STUDIES ON 4S AND 5S RNA OF HeLa CELLS

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To Carol

"Love and Flowers"

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ABSTRACT

SECTION I

Section I is concerned with a partial sequence analysis conducted on 5S RNA from HeLa cells. Analysis of the oligonucleotide pattern after pancreatic ribonuclease digestion of a highly-purified preparation of 5S RNA gave results which were in general agreement with those published for KB cells, both with respect to the identity and the frequency of the partial sequences. However, the presence of a trinucleotide not found in the KB 5S pattern, together with the reproducibly much lower than expected molar yield of the larger oligonucleotides strongly suggested the occurrence of alternate sequences at various sites in the 5S molecules of human cells. The presence of ppGp and pppGp at the 5'-terminus of HeLa 5S RNA was clearly demonstrated. The implications of this finding with regard to the origin of 5S RNA are discussed.

SECTION II

In Section II the proportion of the HeLa cell genome complementary to tRNA was investigated by using RNA-DNA hybridization. The value for saturation of the HeLa DNA by tRNA was found to be 1.1×10^{-5}, which corresponds to about 4900 sites for tRNA per HeLa cell in an exponentially growing culture. Analysis of the nucleotide composition of the hybridized tRNA revealed significant differences from the nucleotide composition of the input tRNA, with the purine to pyrimidine ratio indicating, however, that these differences were

not produced by excessive RNase attack of the hybrid. The size of the hybridized tRNA was only moderately smaller than that of the input RNA; the average S value in formaldehyde was 2.7 (corresponding to a length of about 65 nucleotides), suggesting that a relatively small portion near the ends of the hybridized 4S chains had been removed by RNase.

SECTION III

The proportion of the HeLa cell genome complementary to 5S RNA was investigated by using RNA-DNA hybridization. The value for saturation of the HeLa DNA by 5S RNA was found to be 2.3×10^{-5}, which corresponds to about 7,000 sites for 5S RNA per HeLa cell in an exponentially growing culture. Analysis of the nucleotide composition of the hybridized 5S RNA revealed no significant difference from the nucleotide composition of the input RNA. At the RNA to DNA input ratio of 1:1000, the average S value in formaldehyde of the hybridized 5S RNA corresponded to a polynucleotide chain about two-thirds the size of the input RNA.

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GENERAL INTRODUCTION

GENERAL INTRODUCTION-5S RNA

A low molecular weight RNA, with a sedimentation constant of about 5S, has been found as a component of the ribosomes in all organisms thus far investigated, including bacteria (Rosset and Monier, 1963; Rosset, Monier and Julien, 1964; Marcot-Queiroz, Rosset and Monier, 1966; Schleich and Goldstein, 1966; Morell et al., 1967), aquatic fungus (Comb and Katz, 1964), yeast (Marcot-Queiroz et al., 1965), sea urchins (Comb et al., 1965), clawed toad (Brown and Littna, 1966), and mammalian cells (Galibert et al., 1965; Bachvaroff and Tongur, 1966; Hayward, Legault-Dèmare and Weiss, 1966; Reich et al., 1966; Knight and Darnell, 1967). This 5S RNA is associated with the larger ribosomal subunit, presumably in all organisms, since it is found to be so in E. coli (Rosset et al., 1964), Saccharomyces cerevisiae (Marcot-Queiroz et al., 1965), Blastocladiella emersonii (Comb and Zehavi-Willner, 1967), Xenopus laevis (Brown and Littna, 1966) and HeLa cells (Knight and Darnell, 1967); it is present to the extent of one 5S molecule per large subunit. At low Mg^{++} concentrations ($< 10^{-3}$ M), 5S RNA is much more resistant than transfer RNA to removal from ribosomes of bacteria, as well as of eukaryotic organisms (Rosset et al., 1964; Bachvaroff and Tongur, 1966; Galibert et al., 1966; Schleich and Goldstein, 1966; Aubert et al., 1967; Comb and Sarkar, 1967; Knight and Darnell, 1967). In fact, in order to dissociate 5S RNA completely from ribosomes both of E. coli (Aubert et al., 1967; Sarkar and Comb, 1969) and of Blastocladiella emersonii (Comb and Sarkar, 1967) it is necessary to add EDTA, which permits the

ribosomal subunit to unfold and release 5S RNA, as well as some protein; to recombine the 5S RNA with the EDTA-treated subunit the solubilized protein must be present. Removal of about 50% of the proteins of the E. coli 50S ribosomal subunit with 2 M LiCl also results in loss of 70 to 80% of the 5S RNA (Marcot-Queiroz and Monier, 1967); this effect seems to be restricted to LiCl, since similar concentrations of KCl or NaCl also results in loss of proteins, but not of 5S RNA. The 5S RNA can be restored to LiCl ribosomal "cores" only in the presence of the protein released by LiCl treatment; the 5S binding site of these cores is specific, as tRNA does not affect the 5S binding (Aubert et al., 1967). It was also noted that, when the conformation of 5S RNA was changed (e.g., by gel electrophoresis at high pH), the binding ability to LiCl "cores" was reduced; such binding ability could be restored almost completely by heating the 5S RNA in the presence of Mg^{++}, under the conditions used by Lindahl, Adams and Fresco (1966) for renaturing leucyl tRNA. Taken together, the above results suggest that in all organisms, 5S RNA is a structural component of the larger ribosomal subunit, specifically bound to a site distinct from the tRNA binding sites; this 5S binding depends on the participation of ribosomal protein(s) and requires the proper conformation of 5S RNA.

Isolation of 5S ribosomal RNA has been accomplished by several techniques: chromatography on DEAE-cellulose at 80°C (Comb and Sarkar, 1967), chromatography on methylated albumin-kieselguhr (Marcot-Queiroz et al., 1965), chromatography on protamine-celite columns (Brown and Littna, 1966), gel filtration through Sephadex G-100 (Galibert et al.,

1965), and gel electrophoresis, either on agar (Bachvaroff and Tongur, 1966) or polyacrylamide (Knight and Darnell, 1967). From Table I it can be seen that purified 5S RNA in all organisms examined, from bacteria to humans, has a nucleotide composition similar to that of tRNA but without any pseudouridylic acid or methylated nucleotides; the small amount of pseudouridylic acid or methylated nucleotides detected in some 5S preparations is probably due to contaminating tRNA and/or high molecular weight ribosomal RNA. The table also shows that, unlike tRNA, 5S RNA has no acceptor activity for amino acids. The sedimentation coefficients measured for 5S RNA vary from about 4.5 to 5.3S, and are dependent upon the concentration of 5S RNA used in the sedimentation run (Comb and Zehavi-Willner, 1967).

Although some early work on 5S RNA had suggested that it might be a precursor of tRNA, a large body of evidence has accumulated which totally excludes this possibility. Hybridization studies between DNA and 5S RNA have demonstrated that in bacteria (Zehavi-Willner and Comb, 1966; Morell et al., 1967) and in the clawed toad (Brown and Weber, 1968) the sites in DNA for tRNA and for 5S RNA are distinct. Hybrids formed between 5S RNA and the synthetic RNA complementary to 4S RNA suggest that in rat liver, the base sequences of 5S and tRNA are unrelated (Hayward et al., 1966). Kinetic experiments in E. coli and in KB cells ruled out the possibility of 5S being a tRNA precursor, on the basis of a mathematical analysis of the incorporation of ^{32}P into these RNA species (Galibert et al., 1967).

TABLE I

Characteristics of 5S and transfer RNA of various organisms

Organism	5S RNA nucleotide composition (mole %)						Amino acid acceptor	$S_{20,w}$	tRNA nucleotide composition (mole %)					
	Cp	Ap	Up	Gp	ψp	methylated			Cp	Ap	Up	Gp	ψp	methylated
Escherichia coli[a]	28.9	18.5	16.8	34.8	0.18	n.d.	-	4.5	28.4	18.4	18.8	30.8	2.4	n.d.
Bacillus subtilis[b]	27.1	22.5	19.9	30.3	n.d.	n.d.	n.d.	n.d.	25.8	18.5	22.8	32.9	n.d.	n.d.
Blastocladiella emersonii[c]	24.6	19.2	23.7	32.5	0.6[d]	-	-	5.3[d]	25.5	16.0	25.5	32.9	2.4[d]	n.d.
Saccharomyces cerevisiae[e]	23.8	24.1	23.4	27.4	1.3	0.2	-	n.d.	22.9	19.3	18.6	27.5	4.7	2.1
Rat liver[f]	27.1	18.1	21.6	33.2	0.3	-	-	4.6	28.7	19.5	21.0	30.8	4	3
KB cells[g]	26.4	17.7	24.7	31.0	0.26	-	-	n.d.	25.3	16.5	22.4	31.0	4.8	1.5

(a) Rosset et al. (1964).

(b) Morell et al. (1967).

TABLE I (continued)

(c) Comb and Katz (1964).

(d) Comb and Zehavi-Willner (1967).

(e) Marcot-Queiroz et al. (1965).

(f) Bachvaroff and Tongur (1966).

(g) Galibert et al. (1965).

The primary structure (i.e., the sequence) of 5S RNA from E. coli (Sanger, Brownlee and Barrell, 1967) and from KB cells (Forget and Weissman, 1967) has been determined. The 5S RNA from both species is 120 nucleotides long, and has uridine as its 3'-terminus. However, in E. coli, pUp is the exclusive 5'-terminus found, while in KB cell 5S RNA, pGp is the only 5'-end isolated, appearing in a puzzling half-molar yield. In the present work conducted on 5S RNA of HeLa cells (another cell line of human origin), some pGp was present, but the 5'-terminus was represented mainly by pppGp and ppGp; these ends probably also exist in KB cell 5S RNA, but have not been detected. Comparison of the partial sequences released by T1 or by pancreatic ribonuclease digestion of 5S RNA from E. coli and from KB cells reveals no common sequences larger than tetranucleotides. In E. coli there are two positions in the 5S molecule where alternate bases can occur in the same cell, thereby indicating a certain heterogeneity in the 5S RNA population of these bacteria; the sequence data from KB cells also suggests a limited amount of heterogeneity, which is confirmed by these present partial sequence studies on HeLa 5S RNA.

The secondary structure of 5S RNA has been most extensively investigated in E. coli. Cantor (1968) has demonstrated that E. coli 5S RNA possesses extensive base pairing, as shown by several optical methods, including optical rotatory dispersion (43 base pairs indicated) and ultraviolet difference spectrum (40 to 43 base pairs indicated). From the temperature dependence of optical rotation or ultraviolet absorption, the presence of both AU and GC pairs in 5S RNA

was revealed; the broad melting curve, leveling off at room temperature, implied a large number of double-stranded regions. Optical techniques were also used by Boedtker and Kelling (1967) to explore the secondary structure of E. coli 5S RNA; measurement of the spectrum in the ultraviolet at high and low temperature, before and after reacting with formaldehyde, indicated 63% of the bases were involved in double helices (i.e., 38 base pairs). Comparison of the UV melting profile in neutral buffer to that in 50% methanol buffer solution led to the estimate that double-stranded regions involved 73% of the bases; the UV melting profiles at several different Na^{+} and Mg^{++} concentrations were consistent with a double helical content of 63%. In E. coli 5S RNA the insensitivity of the sedimentation constant to Mg^{++} or Na^{+} suggested a rather rigid three-dimensional structure, which is more asymmetric than the structure for tRNA (Boedtker and Kelling, 1967). The greater asymmetry compared to 4S RNA has also been suggested for Blastocladiella 5S RNA, on the basis of the much larger concentration dependence of the 5S RNA (Comb and Zehavi-Willner, 1967). The 5S RNA of rat liver has a T_m of 55°C in SSC (pH 7.4) and a rather broad UV profile, which levels off at room temperature; all of this is suggestive of a large number of helical regions also in this mammalian RNA (Bachvaroff and Tongur, 1966).

In E. coli (Zehavi-Willner and Comb, 1966) and B. subtilis (Morell et al., 1967) the sites in DNA for 5S RNA are distinct from those for tRNA and for the larger ribosomal RNAs. The number of 5S sites per cell are the same order of magnitude as the number of sites

for the larger ribosomal RNA species, which offers the possibility that the proper stoichiometry of ribosomal RNAs in bacteria may be maintained by the similarity in number of sites for the various ribosomal RNA species. In animal cells, the situation seems to be somewhat different, since it has been demonstrated in Xenopus laevis (Brown and Weber, 1968) that the sites for 5S RNA are located in a different portion of the genome than the sites for the large ribosomal RNA precursor, and that the 5S sites are in great excess over the latter sites. A similar situation with regard to excess 5S RNA sites has been found in the present hybrid studies with HeLa cells. In E. coli, 5S RNA is derived from a precursor molecule, by cleavage of one to four nucleotides from the 5'-end (Monier et al., 1969). The structural studies reported here seem to exclude such a precursor relationship for 5S RNA of HeLa cells, since pppGp and ppGp were found at the 5'-terminus; furthermore, the existence of these latter components impose other strong restrictions on the origin of 5S RNA in HeLa cells.

GENERAL INTRODUCTION-4S RNA

A class of low molecular weight RNA with a sedimentation constant of about 4S has been universally found in the cytoplasm, either free or bound specifically to ribosomes, in all organisms studied. This RNA class, called transfer RNA, has been found to serve as an adaptor molecule, playing an essential role in locating an amino acid in the correct position in a specific polypeptide chain, as determined by the information provided by the nucleotide sequence in mRNA. In brief outline, transfer RNA has been implicated in the following series of reactions:

$$\text{amino acid}_j + \text{ATP} + \text{aminoacyl RNA synthetase}_j \longrightarrow \text{aminoacyl RNA synthetase}_j\text{-AMP-amino acid}_j + \text{PP} \qquad (1)$$

$$\text{aminoacyl RNA synthetase}_j\text{-AMP-amino acid}_j + \text{tRNA}_j\text{pCpCpA} \longrightarrow \text{tRNA}_j\text{pCpCpA-amino acid}_j + \text{aminoacyl RNA synthetase}_j + \text{AMP} \qquad (2)$$

$$\text{NH}_2\cdot(\text{CH}\cdot\text{R}\cdot\text{CO}\cdot\text{NH})_n\cdot\text{CH}\cdot\text{R}_i\text{COO}\cdot\text{ApCpCptRNA}_i \;\vdots\; \text{ribosome-mRNA} \;\vdots\; \text{tRNA}_j\text{pCpCpA}\cdot\text{OOC}\cdot\text{R}_j\text{CH}\cdot\text{NH}_2 \xrightarrow[\text{GTP}]{\text{transfer factors}} \text{NH}_2\cdot(\text{CH}\cdot\text{R}\cdot\text{CO}\cdot\text{NH})_n\cdot\text{CH}\cdot\text{R}_i\text{CO}\cdot\text{NH}\cdot\text{CH}\cdot\text{R}_j\cdot\text{COO}\cdot\text{ApCpCptRNA}_j \;\vdots\; \text{ribosome-mRNA} + \text{tRNA}_i\text{pCpCpA} \qquad (3)$$

More detailed information about these reactions can be found in reviews by Brown (1963) and Attardi (1967). Studies on the specificity of the aminoacylation reaction of tRNA (Reaction 2) in various organisms revealed heterogeneity in the sites which are recognized by the synthetase, within classes of tRNA molecules specific for an amino acid (Berg et al., 1961; Benzer and Weisblum, 1961). In addition, a multiplicity in the codons for a particular amino acid has been demonstrated by the experiments of Weisblum, Benzer and Holley (1962), in which two leucyl tRNAs were separated by countercurrent distribution, one of which responded to poly (U,C) and the other to poly (U,G) in an E. coli incorporation system (Reaction 3). The binding assay of Nirenberg and Leder (1964), which depends on the ability of trinucleotides of known sequence to stimulate specific aminoacyl-tRNA binding to ribosomes, also provided evidence for heterogeneity of tRNAs within classes specific for a particular amino acid.

The published sequence data of yeast transfer RNA specific for alanine (Holley et al., 1965), serine (Zachau, Dütting and Feldman, 1966), tyrosine (Madison, Everett and Kung, 1966), phenylalanine (Rajbhandary et al., 1967) and valine (Bayev et al., 1967) have demonstrated large sequence differences between tRNA species, and small sequence differences within a tRNA species (e.g., there are only three base changes between serine I and serine II tRNA). However, despite such differences in primary structure, a similar secondary structure has been proposed for all of these tRNA species, i.e., a so-called "cloverleaf" model (see Zachau, review, 1967).

RNA-DNA hybridization studies have shown sites complementary to tRNA in the genome of bacteria (Goodman and Rich, 1962; Giacomoni and Spiegelman, 1962; Oishi, Oishi and Sueoka, 1966; Morell et al., 1967; Smith et al., 1968), yeast (Schweizer, MacKechnie and Halvorson, 1969), Drosophila (Ritossa, Atwood and Spiegelman, 1966) and Xenopus (Brown and Weber, 1968). From these studies, it appears that with increased organismic complexity there is an increased number of tRNA genes, varying from 30 to 80 per genome in bacteria, to about 2300 in Xenopus. In the present studies, a large number of sites for tRNA (4,900) has been found in the genome of HeLa cells. The specificity of these hybrids has been assessed according to the procedures used to analyze the 28S and 45S RNA hybrids in HeLa cells (Attardi, Huang and Kabat, 1965b; Attardi and Jeanteur, 1969).

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SECTION I

STRUCTURAL STUDIES ON HeLa 5S RNA

(Manuscript submitted for publication)

This work was done in conjunction with Dr. Francesco Amaldi.

SUMMARY

Highly purified 5S ribosomal RNA has been prepared from HeLa cells. Sedimentation analysis performed in 0.1 M NaCl or under denaturing conditions indicated that the RNA used in the present work was homogeneous in sedimentation properties and was represented by continuous polynucleotide chains. A nucleotide composition analysis showed absence of pseudouridylic acid and methyl groups in 5S RNA, in contrast to high molecular weight ribosomal RNA. An analysis of the oligonucleotide pattern after pancreatic ribonuclease digestion of 5S RNA gave results which were in general agreement with those reported for KB cell 5S RNA, both with respect to the identity and the frequency of the partial sequences. However, a trinucleotide was found in considerable amount among the digestion products of HeLa 5S RNA which was absent in the KB 5S pattern, and which could not be accounted for by the presence of contaminating RNA species. The presence of this trinucleotide, together with the reproducibly much lower than expected molar yield of the larger oligonucleotides strongly suggests the occurrence of alternate sequences at various sites in the 5S molecules of human cells.

The 3'-terminal nucleoside in HeLa 5S RNA has been found to be uridine, as in KB cell 5S RNA. Likewise, as in the latter RNA, guanine nucleotides were found at the 5'-terminus of HeLa 5S RNA; however, in addition to pGp reported for KB cells, the presence of ppGp and pppGp, representing the major portion of the 5'-end, has been clearly demonstrated in HeLa 5S RNA. The implications of this finding with regard to the origin of 5S RNA are discussed.

1. INTRODUCTION

Ribosomes from many, and presumably all organisms contain, in addition to the high molecular weight rRNA species, a low molecular weight RNA component of sedimentation constant about 5S (Rosset and Monier, 1963; Comb and Katz, 1964; Comb, Sarkar, DeVallet and Pinzino, 1965; Galibert, Larsen, Lelong and Boiron, 1965; Marcot-Queiroz, Julien, Rosset and Monier, 1965; Bachvaroff and Tongur, 1966; Brown and Littna, 1966; Knight and Darnell, 1967). This RNA is a constituent of the larger ribosomal subunit (one per particle); its function is unknown.

As to the origin of 5S RNA, the possibility has been suggested (see Perry, 1967) that it may derive, as 18S and 28S, from the 45S rRNA precursor. However, kinetic data (Knight and Darnell, 1967) and RNA-DNA hybridization data (Brown and Weber, 1968; Brown and Dawid, 1968) speak strongly against such a possibility. RNA-DNA hybridization experiments have indicated the presence of several genes for 5S RNA in the bacterial chromosome (about 4 genes in B. subtilis (Morell, Smith, Dubnau and Marmur, 1967), about 10 genes in E. coli (Zehavi-Willner and Comb, 1966)); a redundancy of information for 5S RNA several orders of magnitude higher has been found in the genome of higher organisms (Brown and Weber, 1968; Hatlen and Attardi, in preparation). As in the case of the high molecular weight rRNA species, this multiplicity of genes raises the problem of heterogeneity of 5S RNA. In view of the small size of this rRNA species, an analysis of its primary structure should provide relevant data on this

subject. In the present work, the method of oligonucleotide mapping described in a previous paper (Amaldi and Attardi, 1968) has been applied to HeLa cell 5S RNA. The results obtained suggest the existence of multiple forms of 5S RNA in human cells. Furthermore, the presence of pGp, ppGp and pppGp at the 5'-end of the 5S molecule was demonstrated.

After this work was started, the complete sequence of 5S RNA from E. coli (Sanger, Brownlee and Barrell, 1967) and from human carcinoma (KB) cells (Forget and Weissman, 1967) has been reported. In E. coli several forms of 5S molecules differing from one another by one or more bases have been identified (Sanger et al., 1967); in KB cells the published data are consistent with the existence of a relatively small number of very similar 5S molecules.

2. MATERIALS AND METHODS

(a) Cells and method of growth

For the method of growth of HeLa cells reference is made to a previous paper (Amaldi and Attardi, 1968).

(b) Labeling conditions

For long-term labeling with ^{32}P, exponentially growing HeLa cells were washed twice with phosphate-free modified Eagle's medium plus 5% dialyzed calf serum, then resuspended at a concentration of 4 to 5 x 10^4 cells per ml in medium containing 0.01 mM phosphate and 12 to 20 µCi/ml (^{32}P) orthophosphate. After about 50 hours incubation at 37°, the cells were subjected to a 12 to 13 hour chase with 0.005 M nonradioactive phosphate, in order to reduce the specific activity of the rapidly labeled nuclear and cytoplasmic RNA fractions (Penman et al., 1963; Scherrer et al., 1963; Houssais and Attardi, 1966; Warner et al., 1966).

For short-term labeling with (^{32}P) orthophosphate, cells were washed and resuspended in phosphate-free modified Eagle's medium with 5% dialyzed calf serum (2 x 10^5 cells per ml), incubated for 2 hours at 37°, then exposed for 45 minutes at 37° to (^{32}P) orthophosphate (25 µCi/ml).

For labeling with ^{14}C RNA precursors, HeLa cells were incubated for 48 hours in the presence of 0.12 µCi/ml (8-^{14}C) adenosine (55 mCi/mM), then subjected to a 12 hour chase with 0.001 M unlabeled adenosine; in other experiments, (2-^{14}C) uridine (0.12 µCi/ml; 50 mCi/mM) was utilized, followed by a 12 hour chase with 0.001 M unlabeled uridine.

To label the methyl groups of HeLa cell RNA and DNA, exponentially growing cells were washed twice with methionine-free Eagle's medium plus 5% dialyzed calf serum, resuspended in the same medium at a concentration of 4 to 5 x 10^4 cells per ml; after 2.5 hours incubation at 37°, L-(methyl-^{14}C) methionine (9.3 mCi/mM) was added at 0.20 µCi/ml. This concentration of methionine was sufficient to allow two generations of growth, after which the cells were harvested.

One experiment of double labeling was performed by incubating HeLa cells for 48 hours in modified Eagle's medium (containing 0.01 M phosphate), 5% calf serum, in the presence of 0.06 µCi/ml (8-^{14}C) adenosine (50 mCi/mM) and 6 µCi/ml (^{32}P) orthophosphate. The cells were washed and resuspended in fresh medium, supplemented with 0.001 M unlabeled adenosine, then allowed to grow another 16 hours at 37°; at the end of this chase period, the cells were harvested.

(c) Isolation of the ribosome-polysome fraction and extraction of RNA

All operations described below were carried out at 2 to 4°. The procedure used for isolating the ribosome-polysome fraction was a modification of that described previously (Attardi and Smith, 1962). HeLa cells washed three times in NKM (0.13 M NaCl, 0.005 M KCl, 0.001 M $MgCl_2$) were resuspended in 3 volumes 0.002 M $MgCl_2$, and immediately homogenized in a Potter-Elvehjem homogenizer for 30 seconds (5 to 6 strokes); one-third volume of 4 x Medium A was then added (Medium A is

0.25 M sucrose, 0.025 M KCl, 0.004 M $MgCl_2$, 0.05 M tris buffer, pH 7.4). The homogenate was centrifuged 10 minutes at 600 x g_{av} to sediment nuclei, large debris, and unbroken cells; the resulting supernatant was treated for 30 minutes with 0.5% sodium desoxycholate, and, after a centrifugation at 6,600 x g_{av} for 15 minutes, centrifuged for 2 hours at 105,000 x g_{av} to yield a ribosome-polysome pellet.

The ribosome-polysome pellet was suspended in TKM (0.05 M tris buffer, pH 7.4, 0.025 M KCl, 0.0025 M $MgCl_2$), and the RNA extracted from it as previously described (Attardi et al., 1966). After the final ethanol precipitation, the pellet of RNA was dissolved in 0.1 M NaCl, at a concentration of 0.5 to 10 mg/ml, as determined on the basis of ultraviolet absorption measurements. The value of 214 for $E^{1\,cm}_{1\%}$ at 260 mμ previously used for 18S and 28S RNA (Amaldi and Attardi, 1968) was also employed for 5S RNA.

For the isolation of free polysomes from HeLa cells pulse-labeled with (^{32}P) orthophosphate, the cells were homogenized as described above in 0.002 M $MgCl_2$; one-eighth volume of 2.0 M sucrose in TKM was then added, and the homogenate centrifuged 15 minutes at 6,600 x g_{av}. The resulting supernatant was layered onto a 15 to 30% linear gradient of sucrose in TKM and centrifuged 90 minutes at 25,000 rpm in a Spinco SW-25.1 rotor. Sucrose gradient fractions corresponding to the polysome band were pooled, and the RNA was phenol extracted.

(d) Fractionation of RNA on Sephadex G-100

^{32}P- or ^{14}C-labeled RNA extracted from the ribosome-polysome pellet was fractionated on a 1.8 by 180 cm column of Sephadex G-100 (Galibert et al., 1965), equilibrated with 0.1 M NaCl, at 22°. The flow rate was 30 ml per hour, and 2 ml fractions were collected. The fractions were analyzed by counting a small aliquot (1 to 100 µl) in 15 ml Bray's solution, and/or by determining the optical density at 260 mµ. After the first run through Sephadex, the central fractions of the 5S peak were pooled, precipitated with ethanol, dissolved in a small volume of 0.1 M NaCl, and run on a second column, usually with the addition of unlabeled RNA extracted from the ribosome-polysome pellet; the use of carrier tended to increase the recovery of 5S RNA in the rerun, up to almost 100%. The central fractions of the 5S peak were again pooled, precipitated with ethanol, and dissolved in a suitable medium for the analysis to be performed.

(e) Extraction of DNA

DNA was extracted from the 600 x g_{av} pellet obtained from the total homogenate of L-(methyl-^{14}C) methionine-labeled cells. The extraction was carried out according to the Marmur procedure (Marmur, 1961) with three additional deproteinization steps using phenol containing 0.1% 8-hydroxyquinoline (Kirby, 1962).

(f) Sedimentation analysis of 5S RNA

Sedimentation analysis of the 5S component isolated by Sephadex G-100 chromatography was carried out on a 5 to 20% sucrose gradient in 0.1 M

NaCl, 0.01 M sodium acetate buffer (pH 5.0), in the presence of ^{3}H-4S RNA marker. The gradient was run 11.5 hours at 62,000 rpm in a Spinco SW-65 rotor, at 4°.

For the analysis of sedimentation properties under denaturing conditions, 5S RNA samples mixed with a 4S RNA marker were heated at 80° for 3 minutes in 0.001 M tris buffer (pH 7.0) containing 0.00025 M EDTA, quickly cooled to 0°, and run through a 5 to 20% sucrose gradient in the same buffer; alternatively, samples were heated at 70° for 5 minutes in 18% neutralized formaldehyde containing 0.001 M EDTA, quickly cooled, diluted two times with H_2O, and run on a 5 to 20% sucrose gradient in 0.02 M sodium phosphate buffer (pH 7.4) containing 1% formaldehyde. The conditions of centrifugation for 5S RNA after denaturation were the same as described above. Fractions from the sucrose gradient were collected directly into scintillation vials, and after addition of Bray's solution, were counted in the scintillation counter.

(g) Base composition and sequence analysis

The technique of nucleotide analysis after alkaline hydrolysis has been previously described (Attardi et al., 1966). In some experiments aimed at elucidating the 3'-end of the 5S RNA molecule, an elution step with about 40 ml of 0.005 M formic acid was introduced prior to the 0.02 M formic acid, in order to separate the nucleosides from the 2',3'-cytidylic acid. In other experiments, directed toward determining the nature of the 5'-end of the 5S RNA molecule, after elution of the 2',3'-guanylic acid peak, a linear gradient of 0.5 to 4.0 M formic acid (75 ml of each

concentration) was applied, followed by stepwise elution with 4.0 M formic acid (90 ml), 0.2 M ammonium formate in 4.0 M formic acid (55 ml), 0.4 M ammonium formate in 4.0 M formic acid (70 ml), and 0.8 M ammonium formate in 4.0 M formic acid (125 ml) (modified from Hurlbert et al., 1954). Aliquots were dried on planchets under an infrared lamp (under these conditions the ammonium formate was removed) and counted in a low background gas flow counter. Fractions of the eluate to be further analyzed were lyophilized (this step likewise resulted in removal of virtually all of the ammonium formate).

Oligonucleotide mapping after pancreatic ribonuclease digestion was carried out as previously reported (Amaldi and Attardi, 1968). Elution of the RNase digestion products up to trinucleotides and first tetranucleotides was carried out with a non-linear concentration gradient of NH_4HCO_3 buffer formed by means of a Varigrad 6-chamber system as previously described (Amaldi and Attardi, 1968); after about 800 ml of eluate had been collected (the molarity of NH_4HCO_3 buffer in the effluent being 0.40 to 0.50 M, as determined by a direct conductivity measurement), a new non-linear concentration gradient of NH_4HCO_3 buffer, pH 8.6, was formed by using 4 chambers containing each 150 ml of buffer at the following concentrations: 0.40-0.50 M, 0.60 M, 1.0 M, 1.5 M.

For the identification of the various peaks of the DEAE-cellulose chromatographic patterns, individual components, after removal of NH_4HCO_3 by brief heating at 80° (Amaldi and Attardi, 1968), were hydrolyzed with 0.5 N NaOH for 16 hours at 30° or digested with T1 ribonuclease (Sankyo

Co., Ltd., Tokyo) at a concentration of 6 units per ml in 0.025 M tris buffer, pH 7.4, for 16 hours at 30°; the digestion products were then run through Dowex 1-X8 (formate form). In some cases, the component under investigation was treated with E. coli alkaline phosphatase (Worthington Biochemical Corp., Freehold, N.J.) for 1 hour at 37° at a concentration of 3 units per ml in 0.04 M tris buffer, pH 8.3, in the presence of 2 mg per ml unlabeled 2',3'-ribonucleotides, then extracted with phenol, and digested with snake venom phosphodiesterase (Worthington Biochemical Corp.) for 20 minutes at 22° at a concentration of 0.20 mg per ml in 0.05 M tris buffer, pH 7.4, with 0.15 mg per ml added unlabeled 5'-guanylic acid (to minimize the effects of any possible 5'-nucleotidase activity) (modified from Holley et al., 1964); subsequent to this treatment, the material was analyzed on a Dowex 1-X8 column.

As an aid in the resolution of mixed oligonucleotide peaks, chromatography on columns of DEAE-Sephadex A-25 was employed. The sample was charged onto the column (0.6 x 20 cm), then eluted with a 200 ml linear gradient of 0 to 0.40 M NaCl in 7 M urea, 0.02 M tris (pH 7.6) (Takanami, 1967). Fractions of 2 ml were collected at a flow rate of 11 ml per hour at 22°, then analyzed by scintillation counting of an 0.50 ml aliquot in 15 ml Bray's solution. The nucleotide composition of peaks from the column was determined, after removal of salt and urea on columns of Bio-Gel P-2 (Bio-Rad, Richmond, Calif.), by alkali digestion and Dowex 1-X8 chromatography.

Chromatography on DEAE-Sephadex A-25, as described above, was also utilized to analyze the products larger than mononucleotides produced by alkali digestion of ^{32}P-labeled 5S RNA.

Chromatography on Dowex 1-X2 (Takanami, 1967) was used for the identification of the component eluting in the region of 5'-GDP on Dowex 1-X8. The appropriate fractions from the Dowex 1-X8 column were pooled, lyophilized, incubated 18 hours at 37° in 0.5 N KOH and neutralized with $HClO_4$; the resulting precipitate of $KClO_4$ was removed by centrifugation, washed twice with water (2°) and the washes combined with the first supernatant. The pooled supernatant, together with 0.5 mg each of unlabeled 5'-ADP, 5'-UDP, and 5'-CDP, was loaded on a 0.5 x 17 cm column of Dowex 1-X2 (minus 400 mesh), and 2.5 ml fractions were eluted with a 100 ml linear gradient of NaCl (0-0.25 M) in 0.01 N HCl. After optical density measurement, fractions were dried at 100° in glass scintillation vials, POP-POPOP toluene scintillation mixture was added, and radioactivity in ^{14}C and ^{32}P measured in a scintillation counter.

Liberation of free bases from RNA and DNA by $HClO_4$ treatment, and subsequent analysis on Dowex 50-HCl columns was performed as outlined previously (Brown and Attardi, 1965). The RNA sample was hydrolyzed with 0.1 ml 70% $HClO_4$ at 100° for 1 hour, diluted with 0.3 ml water and neutralized with KOH; the insoluble $KClO_4$ was removed by centrifugation and the pellet washed twice with water (2°); these washings were combined with the original supernatant (final volume about 1 ml). This was brought to 0.5 N HCl and loaded on a 0.55 x 5 cm Dowex-50 column (prepared in a

disposable Pasteur pipette). Elution was carried out with 2.0 N HCl at a flow rate of 5 ml/hr; one ml fractions were collected and analyzed as described for the Dowex 1-X2 column above. This technique was also utilized in the identification of the bases at the 3'- and 5'-ends of the 5S molecule.

3. RESULTS

(a) Purification of 5S RNA

Purification of HeLa 5S RNA by gel filtration through long columns of Sephadex G-100 was carried out as described in Materials and Methods (d). Figure 1a shows a typical elution pattern of ^{32}P-labeled RNA from the ribosome-polysome fraction, with three peaks appearing in this order: high molecular weight rRNA and mRNA, 5S RNA, 4S RNA. A rerun of the central fractions of the 5S peak through Sephadex G-100 resulted in an effective purification of this component (Fig. 1b). Reconstruction experiments performed with labeled high molecular weight RNA added to unlabeled RNA from the ribosome-polysome fraction, showed the absence of any appreciable contamination of the 5S peak by degradation products of high molecular weight rRNA and mRNA arising during the chromatographic run.

In order to investigate the possible contribution of mRNA to the 5S component, RNA extracted from free polysomes of cells labeled with (^{32}P) orthophosphate for 45 minutes (i.e. at a time when about 70% of the labeled RNA heavier than 5S is in mRNA, and the rest in 18S rRNA) was analyzed by Sephadex chromatography. As shown in Figure 2, a small amount of radioactivity (about 4% of that present in the void volume) was eluted in close correspondence with the 5S optical density profile. The nucleotide composition showed that this labeled RNA was markedly different from mRNA extracted from cells labeled for 30 minutes with (^{32}P) orthophosphate (at a time when there is no appreciable 18S labeling)

and rather similar to fully labeled 5S RNA (Table I). Base composition of the high molecular weight RNA was fairly close to that expected for a mixture of mRNA and 18S RNA; the pulse-labeled "4S" material had a base composition somewhat different from that of uniformly labeled 4S RNA.

When centrifuged through a sucrose gradient in sodium acetate-NaCl buffer, the 5S component isolated by two cycles of Sephadex chromatography showed a fairly sharp peak sedimenting about 20% faster than the 4S marker, sometimes with a small amount of trailing material (Fig. 3a). Also after exposure to denaturing conditions (heat (Fig. 3b) or formaldehyde treatment (Fig. 3c)) the 5S RNA sedimented as a homogeneous component, running ahead of the 4S RNA, in general with a small tail. These experiments indicated that the 5S component isolated by gel filtration is substantially homogeneous in sedimentation properties, with the great majority of the molecules being represented by continuous polynucleotide chains.

(b) Nucleotide composition of 5S RNA

Table II shows the nucleotide composition of ^{32}P-labeled and unlabeled 5S RNA and, for comparison, that of 4, 18 and 28S RNA from HeLa cells. No pseudouridylic acid (<0.03%) was detected in 5S RNA. The majority of the radioactive material eluted with 1 N HCl could be accounted for by the 2',5'-3',5'-nucleoside di-, tri- and tetraphosphates released by alkali digestion from the 5'-end of the 5S molecules (see section (d)).

The 5S RNA isolated from cells labeled for two generations in the presence of L-(methyl-^{14}C) methionine showed a small amount of radioactivity

incorporated (Fig. 4). This low level of labeling was previously observed by others, but not further analyzed (Knight and Darnell, 1967); if associated with methyl groups, this would have corresponded to 1 methyl group per 200 nucleotides, or less than 1 methyl group per 5S molecule. Analysis of the distribution of ^{14}C among the bases liberated by perchloric acid treatment showed that the radioactivity was eluted in perfect correspondence with the guanine and adenine marker peaks, and was therefore presumably associated with the purine rings (as a result of the contribution of the ^{14}C-methyl group of methionine to the pool of 1-carbon compounds used in purine biosynthesis (Fig. 5)). Likewise, in a Dowex 1-X8 chromatography run of alkali digested L-(methyl-^{14}C) methionine labeled 5S RNA, the radioactivity profile coincided with the optical density peaks of the marker adenylic and guanylic acids; the 1 N HCl eluate, which would contain any ribose-methylated oligonucleotides, had only 2.5% of the label, i.e. the amount expected to be associated with the 5'-terminal guanosine di-, tri- and tetraphosphates (see section (d)). As a control, the specific activity of adenine and guanine in DNA from the same preparation of labeled cells was determined (HeLa DNA is known not to contain any unusual methylated bases besides 5-methylcytosine (Brown and Attardi, 1965)); by using these specific activity values and the base composition of 5S RNA, essentially all the radioactivity in the 5S component could be accounted for by the labeling of the purine rings (Table III).

(c) Oligonucleotide mapping of 5S RNA after pancreatic ribonuclease digestion

Figure 6 shows a typical DEAE-cellulose chromatographic pattern of the pancreatic RNase digestion products of 5S RNA. The components in the numbered peaks were identified as described in Materials and Methods (g). The data for the alkali and T1 ribonuclease digestion products of fractions corresponding to the various peaks are given in Table IV; Table V summarizes the results of additional tests carried out for the identification of some pancreatic RNase digestion products. Peaks numbered 1 to 8, 11, 12, 14 and 16 were identified in the previously published partial sequence analysis of 18S and 28S RNA (Amaldi and Attardi, 1968); the relevant data are included for the sake of completeness. Two of the other peaks, 19 and 20, could be identified unambiguously as GpGpGpCp and GpGpGpUp, on the basis of the alkali digestion data. The determination of the sequences of oligonucleotides in peaks 9, 18, and 23 (as ApGpCp, GpGpApUp, and GpGpGpApApUp, respectively) required the additional analysis of the T1 ribonuclease digestion products. It should be recalled that digestion with T1 ribonuclease splits bonds following Gp residues, releasing 3'-guanylic acid and oligonucleotides ending in Gp; any oligonucleotides produced, as well as any 5'-terminal nucleoside di-, tri- and tetraphosphates, are eluted with 1 N HCl from Dowex 1-X8.

Peaks 10 and 13 did not correspond to any O.D. marker. After alkaline hydrolysis or T1 RNase digestion of material from peak 10, about twice as much radioactivity was eluted with 1 N HCl as was found in uridylic acid;

these observations suggested pGpUp as the possible main component of peak 10. Material of peak 13 gave a ratio of radioactivity in 1 N HCl eluate to that in uridylic acid of about 3 to 1 after alkaline hydrolysis, and of about 4.5 to 1 after T1 RNase digestion; taking into account the effect of the contamination of peak 13 by adjacent peaks, these results seemed compatible with the identification of this peak as pppGpUp. These tentative assignments of pGpUp and pppGpUp as the main components of peak 10 and 13, respectively, were confirmed by further analysis of the 5'-terminal groups of the 5S RNA molecule (see Results (d)).

Material from peak 15 had the approximate base composition 1 Cp, 2 Ap, 1 Gp upon alkali digestion. When a T1 RNase digest of this material was run on DEAE-Sephadex in urea, with a linear NaCl gradient, a mononucleotide and a trinucleotide peak were obtained, containing 26% and 72% of the radioactivity, respectively. The mononucleotide peak had Cp and Gp in the approximate ratio 1.3 to 1, while the trinucleotide peak had Ap, Gp and Cp in the ratio 4 to 1.3 to 1. This led to the conclusion that peak 15 was a mixture of the two tetranucleotides GpApApCp and ApApGpCp. The apparent slight excess of GpApApCp over ApApGpCp, which is suggested by the base composition data (Tables IV and V) may be due to a partial separation of the two components in the combined peak (fractions corresponding to the left side of peak 15 were used for the analysis).

Material from peak 17, when subjected to alkali and T1 RNase digestion, gave results consistent with either one or both sequences ApGpGpCp or GpApGpCp. In order to resolve this ambiguity, peak 17 material was first

treated with E. coli alkaline phosphatase to remove the terminal phosphate, then digested with snake venom phosphodiesterase to liberate 5' mononucleotides, and finally run on Dowex 1-X8. The results indicated that Ap is the only 5'-terminal nucleotide in the oligonucleotides of peak 17 (Table V). (The higher than expected yield of orthophosphate may be due to a slight nucleolytic activity of the alkaline phosphatase or to incomplete removal of the latter by phenol extraction.) This allowed the unambiguous assignment of ApGpGpCp to these oligonucleotides.

Material from peak 21, both after alkali and T1 RNase digestion, gave a nucleotide composition corresponding to approximately 3 Gp, 1 Ap, thus consistent with the sequence GpGpGpAp. Since ApU (but not ApC) is eluted in the adenylic acid region on Dowex 1-X8 (formate) chromatography (as shown by direct experiments utilizing ^{32}P-labeled, alkaline phosphatase-treated ApUp and ApUp) there was the possibility of the existence of a 3'-terminal uridine nucleoside in the oligonucleotides of peak 21. However, phosphatase treatment of the radioactive material eluted in the adenylic acid region after alkali digestion-Dowex 1-X8 chromatography of peak 21 components released substantially all of the label as orthophosphate (eluted after the adenylic acid peak and before the uridylic acid peak in Dowex 1-X8 chromatography). Therefore, the conclusion was reached that peak 21 results from non-specific action of pancreatic RNase at a bond following Ap (see Discussion).

Peak 22, both after alkali and T1 RNase digestion, appeared to be heterogeneous in composition, with approximately equimolar amounts of

components terminated with Up and Cp. A comparison of the proportion of the individual nucleotide components released by alkali and T1 RNase treatment revealed that about 3 Ap and 2 Gp (taking the terminal Up or Cp as unity) were not eluted in the mononucleotide region upon Dowex 1-X8 chromatography of the T1 RNase digest; this suggested the presence of ApApGp and ApGp among the T1 RNase digestion products. The average length of oligonucleotides in peak 22, estimated from the proportion of pyrimidine nucleotides after alkali and T1 RNase digestion was 5.9 and 5.2, respectively (mean of 5.5), hinting at the possible presence of a pentanucleotide and a hexanucleotide in the peak. This was confirmed by DEAE-Sephadex chromatography in urea of peak 22 material; two peaks of almost equal size were resolved here, one in the pentanucleotide region, and the other in the hexanucleotide region (estimated with reference to the position of a known trinucleotide run in parallel, on the basis of the salt concentration required for elution). This information, together with the approximate 3 to 1 ratio of Gp to 3'-terminal pyrimidine nucleotides in the two components, permitted the partial identification of the sequences as (ApGp,Gp,Gp)Up and (ApApGp,Gp,Gp)Cp.

Peak 24 material after alkali digestion gave a nucleotide composition corresponding to approximately 1 Cp, 2 Ap and 4 Gp, suggesting the presence of a heptanucleotide. After T1 RNase digestion, about 41% of the radioactivity was eluted with 1 N HCl, 47% with Gp, and the rest distributed among the other mononucleotides. DEAE-Sephadex chromatography of the T1 RNase digest gave a mononucleotide and a dinucleotide peak, without

any indication of a trinucleotide peak, thereby tending to exclude the presence of the partial sequences ApApCp and ApApGp (the latter was also incompatible with the T1 RNase digestion data, in view of the low release of Cp). These results suggested the occurrence of ApGp and ApCp in the component of peak 24, therefore permitting the partial identification as (ApGp,Gp,Gp,Gp)ApCp. The low recovery of radioactivity in the 1 N HCl eluate after T1 RNase digestion, relative to that which would be expected on the basis of this assignment, remains unexplained.

(d) Identification of 3'- and 5'-ends of 5S RNA

Identification of the 3'-end of the 5S RNA molecule was performed with RNA extracted from cells exposed for 48 hours to (2-^{14}C) uridine or (8-^{14}C) adenosine. When an alkaline digest of the (2-^{14}C) uridine-labeled 5S RNA was run on Dowex 1-X8 (formate form), about 1.5% of the total radioactivity was eluted with 0.005 M formic acid, almost all in coincidence with the uridine marker (Fig. 7a); in agreement with this latter finding, at least 95% of the radioactivity in the 0.005 M formic acid eluate was recovered as uracil after perchloric acid hydrolysis-Dowex 50-HCl chromatography (Fig. 8a). When 5S RNA doubly labeled with (8-^{14}C) adenosine and (^{32}P) orthophosphate was alkali digested and chromatographed on Dowex 1-X8, only a very small amount of the ^{14}C (0.20 per cent) and ^{32}P (0.06 per cent) label was eluted with 0.005 M formic acid, without any indication of definite peaks (Fig. 7b). These

results pointed to uridine as the 3'-terminal nucleoside of 5S RNA from HeLa cells.

After Dowex 1-X8 chromatography of an alkaline digest of ^{32}P-labeled 5S RNA, about 3.3% of the radioactivity was eluted with 1 N HCl. This eluate was expected to contain, besides any purine-rich alkali-resistant oligonucleotides (Lane and Butler, 1959), any nucleoside di-, tri- and tetraphosphate pertaining to the 5'-end of the molecule. A calculation showed that only 1.7% of the total ^{32}P radioactivity could be accounted for by 5'-ends if these were in the form of nucleoside 3',5'-diphosphates (assuming a length of 120 nucleotides for HeLa 5S RNA, as in 5S RNA from KB cells (Forget and Weissman, 1967) and from E. coli (Sanger et al., 1967)); the likely existence of pGp and pppGp at the 5'-ends of the 5S RNA molecule had been indicated by the results of DEAE-cellulose chromatography of 5S RNA pancreatic RNase digests (Results (c)). After Dowex 1-X8 chromatography of (8-^{14}C) adenosine-labeled 5S RNA, about 2.1% of the radioactivity was eluted with 1 N HCl; base analysis of this material revealed 84% of the radioactivity to be associated with guanine and 10% with adenine (Fig. 8b, Table VI). By contrast, less than 0.1% of the radioactivity was found in the 1 N HCl eluate when 5S RNA labeled with (2-^{14}C) uridine was used (Table VI).

In order to analyze the nature of the components not eluted with the mononucleotides, a system of elution with higher concentrations of formic acid and ammonium formate (as detailed in Materials and Methods, (g)) was applied to the Dowex 1-X8 after elution of 2',3'-guanylic acid.

The pattern obtained (Fig. 9) showed, in addition to some radioactive material eluted with increasing concentration of formic acid before GDP (with a broad peak in the region of ADP), three well-defined labeled peaks: the first (a), rather broad, eluted with 4.0 M formic acid after GDP, the second (b) eluted with 0.4 M ammonium formate in 4.0 M formic acid immediately after GTP, and the third one (c) eluted with 0.8 M ammonium formate in 4.0 M formic acid. The radioactive material eluted before GDP was found in variable amounts in different runs (from 0.5 to 1% of the total ^{32}P label), and could be substantially reduced (by as much as 70%) by prolonging the time of digestion with alkali to 36 hours. The 5S RNA extracted from cells labeled with (8-^{14}C) adenosine showed a considerable amount of radioactivity (0.8 to 1.3%) eluted in this region; base analysis (Table VI) revealed a high proportion of adenine in these components. On the other hand, a negligible amount of radioactivity (<0.05%, Table VI) was found in this region of the chromatographic pattern when 5S RNA from (2-^{14}C) uridine labeled cells was used. These results suggest that the components eluted before GDP consist mainly of purine-rich (in particular, adenine-rich) alkali-resistant oligonucleotides (Lane and Butler, 1959). A series of experiments were carried out to investigate the nature of the components a, b and c. Peak a material was subjected to a second alkali digestion and then chromatographed on a Dowex 1-X2 column; more than 90% of the radioactivity was eluted as two partially resolved peaks between the GDP and UDP markers, in the position expected for 2' and 3' pGp (Takanami, 1967). This indicated

pGp as the main component of peak a. In agreement with this identification, base analysis of the material from peak a isolated from 5S RNA labeled with (8-^{14}C) adenosine showed that 87% of the label was associated with guanine, and 7% with adenine (Table VI); on the contrary, peak a was not labeled when (2-^{14}C) uridine was used as a precursor. As in the case of peak a, peaks b and c were also found to be labeled when (8-^{14}C) adenosine was used as a precursor, but not when (2-^{14}C) uridine was used; base analysis again indicated that the label was in guanine (Table VI). Alkaline phosphatase treatment of material from these two peaks doubly labeled with (8-^{14}C) adenosine and (^{32}P) orthophosphate caused the release of more than 95% of the ^{32}P radioactivity as orthophosphate and of more than 95% of the ^{14}C radioactivity as nucleosides. These results indicated that none of the phosphates in these components were involved in internucleotide linkages. The molar ratios of phosphate to guanine in the peaks a, b and c were found to be 1.6, 2.6 and 4.1, respectively, to be compared to the values of 2, 3 and 4 expected for pGp, ppGp and pppGp. The position of elution of peak b immediately after GTP, analogous to the elution of pGp after GDP, is in agreement with the identification of peak b as ppGp.

Additional evidence in favor of the proposed identification of the 5'-terminal groups of 5S RNA was provided by an analysis by DEAE-Sephadex chromatography of the products larger than mononucleotides produced by alkali digestion of ^{32}P-labeled 5S RNA. As shown in Figure 10, an appreciable amount of radioactivity was eluted in the general region

of the marker di-, tri- and tetranucleotides (provided by a pancreatic ribonuclease digest of (2-^{14}C) uridine labeled 5S RNA). Of the radioactivity which eluted in correspondence with the dinucleotides (peak I), after second alkali treatment-Dowex 1-X8 chromatography, about 50% was recovered as adenylic and guanylic acids, and the rest, presumably represented by alkali-resistant dinucleotides, was eluted in the region preceding GDP (see Fig. 9). Of the ^{32}P label eluted in the region of trinucleotides from DEAE-Sephadex (peak II), about 20% was recovered after a second alkali treatment as guanylic acid, 56% was eluted in the position of the Dowex 1-X8 corresponding to pGp (immediately following the GDP marker, see Fig. 9), and the rest was spread in the region preceding GDP. The elution of nucleoside diphosphates (pXp) with the trinucleotide peak in the DEAE-cellulose chromatographic pattern has been previously reported by Takanami (1966). The last peak of ^{32}P radioactivity in the DEAE-Sephadex pattern (peak III), which was more than twice the size of either peak I or II, was eluted after the tetranucleotide marker in the position previously reported for the elution of pppGp (Roblin, 1968) and pppAp (Takanami, 1966). The small shoulder on the leading edge hinted at the presence of an additional component; it seemed likely that this component was ppGp; in fact the possible elution of ppXp components just prior to pppXp had been suggested by Takanami (1966). The presence of ppGp and pppGp in peak III was confirmed by the analysis of the distribution of ^{32}P radioactivity of this peak after a second alkali treatment and Dowex 1-X8

chromatography; 24% of the label was recovered in the position of elution previously found for ppGp (Fig. 9), while 66% was eluted in correspondence with pppGp.

(e) Frequency of nucleotide sequences in 5S RNA

More than 95% of the original radioactivity associated with 5S RNA was recovered in the DEAE-cellulose chromatographic pattern. The distribution of nucleotides among partial sequences released by pancreatic ribonuclease digestion was calculated from the proportion of ^{32}P radioactivity pertaining to the individual components in the DEAE-cellulose pattern (Table VII). Very similar values for the distribution of nucleotides among partial sequences (up to trinucleotides) were obtained from the ratio of the frequencies of each nucleotide sequence in the 5S RNA to that in the 28S RNA (determined from the specific activity data) and from the distribution of nucleotides among partial sequences in the latter RNA (Amaldi and Attardi, 1968; Jeanteur et al., 1968).

Table VII shows also the average number of each type of sequence per molecule, calculated on the basis of a 5S RNA 120 nucleotides long. An inspection of Table VII shows a reasonably good agreement between the average numbers of different sequences per molecule found in the present work and the corresponding ones previously reported for KB cell 5S RNA (Forget and Weissman, 1967). The lower than expected molar yield of Cp and Up in the 5S RNA pancreatic RNase digest was attributed by the authors to the losses of 2',3'-cyclic nucleoside monophosphates. It

appears from Table VII that the molar yield of a considerable number of the larger RNase digestion products of HeLa 5S RNA is reproducibly lower than unity, to an extent which may not be accounted for by artifacts of the method. In most cases, deviations in the same direction have been reported for the corresponding sequences in KB cell 5S RNA (Table VII).

The available data strongly suggest that the two unresolved components of peak 22 correspond to ApGpGpGpUp and GpGpApApGpCp isolated from KB cell 5S RNA. The component of peak 24, for which the tentative assignment of (ApGp,Gp,Gp,Cp)ApCp was made, probably corresponds to the GpGpGpApGpApCp identified in the pancreatic ribonuclease digest from KB cell 5S RNA. Two components not reported in the analysis of KB cell 5S RNA were detected in the present work among the oligonucleotides released by pancreatic RNase from HeLa 5S RNA. One of these components, GpGpGpAp, present in a molar yield of 0.33, appears to be due to a non-specific RNase attack on a bond following Ap (Beers, 1960). The presence of the other component, ApGpCp, in a molar yield of 0.81 is presumably indicative of the existence of more than one sequence of 5S RNA in HeLa cells (see Discussion).

It appears from Table VIII that uridine accounts for nearly all the 3'-termini of the 5S molecules. A small amount of purine nucleosides was actually found at the 3'-end, presumably as a result of some breakage of the 5S chains very near the end (see Discussion). The three groups found at the 5'-end of the 5S molecule, pGp, ppGp and pppGp, were recovered, after alkali digestion and Dowex 1-X8 chromatography, in an overall amount corresponding to 0.76-0.87 moles per mole of 5S RNA (Table VIII). The recovery figures

for pGp and pppGp were rather close to those obtained for the presumptive corresponding RNase digestion products pGpUp and pppGpUp (peaks 10 and 13) in the DEAE-cellulose chromatographic pattern. The failure to detect ppGpUp in the latter pattern is presumably due to its being hidden under GpApUp or ApGpUp (both recovered in higher than expected yield).

4. DISCUSSION

The 5S RNA utilized in the present work appeared by several criteria to be substantially free from any contamination by known RNA species. The resolution obtained by Sephadex G-100 fractionation, and the results of reconstruction experiments, excluded the presence in the 5S peak of any appreciable amount of high molecular weight rRNA or degradation products thereof, or of 4S RNA; this conclusion was confirmed by the absence in 5S RNA of pseudouridylic acid or methylated nucleotides, which are known components of those RNA species in HeLa cells (Brown and Attardi, 1965; Amaldi and Attardi, 1968). On the basis of the known content of pseudouridylic acid and methyl groups in ribosomal RNA and 4S RNA it can be estimated that the total possible contamination of the 5S preparations by these species was less than 2%. Regarding the possible contamination of 5S by short mRNA chains, the use of a cold chase after the long-term labeling of HeLa cells with an RNA precursor was expected to reduce considerably (from two to eight times) the specific activity of any labile messenger fraction (Penman, Scherrer and Darnell, 1963; Attardi and Attardi, 1967). In order to directly test the possible presence of mRNA in the

5S preparations, RNA was isolated from polysomes of cells subjected to a 45 minute ^{32}P pulse. A small amount of labeled RNA (about 4% of the labeled high molecular weight RNA of polysomes) was eluted from the Sephadex G-100 as a fairly sharp peak, in close correspondence with the 5S optical density profile; this RNA had a base composition which was rather similar to that of fully labeled 5S RNA. The possibility that this pulse-labeled "5S" material represents some precursor of tRNA (Burdon and Clason, 1969), is practically excluded by the difference in base composition from "4S" labeled in a short pulse, and especially by the fact that the RNA was extracted from polysomes which would be expected to contain only mature tRNA. It seems possible that the "5S" material labeled after a 45 minute pulse represents newly synthesized 5S ribosomal RNA. This would imply that under the conditions used in this experiment labeled 5S RNA arrives in the polysomes prior to the appearance of 28S rRNA, a conclusion which is at variance with the results reported by Knight and Darnell (1967). No explanation can at present be given for this discrepancy, although the metabolic imbalance introduced by the phosphate starvation preceding labeling may possibly account for it. The possibility that the pulse-labeled "5S" material represents mRNA seems unlikely both because of the difference in base composition from mRNA and because of the lack of indication of degradation of mRNA which would result in tailing of the high molecular weight peak. If the entire pulse-labeled "5S" material were represented by mRNA, it can be estimated that the contamination of 5S RNA by mRNA in long-term labeled cells would correspond to 10%; this figure would have to be reduced by a factor of two to eight if the contaminating mRNA had the same half-life as the total mRNA population.

The analysis of the sedimentation properties of 5S RNA in 0.1 M NaCl or under denaturing conditions showed the substantial homogeneity in size and the intactness of the RNA molecules used in the present work.

HeLa cell 5S RNA was shown in the present work not to contain any detectable methylation of the bases or of the ribose moieties. The low level of labeling of this RNA species which was found after long-term exposure of the cells to (^{14}C-methyl) methionine, in agreement with a previous report (Knight and Darnell, 1967), was shown here to be completely accounted for by the labeling of purine rings. The lack of methyl groups in HeLa 5S RNA is in agreement with what has been reported for 5S RNA from other sources (Galibert et al., 1965; Comb and Zehavi-Willner, 1967). The absence of methyl groups and of pseudouridylic acid represent a definite structural difference between 5S RNA and the high molecular weight rRNA, the significance of which is at present unknown.

The results obtained on the partial sequence distribution after pancreatic RNase digestion in HeLa cell 5S RNA are generally in good agreement with the published sequence data on 5S RNA from KB cells, another cell line of human origin (Forget and Weissman, 1967). The oligonucleotides of peak 22, which were only partially identified in the present work, presumably correspond to ApGpGpGpUp and GpGpApApGpCp found in KB 5S RNA; similarly, the component of peak 24 is probably GpGpGpApGpApCp. There are, however, some differences which have been found in the partial sequence data between HeLa and KB 5S RNA. Two components have been detected among the pancreatic RNase digestion

products from HeLa 5S RNA which were not reported in the analysis performed with KB material. One of these components, GpGpGpAp, very likely results from a non-specific action of pancreatic RNase on a bond following Ap; such non-specific action of pancreatic RNase has been previously described (Beers, 1960), and may occur to a limited extent when drastic conditions of RNase digestion are used (in order to avoid the accumulation of digestion products containing 2',3'-cyclic phosphates). It is significant in this respect that a region very susceptible to T1 RNase action has been found in KB cell 5S RNA, and this region indeed contains the sequence GpGpGpAp (see the heptanucleotide GpGpGpApGpApCp in the paper on KB 5S RNA and the presumptive corresponding oligonucleotide 24 in HeLa 5S RNA (Table VII)). It may not be a coincidence that, as will be discussed below, the sequence GpGpGpApGpApCp was recovered, both from HeLa and KB cell 5S RNA, with a molar yield considerably lower than unity. However, it should be noticed that no evidence was found of the presence of the fragment GpApCp (resulting from the splitting of the heptanucleotide) in the expected position in the DEAE-cellulose chromatographic pattern, either overlapping or immediately preceding peak 9 (Amaldi and Attardi, 1968). On the other hand, it is not excluded that GpGpGpAp may arise from non-specific splitting of the sequence of GpGpGpApApUp, which is also recovered in molar yield lower than unity, while ApUp is consistently found in a yield significantly higher than unity (1.4).

The other component found among the pancreatic RNase digestion products of HeLa 5S RNA, but not described in the analysis of KB 5S RNA, is the sequence ApGpCp. The possibility that this digestion product

derives from contaminating ribosomal or 4S RNA seems to be excluded on the basis of the purity of the 5S preparations utilized in the present work, as judged by the stringent criteria discussed above. Even for mRNA, the presence of small amounts of which is difficult to exclude, a maximum estimate of the contamination would be 5%; this could not possibly account for the amount of ApGpCp recovered, especially when contrasted to the absence of ApApUp and ApApCp, which would be expected to be found in digests of RNA species with high AU content, as mRNA. The possibility that the occurrence of ApGpCp is related to the presence of the unusual digestion product discussed above, GpGpGpAp, in the sense that they both derive from non-specific cleavage of a hypothetical oligonucleotide, GpGpGpApApGpCp, seems to be excluded by the large difference in molar yield between the two products, and by the fact that the only peak in the DEAE-cellulose pattern containing heptanucleotides (peak 24) was found to consist of oligonucleotides lacking the sequence ApGpCp; furthermore, the difference from unity (0.37) of the molar yield for peak 24 is much too small to account for the appearance of 0.81 moles of ApGpCp. Another possibility to consider is that ApGpCp derives from non-specific splitting of GpGpApApGpCp (which is the presumptive sequence of the hexanucleotide in peak 22, and which is the only digestion product containing ApGpCp recovered in a molar yield lower than unity). This does not seem to be likely, since no evidence of the presence of the other half of the sequence, GpGpAp, was found; this fragment would be expected to be eluted after GpGpUp, by analogy with GpGpGpAp, which is eluted after GpGpGpUp, and should be easily detected because of its amount (identical to that of peak 9).

A more likely explanation for the occurrence of ApGpCp seems to be the presence of alternate sequences in HeLa 5S RNA, resulting from one or more base changes in one or more of the 5S cistrons. To limit attention to the single base changes which could bring about the appearance of this pancreatic digestion product, there are two possibilities:

1) that ApGpCp derives from a different trinucleotide in the 5S chain by a change in one of its bases. This can be excluded by a comparison of the molar yields of the various digestion products for HeLa 5S with those reported for KB cells; this comparison does not show any decrease in the relevant dinucleotides (ApCp, ApUp, GpCp), trinucleotides (ApGpUp, GpGpCp), tetranucleotides (ApGpGpCp), as expected if the base change discussed here had occurred; the pentanucleotide ApGpGpGpUp which was recovered in molar yield lower than unity is also ruled out as a source of ApGpCp by the absence of the required concomitant increase in GpUp.

2) that ApGpCp derives from a purine to pyrimidine change of the base immediately preceding this trinucleotide in the 5S chain. A detailed analysis of the complete sequence of KB 5S RNA and of the partial sequence data obtained in the present work, together with a consideration of the molar yields found for the different RNase digestion products, suggests that the only sequence which could produce by a single mutation the sequence PypApGpCp is the presumptive sequence of peak 22, GpGpApApGpCp; the occurrence of two alternative forms of this sequence, with the first Ap being substituted by Up or Cp, would account at the same time for the molar

yield remarkably lower than unity obtained for GpGpApApGpCp, for the appearance of the new ApGpCp peak, and finally, for the molar yields higher than unity of GpGpCp and GpGpUp. In this respect, it should be noticed that GpGpUp was recovered in molar yield greater than unity also in chromatographic patterns where it was completely resolved from the adjacent peaks. The recovery of GpGpApApGpCp estimated on the basis of the ratio of Cp to Up in the alkaline and T1 digest of material from peak 22 was about 0.45 moles per mole 5S RNA; splitting of the missing 0.55 moles would account for about 70% of the ApGpCp found in the present work.

Aside from the occurrence of ApGpCp in the pancreatic RNase digest of HeLa 5S RNA, another type of evidence suggests the existence of multiple species of 5S RNA in human cells. This evidence is provided by the deviations from unity of the molar yields of GpGpUp (previously discussed) and of all the larger oligonucleotides. Deviations in the same direction were reproducibly observed in the various DEAE-cellulose chromatographic runs of different HeLa 5S RNA preparations; this fact and the fact that similar deviations have also been reported for the molar yields of several corresponding pancreatic RNase digestion products of KB cell 5S RNA, obtained under different conditions of RNase digestion and oligonucleotide fractionation (GpGpGpCp, ApGpGpGpUp, GpGpGpApApUp, GpGpGpApGpApCp), strongly suggest that these discrepancies may reflect the existence of alternate sequences at various sites in the molecule. This need not be a surprising conclusion, since in E. coli strains several forms of 5S molecules differing from one another in one or two bases have been identified (Sanger et al., 1967). On the contrary, it is somewhat surprising that this

heterogeneity is not more pronounced, as expected from the great gene redundancy for 5S RNA in animal cells (more than 50,000 cistrons in Xenopus laevis (Brown and Weber, 1968); about 7,000 cistrons in HeLa cells (Hatlen and Attardi, in preparation)). The relatively low degree of variability found for human 5S RNA may reflect the great restrictions imposed upon evolutionary base sequence changes by the structural requirements for 5S function.

The 3'-terminal nucleoside in HeLa cell 5S RNA is uridine, as has been previously found in KB cell 5S RNA; from the data obtained in the present work it is not possible to say whether two forms of 3'-terminal sequences ($CpUpUpU_{OH}$ and $CpUpU_{OH}$) exist also in HeLa 5S RNA. On the other hand, clear evidence was obtained for the existence of three forms of the 5'-terminal sequences, pGp, ppGp, pppGp, which, taken together, account for 76 to 87% of the 5'-end of the molecule. This is the first instance where the presence of pppXp and ppXp at the 5'-terminus of a naturally-occurring RNA, other than viral, has been reported. In the published sequence work on KB cell 5S RNA, a puzzling half-molar yield of the 5'-terminal fragment after digestion with pancreatic RNase (pGpUp) or T1 RNase (pGp) was reported; it seems possible that the alternative forms of the 5'-terminus, ppGp and pppGp, also exist in KB 5S RNA, but that they were not recognized. The presence of β and γ phosphates at the 5'-terminus of some 5S RNA molecules may be due to incomplete removal of phosphate from the 5'-triphosphate terminus of the nascent 5S molecules; alternatively, their absence may result from degradation occurring in the preparation of 5S RNA. The RNA made *in vitro* with *E. coli* RNA

polymerase (Bremer et al., 1965; Maitra and Hurwitz, 1965) or Qβ RNA polymerase (Banerjee et al., 1967) is known to have pppGp- or pppAp- at its 5'-ends. Among the naturally occurring RNA species, before the present observation, only viral RNA had been reported to contain nucleoside 5'-triphosphate termini (Takanami, 1966; Roblin, 1967; Glitz, 1968). Whether the general absence of 5'-triphosphates in nonviral RNA is due to in vivo enzymatic removal of the terminal phosphates or to the derivation of the RNA molecules by cleavage of longer precursors is not known. Recently, evidence has been presented for the occurrence in E. coli of precursors of 5S RNA containing an extra sequence (one to four nucleotides long) at their 5'-end, which is clipped off after incorporation of the 5S molecule into the structure of ribosomal precursor particles (43S particles)(Monier, Forget, Jordan, Reynier and Varicchio, 1969); the presence of ppGp and pppGp at the 5'-end of the 5S molecules in HeLa cells implies that in these cells there are no precursors of 5S RNA with extra sequences at the 5'-end. The fact that all 5S RNA analyzed in the present work has been isolated from mature ribosomal particles suggests that the presence of a di- or triphosphate at the 5'-end does not interfere with the functional role of 5S RNA; actually, an interesting possibility is that the addition or removal of terminal phosphates may have a regulatory role. The presence of 5' di- and triphosphate groups in 5S molecules indicates that transcription of 5S RNA from the multiple 5S cistrons on the DNA must occur in the form of discrete units and not as polycistronic RNA chains secondarily cut into 5S size. The existence of ppGp and pppGp at the 5'-end of the major part of the 5S molecules would furthermore

restrict any possibility of derivation of 5S RNA from the 45S RNA precursor to one copy per molecule, in correspondence with the 5'-terminal segment; this result, being incompatible with the large excess of 5S sites over 45S sites in HeLa DNA (Hatlen and Attardi, in preparation), brings further evidence against the existence of any relationship between 45S RNA and 5S RNA, in agreement with kinetic data (Knight and Darnell, 1967), and RNA-DNA hybridization data (Brown and Weber, 1968; Brown and Dawid, 1968).

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TABLE I

Nucleotide composition of fractions of free polysomal RNA from ^{32}P pulse-labeled HeLa cells

RNA fraction	Labeling time (min)	Mole %					
		Cp	Ap	Up	Ψp	Gp	GC%
"High molecular weight RNA"	45	26.3	23.3	20.9	2.2	27.4	53.7
"5S RNA"	45	26.4	21.0	24.0	-	28.5	54.9
"4S RNA"	45	33.5	17.0	16.1	4.0	29.6	63.1
mRNA*	30	21.4	24.8	27.9	-	25.8	47.2

RNA was extracted from free polysomes of HeLa cells labeled for 45 minutes with (^{32}P) orthophosphate, as described under Materials and Methods (c), and chromatographed on Sephadex G-100. The ^{32}P-labeled RNA which was eluted in correspondence with the OD_{260} peaks of high molecular weight RNA, 5S RNA and 4S RNA, respectively, was analyzed for nucleotide composition as described in Materials and Methods (g). Sedimentation analysis on a sucrose gradient of 45 minutes ^{32}P pulse-labeled polysomal RNA showed that, after this labeling time, about 30% of the radioactivity sedimenting faster than 5S is in 18S RNA, the balance being associated with mRNA.

*Attardi and Attardi, 1967.

TABLE II

Nucleotide composition of RNA species from HeLa cells

RNA component	Mole %					1 N HCl eluate (% of total	Number of determinations
	Cp	Ap	Up	Ψp	Gp		
^{32}P 4S	28.4	19.0	17.7	3.9	31.0	7.0	(3)
^{32}P 5S	28.7	18.8	22.9	<0.03	29.7	3.3	(5)
Unlabeled 5S	29.0	18.3	22.3	---	30.3	---	(1)
^{32}P 18S*	27.3	20.2	20.5	1.52	30.5	5.1	(4)
^{32}P 28S*	32.3	16.0	15.6	1.10	35.0	2.3	(5)

The nucleotide composition of the unlabeled 5S RNA was determined from the O.D. measurements on the basis of the extinction coefficients of the four 2',3' nucleotides. (The extinction coefficients reported by Beaven et al. (1955) were used, after correction for the different pH of the individual elution media.) The nucleotide composition of the ^{32}P-labeled RNA components was determined from the distribution of label among the 2',3'-nucleotides; the amount of radioactivity eluted with 1 N HCl was not included in this calculation. The proportion of pseudouridylic acid was determined by two successive reruns through Dowex 1-X8 (Amaldi and Attardi, 1968).

*Amaldi and Attardi, 1968.

TABLE III

Specific activity of bases in 5S RNA from L-(methyl-^{14}C) methionine-labeled HeLa cells

Base	Specific activity (dpm/μM)	
	Found	Expected for 100% of label in purine rings*
Adenine	225,000	230,000
Guanine	201,000	210,000

5S RNA was isolated from HeLa cells grown for two generations in the presence of L-(methyl-^{14}C) methionine (Fig. 4) and the distribution of radioactivity among its bases analyzed by perchloric acid treatment-Dowex 50-HCl chromatography (Fig. 5). Since insufficient amounts of 5S RNA were available to permit accurate optical density measurements, the specific activities of adenine and guanine were calculated indirectly. For this purpose, the proportion of ^{14}C radioactivity in adenine (40%) and guanine (58%) in the Dowex 50-HCl pattern was determined. From this information, from the known molar content of adenine (18.7%) and guanine (30.3%) in 5S RNA (Table II; the guanylic acid content was corrected to include the 5'terminal guanosine di-, tri- and tetraphosphates eluted with HCl (see section (d) of Results), and from the specific activity of (methyl-^{14}C) methionine-labeled 5S RNA (105,000 dpm/μM), the specific activities of adenine and guanine could be calculated as follows:

TABLE III (continued)

$$\text{s.a. adenine} = \frac{(105{,}000)(0.40)}{(0.187)} \text{ dpm/}\mu\text{M}$$

$$\text{s.a. guanine} = \frac{(105{,}000)(0.58)}{(0.303)} \text{ dpm/}\mu\text{M}$$

*Determined from the labeling of adenine and guanine in DNA extracted from the same batch of L-(methyl-^{14}C) methionine-labeled cells as used for 5S RNA analysis (see text). The bases were liberated by perchloric acid treatment of DNA and chromatographed on Dowex 50-HCl. The specific activities were determined directly from the radioactivity and OD_{260} measurements by using the extinction coefficients of Beaven et al. (1955), corrected for the pH of the sample.

TABLE IV

Identification of the components in the various peaks of the DEAE-cellulose chromatographic pattern

Peak No.	Alkali digestion cpm(%)					T1 RNase digestion cpm(%)					Major component(s) in peak
	Cp	Ap	Up	Gp	1 N HCl eluate	Cp	Ap	Up	Gp	1 N HCl eluate	
1	98.1	0.3	1.4	0.2	0						Cp
2	<0.1	<0.1	99.8	0.1	0						Up
3	46.0	46.8	0.7	1.9	4.6						ApCp
4	1.0	49.0	47.2	0.9	1.9						ApUp
5	49.4	1.0	2.1	46.5	1.0						GpCp
6	1.0	1.1	47.4	46.2	4.3						GpUp
7	29.3	62.6	1.1	3.2	3.8						ApApCp
8	2.3	55.7	29.5	2.9	9.6						ApApUp
9	30.1	34.2	1.9	30.5	3.4	30.6	1.9	0.1	4.7	62.8	ApGpCp
10	5.1	3.7	32.2	6.4	52.7	2.3	2.3	26.1	4.6	64.6	pGpUp*
11	0.6	32.4	31.8	29.5	5.7	0.1	2.3	2.9	25.0	69.8	GpApUp
12	1.0	31.6	31.4	30.1	5.9	0.2	1.5	26.2	5.9	66.2	ApGpUp
13	3.3	6.3	21.1	8.6	60.7	0.3	0.3	17.7	0.1	81.7	pppGpUp*

TABLE IV (continued)

Peak No.	Alkali digestion cpm(%)					T1 RNase digestion cpm(%)					Major component(s) in peak
	Cp	Ap	Up	Gp	1 N HCl eluate	Cp	Ap	Up	Gp	1 N HCl eluate	
14	31.7	0.7	2.5	62.3	2.8						GpGpCp
15	21.0	44.3	0.9	26.9	6.9	12.7	2.5	0.3	17.0	67.6	ApApGpCp, GpApApCp*
16	0.4	4.3	33.0	53.8	8.5						GpGpUp
17	24.6	25.8	1.1	43.9	4.6	22.4	1.8	1.2	25.1	49.4	ApGpGpCp*
18	1.6	23.9	25.0	44.8	4.7	0.1	1.4	1.3	44.5	52.8	GpGpApUp
19	25.6	3.6	3.2	64.4	3.2						GpGpGpCp
20	0.8	1.5	26.3	68.3	3.1						GpGpGpUp
21	1.0	23.5	0.8	70.2	4.5	2.2	18.3	4.0	62.0	13.3	GpGpGpAp*
22	7.3	24.4	9.7	51.1	7.5	8.6	2.1	10.5	36.0	42.7	(ApGp,Gp,Gp)Up, (Gp,Gp,ApApGp)Cp*
23	1.7	30.6	16.5	46.2	5.3	1.2	2.9	5.5	41.3	59.1	GpGpGpApApUp
24	13.2	26.0	1.9	55.6	3.4	3.6	5.2	2.6	47.2	41.0	(Gp,Gp,Gp,ApGp)ApCp*

TABLE IV (continued)

Fractions of the DEAE-cellulose chromatography of the pancreatic ribonuclease digest of ^{32}P-labeled 5S RNA were subjected to alkaline hydrolysis or T1 ribonuclease digestion as described in Materials and Methods (g). The data pertaining to peaks 1 to 8, 11, 12, 14 and 16 are derived from the previously published sequence analysis of 18 and 28S HeLa cell RNA (Amaldi and Attardi, 1968).

*These assignments required additional tests (see text and Table V).

TABLE V

Analysis of components from peaks 15 and 17 after special treatment

	Peak 15		Peak 17	
	T1 digestion, DEAE-Sephadex*		E. coli alkaline phosphatase, then SVP**	
Alkali digestion products	Mononucleotide peak cts/min (%)	Trinucleotide peak cts/min (%)	Enzymatic digestion products	cts/min (%)
2',3' Cp	50.8	15.5	5' Cp	20.5
2',3' Ap	2.9	61.7	5' Ap	0.9
2',3' Up	6.2	2.5	5' Up	<0.05
2',3' Gp	40.0	20.2	5' Gp	39.8
			orthophosphate	38.5

*Material from peak 15 of the DEAE-cellulose chromatographic pattern was dried down, digested with T1 ribonuclease, and the digest run on DEAE-Sephadex A-25 (see Materials and Methods (g)). A mono- and a trinucleotide peak were eluted; the material from each peak was desalted on Bio-Gel P-2, incubated 16 hours in 0.5 N NaOH at 30°, and chromatographed on Dowex 1-X8.

**Material from peak 17 was dried down, treated with E. coli alkaline phosphatase, phenol extracted, then treated with snake venom phosphodiesterase (SVP; see Materials and Methods (g)); the resulting enzymatic digest was finally run on Dowex 1-X8.

TABLE VI

Analysis of peaks eluted after mononucleotides

in the Dowex 1-X8 chromatographic patterns of 5S RNA alkaline hydrolysates

Fraction	RNA doubly labeled with (^{32}P) orthophosphate and (8-^{14}C) adenosine			RNA labeled with (8-^{14}C) adenosine	RNA labeled with (2-^{14}C) uridine	RNA labeled with (8-^{14}C) adenosine	Probable composition of main component(s)
	Molar ratio of phosphate to guanine*	% ^{32}P released by alkaline phosphatase as orthophosphate	% ^{14}C released by alkaline phosphatase as nucleosides	% of total cpm		% cpm in bases	
A. Total material eluted with 1 N HCl	-	-	-	2.13	<0.1	G 83 A 10	
B. Material eluted with 0.5-4.0 M formic acid before GDP	-	-	-	0.8-1.3	0.05	G 14 A 69	alkali-resistant nucleotides
a	1.6	-	-	0.33	n.d.	G 87 A 7	pGp
b	2.6	>95	>95	0.31	n.d.	G 93 A n.d.	ppGp
c	4.1	>95	>95	0.61	n.d.	G 95 A n.d.	pppGp

TABLE VI (continued)

A. HeLa 5S RNA was isolated from cells grown for two generations in the presence of (8-^{14}C) adenosine or (2-^{14}C) uridine, digested with alkali, and chromatographed on Dowex 1-X8 columns, formate system (Amaldi and Attardi, 1968). The material eluted with 1 N HCl was lyophilized, hydrolyzed with perchloric acid, and the bases separated by Dowex-50, HCl system, as shown in Figure 5.

B. Samples of 5S RNA from HeLa cells labeled with (8-^{14}C) adenosine and (^{32}P) orthophosphate were digested with alkali and chromatographed on Dowex 1-X8, as in (A); after elution of the 2',3'-guanylic acid peak, a system of elution with higher concentrations of formic acid-ammonium formate was applied, as detailed in Materials and Methods (g) (Fig. 9). Aliquots of individual components in the Dowex 1-X8 chromatographic pattern were lyophilized and analyzed for base composition by perchloric acid treatment and Dowex 50-HCl chromatography, as in Figure 5. Alkaline phosphatase treatment of material from peak b and c was carried out after lyophilization, as outlined in Materials and Methods (g); the resulting digest was then chromatographed on Dowex 1-X8, as in (A).

*Determined by taking as a reference the ratio of ^{32}P to ^{14}C cpm in 2',3'-guanylic acid.

TABLE VII

Molar yields of pancreatic RNase digestion products of 5S RNA from HeLa cells

Peak No.	Digestion products	Distribution of nucleotides among digestion products (%) Mean	Range	Molar yield*	Molar yield reported for KB cell 5S RNA**
1	Cp	15.2	15.1-15.4	18.3	13.2***
2	Up	11.7	11.3-12.0	14.1	10.1***
3	ApCp	8.9	8.5-9.2	5.3	4.8
4	ApUp	2.3	2.2-2.5	1.4	1.3
5	GpCp	7.4	7.2-7.5	4.4	4.2
6	GpUp	3.7	3.6-3.7	2.2	2.2
7	ApApCp	0.12	0.06-0.16	<0.1	-
8	ApApUp	0.05	0.03-0.07	<0.1	-
9	ApGpCp	2.0	1.80-2.2	0.81	-
10	pGpUp	0.70	0.67-0.73	0.28	0.5
11	GpApUp	5.7	5.6-5.8	2.3	1.9
12	ApGpUp	3.1	3.08-3.1	1.2	1.1
13	pppGpUp	1.63	1.53-1.73	0.39	-
14	GpGpCp	3.1	2.9-3.3	1.2	0.94
15	ApApGpCp + GpApApCp	7.4	7.0-7.6	2.2	1.03 0.95
16	GpGpUp	3.5	3.3-3.7	1.4	1.15
17	ApGpGpCp	3.6	3.5-3.6	1.1	0.73
18	GpGpApUp	3.6	3.4-3.8	1.1	1.07
19	GpGpGpCp	2.0	1.8-2.2	0.60	0.76
20	GpGpGpUp	2.1	2.0-2.3	0.64	1.01
21	GpGpGpAp	1.09	1.06-1.12	0.33	-
22****	(ApGp,Gp,Gp)Up + (Gp,Gp,ApApGp)Cp	4.6	4.1-5.1	1.0	0.59 0.97
23	GpGpGpApApUp	2.7	2.3-3.1	0.55	0.8
24*****	(Gp,Gp,Gp,ApGp)ApCp	3.7	3.5-3.9	0.63	0.57

TABLE VII (continued)

This table summarizes the data obtained in four different experiments concerning the frequency of partial nucleotide sequences released from 5S RNA by pancreatic RNase digestion, as calculated from the distribution of radioactivity. These experiments involved the use of two different preparations of ^{32}P 5S RNA.

*A length of 120 nucleotides for HeLa 5S RNA has been assumed in this calculation (Forget and Weissman, 1967).

**From Forget and Weissman, 1967.

***The lower than expected values were due to the use of relatively low RNase concentrations, resulting in production of 2',3'-cyclic nucleoside monophosphates, which were not identified in the electrophoresis pattern.

****Corresponding presumably to sequences ApApGpGpUp and GpGpApApGpCp in KB cells.

*****Corresponding presumably to the sequence GpGpGpApGpApCp in KB cells.

TABLE VIII

Molar yields of 3'- and 5'-terminal groups from HeLa 5S RNA

3'-end			5'-end			
Component	RNA labeled with (2-^{14}C) uridine	RNA labeled with (8-^{14}C) adenosine	Component	RNA labeled with (^{32}P) orthophosphate		RNA labeled with (8-^{14}C) adenosine
	Molar yield			% of total cpm	molar yield	molar yield
Uridine	0.94-0.99	-	pGp	0.37	0.22	0.22
Adenosine + Guanosine	-	0.07-0.09	ppGp	0.46	0.18	0.22
			pppGp	1.19	0.36	0.43
					Total 0.76	Total 0.87

The molar yield of 3'-terminal groups was determined from the proportion of radioactivity eluted with 0.005 M formic acid on Dowex 1-X8 chromatography of an alkaline digest of 5S RNA isolated from cells labeled with either (2-^{14}C) uridine or (8-^{14}C) adenosine (see Fig. 7), by assuming a length of 120 nucleotides for the 5S RNA molecule. As reference values for these calculations, the amount of radioactivity and mole % of uridylic acid in 5S in the former case, and the amount of radioactivity and mole % of adenylic acid (lower number) or of guanylic acid (higher number), in the latter case, were used. (After long labeling with (8-^{14}C) adenosine, adenylic acid was found to have 1.4 times the specific activity of guanylic acid.)

TABLE VIII (continued)

The molar yield of the 5'-terminal groups was determined from the proportion of total radioactivity eluted in peaks a, b and c (Fig. 9) after chromatography on Dowex 1-X8 of an alkaline digest of 5S RNA labeled with (^{32}P) orthophosphate or (8-^{14}C) adenosine; in the latter case the amount of radioactivity and mole % of guanylic acid was used as a reference value.

FIGURE LEGENDS

Fig. 1. Purification by gel filtration through Sephadex G-100 of HeLa 5S RNA. (a) RNA was phenol-extracted from the ribosome-polysome fraction of HeLa cells labeled with (^{32}P) orthophosphate, then run through a Sephadex G-100 column equilibrated with 0.1 M NaCl. (b) The fractions indicated by arrows in (a) were pooled, precipitated with ethanol, dissolved in 0.1 M NaCl and rerun through Sephadex G-100.

Fig. 2. Gel filtration through Sephadex G-100 of RNA phenol-extracted from polysomes of HeLa cells labeled for 45 minutes with (^{32}P) orthophosphate. The insert shows on a larger scale the elution pattern in the 5S region. o----o, O.D.$_{260}$; •——•, ^{32}P cpm.

Fig. 3. Sedimentation analysis of 5S RNA under denaturing conditions. (a) Purified ^{32}P-labeled 5S RNA and ^{3}H-labeled 4S RNA were run through a 5 to 20% sucrose gradient in NaCl-acetate buffer in a Spinco SW-65 rotor at 62,000 rpm for 11.5 hours at 4°. (b) ^{32}P-labeled 5S RNA and ^{3}H-labeled 4S RNA were heat denatured in low ionic strength buffer and run through a 5 to 20% sucrose gradient in the same buffer under conditions of centrifugation described in (a). (c) ^{32}P-labeled 5S RNA and ^{3}H-labeled 4S RNA were denatured with formaldehyde and run in a 5 to 20% sucrose gradient in the presence of formaldehyde under the conditions of centrifugation specified in (a). •——•, ^{32}P cpm; o----o, ^{3}H cpm.

Fig. 4. Gel filtration through Sephadex G-100 of RNA phenol-extracted from the ribosome-polysome fraction of HeLa cells labeled for two generations with L-(^{14}C-methyl) methionine. The fractions indicated by arrows were utilized for nucleotide and base analysis (see Fig. 5, Table III, and text). o----o, O.D.$_{260}$; •——•, ^{14}C cpm.

Fig. 5. Separation on Dowex 50-HCl of the products of perchloric acid hydrolysis of 5S RNA isolated from cells labeled with L-(^{14}C-methyl) methionine. Fractions indicated by arrows in the Sephadex G-100 pattern of Figure 4 were pooled, ethanol precipitated, and the final precipitate dissolved in water, then lyophilized. After perchloric acid hydrolysis, the bases were separated on Dowex 50-HCl as detailed in Materials and Methods (g). U, uracil; C, cytosine; G, guanine; A, adenine. o----o, O.D.$_{260}$; •——•, ^{14}C cpm.

Fig. 6. Typical pattern of DEAE-cellulose chromatography of the pancreatic RNase digest of a mixture of ^{32}P-labeled 5S RNA and unlabeled 28S RNA from HeLa cells. See Amaldi and Attardi, 1968, and Materials and Methods (g), for details concerning the conditions of RNase digestion and chromatography. The arrow on the axis of abscissae indicates the position where the second concentration gradient of NH_4HCO_3 buffer was started. For the identification of the numbered peaks see Table IV. ——, O.D.$_{260}$; •----•, ^{32}P cpm.

Fig. 7. Separation on Dowex 1-X8 of nucleosides from mononucleotides in an alkaline digest of 5S RNA extracted from HeLa cells labeled with (2-^{14}C) uridine (a) or doubly labeled with (8-^{14}C) adenosine and (^{32}P) orthophosphate (b). Only the elution pattern of the nucleosides and

the first two mononucleotides is shown. FA, formic acid; o----o, O.D.$_{260}$; o——o, ^{14}C cpm; +····+, ^{32}P cpm.

Fig. 8. Separation on Dowex 50-HCl of the bases released by perchloric acid treatment of the material eluted with 0.005 M formic acid and 1 N HCl on Dowex 1-X8 chromatography of 5S RNA alkaline digests. (a) The material eluted with 0.005 M formic acid on Dowex 1-X8 chromatography of alkali-digested 5S RNA from HeLa cells labeled with (2-^{14}C) uridine was hydrolyzed with perchloric acid in the presence of carrier RNA, then chromatographed on Dowex 50-HCl as discussed in Materials and Methods (g). (b) The material eluted with 1 N HCl on Dowex 1-X8 chromatography of alkali-digested 5S RNA from HeLa cells labeled with (8-^{14}C) adenosine was treated as in (a). o----o, O.D.$_{260}$; o——o, ^{14}C cpm.

Fig. 9. Elution profile after 2',3' guanylic acid in a Dowex 1-X8 chromatography of 5S RNA extensively digested with alkali. HeLa 5S RNA from cells labeled with (^{32}P) orthophosphate was digested for 36 hours in 0.5 N NaOH, chromatographed on Dowex 1-X8; after elution of 2',3'-guanylic acid, elution with high concentrations of formic acid-ammonium formate was carried out as described in Materials and Methods (g). FA, formic acid; AM-F, ammonium formate. o----o, O.D.$_{260}$; o——o, ^{32}P cpm.

Fig. 10. Pattern of DEAE-Sephadex chromatography of a mixture of an alkaline digest of ^{32}P-labeled 5S RNA and a pancreatic RNase digest of (2-^{14}C) uridine-labeled 5S RNA. ^{32}P-labeled 5S RNA was digested with

0.5 N KOH at 30° for 16 hours and neutralized with perchloric acid; after removal of the potassium perchlorate by centrifugation, the hydrolysate was mixed with a pancreatic RNase digest of (2-^{14}C) uridine-labeled 5S RNA (1 mg total RNA, 200 µg pancreatic RNase, in 0.20 ml 0.1 M tris buffer, pH 7.4, 4 hours at 37°), then brought to 7 M urea, 0.02 M tris buffer, pH 7.4, and charged onto a column of DEAE-Sephadex A-25. Conditions of elution and analysis of the various components eluted was performed as described in Materials and Methods (g). o----o, ^{14}C cpm; •——•, ^{32}P cpm.

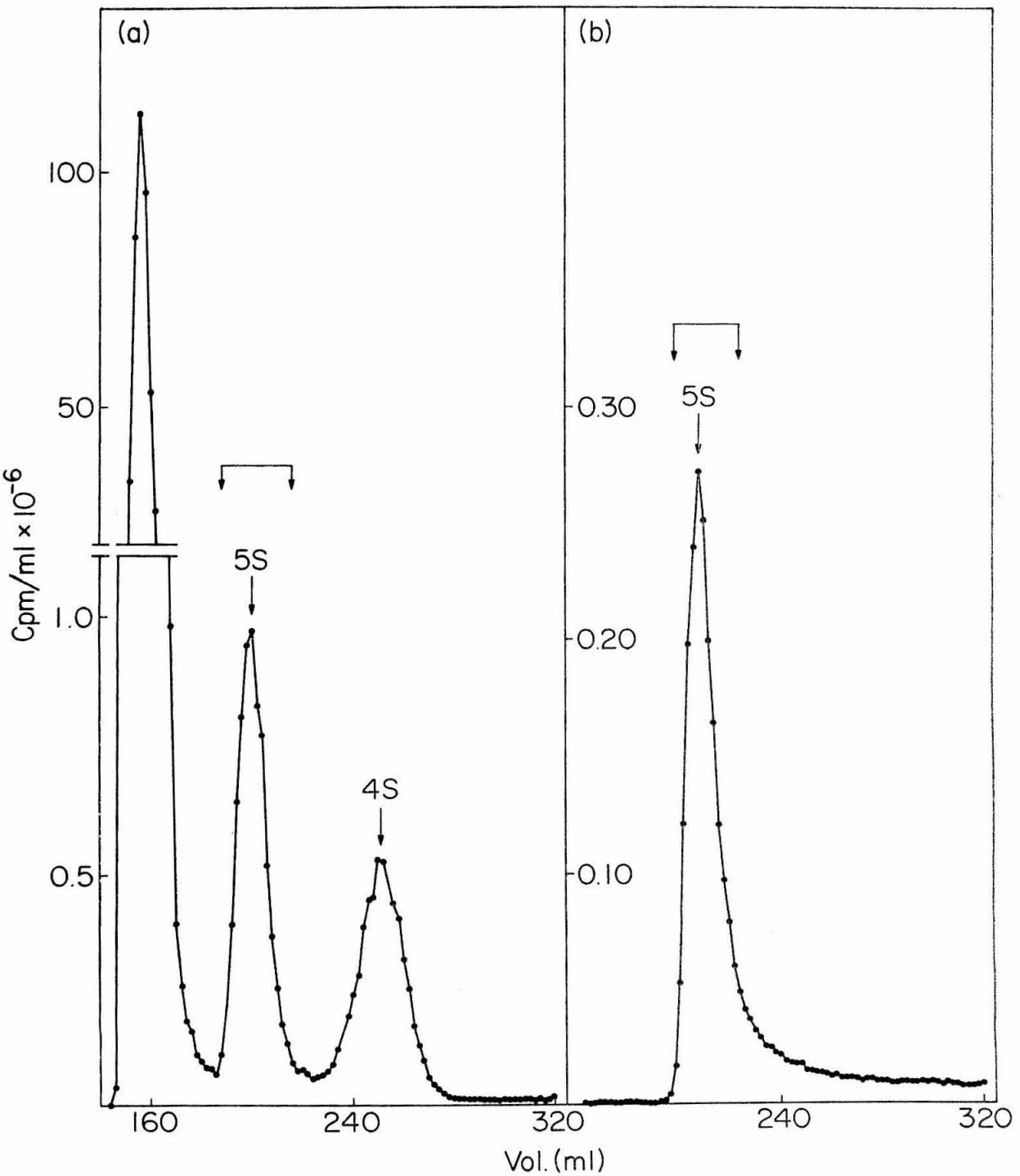
(a)
(b)
100
50
1.0
0.5
0.30
0.20
0.10
5S
4S
5S
Cpm/ml × 10⁻⁶
160
240
320
240
320
Vol. (ml)

Fig. 1

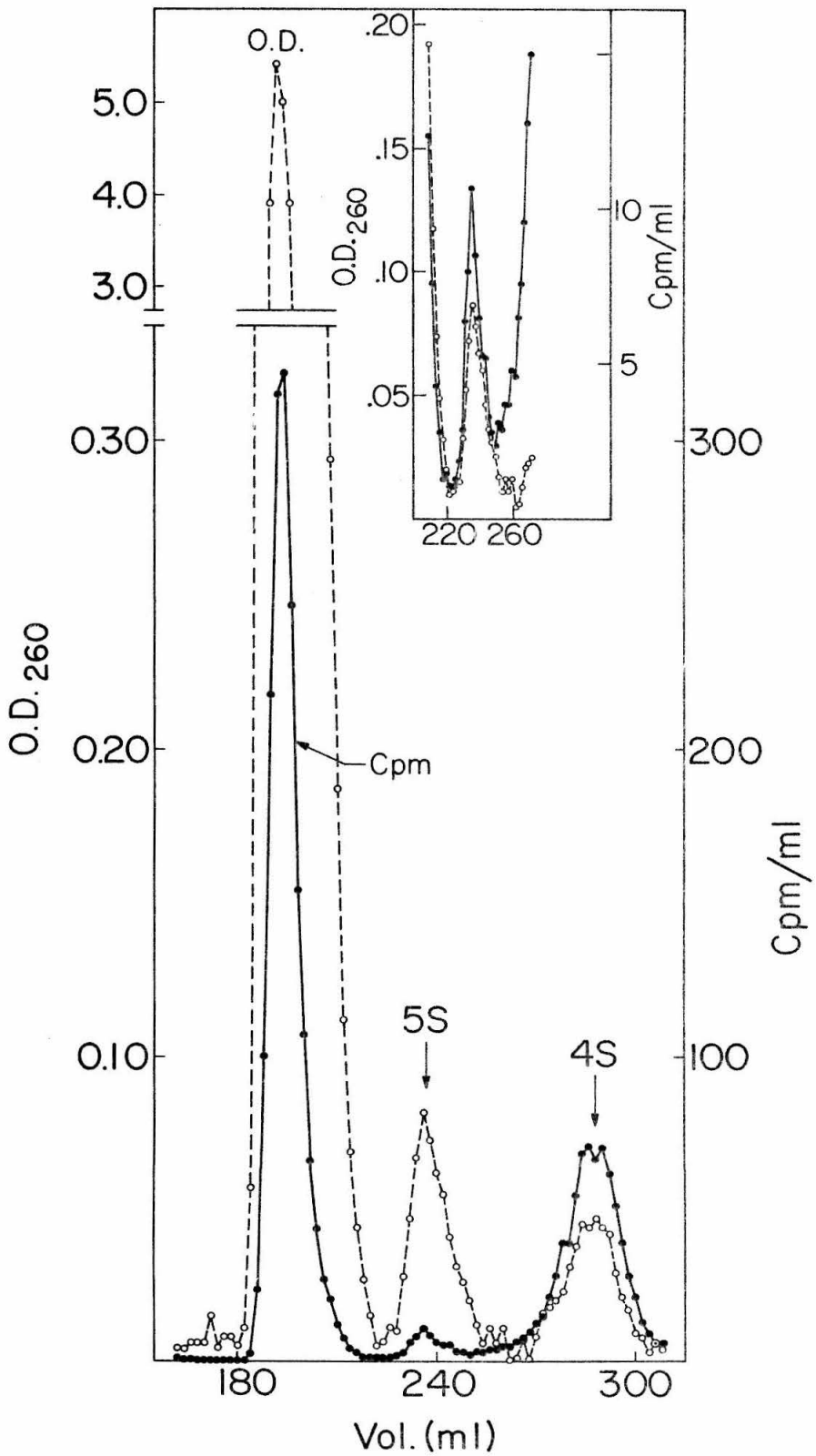
O.D.
5.0
4.0
3.0
.20
.15
.10
.05
O.D.260
10
5
Cpm/ml
220 260
0.30
0.20
0.10
O.D. 260
Cpm
300
200
100
Cpm/ml
5S
4S
180
240
300
Vol. (ml)

Fig. 2

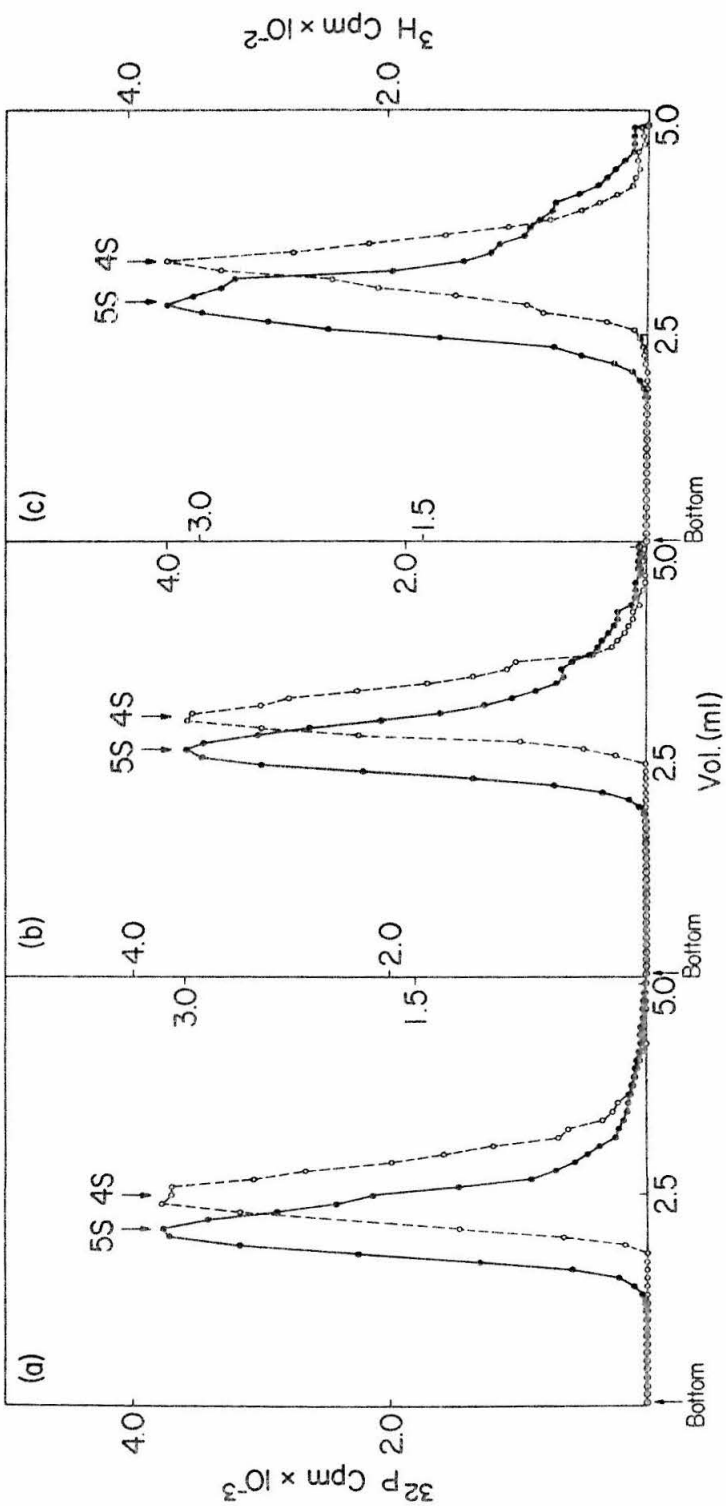

Fig. 3

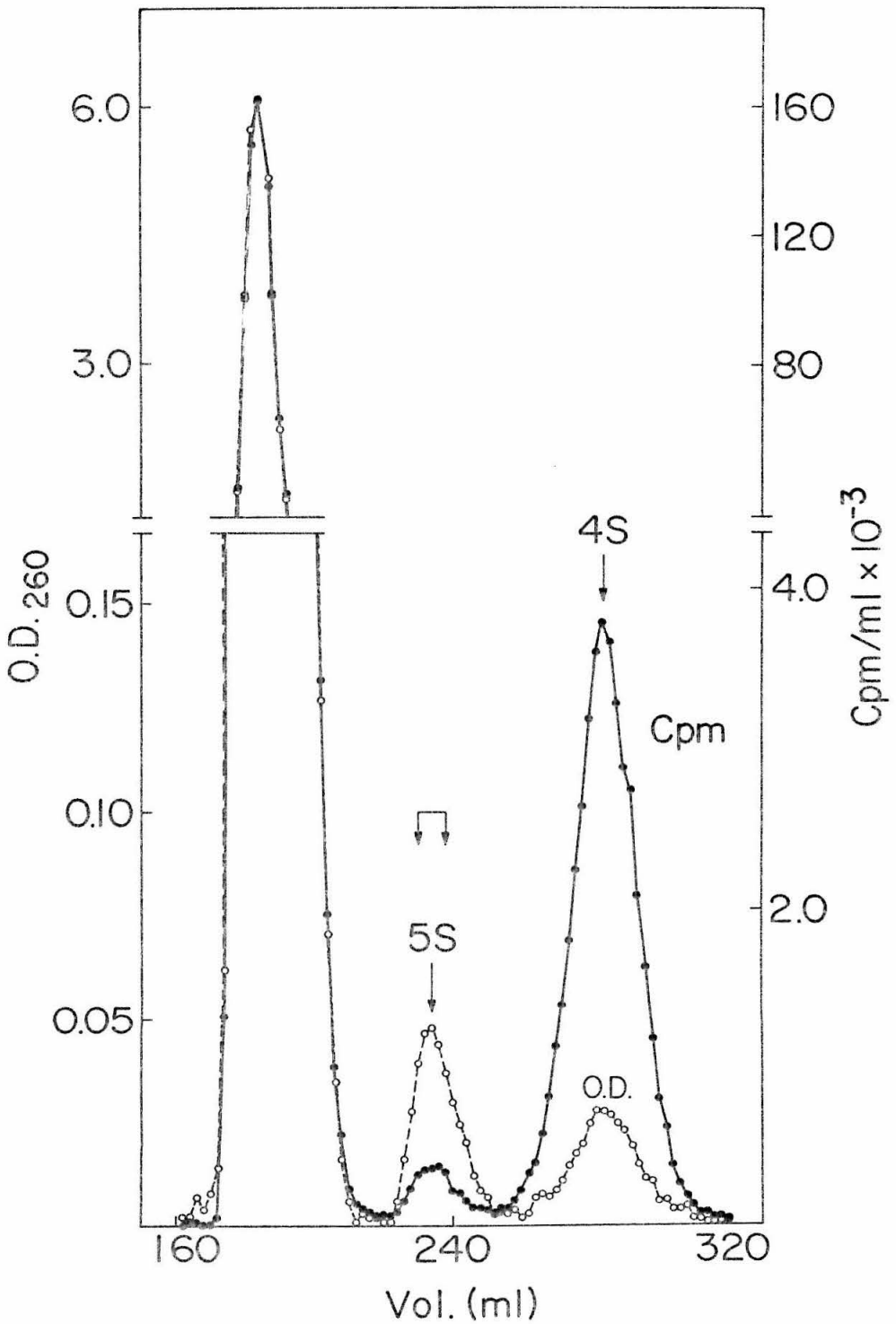
6.0
3.0
0.15
0.10
0.05
O.D. 260
160
120
80
4.0
2.0
Cpm/ml x 10⁻³
4S
5S
Cpm
O.D.
160
240
320
Vol. (ml)

Fig. 4

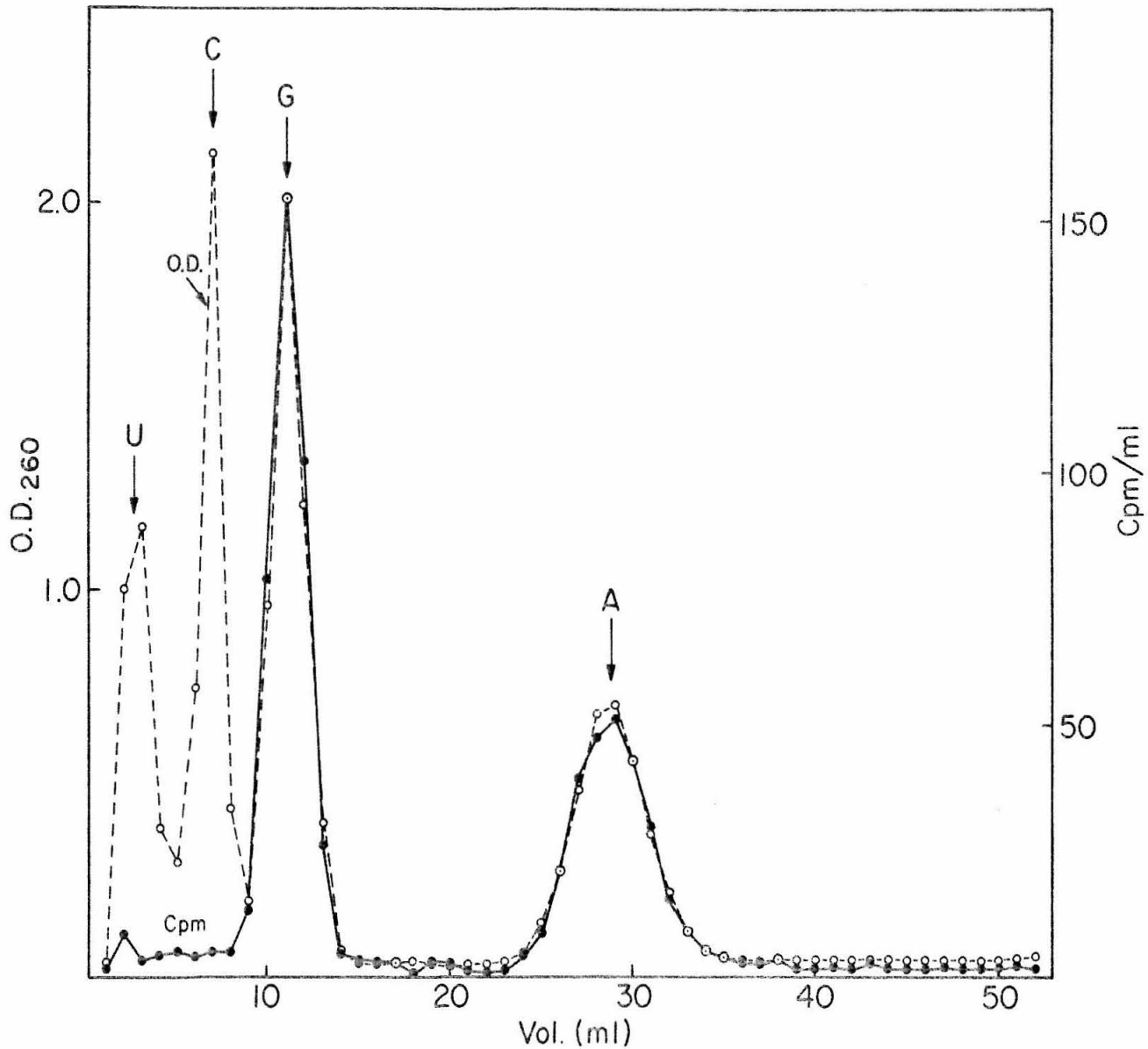

Fig. 5

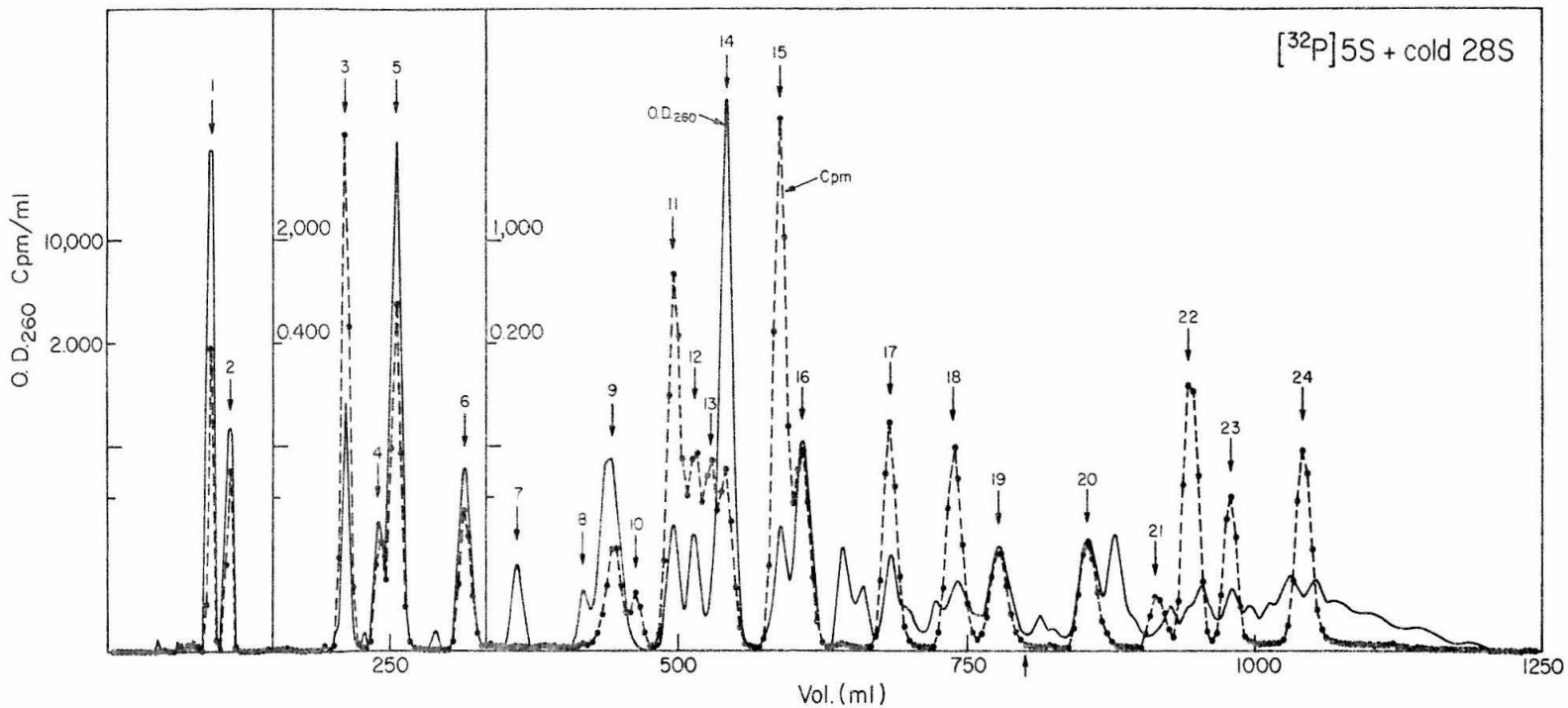
[32P]5S + cold 28S
O. D.260 Cpm/ml
10,000
2,000
2,000
0.400
1,000
0.200
O.D260
Cpm
1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
23
24
250
500
750
1000
1250
Vol. (ml)

Fig. 6

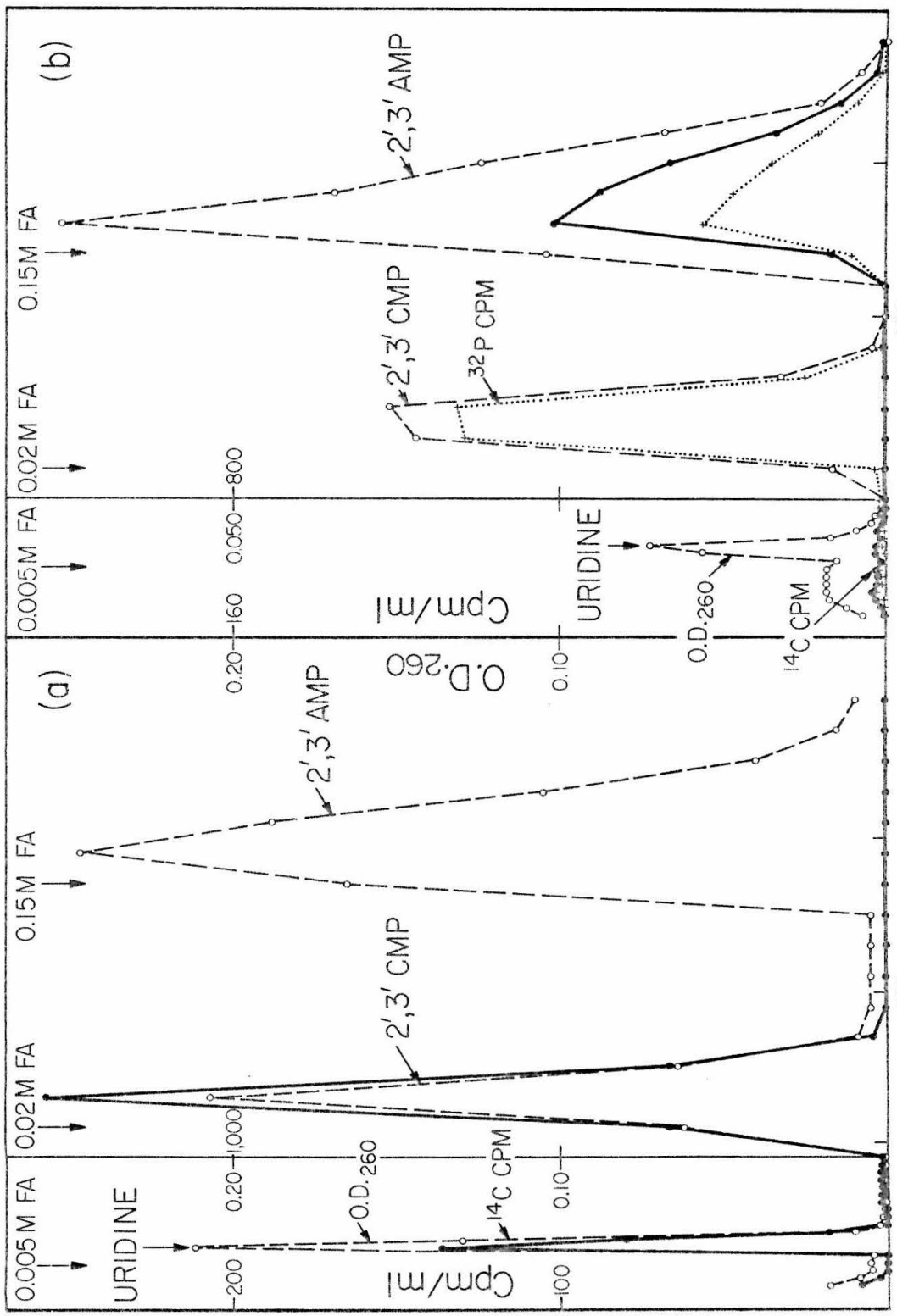
(a)
(b)
0.005 M FA
0.02 M FA
0.15 M FA
URIDINE
2',3' AMP
2',3' CMP
O.D.260
14C CPM
32P CPM
O.D.260
Cpm/ml
Vol. (ml)

Fig. 7

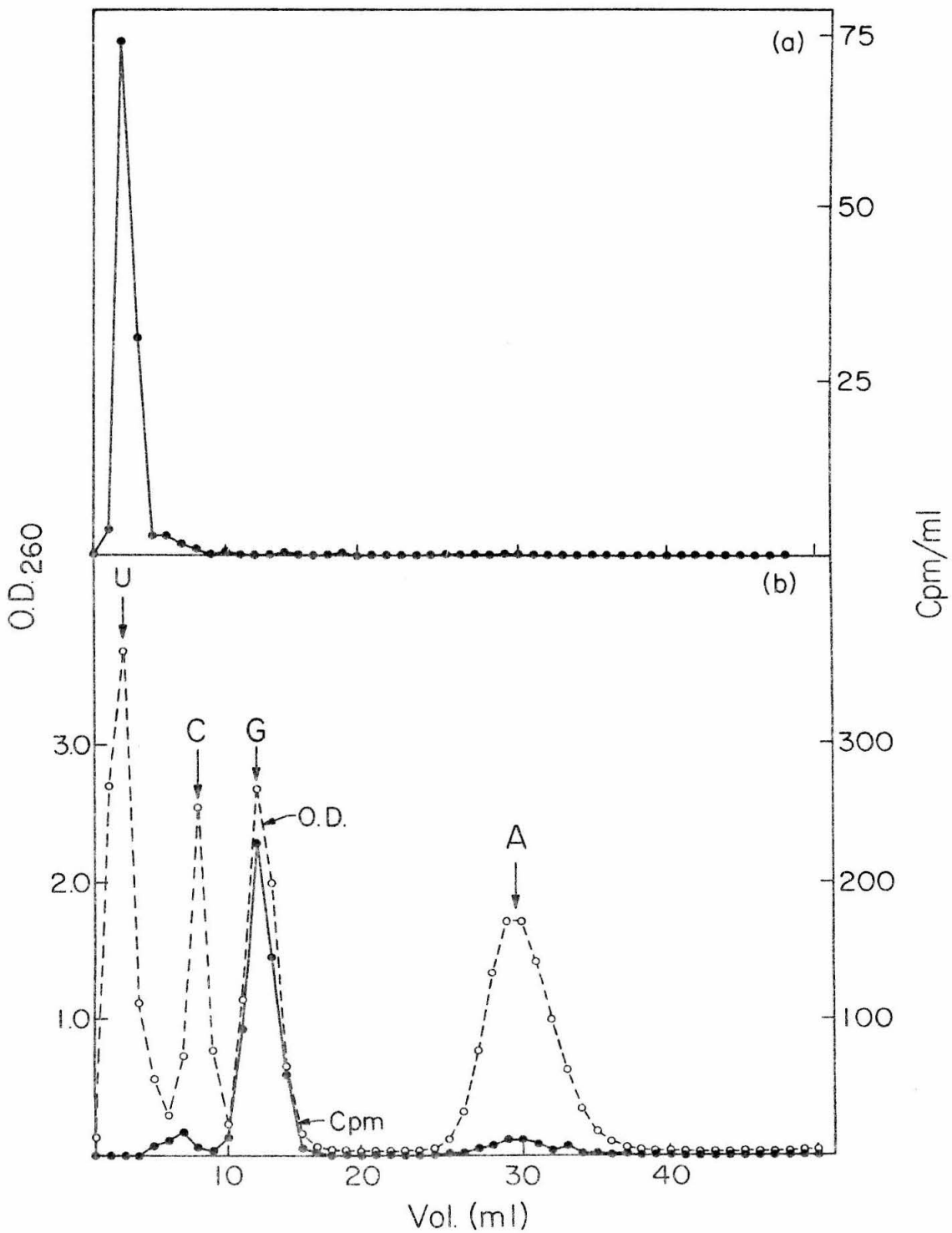
(a)
75
50
25
O.D.260
Cpm/ml
(b)
U
C
G
A
O.D.
Cpm
3.0
2.0
1.0
300
200
100
10
20
30
40
Vol. (ml)

Fig. 8

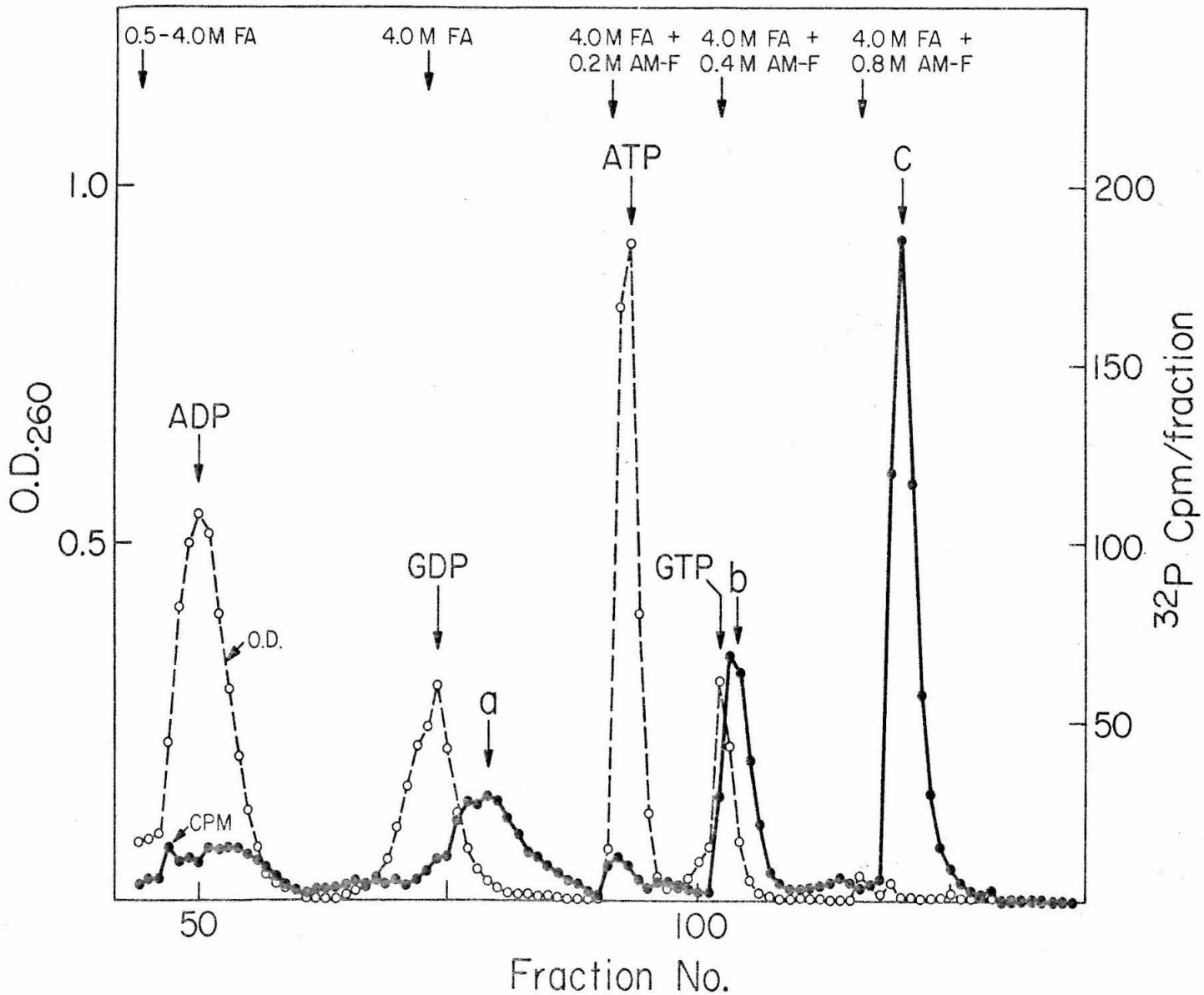
0.5-4.0M FA
4.0M FA
4.0M FA + 0.2M AM-F
4.0M FA + 0.4M AM-F
4.0M FA + 0.8M AM-F
ATP
C
ADP
GDP
a
GTP
b
O.D.
CPM
1.0
0.5
O.D.260
200
150
100
50
32P Cpm/fraction
50
100
Fraction No.

Fig. 9

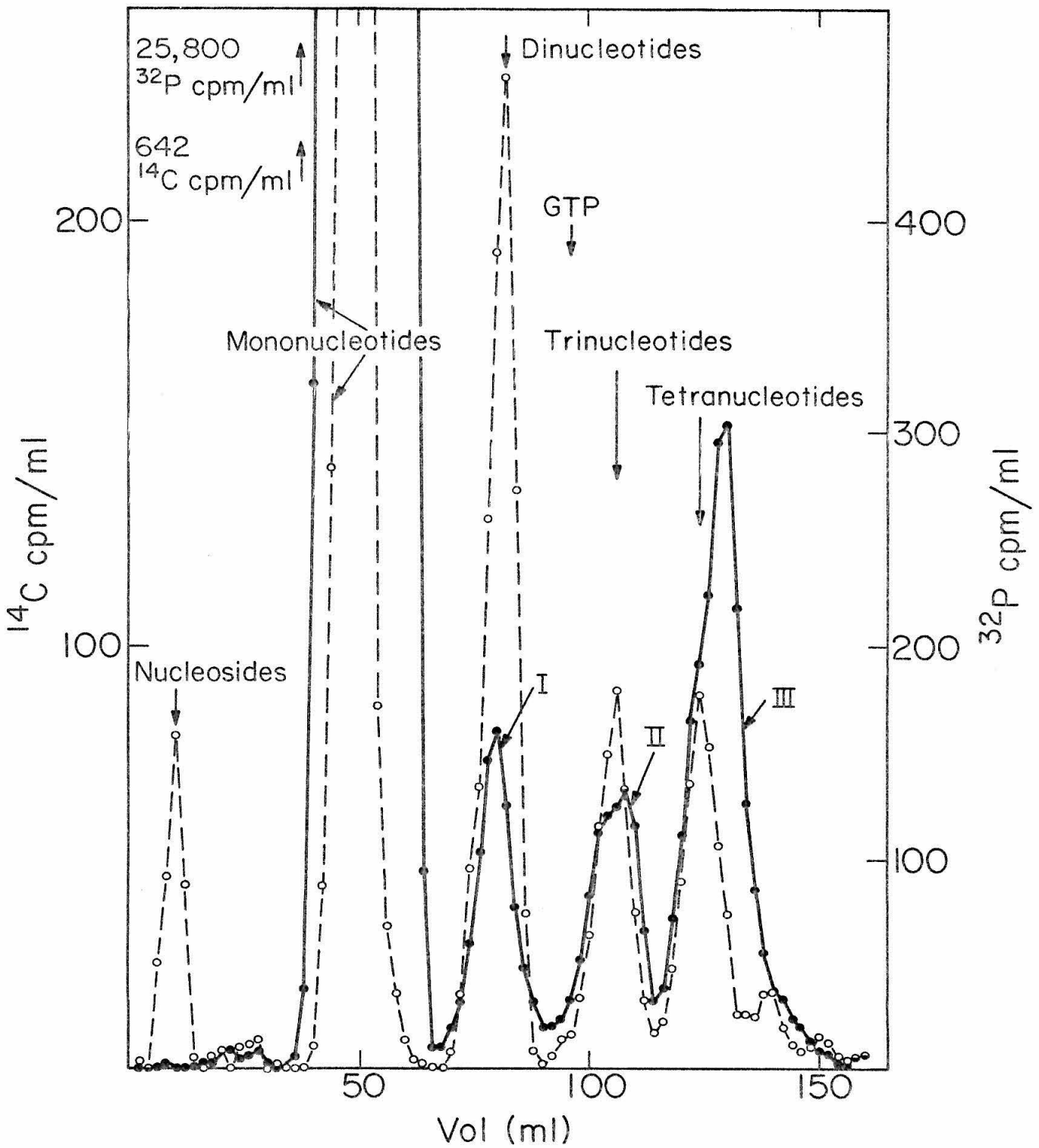
25,800
32P cpm/ml
642
14C cpm/ml
Dinucleotides
GTP
Mononucleotides
Trinucleotides
Tetranucleotides
Nucleosides
I
II
III
200
100
400
300
200
100
50
100
150
Vol (ml)
14C cpm/ml
32P cpm/ml

Fig. 10

SECTION II

HYBRIDIZATION STUDIES WITH HeLa 4S RNA

SUMMARY

The proportion of the HeLa cell genome complementary to tRNA was investigated by using RNA-DNA hybridization. Transfer RNA was phenol-SDS extracted from the cytoplasmic soluble fraction of HeLa cells, and purified by Sephadex G-100 chromatography. These preparations were free from any significant contamination by DNA, mRNA, or 5S, 18S, 28S rRNA, as judged by a number of criteria. Analysis of size and nucleotide composition of the RNA recovered from RNase-treated RNA-DNA hybrids was used to assess the specificity of the hybrids studied.

The value for saturation of the HeLa DNA by tRNA was found to be 1.1×10^{-5}, which corresponds to about 4900 sites for tRNA per HeLa cell in an exponentially growing culture. No competition was detected between HeLa tRNA and 5S, 18S or 28S rRNA. Analysis of the nucleotide composition of the hybridized tRNA revealed significant differences from the nucleotide composition of the input tRNA, with the purine to pyrimidine ratio indicating, however, that these differences were not produced by excessive RNase attack of the hybrid. The size of the hybridized tRNA was only moderately smaller than that of the input RNA; the average S value in formaldehyde was 2.7 (corresponding to an average length of 65 nucleotides), suggesting that a relatively small portion near the ends of the hybridized 4S chains had been removed by RNase; the possible explanations for the differences in nucleotide composition and size of the hybridized RNA from that of the input RNA are discussed.

1. INTRODUCTION

The fraction of the genome which specifies tRNA has been investigated by using the technique of RNA-DNA hybridization in a number of organisms, such as bacteria (Goodman and Rich, 1962; Giacomoni and Spiegelman, 1962; Oishi, Oishi and Sueoka, 1966; Morell, Smith, Dubnau and Marmur, 1967; Smith, Dubnau, Morell and Marmur, 1968), yeast (Schweizer, MacKechnie and Halvorson, 1969), Drosophila (Ritossa, Atwood and Spiegelman, 1966a,b), and Xenopus (Brown and Weber, 1968). From these studies, it appears that with increased organismic complexity there is an increased number of tRNA genes, varying from 30 to 80 per genome in bacteria, to about 2300 in Xenopus. In all eukaryotic cells examined a high degree of redundancy for the genes corresponding to the individual tRNA species has been observed, although not as high as that detected for rRNA genes, which have a redundancy about one order of magnitude higher (see review by Perry, 1967). In the present work, the investigation of tRNA genes has been extended to the genome of HeLa cells, an established cell line of human origin. The stringent criteria of specificity of hybrid formation employed with HeLa rRNA and its precursors (Attardi, Huang and Kabat, 1965b; Jeanteur and Attardi, 1969), has been utilized in analyzing hybrids formed between HeLa DNA and HeLa 4S RNA.* It has been found that a fraction of about

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*In the present work the expressions 4S RNA and tRNA have been used interchangeably, although the assumption that all 4S RNA isolated from the cytoplasm of HeLa cells belongs to the tRNA class has not been proven.

1.1×10^{-5} of HeLa DNA forms specific hybrids with homologous 4S RNA; this corresponds to about 4900 tRNA genes per cell in an exponentially growing culture.

2. MATERIALS AND METHODS

(a) Cells and method of growth

The method of growth of HeLa cells (S3 clonal strain) in suspension has been described previously (Amaldi and Attardi, 1968).

(b) Labeling conditions

Labeling of 4S RNA with ^{32}P was accomplished by exposing cells for 48 hours to ^{32}P-orthophosphate (25 to 50 μc/ml) in modified Eagle's medium (with 5% dialyzed calf serum) containing 4.5×10^{-5} M phosphate. A 12 to 16 hour chase in the presence of 5×10^{-3} M unlabeled phosphate was performed in order to reduce the specific activity of rapidly-labeled nuclear and cytoplasmic RNA species (Penman et al., 1963; Scherrer et al., 1963; Houssais and Attardi, 1966; Warner et al., 1966).

For labeling of 4S RNA with ^{3}H, cells were incubated 48 hours in modified Eagle's medium with 5% dialyzed calf serum in the presence of 0.12 μC/ml (5-^{3}H) uridine.

(c) Buffers

The buffer designations are: (1) TKM: 0.05 M tris buffer (pH 6.7), 0.025 M KCl, 0.0025 M $MgCl_2$; (2) SSC: 0.15 M NaCl, 0.015 M Na citrate; (3) dissociation medium: 0.01 M potassium phosphate buffer

(pH 8.0), 2% formaldehyde, 0.1% SDS; (4) PNF: 0.02 M potassium phosphate buffer (pH 7.4), 0.1 M NaCl, 1% formaldehyde; (5) KT: 0.5 M KCl, 0.01 M tris buffer (pH 7.4).

(d) Isolation and purification of 4S RNA

As described in a previous paper (Hatlen, Amaldi and Attardi, in manuscript) the washed cells were homogenized with a Potter-Elvehjem homogenizer in 0.002 M $MgCl_2$, brought to 0.25 M sucrose, 0.025 M KCl, 0.004 M $MgCl_2$, 0.05 M tris buffer (pH 7.4), and centrifuged at 600 x g_{av}. The resulting supernatant (without deoxycholate treatment) was centrifuged 15 minutes at 6600 x g_{av} to remove membrane components, then centrifuged 2 hours at 105,000 x g_{av} to sediment the ribosome-polysome fraction; the final supernatant was phenol-SDS extracted as detailed elsewhere (Attardi et al., 1965b), then subjected to two passages through Sephadex G-100 columns (1.8 x 180 cm) equilibrated with 0.1 M NaCl (Galibert, Larsen, Lelong and Boiron, 1965). Fractions of 2 ml were collected and analyzed by optical density measurement and/or by plating a small aliquot in Bray's solution, then counting in a scintillation counter. As a check on possible DNA or other ^{32}P-labeled contamination, an aliquot of the 4S RNA was extensively RNase digested (2 hours at 37° with 50 µg/ml heated pancreatic RNase), then chromatographed on Sephadex G-100, equilibrated with 2 x SSC, and the radioactivity eluting in the known position of 4S analyzed. Although very little radioactivity (0.001%) was found in the 4S region, as a further precaution, the final 4S preparation was treated for 1 hour at 0° with

50 μg/ml of chromatographically-purified DNase (Worthington Biochemical Corporation, Freehold, N.J.) in TKM, then phenol extracted, ethanol precipitated, and finally run through Sephadex G-100 (0.9 x 80 cm) equilibrated with 2 x SSC. The 4S peak was located both by radioactivity and by optical density measurements, and the central fractions pooled. In order to remove any possible residual ^{32}P-labeled DNA fragments or other ^{32}P-labeled contaminant (Nygaard and Hall, 1963) a portion of the 4S RNA preparation purified as described above, was heated in 0.1 x SSC for 3 minutes at 80°, quickly cooled, brought to 2 x SSC, then filtered 3 times through nitrocellulose membranes.

Methylated albumin chromatography of the Sephadex-purified 4S RNA was performed according to the procedure of Mandell and Hershey (1960).

^{3}H-labeled and unlabeled 4S RNA were prepared according to the procedures described above. Unlabeled HeLa 5S RNA was extracted from the ribosome-polysome pellet, then purified by 2 passages through Sephadex G-100 (Hatlen, Amaldi and Attardi, in manuscript); unlabeled HeLa 18S and 28S RNA were extracted from the 30S and 50S ribosomal subunits, then purified by 2 cycles of sucrose gradient centrifugation (Amaldi and Attardi, 1968). Unlabeled E. coli 23S rRNA was extracted from the ribosomal pellet of an alumina extract, using phenol-SDS, then purified by 2 cycles of sucrose gradient centrifugation (Attardi et al., 1965a).

(e) Sedimentation properties of 4S RNA under various conditions

Sedimentation analysis of the 4S RNA component isolated by Sephadex G-100 chromatography was carried out in the presence of marker 5S or 18S RNA in a 5 to 20% gradient of sucrose in 0.1 M NaCl, 0.01 M Na acetate (pH 5.0), using the Spinco SW-65 rotor for 11.5 hours at 62,000 rpm, 4°. For the analysis of sedimentation properties under denaturing conditions, the 4S samples (in the presence of 5S RNA marker) were heated at 80° for 3 minutes in 0.001 M tris buffer (pH 7.0), 0.00025 M EDTA, quickly cooled to 0°, and run through a 5 to 20% gradient of sucrose in the same buffer; alternatively, the samples were heated at 70° for 5 minutes in 18% neutralized formaldehyde containing 0.001 M EDTA, quickly cooled, diluted two times with water, and run on a 5 to 20% gradient of sucrose in 0.02 M sodium phosphate buffer (pH 7.4) containing 1% formaldehyde. The conditions of centrifugation for 4S RNA after denaturation were the same as described above. Fractions from the sucrose gradient were collected directly into scintillation vials, and after addition of Bray's solution, were counted in the scintillation counter.

For the analysis of the effects on the size of the 4S RNA of thermal treatment during incubation and dissociation of the hybrid (see section (g)), 4S RNA samples in 2 x SSC were incubated 2 hours at 72°, cooled, then heated 4 minutes at 100°, quickly cooled, and run on a 5 to 20% gradient of sucrose in SSC for 18 hours at 36,000 rpm in a Spinco SW-39 rotor at 4°, in parallel with a nonheated sample. The

combined effects on the size of the 4S RNA of the thermal treatment used for hybrid formation and the formaldehyde treatment used for dissociation of RNA-DNA hybrids (see section (g)) were investigated by incubating the 4S RNA sample for 2 hours at 72° in 2 x SSC, diluting it tenfold with dissociation medium, and heating it again for a total of 6 minutes at 100°, followed by quick cooling. The sample was run on a 5 to 20% gradient of sucrose in PNF, in parallel with an untreated sample, using the Spinco SW-65 rotor for 20 hours at 58,000 rpm, 4°. Sucrose gradients were collected and analyzed for radioactivity as described above.

(f) Isolation and denaturation of DNA

DNA was extracted from total HeLa cells or from *E. coli* by the Marmur procedure (Marmur, 1961) followed by three additional deproteinization steps with phenol.

The DNA was denatured by heating 15 minutes at 100° in 0.1 x SSC, then quick cooling in an ice-salt bath. The final hyperchromicity was about 25%. The denatured DNA solutions were found to be free of any detectable RNase activity, as judged by the observation that no loss of TCA-precipitable radioactivity occurred after incubating 50 μg/ml of DNA with labeled RNA at 37° overnight.

(g) RNA-DNA hybridization and analysis of size and base composition of hybridized RNA

The hybridization experiments were carried out by incubating denatured DNA, at a concentration of 20 μg/ml, and varying amounts of RNA (up to 4 μg/ml) in 2 x SSC for 2 hours at 72°, unless otherwise specified. The reaction was stopped by quick cooling down to 2°. Isolation and analysis of the hybrids was performed according to the procedure described previously (Attardi et al., 1965a), with minor modifications. After pancreatic RNase digestion (5 to 10 μg/ml, 1 hour at 22°), the samples were run through a 0.9 x 80 cm column of Sephadex G-100, equilibrated with 2 x SSC, 22°. Fractions of 2 ml were collected and the $O.D._{260}$ measured; the denatured DNA and RNA-DNA hybrids were eluted as a sharp peak, well separated from the RNase digestion products of the unhybridized RNA. In general, the recovery of DNA was between 70 and 80%. The fractions containing the DNA and the RNA-DNA hybrids were pooled, and after addition of one-ninth volume 5 x KT buffer, filtered through nitrocellulose membrane filters (Bac-T-Flex, type B-6, Schleicher and Schuell (Nygaard and Hall, 1963)); these were washed with about 150 ml of KT buffer at 60°. DNA retention by the filter was tested by measuring the $O.D._{260}$ of the filtrate, and was always greater than 95%. The filters were either dried and analyzed for radioactivity in the scintillation counter or utilized for determination of nucleotide composition and size of the hybridized RNA. For this latter purpose, the filter was inverted in a 20 ml beaker

containing 1.0 ml of dissociation medium and 50 µl of ^{3}H 4S RNA, previously incubated in 2 x SSC under the same conditions as the RNA-DNA mixtures (this ^{3}H RNA served as a control for the size distribution of the input RNA subjected to the same thermal treatment as the hybridized and dissociated RNA). The filter in dissociation medium was heated 2 minutes in a boiling water bath (temperature in the sample was 85 to 90°), then removed with forceps and rinsed on each face with 0.1 ml water. The eluted material (generally containing more than 85% of the original radioactivity) was heated 4 minutes at 100°, quickly cooled, and run through 11.5 ml of a 5 to 20% gradient of sucrose in PNF buffer in the Spinco SW-41 rotor for 51 hours at 37,000 rpm at 4°. Samples were collected directly into scintillation vials, diluted with 1.5 ml water and, after addition of 15 ml Bray's solution, counted in the scintillation counter. For nucleotide composition, a filter was inverted over a small Büchner funnel, and 1.0 ml of 0.5 N NaOH was passed through, followed by an additional 0.5 ml as a rinse; more than 95% of the radioactivity was eluted from the filter by this method. Digestion was carried out for 16 to 18 hours at 30°, then the samples were diluted tenfold with water, brought to pH 8.5, and chromatographed on Dowex 1-X8 as described previously (Attardi, Parnas, Hwang and Attardi, 1966). Samples were dried on aluminum planchets and counted on a Nuclear Chicago low background (2 cpm) gas flow counter.

In each experiment of hybridization, controls were included for nonspecific background, utilizing either E. coli DNA incubated with labeled 4S RNA under the same conditions as those used for hybridization

with HeLa DNA, or employing HeLa DNA and labeled 4S RNA incubated separately, then mixed at room temperature just prior to RNase digestion. In terms of per cent of radioactivity bound to the filters as nonspecific background, these two procedures were equivalent. The background increased in proportion to the input RNA, but within the range of RNA inputs used, was always found to be relatively low. Even at the very high input of 2 μg/ml of labeled RNA, which is very close to the saturation level, the background corresponded to only about 17% of the total radioactivity on the filter (this is equivalent to 0.002% of the radioactivity initially present in the incubation mixture). All the hybridization values reported here have been corrected for the background determined for each point as described above.

(h) Quantitative analysis of RNA-DNA hybrids

The specific activity of the RNA samples was determined by counting in the scintillation counter aliquots directly plated on nitrocellulose membrane filters (previously washed with KT buffer), then dried under the same conditions as the RNA-DNA hybrid filters. For the determination of the concentration of 4S RNA the value 215 for $E^{1\,cm}_{1\%}$ at 258 mμ found for yeast 4S RNA (Lindahl, Henley and Fresco, 1965) was used; the extinction coefficient for HeLa 4S RNA was assumed to be not very different from this figure. The amount of RNA hybridized in each hybridization mixture was determined from the measured radioactivity in the hybrid, on the basis of the specific activity of

the RNA and from the amount of DNA recovered after Sephadex chromatography.

Because difficulty in performing competition experiments at high inputs of RNA had been experienced with high molecular weight ribosomal RNA of HeLa cells (Jeanteur and Attardi, 1969) all competition experiments with unlabeled homologous and heterologous RNA species employed a level of labeled 4S RNA and a range of concentrations of competing unlabeled RNA amounting to 4 μg/ml or less in total. This resulted in competition experiments being done in the rising region of the saturation curve, so that the competition to be expected in the case of homologous 4S RNA had to be calculated on the basis of the dilution of label by unlabeled 4S RNA and of the change in position on the saturation curve. For this latter purpose, a saturation curve was done at the same time with each set of competition experiments.

3. RESULTS

(a) Purification of the 4S RNA

Figure 1a shows a typical elution pattern on Sephadex G-100 of RNA phenol-SDS extracted from the 105,000 x g_{av} supernatant of HeLa cells labeled with ^{32}P-orthophosphate. Some contaminating high molecular weight RNA was eluted in the void volume, followed by a large peak of 4S RNA; the high molecular weight RNA is in general less in amount than the 4S RNA. When the central portion of the 4S peak was rerun (Fig. 1b), substantially less than 1% of the total material was found in the void volume. In reconstruction experiments, labeled high

molecular weight RNA from ribosomes (including mRNA, 18S and 28S RNA) gave negligible tailing into the 4S peak, suggesting that breakdown on the column of high molecular weight RNA to fragments the size of 4S RNA does not occur. On first Sephadex passage, a small shoulder was sometimes found in the region of 5S RNA (Fig. 1a), which was absent in the rerun of the central 4S fractions.

A critical point in the purification of 4S RNA to be utilized for RNA-DNA hybridization is to exclude the presence of labeled DNA fragments in the preparation. In order to minimize such possible contamination, the cells were routinely homogenized very gently (6 to 7 strokes with the homogenizer, which resulted in less than 50% of the nuclei being freed), thereby reducing the breakage of nuclei. Labeled 4S RNA preparations purified by two passages through Sephadex were always found to contain less than 0.001% total radioactivity insensitive to pancreatic RNase. Nonetheless, because the observed hybrid levels for 4S RNA were relatively low, a DNase digestion was routinely performed as a precautionary step on the Sephadex-purified preparation of 4S RNA (Materials and Methods (d)).

(b) Characterization of 4S RNA

4S RNA purified by Sephadex G-100 sedimented as a fairly sharp and symmetrical peak in the region of the sucrose gradient expected for molecules of sedimentation constant of about 4S, as estimated with reference to either a 5S or an 18S RNA marker. Even after heat denaturation and centrifugation through a sucrose gradient in low ionic

strength buffer, 4S RNA sedimented as a sharp peak moving slower than 5S RNA treated in the same fashion, with very little amount of trailing material. Similar results were obtained after formaldehyde treatment and sedimentation in a sucrose gradient in PNF buffer. (See as examples of sedimentation analyses of 4S RNA Fig. 3, Section I.) These results indicated the essential homogeneity in size and the intactness of molecules in the 4S preparations used in these present studies.

Methylated albumin chromatography of the Sephadex-purified 4S RNA showed that this was eluted as a fairly sharp peak at the NaCl range of 0.45 to 0.60 M, which is the region of elution observed for tRNA from various sources, including cells of human origin, KB (Galibert et al., 1965).

The nucleotide composition of HeLa 4S RNA shows a GC content of about 59%, and is fairly close to the nucleotide compositions previously reported for 4S RNA from the human cell lines HeLa and KB (Table I). About 3.9 mole % of pseudouridylic acid was found in HeLa 4S RNA. The extent of methylation of 4S RNA was investigated by labeling HeLa cells for two generations with L-(^{14}C-methyl) methionine (see Section I for details (Fig. 4)) then extracting the RNA from the ribosome-polysome fraction, which should contain only mature, fully-methylated tRNA and no tRNA precursors (Burdon and Clason, 1969). After deducting the labeling of the purine rings, a figure of 1 methyl group per 11.5 nucleotides was found, in good agreement with the figure of 1 per 12 nucleotides found for HeLa 4S RNA extracted from the cytoplasmic soluble fraction (Brown and Attardi, 1965).

(c) Effect of time and temperature of incubation on RNA-DNA hybrid formation

The melting curve in 0.30 M NaCl, 0.01 M tris buffer (pH 7.0) of Sephadex-purified 4S RNA of HeLa cells revealed a rather broad profile, similar to that displayed by 4S RNA from E. coli and yeast (see Brown, review, 1963), with a T_m of 63°. This relatively high T_m, which is in agreement with the fairly high GC content (59%) of HeLa 4S RNA, suggested that a high temperature would be required for hybrid formation with this RNA, as previously reported for bacterial, yeast and Drosophila 4S RNA. The optimum temperature and time of incubation were investigated by using an input ratio of RNA to DNA of 1:100, which is in the early rising portion of the saturation curve. The optimum temperature of hybridization was found to be in the range 68 to 72°, (Fig. 2a) with 72° being chosen for all subsequent experiments. At 72°, maximum hybrid formation already occurred at about 2 hours (Fig. 2b), which was the time used for all later experiments.

(d) Analysis of size and nucleotide composition

As a preliminary to the analysis of size of hybridized RNA, the effect of the thermal and formaldehyde treatment on the sedimentation behavior of 4S RNA was investigated. As shown in Figure 3a, the simple exposure of 4S RNA to the heating steps used in the incubation and dissociation of the RNA-DNA hybrids had very little effect on the sedimentation properties of the RNA, suggesting that there was no appreciable thermal breakage of 4S RNA under these conditions. By

contrast, there was a marked decrease in sedimentation velocity of the 4S RNA when the complete heat-formaldehyde treatment was applied (Fig. 3b); this decrease is presumably due to the denaturation of the 4S RNA as a result of the reaction of formaldehyde with the amino groups of the bases. It should be noticed that the latter sedimentation run was done in a sucrose gradient containing formaldehyde, with which the control 4S RNA may have slowly reacted, with a resulting reduction in its sedimentation rate.

Figure 4 shows the sedimentation profile of RNA recovered from hybrids between 4S RNA and HeLa cell DNA after RNase treatment-Sephadex chromatography. At all ratios of RNA to DNA employed in these experiments, the distribution of the dissociated hybrid RNA is somewhat broader than that of the 4S RNA marker, and with respect to the latter, is displaced towards the meniscus, with a relatively small amount of trailing material. (This trailing material is probably due, at least in part, to short RNA fragments pertaining to the nonspecific background.) By using the approximate value of 3S for the sedimentation constant in formaldehyde of 4S RNA (Boedtker, 1968), the average sedimentation constant of the dissociated RNA was estimated to be about 2.7 S, corresponding to an approximate length of 65 nucleotides (Boedtker, 1968). No obvious effect of the input RNA to DNA ratio on the average sedimentation constant of the dissociated chains was observed.

Nucleotide composition of the RNA retained on the nitrocellulose membrane after RNase treatment and Sephadex chromatography of

the hybridization mixtures was examined at two input ratios of RNA to DNA, 0.05 and 0.10 (at which the background was about 13% and 17%, respectively, of the total radioactivity). As can be seen from Table II, at these two ratios the nucleotide composition is rather similar: it is on the other hand, significantly different from that of the input 4S RNA. The purine to pyrimidine ratio in the hybridized RNA differs by little more than 10% from that of the input RNA, a finding which suggests that the observed changes in nucleotide composition in the hybridized RNA are not due to excessive RNase attack.

(e) Saturation experiments

Figure 5 shows the composite DNA saturation curve obtained with HeLa 4S RNA in three different experiments. It appears that the introduction in the purification of 4S RNA of a prefiltration step through nitrocellulose membranes, in order to eliminate any possible residual traces of DNA contamination, had no effect on the level of hybrids. (In agreement with this observation, more than 98% of the hybridized radioactivity was alkali-sensitive.) It also appears from Figure 5 that the presence of saturating amounts of both 18S and 28S HeLa RNA did not influence at all the hybrid level, excluding the presence of disturbing amounts of fragments of these ribosomal RNA species in the 4S preparations. As can be seen from the figure, the saturation curve for HeLa 4S RNA rises slowly, and finally plateaus at a level corresponding to a fraction of 1.1×10^{-5} of the DNA, around an input RNA to DNA ratio of 0.10.

(f) Competition experiments

Competition experiments performed between labeled HeLa 4S RNA and unlabeled HeLa 4S, 5S, 18S, 28S RNA, and E. coli 23S RNA, are shown in Figure 6. The homologous competition curve (mean of duplicate experiments performed with the same preparation of ^{32}P-4S RNA) coincides with the theoretical curve for 100% competition, as calculated on the basis of dilution and change in position along the saturation curve (Materials and Methods (h)). In the competition experiments carried out with different RNA species, in spite of a certain scatter of the individual points (each representing a single determination), no competition is apparent.

3. DISCUSSION

(a) Purity of the 4S RNA preparations

The resolution obtained in the Sephadex G-100 chromatography and the results of reconstruction experiments excluded any appreciable contamination of the 4S RNA utilized in the present work by high molecular weight RNA or 5S RNA, or by degradation products thereof which might arise from breakage during the chromatography. The final 4S preparations appeared to be homogeneous in size, as judged by the sedimentation properties in 0.1 M NaCl and under denaturing conditions. Concerning the possible contamination by DNA fragments, the sensitivity to RNase of the 4S preparations indicated that it corresponded to less than 1 part in 10^{-5}; more significantly, the complete alkali-sensitivity

of the RNA-DNA hybrids investigated here showed that contamination by DNA fragments of the 4S preparations did not contribute to any appreciable extent to the hybrid formation. The observation that 4S RNA saturated DNA at a level less than one-half that found for 5S RNA (Hatlen and Attardi, in preparation), and also much lower than that observed for 18S and 28S RNA (Jeanteur and Attardi, 1969) and that expected for mRNA, tended to exclude any significant contribution by any of these species to the hybrid level. Further evidence in the same direction comes from the lack of competition between the 4S RNA and the rRNA species (5S, 18S or 28S). The nucleotide composition of the hybridized RNA, which was very different from that expected from mRNA (Wilkie, Houssais and Attardi, in preparation), also spoke against any role of mRNA contamination in the present studies.

(b) Specificity of RNA-DNA hybrids

As discussed above (Discussion (a)), the purity of the RNA preparations used in the present work, the level of DNA saturation observed for 4S RNA, and the nucleotide composition of the hybridized RNA strongly suggest that the hybrids studied here involve, for all practical purposes, only tRNA molecules. Concerning the sites in DNA with which the molecules hybridize, the results of saturation and competition experiments exclude any involvement of either sites for ribosomal RNA (5S, 18S, 28S) or for mRNA. For a more rigorous analysis of the specificity of the RNA-DNA hybrids, with regard to the nature of the DNA sites involved, the stringent criteria previously introduced

for the investigation of hybrids formed with rRNA or its precursor in HeLa cells (Attardi et al., 1965b; Jeanteur and Attardi, 1969) have been applied also in the present study. In the case of the rRNA species, two partially resolved classes of hybridized RNA chains could be recovered from the RNA-DNA hybrids after RNase digestion, a major one which had the same size distribution and nucleotide composition as the input RNA (subjected to the same thermal history), and a minor one consisting of shorter RNA chains with a nucleotide composition markedly different from that of the input RNA. The much higher purine to pyrimidine ratio of these shorter chains suggested that they derived from RNase attack of rRNA or rRNA precursor molecules imperfectly base-paired with DNA. In the present work, no resolution of the dissociated hybrid RNA into two size classes was observed; rather, for all RNA to DNA ratios used, there appeared to be a single distribution somewhat broader than that of the input RNA and slightly displaced towards a smaller size. The nucleotide composition of the hybridized RNA at the two input RNA to DNA ratios examined (at or very near saturation) is different from that of the input RNA. The fact that there is no appreciable increase in the purine to pyrimidine ratio in the hybridized RNA chains, together with the observation that the size of the recovered hybrid was only moderately smaller than that of the input RNA, argue against the possibility of the differences in nucleotide composition from that of the original RNA being due to excessive RNase attack of the hybridized RNA. On the other hand, these differences in nucleotide composition appear to be the same order of magnitude as those observed

between purified tRNA species in yeast (Holley et al., 1965; Zachau et al., 1966; Madison et al., 1966; Rajbhandary et al., 1967). Therefore, it seems likely that at least a part of the differences in nucleotide composition between hybridized and input RNA are due to the proportion of sites for various tRNA species in the genome being different from the proportion of various tRNA species in the cytoplasm, with the result that the overall base composition of the hybridized RNA may not reflect that of the 4S population.

The observation that the average size of the hybridized RNA chains is about 20% smaller than that of the input RNA implies that a portion near the ends of the hybridized tRNA molecules has been cleaved by RNase. This may be due to some mismatching in the hybrids, localized near the ends of the tRNA molecules; this mismatching would be expected if there were sequence variability among the multiple sites in DNA for the same tRNA species and if each tRNA molecule were capable of hybridizing with different sites for the same tRNA species. Another possibility is that the smaller than expected size of the hybridized RNA chains is due to the methyl groups of the 4S RNA bases interfering with hydrogen bonding to DNA, so as to create RNase-sensitive spots. For example, such interference with hydrogen bonding has been described in the case of 6,6-dimethyladenine (but not with 6-methyladenine)(Griffin, Haslam and Reese, 1964). HeLa cell 4S RNA has been shown to contain on the average 7 methyl groups per molecule, of which 64%, or about 4 per molecule, are in methylated bases (Brown and Attardi, 1965), thus providing an ample potential for interference with hydrogen bonding. The observation

made here that the size of the hybridized 4S RNA chains is only moderately smaller than that of the input RNA indicates that any possible hindrance of hydrogen bonding by methyl groups would have to be more frequent in regions close to the ends of the molecules. The shorter RNA segments arising from RNase cleavage at the points of mismatching or location of methylated bases are probably either removed in the washing of the hybrids at 60° (Attardi et al., 1965b), or else spread in the trailing portion of the sucrose gradient distribution of the hybridized RNA.

(c) Fraction of the HeLa genome complementary to 4S RNA

The very slow rising towards saturation observed in the present work for the curve of hybridization of HeLa 4S RNA with homologous DNA is what would be expected for a heterogeneous system with some of the components present in low concentrations. The level of DNA saturation by 4S RNA which was determined here, about 1.1×10^{-5} of the DNA, corresponds to about 4900 sites per cell. If one considers that the HeLa cell genome is equivalent to slightly more than a triploid set of chromosomes, and if one assumes that exponentially growing HeLa cells contain on the average 1.30 times the amount of DNA of resting cells (as L cells, Stanners and Till, 1966), it can be calculated that the amount of tRNA sites per haploid set of chromosomes in a HeLa cell is about 1260, which is fairly close to that determined for a haploid set of chromosomes in Drosophila melanogaster (850) and Xenopus laevis (1150). This relative constancy in number of tRNA sites in different

higher eukaryotic organisms must be contrasted with the large differences in DNA content per cell (for example, a factor of 50 between Drosophila and HeLa cells (Table III)). It is also interesting to compare the ratio of tRNA sites to ribosomal RNA sites in the genome of different organisms (Table III). It appears that in eukaryotic cells this ratio is fairly constant, the reported values varying between 2.3 and 6.5; this ratio may be only slightly higher in bacteria. This relative constancy in the comparative amount of genes complementary to tRNA and rRNA over the whole evolutionary scale may reflect the quantitative requirement for sites of transcription necessary in order to keep the proportion of tRNA and ribosomes invariant for maximum efficiency of protein synthesis.

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TABLE I

^{32}P-Nucleotide composition of 4S RNA from human cell lines

Cell line	Labeling time with ^{32}P-orthophosphate (hours)	Mole %				%GC	Source of 4S
		Cp	Ap	Up+ψp	Gp		
KB[a]	3.0	28.8	18.6	21.6	31.2	60.0	whole cell extract
KB[a]	48	27.8	18.0	21.6	32.6	60.4	ribosomes
HeLa[b]	3.25	30.4	17.4	21.1	31.1	61.5	cytoplasmic extract
HeLa[c]	0.75	33.5	17.0	20.1	29.6	63.1	polysomes
HeLa[c]	48	28.4	19.0	21.6	31.0	59.4	cytoplasmic soluble fraction

(a) Reich, P. R., Forget, B. G. and Weissman, S. M. (1966).

(b) Salzman, N. P., Shatkin, A. J. and Sebring, E. D. (1964).

(c) Hatlen, L. E., Amaldi, F. and Attardi, G. (Section I, Tables I and II).

TABLE II

^{32}P-Nucleotide composition of input RNA and of hybridized RNA recovered from nitrocellulose membranes

RNA analyzed	Mole %					%GC	Pu/Py
	Cp	Ap	ψp	Up	Gp		
Unhybridized input RNA[a]	28.4	19.0	3.9	17.7	31.0	59.4	1.00
Hybridized RNA (0.05 RNA/DNA)	23.8	15.5	4.5	21.1	35.0	58.8	1.02
Hybridized RNA (0.10 RNA/DNA)	20.7	14.4	3.7	22.3	38.8	59.5	1.14

Incubation mixtures containing 300 μg HeLa DNA and 15 μg HeLa ^{32}P-4S RNA or 300 μg HeLa DNA and 30 μg HeLa ^{32}P-4S RNA in a total volume of 15 ml 2 x SSC were incubated 2 hours at 72°; the RNA-DNA hybrids were isolated, and the RNA dissociated from the filters as described in Materials and Methods (g). After 16 hours incubation at 30° in 0.5 N NaOH, the samples, in the presence of alkali-digested yeast RNA carrier, were chromatographed on Dowex 1-X8 as previously described (Attardi et al., 1966). Fractions from the Dowex 1-X8 chromatography were plated on planchets, and counted in a low background gas flow counter.

(a) Hatlen, Amaldi and Attardi (1969), Section I.

TABLE III

Number of sites per genome for tRNA and for the major rRNA component in various organisms

Organism	tRNA sites	Major rRNA sites	tRNA sites / Major rRNA sites	Size of genome used for calculations (daltons)
Escherichia coli	28[a], 50[b]	5[c,d]	6, 10	2.8×10^9 [e]
Bacillus subtilis	40[f], 80[g]	10[f]	4, 8	3.0×10^9 [g,h]
Saccharomyces cerevisiae	320-400[i]	140[i]	2.3-2.9	1.25×10^{11} [i]
Drosophila melanogaster	1700[j]	260[j]	6.5	2.4×10^{11} [j]
Xenopus laevis	2300[k]	970[k]	2.4	3.6×10^{12} [k]
HeLa cells	4900	1400[l]	3.5	1.2×10^{13} [m]

(a) Goodman and Rich (1962).

(b) Morell et al. (1967).

(c) Yankofsky and Spiegelman (1962).

TABLE III (continued)

(d) Attardi et al. (1965).

(e) Corresponding to non-replicating chromosomes (Cairns, 1963).

(f) Smith et al. (1968).

(g) Oishi et al. (1966).

(h) Dennis and Wake (1966). (Corresponds to non-replicating chromosome.)

(i) Schweizer et al. (1969). (These figures pertain to a haploid strain; the size of the genome refers to DNA content of cells in the stationary phase.)

(j) Ritossa et al. (1966a). (These figures refer to a diploid chromosome set; the size of the genome refers to DNA content of adult, non-multiplying cell.)

(k) Brown and Weber (1968). (These figures refer to a diploid chromosome set; the size of the genome refers to DNA content of mature erythrocytes.)

(l) Jeanteur and Attardi (1969).

(m) McConkey and Hopkins (1965). (Refers to DNA content of exponentially growing cells.)

FIGURE LEGENDS

Fig. 1. Purification by gel filtration on Sephadex G-100 of HeLa 4S RNA.

(a) RNA was phenol-SDS extracted from the 105,000 x g_{av} supernatant of HeLa cells labeled with ^{32}P-orthophosphate, and run through a Sephadex G-100 column equilibrated with 0.1 M NaCl.

(b) The fractions indicated by arrows in (a) were pooled, precipitated with ethanol, dissolved in 0.1 M NaCl and rerun through Sephadex G-100.

Fig. 2. Effect of temperature and time of incubation on ^{32}P-4S RNA-HeLa DNA hybrid formation.

(a) Each incubation mixture contained 50 μg HeLa DNA and 0.50 μg HeLa ^{32}P-4S RNA in 2.5 ml of 2 x SSC. After 4 hours incubation at different temperatures, the samples were quickly cooled, and the RNA-DNA hybrids treated for 1 hour at 22° with 10 μg/ml heated pancreatic RNase. One-ninth volume of 5 x KT buffer was added, and the samples were filtered and washed as described in Materials (g). (Plot of duplicate points.)

(b) Incubation mixtures containing 50 μg HeLa DNA and 0.50 μg HeLa ^{32}P-4S RNA in 2.5 ml of 2 x SSC were incubated at 72°. At various intervals, duplicate tubes were removed, quickly cooled, digested with 15 μg/ml heated pancreatic RNase for 1 hour at 22°. One-ninth volume of 5 x KT was added, and the samples were filtered and washed as described in Materials and Methods (g). (Plot of duplicate points.)

Fig. 3. Sedimentation analysis of 4S RNA subjected to heat treatment or heat plus formaldehyde treatment.

(a) HeLa ^{32}P-4S RNA was incubated in 2 x SSC for 2 hours at 72°, cooled, heated 4 minutes at 100°, then quickly cooled. The heat-treated sample was run in parallel with an untreated sample on a 5 ml 5 to 20% gradient of sucrose in SSC for 18 hours at 36,000 rpm in a Spinco SW-39 rotor at 4°.

(b) HeLa ^{32}P-4S RNA was incubated in 2 x SSC for 2 hours at 72°, cooled, diluted tenfold with dissociation medium, heated 6 minutes at 100°, then quickly cooled. The heat and formaldehyde-treated sample was run in parallel with an unheated sample on a 5 ml 5 to 20% gradient of sucrose in PNF for 20 hours at 58,000 rpm in a Spinco SW-65 rotor at 4°.

Fig. 4. Composite diagram showing the sedimentation profile of ^{32}P-4S RNA hybridized with HeLa DNA at different RNA to DNA ratios, as compared to that of the original RNA.

Each incubation mixture contained 120 μg HeLa DNA and varying amounts of ^{32}P-4S RNA in a total volume of 6.0 ml 2 x SSC. After RNase digestion (5 μg/ml, 1 hour at 22°) and Sephadex chromatography, the hybridized RNA was dissociated from DNA, run in sucrose gradients and analyzed as described in Materials and Methods (g). Each gradient contained a ^{3}H-4S RNA marker, incubated and subjected to the same formaldehyde treatment as the hybridized RNA. Assuming a value of 3S for the sedimentation constant in formaldehyde of HeLa 4S RNA (Boedtker, 1968),

the average sedimentation constant of the dissociated RNA chains at all RNA to DNA input ratios was estimated to be about 2.7S.

Fig. 5. Composite saturation curve of HeLa DNA by HeLa ^{32}P-4S RNA in various experiments.

Incubation mixtures containing 20 μg/ml HeLa DNA and various concentrations of HeLa ^{32}P-4S RNA were incubated 2 hours at 72°. After RNase treatment (5 to 10 μg/ml, 1 hour at 22°) the hybrids were isolated as described in Materials and Methods (g). In one experiment (○) the incubation mixtures contained saturating amounts of unlabeled HeLa rRNA (1.0 μg/ml 28S RNA, 0.40 μg/ml 18S RNA). In another experiment (□) the 4S RNA had been subjected to a prefiltration through nitrocellulose membranes, in addition to the usual purification procedure.

Fig. 6. Tests for competition for sites in HeLa DNA between ^{32}P-4S RNA and unlabeled HeLa 4S, 5S, 18S, 28S RNA and *E. coli* 23S RNA.

Each incubation mixture contained 50 μg HeLa DNA, 0.50 μg HeLa ^{32}P-4S RNA and varying amounts of unlabeled RNA. Conditions of incubation and isolation of RNA-DNA hybrids are described in Materials and Methods (g).

○ HeLa 4S RNA

■ HeLa 5S RNA

▲ HeLa 18S RNA

● HeLa 28S RNA

\+ *E. coli* 23S RNA

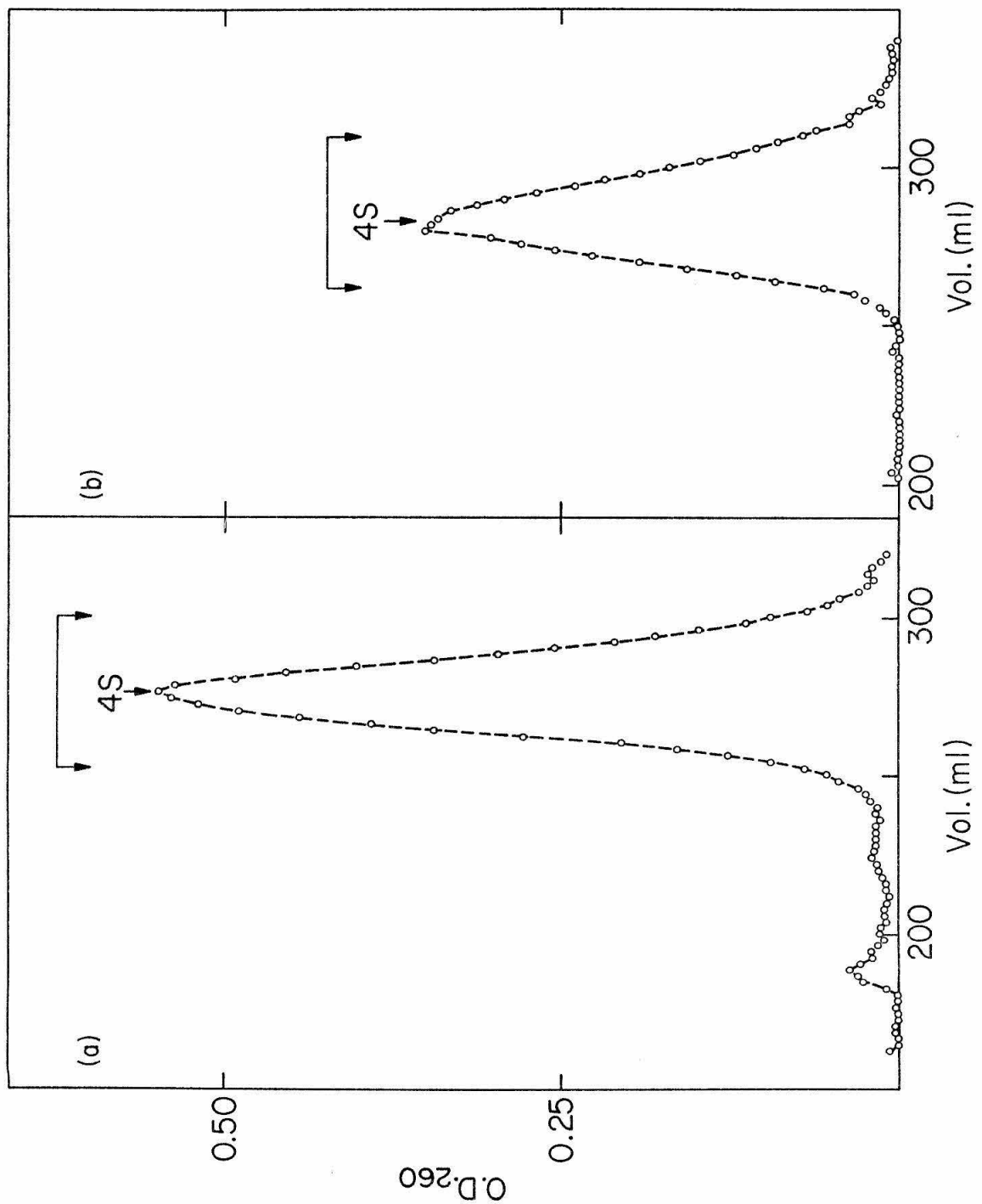

Fig. 1

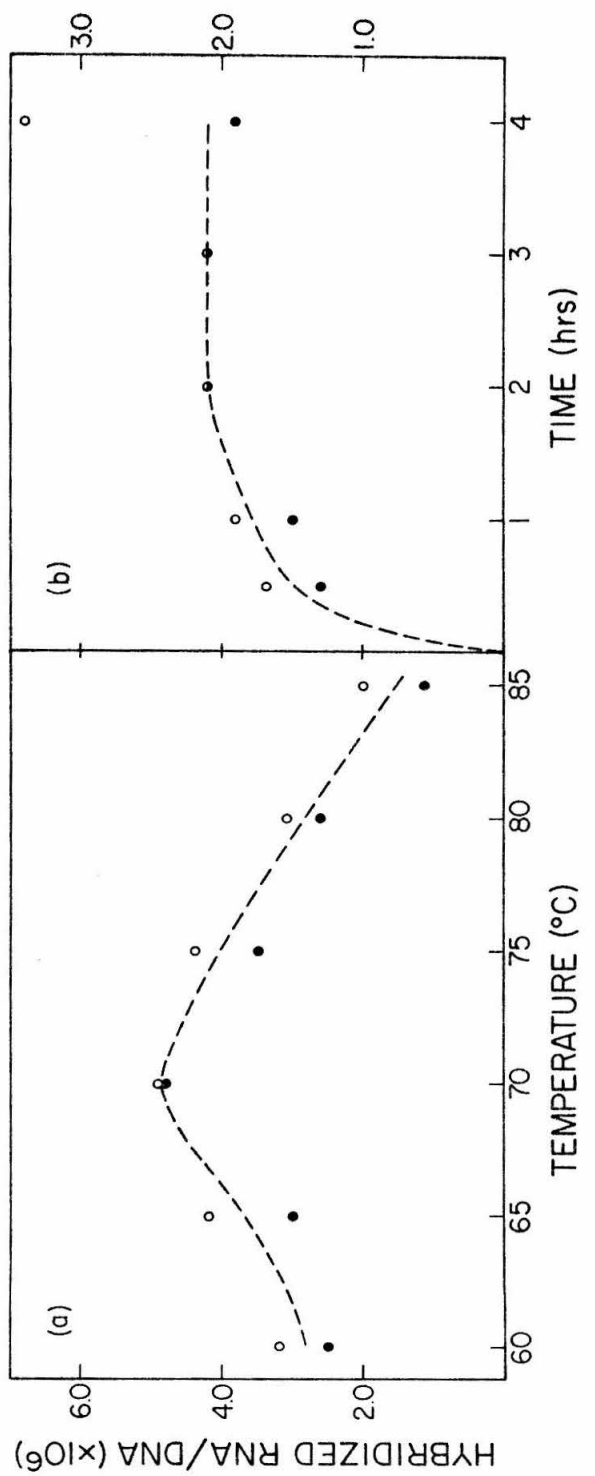
HYBRIDIZED RNA/DNA (×10⁶)
6.0
4.0
2.0
(a)
60
65
70
75
80
85
TEMPERATURE (°C)
3.0
2.0
1.0
(b)
1
2
3
4
TIME (hrs)

Fig. 2

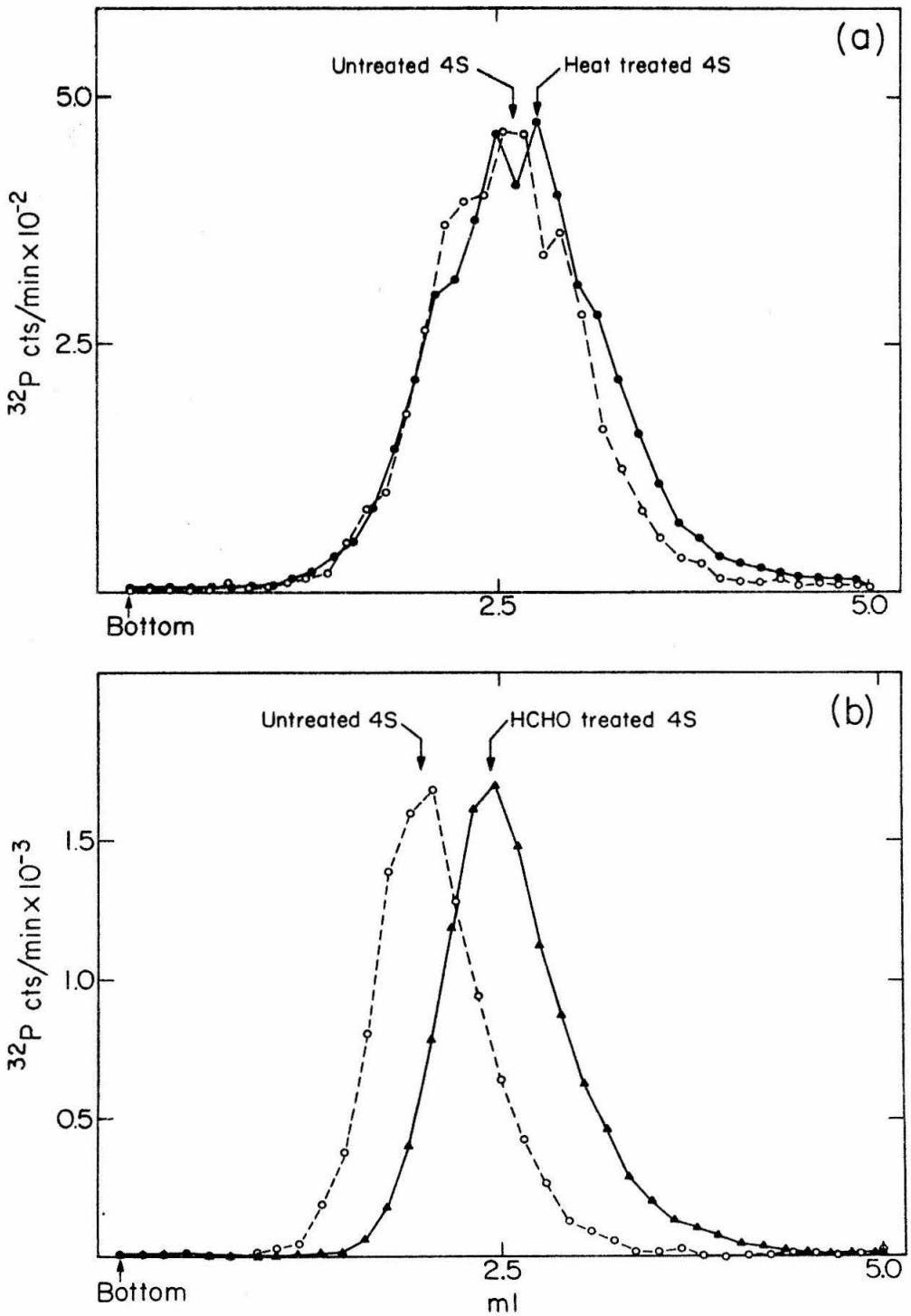
(a)
Untreated 4S
Heat treated 4S
5.0
2.5
^{32}P cts/min x 10^{-2}
Bottom
2.5
5.0
(b)
Untreated 4S
HCHO treated 4S
1.5
1.0
0.5
^{32}P cts/min x 10^{-3}
Bottom
2.5
5.0
ml

Fig. 3

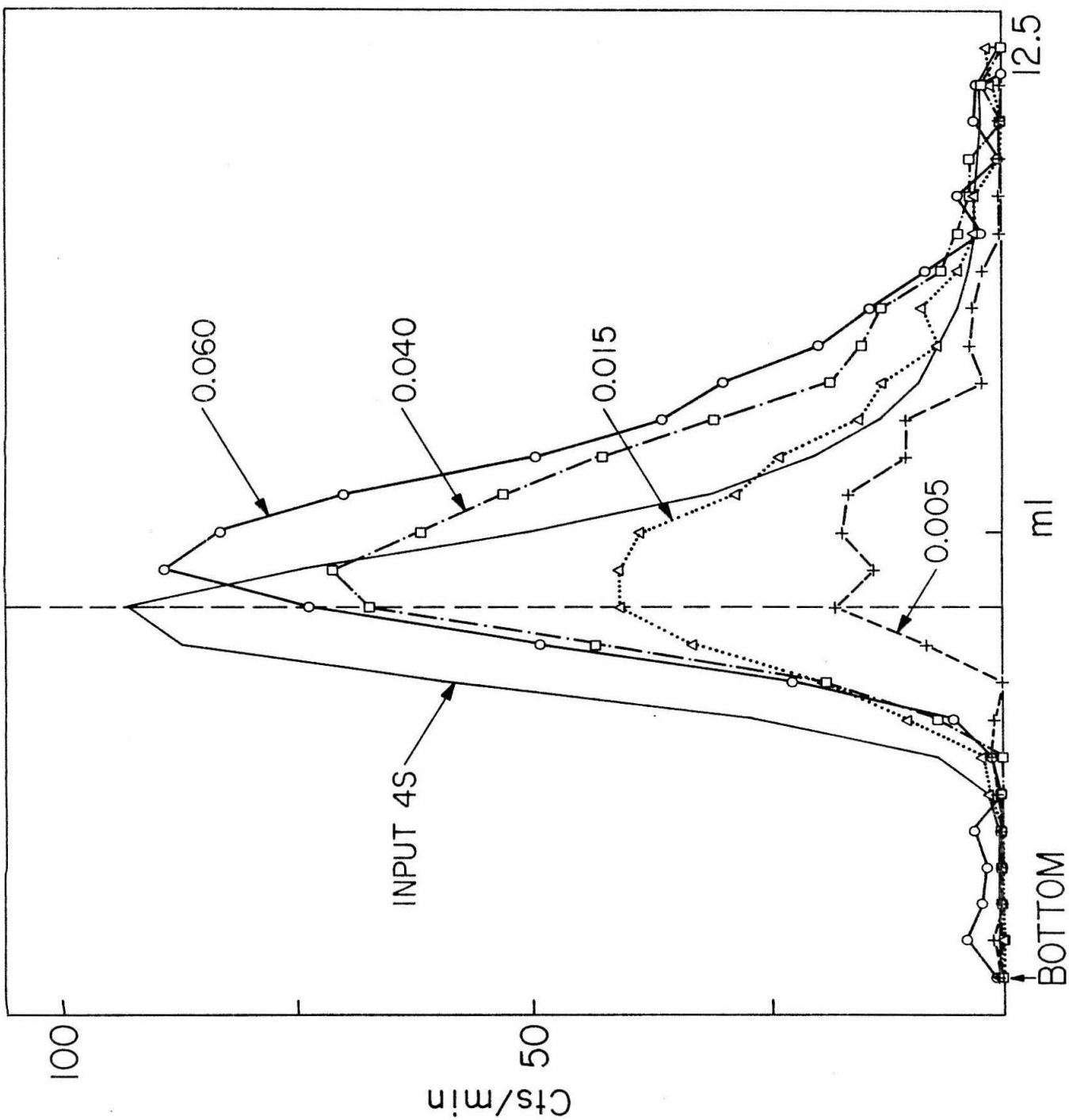

Fig. 4

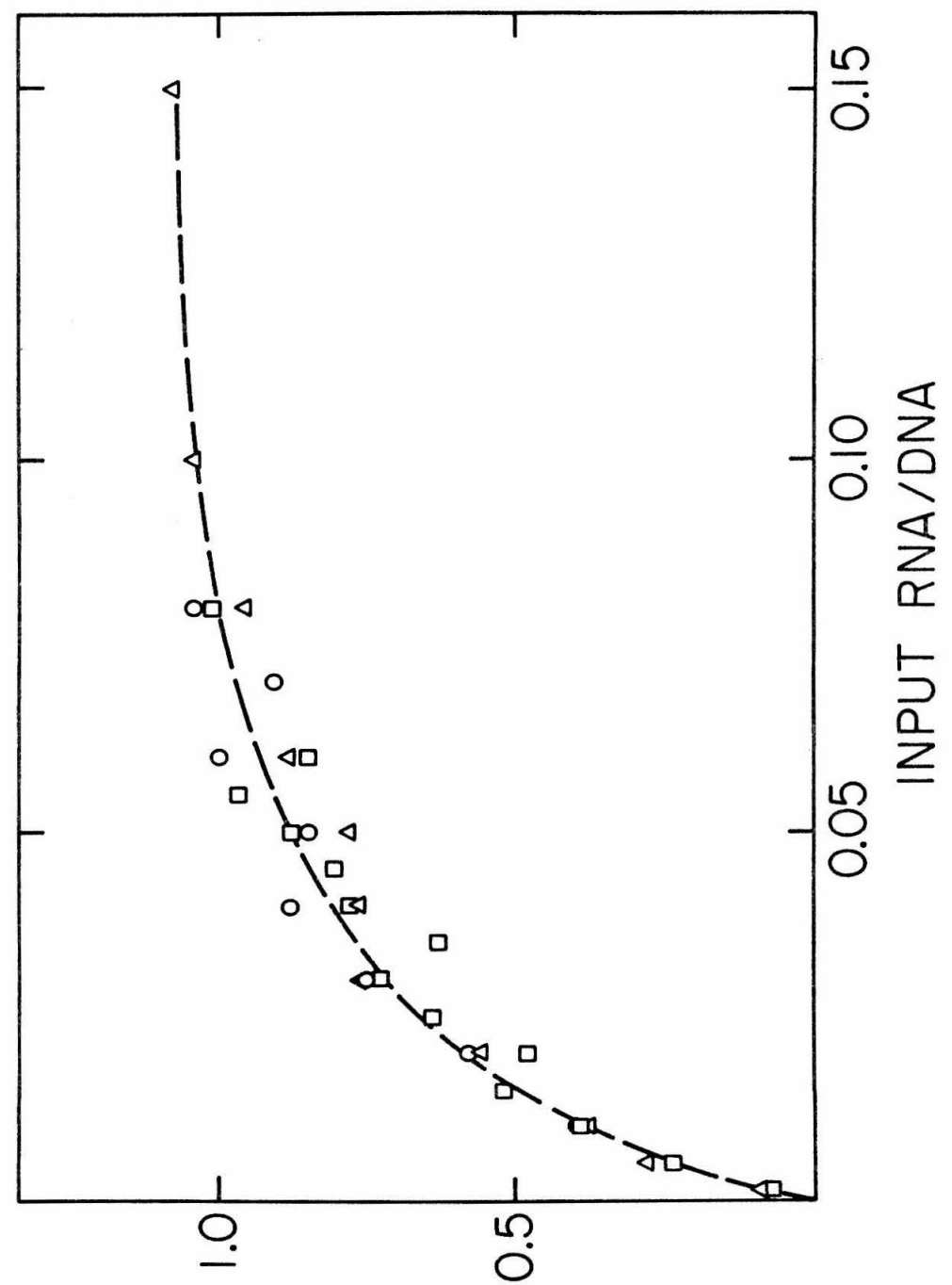

Fig. 5

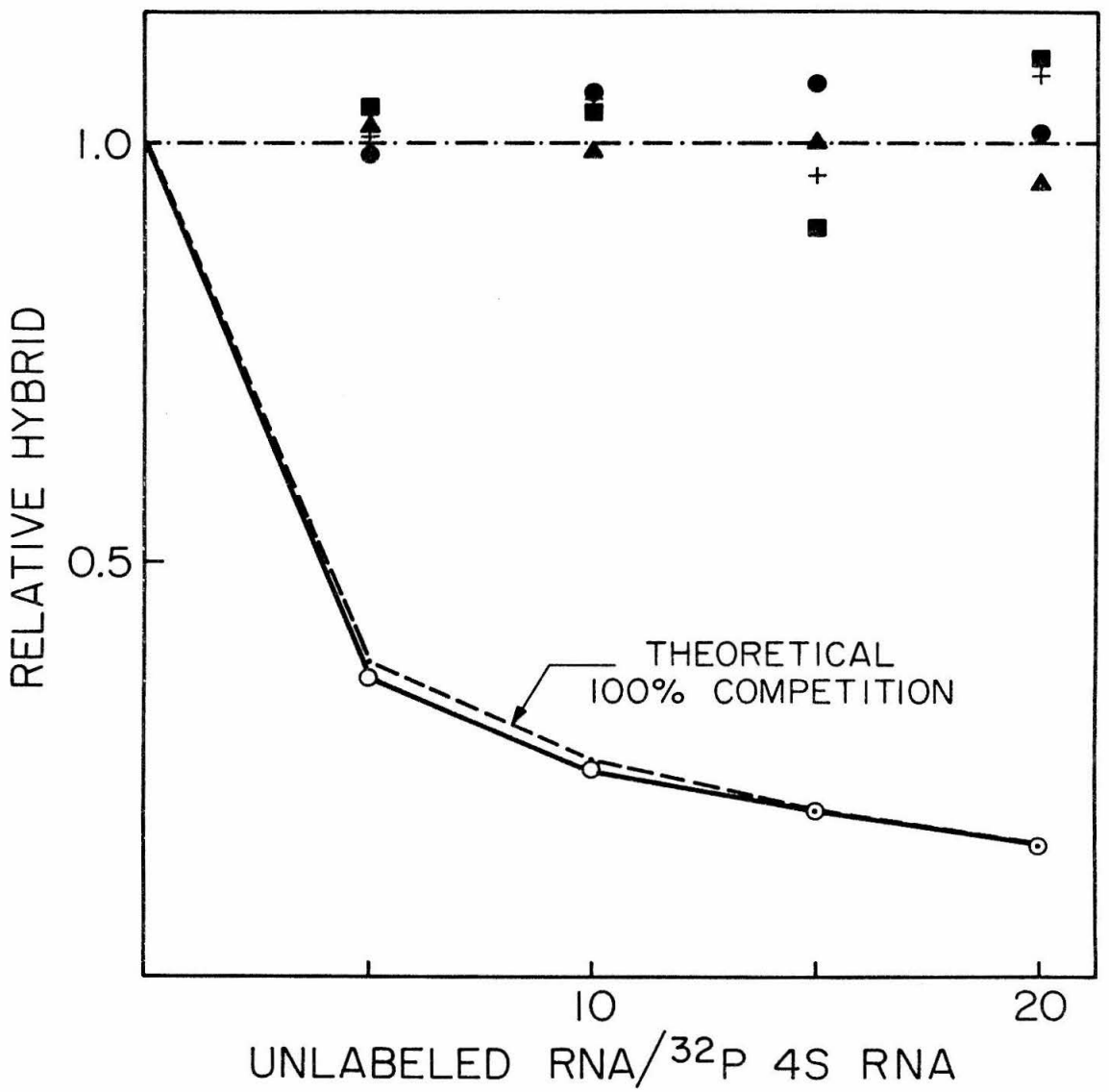
1.0
0.5
RELATIVE HYBRID
THEORETICAL
100% COMPETITION
10
20
UNLABELED RNA/^{32}P 4S RNA

Fig. 6

SECTION III

HYBRIDIZATION STUDIES WITH HeLa 5S RNA

SUMMARY

The proportion of the HeLa cell genome complementary to 5S RNA was investigated by using RNA-DNA hybridization. The 5S RNA was phenol-SDS extracted from the ribosome-polysome fraction of HeLa cells, and purified by Sephadex G-100 chromatography. These preparations were free from any significant contamination by DNA, mRNA, 4S, 18S or 28S RNA, as judged by a number of criteria. Analysis of size and nucleotide composition of the RNA recovered from RNase-treated RNA-DNA hybrids was used to assess the specificity of the hybrids studied.

The value for saturation of the HeLa DNA by 5S RNA was found to be 2.3×10^{-5}, which corresponds to about 7,000 sites for 5S RNA per HeLa cell in an exponentially growing culture. No competition was detected between HeLa 5S RNA and 4S, 18S or 28S RNA. Analysis of the nucleotide composition of the hybridized 5S RNA revealed no significant difference from the nucleotide composition of the input RNA. As determined from the S value in formaldehyde, the size of the hybridized 5S RNA was about two-thirds that of the input RNA at the RNA to DNA input ratio of 1:1000, and about half the size of the input RNA at the RNA to DNA input ratio of 1:50. The significance of these results is discussed.

1. INTRODUCTION

As in the case of the two major ribosomal RNA species, RNA-DNA hybridization studies have revealed the existence of a redundancy of information for 5S RNA in bacteria (about 4 genes in B. subtilis (Morell, Smith, Dubnau and Marmur, 1967), about 10 genes in E. coli (Zehavi-Willner and Comb, 1967)); a redundancy of information for 5S RNA several orders of magnitude higher has been found in Xenopus laevis (about 54,000 genes per diploid chromosome set (Brown and Weber, 1968). Previous work done in this laboratory has shown the existence of more than 1,000 sites for 45S RNA, the large size precursor of 18S and 28S RNA, in HeLa cells (Jeanteur and Attardi, 1969). In the present study the fraction of the HeLa cell genome complementary to 5S RNA has been investigated. It has been found that 5S RNA saturates HeLa DNA at a level which corresponds to about 7,000 5S RNA sites.

2. MATERIAL AND METHODS

(a) Cells and method of growth

For the method of growth of HeLa cells reference is made to a previous paper (Amaldi and Attardi, 1968).

(b) Labeling conditions

The labeling conditions are identical to those described for the preparation of 4S RNA in Section II.

(c) Buffers

The buffer designations are (1) SSC: 0.15 M NaCl, 0.015 M Na citrate; (2) dissociation medium: 0.01 M potassium phosphate buffer (pH 8.0), 2% formaldehyde, 0.1% SDS: (3) PNF: 0.02 M potassium phosphate buffer (pH 7.4), 0.1 M NaCl, 1% formaldehyde.

(d) Purification of RNA

Unlabeled or ^{32}P-labeled 5S RNA was extracted from the ribosome-polysome fraction (with the omission of deoxycholate treatment) and purified by Sephadex G-100 chromatography, as described in Section I. The Sephadex-purified 5S RNA preparation was subjected to a DNase treatment, in order to remove any possible DNA fragments (Section II).

Unlabeled HeLa 18S and 28S RNA were isolated from the ribosome-polysome pellet and purified by two cycles of sucrose gradient centrifugation, as described in Section II. Unlabeled HeLa 4S RNA was prepared from the 105,000 x g_{av} supernatant of a cytoplasmic extract, and purified on Sephadex G-100 (Section II).

(e) RNA-DNA hybridization and analysis of size and nucleotide composition of hybridized RNA

The hybridization experiments were carried out by incubating denatured DNA, at a concentration of 20 μg/ml, and varying amounts of RNA for 2 to 4 hours at 72°, unless otherwise specified. The reaction was stopped by quickly cooling down to 2°. Isolation and analysis of the hybrids was performed as described for 4S RNA in

Section II. Values for non-specific background were determined by incubating labeled 5S RNA with E. coli DNA under the same conditions used for the hybridization with HeLa DNA. At the input RNA to DNA ratio of 1:50, which is in the plateau region, the background corresponded to about 5% of the total radioactivity on the filter. All the hybridization values reported here have been corrected for the background determined for each point as described above.

(f) Quantitative analysis of RNA-DNA hybrids

The quantitative analysis of hybrids with 5S RNA was done in the same way as for 4S RNA (Section II).

3. RESULTS

(a) Purification of the 5S RNA

The purification of HeLa 5S RNA by two passages through Sephadex G-100 has been described in the paper on partial sequence analysis (Section I). In the latter study, a maximum contamination of 2% by 4S, 18S and 28S RNA together, and of 5% of mRNA was estimated. As discussed in Section I, sedimentation analyses performed on 5S RNA after denaturation by heat or formaldehyde showed the essential homogeneity in size and the intactness of molecules in the 5S preparation.

(b) Effect of time and temperature of incubation on RNA-DNA hybrid formation

The investigation of the optimum temperature of hybrid formation between 5S RNA and DNA was carried out at the input RNA to DNA ratio of 1:100. The same hybrid level was obtained after a 2 hour incubation at 70, 72 and 75°; in all subsequent experiments 72° was the temperature used. Figure 1 shows the hybrid levels found after various times of incubation at 72°, for an RNA to DNA ratio of 1:2000; it can be seen that even at this very low input RNA level, the hybridization is practically complete at 3 hours. (In contrast to this result, when 4S RNA was incubated at 72° at the much higher input RNA to DNA ratio of 1:300, it took about 4 hours for reaction to reach a maximum.) In all the experiments described below a 2 to 4 hour incubation was used.

(c) Analysis of size and nucleotide composition of RNA-DNA hybrids

HeLa 5S RNA, when subjected to the same heat and formaldehyde treatment as the hybridized RNA, sedimented on a 5 to 20% sucrose gradient in PNF as a single sharp peak, with a small amount of trailing material, and moving about 20% faster than a 4S RNA marker treated in the same fashion. These results indicated that no appreciable degradation of the RNA occurred as a result of the thermal treatment.

Sedimentation analysis on sucrose gradients in PNF buffer of the hybridized and dissociated RNA, at two different input RNA to DNA ratios (1:1000, which is at about half-saturation, and 1:50, which is in the plateau region), revealed a fairly symmetrical peak, with little trailing material, having an average sedimentation constant lower than the 3.7S expected for 5S RNA denatured by formaldehyde treatment (Boedtker, 1968). At the lower input RNA ratio, the average sedimentation constant was estimated to be 3.2S, with reference to an internal 4S RNA marker subjected to the same heat and formaldehyde treatment as the hybridized RNA, and assumed to have the sedimentation constant of 3S typical of formaldehyde treated 4S RNA (Boedtker, 1968); the value of 3.2S was calculated to correspond to a molecular weight of 27,000, and a chain length of 83 nucleotides (Boedtker, 1968). At the higher RNA to DNA ratio of 1:50, the average sedimentation constant of the hybridized RNA was lower, about 2.8S, corresponding to a molecular weight of 22,000 and a length of 67 nucleotides.

Preliminary experiments showed that, at the input ratio of 5S RNA to DNA of 1:100, which is already in the plateau region of the saturation curve (Fig. 2), the nucleotide composition of the hybrid RNA recovered from the nitrocellulose membrane was quite similar to that of the input RNA (Table I), a finding which strongly suggests that excessive RNase attack of the hybridized RNA had not occurred.

Figure 2 shows the composite DNA saturation curve obtained with HeLa 5S RNA in three different experiments. It appears that after incubation at 72° for 4 hours rather than for 2 hours, saturation of the DNA occurred at lower RNA input levels. It also appears from Figure 2 that the presence of saturating amounts of both 18S and 28S unlabeled HeLa RNA affected only slightly, if at all, the hybrid level.

(e) Competition experiments

Competition experiments performed with labeled HeLa 5S RNA and unlabeled HeLa 4S, 18S or 28S RNA suggested that the possible contribution to the hybrid by any of these species was less than 5% (Table II).

4. DISCUSSION

The results discussed in Section I indicated very little possible contamination of the Sephadex-purified 5S RNA by mRNA, 4S, 18S or 28S RNA. However, levels of contamination which are not detectable by structural studies could affect significantly the results of hybridization experiments. In the present work, the observation that 5S RNA saturated DNA at a level much lower than that found for 18S or 28S RNA (Jeanteur and Attardi, 1969) or expected for mRNA, strongly suggested that these RNA species were not playing any significant role in hybrid formation. Likewise, the results

of competition experiments indicated that the contribution to the hybrid by 4S, 18S or 28S RNA was very little, if any. Finally, the observation that the nucleotide composition of the hybridized RNA was quite similar to that of the input 5S RNA and totally different from that expected for mRNA, argued against any appreciable contribution of the latter species to hybrid formation. With respect to possible contamination of the 5S preparations by fragments of labeled DNA, such contamination was expected to be removed by the DNase treatment of the Sephadex-purified 5S RNA; this expectation was confirmed by the more than 99% sensitivity to alkali of the hybridized RNA.

Sedimentation analysis in a sucrose gradient of the hybridized and dissociated RNA revealed a distribution which, with respect to that of the input 5S RNA, was somewhat broader and displaced toward smaller size. The situation appears to be similar to that described for RNA recovered from hybrids involving 4S RNA (See Section II). In contrast to the latter hybrids, however, the nucleotide composition of the hybrids utilizing 5S RNA is quite similar to that of the input RNA. An explanation for the smaller size distribution of the hybridized and dissociated 5S RNA is suggested by the sequence studies, which have provided evidence for the existence of multiple forms of 5S RNA molecules, presumably different from each other in a few bases (Section I). In fact, any 5S molecule may be capable of hybridizing with any one of the many similar 5S sites in the DNA, forming extensive hydrogen bonds, except in the region(s) of variable

sequence. In such regions, mismatching may occur, creating RNase-sensitive spots; this would result in cleavage of the RNA chains at a limited number of sites. Another possibility, not mutually exclusive with the first, is that multiple occupancy of sites is occurring; this would be consistent with the reduction in average size of the hybridized and dissociated RNA observed at higher input RNA to DNA ratios.

The saturation of DNA at fairly low input levels of RNA which was found in the hybridization of HeLa 5S RNA with homologous DNA (Fig. 2) suggests that 5S RNA possesses only a limited degree of heterogeneity, in agreement with the sequence data (Forget and Weissman, 1967; Section I). In contrast to this result is the slow-rising saturation curve for HeLa 4S RNA (Section II), a system known to possess a large degree of heterogeneity. The fraction of DNA complementary to 5S RNA determined in the present work, i.e., about 2.3×10^{-5}, corresponds to about 7,000 sites for 5S RNA per HeLa cell in an exponentially growing culture. This finding is somewhat surprising if one considers that there are about 1400 sites for the 45S precursors of 18S and 28S rRNA (Jeanteur and Attardi, 1969), and that 5S RNA is present in ribosomes in equimolar amounts with respect to 18S and 28S RNA. A very large number of 5S sites (54,000 per diploid genome) has also been reported in Xenopus laevis, which has a similar number of rRNA genes (about 1,000) as HeLa cells. The large difference in number of sites for

5S RNA and 45S RNA suggests that the 5S RNA sites are transcribed at a reduced rate relative to the 45S sites, or that most of the 5S RNA sites are not being transcribed at all in exponentially growing HeLa cells, or that all the sites are being transcribed at the same rate as the 45S sites, but that much of the transcribed 5S RNA is being degraded.

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Zehavi-Willner, T. and Comb, D. G. (1966), J. Mol. Biol. 16, 250.

TABLE I

^{32}P-Nucleotide composition of input RNA and of hybridized RNA recovered from nitrocellulose membranes

RNA analyzed	Mole %				%GC	Pu/Py
	C_p	A_p	U_p	G_p		
Unhybridized input RNA[a]	28.7	18.8	22.9	29.7	58.4	0.93
Hybridized RNA (0.01 RNA/DNA)	29.3	18.7	23.3	28.8	58.1	0.90

Incubation mixture containing 100 µg HeLa DNA and 1.0 µg HeLa ^{32}P-5S RNA in a total volume of 5.0 ml 2 x SSC was incubated 2 hours at 72°; the RNA-DNA hybrid was isolated, and the RNA dissociated from the filter as described in Section II. After 16 hours incubation at 30° in 0.5 N NaOH, in the presence of alkali-digested yeast RNA carrier, the sample was chromatographed on Dowex 1-X8 as previously described (Attardi et al., 1966). Fractions from the Dowex 1-X8 chromatography were plated on planchets, and counted in a low background gas flow counter.

(a) Hatlen, Amaldi and Attardi (1969), Section I.

TABLE II

Relative hybrid formation between ^{32}P-5S RNA and DNA in the presence of unlabeled heterologous RNA species

Unlabeled RNA	Ratio of unlabeled RNA to ^{32}P-5S RNA	Relative hybrid level
HeLa 4S	5:1	102
	10:1	86
	20:1	97
HeLa 18S	5:1	92
	10:1	95
	20:1	98
HeLa 28S	5:1	102
	10:1	90
	20:1	96

Incubation mixtures containing 50 μg HeLa DNA, 0.50 μg HeLa ^{32}P-5S RNA, and varying amounts (2.5 to 10 μg) unlabeled 4S, 18S or 28S RNA in 2.5 ml 2 x SSC were incubated 2 hours at 72°; the hybrids were isolated and analyzed as described in Materials and Methods (g,h), Section II.

FIGURE LEGENDS

Fig. 1. Effect of time of incubation on ^{32}P-5S RNA-HeLa DNA hybrid formation.

Incubation mixtures containing 50 μg HeLa DNA and 0.025 μg HeLa ^{32}P-5S RNA in 2.5 ml of 2 x SSC were incubated at 72°. At various intervals, duplicate tubes were removed, quickly cooled, digested with 10 μg/ml pancreatic RNase for 1 hour at 22°. One-ninth volume of 2.5 M KCl, 0.05 M tris buffer (pH 7.4) was added, and the samples were filtered and washed as described in Section II. (Plot of duplicate experiments.)

Fig. 2. Composite saturation curve of HeLa DNA by HeLa ^{32}P-5S RNA in various experiments.

Incubation mixtures containing 20 μg/ml HeLa DNA and various concentrations of HeLa ^{32}P-5S RNA were incubated at 72° in 2 x SSC. After RNase treatment (10 μg/ml, 1 hour at 22°) the hybrids were isolated as described in Section II. In one experiment (■) hybridization was performed for 4 hours, rather than 2 hours (o). In another experiment, (Δ) hybridization was carried out for 2 hours, in the presence of saturating amounts of unlabeled HeLa rRNA (1.0 μg/ml 28S RNA, 0.40 μg/ml 18S RNA).

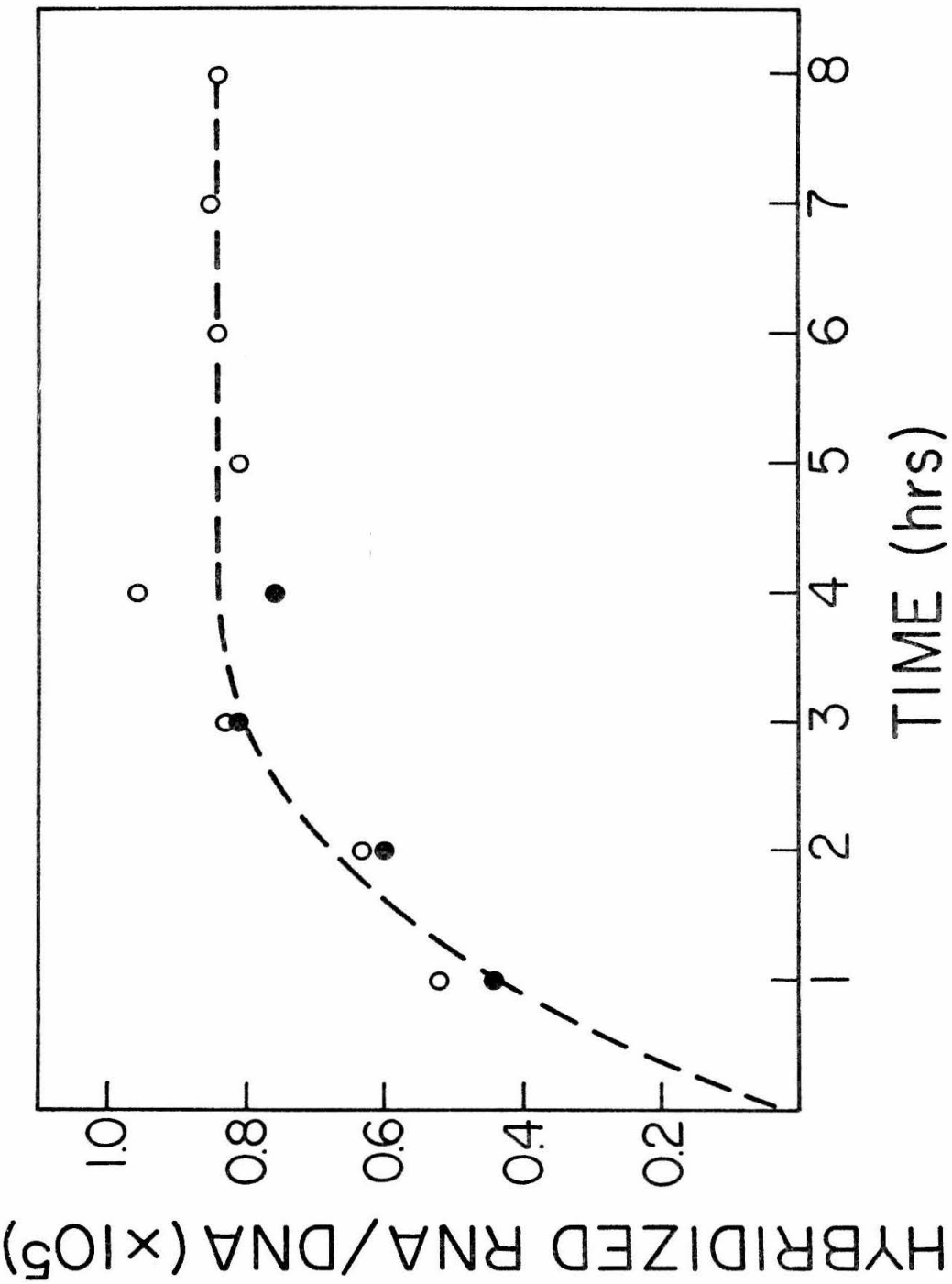

Fig. 1

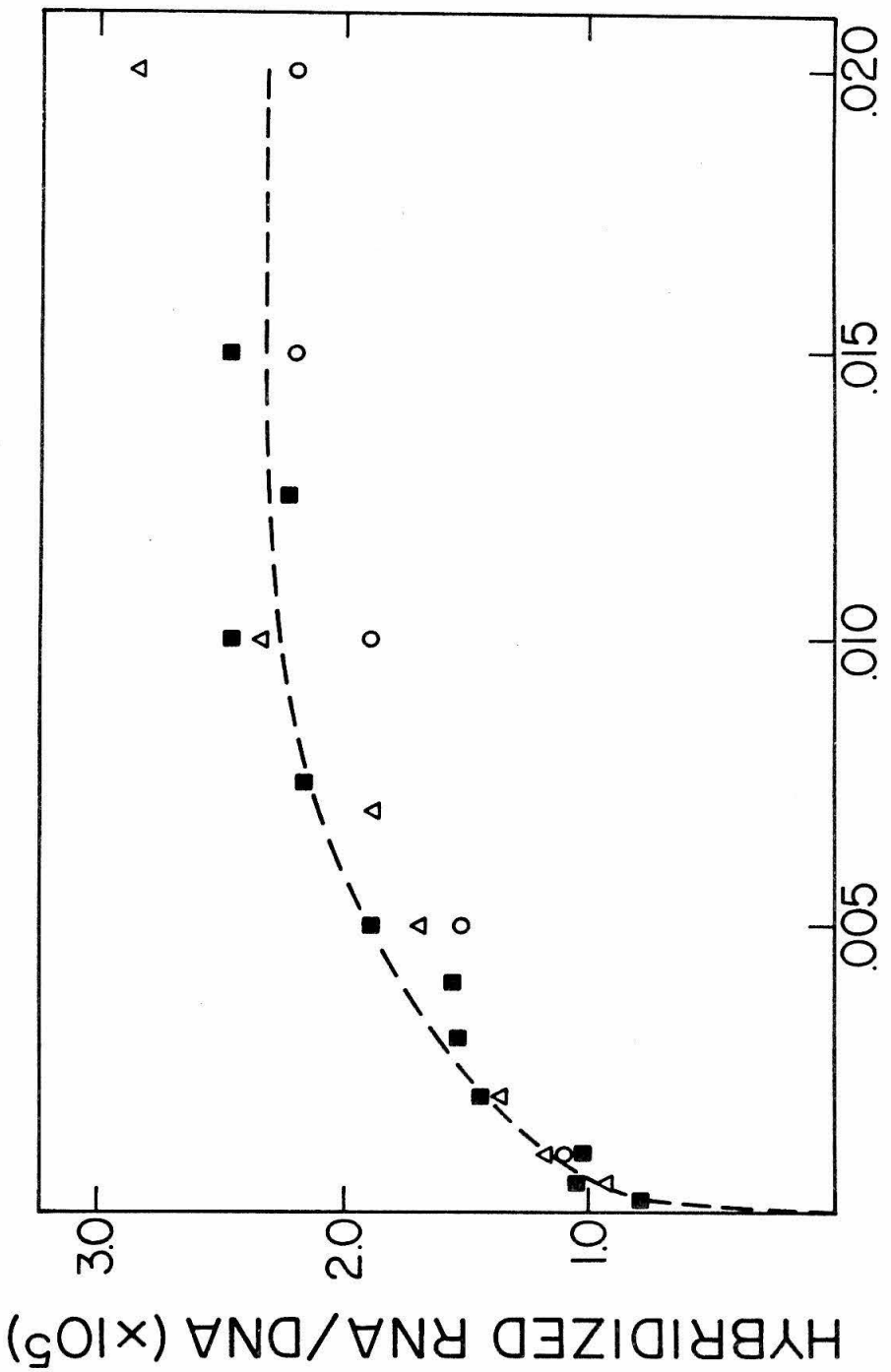

Fig. 2