5. DISCUSSION
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Studies on the impact of cadmium toxicity on vitamin metabolism are scarce (247). A literature survey shows very few reports on the effect of cadmium on the metabolism of B-vitamins. It is needless to state that the coenzymes derived from these vitamins are fundamental in controlling tissue metabolism.

Treatment of rats with the present dose of cadmium for a period of 15 days increased their body weights when compared with controls. A highly significant increase in the wet weight of livers of the treated rats was in conformity with an earlier report (248). This increase in the wet weight of liver may be due to hypertrophy of the tissue as reported by Dudley et al. (78). Although histological examinations of body tissues and behavioral studies were not undertaken in the present investigation, Dudley et al. (249) reported histological studies on liver and kidney tissues of rats chronically exposed to cadmium. Prior to 4 weeks of cadmium treatment, they did not find any cell injury or other morphological changes in liver or in kidney. The dose of cadmium we used in the present investigation did not produce any observable changes in behavioral pattern in animals, although contradictory results have been reported on behavioral effects of cadmium (250, 251).

The significant increase in the hepatic levels of free riboflavin in cadmium treated rats was found to be accompanied
by diminished activity of flavokinase. This indicates an impairment of further utilization of riboflavin.

The present investigation also revealed that cadmium treatment inhibited FMN-phosphatase activity significantly, but the inhibition was much less compared with flavokinase.

Although we did not study the effect of cadmium in vivo on a dose-dependent manner, similar studies were undertaken in vitro. These studies revealed increasing inhibition of both flavokinase and FMN-phosphatase with increasing concentrations of cadmium (Table 5). Like in vivo effects, the in vitro effects of cadmium on FMN phosphatase was less pronounced compared with those on flavokinase. Because zinc inhibits FMN-phosphatase (231), one may think that the effect of cadmium on this enzyme is mediated through modulation of the level of zinc or any other metal in liver. The in vitro effects of cadmium on these two enzymes suggest that cadmium may have a direct effect on flavokinase and FMN-phosphatase, although they do not exclude the possibility that cadmium may compete with zinc in binding to FMN-phosphatase and that zinc metabolism may therefore be a site of action of cadmium.

It is also observed that cadmium treatment leaves the hepatic FMN level unaltered in spite of diminished conversion of riboflavin to FMN. This unaltered FMN level might be due to either diminished degradation of FMN or decreased conversion of FMN to FAD.
A significant increase in the hepatic level of FAD in cadmium-treated rats may be due to increased FAD-pyrophosphorylase activity, which is responsible for the formation of FAD from FMN, or decreased FAD pyrophosphatase which catalyses the hydrolysis of FAD to FMN. We have not measured either of these two enzymes under our present experimental condition. For the same reason it is difficult to explain the unchanged hepatic FMN level in cadmium-treated animals.

The tissue FMN and FAD levels were found to be regulated by the activity of one or more of the riboflavin-metabolizing enzymes in altered physiological situations (203,252). The activities of flavokinase and/or FAD-pyrophosphorylase are sometimes reduced due to decreased availability of free riboflavin in the tissues (203,252). However, in the present investigation, tissue riboflavin level was enhanced. It is therefore possible that the alterations in the levels of riboflavin and FAD in liver as noted in the present investigation may be due to the influence of cadmium on the enzyme activities. However, a better picture can be drawn by studying the rates of incorporation of radioactive riboflavin into various hepatic flavin fractions under our experimental conditions.

Cytosolic flavokinase was first purified to apparent homogeneity from rat liver by affinity chromatography and some of its properties were studied(255). In the present investigation flavokinase from rat liver has been partially purified according to the method of McCormick (233). Purification was carried out
upto the dialysis stage and our results are in close agreement with those reported by McCormick (230,233). The specific activity of 18,500xg supernatant in our hand was 5.78 while in their case the value was 6.0. Similarly, a specific activity of 13.83 was observed for the dialysed enzyme in our preparation which is slightly lower than their preparation. A four fold increase in the specific activity of the ammonium sulphate fraction was observed on dialysis. An inhibitory effect of ammonium ions on the partially purified flavokinase was evident from our studies. Enhancement of enzyme activity after dialysis could be due to removal of ammonium (NH$_4^+$) ions during dialysis. Most of the properties of the enzyme were studied with this dialyzed preparation. However, some of the observations were confirmed with apparently homogeneous enzyme, purified on polyacrylamide gel as described in the text.

Cadmium (Cd$^{2+}$) inhibits dialyzed preparation of flavokinase in a dose-dependent manner. The pattern of inhibition is non-linear indicating an allosteric type of inhibition. This non-linear inhibition was also evident when the dialyzed preparation was assayed in presence of other divalent cations, namely, Cu$^{2+}$ and Hg$^{2+}$. The inhibition of flavokinase with Hg$^{2+}$ and Cu$^{2+}$ was found to be greater than that with Cd$^{2+}$. At $10^{-3}$ M, Hg$^{2+}$ completely inhibited the enzyme activity showing a greater affinity for the enzyme than cadmium. However, this non-linear pattern of inhibition was not exhibited in the
purified preparation. It is difficult for us to explain this discrepancy.

Increasing ATP concentration, a cosubstrate for flavokinase, also shows a non-linear kinetics with an initial lag. In presence of \( \text{Cd}^{2+} \), under similar experimental condition, the enzyme activities were low but still retained the non-linear nature of the activity profile. However, with purified preparation the activity of the enzyme was linear against increasing ATP concentration. Here also we cannot afford any explanation for this discrepancy.

Zinc (\( \text{Zn}^{2+} \)) is an effective activator for flavokinase. It has also been reported to inhibit the contaminating FMN-phosphatase (231). Our studies also supported this observation. Zinc not only stimulates the flavokinase activity of the dialyzed preparation but also abolishes the inhibitory effect of \( \text{Cd}^{2+} \) (Ref. figure 5). The Lineweaver-Burk plot, constructed from this set of data (figure 6) clearly shows a change of \( \text{Km} \) of flavokinase for zinc alone and zinc plus cadmium. On the other hand it shows no change in \( \text{V}_\text{max} \) indicating a clear competition between zinc and cadmium for the enzyme. This experiment further indicates that zinc and cadmium bind at the same site on the enzyme.

This experiment was repeated with the apparently pure flavokinase and the results indicate the same conclusion (figure 15).
Mercury (Hg$^{2+}$) is a very strong inhibitor of flavokinase but unlike cadmium inhibition, mercury inhibition of flavokinase cannot be abolished by zinc indicating that Hg$^{2+}$ has a greater affinity than Cd$^{2+}$ for the enzyme.

Similarly, Cd$^{2+}$ was also found to compete with riboflavin for binding with both the dialyzed preparation as well as purified flavokinase. The Km of riboflavin for the partially purified and purified flavokinase did not differ much — $4.5 \times 10^{-4} \text{M}$ and $4.1 \times 10^{-4} \text{M}$, respectively. Cadmium increased the Km of riboflavin for both the enzyme preparations, while $V_{\text{max}}$ remained unaltered. Thus, a competition between riboflavin and cadmium and that between Cd$^{2+}$ and Zn$^{2+}$ hint at the possibility that Cd$^{2+}$ and Zn$^{2+}$ both bind at the same site on the enzyme molecule where riboflavin binds.

Thus, it is clear from our present investigation that Cd$^{2+}$, Cu$^{2+}$ and Hg$^{2+}$ are strong inhibitors of hepatic flavokinase. Sensitivity to such cations suggests the sulfhydryl nature of this enzyme. Moreover, it has been reported that Cd$^{2+}$ inhibits a large number of enzymes having functional sulfhydryl groups (20). This hints at the possibility that flavokinase may also possess functional sulfhydryl groups at or near its active centre.

Parachloromercuribenzoate (PCMB), a well known thiol blocker, at increasing concentrations inhibit flavokinase activity. Another thiol blocker, N-ethylemaleimide (NEM), also inhibits flavokinase. The inhibition by PCMB was almost
completely reversed by increasing concentrations of glutathione (GSH) and dithiothreitol (DTT). However, as expected, inhibition of flavokinase by NEM could not be reversed by DTT even at much higher concentration. These findings suggest that sulfhydryl group may be involved with flavokinase activity.

The carboxyl group of glutathione may participate more effectively in metal binding. Kinetic analysis of the complexing of glutathione and S-methylglutathione with a series of transition metals led Martin and Edsall (254) to conclude that the gamma-carboxyl group of the glutamyl residue of glutathione successfully competes with sulfur in binding metal ions. This possibility may have biological implications as the mercapto group of GSH would remain free to participate in biochemical processes. However, contrary evidence appears from the study of Zn$^{2+}$ complex of GSH and oxidized GSH. White et al. (255) suggest that Zn$^{2+}$ probably coordinates through the sulfur atom and amino group rather than through amino and carboxyl groups.

Our results show that the inhibition of flavokinase by Cd$^{2+}$ and Hg$^{2+}$ was completely reversed by increasing concentrations of GSH although GSH by itself shows inhibitory effect on the enzyme in a dose-dependent manner. This further strengthens our view that these heavy metal cations bind to an accessible, essential and functional thiol group of the enzyme in bringing about their inhibitory effect.