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Induction of δ-Aminolevulinic Acid Synthetase in Chick Embryo Liver Cells in Culture

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Abstract. δ-Aminolevulinic acid synthetase is induced in chick embryo liver culture by the natural steroid, etiocholanolone, and by the foreign chemical, 3,5-dicarbethoxy-1,4-dihydrocollidine at the level of transcription. Alternatively, inducing chemicals such as allylisopropylacetamide and γ-hexachlorocyclohexane act primarily at the level of translation. Hemin (K_i = 5 μM) inhibits the induction at the level of translation. In liver cell culture, the half-life of δ-aminolevulinic acid synthetase is 3 hr, that of mRNA about 5 hr.

Certain steroids and a large number of chemicals and drugs have been found to induce, in animals and in chick embryo liver cells, an increased porphyrin production in the liver. This "chemical porphyria" was shown to be caused by an increase in the activity of δ-aminolevulinic acid (ALA)-synthetase, the rate-limiting enzyme of heme biosynthesis.[1] The activity of this enzyme not only controls the rate of heme synthesis in the liver but also the rate of hemoglobin formation in erythrocytes.[2] Naturally occurring inducers of this enzyme were found to be 5β-H steroids of the androstane and pregnane series, compounds previously considered to be inactive metabolic products of steroid metabolism *in vivo*. These steroids have been shown to induce an increase in ALA-synthetase not only in the liver[3,4] but also in erythroid cells.[5–7] Unlike the steroids, the drugs and foreign chemicals induce only in the liver and not in other tissues.

The diversity of chemical structures which may induce enhanced ALA-synthetase activity has been puzzling. In this paper we report evidence to suggest that certain compounds may induce the synthesis of ALA-synthetase by affecting the transcriptional process and others by affecting the translational process. Within the last few years many effector sites have become recognized, especially at the translational level (e.g., ref. 8,9). The possibility may now be envisioned that specific inducing chemicals of ALA-synthetase may act at one or another of these sites and indeed may act at hitherto unrecognized sites.

Methods. Previous studies on the inducibility of ALA-synthetase in chick embryo liver culture have depended on the recognition of the activity indirectly, by fluorimetric determination of the porphyrins, in cells or extracts from the cells.[1] To determine ALA-synthetase directly, we used large Petri plates (13 cm diameter) in which cells equivalent to about 5 mg protein can be grown. Two Petri dishes are sufficient for an analysis using modifications of the colorimetric procedure for ALA-pyrrole described previously.[1]

The following experiments were made possible by the fact that in chick embryo liver

in culture, the half-life of the mRNA for ALA-synthetase is about double that of the enzyme itself. Because of the relatively longer half-life of the mRNA, it was possible to study the effect of inducing chemicals on the accumulation of mRNA while protein synthesis was inhibited with cycloheximide, and to study the synthesis of the enzyme from accumulated mRNA while RNA synthesis was inhibited with actinomycin D.

Experiments and their interpretations. The induction of the synthesis of ALA-synthetase by inducing chemicals is specific and not general for all proteins: Tracer studies with radioactive orotic acid and leucine[1] indicated that the addition of inducer did not bring about a detectable change in the overall rates of synthesis of mRNA and protein. More recent studies with radioactive uridine and leucine confirm this result. Therefore, the inducing chemical does not accelerate some limiting step in total RNA or total protein synthesis, but rather accelerates some reactions connected with the synthesis of ALA-synthetase and, perhaps, with several other enzymes functionally related to it.[10]

The half-life of ALA-synthetase in tissue culture is 3 hr: When normal cells were incubated with cycloheximide (0.1 μg/ml) to stop protein synthesis, ALA-synthetase decayed with a half-life of 3 hr. In induced cells, the half-life of the enzyme was shown by the following experiment (Expt. 1 of Table 1). The

TABLE 1. *Actions of inhibitors and inducers on ALA-synthetase formation and decay.**

Experiment	Additions (μg/ml)	Half-lives of ALA-synthetase, (hr)
1	Acetoxycycloheximide (0.06)	3
2	Acetoxycycloheximide (0.06) + AIA (60)	3
3	Acetoxycycloheximide (0.06) + hemin (5)	3
4	Actinomycin D (0.25)	5.2†
5	Actinomycin D (0.25) + etiocholanolone (10)	5.2†
6	Actinomycin D (0.25) + AIA (60)	10.6†
7	Actinomycin D (0.25) + hemin (5)	3.6†

* Chick embryo liver cells grown in monolayers were treated with AIA for 14 hr to induce an increase in ALA-synthetase, then the medium was changed, additions were made as noted, and the decay in activity of the enzyme was determined during the next 4–10 hr.

† Overall half-life for ALA-synthetase. After actinomycin D addition, ALA-synthetase activity increased slightly for 2 hr, remained at a plateau for 3 hr, and finally decayed with a half-life of 3 hr. In experiment 7, the decay of ALA-synthetase took place within 3 hr after additions as noted.

cells in the medium were incubated together with the inducing chemical allylisopropylacetamide (AIA). During a subsequent 14 hr, ALA-synthetase activity increased about 10 times over the basal level. The medium was then changed to remove the AIA, and acetoxycycloheximide was added to block further protein synthesis. ALA-synthetase activity decayed at a first-order rate with a half-life of 3 hr. Thus, in cells in culture, the half-life is 3 hr in both normal and induced cells. In the rate the half-life has been reported to be about 1 hr.[11,12]

Inducing chemicals do not affect the half-life of ALA-synthetase: It was estimated that rat liver synthesizes heme at about one-seventh the rate of bone marrow.[10] Therefore the possibility arose that an inducing chemical might merely act by slowing down the rate of degradation of ALA-synthetase, as was found in the case of catalase by Ganshow *et al.*[13] To test this possibility, the enzyme was first increased by induction with AIA; after 14 hr the inducer was

removed and acetoxycycloheximide added to block further protein synthesis. At the same time an inducing chemical, AIA or etiocholanolone, was added (Expt. 2 of Table 1). It was found that the half-life of the enzyme remained at 3 hr, i.e., the half-life was independent of the presence of inducers. Therefore, inducing chemicals did not act to enhance or decrease the rate of degradation of formed ALA-synthetase. Addition of inducing chemicals to isolated mitochondria affected neither the activity of the ALA-synthetase in the mitochondrian or other limiting reactions for the synthesis of ALA.

Certain inducing chemicals, e.g., AIA and γ-hexachlorocyclohexane (Lindane), act at the translational level to enhance the activity of ALA-synthetase: In this experiment mRNA synthesis was inhibited with actinomycin D in the presence and absence of an inducer. In this way it was possible to recognize whether the inducer, acting on preformed RNA, could cause an increase in ALA-synthetase activity, presumably by causing an enhanced synthesis of the enzyme.

The control experiment was to treat cells with AIA for 14 hr. The AIA was then removed and actinomycin D was added to the cultures to block the synthesis of mRNA (Expt. 4 of Table 1). After the addition of actinomycin D the ALA-synthetase activity increased slightly for 2 hr, then remained at a plateau for the next 3 hr, and finally decayed with a half-life of 3 hr. The apparent half-life of the overall decay rate was about 5.2 hr. This decay rate included the decay of mRNA and of ALA-synthetase, both of which were present at the time of addition of actinomycin D, as well as the decay of the newly synthesized ALA-synthetase formed from the mRNA. In contrast, when AIA was added together with actinomycin D (Expt. 6 of Table 1), the overall apparent half-life of the ALA-synthetase was about 10.6 hr.

These results suggest that after actinomycin D addition, AIA brought about a marked increase in the synthesis of the enzyme from the preformed mRNA. The inducing AIA may have increased either the lifetime of the mRNA for ALA-synthetase or its activity for translation.

In a different type of experiment two kinds of inducers were recognized: one kind (e.g., AIA, Lindane) increased ALA-synthetase activity in the presence of actinomycin D; the other kind (e.g., etiocholanolone, DDC) did not. In this experiment, cells that were not previously treated were given actinomycin-D plus inducer chemical, incubated for 4 hr, and the ALA-synthetase activity then was determined.

Etiocholanolone (5β-androstane-3α-ol,17-one) and DDC, but not AIA or Lindane, induce at the transcriptional level: Cycloheximide, which blocks protein synthesis, was given to the cells in the presence and absence of etiocholanolone for 8 hr (Fig. 1), after which the medium was replaced by one lacking these chemicals. Actinomycin D was added to inhibit further mRNA synthesis, and the activity of ALA-synthetase was measured during the next 6 hr. The accumulation of mRNA was recognized by the increased activity of ALA-synthetase after the removal of the cycloheximide. In similar experiments AIA or Lindane effected small or negligible increases over controls.

Etiocholanolone, however, did not appear to affect the translational process as illustrated in Fig. 2 and experiment 5 of Table 1. Cells were incubated

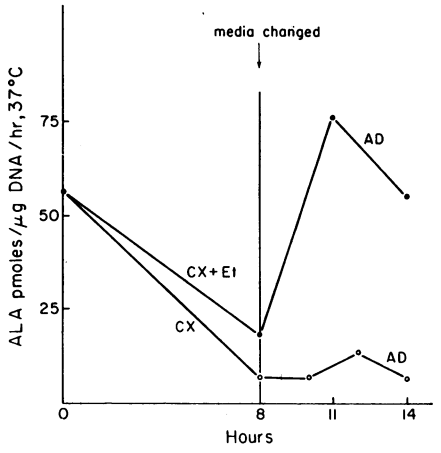

FIG. 1. Increase in ALA-synthetase in the cultured chick embryo liver cells after treatment with cycloheximide and etiocholanolone. Cells were incubated with cycloheximide and etiocholanolone (CX + Et) or with cycloheximide (CX) for 8 hr to accumulate mRNA. Then the medium was replaced with fresh medium and actinomycin D was added. The activity of ALA-synthetase was measured during the subsequent 6 hr. CX (cycloheximide), 0.1 μg/ml; Et (etiocholanolone), 10 μg/ml; AD (Actinomycin D), 0.25 μg/ml.

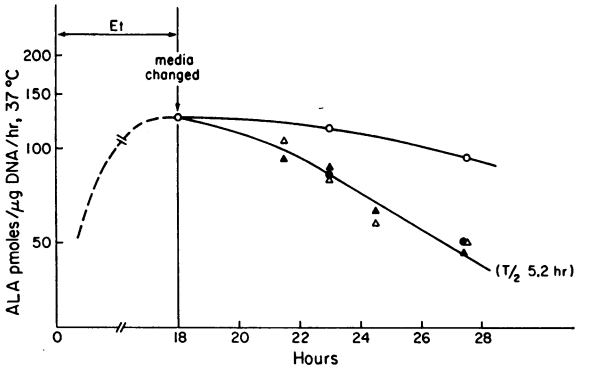

FIG. 2. Effect of etiocholanolone (Et), in presence and absence of actinomycin D, on ALA-synthetase activity in the cultured chick embryo liver cells. Cells were incubated with etiocholanolone (10 μg/ml) for 18 hr, then the medium was replaced with fresh medium and additives. The activity of ALA-synthetase was measured for 10 hr (i.e., between 18 and 28 hr). The upper curve (O—O) represents the activity of ALA-synthetase in the presence of etiocholanolone (10 μg/ml). The lower curve represents the activity: without any addition, (●—●); with actinomycin D (0.25 μg/ml) alone, (△—△); and with actinomycin D (0.25 μg/ml) plus etiocholanolone (10 μg/ml), (▲—▲).

with etiocholanolone for 18 hr, the medium was replaced, and then actinomycin D was added; the apparent half-life of ALA-synthetase (5.2 hr) remained the same in the presence as in the absence of etiocholanolone.

Inhibition by heme occurs at the translational level: Studies with labeled leucine and orotic acid showed that hemin, at 5 μM, did not affect total protein or total RNA synthesis in chick embryo liver cells.[1] These findings were reconfirmed with radioactive leucine and uridine. In addition, it was found that hemin did not affect radioactive thymidine incorporation, which suggested that DNA synthesis was not affected. Our finding that hemin did not stimulate ribonuclease activity also supported the idea that hemin had no effect on nonspecific RNA degradation.

The addition of hemin to isolated mitochondria from induced cells does not directly affect the activity of ALA-synthetase except at concentrations that are, physiologically, abnormally high.[1,10]

When hemin was added to the liver cells in culture, together with acetoxycycloheximide (Expt. 3 of Table 1), the rate of decay of ALA-synthetase was 3 hr, i.e., the same as the control rate without hemin, which indicated that hemin did not affect the rate of decay of the enzyme.

An inhibitory effect of hemin on the synthesis of ALA-synthetase was reported in chick embryo liver cells (K_i 3 μM)[1] and confirmed in whole animal studies by Hayashi *et al.*[14] and by Marver.[15] In the present experiments it was possible to demonstrate that the inhibitory effect is at the translational level. The cells in culture were first pretreated with AIA for 14 hr to increase the level of ALA-synthetase. The medium was then changed twice to remove AIA and replaced with fresh medium to which actinomycin D and hemin were added. It was determined that the average apparent half-life of ALA-synthetase had decreased to 3.6 hr, as compared with 5.2 hr with actinomycin D alone.

These results suggested that hemin inhibited the synthesis of ALA-synthetase at the translational, rather than at the transcriptional level as had been previously hypothesized.[1] Hemin might inhibit by competing with an inducing chemical for a special site, or act at some other site. On the other hand, hemin might cause a decrease in the lifetime of mRNA for ALA-synthetase. The latter idea is disfavored because, as noted above, hemin did not stimulate an increase in ribonuclease activity.

Studies by Marver[15] on the inhibition by hemin of phenobarbital induction of microsomal proteins and enzymes in rats led him to suggest that not only was the synthesis of ALA-synthetase inhibited but also that hemin prevented the phenobarbital enhancement of the smooth endoplasmic reticulum. According to this view a number of other proteins in addition to ALA-synthetase may be induced by drugs and inhibited by hemin.

A tentative interpretation of the present findings is summarized in Fig. 3.

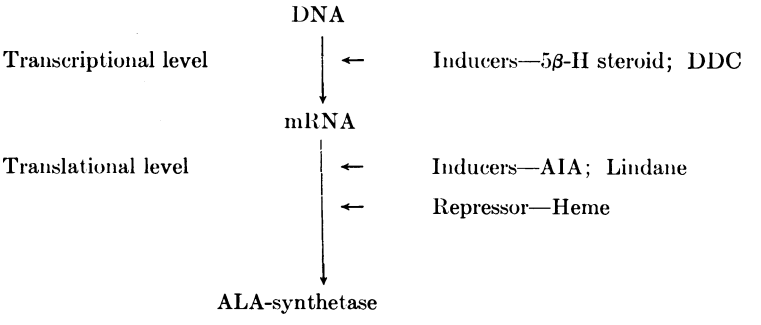

FIG. 3. Controls on ALA-synthetase at the transcriptional and translational levels.

Physiologically occurring 5β-H steroids such as etiocholanolone may act at the transcriptional level, possibly by derepressing an operon to permit the synthesis of the appropriate mRNA for ALA-synthetase. A chemical inducer such as

DDC may also act at this level. In contrast, inducing chemicals like AIA and Lindane appear to act primarily at the translational level. Hemin, at a concentration of 5 μM, appears to be an effective specific repressor at the translational level of the synthesis of ALA-synthetase, acting either by competing with an inducer for a specific site, or by acting at some other site.

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Abbreviations: AIA, allylisopropylacetamide; ALA, δ-aminolevulinic acid; DDC, 3,5-dicarbethoxy-1,4-dihydrocollidine; Lindane, γ-hexachlorocyclohexane.

[1] Granick, S., *J. Biol. Chem.*, **241**, 1359 (1966).
[2] Granick, S., and R. D. Levere, Controls of hemoglobin synthesis, in "International Society of Hematology, XIIth Congress" (1968), p. 274.
[3] Granick, S., and A. Kappas, *J. Biol. Chem.*, **242**, 4587 (1967).
[4] Kappas, A., and S. Granick, *J. Biol. Chem.*, **243**, 346 (1968).
[5] Levere, R. D., A. Kappas, and S. Granick, *Proc. Nat. Acad. Sci. USA*, **58**, 985 (1967).
[6] Gordon, S., E. D. Zanjani, R. D. Levere, and A. Kappas, *Proc. Nat. Acad. Sci. USA*, **65**, 919 (1970).
[7] Necheles, T. F., and U. S. Rai, *Blood*, **34**, 380 (1970).
[8] Summers, W. C., and R. B. Siegel, *Nature*, **223**, 111 (1969).
[9] Dube, S. K., and P. S. Rudland, *Nature*, **226**, 820 (1970).
[10] Granick, S., and S. Sassa, in *Metabolic Regulation*, ed. H. J. Vogel (New York: Academic Press, 1970), in press.
[11] Tschudy, D. P., H. S. Marver, and A. Collins, *Biochem. Biophys. Res. Commun.*, **21**, 480 (1965).
[12] Hayashi, N., B. Yoda, and G. Kikuchi, *Arch. Biochem. Biophys.*, **131**, 83 (1969).
[13] Ganshow, R., and R. T. Schimke, *J. Biol. Chem.*, **244**, 4649 (1969).
[14] Hayashi, N., B. Yoda, and G. Kikuchi, *J. Biochem. (Tokyo)*, **63**, 446 (1968).
[15] Marver, H. S., in *Microsomes and Drug Oxidations*, ed. J. R Gillette *et al.*, (New York: Academic Press, 1969), p. 495.