ACTINOMYCIN INHIBITION OF RNA SYNTHESIS IN RAT LIVER

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Actinomycin D has been used successfully as an analytical tool in virus research and in studies of the synthesis of RNA. Based on the inhibition of cellular RNA synthesis by actinomycin D, Reich, Franklin, Shatkin and Tatum (1962) suggested that the entire complement of RNA (nuclear, ribosomal and transfer) of bacterial and mammalian cells is produced in an actinomycin-sensitive and DNA-dependent reaction. Hurwitz, Furth, Malamy and Alexander (1962) arrived at similar conclusions: actinomycin D blocks RNA synthesis in B. subtilis by inhibiting RNA polymerase but it does not affect RNA pyrophosphorylase, an enzyme which adds CMP and AMP to the end of the transfer RNA chain. Very recently Tamaoki and Mueller (1962) have studied the incorporation of P^{32} orthophosphate into RNA in HeLa cells. Their data show that labeling of all classes of RNA is inhibited by actinomycin D, the 4s RNA being inhibited to a lesser extent than the other types. The three terminal nucleotides of transfer RNA (4s RNA) turn over independently of total synthesis. These authors conclude that the nucleus is the seat of all RNA synthesis in HeLa cells.

The evidence presented below indicates that actinomycin D inhibits the incorporation of P^{32} orthophosphate in all forms of RNA in rat liver. There is also evidence that under these conditions the terminal trinucleotide sequence pCpCpA in the 4s portion of RNA carries all the label.

METHODS

Male albino rats of the Holtzmann strain weighing approximately 250 g. were fasted for 24 hours, then injected intraperitoneally with 1 mg.

actinomycin D dissolved in 0.2 ml. propylene glycol. Control animals received 0.2 ml. of propylene glycol. Three hours later 10 mC P^{32} orthophosphate was injected by the same route and the rats were killed four hours thereafter. The livers were immediately homogenized in a Waring Blendor with 70 ml. of phenol (Mallinckrodt, Analytical Reagent, 88%) and 60 ml. of a solution consisting of 0.01 M phosphate buffer pH 6.8, 1 mM Versene, 1 mM trisodium citrate and 0.05% sodium lauryl sulfate to which 0.05 g. Bentonite was added. Bentonite was prepared according to Fraenkel-Conrat, Singer and Tsugita (1961). The combined use of phenol and 0.05% sodium lauryl sulfate has been shown to cause the release of over 90% of the total RNA keeping its contamination with DNA at a low level (Dingman and Sporn, 1962). All extraction procedures were carried out at 4°. The homogenate was shaken for one hour, the emulsion was broken by centrifugation and the aqueous layer extracted once more with 40 ml. phenol and 0.03 g. Bentonite. The water solution was made 0.2 M with respect to potassium acetate buffer (pH 5.6) and the RNA was precipitated by the addition of 2 volumes of ethanol chilled to -20° with dry ice. The RNA was then reprecipitated three times from the 0.2 M acetate buffer solution with 2 volumes of ethanol. These samples contained less that 1% DNA and 1% protein. The final precipitate was dissolved in 0.15 M NaCl to yield a 1% solution of RNA, which was quickly frozen and stored at -20°.

Three methods were used for the fractionation of the RNA preparations. In the preliminary experiments we applied the method of Harshaw, Brown and Graham (1962) which is based on differential precipitation of RNA components with streptomycin and 1 M NaCl. The fractions were dissolved in 2 ml. 0.15 M NaCl and counted in a Baird Atomic scintillation spectrometer.

Sucrose gradient centrifugation was carried out in a Spinco SW-39 swinging-bucket rotor at 4°. The sample (0.1 ml.) was layered on top of a 4.6 ml. linear gradient from 5-20% and centrifuged for 16 hours at 20,000 rpm. Fractions of 8 drops were collected and 3.0 ml. water added to each. After

measurement of the optical density at 260 mμ the fractions were evaporated on aluminum planchets and counted in a gas-flow counter (covered with Mylar film).

For the chromatographic fractionation of RNA, the methylated albumin-Kieselguhr (MAK) columns were prepared according to a simplified Mandell-Hershey procedure as described by Monier, Maono, Hayes, Hayes and Gros (1962). A sample of 0.8 ml. (8 mg. RNA in 0.3 M NaCl) was applied and eluted under a linear concentration gradient between 0.3 M and 1.3 M NaCl both solutions containing 0.05 M phosphate buffer (pH 6.7). A hydrostatic pressure head of 150 cm. gave a flow rate of 50 ml./hr. Fractions of 3.5 ml. were collected.

Base analyses were carried out by the ionophoresis method of Davidson and Smellie (1952). The paper strips were then scanned under ultraviolet light and in a 4π radio chromatogram scanner. Finally the nucleotide bands on the paper strips were cut out and counted directly in the gas-flow counter.

RESULTS

In preliminary experiments the RNA was fractionated by the streptomycin precipitation technique, both actinomycin C (Sanamycin, Farbenfarbriken Bayer AG) and D were used as inhibitors and the rats were killed one to six hours after the injection of P^{32} orthophosphate. There is no difference between actinomycin C and D in inhibiting RNA synthesis in rat liver or kidney. It can be seen from Table I that actinomycin prevents the labeling of high molecular weight RNA, while the inhibition of labeling of 4s RNA is much less pronounced.

Data from sedimentation experiments (Fig. 1) and from chromatographic separations on a MAK column (Fig. 2) illustrate the almost complete disappearance of label in the ribosomal RNA peaks and the partial blockage of the incorporation of P^{32} into transfer RNA (4s RNA) that result from actinomycin D.

In ionophoretic analysis unfractionated RNA preparations from control animals revealed, under ultraviolet light, bands for the four main nucleotides: cytidylic, adenylic, guanylic and uridylic acids. When scanned for radio-activity, all of these bands showed, as expected, the presence of P^{32}. However, the P^{32} content was greatly reduced in the adenylic, guanylic and uridylic acid

Table I

Incorporation of P^{32} into Rat RNA

Time of labeling hrs.	Tissue		4s RNA CMP/g tissue	4s RNA % of control	High molecular weight RNA CMP/g tissue	High molecular weight RNA % of control
1	liver	control	4050		17925	
		C*	1733	42.8	456	2.5
1	kidney	control	5045		23250	
		C	1500	29.8	535	2.3
6	liver	control	1745		64800	
		C	716	40.6	370	0.6
4	liver	control	2196		31160	
		D*	1030	47.0	930	3.0

*C refers to actinomycin C treated animals; D refers to actinomycin D treated animal.

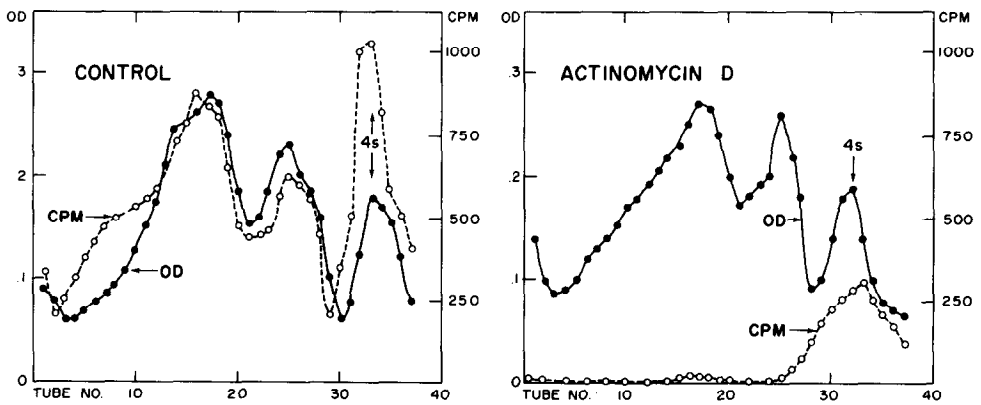

Fig. 1. Sedimentation profiles of rat liver RNA labeled with P 32

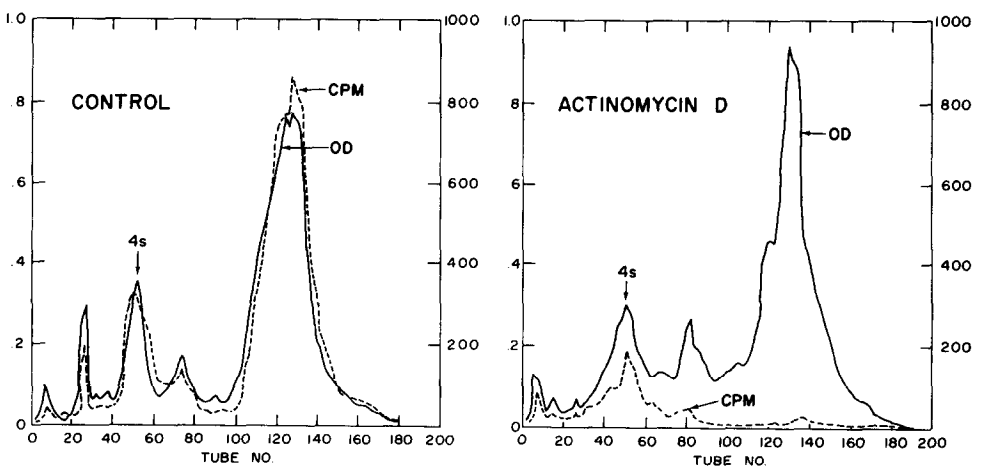

Fig. 2. Chromatographic fractionations of rat liver RNA labeled with P 32

bands after the rats had been treated with actinomycin D, and most of the radioactivity was present in the cytidylic acid (Table II).

Table II

P^{32}-labeled Nucleotide Composition of Total Rat Liver RNA

The values given are moles/100 moles 2'- and 3'-nucleotides

	Cytidylic Acid	Adenylic Acid	Guanylic Acid	Uridylic Acid
Control*	31.3	17.5	32.0	19.2
	29.2	20.0	33.5	17.3
Actinomycin D*	67.6	12.1	9.9	10.4
	63.8	13.1	12.2	10.9

*Values are duplicate determinations from the same alkaline hydrolysates

It is known that rat liver transfer RNA has a nucleotide composition quite similar to that of the total liver RNA. Therefore, the fact that after actinomycin D treatment the unfractionated RNA preparations (of which the 4s part carried 97% of the label) had such a high cytidylic acid content suggests some specific mechanism for labeling. Since the three terminal nucleotides (pCpCpA) of transfer RNA turn over independently from the rest of the RNA chain, they may carry the label. In this case, after alkaline hydrolysis at least 67% of the label should occur in cytidylic acid and the remaining P^{32} should be distributed between the nucleotides next to the pCpCpA sequence. That the pCpCpA sequence indeed carries most, if not all, of the label is indicated by the data in Table II.

In conclusion this investigation shows that in the presence of actinomycin D the synthesis of all forms of RNA except the pCpCpA end groups of transfer RNA is inhibited in rat liver. These findings confirm in the intact mammal observations of others in cell cultures.

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