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SHORT COMMUNICATIONS

Inhibition of NADPH oxidation and oxidative metabolism of drugs in liver microsomes by zinc

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Recent observations that zinc inhibits *in vitro*-induced lipid peroxidation in liver microsomes [1, 2] and protects the liver against the toxic effects of CCl_4 [3] pointed to a possible effect of this metal on the activity of mixed-function oxygenases in the endoplasmic reticulum of the liver. Several authors have stressed the essential role of NADPH as a source of electrons for the microsomal cytochrome P-450 drug-metabolizing enzymes [4, 5]. An NADPH oxidation-linked lipid peroxidation system has also been identified in liver microsomes and first described by Hochstein and Ernster [6]. It was also suggested that both the processes of lipid peroxidation and of drug oxidation depend on the same electron-transport chain [7]. Since CCl_4-induced lipid peroxidation is generally assumed to be initiated by the $\cdot CCl_3$ free radical produced by the NADPH-dependent, cytochrome P-450 enzyme system [8–10], then inhibition of NADPH oxidation may explain the decreased lipid peroxidation and CCl_4-induced hepatotoxicity produced by zinc. In this communication, evidence will be presented that NADPH oxidation is inhibited by zinc and that this reaction results in the inhibition of drug metabolism by liver microsomes.

Rat liver microsomes were prepared by a procedure described in our previous papers [1, 2] and incubated in a medium enriched in NADPH or in NADPH-generating systems, as given in the legend to Fig. 1. The rate of NADPH oxidation was recorded at 340 nm on a Beckman Acta III spectrophotometer at 37° under continuous slow magnetic stirring. Repetitive addition of zinc into the reaction mixture not only slowed down the rate of NADPH oxidation but, at a certain concentration of zinc in the medium, reversed the reaction in the direction of NADP reduction (Fig. 1). The fact that an excess of zinc increases the final content of NADPH above the initial level of NADPH indicates the additional reduction of endogenously present NADP in the microsomal fraction. We have two reasons to believe that the observed inhibition of NADPH oxidation by zinc is related to the effect of this ion on some enzymes present in the microsomal fraction of the liver rather than to a direct interaction of zinc with pyridinenucleotides: (1) heating the reaction mixture at 60° for 10 min abolishes the changes in NADPH content, and (2) there is no spectroscopic evidence on the interaction of Zn^{2+} with NADPH [11].

More rigorous treatment of the effect of various zinc concentrations on NADPH oxidation was carried out under the conditions recommended for the assay of NADPH oxidase [12] and is presented in Fig. 2. The activity of NADPH oxidase, studied at two different concentrations of the substrate and measured as initial velocities during the first min of the reaction, was inhibited by zinc. Only a 10 μM concentration of zinc inhibited 50 per cent of the enzyme activity. Such a low concentration of zinc suggests a possible effective and specific role of zinc in the control of NADPH oxidation within biological systems, even *in vivo*.

Additional evidence indicating that zinc ions at relatively low concentrations interfere with NADPH oxidation-linked reactions was determined by studying the microsomal metabolism of ethylmorphine. The incubation procedure has previously been described by Sipes *et al.* [13]. The only modification was a final protein concentration of 2 mg/ml. The data in Fig. 3 summarize the inhibitory effect of zinc on microsomal ethylmorphine *N*-demethylase activity as related to time. Zinc inhibited the formaldehyde produced by the *N*-demethylation of ethylmorphine throughout an incubation period of 90 min. Since the reaction was still linear at 10 min, this time point was chosen to determine the effect of various concentrations of zinc ions on the metabolism of ethylmorphine. The results are summarized in Fig. 4. It is evident that the activity of the *N*-demethylase, as judged

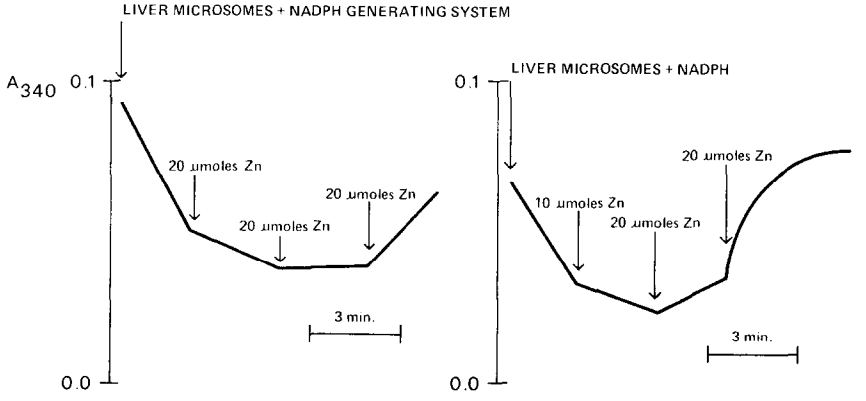

Fig. 1. Effect of zinc on the oxidation of NADPH in rat liver microsomes. A microsomal fraction was prepared and diluted in Tris-KCl buffer (0·05 M, pH 7·4) to obtain 1·5 mg protein/ml. To samples containing 2 ml suspension, 1 ml of either 0·2 mM NADPH or 2 mM of glucose 6-phosphate, 2 units of glucose 6-phosphate dehydrogenase, 5 mM $MgSO_4$ and 20 mM nicotinamide were added and measured at 340 nm at 37° under stirring. Given amounts of zinc in Tris–KCl buffer were added as indicated.

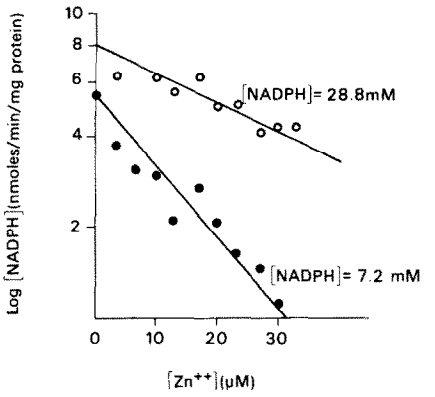

Fig. 2. Effect of zinc on the activity of NADPH oxidase in liver microsomes at low and high substrate levels. Enzyme activity in the microsomal fraction was assayed at pH 5·5 in a system containing 8 μmoles Na_2HPO_4, 54 μmoles KH_2PO_4, 1 μmole $MnCl_2$, 340 μmoles sucrose and 21·6 or 86·4 μmoles NADPH in 3 ml final volume. Control samples did not contain a subcellular fraction. The rate of oxidation of the reduced coenzyme was scanned at 340 nm.

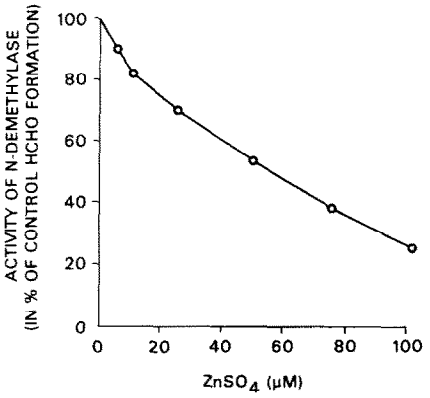

Fig. 4. Effect of zinc concentration on the liver microsomal *N*-demethylase activity. For methodological details, see Fig. 3. Formation of formaldehyde (HCHO) in control samples during a 10-min incubation was 42·3 nmoles/mg of protein.

from the first 10 min of the reaction, is inhibited by zinc in a concentration-related manner. Similar experiments carried out with various zinc salts (gluconate, SO_4^{2-}, Cl^-, CO_3^{2-} and $CH_3CO_2^-$) indicate that the anion has no effect on the magnitude of the inhibition (data not presented).

The experimental evidence presented in this study clearly indicates that, in systems *in vitro* at relatively low concentrations, zinc ions inhibit the oxidation of NADPH and the related metabolism of drugs, as represented in this study by ethylmorphine. The mechanism of zinc interactions with NADPH oxidase is under investigation. It is noteworthy, however, that this enzyme requires Mn^{2+} [12]. There is a possibility that zinc may displace this cation in a manner similar to that suggested by Brunel and Cathala [14] for alkaline phosphatase from bovine brain. In any case, the findings indicating that the activity of NADPH oxidase is stimulated by Mn^{2+} and inhibited by zinc differ from the observation made by May and McCay [15], who showed that peroxidation of microsomal phospholipids dependent on NADPH oxidation was inhibited by Mn^{2+}. We assume that the described inhibition of NADPH oxidation by zinc may explain our observation of the inhibition of lipid peroxidation in the liver by zinc [1, 2] and the protection afforded by zinc against CCl_4-induced hepatotoxicity.

Incubation studies *in vitro* with $^{14}CCl_4$ and liver microsomes have implicated NADPH oxidation in the mechanism of CCl_4 activation to an active intermediate, probably $\cdot CCl_3$. By following the covalent binding of ^{14}C from $^{14}CCl_4$ to microsomal protein as an index of the conversion of $^{14}CCl_4$ to $\cdot{}^{14}CCl_3$, it was reported that covalent binding was prevented by elimination of NADPH from the incubation mixture [16, 17] or by addition of the specific antibody of NADPH-cytochrome *c* reductase [17] (NADPH oxidase) to the incubation medium. If $\cdot CCl_3$ is responsible for the CCl_4-induced lipid peroxidation, then decreasing the formation of it may reduce the peroxidation which is destructive to the liver cells. Therefore, the finding that zinc inhibits NADPH oxidase may be the mechanism by which zinc inhibits CCl_4-induced lipid peroxidation and the subsequent liver damage.

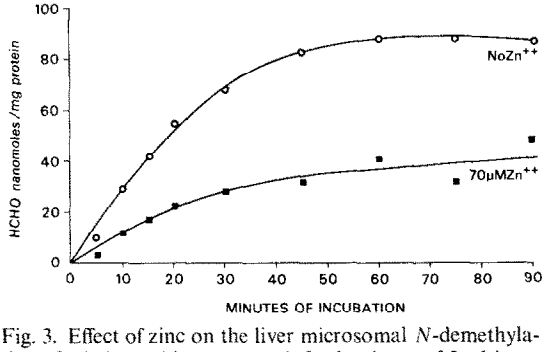

Fig. 3. Effect of zinc on the liver microsomal *N*-demethylation of ethylmorphine *in vitro*. A final volume of 3 ml incubation mixture contained 2 mg/ml of microsomal protein, 10 mM ethylmorphine and NADPH-generating system (NADPH 0·22 mM, glucose 6-phosphate 3·3 mM, nicotinamide 2·0 mM, and glucose 6-phosphate dehydrogenase 1 unit/3 ml). The concentration of zinc sulfate was 70 μM. Incubation was carried out at 37° under slight shaking.

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NOTE ADDED IN PROOF

While studying the mechanism of Zn^{2+} inhibition on NADPH oxidation the concentrations of all reactants were decreased as compared to the conditions of the experiment in Fig. 1. (Only the initial rate of the reaction was used so that Michaelis–Menton mechanisms could be applied.) Under these conditions Zn^{2+} inhibited NADPH oxidation as is shown in Fig. 1, but the increase in absorption at 340 nm was not evident. Repeating the conditions of these previous experiments and scanning the reaction at 340 nm and 600 nm showed an increase in absorption at both these wavelengths when 60 μmoles of Zn^{2+} were added to the reaction vessel. Increasing the temperature showed that this increase in absorption occurred at still lower levels of Zn^{2+}. From these new findings it appears that the increase in absorption at 340 nm in Fig. 1 may be due to micro-precipitation at these high levels of NADPH and Zn^{2+}. Further study is continuing in this laboratory into the possibility of NADPH and Zn^{2+} interactions occurring even though the u.v. spectral analysis which is mentioned in this paper shows no perturbations.

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Mode of action of Withaferin A and Withanolide D

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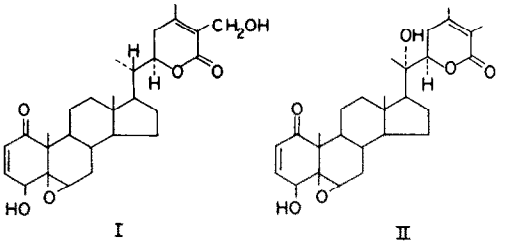
Withaferin A(I) and Withanolide D(II) are steroidal lactones isolated from *Withania somnifera* Dun. Withaferin A was reported to retard growth of various experimental mouse tumours [1, 2]. It produces a mitotic arrest in the metaphase



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of dividing Ehrlich ascites carcinoma cells. Administration of withaferin A to Ehrlich ascites tumour bearing mice resulted in simultaneous disappearance of the tumour and acquisition of immunity towards a subsequent tumour implantation [3]. Chakraborti *et al.* [4] found that withanolide D, the major constituent in the plant occurring in West Bengal, India, has significant antitumour activity against cultured cells derived from human carcinoma of the nasopharynx (KB) and *in vivo* against Sarcoma-180 in mice. Shohat *et. al.* [5] reported from cytological studies that synthesis of DNA and RNA in Ehrlich ascites tumour cells was unaffected by withaferin A. The present study is a biochemical approach to establish the exact mechanism of tumour regression during withaferin A and withanolide D treatment.

DL-^{14}C-Phenylalanine and 2-^{14}C-uracil were obtained from Bhabha Atomic Research Centre, Trombay, India. Withaferin A and Withanolide D were kind gifts from Dr. S. K. Chakraborti, Department of Chemotherapy, of this research centre. Other chemicals used were of analytical grade.

Sarcoma-180 tumour cells were grown in ascites form in 4–6 week old Strain A male mice by intraperitonial transplantation. Seven to ten days following transplantation of the tumour, the animals were sacrificed and cells obtained as a pellet by centrifuging the ascites fluid. Cells were then washed twice with chilled normal saline.

To study the effect of the drugs on incorporation of ^{14}C-phenylalanine into trichloroacetic acid (TCA)-insoluble proteins of Sarcoma-180 cells, the cells were incubated in Medium A (0·25 M sucrose, 0·025 M KCl, 0·005 M $MgCl_2$, 0·05 M Tris-HCL buffer, pH 7·4) with 0·3 μCi of DL-^{14}C-phenylalanine (45·6 mCi/m-mole) in a total volume of 0·1 ml at 37° with constant shaking. The drugs at a concentration of 40 μg/ml were added to one group while another was treated as a control. Four such pairs were incubated for 0 min, 30 min, 1 hr and 2 hr. The incubation mixtures were then applied to 25 mm discs of Whatman 3MM paper, dried and treated with 10% TCA. After 30 minutes, the discs were rinsed twice with 5% TCA, heated at 90°–95° for 15 min in 5% TCA and cooled to room temperature. The discs were then rinsed with 5% TCA followed by alcohol, alcohol–ether (1:1) mixture and finally with ether. The discs were finally dried and counted in a windowless gas flow counter.

To study the effect of the drugs on incorporation of 2-^{14}C-uracil into RNA of Sarcoma-180 cells, the cells were incubated in Medium A with 0·8 μCi of 2-^{14}C-uracil (46·7

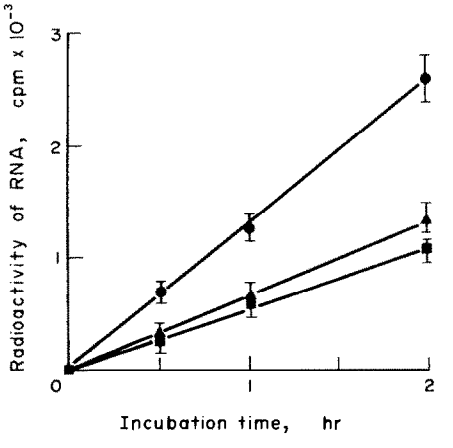

Fig. 1. Incorporation of ^{14}C-uracil into RNA of Sarcoma-180 cells. ●—●- control; ▲—▲-treated with Withaferin A; ■—■-treated with Withanolide D. Mean values ± S.D. of five experiments are given. $P < 0.001$ with respect to the control.