Characterization of the Ribonucleic Acid Synthesized by Isolated Rat-Liver Nuclei

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(Received 18 May 1966)

1. Isolated rat-liver nuclei incorporated [^{14}C]UMP into RNA when incubated in the presence of Mg^{2+} and all four ribonucleoside triphosphates. The addition of bentonite to the system diminished the breakdown of the newly synthesized RNA. 2. AMP and CMP were incorporated in the absence of the other added triphosphates, and in the presence of deoxyribonuclease. 3. RNA synthesized in the presence of Mg^{2+} contained a high proportion of CMP and GMP, and sedimented in the regions of ribosomal RNA and of heavier molecules. About 1% of this RNA hybridized with homologous DNA, and hybrid formation was more effectively inhibited by nuclear RNA than by ribosomal RNA. 4. RNA synthesized in the presence of Mn^{2+} plus ammonium sulphate had a composition intermediate between that of ribosomal RNA and of DNA, and about 4% of this RNA formed hybrids with DNA. 5. Less than 2% of the newly synthesized RNA was capable of forming ribonuclease- and deoxyribonuclease-resistant complexes. 6. It was concluded that the newly synthesized RNA arose as a result of an asymmetric process and included both ribosomal and DNA-like species.

The RNA that is synthesized in the nuclei of intact animal cells *in vitro* includes high-molecular-weight components having base compositions resembling those of ribosomal RNA and of DNA (Scherrer, Latham & Darnell, 1963; Georgiev, Samarina, Lerman, Smirnov & Severtzov, 1963; Yoshikawa-Fukada, Fukada & Kawade, 1965). The ribosome-like RNA has been shown to be the precursor of typical 28s and 18s ribosomal RNA (Scherrer *et al.* 1963; Girard, Penman & Darnell, 1964; Tamaoki & Mueller, 1963; Perry, 1964), whereas the nuclear DNA-like RNA is thought to be the source of the DNA-like messenger RNA associated with cytoplasmic polyribosomes (Penman, Scherrer, Becker & Darnell, 1963; Latham & Darnell, 1965; McConkey & Hopkins, 1965).

Similar observations have been made specifically with liver cells. Thus Steele, Okamura & Busch (1965) have shown that rat-liver nuclei *in vivo* synthesize both high-molecular-weight ribosomal-precursor RNA and DNA-like RNA. The latter probably gives rise to the rapidly labelled DNA-like template RNA found in rat-liver cytoplasm (Munro & Korner, 1963; DiGirolamo, Henshaw & Hiatt, 1964).

Isolated nuclei from tumour cells (Takahashi, Swint & Hurlbert, 1963) and from rat liver (Hurlbert, Liau & Orenge, 1964; Widnell & Tata, 1964b;

Widnell, 1965) have been shown to synthesize both ribosomal RNA and DNA-like RNA when incubated with all four ribonucleoside triphosphates. These results are confirmed in the present work, in which the DNA-like RNA was also characterized by its capacity to form specific complexes with rat-liver DNA.

MATERIALS AND METHODS

Materials. ATP, CTP, GTP and UTP were purchased from Calbiochem Ltd. (Basingstoke, Hants.). Solutions of the required molarity were adjusted to pH 7.0 with 1.0N-NaOH. Creatine phosphate was purchased from Boehringer Corporation Ltd. (Ealing, London, W. 5). Creatine phosphokinase (EC 2.7.3.2) was prepared by the method of Kuby, Noda & Lardy (1954). Crystalline pancreatic deoxyribonuclease and ribonuclease, ribonuclease-free deoxyribonuclease and ribonuclease A were purchased from Worthington Biochemical Corp. (Freehold, N.J., U.S.A.); these enzymes were used as supplied without further purification.

[8- ^{14}C]ATP and [8- ^{14}C]GTP were purchased from Schwartz BioResearch Inc. (Orangeburg, N.Y., U.S.A.). [6- ^{14}C]CTP and [6- ^{14}C]UTP were prepared by the enzymic method of Canellakis, Gottesman & Kammen (1960) from [6- ^{14}C]orotic acid purchased from The Radiochemical Centre (Amersham, Bucks.).

Bentonite was suspended in 0.01M-tris-HCl buffer, pH 7.4, and the coarse particles were removed by centrifuging for 15 min. at 1500g. The supernatant was centrifuged for 20 min. at 10000g and the resulting pellet was washed by

resuspending in the same buffer as before and again centrifuging for 20 min. at 10000g. This procedure was repeated three more times with 0.005 M-MgCl₂-0.01 M-tris-HCl buffer, pH 7.4, as the medium, and the final sediment was stored in suspension in the same medium.

Sucrose solutions for density gradients were boiled for 5 min. and stored at -20°.

Nucleic acids. Rat-liver DNA for hybridization was prepared by a modification of the method of Colter, Brown & Ellem (1962). Liver from rats that had been starved overnight was homogenized with 9 vol. of 1.0 M-NaCl-0.02 M-potassium phosphate buffer, pH 7.4. Sodium deoxycholate was added (final concn. 0.5%) and the homogenate was stirred for 60 min. at 10° with 1 vol. of phenol that had previously been saturated with the homogenizing medium. The mixture was centrifuged for 15 min. at 2000g and the supernatant was re-extracted with phenol and centrifuged as before. The supernatant was mixed with 1 vol. of ethanol, and the DNA was collected as fibres on a glass rod, washed with 75% (v/v) ethanol and dissolved in 0.2 × SSC* (to give approx. 0.4 mg. of DNA/ml.). This solution was incubated with 50 µg. of ribonuclease A/ml. for 30 min. at 37°, adjusted to 1.0 M with respect to NaCl and stirred for 30 min. with phenol. Centrifugation and recovery of DNA were carried out as before, and the treatment with ribonuclease A was repeated. The DNA was re-extracted with phenol (this time in the presence of 0.25% sodium dodecyl sulphate), again recovered and washed with ethanol, and dissolved in 0.2 × SSC to give approx. 1.3 mg. of DNA/ml. This DNA had a sedimentation coefficient of 24 s measured at 20° at a concentration of 100 µg./ml. in 1 × SSC. The solution was diluted to give 200 µg. of DNA/ml. in 0.03 × SSC, heated for 15 min. at 100° to denature the DNA and then cooled quickly in ice.

DNA from *Escherichia coli* was prepared and treated with ribonuclease A as described by Marmur (1961).

Ribosomal RNA was isolated by phenol extraction of ribosomes, which were prepared by deoxycholate treatment of rat-liver microsomes (e.g. Klemperer, 1963).

Nuclear RNA for use as a competitor in hybridization experiments was prepared by phenol extraction of rat-liver nuclei at 60° in the presence of 0.5% sodium dodecyl sulphate as described by Scherrer & Darnell (1962). Nuclei for this purpose were isolated as described by Hymer (1963) by a low-speed centrifugation from a homogenate of liver in 4 vol. of 0.25 M-sucrose-0.003 M-MgCl₂-0.5% Triton X-100.

Estimation of DNA and RNA. DNA was estimated by the method of Burton (1965). RNA was estimated in the presence of DNA as described by Zamenhof (1957).

Preparation of nuclei. Nuclei for incubation were prepared from rat liver by the method of Chauveau, Moulé & Rouiller (1956) as modified by Widnell & Tata (1964a), and were finally suspended in 0.25 M-sucrose-0.003 M-MgCl₂.

Incubation of nuclei. The reaction mixtures were based on those of Allfrey & Mirsky (1959) and of Takahashi *et al.* (1963), and contained in 0.5 ml.: nuclei (containing 1 mg. of DNA), 50 µmoles each of ATP, CTP, GTP and [¹⁴C]UTP, 3.5 µmoles of creatine phosphate, 250 µg. of creatine phosphokinase, 1.6 mg. of bentonite, 2.5 µmoles of MgCl₂,

12.5 µmoles of tris-HCl buffer, pH 7.4, 6 µmoles of NaCl, 10 µmoles of glucose and 85 µmoles of sucrose. During the incubation at 37° the mixture was stirred occasionally with a glass rod to keep the nuclei in suspension.

When the incorporation of nucleotides other than [¹⁴C]UMP was measured, bentonite was omitted since the corresponding nucleosides, produced by degradation during the incubation, are adsorbed by bentonite when acid is subsequently added (Richmond & Cherry, 1964) and so contribute radioactivity to the acid-insoluble reaction product. In incubations for the preparation of labelled RNA the reaction volume was increased 10–20-fold, and in some cases MgCl₂ was replaced by MnCl₂ (5 µmoles/ml.) plus (NH₄)₂SO₄ (to a final concentration corresponding to 5% saturation) and bentonite was omitted.

Estimation of incorporation of radioactivity. After incubation the tubes were cooled in ice and 0.15 ml. of 0.2 M-sodium pyrophosphate (pH 7.5) was added to diminish the non-specific binding of nucleotides (Kammen, Klemperer & Canellakis, 1961). The precipitate that formed on adding 7 ml. of 0.4 N-HClO₄ was collected by centrifugation at 1000g for 10 min. and washed three times by resuspension in 7 ml. of 0.4 N-HClO₄ and centrifugation. The final pellet was transferred in 5.0 N-NH₃ to a stainless-steel planchet (2.5 cm. diam.) and dried in an oven at 80°. Radioactivity was measured at zero thickness in a windowless gas-flow counter (efficiency approx. 30%, background 16 counts/min.). The amount of nucleotide incorporated was calculated from the specific activity of the precursor determined under similar counting conditions.

Extraction of labelled nuclear RNA. No RNA was lost from the nuclei to the medium during short incubations at 37°, but RNA breakdown, as shown by a decrease in the proportion of heavier RNA molecules, was found even at 0°, and the nuclei were therefore not washed after incubation. RNA was extracted from the whole reaction mixture, according to the method of Scherrer & Darnell (1962), with phenol at 60° in the presence of 0.5% sodium dodecyl sulphate and 0.02 M-sodium acetate, pH 5.1. Under these conditions 85–90% of the radioactive RNA was extracted. The RNA, after precipitation with ethanol, was finally dissolved in 0.005 M-MgCl₂-0.02 M-tris-HCl buffer, pH 7.4, to give approx. 250 µg. of RNA/ml. This solution was taken for determination of the composition of the RNA.

DNA in the RNA was removed by treatment with ribonuclease-free deoxyribonuclease, re-extraction with phenol and ethanol precipitation in the presence of 2 M-sodium acetate, pH 7.4, as described by Hiatt (1962). The RNA was finally dissolved in 0.02 M-tris-HCl buffer, pH 7.4, to give approx. 0.5 mg. of RNA/ml. This solution was used for density-gradient studies. It contained 80–85% of the labelled RNA originally present in the nuclei and was contaminated with 15 µg. of deoxyribonucleotide/100 µg. of RNA. For hybridization studies the deoxyribonuclease treatment and phenol extraction were repeated to decrease this contamination to less than 5 µg./100 µg. of RNA, and the final solution contained approx. 150 µg. of RNA/ml. in 0.2 × SSC.

Determination of composition of RNA. RNA extracted from nuclei (approx. 300 µg.) was precipitated in the presence of 1 mg. of carrier ribosomal RNA with 7 ml. of 0.4 N-HClO₄ at 0°. The precipitate was washed twice with 7 ml. of 0.4 N-HClO₄ and dissolved in 0.2 ml. of 0.3 N-KOH (Davidson & Smellie, 1952). After incubation at 37° for

* Abbreviation: SSC, 0.15 M-NaCl-0.015 M-trisodium citrate, pH 7.3, preceded by a number to indicate concentration, e.g. 2 × for double strength (0.30 M-NaCl-0.03 M-trisodium citrate, pH 7.3).

18 hr. the mixture was adjusted to pH 4.0 with 1.0 N-HClO₄ at 0° and the precipitate of KClO₄ was removed by centrifugation for 10 min. at 1000g. A sample of the supernatant (0.05 ml. containing approx. 2500 counts/min.) was applied as a transverse band to a strip of Whatman no. 1 filter paper (2.5 cm. wide), and the 2'(3')-mononucleotides were separated by high-voltage electrophoresis in 0.04 M-sodium citrate, pH 3.5, on a cooled plate (10°) for 2.5 hr. at 50 v/cm. The paper was dried in an oven at 60° and the positions of the nucleotides were located as the absorbing areas under u.v. light.

The paper strip was divided into 2.5 cm. × 2.5 cm. areas, which were placed in 12 ml. of scintillation fluid [toluene containing 4 g. of 2,5-diphenyloxazole and 0.05 g. of 1,4-bis-(5-phenyloxazol-2-yl)benzene/l.] for measurement of radioactivity in a liquid-scintillation counter (background 35 counts/min., efficiency approx. 75%). About 95% of the radioactivity applied to the paper was recovered in the u.v.-absorbing areas. The relative proportions in which the radioactive nucleotides were present were calculated from the relative specific activities of the corresponding ribonucleoside triphosphates.

When the total amount of each nucleotide present was measured, 1 mg. of nuclear RNA was treated as above in the absence of carrier RNA, and the u.v.-absorbing areas were eluted with 3 ml. of 0.1 N-HCl for 3 hr. and the extinctions of the eluates were measured at 260 and 280 mμ (1 cm. light-path). After correction of the readings for blank values obtained from the corresponding areas of a similar strip without RNA hydrolysate, the nucleotides were characterized by the E_{280}/E_{260} ratios, and the amount of each nucleotide was estimated from the millimolar extinction coefficients at 260 mμ: AMP, 14.2, CMP, 6.8, UMP, 10.0; GTP, 11.8 (Beaven, Holiday & Johnson, 1955).

Sucrose-density-gradient centrifugation. Linear 5–20% (w/v) sucrose gradients in 0.1 M-NaCl–0.01 M-tris-HCl buffer, pH 7.4, were prepared by the method of Britten & Roberts (1960). The RNA sample (approx. 200 μg. and 5000 counts/min.) in 0.4 ml. of 0.02 M-tris-HCl buffer, pH 7.4, was layered on top of a 5 ml. gradient and centrifuged at 37000 rev./min. for 4 hr. at 4° in the 3 × 5 ml. swing-out rotor of the MSE preparative ultracentrifuge (Measuring and Scientific Equipment Ltd., London, S.W. 1). The bottom of the centrifuge tube was punctured with a hypodermic needle and the contents were collected serially in 20 equal fractions. Each fraction was diluted with 1 ml. of water and the extinction was measured at 260 mμ (1 cm. light-path). The whole fraction was then transferred to a vial containing 10 ml. of Bray's (1960) scintillation solution, and the radioactivity was measured in a liquid-scintillation counter (efficiency approx. 60%, background 35 counts/min.).

Hybridization of RNA with DNA. The procedure was based on the methods of McConkey & Hopkins (1964) and of Attardi, Huang & Kabat (1965). Labelled RNA (approx. 150 μg./ml. in 0.2 × SSC) was heated before use for 15 min. at 100° and then cooled rapidly in ice. Denatured DNA and RNA were incubated for 16 hr. in a water bath at 60° in a final volume of 1 ml. of 2 × SSC. The water bath was allowed to cool slowly to 25° over a period of 4 hr. Under these conditions the maximum amount of hybrid was formed. Ribonuclease A (10 μg.) was added to each tube and the mixture was incubated for 20 min. at 37°, diluted with 25 ml. of 4 × SSC at 25° and filtered on Millipore filters (Millipore HA, 450 mμ pore size). The filters were then

washed six times in succession with 10 ml. of 4 × SSC at 53° and dried in an oven at 37°. Radioactivity measurements were made in the same way as described above for filter-paper areas containing [¹⁴C]nucleotide, and were corrected for the blank values obtained with mixtures that were not incubated at 60°.

In agreement with McConkey & Hopkins (1964) it was shown that the filters retained 65% of the denatured DNA when quantities up to 120 μg. were applied. This makes it likely that the hybrid retained on the filters represented about 65% of the actual amount of hybrid formed.

Self-annealing of RNA. RNA was heated in 2 × SSC without added DNA for 16 hr. at 60° or 2 hr. at 90°, and then allowed to cool to 25° over a period of 4 hr. (see e.g. Weissmann & Borst, 1963; Colvill, Kanner, Tocchini-Valentini, Sarnat & Geiduschek, 1965). The reaction mixture was subsequently treated with ribonuclease A (10 μg./ml.) for 45 min. at 37°. The ribonuclease-resistant material was then precipitated at 0° with 0.3 ml. of 50% (w/v) trichloroacetic acid in the presence of 100 μg. of ribosomal RNA and 200 μg. of bovine serum albumin. The precipitate was collected by filtration on a Millipore filter (450 mμ pore size) and washed three times in succession with 5 ml. of 5% (w/v) trichloroacetic acid at 0°. The filter was then dried at 37° and the radioactivity was determined as before.

RESULTS

Incorporation of [¹⁴C]UMP into RNA by nuclei. When nuclei were incubated with bentonite, Mg²⁺ and all four ribonucleoside triphosphates, radioactivity was incorporated from [¹⁴C]UTP into acid-insoluble material (Table 1). This incorporation required creatine phosphate as an energy source and showed the typical characteristics of DNA-primed RNA synthesis in that it was dependent on the presence of the four ribonucleoside triphosphates and was inhibited by deoxyribonuclease and actinomycin D. The incomplete inhibition observed in the presence of ribonuclease was attributed to the presence of bentonite in the system.

In the complete system radioactivity was incorporated at a diminishing rate for 30–60 min. (Table 2), but in the absence of bentonite the amount of acid-insoluble radioactivity tended to fall after 10–15 min., suggesting that a greater breakdown of the newly synthesized RNA occurred under these circumstances. Attempts to maintain the rate of synthesis by further additions of ribonucleoside triphosphates were unsuccessful, and it could be shown that fresh nuclei added to the reaction mixture after 10–20 min. incorporated radioactivity at the initial rate. Thus the synthesis of RNA was limited by changes occurring in the nuclei during the incubation.

Composition of the newly synthesized RNA. Although the results in Table 1 show that incorporation of [¹⁴C]UMP was a measure of new RNA synthesis insofar as it was dependent on DNA and

the presence of all four ribonucleoside triphosphates, Table 3 shows that an appreciable incorporation of [^{14}C]AMP and [^{14}C]CMP occurred either in the

Table 1. *Incorporation of UMP into RNA by nuclei*

The complete system contained (in 0.5 ml.): nuclei (containing 1 mg. of DNA), 50 μmoles each of ATP, CTP, GTP and [^{14}C]UTP (7.6×10^6 counts/min./ μmole), 3.5 μmoles of creatine phosphate, 250 $\mu\text{g.}$ of creatine kinase, 1.6 mg. of bentonite, 2.5 μmoles of MgCl_2 , 12.5 μmoles of tris-HCl buffer, pH 7.4, 6 μmoles of NaCl, 10 μmoles of glucose and 85 μmoles of sucrose. These conditions were modified as shown. After incubation at 37° for 10 min. the acid-precipitable radioactivity was collected and counted. The values are corrected for the amount (10 μmoles of UMP) found with unincubated controls.

Incubation conditions	[^{14}C]UMP incorporated (μmoles)
Complete system	384
Creatine phosphate omitted	136
ATP, CTP and GTP omitted	14
Deoxyribonuclease (20 $\mu\text{g.}$) added	42
Actinomycin D (5 $\mu\text{g.}$) added	94
Ribonuclease (50 $\mu\text{g.}$) added	130

Table 2. *Effect of bentonite on incorporation of UMP*

Nuclei were incubated under the conditions described in Table 1, except that bentonite was omitted where indicated. Values are corrected for zero time (10 μmoles of UMP), as in Table 1.

Incubation time (min.)	[^{14}C]UMP incorporated (μmoles)	
	Complete system	Without bentonite
5	310	315
10	396	420
20	530	391
30	565	311
60	585	—

absence of the complementary ribonucleoside triphosphates (column *a*) or in the presence of deoxyribonuclease (column *c*). Therefore ATP and CTP were participating in other reactions in addition to typical DNA-primed RNA synthesis, and the overall composition of the RNA determined from the incorporation of the individual nucleotides need not represent the overall composition of a mixture of typical RNA molecules. On the other hand, the extent to which AMP and CMP were incorporated in other reactions at the same time as in typical RNA synthesis cannot necessarily be measured from the values in columns *a* and *c* (Table 3), since these conditions may favour other reactions, e.g. homopolymer synthesis primed by DNA (Chamberlin & Berg, 1962; Fox & Weiss, 1964) or RNA (Edmonds & Abrams, 1960; Edmonds, 1965).

The overall composition of the RNA calculated from the incorporation of the individual nucleotides (Table 3) shows some resemblance to that of ribosomal RNA, except that the proportion of AMP was very low. A similar composition was found on analysis of RNA isolated from incubation mixtures that contained all four ^{14}C -labelled triphosphates simultaneously (Table 4). When Mn^{2+} plus ammonium sulphate replaced Mg^{2+} (Widnell & Tata, 1964b; Widnell, 1965) the composition of the RNA resembled that of the total nuclear RNA, which has a higher AMP + UMP content and a lower GMP + CMP content than has ribosomal RNA. It can be calculated (e.g. Steele *et al.* 1965) that RNA of this composition could represent a mixture of approximately equal parts of ribosomal RNA and DNA-like RNA.

Sedimentation of RNA in sucrose density gradients. RNA isolated from freshly prepared nuclei sedimented with three peaks (Fig. 1a), which corresponded to the positions occupied under similar conditions by cytoplasmic 28s, 18s and 4s RNA. In addition, the high base line of the peaks indicated the presence of polydisperse material, and there was

Table 3. *Incorporation of labelled nucleotides by nuclei*

Nuclei were incubated for 10 min. under the conditions described in Table 1 with the following modifications. Bentonite was omitted in all cases, [^{14}C]ATP, [^{14}C]CTP and [^{14}C]GTP (all at 3.5×10^6 counts/min./ μmole) were substituted for [^{14}C]UTP as shown, and the other three triphosphates and deoxyribonuclease (20 $\mu\text{g.}$) were added where indicated.

^{14}C -labelled precursor	[^{14}C]Nucleotide incorporated (μmoles)			[^{14}C]Nucleotide incorporated (moles/100 moles)	
	No additions (a)	Other triphosphates added (b)	Triphosphates + deoxyribonuclease added (c)	From (b)	From (b-c)
ATP	95	338	90	15.1	14.4
UTP	6	400	44	17.9	20.7
CTP	129	830	328	37.2	29.2
GTP	44	665	50	29.8	35.7

Table 4. *Composition of nuclear RNA*

Nuclei were incubated as described in Table 1 for 10 min. except that the reaction volume was 1 ml. and all four [^{14}C]ribonucleoside triphosphates were present simultaneously. In (b) bentonite was omitted. In (c) MnCl_2 ($5\mu\text{moles/ml.}$) plus $(\text{NH}_4)_2\text{SO}_4$ (final concn. 5% saturation) were substituted for MgCl_2 , and bentonite was omitted; under these conditions the incorporation of UMP was increased by about 50%. RNA was extracted at 60° with phenol in the presence of 0.5% sodium dodecyl sulphate and hydrolysed with 0.3N-KOH. The nucleotides were separated by paper electrophoresis and taken for measurement of radioactivity (columns a, b and c) or for estimation by the extinction at $260\text{m}\mu$ (total nuclear RNA). The compositions of total rat-liver ribosomal RNA [calculated from Kirby (1965), assuming a 28s/18s weight ratio 2:1] and DNA (Wyatt, 1951) are provided for comparison.

Nucleotide	Composition (moles/100 moles)					
	Radioactive nuclear RNA			Total nuclear RNA	Ribosomal RNA	DNA
	(a)	(b)	(c)			
AMP	15.9	15.0	22.6	20.7	18.5	28.6
UMP	20.8	19.3	23.1	24.3	17.3	28.5*
CMP	34.3	32.9	27.4	26.6	31.4	21.4
GMP	29.0	32.8	26.9	28.4	32.8	21.4

*Thymidine monophosphate.

Fig. 1. Density-gradient sedimentation of nuclear RNA. RNA was extracted (a) from fresh nuclei and (b) from nuclei incubated under the conditions shown in Table 1. The RNA was centrifuged in a 5 ml. sucrose density gradient (5–20%, w/v) for 4 hr. at 37000 rev./min. in the 3×5 ml. swing-out rotor of the MSE preparative centrifuge. The tubes were punctured and fractions were collected for measurement of E_{260} (●) and of radioactivity (○). The first fraction is the lowest part of the gradient.

also evidence for RNA heavier than 28 s. The RNA prepared from nuclei that had been incubated for 10 min. (Fig. 1b) showed evidence of breakdown, since there was an increase in the more slowly sedimenting RNA at the expense of the heavier molecules. The distribution in the same density gradient of RNA labelled with [^{14}C]UMP in the presence of Mg^{2+} showed that the newly synthesized RNA consisted of molecules that sedimented mainly in the regions of ribosomal RNA and of larger molecules.

Hybridization of RNA with DNA. Complementarity between the newly synthesized RNA and rat-liver DNA was demonstrated by the fact that ribonuclease-resistant complexes, which were readily retained by Millipore filters, were formed under conditions that favoured the formation of DNA–RNA hybrids.

Table 5 shows that about 1% of the radioactive RNA synthesized in the presence of Mg^{2+} formed complexes with homologous DNA. These complexes probably represented specific DNA–RNA hybrids, since they were not formed with *E. coli* DNA. Moreover, added ribosomal RNA ($100\mu\text{g.}$), which might be expected to dilute non-specific complexes, had little effect on the amount of complex formed. The amount of hybrid increased as the input of RNA was increased, but the proportion that hybridized was lowered. High concentrations of ribosomal RNA ($800\mu\text{g./ml.}$) inhibited complex-formation but added nuclear RNA was a much more effective inhibitor and at high concentrations inhibited by well over 50%.

The RNA synthesized in the presence of Mn^{2+}

Table 5. *Hybridization of labelled nuclear RNA with DNA*

Nuclei were incubated under the conditions described in Table 1 except that the specific activity of [^{14}C]UTP was 22.8×10^6 counts/min./ μmole and the reaction volume was increased to 10 ml. The RNA was extracted, heated for 15 min. at 100° in $0.2 \times \text{SSC}$ and incubated in the amounts shown with denatured DNA in 1 ml. of $2 \times \text{SSC}$ for 16 hr. at 60° . Denatured *E. coli* DNA, non-radioactive ribosomal RNA or nuclear RNA was present during this incubation as shown. After treatment with ribonuclease A the hybrids were collected by Millipore filtration. The radioactivity retained on the filter was corrected for blank values (2–5 counts/min.) obtained with similar mixtures that were not incubated at 60° .

RNA ($\mu\text{g.}$)	DNA ($\mu\text{g.}$)	Addition	Hybrid	
			(counts/min.)	(% of input)
27	0	—	1	—
27	70	—	47	1.4
54	70	—	65	1.0
81	70	—	82	0.8
27	0	<i>E. coli</i> DNA (65 $\mu\text{g.}$)	2	—
27	70	Ribosomal RNA (100 $\mu\text{g.}$)	37	1.1
27	70	Ribosomal RNA (800 $\mu\text{g.}$)	26	0.8
27	70	Nuclear RNA (100 $\mu\text{g.}$)	21	0.6
27	70	Nuclear RNA (800 $\mu\text{g.}$)	6	0.2

Table 6. *Hybridization of 'DNA-like RNA' with DNA*

The conditions were as described in Table 5, except that the nuclei were incubated in the absence of bentonite and in the presence of $\text{MnCl}_2 + (\text{NH}_4)_2\text{SO}_4$ (see Table 4). The blank values were 3–22 counts/min.

RNA ($\mu\text{g.}$)	DNA ($\mu\text{g.}$)	Addition	Hybrid	
			(counts/min.)	(% of input)
16	0	—	2	—
5	70	—	48	4.1
16	70	—	139	3.6
32	70	—	202	2.6
16	0	<i>E. coli</i> DNA (65 $\mu\text{g.}$)	2	—
16	70	Ribosomal RNA (100 $\mu\text{g.}$)	122	3.1
16	70	Ribosomal RNA (800 $\mu\text{g.}$)	102	2.6
16	70	Nuclear RNA (100 $\mu\text{g.}$)	83	2.1
16	70	Nuclear RNA (100 $\mu\text{g.}$)	22	0.6

Table 7. *Ribonuclease-resistant fraction of RNA*

RNA was prepared as described in Tables 5 and 6 and was heated for 15 min. at 100° in $0.2 \times \text{SSC}$. The ribonuclease-resistant radioactivity was estimated either at once or after the treatment shown. Heating at 60° for 16 hr. or at 90° for 2 hr. was in 1 ml. of $2 \times \text{SSC}$. Treatment with ribonuclease-free deoxyribonuclease (10 $\mu\text{g.}/\text{ml.}$) was for 30 min. at 37° in the presence of 35 μmoles of MgCl_2 ; in this case 20 μmoles of EDTA were added before the subsequent ribonuclease treatment. Ribonuclease-resistant material was collected by precipitation with acid and filtration on Millipore membranes and is expressed as percentage of input, namely 27 $\mu\text{g.}$ and 3360 total counts/min. (Mg^{2+}), or 32 $\mu\text{g.}$ and 7810 total counts/min. (Mn^{2+}).

Treatment of RNA	Ribonuclease-resistant radioactivity (%) in RNA synthesized	
	Mg^{2+} present	Mn^{2+} present
—	4.0	1.6
60° for 16 hr.	6.3	5.3
90° for 2 hr.	6.2	5.4
60° for 16 hr.; then 100° for 15 min.	4.3	1.7
60° for 16 hr.; then deoxyribonuclease	4.5	—
120° for 15 min.*	2.6	—

* In this case prior heating at 100° for 15 min. was omitted, and heating in $0.2 \times \text{SSC}$ was performed in a sealed tube immersed in an oil bath at 120° .

showed a similar behaviour (Table 6), except that about 4% of this RNA formed hybrids.

Tests for the existence of self-complementary RNA. Although Tables 5 and 6 show that without added DNA no ribonuclease-resistant complexes were formed that were retained on Millipore filters, Table 7 shows that complexes that were acid-precipitable were formed under these conditions. Thus heating at 60° for 16 hr. or at 90° for 2 hr., which may favour the formation of double-stranded RNA complexes (Weissmann & Borst, 1963), caused a small increase in resistance to ribonuclease. This increase was, however, due to the formation of DNA-RNA complexes with fragments of DNA present as a contaminant in the RNA preparation, since the increased resistance to ribonuclease was abolished by heating at 100° or by treatment with ribonuclease-free deoxyribonuclease. Essentially similar results were obtained with the RNA synthesized in the presence of Mn^{2+} . It is possible that a small amount of RNA-RNA complex was present in the RNA synthesized in the presence of Mg^{2+} , since heating at 120°, which denatures such complexes (Weissmann & Borst, 1963), lowered the amount of ribonuclease-resistant 'core' of this RNA from the rather high value of 4% to 2.6%.

DISCUSSION

RNA composition and sedimentation. Other workers have previously reported that isolated rat-liver nuclei can synthesize RNA that resembles ribosomal RNA in its composition (see the introduction). In the present work the incorporation of UMP into RNA depended on a typical DNA-primed RNA synthesis, but AMP and CMP could be incorporated by other reactions as well. This causes difficulties in interpreting the overall composition of the newly synthesized RNA, since an unknown proportion of the AMP and CMP may have been incorporated, e.g. as homopolymers. With this reservation, the composition of the newly synthesized RNA, both from the incorporation of the individual nucleotides and from the analysis of the isolated RNA, showed some resemblance to ribosomal RNA, the main difference being that ribosomal RNA has a higher proportion of AMP. A low overall content of AMP has also been reported for the nuclear RNA that is rapidly labelled with [^{32}P]phosphate *in vivo* (Hiatt, 1962), although this value may be open to the criticism that the ^{32}P may not have equilibrated completely with the phosphorus-containing compounds in the cell.

The intact cell synthesizes a large proportion of ribosomal RNA, and much of the RNA labelled in isolated nuclei by [^{14}C]UMP could have been of this type on the basis of its sedimentation in density

gradients. As has also been shown by Widnell (1965), the newly synthesized RNA sedimented in regions that correspond to the positions of ribosomal RNA and of the heavier ribosomal-precursor RNA.

High-molecular-weight nuclear RNA also includes components that are DNA-like in composition and form hybrids readily with DNA (e.g. Scherrer *et al.* 1963) and that have template (messenger) activity for protein synthesis *in vitro* (Sporn & Dingman, 1963; DiGirolamo *et al.* 1964). From the overall composition of the RNA made in the presence of Mg^{2+} such molecules could only constitute a minor component. By contrast, as previously shown by Widnell (1965), a high proportion of DNA-like RNA appeared to be made in the presence of Mn^{2+} . Under these circumstances the composition of the RNA resembled that of the total nuclear RNA, and corresponded to approximately equal proportions of ribosomal-like RNA and DNA-like RNA.

Hybridization of RNA with DNA. The fact that a heterogeneous population of DNA-like (or messenger) RNA molecules has a far larger number of complementary sites on DNA than has ribosomal RNA has made it possible to detect small amounts of such molecules in nuclei and cytoplasm in the presence of an excess of ribosomal RNA (e.g. Scherrer *et al.* 1963; Hoyer, McCarthy & Bolton, 1963; McConkey & Hopkins, 1965). In the experiments described in the present paper the proportion of RNA to DNA was too high for any appreciable fraction of the ribosomal RNA in the nuclear RNA to form specific complexes with DNA, since 100 μg . of DNA has a capacity for not more than about 0.05 μg . of ribosomal RNA (McConkey & Hopkins, 1964; Attardi *et al.* 1965).

In agreement with this, added ribosomal RNA was a less effective inhibitor of hybrid formation than was nuclear RNA, which contains a high proportion of DNA-like RNA (see also Georgiev *et al.* 1963; Steele *et al.* 1965). This suggests that DNA-like RNA was competing with the radioactive RNA for hybrid formation. The inhibition observed at very high concentrations of ribosomal RNA may be attributable to small amounts of associated messenger RNA. Hybridization of the RNA synthesized in the presence of Mg^{2+} therefore depended on the presence of a minor component of DNA-like RNA. This conclusion is supported by the fact that RNA made in the presence of Mn^{2+} contained approx. 50% of DNA-like RNA and formed hybrids much more readily.

It is likely that there is a wide variation in the amounts of the different species of DNA-like RNA in nuclear RNA, as is the case with messenger RNA in intact animal cells (McCarthy & Hoyer, 1964). This could explain the fact that the fraction of

input RNA converted into hybrid decreased with increasing RNA input, since the more abundantly represented species of RNA would already saturate their complementary DNA sites at low RNA inputs.

Hybridization was probably incomplete even at concentrations below that at which the DNA became saturated, because McConkey & Hopkins (1965) under similar conditions found that a maximum of 16% of messenger RNA formed hybrids. Moreover, since only 65% of the denatured DNA was retained on the filters, filter retention of hybrid probably gives a corresponding underestimation of the total amount of hybrid formed. Taking both these facts into consideration and assuming that the hybrids retained by the filters represented only 10% of the readily hybridizable RNA, then 40% of the RNA synthesized in the presence of Mn^{2+} was DNA-like, which is in approximate agreement with the proportion suggested by the overall composition of this RNA. In the same way about 10% of the RNA synthesized in the presence of Mg^{2+} would be of the readily hybridizable type, and this is similar to the proportion of messenger RNA in the rapidly synthesized RNA from intact mouse liver (Hoyer *et al.* 1963).

Asymmetry of RNA. RNA made *in vivo* is asymmetric in that it is a copy of only one strand of the DNA duplex, but in some reconstructed systems 'self-complementary' RNA is copied from both strands (reviewed, e.g., in Geiduschek, Tocchini-Valentini & Sarnat, 1964). On the other hand, in many systems *in vitro* a high proportion of asymmetric RNA is synthesized (e.g. Geiduschek, *et al.* 1964; Green, 1964; Colvill *et al.* 1965; Shenkin & Burdon, 1966). In the present work, the fact that RNA from natural sources inhibited the formation of hybrids with DNA by well over 50% shows that most of this hybridizing RNA was asymmetric. Further, there was no evidence for self-annealing by any of the newly synthesized RNA under conditions that favour the formation of double-stranded RNA complexes. Thus in this respect, and in the fact that both messenger RNA and ribosomal RNA appeared to be present, the RNA synthesized by isolated nuclei resembled that made *in vivo*.

This work was supported in part by a Research Grant from the Medical Research Council.

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