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The Effect of D-Galactosamine and Actinomycin D on Polysomes and Messenger RNA Levels in Rat Liver

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SUMMARY

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The amount of polysomes isolated from rat liver 6 hr after D-galactosamine administration decreased to approximately 50% of that isolated from livers of control rats. This decrease was not found after the administration of actinomycin D, although RNA synthesis was inhibited to a similar extent. No corresponding decrease in total hepatic mRNA after D-galactosamine injection was found upon translation *in vitro* in a reticulocyte cell-free system. The albumin mRNA level measured either by translation *in vitro* and immunoprecipitation or by hybridization to a specific complementary DNA probe was also unchanged after D-galactosamine administration. It is concluded that the disaggregation of polysomes observed after D-galactosamine administration cannot be due to alterations in mRNA but may rather be due to the modification or loss of as yet unknown molecules involved in the binding of ribosomes to mRNA.

INTRODUCTION

It has been reported that repeated administration of large doses of D-galactosamine leads to severe hepatic cell damage in laboratory animals (1). The lesions consist of spotty necrosis of liver cells and periportal infiltration of inflammatory cells (1).

To understand the mechanism underlying these pathological processes, many studies on the biochemical events after D-galactosamine administration have been carried out in several laboratories (reviewed in refs. 2 and 3). As a very early event, a decrease in the levels of UTP has been observed (4). It is likely that this decrease leads to the reduced rates of total RNA synthesis (3), nuclear RNA synthesis (5, 6), and ribosomal RNA synthesis (7) which have been measured. Thus far, no studies on the effect of D-galactosamine on mRNA have been carried out.

In the present paper we describe experiments in which the effects of D-galactosamine and actinomycin D on the levels of total translatable mRNA and on albumin mRNA have been studied.

MATERIALS AND METHODS

Materials. D-Galactosamine-HCl, actinomycin D, sodium deoxycholate, and Triton X-100 were purchased from C. Roth OHG, Karlsruhe, West Germany; sucrose (ribonuclease-free) from Schwarz/Mann, Orangeburg, N. Y.; and heparin (grade I) from Sigma Chemical Company, St. Louis, Mo. L-[3,4,5-^{3}H]Leucine (160.5 Ci/mmole) was obtained from the Radiochemical Centre, Amersham, United Kingdom. All chemicals were of highest purity grade available.

Animals. Male SIV 50 rats were kindly supplied by B. Schreiber, Goedecke AG, Freiburg. Animals had free access to water and a carbohydrate-rich, 20% protein diet (Altromin, from Altromin GmbH, Lage, Germany). Generally, animals weighing 180–200 g were fasted overnight prior to an experiment. The animals received a dose of 400 mg of D-galactosamine or 2 mg of actinomycin D per kilogram of body weight by i.p. injection.

Preparation of polysomes. All glassware and buffers were sterilized by autoclaving. The minced livers were homogenized in 3 volumes of Buffer 1 containing 25 mM Tris-HCl (pH 7.6), 25 mM NaCl, 5 mM magnesium acetate, 0.25 M sucrose, and heparin, 100 mg/liter, and were centrifuged in a Sorvall SS 34 rotor at 15,000 rpm for 10 min. To the postmitochondrial supernatant, a freshly prepared detergent solution containing 5% sodium deoxycholate and 5% Triton X-100 was added to give a final detergent concentration of 0.5%. A portion of this solution (6 ml) was added to a step gradient of 3 ml of 2.0 M sucrose and 2.5 ml of 0.5 M sucrose in Buffer 1 and centrifuged. Polysomes were isolated as a pellet after centrifugation in a Beckman SW 41 rotor at 36,000 rpm for 20 hr.

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Isolation of RNA. Total liver RNA was isolated from freeze-clamped liver according to the method of Bresnick (8). RNA from the postmitochondrial supernatant as well as from polysomes was obtained after cold phenol extraction essentially as described by Zieve and Penman (9). For the translation and hybridization experiments, the precipitated RNA was washed twice with 3 M sodium acetate (pH 6.0) to remove low molecular weight RNA, dissolved in 0.1 M sodium acetate (pH 7.0), and precipitated with 2.5 volumes of ethanol containing 0.1 M NaCl at −20° as described by Palmiter (10).

Translation in vitro. For the *in vitro* translation, the reticulocyte lysate-[^{3}H]leucine Kit (Catalogue No. NEK-002, New England Nuclear Corporation, Boston, Mass.) was used. Protein synthesis was performed in a total incubation volume of 25 μl containing 13 μl of Premix (specified by the supplier), 10 μl of endonuclease-treated rabbit reticulocyte lysate (11), and 2 μl of an aqueous RNA solution. Generally the incubation mixture contained 17 μCi of L-[3,4,5-^{3}H]leucine. Whereas the [^{3}H]-leucine incorporation into total protein was optimal at concentrations of 80 mM K^+ and 1.0 mM Mg^{2+}, the optimal K^+ and Mg^{2+} concentrations required for the translation of albumin mRNA were 90 mM and 2.0 mM, respectively. After incubation at 37° for 1 hr in the case of total protein synthesis or 3 hr in the case of albumin synthesis, translation was stopped by the addition of 5 μl of 0.25 M Tris-HCl (pH 7.7), 10% Triton X-100, 0.75 M NaCl, and 0.02 M leucine. Aliquots of 10 μl were spotted on filter paper (Whatman 3MM) discs, subjected to cold trichloroacetic acid (10%) and boiling trichloro-acetic acid (5%), dried, and counted in a liquid scintillation spectrophotometer.

Immunoprecipitation. Immunoprecipitation of the *in vitro* translation product was performed with an anti-rat serum albumin immunoglobulin G purified by use of an affinity column of rat serum albumin covalently linked to Sepharose. After protein synthesis, 14 μg of rat serum albumin in a volume of 15 μl of 0.14 M NaCl were added, followed by the addition of 75 μg in a volume of 5 μl of purified immunoglobulin G and incubation at 37° for 1 hr and at 4° overnight. The immunoprecipitates were pelleted by sedimentation through a 0.5-ml cushion of 1.0 M sucrose in Buffer 2 (0.05 M Tris-HCl (pH 7.7), 2% Triton X-100, and 0.15 M NaCl). The sediments were washed three times with Buffer 2 and dissolved in 200 μl of 0.05 M Tris-HCl (pH 8.0), 2% SDS,[2] 10% glycerol, 10% 2-mercaptoethanol, and 0.005% bromophenol blue by treatment at 80° for 5 min. Aliquots of 20 μl were trichloroacetic acid-precipitated and used for the determination of radioactivity.

Electrophoresis of immunoprecipitates. A portion of the solubilized immunoprecipitates (180 μl) was added to SDS-polyacrylamide (7%) disc gels. The conditions for electrophoresis were those described by King and Laemmli (12). The gels were cut into 4-mm slices. Radioactivity of the slices was determined after extraction with 1 ml of NCS solubilizer (Amersham/Searle)-water (9:1) for 2 hr at 50°.

Preparation of albumin cDNA. Albumin cDNA was reverse-transcribed from mRNA as described by Hofer *et al.* (13), using 0.5 mM each of dATP, dGTP, and dTTP and 0.08 mM [^{3}H]dCTP. Albumin cDNA was separated from the deoxyribonucleotides by use of a Sephadex G-50 column. After sucrose gradient (4%–20%) centrifugation at 35,000 rpm in an SW 41 rotor for 20 hr, albumin cDNA with an average sedimentation coefficient of 13 S and a specific activity of 15,700 cpm per milligram was isolated.

Hybridization of RNA with albumin cDNA. Capillaries for hybridization were siliconized twice, rinsed with diethylpyrocarbonate (0.1%), and dried. The phosphate buffer for hybridization was passed over Chelex-100, neutralized to pH 7.0. The phenol-extracted RNA as well as the albumin cDNA were desalted by filtration through Sephadex G-25 and Chelex-100. Hybridization was performed in capillaries in 0.12 M phosphate buffer (pH 6.8) containing 1.0 mM EDTA and 0.5% SDS at 65° for 2 days, essentially as described by Krieg *et al.* (14).

High-pressure liquid chromatographic analysis of nucleotides. Nucleotide separation was carried out by high-pressure liquid chromatography as described by Henninger *et al.* (15). Liver samples were obtained by freeze-clamping under ether anesthesia. After extraction with cold 0.7 N perchloric acid and neutralization with potassium bicarbonate, the liver extracts were analyzed on a DuPont (Wilmington, Del.) chromatograph (Model 848) with a single-beam ultraviolet detector operating at 254 nm. Nucleotides were separated on a Partisil column (4.6 mm × 25 cm) (Whatman, Clifton, N. J.) at ambient temperature using a linear gradient from low- to high-concentration eluent within 20 min.

The low-concentration eluent contained KH_2PO_4 (20 mmoles/liter) and was adjusted to pH 4.0; the high-concentration eluent was composed of KH_2PO_4 (250 mmoles/liter) and KCl (0.5 mole/liter) at pH 4.5. The flow rate was 1.5 ml/min. For quantitation, the area under each peak was measured by cutting and weighing on a microbalance; UTP was quantitated by referring to a standard of the highest purity available which was run on the same column.

RESULTS

Polysomes from rat liver have been prepared after D-galactosamine and actinomycin D administration, respectively. The yield of polysomes was found to be decreased to approximately 50% of control values after D-galactosamine administration, whereas essentially no effect of actinomycin D was observed (Table 1). The D-galactosamine effect on the yield of polysomes did not depend on whether polysomal protein or RNA was measured. After incubation of polysomes *in vitro* with 10, 1, or 0.1 mM D-galactosamine for 30 min and subsequent centrifugation on a 17%–35% sucrose gradient, identical polysome profiles were obtained for control and D-galactosamine-treated polysomes (data not shown). Therefore, the effect of D-galactosamine on rat liver polysomes cannot be due to the direct action of the amino sugar on polysomes.

Furthermore, we found no net decrease in polysomes when either total homogenates or postmitochondrial su-

[2] The abbreviation used is: SDS, sodium dodecyl sulfate.

TABLE 1

Effect of actinomycin D and D-galactosamine on rat liver polysomes

Actinomycin D (2 mg/kg) and D-galactosamine (400 mg/kg) were injected i.p. into male rats fasted overnight. The animals were killed 6 hr after the injection. The preparation of polysomes and the extraction of RNA is described under Materials and Methods. The data given are average values ± standard deviation of four and three different experiments with D-galactosamine and actinomycin D, respectively. The numbers in parentheses represent percentage of control.

	Protein			RNA		
	Homogenate	Postmitochondrial supernatant	Polysomes	Postmitochondrial supernatant	Polysomes	Phenol-extracted polysomes
	mg/g liver	*mg/g liver*	*mg/g liver*	*mg/g liver*	*mg/g liver*	*mg/g liver*
Control	190 ± 8 (100)	62 ± 5 (100)	0.99 ± 0.11 (100)	5.1 ± 0.7 (100)	1.13 ± 0.20 (100)	1.13 ± 0.20 (100)
Actinomycin D	198 ± 8 (104)	65 ± 7 (104)	0.96 ± 0.13 (97)	5.47 ± 0.5 (107)	1.17 ± 0.10 (104)	1.42 ± 0.03 (113)
D-Galactosamine	190 ± 9 (100)	57 ± 4 (92)	0.55 ± 0.06 (56)	5.6 ± 0.6 (109)	0.6 ± 0.06 (53)	0.63 ± 0.01 (50)

pernatants from control and D-galactosamine-treated animals were mixed and used for the isolation of polysomes. Whereas the yield of polysomes isolated from total homogenates of D-galactosamine-treated rats was 58%, the yields of polysomes obtained from 1:1 mixtures of either total homogenates or postmitochondrial supernatants of control and D-galactosamine-treated animals were 76% and 77%, respectively.

It is also demonstrated in Table 1 that the yield of either protein or RNA in the postmitochondrial supernatant is unchanged after the administration of D-galactosamine.

To answer the question whether the marked reduction in the yield of polysomes after D-galactosamine administration (as compared with that after actinomycin D administration) is due to a different inhibition of RNA synthesis, we determined the incorporation of [^{3}H]UMP into total liver RNA after injection of [^{3}H]orotate. RNA synthesis was drastically reduced after the injection of both drugs (Table 2). It should be emphasized, however, that measurements and comparison of RNA synthesis are meaningful only when the precursor pools are comparable. We therefore determined the nucleotide pools in $HClO_4$ extracts of freeze-clamped livers 6 hr after the administration of the different drugs. Whereas an increase in the UTP pool size was found after actinomycin D injection, the well-known decrease in the UTP level after D-galactosamine administration was found. We have therefore used the specific radioactivities of UTP to correct the value for RNA synthesis after the administration of actinomycin D and D-galactosamine (Table 2, last column).

The corrected data show that the inhibition values for RNA synthesis after the administration of both inhibitors are very similar. Therefore, the effect of D-galactosamine on the yield of polysomes cannot be explained by different degrees of inhibition of mRNA synthesis.

In the following experiments the effect of both drugs on total mRNA and mRNA for albumin were studied by means of translation *in vitro* and hybridization with albumin-cDNA. To compare the translatable mRNA from polysomes of D-galactosamine- and actinomycin D-treated animals, complicated steps during the preparation of poly(A)$^+$ mRNA from polysomes, such as binding to and elution from a poly(U)-Sepharose- or oligo(dT)-cellulose column were avoided. Since total RNA obtained after phenol extraction of polysomes contains some components of low molecular weight which inhibit translation *in vitro,* the RNA was extracted with 3.0 M sodium acetate prior to translation as described by Palmiter (10).

Total RNA from polysomes of control, D-galactosamine-, and actinomycin D-treated animals was tested for the ability to direct protein synthesis in a reticulocyte lysate system. In all cases the [^{3}H]leucine incorporation into total protein was linear up to a concentration of 80 μg/ml of assay mixture. No significant differences with respect to [^{3}H]leucine incorporation between RNA isolated from polysomes of D-galactosamine-treated animals and controls were detected (Fig. 1a). In the case of actinomycin D, however, a slightly higher rate of [^{3}H]-leucine incorporation was observed.

TABLE 2

Specific radioactivity of UTP and inhibition of RNA synthesis after administration of actinomycin D and D-galactosamine

Actinomycin D (2 mg/kg) and D-galactosamine (400 mg/kg) were injected i.p. into animals fasted overnight. The animals were killed 6 hr after the injection. Thirty minutes before the animals were killed, 0.5 mCi 5-[^{3}H]orotate/kg was injected i.p. The experimental details for the determination of the UTP pools, the specific radioactivities, and the incorporation of [^{3}H]UMP into RNA are given under Materials and Methods. Total liver RNA was isolated according to the method of Bresnick (8). The data given as nanomoles or counts per minute per gram of freeze-clamped liver are means ± standard deviation of three determinations from three different animals; percentages of the corresponding control values are shown in parentheses.

	UTP			RNA synthesis	
	Content	Radioactivity	Specific radioactivity	Found	Corrected
	nmoles/g	*(cpm/g) × 10^{-3}*	*cpm/nmole*	*cpm/g*	*nmoles/g*
Control	177 ± 10	266	1,516	18,400 (100)	12.1 (100)
Actinomycin D	285 ± 22	502	1,755	4,600 (25)	2.6 (22)
D-Galactosamine	28 ± 13	29.7	1,058	2,024 (11)	1.9 (16)

When the proteins synthesized *in vitro* were analyzed with respect to the amount of albumin by immunoprecipitation with an albumin-specific immunoglobulin, it was found that the mRNA isolated from polysomes of D-galactosamine- and actinomycin D-treated animals did not differ in its abilities to direct albumin synthesis (Fig. 1b).

The immunoprecipitates were further characterized by SDS-polyacrylamide gel electrophoresis; one radioactive peak which co-migrated with albumin was detected. RNA isolated from polysomes of D-galactosamine-treated and of control animals and translated in a reticulocyte system led to essentially the same amounts of albumin as demonstrated in Fig. 2.

To corroborate the *in vitro* translation data by an independent method, hybridization assays were performed on RNA preparations from polysomes of D-galactosamine- and actinomycin D-treated animals. The results of these assays are presented in Fig. 3. Hybridization reactions were complete over a range of 2 log units, and they went to approximately 90% completion. As is shown in Fig. 3a and 3b there was essentially no difference with respect to the saturation hybridization curves obtained with albumin-cDNA and RNA from polysomes of D-galactosamine- or actinomycin D-treated animals. Since the hybridization curves varied between controls of different experiments, it was necessary to run individual controls in order to obtain comparable results.

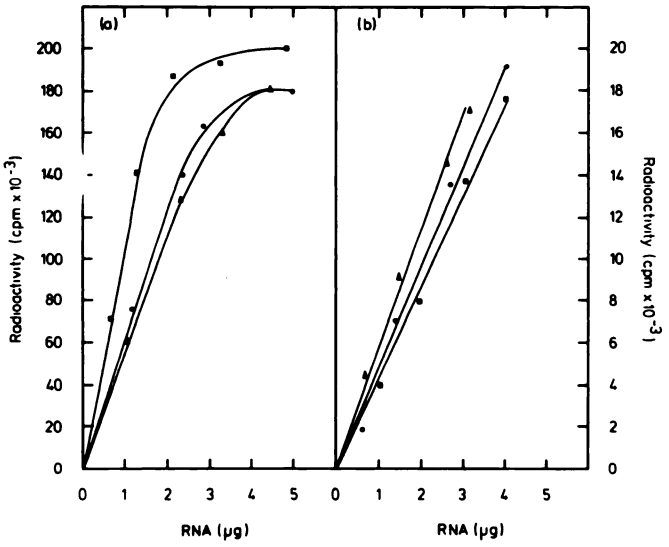

FIG. 1. *In vitro translation of polysomal RNA in a reticulocyte-lysate system*

Actinomycin D (2 mg/kg) and D-galactosamine (400 mg/kg), respectively, were injected i.p. into male rats fasted overnight. The animals were killed 6 hr after injection. The preparations of RNA from polysomes and the details of translation are described under Materials and Methods. Phenol-extracted RNA from polysomes of control (○), D-galactosamine-treated (△), and actinomycin D (□)-treated animals were used in the translation assay.

a. [^{3}H]Leucine incorporation into total proteins. The samples containing 17 µCi of [^{3}H]leucine and RNA as indicated above were incubated at 37° for 1 hr. The reaction was stopped and the radioactivity was determined as described under Materials and Methods.

b. [^{3}H]Leucine incorporation into albumin. The details for immunoprecipitation are described under Materials and Methods.

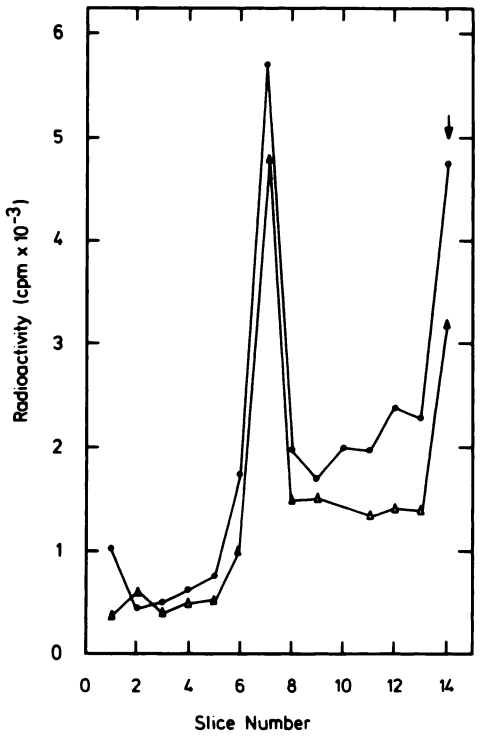

FIG. 2. *SDS-polyacrylamide gel electrophoresis of immunoprecipitated albumin*

Phenol-extracted RNA (13 µg) was translated into protein. The details for translation *in vitro* and the conditions for immunoprecipitation are described under Materials and Methods. SDS-polyacrylamide (7%) gel electrophoresis was carried out according to the method of King and Laemmli (12). The cylindrical gels were cut into 4-mm slices and the radioactivity was determined in each slice. ○, Control; △, D-galactosamine-treated.

From the experiments described above, it became clear that RNA isolated from polysomes of D-galactosamine- and actinomycin D-treated animals did not differ from that of controls in its ability to direct total protein as well as albumin synthesis in an *in vitro* reticulocyte system.

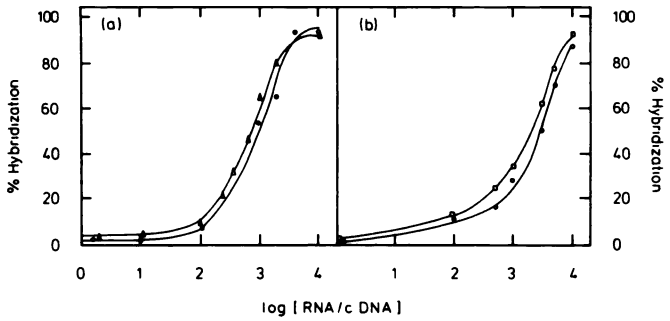

FIG. 3. *Titration curves of different polysomal RNAs with albumin-cDNA*

The experimental details for the hybridization are given under Materials and Methods. Increasing amounts of polysomal RNA were hybridized to 0.1 ng of albumin-cDNA per 1 µl of reaction aliquot.

a. Polysomal RNA from controls (○) and D-galactosamine-treated animals (△).

b. Polysomal RNA from controls (○) and actinomycin D-treated animals (□).

TABLE 3

RNA contents of postmitochondrial supernatants from livers of D-galactosamine-treated and untreated rats

D-Galactosamine was administered as described in legend to Table 1. The phenol extraction of RNA and the RNA determination are described under Materials and Methods.

	RNA of postmitochondrial supernatants	
	Before phenol extraction	After phenol extraction
	mg/g	*mg/g*
Control	5.10	3.25
	(100)	(100)
D-Galactosamine	5.60	2.96
	(109)	(91)

To determine whether the 50% decrease in yield of polysomes after D-galactosamine administration was due to a loss of mRNA or to some other disturbance(s) of polysomal structure, we isolated, translated, and hybridized total RNA from the postmitochondrial supernatants of D-galactosamine-treated and control animals. Whereas the RNA content of the postmitochondrial supernatants from both groups was the same, there was a decrease (although a small one) in total RNA after phenol extraction (Table 3). The RNA obtained from the postmitochondrial supernatant after D-galactosamine administration did not differ from that of controls in its ability to direct total protein synthesis (Fig. 4a) as well as albumin synthesis (Fig. 4b) in the reticulocyte system. Figure 5 shows the hybridization of albumin-cDNA to total liver RNA extracted from the postmitochondrial supernatant 6 hr after D-galactosamine administration. Because no differences in translatability (Fig. 4a and b) and the amount of albumin-mRNA (Fig. 5) were observed for the total RNA isolated from postmitochondrial supernatants of D-galactosamine-treated and untreated animals, it must be concluded that the drastic decrease in the yield of polysomes after D-galactosamine administration cannot be due to a loss of mRNA.

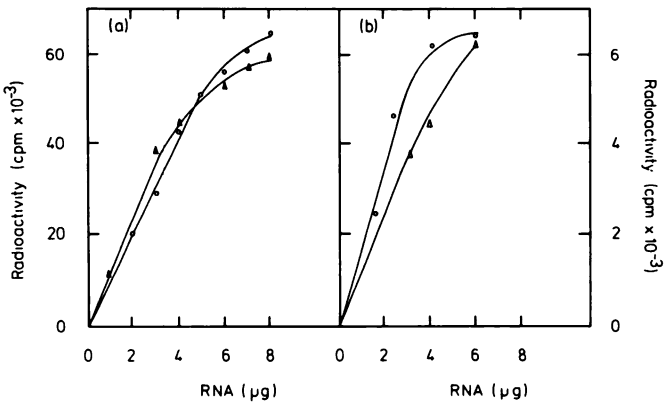

FIG. 4. *Translation in vitro of total RNA from postmitochondrial supernatants*

The experimental conditions are the same as those described in legend to Fig. 2, except that phenol-extracted RNA from postmitochondrial supernatants was used.

a. [^{3}H]Leucine incorporation into total proteins.

b. [^{3}H]Leucine incorporation into albumin RNA from D-galactosamine-treated (△) and control (○) animals.

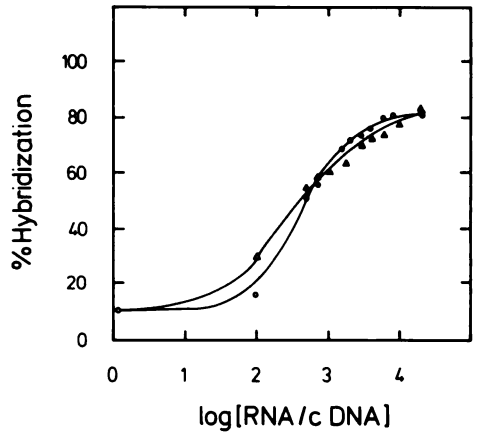

FIG. 5. *Titration curves of RNA from postmitochondrial supernatants with albumin-cDNA*

The experimental details are the same as those described in legend to Fig. 4, except for the RNA, which was isolated from postmitochondrial supernatants of livers from D-galactosamine-treated (△) and control (○) animals.

DISCUSSION

It has been suggested that the impaired RNA and protein synthesis observed after D-galactosamine administration are the cause for the liver cell death (2). In the present studies we administered actinomycin D to rats at concentrations whereby the RNA synthesis was inhibited to an extent similar to that after D-galactosamine injection. Under these conditions of inhibition of RNA synthesis, no liver cell necrosis was detectable after actinomycin D administration. The two single injections of 2 mg of actinomycin D per kilogram as used in the present studies did not lead to overt effects during the first 6 hr. Thereafter, diarrhea began, and the animals became depressed and moribund with slow, dyspneic respiration. Thus, inhibition of RNA synthesis per se does not produce cell death. Additional alterations must therefore occur after D-galactosamine administration which, in combination with the inhibition of transcription, may cause liver cell death.

As shown in the present studies a remarkable difference in the action of D-galactosamine on one hand and actinomycin D on the other was the effect of D-galactosamine on the polysomes. The administration of D-galactosamine led to a disaggregation of polysomes, an effect which has also been noted by other authors (3). From our studies on the levels of total mRNA and on albumin mRNA by means of hybridization and translation *in vitro*, it must be concluded that the drastic polysome disaggregation which is observed after D-galactosamine administration is not due to alterations in mRNA levels. Besides the decrease in the UTP pools, an accumulation of UDP-hexosamines has been observed after the administration of D-galactosamine (16). The latter effect may lead to disturbances in glycoprotein synthesis, although the mechanism of glycoprotein synthesis is not yet fully understood. Thus, it may be speculated that glycoproteins are involved in polysome formation and that D-galactosamine may exert a direct effect on certain ribosomal proteins responsible for the attachment of ribosomes to mRNA. Several ribosomal proteins have re-

cently been identified as glycoproteins (17). Furthermore, it should be realized that mRNA is always found as a ribonucleoprotein complex, and some of the proteins associated with the mRNA may be glycoproteins; these proteins may also be involved in the binding of ribosomes. Thus it may be possible that alterations or even losses of particular glycoproteins may result in the disaggregation of polysomes and in turn in an inhibition of protein synthesis observed after D-galactosamine administration. It is interesting in this connection that Endo and Natori (18) found a breakdown of hepatic polysomes after the administration of ethionine to rats. However, the mRNA in the liver cytoplasm of ethionine-treated rats remained intact and attached to rough endoplasmic reticulum membranes.

Although our experiments have shown that inhibition of RNA synthesis after D-galactosamine administration does not result in a decrease of over-all mRNA and albumin mRNA levels, it is clear that an inhibition of the synthesis of short-lived mRNA species present in very low amounts after D-galactosamine treatment might have escaped detection. The synthesis of these mRNA species should, of course, also be affected after actinomycin D administration. Experiments are in progress in order to determine whether D-galactosamine and actinomycin D exert different effects on short-lived mRNA. In this connection it should be mentioned that D-galactosamine greatly depressed the dexamethasone-induced increase of tyrosine aminotransferase (19). These findings have been interpreted as an effect of D-galactosamine on the synthesis of mRNA for tyrosine aminotransferase.

It is evident from the present studies that both actinomycin D and D-galactosamine strongly inhibit RNA synthesis. However, in contrast to actinomycin D, D-galactosamine leads to a rapid disaggregation of polysomes which in turn is followed by an inhibition of protein synthesis. The biochemical basis for the breakdown of polysomes remains to be elucidated. The administration of D-galactosamine to rats and its effect on the polysomes may thus be regarded as a suitable system with which to study the factor(s) involved in polysome structure and stabilization.

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