Nucleolar 4s Ribonucleic Acid in Dipteran Salivary Glands in the Presence of Inhibitor

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1. Salivary glands of insect larvae accumulate newly made transfer RNA in the nucleolus when maintained in the presence of nucleoside antagonists that inhibit RNA synthesis preferentially at the chromosome. 2. The nucleus contains precursor transfer RNA, which, on the basis of the general evidence, may originate in the chromosome and then be methylated in the nucleolus. 3. The maturation of precursor ribosomal RNA is blocked in the nucleolus during inhibition. 4. The transport of nuclear RNA to cytoplasm is also blocked. 5. It is suggested that, if the transfer RNA accumulated in the nucleolus does indeed originate in the chromosome, the accumulation may result from the blockage of an obligatory transient association of the RNA with the nucleolus.

During recent years several lines of investigation on various cell types seem to point in general to a chromosomal origin of transfer RNA (reviewed by Sirlin, 1968). On the other hand, as described in this paper, newly synthesized 4s RNA with the characteristics of transfer RNA is found mainly in nucleoli of salivary glands when these are maintained *in vitro* in the presence of substituted benzimidazoles.

MATERIALS AND METHODS

Biological material. The pre-metamorphic larvae of the chironomid *Smittia parthenogenetica* were used. Breeding and characterization of the stage III were as described previously (Birnstiel, Jacob & Sirlin, 1965; Sirlin, Jacob & Birnstiel, 1966; Sirlin, Jacob & Tandler, 1963).

Chemicals. DRB,† TRB and actinomycin D were gifts from Merck, Sharp and Dohme Inc., Rahway, N.J., U.S.A. Puromycin was presented by Lederle Laboratories, Pearl River, N.Y., U.S.A. *Escherichia coli* B soluble RNA was obtained from Schwarz BioResearch, Mount Vernon, N.Y., U.S.A. 'Highly polymerized' yeast RNA (4–10s, by chromatographic analysis on MAK column) was from British Drug Houses Ltd., Poole, Dorset. Tracers purchased from The Radiochemical Centre, Amersham, Bucks., were: [nominal 8-³H]guanosine (4500 mc/m-mole), DL-[1-¹⁴C]-leucine (36.6 mc/m-mole), L-[Me-¹⁴C]methionine (25.0–29.1 mc/m-mole), [2-¹⁴C]uridine (36.9 mc/m-mole), [5-³H]-

uridine (may contain about 2% of [6-³H]uridine) (22 400–31 000 mc/m-mole) and U-¹⁴C-labelled *Chlorella* protein hydrolysate (145 mc/g).

Incubation of glands. The incubation in synthetic medium was as described previously (Birnstiel *et al.* 1965; Sirlin *et al.* 1963, 1966), and particulars are given for each experiment. In more recent experiments, temperatures were maintained close to 25° in the thermal blanket underneath the sealed incubation chambers, and to 23° in the ambient, to prevent concentration of medium through excessive condensation.

According to several criteria the glands remain functional during incubation (Sirlin *et al.* 1963, 1966). Damaged glands lost their transparency and were discarded.

Radioautography. Radioautographs were prepared and measured quantitatively as described previously (Sirlin *et al.* 1963, 1966). Incorporation in batches prepared for chemical analysis was monitored visually in radioautographs made from a few glands. Though acetic acid does not solubilize RNA, as shown below, the squashing was done within 40 sec. as a precaution against mechanical loss.

When removal of RNA from glands was required before radioautography, ribonuclease (five-times crystallized; L. Light and Co. Ltd., Colnbrook, Bucks.) was dissolved (0.2 mg./ml.) at 37° in half-strength sodium barbiturate-sodium acetate buffer (Michaelis, 1931), pH 7 at room temperature, without NaCl, and immediately used on the preparations for 15–45 min. Controls were treated similarly but without enzyme. After digestion the slides were passed to ice-cold 5% (w/v) trichloroacetic acid for 5 min. Lanthanum was omitted when possible from the fixatives used for these glands.

Recovery of RNA in radioautography. (1) Squashes. The recovery of radioactivity in radioautographs of squashes as used for the measurements in Tables 2 and 3 was examined chemically in duplicate experiments. The results were identical. After radioactive incubation the glands were passed through the complete radioautography procedure:

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† Abbreviations: DRB, 5,6-dichloro-1-(β -D-ribofuranosyl)benzimidazole; TRB, 4,5,6-trichloro-1-(β -D-ribofuranosyl)benzimidazole; MAK, methylated albumin-kieselguhr; SDS, sodium dodecyl sulphate.

(Sirlin *et al.* 1963) up to film exposure, as follows. (a) The incubation medium was flooded with 5% (w/v) trichloroacetic acid (A.R.; British Drug Houses Ltd.) containing 1.6% (w/v) of $\text{LaCl}_3 \cdot 7\text{H}_2\text{O}$ (Hopkin and Williams Ltd., Chadwell Heath, Essex). The glands were promptly transferred to fresh acid for 20 min. and then passed to: (b) 45% (v/v) acetic acid (A.R.; British Drug Houses Ltd.) containing 1.6% (w/v) of $\text{LaCl}_3 \cdot 7\text{H}_2\text{O}$ for about 2 min. (during this time squashes were prepared on gelatin-subbed slides precooled to -10°) [slides were then taken through (e)]; (c) 5% trichloroacetic acid containing 1.6% (w/v) of $\text{LaCl}_3 \cdot 7\text{H}_2\text{O}$ for 30 min.; (d) sodium barbiturate-sodium acetate buffer (Michaelis, 1931), pH 5.1, without NaCl, for 30 min.; (e) water (pH 5.2) for 15 min. The glands were then (f) scraped off in water into a glass homogenizer kept in acetone-solid CO_2 . Homogenization was performed in an ice bath with ten strokes of a motor-driven tight-fitting Teflon pestle. All solutions contained uridine (3 mg./ml.). All solutions were at $2-4^\circ$ except (e), which was at about 21° .

The above fractions after removal of the glands were examined for RNA lost from the glands, by using the following techniques carried out in the cold, except where otherwise stated.

(a) The fraction (15 ml.) was made up to 5% trichloroacetic acid-1.6% $\text{LaCl}_3 \cdot 7\text{H}_2\text{O}$ (100 ml.), 2 mg. of carrier RNA (yeast, sodium salt; L. Light and Co. Ltd.) in 0.2 ml. of water was added slowly with stirring, and the mixture was left standing for 1 hr. The precipitated RNA was spun down, washed twice with ethanol and dissolved in 5 ml. of 0.05 M-tris (Trizma base; Sigma Chemical Co., St Louis, Mo., U.S.A.) containing uridine (3 mg./ml.), pH 9.0 at 20° . The small residue of gland debris was removed by centrifugation (it contained 4–8 μg . of RNA in this and following fractions, as determined spectrophotometrically after hot- HClO_4 hydrolysis). The supernatant was made to 5% trichloroacetic acid, and left standing for 1 hr. with occasional stirring. The RNA that is precipitated was spun down, washed twice with ethanol, once with ethanol-ether (3:1, v/v) for 30 min. with occasional stirring, and deep-frozen for later analysis.

(b) The fraction (5.5 ml.) was dialysed for 6 hr. against three changes of water (total more than 6 l.), made to 5% trichloroacetic acid- LaCl_3 (final vol. 100 ml.), and thereafter treated as (a).

(c) This fraction (90 ml.) was treated as (a).

(d) and (e) Portions (45 ml.) of each fraction (95 and 135 ml.) were pooled, made to 5% trichloroacetic acid- LaCl_3 (final vol. 100 ml.), and then treated as (a).

(f) The gland homogenate (12.5 ml.) was made to 5% trichloroacetic acid- LaCl_3 (final vol. 25 ml.), 0.5 mg. of RNA in 0.05 ml. of water was added, and the mixture was left standing for 1 hr. The precipitate was collected by centrifugation, washed twice with ethanol, once with ethanol-ether for 30 min., and deep-frozen.

Tests to determine the effectiveness of the above procedure were carried out, and lead to the following conclusions. (1) Loss of carrier yeast RNA as a result of treatment with ethanol after trichloroacetic acid, as found in cell homogenates by Steele & Busch (1964), was negligible. (2) Treatment with trichloroacetic acid- LaCl_3 for up to 6 hr., as in fraction (a), did not increase loss of RNA in the previous test. (3) With *Xenopus laevis* total RNA and with *E. coli* soluble RNA, no loss caused by treatment with 45% acetic acid- LaCl_3 was measurable by orcinol reaction or u.v.

absorption in total or acid-precipitable dialysis residue as in fraction (b). Cells swell in acetic acid as the result of protein hydration (Baker, 1958). (4) With highly [^3H]-uridine-labelled *E. coli* soluble RNA prepared by the method of Ehrenstein & Lipmann (1961), loss of radioactivity in the tris residue was negligible.

The RNA pellets were analysed essentially by the procedure of Loening (1965). Hydrolysis was overnight in 0.25 ml. of 0.33 N-KOH at 37° , under a layer of heptane. The digest was acidified to about pH 2.5 with 2 N- HClO_4 , then chilled on ice, and the precipitated KClO_4 centrifuged down at 0° . The supernatant was transferred to glass depressions, 0.1 ml. of 50 mM-potassium citrate buffer, pH 3.5, containing 50 μg . of uridine was added and the mixture was dried. The samples were dissolved in 0.1 ml. of water by rubbing with a glass rod, and divided into half-samples for electrophoresis. Nucleotides were separated at 40 v/cm. on Whatman no. 3MM paper in potassium citrate buffer cooled in carbon tetrachloride. The strips were monitored under u.v. until adequate separation was obtained (about 1 hr.) and dried. Individual spots and a blank ahead of them were cut out as ribbons into centrifuge tubes, and eluted overnight in 5 ml. of 0.02 N-HCl at room temperature. After removal of the paper, the samples were dried overnight at 58° , taken up in 0.4 ml. of water, mixed with 15 ml. of scintillation medium and counted in a Packard Tri-Carb spectrometer. The results are shown in Table 1. Blank counts were 1.5% of total nucleotide counts in the gland homogenate (f) and negligible elsewhere. The u.v. spectra of samples of the initial digest and separated nucleotides were scanned with a Unicam SP.800 spectrophotometer. The combined nucleotide peaks at 258 m μ showed a recovery of $66.8 \pm 2.8\%$. The recovered marker uridine was the same in fractions (a), (b), (c) and (d+e). Molar ratios for the major nucleotides in these fractions were similar. [The homogenate (f), with the bulk of gland RNA, had different values.]

Only fraction (a) has significant radioactivity, which amounts to 2.2% of that of (a) plus (f). The loss of RNA during the radioautography procedure was therefore negligible: about 98% of the labelled gland RNA was recovered.

(2) Sections. Radioautographs of sectioned glands incubated with [^3H]uridine and DRB-TRB show visually the same preponderant nucleolar radioactivity that is found in squashed glands (Table 2). After withdrawal of medium, 81 pairs of glands were immediately flooded with Carnoy's fixative (ethanol-chloroform-acetic acid; 6:3:1, by vol.) for 10 min., with fresh fixative for 10 min., passed through ethanol and benzene, and embedded for 1 hr. at 56° . Sections were cut at 2μ and spread at 45° on gelatin-subbed slides with ethanol. Treatment of slides was: benzene, ethanol, aq. 95% (v/v) ethanol, aq. 75% ethanol-3.6% (w/v) formaldehyde (saturated with CaCO_3 , pH 7.8) for 6 hr., aq. 75% and 50% ethanol, ice-cold 5% trichloroacetic acid for 5 min., and several changes of ice-cold aq. 1.6% $\text{LaCl}_3 \cdot 7\text{H}_2\text{O}$ for 1 hr. Radioautographs were coated directly on cold slides from the last solution. Some of the slides were digested with ribonuclease before acid treatment. The radioautographs from duplicate experiments were identical and are illustrated elsewhere (Sirlin, 1966).

In similar experiments with [^3H]guanosine, 15 pairs of glands were incubated with tracer and DRB-TRB as described in Fig. 6. The histological procedure starting with

Table 1. *Recovery of radioactive RNA after radioautography procedure*

Glands (294 pairs), in batches of 20 pairs, were first incubated for 110 min., and then with [5-³H]uridine (91 μ C/ml.) for 45 min. Fractions obtained during radioautography procedure after removal of the glands are described in the Materials and Methods section. Fraction (a), including the radioactive incubation medium, originally contained approx. 4×10^9 counts/20 min. as tracer, and possibly acid-soluble nucleotides released from the glands: in this fraction the radioactivities for nucleotides other than uridylic acid (given in parentheses) were calculated by taking the actual radioactivity in uridylic acid to be 84% of total nucleotide radioactivity as in the gland homogenate (f). This value accords with several determinations (see the Results section).

Fraction	Total net radioactivity (counts/20 min.)				
	(a)	(b)	(c)	(d) + (e)	(f) (gland homogenate)
Adenylic acid	(3)	3	0	2	155
Cytidylic acid	(6)	0	0	0	244
Guanylic acid	(54)	11	6	4	2360
Uridylic acid	331	0	0	0	14493
Total	394				17252

Carnoy's fixative was as the previous one, except that (a) benzene (before wax) was replaced by terpineol and (after wax) by xylene, (b) sections were spread with aq. LaCl_3 , (c) the ethanol series without formaldehyde was in the cold, and (d) radioautographs were coated in 1.5% (w/v) lanthanum triacetate (British Drug Houses Ltd.) to further immobilize RNA. Another 15 pairs were incubated similarly and flooded with ice-cold 10% trichloroacetic acid–3.2% $\text{LaCl}_3 \cdot 7\text{H}_2\text{O}$ (which might displace cell RNA less than Carnoy's mixture). These glands were quickly passed to 5% trichloroacetic acid for 30 min., washed with 0.2M-acetate buffer, pH 4.9, treated with aq. 70% and 95% (v/v) ethanol followed by ethanol, and thereafter as the Carnoy-fixed glands. All aqueous solutions contained 1.6% $\text{LaCl}_3 \cdot 7\text{H}_2\text{O}$ and were ice-cold. Radioautographs showed a qualitatively identical intracellular distribution of [³H]-guanosine with the two fixatives. This resembled in its mainly nucleolar localization the distribution of [³H]uridine (Table 2). Incorporation was into RNA, as shown by ribonuclease digestion.

Loss of RNA during the procedure used for the uridine-labelled glands was examined spectrophotometrically. A number of glands (216 pairs) were incubated for 45 min. in sucrose medium, pH 7.6 (W. Beermann, cited by Clever, 1964), to replace the standard incubation medium, which contains u.v.-absorbing ingredients. The glands were processed in bulk as described, except that LaCl_3 and trichloroacetic acid were omitted, and water at 26° (the temperature at coating of the radioautographs) for 5 min. was added. (Mechanical loss from embedding onwards was 42% of the input $2.5 E_{260}^{1\text{cm}}$ units, estimated from homogenates hydrolysed in hot HClO_4 .) Extraction or concentration (or both) of pre-embedding fluids and water indicated that only traces of nucleotide could have escaped detection in Carnoy's fixative owing to blank absorption, in agreement with previous investigation of this fixative (Edström, Grampp & Schor, 1961). The other fluids showed no nucleotide absorption.

Chemical analysis of label in fixed glands. The nature of the predominant uridylic acid label of the glands in radioautographs (Table 1) was examined after alkaline hydrolysis.

If the label was in RNA it would be found in the 2',3'-isomers. This was demonstrated in the following experiment. A number of glands (380 pairs) were incubated as indicated but with DRB and TRB present (as in Table 2). After transfer to a centrifuge tube, the procedure followed closely that described for radioautography of squashes. Towards the end of each step the glands were packed down in a Sorvall RC2-B centrifuge. Squashing was reproduced by homogenization in acetic acid (which unavoidably took 10 min., mostly at -8°). RNA was finally solubilized from the glands with 0.3N-KOH at 37° for 1 hr., the residue was centrifuged off, and hydrolysis continued for 15 hr. Preparation of the digest for electrophoresis was as before, with uridine 3'-phosphate, uridine 5'-phosphate and uridine added as markers. Electrophoresis was in 0.05M-sodium borate buffer, pH 9.2 (Jaenicke & Vollbrechtshausen, 1952), at 900 v and 10–20 mA for 105 min. The order of separation from the origin is: uridine, adenylic acid, cytidylic acid, guanylic acid, uridine 3'-phosphate and uridine 5'-phosphate. After elution uridine 3'-phosphate (about 800 counts/min.) had 84% and 96.2% of total nucleotide and uridine 3'-phosphate + uridine 5'-phosphate radioactivity respectively. Uridine had 2.4% of the nucleotide radioactivity. A blank ahead of uridine 5'-phosphate had 16.5% of the uridine + nucleotide radioactivity. The alkaline residue (DNA) had very little radioactivity, as expected. Total recovered radioactivity was 88% of the digest. From these results, at least 70% of the acid-fixed radioactivity in the DRB-TRB-treated cell that is recovered in radioautographs (see above) is in RNA. A preliminary experiment gave similar results.

Preparation of RNA. For sedimentation analysis the RNA from glands frozen on solid CO_2 was extracted with phenol and aq. 1% (w/v) SDS ('purified'; British Drug Houses Ltd.) followed by phenol at 60° (Birnstiel *et al.* 1965). *X. laevis* RNA extracted by the same procedure was used as carrier. The yield of RNA was better than 80% of acid-precipitable radioactivity, and did not materially improve with re-extraction.

For gel electrophoresis and MAK chromatography several extraction procedures were used. (a) One vol. of 2% SDS

was added to the incubation medium at room temperature (final pH 8.0). (b) In other experiments 0.6% SDS (final concn.) was used with 0.23 M-NaCl to stabilize secondary structure of RNA (Kirby, 1966). (The incubation medium contains 0.12 M-NaCl.) (c) Washed bentonite (Saponara & Enger, 1966) was added at 2–4 mg./ml. with or soon after 1% SDS (final concn.; pH 8.0). (d) Two vol. or more of cold 2% (w/v) tri-isopropylmethylsulphonic acid (sodium salt; Eastman Kodak Co., Rochester, N.Y., U.S.A.) in 0.06 M-sodium acetate was added to the glands previously quick-cooled over ice-salt (final pH 6.0). (e) One vol. of SDS was followed by 1 vol. of tri-isopropylmethylsulphonic acid. After these procedures the lysed glands were frozen inside a glass homogenizer on solid CO₂. The resulting RNA separations after these treatments were qualitatively similar.

The lysed glands were thawed by homogenization in one of the above detergent mixtures with a Teflon pestle in ice, and, after addition of an equal volume of phenol-cresol-hydroxyquinoline (Kirby, 1966; *m*-cresol was redistilled), the homogenization was repeated for a few minutes with the homogenizer warmed by the hand. After shaking for 30 min. at room temperature, the aqueous phase and interphase were collected by centrifugation and shaken with fresh phenol mixture. [Failure to re-extract the interphase caused a loss of 30s ribosomal RNA (by extinction), without affecting the radioactive profile shown in Fig. 2.] The aqueous phase was extracted once or twice more, and made to 0.5 M-NaCl for the last extraction. After adding 2% (w/v) sodium acetate, pH 5.0, and carrier ribonucleoside or methionine (generally 500 µg.) the RNA was precipitated with ethanol. To eliminate opalescence and low-molecular-weight ³H-labelled impurities (observed with gel electrophoresis) the samples were dissolved in a minimum volume of 0.15 M-sodium acetate–0.5% SDS, pH 5.8, precipitated with ethanol, and the precipitation in the presence of SDS was repeated two or three times. When required, carrier *E. coli* soluble RNA was added before the precipitation.

To check for radioactivity lost during extraction, the re-extracted interphase and (second) phenol phase of a double-labelling experiment with [³H]uridine and [*Me*-¹⁴C]-methionine (Fig. 10) were extracted at 60° in the presence of carrier uridine and methionine and 1% SDS. No further ¹⁴C or ³H radioactivity was obtained in RNA.

Analysis of RNA. Particulars of sucrose-gradient sedimentation and radioactive measurement have been given previously (Birnstiel *et al.* 1965). The [¹⁴C]methylated 4s RNA had less than 2% of the radioactivity as methionyl-RNA. As a routine the amino acid was completely cleaved by heating at 37° in 0.1 M-tris, pH 9.4, for 30–60 min. The protein contamination in this region of the sedimentation was about 5% by radioactivity measurements.

Polyacrylamide-gel electrophoresis was by the procedure of Loening (1967). The buffers used were: (a) 40 mM-tris-acetic acid (pH 7.6)–20 mM-sodium acetate–2 mM-EDTA–0.2% SDS and (b) 36 mM-tris-HCl (pH 7.7)–30 mM-sodium phosphate–1 mM-EDTA–0.2% SDS. The separations were run at 40–50 v and 5–6.5 ma for up to 1.5 hr. at room temperature. The gels were scanned at 280 mµ with a Joyce-Loebl Chromoscan instrument; under these conditions the measured extinction is not proportional to RNA concentration. Gel slices were obtained as previously described and then individually placed in scintillation vials. To hydrolyse the RNA the gel slices were heated at 60° in

0.5 ml. of 10% (v/v) piperidine for several hours, and then allowed to dry. Then 0.5 ml. of water was added, which causes the gel slices to swell to several times their normal volume, followed by 15 ml. of a water-miscible scintillation fluid. The radioactivity of RNA was measured. Low-radioactivity samples were counted until the profile was reproducible.

MAK chromatography was carried out by the method of Mandell & Hershey (1960), except that methylation of the albumin was prolonged to 5 days at 37° to ensure a more basic product. To improve resolution of RNA peaks, a bottom layer (1 ml.) was prepared by mixing 0.5 ml. of aq. 1% (w/v) methylated albumin with 1.0 g. of kieselguhr suspended in 5 ml. of 0.1 M-NaCl–0.05 M-sodium phosphate buffer (pH 6.25). The remainder was mixed with 0.6 g. of kieselguhr suspended similarly and used for the top layer. The columns were 1.1 cm. × 6.0 cm. The RNA was applied and then washed with 35 ml. of 0.3 M-NaCl in buffer (2–5 ml. was left above the column); elution was with a linear 0.3–1.2 M-NaCl gradient (total 100 ml.). After measurement of the extinction of each fraction (2.4 ml.) indicated, 15 µg. of 'highly polymerized' yeast RNA was added to each fraction, precipitated with acid, collected and rinsed with ethanol and ether on Whatman GF/A disks. These were counted in a Beckman LS-200 spectrometer to at least ± 5% (2σ) error. In double-labelling experiments the counts were corrected for 'spill' by using the pre-set and adjustable modules of the instrument calibrated against standards. The washes contained little acid-precipitable radioactivity, but as much non-precipitable radioactivity as the RNA recovered from the column; this possibly corresponds to the impurities found with gel electrophoresis. Sometimes the gradient was followed by 1.2 M-NaCl in buffer or by *N*-NaOH (12 ml.), without release of significant radioactivity. About 88% of ¹⁴C-labelled amino acid mixture associated with 4s RNA in MAK separations (Fig. 10) was released at pH 8.8 (at 37° for 45 min.) with little loss of [³H]RNA. To correct for radioactive DNA in experiments with [8-³H]guanosine, the counted samples were solubilized with 0.3 N-KOH at 37° for 1 hr. The radioactivity precipitated with acid in the presence of carrier RNA is in DNA (Hutchison & Munro, 1961).

For pseudouridylic acid analysis in MAK separation, the [²⁻¹⁴C]uridine-labelled RNA was mixed with 1.35 mg. of *E. coli* soluble RNA as carrier, precipitated with acid and washed twice with aq. 80% (v/v) ethanol. Nucleotides obtained by alkaline hydrolysis were separated by electrophoresis in 0.1 M-ammonium formate (pH 3.5) on Whatman no. 3MM paper cooled in carbon tetrachloride (Bergquist, 1966). Second-dimension (descending) chromatography was in aq. 70% (v/v) propan-2-ol with 5% (v/v) of aq. NH₃ soln. (sp.gr. 0.88) in the solvent at the bottom of the tank (Markham & Smith, 1952) for 18 hr. The spots were eluted with water, dried with 1 drop of detergent on 2 in. planchets and counted in a Beckman Lowbeta instrument. The position of pseudouridylic acid (Watson & Ralph, 1966) was confirmed by u.v.-absorption spectrum.

Sedimentation coefficients for *Smittia* (larvae at post-feeding stage) were determined in the Spinco model E analytical ultracentrifuge, with *X. laevis* 18s and 28s RNA as internal controls. The RNA was extracted with phenol-SDS, at high ionic strength (0.2 M-NaCl) and low temperature to prevent loss of secondary structure (Kirby, 1966). The 19s and 30s *S*_{20,w} values found for ribosomal RNA agree

Table 2. *Radioautography of RNA synthesis*

Glands were incubated singly first for 110 min., and then with [^3H]uridine (91 $\mu\text{C}/\text{ml.}$) for 45 min., either with or without DRB and TRB (40 $\mu\text{g.}/\text{ml.}$ each) present throughout incubation. Radioautographs were prepared from squashes. Cell radioactivity is removed by digestion at 37° with ribonuclease (0.2 mg./ml.), buffered to pH 7, for 30 min. (see the Materials and Methods section), except for sporadic traces in chromosomes that probably are in DNA (see the text). Results are given as means $\pm$ s.e.m. of the numbers of glands given in parentheses.

	Net radioactivity (grain counts)		Inhibition (%)
	Controls	DRB-TRB	
Nucleolus*	360.3 $\pm$ 39.5 (12)	232.4 $\pm$ 34.7 (11)	35.5
Chromosomes*	185.2 $\pm$ 38.7 (12)	20.9 $\pm$ 3.7 (11)	88.7
Cytoplasm†	(157.6 $\pm$ 16.4) (12)	(8.0 $\pm$ 4.0) (11)	94.9

* Total grain count.

† Grain count over about one-quarter of cytoplasm, multiplied by 4.

Table 3. *Radioautography of RNA methylation*

Glands were incubated singly first for 30 min., and then with [$\text{Me-}^{14}\text{C}$]methionine (50 $\mu\text{C}/\text{ml.}$) for 30 min. Puromycin (300 $\mu\text{g.}/\text{ml.}$) was present throughout incubation. Squash preparations of the glands were (a) not treated, or were treated (b) with buffer and (c) with buffer and ribonuclease as in Table 2, before radioautography. Decrease of cytoplasmic radioactivity in buffer alone is caused by loss of ^{14}C -labelled protein (Birnstiel *et al.* 1965). Most nucleolar counts after ribonuclease are due to nearby cytoplasm. Chromosomal radioactivity (not included) behaves as that in cytoplasm, which excludes significant DNA methylation. Net grain counts $\pm$ s.e.m. are for total nucleoli, and for 8550 μ^2 of cytoplasm. Figures in parentheses indicate the numbers of glands used.

	Net radioactivity (grain counts)			Inhibition from (a) to (c) (%)
	Untreated (a)	Buffer (b)	Buffer + ribonuclease (c)	
Nucleolus	91.8 $\pm$ 4.7 (46)	102.6 $\pm$ 7.4 (31)	10.0 $\pm$ 0.2 (58)	89.1
Cytoplasm	328.7 $\pm$ 18.7 (46)	242.1 $\pm$ 10.1 (30)	299.9 $\pm$ 7.1 (58)	8.8

with those in *Drosophila* (Kirby, 1966). In carrier-free separations the conventional values 4 and 5 s are taken from other organisms.

RESULTS

Nucleolar RNA synthesis in intact glands

Experiments with [^3H]uridine. Treatment with chlorine-substituted benzimidazoles results in inhibition of RNA synthesis as shown in Table 2. In terms of radioactive RNA, the treated intact gland virtually represents a sample of the nucleolus. Since, as discussed below, the sampling with radioautography is as complete as with chemical extractions, the total radioactive RNA extracted from these glands can be regarded as 'nucleolar' RNA; it contains 11% of non-nucleolar RNA. Radioautographs of treated cells are illustrated elsewhere (Sirlin, 1966).

The total RNA and 'nucleolar' RNA measured in the radioautographs each contains 84% of the radioactivity in UMP as determined by two independent methods, of which 96.2% is present as

the 2',3'-isomers after alkaline hydrolysis (see the Materials and Methods section). The first value compares well with 84.7% and 94.6% UMP by radioactivity found for the extracted 'nucleolar' RNA (Birnstiel *et al.* 1965). The results indicate RNA synthesis.

Nucleolar RNA synthesis is qualitatively identical in radioautographs of squashes or of sections with two fixatives, as described in the Materials and Methods section. The technique has a recovery of RNA of the same order as chemical extractions with phenol-SDS or phenol-tri-isopropyl-naphthalene-sulphonic acid (see the Materials and Methods section). It is therefore possible to correlate the chemical data with the distribution of RNA in the cell.

DRB and TRB act at the level of RNA precursor pools by virtue of their antagonism to purine ribosides. They do not interfere with DNA transcription (Sirlin *et al.* 1966), or directly affect protein synthesis (Allfrey, Mirsky & Osawa, 1957). The reason for the selective chromosomal inhibition remains unknown, but has also been observed in the starfish oocyte (Geuskens, 1961, see p. 159).

DRB and TRB finally inhibit cell RNA synthesis completely, and de-inhibition follows their withdrawal (Sirlin & Jacob, 1964). A dramatic 'rebound' of synthesis above the normal is then found.

Experiments with [Me-¹⁴C]methionine. Puro-mycin inhibits protein synthesis without altering the radioactive profile of sedimented RNA (Birnstiel *et al.* 1965). This allows a demonstration of radioactivity located in the nucleolus that is insensitive to the antibiotic, high pH or neutral hydroxylamine (Sirlin *et al.* 1963), but is sensitive to hot-trichloro-acetic acid hydrolysis (Birnstiel *et al.* 1965) or ribonuclease. Only the nucleolar radioactivity is sensitive to the enzyme (Table 3), indicating that this radioactivity is in RNA. After alkaline hydrolysis the radioactivity behaves chromatographically as methylated purine and pyrimidine nucleotides (Sirlin *et al.* 1963), and therefore represents RNA methylation. Radioautographs are illustrated elsewhere (Sirlin *et al.* 1963). The total radioactive RNA extracted from these glands is therefore taken as 'nucleolar' RNA.

Radioactive profile of low-molecular-weight RNA

The newly synthesized 'nucleolar' RNA is shown by sedimentation analysis to consist of high- and low-molecular-weight RNA (Fig. 1), with little of the ribosomal RNA and polydisperse RNA present in the whole cell.

A similar profile is obtained with gel electrophoresis (Fig. 2). The first optical peak corresponding to DNA has a little label (possibly due to contaminant [6-³H]uridine) as a shoulder on RNA label.

Fig. 3 shows the low-molecular-weight RNA fractionated at a higher gel concentration. The bulk of radioactivity coincides with the 4s optical peak. Some radioactivity trails behind, but little is associated with the 5s peak. Two minor unlabelled optical peaks derived from whole cell RNA have been consistently observed in these separations.

An MAK profile is shown in Fig. 4. The optical peaks corresponding to the marker RNA are in order of elution: 4s, 5s (confirmed by gel separation) and DNA (0.11%, according to Schwarz Bio-Research specification). Usually, the 4s RNA peak trails more than in gel separations. Peaks of radioactivity accompany the 4s and 5s RNA, with an intermediate peak at 0.65M-sodium chloride that is discussed below. DNA is not labelled. High-molecular-weight radioactivity shows a peak behind ribosomal RNA; it has a radioactive shoulder at the tail. It should be noted that polyuridylic acid (4-6s) is not eluted from the MAK column with M-sodium chloride (Asano, 1965), and that in the present experiments no radioactivity is retained in the column. This excludes the presence of poly-

Fig. 1. Sucrose-gradient sedimentation of uridine-labelled 'nucleolar' RNA. Control glands (84 pairs) were incubated first for 110 min., and then with [5-³H]uridine (91 μ C/ml.) for 45 min. Other glands (199 pairs) were incubated as the controls, except that DRB and TRB (40 μ g./ml. each) were present throughout incubation. In the presence of DRB and TRB about 90% of the radioactive RNA is nucleolar (Table 2). The number written on each control peak represents the percentage of radioactivity resisting inhibition calculated on a per gland basis. The RNA was extracted with phenol-SDS as described in the Materials and Methods section and sedimented in 5-20% (w/v) sucrose gradient in 10 mM-tris (pH 7.2)-1% (v/v) water-saturated phenol. Sedimentation coefficients correspond to those of carrier *X. laevis* RNA.

uridylic acid in, and the absence of any RNA species from, the separations.

The content of pseudouridine is higher in the region of 4s RNA, as shown by the following experiment. A number of glands (740 pairs) were incubated first with DRB and TRB as in Fig. 2, and then in similar medium with [2-¹⁴C]uridine (20 μ C/ml.) for 45 min. The intracellular distribution of [¹⁴C]RNA by radioautography is qualitatively identical with that in Table 2. The extracted RNA was separated on an MAK column together with 50 μ g. of *E. coli* soluble RNA, and fractions were pooled in two groups of six each comprising the bulk of 4s and 5s RNA respectively. The ratios ($10 \times \psi$ UMP counts/UMP counts) (see the Materials and Methods section) were 1.54 and 0.12. UMP in 4s RNA had $5.6 \pm 5\%$ (2σ) counts/min.

Insect soluble RNA contains considerably more guanosine + cytosine than ribosomal RNA, i.e. 60%

Fig. 2. Polyacrylamide-gel electrophoresis of uridine-labelled 'nucleolar' RNA. Glands (502 pairs) were incubated with DRB, TRB and [^3H]uridine as indicated in Fig. 1. One-fifth of the RNA, extracted as described in the Materials and Methods section, was fractionated carrier-free in a 2.4% (w/v) polyacrylamide gel. Fractions 11–36 were counted in pairs. —, E_{280} ; ----, radioactivity.

Fig. 3. Polyacrylamide-gel electrophoresis of low-molecular-weight uridine-labelled 'nucleolar' RNA. A same amount of the sample in Fig. 2 was fractionated carrier-free in a 7.5% gel. Fractions 1–24 were counted in triplets. At this gel concentration the ribosomal RNA does not leave the origin. Arrows point to unlabelled optical peaks derived from whole-cell RNA that are encountered in this region. —, E_{280} ; ----, radioactivity.

Fig. 4. MAK-column chromatography of uridine-labelled 'nucleolar' RNA. One-and-a-half times the amount of the sample in Fig. 2 was fractionated by MAK as described in the Materials and Methods section. A sample (200 μg .) of authentic *E. coli* soluble RNA ($S_{20,w}$ 3.9s) was included. The two major ribosomal RNA species were eluted as a single optical peak. —, E_{280} ; ----, radioactivity.

Fig. 5. MAK-column chromatography of guanosine-labelled 'nucleolar' RNA. Glands (350 pairs) were incubated first with DRB and TRB as in Fig. 2, and then in similar medium with [^3H]guanosine (57 $\mu\text{C}/\text{ml}$.) for 45 min. The RNA was fractionated on a MAK column with 50 μg of *E. coli* soluble RNA. The approximate position of 5s RNA is indicated. —, E_{280} ; ----, radioactivity.

and 43% (Kirby, 1966). Fig. 5 shows that [^3H]guanosine in effect labels low-molecular-weight RNA relatively more than does [^3H]uridine (cf. Fig. 4). Most of the radioactivity is eluted at about 0.5M-sodium chloride with 4s RNA. A minor peak of radioactivity is eluted at about 0.7M-sodium chloride with 5s RNA. The radioactive shoulder at 0.8M-sodium chloride includes DNA (see below). In radioautographs the nucleolar proportion of radioactivity in these cells is as high as or higher than

Fig. 6. Polyacrylamide-gel electrophoresis of guanosine-labelled 'nucleolar' RNA. Glands (530 pairs) were incubated first with DRB and TRB, and then with $[8\text{-}^3\text{H}]\text{guanosine}$ as in Fig. 5 for 40 min. Half of the RNA was separated carrier-free in a 7.5% gel. Fractions 1–40 were counted in groups of four. At this gel concentration DNA does not leave the origin. —, E_{280} ; ----, radioactivity.

Fig. 7. MAK-column chromatography of guanosine-labelled 'nucleolar' RNA. The other half of the sample in Fig. 6 was fractionated on an MAK column with 50 μg . of *E. coli* soluble RNA. Analysis for DNA as described in the Materials and Methods section showed that fraction B contained about 40% of DNA by radioactivity, fraction A none. —, E_{280} ; ----, radioactivity.

shown in Table 2 with DRB–TRB, i.e. the radioactivity is practically all nucleolar. This radioactivity is ribonuclease-sensitive, in contrast with some residual radioactivity (DNA) in chromosomes.

The electrophoresis and chromatography profiles of $[^3\text{H}]\text{guanosine}$ -labelled RNA were compared. In the low-molecular-weight region, electrophoresis (Fig. 6) shows a single trailing radioactive peak at 4s. The chromatography profile (Fig. 7) is in substantial agreement, since there is little radioactive 5s RNA in the separation; the second radioactive peak includes a substantial amount of DNA. Fig. 6 provides also a comparison with uridine label in the low-molecular-weight RNA region (cf. Fig. 3).

Fig. 8. Radioactivity in low-molecular-weight RNA after brief incubation with uridine. (a) Glands (490 pairs) were incubated first with DRB and TRB as in Fig. 2, and then in similar medium with $[5\text{-}^3\text{H}]\text{uridine}$ (182 $\mu\text{C}/\text{ml}$.) for 15 min. (b) Glands (325 pairs) were incubated as in (a) except that the radioactive incubation (91 $\mu\text{C}/\text{ml}$.) was for 35 min. The RNA species were separated carrier-free on MAK columns. —, E_{280} ; ----, radioactivity.

Time-course of incorporation

An approximate evaluation of the kinetics of RNA synthesis was obtained from the relative rates of accumulation of (peak) radioactivity into the different components. Specific activities could not usually be obtained since the amounts of RNA were too small.

The radioactivity eluted from MAK column is higher at about 0.6M-sodium chloride than it is in 4s RNA at 15min. incubation with $[^3\text{H}]\text{uridine}$ (Fig. 8a). At 35min. the two radioactivities are equal (Fig. 8b). At 45min. the 4s RNA radioactivity is higher (Fig. 4).

A similar pattern is seen after labelling with $[^3\text{H}]\text{guanosine}$. The radioactivity profile at 30min. (Fig. 9) is higher at about 0.6M-sodium chloride relative to 4s RNA than it is at 40min. (Fig. 7) or 45min. (Fig. 5).

The radioactivity in 5s RNA shows no relationship to either the 4s or to the 0.6M-sodium chloride RNA radioactivity from 15 to 45min. incubation with $[^3\text{H}]\text{uridine}$. On the other hand, the 5s RNA radioactivity remains at a value corresponding to

Fig. 9. Radioactivity in low-molecular-weight RNA after brief incubation with guanosine. Glands (350 pairs) were incubated first with DRB and TRB, and then with $[8\text{-}^3\text{H}]\text{-guanosine}$ as in Fig. 5 for 30 min. The RNA was separated on an MAK column with 100 $\mu\text{g.}$ of *E. coli* soluble RNA. —, E_{260} ; ----, radioactivity.

about one-half of the radioactivity eluted behind the ribosomal peak. This is not clear with guanosine labelling because of radioactivity present in DNA.

These observations suggest that the RNA eluted at about 0.6M-sodium chloride is a precursor of 4s RNA. In contrast, 5s RNA behaves independently, but co-ordinately with the high-molecular-weight RNA.

That the analysis of *Smittia* RNA with marker *E. coli* RNA is substantially correct was shown later with mealworm (*Tenebrio molitor*) RNA. The optical profile of *Tenebrio* RNA in MAK separations identical with the present ones resembles closely that of *E. coli* RNA. The optical peaks are at: 4s RNA (0.55M-sodium chloride); 5s RNA (0.66M-sodium chloride); DNA (0.85M-sodium chloride).

Substitution of 4s RNA

Aminoacylation of 4s RNA. Amino acids are incorporated only in 4s RNA (Fig. 10). About 88% of the radioactivity in fractions 11–17 was released at pH 8.8 as described in the Materials and Methods section. This indicates aminoacylation of RNA. (The ^{14}C -labelled protein hydrolysate does not contain methionine.)

Methylation of 4s RNA. RNA methylation takes place in the nucleolus (Table 3) within 5 min. of incubation (Sirlin *et al.* 1966). Sedimentation analysis showed that methylation as followed by incorporation of label from $\text{L-[Me-}^{14}\text{C]methionine}$ is almost exclusively in 4s RNA. This is also seen in MAK and electrophoresis separations (Fig. 10). As expected, 5s RNA is not methylated. DNA is also not methylated. In both types of separation the methylation profile trails as a shoulder into the precursor (about 0.6M-sodium chloride) region; the specific activity of RNA in this region is less than in 4s RNA. Some methylation occurs in the high-

molecular-weight RNA behind the ribosomal peak, which itself is practically not methylated. More than 51% of ^{14}C in fractions 12–16 in the MAK separation remained acid-precipitable after pH 8.8 treatment. This indicates that a substantial amount of label is in RNA and not as methionyl-RNA. The preponderance of ^{14}C in 4s RNA eliminates the possibility that C_1 units were directly incorporated into nucleosides, as first shown by chromatography (Sirlin *et al.* 1963).

The profile after DRB and TRB incubation in Fig. 10 is very similar to that after incubation without DRB and TRB (Sirlin *et al.* 1966), except perhaps for a probably greater methylation of high-molecular-weight RNA without DRB and TRB.

Effect of actinomycin on synthesis and methylation of 4s RNA. Actinomycin, which inhibits nucleolar RNA synthesis almost selectively (Sirlin, Jacob & Kato, 1962), gradually cancels the nucleolar RNA methylation as well (Sirlin *et al.* 1963). From these radioautography observations it was expected that the antibiotic could also be used in studies on the extracted RNA but in the direction opposite to that with DRB and TRB. This proved impossible because of the massive degradation of newly labelled high-molecular-weight RNA observed in the sedimentation profile after incubation with actinomycin at a concentration as low as 0.1 $\mu\text{g./ml.}$ (Sirlin *et al.* 1966). This degradation is paralleled by an excessive accumulation of radioactivity in the cytoplasm as seen with radioautography. At higher concentrations of actinomycin (0.2 and 0.5 $\mu\text{g./ml.}$) the sedimented radioactivity that is incorporated into RNA is greatly decreased; many nucleoli are destroyed. Degradation of RNA in the presence of the antibiotic is known (Harris, 1965; Reich & Goldberg, 1964), and may be caused by a Mg^{2+} -dependent nuclease (Mandal & Abrams, 1967).

However, with methylation actinomycin provided information that is the expected counterpart of DRB and TRB. The inhibition of methylation observed in the sedimentation profile parallels approximately that of RNA synthesis, but it is relatively more severe at low doses (Sirlin *et al.* 1966). This reinforces the inference from radioautography observation that little of the actinomycin-treated RNA remaining at the top of the sedimentation can be 4s RNA capable of methylation. (The amount of 4s RNA in the sedimentation cannot be estimated in the presence of degraded high-molecular-weight RNA.) These findings indicate the rapid exhaustion of an RNA substrate available for immediate methylation. It follows that methylation depends closely on synthesis. Direct evidence for this is the recovery of $[^{14}\text{C}]\text{uridine}$ as $[^{14}\text{C}]\text{ribothymidine}$ in 'nucleolar' RNA (Birnstiel *et al.* 1965).

Fig. 10. Aminoacylation and methylation of 4s RNA. Glands (825 pairs) were incubated first with DRB and TRB for 110 min., and then in similar medium with $[1\text{-}^{14}\text{C}]$ leucine ($23\text{ }\mu\text{C}/\text{ml.}$) and ^{14}C -labelled *Chlorella* protein hydrolysate ($60\text{ }\mu\text{C}/\text{ml.}$) for 45 min. Glands (730 pairs) were similarly incubated, but with only $[Me\text{-}^{14}\text{C}]$ methionine ($45\text{ }\mu\text{C}/\text{ml.}$) in the second incubation. Both experiments included $[^3\text{H}]$ uridine as reference (not illustrated). The first RNA was extracted with phenol-tri-isopropyl-naphthalenesulphonic acid, pH 5.4. The RNA samples were fractionated carrier-free on separate MAK columns, and the radioactive profiles were aligned by the optical profiles. One-third of the methionine-labelled RNA was separated in a 7.5% gel (insert). —, E_{260} or E_{280} ; ·····, methionine radioactivity; ----, amino acid radioactivity.

Since methylation occurs only in newly synthesized RNA, the observation that 4s RNA methylation continues after suppression of practically all non-nucleolar RNA synthesis with DRB and TRB (Fig. 10) in effect establishes that 4s RNA methylation and nucleolar RNA methylation (Table 3) are equivalent. Nucleolar methylation is not affected visually in radioautographs by DRB and TRB.

4s RNA is not a degradation product

Several lines of evidence indicate that the observed 4s RNA is not the result of degradation of larger RNA molecules. The total radioactivity profile conforms to the optical profile after several extraction procedures whereby the glands are directly brought in contact with detergents that inhibit nucleases (Kirby, 1966). The procedure eliminates nuclease activity (Sirlin *et al.* 1966).

DRB and TRB act at the precursor pool level and on this basis should not cause (faulty) synthesis of an abnormally small molecule. In these respects, DRB and TRB differ radically from actinomycin, which acts at the template level and results in extensive degradation at lower concentration. 4s RNA is not a degradation product since whole-cell

radioactive RNA is not degraded in the presence of DRB and TRB after synthesis (Birnstiel *et al.* 1965).

As described above, methylation depends on synthesis and is almost all in 4s RNA, even after inhibition of practically all RNA synthesis. It follows that 4s RNA does not derive from larger molecules. This is also clear from the compositional shift of the radioactive profile with tritiated uridine and guanosine (compare Figs. 4 and 5). The time-course of radioactivity incorporation into high-molecular-weight RNA indicates stability of the molecules during the incubation periods used.

That the RNA is transfer RNA is shown by these criteria: (a) it is eluted from MAK column indistinguishably from aminoacyl-transfer RNA; (b) it contains pseudouridine, ribothymidine and other methylated nucleosides; (c) it shows a different kinetics from 5s ribosomal RNA and high-molecular-weight RNA (i.e. ribosomal precursor; see the Discussion section).

DISCUSSION

The salivary glands were maintained at a temperature (25°) used as a routine in insect tissue culture (Jones & Cunningham, 1961). On several

functional and structural criteria the cells remain normal for several hours (Jacob & Sirlin, 1964; Sirlin *et al.* 1966), and as shown here synthesize all types of RNA. After removal of DRB and TRB from the incubation medium the synthesis in the cell is totally restored (Sirlin & Jacob, 1964). For these reasons, a nucleolar lesion such as reported by Simard & Bernhard (1967) in mammalian cells exposed to 42° does not seem to explain the accumulation of RNA that has been found.

In the normal cell about one half of the RNA radioactivity is found in the nucleolus and the other half in the chromosomes and cytoplasm (Table 2). The sedimentation profile in Fig. 1 shows that all RNA species are represented. The DRB-TRB-treated cell has 37% of the normal radioactivity, and the loss is mainly borne by the chromosomes and cytoplasm. On a per gland basis, this loss is almost quantitatively reflected in the disappearance of radioactive RNA species in the intermediate sedimentation range (cf. Sirlin, Jacob & Birnstiel, 1965).

A precursor of transfer RNA was put forward (Comb & Silver, 1966; Burdon, Martin & Lal, 1967) on the basis of its kinetics. Compared with transfer RNA, it has a similar composition, is eluted from MAK column at higher salt concentration, is less retarded by Sephadex gel filtration, and is sub-methylated. The RNA in *Smittia* that is eluted from MAK column at about 0.6M-sodium chloride has these characteristics. It is less distinguishable by gel electrophoresis. It accepts little amino acid (Fig. 10), which suggests that it cannot as yet interact with the cognate enzymes. With gel filtration the region of precursor transfer RNA shows several early radioactive peaks (Burdon *et al.* 1967). It was suggested that the RNA in these peaks could have a looser secondary structure than mature transfer RNA, or a greater susceptibility to denaturation resulting in conformational changes that determine a faster rate of filtration, or both (cf. Gartland & Sueoka, 1966; Lindahl, Adams & Fresco, 1966). Contrary to observations with the Krebs ascites tumour (Burdon *et al.* 1967), we have no evidence in *Smittia* for methylation of precursor other than in the nucleolus. At the larval stage investigated, further methylation elsewhere in the cell is possible but can only be a minor one. However, these authors did not find the expected newly synthesized precursor transfer RNA in the isolated Krebs-cell nucleus, so that leakage to cytoplasm was not excluded from their experiments.

Synthesis of 5s RNA is co-ordinated with ribosomal RNA synthesis (Brown & Littna, 1966; Galibert, Lelong, Larsen & Boiron, 1967; Rosset, Monier & Julien, 1964), though there is a preformed nuclear pool of this RNA (Knight & Darnell, 1967). In the *Smittia* cell it co-ordinates with the high-

molecular-weight RNA, which includes ribosomal RNA precursor (see below). In other cells, as in the present one, 5s RNA can be initially more radioactive than transfer RNA (Galibert *et al.* 1967; Ingle, Key & Holm, 1965; Watson & Ralph, 1966), though it may possibly include some transfer RNA precursor. The 5s RNA appears to be chromosomal since, though it is missing from the enucleolate *Xenopus* (Brown & Littna, 1966), the governing DNA is present as shown by DNA-RNA hybridization (D. D. Brown, personal communication). As the mutant also lacks synthesis of ribosomal RNA, the observation stresses the closely co-ordinated synthesis of ribosomal RNA and 5s RNA.

How much transfer RNA was found in the nucleolus of this cell can be answered as follows. (a) In the DRB-TRB-inhibited cell 89% of total label is nucleolar and 8% is chromosomal (Table 2); with shorter incubation times the relative nucleolar label is greater. (b) The labelling of 4s RNA represents synthesis, as shown by the recovery of 2',3'-uridylic acid. The elution and kinetic behaviour, and the content in methylated nucleosides (including ribothymidine) and pseudouridine, further support the evidence that true transfer RNA is made. (c) An estimated 40% of extracted uridine-labelled RNA in the inhibited cell is transfer RNA by gel and MAK separations (Figs. 3 and 4). A higher percentage of guanine-labelled RNA is transfer RNA (Fig. 5) (the label remains mostly nucleolar). (d) Two assumptions are made with respect to uridine label in the inhibited cell: first, that all the 30% non-RNA radioactivity registered in radioautographs (see the Materials and Methods section) is present exclusively in the nucleolus, and, secondly, that all non-transfer RNA species are also present exclusively in the nucleolus. It is then calculated, on the basis that 40% of the radioactivity per separation is in transfer RNA, that 60% of newly made transfer RNA in the inhibited cell would have been detected in the nucleolus. The corresponding value for chromosomal transfer RNA is 30%. Even if only 30% of the radioactivity were in transfer RNA, 47% of this transfer RNA would have been found in the nucleolus and 40% in the chromosomes. It is therefore considered that a substantial proportion of new transfer RNA in the inhibited cell is found in the nucleolus.

The evidence so far from several organisms tends to support the view that transfer RNA derives from the chromosomes (Brown & Gurdon, 1964; Edström & Daneholt, 1967; Perry, 1962; Ritossa, Atwood & Spiegelman, 1966; Woods & Zubay, 1965). Our finding that transfer RNA is present in the nucleolus is not necessarily in conflict with this general view. In that case, however, the amount of transfer RNA

involved would suggest a nucleolar accumulation. That this accumulation depends on the cell inhibition is made likely by the concomitant blockage of ribosomal RNA maturation (see below) and the 95% decrease of RNA transported to cytoplasm (Table 2). (This drastic decrease argues against a mere slowing-down of cell metabolism.) The significance of the accumulation is not understood, but the methylation practically restricted to the newly made transfer RNA (Fig. 10) present in the normal nucleolus (Table 3) is a reasonable indication for an underlying physiological basis. The most probable substrate for methylation is the sub-methylated transfer RNA precursor eluted at about 0.6M-sodium chloride in Figs. 4 and 10. Accepting the view that transfer RNA derives from the chromosomes, we suggest as a possible interpretation of our results that the accumulation in the presence of drugs represents a blockage in an obligatory transient association of transfer RNA with the nucleolus that is perhaps related to maturation of the molecule. However, if future work revealed that transfer RNA originates also in the nucleolus, the relevance of our observations could possibly be in connexion with some aspect of this nucleolar synthesis.

The radioactive profile encountered with DRB and TRB, i.e. low- and high-molecular-weight RNA but absence of ribosomal RNA, has been found in yeast cells treated with cycloheximide (Fukuhara, 1965; de Kloet, 1966) or 5-fluorouracil (de Kloet, 1968), and in mammalian cells treated with puromycin (Holland, 1963; Tamaoki & Mueller, 1963) or subjected to a nutritional shift (Watson & Ralph, 1966).

In *Smittia* the radioactive RNA with apparent molecular weight higher than that of ribosomal RNA was found in all three kinds of separation. This RNA from MAK columns is composed mainly of ribosomal precursor and, tailing behind it, some DNA-like RNA (Yoshikawa, Fukada & Kawade, 1964). Since no radioactive RNA was retained in our columns as has been found for the major part of DNA-like RNA (Ellem, 1966), it would appear that the severe chromosomal inhibition (Table 2) has decreased this synthesis. However, DNA-like RNA may be present in the tailing radioactive shoulders in Figs. 4, 8(b) and 9. On the other hand, that the post-ribosomal RNA represents mainly ribosomal precursor is indicated by the coincident methylation profile in Fig. 10 (cf. Greenberg & Penman, 1966). This precursor formed in the presence of DRB and TRB probably is submethylated compared with non-treated controls (Sirlin *et al.* 1966). Owing to its lability in insects, the actual size would depend on the conditions of extraction (Kirby, 1966). The ribosomal RNA precursor is known to be assembled (Perry, 1962) and methylated (Greenberg &

Penman, 1966) in the organelle. It is implicit in the calculations made above that the nucleolar radioactivity observed in DRB-TRB-treated cells would fully accommodate the ribosomal RNA precursor (together with transfer RNA as discussed). In our material the nucleolar maturation of precursor is blocked by DRB and TRB, since it forms ribosomal RNA only in non-treated controls (as detected with uridine and methylation). Presumably this is caused by the action of these drugs at the nucleoside level. Blocked ribosomal RNA precursor is also characteristic of the other inhibited systems mentioned above, where it is similarly found to be sub-methylated (de Kloet, 1966; see also Vaughan, Soeiro, Warner & Darnell, 1967).

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