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DIFFERENTIAL SENSITIVITY OF RAT LIVER AND RAT HEPATOMA CELLS TO α-AMANITIN*

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Abstract—α-Amanitin, an inhibitor of RNA polymerase derived from the mushroom *Amanita phalloides*, was shown to be about five times more potent than actinomycin-D in inhibiting rat liver tyrosine aminotransferase induction by cortisol. Rat hepatoma cells grown in culture, however, were resistant to these inhibitory effects of α-amanitin, while actinomycin-D effectively inhibited enzyme induction in these cells. The insensitivity of the hepatoma cells could not be accounted for by: (a) specific binding of α-amanitin to fetal calf serum protein present in the tissue culture medium; (b) metabolic conversion of this bicyclic octapeptide to an active compound in rat liver which does not occur in hepatoma cells; or (c) rapid inactivation of this substance by the hepatoma cells. RNA polymerase studies with isolated nuclei indicated that inhibition of cortisol induction of rat liver tyrosine aminotransferase by α-amanitin was accompanied by an inhibition of $Mn^{2+}/(NH_4)_2SO_4$-activated RNA polymerase. No effect on the Mg^{2+}-activated polymerase was noted. Similar experiments with hepatoma cells, in conjunction with [^{14}C]-uracil and [$^{3}H_5$]-orotic acid uptake studies, indicated that the RNA polymerase of these cells was sensitive to α-amanitin only when the cell membrane was broken. The two major conclusions from these data are: (1) hepatoma cells are insensitive to α-amanitin because they are impermeable to this substance, and (2) the correlation between inhibition of the $Mn^{2+}/(NH_4)_2SO_4$-activated RNA polymerase and inhibition of tyrosine aminotransferase induction by cortisol strongly suggests that modification of transcription by this hormone is the primary stimulus for increased synthesis of this enzyme.

THE TRANSCRIPTION of DNA in eukaryotic cells appears to result from multiple forms of RNA polymerase.[1,2] Present evidence indicates that two such enzymes are located in the nucleolus (referred to as RNA polymerase IA and IB) and are responsible for ribosomal RNA synthesis; and at least one is located in the nucleoplasm (referred to as RNA polymerase II), and is responsible for chromatin transcription.[1–6] In 1966, Widnell and Tata[7] discovered that RNA polymerase can be preferentially activated by Mn^{2+} in the presence of ammonium sulfate, and by Mg^{2+} in the absence of this salt. Nearest neighbor analysis of reaction products indicated that the base composition of RNA synthesized by the Mg^{2+}-activated enzyme was similar to ribosomal RNA, and that of the $Mn^{2+}/(NH_4)_2SO_4$-activated enzyme similar to messenger RNA. Such a definitive delineation of enzyme function is an oversimplification since it has been shown that both the nuclear and nucleolar polymerases are activated by Mn^{2+} in the presence of ammonium sulfate.[6] However, the nuclear enzyme demonstrates a significantly greater degree of activation than the nucleolar enzyme. On this basis, determination of Mg^{2+} and $Mn^{2+}/(NH_4)_2SO_4$-activated RNA polymerase activity can be

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assumed to approximate transcription of ribosomal and messenger RNA respectively.

The $Mn^{2+}/(NH_4)_2SO_4$-activated polymerase is inhibited by α-amanitin,[4,8,9] a bicyclic octapeptide isolated from the poisonous mushroom *Amanita phalloides*.[10–12] The observations that α-amanitin has a more selective inhibitory effect on the $Mn^{2+}/(NH_4)_2SO_4$-activated polymerase, and that varying DNA concentration with a solubilized rat liver RNA polymerase preparation does not alter the inhibitory effects of α-amanitin, suggest that this substance acts directly on the enzyme and not on the template as does actinomycin-D.[13] Based on this rather specific action on RNA polymerase, we investigated the effects of α-amanitin on induction of tyrosine aminotransferase by cortisol.

As was reported by Sekeris *et al.*,[14] α-amanitin was found to inhibit cortisol induction of rat liver tyrosine aminotransferase. The present data indicate that it is about five times more potent than actinomycin-D. However, when rat hepatoma cells grown in culture were exposed to these inhibitors, α-amanitin was without effect, while actinomycin retained its capacity to inhibit tyrosine aminotransferase induction. An analysis of this differential sensitivity to α-amanitin indicated that, despite a lower molecular weight than actinomycin-D, the hepatoma cell membrane is relatively impermeable to α-amanitin.

MATERIALS AND METHODS

Animals. Adrenalectomized male rats of the Sprague–Dawley strain weighing about 200 g were obtained from Charles River Laboratories (Wilmington, Mass.). Animals were maintained for at least 2 days after arrival on normal rat pellet diets supplemented with drinking water containing 0·9% NaCl. For liver analysis, each rat was anesthetized with pentobarbital (30 mg/kg, intraperitoneally), and the upper abdominal cavity exposed by a 1-in. midline incision. Biopsies were taken by cutting wedge-shaped samples from the edges of the liver lobes as indicated previously.[15] The biopsy sample was weighed, homogenized in 1 ml of cold (4°) 0·1 M sodium phosphate buffer (pH 7·5) with glass tissue homogenizers, and then centrifuged at about 18,000 *g* at 4° for 20 min. The supernatant was assayed for tyrosine aminotransferase[16] and protein.[17]

Isolation of liver nuclei. Animals were sacrificed by cervical dislocation. The livers were perfused with cold (4°) 0·32 M sucrose containing 0·003 M $MgCl_2$ (SM solution), removed, and approximately 6 g was minced with scissors and homogenized in 23 ml of the same solution using an electrically operated Teflon–glass tissue homogenizer (about 10–15 strokes). The volume of the homogenate was brought to 36 ml with SM solution and then to about 48 ml with distilled water. After dividing the homogenate, which was now approximately 0·2 M with respect to sucrose, about 15 ml of SM solution was layered carefully beneath each half and then centrifuged for 10 min at 700 *g*. The residues, which contained the nuclei, were suspended in 2·4 M sucrose containing 0·001 M $MgCl_2$ and centrifuged for 90 min at 39,000 rev/min in a SW 41 head (Beckman Instruments, Inc.). As indicated by Widnell and Tata,[18] the nuclei sediment to the bottom while whole cells and cellular particles float to the top forming a solid plug. The latter was readily removed with a spatula and the underlying fluid carefully decanted. Remaining droplets were removed by inserting the edge of an absorbant tissue into the tube with forceps. Distilled water was carefully run down the wall of the tube and then discarded by gentle decantation. After such a wash the tube containing the nuclei was placed in an ice bath until ready for use (about 10–20 min

later). At this time they were suspended in appropriate reaction mixtures and assayed for Mg^{2+} and Mn^{2+} activated RNA polymerase by the methods of Widnell and Tata.[7]

Hepatoma cells. Detailed procedures for culturing and collecting hepatoma cells (Reuber H-35) for analysis have been described previously.[19,20] Additional details of the experiments presented are indicated in legends of the appropriate figures and tables.

RNA polymerase assays of hepatoma cell nuclei were carried out by combining the cells of 10–12 similarly treated cultures. At the time of harvest each culture bottle contained about 25 mg cells wet wt. After washing the hepatoma cells three times with 0.1 M sodium phosphate buffer (pH 7·5) and once with SM solution, they were suspended in 2 ml of the latter solution and sonicated for 5 sec with a Bronson sonifier. The broken cells were centrifuged for 5 min at about 4000 *g*. The residue, which was considered the hepatoma cell nuclear fraction, was suspended in appropraite reaction mixtures and assayed for Mg^{2+} and $Mn^{2+}/(NH_4)_2SO_4$-activated RNA polymerase as above. Incorporation of [U-^{14}C]-UTP Na_4 was proportional to DNA content within the range employed (60–130 μg of DNA in 0·5 ml incubation mixture).

Drugs. Cortisol (hydrocortisone sodium succinate, Solu-Cortef) was obtained from the Upjohn Company, actinomycin-D from the Merck Institute, and α-amanitin was generously supplied by Prof. T. Wieland of the Max Planck Institute. Radioactive uridine triphosphate [U-^{14}C]-UTP Na_4, [$^{14}C_2$]-uracil and cortisol ([$^{14}C_4$]-hydrocortisone) were obtained from New England Nuclear Corp. (Boston, Mass.) and [$^{3}H_5$]-orotic acid from Nuclear Chicago (Chicago, Ill.).

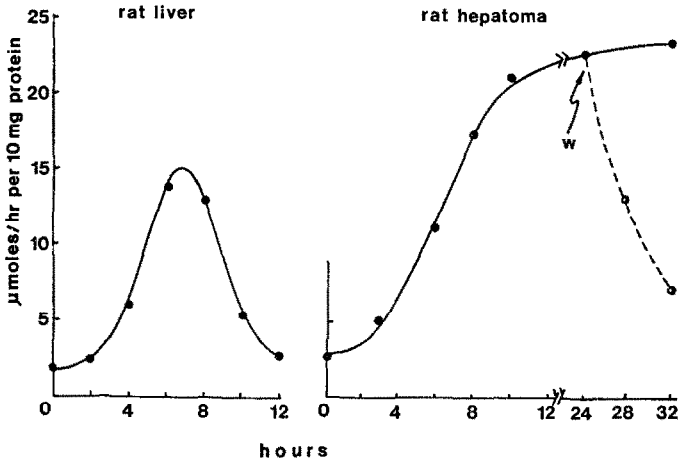

FIG. 1. Changes in tyrosine aminotransferase activity in rat liver and rat hepatoma cells after cortisol administration. Rat liver: after anesthetizing a group of animals with pentobarbitol (30 mg/kg, intraperitoneally), liver biopsy samples were taken to determine basal tyrosine aminotransferase activity. Subsequent biopsy samples were taken at 2-hr intervals after the intraperitoneal administration of 10 mg of cortisol (hydrocortisone sodium succinate). Each point represents the average enzyme activity of several animals. Rat hepatoma: a series of cultures were grown to a density of approximately 3×10^7 cells. Three cultures were harvested after adding saline and the others collected at various intervals after the addition of cortisol (final concentration, 10^{-6} M). The elevated level of enzyme activity is maintained for an extended time period due to little, if any, hormone metabolism by these cells. When the hormone is washed out (W) enzyme activity rapidly returns to basal levels. Each point represents the average enzyme activity of at least two cultures. Tyrosine aminotransferase activity is expressed as micromoles of *p*-hydroxyphenylpyruvate formed per hour per 10 mg of protein.

RESULTS

The induction of tyrosine aminotransferase by cortisol in both rat liver and rat hepatoma cells (Reuber H-35) is shown in Fig. 1. When enzyme activity is expressed on a protein basis tyrosine aminotransferase (TA) activity in hepatoma cells reached levels about 50–100 per cent higher than the peak activity observed in liver. This difference is due almost entirely to the rapid metabolism of cortisol in rat liver. Continuous cortisol administration (hourly intraperitoneal injections) produces a similar type of induction pattern in rat liver as that shown for the hepatoma cells.[15] Previous experiments have shown that neither diurnal rhythm nor the biopsy technique significantly affect rat TA activity under the conditions employed.[15]

After the administration of 300 μg pF actinomycin-D (mol. wt 1254), induction of tyrosine aminotransferase was completely inhibited (Fig. 2). An intraperitoneal injection of 50 μg produced no observable effect, while lowering the dose of actinomycin-D to 10 μg markedly potentiated cortisol induction. This low dose of actinomycin-D had no effect on tyrosine aminotransferase activity when tested in the absence of cortisol. As shown in Fig. 2, complete inhibition of tyrosine aminotransferase induction occurred with 50 μg of α-amanitin (mol. wt 916). Taking into account the difference in molecular weight, α-amanitin was about five-times more potent than actinomycin-D.

In hepatoma cells, 50 per cent inhibition of tyrosine aminotransferase induction was attained with about 0·05 μg/ml of actinomycin-D and complete inhibition of enzyme induction occurred at a concentration of 6 μg/ml. No effect was observed with

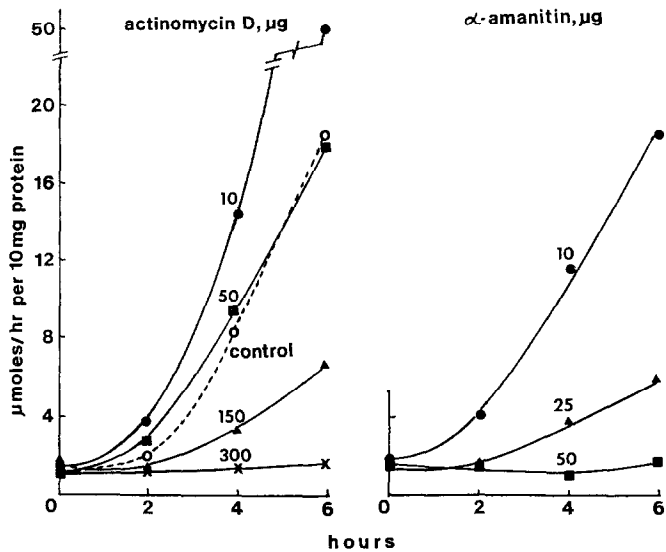

FIG. 2. Inhibition of tyrosine aminotransferase induction in rat liver by actinomycin-D and α-amanitin. Animals were treated initially as indicated in the legend of Fig. 1. After taking control biopsy samples, actinomycin-D or α-amanitin was administered intraperitoneally in the doses indicated. Thirty min later 10 mg of cortisol (hydrocortisone sodium succinate) was injected in the same manner. Additional liver biopsies were taken at the intervals indicated. Each point represents the average of at least two animals. Enzyme activity is expressed as micromoles of *p*-hydroxyphenylpyruvate per hour per 10 mg of protein.

as much as 10 μg/ml of α-amanitin (Fig. 3). Our first impression of this complete lack of sensitivity to α-amanitin (and the simplest possibility for us to test experimentally) was that fetal calf serum protein in the tissue culture medium preferentially bound α-amanitin. The data in Table 1 indicate that even in the absence of fetal calf serum 5 μg/ml of α-amanitin produced little effect compared to the inhibition observed with 0·125 μg/ml of actinomycin-D. The data in Table 1 also indicate that lowering the

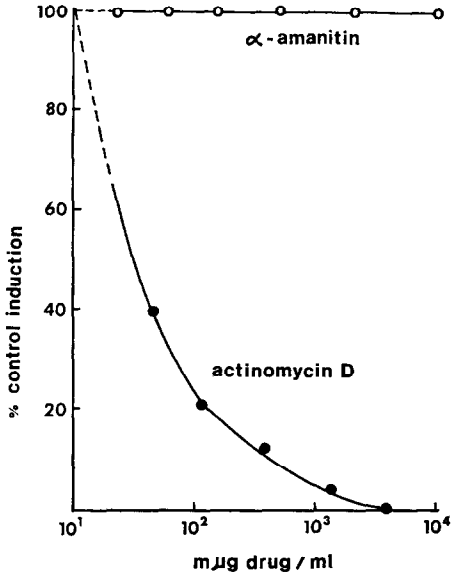

FIG. 3. Effects of actinomycin-D and α-amanitin on tyrosine aminotransferase induction in rat hepatoma cells. A series of hepatoma culture bottles containing approximately 3×10^7 cells each were exposed to various concentrations of actinomycin-D and α-amanitin. Thirty min later cortisol (10^{-6} M) was added and incubation continued for an additional 7 hr. After harvesting the cells and analyzing them for TA activity, the effects of actinomycin-D and α-amanitin were expressed in terms of hormone induction in the absence of these substances. Each point represents the average enzyme activity of duplicate cultures.

TABLE 1. EFFECTS OF ACTINOMYCIN-D AND α-AMANITIN ON TYROSINE AMINOTRANSFERASE INDUCTION IN RAT HEPATOMA CELLS IN THE PRESENCE AND ABSENCE OF FETAL CALF SERUM IN THE INCUBATION MEDIUM*

Additions	Tyrosine aminotransferase activity	
	With fetal calf serum	Without fetal calf serum
None	0·6	1·2
Cortisol, 10^{-6} M	2·5	3·5
α-Amanitin, 5 μg/ml plus cortisol, 10^{-6} M	2·5	3·2
Actinomycin-D, 0·125 μg/ml plus cortisol, 10^{-6} M	0·9	1·5

* A series of hepatoma cultures were grown to a cell density of about 3×10^7 cells. Half the cultures were washed with Dulbecco Modified Eagle Medium containing fetal calf serum and the other half with the same medium lacking fetal calf serum. Actinomycin-D and α-amanitin were added to some cultures in each group. Thirty min later cortisol (10^{-6} M) was added, and the cultures were incubated for an additional 7 hr. All cultures were then harvested and analyzed for tyrosine aminotransferase activity (micromoles of *p*-hydroxyphenylpyruvate formed per hour per 100 μg of DNA). Each value is the average of duplicate cultures.

protein content of the incubation medium stimulated tyrosine aminotransferase activity. Although this effect is of itself quite interesting, a discussion of this observation is beyond the scope of the present report. Despite the elevation of enzyme activity in a protein-free medium, it is clear that lack of inhibition with α-amanitin was not due to protein binding.

A possible mechanism by which hepatic cells would be unresponsive to cortisol in the presence of α-amanitin is by inhibition of hormone uptake. Using [$^{14}C_4$]-hydrocortisone, no difference in hormone uptake was noted between control and α-amanitin-treated animals. An alternative explanation for α-amanitin inhibition of tyrosine aminotransferase induction in rat liver and not in hepatoma cells is that the drug is metabolized in the liver to an active form, while no such transformation can occur in hepatoma cells. To test this possibility, α-amanitin (0·2 ml, 500 μg/ml) and actinomycin-D (0·2 ml, 500 μg/ml) were incubated separately in 0·8 ml of a 30 per cent liver homogenate for 1 hr at 37°. It was observed that after such treatment α-amanitin was still capable of inhibiting induction of tyrosine aminotransferase in rat liver but not in hepatoma cells, while actinomycin-D retained its inhibitory effect on enzyme induction in both systems. To demonstrate that α-amanitin in the liver homogenate was still active, 0·5 ml was injected into one rat and 0·5 ml of the saline homogenate into another. Thirty min later 10 mg of cortisol was administered i.p. to both animals. TA induction was inhibited in the former but not in the latter. To determine whether hepatoma cells rapidly metabolize α-amanitin, a concentrated hepatoma cell homogenate (cells of 10 cultures) was incubated for 1 hr at 37° with 100 μg of α-amanitin in a total volume of 1 ml. A 0·5-ml aliquot of the α-amanitin homogenate was still capable of inhibiting cortisol induction of tyrosine aminotransferase in rat liver. Despite the fact that such experiments do not demonstrate whether or not intact liver or hepatoma cells metabolize α-amanitin, the above data suggest that alternative explanations for hepatoma insensitivity to this drug are more likely. For example, RNA polymerase in hepatoma cells may have mutated so that it is no longer sensitive to α-amanitin, or the hepatoma cell membrane differs from that of the hepatocyte in that it is impermeable to this substance. Accordingly, RNA polymerase activity in nuclei isolated from rat liver and rat hepatoma cells was tested for their sensitivity to α-amanitin and actinomycin-D both *in vivo* and *in vitro*. Mg^{2+}- and $Mn^{2+}/(NH_4)_2SO_4$-activated RNA polymerase activities were determined in all experiments to approximate the ribosomal and messenger RNA synthesizing capacitities of these nuclei.[6,7,21]

The data in Table 2 indicate 610 and 2170 μμmoles of UTP were incorporated/mg of DNA in control rat liver nuclei for the Mg^{2+}- and $Mn^{2+}/(NH_4)_2SO_4$-activated enzymes respectively. (These values are in good agreement with those reported for rat liver nuclei by Jacob *et al.*[21] who observed 1180 and 2865 μμmoles of UTP incorporated/mg of DNA.) Radioactive UTP incorporation was markedly reduced when incubation was carried out at 0°, or when non-radioactive nucleotides (ATP, CTP and GTP) were omitted from reaction mixtures incubated at 37°. These control experiments indicate that most of the ^{14}C-UTP incorporation resulted from an enzyme reaction and not from nonspecific binding or trapping of the radioactive precursor in the perchloric acid precipitate, and also that the likely product of the reaction was RNA. Addition of actinomycin-D (500 ng g/ml) inhibited the Mg^{2+}-and $Mn^{2+}/(NH_4)_2SO_4$-activated RNA polymerase about 60 and 50 per cent respectively. Incubation with α-amanitin (50 ng g/ml) caused only a 13 per cent decrease in the Mg^{2+}-activated

TABLE 2. INHIBITION OF Mg^{2+}- AND $Mn^{2+}/(NH_4)_2SO_4$-ACTIVATED RNA POLYMERASE IN RAT LIVER NUCLEI AFTER TREATMENT WITH α-AMANITIN AND ACTINOMYCIN-D *in vitro* AND *in vivo**

Treatment (*in vivo*)	Treatment (*in vitro*)	Temp. (°C)	Mg^{2+} ($\mu\mu$moles UTP incorporated/mg DNA)	$Mn^{2+}/(NH_4)_2SO_4$ ($\mu\mu$moles UTP incorporated/mg DNA)	Relative TA activity
		37	610	2170	1·0
		0	40	223	
	Minus cold nucleotides	37	12	43	
	Actinomycin-D (500 ng g/ml)	37	231	1069	
	α-Amanitin (50 ng g/ml)	37	531	826	
Cortisol, 10 mg		37	634	1630	4·2
Cortisol, 10 mg		0	41	150	
Actinomycin-D, 300 μg + cortisol, 10 mg		37	153	514	1·1
Actinomycin-D, 300 μg + cortisol, 10 mg		0	23	62	
α-Amanitin, 50 μg + cortisol 10 mg		37	735	694	0·9
α-Amanitin, 50 μg + cortisol, 10 mg		0	79	88	
α-Amanitin, 50 μg + cortisol, 10 mg	α-Amanitin (50 ng g/ml)	37	607	316	

* Control rats were injected with saline. Rats treated with hormone were injected intraperitoneally with 10 mg of cortisol and sacrificed 3 hr later. Those receiving actinomycin-D (300 μg) or α-amanitin (50 μg) were injected intraperitoneally with these substances 30 min prior to the administration of cortisol (as above). These animals were then sacrificed 3 hr later. Nuclei were isolated from the liver by methods outlined by Widnell and Tata[18] and RNA polymerase activity determined essentially as described by these investigators.[7] In the present experiments incorporation of radioactive uridine triphosphate was measured ([U-^{14}C]-UTP Na_4, sp. act. 16·6 μCi/μmole, 2·5 μCi/ml). Each incubation mixture contained 0·25 μCi in a total volume of 0·5 ml. Assays for Mg^{2+}- and $Mn^{2+}/(NH_4)_2SO_4$-activated RNA polymerase were carried out for 20 and 45 min respectively. Initially all tubes were kept at 0° for 5 min to allow for adequate mixing of the incubation components. After this period (zero time) some tubes were placed in a Dubnoff incubator at 37° and others kept at 0°. Reactions were terminated by the addition of 2 ml of 0·5 N perchloric acid. After 30 min at 0° the precipitates were collected by centrifugation and washed three times with 3 ml of 0·25 N perchloric acid. The final residues were dissolved in 0·2 ml of NCS (Amersham/Searle) and quantitatively transferred to counting vials using a toluene–alcohol scintillation system.[22] Each preparation of nuclei was analyzed for DNA content by the procedure of Burton[23] as modified by Grossman and Mavrides.[15] The supernatant of the nuclear fraction was centrifuged at 18,000 *g* and assayed for tyrosine aminotransferase (TA) activity. Each value represents the average of duplicate assays. The data are representative of those obtained from several animals.

enzyme, while the $Mn^{2+}/(NH_4)_2SO_4$-activated enzyme was inhibited about 60 per cent. On a molar basis the $Mn^{2+}/(NH_4)_2SO_4$-activated polymerase was about nine times more susceptible to inhibition by α-amanitin than to actinomycin-D. This correlates well with the data obtained from the dose–response curve illustrated in Fig. 2 which indicates α-amanitin was about five times more effective than actinomycin-D in inhibiting tyrosine aminotransferase induction by cortisol.

TABLE 3. INHIBITION OF Mg^{2+}- AND $Mn^{2+}/(NH_4)_2SO_4$-ACTIVATED RNA POLYMERASE IN RAT HEPATOMA CELL NUCLEI AFTER TREATMENT WITH α-AMANITIN AND ACTINOMYCIN-D *in vitro* AND *in vivo* (IN CULTURE)*

Treatment (*in vivo* in culture)	Treatment (*in vitro*)	Temp. (°C)	Mg^{2+} ($\mu\mu$moles UTP incorporated/mg DNA)	$Mn^{2+}/(NH_4)_2SO_4$ ($\mu\mu$moles UTP incorporated/mg DNA)	Relative TA activity
		37	1080	2043	1·0
		0	61	186	
	Minus cold nucleotides	37	22	42	
	Actinomycin-D (500 ng g/ml)	37	453	924	
	α-Amanitin (50 ng g/ml)	37	728	273	
Cortisol, 10^{-6} M		37	1070	1820	4·5
Cortisol, 10^{-6} M		0	46	218	
Actinomycin-D, 1·7 μg/ml + cortisol, 10^{-6} M		37	130	287	1·2
Actinomycin-D, 1·7 μg/ml + cortisol, 10^{-6} M		0	29	46	
α-Amanitin, 10 μg/ml + cortisol, 10^{-6} M		37	924	1272	4·0
α-Amanitin, 10 μg/ml + cortisol, 10^{-6} M		0	42	155	
α-Amanitin, 10 μg/ml + cortisol, 10^{-6} M	α-Amanitin (15 ng g/ml)	37	543	329	

* Cells from 25 untreated cultures were combined and the nuclei isolated as indicated in the Materials and Methods section. In other experiments, cells from 10–12 similarly treated cultures were combined after treatment with: cortisol (10^{-6} M); α-amanitin (10 μg/ml culture medium) for 30 min followed by addition of cortisol (10^{-6} M); or actinomycin-D (1·7 μg/ml culture medium) for 30 min followed by addition of cortisol (10^{-6} M). In each experiment the cells were harvested 7 hr after cortisol addition. After washing three times with 5-ml vol. of 0·1 M sodium phosphate buffer, pH 7·5, the cells were sonicated for about 5 sec and the nuclei collected as indicted above. After obtaining the nuclei the procedures followed were the same as that indicated in the legend of Table 2. The supernatant of the nuclear fraction was centrifuged at 18,000 *g* and assayed for tyrosine aminotransferase TA) activity.

Nuclei isolated from livers of animals previously treated with cortisol demonstrated the same Mg^{2+}-activated polymerase activity as controls, but a somewhat reduced level of $Mn^{2+}/(NH_4)_2SO_4$-activated enzyme (Table 2). Administration of actinomycin (300 μg) prior to cortisol treatment inhibited both polymerases by about 70–75 per cent. α-Amanitin, however, had no inhibitory effect on the Mg^{2+}-activated polymerase but inhibited the $Mn^{2+}/(NH_4)_2SO_4$-activated enzyme by about 55 per cent. To demonstrate more conclusively that the Mg^{2+}-activated enzyme was relatively refractory to α-amanitin, 50 ng g/ml of this substance was added to reaction mixtures containing nuclei from α-amanitin-treated animals. The data in Table 2 indicate that

the Mg^{2+}-activated enzyme remained as active as enzyme derived from the cortisol-treated animals, but the $Mn^{2+}/(NH_4)_2SO_4$-activated enzyme was inhibited now by about 80 per cent. These data imply that the $Mn^{2+}/(NH_4)_2SO_4$-activated RNA polymerase is required for induction of tyrosine aminotransferase by cortisol.

If the insensitivity of hepatoma cells to α-amanitin is due to an altered polymerase, then nuclei isolated from these cells should be refractory whether treated either *in vitro* or *in vivo* with this substance. The data in Table 3 indicate that both polymerases were, if anything, more sensitive to α-amanitin (50 ng g/ml) than the liver enzymes. The Mg^{2+}- and $Mn^{2+}/(NH_4)_2SO_4$-activated enzymes were inhibited about 33 and 87 per cent, respectively, while inhibition by actinomycin-D (500 ng g/ml) was about the same as in rat liver nuclei (55–60 per cent). However, nuclei from hepatoma cells treated in culture with α-amanitin (10 μg/ml) demonstrated considerably less inhibition. Most likely, this observed inhibition of the Mg^{2+}- and $Mn^{2+}/(NH_4)_2SO_4$-activated enzymes (14 and 30 per cent) was an artifact and grossly overestimates the degree of polymerase inhibition in these nuclei, if indeed, there was truly any inhibition at all. The concentration of α-amanitin in the culture medium was 10,000 ng g/ml. Despite our efforts to wash the cells free of α-amanitin, some probably remained membrane-bound. Upon brief sonication to break the cells, the exposed nuclei probably reacted with the α-amanitin that dissociated as a result of membrane rupture. The amount of α-amanitin made available did not have to be very large since as little as 15 ng g/ml inhibited the Mg^{2+}- and $Mn^{2+}/(NH_4)_2SO_4$-activated enzymes 50 and 82 per cent, respectively (Table 3).

To demonstrate that RNA synthesis in nuclei of intact hepatoma cells was not inhibited significantly by α-amanitin and, therefore, that these cells are impermeable to

TABLE 4. EFFECT OF α-AMANITIN AND ACTINOMYCIN-D ON [$^3H_5$]-OROTIC ACID INCORPORATION INTO RAT LIVER RNA*

	TA activity (μmoles/hr/10 mg protein)	$HClO_4$ soluble (counts/min/10 mg protein)	$HClO_4$ insoluble (counts/min/10 mg protein)
Control	1·16	33,800	3180
Cortisol (10 mg)	8·12	34,600	3630
α-Amanitin (50 μg) + cortisol (10 mg)	0·89	42,000	930
Actinomycin-D (300 μg) + cortisol (10 mg)	1·32	42,800	1300

* Animals were anesthetized with pentobarbital (30 mg/kg) and injected with: saline (control); cortisol, 10 mg; α-amanitin, 70 μg ,then cortisol, 10 mg, 30 min later; and actinomycin-D, 300 μg, then cortisol, 10 mg, 30 min later. Three hr after cortisol administration 50 μCi of [$^3H_5$]-orotic acid (2·14 Ci/m-mole, 50 μCi/ml) was injected. All substances were given intraperitoneally. One hr later, 500 mg of liver was homogenized in 4·5 ml of saline. To 0·5 ml of whole homogenate was added 0·5 ml of 1 N perchloric acid. After 30 min at 0° the precipitate was collected by centrifugation. A 0·2-ml aliquot of the supernatant was counted ($HClO_4$ soluble counts) and the residue washed three times with 5-ml vol. of 0·25 N perchloric acid. The final residue was dissolved in Protosol (New England Nuclear Corp.) and counted ($HClO_4$ insoluble counts). Another aliquot of the original homogenate was centrifuged at 18,000 *g* for 20 min and the supernatant assayed for tyrosine aminotransferase activity (micromoles of *p*-hydroxyphenylpyruvate formed per hour per 10 milligram of protein). The values presented are averages of duplicate experiments.

this substance, incorporation of [^{3}H]-orotic acid and [^{14}C]-uracil into RNA of rat liver and rat hepatoma cells was determined. The data in Table 4 indicate that in rat liver both α-amanitin and actinomycin-D inhibited [^{3}H]-orotic acid incorporation into perchloric acid insoluble material (RNA). This effect cannot be ascribed to inhibition of [^{3}H]-orotic acid uptake, since the perchloric acid soluble counts (an index of cell uptake) was even greater than in the control. The elevated $HClO_4$ soluble counts in the inhibited cultures probably reflect reduced utilization of this RNA precursor. The per cent inhibition of [^{3}H]-orotic acid uptake, about 70 and 50 per cent for α-amanitin and actinomycin-D, is in good agreement with observed inhibitory effects on RNA polymerase. In hepatoma cells, there was little, if any, inhibition of [^{14}C]-uracil incorporation into RNA by α-amanitin, while actinomycin-D inhibited incorporation by about 90 per cent (Table 5). The latter observation is in good agreement with the

TABLE 5. EFFECT OF α-AMANITIN AND ACTINOMYCIN-D ON [^{14}C]-URACIL INCORPORATION INTO RAT HEPATOMA RNA*

	TA activity (μmoles/hr/10 mg protein)	$HClO_4$ soluble (counts/min/10 mg protein)	$HClO_4$ insoluble (counts/min/10 mg protein)
Control	0·78	1490	420
Cortisol (10^{-6} M)	2·48	1370	418
α-Amanitin (10 μg/ml) + cortisol (10^{-6} M)	2·32	1800	390
Acintomycin-D (1·7 μg/ml) + cortisol (10^{-6} M)	0·83	1600	34

* Triplicate cultures were employed for each of the following experimental conditions: saline (control); cortisol, final concentration 10^{-6} M; α-amanitin, 10 μg/ml, followed 30 min later by cortisol (10^{-6} M); and actinomycin-D, 1·7 μg/ml, followed 30 min later by cortisol (10^{-6} M). Three hr after cortisol addition 2 μCi of [^{14}C]-uracil was added (33·3 μCi/μmole, 50 μCi/ml) and incubation continued for 30 min. At this time the cells were harvested, washed three times with 5-ml vol. of 0·1 N sodium phosphate buffer, pH 7·5, and sonicated for 10 sec in 1 ml of saline. To 0·5 ml was added 0·5 ml of 1 N perchloric acid. After 30 min at 0° the precipitate was separated by centrifugation. A 0·2-ml aliquot of the supernatant was counted ($HClO_4$ soluble counts) and precipitate washed three times with 5-ml vol. of 0·25 N perchloric acid. The final residue was dissolved in Protosol (New England Nuclear Corp.) and counted ($HClO_4$ insoluble counts). Another aliquot of the original sonicated extract was centrifuged at 18,000 *g* for 20 min and the supernatant assayed for tyrosine aminotransferase activity (micromoles of *p*-hydroxyphenylpyruvate formed per hour per 10 mg of protein).

observed inhibition of RNA polymerase (Table 3), and the former result confirms the idea that α-amanitin does not inhibit RNA polymerase in intact hepatoma cells. The inhibition of RNA polymerase observed above (Table 3) most likely occurred after the cells were broken. Therefore, the insensitivity of hepatoma cells to α-amanitin appears to be due to impermeability.

DISCUSSION

The data in Table 2 indicate that α-amanitin was about nine times more potent than actinomycin-D in inhibiting $Mn^{2+}/(NH_4)_2SO_4$-activated RNA polymerase but, unlike actinomycin-D, was essentially without effect on the Mg^{2+}-activated enzyme. Since

the mechanisms giving rise to differences in RNA polymerase activity in the presence of Mg^{2+}, or Mn^{2+} plus ammonium sulfate, are not under stood, it is difficult to assess fully the meaning of the above observations. However, assuming that $Mn^{2+}/(NH_4)_2SO_4$-activated polymerase activity approximates messenger RNA synthesis, the simultaneous inhibition by α-amanitin of this enzyme and cortisol induction of tyrosine aminotransferase suggest that both effects are causally related. That is, cortisol in some specific manner activates synthesis of tyrosine aminotransferase-messenger RNA and this effect is inhibited by α-amanitin. Since cortisol induces synthesis of relatively few proteins, while depressing synthesis of others as evidenced by no net increase or even decline in total liver protein,[24] overall changes in $Mn^{2+}/(NH_4)_2SO_4$-activated polymerase need not occur, or may even decline in activity. The results in Table 2 indicate that the $Mn^{2+}/(NH_4)_2SO_4$-activated polymerase decreased in activity after cortisol administration. Reports that total RNA polymerase activity of rat liver increases after cortisone administration[25,26] probably reflects improved template activity and not an increase in polymerase molecules.[27] Apparently, under the assay conditions employed, no such facilitation of polymerase activity was observed.

The ineffectiveness of α-amanitin in heptoma cells in culture was not due to an insensitive $Mn^{2+}/(NH_4)_2SO_4$-activated enzyme. As shown in Table 3, this polymerase in hepatoma nuclei was readily inhibited by α-amanitin *in vitro*. The relative resistance of the enzyme to α-amanitin after treatment of whole cells is attributed to poor penetration. Whatever inhibition did occur probably reflects interaction between α-amanitin and nuclei after the cells were broken. This is borne out by the data in Tables 4 and 5 which demonstrate RNA synthesis is inhibited by α-amanitin in rat liver but not in hepatoma cells. Actinomycin-D inhibited RNA synthesis in both systems.

Recently, Yu and Feigelson[28] and Sajdel and Jacob[3] reported that cortisone acetate or cortisol enhances the nucleolar RNA polymerases of rat liver nuclei. The latter group suggests that this effect is brought about by an allosteric alteration of the enzyme. In the present work no increase in the Mg^{2+}-activated RNA polymerase was observed 3 hr after cortisol treatment (Table 2). Since α-amanitin caused little or no change in the activity of this enzyme compared to its marked inhibitory effect on the $Mn^{2+}/(NH_4)_2SO_4$-activated polymerase, it is likely that inhibition by α-amanitin of tyrosine aminotransferase induction is more closely related to changes in messenger RNA synthesis than to changes in ribosomal RNA synthesis.

Analysis of the differential sensitivity of rat liver and rat hepatoma cells to α-amanitin has led to two significant conclusions: (1) rat hepatoma cells and rat hepatocytes appear to have different permeability characteristics; and (2) a positive correlation exists between inhibition of $Mn^{2+}/(NH_4)_2SO_4$-activated RNA polymerase and inhibition of tyrosine amino transferase by cortisol. The latter is taken as evidence that stimulation of transcription is a requisite for induction of tyrosine aminotransferase by cortisol.

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