THE EFFECT OF MENGOVIRUS INFECTION ON THE ACTIVITY OF THE DNA-DEPENDENT RNA POLYMERASE OF L-CELLS

BY D. BALTIMORE AND R. M. FRANKLIN

THE ROCKEFELLER INSTITUTE

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Mengovirus is a small RNA virus, approximately 27 m\(\mu\) in diameter,\(^1\)\(^2\) containing only RNA and protein, according to the analyses of Faulkner \textit{et al.} on the closely related encephalomyocarditis (EMC) virus.\(^2\) It is a member of the Columbia SK group of viruses which we have been studying in detail in recent years.\(^3\)\(^4\)

Cytochemical studies on the development of Mengovirus in L-cells, a permanent cell line derived from mouse connective tissue, have shown the localization of viral protein in the cytoplasm along with increased concentrations of RNA in a perinuclear area.\(^4\)\(^5\) Early morphological changes are chiefly cytoplasmic, whereas excessive nuclear degeneration occurs only relatively late in viral development.\(^1\)\(^4\)

From these studies, it may be concluded that the infective particle makes its appearance in the cytoplasm in a perinuclear region. It further seems probable that the viral protein is synthesized in the cytoplasm.

Localization of the site of viral RNA synthesis was attempted by autoradiographic procedures.\(^6\) Whereas in the normal cell the usual pattern of early nuclear and nucleolar uptake of tritiated ribonucleosides into acid-insoluble material was seen, in virus-infected cells only cytoplasmic incorporation was evident, even with a four-min pulse of tritiated uridine.\(^6\) It appears that two events occur: nuclear RNA synthesis is inhibited, and a system for synthesizing RNA appears in the cytoplasm. In this paper, the inhibition of nuclear RNA synthesis is investigated on the enzymatic level and, indeed, a marked decrease in the activity of the DNA-dependent RNA polymerase of the infected cell nucleus was found.

Using cytochemical and biochemical techniques, actinomycin has been shown to be a specific inhibitor of total cellular RNA synthesis.\(^7\)\(^8\) Studies with appropriate viruses indicate that actinomycin inhibits DNA-dependent RNA synthesis.\(^7\) In the course of the present study, we will show that actinomycin inhibits DNA-dependent RNA synthesis in an enzyme fraction obtained from L-cells. Similar observations have been made on purified enzyme preparations isolated from \textit{Escherichia coli} and on an enzyme preparation from another mammalian cell line.\(^10\)

Materials and Methods.—Biological techniques: L-cells (strain 929) were maintained in exponential growth in suspension cultures\(^11\) and harvested when the cell density reached about 5 \(\times\) 10\(^6\) cells/ml. Eagle’s medium,\(^12\) with salts for spinner culture plus non-essential amino acids,\(^13\) was supplemented with 7\% fetal calf serum (Microbiological Associates, Bethesda, Md.).

A description of the strain of Mengovirus used and its interaction with stationary (monolayer) cultures of L-cells has been published.\(^4\)\(^\text{13}\) A similar development of virus has been observed in L-cells in suspension.\(^5\)

Cells were infected with virus as follows: bottles containing a total of 7–8 \(\times\) 10\(^7\) cells were centrifuged. The cells were resuspended in 2.0 ml of stock Mengovirus at a titer of 1–2 \(\times\) 10\(^8\) plaque-forming units (PFU)/ml. Thus, each cell was exposed to 10 or more infectious virus particles, insuring a rapid infection of all cells. After incubation with intermittent shaking for 30 min at room temperature, medium was returned to the bottles and the cells incubated at 37\(^\circ\)C on the roller machine until harvest time. In these experiments, times after infection refer to times after the addition of medium.
Preparation of the enzyme: For an experiment, from $5 \times 10^5$ to $2 \times 10^5$ of control or infected cells were harvested by centrifugation and washed twice in 0.25 M sucrose plus 0.001 M magnesium chloride (sucrose-Mg). Washing and all subsequent steps were performed at 4°C. All reagents contained 0.005 M mercaptoethanol.

The following procedures are essentially those of Weiss and of Goldberg. To prepare enzyme, the packed cells were suspended in 2 volumes of sucrose-Mg, and 3 ml aliquots were mixed with one gm of washed glass beads (Superbrite 110-5005, Minnesota Mining and Manufacturing Company) and homogenized in a Virtis "45" homogenizer for 3 min at a setting of 70. The nuclei were then spun down at 600 × g for eight min, washed twice in 0.25 M sucrose, and then disrupted osmotically by adding 0.05 M Tris buffered at pH 7.2. A short burst of sonic energy was given to the preparation in early experiments, but later results indicated that sonication was unnecessary and decreased the resulting enzymatic activity. To give a final concentration of 0.4 M, 2 M KCl was added to the lysate and the resulting precipitate of DNA and protein was wound up on a glass rod. This DNA-protein aggregate was washed twice in 0.4 M KCl, buffered with 0.05 M Tris, pH 7.2, and resuspended in 0.05 M Tris buffer, pH 7.9, with the aid of a Dounce homogenizer. Following Weiss, this preparation will be referred to as the aggregate-enzyme.

Aggregate-enzyme preparations from either uninfected or infected cells have approximately equal quantities of DNA and protein. RNA is also present at about one third the concentration of DNA. The reaction shows linear kinetics for at least 20 min, whether the enzyme was isolated from uninfected or infected cells.

Preparation of nuclei: Since preparation of the "aggregate-enzyme" as described above is a laborious procedure requiring large numbers of cells, a simpler method for the isolation of the enzyme system was developed. About 10⁹ cells were taken up in 20 ml of distilled water and the cells were homogenized in a Dounce homogenizer by 10 strokes of the loose-fitting pestle and 20 strokes of the tight-fitting pestle. This produced clean nuclei as demonstrated by phase microscopy. 4 ml of a 5× concentrate of sucrose-Mg was added; the nuclei were centrifuged at 600 × g, washed once in sucrose-Mg, and taken up in about 4 ml of sucrose-Mg. The properties of these nuclei are described under Results.

Incorporation experiments: Studies of incorporation of H⁻uridine and H⁻thymidine into RNA and DNA were done by a "coverslip technique" as for autoradiography. Sterile 18 mm square microscopic coverslips (Corning, No. 1) were placed in 60 × 15 mm plastic Petri plates (Falcon Plastics) and seeded with $5 \times 10^6$ cells per plate. The cells, which remain in logarithmic growth as a monolayer, were infected one to two days after inoculation and, at various times after infection, exposed to a tracer compound dissolved in growth medium (H⁻uridine for measuring RNA synthesis and H⁻thymidine for DNA synthesis). Incorporation was stopped after 30 min by rapid fixation of the cells in cold acetic alcohol (3 parts ethyl alcohol to 1 part glacial acetic acid). The preparations were digested with DNAse if RNA synthesis was under study, extracted with 0.5 M perchloric acid in either case, dried, placed on aluminum planchets, and counted in a windowless gas-flow counter.

Analytical methods: Protein was determined by the method of Lowry et al. using Fraction V bovine albumin powder (Armour Pharmaceutical Company, Kankakee, Illinois) as a standard. RNA was estimated by the orcinol reaction, making appropriate corrections for DNA. Standard RNA was prepared from chick embryos by phenol extraction, followed by precipitation with 1 M NaCl. The concentration of standard RNA was determined by its optical density at 260 mµ using the factor: 1 μg RNA-nitrogen/ml equivalent to an optical density at 260 mµ of 0.155 (1 cm path length). DNA was estimated by the diphenylamine reaction. Standard DNA was a commercial preparation of highly polymerized calf thymus DNA. This was hydrolyzed for 40 min at 70°C in 0.5 M perchloric acid and the concentration was then determined by optical density at 267 mµ using the factor OD₅₂₀ × 32.94 = μg DNA/ml.

RNA used in these experiments was prepared from rat liver microsomes by phenol extraction followed by precipitation in 1 M NaCl. DNA was prepared from calf thymus using the duonpol procedure of Kay, Simmons, and Dounce. Actinomycin D was kindly provided by Karl Folkers, Merck, Sharp and Dohme Research Laboratory, Rahway, New Jersey. ATP-8-C was obtained from Schwarz BioResearch, Inc.,
Orangeburg, New York. Nonlabeled ribonucleoside triphosphates were obtained from Sigma Chemical Company, St. Louis, Mo.

Results.—Characteristics of the enzyme and the actinomycin effect: By numerous criteria, the aggregate-enzyme preparation described here has the characteristics of the DNA-dependent RNA polymerase of mammalian cells described by Goldberg. As can be seen in Table 1, the enzymatic activity is dependent on DNA since addition of DNase abolished activity. The product is hydrolyzed by a 16 hr treatment at 37°C with 0.3 N NaOH. Incorporation is not terminal as was shown by treating the product at pH 6.8 for 1 hr at room temperature with sodium periodate (0.001 M) and cleaving the oxidized terminal residue by incubation for 1 hr at 60°C at pH 9.9 (glycine buffer).

<table>
<thead>
<tr>
<th>TABLE 1</th>
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<td>CHARACTERISTICS OF THE AGGREGATE-ENZYME FROM L-CELLS AND THE EFFECT OF ACTINOMYCIN</td>
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</table>

Experiment | 
| Complete | μmoles C¹⁴-ATP incorporated per mg protein |
| 1 | 455 |
| + DNase, 10 μg/ml | <15 |
| - UTP, - GTP | <15 |
| after periodate oxidation | 380 |
| after 0.3 N NaOH hydrolysis | <15 |
| 2 | 398 |
| Complete | |
| + actinomycin, 8 μmoles/ml | 24 |

The complete reaction mixture contained the following constituents in a total volume of 0.5 ml: 50 μmoles Tris, pH 7.9; 1.5 μmoles manganese chloride; 10 μmoles sodium fluoride; 2.5 μmoles mercaptoethanol; 0.1 μC¹⁴-ATP (2.05 or 3.5 μC/mg); 60 μg each of GTP, UTP, and CTP; 0.05 ml of 4°C-saturated ammonium sulfate; and 0.2 ml of aggregate-enzyme (approximately 0.30 mg). After 20 min incubation at 37°C, the reaction tube was cooled to 4°C, 0.5 ml of 0.1 M sodium pyrophosphate was added to release adsorbed ATP and 1 mg of bovine serum albumin added as carrier. 0.5 M perchloric acid was added, and the precipitate was centrifuged off, washed three times with 0.5 M perchloric acid and once with ethanol-ether (1:1), taken up in hydroxide of hyamine (Packard Instruments Company), and counted in a Packard Tri-Carb liquid scintillation counter.

Per milligram of "aggregate" protein, our preparations possess an incorporating activity very similar to that described by Goldberg. The enzyme shows a four-fold decrease in activity when manganese is replaced by magnesium and is activated by high salt concentrations as reported by Goldberg.

We have seen no indications of polyadenylic acid formation as reported by Chamberlin and Berg for a purified DNA-dependent incorporating system from E. coli. This was studied, as in their system, by addition of C¹⁴-ATP without the other triphosphates. Under these conditions, as shown in Table 1, there is no ATP incorporation.

Table 1 also shows that actinomycin can inhibit at least 90 per cent of the enzymatic incorporation of ATP into the acid-insoluble residue.

Effect of virus infection: The aggregate-enzyme isolated from Mengovirus-infected cells has much less activity per unit of protein than that isolated from uninfected cells (Table 2). Since preparation of the aggregate-enzyme requires large numbers of cells, only isolated points in the infectious cycle have been studied in any one experiment. It is evident that activity falls soon after infection and that the inhibition is permanent. The residual activity is sensitive to actinomycin.

Studies with isolated nuclei: Activity of the aggregate-enzyme can be studied in nuclei prepared by the distilled water technique described under Methods. These nuclei are freely permeable to triphosphates, and their properties can be seen from Table 3 to be identical to the properties of the isolated aggregate-enzyme. They show the same dependence on manganese, the same activation by high ionic...
TABLE 2
C14-ATP INCORPORATION BY THE AGGREGATE-ENZYME ISOLATED FROM MENGOVIRUS-INFECTED L-CYTOPHAGES

<table>
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<tr>
<th>Exp. no.</th>
<th>Hr after infection</th>
<th>μmoles C14-ATP incorporated per mg protein</th>
</tr>
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<tbody>
<tr>
<td>6</td>
<td>0</td>
<td>440</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>358</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>282</td>
</tr>
<tr>
<td></td>
<td>9</td>
<td>104</td>
</tr>
<tr>
<td>15</td>
<td>0</td>
<td>446</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>182</td>
</tr>
<tr>
<td></td>
<td>8</td>
<td>46</td>
</tr>
<tr>
<td>23</td>
<td>0</td>
<td>375</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>44</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>77</td>
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Assay conditions are as described in Table 1.

strength, the same sensitivity to actinomycin and DNase, and the same dependence on UTP and GTP.

Figure 1 shows the decrease in activity of aggregate-enzyme in nuclei isolated from infected cells. Within 1 hr after infection with Mengovirus, the enzyme activity begins to decrease, reaching a minimum at about 2 1/2 hours after infection. It should be recalled that virus production starts only 3 hr after infection. Virus which has been inactivated with ultraviolet light does not produce this effect.

TABLE 3
CHARACTERISTICS OF L-CYTOPHAGE NUCLEI ISOLATED BY DISTILLED WATER HOMOGENIZATION
rpm C14-ATP incorporated per mg protein

<table>
<thead>
<tr>
<th>Condition</th>
<th>Incorporation</th>
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<tbody>
<tr>
<td>Complete</td>
<td>485</td>
</tr>
<tr>
<td>-UTP, -GTP</td>
<td></td>
</tr>
<tr>
<td>+ DNase, 10 μg/ml</td>
<td>56</td>
</tr>
<tr>
<td>+ Actinomycin, 8 μmoles/ml</td>
<td>&lt;20</td>
</tr>
<tr>
<td>Manganese, + 1.5 μmoles magnesium chloride</td>
<td>170</td>
</tr>
<tr>
<td>(NH4)2SO4</td>
<td>12</td>
</tr>
</tbody>
</table>

Incubation was for 10 min at 37°C. Other conditions as in Table 1 except that the ethanol-ether washed pellet was dissolved in formic acid, placed on planchete, and counted in a windowless gas flow counter.

In vivo studies: The decrease of nuclear RNA synthesis in L-cells after infection has been described. Since all initial RNA synthesis in these cells is nuclear, the decreased activity of the RNA polymerase can be demonstrated by feeding cells pulses of H3-uridine at various times after infection. Such an experiment has been done using cells grown on coverslips. There is a dramatic decrease of activity of cellular RNA synthesis soon after infection (Fig. 1). This precedes the decrease in synthetic activity of isolated nuclei. Furthermore, there is an increase in RNA synthesis starting at the time of virus maturation. This synthesis is localized in the cytoplasm and is insensitive to actinomycin. Further data on this synthetic process will be published.

Nature of the inhibition of the aggregate-enzyme: Inhibition could be due to a specific or nonspecific effect on the enzyme or to a change or degradation of primer DNA. To study this latter possibility, DNA was isolated by phenol extraction from nuclei of uninfected L-cells and cells infected for 4 and 8 hr. The sedimentation constants and melting profiles were determined. For sedimentation analysis, each preparation was centrifuged in 0.1 M NaCl, 0.1 M phosphate buffer, pH 7.2, at a concentration of 20 μg/ml. UV absorption optics were used on the Spinco Model E centrifuge. The sedimentation constants (S20) of the control and 4- and 8-hr-infected preparations were 23, 29, and 30 respectively. The same preparations
DNA synthesis

RNA synthesis

RNA polymerase activity in nuclei

Time after infection in hours

Per cent of initial incorporation rate

100
80
60
40
20
0

0
1 2 3 4 5 6 7 8

Fig. 1.—Comparison of in vivo RNA and DNA synthesis and activity of the RNA polymerase of isolated nuclei after infection of L-cells with Mengovirus. OOOO = H\textsuperscript{3}-uridine incorporation measured by the "coverslip technique." XXXX = H\textsuperscript{3}-thymidine incorporation measured by the "coverslip technique." --- = C\textsuperscript{14}-ATP incorporation by isolated nuclei. Conditions of assay as in Table 1.

were diluted threefold into distilled water and slowly heated to 95°C while absorption at 260 nm was measured. A melting temperature of approximately 82°C was given by each preparation and no change in the shape of the melting curve was observed. Thus, there seems to be little or no gross change in the hydrodynamic properties, size, or secondary structure of the extractable DNA, even at the end of the infectious cycle when little or no aggregate-enzyme activity is measurable.

In order to test for degradation of DNA, highly polymerized calf thymus DNA was added to aggregate-enzyme preparations from control cells and from cells infected for 2 and 6 hr. The concentration of added DNA in the reaction mixture was 300 μg/ml. The added DNA actually caused a decrease of about 20 per cent in the incorporation of ATP, possibly due to binding of manganese. Since added DNA did not increase enzymatic activity in the control preparations, the enzyme system from both control and infected cells is probably "saturated" with respect to DNA. The similar ratios of DNA to protein in the aggregate-enzyme of control and infected cells further suggest that gross changes in the DNA template cannot be implicated in the loss of activity reported here. This is also shown in experiments where cells were prelabeled with H\textsuperscript{3}-thymidine and the effect of infection on DNA breakdown was studied.\textsuperscript{6} No significant loss of label from the DNA could be demonstrated.

Aggregate-enzyme preparations from control cells and cells infected 4 and 8 hr were assayed for DNase and RNase activity. 0.005 ml aliquots of aggregate-enzyme were incubated with 10\textsuperscript{-3} M EDTA, 0.02 M phosphate buffer (pH 7), and 70 mg rat liver microsomal RNA in 0.5 ml for RNase assay and with 10\textsuperscript{-3} M magnesium chloride, 0.02 M phosphate buffer (pH 7), and 150 μg DNA in 0.5 ml for DNase assay. After 60 min, acid-insoluble material was precipitated with 0.5 M perchloric acid and the OD\textsubscript{260} of the supernatant was read. No DNase activity could be detected. The slight amount of RNase activity was the same in both infected and control preparations. Furthermore, the relative amount of RNA in the
isolated aggregate-enzyme did not change markedly throughout the course of infection. Also, cytochemical and biochemical studies did not suggest any marked change in the amounts of RNA present in the nucleus or in the whole cell until rather late in infection.4, 6

According to these preliminary data, therefore, it does not seem likely that activation of an enzyme which may destroy either the DNA template or the RNA product could be responsible for the inhibition.

Figure 1 shows that DNA synthesis is normal for more than three hr after infection. Thus, the inhibition of RNA synthesis is not an inhibition of all DNA-dependent reactions but is limited to the RNA-polymerase. Also, the acid and alkaline phosphatase activities of a 600 × g cytoplasmic supernatant were shown to be unaffected up to 8 hr after infection, further indicating that there is no non-specific diminution of the cell's enzymatic capacities.

Thus, our data, at present, would suggest that the inhibition may be due to a direct effect on one or, at most, a few enzymes. Experiments to test these hypotheses are in progress.

Discussion.—The above data show that infection of L-cells with Mengovirus causes a marked decrease in the activity of the DNA-dependent RNA polymerase (aggregate-enzyme). A decrease is seen in in vivo nuclear RNA synthesis and in the activity of isolated nuclei or aggregate-enzyme. These results correlate well with the autoradiographic studies which show a marked decrease of nuclear incorporation after infection.5 It should be emphasized that the enzyme inhibition seen in these studies occurs before mature virus or viral RNA is demonstrable. The difference between the kinetics seen in vivo and in vivo is almost certainly a reflection of the different manner in which suspension and monolayer cultures are infected. The lower final plateau of the coverslip cultures could well be an artifact since an inhibition would be magnified by dilution of exogenous label caused by an intracellular accumulation of unlabeled precursor.29

Studies on the biochemical events associated with the multiplication of EMC virus in mouse ascites cells also are supported by our findings.27 Synthesis of RNA and DNA was followed in infected and control cells using C14-orotic acid label. After exposure to the tracer, cells were homogenized and fractionated. Nuclear RNA synthesis decreased rapidly after infection, following approximately the kinetics seen in the present study. For example, 4 hr after infection, only 20 per cent of control activity was found in nuclear RNA. These data further showed that DNA synthesis was normal up to about 4 hr after infection, decreasing slowly thereafter.27

Neither the synthesis of cellular DNA nor its integrity are necessary for the replication of Mengovirus.28, 29 Employing actinomycin-inhibited cells, Reich et al.7 showed that DNA function, as expressed by nuclear RNA synthesis, is not a prerequisite for the multiplication of Mengovirus. The present study shows that, in fact, the virus infection directly inhibits the DNA-dependent RNA synthesis. In this fashion, RNA precursors may be conserved for use in synthesizing viral RNA rather than being used for making cellular RNA.

The present findings may help to explain how a virus kills a cell. Inhibition of nuclear RNA synthesis may be expected to interfere with protein synthesis and lead to eventual cell death. On the other hand, this is by no means the complete
explanation of viral cytopathogenesis since an actinomycin-inhibited cell, which is also deprived of all nuclear RNA synthesis, undergoes much less drastic morphological changes.4, 7, 8

Cellular RNA synthesis stops soon after infection of E. coli with T2 bacteriophage.30 DNA breakdown also begins immediately after infection,21 and this seems to explain the rapid cessation of RNA synthesis. Although the breakdown of cellular DNA is dependent on prior protein synthesis,21 some data suggest that the breakdown is due to the activation of a cellular enzyme.32 Our results with Mengovirus-infected cells are reminiscent of these studies with bacteriophage. However, although a granulation of the chromatin is seen soon after infection,4 there is no direct indication of extensive DNA breakdown. DNA, isolated from cells 8 hr after they have been infected with Mengovirus, has a high sedimentation coefficient. Further, no loss of DNA is demonstrable after infection. Therefore, although the two processes appear to be analogous, they may involve fundamentally different mechanisms: in one case DNA breakdown and in the other case inhibition of the enzyme responsible for RNA synthesis (cf. note added in proof).

A further analogy with bacteriophage infection is possible. Volk and Astrachan24 showed that although total RNA synthesis is inhibited after bacteriophage infection, there is a small fraction of rapidly-turning-over RNA that is synthesized. This fraction has the same base composition as the DNA of the virus and it is felt that this RNA is messenger RNA. After infection of L-cells with Mengovirus, again cellular RNA synthesis stops and a new fraction of RNA is synthesized, as is seen by its intracellular localization. It has yet to be demonstrated that this RNA is viral RNA, although this conclusion is probable. Since the virus directs the synthesis of new protein, it can be imagined that this RNA bears the function of a messenger directing the synthesis of new protein species.

Note added in proof.—Using puromycin as an inhibitor of protein synthesis, the inhibition of normal RNA synthesis in Mengovirus-infected L-cells has been shown to be dependent on a virus-induced "early" protein synthesis (R. M. Franklin and D. Baltimore, in Basic Mechanisms in Animal Virus Biology, Cold Spring Harbor Symposia on Quantitative Biology, vol. 27 (1962), in press). There is also clear indication of a specific T4 bacteriophage-induced early protein synthesis mediating the inhibition of E. coli host cell RNA synthesis (M. Nomura, K. Okamoto, and K. Asano, J. Mol. Biol., 4, 376 (1962)). It may be that inhibition of normal RNA synthesis does precede breakdown of host cell DNA, at least in some phage-infected cells. Therefore the analogy between inhibition of normal RNA synthesis by a mammalian RNA virus and some DNA bacteriophages may be more than superficial.

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A GENERAL METHOD FOR THE ISOLATION OF RNA COMPLEMENTARY TO DNA

BY E. T. BOLTON AND B. J. McCARTHY

DEPARTMENT OF TERRESTRIAL MAGNETISM, CARNEGIE INSTITUTION OF WASHINGTON

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During the past few years evidence has accumulated that a class of RNA molecules which resemble DNA in base composition is synthesized in living cells. It has been proposed that this RNA is an intermediary in the transfer of genetic information from DNA to protein. It is therefore of importance to have available a general method for the purification of such RNA molecules in order to study their chemical character and information content. Hall and Spiegelman showed that T2-specific RNA could be hybridized with heat-denatured T2 DNA and the hybrid isolated by cesium chloride density gradient centrifugation. With this method Hayashi and Spiegelman were able to demonstrate small amounts of DNA-like RNA in nongrowing bacteria. Base analyses of rapidly labeled RNA strongly suggest the existence of complementary RNA in several species of growing bacteria but attempts to purify such molecules have been only partially successful.

Bautz and Hall have made a notable advance toward purifying RNA molecules with a base sequence complementary to DNA. They used phospho-cellulose ace-