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The Effect of Thioacetamide on the Maturation of High-Molecular-Weight Ribonucleic Acid in Tumour Cells

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1. Although thioacetamide treatment of Krebs II ascites-tumour cells did not markedly affect the rate of RNA synthesis *in vivo*, it caused the formation of an unusual single-stranded RNA component sedimenting at approx. 26s. 2. The maturation process leading to the formation of methylated RNA was examined by following the kinetics of incorporation into RNA of radioactivity from [G-^{3}H]uridine and L-[*Me*-^{14}C]methionine. In treated and untreated tumour cells extensive methylation was observed, not only of the ribosomal RNA species, but also of their precursors, especially the precursor species sedimenting at 35s. 3. Evidence is also presented to suggest that methylation of low-molecular-weight RNA species occurs both in the nucleus and in the cytoplasm of these tumour cells. 4. Thioacetamide did not appear to have an effect on RNA methylation *in vivo*, and in thioacetamide-treated cells the 26s RNA accumulated within the nucleus, where it was methylated. 5. It is postulated that the 26s RNA is most likely to arise as a result of a fault in the scission process that gives rise to the ribosomal RNA components from their high-molecular-weight precursors.

Previous studies carried out *in vivo* on the methylation of RNA in Krebs II ascites-tumour cells by using L-[*Me*-^{14}C]methionine as donor of methyl groups have demonstrated that only nascent RNA is methylated (Burdon, 1966), and that of the high-molecular-weight RNA species present in these cells, both the 30s and 19s ribosomal RNA components become methylated. It has been proposed that in mammalian cells ribosomal RNA is derived from precursors that sediment at approx. 45s and 35s (Scherrer, Latham & Darnell, 1963; Rake & Graham, 1964; Penman, 1966; Muramatsu, Hodnett, Steele & Busch, 1966), and preliminary studies (Burdon, 1966) have indicated that of these precursors the 35s species is extensively methylated.

The maturation process leading to the formation of the 30s and 19s ribosomal RNA components has now been studied in Krebs II cells in more detail by following the kinetics of incorporation of [^{3}H]-uridine and methyl groups from L-[*Me*-^{14}C]methionine into RNA components of nuclear and cytoplasmic fractions. By this means a more precise indication of the stage (or stages) at which methylation of RNA occurs, and its extent, was obtained.

In addition, experiments were carried out to determine the effect of thioacetamide on this process. Thioacetamide is a weak hepatic carcinogen that, when administered to rats, leads to increased nucleolar RNA synthesis in their livers (Steele, Okamura & Busch, 1965) and appears to block the formation of ribosomal RNA from its rapidly sedimenting precursors (Steele & Busch, 1966). The experiments carried out with Krebs II cells indicate that, although thioacetamide does have some effect on the maturation process leading to the formation of ribosomal RNA, its effect is different from that encountered in rat livers. A portion of the rapidly sedimenting precursor RNA is degraded in some manner to give rise to an unusual single-stranded RNA component, sedimenting at approx. 26s, which becomes methylated and accumulates within the nucleus.

EXPERIMENTAL

Materials. [G-^{3}H]Uridine (3·33c/m-mole), [6-^{3}H]thymidine (1·9c/m-mole), [^{32}P]orthophosphate (57c/mg. of phosphorus) and L-[*Me*^{14}C]methionine (29·5mc/m-mole) were obtained from The Radiochemical Centre, Amersham, Bucks. Sodium dodecyl sulphate, bentonite and thioacetamide were purchased from British Drug Houses Ltd., Poole, Dorset. Tween 80 was obtained from Koch-Light Laboratories Ltd., Colnbrook, Bucks.

Actinomycin D was a gift from Merck, Sharp and Dohme Inc., Rahway, N.J., U.S.A.

Horse serum (no. 3, natural clot, unheated) was purchased from Burroughs Wellcome and Co., London, N.W. 1.

2,5-Diphenyloxazole, 1,4-bis-(5-phenyloxazol-2-yl)-

benzene, Scinstant NE572 and scintillation-grade dioxan were obtained from Nuclear Enterprises (G.B.) Ltd., Edinburgh.

Deoxyribonuclease I (electrophoretically purified from bovine pancreas, ribonuclease-free), ribonuclease A (from bovine pancreas, protease-free) and thymidine were purchased from Sigma (London) Chemical Co. Ltd., London, S.W. 6.

Both deoxyribonuclease I and ribonuclease A were dissolved in 20mM-tris–HCl buffer, pH7·1, and before use the solution of ribonuclease A was held at 100° for 10min. to inactivate traces of deoxyribonuclease.

Cellulose ester membrane filters (0·45μ and 0·22μ pore sizes) were obtained from Millipore (U.K.) Ltd., Wembley, Middlesex (HAWP02500 and GSWP04700 respectively).

Media and other solutions. Phosphate-buffered saline was prepared as described by Martin, Malec, Sved & Work (1961) and was sterilized by filtration through Millipore filters of 0·22μ pore size.

Hanks medium, containing six times the normal amount of glucose, was constituted as follows: NaCl (8·00g.), KCl (0·40g.), $CaCl_2$ (0·14g.), $MgSO_4,7H_2O$ (0·10g.), $MgCl_2,6H_2O$ (0·10g.), $Na_2HPO_4,2H_2O$ (0·06g.), KH_2PO_4 (0·06g.), glucose (6·00g.), $NaHCO_3$ (0·35g.), phenol red (0·02g.), benzylpenicillin (10^5i.u.; Glaxo Laboratories, Greenford, Middlesex) and streptomycin sulphate (0·06g.; Glaxo Laboratories) plus glass-distilled water to a final volume of 1l. As with phosphate-buffered saline this solution was sterilized by filtration. Phosphate-free Hanks medium was prepared as above but without $Na_2HPO_4,2H_2O$ and KH_2PO_4.

Standard saline–citrate was as follows: 0·15M-NaCl–15mM-sodium citrate adjusted to pH7·1.

Biological materials. Krebs II ascites-tumour cells were maintained as described by Martin *et al.* (1961) by serial transplantation in mice (Porton strain). The ascitic fluid containing 1×10^8–2×10^8 cells/ml. was harvested under sterile conditions 7–9 days after inoculation and the tumour cells were washed thoroughly with phosphate-buffered saline by the procedure of Martin *et al.* (1961). Finally the washed cells were transferred to Hanks medium, centrifuged down at 600*g* for 5min. and resuspended in Hanks medium supplemented with 50% (v/v) of horse serum (McNamara, 1964) at a concentration of 10^6 cells/ml. The cells were counted and stained with nigrosin (E. Gurr Ltd., London, S.W. 14) to assess the numbers of dead and live cells as described by Martin *et al.* (1961). During the experiments described in the present paper, the number of dead cells never rose above 5% of the total number even in the presence of thioacetamide.

Incubation of Krebs II ascites-tumour cells. For the estimation of [G-^{3}H]uridine incorporation into both RNA and the acid-soluble fraction, 3ml. portions of the tumour cells in Hanks medium plus horse serum were incubated in an atmosphere containing 5% of CO_2 at 37° with appropriate additions in small plastic Petri dishes (45mm. diam.) from Sterilin Ltd., Richmond, Surrey. After incubation the cells and medium were removed, added to an equal volume of ice-cold phosphate-buffered saline and centrifuged down at 600*g* for 2min. at 0°, then washed twice with further portions of ice-cold phosphate-buffered saline to remove any excess of radioactive material.

Where a larger number of cells was required so that labelled RNA could be isolated and analysed by density-gradient centrifugation, 10ml. batches of tumour cells were incubated in larger plastic Petri dishes (90mm. diam.) with appropriate additions. After incubation, these cells were washed with phosphate-buffered saline as described above.

Measurement of [G-^{3}H]*uridine uptake into RNA and the acid-soluble fraction of Krebs II ascites-tumour cells.* The cells from a small Petri dish (3×10^6 cells), after incubation in the presence of [G-^{3}H]uridine, were washed with phosphate-buffered saline as described above, suspended in 0·25ml. of 0·2N-$HClO_4$ and kept at 0° for 15min. The precipitate was then collected by centrifugation at 600*g* for 15min. at 0° and the supernatant fluid removed. The precipitate was extracted twice more with further 2·5ml. portions of ice-cold 0·2N-$HClO_4$ in the same manner, and these extracts were combined with the first. The total $HClO_4$ extract was neutralized with KOH to pH7, and after 20min. at 0° the precipitate of $KClO_4$ was removed by centrifugation at 600*g* for 20min. at 0°. The neutralized extract was adjusted to a final volume of 10ml. and is henceforth referred to as the acid-soluble fraction. The concentration of nucleotides in this fraction was estimated from E_{257} assuming $E^{1\,cm.}_{257}$ 0·330 for 1μg. of nucleotide phosphorus/ml. (Martin *et al.* 1961). A sample (0·5ml.) was taken for assay of radioactivity after addition of 4ml. of dioxan-based scintillator fluid (Scinstant NE572 in scintillation-grade dioxan) with a liquid-scintillation spectrometer (either Nuclear–Chicago 725 or Packard Tri-Carb series 4000). By this means a measure of the specific radioactivity of the acid-soluble nucleotide fraction was obtained.

The RNA, present in the acid-insoluble precipitate that remained after the removal of the acid-soluble fraction, was then hydrolysed by incubation at 37° for 1hr. in 5ml. of 0·3N-KOH according to the procedure outlined by Munro & Fleck (1966). After incubation, the DNA and protein remaining acid-insoluble were precipitated by the addition of 2·5ml. of 1·2N-$HClO_4$ and removed by centrifugation at 600*g* for 20min. The supernatant fluid, which contained the hydrolysed RNA, was neutralized to pH7 with KOH, and after 20min. at 0° the precipitate of $KClO_4$ was removed by centrifugation at 600*g* for 20min. at 0°. The volume of the supernatant fluid was adjusted to 10ml. and the concentration of hydrolysed RNA present was estimated from E_{268} assuming $E^{1\,cm.}_{268}$ 0·280 for 1μg. of RNA phosphorus/ml. (Burdon & Smellie, 1961). A sample was removed for assay of radioactivity by liquid-scintillation spectrometry as described above for the acid-soluble fraction. By this means an estimate of the specific radioactivity of the RNA fraction of these cells under various conditions was obtained.

The relative specific radioactivity of the RNA fraction is defined as the ratio of the specific radioactivity of the RNA fraction to the specific radioactivity of the acid-soluble nucleotide fraction.

Extraction of RNA from intact Krebs II ascites-tumour cells. After incubation with appropriate additions and washing with ice-cold phosphate-buffered saline, as described above, the cells from five large Petri dishes (5×10^7 cells) were suspended in 5ml. of ice-cold phosphate-buffered saline containing 0·25% of bentonite (treated according to the procedure described by Fraenkel-Conrat, Singer & Tsugita, 1961) and sodium dodecyl sulphate was added to a final concentration of 1% (w/v). After standing at room temperature for 5min., the suspension was extracted with an equal volume of 80% (w/v) phenol in 20mM-tris–HCl

buffer, pH 7·1, with vigorous shaking for 10 min. at room temperature. The resulting emulsion was separated by centrifugation at 10000*g*, and the aqueous phase was removed, re-extracted with phenol and then three times with ether to remove traces of phenol. After removal of ether with a stream of N_2 the addition of 2 vol. of ethanol precipitated the cell DNA and RNA. The mixture was kept for 1 hr. at −20° and the nucleic acids were then collected by centrifugation at 600*g* for 20 min. at −10° and dissolved in 3 ml. of 5 mM-$MgCl_2$–20 mM-tris–HCl buffer, pH 7·1, containing bentonite (0·1%) and 30 μg. of deoxyribonuclease I. After incubation at 37° for 30 min. the mixture was extracted twice with equal volumes of 80% (w/v) phenol in 20 mM-tris–HCl buffer, pH 7·1. The final aqueous phase was removed, extracted three times with ether to remove traces of phenol and after removal of the ether with a stream of N_2 the resulting solution was dialysed against 4 l. of 20 mM-tris–HCl buffer, pH 7·1, for 4 hr. After dialysis the RNA-containing solution was removed, 2 vol. of ethanol added and the RNA allowed to precipitate at −20° for 1 hr., after which it was collected by centrifugation at 10000*g* for 20 min. and suspended in 0·2 ml. of 20 mM-tris–HCl buffer, pH 7·1.

Fractionation of Krebs II ascites-tumour cells into nuclei and cytoplasm. This was achieved for batches of 5×10^7 cells by using the Tween 80 method of Fisher & Harris (1962) as modified by McNamara (1964) for Krebs II cells.

Extraction of RNA from nuclear and cytoplasmic fractions of Krebs II ascites-tumour cells. The cytoplasmic fraction obtained from above was treated directly with sodium dodecyl sulphate, bentonite and phenol as described above for the isolation of RNA from intact cells, and the nuclear pellet obtained was suspended in 5 ml. of ice-cold phosphate-buffered saline containing bentonite (0·25%) before the addition of sodium dodecyl sulphate and phenol as described above.

Sucrose-density-gradient analysis of RNA preparations. A 0·1 ml. sample of the appropriate RNA preparation was layered on top of a linear 5–20% (w/v) sucrose gradient (Britten & Roberts, 1960) in 1 mM-$MgCl_2$–20 mM-tris–HCl buffer, pH 7·1, containing sodium dodecyl sulphate (0·01%), and kept at 4°. The sucrose solutions used to form the gradient had been previously autoclaved at 15 lb./in.^{2} for 10 min. to remove any nuclease contamination.

After centrifugation at 40000 rev./min. in either the SW 50 rotor of the Spinco model L ultracentrifuge or in the SW 40 rotor of the Griffin–Christ Omega II ultracentrifuge, 3-drop fractions were collected, after puncture of the cellulose nitrate tube with a hypodermic needle, into 2 ml. portions of 20 mM-tris–HCl buffer, pH 7·1, containing sodium dodecyl sulphate (0·01%). Each fraction was examined for E_{260} and then for acid-insoluble radioactivity by collection of 5%-(w/v)-trichloroacetic acid-insoluble material on Millipore filters (0·45 μ pore size), and counting these filters, after drying, in 5 ml. of toluene-based scintillator fluid [5% 2,5-diphenyloxazole plus 0·3% 1,4-bis-(5-phenyloxazol-2-yl)-benzene in A.R. toluene] in a liquid-scintillation spectrometer.

In certain experiments where L-[*Me*-^{14}C]methionine was used as a source of labelled methyl groups each fraction was assayed for E_{260}, adjusted with tris–HCl buffer to pH 8·5 (final concn. 0·5 M), incubated at 37° for 30 min. to hydrolyse the aminoacyl ester bond of any [*Me*-^{14}C]methionyl-transfer RNA that might be present (Burdon, 1966), acidified with trichloroacetic acid and the acid-insoluble radioactivity collected and measured as described above.

In other experiments the ribonuclease-resistance of the radioactive RNA present in each fraction from the gradient was determined. In these cases the fractions were collected into standard saline–citrate (2 ml.) and to each sample was added 50 μg. of ribonuclease A. After incubation for 30 min. at 25° each sample was acidified with trichloroacetic acid and the acid-insoluble ribonuclease-resistant radioactivity was collected on Millipore filters, which were then assayed for radioactivity in the manner described above.

Finally it was necessary in certain cases to change the composition of the sucrose solutions used for density-gradient analysis. The alternative used was as follows: 1 mM-$MgCl_2$–0·15 M-NaCl–20 mM-tris–HCl buffer, pH 7·1, containing sodium dodecyl sulphate (0·01%). To facilitate subsequent discussions approximate sedimentation coefficients were assigned to various RNA species by the method of Martin & Ames (1961) after comparison with Krebs II-cell ribosomal RNA components whose $S_{20,w}$ values have been obtained by Eason, Cline & Smellie (1963).

Determination of the base composition of 26s RNA in thioacetamide-treated Krebs II ascites-tumour cells. For this purpose Krebs II cells were incubated for 24 hr. at 10^6/ml. in phosphate-free Hanks medium supplemented with 50% (v/v) of horse serum containing [^{32}P]orthophosphate (20 μc/ml.) and thioacetamide (300 μg./ml.). After incubation RNA was isolated from the nuclear fraction and subjected to sucrose-density-gradient analysis to locate the acid-insoluble radioactivity corresponding to the 26s RNA component. After assay for radioactivity, the appropriate Millipore filters were removed from the scintillator fluid and the precipitated RNA was removed from the filters and hydrolysed in alkali as described by Brown & Littna (1964). The hydrolysate after neutralization was then subjected to two-dimensional paper chromatography along with a suitable quantity of an alkali hydrolysate of yeast soluble RNA (Calbiochem Ltd., Basingstoke, Hants.) to act as marker so that regions corresponding to the 2′(or 3′)-monophosphates of adenosine, guanosine, cytidine and uridine could be detected by examination of the chromatogram in ultraviolet light. The solvent systems used were those employed by Y. Hayashi & S. Osawa (personal communication) and were as follows: first dimension (ascending for 17 hr.), isobutyric acid–0·5 N-NH_3 (5:3, v/v); second dimension (ascending for 23 hr.), propan-2-ol–6 N-HCl (13:7, v/v). The regions corresponding to the 2′(or 3′)-monophosphates of adenosine, guanosine, cytidine and uridine were cut out, shredded into glass scintillator vials and assayed directly for ^{32}P radioactivity by immersion in 5 ml. of toluene-based scintillator [5% 2,5-diphenyloxazole plus 0·3% 1,4-bis-(5-phenyloxazol-2-yl)benzene in A.R. toluene], so that the RNA base composition can be assessed by comparison of the ^{32}P radioactivity in each of the separated 2′(or 3′)-ribonucleotides.

Precautions taken when handling RNA. All equipment and solutions coming in contact with RNA at any stage were sterilized to remove contaminating nucleases.

Sterile glassware was flamed before use. Solutions were autoclaved at 15 lb./in.^{2} for 20 min.

Visking tubing (8/32 in. diam.) for dialysis was heated at 80° in 0·1% sodium dodecyl sulphate–0·1% disodium EDTA, pH 7, for 10 min. and then rinsed thoroughly with sterile distilled water.

RESULTS

Krebs II cells in Hanks medium supplemented with horse serum were incubated initially for various times in the presence and absence of thioacetamide (300μg./ml. of medium) and then with [G-^{3}H]uridine for 20min. to assess the capacity of these cells to carry out synthesis of RNA under these conditions. Examination of the specific radioactivity of the RNA fractions from cells 'pulse'-labelled after various times indicated that thioacetamide did not have pronounced effect on RNA-synthetic ability (Fig. 1*a*). However, thioacetamide did have an inhibitory effect on the uptake of [G-^{3}H]uridine into the acid-soluble fraction (Fig. 1*b*). This inhibitory effect increases markedly as the concentration of thioacetamide is increased in the medium. Because of this effect, a calculation of the relative specific radioactivity (Martin *et al.* 1961) was carried out as described in the Experimental section and on this basis it appeared that the rate of RNA synthesis was only marginally greater in the thioacetamide-treated cells as compared with untreated cells (Fig. 1*c*). Under these conditions there was no net increase in the amount of RNA per cell either in the presence or absence of thioacetamide.

The tumour cells were then incubated with [G-^{3}H]uridine in the presence and absence of thioacetamide for 24hr. The total RNA was extracted from the cells with sodium dodecyl sulphate and phenol and initially analysed by density-gradient centrifugation in sucrose solutions in 1mM-magnesium chloride–0·15M-sodium chloride–20mM-tris–hydrochloric acid buffer, pH7·1. Figs. 2(*a*) and 2(*b*) show that RNA from both thioacetamide-treated and untreated cells gives three main E_{260} peaks corresponding to the 30s, 19s and approx. 4–5s RNA species (Eason *et al.* 1963). However, whereas the acid-insoluble ^{3}H radioactivity incorporated into the RNA from untreated cells corresponds closely to the E_{260} peaks (Fig. 2*b*), that from the thioacetamide-treated cells corresponds less well with the E_{260} profile. A large portion sediments in advance of the large ribosomal RNA component (30s) and in the region 35–37s. In addition, a smaller portion sediments between the 30s and 19s ribosomal components at a position corresponding to about 24–27s. When sodium chloride was omitted from the density gradient a major portion of the acid-insoluble ^{3}H radioactivity sediments at about 26s as a discrete peak, while the remainder corresponds more closely to the peaks of the E_{260} profile. For this reason all subsequent density-gradient analyses were carried out with sucrose solutions containing no sodium chloride as just described so that possible aggregations were minimized.

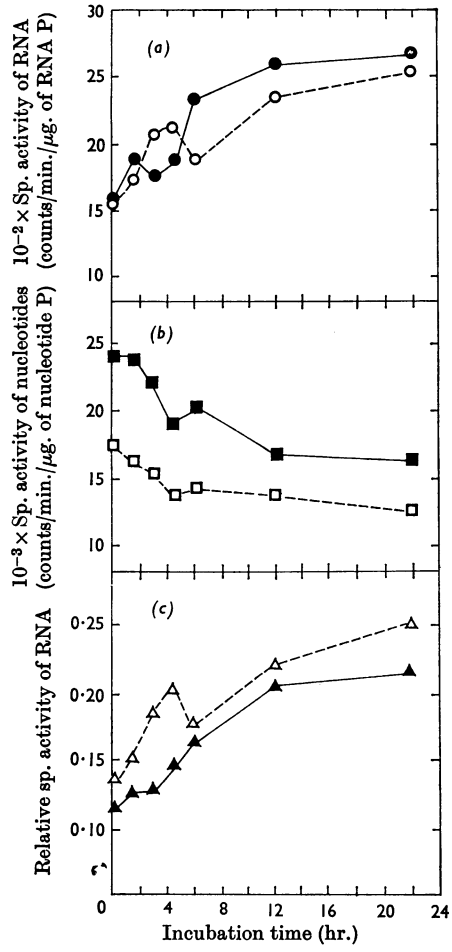

Fig. 1. Effect of treating Krebs II ascites-tumour cells with thioacetamide on their ability to synthesize RNA. Batches (3ml.) of Krebs II cells were incubated at 10^6 cells/ml. in Hanks medium containing 50% (v/v) of horse serum (as described in the Experimental section) for 20min. with 20μc of [G-^{3}H]uridine after prior incubation for various times in the presence or absence of thioacetamide (300μg./ml. of medium). The cells were then removed from the Petri dishes and the uptake of [G-^{3}H]uridine into RNA and the acid-soluble fraction was measured as described in the Experimental section. (*a*) Specific radioactivity of total cell RNA in control cells (●) and in thioacetamide-treated cells (○); (*b*) specific radioactivity of nucleotides in the acid-soluble fraction in control cells (■) and in thioacetamide-treated cells (□); (*c*) relative specific radioactivity of RNA (calculated as described in the Experimental section) in control cells (▲) and in thioacetamide-treated cells (△). Each point in this Figure represents the mean of three separate determinations.

The resistance to ribonuclease A of the [^{3}H]-uridine-labelled RNA isolated from cells labelled in the presence of thioacetamide for 24hr. was

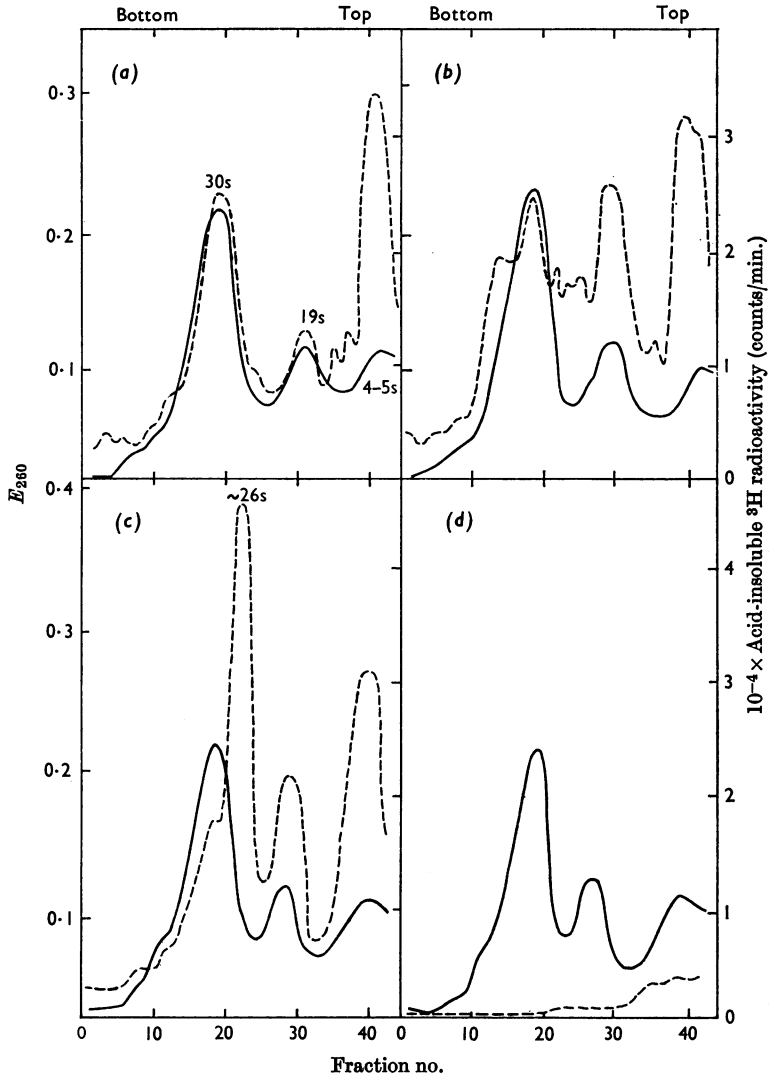

Fig. 2. Effect of thioacetamide on the synthesis of high-molecular-weight RNA in Krebs II ascites-tumour cells. ^{3}H-labelled RNA was isolated from Krebs II cells incubated for 24hr. in 10ml. batches (at 10^6 cells/ml. of medium as in Fig. 1) each with 20μc of [G-^{3}H]uridine and analysed by sucrose-density-gradient centrifugation. (*a*) RNA from control cells incubated with [^{3}H]uridine alone and sedimented in sucrose solutions in 1mM-$MgCl_2$–0·15M-NaCl–20mM-tris–HCl buffer, pH7·1, containing sodium dodecyl sulphate (0·01%); (*b*) RNA from cells incubated with [^{3}H]uridine in the presence of thioacetamide (300μg./ml. of medium) and sedimented in a sucrose medium identical with that used in (*a*); (*c*) the same RNA sample as in (*b*), but sedimented in sucrose in 1mM-$MgCl_2$–20mM-tris–HCl buffer, pH7·1, containing sodium dodecyl sulphate (0·01%); (*d*) the same as (*c*), but each fraction from the gradient was collected in standard saline–citrate and treated with ribonuclease A as described in the Experimental section. ——, E_{260}; – – – –, acid-insoluble ^{3}H radioactivity. Approximate sedimentation coefficients have been assigned to the E_{260} peaks to facilitate subsequent discussion (see Fig. 2*a*). Further, in these and subsequent density-gradient analyses fraction 1 represents the first fraction taken from the bottom of the gradient.

determined. The results given in Fig. 2 indicate that the only acid-insoluble material resistant to ribonuclease A in standard saline–citrate sedimented in the region corresponding to 4–5s. Thus on this basis (Weissmann, Borst, Burdon, Billiter & Ochoa, 1964) it seemed likely that the 26s RNA was single-stranded.

Incubation of the cells with 50μM-[6-^{3}H]thymi-

dine for 24hr. followed by extraction of the RNA by the usual method revealed no acid-insoluble radioactivity sedimenting in the 26s region. This suggests that the acid-insoluble radioactivity in the 26s region represents RNA that is not associated with DNA molecules, as has been found under certain conditions in tumour tissues by Wilkinson & Kirby (1966).

Krebs II cells were incubated with [G-^{3}H]uridine and L-[*Me*-^{14}C]methionine in the presence of large amounts of non-radioactive sodium formate (to prevent equilibration of [^{14}C]methyl groups from the methionine with the C_1 unit pool as recommended by Winocour, Kaye & Stollar, 1965) and the kinetics of incorporation of [^{3}H]uridine and [^{14}C]methyl groups into RNA of both nuclear and cytoplasmic fractions were studied by using sucrose-density-gradient centrifugation. The results of this study are presented in Figs. 3–6. Fig. 3(*a*) shows that where the cells were incubated for 20min. with the radioactive RNA precursors very little acid-insoluble ^{14}C radioactivity (representing incorporation of methyl groups) was associated with the 45s RNA species of the nucleus, which on the other hand rapidly incorporates

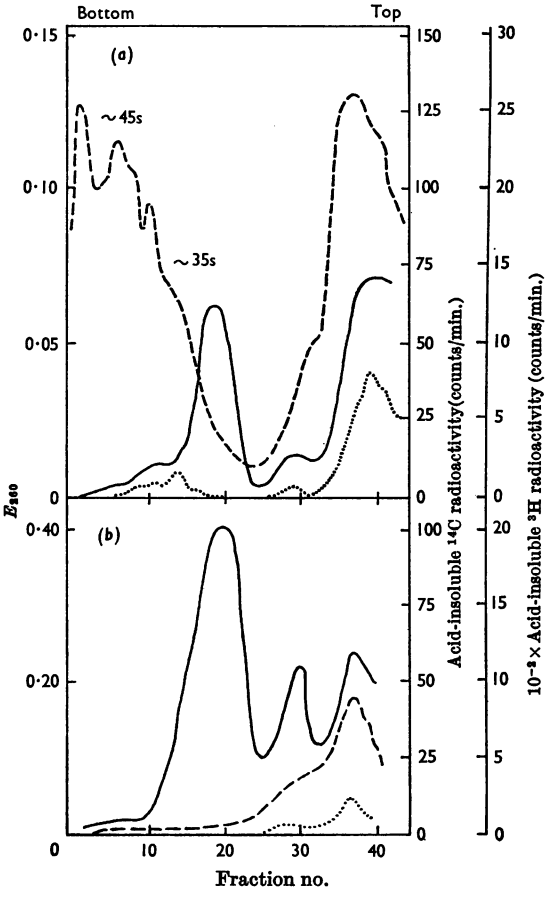

Fig. 3. Sucrose-density-gradient analysis of nuclear and cytoplasmic RNA from Krebs II ascites-tumour cells after incubation with [G-^{3}H]uridine and L-[*Me*-^{14}C]methionine for 20min. Batches (10ml.) of Krebs II cells were incubated as described for Fig. 2 for 20min. with 20μc of [^{3}H]uridine and 10μc of L-[*Me*-^{14}C]methionine in the presence of 20mM-sodium formate and the RNA from the nuclear fraction (*a*) and cytoplasmic fraction (*b*) examined by sedimentation in a sucrose medium, identical with that used in Fig. 2(*c*), in 1mM-$MgCl_2$–tris–HCl buffer, pH7·1, containing sodium dodecyl sulphate (0·01%). After collction, each fraction was incubated at pH8·5 as described in the Experimental section to hydrolyse any [^{14}C]methionyl-transfer RNA that might be present before precipitation with acid. ——, E_{260}; – – –, acid-insoluble ^{3}H radioactivity; ····· acid-insoluble ^{14}C radioactivity.

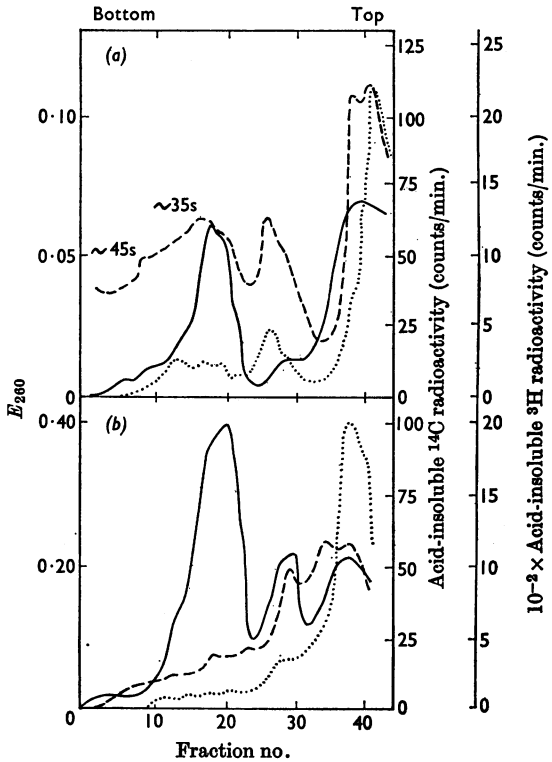

Fig. 4. Sucrose-density-gradient analysis of nuclear and cytoplasmic RNA from Krebs II ascites-tumour cells after incubation with [G-^{3}H]uridine and L-[*Me*-^{14}C]methionine for 1hr. The conditions of incubation and analysis employed were similar to those used in Fig. 3. (*a*) RNA from the nuclear fraction; (*b*) RNA from cytoplasmic fraction. ——, E_{260}; – – –, acid-insoluble ^{3}H radioactivity; ·····, acid-insoluble ^{14}C radioactivity.

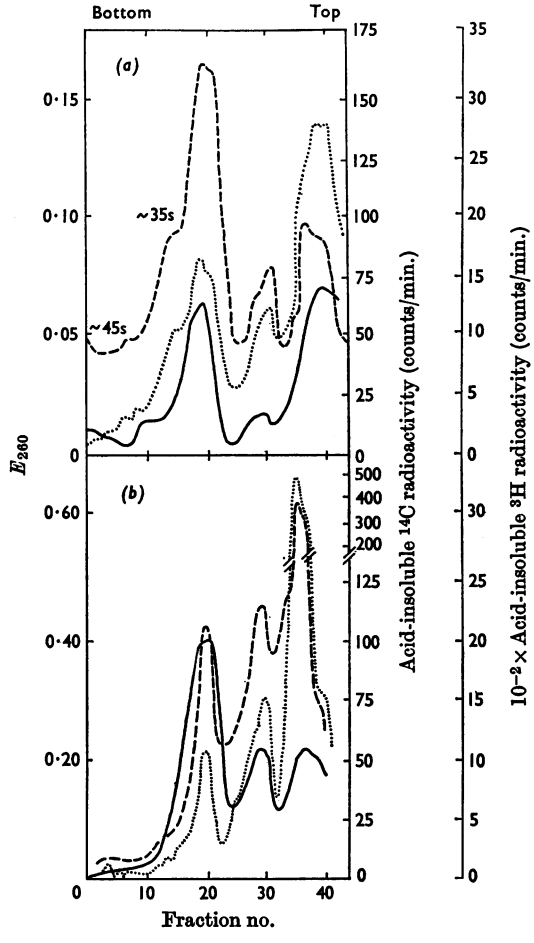

Fig. 5. Sucrose-density-gradient analysis of nuclear and cytoplasmic RNA from Krebs II ascites-tumour cells after incubation with [G-^{3}H]uridine and L-[*Me*-^{14}C]methionine for 2hr. The conditions of incubation and subsequent analysis were similar to those used in Fig. 3. (*a*) RNA from the nuclear fraction; (*b*) RNA from the cytoplasmic fraction. ——, E_{260}; – – –, acid-insoluble ^{3}H radioactivity; ····· acid-insoluble ^{14}C radioactivity.

[^{3}H]uridine. However, some ^{14}C radioactivity was detected in the region of the 35s species of the nuclear RNA. At this stage the acid-insoluble ^{3}H radioactivity occurring in the cytoplasmic RNA is predominantly of low molecular weight, sedimenting in the region 4–5s, and although there is some ^{14}C radioactivity corresponding to this fraction its amount is still low (Fig. 3*b*). After 60min. incubation (Fig. 4*a*) considerable ^{14}C radioactivity was associated with the [^{3}H]uridine-labelled RNA fraction sedimenting at 35s, but there was still little evidence of ^{14}C radioactivity in the 45s region of the nuclear fraction. Also, more ^{3}H radioactivity appeared in the 30s, 19s and 4–5s regions of the nuclear RNA as well as in the 19s and 4–5s regions of the cytoplasmic RNA (Fig. 4*b*). By 120min. (Fig. 5*a*) the regions of the gradients corresponding to the ribosomal RNA components of the cytoplasm were extensively labelled with ^{3}H, and ratios of ^{14}C radioactivity (representing methylation of nascent RNA) to ^{3}H radioactivity (representing the synthesis of new RNA) in the 30s and 19s regions have reached values similar to those for the corresponding 30s and 19s fraction of nuclear RNA (Figs. 5*b* and 7), and remain constant at least until 22hr. of incubation with the radioactive precursors (Figs. 6*b* and 7). However, the ratio of ^{14}C radioactivity to ^{3}H radioactivity for the 4–5s region of nuclear RNA has by this time increased to a value roughly twice (Figs. 5*a* and 7) that found for 19s RNA isolated from either the nucleus or the cytoplasm, whereas the ratio for the 4–5s region from the cytoplasm is considerably higher and after 22hr. incubation reached a value roughly four times that found for cytoplasmic 19s RNA (Figs. 5*b*, 6*b* and 7). This type of observation can only be approximate, owing to the incomplete separation achieved, but it suggests that methylation of RNA, at least in Krebs II cells, may occur not only in the nucleolus (Sirlin, Jacob & Tandler, 1963; Birnstiel, Fleissner & Borek, 1963; Sirlin, Jacob & Birnstiel, 1966) but also in the cytoplasm. However, these results do not exclude the possibility of a more rapid transfer of fully methylated 4–5s RNA from the nucleus into the cytoplasm.

Similar experiments were carried out on the Krebs II cells in the presence of thioacetamide, and from the situation after incubation for 22hr. it is evident that the 26s RNA, previously observed, accumulated within the nucleus (Fig. 6*c*) and the ratio of ^{14}C radioactivity to ^{3}H radioactivity in that region was similar to that found in the 30s region of the cytoplasmic RNA (Fig. 6*d*). Moreover, it appeared that the RNA present in the cytoplasm was normal, having ratios of ^{14}C radioactivity to ^{3}H radioactivity (0·028 for 30s component and 0·041 for 19s component) similar to those from cytoplasmic fraction of untreated cells incubated with the same precursors for a similar time (Fig. 7).

To decide whether or not the 26s RNA species arose in some manner from the ribosomal precursors, the Krebs II cells were first incubated for 20min. with [G-^{3}H]uridine after pretreatment with thioacetamide for 3hr. Examination of the RNA from the intact cells labelled in this experiment showed that both the 45s and 35s regions were extensively labelled as well as the 26s region (Fig. 8*a*). However, it was already clear from previous experiments (Fig. 3*a*) that no significant peak of ^{3}H radioactivity occurred in the region of 26s in the absence of thioacetamide. Further, if the cells were pre-

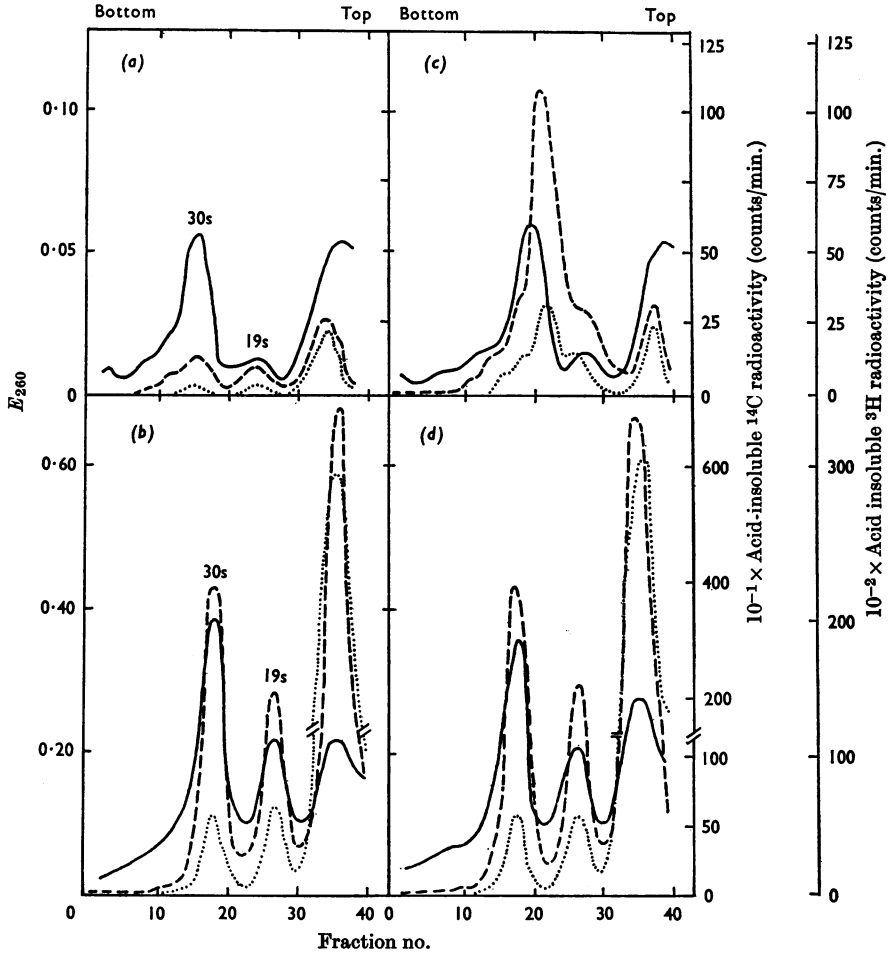

Fig. 6. Sucrose-density-gradient analysis of nuclear and cytoplasmic RNA from Krebs II ascites-tumour cells after incubation for 22hr. with [G-^{3}H]uridine and L-[*Me*-^{14}C]methionine in the presence and absence of thioacetamide. (*a*) RNA from the nuclear fraction of control cells; (*b*) RNA from the cytoplasmic fraction of control cells; (*c*) RNA from the nuclear fraction of cells incubated in the presence of thioacetamide (300μg./ml.); (*d*) RNA from the cytoplasmic fraction of cells incubated in the presence of thioacetamide (300μg./ml.). The conditions of incubation and analysis were similar to those employed in Fig. 3. ——, E_{260}; – – – –, acid-insoluble ^{3}H radioactivity; ·····, acid-insoluble ^{14}C radioactivity.

incubated with thioacetamide for 3hr. and then incubated with [G-^{3}H]uridine in the presence of actinomycin D (1μg./ml. of medium), no significant amount of radioactivity appeared in the 45s, 35s or 26s regions, although a small amount appeared in the 4–5s region (Fig. 8*b*). This suggested that the 26s RNA arose *in vivo* as a result of DNA transcription occurring in the tumour cells.

However, it remains to be seen whether it arose independently of ribosomal RNA, or as a result of some fault, induced by the thioacetamide, in the maturation process leading to the formation of ribosomal RNA. To examine these alternatives, Krebs II cells were preincubated with thioacetamide and then the incubation was continued for 5min. in the presence of [G-^{3}H]uridine. When the labelled RNA from these cells was examined the 45s region was considerably more labelled than was the 35s region (Fig. 8*c*). This observation was compatible with the model proposed by Penman (1966) for mammalian cells whereby the 45s species is first synthesized and then degraded to give rise to the 35s species. However, if actinomycin D (1μg./ml. of medium) was added after the short 5min.

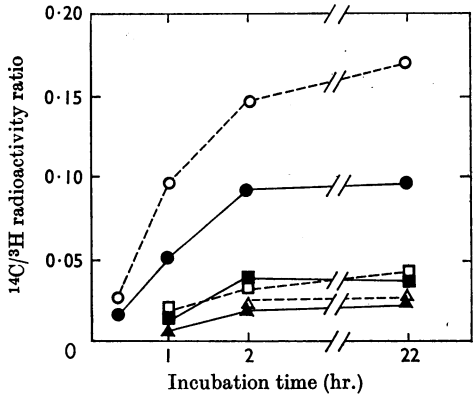

Fig. 7. Extent of methylation of newly synthesized RNA in the nuclear and cytoplasmic fraction of Krebs II ascites-tumour cells. The ratio of ^{14}C radioactivity (representing the degree of RNA methylation) to ^{3}H radioactivity (representing RNA synthesis) is plotted, from the data from Figs. 3–6, as a function of incubation time for the nuclear 30s region (▲), cytoplasmic 30s region (△), nuclear 19s region (■), cytoplasmic 19s region (□), nuclear 4–5s region (●) and cytoplasmic 4–5s region (○).

incubation of the thioacetamide-treated cells with [G-^{3}H]uridine and the incubation continued for a further 15min., it was clear that in addition to the increase in acid-insoluble ^{3}H radioactivity in the 35s region there was now a peak of radioactivity in the 26s region (Fig. 8*d*). Thus, although on this basis it could not be ruled out that the nuclear 26s RNA induced in the presence of thioacetamide arises independently of ribosomal RNA, yet at a relatively low rate, it appeared more likely that it arose as a result of some disorder of the maturation process leading to the formation of ribosomal RNA.

The base composition of the 26s RNA was determined by examining the distribution of ^{32}P in the 2′(or 3′)-ribonucleotides, separated after alkaline hydrolysis, of ^{32}P-labelled 26s RNA isolated from the nuclear fraction of thioacetamide-treated Krebs II cells incubated in phosphate-free Hanks medium supplemented with horse serum in the presence of [^{32}P]orthophosphate. However, much has been written about the value of this technique and at best it must only be considered to give the 'apparent' base composition (e.g. Attardi, Parnas, Hwang & Attardi, 1966). Nevertheless it was evident (Table 1) that although the 'apparent' base ratio of the 26s RNA was not precisely similar to the base composition of either ribosomal RNA species, previously determined by Montagnier & Bellamy (1964), it resembled more closely that of the smaller 19s species.

DISCUSSION

The observation that thioacetamide did not appear to have a particularly marked effect on the rate of RNA synthesis in Krebs II cells (Fig. 1) is in contrast with the results obtained from the livers of rats injected intraperitoneally with the drug (Steele *et al.* 1965). However, considerable differences exist between experiments carried out on whole animals and the tumour cells suspended in culture media as employed in the present studies.

From studies on livers of thioacetamide-treated rats, Steele & Busch (1966) considered that thioacetamide exerted a stimulatory effect on the process leading to the synthesis of ribosomal RNA, since they observed increases in the amount of the high-molecular-weight ribosomal precursor-type RNA species in the nuclear fractions. From the present results it would appear that thioacetamide does exert an effect on the ribosomal RNA maturation process that occurs in Krebs II cells. However, by using isotopic-labelling techniques, the presence of an unusual single-stranded RNA species sedimenting in a region corresponding to 26s was detected in cells treated with thioacetamide. Although this component was not detected in the livers of thioacetamide-treated rats (Steele & Busch, 1966), its sedimentation coefficient varied with the ionic strength of the medium used for density-gradient centrifugation, a value of approx. 37s being observed when the medium was similar in ionic strength to those used by Busch and his collaborators. On the other hand, the normally occurring 35s component that becomes labelled in the process leading to the formation of ribosomal RNA (Figs. 3–5) does not show a very marked response to a similar increase in ionic strength. It is conceivable, however, that the accumulation of high-molecular-weight ribosomal precursor RNA observed by Steele & Busch (1966) might include some of this 26s type of RNA by virtue of the higher ionic strength used in their gradient analyses, since under these conditions it would be impossible to distinguish one from the other.

The mechanism of the process that gives rise to the 26s RNA cannot yet be determined. However, from an examination of the maturation process occurring in untreated Krebs II cells leading to the formation of methylated ribosomal RNA, it was clear that no well-defined 26s component arises at any time during the process as followed by density-gradient centrifugation in low-ionic-strength media. This study also revealed that of the ribosomal precursor RNA components the 35s species was extensively methylated, as already found (Burdon, 1966; Saporana & Enger, 1966), but the extent of the methylation was close to that found for the mature 30s ribosomal RNA, thus lending some further

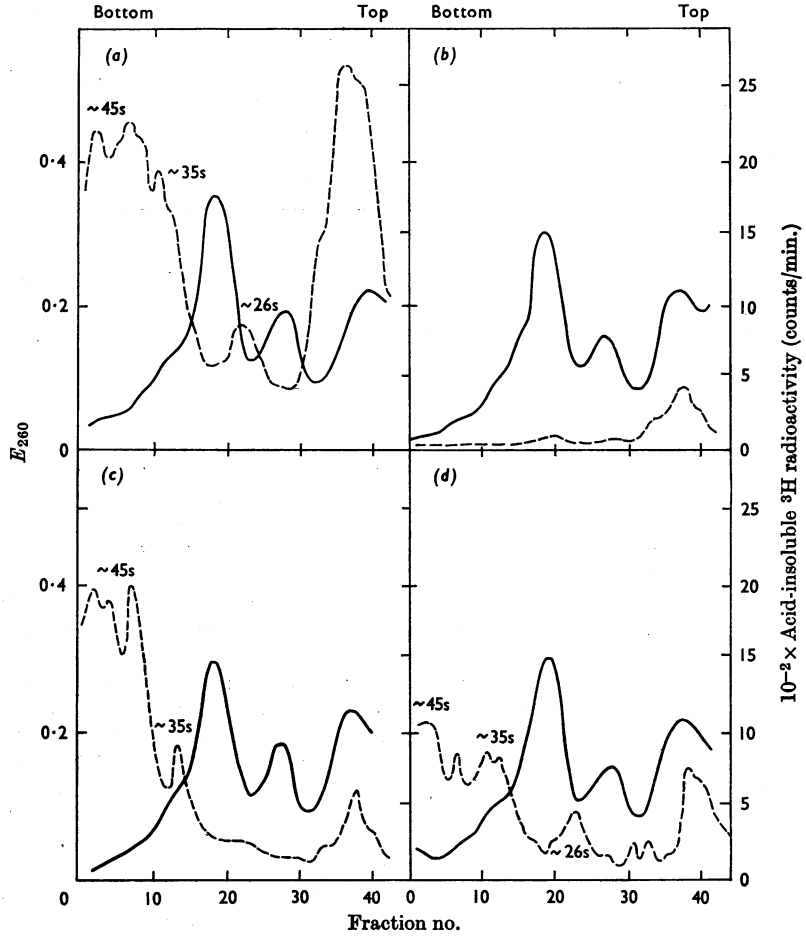

Fig. 8. Effect of thioacetamide on RNA synthesis in Krebs II ascites-tumour cells in the presence and absence of actinomycin D. Krebs II cells were preincubated for 3hr. in the presence of thioacetamide (300μg./ml.) in 10ml. batches (10^6 cells/ml.). RNA was then isolated from the cells and subjected to density-gradient centrifugation in 5–20% (w/v) sucrose solution in 1mM-$MgCl_2$–20mM-tris–HCl buffer, pH7·1, containing sodium dodecyl sulphate (0·01%), after: (*a*) incubation of each batch for a further 20min. with 20μc of [G-^{3}H]uridine without removing the thioacetamide; (*b*) incubation of each batch for a further 20min. with 20 μc of [G-^{3}H]uridine and actinomycin D (1μg./ml.) without removing the thioacetamide; (*c*) further incubation of each batch as described for (*a*), but only for 5min.; (*d*) further incubation of each batch as described for (*c*), but followed by the addition of actinomycin D (1μg./ml.) and a further 15min. incubation without removal of the thioacetamide. ——, E_{260}; – – – –, acid-insoluble ^{3}H radioactivity.

support to the model proposed for mammalian cells by Penman (1966) whereby the 35s species gives rise directly to the larger ribosomal RNA component. On the other hand, with the techniques employed in the present study there is some difficulty in estimating the extent of methylation of the precursor RNA sedimenting in the 45s region. Although the [^{14}C]methyl radioactivity there is always extremely low it cannot be excluded that a portion of the ^{3}H radioactivity sedimenting in this region might be attributable to the polydisperse 'DNA-like' RNA that has been found in mammalian cells (Attardi *et al.* 1966; Warner, Soeiro, Birnboim Girard & Darnell, 1966; Houssais & Attardi, 1966). The observations that suggest that methylation might occur in both nucleus and cytoplasm of these tumour cells is noteworthy since it has been found that enzymes capable of methylating 'methyl-deficient' *Escherichia coli* transfer RNA are considerably more active in tumour-cell extracts,

Table 1. *Base composition of the* ^{32}P-*labelled* 26s *RNA species formed in Krebs II ascites-tumour cells treated with thioacetamide*

The base composition of the 26s component was determined by the method described in the Experimental section and the values presented are means ± S.E.M. of six separate determinations. The base compositions of the ribosomal components were taken from the data of Montagnier & Bellamy (1964) and that of Krebs II-cell DNA was obtained by R. H. Burdon & T. A. Anderson (unpublished work).

	Base composition (moles/100 moles)			
Nucleic acid	CMP	AMP	GMP	UMP (or dTMP)
^{32}P-labelled 26s RNA	26·0 ± 1·2	19·1 ± 0·9	27·5 ± 0·8	27·4 ± 0·4
Krebs II-cell 19s RNA (ribosomal)	27·0	21·2	29·4	21·4
Krebs II-cell 30s RNA (ribosomal)	30·7	16·1	36·1	17·1
Krebs II-cell DNA	20·6	28·9	20·9	29·4

which are essentially cytoplasmic, than in extracts from corresponding normal tissues (Srinivasan & Borek, 1966).

Further experiments demonstrated that, although the 26s RNA species induced by thioacetamide accumulated in the nucleus and was methylated to an extent similar to that of ribosomal RNA (ratio of ^{14}C radioactivity to ^{3}H radioactivity approx. 0·029; cf. Fig. 7), the ribosomal RNA that did appear in the cytoplasm in the presence of thioacetamide was methylated to the normal extent (ratio of ^{14}C radioactivity to ^{3}H radioactivity for 30s RNA 0·028 and that for 19s 0·041; cf. Fig. 7). Thus although thioacetamide affects the maturation process in some manner the stage of maturation involving the insertion of methyl groups into the RNA chain is unaffected by the drug.

Short-term labelling experiments, coupled with the use of actinomycin D and base ratio analyses, suggest that, if the 26s species induced by thioacetamide is the result of a fault arising in the process proposed by Penman (1966), it is more probable that the fault lies in the conversion of the 45s precursor RNA into the smaller ribosomal RNA component. However, it is not clear how this situation could arise, although Villalobos, Steele & Busch (1964) reported an increase in the general level of nuclear ribonuclease activity in the livers of thioacetamide-treated rats. On the other hand, the results of Steele *et al.* (1965) suggest that an effect of thioacetamide treatment is to suppress the activity of a particular ribonuclease in rat liver that cleaves the precursors of ribosomal RNA. However, there may be a number of nuclear ribonucleases with different specificities associated with various regions of the nucleus.

On this basis it could be proposed, for the thioacetamide-treated Krebs II cells, that in addition to the normal nuclear ribonuclease responsible for the cleavage of the 45s precursor species on the scheme proposed by Penman (1966) either (*a*) a small amount of a latent ribonuclease with a different specificity is activated which degrades some of the 45s precursor RNA to give rise to the 26s component, or (*b*) there is a disruption of the normal nuclear organization such that a ribonuclease which normally does not come into contact with ribosomal precursor RNA, owing to nuclear compartmentalization, is allowed to degrade some 45s material, giving rise to a small amount of 26s RNA. Further, it seems reasonable to propose that the 26s RNA thus formed now cannot combine in the normal manner with protein sub-units to become a constituent part of cytoplasmic ribosomes, and thus can only accumulate in the nucleus.

At present, since thioacetamide affects the maturation of ribosomal RNA, both in rat liver and ascites-tumour cells, but in different ways, it is difficult to decide whether or not this effect has any connexion with the drug's action as a carcinogen.

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