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INACTIVATION OF 3-HYDROXY-3-METHYLGLUTARYL CoA REDUCTASE BY Fe^{++} AND A CYTOSOLIC PROTEIN

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Summary: Rat liver microsomal HMGCoA reductase was inhibited by Fe^{++} and this effect was enhanced by a cytosolic protein.

The successful assay of the hepatic microsomal enzyme, 3-hydroxy-3-methylglutaryl CoA (HMGCoA) reductase, depends on the use of large excess of EDTA (30 mM), first introduced by Linn (1), and a sulfhydryl compound, dithiothreitol (DTT) in the homogenizing medium for preparing microsomes as well as in the reaction mixture for the assay (2). The reasons pointed out for the use of EDTA were "the desire to have the reaction stop at mevalonate" (1) by chelating with Mg^{++} and preventing the action of mevalonate kinase and also to "preserve the substrate" by inhibiting the cleavage enzyme (3). Sufficient substrate is always available in the assay mixture since it is used at 4-fold excess of the saturating concentration. The routine assay does not have Mg^{++} or ATP and mevalonate-utilizing enzymes, being cytosolic soluble proteins, are unlikely to be present in significant concentration in isolated microsomes. Therefore the need for EDTA must be different from preserving either the substrate or the product of the reductase.

We now find that $FeSO_4$ inactivates irreversibly HMGCoA reductase. In washed microsomes the Fe-inhibition of the reductase was obtained only in presence of cytosol. This effect of Fe^{++} not only explains the indispensability of EDTA in the homogenizing medium to obtain

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the enzyme in active state from the liver (the iron-storage tissue) but also opens a new possibility of regulation of this enzyme.

Experimental: Rats were killed by guillotining and the livers were used for preparing microsomes by differential centrifugation. The homogenizing medium used consisted of sucrose (0.25 M), potassium phosphate buffer (0.1 M, pH 7.3), nicotinamide (30 mM), DTT (5 mM) and EDTA (1 mM). For each gram of liver, 4 ml of the homogenizing medium was used and the microsomal sediment was obtained by centrifuging post-mitochondrial supernatnat at 95,000 x g for 60 min. The sediment was used as the "unwashed microsomes". Where washing was carried out, the sediment was suspended in the original volume of the homogenizing medium omitting EDTA and the centrifugation was repeated. The microsomal pellets were resuspended in 0.1 M phosphate buffer (pH 7.3) at a concentration of about 10 mg protein/ml. The assay of HMGCoA reductase was carried out according to Shapiro *et al* (2). The reaction mixture contained phosphate buffer (0.1 M, pH 7.3), DTT (10 mM), G-6-P (45 mM), G-6-P dehydrogenase (1 unit), NADP (4.5 mM), HMGCoA (40 nmoles, 1700 dpm/nmole) and microsomes (about 0.3 mg protein) in a total volume of 0.1 ml. The mevalonate formed was separated in its lactone form on t.l.c. on silica gel G and estimated by the radioactivity. The activity of the enzyme is expressed as picomoles of mevalonate formed/min per mg. protein.

Results: Omitting EDTA from the homogenizing medium leads to microsomal preparations low in HMGCoA reductase activity. Once microsomes were prepared in EDTA-medium, they could be washed with EDTA-free medium and assayed in the absence of EDTA without loss of activity. The other essential reagent, DTT could be omitted from the homogenizing medium but must be present during the assay at 5 mM or above to give maximal activity. Thus, EDTA is essential during the preparation of microsomes to obtain active enzyme and DTT is necessary during the assay to realize its optimal activity. As an extension of the earlier observation that Fe^{++} could partially substitute for Mg^{++} in the inhibition by ATP (4) we now find that $FeSO_4$ alone could also inhibit HMGCoA reductase (Table 1) whether NADPH or NADPH-generating system (G-6-P + G-6-P dehydrogenase) was used. The inhibitory effect of Fe^{++} was not due to decreased availability of either of the two substrates, HMGCoA and NADPH, as both were present at the end of the reaction at concentrations higher than required for saturation.

Table 1. Effect of Fe^{++} on HMGCoA Reductase Activity

Reducing Source	Picomoles/min per mg protein		% Control
	no $FeSO_4$	+ $FeSO_4$ (5 mM)	
G-6-P dehydrogenase NADP + G-6-P	182	44	24
NADPH	194	34	18

Unwashed microsomes were used. The NADPH-regenerating system used was the same as described in the Experimental and in the second set of experiments NADPH (4.5 mM) replaced this.

Similar inhibition was obtained with ferric chloride and ferric nitrate. In view of DTT being present in the assay medium these will be reduced to Fe^{++} ions which then are the active species. Inhibition was also obtained in Tris buffer as well as in phosphate buffer although in the latter, the Fe-salts were precipitated.

The inhibition by $FeSO_4$ was not affected by the addition of D-mannitol (0.1 M), catalase (50 ug/ml), Ca^{++} (10 mM), Mg^{++} (10 mM), KCN (5 mM) or transferrin (2 mg/ml). The Fe-storage product in the liver, ferritin (0.1 mg/ml) also inhibited and this was apparently due to Fe released in presence of the reducing agent, DTT, present in the assay. Addition of α,α-dipyridyl to the reaction mixture even at 10 mM concentration gave only a partial reversal (Table 2)

Table 2. Effect of α,α-Dipyridyl and EDTA on Fe-inhibition

Addition	Picomoles/min per mg protein		% Control
	no $FeSO_4$	+ $FeSO_4$ (5 mM)	
None	148	70	47
α,α-dipyridyl (10 mM)	123	80	65
None	178	53	30
EDTA (20 mM)	145	132	91

The reagents α,α-dipyridyl and EDTA were added to microsomes in the buffer and the NADPH-regenerating system, before $FeSO_4$ was added.

Table 3. Effect of dialysis and washing microsomes treated with $FeSO_4$

Test system	picomoles/min per mg protein		% Control
	no treatment	$FeSO_4$-treated	
Direct assay	180	-	
After dialysis	116	16	14
Direct assay	153	-	
After washing	91	54	60

Suspensions of microsomes (10 mg/ml in 50 mM Tris buffer, pH 7.3) were treated with $FeSO_4$ (5 mM) and incubated for 5 min at 37°C. The control and experimental samples (0.2 ml each) were then dialyzed against phosphate buffer (50 mM, pH 7.3) containing 1 mM each of EDTA and DTT (250 ml, 3 changes for 90 min with stirring). Another set of samples similarly treated with $FeSO_4$ were diluted with 10 ml medium containing phosphate buffer (50 mM), NaCl (70 mM), EDTA (30 mM) and DTT (5 mM) and the mixture centrifuged at 95,000 x g for 60 min. The sediments were suspended in phosphate buffer (50 mM) and assayed for HMGCoA reductase activity. Note the loss in specific activity after the dialysis or washing procedure in the control samples.

while the complexing of Fe^{++} with the excess chelator was obvious by the red color formed. Addition of EDTA (20 mM) prevented the inhibition only when mixed with Fe^{++} before microsomes were added (Table 2). However, if microsomes and $FeSO_4$ were first mixed and then EDTA was added inhibition of the enzyme was found. Once the enzyme was affected by Fe^{++}, its presence during the reaction seems unnecessary for the inhibition as indicated by the following two experiments. Firstly, the activity of the reductase in $FeSO_4$-treated microsomes was not restored after dialysis against 0.05 M phosphate buffer containing 1 mM each of EDTA and DTT (Table 3). Secondly, washing with a solution containing phosphate buffer (50 mM), NaCl (70 mM), EDTA (30 mM) and DTT (5 mM) to remove added $FeSO_4$ had yielded microsomes with much lower activity than the similarly treated sample having no $FeSO_4$ (Table 3). These experiments also show that complete reversal of inhibition was not possible even after removal of $FeSO_4$. It is however uncertain whether the non-reversal is due to Fe-induced modification or due to sufficient Fe

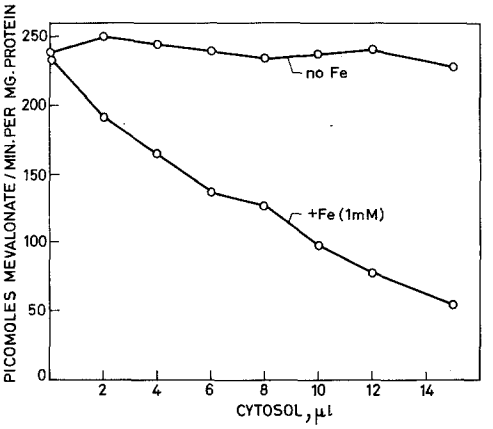

Fig.1. Inhibition of HMGCoA reductase by cytosol in presence of $FeSO_4$.

being firmly bound to microsomes that resists the processes of dialysis and washing despite the presence of EDTA.

A critical concentration of $FeSO_4$ seems to be required for inhibition. With a number of microsomal preparations this was about 0.3 mM. Inhibition was not progressive with the concentration of Fe and concentrations higher than the critical one (upto 5 mM) did not increase the inhibition. The extent of inhibition by $FeSO_4$ varied with different microsomal preparations. In unwashed microsomes the maximum inhibition was in the range of 50-80% but it was no more than 20% in once-washed microsomes, suggesting a clue that another factor variably attached in microsomes is responsible for eliciting Fe-inhibition. This was experimentally verified. Inhibition by $FeSO_4$ (1 mM) was found to be restored in washed microsomes on addition of cytosol (95,000 x g supernatant, 21.4 mg protein/ml) and the concentration of the cytosolic factor seemed to determine the extent of inhibition of the reductase, as increasing its concentration increased the inhibition (Fig.1). The cytosolic factor was found to be labile when heated at 60°C for 5 min or 100°C for 1 min or on addition of perchloric acid (1N) followed by neutrali-

zation. It was non-dialyzable, precipitable by ammonium sulfate and was not found in the ultrafiltrate. All these indicated that the cytosolic factor is a protein.

Discussion: Lipid peroxidation occurs in microsomes in the presence of NADPH and can destroy the membrane structure and activities of the associated enzymes (5). This is enhanced by Fe-salts (6). These being common with the present system the inhibitory effects of Fe must be examined in the light of lipid peroxidation. This is unlikely in the present experiments because at the high microsomal protein concentration of about 3 mg/ml used in our assay lipid peroxidation occurs at very low rate and also thiol compounds further inhibit it (5). Thus the essential requirement of DTT in the assay may indeed be to prevent lipid peroxidation and inactivation of HMGCoA reductase through lipid peroxidation.

"Feedback" regulation of HMGCoA reductase by dietary cholesterol occurs almost exclusively in the liver - the major cholesterol-producing as well as Fe-storage tissue, and this makes the Fe-inhibition highly significant. Since the cytosolic protein seems abundant the inhibition of the reductase may have been caused by the release of Fe from ferritin under conditions wherein the reductase decreases to very low levels. The Fe-inhibition effect, being obtained by naturally-occurring cytosolic components, offers a new mechanism of regulating cholesterol synthesis.

In an excellent series of investigations Gaylor and coworkers (7) described a coordinated activation of two microsomal enzymes in cholesterol biosynthesis, HMGCoA reductase and methyl sterol oxidase. This was achieved by a heat-stable cytosolic protein, identical to Z-protein (8). More interestingly they described end-product, inhibition of these two enzymes by cholesterol-derived oxidized products known to inhibit cholesterol synthesis (9), only in the presence of a heat-

stable cytosolic protein (8). This apparently first demonstration of *in vitro* inhibition of the "feedback type" had not received the due attention. The cytosolic protein in our studies being heat-labile represents probably another type of regulation of the reductase. These two proteins, interacting with a number of effectors, offer immense potential of regulating HMGCoA reductase known to occur under a wide variety of conditions.

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References

1. Linn, T.C. J. Biol. Chem. 242, 984-989 (1967)

2. Shapiro, D.J., Nordstrom, J.L., Mitschelen, J.J. and Rodwell,V.W. Biochem. Biophys. Res. Communs., 370, 369-377 (1974)

3. Bachhawat, B.K., Robinson, W.G. and Coon, M.J. J. Biol. Chem. 216, 727-732 (1955)

4. Rajan George, Satish Menon, A. and Ramasarma, T. Indian J. Biochem. Biophys. 17, 1-7 (1980)

5. Wills, E.D. Biochem. J. 113, 315-324 (1969)

6. Wills, E.D. Biochem. J. 113, 325-332 (1969)

7. Spence, J.T. and Gaylor, J.L. J. Biol. Chem. 252, 5852-5858 (1977)

8. Billheimer, J.T. and Gaylor, J.L. J. Biol. Chem. 255, 8128-8135 (1980)

9. Kandutsch, A.A. and Chen, H.W. J. Biol. Chem. 248, 8408-8417 (1973)