

**PURIFICATION AND PROPERTIES OF GLUTATHIONE PEROXIDASE**

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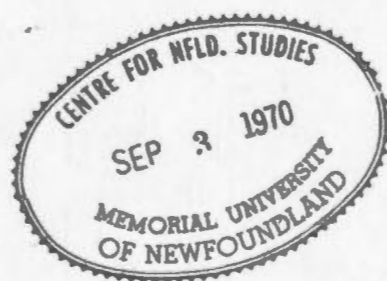
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PURIFICATION AND PROPERTIES OF GLUTATHIONE PEROXIDASE

A thesis

by

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Department of Biochemistry  
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## ABSTRACT

Glutathione peroxidase catalyses the decomposition of a wide range of hydroperoxides. In the present work this enzyme was purified approximately 2,500-fold from pig's blood.

The kinetics of glutathione peroxidase were shown to be rather complex. Using glutathione as substrate, a linear Lineweaver-Burk plot was obtained with an approximate  $K_m$  value of 3 mM.

Using cumene hydroperoxide as substrate kinetic analysis on the purified enzyme gave non-linear Lineweaver-Burk plots.

A wide range of nucleotides inhibited the enzyme, pyridine nucleotides exerting the strongest inhibition. Also, inhibitory effectiveness increased with the number of phosphate groups in the nucleotide. Nucleotide inhibition was competitive with respect to GSH and of a mixed nature with respect to hydroperoxides. In addition, increased levels of hydroperoxides enhanced nucleotide inhibition.

The inhibitory action of nucleotides on enzyme activity could be substantially decreased by x-ray, ethanol or trypsin treatment with a lesser decrease of catalytic activity. Conversely, heat, pCMB, or sodium lauryl sulfate preferentially abolished the catalytic function with a lesser effect on the nucleotide inhibition.

It was concluded that nucleotides interact with the enzyme at a site other than the active center and hence that glutathione peroxidase is an allosteric enzyme.

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## INTRODUCTION

### 1. Peroxidation in non-biological systems

Apparently in the following phenomena: production of brown oils, film formation in applied protective oil coatings, rancidification of fats, there is a spontaneous reaction between atmospheric oxygen and some organic compounds. The main reaction is the autoxidation of unsaturated lipids. It is generally accepted that the initial phases of this reaction result in the formation of hydroperoxides of unsaturated fatty acids.

The mechanism of peroxidation in vitro is now well understood and extensively reviewed (1, 2). The initiation of the reaction depends on the formation of free radicals. As oxygen is a diradical it reacts with initial radical species to form a peroxy radical which can then propagate by a chain reaction. The chain reaction is terminated by radical scavengers such as antioxidants or by radical-radical combination.

The peroxidation of unsaturated lipids is affected by light, heat, concentration of oxygen and the presence of catalysts or inhibitors. Autoxidation of hydrogen donors such as dihydric phenols, ascorbic acid, hydroquinones, etc., results in  $H_2O_2$  formation, the reaction being catalysed by metals. Heavy metals, especially those having two valence states (Fe, Cu, Co) generally stimulate lipid peroxidation.

Hemoglobin and other haematin compounds act as catalysts by decreasing the activation energy of the reaction and shortening the induction period (3).

## 2. Peroxide formation in biological systems

Peroxides in biological systems may be dietary in origin or generated intracellularly. Living cells contain unsaturated fatty acids such as linoleic, linolenic and arachidonic acids, which, as constituents of phospholipids, form important parts of the structure of the cells, especially membranes.

The types of peroxides produced in the animal or plant cells include hydrogen peroxide and hydroperoxides of unsaturated lipids and to a small extent, of nucleic acids (4), squalene (5), flavins and pteridines, certain amino acids (6) and steroids (7).

The formation of peroxides in the cell is governed by the availability of peroxidisable substances, oxygen tension and the presence of metal or enzymic catalysts, antioxidants and hydrogen donors.

## 3. Factors affecting peroxidation of organic compounds

### a. Metal catalysed peroxidation

The influence of metal catalysis on lipid peroxidation has been known for a long time (8). The most active metal

catalysts are those which have two oxidation states interconvertible by a single electron transfer (Fe, Cu, Co).

Wills (9) found that only a relatively few metals:  $\text{Co}^{2+}$ ,  $\text{Mn}^{2+}$ ,  $\text{V}^{2+}$ ,  $\text{Fe}^{3+}$  and  $\text{Fe}^{2+}$  can produce a rapid rate of peroxidation. The most active catalysts are  $\text{Co}^{2+}$  and  $\text{Mn}^{2+}$  in concentration range  $10^{-4} - 10^{-3}\text{M}$ , whilst  $\text{Cu}^{2+}$ ,  $\text{Fe}^{2+}$  and  $\text{Fe}^{3+}$  are weakly active. The catalytic activity of  $\text{Fe}^{3+}$  can be strongly stimulated by addition of hydrogen donors such as ascorbic acid or cysteine which reduce  $\text{Fe}^{3+}$  to the more active  $\text{Fe}^{2+}$  ion.

In vivo, most metal ions are complexed with amino acids or proteins; the effect of chelation on metal-catalysed fatty acid peroxidation is very important. In tissue homogenates it is more likely that lipid peroxide formation is catalysed by the most abundant of the catalytic metals, namely iron, either in the free state or bound in a porphyrine complex.

The tissues isolated from animals contain very little lipid peroxide (9), but the level increases markedly after incubation in vitro. In tissue homogenates, lipid peroxides are formed most readily at pH 5.5 and the rate of formation is increased by addition of ascorbate or  $\text{Fe}^{2+}$ . Bernheim (10) observed that the addition of  $1\text{ }\mu\text{M}$   $\text{Fe}^{2+}$ , which represents the iron concentration in mitochondria, is involved in the ascorbate catalysis since  $5\text{ }\mu\text{M}$  EDTA completely inhibits the reaction.

#### b. Hematin catalysed peroxidation

Wills (11) reported that the conversion of iron to an iron chelate can greatly increase its catalytic activity and

indeed haemoproteins are very effective catalysts (12). Tappel (12) noted no changes in Fe redox state during hematin-induced peroxidation. Once formed the heme-lipid peroxide complex decomposes intramolecularly with scission of the o-o bond to form two free radicals. The last hypothesis about lipid peroxidation catalysed by haemin compounds was presented by Tarladgis (13), who claimed that the spin state of Fe in the heme compounds governs the catalytic activity and not the oxidation state of the metal. The high spin state is the active one and any treatment (e.g. suitable ligands) which converts this state to the low spin one, delays the onset of lipid peroxidation.

c. Enzymic catalysis of peroxidation

Lipoxidase is an enzyme which catalyses the direct oxidation of unsaturated fatty acids containing cis 1,4 penta-diene systems. This enzyme was found only in legumes, some cereal grains and oil seeds (14).

In 1963 Hochstein and Ernster (15) reported an enzymic system which catalyses lipid peroxidation in liver microsomes. This complex reaction requires the presence of oxygen, NADH oxidase, non-heme iron and nucleoside di- or tri-phosphate.

It is known that free reduced flavins are reoxidized very rapidly by molecular oxygen to form  $H_2O_2$ . Flavoenzymes are involved in metabolism of amino acids, purines, alcohol,

glycolytic intermediates, etc. Peroxisomes, a microbody subcellular fraction, are thought to be the site of hydrogen peroxide metabolism in the cell, since many flavoproteins are located there.

Phagocytosis by polymononuclear leukocytes is accompanied by an increase in oxygen uptake, pentoso phosphate shunt activity, and NADH oxidase activity (16). The  $H_2O_2$  formed during phagocytosis may be involved in the killing of bacteria by leukocytes.

#### d. Irradiation

Because there are similarities between the biological effects of peroxides and of ionizing radiation, the role of peroxides in radiation damage of tissues has been widely investigated.

Hydroperoxides are obtained following irradiation of aqueous solutions of amino acids, nucleic acids and steroids (17). But in vivo, there is no direct evidence for peroxide presence in the tissues of irradiated animals (18, 19).

All the controversial results did not exclude the possibility for a role of peroxides in early stages of irradiation, but they probably have no marked role in the irradiation injury.

#### e. Drug-catalysing peroxide formation

The high concentration of oxyhemoglobin in erythrocytes can be a powerful source of oxygen for the oxidation of various H donors, e.g. hydroxylated drug metabolites. In some individuals with certain hereditary deficiencies, such as

glucose-6-phosphate dehydrogenase deficiency, some drugs, such as antimalarials (primaquine), vitamin K<sub>3</sub>, acetyl-phenylhydrazine, etc., produce hemolysis in vivo as a result of the high concentration of H<sub>2</sub>O<sub>2</sub> formed in the erythrocytes (20, 21). Most drugs are metabolized in liver microsomes, the hydroxylating enzymes requiring NADPH and O<sub>2</sub> for optimal activity. During enzymatic oxidation of NADPH, which occurs in liver microsomes, lipid peroxidation was noted (22).

Slater (23) reported that a single dose of carbon tetrachloride administered to rats produced a considerable accumulation of fats in liver due to the metabolism of the solvent to a more toxic compound, which interacts with the NADPH-ADP-Fe<sup>2+</sup> system from microsomes producing free radicals and lipid peroxides. Phenatiazine and chlorpromazine, acting as free radical scavengers, inhibit the peroxidation.

#### 4. Lipid peroxidation in subcellular fractions

There is insufficient evidence for the presence of lipid peroxides in normal tissues, probably because if they are formed they would probably be rapidly metabolized so that no detectable amounts can accumulate. Peroxidation occurs very rapidly in many organs as soon as the cells are damaged, e.g. during homogenisation. The extent of lipid peroxidation occurring in any subcellular fractions depends on several factors including: the unsaturated fatty acids content of the

lipids, the availability of catalysts (metal ions or hemo-proteins) and antioxidants such as  $\alpha$ -tocopherol, selenium (24).

Since small amounts of EDTA inhibit the peroxidation in subcellular fractions and a trace of  $\text{Fe}^{2+}$  or  $\text{Fe}^{3+}$  reversed the inhibition most workers agreed that this metal in a non-heme state is apparently the catalyst (25). A second factor is the presence of ascorbate. With microsomes NADPH can substitute for ascorbate. Lipid peroxidation is associated with ascorbate or GSH + GSSG-induced swelling of mitochondria (26).

Christophersen (27) found that in liver homogenates lipid peroxidation starts as soon as the supply of endogenous GSH is exhausted by oxidation. Glutathione is therefore an important antioxidant but only in the reduced state.

Most of the controversial results concerned with lipid peroxidation in vivo are due to the lack of a satisfactory test. The most widely used method for measuring lipid peroxidation is the thiobarbituric acid test (TBA) based on the color development on reaction of TBA with certain decomposition products of lipid peroxides mainly malonic dialdehyde. Metal contaminants greatly influenced this method (28). Niehaus and Samuelson (29) demonstrated that most of the malonal aldehyde produced during microsomal lipid peroxidation came from the arachidonic acid of tissue phospholipids.

In general, it seems that there is no completely satisfactory test for lipid peroxides in biological systems. It

is therefore possible that lipid peroxides may be of much greater importance and occurrence in biological systems than is currently realized.

#### 5. Consequence of peroxidation

Peroxides are very powerful oxidising agents, which may damage or destroy many biological compounds; their effects might be partly explained by reaction with protein SH groups, methionine, tyrosine and heme compounds (30).

Lipid peroxides in subcellular fractions lead to increased membrane permeability, lysis and formation of membrane ghosts (26). Lipid peroxides were reported to damage cytochromes (30) and different enzymes. Lipid peroxidation occurs in erythrocytes of patients with paroxysmal nocturnal hemoglobinuria (31).  $H_2O_2$  and organic peroxides were reported as mutagenic agents (32) and to be involved in the formation of prostaglandins (33).  $H_2O_2$  and lipid peroxides were claimed to be involved in many pathological states such as drug-induced methemoglobinemia, ageing, hyperoxia, atherosclerosis, cancer (reviewed by Barber (34)).

#### 6. Peroxide decomposition

$H_2O_2$  is produced normally in the cells through the metabolic processes in an amount ranging from 1 - 10 m moles  $H_2O_2$ /kg of wet tissue/hr (35) but it is maintained in a non-toxic level by the combined action of catalase and glutathione peroxidase.

a. Non-enzymic factors

The transition metals can catalyse not only lipid peroxide formation but also their decomposition. O'Brien (36) found that the effectiveness for LAHPO decomposition was  $\text{Fe}^{2+} > \text{Fe}^{3+} > \text{Cu}^{2+} > \text{Co}^{2+}$ . EDTA and cyanide completely inhibit the reaction. Hydrogen donors such as ascorbate and cysteine markedly stimulate LAHPO decomposition by  $\text{Fe}^{3+}$  or  $\text{Cu}^{2+}$ .

Since hemoproteins catalyse lipid peroxide formation, their ability is dependent on their concentration. Banks and colab (37) and Lewis and Wills (38) showed that haem compounds in higher concentration ( $> 10^{-4}$  M) inhibit lipid peroxidation. O'Brien (36) found that hematin compounds were more efficient than inorganic iron in LAHPO decomposition.

The order of effectiveness of hemoproteins at decomposing LAHPO was methemoglobin > cytochrome c > oxyhemoglobin > myoglobin. O'Brien (36) also found that LAHPO could be decomposed by nucleophiles. The order of efficiency was: dimercaptopropanol > cysteine > ascorbate > thiourea. Caldwell and Tappel (39) reported hydroperoxide decomposition by selenocysteine, since selenium compounds can mimic antioxidant properties of vitamin E in vivo.

b. Products of lipid peroxide decomposition

Organic peroxide decomposition involves the scission of the o-o bond to form free radicals and also 1,2 diene conjugation. Due to biological conditions, the products of lipid

peroxide decomposition will give a complex range of products. As O'Brien (36) and Christophersen (40) reported the main products of enzymatic decomposition of LAHPO or decomposition catalysed by nucleophiles were 9-hydroxy 10,12 and 13 hydroxy 9, 11 octadecadienoic acids. The decomposition of LAHPO by metals or hemoproteins give more complex products, presumably because of the free radical nature of the reaction (36).

### c. Enzymic Decomposition

Considerable lipid peroxidation occurs in tissue homogenates and subcellular fractions. Wills (11) reported that tissue homogenates at high concentrations also catalyse peroxide decomposition.

O'Brien and Little (41) reported the decomposition of LAHPO in vitro was catalysed by subcellular fractions. They found that the amount of LAHPO decomposed per unit time was proportional to the amount of the fraction in the system. Since cyanide and ascorbate had no effect on LAHPO decomposition by the supernatant fraction, hematin compounds or transition metal ions seemed not to be involved. The catalytic ability of mitochondria and microsomes in decomposing LAHPO was slightly affected by previous boiling or azide incubation, suggesting that no enzymic peroxidase or catalase was involved. The decomposition of LAHPO by supernatant gave different results since boiling and dialysis inhibited the reaction. Glutathione was found to be the dialysable cofactor and the products formed were similar to that found for the decomposition of LAHPO by nucleophiles.

The decomposition of LAHPO by supernatant is inhibited by SH reagents (pCMB, iodoacetamide) proportional to the amount of supernatant fraction present, stimulated by NADPH and has an alkaline pH optimum. The kinetics and properties of this system were similar to those of glutathione peroxidase discovered by Mills and Randall in erythrocytes (42). Further evidence that supernatant peroxidase was identical to erythrocyte glutathione peroxidase was the discovery that the purified enzyme could utilize  $H_2O_2$  as well as LAHPO as substrate for the oxidation of GSH. Glutathione peroxidase, unlike hemoprotein peroxidases, was not inhibited by cyanide, CO or azide.

Glutathione peroxidase was located in the mitochondria and supernatant fraction but not in the microsomal fraction.

Neubert et al (43) have showed that contraction factor II of mitochondria is glutathione peroxidase and that during glutathione-induced lipid peroxidation, as a result of mitochondrial swelling, the enzyme is released. Green and O'Brien (44) have recently shown that glutathione peroxidase was released from mitochondria during swelling induced by several different agents and is located in the matrix.

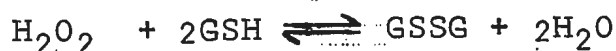
The capacity of different organs or tissues in removing intracellular hydroperoxides probably depends on the local levels of glutathione peroxidase as well as on non-enzymic factors (presumably hemoproteins).

Glutathione Peroxidase (GSH: H<sub>2</sub>O<sub>2</sub> oxidoreductase, EC 1.11.1.9)

Glutathione peroxidase was first detected in erythrocytes as a consequence of its ability to protect hemoglobin from oxidative breakdown (45). Mills and Randell (42) used ascorbate as a stressor agent and oxidation of hemoglobin was attributed to H<sub>2</sub>O<sub>2</sub> generated from a reaction between ascorbic acid and oxyhemoglobin. In red cells incubated with azide in order to inhibit catalase, marked formation of methemoglobin and choleglobin occurred. Hemoglobin could be protected by adding glucose.

Cohen and Hochstein (46) established that the oxidation of glutathione is the major pathway of H<sub>2</sub>O<sub>2</sub> metabolism in the intact erythrocyte and that glutathione peroxidase activity is linked to pentose monophosphate shunt. These authors have also reported the generation of H<sub>2</sub>O<sub>2</sub> in erythrocytes following incubation with some drugs such as phenylhydrazine, hydroquinone, antimalarials (21, 47).

The catalytic reaction has been represented by the following equation:

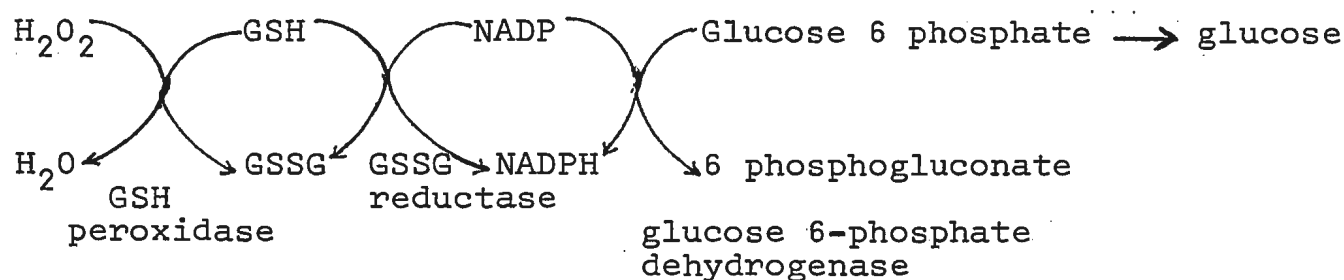


This protective system has a lasting effect only when oxidized glutathione is continuously reduced. In erythrocytes the NADP-linked reactions of the pentose monophosphate shunt in connection with glutathione reductase maintains glutathione in reduced form.

An alternate source, in other tissues is from malate

and isocitric dehydrogenases, but not in erythrocytes, where an intact Krebs cycle is missing. Jacob and Jandl (48) showed that the rate of glucose metabolism by the pentose monophosphate shunt pathway depends on the ratio of oxidised to reduced glutathione; an increased metabolic rate results from an increase of NADP produced by GSSG reductase. Hydrogen peroxide stimulated this pathway by GSSG formation via glutathione peroxidase.

The relationship of these reactions and the place of glutathione peroxidase in the intermediary metabolism is shown in the scheme:



The enzyme has been purified from bovine erythrocytes and shown to be distinct from both catalase and hemoglobin (49). Its presence was noted in normal rat and mouse tissues and in certain tumor cells (50).

In the lens, where catalase activity is weak, glutathione peroxidase decomposes  $\text{H}_2\text{O}_2$  in a reaction coupled with the autoxidation of ascorbic acid (51).

Glutathione peroxidase was partially purified from erythrocytes by Mills (49), Paglia and Valentine (52), Schneider and Flohé (53) and from liver by Mills (54), Little

and O'Brien (55) and from lens by Holmberg (56).

Some of the kinetics of glutathione peroxidase were reported by Paglia (52) and Schneider and Flohé (53).

In the following study the kinetics, the regulatory properties and the effects of inhibitors are presented.

## MATERIALS AND METHODS

### 1. Materials

All biochemicals used in this work were purchased from Sigma Chemical Company, St. Louis, Mo. Cumene hydroperoxide was obtained from Matheson, Coleman and Bell, Ohio. All chemical reagents used were of the analytical grade of purity.

### 2. Methods

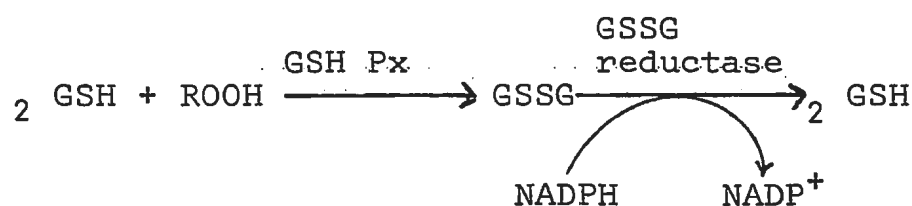
#### The method of assay

The first procedure assay for glutathione peroxidase was described by Mills (42) and was based on the ability of the enzyme to prevent the oxidative breakdown of hemoglobin. The amount of enzyme producing a 50% inhibition of choleglobin formation was designated to represent 1 unit of enzyme activity. As Mills himself reported, this procedure could be used only as a qualitative assay.

The second procedure was also described by Mills (42); the rate of oxidation of GSH by hydrogen peroxide was taken as a measure of glutathione peroxidase activity. This method is more accurate, but is rather laborious since the rate of GSH oxidation is obtained by titrating the unreacted GSH at different times during the reaction using the method of Boyer (57), or polarographically (53).

The third procedure is derived from the second in that it measures the rate of GSH oxidation by  $H_2O_2$  or lipid

hydroperoxides as catalysed by glutathione peroxidase, but instead of measuring progressive loss of GSH, the latter is maintained at a constant level by addition of exogenous glutathione reductase and NADPH. So, this procedure mimics to a certain extent the physiological role of GSH peroxidase:



According to this procedure glutathione peroxidase activity is estimated from the rate of GSSG formation by following the decrease in absorbance of the reaction mixture at 340 mμ as NADPH is converted to NADP<sup>+</sup>.

This is also the method of assay which was normally used in this work. The concentrations of components used were: 0.3 mM GSH, 0.12 mM NADPH, 1.2 mM EDTA, 0.086 M Tris-HCl buffer pH 7.0 or 8.5 and H<sub>2</sub>O to 1 ml. As substrate, cumene hydroperoxide 0.15 mM was used.

One unit of enzyme activity was defined as causing a change in E<sub>340</sub> of 1 optical density unit/minute under the above conditions of pH at 22°C.

As GSSG reductase is known to be inhibited by high concentration of glutathione, frequent checks were made to ensure that the peroxidase and not the reductase was rate limiting. Large excesses of reductase were therefore used. Cumene hydroperoxide was used because it is not affected by

catalase, is more stable and is more readily available than LAHPO.

For experiments where GSSG effects on the enzyme were studied, the enzyme activity was measured by titration of residual GSH at different times during the reaction using the method of Boyer (57). The reaction mixture was 0.1 M Tris-HCl pH 8.5, GSH 0.035 - 0.2 mM, CuOOH 0.03 - 0.2 mM. In order to stop the reaction 0.25 mM pCMB was added.

From the introduction it was obvious that certain metal ions especially  $\text{Fe}^{2+}$  and  $\text{Fe}^{3+}$  and also hemoproteins could be competitive with glutathione peroxidase for decomposing hydroperoxides. Furthermore, these compounds are very effective catalysts of thiol (58) and NADPH oxidation by hydroperoxides.

It was important to know if EDTA in concentrations used in the assaying method (1.2 mM) was enough to keep non-enzymic reaction (GSH oxidation) at a low level.

From the results presented in Table I the influence of  $\text{Fe}^{2+}$  of  $\text{Fe}^{3+}$  in assaying method might exceed chelating capacity of EDTA used from a concentration higher than 0.5 mM which is very unlikely to obtain during enzyme purification. However, EDTA will not effectively chelate heme iron.

As Lewis and Wills (38) and O'Brien showed, hemoproteins are powerful catalysts of lipid peroxide decomposition. As hemoproteins are present in great concentration in erythrocytes (95% of total proteins) and tissues, it was necessary to study their effect on assaying procedure.

TABLE I

THE INFLUENCE OF  $\text{Fe}^{2+}$  AND  $\text{Fe}^{3+}$  ON THE ASSAYING PROCEDURE\*

<u>Concentration</u> <u>mM</u>	<u><math>\text{Fe}^{2+}</math></u>	<u><math>\text{Fe}^{3+}</math></u>
0	0.35	0.35
0.025	0.358	0.35
0.050	0.386	0.35
0.1	0.395	0.35
0.5	0.43	0.40
1	0.48	0.53
2.5	0.64	0.69

\* The results were expressed as  $\Delta E_{340}/\text{min}$

TABLE II

THE INFLUENCE OF HEMOPROTEINS ON THE ASSAYING PROCEDURE\*

<u>Concentration</u> <u>mM</u>	<u>HbO<sub>2</sub></u>	<u>MetHb</u>
0	0.35	0.35
1	0.35	0.40
2	0.35	0.54
5	0.40	0.95
50	0.44	--

\* The results are expressed as  $\Delta E_{340}/\text{min.}$  The experiment was carried out at pH 7.0.

Oxyhemoglobin was prepared from pig blood by DEAE-cellulose column chromatography.

The results presented in TABLE II showed that oxyhemoglobin does not interfere strongly in the assay. Hydroperoxides oxidize oxyhemoglobin to methemoglobin which, it can be seen, is a much better catalyst. However, in the assay system used, oxyhemoglobin oxidation was relatively slow and significant conversion did not occur during the assay time.

As hemoglobin in erythrocytes and tissues is kept tightly in reduced form it could interfere in the assay only in hemolysate or in the homogenate of a tissue. The possible interference is of course lost during purification procedure. Anyway its possible influence on kinetics and inhibitors action was studied and will be presented later.

The assays were performed at a UNICAM SP800 spectrophotometer with a YEW laboratory recorder (Japan).

Unless otherwise stated all operations were carried out at 2 - 5°C.

Electrophoresis was performed in a Gelman apparatus using cellulose acetate strips. The procedure was carried out using 0.05 M Tris-barbital buffer, pH 8.5, at 1.25 mA per strip for 2 hours.

The enzyme was irradiated in glass tubes in the presence of the air at 0°. The irradiation source was a

Philips MG100 x-ray machine. The radiation parameters were 100 kv, 10 mA with 0.5 mm Be filtration. The dose rate was 2 kR/min.

### Abbreviations

The following abbreviations are used in this work:

GSH = reduced glutathione; GSSG = oxidized glutathione; GSSG red = glutathione reductase; GSH Px = glutathione peroxidase; LAHPO = linoleic acid hydroperoxide; CuOOH = cumene hydroperoxide; PPP = pentose monophosphate pathway; HbO<sub>2</sub> = oxyhemoglobin; MetHb = methemoglobin; pCMB = para chloro-mercuribenzoate; NEM = N-ethylmaleimide; NBS = N-bromosuccinimide.

## RESULTS

### 1. Purification of glutathione peroxidase -

#### Preparation of glutathione peroxidase from pig erythrocytes

According to Little and O'Brien (55) blood is the richest source of glutathione peroxidase and most of the previous preparations were obtained from erythrocytes. The main problem in preparation of an enzyme from erythrocytes is the removal of hemoglobin which takes account of more than 95% of total erythrocyte proteins. There are several procedures for removing the hemoglobin but neither is perfect. Mills (40) used Keilin's procedure (59) involving ethanol and acetone precipitation. Paglia and Valentine (52) used ammonium sulfate precipitation and Schneider and Flohé (53) used batch-wise adsorption of DEAE-Sephadex. Because there is not a standard procedure for removing the hemoglobin and for separating it from GSH peroxidase, the best procedure had to be found.

A study performed to find the best conditions for enzyme precipitation gave the following results: glutathione Px is precipitated almost completely by 40% acetone or 55% saturation  $(\text{NH}_4)_2\text{SO}_4$ . In Table III, the results obtained with different procedures for separating glutathione peroxidase from hemoglobin are reported.

According to the results presented above all the procedures cause losses of enzyme activity, but we must not forget the possibility of hemoglobin interference in the

\*

TABLE III

Concen- tration %	% GSH Px activity			% Protein		% GSH Px. inhibition before cen- trifugation	% specific activity in pre- cipitate
	<u>Before centrifuging</u>	<u>Super- natant</u>	<u>Precip- itate</u>	<u>Super- natant</u>	<u>Precip- itate</u>		
(NH <sub>4</sub> ) <sub>2</sub> SO <sub>4</sub> (saturation) 55	89.3	6.1	77.4	56.4	32.7	10.7	2.3
Ethanol 50	82.5	15.6	12.0	16.3	80.5	17.5	-
Acetone 40	88.3	10.8	73.8	67.8	32.9	11.7	2.3
Ethanol + Acetone 50 + 40	86.8	3.8	82.5	69.8	26.5	14.2	3.1
DEAE cellulose 0.8mg/10ml	-	72.8		38.3			

\* The initial hemolysate values for GSH Px activity and for total protein were taken as 100%.

enzyme assay. Of the procedures used, the most suitable seems to be the solvent precipitation by Keilin's method. This procedure gives the highest recovery of enzyme activity and the highest specific activity in the precipitate and also removes most of the hemoglobin.

Batchwise adsorption on DEAE-cellulose is a rather new method and its use is widespread. A disadvantage of this method is the compulsory prior removal of salts, but is unlikely to damage the enzyme.

a. Preparation of hemolysate

After several attempts the following procedure was found to be the most suitable: Pig blood (3.5 l.) containing approximately 5 gm EDTA as anticoagulant, was allowed to stand overnight. The plasma was removed by suction and the sedimented erythrocytes were centrifuged at 1500 x g for 30 minutes and then washed twice with equal volumes of isotonic saline and centrifuged as before. The buffy coat layer which contained all leukocytes and platelets was sucked off. The erythrocytes were then hemolysed by one hour exposure to a solution of aqueous saponine (0.05% w/v) in equal volume. The mixture was centrifuged at 10,000 x g for 15 minutes and the precipitate discarded.

b. Solvent precipitation

The hemolysate was adjusted at pH 6.5 with acetic acid and a 50% (v/v). Ethanol at -20° was added. After

standing at  $-20^{\circ}$  one-half hour, the mixture was centrifuged at  $-5^{\circ}$  with 10,000 x g for 10 minutes and the denatured hemoglobin was discarded. To the supernatant a 40% acetone (v/v) was added at  $-20^{\circ}$  and let stand for 30 minutes and then centrifuged at 10,000 x g for 10 minutes. The precipitate was then dissolved in 0.05 M potassium phosphate buffer pH 6.8.

c. Sephadex CM-50 column

As the removal of hemoglobin by solvent precipitation was not 100% effective, it was found necessary to run a chromatographic column in order to separate some denatured hemoglobin which was not precipitated by solvents. This denatured hemoglobin stuck on the top of the column so very tightly that it could be removed only with a very strong solution of NaCl (2M) or by regenerating procedure.

The solution from previous step was applied to a column (5 x 40 cm) of Sephadex CM-50 ion exchange resin and eluted with the above buffer; the GSH Px did not adhere to the column. The colourless fractions containing GSH Px activity were collected, pooled, and concentrated by pressure filtration through DAAFL0 (Amicon) P-30 ultrafiltration membrane.

d. Sephadex G-100 column

The concentrated enzyme solution was applied to a column (10 x 90 cm) of Sephadex G-100 and eluted with 0.02 M Tris-HCl buffer pH 7.0. The fractions with GSH Px activity were collected and pooled.



Fig. 1

Purification of GSH Peroxidase on Sephadex G100 column (10 x 90 cm)

●—●—● activity of enzyme  
○—○—○ protein

In Fig. 1 a typical elution pattern is shown. The enzyme was eluted before the highest peak of protein which had a yellow colour and a marked absorpition at 410 m $\mu$ .

e. DEAE-cellulose column

The pooled fractions from the previous step were loaded into a column 5 x 40 cm, of DEAE-cellulose and a continuous gradient of potassium phosphate buffer, pH 6.8, from 0.005M to 0.1M was applied. Glutathione peroxidase stuck to the column initially and began to be eluted at approximately 0.07M phosphate. The elution behaviour is presented in Fig. 2. The fractions containing GSH peroxidase activity were pooled and concentrated by pressure filtration as above.

The results obtained with this standardized procedure are exemplified in Table IV.

As can be seen in Table IV the apparent overall purification factor for the enzyme was approximately 10,000. However, the purification procedure presents an unusual facet. After G100 chromatography anomalous high recoveries were always obtained. Since the protein content has decreased slightly, after this step, the activity of GSH peroxidase doubled and the specific activity increased very much. This surprising behaviour, which was always observed, can be explained only by removal of an inhibitor.

TABLE IV

Summary of GSH peroxidase purification

<u>Steps</u>	<u>Volume</u> <u>ml</u>	<u>Total activity</u> <u>GSH Px units</u>	<u>Total protein</u> <u>mg</u>	<u>Specific</u> <u>Activity</u> <u>units/mg</u>	<u>Yield of</u> <u>activity</u> <u>%</u>
Hemolysate	1100	357,000	275,000	1.3	100
Solvent precipitation	160	168,000	928	181	47
CM-50 column	150	120,000	135	890	33
Sephadex G100	820	307,000	114.8	2678	86
DEAE-cellulose	50	205,000	20	10,000	57

Indeed the fractions pooled from the region where protein level was higher exerted an inhibition effect. The nature of this inhibitor was not established yet. Since the increased specific activity obtained after the above purification scheme may be due partly to inhibitor removal in addition to removal of other proteins, no real meaningful purification factor can be given.

Considerable variations were noted in the specific activity of hemolysates from different samples of pigs' blood. These variations could be explained by some hormonal and sex effects on the levels of glutathione peroxidase (60).

#### Estimation of purity

The final preparation of GSH-peroxidase did not exhibit any absorption in visible spectrum, but only a protein characteristic peak at 250 m $\mu$ . That is in agreement with previous work (40, 52, 53, 55).

In an attempt to examine the purity of the present final enzyme preparation electrophoresis on cellulose acetate strips was performed. In Fig. 3 a pattern of electrophoresis behaviour is shown. Similar results were obtained with disc electrophoresis on starch gel. From the results obtained the major protein band showed GSH peroxidase activity. It thus seems possible that the enzyme preparation was substantially pure.

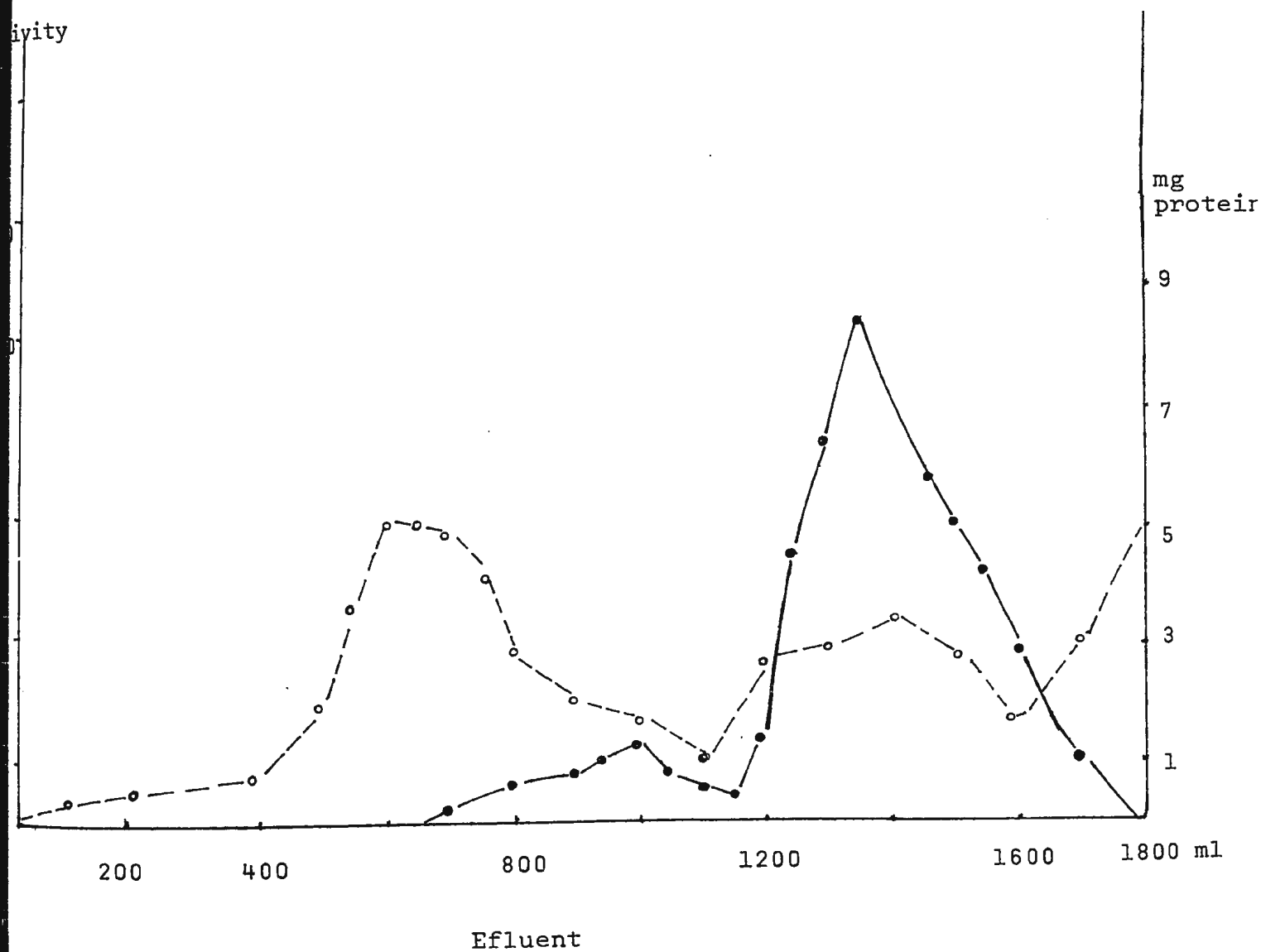


Fig. 2

Purification of GSH-Peroxidase on DEAE-Sephadex column

○—○—○ Activity of enzyme  
 ●- -●- -● protein

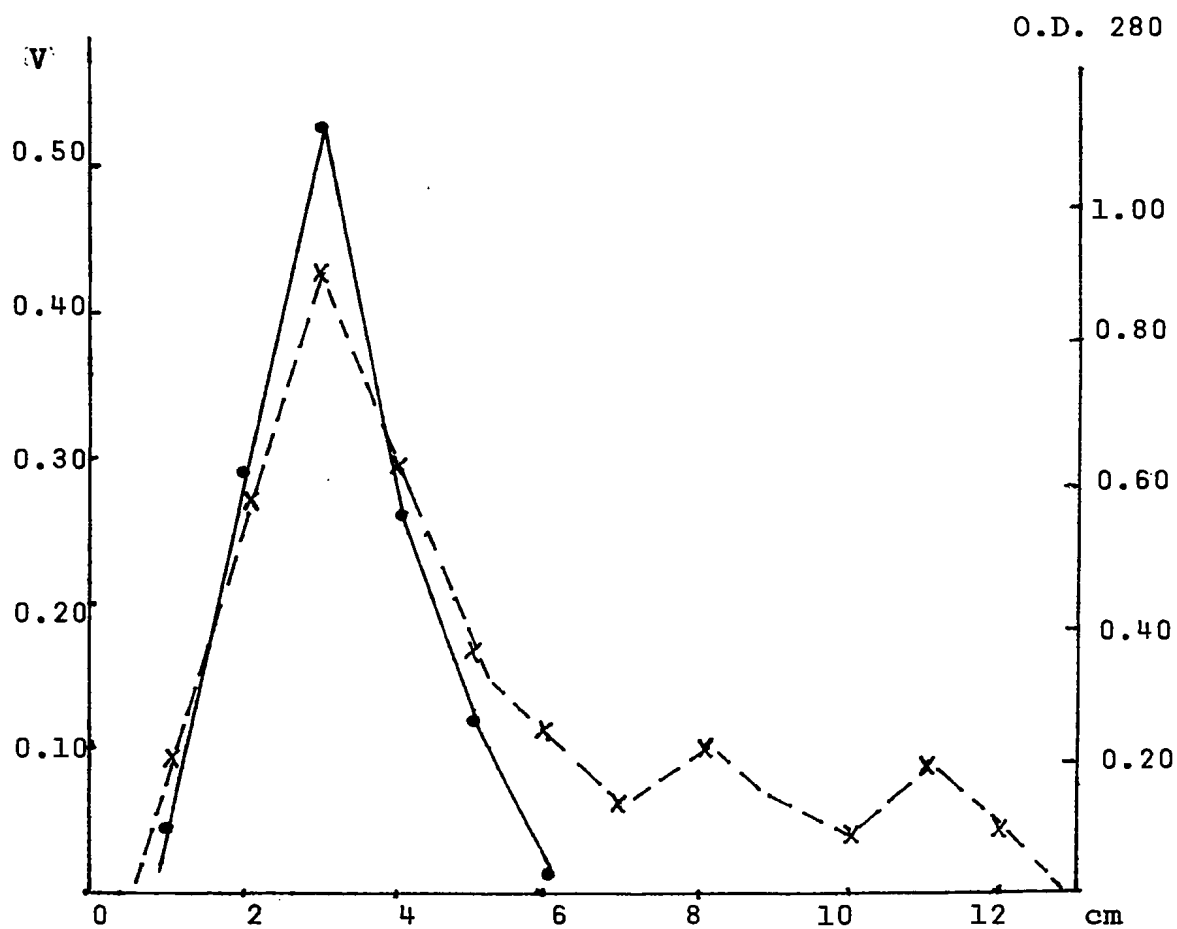


Fig. 3

Electrophoresis pattern of GSH Peroxidase

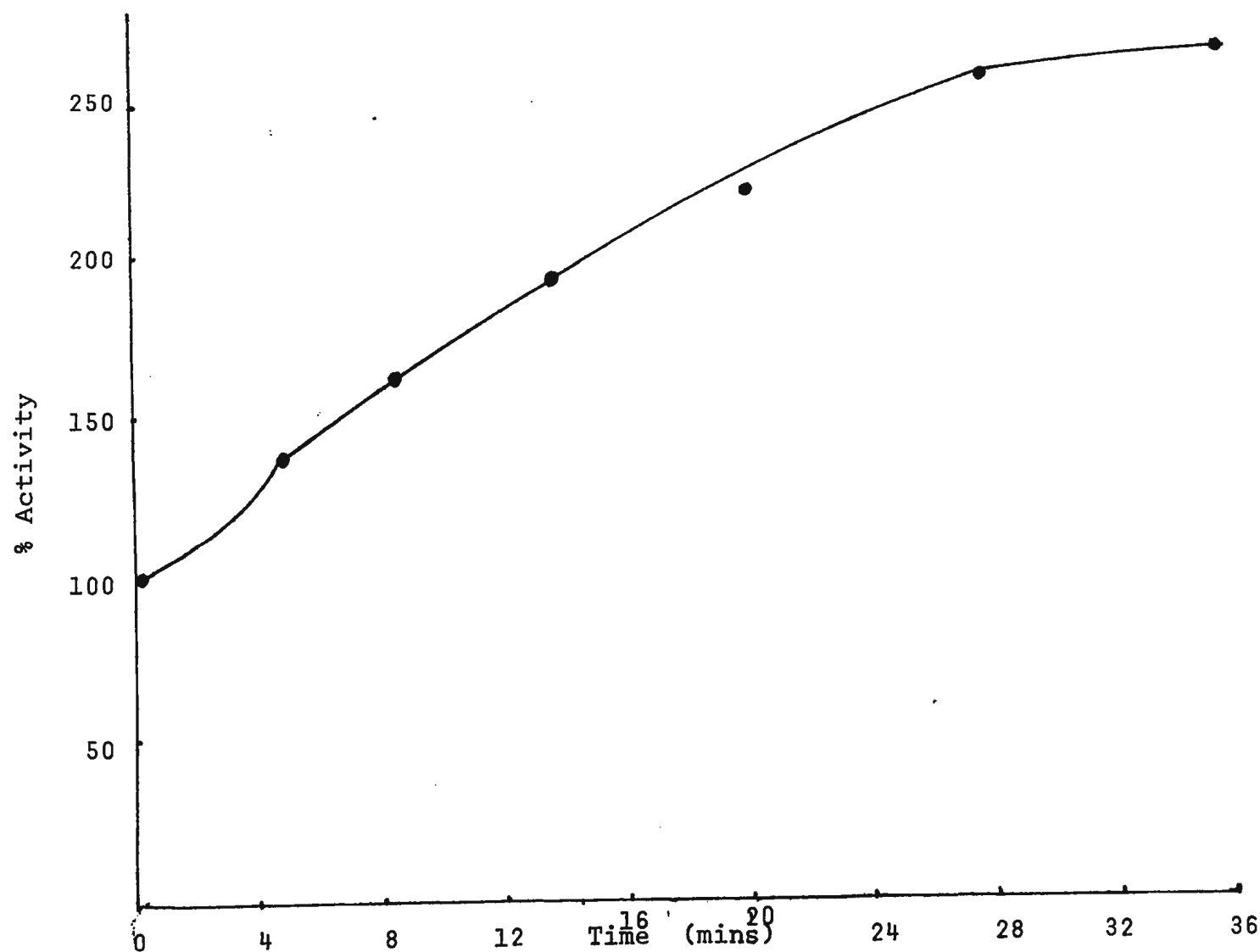


Fig. 4

Effect of GSH Preincubation on GSH Px Activity  
GSH peroxidase (3 mg/ml) was incubated at pH 7.0 and 30° in 5 mM GSH.

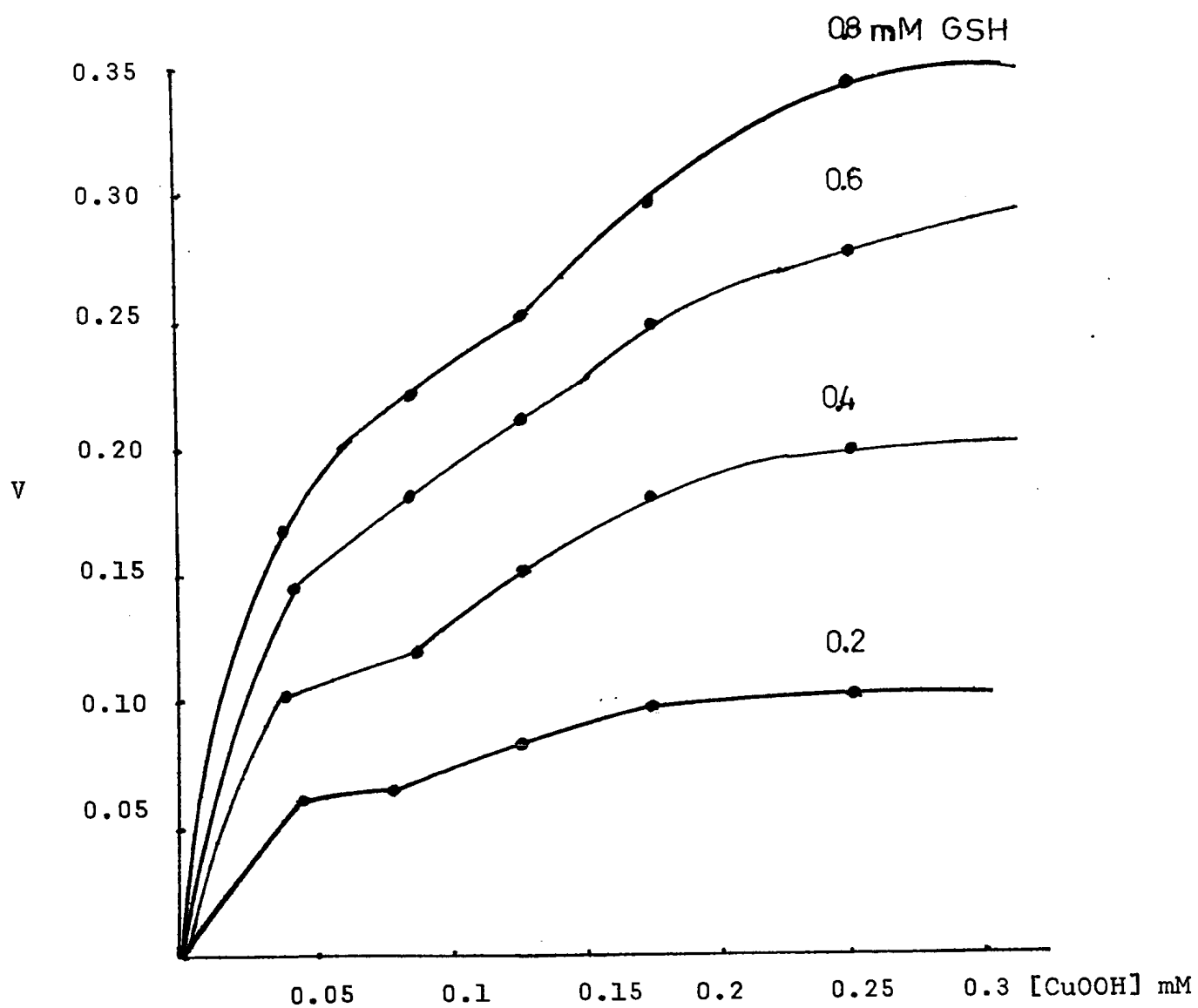


Fig. 5

Effect of CuOOH on GSH Peroxidase Activity in the Presence of Different Concentrations of GSH.

### Stability

Highly purified glutathione peroxidase seems to be an unstable enzyme since considerable inactivation was noted, especially in dilute solution. The same instability was also reported by Mills (42) and recently by Flohé and Brand (61).

In an attempt to find the best procedure to store the enzyme activity, samples of GSH peroxidase were kept with different compounds added at 5° for different periods of time. The results of this experiment are presented in Table V.

The results presented in Table V show it is possible to keep the catalytic activity for 4 weeks with approximately 40% loss if 20% glycerol or 65% ammonium sulfate were added. The first property of the GSH peroxidase lost on storage was its sensitivity to nucleotide inhibition.

With fresh preparations, preincubation of the enzyme with high levels of GSH caused significant increases in activity. Aged samples did not exhibit this behaviour. The results of this experiment are presented in Fig. 4.

TABLE V

Attempts to store GSH peroxidase activity \*

<u>Storage conditions</u> <u>[protein] = 2.5 mg/ml</u>	<u>GSH activity after 4 weeks</u>	
	<u>% Remaining</u> <u>catalytic activity</u>	<u>% Remaining</u> <u>sensititivity to nucleotides</u> <sup>+</sup>
No addition	43	0
1 mM GSH	55	12
1 mM ATP	45	5
20% Glycerol	63	35
65% Ammonium Sulfate	68	16
Frozen	55	10

\* The activity of GSH peroxidase without any addition at the beginning of the experiment was taken as 100% for both catalytic and allosteric activities.

<sup>+</sup> See p. 49.

## 2. Enzyme Kinetics

### a. Peroxide as substrate

The kinetics of glutathione peroxidase with respect to  $\text{H}_2\text{O}_2$  concentration have been reported (52, 53). In the range of  $\text{H}_2\text{O}_2$  concentration  $1 \cdot 10^{-5}$  to  $5 \cdot 10^{-3}$  moles, the reaction was found to be zero order with respect to  $\text{H}_2\text{O}_2$  concentration. An apparent  $K_m$  for  $\text{H}_2\text{O}_2$  has been reported as  $10^{-5}\text{M}$  (52).

Little and O'Brien (55) working with a liver supernatant and with LAHPO as substrate, also reported a zero order reaction type above  $10 \mu\text{M}$ . The apparent  $K_m$  of the GSH peroxidase for LAHPO was estimated to be  $2 \cdot 10^{-6}\text{M}$ . So it is possible to observe that with either  $\text{H}_2\text{O}_2$  or with LAHPO, very low substrate concentration ( $< 10^{-5}\text{M}$ ) were required before these compounds became rate limiting and this makes the kinetic studies difficult.

Using cumene hydroperoxide as substrate, higher concentrations were rate limiting and therefore all the studies on GSH peroxidase were carried out using this substrate.

In Fig. 5 and Fig. 6 the kinetics of GSH peroxidase are presented. Cumene hydroperoxide curves for the enzyme are shown as double reciprocal plots after the fashion of Lineweaver and Burk. The influence of GSH concentration on the hydroperoxide curves is also shown.

It is apparent that significant deviations from the classical linear pattern results. Also, as the concentration of

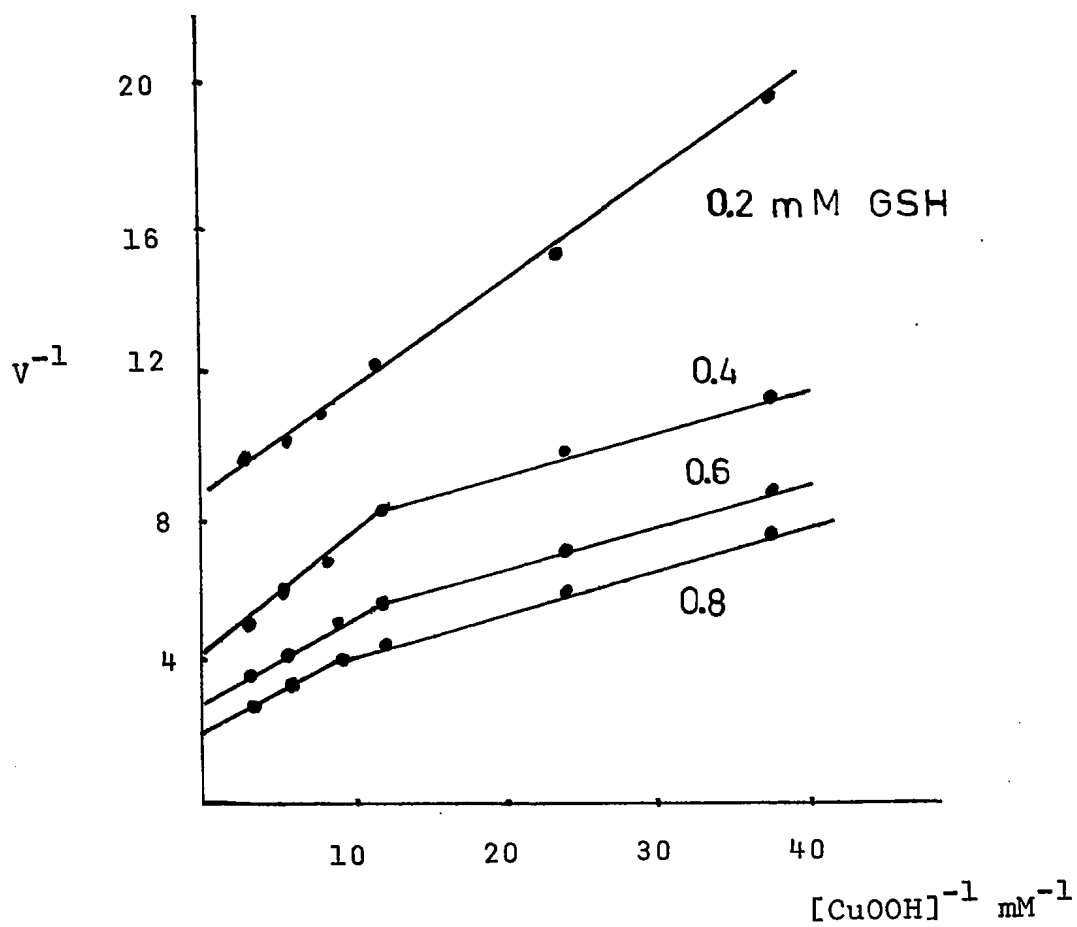


Fig. 6

Effect of GSH concentration on GSH Peroxidase activity with CuOOH as substrate (Lineweaver-Burk plot)

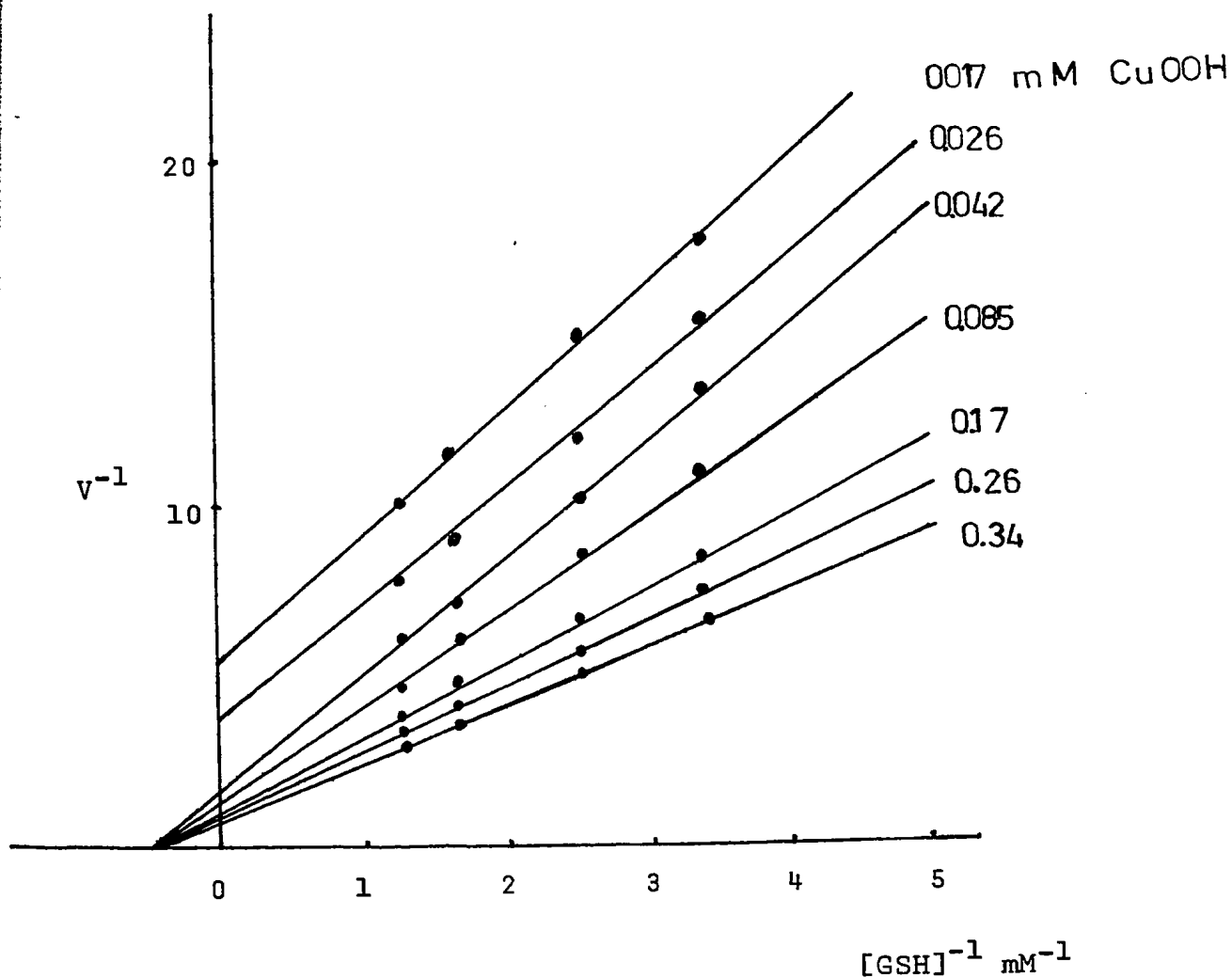


Fig. 7

Lineweaver-Burk plot of GSH Peroxidase for GSH as substrate in the presence of different concentrations of CuOOH.

GSH increases, the apparent  $K_m$  of GSH peroxidase is also increased.

It is also possible to see from Fig. 5 that the curves describing peroxide concentration dependence have two points of inflection with an intermediary plateau. The kinetic behaviour of the enzyme could be explained by the presence of two enzymes with quite different values of  $K_m$  and  $V_{max}$ . It is also possible that the enzyme may exist in two or more forms with the relative amounts of each depending on the relative levels of GSH and CuOOH in the assay.

b. GSH as substrate

The kinetics of GSH peroxidase activity with respect to GSH have been reported (52, 53). Over a wide range of GSH concentrations Hochstein and Utter (61) described deviations from the first order behaviour at high GSH concentrations and attributed this to inhibition of GSSG reductase used in the assay by high levels of GSH.

Since high levels of GSH concentration increase the apparent  $K_m$  value of the enzyme for CuOOH, apparent saturation kinetics may be exhibited to GSH if less than saturation levels of CuOOH are used. However, Fig. 7 shows linear Lineweaver-Burke plots for GSH as substrate. It is apparent that at low CuOOH concentration a parallel family of lines results. As CuOOH is increased, the slopes change and converge on the abscissa. A  $K_m$  value for GSH of about 3 mM may be calculated.

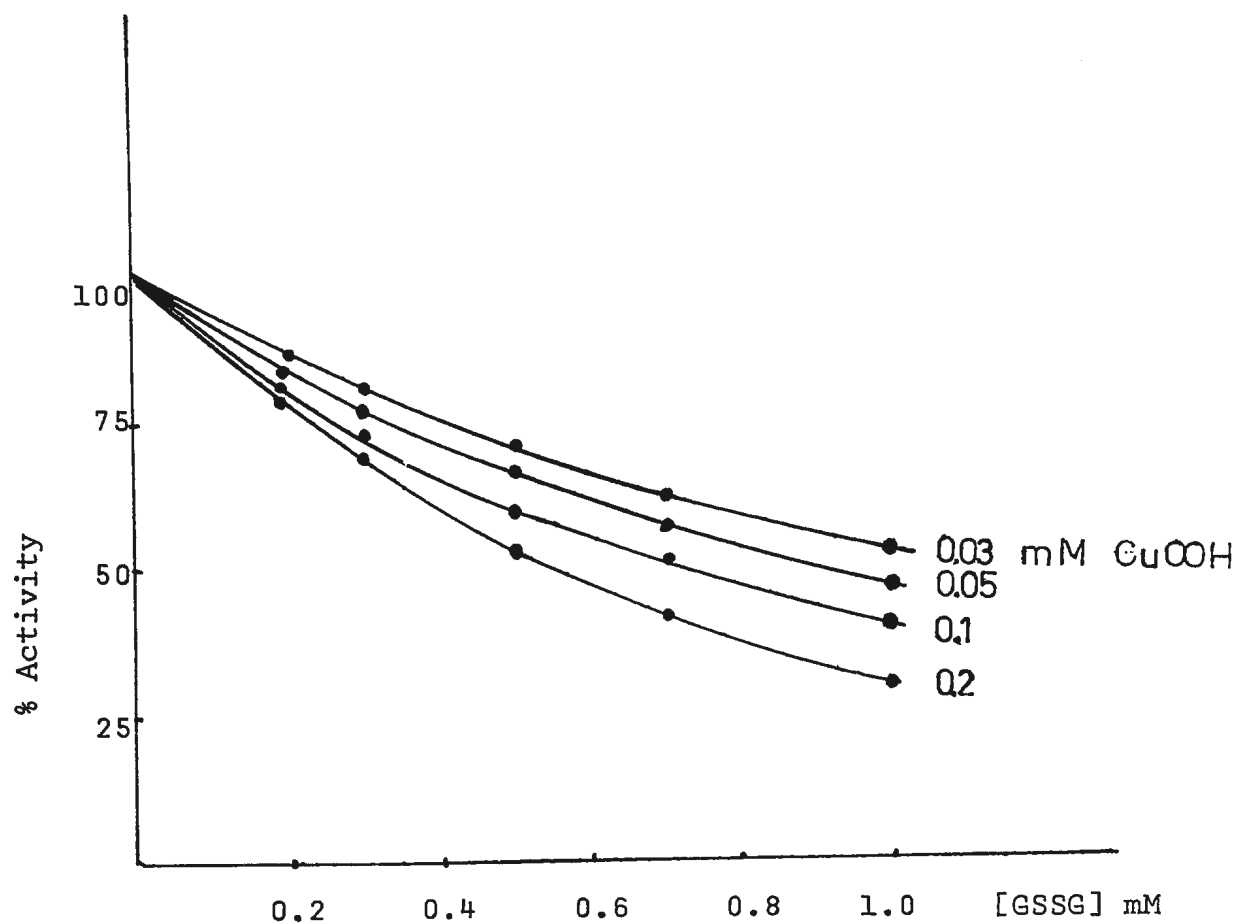


Fig. 8

Effect of CuOOH concentration on inhibition of GSG Peroxidase by GSSG.

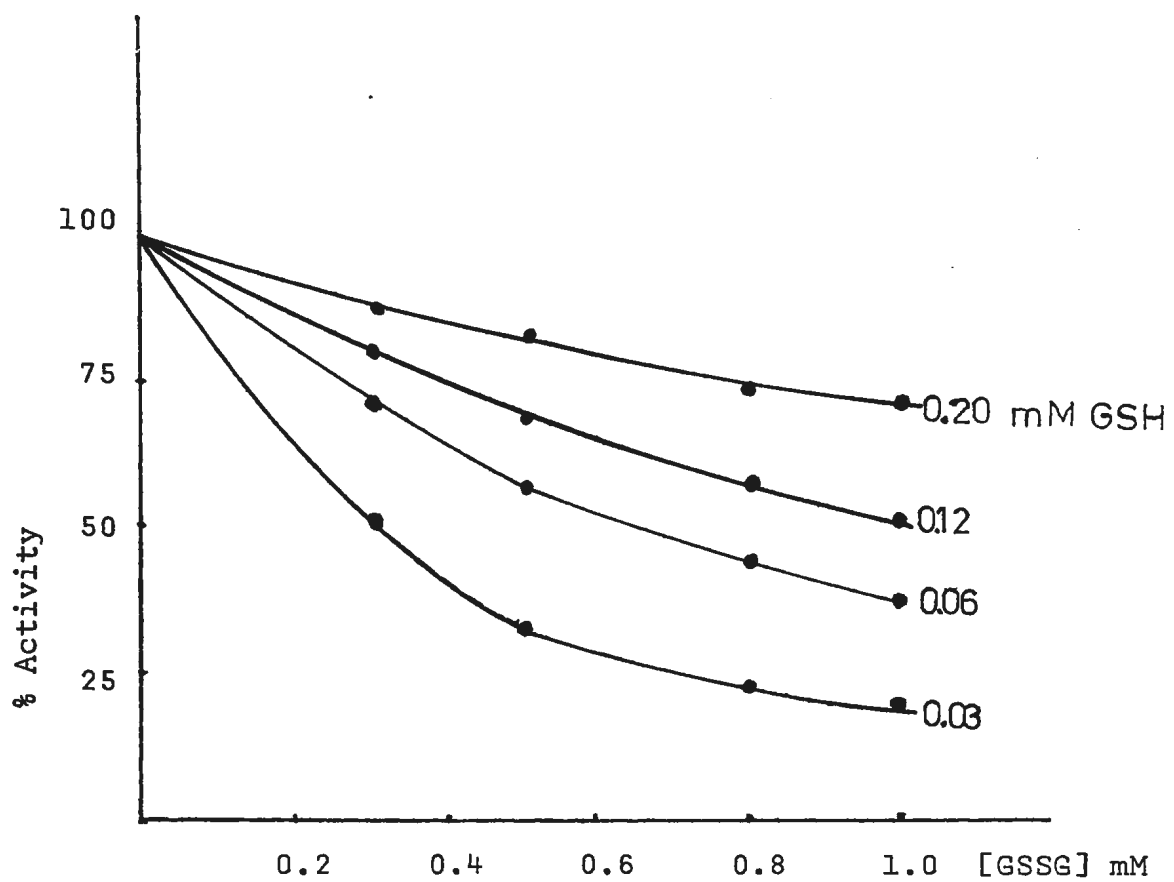


Fig. 9

Effect of GSH concentration on inhibition of GSH peroxidase by GSSG.

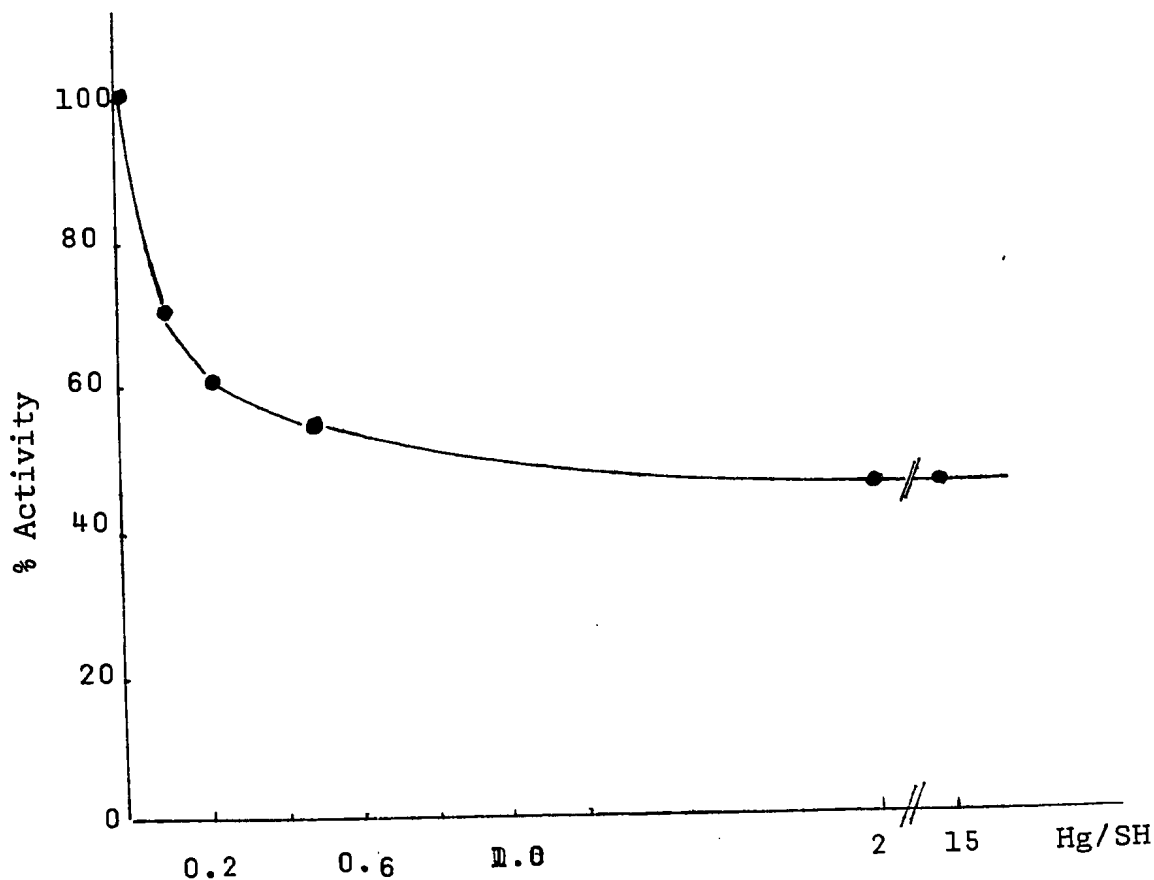


Fig. 10

The effect of pCMB on GSH peroxidase activity.

GSH peroxidase (8 mg/ml) was preincubated with various levels of pCMB at pH 7.0 and 25° for 10 minutes before assay.

The present saturation kinetics are not explained by GSH inhibition of GSSG reductase since the latter was never rate limiting. Nor may they be attributed to GSH induced changes in the enzyme bindings of CuOOH. 3 mM seems to be the limiting value of the  $K_m$  for GSH at high CuOOH levels.

According to Little and O'Brien (41, 55) the stoichiometric calculations showed one equivalent of LAHPO oxidized 2 molar equivalents of GSH and the same was reported for  $H_2O_2$  (53).

#### c. Products inhibition

It is interesting to mention that, while GSH acts as substrate and enhances GSH peroxidase activity, GSSG inhibits strongly.

The inhibition by GSSG depends on peroxide levels, since increased concentrations of CuOOH increase GSSG inhibition (Fig. 8). Furthermore, increased levels of GSH decrease the inhibition by GSSG (Fig. 9). That suggests the oxidation state of glutathione could modulate the enzyme catalytic activity.

GSH peroxidase decomposition of CuOOH would be expected to form cumene hydroxide. However, this latter compound did not affect the enzyme activity up to a concentration of 30 mM.

#### Chemical inhibitors

It has been suggested (41, 42, 53, 55) that GSH peroxidase was not inhibited by  $CN^-$ , azide, but only by certain SH reagents; quinacrine, a flavin analog did not inhibit the enzyme either (55).

TABLE VI

The inhibition of GSH peroxidase by some SH reagents and oxidants \*

<u>Inhibitor</u>	<u>Concentration of inhibitor required for 30% inhibition mM</u>	<u>Time of incubation (minutes)</u>
N-bromosuccinimide (NBS)	0.010	10
p-Chloromercuribenzoate (pCMB)	0.020	10
Iodine	0.1	15
Iodoacetic acid	0.7	30
Sodium sulfite	0.5	30
N-ethylmaleimide	1.5	15
Penicillamine	0.030	15
Cystamine	0.6	15

\* The experiment was carried out at pH 7.0, using GSH peroxidase (0.3 mg/ml) incubated with inhibitors for different periods of time.

The inhibition exerted by thiol reagents has some peculiarities, since not all these compounds inhibit the enzyme activity. O'Brien and Little (55) did not observe any inhibition by 3 mM iodoacetate, iodoacetamide, arsenite or cadmium chloride. For that reason the effects of chemical inhibitors were studied in greater detail. In Table VI the inhibition produced by various SH reagents (pCMB, iodoacetic acid) and oxidants on enzymic activity are presented.

According to Table VI pCMB and NBS are the most powerful inhibitors since  $< 0.03$  mM concentration inhibits 30% of enzyme activity. Iodoacetic acid and sulfite produced a 30% inhibition of enzymic activity only at a concentration higher than 0.5 mM. Iodine has an intermediary action since it inhibits 30% of enzymic activity at a concentration of 0.1 mM.

Cumene hydroperoxide in excess concentration could alter the inhibition produced by iodoacetate or pCMB. Also GSH only partially decreased iodoacetate inhibition.

As these SH inhibitors and oxidants have a great affinity for SH groups and the time of incubation was rather short, it is very likely that mainly SH groups were reacting.

Some estimations of present SH groups according to Boyer's method gave an average of 0.5 to 1 SH group per 100,000 gm protein.

It is also significant that pCMB gave essentially

maximal inhibition when present in the ratio of 1 pCMB/S<sub>H</sub> group in the enzyme preparation (See Fig. 10). At this point approximately 50% of the original enzyme activity remained. For further inactivation by pCMB enormous excesses were needed.

The inhibition by cystamine, panicillamine is probably similar in mechanism with that of GSSG, which was reported above. It is interesting that since GSSG and penicillamine inhibit at rather the same concentration, 0.2 - 0.3 mM, the inhibition of GSH peroxidase by cystamine is three times weaker.

Preincubation of enzyme with 1 mM GSH and subsequent removal of GSH by Sephadex G50 column did not significantly affect the action of S<sub>H</sub> inhibitors.

All these facts suggest that S<sub>H</sub> groups may be partly involved in the catalytic activity of GSH peroxidase. Glutathione can reverse this inhibition only partly.

The effects of chemical inhibitors will be studied further in connection with enzyme regulators.

GSH peroxidase activity was not inhibited by Fe chelating reagents such as o-phenantroline,  $\alpha$ - $\alpha'$ -dipyridyl and o-tolidine in concentrations ranging up to 3 mM.

As GSH peroxidase has unusual kinetics, it was useful to study its temperature curve. Most enzymes have a linear Arrhenius plot which shows the presence of a simple species of enzyme. According to Fig. 11 GSH peroxidase presented an anomalous behaviour with a rather sharp transition at 10°

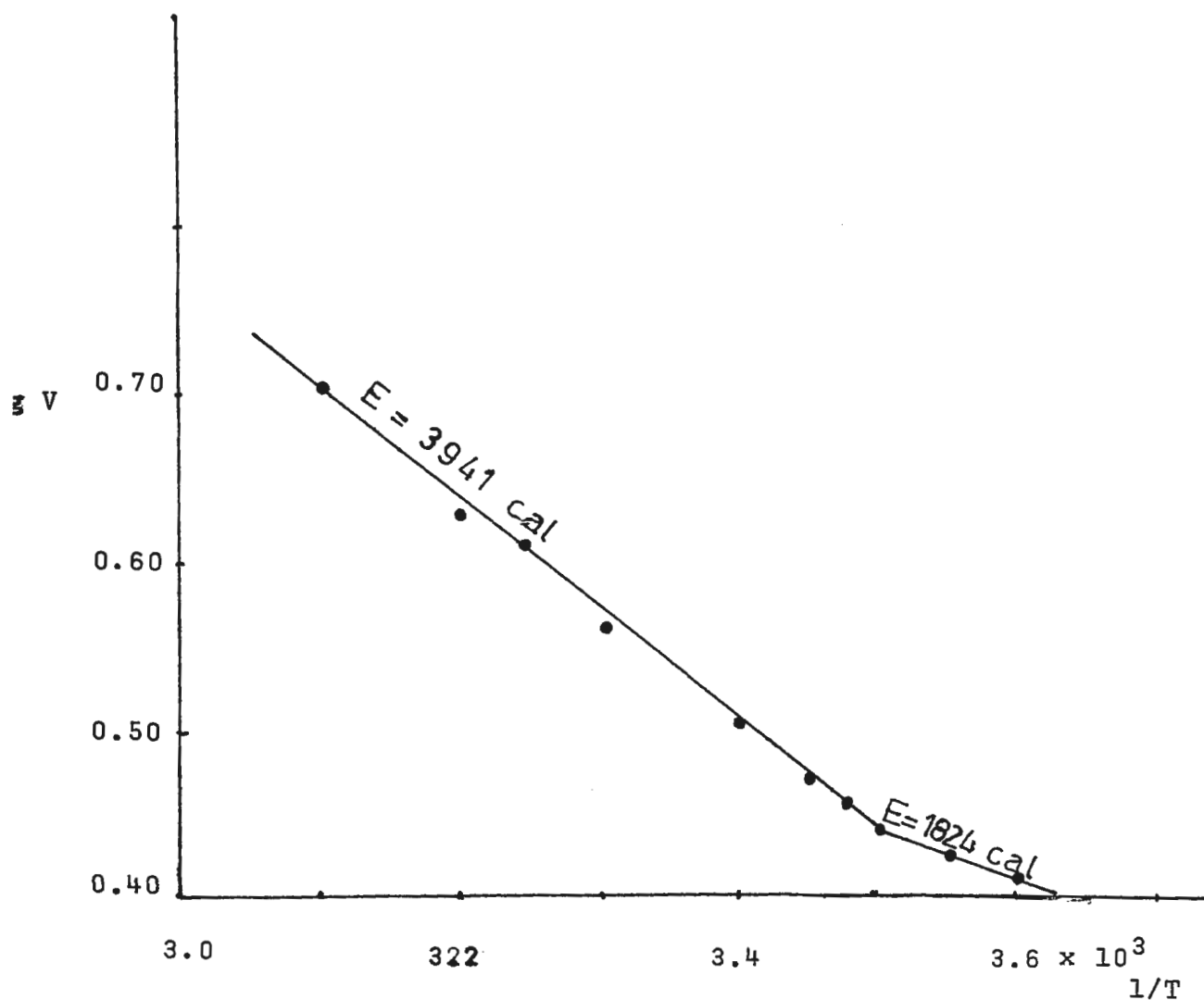


Fig. 11

Effect of temperature on GSH Peroxidase activity (Arrhenius plot)

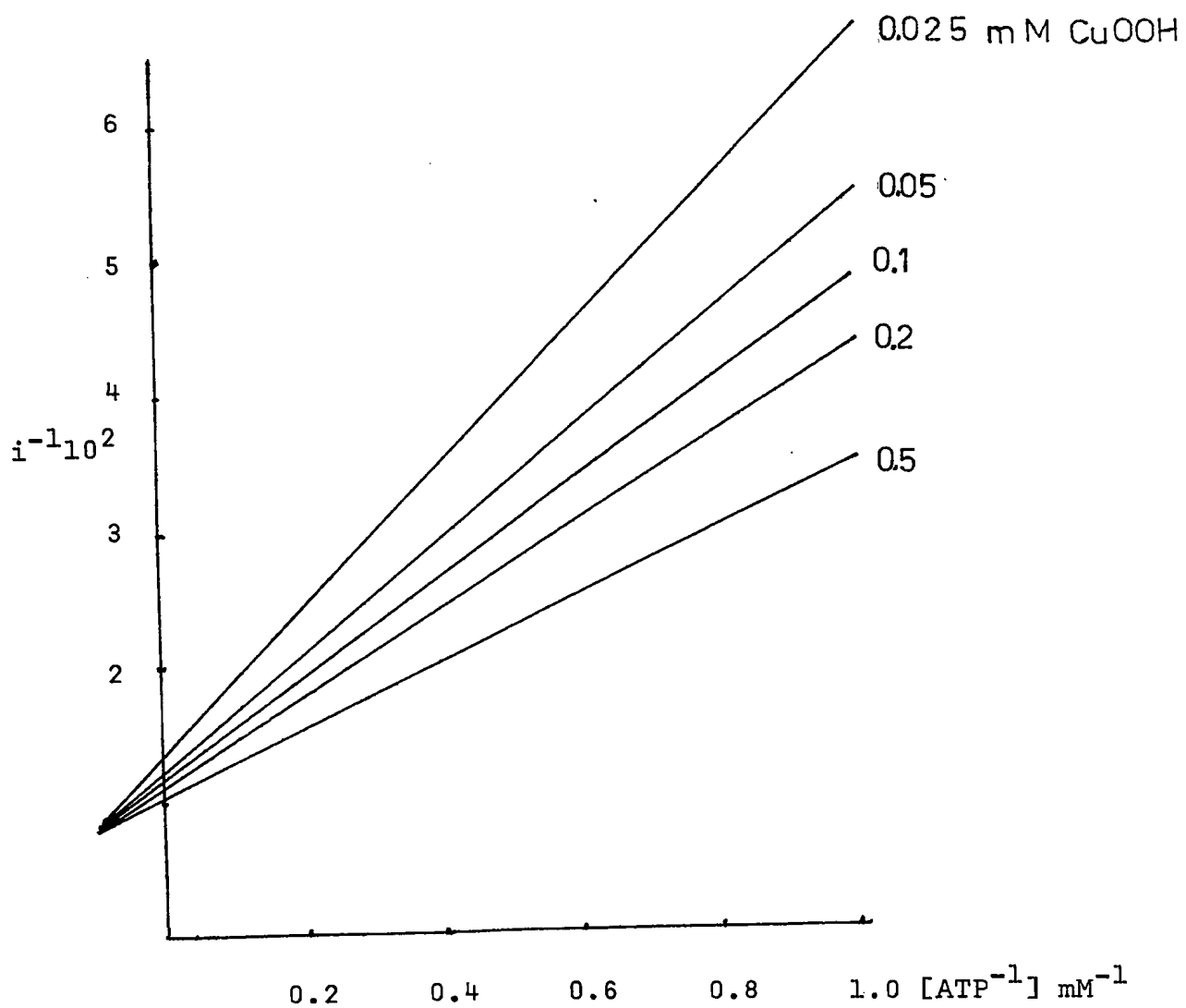


Fig. 12

Effect of CuOOH on GSH Peroxidase inhibition by ATP (Webb plot)

between 2 linear regions of the Arrhenius plot. That is additional evidence to support the previous conclusions with enzyme kinetics.

The most plausible explanation is that the enzyme is capable of existing in 2 stable forms over different temperature ranges, each form possessing catalytic activity, but displaying different energies of activation of the catalysed reaction. The transformation from one form to another form of the enzyme could involve a conformational change.

A similar behaviour of Arrhenius plot and of unusual kinetics is also shown by some flavoenzymes such as glutathione reductase, L-amino acid oxidase.

### 3. Enzyme Regulation

Considerable work was done to find possible natural regulators. Amino acids other than cysteine or cystine or glucose phosphate esters, did not alter enzyme activity. Since there is an increasing number of enzymes whose activity is regulated by nucleotides, action of nucleotides on GSH peroxidase activity was investigated. It was found that this enzyme was sensitive to inhibition by a wide range of nucleotides.

The effect of different purine and pyrimidine nucleotides was investigated. As adenosine nucleotides seemed to be more efficient, a wide range of adenosine bounded compounds was studied and the results are presented in Table VI.

According to these results and taking account of the

TABLE VII

Inhibition of GSH peroxidase by different nucleotides

<u>Nucleotide</u>	<u>[Nucleotide] required for 30% inhibition</u>
NADPH	0.26
5'adenosine tetraphosphate	0.5
NADH, NADP <sup>+</sup> , 5'ATP	0.7
3'-AMP	1.0
5'ADP	1.5
3'-5'AMP, GTP, ITP	2.4
2'AMP	3.0
CTP, 5'AMP	7.5
NAD <sup>+</sup>	9.0
Adenosine	12.0

physiological range of concentrations only 5'ATP, 5'ADP and NADPH could exert a natural regulatory action. For that reason their action, especially that of ATP, was studied in more detail.

a. ATP inhibition

The inhibition of GSH peroxidase by ATP is affected by GSH and cumene hydroperoxide concentrations as it is presented in Fig. 12 and Fig. 13.

According to these figures, ATP inhibition is enhanced by increasing CuOOH levels. Furthermore, the inhibition seems to be purely competitive with GSH (See Fig. 12 where data are plotted after the fashion of Webb). A  $K_i$  value of 2.9 mM can be calculated for the interaction between GSH and ATP.

Because of the complex basic kinetics no further studies were made on the kinetics of inhibition by nucleotides.

b. NADPH effect

NADPH was found to be one of the most efficient nucleotide inhibitors. The kinetics of NADPH inhibition are also complex since an apparent stimulatory effect was observed at low concentrations (0.1 - 0.2 mM) but only with NADPH and to a smaller extent with NADH. This stimulatory effect is greater in the first stages of purification and is lost after the last step. This stimulatory effect is slightly affected by SH reagents but completely abolished by oxidants and x-rays.

c. Coenzyme A inhibition

During investigation of the effects of different

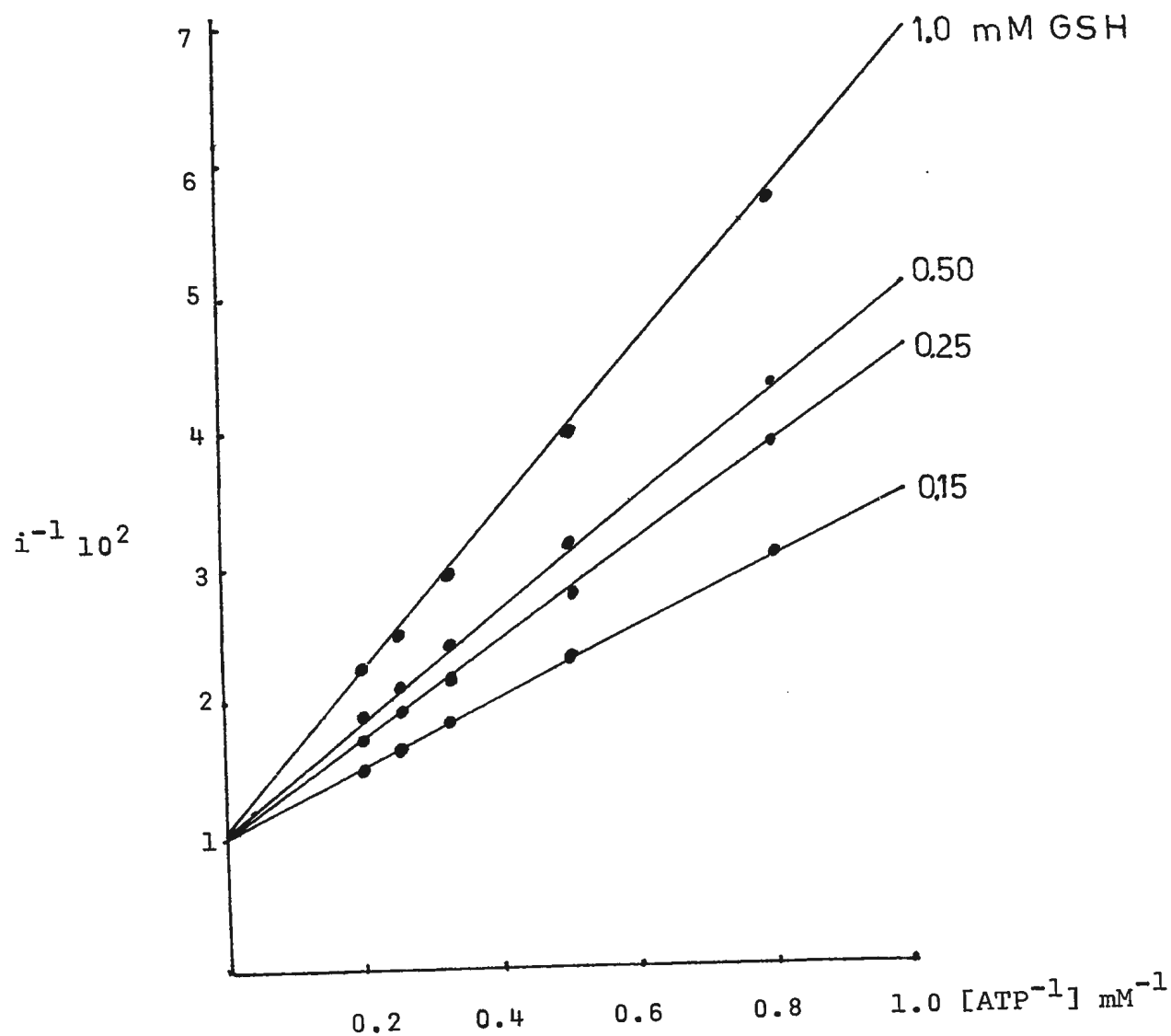


Fig. 13

Effect of GSH on GSH peroxidase inhibition by ATP (Webb plot)

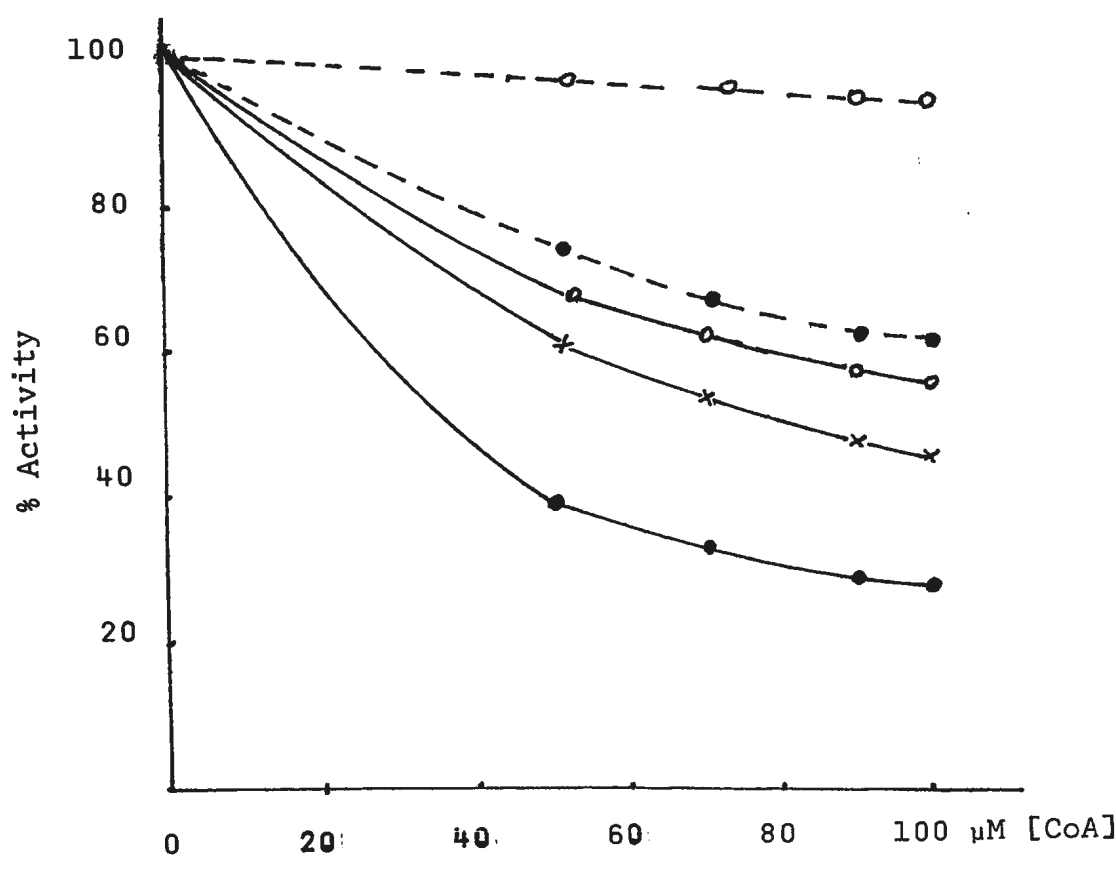


Fig. 14

Inhibition of GSH Peroxidase by CoA and related compounds  
in the presence of some SH reagents.

- o --- o --- o panthotenate
- --- ● --- ● CoA + pCMB
- o — o — o CoA + NEM
- x — x — x Acetyl CoA
- — ● — ● CoA

nucleotides on GSH peroxidase activity it was discovered that coenzyme A exerted an extremely powerful inhibition. In Fig. 14 the inhibition by coenzyme A and related compounds is presented.

Coenzyme A is the strongest in this group of compounds since it produces a 50% inhibition at 30  $\mu$ M concentration.

The action of GSH and CuOOH on the inhibition by CoA was similar to the effects on ATP inhibition. The inhibition was enhanced by increased CuOOH concentrations and decreased by increased GSH concentrations with the interaction between CoA and GSH being purely competitive.

The inhibitory nature of coenzyme A, a more complex molecule than that of ATP, could be attributed to its nucleotide nature, its pantothenate residue or its sulphhydryl group. Attempts were made in order to establish which residue in CoA was mainly responsible for the inhibitory action. GSH peroxidase was not inhibited by pantothenate up to 2 mM concentration.

From Fig. 15 it is possible to see that by blocking the sulphhydryl group of CoA either by treatment with NEM or by acetylation only a slight decrease of inhibitory effectiveness occurred. Presumably therefore, the nucleotide residue of CoA is responsible for the inhibition. In view of this conclusion it would be likely that ATP and CoA would interact with the enzyme at the same site. Fig. 4 shows that this is indeed

% Activity

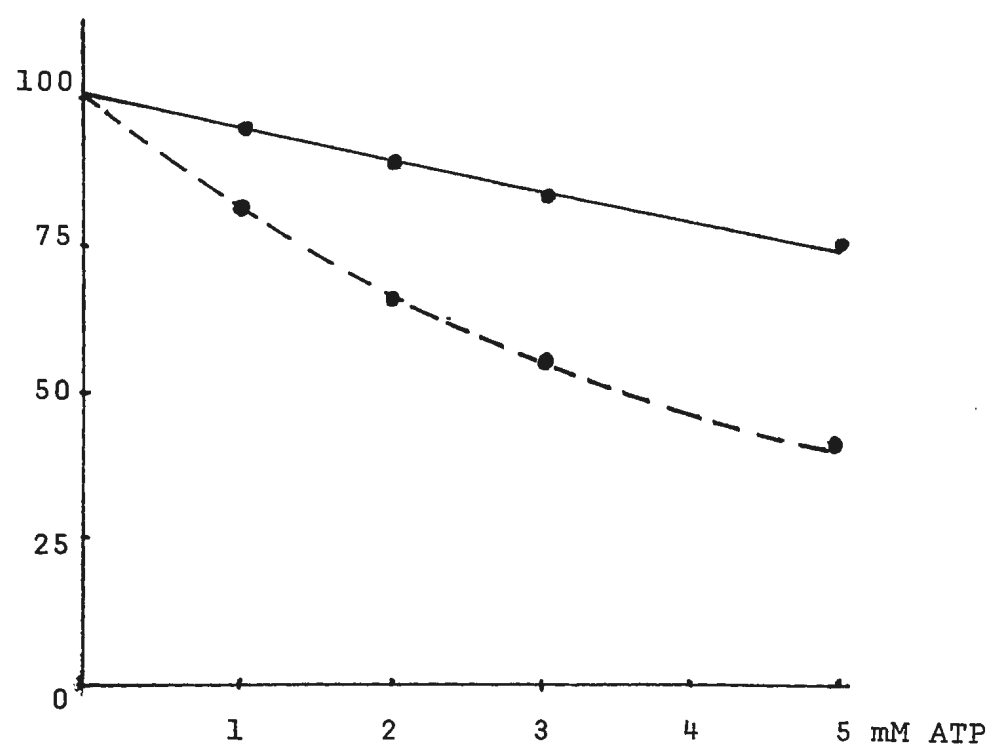


Fig. 15

Effect of CoA on GSH peroxidase inhibition by ATP.

- — ● — ● 16 μM CoA + 3 mM ATP
- - - ● - - 3 mM ATP

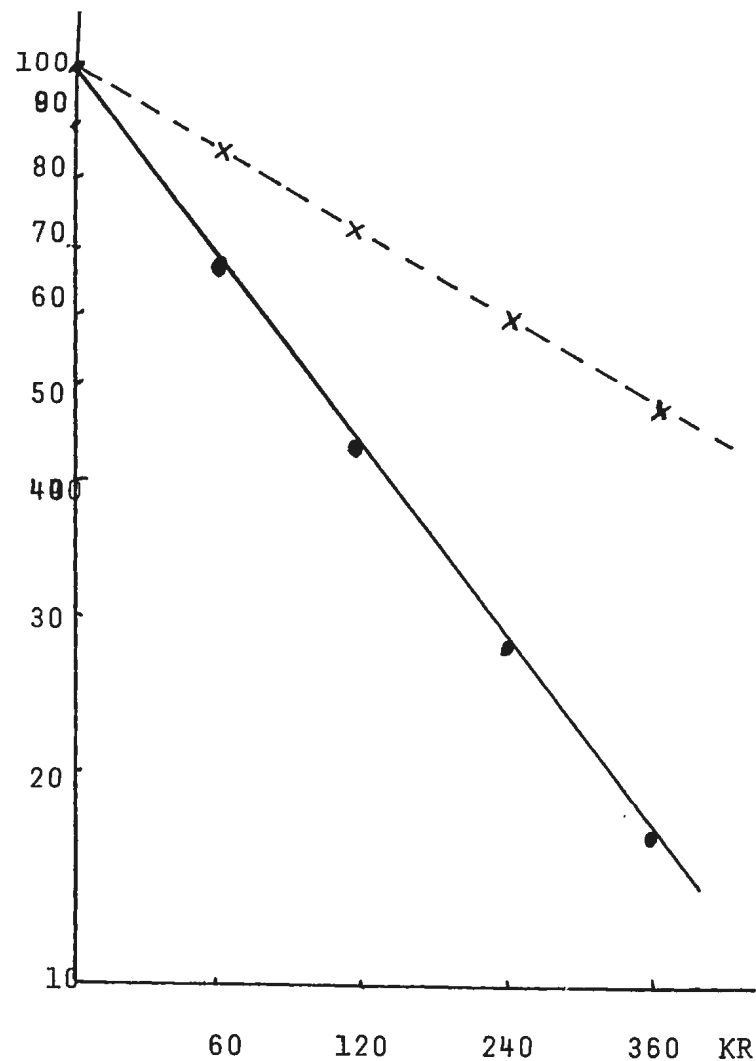
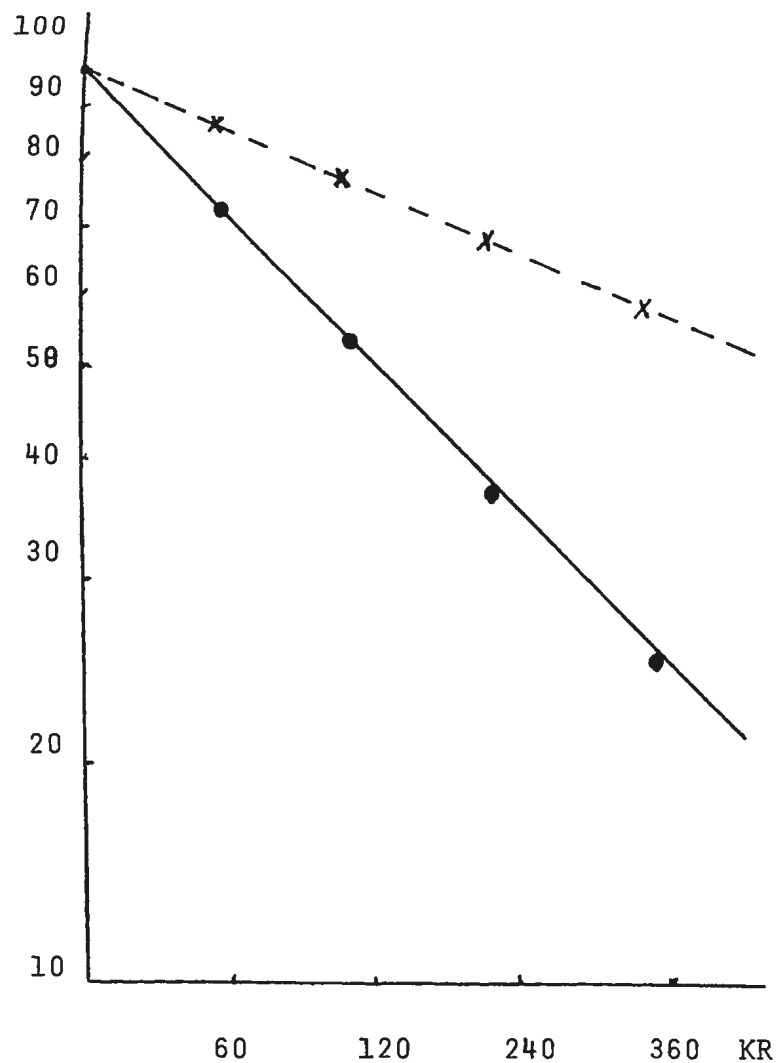


Fig. 16 and 17

Effect of x-irradiation on catalytic (Fig. 16) and allosteric (Fig. 17) functions of GSH peroxidase

●—● Native Enzyme  
x----x Enzyme + 50  $\mu$ M CuOOH

the case, i.e. preincubation of the enzyme with ATP lessens the subsequent inhibition by CoA and vice versa.

#### Physical and chemical modifications

It was important to know if the inhibitory action of these nucleotides is exerted either by interacting at the active center of glutathione peroxidase or at another site. In order to gain more information on the site of nucleotide interaction the enzyme was subjected to various physical and chemical treatments. The catalytic activity and ATP inhibition were then measured and the results are presented in Table VIII.

#### 4. Liver glutathione peroxidase

In previous work it was shown that rat liver contains a highly active glutathione peroxidase that catalyses GSH oxidation by  $H_2O_2$  (54) or by linoleic acid hydroperoxide (55, 40).

Mills (54) used a similar solvent precipitation (a mixture of ethanol, chloroform and acetone) with that used for blood. Little and O'Brien (55) used also an ethanol precipitated enzyme. Christophersen (40) worked with a rat liver supernatant and subjected it to gel-filtration on Sephadex G25.

Since the liver has many more metabolic processes than the red cells and all subcellular fractions are present, the purification of an enzyme is more difficult. The procedure used for liver glutathione peroxidase is presented below.

TABLE VIII

a, b

Modification of GSH Px catalytic and allosteric activity by physical and chemical agents

<u>Treatment</u>	<u>Residual catalytic activity %</u>	<u>Residual ATP sensitivity %</u>	<u>% loss of ATP response % loss of catalytic activity</u>
None	100	100	--
Heat 48°	32	56	0.64
Sodium lauryl sulphate 20 mM	58	68	0.76
pCMB (5 mM)	40	56	0.69
Trypsin 4 mg/ml	60	50	1.25
Ethanol 30% w/v	73	63	1.37
X-rays (60 kR)	45	23	1.90

<sup>a</sup> GSH Px (0.3 mg/ml) in 0.05 M phosphate buffer pH 7.0 was treated for 10 minutes at 22° as indicated above. In the x-rays irradiation experiments GSH Px (0.1 mg/ml) in 0.017 M phosphate buffer, pH 7.0, was irradiated for 30 minutes at 0°. For the assay the treated enzyme was diluted 600 times. Glutathione reductase was not affected by these inhibitors.

<sup>b</sup> Native control enzyme was inhibited 60% by 3 mM ATP and this was defined as full (100%) ATP sensitivity. The amount of inhibition of the treated GSH Px samples by 3 mM ATP was measured and expressed as % of that of the control.

TABLE IX

The purification of liver glutathione peroxidase

<u>Steps</u>	<u>Volume ml</u>	<u>Total activity GSH Px units</u>	<u>Total protein mg</u>	<u>Specific Activity units/mg</u>	<u>Yield of activity - %</u>
Homogenate	500	650,000	24,700	26.3	100
Ethanol precipitate	200	480,000	7,500	64	73
(NH <sub>4</sub> ) <sub>2</sub> SO <sub>4</sub> 60% saturation	120	310,000	1,800	173	47
Sephadex G100	300	330,000	1,600	206	59
DEAE cellulose	270	225,000	1,015	221	34

a. Homogenization:

The perfused pig liver (150 g) was homogenized in a Waring blendor in cold saline or with a Potter-Elvehjem homogenizer in sucrose 0.25M. The last treatment was used to prepare mitochondrial and supernatant subcellular fractions by the classical differential centrifugation procedure. Mitochondrial fraction was homogenized (solubilized) in Tris-HCl 0.1M buffer, pH 7.0, and subjected to ultrasonication. When liver homogenate was obtained by Waring blendor treatment it was further centrifuged for 90 minutes at 48,000 g.

b. Solvent precipitation:

Both mitochondrial and supernatant fractions were precipitated by 2 volumes ethanol at  $-20^{\circ}$  and the precipitate was centrifuged and extracted with the same buffer. The mitochondrial fraction was studied as it was obtained after this treatment. Because the supernatant contained a great amount of hemoproteins a  $(\text{NH}_4)_2\text{SO}_4$  60% precipitation was necessary.

The further purification was similar with that employed for blood enzyme consisting of Sephadex G100 and DEAE-cellulose columns. The results obtained with this procedure are presented in Table IX.

Compared with blood enzyme the purification of liver enzyme was not so successful since the specific activity has increased only ten times. The final preparation had a yellow-greenish colour with a maximum absorption peak at 415 m $\mu$  suggesting a hemin contamination. By replacing the last step

(DEAE cellulose) with a hydroxyl apatite column the yield was not much better. An attempt was made to improve this purification by avoiding solvent treatment and replacing it with DEAE-cellulose selective adsorption but this was not successful.

c. The properties of liver glutathione peroxidase

Both mitochondrial and supernatant enzymatic preparations were precipitated almost completely by 60% saturation  $(\text{NH}_4)_2\text{SO}_4$  as with the blood enzyme.

Since the liver enzyme was less purified than blood enzyme, it presented the same properties, optimum pH, temperature, with differences within the experimental error. The liver enzyme had identical kinetics for GSH and CuOOH. The liver enzyme is inhibited by nucleotides in an identical manner to blood enzyme.

The single difference was exhibited by GSSG inhibition. The liver enzyme seems to be more sensitive to GSSG than blood enzyme. But because the degree of purification is poorer for liver enzyme it is difficult to be sure that this excess of sensitivity is meaningful. Despite the fact that red cells do not contain CoA blood glutathione peroxidase is as sensitive to CoA as the enzyme from liver. As the concentration of CoA needed to inhibit glutathione peroxidase is in the physiological range [200 - 500  $\mu$  moles/g fresh liver (59)] it is very likely that for liver enzyme the levels of CoA and

acetyl CoA may be one of the natural regulators.

Attempts to find some of the intermediates of the fatty acid oxidation (such as oxaloacetate) could modify GSH Px activity were negative.

### 5. Irradiation

Although direct evidence for peroxides in tissue of irradiated animals is lacking, the role of peroxides in radiation damage in vivo is still not understood.

An important factor in this problem could be GSH peroxidase, due to its ability to decompose efficiently a wide range of peroxides.

In this aim, blood purified GSH peroxidase was subjected to x-irradiation alone or in the presence of its substrates. Fig. 16 shows that the enzyme, upon x-irradiation, exhibited the usual exponential relationship of inactivation to dose. It is apparent also that the presence of CuOOH causes a significant protection of the catalytic function.

It was mentioned above (enzyme regulation) that the allosteric function of the enzyme was more radiosensitive than the catalytic function. This is also shown in the data from Figs. 16 and 17. It is apparent that the  $D_{37}$  from the catalytic activity is 290 kR under the experimental conditions and that for the allosteric activity it is about 170 kR. It is seen that CuOOH causes radioprotection of the allosteric site. It was also observed that ATP causes no protection of either the catalytic or the allosteric functions.

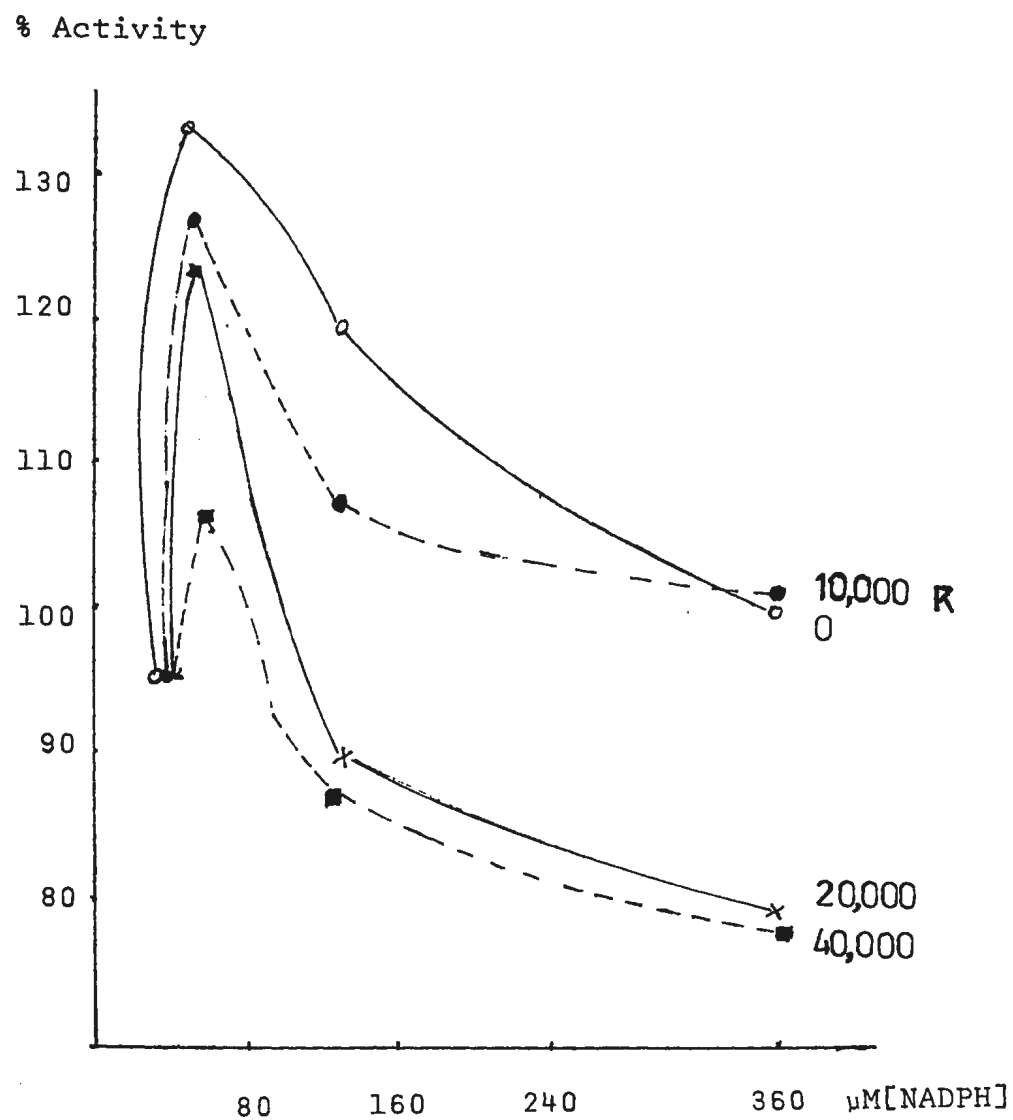


Fig. 18

The influence of irradiation on NADPH stimulation.

It was reported (enzyme regulation) that NADPH in low concentration enhanced GSH peroxidase activity. Irradiation of the enzyme abolished this stimulation. The destruction of NADPH-induced stimulation was proportional with the irradiation dose (Fig. 18). This destruction of NADPH-induced stimulation by irradiation was diminished by cumene hydroperoxide.

The radioprotection offered by the hydroperoxide was proportional with its concentration. The same proportional relationship between the concentration of  $\text{CuOOH}$  and the radioprotection effect was observed for the destruction of allosteric site.

All these experiments suggest that the catalytic site of GSH peroxidase and the allosteric site are different.

Secondly, the allosteric site seems to be more radiosensitive than the catalytic one.

Thirdly, as the peroxide affords some protection on the allosteric site, that would indicate a proximal vicinity between these two sites or some form of interaction between them.

Finally, the liver enzyme preparation was not as radiosensitive as the blood enzyme in both catalytic and allosteric properties. This difference in radiosensitivity is presumably due to protein impurities from the liver enzyme. This explanation is very likely as there are only minor differences in allosteric properties between both enzymes.

These facts may suggest that the allosteric site is more exposed than the catalytic one or that it contains more radiosensitive amino acid residues.

## DISCUSSION

According to previous results and those presented above, glutathione peroxidase does not seem to possess any prosthetic group.

Hypotheses on the mechanism of GSH peroxidase have been presented by Schneider and Flohé (53) and recently by Flohé and Brand (62). These authors presented firstly a SH-SS catalysis mechanism which assumes that at the catalytic site two SH groups are involved. The enzyme in the reduced form is assumed to react very rapidly with the hydroperoxide and an SS bridge is formed. GSH has the role of regenerating the enzyme SH groups. Flohé and Brand (62) proposed another mechanism based on the apparent similarity with haemin peroxidases. Both hypotheses are based on limited experimental evidence (and especially for the second, they did not reach a saturation state of the enzyme by GSH). Moreover, the similarity of GSH peroxidase with haemin peroxidases is not particularly strong since GSH peroxidase seems to possess no haem group and shows different kinetics.

The presence of an SH group, at least at the catalytic center, is very likely and in agreement with the results presented here. But, according to the results presented above, which show complex behaviour in the kinetics with respect to hydroperoxide and those regarding nucleotide inhibitions, Schneider and Flohé's hypotheses are not adequate.

The reason why other workers have not reported the anomalous kinetics is probably that they have used  $\text{H}_2\text{O}_2$  as substrate. Due to the very low ( $\sim 1 \mu\text{M}$ )  $K_m$  for  $\text{H}_2\text{O}_2$ , it is experimentally very difficult to obtain kinetic data for the enzyme under conditions where  $\text{H}_2\text{O}_2$  is rate limiting. The binding of cumene hydroperoxide to the enzyme seems to be about 100-fold weaker than  $\text{H}_2\text{O}_2$ . Much higher levels of this substrate are rate limiting and hence kinetics data is easier to obtain.

GSH peroxidase has been reported to follow first order kinetics with regard to GSH concentration (47, 55, 52, 53).

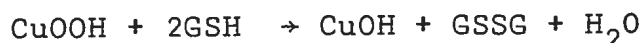
Since Paglie and Valentine (52), Hochstein and Utley (61) described a saturation of the enzyme, Flohé and Brand (62) were not able to confirm this saturation. According to our results there is a strong implication of interaction between GSH and  $\text{CuOOH}$  binding sites, as increased hydroperoxide concentrations increase the  $K_m$  of the enzyme for GSH which was estimated as approximately 3 mM GSH (limiting value). In Fig. 6 it is shown clearly that at low  $\text{CuOOH}$  concentrations parallel lines were obtained. As the concentrations of  $\text{CuOOH}$  is increased the slopes change from parallel to converging lines suggesting strong interactions.

It must be realized that the GSH kinetics studies, as carried out by Flohé et al with  $\text{H}_2\text{O}_2$ , used hydroperoxide.

levels of  $>10^3 \times K_m$  for hydroperoxide. The present studies involved considerably lower levels of hydroperoxide and since cumene OOH becomes rate limiting at levels of  $10^2 >$  levels, where  $H_2O_2$  is rate limiting; the present data for GSH might possibly be expected to be somewhat different from the data of Flohé et al (62).

GSH peroxidase presents non linear Lineweaver-Burk plots with respect to CuOOH as substrate. The kinetic pattern is complex as high levels of CuOOH apparently enhance the enzyme activity. This behaviour may suggest the existence of the enzyme in two or more forms with different activities. This possibility received additional evidence from the Arrhenius plot (Fig. 11) which shows an inflection point.

As the stoichiometric equation for the enzyme reaction pointed out



any proposed mechanism must indicate if there are one or two separate binding sites for GSH and if there is an ordered reaction between CuOOH and the two molecules of GSH. If there is an ordered sequence of binding, as in a ping-pong mechanism, one GSH and one CuOOH would react with GSH peroxidase yielding an enzyme intermediate which will react better with the second molecule of GSH.

Glutathione peroxidase does not exhibit signoidal

relationship of the rate to substrate concentration or in the degree of inhibition vs inhibitor concentration as is shown by most allosteric enzymes. Therefore, GSH peroxidase may not fit the classical concept of a multisited allosteric enzyme and several allosteric enzymes present a similar behaviour. A similar anomalous type of kinetics was also reported for glutathione reductase, phosphoenol pyruvate carboxylase, glutamate dehydrogenase; these enzymes have complex kinetics but all possess more than two substrate binding sites and are composed of four or more subunits (81).

In our results it was noted that the catalytic activity and the sensitivity of the enzyme to inhibition by ATP could be modified selectively by various chemical or physical treatments of GSH peroxidase (Table VIII). This is good evidence that the nucleotides interact with enzymes at a site distinct from the catalytic and hence GSH peroxidase could be an allosteric enzyme. The fact that the allosteric site is very sensitive to physical factors (irradiation, storage) may indicate possible conformational changes limited to this region.

GSH peroxidase possesses very low  $K_m$  values for hydroperoxide as substrate, ranging between  $10^{-6}$  to  $10^{-5}$  M, indicating a high affinity of the enzyme for hydroperoxides and that intracellular levels of hydroperoxides may be adequate to fully saturate the enzyme. By working with cumene hydroperoxide which has a greater  $K_m$ , instead of  $H_2O_2$  or LAHPO, it

was possible to study the kinetics in a greater range of concentrations and to find the interactions between hydroperoxide and GSH.

Since a true  $K_m$  value for GSH was difficult to obtain an average value of  $3 \cdot 10^{-3} M$  was given. This value is in the physiological intracellular range of concentration and also indicates that the existing amounts of GSH will substantially saturate the enzyme. The value of 3 mM for a  $K_m$  for GSH was found with  $CuOOH$ . It may be quite different for  $HOOH$  on lipid peroxides. We cannot, then, say very much about the physiological implication of this value of 3 mM. As both substrates, within the cell, are near saturating levels, it is unlikely that a small change in their intracellular levels will markedly affect the catalytic activity.

GSSG was shown to possess a strong inhibition effect which might suggest that GSH/GSSG ratio could be one of the natural regulators of catalytic activity. However, because in vivo most of the glutathione is in the reduced form due to the efficient reduction by NADPH-related glutathione reductase, and because GSH peroxidase is working at the saturating levels of GSH, the role of GSH/GSSG ratio may not be so important for enzyme regulation.

Thus, there is good evidence that GSH peroxidase activity is essentially regulated by the levels of the various nucleotides. The inhibitory effect of different nucleotides was observed at their physiological levels. Thus, the

elevated amounts of ATP occurring during gluconeogenesis may mean that hydroperoxide detoxification will be more efficient during glycolysis.

Erythrocyte is one of the richest sources of GSH peroxidase. As it was presented in the Introduction, the enzyme activity is closely associated with the pentose phosphate pathway. As Jacob and Jandl (63) pointed out, the activity of pentose phosphate pathway reflects the relative amount of  $\text{NADP}^+$ . Since GSSG reductase specifically requires NADPH as its cofactor and since GSH is present in relatively large amounts in the red cells, the reduction of GSSG to GSH represents an important potential source of NADPH. Pentose phosphate pathway activity is regulated primarily by GSH. Increasing the ratio of oxidized to reduced glutathione (either by oxidizing or by partially blocking GSH with NEM) increased the rate of pentose phosphate pathway. Complete blockage of cellular GSH by NEM depressed the pentose phosphate pathway activity despite little or no effect on the Embden Meyerhoff pathway.

Sustained low levels of  $\text{H}_2\text{O}_2$ , whether generated by aerobic oxidases or by the coupled oxidation of ascorbate and oxyhemoglobin stimulates the pentose phosphate pathway. This stimulation was potentiated by blocking catalase (by azide) and was prevented by blocking GSH.

Since erythrocyte GSSG reductase requires NADPH or NADH as its cofactor, and since GSH is present in relatively large

amounts in the red cells, the oxidation of GSH to GSSG by hydroperoxides will normally result in an enhanced pentose phosphate pathway activity.

Furthermore, GSH maintains other thiol groups of the cell in the reduced form through redox interactions (64). Failure of maintenance of thiol activity due to the action of  $H_2O_2$  or lipid peroxides may result in hemolysis with oxidative denaturation of hemoglobin. Jacob and Jandl (63) found that GSH is oxidized more rapidly than hemoglobin, when the red cells are exposed to a variety of oxidant compounds and methemoglobin appears only after the complete depletion of GSH.

In normal red cells, in acatalatic and in catalase-inhibited erythrocytes exposed to low and continuous generation of  $H_2O_2$ , pentose phosphate pathway is always enhanced. This suggests that GSH peroxidase may be more important than catalase in decomposing  $H_2O_2$  in the cell. According to Cohen and Hochstein (47), Hochstein and Utley (61) and Flohé and Brand (62), GSH peroxidase is the primary mechanism for removal of  $H_2O_2$  at low concentrations. They gave evidence that catalase will have no significant catalatic function intracellularly at concentrations below  $3 \mu M H_2O_2$ . The experimental evidence was given by experiments where catalase was inhibited by azide and in conditions of low concentrations of  $H_2O_2$  1-10  $\mu M$ , mainly obtained by diffusion.

The same experiment was repeated by Panicker and Iyer (65) using erythrocytes with high content of catalase (man)

intermediate (rat) and very low (dog). They showed that in the presence of azide and  $H_2O_2$ , methemoglobin formation is inversely proportional to the content of catalase. Catalase can therefore protect the hemoglobin at low levels of  $H_2O_2$  (1 - 3  $\mu M$ ).

Nicholls (66) tried to solve this problem by comparing the molecular reaction rates of catalase and GSH peroxidase. Nicholls calculated that for a wide range of concentrations of  $H_2O_2$ , catalase reacts with peroxide at a rate of  $6 \cdot 10^6 M^{-1} sec^{-1}$  and that GSH peroxidase is able to remove  $H_2O_2$  at a rate of  $10^4 M^{-1} sec^{-1}$ . This suggests that catalase is the primary agent in removing  $H_2O_2$  in normal, physiological conditions and GSH peroxidase may give additional support.

Jacob and Jandl (63) observed that always when catalase is inhibited, the pentose phopshate pathway is increased, thereby providing more NADPH for decomposition of  $H_2O_2$  by the GSH peroxidase system.

According to the results presented in Table VII, there are significant differences in inhibitory action between reduced and oxidized forms of nucleotides. That may suggest that modifications of NADPH/NADP ratio could have regulatory effects on GSH peroxidase activity. In addition, ATP/ADP could have similar effect.

However, ATP is necessary for the red cell viability

since the hemolytic process is accompanied by ATP depletion (67) but its role is far from understood. A hereditary disease has been described (68) where erythrocyte ATP levels are increased. An inhibition of GSH peroxidase could be partly responsible for their increased fragility. The ATP concentration in erythrocytes is around 1 mM and may therefore be able to regulate the GSH peroxidase activity.

Lipid peroxides may appear in a congenital disease, paroxysmal nocturnal hemoglobinuria (69), due to an abnormal composition of lipids in the erythrocyte membrane. Lipid peroxides were also found when normal erythrocytes were incubated with  $H_2O_2$  and GSH (70) or exposed to ozone (71) or hyperoxia (72).

The effect of  $H_2O_2$  and GSH could be explained if GSH peroxidase was not located in the erythrocyte membrane. In conditions when the oxidizing agent acts on the exterior of erythrocyte membrane, GSH peroxidase would then be ineffective. The peroxidase would be efficient only within the red cell and in connection with the metabolism of the erythrocytes.

Liver glutathione peroxidase from liver mitochondria and supernatant, although not in the same degree of purity, was found to possess the same properties as the blood enzyme.

Hunter et al (28) reported that lipid peroxide formation was associated with GSH + GSSG-induced mitochondrial swelling. Neubert et al (43) showed that the glutathione

peroxidase accounted for 80% of the activity of c-factor necessary for mitochondrial contraction. Green and O'Brien (71) recently reported that glutathione peroxidase was released from the mitochondria during the swelling initiated by GSH, GSH + GSSG, ascorbate and oleate, but not during the swelling induced by agents which do not form lipid peroxides such as phosphate or calcium. The peroxidase is released only during the swelling process and in an amount directly proportional to the extent of the swelling. The stimulatory effect of GSSG and certain nucleotides, such as ATP, on swelling may arise from their inhibitory action on glutathione peroxidase. The inhibitory action of GSH peroxidase on mitochondrial swelling may result from its effectiveness in catalysing the restoration of essential disulfide groups produced by lipid peroxides.

The peroxisomes are known to be the site of much of the  $H_2O_2$  metabolism within the liver cell and contain catalase but not glutathione peroxidase. This may suggest that for liver, catalase action is restricted to decomposition of  $H_2O_2$  formed by flavin enzymes within the peroxisomes. It may also be involved in the oxidation of cytoplasmic NADH necessary for the maintenance of glycolysis. Glutathione peroxidase may function in the decomposition of  $H_2O_2$  produced by flavin enzymes in the cytosol and mitochondrial matrix. Glutathione peroxidase may also be responsible for the decomposition of lipid peroxides formed in membranes bathed by the cytosol or lipid peroxides formed on the inner surface of the inner

membrane of the mitochondria (73).

It has to be mentioned that Christophersen (40) found a correlation between the rate of aerobic oxidation of GSH and GSH peroxidase activity. It seems that GSH peroxidase can prevent lipid peroxidation and thus may have a vital role in maintaining cell integrity.

The lack of peroxidase in the microsomal fraction (74) and the presence of an enzyme catalysing lipid peroxidation may partly explain why isolated microsomes are particularly susceptible to lipid peroxidation.

Glutathione peroxidase from the cytosol could be involved in several metabolic processes, especially in glucose oxidation in connection with the pentose phosphate pathway which in liver may take account of approximately 50% of glucose metabolism (63).

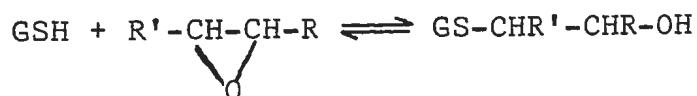
The GSH concentration could be very important for controlling the concentration of free CoA in the liver through the reaction



Since the equilibrium constant of this reaction is near unity (75) changes in the GSH/GSSG concentration ratio are expected to produce equal changes in the CoASH/GSSCoA ratio. Such changes in the liver content of free CoA may have important effects on fatty acid metabolism, e.g. increased concentration of Free CoA might accelerate lipogenesis and decreased level

might increase the concentration of free fatty acids and favour peroxidation.

Glutathione peroxidase, by influencing the GSH concentrations, might also be involved in the regulation of the ratio of saturated to unsaturated fatty acids. Indeed, Bayland and Chasseaud (76) described an enzyme in rat liver supernatant which catalysed the addition of GSH to double bonds. Bayland and Williams (77) also demonstrated that liver supernatant catalysed the conjugation of some epoxides with GSH according to the reaction



GSH may also be necessary for the intracellular transport of steroids because of its ability to form soluble conjugates with steroids.

Pinto and Bartley (78) showed that the female sex hormones (oestradiol and progesterone) can affect the GSH peroxidase activity of the liver and the rates at which rat liver oxidizes GSH or peroxidises lipids. Also, it is possible that changes in GSH peroxidase activity during the oestrus cycle and in pregnancy are associated with hormone changes.

The 20-fold higher rate of lipid peroxidation found in the female liver compared to the male, was partly due to the higher content of unsaturated lipids (79). Lipid peroxidation and the metabolism of oestrogens appear to be

competing processes (80) and suggest a relationship between GSH oxidation, lipid peroxidation, and oestrogen metabolism. The common factor in this relationship may be NADPH required for hydroxylation of oestrogens, for GSH reduction, and for fatty acid synthesis.

The action of sex hormone may also result in a possible GSH peroxidase induction, as a result of their action in enhancing lipid peroxidation. It is interesting that in the intestinal mucosa, which does not appear to undergo lipid peroxidation, GSH peroxidase level is also very low.

Further studies need to be done, to find out if GSH peroxidase is responsible for the very efficient protection against lipid peroxidation in vivo since no lipid peroxides have been found under normal conditions.

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