9). The possible involvement of this latter chelation mechanism is indicated by the decrease in inhibitor activity observed when this chelation is prevented by the shift in the substituent from position 1 in the ring system to position 2. Chelation by the amine groups of the side chain appears to be an unlikely mechanism since compound 3, which has high activity, would give rise to a sterically unfavourable 7-membered chelate ring as compared to compounds 1 and 2 which are of lower activity, but which would yield favourable 5- or 6-membered rings.

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δ-Aminolaevulinate synthase: mechanism of its response to malathion

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Aminolaevulinate (ALA)-synthase is the first and rate-limiting enzyme of hepatic heme biosynthesis [1]. ALA-synthase is induced by several drugs and steroids in the liver [1–4]. This experimentally induced porphyria resembles the human acute hepatic porphyrias in its biochemistry [5]. 2-Allyl-2-isopropylacetamide (AIA) and 3,5-diethoxycarbonyl-1,4-dihydrocollidine (DDC) also have been studied as potent inducers of ALA-synthase [6, 7]. In animals treated with these drugs a considerable amount of the enzyme accumulates in mitochondria, as well as cytosol, of the liver [7]. The enzyme is synthesized on free polyribosomes as a cytosolic precursor which is processed during incorporation into mitochondria to give the mature form [8–10].

Heme regulates the activity of ALA-synthase in the liver [1]. Ferrochelatase catalyzes the insertion of ferrous iron

into protoporphyrin IX to form heme, the end product of this pathway.

Malathion is an organophosphorous pesticide used in agriculture. We have reported previously that malathion enhances ALA-synthase activity as well as the content of porphyrins in chick embryo liver *in ovo* [11].

In the present paper, the mechanism of the increase of ALA-synthase activity induced by malathion was investigated using (a) inhibitors of protein synthesis, (b) hemin and (c) phenobarbital, an inducer of drug-metabolizing systems [12]. We also studied the effects of malathion addition *in vitro* on the activity of ALA-synthase from normal chick embryo liver, as well as the effect of this pesticide on ferrochelatase activity and on subcellular distribution of ALA-synthase.

Materials and methods

Chemicals. Malathion, technical grade, was a gift from Syntial S.A., Buenos Aires, Argentina. The purity of the sample was 96%, w/w. Hemin and mesoporphyrin IX dihydrochloride were obtained from Porphyrin Products, Logan, UT.

Animals. Chick embryos (18- to 19-days-old) of the New Hampshire strain were used. Drugs were injected through chorioallantoic membrane, under sterile conditions, into the fluids surrounding the embryo. At the end of the incubation period the embryos were killed by decapitation. The livers, removed and carefully separated from the gallbladder, were immediately cooled on ice and washed with cold saline.

Malathion (60 mg/embryo), hemin (5 mg/embryo) and actinomycin D (300 μg/embryo) were injected in 0.2 ml of dimethyl sulfoxide (DMSO). Cycloheximide (10 μg/embryo) and phenobarbital (5 mg/embryo) were injected in saline.

Liver fractionation. Livers were homogenized with 0.25 M sucrose (pH 7.2). Homogenates were centrifuged at 11,000 *g* for 20 min. The pellet obtained was resuspended in 0.25 M sucrose and again centrifuged as above to obtain the mitochondrial fraction. The combined supernatant fractions were centrifuged at 105,000 *g* for 1 hr to obtain microsomes and the supernatant corresponding to the cytosol fraction.

Enzyme activities. ALA-synthase activity was assayed in whole homogenates by the method of Marver *et al.* [13]. For the chick embryo liver assay, mixtures containing 200 μmol glycine, 20 μmol EDTA, 100 μmol Tris–HCl (pH 7.2), 0.3 μmol pyridoxal phosphate and the amount of homogenate corresponding to 0.125 g of liver, in a final volume of 2 ml, were incubated at 37° for 40 or 60 min.

When subcellular distribution of ALA-synthase was measured, the same method was employed except that a succinil-CoA-generating system was added to the incubation mixture. This mixture contained in addition to the above: 10 μmol ATP, 0.2 μmol coenzyme. A, 40 μmol $MgCl_2$, 10 μmol succinate and 0.05 units succinil-CoA-synthase prepared from *Escherichia coli* according to Ohashi and Kikuchi [14]. One unit of succinil-CoA-synthase was defined as the amount of protein that formed 1 μmol succinil-CoA in 1 min when assayed by the method of Kaufman [15].

Succinate dehydrogenase (SDH) was assayed by the method of Pennington [16]. One unit of SDH was defined as the amount of protein that catalyzed the reduction of 2-(*p*-iodophenyl)-3-(*p*-nitrophenyl)-5-phenyl tetrazolium chloride to give an increase of 1 in absorbance at 490 nm under the assay conditions employed.

Glutamate dehydrogenase (GDH) was assayed according to the method of Beaufay *et al.* [17]. One enzyme unit was defined as the amount of protein that produced a decrease of 0.01 in the absorbance at 340 nm in 10 sec.

Ferrochelatase was measured in the 11,000 *g* pellet by the method of Cole *et al.* [18].

Statistical treatment of results. Results are expressed as the arithmetic mean ± SEM. To analyze the level of significance of the data, a non-parametric method, the Wilcoxon two-samples test, was used [19]. The level of significance was set at $P < 0.05$.

Results and discussion

Malathion markedly elevated ALA-synthase activity in the chick embryo liver *in ovo*. The enzyme activity continuously increased from 2 to 9 hr after injection of the drug.

Cycloheximide as well as actinomycin D prevented the increase in ALA-synthase in response to malathion (Table 1). If the embryos were first treated with the pesticide for 3.5 hr to increase ALA-synthase activity and then cycloheximide was injected, the enzyme activity fell rapidly, with a half-life of 105 min (Fig. 1). Different half-lives of hepatic ALA-synthase in chick embryos of the White Leghorn strain have been reported [6, 20, 21]. Our results are not in close agreement with these data but we employed chick embryos of the New Hampshire strain and, furthermore, the half-life of ALA-synthase seems to vary according to the drugs used as inducers [6].

When actinomycin D was injected 3.5 hr after the administration of the pesticide, the enzyme activity continued to increase for 3 hr and then began to decrease (Fig. 2). Therefore, the inhibitor does not act immediately and new ALA-synthase mRNA is synthesized for some time.

When homogenates from normal chick embryo livers were incubated *in vitro* with malathion, no direct effect on normal ALA-synthase activity was seen.

Table 1. Effect of cycloheximide, actinomycin D and hemin on the increase of ALA-synthase produced by malathion

Treatment	ALA-synthase activity [nmol ALA formed·(g liver)$^{-1}$·(40 min)$^{-1}$]
Malathion	33.8 ± 3.9 (5)
Malathion + cycloheximide	5.9 ± 0.9* (5)
Malathion + actinomycin D	8.6 ± 2.4* (7)
Malathion + hemin	13.4 ± 3.8† (7)

Chick embryos were treated with malathion (60 mg/embryo) for 3.5 hr. Cycloheximide (10 μg/embryo) was injected 1 hr before and 1 hr after the injection of the pesticide. Actinomycin D (300 μg/embryo) or hemin (5 mg/embryo) was injected simultaneously with malathion. Hepatic ALA-synthase was measured by the method of Marver *et al.* [13] as described in Materials and Methods. Values are means ± SEM. The numbers in parentheses represent the number of individual determinations used in calculating the mean.

*† Significantly different when compared with malathion: * $P < 0.01$, and † $P < 0.05$.

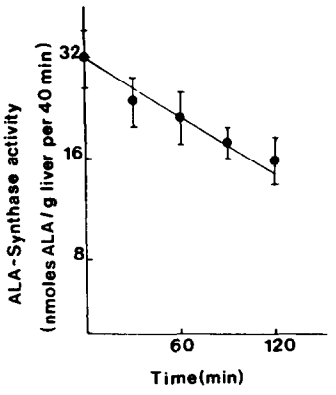

Fig. 1. Decay of ALA-synthase after the injection of cycloheximide in liver of chick embryos treated with malathion. Cycloheximide (10 μg/embryo) was injected into the embryos 3.5 hr after the administration of malathion (60 mg/embryo), and then the decay of enzyme activity was followed. The time of cycloheximide injection was taken as zero time. Hepatic ALA-synthase was measured by the method of Marver *et al.* [13] as described in Materials and Methods. Each point is the mean ± SEM for seven individual determinations.

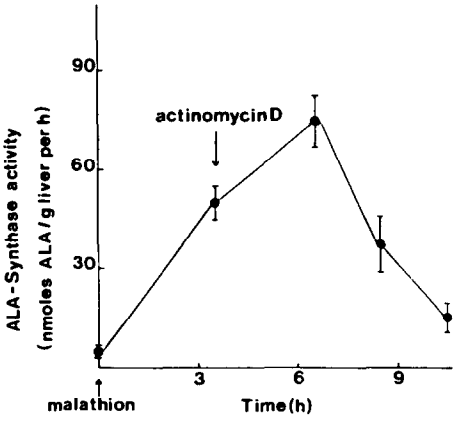

Fig. 2. Effect of the administration of actinomycin D in the middle of the increase of ALA-synthase produced by malathion. Chick embryos were treated with malathion (60 mg/embryo) for 3.5 hr and then actinomycin D (300 μg/embryo) was injected. To assure the persistence of the inhibitor action during the period studied, a second injection of actinomycin D (50 μg/embryo) was given 3 hr after the first one. Hepatic ALA-synthase was measured by the method of Marver *et al.* [13] as described in Materials and Methods. Each point is the mean ± SEM for three individual determinations.

To determine if the increase of ALA-synthase activity might be attributed to a metabolite of the pesticide, the effect of the pretreatment of the embryos with phenobarbital for 1 hr prior to the injection of malathion was studied. Taking into account the increase of activity produced by phenobarbital and malathion separately, the administration of both drugs had no synergistic effect (Table 2). We also noted that pretreatment with phenobarbital did not improve the hepatic accumulation of porphyrins promoted by malathion (results not shown). Thus, the action of malathion is not mediated through a metabolite, and the stimulation of drug-metabolizing systems by phenobarbital is not involved in the formation of a metabolite responsible for the effect of the pesticide. In addition, the pesticide had no direct effect on ALA-synthase activity. These observations coupled with the data obtained with cycloheximide and actinomycin D support an inducer effect of malathion on ALA-synthase. Observations that AIA and DDC are potent inducers of this enzyme [7] are in agreement with this.

Injection of the pesticide with hemin produced a partial inhibition of the increase of ALA-synthase (Table 1). When hemin was injected midway in the increase of ALA-synthase by malathion (at 3 hr), it prevented a further increase of enzyme activity (Fig. 3). The effect of hemin on the induction of ALA-synthase by AIA and DDC [6] is similar to the one reported here.

It was found that hepatic ferrochelatase activity (142 ± 20 nmol mesoheme/g liver per hr) was decreased significantly in chick embryos treated with malathion with respect to DMSO controls (245 ± 19 nmol mesoheme/g liver per hr). If this decrease of activity affects the regulatory heme pool, this event may be involved in the induction of ALA-synthase. However, this seems to be unlikely because it has been reported that inhibition of ferrochelatase is not enough to induce ALA-synthase in chick embryo liver [18, 22, 23].

In control embryos (treated with DMSO), the total ALA-synthase activity in the homogenate was recovered in the mitochondrial fraction. In chick embryos treated with malathion, most of the activity was recovered in the mitochondrial fraction, although part of it appeared in the cytosol. The percentage of cytosolic activity varied from 10 to 20%. The greater the induction obtained, the more

Table 2. Effect of phenobarbital on the increase of ALA-synthase produced by malathion

	ALA-synthase activity [nmol ALA formed·(g liver)$^{-1}$·hr^{-1}]	
Treatment	2.5 hr	4.5 hr
DMSO	5.1 ± 0.9 (3)	5.4 ± 1.5 (3)
DMSO + phenobarbital	18.4 ± 3.6 (4)	48.1 ± 5.0 (10)
Malathion	27.6 ± 3.4 (5)	33.6 ± 5.8 (6)
Malathion + phenobarbital	45.3 ± 18.9 (3)	76.7 ± 15.1 (8)

Chick embryos were injected with phenobarbital (5 mg/embryo) 1 hr prior to the administration of malathion (60 mg/embryo) or solvent (0.2 ml of DMSO/embryo). The embryos were killed 2.5 hr or 4.5 hr after the second injection. Hepatic ALA-synthase was measured by the method of Marver *et al.* [13] as described in Materials and Methods. Each value is the mean ± SEM. The numbers in parentheses represent the number of individual values used in calculating the mean.

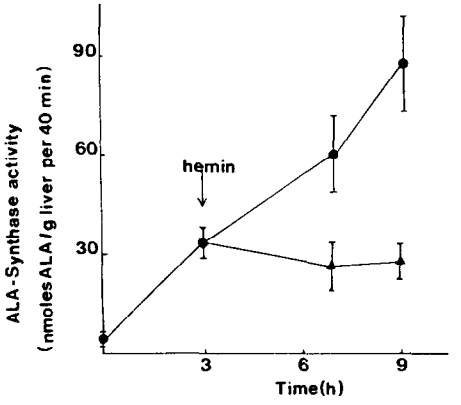

Fig. 3. Effect of the administration of hemin in the middle of the increase of ALA-synthase produced by malathion. Chick embryos were treated with malathion (60 mg/embryo) for 3 hr; then hemin (5 mg/embryo) was injected and hepatic ALA-synthase was measured at the times indicated using the method of Marver *et al.* [13] as described in Materials and Methods. Key: (●) malathion alone; and (▲) after the injection of hemin. Each point is the mean ± SEM for five individual determinations.

cytosolic activity found (Table 3). This cytosolic activity cannot be attributed to contamination with mitochondrial matrix because only 2% of total GDH activity appeared in this fraction.

It has been proposed [7] that ALA-synthase may accumulate in the cytosol when the rate of synthesis of the enzyme is greater than the rate of incorporation into the mitochondria, and enzyme incorporation into the mitochondria may be limited to a certain level of enzyme. Our results are in agreement with this proposal because the higher the increase in enzyme produced by malathion, the more noticeable is the level of cytosolic enzyme.

It has been reported that, in contrast to mammals and adult chicken, in the chick embryo liver, whether cultured or *in ovo*, ALA-synthase activity does not accumulate appreciably in the soluble fraction after treatment with AIA or DDC [6, 24]. However, since the precursor of the enzyme has been observed in chick embryo liver cytosol, it has been postulated that the level of this precursor may be too low to be detected or may be inactive [10]. That the precursor of chick embryo liver ALA-synthase is inactive seems unlikely because the precursor of the enzyme from rat liver is active and it seems to have a high degree of amino acid homology with the chick embryo enzyme [25]. Besides, in the adult chicken the active center of the enzyme is not influenced by the presence of the extra amino acid sequence, and both mitochondrial and cytosolic enzymes are active [26].

In the present study soluble ALA synthase activity could not be attributed to leakage of the enzyme from mitochondria. This suggests that the cytosolic precursor of the enzyme was active.

Thus, malathion seems to induce ALA-synthase in the chick embryo liver *in ovo* similar to AIA and DDC.

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