IN VITRO SYNTHESIS OF SOLUBLE RIBONUCLEIC ACID

A THESIS

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ABSTRACT

Preliminary observations were made on the biosynthesis of soluble RNA (sRNA) in the mouse Ehrlich ascites tumor cell. The incorporation of uridine-2-^{14}C into sRNA in the intact cells and in an "intact nuclear preparation" has been demonstrated. Actinomycin D inhibited the incorporation of uridine-2-^{14}C into sRNA in both intact cells and in the "intact nuclear preparation" DNAase inhibited the incorporation of uridine-2-^{14}C into the nuclear sRNA of the "intact nuclear preparation". The nucleotide composition of cytoplasmic acceptor RNA (purified on an MAK column) was found to be almost the same as that of the nuclear sRNA.

DNA-dependent RNA polymerase was prepared following the method of Chamberlin and Berg (7S). Dr. E.S. McFarlane prepared the DNA primer specific for sRNA synthesis from DNA-sRNA hybrids. With this purified RNA polymerase and the DNA primer specific for sRNA biosynthesis the incorporation of UTP-2-^{14}C into acid insoluble material was demonstrated. This incorporation was inhibited by actinomycin D and DNAase. The acid insoluble product of this reaction was shown to be unstable in alkali and sensitive to pancreatic ribonuclease indicating that this product is "RNA-like". Chromatography on an MAK column of this "RNA-like" product indicated that it was similar to acceptor RNA. Many attempts were made to demonstrate amino acid binding capacity of this "RNA-like" product. Although no definite demonstration of this capacity was obtained it is felt that such a demonstration is now possible with certain adjustments to the system.

The activities of the amino acid activating enzymes, the nucleotide incorporating enzymes and inorganic pyrophosphatase were studied in the "pH 5 enzymes" fraction, the 105,000 x g supernatant fraction, in protamine sulfate treated samples of these two fractions and in "pH 5 enzymes" fraction chromatographed on DEAE-cellulose.

Pancreatic ribonuclease was removed from purified commercial pancreatic DNAase by filtration on a G-50 Sephadex column.

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INTRODUCTION

The Role of sRNA in Protein Biosynthesis

In 1957 Hoagland (1) first demonstrated a covalent association of a low molecular weight RNA (soluble RNA or sRNA) with amino acids in the so-called "pH 5 enzymes" fraction prepared from 105,000 x g supernatant of rat liver homogenate. Formation of this linkage required ATP and was catalyzed by amino acid activating enzymes. These enzymes were known to activate the carboxyl groups of amino acids in the presence of ATP. The amino acyl-RNA subsequently transferred its amino acid to ribosomal protein in the presence of GTP and a nucleoside triphosphate generating system (1). These two functions have given more precise names to this RNA, namely "amino acid acceptor RNA" and "transfer RNA".

The synthesis of proteins appears to proceed in three steps:

(i) Activation of the amino acid

$$\text{Activating Enzyme} + \text{amino acid (aa)} + \text{ATP} \underset{}{\overset{Mg^{++}}{\rightleftharpoons}} \text{Activating Enzyme-(aa~AMP)} + PP_i$$

(ii) Reaction of activated amino acid with sRNA

$$\text{Activating Enzyme-(aa~AMP)} + \text{sRNA} \overset{Mg^{++}}{\rightleftharpoons} \underbrace{\text{sRNA~aa}}_{\text{amino acyl-RNA}} + \text{AMP} + \text{Activating Enzyme}$$

(iii) Transfer of amino acid from amino acyl-RNA into ribosomal protein

$$(n)\text{sRNA~aa} + \text{polyribosomes} \xrightarrow[2 \text{ enzymes}]{Mg^{++}\ GTP} \text{polyribosome-(sRNA~polypeptide)} + (n-1)\text{sRNA}.$$

There is a great deal of experimental evidence for the first two steps involving the activating enzymes. Tracer studies using labelled amino acids have demonstrated the formation of an enzyme-bound adenylate intermediate, its reaction with sRNA, and the transfer of the activated amino acid into peptide linkages of ribosomal protein (1-7). The

enzymes which catalyze amino acyl-RNA formation also catalyze amino acid-dependent ATP-^{32}PPi exchange (1, 3, 5, 7, 8), ATP-dependent formation of amino acid hydroxamates (9-13) and amino acid- and RNA-dependent exchange of ^{14}C-AMP with ATP (2). Activating enzymes specific for one amino acid have been purified (14-17). Amino acid adenylates have been detected in the presence of large amounts of purified activating enzymes (18-20) and have been chemically synthesized.

The third step is not yet fully understood. It is believed that messenger RNA (21) carries the information necessary to direct protein synthesis. This information is contained in specific sequences of nucleotides which correspond to specific sequences of amino acids in protein molecules. Messenger RNA attaches itself to the ribosomes to form polyribosomes. Here the "message" is translated into the amino acid sequence in the protein molecule. Each of the 20 amino acids is bound to its own specific sRNA which has a particular sequence of three bases that hydrogen bond with three complementary bases (the "codon") in the messenger RNA. Thus the various amino acyl-RNA molecules line up along the template messenger RNA in the sequence in which the amino acids occur in the polypeptide chain.

Peptide bond formation begins at the N-terminal end of the polypeptide (22). The transfer of the amino acids from amino acyl-RNA requires GTP and at least two transfer enzymes (23). One of these has GTPase activity and appears to catalyze the binding of the amino acyl-RNAs to the ribosomes (24). The other enzyme probably catalyses peptide bond formation (23). As each peptide bond is formed one free

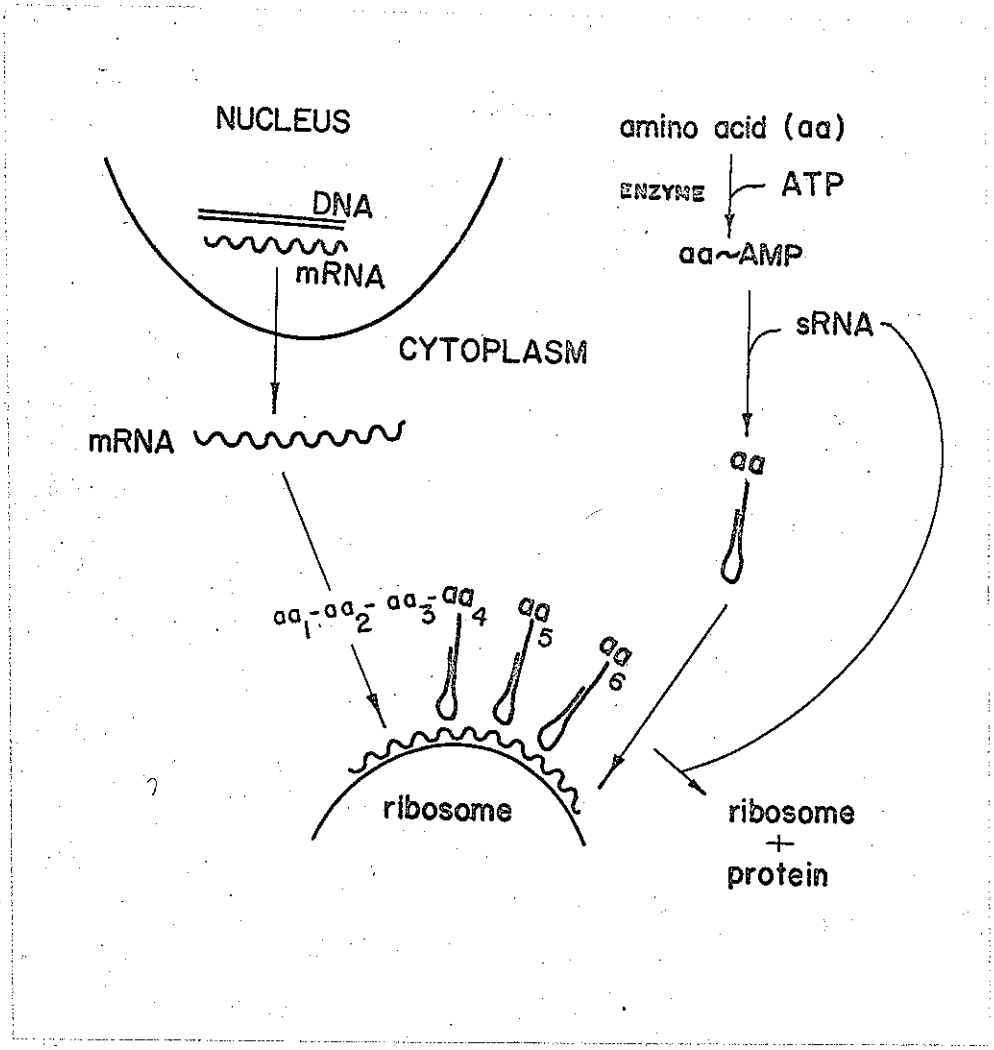


Figure 1. Diagrammatic representation of the mechanism of protein synthesis.

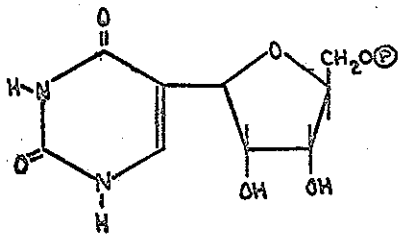
sRNA molecule is released as the elongating peptide chain is transferred to the incoming amino acyl-RNA (21). Thus the growing chain of amino acids is apparently held by sRNA to the ribosome until the last amino acid is in place. See Fig. 1 for a diagrammatic representation of protein synthesis.

Physical and Chemical Properties of sRNA

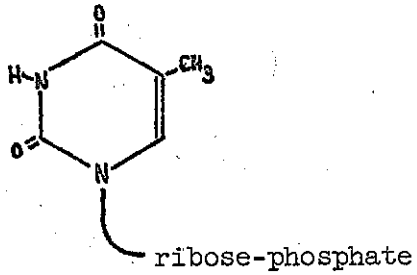
sRNA is a single-stranded polynucleotide since only one nucleoside diphosphate and only one nucleoside are released per mole on alkaline digestion (25-29). It is homogeneous in the ultracentrifuge (30) and has a molecular weight of 24000 ± 1000 which corresponds to an average chain length of 70 nucleotides (30-33). sRNAs from all sources have a near equivalence of complementary bases which suggests base-pairing as occurs in DNA (34, 35). Evidence for a secondary structure is given by studies of the hyperchromic effect, the amount of which indicates that 50-75% or more of the bases in the nucleotide chain are paired (33, 36-39). Luborsky and Cantoni (30) using ultracentrifugation procedures and viscosity measurements showed that sRNA is asymmetrical with an axial ratio of 9:1, thus a rigid rod shape similar to DNA. Nihei and Cantoni (40) found that when snake venom phosphodiesterase acted on sRNA the maximum hyperchromic effect was obtained when only 40-50% of the chain had been digested, while, on the other hand, alkali treatment resulted in an increase in optical density parallel with the degree of hydrolysis. This provides strong evidence for the existence of one long base paired region. Another

indication of secondary structure is that sRNA in dilute solution with Mg^{++} is resistant to pancreatic ribonuclease (41-44).

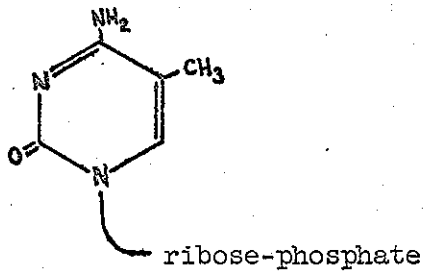
All active amino acid-acceptor RNA chains contain the identical sequence ....pCpCpA at the acceptor end (25, 27, 45, 46) and a pOp at the other end (29). However the internal nucleotide sequences are not all the same. Berg et al (47) has shown heterogeneity in the nucleotide sequence adjacent to the ....pCpCpA end, and Cantoni et al (48) from analysis of T_1 RNAase and pancreatic RNAase digests of sRNA, concluded that the base sequence of sRNA was non-random. This implies that sRNA from any given source is a mixture of more than one kind of molecule, each of which probably has a specific sequence of nucleotides. That each amino acid has its own acceptor RNA is a prerequisite of the adaptor role which sRNA plays in the arranging of amino acids in protein. In keeping with this hypothesis, the attachment of an amino acid to sRNA is independent of the attachment of other amino acids (1). Specific sRNAs have been partially purified by chemical and/or chromatographic procedures. Valine acceptor RNA has been purified more than 90% by a combination of these methods (49). However countercurrent distribution appears to be the best general method of the future. Holley et al (50) have purified alanine-, tyrosine-, valine-, and also leucine-specific RNAs (51) by countercurrent distribution and have found two leucine- (51) and two tyrosine- specific RNAs (50). The finding of more than one sRNA specific for a given amino acid supports the hypothesis that the code is "degenerate". This could mean that there is a different acceptor RNA corresponding to every possible nucleotide triplet. That



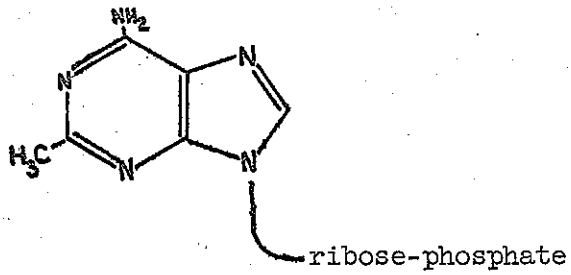
pseudouridylic acid



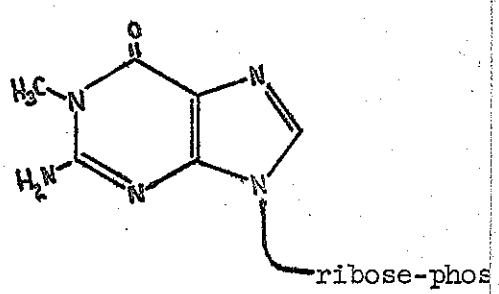
ribothymidylic acid



5-methyl cytidylic acid



2-methyl adenylic acid



1-methyl guanylic acid

Figure 3. "Odd" nucleotides present in sRNA.

Note: The following methylated bases as well as the five examples above have been found in sRNA of different sources: 6-methylamino-purine (123, 125), 6-dimethylamino-purine (124, 125), 6-amino-1-methyl-purine (125), 6-hydroxyl-2-methylamino-purine (123-125), 6-hydroxyl-2-dimethylamino-purine (123-125) and 6-hydroxyl-1-methyl-purine (126).

is 4 x 4 x 4 or 64 different acceptor RNAs (52).

Only one amino acid is bound per sRNA molecule (34, 53). It is linked through an ester bond to the 2' or 3' hydroxyl group of the ribose in the terminal adenylate (34, 53). Studies of the inhibition of protein synthesis by puromycin and its 2' analogue indicate that the amino acid is probably attached to the 3' position of the ribose. Puromycin closely resembles an amino acyl-adenylate (Fig. 2) and inhibits protein synthesis by binding to the C-terminal end of the nascent protein in place of the sRNA (54) and thereby prevents elongation of the peptide chain. The puromycin does not have a polynucleotide chain to bind with the ribosome and hence the polypeptide chain dissociates from the ribosome (55, 56). Since the 2' analogue does not inhibit, the enzyme responsible for linking the polypeptide chain to sRNA is probably specific for an amino acid at the 3' position.

A very unique feature of sRNA is the relatively high content of "odd" nucleotides that are not found in other types of RNA (57). Pseodouridylic acid, ribothymidylate and other methylated purines and pyrimidines are present (Fig. 3). Although in unfractionated sRNA there is an average of three residues of pseodouridylate and one residue of ribothymidylate per molecule of sRNA (57) there is not enough of the other methylated bases to account for even one residue per molecule (57). Different sRNA molecules must contain different methylated bases. This has been shown to be the case (50, 58).

Sequence studies on unfractionated sRNA indicate that the "odd" nucleotides may occur concentrated near the middle of the polynucleotide chain (59-61). This would be in the "loop" region

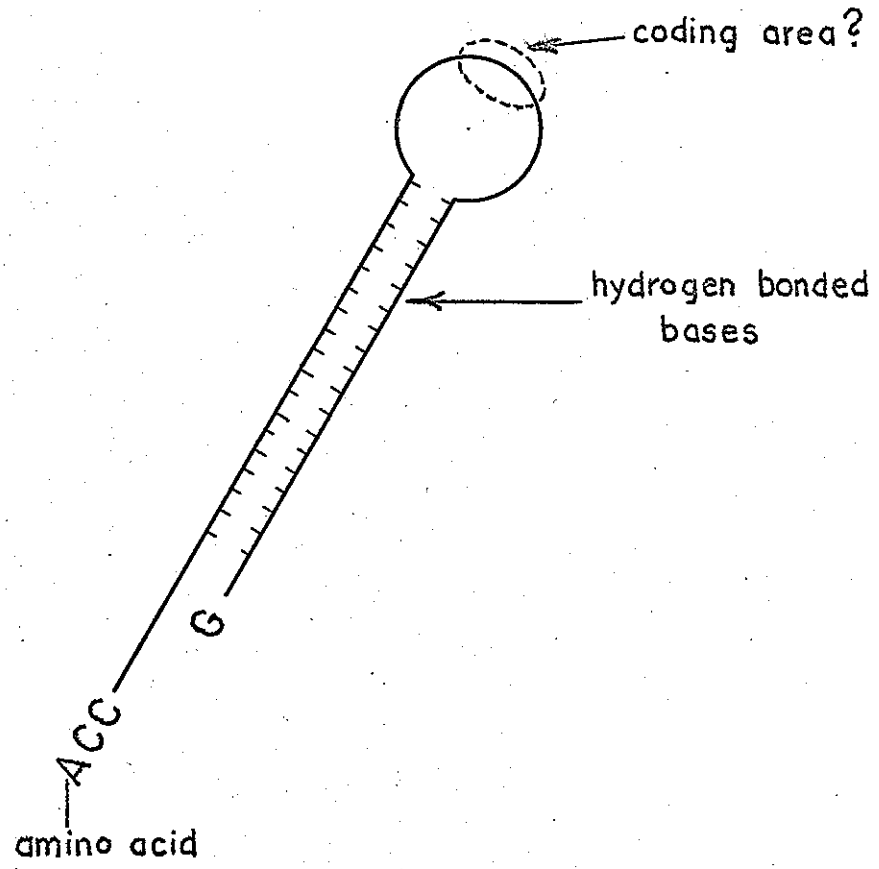


Figure 4. Diagrammatic representation of the sRNA molecule.

of unpaired bases (Figure 4). Cantoni has confirmed this and now claims that the loop region of serine acceptor RNA contains a trinucleotide sequence which is complementary to the code word for serine and further that this region is bounded by pseudouridylic acid residues (62). Miura (63) found that unfractionated yeast transfer RNA contained a small fraction having the sequence of pApApA and that this sequence appeared to be bounded by uridylic acid and/or its homologues (Up, ψp, Tp). This could be the coding area of the phenylalanine sRNA. If the methylated bases are present in the "loop" region they may facilitate the attachment of sRNA to messenger RNA (64). In support of this hypothesis Littauer (65) has demonstrated that "methyl poor" sRNA from a methionine starved methionineless mutant of E. coli will function as efficiently as sRNA from wild type E. coli as acceptor RNA but less efficiently as transfer RNA.

It may be noted that Holley et al (66) find that the methylated bases are not concentrated near the centre of the yeast alanine acceptor RNA molecule.

Littauer (65) also found that the "methyl poor" sRNA is twice as susceptible to the E. coli K^+-activated diesterase as is wild-type sRNA. Thus methylation may be important to the conservation of sRNA in vivo.

In addition to the large group of minor nucleosides in which methyl groups are attached to the heterocyclic base, another group of minor RNA nucleosides exists in which the methyl groups are attached to the 2-O position of the ribose. Hall (67) has isolated these 2'-O-methylribonucleosides from sRNA and from ribosomal RNA. Twelve of

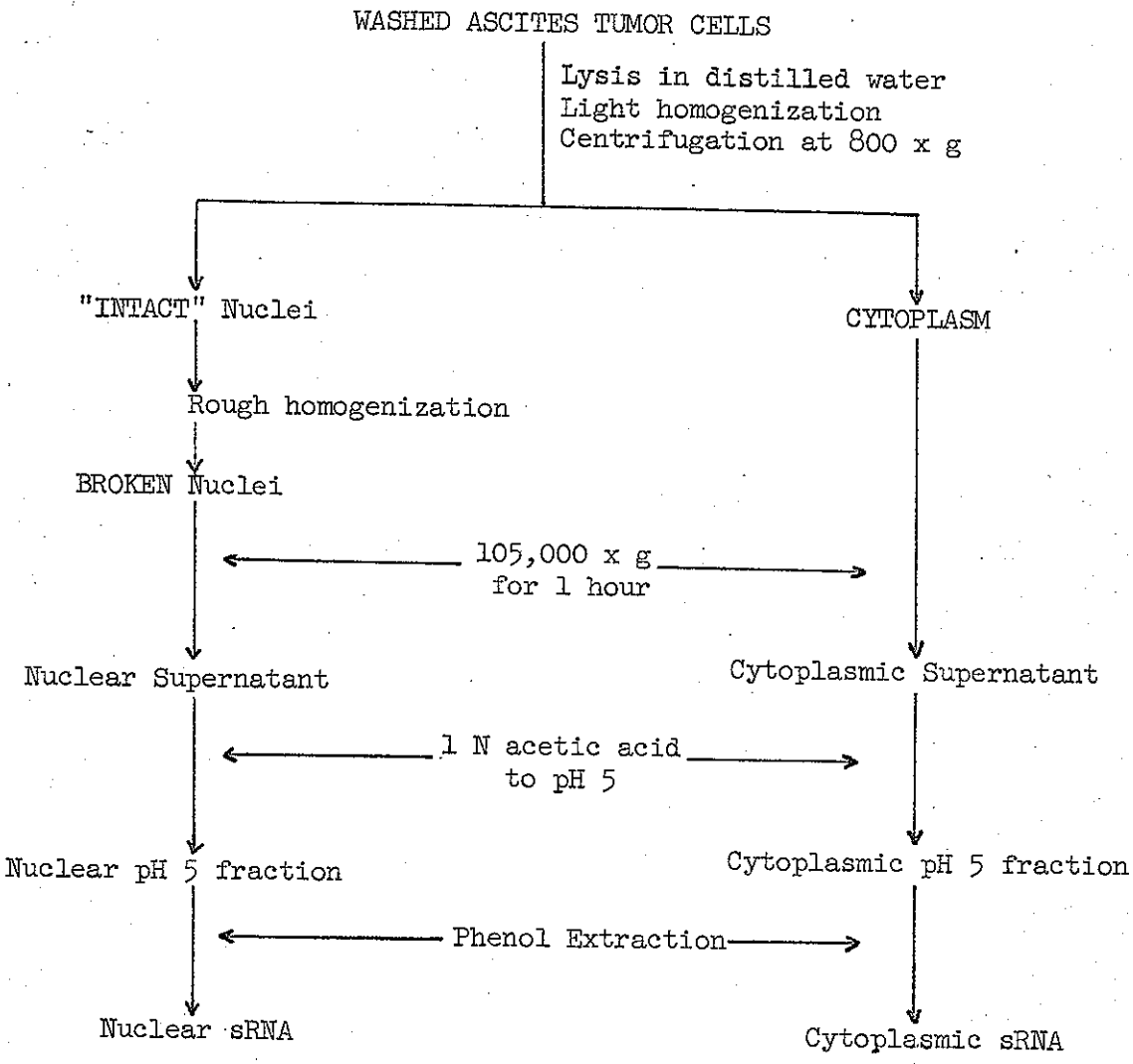


Figure 5. Fractionation scheme employed in preparation of cell fractions of ascites tumor cells.

thirteen samples from yeast, bacterial and mammalian systems yielded about 1% of their ribonucleoside content as methylribonucleosides. As a result of the presence of a methyl group at the 2' position, pancreatic ribonuclease cannot act at the 3' position of the methylribonucleotide. Also the physical properties of a molecule may be altered. At present however the function of these methylribonucleotides is unknown.

Enzymatic Synthesis of RNA

Four distinct enzymatic incorporations of nucleoside monophosphates into RNA have been reported: (i) nucleotide incorporating enzyme, (ii) polynucleotide phosphorylase, (iii) RNA-dependent RNA polymerase and (iv) DNA-dependent RNA polymerase.

Nucleotide incorporating enzyme (33, 68) reversibly catalyses the incorporation of the two CMPs and AMP of the ....pCpCpA end of amino acid acceptor RNA. It utilizes CTP and ATP for this incorporation. In the reverse reaction in the presence of a high concentration of PPi it acts as a pyrophosphorylase (27). Its apparent role in vivo is to restore the terminal end of amino acid acceptor RNA to its functional form (69, 70).

Polynucleotide phosphorylase (71) catalyses the formation of high molecular weight polynucleotides from ribonucleoside diphosphates releasing orthophosphate. It has been found in plant, animal and bacterial cells. Homopolymers, poly A, poly C, poly U, poly I, etc., copolymers and mixed polymers have been synthesized with this enzyme. The composition of the polymers reflects the concentrations of the nucleoside diphosphates used for the synthesis. There is evidence that

this incorporation is a random process (71). The role of this enzyme *in vivo*, however, is likely catabolic since the concentration of nucleoside diphosphates required for the synthetic process is probably never attained.

RNA-dependent RNA polymerase is responsible for viral RNA replication. This replication has been demonstrated to be independent of DNA (72-74). The viral RNA itself serves as a template for the formation of the "replicase" and is conserved in both translation and replication (73). The RNA-polymerase has been isolated and purified from MSϕ2 phage-infected *E. coli* Hfr. It has been found to require RNA primer and all four nucleoside triphosphates. It is partially active with other viral RNA but completely inactive with host sRNA and ribosomal RNA (73). No RNA-dependent RNA polymerase has been reported in cells uninfected by RNA viruses.

DNA-dependent RNA polymerase has been partially purified from plant, animal and bacterial sources (75-79). The reaction catalyzed by this enzyme is:

$$n_1ATP + n_2GTP + n_3CTP + n_4UTP \xrightarrow[\text{DNA Primer}]{Mg^{++} \text{ or } Mn^{++}}$$

$$(AMP_{n_1}\text{-}GMP_{n_2}\text{-}CMP_{n_3}\text{-}UMP_{n_4}) + (n_1 + n_2 + n_3 + n_4)PPi$$

It is completely dependent upon and specific for all four nucleoside triphosphates, a divalent cation and DNA primer (82). However some reports indicate that RNA can prime the reaction (80, 81). The reaction is reversible (82). The primer can be either native DNA, or heat-denatured (single-stranded) DNA, or small oligodeoxynucleotide

fragments (82). The primer acts catalytically and is not altered during the reaction (83). Most important the RNA synthesized appears to be a copy of the DNA used as primer both in composition (78, 83-85) and sequence (84, 85). In vitro the enzyme copies both strands of DNA (78), although probably only one strand is copied in vivo (86, 87). In vitro, if single-stranded DNA is used, DNA-RNA hybrids are formed (88), whereas double-stranded primer is copied conservatively (88).

On the basis of DNA-RNA hybrid formation experiments (see below) it appears that messenger RNA, ribosomal RNA and soluble RNA are all copied from the DNA of the cell (42, 89, 90). Thus the DNA-dependent RNA polymerase probably catalyses this copying.

Biosynthesis of sRNA

Since each type of sRNA is apparently a highly specific macromolecule the most likely mechanism for its biosynthesis would involve a "template". This template would presumably be either DNA, upon which messenger RNA is made, or RNA, such as is involved in viral replication. In the first case the sRNA might be considered a special kind of "messenger" RNA. Brown and Zubay (91) postulated that RNA is the template for sRNA biosynthesis and McCully and Cantoni postulate that sRNA serves as its own template (92). However, an increasing body of evidence indicates that DNA is involved.

Giacomoni and Spiegelman (42), Goodman and Rich (89) and McFarlane and Fraser (90) have demonstrated that sRNA forms specific DNA-sRNA hybrids when annealed with homologous, heat-denatured DNA. No hybrids are formed with heterologous DNA. The DNA is saturated at concentrations of sRNA which indicate that only a small portion (0·02-0·03%) of the DNA is complementary to the sRNA molecule. Thus

there are regions in the homologous DNA that are complementary to sRNA and these regions are restricted to a very small portion of the DNA genome. This is consistent with the idea that DNA is the template for sRNA synthesis.

Other types of evidence are available. The incorporation of ^{32}P orthophosphate into sRNA of the nucleus of Hela cells is partially sensitive to actinomycin (93). Actinomycin is known to inhibit DNA-dependent RNA synthesis *in vivo* and *in vitro* with mammalian and bacterial systems, but does not affect the replication of RNA viruses (94). Kirk (95) has shown that actinomycin and DNA combine chemically resulting in a spectral change of the actinomycin, while actinomycin and RNA do not interact. Goldberg *et al* (94) demonstrated that the binding was absolutely dependent on guanine residues in the DNA. Thus sensitivity of sRNA synthesis to actinomycin also indicates a DNA template.

Perry (96) studied the effect of actinomycin on RNA synthesis in tissue cultures of L-strain mouse fibroblasts with autoradiographic techniques. He demonstrated that 4S RNA (one may assume that a significant portion of this is sRNA) is first synthesized in the chromatin region of the nucleus and later appears in the cytoplasm.

Chipchase and Birnstiel (97) have recently shown that the incorporation of tritiated uridine into nuclear sRNA of isolated pea nuclei is sensitive to DNAase and actinomycin. This also indicates that sRNA is made on a DNA template in the nucleus.

Considerable evidence has accumulated which indicates that the "odd" nucleotides found in sRNA result from modifications of a "precursor polyribonucleotide" which is synthesized with the four

unmodified bases, A, G, U and C. Certain of these bases are subsequently methylated by specific enzymes (98-100). Robbins et al (101) have obtained some evidence that the pseudouridylic acid residues of sRNA likewise originate at the polyribonucleotide level. If these interpretations are correct then the problem of sRNA biosynthesis becomes a problem of the biosynthesis of "precursor polyribonucleotide".

It seems likely that "precursor polyribonucleotide" and "messenger" RNA are synthesized by similar mechanisms. Hence the *in vitro* synthesis of sRNA might be possible with a DNA template specific for sRNA formation, prepared from DNA-sRNA hybrid material, and DNA-dependent RNA polymerase purified by the method of Chamberlin and Berg (88). The present work was undertaken to investigate this possibility.

MATERIALS AND METHODS

Radioisotopes

Uridine-2-^{14}C was obtained from the New England Nuclear Corporation and was made up in distilled water. The preparation used had a specific activity of 123 μc/mg; 1 c.p.m. was equivalent to 56·5 mμ moles of uridine (automatic counter, see below). UTP-2-^{14}C was obtained from Schwarz Bioresearch Inc., Orangeburg, N.Y., 50 μc in 5 ml of aqueous 50% ethanol. This preparation had a specific activity of 27 mc/mMole; 1 c.p.m. was equivalent to 72·0 mμ moles of UTP-2-^{14}C with the automatic counter and equivalent to 44·6 mμ moles of UTP-2-^{14}C with the manual counter (see below). The solution was used undiluted. ^{14}C-algal hydrolysate was obtained from Merck and Co. Limited, Merck Radiochemicals for Research and Industry. It was dissolved in distilled water. The specific activity of the preparation was 0·1 mc/0·77 mg; 1 c.p.m. was equivalent to 120 mμ moles of amino acids on the automatic counter and 74·1 mμ moles on the manual counter, assuming that the average molecular weight of the constituent amino acids was 125.

All preparations were stored at -15°C.

Chemicals

Nucleoside triphosphates, Tris buffer (Trizma Base) and Protamine sulfate (salmine) were obtained from Sigma Chemical Company, 3500 DeKalb St., St. Louis 18, Missouri, U.S.A. Actinomycin D was obtained from Merck Sharpe and Dohme of Canada Ltd; glass "Superbrite 100" beads from Minnesota Mining and Manufacturing Company; DEAE-cellulose from Brown and Company; bovine serum albumen (Fraction V) from Nutritional Biochemicals Corporation, Cleveland, Ohio. Crystalline pancreatic ribonuclease and deoxyribonuclease was obtained from Worthington Biochemical Corporation, Freehold, N.J., U.S.A.

All other chemicals were "Analar" grade, obtained from the British Drug Houses (Canada) Ltd., Barclay Ave., Queensway, Toronto 14, Ontario.

Tissue Fractions

Experimental results were obtained using mouse Ehrlich ascites carcinoma cells except for the preparation of the DNA-dependent RNA polymerase from E. coli B cells. The tumors were propagated by transplantation every seventh day in female CF_1 Swiss white mice. The mice were supplied by the Lemberger Co., 1436 South Park Ave., Oshkosh, Wis., U.S.A. The tumors were originally obtained in the solid form from Dr. P.G. Scholefield of the McGill-Montreal General Hospital Research Institute, 3619 University St., Montreal, Que. The tumors were initiated by interperitoneal injection and harvested after seven days. Injection of 0·2-0·4 cc of tumor resulted in a 5·0-12·0 cc tumor after seven days. Only cells from first and second liquid-to-liquid transplantations were used. The liquid tumors were sometimes haemorrhagic and blood was collected along with the tumor. Tumors were discarded when haemorrhaging was extensive. After sacrifice of the animal by cervical dislocation the tumors were immediately collected with a hypodermic syringe and No. 18 needle. All subsequent operations were carried out at 0-4° C.

The suspension of ascites cells in ascitic fluid will be referred to as the "intact cell preparation".

When further cell fractions were required the cells were washed several times with "ascites wash fluid" (0·14 M NaCl, 0·02 M glucose, 0·04 M Tris buffer pH 8·5) until all blood cells and ascitic

fluid were removed (102). The cells were then packed in graduated glass centrifuge tubes by centrifugation at 2500 r.p.m. for two minutes in a clinical centrifuge. This volume of packed cells was suspended in 10 volumes of cold, distilled water and allowed to stand lysing for 10 minutes (102). The suspension was homogenized twice with a large, loose-fitting Potter homogenizer. The lysing was then terminated by adding 1/4 volume of "concentrated salts" (102)(or other concentrated salt solution as indicated) which contained 0·25 M sucrose, 0·025 M KCl and 0·005 M $MgCl_2$.

The cells were examined under the light microscope at 888 X magnification after staining with either methylene blue or acetoorcein-fast green. It was found that this lysing procedure broke the cell walls, but left the nuclei morphologically intact.

The nuclei were collected by centrifugation at 800 x g for 10 minutes (2500 r.p.m.) in a Servall refrigerated centrifuge. The nuclei were suspended in a diluted "concentrated salts" (or other concentrated salts solution as indicated) solution as described above. This suspension is referred to as the "intact nuclear preparation".

Preparation of Nuclear and Cytoplasmic sRNAs

To isolate sRNA from the nucleus the "intact nuclear preparation" was subjected to rough homogenization (7-9 passes) in a tight-fitting Potter homogenizer. Microscopic examination indicated the nuclei were completely disrupted by this treatment. The suspension was then centrifuged at 800 x g for 10 minutes to remove nuclear debris and unbroken nuclei. Ribosomes were sedimented by centrifugation at 105000 x g for 60 minutes in the Spinco Model L ultracentrifuge.

The supernatant was adjusted to pH 5·0 with 1 N acetic acid and a "nuclear pH 5 fraction" was collected by centrifugation for 10 minutes at 10000 r.p.m. in the Servall refrigerated centrifuge. The precipitate was suspended in cold Medium A (0·25 M sucrose, 0·025 M KCl, 0·005 M $MgCl_2$ and 0·05 M Tris buffer, pH 7·6) (102). The suspension was shaken for one hour at room temperature with an equal volume of 90% phenol to remove protein following the method of Kirby (103). The resulting mixture was centrifuged at 11000 r.p.m. (14500 x g) for 15 minutes. The top aqueous layer was removed carefully with a Pasteur pipette, leaving the interfacial layer of denatured protein undisturbed. The aqueous layer, containing the sRNA, was extracted three times with ether to remove contaminating phenol and 2·5 volumes of cold 95% ethanol plus 1/10 volume of 20% KAc, pH 5·0, were added to precipitate the sRNA. The suspension was allowed to stand overnight in the deep freeze at -20°C to complete precipitation.

sRNA of the cell cytoplasm was prepared from the 800 x g supernatant fraction. This supernatant fraction was centrifuged at 105000 x g on the Spinco Model L ultracentrifuge to sediment the ribosomes. The 105000 x g supernatant from this spin was adjusted to pH 5·0 with 1 M acetic acid and a "cytoplasmic pH 5 fraction" or "pH 5 enzymes" fraction precipitated. To isolate the cytoplasmic sRNA this precipitate was collected and extracted with phenol in the same way as the "nuclear pH 5 fraction" (see above).

A summary of the fractionation scheme is represented in Figure 5.

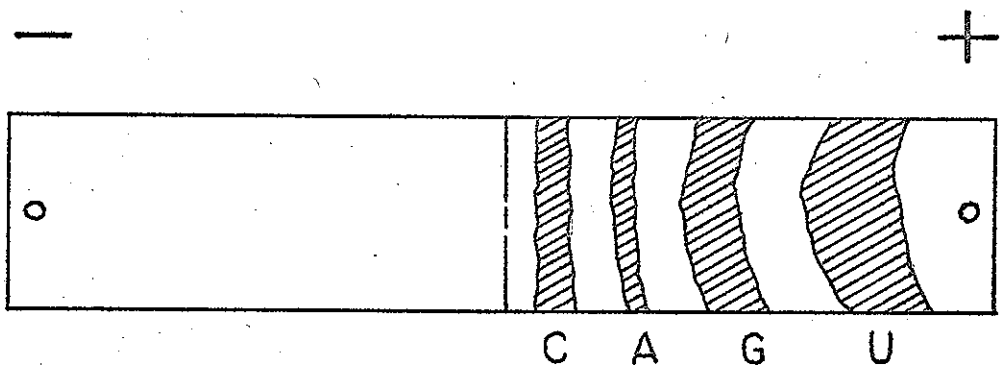


Figure 6. Diagrammatic representation of the separation obtained by paper electrophoresis of CMP (C), AMP (A), GMP (G), and UMP (U), in an 0·02 M citrate buffer pH 3·5. A potential of 500 volts was applied to the paper for 3 hours.

used for each determination. Duplicate determinations of two different amounts of hydrolysate (corresponding to 200 µg and 400 µg of sRNA) were run simultaneously along with blank strips. The electrophoretic separation obtainable is represented in Figure 6.

Preparation of DNA-dependent RNA polymerase

DNA-dependent RNA polymerase was prepared from E. coli B cells. The first preparations were with cells grown in a medium containing yeast extract as a nitrogen source (110). The harvested cells were washed twice with an aqueous medium containing 0·5 % NaCl and 0·5 % KCl, collected by centrifugation at 11000 r.p.m. (14500 x g) on the Servall refrigerated centrifuge, and stored in the deep freeze at -15°C. Later frozen cells were purchased from General Biochemicals, Laboratory Park, Chagrin Falls, Ohio.

The enzyme was prepared according to the method of Chamberlin and Berg (78). All operations were carried out at 4°C unless otherwise noted.

(I) Preparation of "Initial Extract". Glass beads were washed in 2 N HCl followed by distilled water until the pH of the wash was that of the distilled water. Either 140 gm of harvested E. coli B cells or 100 gm of the frozen commercially obtained cells were used for each preparation. The volume of buffer and the weight of glass beads used were the same in each case. The cells were disrupted in a Waring Blendor during twelve one minute periods. The temperature was not allowed to exceed 10°C. In the intervals between the blending periods the temperature of the suspension was lowered to at least 4°C by placing

the blending flask in an ice bath or in methylcellosolve kept at -20°C by the addition of dry ice. The latter cooling procedure required stirring of the suspension to prevent freezing. The "initial extract" was then collected as described (78).

(II) The Streptomycin-Protamine Fractionation. Streptomycin sulfate was obtained from British Drug Houses, Toronto, Ontario. An 8% w/v solution was used in later preparations instead of the 10% w/v solution designated (78). Protamine sulfate was used as described (78).

(III) Ammonium Sulfate Fractionation. This was carried out as described (78).

(IV) Adsorption and Elution from DEAE-Cellulose. It was found that a column with a needle valve control was necessary to maintain the proper flow rate (0·5 ml/min). Twenty 2·0 ml fractions were collected and assayed for polymerase activity as described below.

Fractions III and IV were stored at 0-4°C. Fraction III stored at this temperature retained 50% of its activity after 10 days. Fraction IV retained 90% of its activity after two weeks and 40-60% after one month as stated by Chamberlin and Berg (78).

Fraction IV enzyme was used in all experiments unless otherwise indicated.

Standard Assay for DNA-dependent RNA polymerase

The procedure of Chamberlin and Berg (78) was used to assay DNA-dependent RNA polymerase. The reaction mixture (0·25 ml) contained: 10 μmoles of Tris buffer, pH 7·9, 0·25 μmole of $MnCl_2$, 1·0 μmole of $MgCl_2$, 100 mμmoles each of ATP, CTP, GTP and UTP, 250 mμmoles of calf thymus DNA (obtained from Worthington Biochemicals Corporation), 3·0 μmoles of β-mercaptoethanol, plus 5-25 units of enzyme. UTP-2-^{14}C was added such that the specific activity of the total UTP was approximately

500 c.p.m. per mμ mole. After incubation at 37°C for 10 minutes the reaction mixture was chilled in ice and 1·2 mg of serum albumen (0·06 ml) was added and mixed, followed by 3 ml of cold 3·5% perchloric acid (PCA). The precipitate was dispersed with a Vortex mixer and collected by centrifugation for 5 minutes at 15000 x g (11000 r.p.m. in the SM-24 rotor of the Servall refrigerated centrifuge). The precipitate was washed twice with 3·0 ml portions of PCA. The residue was suspended in 0·5 ml of 2 N ammonium hydroxide, transferred to an aluminum planchet, dried and counted.

In the present work one unit of enzyme activity corresponds to an incorporation of 1·0 mμ mole of ^{14}C-UMP per hour under the conditions described above.

Preparation of DNA template specific for sRNA biosynthesis

This method was developed by Dr. E.S. McFarlane. Primer DNA was prepared from ascites tumor cells by the method of Marmur (109). Cytoplasmic sRNA was prepared from ascites tumor cells and fractionated on MAK columns as described above. The first component eluted from the column (amino acid acceptor RNA) was retained. To prepare the mouse Ehrlich ascites tumor DNA-sRNA hybrids, 1 mg of heat-denatured ascites tumor DNA (heated 15 minutes at 90° and rapidly cooled) and 0·05 mg of purified sRNA in 10 mls of a buffered salt solution (0·01 M Tris pH 7·3, 0·3 M NaCl and 0·001 M $MgCl_2$) were heated together at 72°C for 2 hours and then quickly cooled in an ice bath. The solution was then incubated with E. coli B phosphodiesterase (prepared following the method of Lehman (110-112)) for 30 minutes at 30°C to destroy the denatured DNA. The resulting solution was cooled and placed on an

MAK column. A linear gradient was established with 120 mls of 0·4 M NaCl and 120 mls of 1·2 M NaCl both buffered with 0·05 M sodium phosphate buffer pH 6·5. Three ml fractions were collected. From preliminary experiments employing purified ^{32}P-sRNA annealed with ascites tumor DNA it was known that tubes 34-44 contained the hybrid. These fractions (34-44) from several MAK column chromatograms were pooled, dialysed against water, and concentrated to approximately 20 mls by flash evaporation.

This concentrated solution containing hybrid material was heated for 15 minutes at 90°C (heat-denatured) and then cooled quickly in an ice-bath. The heat-denatured hybrid was then re-chromatographed on an MAK column with the same buffered salt gradient as above. It was shown previously (90) that the sRNA contained in the heat-denatured hybrid solution eluted from the column at low salt concentrations (0·4-0·5 M), while the DNA eluted at high NaCl concentrations (0·8-0·9 M). Thus fractions 34-44 were again collected and concentrated by flash evaporation. This concentrated solution then contained only the DNA of the DNA-sRNA hybrid material, (single-stranded).

The above procedure is both laborious and time-consuming. A shorter method for mass isolation of this DNA template is now used.

Ascites tumor DNA and sRNA were isolated as above. Hybridization was carried out with ten tubes, each containing 1 mg of DNA and 0·05 mg of purified sRNA. Each was incubated for 2 hours at 72°C, cooled quickly, and then incubated with E. coli B phosphodiesterase for 30 minutes at 30°C. The contents of all ten tubes were placed on a MAK column which had been previously equilibrated with 0·5 M NaCl

buffered with 0·05 M phosphate, pH 6·5. The column was washed with the above buffered salt solution until the effluent was free of U.V.-absorbing material (sRNA and deoxynucleotides). Following this buffered 1·2 M NaCl was applied to the column and 6 ml fractions were collected. The fractions containing U.V.-absorbing material (4-11) were pooled, dialysed against water, and concentrated in 15 ml by flash evaporation. The concentrated solution containing the hybrid was heated at 90°C for 15 minutes (heat-denatured), cooled quickly, and applied to a MAK column previously equilibrated as above. The column was washed with buffered 0·5 M NaCl until no U.V.-absorbing material appeared in the eluant (sRNA). Buffered 1·2 M NaCl was then placed on the column and 6 ml fractions were collected. The fractions containing U.V.-absorbing material (6-10) were pooled, dialysed against water and concentrated by flash evaporation. The concentrated solution then contained the DNA of the DNA-sRNA hybrid.

Activity of the Nucleotide Incorporating in "pH 5 enzymes" Fraction and the 105000 x g Supernatant Fraction

Preparation of pyrophosphorylyzed sRNA was carried out by incubating the "pH 5 enzymes" fraction with a relatively high concentration of PPi (27). In this method 5·0 ml of "pH 5 enzymes" fraction were incubated in 0·05 M KF (117) and 0·05 M NaPPi for 30 minutes at 37°C. The sRNA was then isolated by the phenol extraction method of Kirby (103) and stored frozen in aqueous solution.

The activity of the nucleotide incorporating enzyme in the "pH 5 enzymes" fraction and the 105000 x g supernatant fraction of Ehrlich ascites tumor cells was measured by its ability to catalyze the restoration of amino acid binding capacity of pyrophosphorylyzed sRNA.

This was tested by measuring the incorporation of ^{14}C amino acids (^{14}C-algal hydrolysate) into sRNA in the absence and presence of CTP. The incubation mixture (1·0 ml) contained 5 µmoles ATP, pH 7·5, 10 µmoles mercaptoethanol, 5 µmoles $MgCl_2$, 25 µmoles of Tris buffer, pH 7·5, 500000 c.p.m. of ^{14}C amino acids, 0·2 mg of pyrophosphorylysed sRNA, 1·0 µmole of CTP and 0·1 ml of "pH 5 enzymes" fraction or 105000 x g supernatant fraction. Control incubation mixtures lacked CTP and/or sRNA. Blanks were run with heated "pH 5 enzymes" fraction (heated for 3 minutes at 77°C).

All incubation mixtures were preincubated without ^{14}C-amino acids for 20 minutes at 37°C. After the addition of ^{14}C-amino acids these mixtures were incubated a further 10 minutes at 37°. The reactions were stopped by cooling in an ice bath, adding 1·2 mg of serum albumen (0·06 ml) followed by 4·0 ml of 3·5% PCA containing 1% lactalbumen hydrolysate. The precipitate obtained was sedimented in a clinical centrifuge for 5 minutes and then washed twice with the PCA-lactalbumen mixture. The precipitate was then resuspended in 0·5 ml of 4 N NH_4OH, plated, dried and counted.

Pyrophosphatase Activity of "pH 5 enzymes" Fraction and 105000 x g Supernatant Fraction

Inorganic pyrophosphatase activity was estimated essentially by the method of Kunitz (113). Inorganic phosphate was determined by the method of King (114). The incubation mixture (10·0 ml) contained 500 µmoles of Tris buffer pH 7·5, 200 µmoles NaPPi, 80 µmoles of $MgCl_2$ and 1·0 ml of "pH 5 enzymes" fraction or 105000 x g supernatant fraction. Two controls, one without NaPPi and one without enzyme were incubated

simultaneously. The mixtures were incubated at 37° and aliquots (1·0 ml) were removed at 0, 5, 10 and 20 minutes and added to 1·0 ml of 60% PCA. The samples were centrifuged for 2 minutes with a clinical centrifuge to remove the precipitated protein and then the concentration of inorganic phosphate in the clarified supernatant was determined. The removal of protein was accomplished as quickly as possible to minimize the hydrolysis of PPi by the PCA.

Treatment of "pH 5 enzymes" Fraction with Protamine Sulfate

The "pH 5 enzymes" fraction was treated with protamine sulfate to remove sRNA (115). Different concentrations of protamine sulfate were used to determine the concentration which would precipitate most of the sRNA without removing pyrophosphatase activity, nucleotide incorporating enzyme activity, or, and most important of all, amino acid activating enzymes.

The protamine sulfate (1% w/v) was always freshly prepared as suggested by Hele (115), filtered and kept cool in an ice bath. The protamine sulfate (0·1, 0·2, 0·4 and 0·8 ml) was added to 2·0 ml fractions of "pH 5 enzymes" fraction. The treated fractions were then centrifuged for 5 minutes with the clinical centrifuge. The supernatants were decanted and assayed for the various enzymic activities listed above and for sRNA content (116).

Chromatography of "pH 5 enzymes" Fraction on DEAE-cellulose

The "pH 5 enzymes" fraction was chromatographed on DEAE-cellulose to remove sRNA (118). The DEAE-cellulose was previously washed with 1 N NaOH and 1 M KH_2PO_4 buffer and equilibrated with 6 mM mercaptoethanol buffered with 0·10 M Tris buffer pH 7·5.

A column (6 x 1·1 cm) was prepared and 10 ml of "pH 5 enzymes" fraction (previously diluted 1:4·5 with Tris-mercaptoethanol buffer) were run through with the Tris-mercaptoethanol buffer as eluent. The first 20 ml were retained. The activity of nucleotide incorporating enzymes and amino acid activating enzymes, and the sRNA content of the chromatographed fraction was measured.

Treatment of DNAase on G-50 Sephadex

The commercial crystalline DNAase was passed through a G-50 Sephadex column to remove residual RNAase activity as suggested by Hiatt (119). The G-50 Sephadex was allowed to swell in distilled water for 3 days and the fines were decanted. Glass beads were placed in the column to a height of 1 cm to prevent residual fines from plugging the sintered glass disk of the column (120). G-50 Sephadex column, 17 cm x 1·1 cm, was prepared on top of this. The following operations were carried out in a cold-room ($4^{o}C$). The level of the column was stabilized by eluting with water for 36 hours (120). Following this 6 mg of DNAase dissolved in 0·5 mls of H_2O were placed on the column and the column eluted with water. Fractions of 1·0 ml were collected and the optical densities at 280 mμ measured on a Beckman DU spectrophotometer. The first fraction in which a large optical density at 280 mμ appeared was used in subsequent experiments. It is to be noted that all experiments involving DNAase employed the unfractionated DNAase unless otherwise indicated.

Determination of Radioactivity

Incorporation of uridine-2-^{14}C into sRNA was estimated by plating samples of the RNA-containing solutions on stainless steel planchets, evaporating to dryness under an infrared lamp and counting.

Incorporation of UTP-2-^{14}C and ^{14}C-amino acids (^{14}C-algal hydrolysate) into acid insoluble material ("RNA-like" material) was estimated by dissolving the washed precipitate in 0·5 ml of 2 N NH_4OH or 4 N NH_4OH, plating on stainless steel planchets, drying and counting.

All counting was done on a Nuclear Chicago micromil window gas-flow counter. All determinations were made at infinite thinness. Samples that counted less than 100 c.p.m. above background were counted on a Model C-110B low background automatic counter (background less than 2·0 c.p.m.). More active samples were sometimes counted on a manual counter with a background of 10·0 c.p.m. All samples were corrected for background.

Analytical Determinations

Protein estimations were made by the method of Lowry et al (121) using 5 x crystallized egg albumin (Sigma Chemical Co.) as a standard.

sRNA was estimated from the 260 mμ and 280 mμ absorption by the method of Warburg and Christian (122).

TABLE 1

The effect of actinomycin D on the incorporation of uridine-2-^{14}C into cytoplasmic sRNA in an "intact cells preparation"

Addition	Specific Activity (c.p.m./mg sRNA)
None	140·0
Actinomycin	4·0

Note: sRNA was labelled in the presence of 10·5 ml of "intact cells preparation", 125 mmoles of sucrose, 12·5 mmoles of Tris buffer, pH 7·7, 0·5 mmoles of $CaCl_2$, 0·05 mmoles of $MgSO_4$ and 50 mμmoles of uridine-2-^{14}C (950,000 c.p.m.) in a final volume of 15 ml. The cells were preincubated with and without actinomycin D (6 μg/ml) for 20 minutes at 37° and then for a further 60 minutes after the addition of the uridine-2-^{14}C. The reaction was stopped by cooling in an ice bath. sRNA was extracted by the phenol method and counted on the automatic counter.

TABLE 2

The effects of actinomycin D and DNAase on the incorporation of uridine-2-^{14}C into nuclear sRNA in an "intact nuclear preperation"

Addition	Specific Activity (c.p.m./mg sRNA)	
	Expt. 1	Expt. 2
None	42·0	27·6
Actinomycin	11·5	3·6
DNAase	7·3	--

Note: The nuclei were preincubated with and without actinomycin (0·6 µg/ml in Expt. 1 and 2·5 µg/ml in Expt. 2) and with and without DNAase (18 µg/ml in Expt. 1) for 25 minutes (Expt. 1) and 20 minutes (Expt. 2) followed by the addition of uridine-2-^{14}C (50 mµmoles) and a further incubation for 4 hours at 37° in a final volume of 15 ml. The pH was checked every 30 minutes. The sRNA was extracted by the phenol method and counted on the automatic counter.

RESULTS

Preliminary study of sRNA biosynthesis in mouse Ehrlich ascites tumor cells

The data presented in Table 1 show that the incorporation of uridine-2-^{14}C into cytoplasmic sRNA of an "intact cells preparation" is inhibited approximately 97% by actinomycin D (6 µg/ml, preincubated for 20 minutes). All flasks were incubated in the dark because actinomycin D is light-sensitive.

In Table 2 results are presented which show the effects of actinomycin D and DNAase on the incorporation of uridine-2-^{14}C into nuclear sRNA of an "intact nuclear preparation". The "intact nuclear preparation" was prepared as described above (see p. 14) except; (i) a concentrated salts buffer giving a final concentration of 0·25 M sucrose, 0·075 M Tris buffer, pH 7·7, 0·001 M $CaCl_2$ and 0·0001 M $MgSO_4$ was added to stop lysis of the cells and (ii) the nuclei collected by centrifugation were suspended in 3 volumes of a buffered salts solution resulting in a final concentration of materials as given in (i) above. It may be seen that preincubation with actinomycin inhibited the incorporation of uridine-2-^{14}C into nuclear sRNA of this "intact nuclear preparation" by 72% (0·6 µg/ml) and 87% (2·5 µg/ml) and preincubation with DNAase (18 µg/ml) inhibited 83%. The pH of the reaction mixtures was checked periodically because of the long incubation time (4 hours).

The two major components of mouse Ehrlich ascites tumor sRNA obtained by MAK column chromatography

Nuclear and cytoplasmic sRNAs were fractionated on methylated

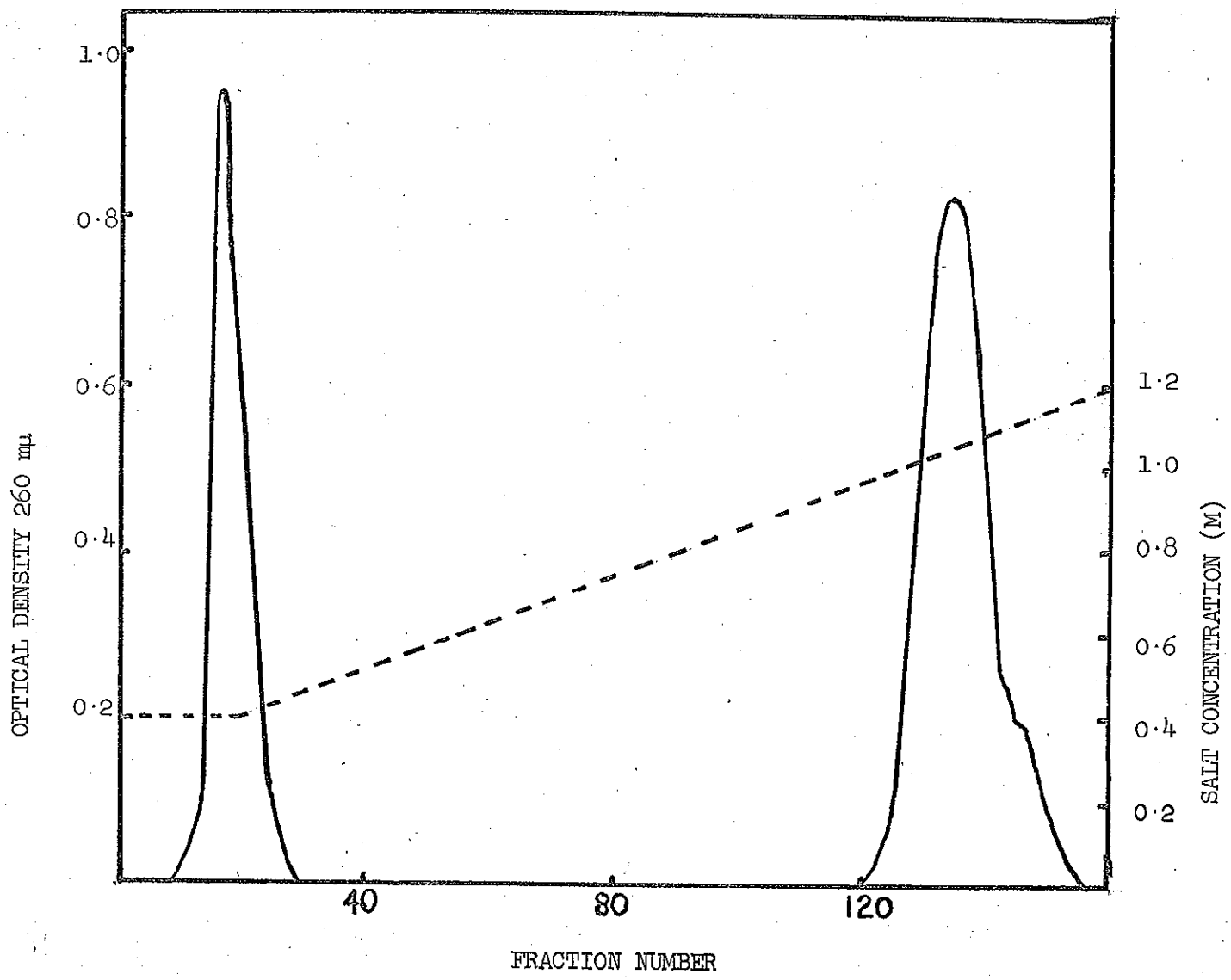


Figure 7. Fractionation of nuclear and cytoplasmic sRNA of mouse Ehrlich ascites tumor cells on a methylated albumen-kieselguhr (MAK) column according to the method of Mandell and Hershey (104).

TABLE 3

Nucleotide compositions of the first components (acceptor RNA fractions) of nucleus and cytoplasmic sRNAs fractionated on MAK columns

Source of acceptor RNA fraction	Nucleotides (moles %)				Ratio of Purines/Pyrimidines
	AMP	UMP	CMP	GMP	
Nucleus	16·9	13·1	39·0	31·5	0·093
Cytoplasm	17·6	14·1	38·3	30·1	0·091

albumen columns as described above (see p. 16). The elution patterns indicated that two major components were present (see Fig. 7). The recovery of U.V.-absorbing material from the chromatography of either nuclear or cytoplasmic sRNA varied between 85-95% with the RNA in the first peak (eluted at 0·4-0·5 M NaCl) accounting for 35% of the total U.V.-absorbing material placed on the column. This first component is amino acid acceptor RNA (105).

The nucleotide compositions of the acceptor RNA fractions of nuclear and cytoplasmic sRNAs fractionated on MAK columns were determined by methods described above (see p. 16). It may be seen that the base composition of acceptor RNA isolated from the nucleus is very nearly the same as the base composition of cytoplasmic acceptor RNA. Furthermore the per cent of AMP (16·9) and of UMP (13·1) indicates that approximately 87% of these two nucleotides could be base-paired in the nuclear acceptor RNA. Similarly 89% of the CMP and GMP of the nuclear acceptor RNA would be base-paired. The same can be said of the cytoplasmic sRNA.

The second peak (eluted at 0·8-0·9 M NaCl) accounted for 65% of the total U.V.-absorbing material placed on the column. The protein composition of this peak was shown to be less than 1% for both nuclear and cytoplasmic sRNA. Treatment of this second component with DNAase (20 μg/ml for 18 hours at 37° in 0·05 M Tris, pH 7·7), followed by dialysis against water, resulted in no loss of U.V.-absorbing material. Therefore this second component contains little, if any, DNA material. Treatment with 0·3 N KOH for 18 hours at 37° followed by dialysis against water showed a loss of 55-65% of the U.V.-absorbing material after the dialysis. Pancreatic ribonuclease treatment (20 μg/ml for 6 hours at 37° in 0·05 M Tris buffer, pH 7·7)

TABLE 4

Results of preparations of DNA-dependent RNA polymerase from E. coli B (78)

Preparation	FRACTION					
	I Initial Extract		II Streptomycin-Protamine		III Ammonium Sulfate	
	Total Activity units	Specific Activity units/mg	Total Activity units	Specific Activity units/mg	Total Activity units	Specific Activity units/mg
1	125,500	19·1	11,900	79·3	12,100	228·3
2	141,100	17·3	14,200	90·0	20,900	402·0
3	163,000	22·2	24,800	147·6	26,000	542·7
4	106,000	----	------	----	36,000	-----
5	106,800	18·6	20,500	161·4	25,500	520·4
Chamberlin and Berg (78)	370,000	40	205,000	1,600	200,000	2,500

Note: One unit of enzyme activity in preparations 1-5 corresponds to an incorporation of 1·0 mμmole of ^{14}C-UMP per hour under the conditions described in the assay procedure (see p. 19). One unit of enzyme activity in the preparation of Chamberlin and Berg (78) corresponds to an incorporation of 1·0 mμmole of ^{14}C-CMP per hour under the same conditions.

followed by dialysis against water showed a loss of 22-30% of the U.V.-absorbing material. This sensitivity to alkali and pancreatic ribonuclease indicates that some of this second component of both nuclear and cytoplasmic sRNA is RNA. Pancreatic ribonuclease treatment (100 µg/ml for 22 hours at 37° in 0·05 M Tris buffer, pH 7·7) of the second component of cytoplasmic sRNA, followed by dialysis against 2 M NaCl showed a loss of 94% of the U.V.-absorbing material. This indicates that most of the second peak of cytoplasmic sRNA is RNA material.

Preparation of DNA-dependent RNA polymerase from E. coli B

The preparation of DNA-dependent RNA polymerase was carried out as described above (see p. 17). Commercially obtained E. coli B cells were used in preparations 4 and 5. Preperations 2, 3, 4 and 5 were carried out with the 8% w/v solution of streptomycin sulfate while in preparation 1, the 10% w/v solution was used (78). It may be seen that this change in the procedure approximately doubled the specific activity of the Fraction III enzyme. The low specific activity of the "Initial Extract" in our preparations as compared to that obtained by Chamberlin and Berg (78) may simply be a result of the different unit of enzyme activity used. It may also be noted that the specific activity of the extract after the streptomycin protamine step is much lower in our preparations than the specific activity reported by Chamberlin and Berg.

Preliminary studies of the DNA-dependent RNA polymerase reaction

The data presented in Table 5 show that actinomycin D (2·0 µg/ml), and DNAase (160 µg/ml) inhibit the incorporation of

TABLE 5

The effect of DNAase and actinomycin D on the incorporation of UTP-2-^{14}C into "acid-insoluble" material catalysed by DNA-dependent RNA polymerase

Additions	C.p.m. ("Acid-Insoluble" Material)	
	Preincubated 3 min.	Preincubated 10 min.
None	440·0	480·0
Actinomycin	107·0	11·0
DNAase	0·0	1·0

Note: Incubating medium and wash procedure was the same as in the standard assay procedure for activity of RNA polymerase (see p. 18). Samples were preincubated with and without actinomycin D (2·0 μg/ml) and DNAase (160 μg/ml) for 3 minutes or for 10 minutes before addition of DNA-dependent RNA polymerase and further incubated for 10 minutes at 37°. The reactions were stopped by cooling in an ice bath and the "acid-insoluble" material was washed and counted with the manual counter. Blanks (lacking RNA polymerase) were run simultaneously and assayed for radioactivity (10·0 c.p.m.). The results in this table were corrected for the blanks.

TABLE 6

The effect of RNAase and alkali on the incorporation of UTP-2-^{14}C into "acid insoluble" material with RNA polymerase reaction and the DNA primer specific for sRNA synthesis

Treatment	C.p.m. ("Acid Insoluble" Material)
None	1650
RNAase	280
Alkali	31

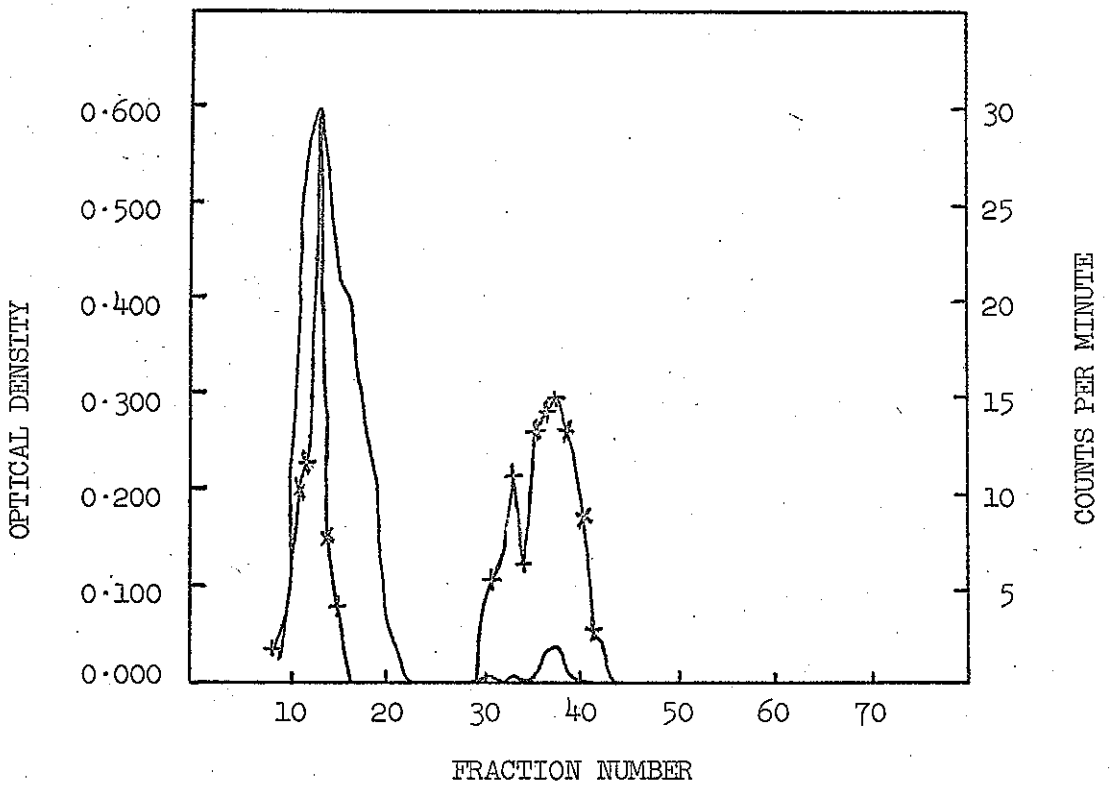
Note: The reaction mixture (0·5 ml) contained 40·0 mM of Tris buffer, pH 7·9, 1·0 mM of $MnCl_2$, 4·0 mM $MgCl_2$, 400 mμmoles each of ATP, CTP, GTP and UTP, 15 mμmoles of specific DNA primer, UTP-2-^{14}C such that the specific activity of the total UTP was 250 c.p.m. per mμmole, and 12 mM mercaptoethanol and RNA polymerase. Polymerase was added every 20 minutes (total of 60 units). After incubation at 37° for 2 hours the reaction mixtures were treated for 1 hour at 37° with RNAase (3 μg/ml) or in KOH (1 N), then chilled in ice and the "acid insoluble" material assayed for radioactivity as in the standard assay procedure for RNA polymerase (see p. 19). Blanks (preincubated for 5 minutes with 150 μg/ml DNAase before addition of RNA polymerase) were run simultaneously and assayed for radioactivity (12·0 c.p.m.). The results in this table were corrected for the blanks.

UTP-2-^{14}C into "acid-insoluble" material catalysed by DNA-dependent RNA polymerase with calf thymus DNA as the primer DNA (DNA template). The DNAase (160 µg/ml) inhibited the UTP-2-^{14}C incorporation more than 99%, therefore, samples preincubated with DNAase were subsequently used as blanks in the following experiments.

In Table 6 results are presented which show the effects of pancreatic ribonuclease and alkali on the radioactivity of the "acid insoluble" product of the RNA polymerase reaction primed with single-stranded DNA specific for sRNA synthesis. It may be seen that the pancreatic ribonuclease (3 µg/ml for 60 minutes at 37°) solubilized 83% of the counts in the "acid insoluble" product and the alkali treatment (1 N KOH for 60 minutes at 37°) solubilized more than 98% of these counts. This was taken as an indication that the "acid insoluble" counts are incorporated into an "RNA-like" material. It should be noted that the amount of nucleoside triphosphates and RNA polymerase added, and the long incubation period (2 hours) resulted in a synthesis of 47 µmoles of sRNA-phosphate. (Calculated by assuming that 14 moles% of sRNA is UMP (see Table 3).) This is more than three times the amount of DNA-phosphate used as primer in the reaction mixture.

When the "RNA-like" product was chromatographed on an MAK column the graph shown in Figure 8 was obtained. (This chromatograph was provided by Dr. E.S. McFarlane.) The total reaction mixture for the synthesis of the "RNA-like" product was placed on the column after a 3 hour incubation. Purified mouse Ehrlich ascites tumor sRNA (acceptor RNA) was also placed on the column at the same time to act as a marker. The high peak of optical density (maximum at fraction 13) indicates that the added purified sRNA is eluting from the column. It may be seen that a peak of radioactivity corresponding to this

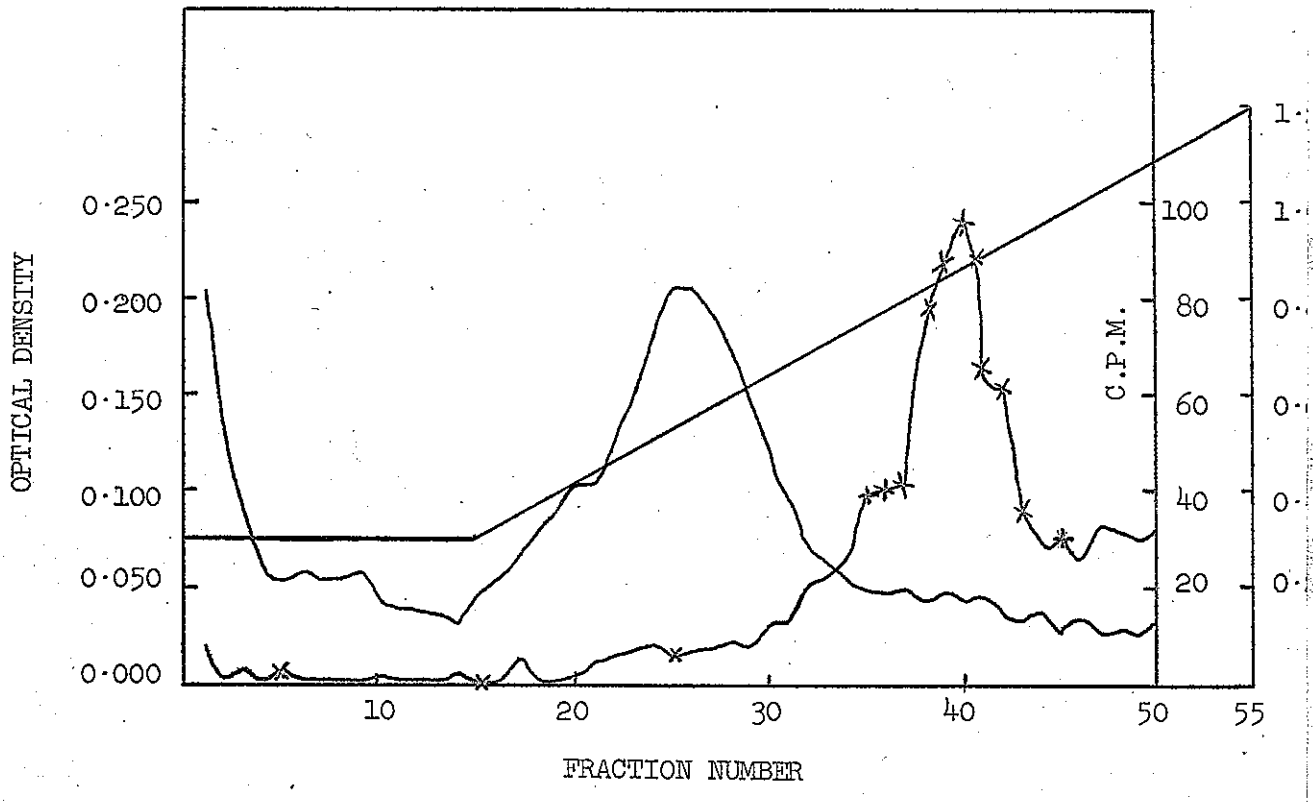
Figure 8.



Note: The crosses (+) represent counts per minute and the unmarked line represents optical density measured at 260 mμ.

Chromatography on an MAK column of the "RNA-like" product of DNA-dependent RNA polymerase reaction primed with DNA specific for sRNA biosynthesis (single-stranded). The reaction mixture was the same as that in Table 6 except 24·4 mμmoles of DNA-phosphate was used as primer. The reaction mixture was incubated for 3 hours at 37° and was placed directly on an MAK column along with 0·54 mg of purified (chromatographed) ascites tumor cytoplasmic sRNA as a marker. This column had been previously equilibrated with 0·3 M NaCl buffered with 0·05 M phosphate buffer, pH 6·5. The column was washed with buffered 0·3 M NaCl until the optical density of the eluant was 0·000. A linear gradient was established with 120 ml each of buffered 0·4 M NaCl and 1·2 M NaCl at pH 6·5. Three ml fractions were collected and the optical densities measured at 260 mμ in a Beckman DU spectrophotometer. Serum albumen (1·0 mg) was added to 1 ml samples of each of these fractions, followed by the addition of 3 ml of 3·5% PCA. The precipitate, collected by centrifugation (5 minutes at 14500 x g), was dissolved in 2 N NH_4OH, plated and counted on the manual counter. A control reaction mixture was incubated simultaneously. The reaction was stopped by cooling in ice. Serum albumen (1 mg) was added followed by 3 ml of 3·5% PCA. The precipitate was collected by centrifugation as above and washed twice with 3·5% PCA before dissolving in 2 N NH_4OH, plating and counting. The control sample counted 589 c.p.m. which corresponds to 17 mμmoles of sRNA-phosphate, calculated as described in Results. Total counts recovered from the column as acid-insoluble counts were 582 c.p.m.

Figure 9.



Note: The crosses (+) represent counts per minute and the unmarked line represents optical density measured at 260 mμ.

Chromatography on an MAK column of the "RNA-like" product of the DNA-dependent RNA polymerase reaction primed with DNA specific for sRNA biosynthesis (single-stranded). The reaction mixture was the same as that in Table 6 except 122·0 mμmoles of DNA-phosphate was used as primer, and 2·4 x 10^6 c.p.m. of UTP-2-^{14}C was added. The reaction mixture was incubated for 2 1/2 hours at 37° and placed directly on an MAK column along with 0·8 mg of purified (chromatographed) ascites tumor cytoplasmic sRNA added as a marker. The column had been previously equilibrated with 0·3 N NaCl buffered with 0·05 M phosphate buffer pH 6·5. The column was washed with buffered 0·3 M NaCl until the optical density (measured at 260 mμ) of the eluant was 0·000. (Fractions 1-15 were the last 15 fractions of the wash procedure.) A linear NaCl gradient was established with 110 ml each of 0·3 M NaCl and 1·2 M NaCl buffered with phosphate buffer pH 6·5. Four ml fractions were collected and the optical densities read at 260 mμ. Serum albumen (1·2 mg in 0·06 ml) was added to 1 ml samples of each of these fractions. Three ml of 3·5% PCA was then added. The precipitate, collected by centrifugation (5 minutes at 14500 x g), was dissolved in 2 N NH_4OH, plated and counted on the manual counter. Total counts recovered from the column as acid-insoluble counts were 3994 c.p.m. This corresponds to 45·7 mμmoles of sRNA-phosphate, calculated as described in Results.

sRNA peak is present. A second peak of radioactivity was eluted at a NaCl concentration similar to that which elutes DNA-sRNA hybrid (90). A small peak of U.V.-absorbing material also was eluted at this NaCl concentration and may be due to DNA-sRNA hybrid material. It should be noted that the incorporation of UTP-2-^{14}C into "RNA-like" (acid insoluble) material indicates a synthesis of 17·0 mμmoles of sRNA-phosphate while the amount of primer DNA used in the reaction was 24·4 mμmoles of DNA-phosphate.

Figure 9 shows the results of another experiment where the "RNA-like" product was chromatographed on an MAK column. (The total reaction mixture was placed on the column as in Table 8.) The high optical density of the first few fractions (fractions 1, 2 and 3 of the wash procedure) is due to the nucleoside triphosphates in the reaction mixture. The optical density peak obtained in fractions 21-33 is the purified sRNA eluting from the column. It may be seen that the peak of radioactivity (fractions 34-44) was eluted from the column at the NaCl concentration which elutes DNA-sRNA hybrids (0·8-0·9 M NaCl, (90)), not with the sRNA. Total counts recovered from the column as acid-insoluble counts were 3994 c.p.m. This corresponds to 45·7 mμmoles of sRNA-phosphate synthesized (see page 29). It should be noted that 122·0 mμmoles of DNA-phosphate was used as primer.

<u>Measurement of the Amino Acid binding capacity of the "RNA-like" product of the DNA-dependent RNA polymerase reaction primed with DNA specific for sRNA biosynthesis (single-stranded)</u>

The amino acid binding capacity of the "RNA-like" product was tested by measuring the incorporation of ^{14}C-amino acids

Table 7

The incorporation of ^{14}C-amino acids (^{14}C-algal hydrolysate) into the "RNA-like" product of the DNA-dependent RNA polymerase reaction primed with DNA specific for sRNA synthesis

Preincubation (without ^{14}C-amino acids)	C.p.m. incorporated into "RNA-like" product					
	Expt. 1	Expt. 2	Expt. 3	Expt. 4	Expt. 5 (i)	Expt. 5 (ii)
A. None (Blank)	769	407	451 419	544 513	186	165
B. None	949	461	422 443	661 545	---	---
C. 10 minutes	---	640	452 405	--- ---	---	---
D. 20 minutes	---	---	410 399	300 217	178	184

Note: Reaction mixtures for synthesis of "RNA-like" material were as in Table 6, except more DNA primer was added (20-80 mμmoles of DNA-phosphate, see Table 8). The samples were incubated for 2-3 hours. RNA polymerase was added every 20-30 minutes to a total of 50-80 units of enzyme activity (experiment 5, (i) Fraction III enzyme, (ii) Fraction IV enzyme). Following this 2-3 hour incubation a second reaction mixture (amino acid activating enzymes system, 0·5 ml) containing 5 μmoles of ATP, pH 7·5, 500,000 c.p.m. of ^{14}C-algal hydrolysate, 10 μmoles of mercaptoethanol, 5 μmoles of $MgCl_2$, 25 μmoles of Tris buffer, pH 7·5 and 0·1 ml of "pH 5 enzymes" fraction or other source of activating enzymes (experiment 1, 0·1 ml of diluted "pH 5 enzymes" fraction (diluted 1:20); experiment 2, 0·1 ml of diluted "pH 5 enzymes" fraction (diluted 1:50); experiment 3, 0·1 ml diluted "pH 5 enzymes" fraction (diluted 1:40); experiment 4, 1·0 ml protamine sulfate treated "pH 5 enzymes" fraction (undiluted); experiment 5, 0·25 ml of DEAE-cellulose treated "pH 5 enzymes" fraction) was added. A and B were incubated for a further 5 minutes. C and D were incubated without ^{14}C-algal hydrolysate for 10 and 20 minutes respectively, followed by the addition of the ^{14}C-algal hydrolysate and a further 5 minutes incubation. Reactions were stopped by cooling in an ice bath and adding 1-2 mg (0·06 ml) serum albumen and 4 ml of 3·5% PCA containing 1% lactalbumen hydrolysate. The resulting precipitate was collected by centrifugation (5 minutes at 15,000 x g) and washed twice with 4 ml of the PCA-lactalbumen solution. The residue was dissolved in 4 N NH_4OH, plated and counted on the manual counter. The blanks were incubated with DNAase (150 μg/ml DNAase for 3 minutes in experiments 1 and 2 and 150 μg/ml for 5 minutes in experiments 3, 4 and 5) before the addition of RNA polymerase. Therefore the blanks contained no "RNA-like" product (no DNA primer for the reaction).

Table 8

Amounts of DNA-phosphate used as primer and the amounts of sRNA-phosphate synthesized in experiments 1, 2, 3, 4 and 5 (see Table 7)

Expt. No.	DNA primer (µmoles of DNA-p)	C.p.m. UTP-2-^{14}C incorporation	sRNA synthesized (µmoles of DNA-p)
1	80·5	2740	96·7
2	21·0	800	20·8
3	24·4	1361	38·4
	24·4	1431	40·4
4	24·4	885	25·0
5(i)	24·4	4950	139·7
(ii)	24·4	3300	93·0

Note: The DNA primer used in experiments 1 and 2 was not purified by MAK column chromatography of heat-denatured DNA-sRNA hybrid (see p. 20). The hybrid (heat-denatured, 15 minutes at 90°) itself was used and the amount of nucleic acid in this hybrid was calculated by the method of Warburg and Christian (122). The amount of DNA-phosphate in this added hybrid was taken to be 1/2 of the nucleic acid content. The other 1/2 of the hybrid would be sRNA. In experiments 3, 4 and 5 the DNA primer added was purified by MAK column chromatography which removed the sRNA (see p. 20). The amount of DNA-phosphate was calculated by the method above (122) assuming that all the nucleic acid present was DNA.

The amount of sRNA formed was calculated by assuming that 14% of the nucleotides in the sRNA are UMP (see Table 3).

Table 9

Measurement of nucleotide incorporating enzyme activity

Additions	C.p.m. incorporated into sRNA (Incorporation of ^{14}C-amino acids into acid-insoluble materi		
	105,000 x g supernatant		"pH 5 enzymes"
	Untreated	Protamine sulfate treated	Protamine sulfate trea
None (Blank)	153	133	345
None	189	182	650
Pyrophosphorylysed sRNA	361	253	767
Pyrophosphorylysed sRNA and CTP	929	535	986

Note: See Materials and Methods. Heat-denatured enzyme was used in the blanks.

(^{14}C-algal hydrolysate) into "acid insoluble" material (see Table 7). The reaction mixture for the synthesis of the "RNA-like" product was incubated at 37° for 2-3 hours. The amino acid activating enzymes system was then added to this reaction mixture and incubated for a further 5 minutes at 37°. The reaction was stopped by cooling and the "acid-insoluble" material was collected, washed, plated and counted as described in Table 7. Blanks were run simultaneously. The blanks were samples preincubated at 37° for 3 or for 5 minutes with 150 µg/ml of DNAase to destroy the DNA primer. Thus no "RNA-like" product would be formed and any ^{14}C-amino acid incorporation found could not be incorporation into the "RNA-like" product. However, incorporation above the blank would indicate that the "RNA-like" product was incorporating amino acids. In some experiments the amino acid activating enzymes system was incubated at 37° for 10 or for 20 minutes before adding ^{14}C-amino acids (see Table 7). Following the addition of the ^{14}C-amino acids the incubation was continued for a further 5 minutes. Thus <u>all</u> samples were incubated with radioactive amino acids for exactly 5 minutes.

It may be seen in Table 7 that in experiments 1 and 2 the incorporation of ^{14}C-amino acids into acid insoluble material is higher in the controls than in the blanks. Also in experiment 2 the control preincubated for 10 minutes with the amino acid activating enzymes system incorporated more amino acids than the control that was not preincubated. In experiments 3, 4 and 5 the controls, pre-incubated or not, do <u>not</u> show a higher incorporation than the blanks.

In experiment 4 the controls preincubated for 20 minutes have a much lower incorporation than the blanks. Fraction III RNA polymerase was shown to behave similarly to Fraction IV RNA polymerase with respect to the incorporation of ^{14}C-amino acids (experiment 5).

It should be noted that in experiments 2, 3, 4 and 5 DNAase (150 µg/ml) was incubated with the "RNA-like" product after its synthesis, just before the amino acid activating enzymes system was added. This was done to release the "RNA-like" product from the expected DNA-RNA hybrid. (Since single-stranded DNA was used as template DNA-RNA hybrids will be formed (88).) Also in experiments 1 and 2 the DNA used as primer was subjected to MAK column chromatography only once, and as a hybrid. (The hybrid was heat-denatured before using a primer DNA.) However, the DNA used as primer in experiments 3, 4 and 5 was chromatographed on an MAK column a second time (after heat-denaturation of the hybrid) to further purify the DNA by removing the sRNA of the hybrid (see p. 20). Thus the DNA is in the single-stranded form during the second MAK column chromatography.

The incorporation of ^{14}C-amino acids into the blanks (see Table 7, A) is due mainly to endogenous acceptor RNA of the activating enzymes added. Since the "pH 5 enzymes" fraction added in experiments 2 and 3 was diluted 1:50 and 1:40 respectively, the blanks in these experiments were markedly less than the blank in experiment 1 where the "pH 5 enzymes" fraction was diluted 1:20. In experiment 4 the "pH 5 enzymes" fraction was treated with protamine sulfate (see p. 23 and p.34) to remove the sRNA and hence even when undiluted enzyme was added the blank was not as high as that in experiment 1. It may be seen that the lowest blanks were obtained using the DEAE-cellulose treated "pH 5 enzymes" fraction (see p. 24 and p.35).

Study of the activity of nucleotide incorporating enzyme, pyrophosphatase and amino acid activating enzymes in the "pH 5 enzymes" fraction and the 105,000 x g supernatant fraction of mouse Ehrlich ascites tumor cells and the effect of protamine sulfate treatment and DEAE-cellulose chromatography on these activities.

It seemed probable that "precursor polyribonucleotide" (see p. 11) was actually synthesized in the DNA-dependent RNA polymerase reaction primed with DNA specific for sRNA biosynthesis. Therefore the difficulty in obtaining a definite incorporation of ^{14}C-amino acids into the "RNA-like" material synthesized by the RNA polymerase reaction might be due to some difference between "precursor polyribonucleotide" and amino acid acceptor RNA.

Since unmethylated sRNA can accept amino acids (65) the methylating enzymes are not likely necessary. However, the pCpCpA end of an sRNA molecule is essential for the binding of an amino acid. Since all sRNA molecules have this same terminal trinucleotide sequence, this sequence may not be coded for in the DNA genome. The nucleotide incorporating enzyme may therefore be required in order to complete the molecule.

One of the products of the RNA polymerase reaction is inorganic pyrophosphate. The products of the nucleotide incorporating enzyme are inorganic pyrophosphate and the pCpCpA end of sRNA molecules. It is possible then that inorganic pyrophosphatase is necessary to lower the concentration of PPi in the incubation mixture in order to complete the pCpCpA ends. Thus the activities of the nucleotide incorporating enzyme and of pyrophosphatase may be important for the formation of amino acyl RNAs with the "RNA-like" product.

The presence of sRNA in the "pH 5 enzymes" fraction results

in high blanks. If these blanks could be lowered then a small increase in incorporation of ^{14}C-amino acids (300-400 c.p.m.) in the controls over the blanks would be much more significant. The 105,000 x g supernatant fraction is 13-14 times more dilute than the "pH 5 enzymes" fraction and has a high amino acid activating enzymes activity. Protamine sulfate treated "pH 5 enzymes" fraction and DEAE-cellulose chromatographed "pH 5 enzymes" fraction both contain much less sRNA than the untreated "pH 5 enzymes" fraction and consequently the treated enzymes give lower blanks. Therefore the effects of protamine sulfate treatment and DEAE-cellulose chromatography on the activities of the nucleotide incorporating enzyme, pyrophosphatase, and the amino acid activating enzymes were studied.

Preliminary experiments with protamine sulfate indicated that the addition of 0·1 ml of a 1% w/v solution of protamine sulfate to 2·0 ml of "pH 5 enzymes" fraction (see p. 23) removed more than 90% of the sRNA content while less than 50% of the amino acid activating enzymes activity was lost. Further addition of protamine sulfate precipitated more sRNA but the activating enzymes were also precipitated. Thus the protamine sulfate treatment used in all experiments was that indicated above.

In Table 9 are shown the results of an experiment designed to measure the activity of the nucleotide incorporating enzyme in "pH 5 enzymes" fraction and in the 105,000 x g supernatant fraction (with and without protamine sulfate treatment). It may be seen that the 105,000 x g supernatant fraction is a better source of this enzyme than is the protamine sulfate treated "pH 5 enzymes" fraction.

Table 10

Measurement of pyrophosphatase activity

Incubation time	Pi released by pyrophosphatase (mg)		
	105,000 x g supernatant	"pH 5 enzymes"	
	Untreated	Untreated	Protamine sulfate treated
0 minutes	0·000	0·000	0·000
5 minutes	> 0·131	0·007	0·002
10 minutes	> 0·131	0·011	0·005
20 minutes	> 0·131	0·017	0·012

Note: See Materials and Methods. Blanks containing Na-PPi only and enzyme only were run simultaneously and the above results were corrected for these blanks.

Table 11

The effect of DNAase and heat on the "RNA-like" product of the RNA polymerase reaction

Treatment	C.p.m. (Incorporation of UTP-2-^{14}C into acid insoluble material)					
	Expt. 1	Expt. 2	Expt. 3	Expt. 4	Expt. 5	Expt. 6
A. None	1614	1566 1550	402	317	174	438 477
B. DNAase	390	366 358	24	15	--	--
C. DNAase (purified)	--	--	--	--	107	--
D. Heat	--	--	--	--	--	447 422

Note: The reaction mixtures were the same as those in Table 6 except that 20-40 mumoles of DNA-phosphate was used as primer. After incubating the reaction mixtures for 2-3 hours, DNAase was added (final concentration 150 µg/ml) and incubated for 5 minutes at 37°, or the reaction mixture was heated for 15 minutes at 90°. In C the DNAase was previously purified on G-50 Sephadex as in the Materials and Methods. Following this the acid insoluble material was assayed for radioactivity as in the standard assay procedure for RNA polymerase (see p. 19).

Furthermore the blanks are lower with the 105,000 x g supernatant fraction than with the protamine sulfate treated "pH 5 enzymes" fraction indicating less sRNA in the 105,000 x g supernatant fraction.

In Table 10 the results of an experiment designed to test the activity of inorganic pyrophosphatase in "pH 5 enzymes" fraction (with and without protamine sulfate treatment) and in 105,000 x g supernatant fraction are shown. It may be seen that the 105,000 x g supernatant is a much better source of the inorganic pyrophosphatase than is the "pH 5 enzymes" fraction.

DEAE-cellulose chromatography of the "pH 5 enzymes" fraction was found to remove over 80% of the sRNA content. The sRNA was calculated by the method of Fraser et al (116). Measurement of the amino acid activating enzymes activity showed that only 25% of this activity was lost in treatment. Dilution of the DEAE-cellulose treated enzyme resulted in further losses (about 50% at 1:5 dilution).

Effect of DNAase and heat on the "RNA-like" product of the RNA polymerase reaction

In Table 11 the results of several experiments designed to test the effect of DNAase and heating on the "RNA-like" product of the RNA polymerase reaction are shown. It may be seen that DNAase (not purified by G-50 Sephadex filtration) solubilized 86-95% of the acid insoluble counts, while the purified DNAase solubilized only 39% of the acid insoluble counts. The heat treatment (15 minutes at 90° which separates the strands of the DNA-sRNA hybrid) solubilized only a few percent of the acid insoluble counts.

DISCUSSION

The incorporation of uridine-2-^{14}C into sRNA is probably a measure of sRNA synthesis since uridine is incorporated into the polynucleotide chain (uridine does not add onto the end of existing RNA molecules). The inhibition of this incorporation in intact mouse Ehrlich ascites tumor cells by actinomycin (Table 1) indicates that the sRNA biosynthesis is DNA-dependent. This is in agreement with similar experiments done in collaboration with Cook *et al* (105). Also, results obtained by Cook *et al* (105) with ^{14}C-methyl methionine incorporation into nuclear sRNA with intact and broken nuclear preparations indicated that the methylation of sRNA of mouse Ehrlich ascites tumor cells occurred in the nucleus.

The inhibition of uridine-2-^{14}C into nuclear sRNA by both DNAase and actinomycin in an "intact nuclear preparation" (Table 2) indicate that sRNA has been synthesized in the nuclear extract and that this synthesis is DNA-dependent. It may be seen in Table 2 that the counts, even in the control samples, were low. Several experiments were done to improve the nuclear preparation without success. The nuclei appear morphologically intact when examined with a microscope but it is likely that enzymes could leak out through the nuclear membrane during the preparation. Also nucleases may be released in the nuclei when they are removed from their cytoplasmic environment. An improved nuclear preparation is probably possible but this was not our objective.

Fractionation of phenol-extracted sRNA (103) on MAK columns (Figure 7) showed two major peaks of U.V.-absorbing material at 260 mμ. These two components of the sRNA were tested by Cook et al (105) for amino acid binding capacity using l-^{14}C-glycine. The component which eluted at low salt concentration (0·4-0·5 M NaCl) was shown to be amino acid acceptor RNA. The second component (eluting at 0·8-0·9 M NaCl) appears to be RNA as well (see p. 28), although not acceptor RNA. The second component was incubated with pancreatic ribonuclease following the method of Bell et al (127). The long incubation period (22 hours) results in the hydrolysis of all non-resistant phosphodiester bonds. There are certain resistant phosphodiester bonds however which are not hydrolysed. These would involve a 2'-O-methyl ribotide or possibly other normal nucleotides such as are resistant to alkaline hydrolysis (128, 129). By dialysing against 2 M NaCl (130) resistant di-, tri- and tetranucleotides can be removed from the ribonuclease-treated solution (against water only mononucleotides are removed). Thus the long incubation period with pancreatic ribonuclease followed by dialysis against 2 M NaCl showed a loss of 94% of the U.V.-absorbing material (of the second component) indicating that the second component is RNA. This second component may be breakdown products of "messenger" RNA and/or ribosomal RNA which are similar enough to acceptor RNA to be extracted by the same procedure (phenol extraction).

The first component of the MAK column chromatography was also shown to be the component which is methylated by ^{14}C-CH_3-methionine, and which is labelled with uridine-2-^{14}C in intact cell preparations (105). Thus the conclusions drawn from the ^{14}C-methyl methionine and uridine-2-^{14}C incorporation experiments were warranted

for these two radioisotopes were incorporated into amino acid acceptor RNA (sRNA).

Incorporation experiments (^{14}C-methyl methionine, uridine-2-^{14}C and 1-^{14}C-glycine) with whole mouse Ehrlich ascites tumor cells showed that the nuclear sRNA had a higher specific activity than the cytoplasmic sRNA (105). This indicates a possible precursor relationship between nuclear sRNA and cytoplasmic sRNA.

It was shown (Table 3) that the first components of nuclear and cytoplasmic sRNAs fractionated on MAK columns have a very similar base composition. This is necessary if nuclear sRNA is in fact a precursor of cytoplasmic sRNA. It was also shown that the base composition of both nuclear and cytoplasmic sRNA is such that more than 67% of the complementary bases could be base-paired. This is in agreement with the studies of the hyperchromic effect which indicated that at least 50-75% of the bases in sRNA are paired (see p. 3).

McFarlane *et al* (90) have demonstrated that DNA-sRNA hybrids can be formed with the mouse Ehrlich ascites tumor DNA and amino acid acceptor RNA. Thus it seems likely that extensive regions exist in the DNA having nucleotide sequences complementary to the sequences of nucleotides in sRNA.

In summary, it seems probable that in the mouse Ehrlich ascites tumor cell, sRNA is made in the nucleus under control of the DNA, is methylated, and is subsequently transferred to the cytoplasm. Further evidence for this DNA control in mammalian and other systems is discussed in the Introduction.

If then, the ascites tumor sRNA is made on a DNA template, it might be possible to synthesize it *in vitro* using DNA that forms hybrids with the sRNA (i.e. DNA that is complementary to the sRNA) as primer and the DNA-dependent RNA polymerase purified by Chamberlin and Berg (78). (This RNA polymerase will use any DNA as primer.)

DNA-dependent RNA polymerase was purified following the method of Chamberlin and Berg (78). The results of several preparations including those of Chamberlin and Berg, for purposes of comparison, are given in Table 4. It may be seen that the specific activity (units/mg) of the initial extract of our preparations is approximately one-half that of Chamberlin and Berg (78). However, since calf thymus DNA was used as primer instead of the salmon sperm DNA used by Chamberlin and Berg (78), and since the units of activity were different (see Table 4) as well, similar results could not be expected. It may also be seen in Table 4 that the streptomycin-sulfate fractionation step provides a 4-9-fold purification while Chamberlin and Berg reported a 40-fold purification. Several unsuccessful attempts were made to locate the source of this loss of enzyme activity (75-90%).

The dependence of the RNA polymerase reaction on DNA is shown in Table 5. Both actinomycin and DNAase, when preincubated with the DNA primer, strongly inhibit the incorporation of UTP-2-^{14}C into acid-insoluble material.

The acid insoluble product, prepared with RNA polymerase and the DNA primer specific for sRNA synthesis, was rendered acid soluble by incubation with alkali (1 N) or pancreatic ribonuclease. We therefore refer to the acid insoluble product as "RNA-like" material. Similar systems have been shown to synthesize RNA in this manner and in fact the RNA is a copy of the DNA template (see Introduction).

Chamberlin and Berg (108) have shown that when single-stranded DNA (ϕx 174) is used to prime the RNA polymerase reaction, DNA-RNA hybrids are formed and no free RNA appears until the amount of RNA product is equal to the amount of template DNA (i.e. all of the DNA template is copied). The results of fractionation of the "RNA-like" product on an MAK column are shown in Figure 9. In this experiment 45·7 mμmoles of sRNA-phosphate (calculated) were synthesized with 122·0 mμmoles of DNA-phosphate (approximately one-third of the DNA is copied) as primer (single-stranded). Therefore we would expect all the RNA product to be in the form of a hybrid with its template DNA. In the light of the foregoing information the finding that all of the "RNA-like" product eluted at the same salt concentration that elutes DNA-sRNA hybrids (0·8-0·9 M NaCl (90)) seems to indicate that the "RNA-like" product formed is similar to sRNA for it forms a similar hybrid with the DNA of the DNA-sRNA hybrid.

Figure 8 showed the results of an experiment similar to that shown in Figure 9 except more of the DNA template was copied (17·0 mμmoles of sRNA-phosphate synthesized with 24·4 mμmoles of DNA template). Some of the "RNA-like" product formed was eluted at the same salt concentration that eluted DNA-sRNA hybrids as in Figure 9. However, the remainder of this product eluted with the purified acceptor RNA that was added as a marker. This seems to indicate that some of the "RNA-like" product is free of its DNA template and is similar to sRNA. Since according to our calculations the DNA template has not all been copied (only approximately 75%), this does not agree with the findings of Chamberlin

and Berg (78).

The possibility of demonstrating that the "RNA-like" product is amino acid acceptor RNA was considered. When 0·23 mg of sRNA (phenol extracted) of mouse Ehrlich ascites tumor cells was fully labelled with ^{14}C-amino acids (^{14}C-algal hydrolysate) it incorporated 4166 c.p.m. (manual counter). Therefore 1 mμmole of this sRNA would incorporate 1350 c.p.m. of ^{14}C-amino acids. (Since one mole of sRNA weighs approximately 25000 grams, one mμmole of sRNA weighs 25 μg. If 0·23 mg of sRNA will incorporate 4166 c.p.m. then 1 mg will incorporate 18112 c.p.m. Therefore 1 mμmole of sRNA will incorporate 25/1000 x 18112 = 452·4 c.p.m. However, it was shown previously (105) that less than 35% of the phenol extracted sRNA has the capacity to bind amino acids. Therefore 1 mμmole of sRNA will bind 3 x 452·4 or 1350 c.p.m. of ^{14}C-amino acids.) Finally since there are 70 nucleotides per sRNA molecule, there are 70 mμmoles of sRNA-phosphate per mμmole of sRNA. It is shown in Table 8 that in the five experiments in which the biological activity of the "RNA-like" product was tested, by incubating this product with amino acid activating enzymes, ATP, Mg^{++} and the ^{14}C-amino acids, enough "RNA-like" product was synthesized to be detected.

In experiment 1 an increase of 180 c.p.m. above the blank is shown. Because of the high blank however this count is not very significant (the increase is only approximately 23% of the blank). The "pH 5 enzymes" fraction was diluted 1:50 rather than 1:20 in the next experiment and the blank was decreased to 407. The test sample, preincubated for 10 minutes with ^{14}C-amino acids and then for 5 minutes with amino acids, incorporated 640 c.p.m. for an increase above the blank (407 c.p.m.) of 233 c.p.m. This indicated that the "RNA-like"

product did have biological activity. However the results of this experiment could not be repeated as can be seen in experiments 3, 4 and 5. Furthermore it was later found that the purified commerical pancreatic DNAase, used in this experiment and in experiments 3, 4 and 5 to destroy the primer DNA and thereby free the sRNA for binding with amino acids, was contaminated with pancreatic ribonuclease. Thus during the 3 or 5 minute preincubation with DNAase and for the 5, 15 or 25 minute incubation with the amino acid activating enzymes, the "RNA-like" product was being attacked by ribonuclease. Thus one could not expect to demonstrate amino acid accepting capacity of the newly synthesized "RNA-like" material for it was degraded.

In Table 11 the results of a study of the effect of DNAase and heat on the "RNA-like" product are shown. It may be seen that the DNAase that was passed through the G-50 Sephadex column appeared to have less ribonuclease activity (50% less). Thus by using a larger column of G-50 Sephadex the ribonuclease can likely be completely removed. The effect of heat (15 minutes at 90°) was measured to test the possibility that only short regions of the DNA template were being copied. Thus once the DNA is hydrolysed the "RNA-like" product would be too small to be acid insoluble. However it was found that the heat treatment did not solubilise any of the counts.

The preparation of the DNA primer is another problem. Mandell and Hershey report that with the MAK columns slight channelling or too high an elution rate will mechanically break up DNA. The single-stranded DNA (after sRNA has been separated by heating) would be particularly susceptible to degradation. Thus a very careful preparation of primer is necessary.

The studies of the various sources of amino acid activating enzymes indicated that the most active source with the lowest blank (for sRNA) was the 105,000 x g supernatant or the DEAE-cellulose chromatographed "pH 5 enzymes" fraction.

In summary it is felt that with a careful preparation of primer, using DNAase free of ribonuclease activity, and with DEAE-cellulose chromatographed "pH 5 enzymes" fraction as a source of activating enzymes, the demonstration of biological activity of the "RNA-like" product of the RNA polymerase reaction will likely be possible.

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