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STIMULATORY AND INHIBITORY EFFECTS OF MANGANOUS AND FERROUS IONS ON EPINEPHRINE OXIDATION

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Abstract—Low concentrations of manganous ions stimulate the oxidation of epinephrine to adrenochrome by rat liver microsomes, or by isolated NADPH-cytochrome *c* reductase, 4 fold. In contrast, much higher manganous ion concentrations are required for the chemical oxidation of epinephrine. Whereas the microsomal manganese stimulated epinephrine oxidation is inhibited by superoxide dismutase, as is the non-stimulated enzymatic oxidation, superoxide dismutase has no inhibitory effect on the oxidation of epinephrine in the absence of enzymes.

Ferrous ions inhibit the manganous ion stimulated chemical oxidation of epinephrine more effectively than the similarly stimulated enzymatic oxidation of epinephrine. Based upon these observations different mechanisms for chemical and enzymatic manganous ion-induced epinephrine oxidation are suggested. Furthermore, possible implications of the metal ion-stimulated or inhibited oxidation of catechols *in vivo* are discussed.

Catechol compounds including catecholamines are physiologically active chemical compounds. Furthermore, compounds with catechol moitey occur in the body during metabolism of many xenobiotics. Several catechols are suspected to exert toxic effects in the body, because some studies showed they can be covalently bound to cell constituents after oxidation [1–10]. Hepatic microsomal enzymes are especially able to catalyze covalent protein binding of catechols *in vitro* [1–5]. However, relatively few such molecules are found bound to proteins after administration of catechols or their precursors to animals *in vivo* [10].

On the other hand, catechol compounds are efficient inhibitors of lipid peroxidation in hepatic microsomes *in vitro* [11] and probably also in animals *in vivo* [12]. Lipid peroxidation can cause cell damage and other toxic reactions in the organism whether induced by chemicals or induced by pathogens [13, 14]. Therefore, in order to know whether catechol compounds are potentially harmful or advantageous, it is necessary to have a knowledge of the fate of the catechols in the whole organism.

For example, metal ions normally present in the body can form chelation complexes with catechols [15–17] presumably thereby modifying the catechol properties and functions in the organism. This metal chelating capacity of catechols was intensively studied for the first time in 1912 [18, 19]. It is now well established that the autoxidation of catechols at physiological pH is caused by catechol–chelate complexes with trace metal ions present in most chemicals and incubation vials [20, 21]. But the molecular mechanism of this chemical oxidation process still remains unclear [20–22].

Furthermore, information is also lacking as to whether transition metal ions influence the enzyme catalyzed oxidation of catechols. For these reasons we were interested in the effects of transition metals on catechol oxidation catalyzed by hepatic microsomal enzymes, and in a comparison of this with the oxidation catalyzed by the metal ions alone. We selected epinephrine as the catechol compound because its readily measurable oxidation product adrenochrome [23] is formed rapidly by liver microsomes [24–28]. The metal ions manganous (Mn^{2+}) and ferrous (Fe^{2+}) were chosen since several studies on the mechanism by which they influence the chemical oxidation of catechols have been undertaken previously [20–22, 29, 30]. Furthermore, manganous ions reportedly influence the metabolism of drugs and other xenobiotics in liver microsomes [31–36], and inhibit microsomal lipid peroxidation [37].

Our results indicate that the manganese stimulated chemical oxidation of epinephrine to adrenochrome, and that mediated by microsomal enzymes and supported by NADPH, differ qualitatively and quantitatively.

MATERIALS AND METHODS

All chemicals including $MnCl_2 \cdot 4H_2O$ and $FeCl_2 \cdot 4H_2O$ were of analytical grade and were obtained from Merck Co., West Point, PA, U.S.A. L-Epinephrine·HCl and all other biochemicals were of the purest grade available and were purchased from Sigma Chemicals, St. Louis, MO, U.S.A., except superoxide dismutase (erythrocuprein) which was obtained from Miles Labs, Elkhart, TN, U.S.A.

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Rat liver microsomes were prepared from male Sprague–Dawley rats fed *ad lib.* Rats were decapitated, the livers removed, and microsomes prepared and washed by the calcium aggregation technique [38].

Cytochrome *c* reductase was isolated and purified from rat liver microsomes, and the activity was determined as described elsewhere [39].

Epinephrine oxidation to adrenochrome was measured at 37° using a double beam Aminco DW 2 spectrophotometer at 480 nm. Both the reference and the sample cuvette contained 0.1 M Tris–HCl-buffer (pH 7.4), 5 mM $MgCl_2$, 0.2 mM epinephrine·HCl and either 2.0 mg/ml microsomal protein or 18.4 mU/ml NADPH–cytochrome *c* reductase (total volumes 2.5 ml). In our experiments epinephrine was present in both the sample and the reference cuvette in order to cancel the low, non-enzymatic autoxidation of epinephrine at pH 7.4 described by several authors [20–23].

After 3 min preincubation at 37° in the cuvettes, the non-enzymatic epinephrine oxidation was initiated by adding 10 µl of an appropriate $MnCl_2$-solution (in H_2O) to the sample cuvette. The enzymatic epinephrine oxidation was started by the addition of 10 µl of an NADPH-solution (in 1% $NaHCO_3$) to the sample cuvette, which resulted in a final NADPH-concentration of 1 mM.

When the effect of $MnCl_2$ on the enzymatic adrenochrome formation was measured, the $MnCl_2$ solution was added simultaneously with the NADPH solution to the sample cuvette to initiate the epinephrine oxidation. When the inhibitory effect of ferrous ions on epinephrine oxidation was determined, appropriate $FeCl_2$ concentrations were present in both cuvettes during the preincubation period in order to cancel the absorbance of the purple colored ferrous ion epinephrine chelate complex [17–22].

The non-enzymatic adrenochrome formation rate was calculated from the initial velocity using an extinction coefficient of 4.02 $mM^{-1} \times cm^{-1}$ as described by Green *et al.* [40]. The enzyme-dependent adrenochrome formation was calculated as described by Aust *et al.* [24].

The red color of the manganous epinephrine chelate complex [20–22] did not influence the adrenochrome determination, because this red complex occurred immediately (within 1 sec) after the addition of the manganous ions. This resulted in a higher absorbance in the sample cuvette at zero time which was cancelled from our calculations.

RESULTS

Figure 1 shows two representative time-dependence curves of the non-enzymatic adrenochrome formation induced by two different $MnCl_2$ concentrations in the presence of liver microsomes, but without NADPH. At all $MnCl_2$ concentrations used, a plateau of about 145 nmoles/ml adrenochrome is obtained. This value is lower than the theoretical amount of 200 nmoles/ml adrenochrome. However, simultaneously with the manganous ion (Mn^{2+})-induced adrenochrome formation, some autoxidation of epinephrine occurs in both cuvettes, and this cancels some of the adrenochrome in the sample cuvette. With time the curve decreases from the plateau (not shown in Fig. 1), because of the further

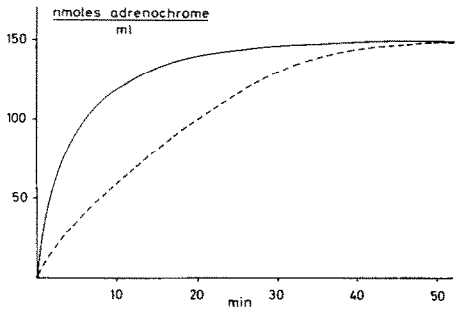

Fig. 1. Time curves of the non-enzymatic Mn^{2+}-ion-stimulated adrenochrome formation in presence of liver microsomes (2 mg prot./ml) and 2×10^{-4} M epinephrine, but in absence of NADPH. Two different concentrations of $MnCl_2$ are shown:– – – 5×10^{-3} M $MnCl_2$; ——— 5×10^{-2} M $MnCl_2$. The two curves represent typical tracings of 4 similar experiments performed with microsomal preparations from different rats.

autoxidation of epinephrine in the reference cuvette. These data agree with findings of other authors who studied the effect of manganous ions on epinephrine in buffer alone [23, 29, 30, 41].

The time course of adrenochrome formation in microsomes at two different $MnCl_2$ concentrations with NADPH is shown in Fig. 2. From the shapes and rates of the two representative time curves it is obvious that Mn^{2+}-induced, enzyme-dependent adrenochrome formation in microsomes differs from the non-enzymatic epinephrine oxidation. The shapes of the curves of the enzymatic adrenochrome formation in presence of $MnCl_2$ are similar to that obtained with microsomes plus epinephrine and NADPH alone [24–28]. As with the non-enzymatic oxidation, the rate was dependent upon the $MnCl_2$ concentration.

In the presence of $MnCl_2$ the adrenochrome level decreased eventually with incubation time (Fig. 2), as seen by others with microsomes in the absence of $MnCl_2$ [24–28]. It is assumed that this decrease is due to leuko-adrenochrome formation by microsomal ad-

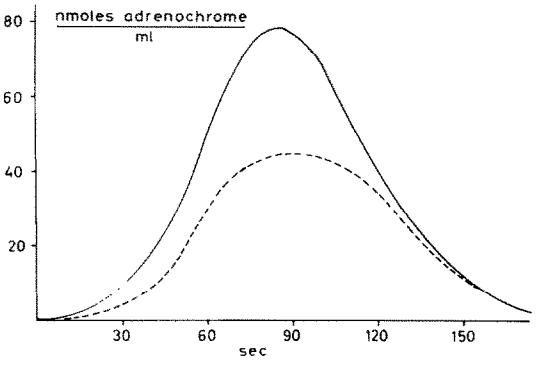

Fig. 2. Time curves of the enzymatic Mn^{2+}-ion-stimulated adrenochrome formation catalyzed by microsomes (2 mg prot./ml) in the presence of 2×10^{-4} M epinephrine and NADPH. Two different concentrations of $MnCl_2$ are shown: – – – – 1×10^{-5} M $MnCl_2$; ——— 1×10^{-4} M $MnCl_2$. The two curves represent typical tracings of 4 similar experiments performed with microsomal preparations from different rats.

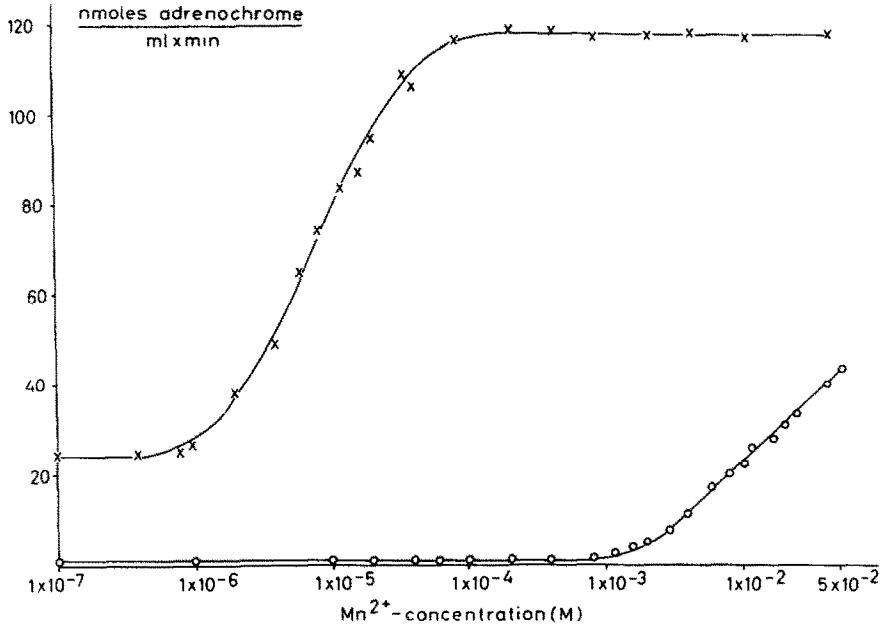

Fig. 3. Concentration dependence of the rates of Mn^{2+}-ion-stimulated adrenochrome formation in presence of microsomes (2 mg prot./ml) and 2×10^{-4} M epinephrine and in presence (x-x-x) or in absence (O-O-O) of NADPH. The semilogarithmic plot shows the mean values of 4 experiments with microsomal preparations from different rats.

renochrome reduction [24–28]. Since the absorbance of the zero time point is actually greater than zero due to the red colored Mn^{2+}–epinephrine complex as pointed out above, and since the adrenochrome plot of Fig. 2 stops at this absorbance, we assume that leuko-adrenochrome, formed by adrenochrome reduction, can also complex manganous ions leading to the same absorbance of colored complexes as the manganous ion–epinephrine complexes present at zero time.

The rates of adrenochrome formation as a function of $MnCl_2$ concentrations is shown in Fig. 3. In the absence of NADPH (lower curve) very high $MnCl_2$ concentrations are necessary to form measurable epinephrine oxidation rates. On the other hand, with NADPH a basal rate of about 24 nmoles adrenochrome/ml is formed per min by the microsomes. This rate increases rapidly with increasing $MnCl_2$ concentrations to a maximal (4 fold) at 1×10^{-4} M $MnCl_2$ (Fig.

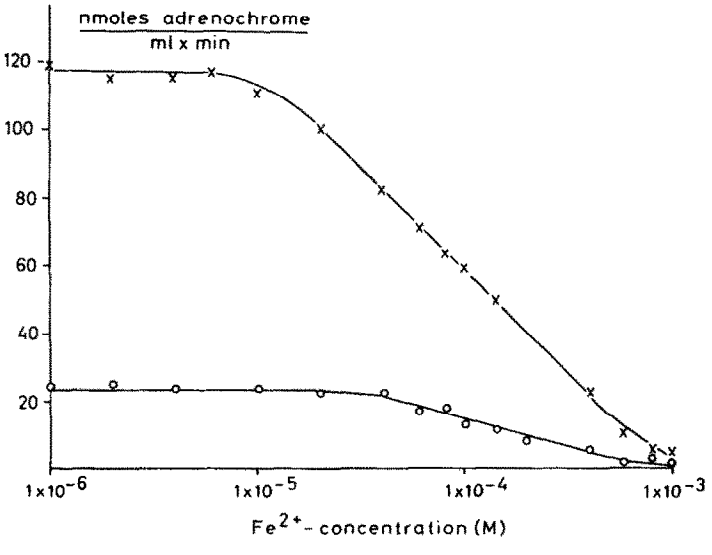

Fig. 4. Inhibition of Mn^{2+}-ion-stimulated enzymatic adrenochrome formation rate by different concentrations of $FeCl_2$ in microsomes (2 mg prot./ml) with NADPH, in presence of 2×10^{-4} M epinephrine and 1×10^{-4} M $MnCl_2$ (x-x-x) or in absence of $MnCl_2$ (O-O-O). The semilogarithmic plot shows the mean values of 4 experiments with microsomal preparations from different rats.

Table 1. Influence of superoxide dismutase on Mn^{2+}-ion-stimulated adrenochrome formation catalyzed by microsomes or by NADPH–cytochrome *c* reductase

	nmoles Adrenochrome/ml/min	
	With microsomes (2 mg prot./ml)	With cytochrome *c* reductase (18.4 mU/ml)
Without NADPH	0.00*	0.00*
Without NADPH, with SOD	0.00*	0.00*
Without NADPH, with $MnCl_2$	1.78 ± 0.01*	1.77 ± 0.01*
Without NADPH, with $MnCl_2$, with SOD	1.87 ± 0.02*	1.87 ± 0.02*
With NADPH	23.8 ± 1.42	0.95 ± 0.05
With NADPH, with SOD	3.6 ± 0.21	0.08 ± 0.005
With NADPH, with $MnCl_2$	118.2 ± 4.63	3.74 ± 0.15
With NADPH, with $MnCl_2$, with SOD	17.8 ± 0.89	0.46 ± 0.02

Epinephrine in a concentration of 2×10^{-4} M was used. SOD (= superoxide dismutase) was present in an activity of 10 U/ml. The $MnCl_2$ concentration was 2×10^{-3} M. Mean values ± S.D. of 4 experiments with microsomal preparations from different rats or of 4 different experiments with NADPH–cytochrome *c* reductase are shown.

* Similar values were obtained when microsomes or NADPH–cytochrome *c* reductase were absent.

3). At this $MnCl_2$ concentration a significant non-enzymatic $MnCl_2$-induced adrenochrome formation is not detected (Fig. 3). The latter starts at about 1×10^{-3} M $MnCl_2$ and remains linear with increasing $MnCl_2$ concentrations in the semilogarithmic plot. $MnCl_2$ concentrations higher than 5×10^{-2} M were not used in the experiments described.

Superoxide dismutase at 18.4 mU/ml is unable to inhibit the $MnCl_2$-induced non-enzymatic oxidation of epinephrine to adrenochrome (Table 1). On the other hand, superoxide dismutase is an effective inhibitor of epinephrine oxidation catalyzed by liver microsomes with or without $MnCl_2$ stimulation (Table 1).

Similar effects are obtained with isolated NADPH–cytochrome *c* reductase, which is able to catalyze the oxidation of epinephrine [24, 27, 29]. As with microsomes, the NADPH–cytochrome *c* reductase-dependent epinephrine oxidation to adrenochrome is increased in the presence of $MnCl_2$ 4–5 fold (Table 1).

Because ferrous ions form complexes with epinephrine which are relatively stable against autoxidation [23, 29, 30, 41], we were interested to see whether $FeCl_2$ inhibits the NADPH-dependent microsomal epinephrine oxidation. Figure 4 demonstrates that $FeCl_2$ concentrations between 1×10^{-6} M and 3×10^{-5} M do not influence adrenochrome formation, although a complex of ferrous ion and epinephrine does form as evidenced by its purple color. Higher $FeCl_2$ concentrations, however, do inhibit adrenochrome formation. From the data in Fig. 4 an I_{50}-value ($FeCl_2$ concentration which inhibits adrenochrome formation to 50 per cent) of $1–2 \times 10^{-4}$ M could be calculated.

Ferrous ions are also able to inhibit the microsomal NADPH-dependent, $MnCl_2$-stimulated epinephrine oxidation (Fig. 4). An I_{50}-value of about 1×10^{-4} M $FeCl_2$ could be calculated from the data of Fig. 4, a value similar to the $MnCl_2$ concentration.

Furthermore, $FeCl_2$ is also a potent inhibitor of the $MnCl_2$-induced non-enzymatic epinephrine oxidation, as suggested by other authors [23, 29, 30, 41]. Ferrous ions are much more effective inhibitors of the $MnCl_2$-induced non-enzymatic epinephrine oxidation (Fig. 5; $I_{50} = 3.7 \times 10^{-5}$ M) than of the enzymatic reaction (Fig. 4; $I_{50} = 1 \times 10^{-4}$ M). In the studies shown in Fig. 5 it was necessary to use a higher concentration of $MnCl_2$ than in Fig. 4, in order to obtain sufficient rates of adrenochrome to study. With respect to the I_{50}-value obtained, therefore, an even higher comparable I_{50}-value in Fig. 5 would be expected than the 3.7×10^{-5} M $FeCl_2$ obtained.

DISCUSSION

The metal catalyzed, non-enzymatic oxidation of phenols and catechols is a well known phenomenon [20–23, 29, 30]. Gillette *et al.* [41] were the first to report a stimulation of catecholamine oxidation by transition metals. Several subsequent publications deal with the mechanism of this metal ion-mediated non-enzymatic catechol oxidation, but few studies have been concerned with the influence of transition metals on enzymatic catechol oxidations.

Our results demonstrate that manganous and ferrous ions markedly influence NADPH-dependent epinephrine oxidation catalyzed by liver microsomes or by isolated NADPH–cytochrome *c* reductase. The approximately 4 fold stimulation by 1×10^{-4} M Mn^{2+}-ions, concentrations which are not high enough to stimulate non-enzymatic epinephrine oxidation significantly, suggests that different mechanisms are responsi-

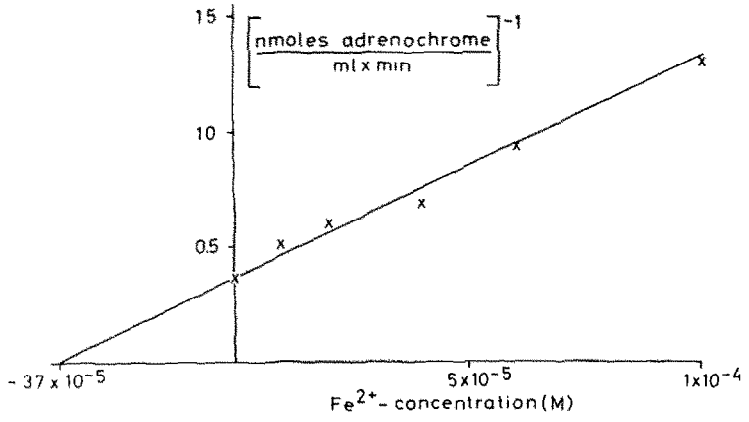

Fig. 5. Dixon-plot of the inhibition of Mn^{2+}-ion-stimulated non-enzymatic adrenochrome formation rate by $FeCl_2$ in the presence of microsomes (2 mg prot./ml), 2×10^{-4} M epinephrine and 2×10^{-3} M $MnCl_2$. The data represent the mean values of 4 experiments with microsomal preparations from different rats.

ble for both oxidation reactions. These differences include a greater $MnCl_2$ sensitivity for the enzymatic reaction and extend to different sensitivity to superoxide dismutase. Further, the oxidation of epinephrine induced by Mn^{2+}-ions alone (Fig. 1) is self-propagating, i.e. continues to completion. The NADPH-driven microsomal reaction requires a constant source of NADPH.

Therefore, the data is consistent with the concept that superoxide anion (O_2^-) is not involved in the oxidation of epinephrine catalyzed by Mn^{2+}-ions alone. This agrees with the oxidation mechanism of catechols which has been designed by Martell *et al.* [20–22]. They assume that during the Mn^{2+}-ion-catalyzed oxidation of catechols, a chelate complex of two catechols and one metal ion is formed which is able to bind molecular oxygen. This complex might then transfer two electrons directly to the oxygen molecule. This would lead to O_2^{2-} (= H_2O_2) and one quinone molecule, a reaction which has been observed [20–22].

Such a catechol–metal complex is presumably also formed during the enzyme-driven Mn^{2+}-ion-stimulated oxidation of epinephrine observed during our experiments (Fig. 3, Table 1). This is supported by the maximal stimulation by 1×10^{-4} M $MnCl_2$, at an epinephrine concentration of 2×10^{-4} M (Fig. 3).

The inhibitory effects of Fe^{2+}-ions (Fig. 4) support this concept of complex formation between Mn^{2+}-ions and the catechol. In the enzymatic reaction, the approximate Fe^{2+}-ion concentration which inhibits the NADPH-mediated microsomal enzyme-dependent epinephrine oxidation to 50 per cent ($1–2 \times 10^{-4}$ M $FeCl_2$) also inhibits the Mn^{2+}-ion-stimulated enzyme-dependent adrenochrome formation to 50 per cent (1×10^{-4} M $FeCl_2$). The non-enzymatic oxidation of epinephrine, mediated by Mn^{2+}-ions alone, exhibits a sensitivity to iron ions some 2.5 times that of the enzymatic reaction, perhaps because the latter reaction is saturated with respect to Mn^{2+}-ions at the levels used (Fig. 4; 1×10^{-4} M) while the non-enzymatic reaction still exhibits a direct activity dependence on Mn^{2+}-ions at the levels used (Fig. 5; 2×10^{-3} M), as shown in Fig. 3.

The oxidation of epinephrine and other catechols can be stimulated in the endoplasmic reticulum (microsomes) by Mn^{2+}-ions in concentrations comparable to that present in human hepatocytes *in vivo* ($1–10 \times 10^{-6}$ M Mn^{2+}-ions; [42]). A higher oxidation rate of catechols in cells could lead to more catechols covalently bound to cell constituents. However, although some evidence exists from *in vitro* experiments [1–5], such a relationship has not yet been proven. But if both processes are really related, a stimulation or an inhibition of the catechol oxidation process by metal ions would give rise to a change in the covalent binding of the catechols to proteins. A change in the amount of catechol molecules covalently bound to proteins *in vivo* could influence the toxicity of any catechol compound. Whether metal ions are able to influence these parameters *in vivo* will be studied in the near future.

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