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INHIBITORY EFFECT OF SELENIUM ON ENZYMES INVOLVED IN HEME BIOSYNTHETIC PATHWAY IN CHICK EMBRYOS

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Effect of different concentrations of selenium (Se) on heme biosynthesis was studied at different developmental stages of chick embryo. The first rate limiting enzyme ALA-synthase (ALA-S; E.C. 2.3–1.37) activity was enhanced by selenium, while hepatic and blood ALA-dehydratase activity (ALA-d; E.C. 3.2.1.24) was decreased. Hepatic and blood free-sulfhydryl (–SH) group contents were significantly decreased by Se. Further, hepatic aminolevulinic acid (ALA) and total blood porphyrin levels were enhanced and hepatic heme levels were depleted by selenium exposure. Heme biosynthesis was maximally inhibited in the E4 (4th day injected embryos) when compared to later periods.

Keywords: Selenium, ALA-S, ALA-d, SH-groups, porphyrins, heme

INTRODUCTION

Selenium is an essential micronutrient that has an important role in health and disease.[1] Most reviews on Se biochemistry emphasize primarily on the aspects of Se deficiency, rather than Se toxicity.[2] In recent years the toxic effects associated with nutritional over exposure to Se in humans and animals have been described.[3] Biochemical studies of heme biosynthesis in experimental animals are useful to elucidate the mechanism underlying the different porphyrias.[4] This pathway is sensitive to the action of metal ions, because most of the enzymes are SH dependent.[5] Heavy metals like lead, cadmium and mercury are known to interfere with heme metabolism in experimental animals and in man causing induced chemical porphyria.[6–8] Excretion of urinary porphyrins was also reported in cases of occupational exposure to various environmental pollutants.[9] Here we describe the effect of Se exposure on the heme biosynthetic pathway in chick embryos.

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MATERIALS AND METHODS

Freshly laid, Bobcock strain zero day old eggs were obtained from the Government Veterinary College, Tirupati, Chittoor Dist. (A.P). They were placed in an egg incubator at 37°C, rotated once in a day during incubation. Different doses of selenium (12.5, 25 and 37.5 μmoles) in a total volume of 10 μl were injected directly into the yolk sac through a small hole on the 4, 7 and 11th day of embryonic development. The hole was covered with a surgical tape to prevent infection. Embryos were opened on the 19th day of incubation to collect blood and liver for the determination of 5-ALA synthase, 5-ALA dehydratase, 5-ALA, free-SH groups, porphyrin and hepatic heme levels.

Hepatic ALA levels were estimated according to the method of Mauzerall *et al.*[10] ALA synthase catalyzes the condensation of glycine and succinyl Co-A to form ALA and was assayed using the procedure of Lein *et al.*[11] The assay of hepatic ALA dehydratase was based on the measurement of the amount of porphobilinogen produced from the substrate ALA with the help of modified Ehrlich reagent as described by Mauzerall *et al.*[12] Blood ALA dehydratase activity was measured by the method of Granick *et al.*[13] Hepatic and blood free sulfhydryl groups were estimated according to the method of Ellman.[14] Blood porphyrin levels were estimated by the method of Heller *et al.*[15] Hepatic heme was extracted according to Gerber and Maes[16] and estimated spectrophotometrically by the method of Christian.[17] Protein content was measured according to Lowry's method[18] using bovine serum albumin as a standard. Statistical evaluation was done by the Student's *t*-test.

RESULTS

Significant accumulation of ALA (Figure 1) was observed in E4 than in E11. In E4 about 1.7 and 1.9 fold induction was observed with 12.5 and 37.5 μmoles of Se treatment, respectively. Significant induction of ALA-S activity was observed in E4 than in E11 (Figure 2). In E4 about 1.6, 2.2 and 3.4 fold induction of ALA-S activity was observed with 12.5, 25 and 37.5 μmoles of Se treatment, respectively.

Selenium treatment decreased the ALA-d activity in a dose dependent manner (Tables I and II). The inhibition of blood ALA-d activity was more pronounced in E7. Blood ALA-d activity was inhibited to 34, 38 and 25% with 37.5 μmoles of Se treatment on E4, E7 and E11 respectively. Hepatic ALA-d activity was reduced to 39, 43 and 54% in E4 and to 9, 16 and 25% in E11 with 12.5, 25.0 and 37.5 μmoles of Se treatment, respectively.

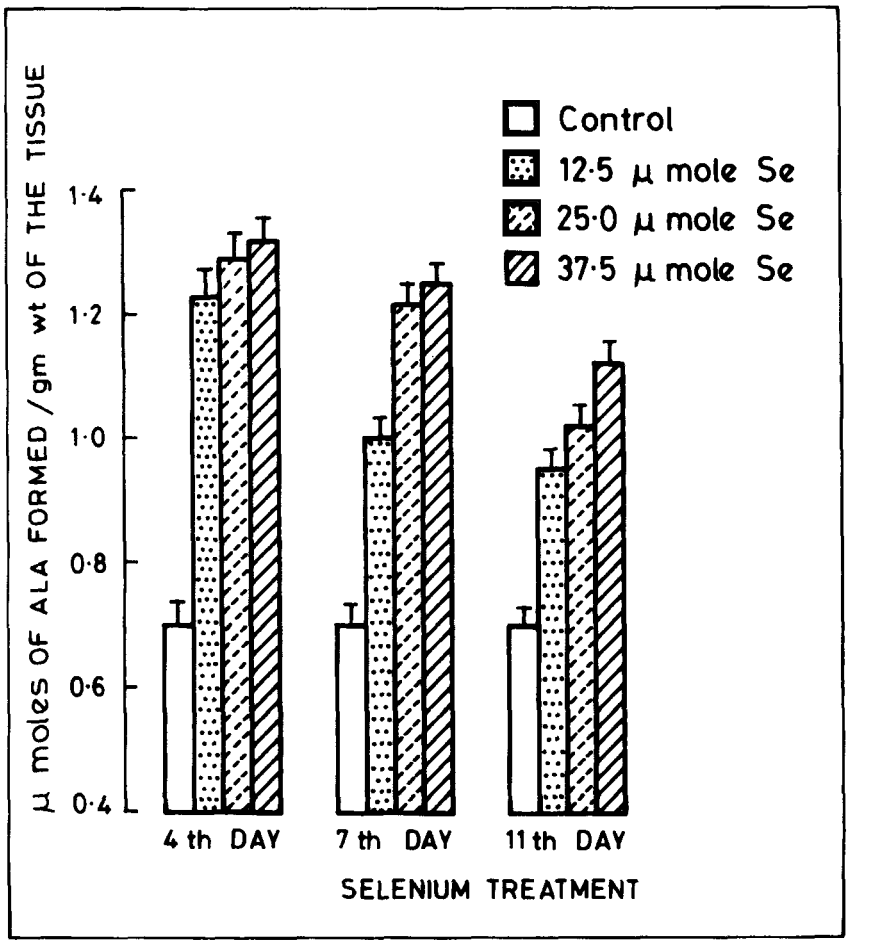

FIGURE 1 Hepatic ALA levels in Se treated chick embryos. One milliliter (1.0 ml) of sample containing ALA was treated with 1 ml of 10% trichloracetic acid (TCA). The mixture was kept in boiling water bath for 10 min and centrifuged for 10 min at 2000 rpm. The supernatant was collected, and to the precipitate was added 1 ml of distilled water. This sample was kept in boiling water bath and centrifuged at 2000 rpm as mentioned above and the supernatant was collected. The two supernatants were mixed, and to this mixture added 2 ml of 0.2 M phosphate buffer (pH 6.8) and 0.2 ml of ethyl acetoacetate.The sample was kept in boiling water bath for 15 min. After cooling, 2 ml of this solution was treated with an equal volume of modified Ehrlich reagent, and the absorbance was measured at 553 nm after 10 min against a blank. ALA levels were elevated in Se treated chick embryos. Results are expressed as μ moles of ALA formed/gm of the tissue. Values are mean±S.D ($N = 10$).

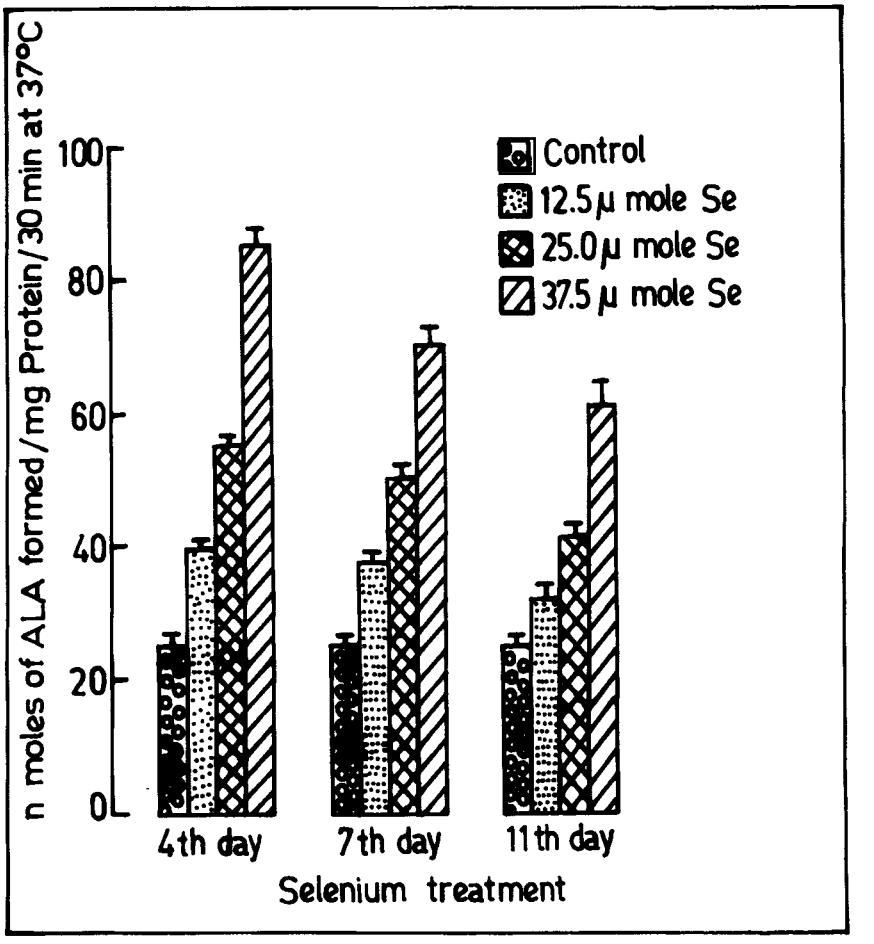

FIGURE 2 Hepatic ALA-S activity in chick embryos treated with Se. The assay mixture contained 250 μl of 60 mM Tris-HCl buffer, (pH 7.4) and each 50 μl of glycine, Na-succinate, $MgCl_2$, EDTA, pyridoxal phosphate and 500 μl of liver homogenate (in Tris-HCl buffer (pH 7.4) containing 10 mM pyridoxal phosphate) in a final volume of 1 ml. The tubes were incubated in a shaking water bath at 37°C for 30 min. The reaction was arrested by the addition of 0.2 ml of 25% TCA to the incubation mixture. For the blank, buffer was added instead of glycine and Na-succinate. After centrifuging for 10 min at 3000 rpm, 1 ml of the clear supernatant was further assayed for ALA formed as described previously.

ALA-S activity increased with Se treatment. Results are expressed as nmoles of ALA formed/mg Protein/30 min. Values are mean±S.D ($N = 10$).

TABLE I Effect of selenium on blood ALA dehydratase activity

Treatment with Se (*μ moles/kg egg wt.*)	*ALA-D activity (n moles of PBG/ml RBC/h at 37°C)* *Day on which embryos were injected*		
	4th day	*7th day*	*11th day*
Control (–Se)	1675 ± 127	1675 ± 127	1675 ± 127
12.5	1218 ± 109**	1376 ± 121*	1493 ± 118
25.0	1187 ± 100***	1061 ± 94***	1349 ± 94**
37.5	1103 ± 97***	1044 ± 86***	1262 ± 93***

Values are average of four separate experiments; mean±S.D. $n = 10$ in each group. *** $P < 0.001$; ** $P < 0.01$; * $P < 0.02$ vs control.

TABLE II Effect of selenium on hepatic ALA dehydratase activity

Treatment with Se (*μ moles/kg egg wt.*)	*ALA-D activity (n moles of PBG/mg protein/h at 37°C)* *Day on which embryos were injected*		
	4th day	*7th day*	*11th day*
Control (–Se)	42.99 ± 4.0	42.99 ± 4.0	42.99 ± 4.0
12.5	26.00 ± 2.1***	27.83 ± 2.3**	39.30 ± 2.9
25.0	24.38 ± 0.8***	25.33 ± 1.4***	36.31 ± 2.7*
37.5	20.00 ± 0.9***	22.90 ± 0.8***	32.41 ± 2.4**

Values are average of four separate experiments; mean±S.D. $n = 10$ in each group. *** $P < 0.001$; ** $P < 0.01$; * $P < 0.02$ vs control.

The results in Tables III and IV show the reduction in sulfhydryl groups with Se treatment. The hepatic sulfhydryl groups were significantly reduced to about 46, 41 and 36% with 25 μmoles of Se concentration in E4, E7 and E11 respectively. However, in blood the free –SH content was not decreased considerably as in hepatic tissues. Significant reduction of SH groups (27, 36 and 42%) was observed in E4 with 12.5, 25 and 37.5 μmoles of Se treatment, respectively.

In chick embryos, blood porphyrin levels were increased with an increase in Se concentration (Figure 3). Significant accumulation was observed in E7 than in E11. In E4 1.5, 1.6 and 2.6 fold induction was observed with 12.5, 25 and 37.5 μmoles of Se, respectively. Whereas hepatic heme levels (Figure 4) were reduced to 16, 44 and 56% in E4 and to 9, 19 and 34% in E11 with 12.5, 25 and 37.5 μmoles of Se treatment, respectively.

TABLE III Effect of selenium on hepatic sulphydryl content

Treatment with Se (*μ moles/kg egg wt.*)	*Sulphydryl content (m moles/g wt.) Day on which embryos were injected*		
	4th day	*7th day*	*11th day*
Control (–Se)	0.39 ± 0.03	0.39 ± 0.03	0.39 ± 0.03
12.5	0.23 ± 0.02*	0.25 ± 0.02*	0.27 ± 0.02*
25.0	0.21 ± 0.02*	0.23 ± 0.02*	0.25 ± 0.02*
37.5	0.19 ± 0.01*	0.20 ± 0.01*	0.13 ± 0.01*

Values are mean±S.E. of four separate experiments. $n = 10$ in each group. * $P < 0.001$ vs control.

TABLE IV Effect of selenium on blood sulfhydryl content

Treatment with Se (*μ moles/kg egg wt.*)	*Sulphydryl content (m moles of SH/L blood) Day on which embryos were injected*		
	4th day	*7th day*	*11th day*
Control (–Se)	3.44 ± 0.30	3.44 ± 0.30	3.44 ± 0.30
12.5	2.50 ± 0.20**	2.80 ± 0.26*	3.10 ± 0.25
25.0	2.20 ± 0.16***	2.50 ± 0.22**	2.60 ± 0.22**
37.5	2.00 ± 0.14***	2.10 ± 0.19***	1.90 ± 0.16***

Values are average of four separate experiments; mean±S.D. $n = 10$ in each group. * $P < 0.02$; ** $P < 0.001$; *** $P < 0.001$.

DISCUSSION

The present study showed that the trace element Se is capable of mediating remarkable alterations in the activities of various enzymes of the heme biosynthetic pathway in chick embryos. It was observed that the effect of Se was more prominent in the early stages of development of the embryo. One possible explanation for this is that proper homeostatic mechanism may not be available during early developmental stages of the embryo. A major site for control of hepatic heme biosynthesis was proposed at the level of ALA formation, hence the primary focus appears to be the site of ALA-synthase. The ALA-S activity was induced leading to the accumulation of ALA and porphyrins with increasing Se concentration. It was assumed that porphyrins accumulate as a result of the over production of ALA and the inability of enzymes other than ALA-S to cope with an increased flux

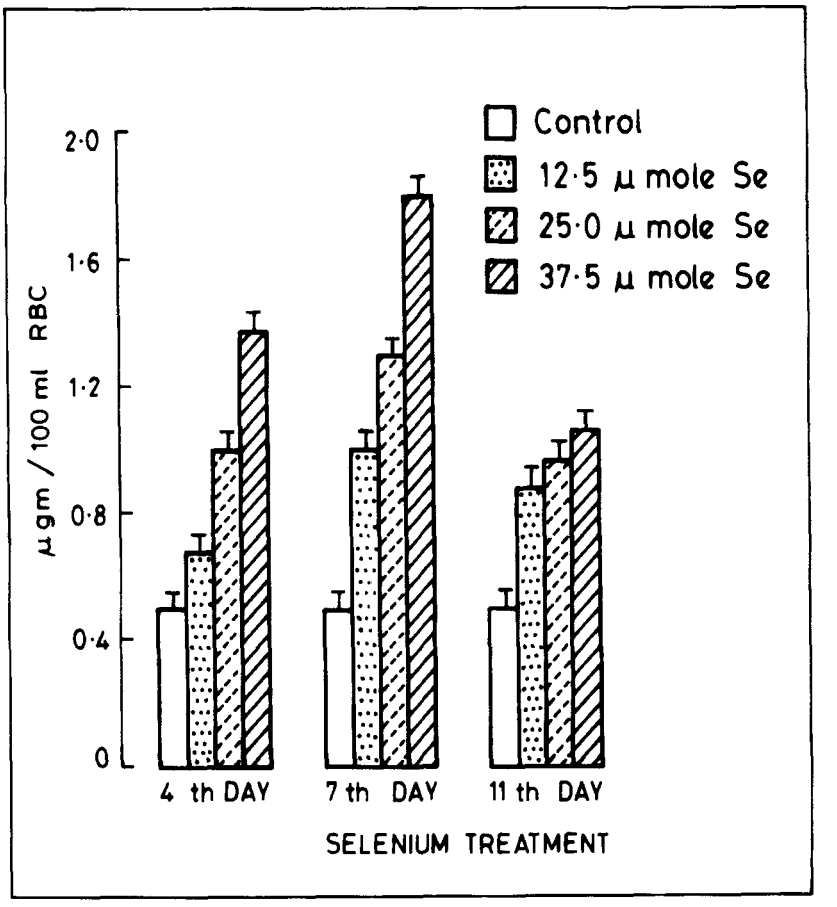

FIGURE 3 Blood porphyrin levels in selenium treated chick embryos. To 2 ml of whole blood, 2 ml of acetone and ethyl acetate (1:9) was added, and the mixture was stirred vigorously with a glass rod for about 1 min. Then 4 ml of formic acid and ether (1:9) was added, and the mixture was again stirred vigorously for 1 min. The mixture was centrifuged. The precipitate was further extracted with 4 ml of formic acid and ether (1:9), and the supernatant was combined with the first extraction. To this 2 ml of 1.5 N HCl was added, and the extract, stirred vigorously. The volume of the lower layer containing HCl was noted and transferred to a separate tube. The absorbance of the solution was recorded at 380, 407 and 430 nm using 1.5 N HCl as blank.

Blood porphyrin levels increased with selenium treatment. Results are expressed as μgm/100 ml RBC. Values are mean±S.D. ($N = 10$).

$$\frac{2 \times (A_{407}) - (A_{380} + A_{430}) \times 1.28 \times \text{ml HCl} \times 100}{\text{Hematocrit value} \times \text{ml of whole blood}}$$

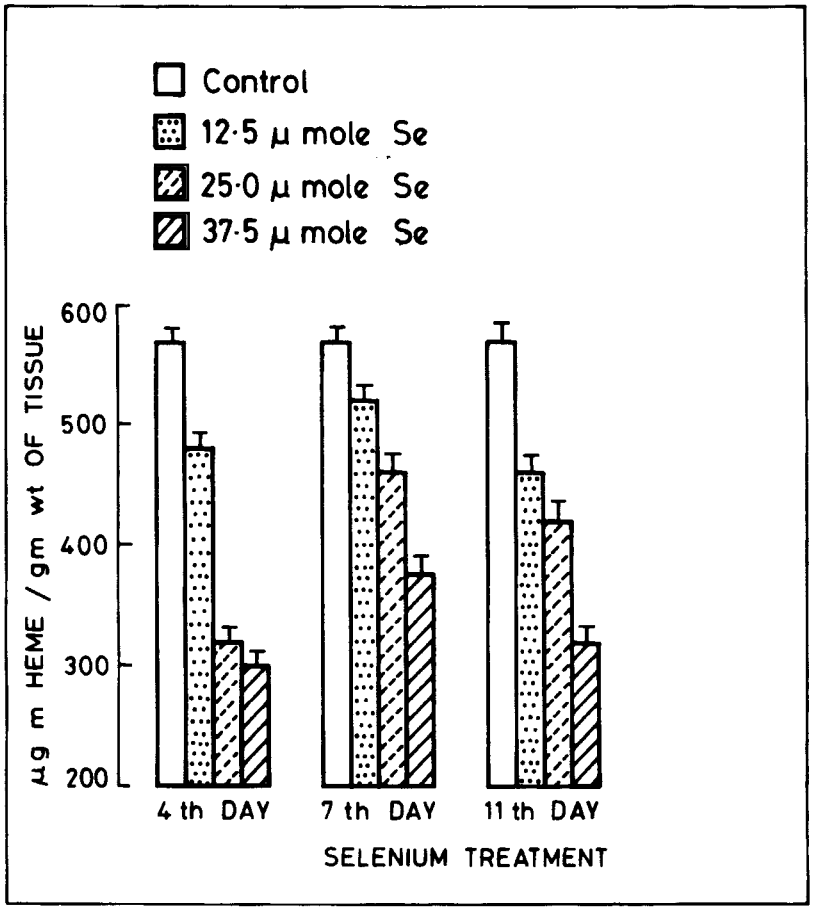

FIGURE 4 Hepatic heme levels in selenium treated chick embryos. Livers were homogenized in a mixture of chloroform and methanol (2:1) and centrifuged. The supernatant was discarded, and to the precipitate was added 1 ml of 50% ethanol. The mixture was stirred well and centrifuged. Again the supernatant was discarded, and the precipitate from this fraction was extracted with 1 ml of acidic acetone and used for heme estimation by forming pyridine haemochromogen complex with the use of 3 ml of standard alkaline pyridine solution. Prior to taking the reading, a small excess of sodium dithionate was added to reduce the pyridine haemochromogen complex, and the optical density at 557.5 nm was noted within 5 min, since the reduced pyridine haemochromogen is quite an unstable compound. Hepatic heme levels were expressed as μg of heme/g wet wt. of tissue using heme standard curve.

Heme levels decreased with selenium treatment. Results were expressed as μg of heme/g weight of tissue. Values are mean±S.D. ($N = 10$).

TABLE V Effect of selenium, selenium + actinomycin-D on ALA-S activity

(ALA-S activity)	
Treatment with Se *μ moles/kg egg wt.)*	*n moles of ALA formed/mg protein/1/2 h at 37°C*
Control (–Se) (–Act-D)	17.89 ± 1.50
(+ Act-D)	6.63 ± 0.50
Se 12.5	23.15 ± 2.00
Se 12.5 + Act-D	11.00 ± 0.90
Se 25.0	26.52 ± 2.23
Se 25.0 + Act-D	9.00 ± 0.78
Se 37.5	34.20 ± 3.00
Se 37.5 + Act-D	8.73 ± 0.80

Values are average of four separate experiments; mean±S.D. $n = 10$ in each group.

of intermediates through the pathway.[19] The decreased hepatic heme levels may be due to the increase in ALA-S activity and corresponding increase in hepatic ALA-levels. Studies with Actinomycin-D (Table V) suggest that the induction of ALA-S by Se may be regulated at the transcriptional level.

The dose-dependent inhibition of ALA-d activity and decreased –SH groups observed with Se treatment suggest that the inhibition of ALA-d may be due to Se binding the active site of the enzyme to –SH groups. This possibly causes a configurational change of the enzyme leading to inhibition of the enzyme. These results are in agreement with those of Fujita *et al.*,[20] who reported that the inhibition of blood ALA-d most likely represents the direct reaction with the –SH groups of the enzyme. As shown earlier, Se reacts with intracellular –SH compounds to form seleno-trisulfides,[21] thus removing –SH groups that are required for enzyme activity. However, the relatively less sensitivity of blood ALA-d activity to Se administration suggests the possibility that some –SH groups of the enzyme are inaccessible to selenium. Increased levels of porphyrins with concomitant inhibition of ALA-d activity in erythrocytes were observed by the administration of tin,[22] whereas in the case of lead,[23] ferrochelatase was found to be affected.

The present results indicate that not only lead and tin but also Se interferes with the heme biosynthesis leading to the accumulation of intermediates like porphyrins, ALA and depleted heme levels. Also, the control of heme synthetic enzymes in certain tissues appears to be distinct at the gene level, which would explain the varied effect of Se on hepatic and blood ALA-d activities.[24] The decreased heme levels upon exposure to Se play an important biological role, since the cytochromes

contain heme as their prosthetic moiety. As reported by Dailey[25] Se may modify the –SH groups and inhibit the activity of the enzyme ferrochelatase leading to the accumulation of porphyrins. Increased blood porphyrin levels may be due to the increased activity of ALA-S or a decreased activity of ferrochelatase which utilizes protoporphyrin as the substrate to synthesize heme in a limited manner, leading to the depletion of heme.

Reduction of the regulatory heme pool may be by one or more of the following mechanisms: (1) accelerated destruction of heme, (2) inhibition of ferrochelatase (which catalyses the insertion of iron into protoporphyrin to form heme) by selenium,[26] and (3) induction of heme oxygenase by Se treatment.[27] Hence, the elevation of heme oxygenase activity elicited by the Se as reported earlier may also be responsible for the decrease in cellular heme content in chick embryos. These facts follow the biochemical principle that the first enzyme (ALA-S) in an unbranched biosynthetic pathway controls the flux of substrates down the pathway.

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