MOLECULAR PHARMACOLOGY, 13, 196-215

Interaction of Epinephrine Metabolites with the Liver Microsomal Electron Transport System

T. UEMURA,[1] E. CHIESARA, AND D. COVA

Institute of Pharmacology, University of Milan, and Consiglio Nazionale delle Ricerche Center of Cytopharmacology, Milan, Italy

(Received January 9, 1976)
(Accepted October 7, 1976)

SUMMARY

UEMURA, T., CHIESARA, E. & COVA, D. (1977) Interaction of epinephrine metabolites with the liver microsomal electron transport system. *Mol. Pharmacol.*, 13, 196-215.

3,4-Benzpyrene hydroxylase activity, NADPH-neotetrazolium reductase activity, and the NADPH-dependent lipid peroxidation reaction were inhibited by 100 μM epinephrine about 70%, 50%, and 70%, respectively, using either NADPH or an NADPH-generating system consisting of NADP and glucose 6-phosphate dehydrogenase. On the other hand, inhibition of benzpyrene hydroxylase and NADPH-neotetrazolium reductase activity by epinephrine was abolished using NADP and the soluble fraction as NADPH-generating system. Epinephrine itself did not have an inhibitory effect on these two enzyme activities; inhibition was due partly to electron transfer to epinephrine through superoxide-anion radical ($O_2^{\cdot-}$) at the level of NADPH-cytochrome *c* reductase and partly to the adrenochrome produced by epinephrine oxidation. Moreover, it was proved that adrenochrome itself was metabolized by liver microsomes consuming NADPH and transformed into leucoadrenochrome, probably via adrenochrome semiquinone, which may also have an inhibitory effect on benzpyrene hydroxylase even though it was much less effective than adrenochrome. It was found that the soluble fraction contained a heat-stable protein factor that blocked adrenochrome formation, thus reducing the inhibition of benzpyrene hydroxylase and NADPH-neotetrazolium reductase activities by epinephrine. This heat-stable protein factor was purified by a fairly simple method and identified as superoxide dismutase (hepatocuprein) from its spectrophotometric and electron paramagnetic resonance spectra and its capacity to scavenge $O_2^{\cdot-}$. The purified hepatocuprein alone, like the crude heated soluble fraction, had no inhibitory effect on the microsomal hydroxylation reaction and could not prevent the inhibitory action of adrenochrome. Like the heated soluble fraction, it only reduced the inhibitory action of epinephrine on microsomal benzpyrene hydroxylase activity. The addition of purified hepatocuprein alone significantly inhibited the microsomal NADPH-dependent lipid peroxidation reaction at high ionic strength. However, it could not block the inhibitory effect of epinephrine on the lipid peroxidation reaction as did the crude soluble fraction. Thus it is suggested that another mechanism is operative for the lipid peroxidation reaction, because inhibition was not affected by either the soluble fraction or the purified hepatocuprein. The inhibitory mechanism of epinephrine metabolites toward

This paper is dedicated to Professor Emilio Trabucchi on the occasion of his 70th birthday. This research was supported by grants from the Consiglio Nazionale delle Ricerche and the Ministero degli Affari Esteri.

[1] On leave from the Department of Biochemistry, University of Osaka Medical School, Osaka, Japan.

ISSN 0026-895x

the microsomal mixed-function oxidase system and the mode of action of hepatocuprein are discussed.

INTRODUCTION

NADPH-linked peroxidation of microsomal lipids was first reported by Hochstein and Ernster (1, 2). The relationship between the lipid peroxidation reaction and the NADPH-linked drug-hydroxylating system is not completely understood. It was suggested by Ernster *et al.* (3–5) and by Wills (6) that the two reactions involved a common NADPH-oxidizing enzyme, NADPH–cytochrome *c* reductase, which contributes reducing equivalents to both reactions. It was also observed that several substrates that are hydroxylated or demethylated through the microsomal mixed-function oxidase system inhibit the lipid peroxidation reaction of liver microsomes. More recently, however, these observations have been interpreted assuming that such inhibition is due to the antioxidant properties of the substrate or its metabolites (7) and not to competition for reducing equivalents.

On the other hand, it was reported that superoxide anion radical produced by NADPH–cytochrome *c* reductase participates in the lipid peroxidation reaction and in epinephrine oxidation (8, 9). It is also known that drug hydroxylations and demethylations are inhibited by physiological substances such as epinephrine and norepinephrine (10). However, Cooper and Rosenthal (11) reported that the addition of norepinephrine or epinephrine to bovine adrenal homogenates accelerated the hydroxylation of steroids.

The present study was undertaken to examine the effect of epinephrine *in vitro* on the microsomal electron transport system, comparing the effects of different NADPH-generating systems, and to clarify the mode of action of epinephrine and its metabolites on drug hydroxylation and the lipid peroxidation reaction. This paper reports the existence of a heat-stable factor in the soluble fraction, a fairly simple method for its purification, its identification as hepatocuprein (superoxide dismutase), and its interaction with the liver microsomal mixed-function oxidase system in the presence and absence of epinephrine and its metabolites.

MATERIALS AND METHODS

Materials

NADPH and cytochrome *c* were obtained from Boehringer/Mannheim or Sigma Chemical Company. *l*-Epinephrine bitartrate and adrenochrome were obtained from Sigma; xanthine oxidase, from Boehringer/Mannheim; and xanthine and Tiron, from Merck-Schuchardt. Other chemicals used were obtained from Bracco S.p.A. and were of reagent grade.

Preparation of Microsomes

Microsomes were prepared from the livers of fasted male Sprague-Dawley rats (300–500 g) by a modification of the procedure of Mitoma *et al.* (12). The livers were first perfused through the main blood vessels with an ice-cold 1.15% KCl solution to remove as much hemoglobin as possible. The microsomal pellets, washed once with 1.15% KCl solution, were suspended in 0.1 M Tris-HCl buffer (pH 7.5) at a protein concentration of 15–20 mg/ml. This microsomal preparation was found to be practically free of hemoglobin on spectrophotometric analysis in the presence of CO.

Enzyme Assays

3,4-Benzpyrene hydroxylase activity was determined by the method of Nebert and Gelboin (13) in an Aminco-Bowman spectrophotofluorometer. The fluorescence of a known concentration of quinine sulfate was used to calculate the number of nanomoles of 3-hydroxybenzpyrene formed (14).

Epinephrine oxidation was assayed by the method of Mazur *et al.* (15). Because epinephrine is also metabolized by cytochrome oxidase (16), 1.0–1.5 mM KCN was added to the reaction mixture to inhibit the cytochrome oxidase activity derived from mitochondrial contamination, al-

though KCN was not added in the original method (15). A millimolar extinction coefficient of 4.06 mM^{-1} cm^{-1} obtained from our 0.1 mM adrenochrome solution in distilled water at 480 nm was used to calculate the amount of adrenochrome formed. This extinction coefficient is in good agreement with the value of 4020 M^{-1} cm^{-1} reported by Green *et al.* (17).

Superoxide dismutase activity was assayed by the method of Misra and Fridovich (18), which is based on the fact that the nonenzymatic oxidation of epinephrine to adrenochrome at elevated pH (pH 10.2) is inhibited by superoxide dismutase. We confirmed their observation that 1 xanthine–xanthine oxidase unit is nearly equivalent to 2–3 adrenochrome units of superoxide dismutase activity. The spectrophotometric studies were performed on a Unicam SP 1800 spectrophotometer.

NADPH-NT[2] reductase activity was assayed by the methods of Williams and Kamin (19) and Mazel (20). A millimolar extinction coefficient of 14 mM^{-1} cm^{-1} at 500 nm was used to determine the amount of formazan formed (20, 21). Microsomal NADPH-dependent lipid peroxidation activity was measured by determining the amount of malondialdehyde formed in the thiobarbituric acid reaction (22). The millimolar extinction coefficient quoted by Sinnhuber, Yu, and Yu (23), 156 mM^{-1} cm^{-1} at 530 nm, was used. Protein concentration was determined by the method of Lowry *et al.* (24) with crystalline bovine serum albumin as standard. The colorimetric determinations employed a Saitron Monospec B spectrophotometer.

Heat and Acid Treatment of Soluble Fraction

The soluble fraction was heated at 97° for 5 min, followed by centrifugation. Acid treatment of the heated supernatant was performed in 3 N HCl at 97° for 30 min, followed by adjustment to pH 6.5–7.0 with 12 N NaOH. In order to compare the effect of the heat-stable factor(s) in the heated supernatant with that of GSH or ascorbate, the same acid treatment was performed for the latter two compounds.

Disc gel electrophoresis was performed on 7.5% polyacrylamide gels in Tris-glycine buffer, pH 8.3, essentially as described by Davis (25), with a Buchler instrument. Atomic absorption was measured with a Perkin-Elmer model 403 atomic absorption spectrophotometer. EPR spectra were measured with a JEOL JES ME 1x X-band EPR spectrometer, utilizing a 100-kc field modulation with a 9.5-GHz microwave bridge assembly at a sample temperature of −150°.

Purification of Heat-Stable Factor from Soluble Fraction

Step. 1: Preparation of soluble fraction from rat liver. The soluble fraction was prepared from rat livers (570–640 g from about 50 rats) perfused extensively with ice-cold 1.15% KCl solution to remove as much hemoglobin as possible.

Step. 2: Heat treatment. The soluble fraction was heated directly in a boiling water bath at 97° for 15 min, and the denatured protein was discarded by centrifugation and filtration. During the heat treatment, the flask was shaken only once, after 7 min. The 15-min period was chosen to denature other proteins as much as possible with only a small loss of enzyme blocking activity of red color formation.

Step. 3: Ammonium sulfate fractionation and dialysis. The clear, faintly yellow fluid obtained was fractionated with solid $(NH_4)_2SO_4$ and dialyzed according to Fried *et al.* (26).

Step. 4: Chromatography on DEAE-cellulose. The pale yellow fluid, clarified by centrifugation, was adsorbed on a column (2.5 × 62 cm) of DE-52 equilibrated with 12.5 mM ammonium bicarbonate (27). The column was washed with 400 ml of the same solution and eluted with an increasing linear concentration gradient of KCl from 0 to 0.34 M in the same medium.

RESULTS

Effect of Epinephrine on BP Hydroxylase, NADPH-NT Reductase, and NADPH-Dependent Lipid Peroxidation

Table 1 summarizes the inhibitory effect of epinephrine on microsomal BP hydrox-

[2] The abbreviations used are: NT, neotetrazolium chloride; BP, 3,4-benzpyrene; MDA, malonyl dialdehyde.

TABLE 1

Effect of epinephrine on BP hydroxylase, NADPH-NT reductase, and NADPH-dependent lipid peroxidation reaction

The BP hydroxylase reaction mixture contained 0.3 mg of microsomal protein, 0.1 M Tris-HCl (pH 7.5), 5 mM $MgCl_2$, 5 mM glucose 6-phosphate, 190 μM BP (added in 20 μl of acetone), 0.25 ml of the soluble fraction or 7.2 units of glucose 6-phosphate dehydrogenase, 100 μM epinephrine (added in 0.001 N HCl), and 1.2 mM NADP(H) in a final volume of 1.0 ml. Reactions were carried out at 37° for 10 min with mechanical shaking in the dark. The NADPH-NT reductase reaction mixture contained 0.2 mg of microsomal protein, 0.2 M potassium phosphate buffer (pH 7.5), 0.375 mM NT, 5 mM $MgCl_2$, 5 mM glucose 6-phosphate, 0.2 ml of the soluble fraction or 3.6 units of glucose 6-phosphate dehydrogenase, 100 μM epinephrine, and 0.8 mM NADP(H) in a final volume of 0.8 ml. Reactions were carried out at 37° for 10 min with mechanical shaking. For the NADPH-dependent lipid peroxidation reaction, the mixture contained 0.4 mg of microsomal protein, 0.1 M Tris-HCl (pH 7.5), 5 mM $MgCl_2$, 5 mM glucose 6-phosphate, 2 mM ADP, 40 μM $FeCl_3$, 0.2 ml of the soluble fraction or 3.6 units of glucose 6-phosphate dehydrogenase, 100 μM epinephrine, and 0.6 mM NADP(H) in a final volume of 2.0 ml. Reactions were carried out at 37° for 15 min with mechanical shaking.

NADPH-regenerating system	BP hydroxylase[a]	NADPH-NT reductase[b]	NADPH-dependent lipid peroxidation[c]
	nmoles product/min/mg protein (%)		
NADPH			
Control	2.14 (100)	165 (100)	5.54 (100)
Epinephrine	0.53 (25)	69 (42)	1.43 (25)
NADP, glucose-6-P, glucose-6-P dehydrogenase			
Control	2.82 (100)	162 (100)	7.94 (100)
Epinephrine	0.98 (35)	94 (58)	1.31 (16)
NADP, glucose-6-P, soluble fraction			
Control	4.65 (100)	230 (100)	4.97 (100)
Epinephrine	4.65 (100)	226 (100)	1.00 (20)

[a] The formation of 3-hydroxybenzpyrene was measured.

[b] The formation of formazan was measured.

[c] The formation of MDA was measured.

ylase, NADPH-NT reductase activity, and the NADPH-dependent lipid peroxidation reaction supported by three sources of reducing electrons. As expected, BP hydroxylase and NADPH-NT reductase activities and the NADPH-dependent lipid peroxidation reaction were inhibited by 100 μM epinephrine about 70%, 50%, and 70%, respectively, using NADPH or the NADPH-glucose 6-phosphate-glucose 6-phosphate dehydrogenase NADPH-generating system. A similar result was reported by Ichikawa *et al.* (10), who observed the inhibition of *p*-hydroxylation of *o*-chloroaniline by 500 μM epinephrine.

As shown in Table 1, however, with NADP, glucose 6-phosphate, and the soluble fraction as NADPH-generating system, the BP hydroxylase and NADPH-NT reductase activities were not inhibited by epinephrine whereas inhibition of the lipid peroxidation reaction was maintained. The specific activity of BP hydroxylation supported by glucose 6-phosphate dehydrogenase was about 60–70% of that supported by the soluble fraction. The reason is at present unknown. The soluble fraction may contain some factor that activates the metabolism of type I substrates (28, 29).

Using NADPH or the NADPH-glucose-6-phosphate-glucose-6-phosphate dehydrogenase system, the red color derived from the epinephrine oxidation product, adrenochrome, was observed in the reaction mixture after 15 min of incubation (the rate of adrenochrome formation was about 36 nmoles/min/mg of protein), and BP hydroxylase activity was inhibited at all times. Using the NADPH-glucose-6-phosphate-soluble fraction NADPH-generating system, however, the red color of adrenochrome and the inhibition of BP hydroxylation were not seen. Therefore, to examine the relationship between the presence of adrenochrome and the inhibition of these two enzyme activities, adrenochrome was added directly to the reaction mixture for the BP hydroxylase assay.

As shown in Table 2, BP hydroxylase activity was inhibited by adrenochrome using either NADPH-generating system. In contrast to epinephrine, 100 μM adrenochrome inhibited BP hydroxylase activity by about 45% despite the use of the soluble fraction. By adding NADPH directly as the electron donor, the possibility that glucose 6-phosphate dehydrogenase was inhibited by adrenochrome was excluded. For these observations, it is clear that adrenochrome itself acts as an inhibitor of the microsomal mixed-function oxidase. The time course of the inhibitory effects of epinephrine and adrenochrome on BP hydroxylase activity showed that adrenochrome was the better hydroxylase inhibitor. Inhibition persisted even after the final incubation period (30 min), despite the further addition of NADPH during the course of the reaction. The red color of adrenochrome disappeared within a few minutes, but the inhibition remained. Therefore it seems likely that not only adrenochrome but also its metabolite acts as inhibitor. To exclude a possible insufficiency of oxygen as the reason for competition between epinephrine and benzpyrene, oxygen was bubbled through the incubation mixture. Under this condition BP hydroxylase activity was inhibited very strongly by 100 μM epinephrine, with the simultaneous appearance of the intense red color. Therefore inhibition was not due to an insufficiency of oxygen but to the strong electron withdrawal and the large amount of adrenochrome formed.

TABLE 2

Inhibition of BP hydroxylase activity by adrenochrome

Enzyme activity was assayed as described in Table 1, except for the use of 0.65 mg of microsomal protein and the addition of the indicated amount of adrenochrome. Reactions were carried out at 37° for 15 min.

NADPH-generating system	Adrenochrome	BP hydroxylase activity
	μM	*nmoles/min/mg protein (%)*
NADP, glucose-6-P, soluble fraction	0	4.26 (100)
	50	3.88 (91)
	100	2.34 (55)
NADP, glucose-6-P, glucose-6-P dehydrogenase	0	2.32 (100)
	50	1.35 (58)
	100	0.51 (22)

Effect of Heated Soluble Fraction on BP Hydroxylase Activity

It was of interest to clarify the mechanism of prevention of inhibition by epinephrine and of red color formation, using the soluble fraction as NADPH-generating system. The soluble fraction was heated at 95° for 5 min. As shown in Table 3, the existence of a heat-stable factor(s) in the soluble fraction was found, since the heated supernatant was still effective in blocking the inhibitory effect of epinephrine and the red color derived from epinephrine oxidation was not seen. Since this heat-stable factor(s) must possess a lower oxidation-reduction potential than epinephrine, it was compared with the physiological oxidoreductants GSH and ascorbate. As expected, neither compound produced the inhibitory effect on BP hydroxylase activity and the simultaneous adrenochrome formation observed with epinephrine (Table 3).

In order to characterize this heat-stable factor(s) further, the heated soluble fraction was treated with acid to determine whether it could still reduce the inhibitory effect of epinephrine on BP hydroxylase activity. The heated soluble fraction treated with acid could no longer reduce the inhibitory effect of epinephrine (Table 3), whereas GSH and ascorbate retained the ability to abolish the inhibitory effect of epinephrine even after acid treatment under the same conditions. Therefore it seems unlikely that this factor(s) may be GSH or ascorbate.

Effect of Heated Soluble Fraction on Adrenochrome Formation

In order to clarify the mode of action of the heat-stable factor(s) in preventing the inhibition of hydroxylase activity by epinephrine and in suppressing red color formation, the influence of the heat-treated

TABLE 3

Effect of heat-stable factor(s), ascorbate, and GSH, with and without acid treatment, on inhibition of BP hydroxylase activity by epinephrine

The experimental conditions were the same as described in Table 1, except for the addition of the heated supernatant (HS; 0.2 ml in experiment 1 and 0.25 ml in experiment 2), 2 mM ascorbate, or 2 mM GSH. Acid treatment was performed as described in the text. In experiment 2 NADPH alone was added; in experiment 1 the NADPH-regenerating system was NADP, glucose 6-phosphate, and glucose 6-phosphate dehydrogenase.

Additions	BP hydroxylase activity	Adrenochrome formation
	nmoles/mg protein/min (%)	
Experiment 1		
None (control)	2.72 (100)	
HS alone	2.58 (95)	
Epinephrine (100 μM)	0.87 (32)	+
Epinephrine + HS	2.31 (85)	–
Ascorbate	2.20 (81)	
Epinephrine + ascorbate	1.80 (66)	–
GSH	2.40 (88)	
Epinephrine + GSH	2.86 (108)	–
Experiment 2		
None (control)	2.02 (100)	
Epinephrine (100 μM)	0.32 (16)	+
Epinephrine + HS	1.56 (78)	–
Epinephrine + acid-treated HS	0.15 (8)	+
Epinephrine + GSH	1.78 (88)	–
Epinephrine + acid-treated GSH	1.88 (93)	–
Epinephrine + ascorbate	1.03 (51)	–
Epinephrine + acid-treated ascorbate	1.28 (63)	–

soluble fraction on adrenochrome formation was investigated. Figure 1 shows the time course of adrenochrome formation by microsomes and the effect of the heat-treated soluble fraction. As reported by Aust *et al.* (9), an unexplained lag period was seen, which was lengthened by adding the heated soluble fraction. NADPH was not consumed during this lag time (Fig. 1). These results indicate that the heated supernatant contains some factor(s) that interferes with epinephrine oxidation.

When adrenochrome formation after addition of the heated supernatant treated with acid was measured (Fig. 2), no further lengthening of the lag time was observed, while GSH or ascorbate treated with acid under the same conditions increased the lag time. This spectrophotometric result is in accordance with the data for BP hydroxylase inhibition (Table 3). Therefore it can be concluded that the heat-stable factor(s) in the soluble fraction is neither GSH nor ascorbate.

Another possible explanation for the colorless reaction mixture is the proposal of Imaizumi (28) that epinephrine quinone reacts with an amino acid which is oxidatively deaminated, liberating hydrogen, which reduces epinephrine quinone to epinephrine. This possibility was ruled out by testing glycine and L-histidine at a concentration of 1 mM.

Identification of Purified Heat-Stable Factor as Hepatocuprein (Superoxide Dismutase)

Since adrenochrome formation was inhibited approximately 90% even after the soluble fraction was heated at 97° for 15 min, this fraction probably contains a heat-stable protein. In this respect it is similar to the neotetrazolium reductase inhibitor recently isolated from beef liver (26, 31), which may be the same as hepatocuprein (27), hen liver cytoplasmic superoxide dismutase (32), and bovine erythrocyte superoxide dismutase (33). Forman and Fridovich (34) reported that the bovine erythrocyte holoenzyme was not perceptibly inactivated at 70°. On the basis of these data, the heat-stable protein factor was purified according to published isolation methods (26, 27, 31, 32), except for heat treatment as described in MATERIALS AND METHODS. A typical elution pattern is presented in Fig. 3. Fractions having an A_{259}:A_{280} absorbance ratio higher than 1.36 were pooled (35)[3] and concentrated by ultrafiltration using a Diaflow PM-10 membrane (32). The concentrated solution was clarified by centrifugation and used for spectral as well as activity assays. Typical

[3] The A_{259}:A_{280} absorbance ratio of 1.0 or more, used by Sawada *et al.* (35), is a good, simple criterion, since almost no activity could be detected in fractions where this ratio was less than 1.0.

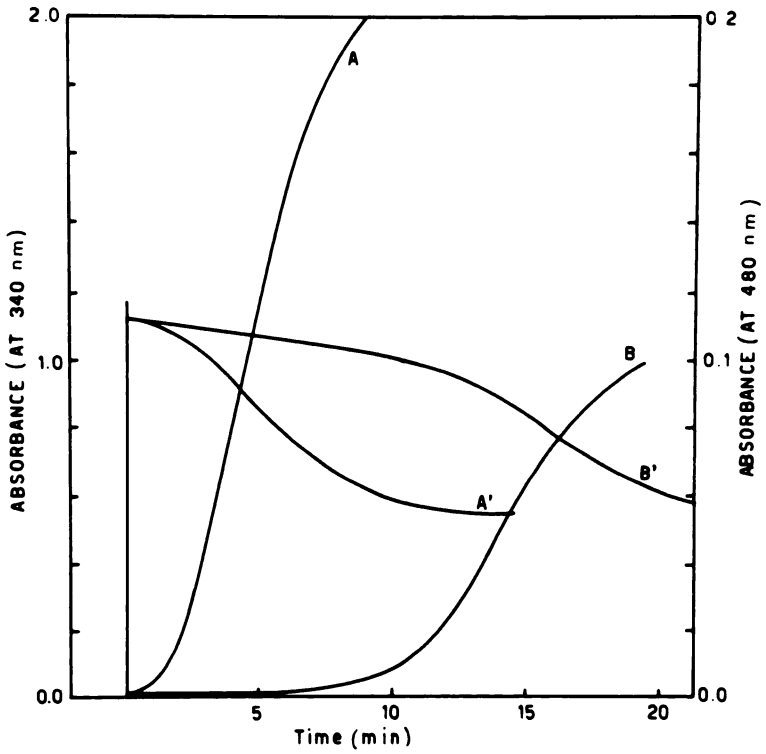


FIG. 1. *Relationship between adenochrome formation and NADPH oxidation by rat liver microsomes*
The reaction mixture, in a final volume of 1.5 ml, contained 0.30 mg of microsomal protein, 0.1 M Tris-HCl buffer (pH 8.1), 0.5 mM epinephrine, 1 mM KCN, 0.2 mM NADPH, and 0.1 ml of the heated supernatant (1.5 times diluted with H_2O). The reaction was started by the addition of NADPH, and optical density was recorded at 480 nm and at 340 nm against a reference cuvette which contained all components except NADPH. All the assays were carried out at room temperature (29°). Curves *A* (at 480 nm) and *A'* (at 340 nm), controls; *B* (at 480 nm) and *B'* (at 340 nm), with heated supernatant.

purification data are summarized in Table 4.

Spectral properties. The optical absorption spectrum of the purified heat-stable protein factor from boiled rat liver supernatant (Fig. 4A) is very similar to those reported for erythrocuprein (33), hepatocuprein (27, 36), and cerebrocuprein (36). The broad absorption peak at about 670 nm and the blue-green color of our purified sample indicate the presence of copper. The molar extinction coefficients obtained from our ultraviolet and visible spectra (ϵ_{259} = 5330 and ϵ_{670} = 129 M^{-1} cm^{-1}) are somewhat lower than those reported by others (33, 36). A molar absorptivity in the ultraviolet region about 50% lower than other reports (33, 37) coincides with an approximately 50% lower content of phenylalanine in our samples. The EPR spectra of oxidized and sodium borohydride-reduced samples are presented in Fig. 4B. The oxidized EPR spectrum is again similar to those of erythrocuprein, hepatocuprein, cerebrocuprein (36), bovine heart superoxide dismutase (38), and green pea superoxide dismutase (35).

Metal analysis. Metal analyses performed on three separate preparations by atomic absorption spectrophotometry demonstrated that our purified samples contained 0.70–0.88 atom of copper and 1.20–1.45 atoms of zinc per molecule of enzyme, assuming a molecular weight of about 33,000 (32, 36, 37). Manganese and iron could not be detected. The proportion of copper relative to zinc is somewhat lower than reported for human hepatocuprein (36), bovine erythrocuprein (33), and bovine heart superoxide dismutase (38), in

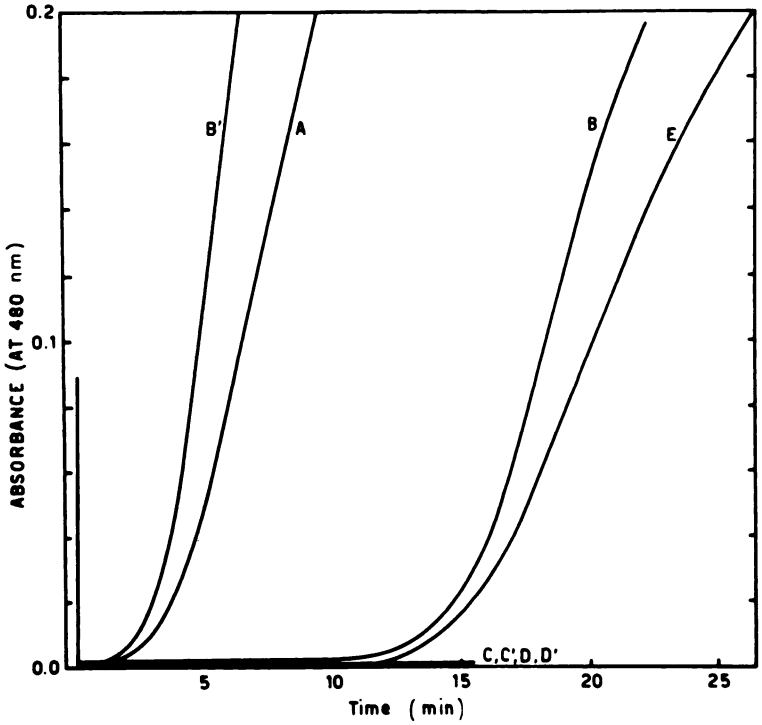


FIG. 2. *Effect of heated supernatant, GSH, and ascorbate, with and without acid treatment, on oxidation of epinephrine catalyzed by rat liver microsomes*

The reaction mixture, in a final volume of 1.5 ml, contained 0.32 mg of microsomal protein, 0.1 M Tris-HCl buffer (pH 8.1), 0.5 mM epinephrine, 1.0 mM KCN, 0.24 mM NADPH, and 0.15 ml of the heated supernatant (diluted 1.5 times) GSH as indicated, or 1 mM ascorbate, either untreated or treated with acid. The reaction was started by the addition of NADPH, and the time course of the change in absorbance at 480 nm was determined at room temperature (29°) against a reference cuvette which contained all components except NADPH. Curve *A*, control; *B*, heated supernatant; *B'*, acid-treated *B*; *C*, 1 mM GSH; *C'*, acid-treated *C*; *D*, 1 mM ascorbate; *D'*, acid-treated *D*; *E*, 0.2 mM GSH.

accordance with the low extinction coefficient of our sample at 670 nm. The reason was not further studied in the present work, but it may be due to the presence of either a natural apoprotein in rat liver cytoplasm or an apoprotein produced by the fairly drastic heat treatment.

Enzymatic activity. With respect to its enzymatic activity as an O_2^- scavenger, our purified heat-stable factor inhibited both cytochrome *c* reduction in the xanthine–xanthine oxidase system and adrenochrome formation at alkaline pH, both of which require O_2^- (18). It was approximately 3 times more active in inhibiting adrenochrome formation than cytochrome *c* reduction (Fig. 5), in agreement with Misra and Fridovich (18), who found the former method to be twice as sensitive as the latter for bovine erythrocuprein. The catalytic activity of our preparation was also similar to that of Misra and Fridovich (18), who used 46 ng/ml of bovine erythrocyte superoxide dismutase to achieve 50% inhibition of adrenochrome formation, while we obtained it with 71 ng/ml of our purified preparation (Fig. 5).

Purity. As shown in Fig. 6, our sample was separable into three bands by electrophoresis on polyacrylamide gel, even in the absence of sodium dodecyl sulfate and mercaptoethanol. Bannister *et al.* (40) reported that bovine erythrocyte cupric-zinc protein showed two components on polyacrylamide gel electrophoresis, and Weisiger and Fridovich (32) observed a major and a minor band when the chicken liver cytosol enzyme was subjected to polyacryl-

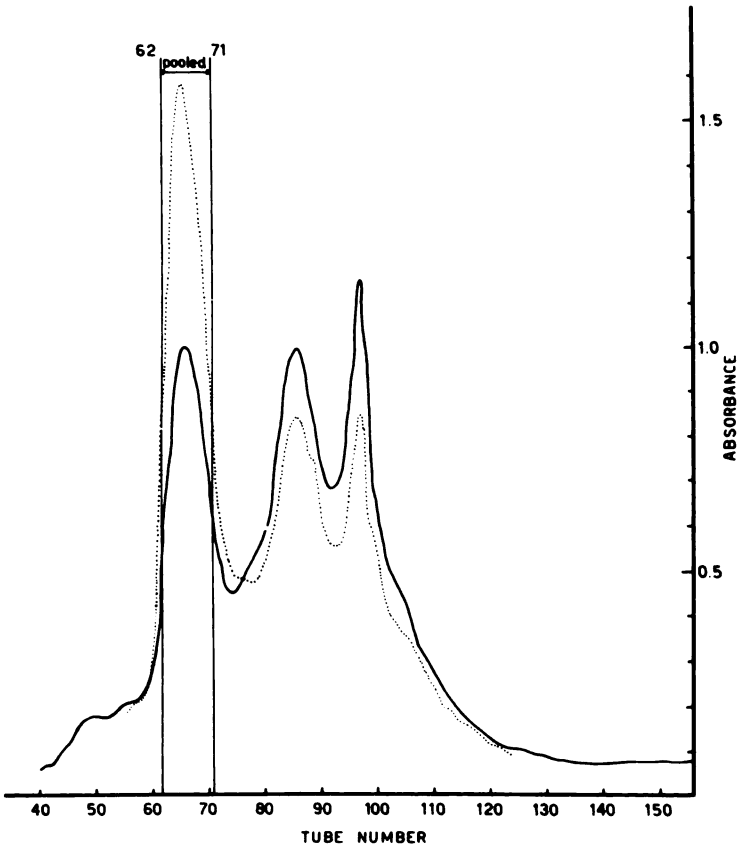


FIG. 3. *Adsorption chromatography on DE-52*

The sample was adsorbed on a column, 2.0 × 62 cm, of DE-52 equilibrated with 12.5 mM ammonium bicarbonate and then eluted with a linear KCl gradient from 0 to 0.34 M in ammonium bicarbonate. Fractions were analyzed for absorbance at 280 nm (——) and 259 nm (· · · ·).

TABLE 4

Purification of rat liver cytoplasmic superoxide dismutase

Step	Total volume	Protein		Activity		Yield	Purification
		Concentration	Total	Total	Specific		
	ml	*mg/ml*	*mg*	*units*	*units/mg protein*	*%*	*-fold*
1. Native supernatant	1690	15.84	26,800	2,115,000	79	100	1.0
2. Boiled supernatant	1550	1.45	2,240	1,995,000	893	94	11.3
3. $(NH_4)_2SO_4$ and dialysis	32.5	15.10	441	1,340,000	3,025	63	38.3
4. DEAE-cellulose	20	6.04	128	1,030,000	8,040	39	102

amide gel electrophoresis in the presence of 1% sodium dodecyl sulfate and 0.1 M mercaptoethanol. Figure 6 shows, however, that all bands were active when stained and tested photochemically (39). Thus apparently no other protein components were present which did not exhibit superoxide dismutase activity. The presence of three active bands was probably due to polymer formation, as was observed for chicken liver cytoplasmic superoxide dismutase which, although freshly prepared, separated into two peaks on Sephadex G-75 (32).

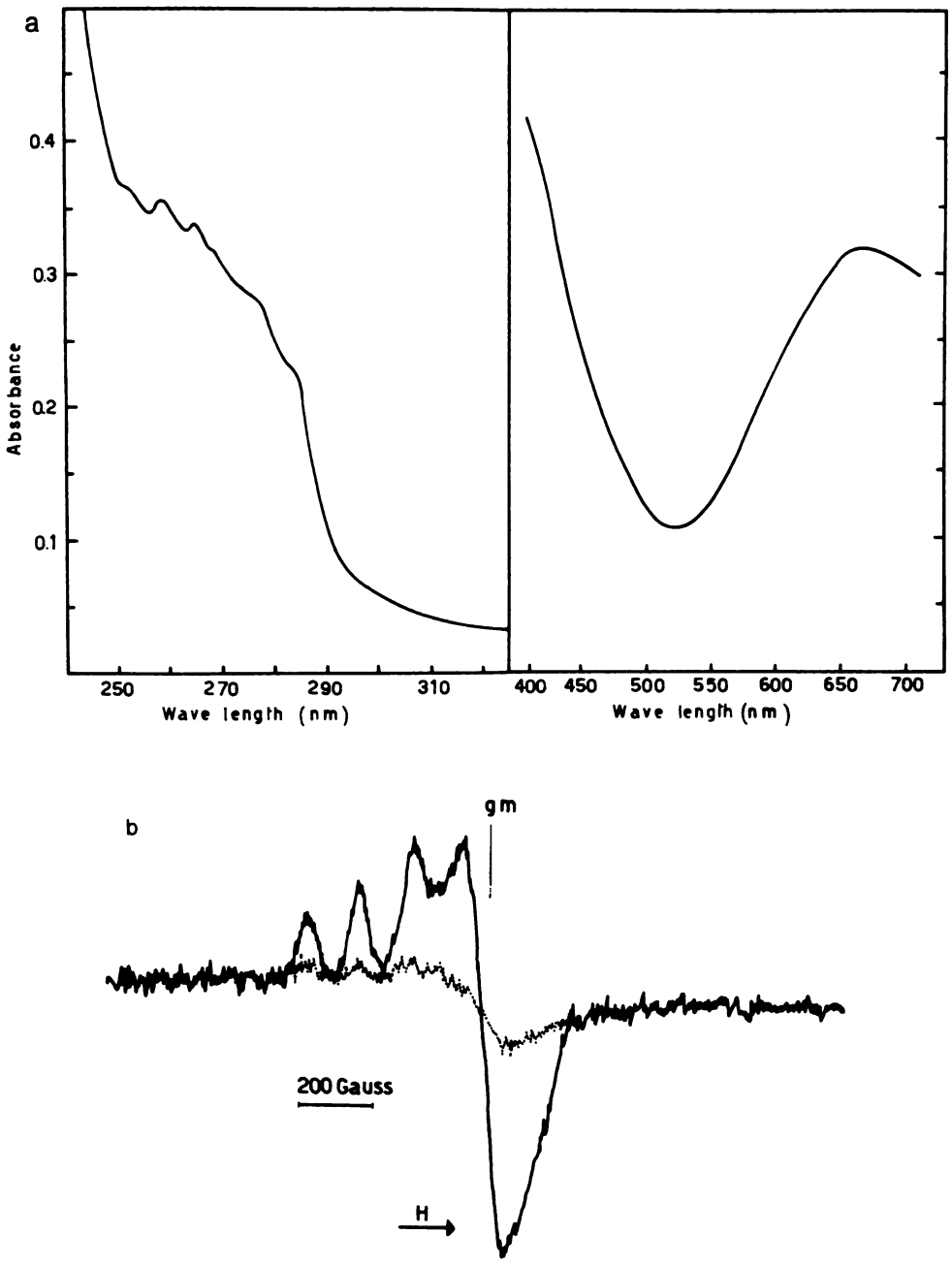


FIG. 4. *Spectral analyses of superoxide dismutase from rat liver cytoplasm*

a. Ultraviolet and visible absorption spectra. The concentration of the enzyme was 2.0 mg/ml for the ultraviolet region (left) and 79.2 mg/ml for the visible region (right), in 12.5 mM ammonium bicarbonate. The light path was 10 mm.

b. EPR spectra of oxidized (——) and sodium borohydride-reduced (· · · ·) superoxide dismutase. The enzyme concentration was 67.6 mg/ml in 12.5 mM ammonium bicarbonate. The microwave frequency was 9.215 GHz; microwave power, 4 mW; modulation amplitude, 10 G; scan rate, 80 G/min; time constant, 1.0 sec; and sample temperature, −150°. The values of spectral parameters were estimated by comparison with the DPPH (α,α-diphenyl-β-picryl-hydrazyl) signal and were: $g_{\parallel} = 2.24$, $g_{m} = 2.04$, and $A_{\parallel} = 0.014$ cm^{-1}.

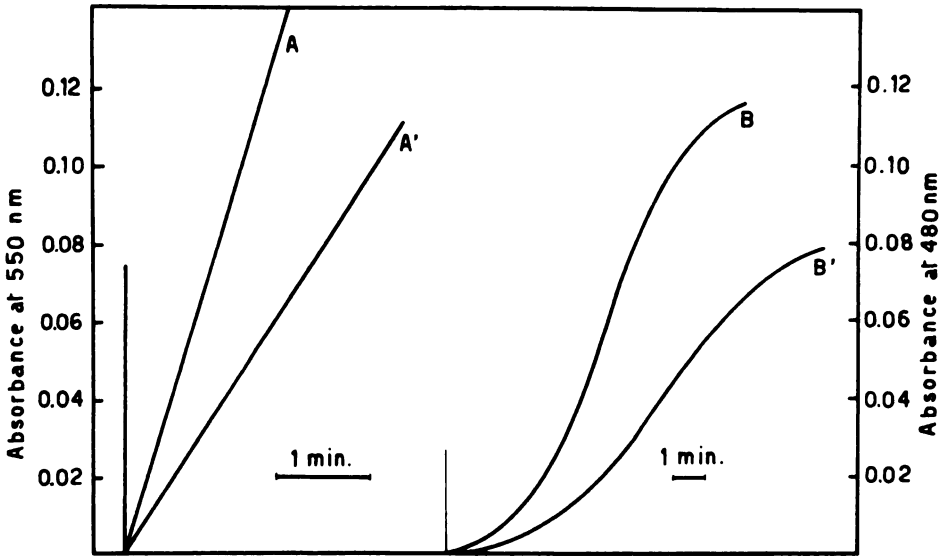


FIG. 5. *Comparison of two different systems for superoxide dismutase activity*

The cuvettes contained 20 μM ferricytochrome *c*, 66 μM xanthine (dissolved in 0.05 M sodium carbonate, pH 10.2), 10 nM xanthine oxidase (milk), 100 μM EDTA, 0.05 M sodium carbonate (pH 10.2), and the indicated amounts of purified hepatocuprein in a total volume of 1.52 ml. The reference cuvette contained all components except xanthine oxidase. The increments in absorbance at 550 nm were recorded at 30°. When adrenochrome production was measured, cuvettes contained 500 μM epinephrine, 100 μM EDTA, 0.05 M sodium carbonate (pH 10.2), and the indicated amounts of purified hepatocuprein in a total volume of 1.50 ml. The reference cuvette contained all components except epinephrine. The increase in absorbance at 480 nm was measured at 30°. Curve *A*, cytochrome *c* reduction by the xanthine oxidase system; *A'*, *A* + 286 ng of purified hepatocuprein; *B*, adenochrome formation; *B'*, *B* + 107 ng of purified hepatocuprein.

Amino acid analysis. Two preparations were analyzed separately for their amino acid content after hydrolysis for 22 hr in 6 N HCl in sealed tubes freed of air. Like other superoxide dismutases (37), our preparations contained little or no methionine, tyrosine, or tryptophan (Table 5). In its low content of lysine and phenylalanine our sample resembles a *Neurospora* superoxide dismutase (42), while the contents of other amino acids, such as glycine, are similar to those of the mammalian erythrocyte enzyme (37).

All these results indicate that our heat-stable protein factor is identical with hepatocuprein which acts as superoxide dismutase; thus it abolished the inhibitory effect of epinephrine on BP hydroxylase activity by blocking its transformation to adrenochrome.

It seems likely that native rat liver cytoplasm contains some stabilizer(s) against heat denaturation of superoxide dismutase, since the cytoplasmic enzyme molecule itself varies only slightly from organ to organ and from animal to animal except for small variations in amino acid composition (37). It is possible, however, that amino acid substitutions have resulted in thermostability, as in mouse liver superoxide dismutase (43). Marnett *et al.* (44) reported that the supernatant of sheep vesicular gland easily loses its superoxide dismutase activity on boiling.

Effect of Purified Hepatocuprein (Superoxide Dismutase) on Microsomal BP Hydroxylase Activity and NADPH-Dependent Lipid Peroxidation Reaction

The possible participation of O_2^- in the microsomal hydroxylation reaction, by serving either as an activated form of oxygen or as a reducing agent, is now the subject of some controversy (45–49). Coon *et al.* (45) observed slight but significant inhibition of benzphetamine hydroxylation by superoxide dismutase in an intact microsomal suspension and quite strong

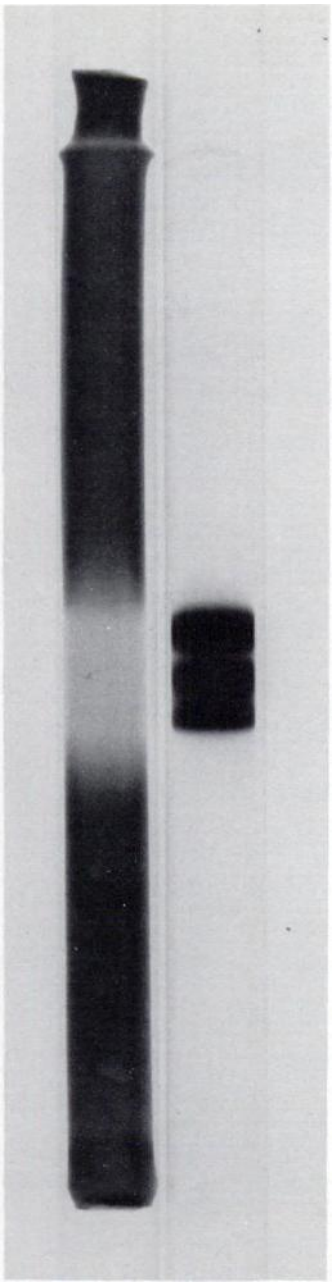


FIG. 6. *Polyacrylamide gel electrophoresis at pH 8.3 of purified hepatocuprein isolated from rat liver cytoplasm*

The large amount of enzyme (145 μg) was purposely charged in order to detect impurities. Electrophoresis was performed at 70–150 V and 1.5 mamp/tube for about 4 hr. Gel 1 (right) was stained with Amido black. Gel 2 (left) was stained for enzymatic activity according to the photochemical method of Beauchamp and Fridovich (39).

inhibition by Tiron in their reconstituted system. As shown in Table 6, BP hydroxylase activity was not inhibited even in the presence of large amounts of purified hepatocuprein at high salt concentrations (0.6 M NaCl) with a molar ratio of superoxide dismutase to P-450 of about 130:1, which is about 5.4 times higher than that used by Coon *et al.* (45, 46). As shown below, one can exclude the possibility that the decrease in superoxide dismutase activity at high ionic strength (50) was responsible for the lack of effect on the hydroxylation reaction. Furthermore, Tiron, a powerful superoxide anion scavenger, had only a slight effect on microsomal BP hydroxylase activity at a concentration of 2 mM at normal ionic strength. These results suggest that $O_2^{\cdot-}$ is not a direct hydroxylating species, even though one cannot rule out the possibility that superoxide dismutase is incapable of attacking cytochrome P-450–heme-bound active oxygen ($O_2^{\cdot-}$) as suggested by Kimura *et al.* (48), or that, prior to the action of superoxide dismutase, the ferrous enzyme–superoxide intermediate decomposes very rapidly to a ferric enzyme–monooxygen species in the presence of substrate for cytochrome P-450 as proposed more recently by Hrycay *et al.* (51).

In the presence of epinephrine, which consumes $O_2^{\cdot-}$ at the level of NADPH–cytochrome *c* reductase, however, the addition of hepatocuprein abolished the inhibitory effect of epinephrine on BP hydroxylase activity, as did the crude soluble fraction (Tables 3 and 6). In contrast, the inhibitory effect of adrenochrome was not abolished by the addition of hepatocuprein (Tables 2 and 6), indicating that adrenochrome unlike epinephrine does not use $O_2^{\cdot-}$. These conclusions are supported by the effect of added hepatocuprein on NADPH oxidation, as reported in the next section.

In the NADPH-dependent microsomal lipid peroxidation reaction the addition of a small amount of hepatocuprein (10 μg) did not inhibit MDA formation, as reported by King *et al.* (52). Increasing the amount of added hepatocuprein, however, inhibited MDA formation (Table 7). Even

TABLE 5

Amino acid composition of rat hepatocuprein

Amino acid[a]	Residues			Calculated No. of residues per 33,000 mol wt (nearest integer)
	Preparation I	Preparation II	Mean	
	g/100 g protein			
Lysine	5.24	5.58	5.41	12
Histidine	7.24	6.44	6.84	15
Arginine	4.31	4.22	4.26	8
Aspartic acid[b]	11.83	12.05	11.94	30
Threonine	6.05	6.06	6.05	17
Serine	5.62	5.17	5.39	17
Glutamic acid[b]	15.19	11.90	13.54	30
Proline	4.24	4.30	4.27	12
Glycine	11.89	11.43	11.66	51
Alanine	6.20	6.04	6.12	23
Valine	8.50	8.56	8.53	24
Methionine	0	0	0	0
Isoleucine	5.13	5.33	5.23	13
Leucine	4.93	4.71	4.82	12
Tyrosine	0	0	0	0
Phenylalanine	2.50	2.58	2.54	5
Tryptophan[c]	0.12	0.48	0.30	0

[a] Half-cystine and cysteine were not determined.

[b] Amide content was not determined.

[c] Determined by the method of Spies and Chambers (41).

TABLE 6

Effect of superoxide anion scavengers on microsomal BP hydroxylase activity in the presence and absence of epinephrine and adrenochrome

The reaction mixtures contained 1.2 mM NADPH as electron donor and were the same as described in Table 1, except for the addition of the indicated amount of purified hepatocuprein in the presence of 0.6 M NaCl or the addition of 2.0 mM Tiron without 0.6 M NaCl. The reactions were carried out at 30° for 10 min with mechanical shaking in the dark.

O_2^- scavengers	Control	Epinephrine (100 μM)	Adrenochrome (100 μM)
		nmole/mg protein/min (%)	
None	0.40 (100)	0.14 (35)	0.13 (32)
Hepatocuprein (856 μg)	0.47 (117)	0.41 (102)	0.15 (37)
Hepatocuprein (1712 μg)	0.42 (105)	0.42 (105)	0.13 (32)
None	0.82 (100)	0.24 (24)	0.08 (9)
Tiron (2 mM)	0.73 (89)	0.21 (25)	0.09 (11)

large amounts of hepatocuprein could not abolish the inhibitory effect of catecholamines on the microsomal lipid peroxidation reaction, as did the native supernatant (Tables 1 and 7). Adrenochrome is a stronger lipid peroxidation inhibitor than epinephrine. Our native soluble fraction contained about 88 μg/ml of superoxide dismutase, which is approximately equal to 0.24 mg/g of liver (calculated from Table 4 and Fig. 5).

Our finding that superoxide dismutase itself inhibits the microsomal NADPH-dependent lipid peroxidation reaction is in accordance with that obtained in the purified NADPH–cytochrome *c* reductase system by Pederson and Aust (8) but does not agree with that of King *et al.* (52), who reported no inhibition of lipid peroxidation in whole microsomes by superoxide dismutase. The discrepancy may be related to the ratio of microsomal protein to superoxide dismutase or to differences in ionic strength. It seems likely that the destruc-

TABLE 7

Effect of purified hepatocuprein on microsomal NADPH-dependent lipid peroxidation reaction

The reaction mixture contained 0.21 mg of microsomal protein, 0.1 M Tris-HCl buffer (pH 8.1), 2 mM ADP, 40 μM $FeCl_3$, 0.6 M NaCl, 1.8 mM NADPH, and the indicated amounts of epinephrine, adrenochrome, or hepatocuprein in a final volume of 2.0 ml. Blank test tubes contained all components except NADPH. Reactions were carried out at 30° for 10 min with mechanical shaking.

Hepatocuprein	Control	Epinephrine (50 μM)	Adrenochrome (50 μM)
μg	*nmoles MDA formed/mg protein/min (%)*		
0	6.30 (100)	4.52 (72)	0.75 (12)
10	6.06 (96)	4.72 (75)	0.88 (14)
100	5.06 (80)	4.72 (75)	0.88 (14)
1000	4.04 (64)	3.66 (58)	0.69 (11)

tion of microsomal membranes by high ionic strength and the addition of large amounts of superoxide dismutase are necessary to see the inhibitory effect of the latter on the lipid peroxidation reaction in whole microsomes.

Metabolism of Adrenochrome by Liver Microsomes, and Effect of Purified Hepatocuprein (Superoxide Dismutase)

In order to clarify the inhibitory mechanism of adrenochrome on microsomal BP hydroxylase activity and to determine whether adrenochrome is oxidized to oxoadrenochrome via adrenolutin or reduced to leucoadrenochrome via adrenochrome semiquinone, thus indicating whether or not $O_2^{\cdot-}$ participates in the metabolism of adrenochrome in hepatic microsomes, the following experiments were performed.

Figure 7 shows the enzymatic formation and disappearance of adrenochrome by liver microsomes. Once formed, adrenochrome disappeared when NADPH was added; subsequent aeration caused the red color to reappear, although not to its initial intensity.[4] This result suggests that adrenochrome was transformed to leucoadrenochrome, consuming NADPH. Indeed, NADPH was aerobically oxidized very slowly in the presence of microsomes (Fig. 8), and the addition of adrenochrome stimulated this oxidation very strongly, as reported for vitamin K_3 (54). Moreover, the consumption of NADPH in the presence of adrenochrome was not inhibited by the addition of either a large amount of purified hepatocuprein or a chemical superoxide anion scavenger, Tiron (Fig. 8).

The addition of adrenochrome or vitamin K_3 to microsomes resulted in the increased formation of $O_2^{\cdot-}$, detected by the acceleration of cytochrome *c* reduction, as in the case of ferredoxin-NADP reductase (55) or the xanthine oxidase system (55, 56). This result indicates that adrenochrome and vitamin K_3 receive 1 electron and are transformed to semiquinones, which react easily with oxygen and consequently provoke the increased formation of $O_2^{\cdot-}$ as well as leakage of electrons, as demonstrated by Nishibayashi *et al.* (57, 58) and Iyanagi and Yamazaki (59) with naphthoquinone derivatives. These observations indicate that liver microsomes reduce adrenochrome to leucoadrenochrome, probably via adrenochrome semiquinone, consuming NADPH,[5] and that the reduction is not mediated indirectly by $O_2^{\cdot-}$ but

[4] According to the work of Harley-Mason (53), leucoadrenochrome immediately undergoes irreversible dehydration to 5,6-dihydroxy-*N*-methylindole. This may be why the red color could not be completely restored.

[5] Adrenochrome is not very stable and is itself subject to further nonenzymatic oxidation in strongly alkaline solution as well as at physiological pH. It is therefore necessary to determine whether the disappearance of red color observed in microsomal system represents nonenzymatic oxidation of adrenochrome. The oxidation product of adrenochrome is oxoadrenochrome (nonfluorescent), which is formed via adrenolutin (strongly fluorescent), whose formation from adrenochrome in alkaline solution is irreversible. The reduction product of adrenochrome is colorless leucoadrenochrome (strongly fluorescent), and this reaction is reversible. We observed that nonenzymatic oxidation of adrenochrome at alkaline pH (pH 10.2) produced a faint yellow color, which did not return to red on aeration, whereas NADPH-dependent disappearance of the red color of adrenochrome at pH 7.5 produced a colorless solution that partially resumed its red color on aeration. Moreover, the disappearance of red color did not occur without the addition of NADPH. Therefore the disappearance of adrenochrome was truly due to its enzymatic reduction to the leuco form and not to nonenzymatic oxidation (see ref. 60).

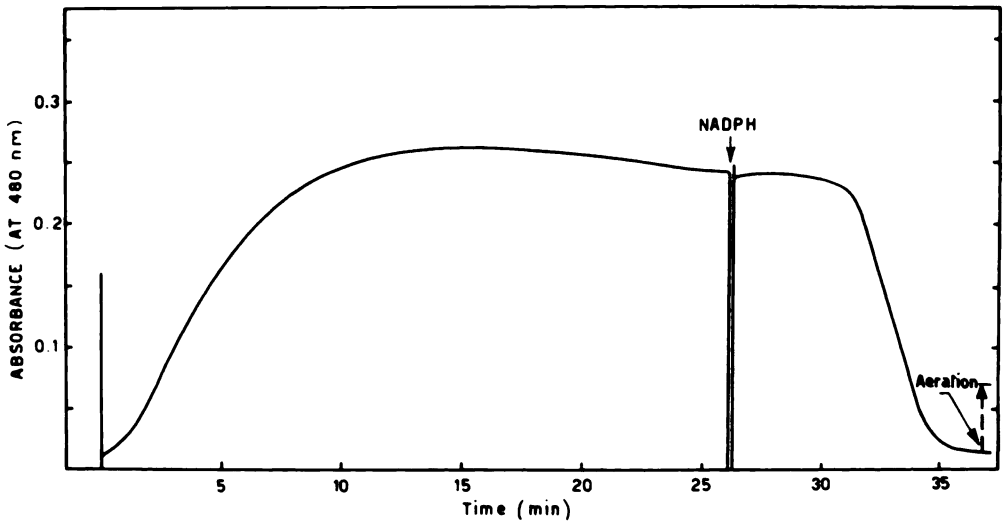


FIG. 7. *NADPH-dependent formation and disappearance of adenochrome by rat liver microsomes*
The reaction mixture, in a final volume of 1.5 ml, contained 0.30 mg of microsomal protein, 0.1 M Tris-HCl buffer (pH 8.1), 0.5 mM epinephrine, 1 mM KCN, and 1.2 mM NADPH. The reaction was initiated by the addition of NADPH, and the time course of the change in absorbance at 480 nm was determined against a reference cuvette which contained all components except NADPH. As indicated, 0.625 mM NADPH was added during the course of the reaction.

directly by electrons, since neither purified hepatocuprein nor Tiron affected the transformation of adrenochrome to leucoadrenochrome. This explanation contradicts that of Augusto *et al.* (61), who reported indirect adrenochrome reduction by O_2^- in liver microsomes.

DISCUSSION

Superoxide anion radical produced by NADPH-cytochrome *c* reductase can catalyze many reactions—epinephrine oxidation (9), cytochrome *c* reduction (62), and lipid peroxidation (7)—even though recent work has demonstrated that the radical species causing direct lipid peroxidation in microsomes during NADPH oxidation is not O_2^- but a hydroxyl radical derived from O_2^- and H_2O_2 (63).

In the absence of epinephrine, and by using the soluble fraction as NADPH-generating system, the microsomal NADPH-dependent lipid peroxidation reaction was inhibited and BP hydroxylase activity was accelerated about 2-fold compared with the reaction supported by NADPH alone or by the glucose 6-phosphate dehydrogenase system (Table 1). Thus there appears to be an inverse relationship between lipid peroxidation and monooxygenase activity, as pointed by Kamataki *et al.* (64) and Kotake *et al.* (65). The heat-stable factor in our soluble fraction, identified as hepatocuprein, however, is similar neither to the heat-labile factor of Kotake *et al.* (65) nor to the factor(s) of Kamataki *et al.* (64). The factor(s) in their soluble fraction stimulated the monooxygenase system by reversing the inhibitory effect of NADPH-supported lipid peroxidation, whereas hepatocuprein itself had no effect on monooxygenase activity even though it significantly inhibited the microsomal lipid peroxidation reaction (Tables 6 and 7). EDTA could partially mimic the action of their factor(s) (64, 65) but it could not duplicate the action of either the soluble fraction or purified hepatocuprein, since it did not inhibit the microsomal enzymatic oxidation of epinephrine to adrenochrome.

In the presence of epinephrine the monooxygenase activity was inhibited, but addition of the heated supernatant decreased

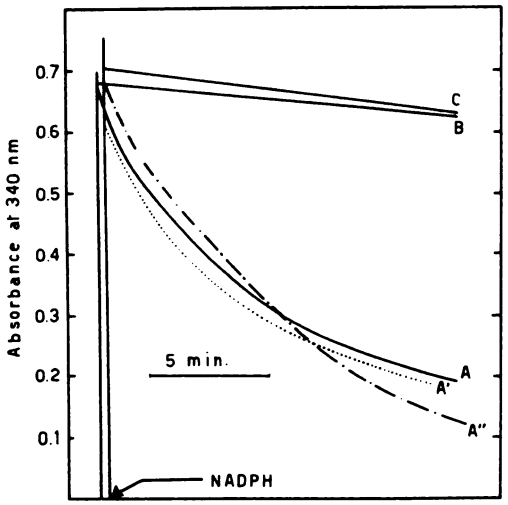


FIG. 8. *Effect of adrenochrome on microsomal oxidation of NADPH in the absence and presence of superoxide anion scavenger*

The reaction mixture, in a final volume of 1.5 ml, contained 0.35 mg of microsomal protein, 0.1 M Tris-HCl buffer (pH 8.1), 60 μM adrenochrome, and 120 μM NADPH. KCN was omitted because of its inhibitory effect on cytoplasmic superoxide dismutase (32). The reaction was started by the addition of NADPH, and the optical density was recorded at 340 nm at a room temperature of approximately 29° against a reference cuvette which contained all components except NADPH. Curve *A*, microsomes + NADPH + adrenochrome; *A'*, *A* + 1352 μg of purified hepatocuprein; *A"*, *A* + 4 mM Tiron; B, microsomes + NADPH; *C*, NADPH + adrenochrome.

the inhibition of BP hydroxylase without transformation of epinephrine to adrenochrome. Therefore epinephrine itself has no inhibitory effect on BP hydroxylase activity. The inhibitory effect of epinephrine on lipid peroxidation, however, persisted despite addition of the soluble fraction (Table 1). Therefore the inhibitory mechanism of epinephrine for lipid peroxidation is not the same as for BP hydroxylase and NADPH-NT reductase. Possibly the inhibition of lipid peroxidation by epinephrine may depend on the epinephrine molecule itself, as suggested by Kohn and Liversedge (66), whereas inhibition of BP hydroxylase and NADPH-NT reductase activities is due partly to electron transfer to epinephrine through O_2^- at the level of NADPH–cytochrome *c* reductase and partly to the epinephrine oxidation product, adrenochrome, because of the close relationship between adrenochrome formation and inhibition of BP hydroxylase activity (Tables 2 and 3). Augusto *et al.* (61) reported that lipid peroxidation was not affected by 10 μM epinephrine. We confirmed their result but did achieve inhibition at higher concentrations of epinephrine (Table 1).

Walaas *et al.* (67) pointed out the probable formation of a semiquinone form of epinephrine by complex formation with cupric ions or interaction with a cupric atom in ceruloplasmin in the presence of oxygen; the latter complex on dehydrogenation forms indole. Therefore it is unlikely that epinephrine quinone is a true catalytic intermediate in the transfer of electrons between NADPH–cytochrome *c* reductase and oxygen as suggested by Augusto *et al.* (61). Even though epinephrine quinone receives 1 electron from the flavoprotein and is converted to a 1-electron reduction product, epinephrine semiquinone as the epinephrine–cupric ion complex (67), it must subsequently be converted to an indole in the presence of oxygen (67). We propose that adrenochrome, not epinephrine quinone, is the true catalytic species. This proposal is based on the following experimental evidence. First, the finding that NADPH consumption was never seen until adrenochrome formation was initiated does not support epinephrine quinone as the active intermediate. Second, the addition of superoxide dismutase after the lag time did not suppress epinephrine-stimulated NADPH oxidation according to the work of Augusto *et al.* (61). If epinephrine quinone is the true catalytic species, partial or complete cessation of NADPH oxidation would be expected, because the transformation of epinephrine to epinephrine quinone would be blocked by superoxide dismutase, without subsequent conversion of the quinone to adrenochrome. This situation can be explained instead by considering adrenochrome as the true catalytic species. Third, the addition of adrenochrome to microsomes resulted in the increased formation of O_2^-, which indicates semiquinone formation, as observed with vitamin K_3 (Fig. 9).

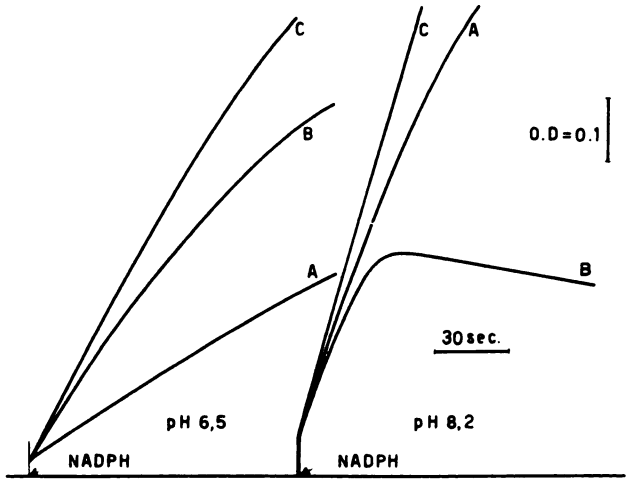


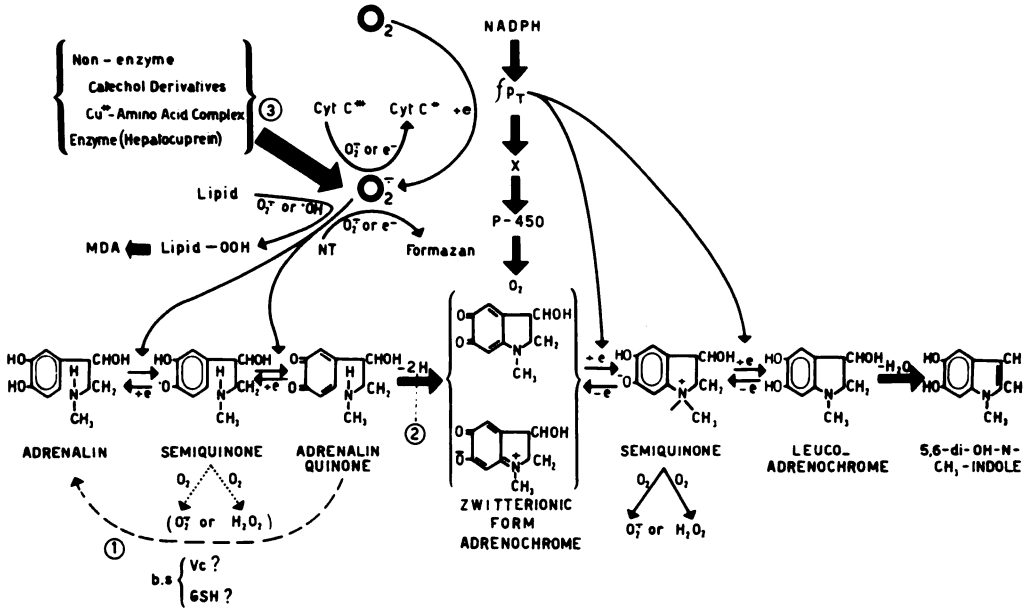
FIG. 9. *Effect of addition of adrenochrome or vitamin K_3 on microsomal NADPH-cytochrome c reductase activity at various pH values*

The reaction mixture contained, in a final volume of 1.5 ml, 0.30 mg of microsomal protein, 0.1 M phosphate buffer (pH 6.5 or 8.2), 50 μM yeast cytochrome *c*, 1 mM KCN, and 200 μM NADPH in the sample cuvette (A). Adrenochrome 60 μM (0.1 ml of aqueous solution) (B) or vitamin K_3 60 μM (added in 10 μl of acetone) (C) was added to both cuvettes. The reaction was started by the addition of NADPH, and the optical density increments at 550 nm were recorded at a room temperature of approximately 29° against a reference cuvette which contained all components except NADPH.

These considerations indicate that adrenochrome is the true active intermediate and is directly reduced by an electron derived from the flavoprotein, and that its inhibitory mechanism on BP hydroxylase activity as well as its stimulatory effect on NADPH oxidase activity (Table 6 and Fig. 8) is due to electron transfer, producing adrenochrome semiquinone. However, it is still unclear whether epinephrine metabolites having a quinonoid structure function in regulating microsomal electron flow under physiological conditions, since small amounts of adrenochrome have been reported to stimulate NADH oxidation in heart muscle (68) and NADPH oxidation in thyroid gland (69).

With regard to the mechanism(s) of the reduction of the inhibitory effect of epinephrine on BP hydroxylase activity by the heated supernatant, at least two possibilities must be taken into consideration (Scheme 1). The first possible candidate(s) and its active site (site 1 or 2 in Scheme 1) was excluded by our experimental results. The second candidate(s) is an O_2^- scavenger(s) which is either an enzyme or a non-protein factor(s) such as a catechol derivative (62) or a copper–amino acid complex (70) (site 3 in Scheme 1). The rat liver soluble fraction contains a very heat-stable protein factor which can indeed scavenge O_2^- (Fig. 2) and was identified as hepatocuprein from its ultraviolet, visible, and EPR spectra (Fig. 4A and B) as well as from its capacity to scavenge O_2^- (Fig. 5).

The blocking capacity of inhibitory effect of epinephrine on BP hydroxylase as well as NADPH-NT reductase activity in cytosol, and the absence of adrenochrome formation, were not due to catechol *O*-methyltransferase activity, which is the main metabolic pathway of epinephrine, because *S*-adenosylmethionine was not added to the reaction medium. Even though endogenous *S*-adenosylmethionine and catechol *O*-methyltransferase may have supported some conversion of epinephrine to metanephrine, the reproducible failure of epinephrine to inhibit the reactions, using either the heated supernatant or purified hepatocuprein, excludes any significant loss of epinephrine via the catechol *O*-methyltransferase pathway,



SCHEME 1. *Mechanism of inhibitory effect of epinephrine ("Adrenalin") and its metabolites, and mode of action of hepatocuprein*

f_{PT}, NADPH–cytochrome *c* reductase.

since the fairly drastic heat treatment of the soluble fraction should have destroyed catechol *O*-methyltransferase activity in the purified hepatocuprein preparation.

Our purified hepatocuprein is functionally very similar to the tetrazolium reductase inhibitor from beef liver (26, 31). The former inhibits adrenochrome formation, protecting BP hydroxylase activity from the blocking action of epinephrine, while the latter inhibits formazan formation, protecting oxygen consumption from the blocking action of tetrazolium (26). Therefore it seems very likely that the hepatocuprein isolated here is analogous to the tetrazolium reductase inhibitor of Fried *et al.* (26, 31). There is, however, an important difference between our purified hepatocuprein and their tetrazolium reductase inhibitor. Their active preparation showed no blue color, while our preparation showed an absorbance peak at about 670 nm, like other hepatocupreins or erythrocupreins (27, 32, 33, 36), which indicates the presence of copper atoms. Fried *et al.* concluded that their tetrazolium reductase inhibitor was a member of the superoxide dismutase class, probably a nonblue cupric protein (31). It is important to keep in mind, however, that the molar absorptivity of this protein at about 670 nm is generally very low compared with other "blue" proteins, whose extinction coefficients are in the range of 3500–5600 M^{-1} cm^{-1} (71), while those of superoxide dismutase lie in the range of 150–490 (33, 34, 42, 72, 73), which is about 10–20 times lower. Accordingly, extensive concentration of the enzyme preparation is necessary to visualize its "blue" color. Further studies should reveal the differences between our hepatocuprein preparation and the tetrazolium reductase inhibitor of Fried *et al.* (26, 31).

ACKNOWLEDGMENTS

We are grateful to Dr. Fabio Campadelli at the Research Center Bollate, Montecatini Edison, S.p.A., Milan, for performing the EPR analysis, and to Dr. Sergio Saracchi of the Faculty of Agriculture, Milan University, for amino acid analyses. We would like to thank Mr. Mario Fittipaldi and Miss Paola Adamoli at the Provincial Laboratory of Public Health, Milan, for their kind help in the atomic absorption studies. We also thank Professor Toshio Yamano of the Department of Biochemistry, Osaka University Medical School, for many valuable suggestions. The expert assistance of Misses Alessan-

dra Favero and Clara Varicchio is gratefully acknowledged.

REFERENCES

1. Hochstein, P. & Ernster, L. (1963) *Biochem. Biophys. Res. Commun.*, 12, 388–394.
2. Hochstein, P., Nordenbrand, K. & Ernster, L. (1964) *Biochem. Biophys. Res. Commun.*, 14, 323–328.
3. Orrenius, S., Dallner, G. & Ernster, L. (1964) *Biochem. Biophys. Res. Commun.*, 14, 329–334.
4. Ernster, L., Nordenbrand, K., Orrenius, S. & Laldas, M. (1968) *Hoppe-Zeyler's Z. Physiol. Chem.*, 349, 1604–1605.
5. Ernster, L. & Nordenbrand, K. (1967) *Methods Enzymol.*, 10, 574–580.
6. Wills, E. D. (1968) in *Biochemical Aspects of Antimetabolites and of Drug Hydroxylation* (Shugar, D., ed.), pp. 273–278, Academic Press, New York.
7. Pederson, T. C. & Aust, S. D. (1972) *Fed. Proc.*, 31, 894.
8. Pederson, T. C. & Aust, S. D. (1972) *Biochem. Biophys. Res. Commun.*, 47, 789–795.
9. Aust, S. D., Roering, D. L. & Pederson, T. C. (1972) *Biochem. Biophys. Res. Commun.*, 47, 1133–1137.
10. Ichikawa, Y., Yamano, T. & Fujishima, H. (1969) *Biochim. Biophys. Acta*, 171, 32–46.
11. Cooper, D. Y. & Rosenthal, O. (1962) *Arch. Biochem. Biophys.*, 96, 327–330.
12. Mitoma, C., Posner, H. S., Reit, H. C. & Udenfriend, S. (1956) *Arch. Biochem. Biophys.*, 61, 431–441.
13. Nebert, D. W. & Gelboin, H. V. (1968) *J. Biol. Chem.*, 243, 6242–6249.
14. Rickert, D. E. & Fouts, J. R. (1970) *Biochem. Pharmacol.*, 19, 381–390.
15. Mazur, A., Green, S. & Shorr, E. (1956) *J. Biol. Chem.*, 220, 227–235.
16. Bacq, Z. M. (1949) *Pharmacol. Rev.*, 1, 1–26.
17. Green, S., Mazur, A. & Shorr, E. (1956) *J. Biol. Chem.*, 220, 237–255.
18. Misra, M. P. & Fridovich, I. (1972) *J. Biol. Chem.*, 247, 3170–3175.
19. Williams, C. H. & Kamin, H. (1962) *J. Biol. Chem.*, 237, 587–595.
20. Mazel, P. (1971) in *Fundamentals of Drug Metabolism and Drug Disposition* (La Du, B. N., Mandel, H. G. & Way, E. L., eds.), pp. 577–578, Williams & Wilkins, Baltimore.
21. Hernandez, P. H., Gillette, J. R. & Mazel, P. (1967) *Biochem. Pharmacol.*, 16, 1859–1875.
22. Wilbur, K. M., Berheim, F. & Shapiro, O. W. (1949) *Arch. Biochem. Biophys.*, 24, 305–313.
23. Sinnhuber, R. O., Yu, T. C. & Yu, T. C. (1958) *Food Res.*, 23, 626–634.
24. Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951) *J. Biol. Chem.*, 193, 265–275.
25. Davis, B. J. (1964) *Ann. N. Y. Acad. Sci.*, 121, 404–427.
26. Fried, R., Fried, L. W. & Babin, D. (1970) *Eur. J. Biochem.*, 16, 339–406.
27. Porter, H., Sweeney, M. & Porter, E. M. (1964) *Arch. Biochem. Biophys.*, 105, 319–325.
28. Kuntzman, K., Levin, W., Shilling, G. & Alvares, A. (1969) in *Microsomes and Drug Oxidations* (Gillette, J. R., Conney, A. H., Cosmides, G. J., Estabrook, R. W., Fouts, J. R. & Mannering, G. J., eds.), pp. 349–369, Academic Press, New York.
29. Nelson, D. O., Lorusso, D. J. & Mannering, G. J. (1973) *Biochem. Biophys. Res. Commun.*, 53, 995–1001.
30. Imaizumi, R. (1962) *Protein, Nucleic Acid Enzyme*, 7, 316–327 (in Japanese).
31. Fried, R., Fried, L. W. & Babin, D. R. (1973) *Eur. J. Biochem.*, 33, 439–445.
32. Weisiger, R. & Fridovich, I. (1973) *J. Biol. Chem.*, 248, 3582–3592.
33. McCord, J. M. & Fridovich, I. (1969) *J. Biol. Chem.*, 244, 6049–6055.
34. Forman, H. J. & Fridovich, I. (1973) *J. Biol. Chem.*, 248, 2645– 2649.
35. Sawada, Y., Ohyama, T. & Yamazaki, I. (1972) *Biochim. Biophys. Acta*, 268, 305–312.
36. Carrico, R. J. & Deutsch, H. F. (1969) *J. Biol. Chem.*, 244, 6087–6093.
37. Albergoni, V. & Cassini, A. (1974) *Comp. Biochem. Physiol.*, 47B, 767–777.
38. Keele, B. B. J., McCord, J. M. & Fridovich, I. (1971) *J. Biol. Chem.*, 246, 2875–2880.
39. Beauchamp, C. O. & Fridovich, I. (1971) *Anal. Biochem.*, 44, 276–287.
40. Bannister, J., Bannister, W. & Wood, E. (1971) *Eur. J. Biochem.*, 18, 178–186.
41. Spies, J. R. & Chambers, D. C. (1949) *Anal. Chem.*, 21, 1249–1266.
42. Misra, H. P. & Fridovich, I. (1972) *J. Biol. Chem.*, 247, 3410–3414.
43. Tegelström, H. (1975) *Hereditas*, 81, 185–198.
44. Marnett, L. J., Wlodawer, P. & Samuelsson, B. (1974) *Biochem. Biophys. Res. Commun.*, 60, 1286–1294.
45. Coon, M. J., Strobel, H. W., Heidema, J. K., Kaschnitz, R. M., Autor, A. P. & Ballou, D. P. (1972) in *The Molecular Basis of Electron Transport, Miami Winter Symposia*, Vol. 4 (Schultz, J. & Cameron, B. F., eds.), pp. 231–250, Academic Press, New York.
46. Coon, M. J., Strobel, H. W., Autor, A. P., Heidema, J. & Duppel, W. (1972) *Biochem. Soc. Symp.*, 34, 45–54.
47. Ichihara, K., Kusunose, E. & Kusunose, M. (1973) *Eur. J. Biochem.*, 38, 463–472.

48. Kimura, T., Nakamura, S., Huang, J. J., Chu, J. W., Wang, H. P. & Tsernoglou, D. (1973) *Ann. N. Y. Acad. Sci.*, 212, 94–106.
49. Estabrook, R. (1973) *Ann. N. Y. Acad. Sci.*, 212, 463–467.
50. Rigo, A., Viglino, P., Rotilio, G. & Tomat, R. (1975) *FEBS Lett.*, 50, 86–88.
51. Hrycay, E. G., Gustafsson, J. A., Ingelman-Sundberg, M. & Ernster, L. (1975) *Biochem. Biophys. Res. Commun.*, 66, 209–216.
52. King, M. M., Lai, E. K. & McCay, P. B. (1975) *J. Biol. Chem.*, 250, 6496–6502.
53. Harley-Mason, J. (1948) *Experientia*, 4, 307–308.
54. Sato, R., Nishibayashi, H. & Omura, T. (1962) *Biochim. Biophys. Acta*, 63, 550–552.
55. Misra, H. P. & Fridovich, I. (1972) *J. Biol. Chem.*, 247, 188–192.
56. McCord, J. M. & Fridovich, I. (1970) *J. Biol. Chem.*, 245, 1374–1377.
57. Nishibayashi, H., Nakai, N. & Sato, R. (1967) *J. Biochem. (Tokyo)*, 62, 215–222.
58. Nishibayashi-Yamashita, H. & Sato, R. (1970) *J. Biochem. (Tokyo)*, 67, 199–210.
59. Iyanagi, T. & Yamazaki, I. (1969) *Biochim. Biophys. Acta*, 172, 370–381.
60. Heacock, R. A. (1959) *Chem. Rev.*, 59, 181–237.
61. Augusto, O., Bechara, E. J. H., Sanioto, D. L. & Cilento, G. (1973) *Arch. Biochem. Biophys.*, 158, 359–364.
62. Miller, R. W. (1970) *Can. J. Biochem.*, 48, 935–939.
63. Fong, K. L., McCay, P. B., Poyer, J. L., Keele, B. B. & Misra, H. (1973) *J. Biol. Chem.*, 248, 7792–7797.
64. Kamataki, T., Ozawa, N., Kitada, M., Kitagawa, H. & Sato, R. (1974) *Biochem. Pharmacol.*, 23, 2485–2490.
65. Kotake, A. N., Deloria, L. B., Abbott, V. S. & Mannering, G. J. (1975) *Biochem. Biophys. Res. Commun.*, 63, 209–216.
66. Kohn, H. I. & Liversedge, M. (1944) *J. Pharmacol. Exp. Ther.*, 82, 292–300.
67. Walaas, E., Walaas, O. & Haavaldsen, S. (1963) *Arch. Biochem. Biophys.*, 100, 97–109.
68. Green, D. E. & Richter, D. (1937) *Biochem. J.*, 31, 596–616.
69. Pastan, I., Herring, B., Johnson, P. & Field, J. B. (1962) *J. Biol. Chem.*, 237, 287–290.
70. Joester, K. E., Jung, G., Weber, U. & Weser, U. (1972) *FEBS Lett.*, 25, 25–28.
71. Malkin, R. & Malmström, B. G. (1971) *Adv. Enzymol.*, 33, 177–244.
72. Weser, U., Fretzdorff, A. & Prinz, R. (1972) *FEBS Lett.*, 27, 267–269.
73. Goscin, S. A. & Fridovich, I. (1972) *Biochim. Biophys. Acta*, 289, 276–283.