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Enhancement of RNA Synthesis in Avian Liver Cell Cultures by a 5β-Steroid Metabolite During Induction of δ-Aminolevulinate Synthase

(3α-hydroxy-5β-androstan-17-one and 2-allyl-2-isopropylacetamide action/porphyrin-heme pathway/RNA polymerases/regulation/protein synthesis)

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ABSTRACT **The porphyrin-heme pathway is controlled in the liver at the level of the mitochondrial enzyme δ-aminolevulinate synthase (EC 2.3.1.37), a protein inducible in cultured avian hepatocytes by a variety of chemicals including certain 5β-metabolites of steroid hormones. The great sensitivity of the induction process to inhibition by agents known to block transcriptional activity of genetic material suggests that some control mechanism may be operating at this level to regulate the formation of the enzyme. We report here enhancement of nuclear RNA synthesis and of Mn^{2+}–$(NH_4)_2SO_4$-stimulated DNA-dependent RNA polymerase (EC 2.7.7.6) activities by the 5β-steroid metabolite, 3α-hydroxy-5β-androstan-17-one (etiocholanolone), in cultured avian hepatocytes during induction of the enzyme. These changes were demonstrated in the G_1 phase of the hepatocyte cell cycle at a time when DNA synthesis is constant. Our findings support the view that one of the early steps in the process of induction of δ-aminolevulinate synthase by steroid metabolites requires new RNA synthesis, very probably messenger RNA, suggesting a 5β-steroid transcriptional control mechanism for induction of this protein.**

The rate-controlling step in porphyrin-heme biosynthesis is localized at the level of the mitochondrial enzyme δ-aminolevulinate synthase (ALA-synthase) [EC 2.3.1.37; succinyl-CoA:glycine *C*-succinyltransferase (decarboxylating)] (1–4). This protein is inducible by many chemicals *in vivo* in chick embryo (4, 5) and in avian hepatocytes grown in primary cultures (3, 6). These chemicals include 5β-steroid metabolites, such as 3α-hydroxy-5β-androstan-17-one (etiocholanolone), derived from the biotransformation of sex hormones in man (3, 7), a class of hormone derivatives previously thought to be inert. The great sensitivity of the induction process to various inhibitors, particularly those preventing RNA synthesis (1, 8), implies that some control mechanism is operating at the transcriptional level to regulate the formation of the enzyme. The importance of new mRNA formation prior to induction of this specific protein in the liver has also been suggested by our previous demonstration that α-amanitin, a potent inhibitor of the nucleoplasmic DNA-dependent RNA polymerase, blocks induction of ALA-synthase *in vivo* by the barbiturate analogue, 2-allyl-2-isopropylacetamide (ALA), and the steroid etiocholanolone (1).

Abbreviations: ALA-synthase, δ-aminolevulinate synthase; AIA, 2-allyl-2-isopropylacetamide; etiocholanolone, 3α-hydroxy-5β-androstan-17-one.

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We report here a study in primary cultures of chick embryo hepatocytes *in vitro*, showing enhancement of total and nuclear RNA synthesis and of nucleoplasmic DNA-dependent RNA polymerase (EC 2.7.7.6 nucleosidetriphosphate:RNA nucleotidyltransferase) activities by the 5β-steroid, etiocholanolone, during the induction period of ALA-synthase. This is a novel biological activity of 5β-steroid metabolites and indicates that these hormone degradation products can act in liver cells by molecular mechanisms analogous to those characterizing the action of primary sex hormones in classical genital target tissues. These results also support the view that one of the early 5β-steroid responsive steps controlling induction of ALA-synthase is operating at the transcriptional level in hepatic cells.

MATERIALS AND METHODS

Unlabeled ribonucleoside triphosphates were purchased from Sigma Biochemicals; [2-^{14}C]thymidine (50 mCi/mmol), [^{3}H]-uridine (5–15 Ci/mmol), [8-^{14}C]ATP (35 mCi/mmol), and Omnifluor were obtained from New England Nuclear Corp.; Soluene was from Packard Instrument.

Hepatic Cell Cultures. The technique has been described (6). Briefly, liver cells from 15-day-old chick embryos were grown in primary cultures either in small vials that contained a 16-mm coverslip and 1.0 ml of Eagle's basal medium supplemented with amino acids, glutamine, 5% fetal-bovine serum, and antibiotics; or in Falcon plastic petri dishes (100 and 150 mm diameter) containing, respectively, 20 and 40 ml of the same medium. Cells were incubated initially at 37° in the presence of 95% O_2–5% CO_2 for 16–24 hr to permit a cell monolayer to form. After change of medium, 5 μg of etiocholanolone or 30 μg of AIA were added dissolved in 5 μl of propylene glycol per ml of medium, α-amanitin was in 0.15 M NaCl, and incubation was continued for 24 hr.

To assess the magnitude of the induction response, porphyrin contents of cells and medium were quantitated in triplicate by a fluorometric procedure (9) utilizing a Perkin–Elmer Fluorescence Spectrophotometer (model MPF-3) equipped with a red-sensitive R-136 photomultiplier tube. If cells grown in large petri dishes were used for isolation of nuclei (10) and for determination of protein (11) or DNA (12), medium from two dishes was removed quantitatively with a pasteur pipette prior to harvesting of the cells and porphyrin content of an aliquot was determined.

Labeling Conditions of Hepatocytes in Cultures. To determine possible changes in RNA synthesis due to induction, cells were labeled with 5 μCi of [^{3}H]uridine per ml of medium for exactly 15 min in a CO_2 incubator at 37° prior to each time studied. At the end of the incubation period, medium was aspirated off quickly and cold 0.32 M sucrose–3 mM $MgCl_2$ was added. The cell monolayer was rinsed, scraped off the bottom of the petri dishes, and homogenized in a Potter–Elvehjem homogenizer. Homogenates from duplicate dishes were made up to 30 ml with the same sucrose solution, and 4 aliquots (0.5 ml each) were taken for determination of [^{3}H]-uridine incorporation; the remaining volume was used for nuclei isolation (10). Nucleic acids and proteins were precipitated with 0.5 ml of 10% trichloroacetic acid in the cold, and two of the aliquots were heated for 20 min at 90° to hydrolyze RNA; this permits a correction for [^{3}H]uridine not incorporated into RNA (13), less than 20% in all experiments. All aliquots were washed three times with 5% trichloroacetic acid by centrifugation and the final pellets, solubilized in 0.5 ml of Soluene, were transferred quantitatively with 10 ml of Omnifluor–toluene to a counting vial; radioactivity was determined in a Packard liquid scintillation Spectrometer.

For [^{14}C]thymidine incorporation into DNA by avian hepatocytes in cultures, cells were grown on coverslips in small vials (6) for the times indicated; medium was changed at 24, 48, and 72 hr. Cells were labeled 20 min in a CO_2 incubator at 37° prior to each time point indicated on the graph of Fig. 2. At the end of the labeling period, each coverslip containing the cell monolayer was washed twice in ice-cold Na phosphate–saline buffer and once in glacial acetic acid–95% ethanol (1:2), fixed (14), and solubilized in Soluene. Radioactivity was determined as described for [^{3}H]uridine.

Enzyme Assays. DNA-dependent RNA polymerase activities of nuclei, isolated from induced cultured hepatocytes, were measured in the presence of Mg^{2+} or Mn^{2+} ions and 0.3 M $(NH_4)_2SO_4$ (15) with the following modifications: 75 nmol each of GTP, CTP, and UTP and nmol of [8-^{14}C]ATP, and 0.1 ml of nuclear preparation containing 5–15 μg of DNA; the other components remained unchanged. α-Amanitin and other inhibitors were added *in vitro* to the RNA polymerase assay mixture at the beginning of the incubation. For the Mn^{2+}–$(NH_4)_2SO_4$ and Mg^{2+} assays, incubations were 45 and 10 min, respectively, at 36°. RNA polymerase activities are expressed as cpm of [8-^{14}C]AMP incorporated into RNA per μg of DNA. Radioactivity incorporated into newly synthesized RNA was determined as described (10).

RESULTS

Inducibility of ALA-Synthase During the Hepatocyte Cell Cycle in Cultures. Etiocholanolone, a 5β-steroid derived from the endogenous metabolic transformation of testosterone in man (16), induces ALA-synthase formation and a marked porphyrinogenesis in avian liver cell cultures (6, 17). An example of this induction response is shown in Fig. 1, and is compared with that produced by AIA. The toxin, α-amanitin, inhibits markedly both induction responses during the 24-hr incubation period.

Inducibility of ALA-synthase in cultured hepatocytes was observed only at certain times of the cell cycle. The greatest induction response was elicited when etiocholanolone or AIA was added to the cultures after an initial growth of 15–25 hr; little induction occurred after 46 hr, even with two prior changes of medium. An attempt was therefore made to study the characteristics of the generation cycle of chick-embryo liver cells in cultures. By use of [^{14}C]thymidine uptake, the rate of DNA synthesis was studied over a period of 72 hr, as shown in Fig. 2. Increases in DNA synthesis started at 6, 31, and 50 hr after the beginning of the cultures and lasted several hours prior to cell division; the latter was following by a post-mitotic resting phase during which time [^{14}C]thymidine uptake was constant. In addition, it was observed that 10 hr

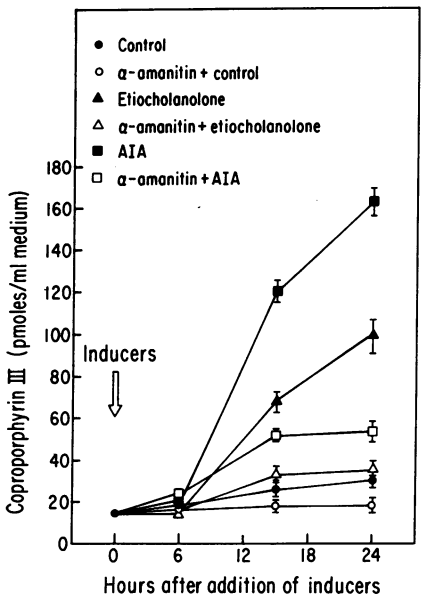

FIG. 1. Time course for porphyrin biosynthesis in cultured avian hepatocytes after induction of ALA-synthase by etiocholanolone and AIA. Liver cells were grown in small vials as described in *Methods*. The culture medium was changed, and α-amanitin (1.0 μg/ml of medium) was added 1 hr prior to inducing agents. The cells were then incubated for the times indicated. Coproporphyrin contents were determined as described in *Methods*. Each point represents the mean and standard deviation of 6 to 12 replicate determinations.

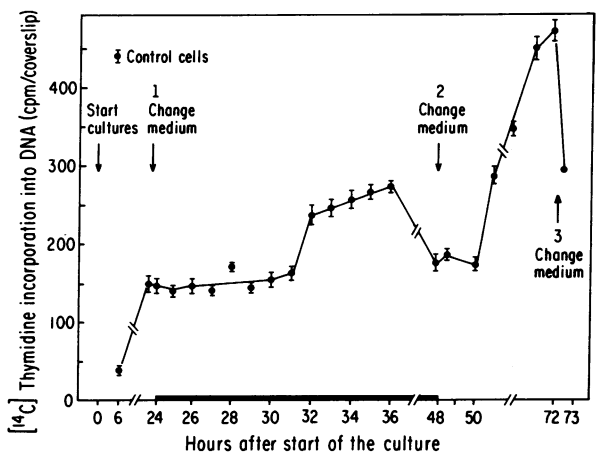

FIG. 2. Incorporation of [^{14}C]thymidine into DNA by cultured avian hepatocytes. Cells from control chick-embryo livers were grown on coverslips in small vials for the times indicated and labeled for 20 min as described in *Methods*. Each point represents the mean and standard deviation of five replicate determinations.

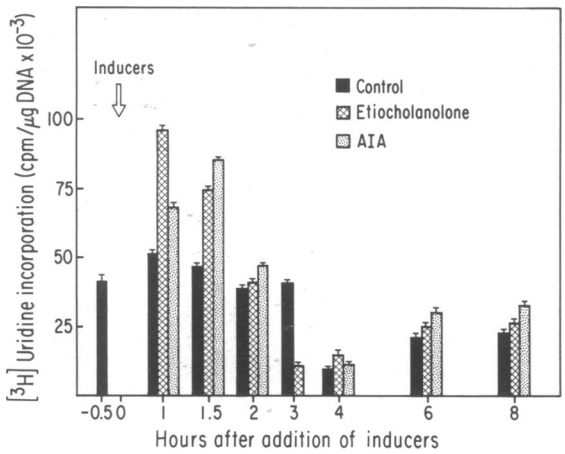

FIG. 3. Effect of a 5β-steroid and a drug inducing agent on RNA synthesis in avian hepatocyte cultures. Cells from chick-embryo livers were grown as described in *Methods*. The medium was changed and the inducing agents, etiocholanolone and AIA, were added. The cells were then incubated for the times indicated. Cells were labeled for 15 min with [^{3}H]uridine; when labeling was terminated, the dishes were quickly chilled over crushed ice and the medium was removed. [^{3}H]Uridine incorporation into total cellular RNA was determined as described in *Methods*. Values for cpm have been multiplied by 10^{-3}.

after induction was initiated, the 5β-steroid-treated cultures consistently incorporated approximately 30% more DNA precursor than controls, whereas AIA-treated cultures increased [^{14}C]thymidine incorporation only 10–15% over controls.

Enhancement of RNA Synthesis by a 5β-Steroid. The stimulatory effect of etiocholanolone on ALA-synthase activity was accompanied by an increase in RNA synthesis (2), a novel observation for 5β-steroid metabolites. A typical time course experiment is shown in Fig. 3, where the rate of incorporation of [^{3}H]uridine into total cellular RNA was measured after a 15-min pulse of [^{3}H]uridine at the times indicated. As can be seen, enhancement of RNA synthesis is most pronounced in the steroid-treated cultures, reaching peak stimulation 1 hr after addition of the steroid, whereas in AIA-treated cells this was observed at 1$^1/_2$ hr, reaching 89% and 81% over control values, respectively, at these times. Peak enhancement for [^{3}H]uridine incorporation was always observed at the beginning of the induction period, and increase in incorporation could be detected as early as 15 min after addition of the steroid. After 3 hr of induction, [^{3}H]uridine incorporation had fallen below control levels in the steroid-treated cells but rose again at 4, 6, 8, and even 24 hr. Similar results with the steroid and AIA were obtained originally when cells were grown on coverslips in small vials, under the same conditions as here in petri dishes.

In an initial attempt to determine if any changes could be detected in the rapidly labeled RNA present in the cytoplasm of induced hepatocytes, cytoplasmic fractions were isolated from cell homogenates after the hepatocytes had been exposed to a 10-min pulse of [^{3}H]uridine. Significant increases in specific activity of 31% and 53% over control values were observed in the steroid and AIA-treated cells, respectively, within 2 hr after induction. These findings correspond to the changes reported in total cellular RNA shown in Fig. 3; however, the amounts of isotope detected in the cytoplasmic fractions were quite small when compared to the total labeled RNA in the cells and difficult to analyze further.

TABLE 1. *Requirements for DNA-dependent RNA polymerase activity in nuclei isolated from cultured avian hepatocytes*

Reaction mixture	Mn^{2+}–$(NH_4)_2SO_4$ assay		Mg^{2+} assay	
	cpm/μg of DNA	[^{14}C]-AMP incorporated (%)	cpm/μg of DNA	[^{14}C]-AMP incorporated (%)
Complete incubation medium	32.0	100	21.0	100
− CTP, UTP, GTP	6.9	22	2.3	11
− CTP	3.4	11	2.4	12
− UTP	1.3	4	3.9	18
− GTP	8.0	25	12.0	57
+ 50 μg of DNase	6.1	19	1.9	0.09
+ 200 μg of RNase	0.23	0.7	3.6	17
+ 5 μg of actinomycin D	2.1	6.6	13.0	41
+ 0.2 μg of α-amanitin	4.4	14	21.0	100

Data represent mean of duplicate determinations.

Effect of Etiocholanolone on DNA-dependent RNA Polymerase Activities. Since [^{3}H]uridine was found to be predominantly incorporated into nuclear RNA in other experiments and as shown in Table 3, it was of interest to determine if any stimulatory effect could be detected in the activities of the nuclear DNA-dependent RNA polymerase of the induced cells in culture. Because RNA polymerase activities have not been studied before in chick-embryo hepatocytes grown in primary cultures, probably because of the restriction imposed by the small numbers of cells available in this culture system, it was necessary to ascertain first the basic properties of these enzymes in controls prior to our studies in the steroid or AIA-induced cells. It was found that requirements for Mg^{2+}, Mn^{2+}, and $(NH_4)_2SO_4$ resemble those of RNA polymerases of other species, including whole avian liver (0, 18–20), showing a marked stimulation by Mn^{2+} in the presence of $(NH_4)_2SO_4$ at 2 mM and exceeding markedly that found for Mg^{2+}. Four ribonucleoside triphosphates and a DNA template were necessary for maximum activity and both types of reactions were inhibited by DNase, RNase, and actinomycin D, but only the Mn^{2+}–$(NH_4)_2SO_4$-stimulated enzyme was inhibited by α-amanitin (Table 1). This marked inhibition *in vitro* of the Mn^{2+}–$(NH_4)_2SO_4$-stimulated enzyme by α-amanitin and resistance to this toxin by the Mg^{2+} enzyme showed characteristics already observed in other systems (21) but not previously reported for avian liver. In addition, synthesis of RNA by both enzymes was found to be linear between 2 and 15 μg of DNA, concentrations used in our assay system.

To correlate with the enhancement of RNA synthesis observed with [^{3}H]uridine labeling, we studied the nuclear activities of DNA-dependent RNA polymerases in nuclei of 5β-steroid-treated cultured hepatocytes and compared it to AIA-treated and control hepatocytes to determine which enzymes were stimulated by the inducers. Table 2 shows that the Mn^{2+}–$(NH_4)_2SO_4$-stimulated enzyme of etiocholanolone

TABLE 2. *Specific activities of DNA-dependent RNA polymerase in nuclei of steroid- and AIA-treated cultured avian hepatocytes*

Additions*	Induction time (hr)	[8-^{14}C]AMP incorporated into nuclear RNA: Mn^{2+}–$(NH_4)_2SO_4$ assay: cpm/μg of DNA	Mn^{2+}–$(NH_4)_2SO_4$ assay: % of control	Mg^{2+} assay: cpm/μg of DNA	Mg^{2+} assay: % of control
1. None	3	15.2 ± 0.7	100	22.3 ± 1.4	100
Etiocholanolone	3	19.6 ± 1.4	130	19.7 ± 1.4	88
AIA	3	20.6 ± 0.9	136	24.4 ± 1.6	110
2. None	8	38.7 ± 2.3	100	14.5 ± 1.0	100
Etiocholanolone	8	55.3 ± 4.8	142	13.8 ± 1.1	95
AIA	8	48.1 ± 1.6	124	13.5 ± 2.4	93
3. None	21	36.0 ± 0.5	100	18.1 ± 1.8	100
Etiocholanolone	21	47.4 ± 1.2	132	24.6 ± 1.3	136
AIA	21	44.2 ± 4.4	123	22.2 ± 1.0	123

Data represent mean and standard deviation of duplicate determinations.

* Etiocholanolone (5 μg) and AIA (30 μg) were added per ml of medium and the cells were incubated for the times indicated.

and AIA-treated cells was stimulated at 3, 8, and 21 hr after induction; the stimulation was statistically significant ($P \leq 0.05$) in the nuclei of steroid-treated cultures whereas activities of the Mg^{2+}-stimulated polymerase were significantly increased only at 21 hr. Similar results were obtained 24 hr after induction for both enzyme activities as were observed at 21 hr. The effect of α-amanitin on nuclear RNA synthesis is shown in Table 3; such synthesis was markedly inhibited, and only the Mn^{2+}–$(NH_4)_2SO_4$-stimulated RNA polymerase was inhibited *in vivo* by the toxin. Actinomycin D (0.1 μg/ml) was also found to inhibit the early increase in RNA synthesis evoked by both inducers.

DISCUSSION

The marked porphyrinogenesis observed in avian liver-cell cultures after induction of ALA-synthase is an effect produced, among endogenous substances, only by steroid metabolites of the 5β-type, but common to many exogenous chemicals such as drugs and other foreign agents (3, 6). The possibility that a similar mechanism is involved in the induction process elicited by all of these structurally diverse substances is of considerable interest, because ALA-synthase controls the rate-limiting step of the heme pathway in the liver.

A search was made for selective accumulation of DNA, RNA, or protein in cultured liver cells treated with prototype chemicals of the steroid and barbiturate categories of inducers during the 24-hr induction period, and our findings indicated that neither produced marked changes in these parameters. These findings contrast with the effects produced by primary steroid hormones in other test systems, such as genital and other tissues, in which pronounced changes in DNA and RNA content (22) and protein and RNA synthesis occur (23). They also contrast with those in whole animals, where the production of porphyria in rats with AIA is accompanied by substantial hypertrophy of the liver (24, 25). In our cell culture, the 5β-steroid, etiocholanolone, enhanced DNA synthesis only slightly, but did not affect the total protein content of the cells during this time. It is possible that more pronounced steroid-induced changes could be observed in the cytoplasm of treated liver cells if porphyrins, which accumulate in the cultures, did not significantly inhibit protein synthesis (26). ALA-synthase is the only protein known to be significantly induced during the 24-hr induction period, and the increase in this enzyme activity undoubtedly represents only a small fraction of the total protein content in the cytoplasm of induced hepatocytes.

When DNA synthesis was studied in cultures during a continuous 72-hr period, it was found that peak induction for ALA-synthase occurred only during the first 16–26 hr after the start of the cultures, during a resting phase for the cells, when the rate of DNA synthesis was constant. The enzyme is therefore most inducible when avian hepatocytes are in the G_1 phase of their cell cycle, an observation similar to that made for induction of tyrosine aminotransferase by dexamethasone in HTC cells, a mammalian hepatoma cultured cell line (27).

To ascertain whether there is a transcriptional control for induction of ALA-synthase by the 5β-steroid, etiocholanolone, we studied the possibility of selective stimulation of RNA synthesis during the induction period, and compared it to AIA-treated and control cells using short pulses of [^{3}H]uridine to exclude ribosomal RNA labeling in the cytoplasm (28), thus preferentially labeling heterogeneous RNA in the nucleus. A

TABLE ?. *Effect of α-amanitin on nuclear RNA synthesis in steroid- and AIA-treated cultured avian hepatocytes*

Additions*	[^{3}H]Uridine incorporation into nuclear RNA: cpm/μg of DNA	Incorporation (%)	Inhibition (%)	DNA-dependent RNA polymerase activities of nuclei: Mn^{2+}–$(NH_4)_2SO_4$ assay: cpm/μg of DNA	Mn^{2+}–$(NH_4)_2SO_4$ assay: Inhibition (%)	Mg^{2+} assay: cpm/μg of DNA	Mg^{2+} assay: Inhibition (%)
None	661	100	—	19.0	—	16.9	—
α-Amanitin	203	31	69	4.5	76	20.4	None
Etiocholanolone	836	100	—	29.4	—	15.3	—
α-Amanitin + etiocholanolone	246	29	71	5.3	82	16.0	None
AIA	743	100	—	20.6	—	14.8	—
α-Amanitin + AIA	179	24	76	5.1	75	22.8	None

* α-Amanitin (0.5 μg) was added after the culture medium was changed and 1 hr prior to addition of etiocholanoline (5 μg) and AIA (30 μg), all additions being per ml of medium. After addition of inducers, cells were incubated 6.5 hr and nuclei were isolated as described in *Methods*. All determinations were done in duplicate.

major increase was observed in steroid-induced hepatocytes immediately after addition of inducers. The small stimulation present in control cultures at these times is due to the change of medium, since no stimulation was obtained in the control cultures of cells induced 4 hr after the change of medium. The sequential patterns of [^{3}H]uridine incorporation by hepatocytes in culture depicted in Fig. 3 were observed in eight other experiments, some of them when cells were grown in small vials instead of petri dishes; the times for peat stimulation were slightly different but always between 1 and 6 hr, and the overall patterns during the entire induction period were similar. Therefore, the early stimulation in RNA synthesis observed in the nucleus and cytoplasm of induced cells prior to ALA-synthase induction is probably due to new mRNA formation triggered by the action of the inducers, since this effect is inhibited by α-amanitin. From these studies it is not possible to know if this new mRNA codes for ALA-synthase itself or for some other protein factor required for induction of the enzyme. The pattern of RNA synthesis observed later in the induction period points toward events in DNA and RNA synthesis known to occur prior to mitosis (29), the induced cells being stimulated more than controls at these times.

To correlate with our findings regarding RNA synthesis in induced cultured cells, the effects of the inducers on the DNA-dependent RNA polymerase activities in liver cells were also studied. These experiments showed that the activity of the nuclear Mn^{2+}–$(NH_4)_2SO_4$-stimulated enzyme was enhanced more markedly after treatment of the cultures with the 5β-steroid than with the barbiturate, whereas the Mg^{2+} enzyme was not stimulated except by the steroid at 21 hr. In addition, with both inducers, only the Mn^{2+}–$(NH_4)_2SO_4$-stimulated enzyme was inhibited by α-amanitin when the cells were treated in cultures with this toxin prior to addition of inducers. The Mg^{2+}-stimulated enzyme, responsible for ribosomal RNA synthesis in the nucleolus, was not inhibited by α-amanitin under similar conditions.

These observations support the idea that mRNA synthesis is a primary requirement in the mechanism of induction of ALA-synthase by these inducers and that new nuclear RNA synthesis is clearly required for the induction effect to proceed. The fact that 5β-steroid metabolites, a class of hormone degradation products not previously shown to stimulate nucleic acid synthesis, have such an action is of special interest and broadens the variety of chemical moieties derived from endocrine secretions that can potentially regulate cell function.

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