EFFECTS OF α-AMANITIN ON IN VITRO LABELING OF RNA FROM DEFINED NUCLEAR COMPONENTS IN SALIVARY GLAND CELLS FROM *CHIRONOMUS TENTANS*

E. EGYHÁZI, B. D'MONTE, and J.-E. EDSTRÖM

From the Department of Histology, Karolinska Institutet, S-104 01 Stockholm, Sweden.
Dr. D'Monte's permanent address is the Department of Biochemistry, All-India Institute of Medical Sciences, New Delhi 16, India.

ABSTRACT

The effect of α-amanitin on nucleoside labeling of RNA in nucleoli, chromosomes, nuclear sap, and cytoplasm from *Chironomus tentans* salivary gland cells was investigated by radioautography and gel electrophoresis. Preribosomal RNA formation and processing in the nucleolus was not measurably influenced by the drug, and both 28 S and 18 S ribosomal RNA were transferred to the cytoplasm. In the chromosomes the heterogeneous RNA labeling was completely inhibited for the large size range (above 45–50 S) and partially for the low range. The labeling of 4–5 S chromosomal RNA was only moderately reduced. Most of the chromosomes showed radioautographically a disappearance of the normal band pattern, but some retained a pattern of weakly labeled bands. The electrophoretic results for the nuclear sap paralleled those for the chromosomes. The effect of α-amanitin on RNA labeling in these cells is similar but not identical to that of the substituted benzimidazole 5,6-dichloro-1(β-D-ribofuranosyl) benzimidazole (DRB).

INTRODUCTION

The mushroom poison α-amanitin characterized by Wieland (1) has been shown to inhibit the RNA polymerase II (the nucleoplasmic form) but to have little effect on the polymerase I (the nucleolar form) (2–7). The RNA polymerase III (7–9) is, like the polymerase I, resistant to α-amanitin (6, 7). One would expect the synthesis of nucleolar RNA to remain unaffected by α-amanitin. In recent in vivo experiments (10, 11), inhibitory effects were, however, observed on both ribosomal and nuclear heterogeneous RNA synthesis, in apparent contrast to the in vitro experiments. It would therefore be desirable to study the effect of α-amanitin on cellular RNA synthesis in less complex systems, but its permeability through the plasma membrane is low. The explanted salivary glands of chironomids are, however, sensitive to the drug (12, 13) and offer a convenient system for the study of its effect on the labeling of various RNA fractions.

MATERIALS AND METHODS

Late fourth larval instar animals of the species *Chironomus tentans* were used, cultured as described by Beermann (14). The incubation solution (15) is a modification of a medium earlier described by Cannon (16). Glands were preincubated for 60 min with or without the drug followed by incubations with or without the drug in the presence of tritiated nucleosides for 45 min or 180 min at 18°C. As a rule four glands were incubated in a 50 μl solution containing
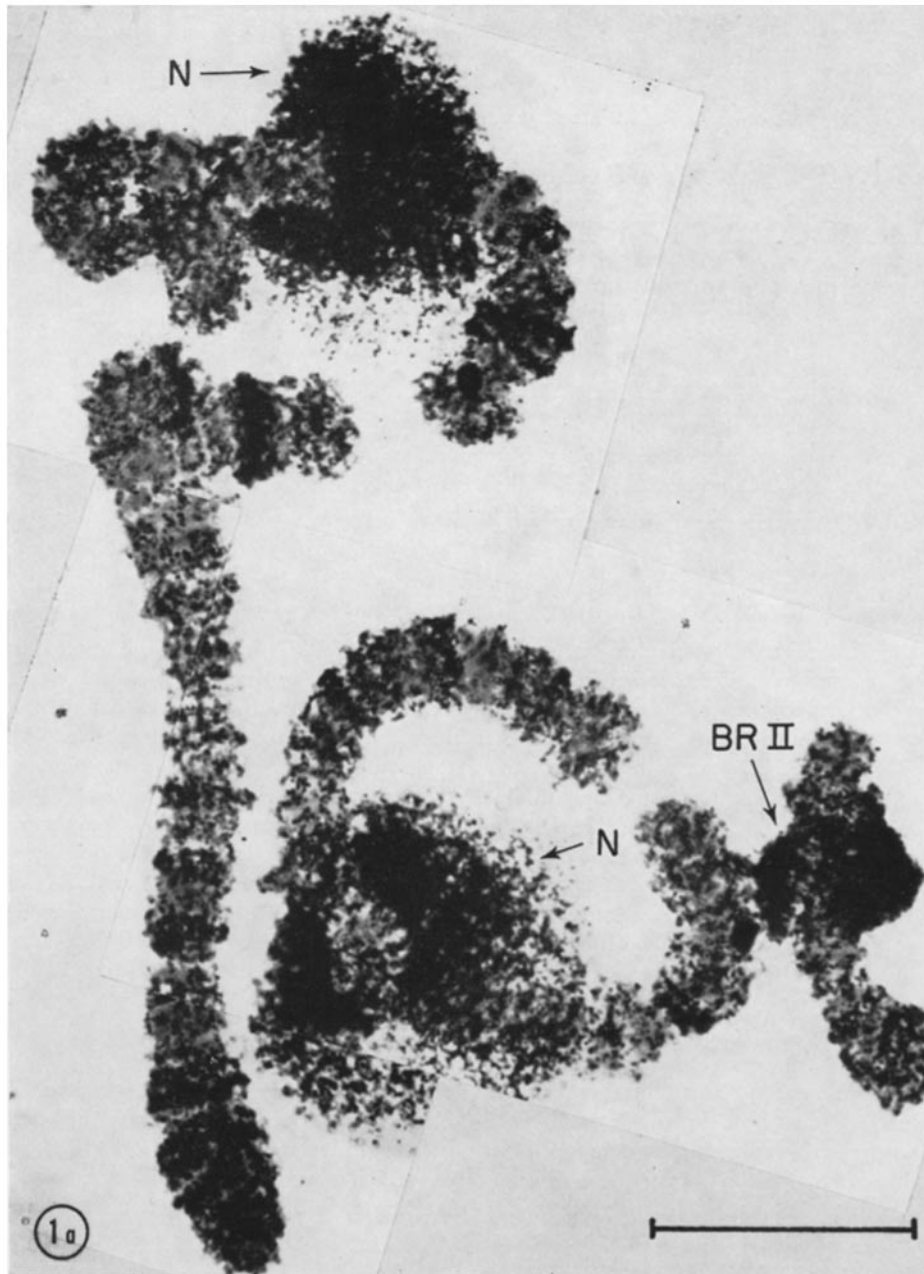
FIGURE 1   Radioautograms of chromosomes from salivary glands incubated in vitro in the absence (Fig. 1 a) and presence (Fig. 1 b) of α-amanitin. Control glands were incubated in vitro for 45 min at 18°C with 60 μCi tritiated uridine per ml medium after preincubation for 60 min in the absence of isotope. Drug-treated glands were incubated for 45 min at 18°C with 120 μCi tritiated uridine per ml medium after preincubation for 60 min in the absence of isotope. The α-amanitin concentration was 16 μg per ml during incubation and preincubation. Exposure time: 4 days, Kodak AR10 film. The scale indicates 25 μ. N and BR II denote nucleoli and the second Balbiani ring, respectively. $\times$ 1400.
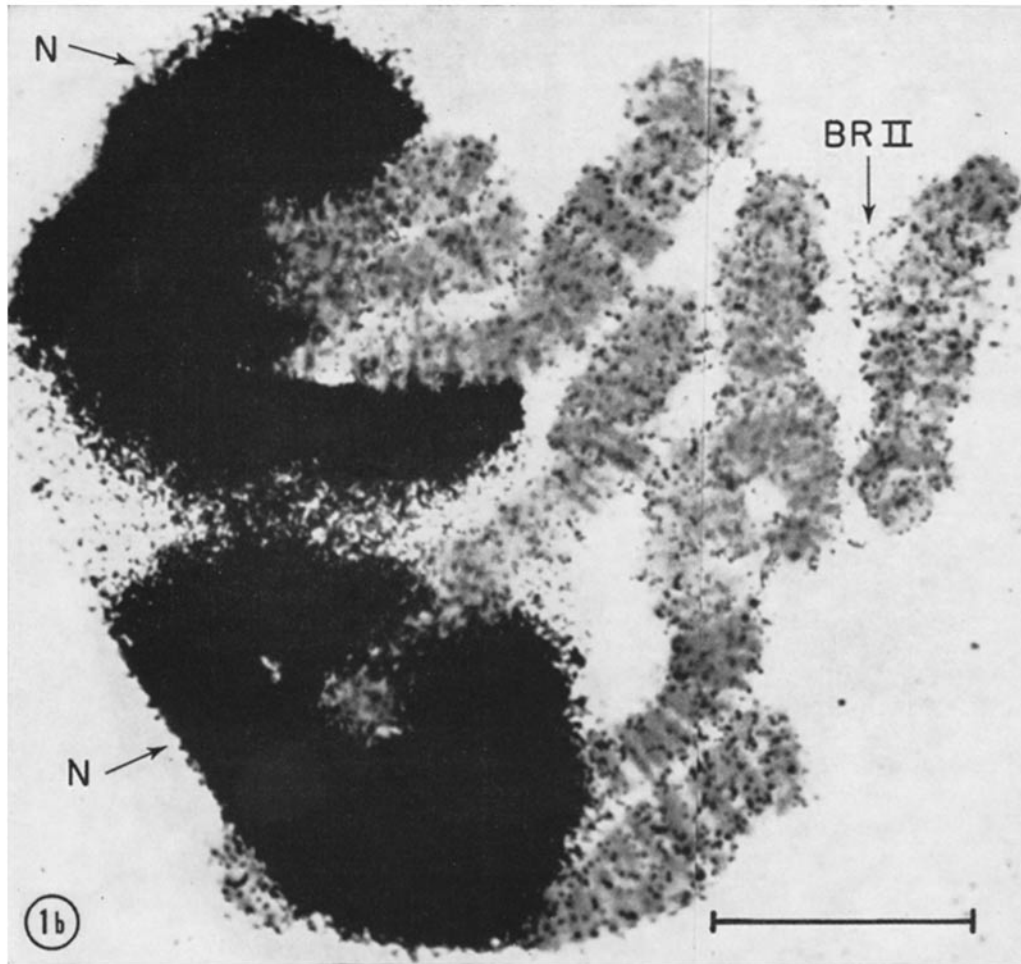
α-amanitin, and the four sister glands were incubated in a 50 μl volume without the drug. Usually a concentration of α-amanitin of 16 μg per ml was used. Glands were either radioautographed as described previously (17) or else RNA was extracted from fixed microdissected glands and analyzed on 2% agarose gels or 9% polyacrylamide gels (18, 19). The isotopes used were tritiated nucleosides obtained from Amersham England at specific activities of 25–30 Ci/mmole. One sample of α-amanitin used in initial work was obtained from Dr. W. Rutter, and a second sample was a gift from Dr. T. Wieland.

RESULTS

Radioautography

Radioautographs of control glands labeled for 45 min showed label over nucleoli and a large number of chromosome bands (Fig. 1 a). In particular the large puff in the fourth chromosome, the Balbiani ring II, was strongly labeled. In α-amanitin treated glands, nucleoli retained the label, but in most cases band labeling was absent from the chromosomes which, however, exhibited a diffuse labeling distinctly above the background levels of label (Fig. 1 b). In several glands all cells showed this type of labeling. It was more usual, however, that the response was varied and that the chromosomes in a minority of the cells retained some, decreased band labeling (cf. ref. 12 and 13). The reason for an absence of band patterns in the chromosomes of the majority of the cells was not simply a general decreased level of incorporation. If isotope doses were increased four to five times over controls, nucleolar label and chromosomal background label increased but radioautographically banded chromosomes were not more frequent. The possibility that remaining band patterns in a minority of cells could be due
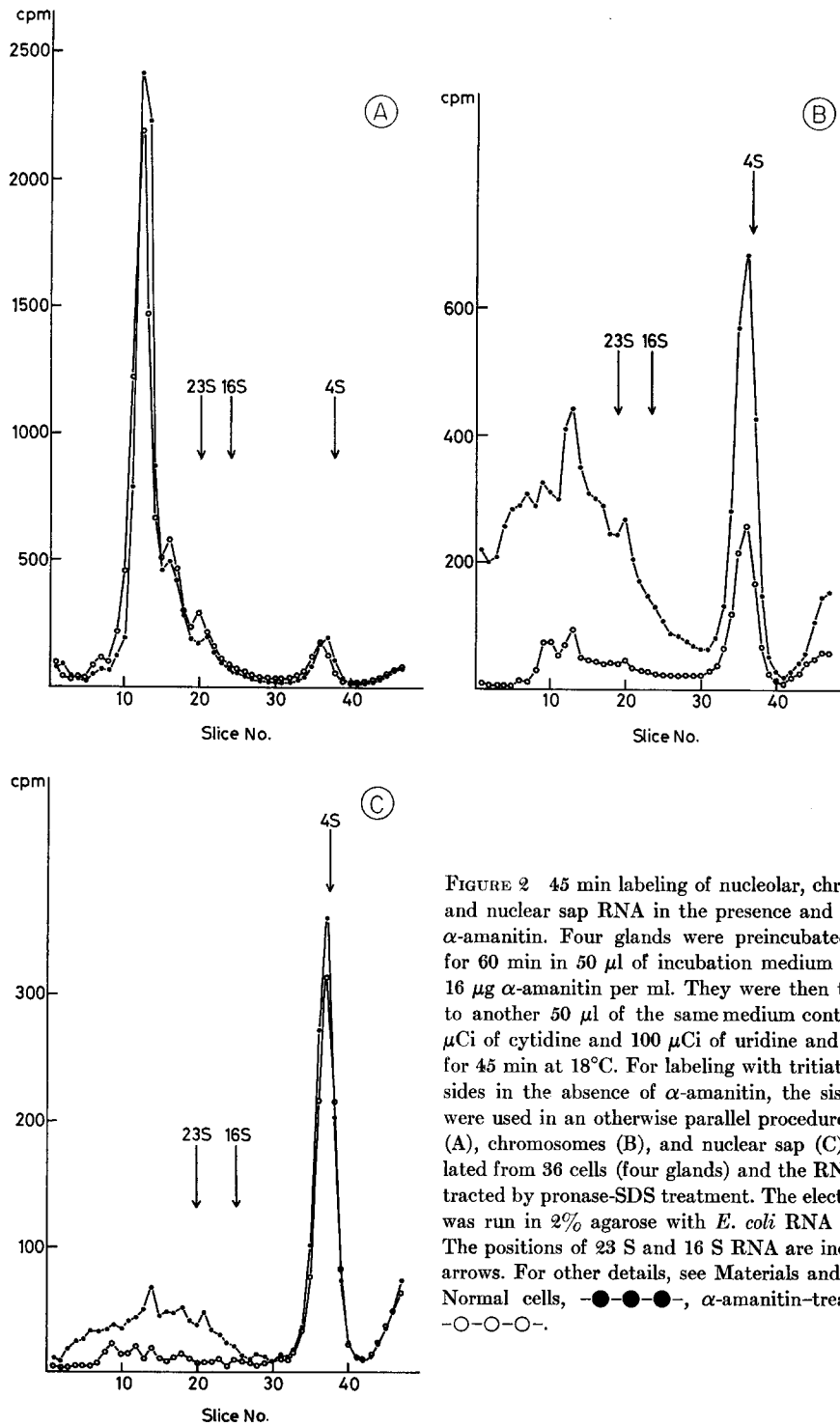
FIGURE 2 45 min labeling of nucleolar, chromosomal, and nuclear sap RNA in the presence and absence of α-amanitin. Four glands were preincubated at 18°C for 60 min in 50 μl of incubation medium containing 16 μg α-amanitin per ml. They were then transferred to another 50 μl of the same medium containing 100 μCi of cytidine and 100 μCi of uridine and incubated for 45 min at 18°C. For labeling with tritiated nucleosides in the absence of α-amanitin, the sister glands were used in an otherwise parallel procedure. Nucleoli (A), chromosomes (B), and nuclear sap (C) were isolated from 36 cells (four glands) and the RNA was extracted by pronase-SDS treatment. The electrophoresis was run in 2% agarose with *E. coli* RNA as carrier. The positions of 23 S and 16 S RNA are indicated by arrows. For other details, see Materials and Methods. Normal cells, -●-●-●-, α-amanitin–treated cells, -○-○-○-.

to inadequate concentrations of α-amanitin in the incubation medium was ruled out in experiments in which the dose was increased five times, to 80 μg per ml.

Gel Electrophoretic Analyses

In glands labeled for 45 min after preincubation for 60 min, the nucleolar labeling pattern was not markedly influenced by α-amanitin, i.e. both the 38 S and 4 S RNA fractions label and there is a beginning conversion of 38 S RNA to 30 S and 23 S RNA (Fig. 2 a). The chromosomal analyses indicated a considerable suppression of the labeling of heterogeneous RNA (H RNA) (Fig. 2 b), to 10–15% of the normal amount of label. The drug inhibited labeling in the high molecular weight range of the H RNA spectrum rather completely, while the region below 45–50 S showed less inhibition of isotope incorporation. In controls the label in the H RNA region amounted to 80–90% of the total label; in drug-treated glands the

corresponding value was 25–35%. It is likely that there is some effect also on the labeling of 4–5 S RNA which in most cases was decreased. The results of the nuclear sap analyses paralleled those of the chromosomes in showing a differential suppression of the H RNA label, in particular in the high molecular weight range (Fig. 2 c). Quantitative comparisons between sap from controls and sap from experimental glands are less meaningful since the nuclear sap was not collected quantitatively. The label remaining in the H RNA range smaller than 45–50 S could be due either to aberrant RNA synthesis (i.e. premature termination of RNA synthesis) or to the existence of a special class of relatively low molecular weight chromosomal H RNA synthesized by an α-amanitin-insensitive RNA polymerase.

Since the low molecular weight RNA consists of different RNA species, this range was resolved by analyses in high per cent polyacrylamide gels. Peaks were present at 8 S, 5 S, the region between
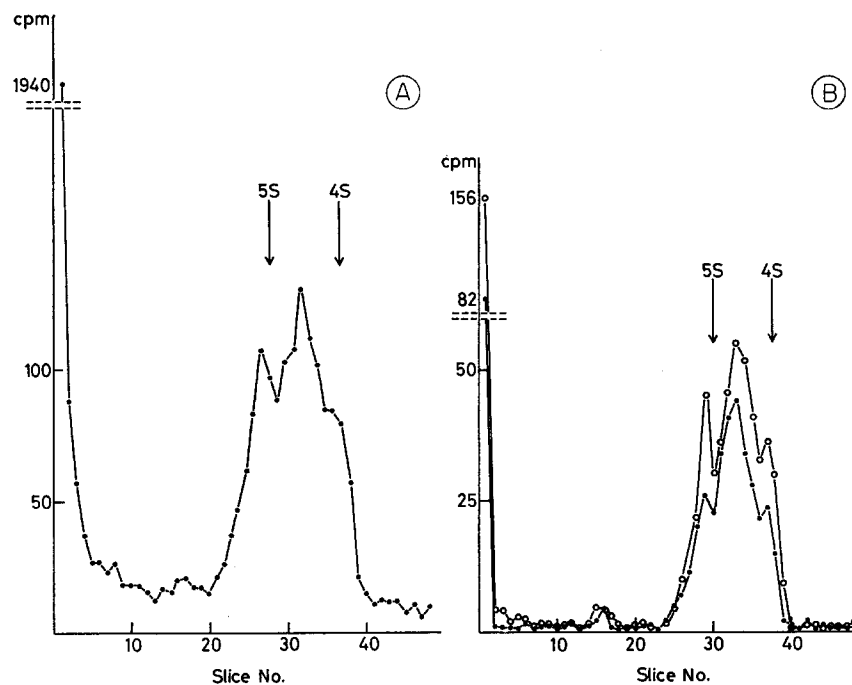

Figure 3   45 min labeling of low molecular weight chromosomal RNA in α-amanitin-treated, DRB-treated, and normal cells. In each experiment four glands were preincubated at 18°C for 60 min in 50 μl of incubation medium in the presence of α-amanitin (16 μg/ml), DRB (30 μg/ml), or without any drugs. They were then transferred to another 50 μl of the same medium, containing 100 μCi of cytidine and 100 μCi of uridine, and incubated for 45 min at 18°C. Chromosomes were isolated from 40 normal cells (four glands) (A); and from drug-treated cells (40 of each) (four glands) (B). α-amanitin, -O-O-O-; DRB -●-●-●-●-. For other data, see Fig. 2.
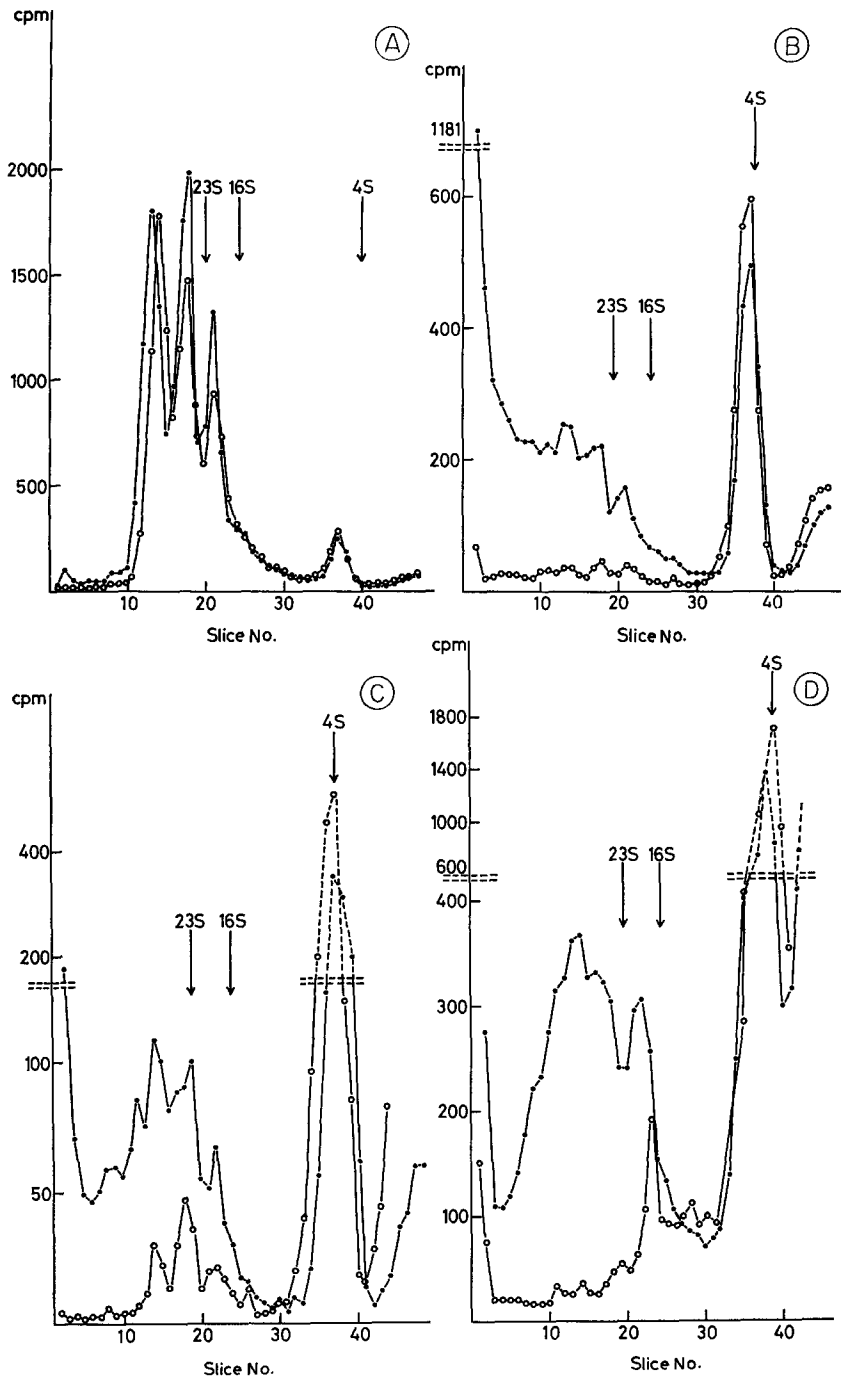
FIGURE 4 180 min labeling of nucleolar, chromosomal, nuclear sap, and cytoplasmic RNA in the presence and absence of α-amanitin. Four glands were preincubated at 18°C for 60 min in 50 μl of incubation medium containing 16 μg α-amanitin per ml. They were then transferred to another 50 μl of the same medium containing 100 μCi of uridine, 100 μCi of cytidine, and incubated for 180 min at 18°C. For labeling with tritiated nucleosides in the absence of α-amanitin, the sister glands were used in an otherwise parallel procedure. Nucleoli (A), chromosomes (B), nuclear sap (C), and cytoplasm (D) were isolated from 32 cells (four glands). Normal cells, -●-●-●-; α-amanitin-treated cells, -O-O-O-.

4–5 S, and at 4 S in controls (Fig. 3 a) as well as in inhibited glands (Fig. 3 b).

Since the radioautographic labeling pattern in chromosomes differs after incorporation in the presence of DRB (17) and α-amanitin and since in both cases most of the labeled RNA is of low molecular weight, the RNA profiles in the low molecular weight range were compared after the two types of treatment. No significant differences were recorded (Fig. 3 b).

In glands labeled for 180 min after preincubation for 60 min, nucleolar RNA in controls as well as in drug-treated animals showed distinct 30 S and 23 S RNA fractions (Fig. 4 a) in addition to the rapidly labeled 38 S and 4 S RNA peaks. The 30 S and 23 S nucleolar RNA fractions are known to be products of 38 S (20, 21), a conversion which consequently occurs also in the presence of α-amanitin. The chromosomal (Fig. 4 b) and nuclear sap (Fig. 4 c) profiles were influenced in much the same way as during a 45 min treatment. In the cytoplasm the drug inhibited labeling of most of the H RNA but permitted entry of label into 18 S RNA (Fig. 4 d). It could not be decided after 180 min incubations whether the cytoplasmic appearance of 28 S RNA is affected. Only little material is present at 28 S in cytoplasm from treated glands. The large amount of H RNA in controls in this range made it impossible to judge whether 28 S RNA was present or not. In analyses after 6 hr of incubation it was found, however, that 28 S RNA appears in the cytoplasm during α-amanitin treatment. Overlapping H RNA in controls in this range precluded quantitative comparisons.

DISCUSSION

Although the labeling and not the synthesis of RNA was measured, it is unlikely that the inhibitory effect of α-amanitin on RNA labeling could be explained as effect mainly on precursor pools. The nucleolar RNA labeling patterns were similar in controls and treated glands. Therefore, unless nucleoli and chromosomes are labeled from different pools, the labeling by nucleosides gives a measure of the relative rates of synthesis.

In agreement with Wobus et al., (12), who investigated another chironomid, and with Beermann (13), who investigated Chironomus tentans, we found in radioautographs that the normal band labeling of the polytene chromosomes was usually abolished by α-amanitin. Our biochemical data were in agreement with these results. They showed a 80–90% reduction of labeled H RNA by α-amanitin. The radioautographs showed a minority

of chromosome sets with remaining band labeling although of reduced intensity. Such cells must have been affected by the drug treatment since the biochemical analyses consistently showed an absence of label in the range above 45–50 S after α-amanitin treatment. Labeled bands, when present, may indicate a partial synthesis of H RNA molecules, a special class of H RNA of relatively low molecular weight (e. g. 15–45 S), the synthesis of which is less sensitive to α-amanitin, or they could be caused by low molecular weight RNA species (e. g. 4–8 S) or by preribosomal RNA (23).

The biochemical data showed that 4–5 RNA became labeled to a considerable extent during α-amanitin treatment. In most cases there was some reduction of the chromosomal 4–5 S RNA label. Judging by the radioautographic data, the chromosomal label in inhibited glands is largely due to diffusely distributed RNA and has no relation to the band pattern. A different situation obtained in glands labeled during treatment with the substituted benzimidazole DRB (17, 22). Here a dominating fraction of the chromosomal label is contained in low molecular weight RNA, and the chromosomes display in radioautographs a distinct pattern of band labeling. The differences in results are not related to any obvious differences in the labeling profiles of RNA in the low molecular weight RNA range, but it cannot be excluded, of course, that α-amanitin inhibits the labeling of low molecular weight RNA species present during DRB treatment. Another possibility is that the difference in labeling patterns is caused by different processing of low molecular weight RNA after its synthesis. We found the effects of α-amanitin and DRB to be different in at least two more respects. DRB prevents export of 18 S RNA but not 28 S RNA to the cytoplasm and also the normal appearance of 18 S RNA in the chromosomes and nuclear sap (15). During α-amanitin treatment the 18 S RNA is exported. DRB leaves only little label in the H RNA range of chromosomes (17, 22) while fractions were consistently present in the low molecular weight part of the H RNA pattern after α-amanitin treatment.

Chromosomal H RNA can be assumed to be synthesized in the extranucleolar part of the chromosomes. Therefore, our data which show suppression of its labeling support the view that a nucleoplasmic (i. e. extranucleolar) RNA polymerase is inhibited by α-amanitin. It is clear, on the other hand, that the response of extranucleolar RNA labeling to α-amanitin is not uniform since

not only did the 8 S, 5 S, and 4–5 S RNA fractions remained labeled, but also RNA molecules less than 45–50 S were suppressed comparatively little. At least part of the residual label may be due to preribosomal RNA which migrates from the nucleoli to the chromosomes (23). This cannot explain all residual label, and it is possible that there exist polymerases for chromosomal H RNA in the range between 15 S and 45 S with little or no sensitivity to α-amanitin. Another possibility is that the results are caused by premature termination of H RNA as a result of an action of the drug on elongation, possibly in cells which showed residual band labeling in the radioautographs. The labeling of 8 S, 5 S, and 4 S RNA indicates the existence of chromosomal α-amanitin–insensitive polymerases since the corresponding RNA fractions are likely to originate in the extranucleolar part of the chromosomes in the diptera (24–27).

The biochemical results give a clear indication of an absence of a measurable influence on the labeling of nucleolar RNA. They furthermore show that the normal conversion within the nucleolus of 38 S RNA to 30 S and 23 S RNA occurs during α-amanitin treatment and that both 28 S and 18 S RNA will appear in the cytoplasm. These results are understandable in the light of the reported nucleolar localization of an α-ama nitin–resistant RNA polymerase (5). They also show that the nucleolar processing of preribosomal RNA and ribosomal RNA export are independent of the synthesis of a large proportion of chromosomal H RNA. These results are in contrast to those obtained in experiments with intact animals where α-amanitin exerted an effect not only on the heterogeneous rapidly labeled RNA, but also on the formation of ribosomal RNA (10, 11). Our results argue against an interpretation which could be given to these findings, namely that the ribosomal RNA synthesis is adapted to that of H RNA by some kind of direct feedback mechanism.

In conclusion, parallels can be drawn between the undisturbed labeling of RNA in nucleoli and their content of an α-amanitin–insensitive RNA polymerase on one hand, and between the inhibited chromosomal H RNA labeling and the reported nucleoplasmic localization of a sensitive RNA polymerase on the other. It is clear, finally, that one or several insensitive RNA polymerases exist in the chromosomes which are responsible for the synthesis of a number of mainly low molecular weight RNA species.

REFERENCES

1. WIELAND, T. 1968. Poisonous principles of mushrooms of the genus Amanita. *Science (Washington).* 159:946.
2. STIRPE, F., and L. FIUME. 1967. Studies on the pathogenesis of liver necrosis by α-amanitin. *Biochim. J.* 105:779.
3. SEIFART, K. H., and C. E. SEKERIS. 1969. α-amanitin, a specific inhibitor of transcription by mammalian RNA-polymerase. *Z. Naturforsch.* 24 b:1538.
4. NOVELLO, F., and F. STIRPE. 1969. Experimental conditions affecting ribonucleic acid polymerase in isolated rat liver nuclei. *Biochem. J.* 112: 721.
5. JACOB, S. T., E. M. SAJDEL, and H. N. MUNRO. 1970. Different responses of soluble whole nuclear RNA polymerase and soluble nucleolar RNA polymerase to divalent cations and to inhibition by α-amanitin. *Biochem. Biophys. Res. Commun.* 38:765.
6. LINDELL, T. J., E. WEINBERG, P. W. MORRIS, R. G. ROEDER, and W. J. RUTTER. 1970. Specific inhibition of nuclear RNA polymerase II by α-amanitin. *Science (Washington).* 170:447.
7. ROEDER, R. G., R. H. REEDER, and D. D. BROWN. 1970. Multiple forms of RNA polymerase in *Xenopus laevis*: Their relationship to RNA synthesis *in vivo* and their fidelity of transcription *in vitro. Cold Spring Harbor Symp. Quant. Biol.* 35:727.
8. REEDER, R. G., and W. J. RUTTER. 1969. Multiple forms of DNA-dependent RNA polymerase in eukaryotic organisms. *Nature (London).* 224:234.
9. ROEDER, R. G., and W. J. RUTTER. 1970. Specific nucleolar and nucleoplasmic RNA polymerases. *Proc. Nat. Acad. Sci. U. S. A.* 65:675.
10. NIESSING, J., B. SCHNIEDERS, W. KUNZ, K. H. SEIFART, and C. E. SEKERIS. 1970. Inhibition of RNA synthesis by α-amanitin *in vivo. Z. Naturforsch.* 25 b:1119.
11. JACOB, S. T., W. MUECKE, E. M. SAJDEL, and H. N. MUNRO. 1970. Evidence for extranucleo-

lar control of RNA synthesis in the nucleolus. *Biochem. Biophys. Res. Commun.* **40:**334.

12. Wobus, U., R. Panitz, and E. Serfling. 1971. α-amanitin: its effect on RNA synthesis in polytene chromosomes. *Experientia (Basel).* **27:**1202.

13. Beermann, W. 1971. Effect of α-amanitin on puffing and intranuclear RNA synthesis in *Chironomus* salivary glands. *Chromosoma.* **34:**152.

14. Beermann, W. 1952. Chromomerenkonstanz und spezifische Modifikationen der Chromssomen Struktur in der Entwicklung und Organdifferenzierung von *Chironomus tentans.* *Chromosoma.* **5:**139.

15. Ringborg, U., and L. Rydlander. 1971. Nucleolar derived RNA in chromosomes, nuclear sap and cytoplasm of *Chironomus tentans* salivary gland cells. *J. Cell Biol.* **51:** 355.

16. Cannon, G. B. 1964. Culture of insect salivary glands in a chemically defined medium. *Science (Washington).* **146:**1063.

17. Edström, J.-E., E. Egyházi, B. Daneholt, B. Lambert, and U. Ringborg. 1972. Localization of newly synthesized low molecular weight RNA in polytene chromosomes from *Chironomus tentans.* *Chromosoma.* **35:**431.

18. Daneholt, B., J.-E. Edström, E. Egyházi, B. Lambert, and U. Ringborg. 1969. Chromosomal RNA synthesis in polytene chromosomes of *Chironomus tentans.* *Chromosoma.* **28:**399.

19. Egyházi, E., B. Daneholt, J.-E. Edström, B. Lambert, and U. Ringborg. 1969. Low molecular weight RNA in cell components of *Chironomus tentans* salivary glands. *J. Mol. Biol.* **44:**517.

20. Edström, J.-E., and B. Daneholt. 1967. Sedimentation properties of the newly synthesized RNA from isolated nuclear components of *Chironomus tentans* salivary gland cells. *J. Mol. Biol.* **28:**331.

21. Ringborg, U., B. Daneholt, J.-E. Edström, E. Egyházi, and B. Lambert. 1970. Electrophoretic characterization of nucleolar RNA from *Chironomus tentans* salivary gland cells. *J. Mol. Biol.* **51:**327.

22. Egyházi, E., B. Daneholt, J.-E. Edström, B. Lambert, and U. Ringborg. 1970. Differential inhibitory effect of a substituted benzimidazole on RNA labeling in polytene chromosomes. *J. Cell Biol.* **47:**516.

23. Ringborg, U., B. Daneholt, J.-E. Edström, E. Egyházi, and L. Rydlander. 1970. Evidence for transport of preribosomal RNA from the nucleolus to the chromosomes in *Chironomus tentans* salivary gland cells. *J. Mol. Biol.* **51:**679.

24. Ritossa, F. M., K. C. Atwood, and S. Spiegelman. 1966. On the redundancy of DNA complementary to amino acid transfer RNA and its absence from the nucleolar organizer region of *Drosophila melanogaster.* *Genetics.* **54:**663.

25. Tartof, K. D., and R. P. Perry. 1970. The 5 S RNA genes of *Drosophila melanogaster.* *J. Mol. Biol.* **51:**171.

26. Wimber, D. E., and D. M. Steffensen. 1970. Localization of 5 S genes on *Drosophila* chromosomes by RNA-DNA hybridization. *Science (Washington).* **170:**639.

27. Quincey, R. V. 1971. The number and location of genes for 5 S ribonucleic acid within the genome of *Drosophila melanogaster.* *Biochem. J.* **123:**227.