl-2-mercaptoimidazole (Tapazole) was kindly supplied by Eli Lilly and Co. Ergothioneine disulfide, prepared as described by HEATH AND TOENNIES[10], was freshly dissolved in absolute alcohol, and its concentration was determined by the optical density at 258 mμ after reduction by glutathione. Other special reagents were obtained as previously described[11,5]. Thyroxine was dissolved in 0.002 *N* sodium hydroxide and an aqueous solution of estradiol was prepared as described by MCSHAN AND MEYER[12].

RESULTS

Oxidation of ergothioneine by peroxidase

Oxidation by added H_2O_2

Ergothioneine is oxidized to ergothioneine disulfide by an equivalent amount of H_2O_2 in strongly acid solution[10]. Fig. 1 demonstrates that the rate of oxidation of ergothioneine by H_2O_2 at neutral pH is extremely slow (see ref. 10) and is only slightly increased by the addition of horseradish peroxidase. A number of peroxidase-catalyzed reactions are stimulated by thyroxine or estradiol[11,6,5,13]. A rapid oxidation of ergothioneine was observed when thyroxine was added to the H_2O_2–peroxidase system under the conditions employed in Fig. 1. Estradiol also stimulated this oxidation. Treatment of the oxidation product with reduced glutathione (or cysteine) resulted in a rapid increase in absorption at 258 mμ, and the spectrum of ergothioneine was again observed (Fig. 1). The reduction of ergothioneine disulfide by glutathione and

cysteine was reported by HEATH AND TOENNIES[10]. Added H_2O_2 could be replaced by a H_2O_2 generating enzyme system (glucose–glucose oxidase), and myeloperoxidase or lactoperoxidase could be employed instead of horseradish peroxidase.

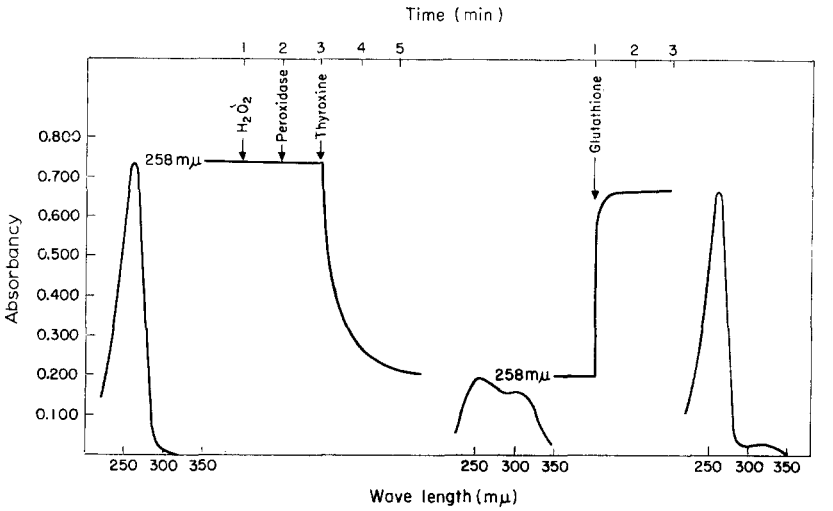

Fig. 1. Effect of thyroxine on the oxidation of ergothioneine by hydrogen peroxide and peroxidase. The reaction mixture contained initially 200 μmoles of phosphate buffer pH 7.0, 0.15 μmole of ergothioneine and water to a final volume of 3.0 ml. Hydrogen peroxide (0.1 μmole), horseradish peroxidase (100 μg), sodium-L-thyroxine (0.05 μmole) and reduced glutathione (0.5 μmole) were added as indicated. The absorption spectrum of the solution was determined where indicated. The wavelength was maintained at 258 mμ between spectra. The blank contained all components except ergothioneine.

Aerobic oxidation

Since the demonstration by THEORELL AND SWEDIN[14] that peroxidase can act as an aerobic oxidase for dihydroxyfumaric acid, a number of substances have been found to be oxidized aerobically by peroxidase (see ref. 3). These oxidations require Mn^{2+}, are stimulated by certain phenolic compounds and are inhibited by catalase. A number of substances which are not ordinarily oxidized by peroxidase in the absence of added H_2O_2 may be oxidized aerobically if DPNH (or TPNH) is added to the reaction mixture in addition to Mn^{2+} and a phenolic catalyst (*e.g.* thyroxine or estradiol), and this type of aerobic oxidation also is inhibited by catalase[13]. It has been suggested that the oxidation of DPNH by peroxidase, Mn^{2+}, oxygen and a phenolic catalyst results in the formation of an oxidant which is either H_2O_2 itself or a closely related substance, and that this oxidant can be utilized in a coupled oxidation[13]. Ergothioneine was not oxidized by peroxidase, Mn^{2+} and oxygen either in the presence or absence of a phenolic catalyst (thyroxine or estradiol). The aerobic oxidation of ergothioneine by peroxidase was observed however if DPNH (or TPNH) was added to the reaction mixture in addition to Mn^{2+} and thyroxine (Fig. 2). Spectrum (———) represents the absorption spectrum of ergothioneine in a reaction mixture which contained Mn^{2+}, thyroxine, oxygen and peroxidase in phosphate buffer pH 7.0. DPNH (or TPNH) was added to both the experimental flask and the blank and a second spectrum (– – –), taken after 5.0 min, indicated the complete oxidation

of ergothioneine. No oxidation of ergothioneine was observed during this period in the absence of DPNH (or TPNH). A 5 min delay was required before the second spectrum was taken since DPNH was oxidized during this period. The oxidation of DPNH, which was complete at the end of the 5 min period in both the experimental flask and the blank, proceeded at a more rapid rate in the former than in the latter. An absorption spectrum taken after the addition of glutathione to the oxidation products of ergothioneine (– – – –) suggested that ergothioneine had been regenerated. However, some nonreversible oxidation had occurred. The aerobic oxidation of ergothioneine by peroxidase under the conditions employed in Fig. 2 was inhibited by catalase.

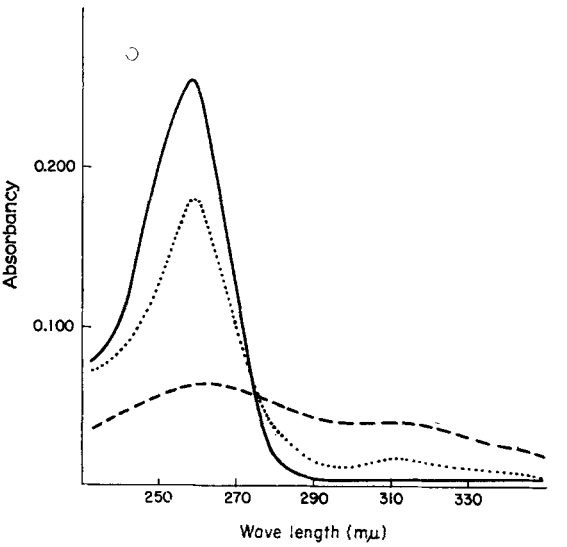

Fig. 2. Aerobic oxidation of ergothioneine by peroxidase. The reaction mixture contained initially 200 μmoles of phosphate buffer pH 7.0, 0.05 μmole of ergothioneine, 0.5 μmole of $MnCl_2$, 0.05 μmole of thyroxine, 10 μg of peroxidase and water to a final volume of 3.0 ml. The absorption spectrum of the solution was determined (———) and DPNH (0.1 μmole) was added. After a 5 min delay a second spectrum was taken (– – – –). Reduced glutathione (0.5 μmole) was added and a third spectrum was taken (-------). The blank contained all components except ergothioneine.

Stimulation of DPNH oxidation by ergothioneine

Fig. 3 demonstrates the stimulatory effect of ergothioneine on the oxidation of DPNH by horseradish peroxidase in the presence of Mn^{2+}, phosphate buffer pH 7.0 and oxygen. The oxidation of TPNH also was stimulated by ergothioneine under these conditions. The requirement for peroxidase, ergothioneine, Mn^{2+}, and oxygen is demonstrated in Fig. 3a. The initial activity which remained in the reaction mixture flushed with nitrogen was probably due to the small amount of dissolved oxygen which had not been displaced by the flushing procedure. The oxygen uptake approached 0.5 mole/mole of DPNH oxidized. Fig. 3b indicates the dependence on certain buffers. Phosphate was the most effective buffer under the conditions employed. Of the 3 buffers which were relatively ineffective (glycylglycine, Tris and imidazole), only imidazole buffer had a strong inhibitory effect on DPNH oxidation when employed in conjunction with phosphate buffer. Myeloperoxidase or lactoperoxidase could replace horseradish peroxidase in this reaction.

The concentration of ergothioneine employed in Fig. 3a was $3.3 \cdot 10^{-4}$ *M*. 2-Mercaptohistidine, 2-mercaptoimidazole and 1-methyl-2-mercaptoimidazole also were active at this concentration, whereas histidine and imidazole were inactive at a final concentration of $3.3 \cdot 10^{-3}$ *M*. Ergothioneine disulfide had 3–4 % of the activity of ergothioneine. Ergothioneine disulfide decomposes slowly in aqueous solution to

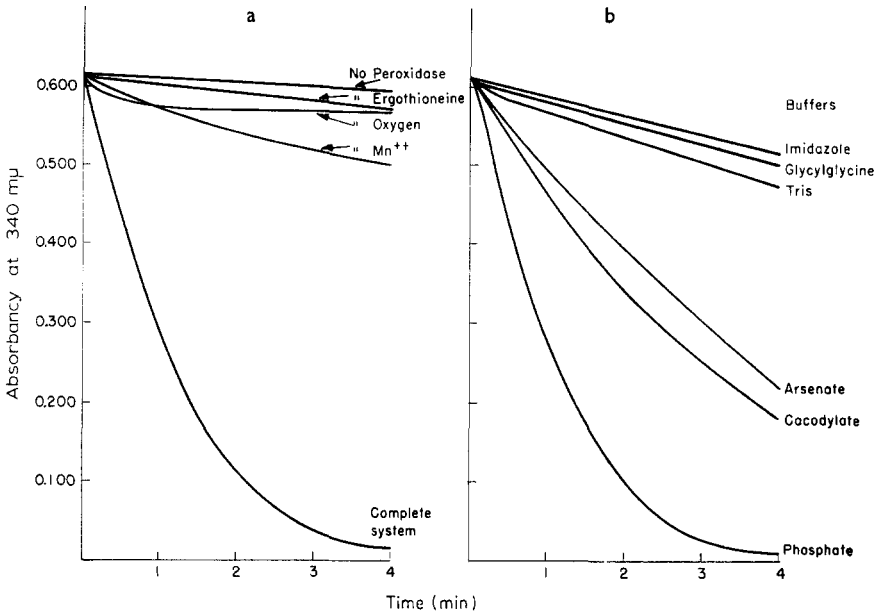

Fig. 3. Effect of ergothioneine on the oxidation of DPNH. (a) The complete system contained 200 μmoles of phosphate buffer pH 7.0, 0.3 μmole of DPNH, 0.5 μmole of $MnCl_2$, 100 μg of horseradish peroxidase, 1.0 μmole of ergothioneine and water to a final volume of 3.0 ml. Nitrogen was bubbled through the reaction mixture designated "No oxygen" for 5 min prior to and 30 sec following the addition of the last component, peroxidase. (b) As in (a) except that 200 μmoles of the buffers indicated (pH 7.0) were employed.

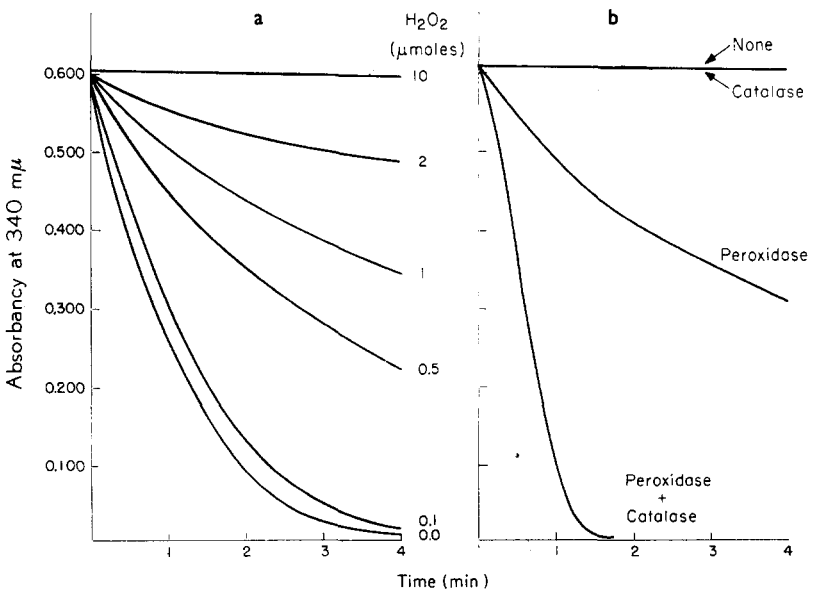

Fig. 4. Effect of H_2O_2 and catalase. (a) As for the complete system in Fig. 3a except that H_2O_2 was added as indicated. (b) The reaction mixture contained 200 μmoles of phosphate buffer pH 7.0, 0.3 μmole of DPNH, 0.5 μmole of $MnCl_2$, 0.1 μmole of ergothioneine and water to a final volume of 3.0 ml. Horseradish peroxidase (100 μg) and catalase (7500 units) were added as indicated. The reaction mixture designated "None" contained neither peroxidase or catalase.

yield 60–70% of the theoretical amount of ergothioneine[10]. This suggests that the slight stimulatory effect of ergothioneine disulfide on the oxidation of DPNH by peroxidase may be due to its partial degradation to ergothioneine.

The oxidation of DPNH by Mn^{2+}, peroxidase, ergothioneine and oxygen was inhibited by H_2O_2 under the conditions employed in Fig. 4a. A concentration of H_2O_2 equimolar to that of ergothioneine produced a 50–75% inhibition of DPNH oxidation. This relationship was maintained at different ergothioneine concentrations. Crystalline beef liver catalase was found to have a marked stimulatory effect on the oxidation of DPNH by Mn^{2+}, peroxidase, ergothioneine and oxygen under the conditions employed in Fig. 4b. Catalase itself in high concentrations can catalyze the aerobic oxidation of DPNH in the presence of Mn^{2+} and dichlorophenol and this reaction is stimulated by sulfite ions or ergothioneine (S. J. KLEBANOFF, unpublished data). However, no oxidation of DPNH by catalase was observed in the absence of peroxidase under the conditions employed in Fig. 4b. When the catalase concentration was increased, an initial lag period was observed during which the oxidation of DPNH occurred at a slower rate in the presence of catalase than in its absence.

Sulfite ions stimulate the oxidation of DPNH by peroxidase, Mn^{2+} and oxygen[8,9]. One of the most striking chemical properties of ergothioneine is the ease of the oxidative removal of the sulfur atom by ferric chloride or bromide water and its oxidation to sulfuric acid[15]. Ergothioneine disulfide is unstable in aqueous solution, particularly at alkaline pH[10], and it has been suggested[1] that this instability may be due to the hydrolytic cleavage of the disulfide bond as observed by BALABAN AND KING[16] for a thioimidazole disulfide. Sulfur dioxide is one of the products of this reaction. The possibility that the stimulatory effect of ergothioneine on DPNH oxidation by peroxidase, Mn^{2+} and oxygen may be due to the cleavage of the sulfur atom from ergothioneine and its stepwise oxidation through a sulfite ion intermediate prompted a comparison of the stimulatory effect of ergothioneine and sulfite ions on DPNH oxidation under identical conditions. Ergothioneine was found to stimulate the oxidation of DPNH in catalytic concentrations whereas sulfite ions were effective in stoichiometric concentrations under the conditions employed in Fig. 5. Approximately 1.0 μmole of sulfite was required for the complete oxidation of 0.3 μmole of DPNH. A second aliquot of DPNH (0.3 μmole) added after the complete oxidation of 0.3 μmole of DPNH by peroxidase, Mn^{2+}, oxygen, catalase and sulfite ions (1.0 μmole) was oxidized at the same rate as in the absence of sulfite ions. On the other hand, a second aliquot of DPNH was oxidized at 80% of the rate of the initial aliquot when the sulfite ions were replaced by ergothioneine (0.1 μmole). This suggests that sulfite ions rapidly disappear from the reaction mixture, whereas ergothioneine is only partially destroyed under the conditions employed in Fig. 5.

The stimulatory effect of a combination of ergothioneine and sulfite ions on the oxidation of DPNH by peroxidase was very much less than the effect of either ergothioneine or sulfite ions alone under the conditions employed in Table I. The aerobic oxidation of sulfite ions is initiated by peroxidase in the presence of Mn^{2+} or certain phenolic compounds such as thyroxine or estradiol and this reaction is inhibited by a number of substances presumably as a result of their ability to react with free radical intermediates in the chain reaction[9]. The oxidation of sulfite ions under these conditions is inhibited also by ergothioneine.

The stimulatory effect of a combination of ergothioneine and either thyroxine or

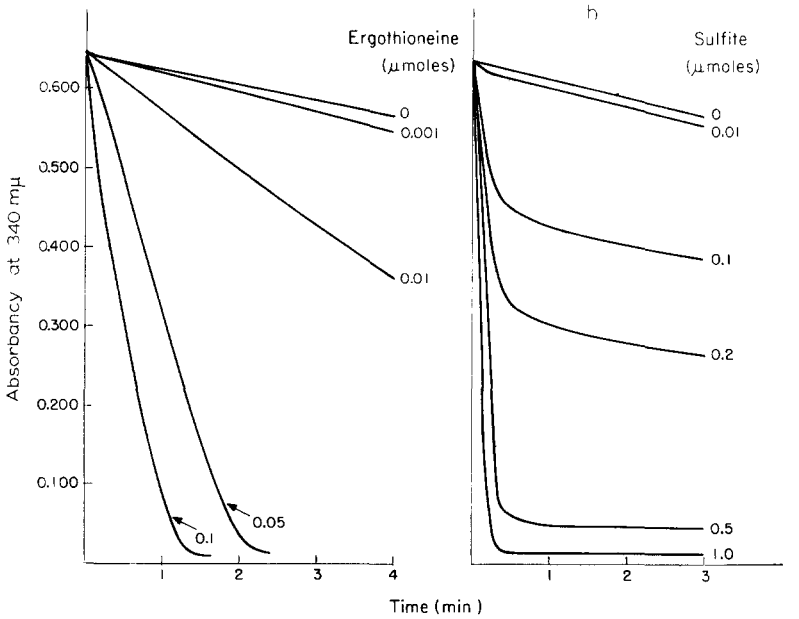

Fig. 5. Effect of variation of ergothioneine and sulfite concentration. The reaction mixture contained 200 μmoles of phosphate buffer pH 7.0, 0.3 μmole of DPNH, 0.5 μmole of $MnCl_2$, 100 μg of horseradish peroxidase, 7500 units of catalase, and water to a final volume of 3.0 ml. Ergothioneine (a) or sodium bisulfite (b) were added in the amounts indicated.

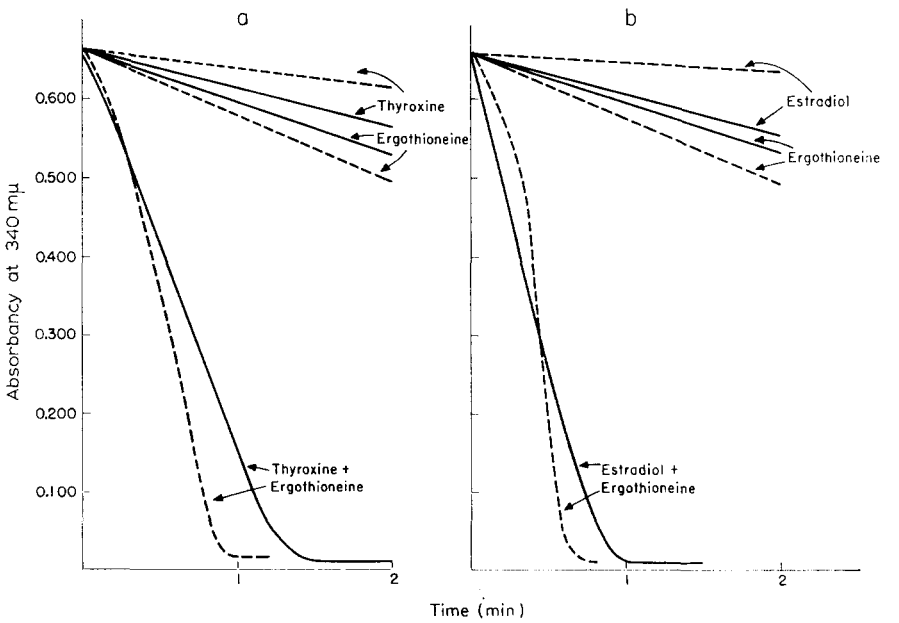

Fig. 6. Effect of a combination of thyroxine and ergothioneine, or estradiol and ergothioneine. The reaction mixture contained 200 μmoles of phosphate buffer pH 7.0, 0.3 μmole of DPNH, 0.5 μmole of $MnCl_2$, 5 μg of horseradish peroxidase and water to a final volume of 3.0 ml. The reaction mixtures represented by the dotted lines contained, in addition, 7500 units of crystalline catalase. Thyroxine (0.05 μmole), estradiol (0.05 μmole) and ergothioneine (1.0 μmole) were added as indicated.

estradiol on the Mn^{2+}-dependent aerobic oxidation of DPNH by peroxidase was considerably greater than additive (Fig. 6). In this experiment, the peroxidase concentration was decreased until thyroxine, estradiol or ergothioneine alone had relatively little effect at the concentrations employed. Catalase, in the concentration employed in Fig. 6, inhibited the oxidation of DPNH in the presence of thyroxine or estradiol alone, as previously described[5,6] and increased the rate of oxidation of DPNH in the presence of ergothioneine alone (see also Fig. 3). In the presence of a combination of thyroxine and ergothioneine, or estradiol and ergothioneine, catalase produced an initial inhibition of DPNH oxidation which was followed by a stimulation.

TABLE I

EFFECT OF A COMBINATION OF ERGOTHIONEINE AND SULFITE IONS

As for the complete system in Fig. 3a except that ergothioneine (1.0 μmole) and sodium bisulfite (1.0 μmole) were added as indicated.

Additions	*Absorbancy change per min at 340 mμ*
—	0.030
Ergothioneine	0.385
Sulfite ions	0.500
Ergothioneine + sulfite ions	0.045

DISCUSSION

Certain phenolic compounds[3,5,6] and aromatic amines[7] stimulate the Mn^{2+} dependent aerobic oxidation of DPNH (or TPNH) by peroxidase. These compounds are believed to act as oxidation–reduction catalysts, being alternately oxidized, probably to a free radical intermediate, by the peroxidase system and reduced by DPNH (or TPNH). Sulfite ions also stimulate the oxidation of DPNH by peroxidase, Mn^{2+} and oxygen[8,9]. Stoichiometric concentrations of sulfite ions are required, and it has been proposed[9] that DPNH is oxidized by highly reactive intermediates formed during the aerobic oxidation of sulfite ions. The latter is a chain reaction initiated by free radicals generated by certain peroxidase catalyzed reactions[8,9,17]. The present paper reports on the stimulatory effect of ergothioneine on the Mn^{2+}-dependent aerobic oxidation of DPNH by peroxidase. Ergothioneine, when adequately protected by the addition of catalase, is effective in catalytic concentrations (Fig. 5). The ease of oxidation of ergothioneine by peroxidase under certain conditions (see Figs. 1 and 2) suggests that ergothioneine acts as an oxidation–reduction catalyst in this reaction. The stimulatory effect of ergothioneine on DPNH oxidation is shared by 2-mercaptohistidine, 2-mercaptoimidazole and 1-methyl-2-mercaptoimidazole, but not by histidine or imidazole. These observations implicate the thiol (or thione) group. The sulfhydryl radical is the initial oxidation product of ergothioneine, and the operation of an ergothioneine–ergothioneine sulfhydryl radical oxidation reduction system would be analogous to the systems proposed for the catalytic effect of certain monophenols[3,6,9] and aromatic amines[7] on the aerobic oxidation of DPNH by peroxidase.

The oxidation of ergothioneine to the disulfide form by equivalent amounts of H_2O_2 was reported by HEATH AND TOENNIES[10]. That ergothioneine disulfide is one of the products of ergothioneine oxidation by peroxidase under the conditions employed

in Figs. 1 and 2 is suggested by the spectral changes and by its reduction to ergothioneine by glutathione or cysteine. Several observations suggest, however, that the stimulation of DPNH oxidation by ergothioneine is not due to the operation of an ergothioneine–ergothioneine disulfide oxidation–reduction system. Ergothioneine disulfide is very much less effective than ergothioneine as a stimulant of the Mn^{2+}-dependent aerobic oxidation of DPNH by peroxidase. The instability of ergothioneine disulfide in aqueous solution[10] suggests that the slight stimulatory effect of ergothioneine disulfide may be due to its partial degradation to ergothioneine. Furthermore, H_2O_2 which would be expected to promote the oxidation of ergothioneine to the disulfide form, decreases the stimulatory effect of ergothioneine on DPNH oxidation (Fig. 4a), whereas catalase potentiates the effect of ergothioneine on DPNH oxidation in concentrations which inhibit the aerobic oxidation of ergothioneine to the disulfide form by peroxidase Mn^{2+}, DPNH and thyroxine (Fig. 4b).

The oxidative degradation of ergothioneine with the release of sulfite ions into the solution also does not appear to be the mechanism for the stimulation of DPNH oxidation since the stimulatory effect of ergothioneine and sulfite ions differ in several respects. Ergothioneine is active in catalytic concentrations with little loss in stimulatory activity, whereas sulfite ions are effective in stoichiometric concentrations with a complete loss of stimulatory activity. Furthermore, a combination of sulfite ions and ergothioneine had little stimulatory activity under conditions in which either alone has a marked stimulatory effect on DPNH oxidation (Table I). This mutual antagonism between sulfite ions and ergothioneine may result from the formation of an addition compound similar to that proposed by BALABAN AND KING[16] to account for the absorption of sulfur dioxide by 2-thiolglyoxalines. It is of interest that both the sulfite stimulated[9] and the ergothioneine stimulated (Fig. 3) oxidation of DPNH by peroxidase show a similar preference for certain buffers.

Ergothioneine stimulates the oxidation of reduced pyridine nucleotides by a number of mammalian peroxidases. Myeloperoxidase and lactoperoxidase were employed in the present study. Ergothioneine also has been found to stimulate the oxidation of DPNH by an enzyme preparation from beef thyroid and from rat uterus, stomach, small intestine, large intestine, spleen and lung (S. J. KLEBANOFF, unpublished data). The concentration of ergothioneine required to stimulate the oxidation of DPNH *in vitro* is well within the concentration found in a number of mammalian tissues (see ref. 1) particularly when ergothioneine is combined with either catalase or a phenolic catalyst such as thyroxine or estradiol. This suggests that ergothioneine may function in certain tissues as a stimulant of the oxidation of reduced pyridine nucleotides by a peroxidase present in those tissues. The observation that the combined effect of ergothioneine and thyroxine, or ergothioneine and estradiol on the oxidation of DPNH is considerably greater than additive raises the question of a possible relationship between the actions of peroxidase, ergothioneine and thyroxine or estradiol in certain mammalian tissues. This possibility is under investigation.

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SOME CHARACTERISTICS OF THE PYRIMIDINE TRANSPORT PROCESS OF THE SMALL INTESTINE*

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SUMMARY

Uracil is actively transported across the wall of the rat, hamster, and frog small intestine *in vitro*. The transport process requires oxygen, is blocked by a number of substances which interfere with cell metabolism, and is inhibited by pyrimidines with chemical structures similar to that of uracil.

INTRODUCTION

An earlier study of pyrimidine absorption in the intact rat revealed that uracil and thymine cross the intestinal epithelium by both active transport and passive diffusion[1]. Active transport is the predominant mode of absorption at low concentrations of the pyrimidines, whereas passive diffusion predominates at high concentrations at which the active transport process is saturated. The transport mechanism appears to be different from those responsible for the active transfer of sugars and amino

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