CHROMATOGRAPHIC SEPARATION OF ANNEALED AND ENZYMATICALLY SYNTHESIZED RNA-DNA HYBRIDS

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ABSTRACT A procedure is described for the chromatographic detection and isolation of DNA-RNA hybrids on columns of methylated albumin coated on kieselguhr (MAK). Its use is illustrated with both annealed and enzymatically synthesized hybrids. The method has the advantage of a wide range in capacity and resolution and permits actual isolation of the hybrid structure. It is uniquely effective in experiments involving hybridization with small DNA fragments.

INTRODUCTION

Isotopic labeling, combined with centrifugation in cesium chloride density gradients, permitted (Hall and Spiegelman, 1961) the demonstration of specific hybrid formation between T2-RNA and homologous DNA. In these investigations, swinging bucket rotors were used to allow the isolation and characterization of the hybirds formed during the annealing process. This method of testing for sequence complementarity was subsequently successfully employed in a number of investigations (Gros *et al.*, 1961; Geiduschek *et al.*, 1961; Hayashi and Spiegelman, 1961; Scherrer *et al.*, 1963).

The sensitivity of hybrid detection was increased (Yankofsky and Spiegelman, 1962*a* and *b*) by taking advantage of the ribonuclease resistance of DNA-RNA hybrids to eliminate adventitious pairing. The specificity of the detection was further refined (Yankofsky and Spiegelman, 1962*b* and 1963) by testing for competitive interactions during annealing, using two identifying isotopic labels on the competing RNA molecules. These extensions made possible the identification of complementary sequences corresponding to approximately 0.01 per cent of the total genome of *Escherichia coli*. With the aid of these devices, Yankofsky and Spiegelman showed (1962*a* and *b*, 1963) that sequences existed in homologous DNA complementary to the two ribosomal RNA components and that the two sets of sequences were different. Identical procedures were successfully employed in establishing DNA sequences complementary to ribosomal RNA in plants (Chip-

chase and Birnstiel, 1963) and to s-RNA in bacteria (Goodman and Rich, 1962); Giacomoni and Spiegelman, 1962).

The studies noted all used the time-consuming separation by equilibrium centrifugation in CsCl gradients. The obvious potential usefulness of the hybridization test stimulated a search for more convenient methods for detecting hybrids. Bautz and Hall (1962) introduced a new approach by seeking to bind the DNA covalently to columns of phosphocellulose acetate. They used glucosylated DNA and diclohexylcarbodiimide as the coupling agent. Further investigation of this procedure by Bolton and McCarthy (1962) revealed that covalent linkage was unnecessary since most of the DNA was physically trapped even on omission of the chemical coupling step. Recognizing that any process of physical immobilization would do, they developed the very useful agar column procedure. Using the same principle, Nygaard and Hall (1963) reported a conveniently rapid detection of DNA-RNA hybrids which depends on selective adsorption of single-stranded DNA on nitrocellulose membrane filters.

The original density centrifugation method and the newer procedures mentioned depend on the existence of relatively large DNA fragments. In the course of investigations with the single-stranded ϕX174 bacteriophage, it was found that none of these procedures could be used with certainty. The difficulties stemmed primarily from the small size (1×10^6 and less) of the DNA which had to be employed in the hybridizations. These fragments were not effectively trapped in either agar gels or on membrane filters. As a result, we were led to develop a new procedure which emerged from the observation (Hayashi *et al.*, 1963*a* and *b*) that single-stranded DNA of the bacteriophage, ϕX174, is readily separated from its double-stranded "replicating form" (RF-DNA) on columns of methylated albumin. It was presumed that RNA hybridized to DNA would chromatograph in approximately the same position as denatured DNA to which it is complexed, the position depending on the percentage of the DNA hybridized.

It is the purpose of the present paper to provide details of this method and illustrate its applicability to the separation of DNA-RNA hybrids formed by both annealing as well as by enzymatic synthesis. The procedure is of value in any experiment which is forced to deal with hybrids involving small DNA fragments. In addition, it has the added advantage of the flexibility easily attainable by variation in the kind of salt gradients employed during elution from the columns.

MATERIALS AND METHODS

Preparation of Nucleic Acids. ϕX174 RF-DNA and the corresponding single-stranded DNA were isolated and purified chromatographically as described by Hayashi, Hayashi, and Spiegelman (1963*a* and *b*). T2-DNA was isolated by the procedure of Grossman *et al.* (1961) and bacterial DNA was purified according to Marmur (1961). All DNA preparations used were tested (Hayashi *et al.*, 1964) for the presence of nuclease by examining for acid soluble with radioactive substrates after extensive (20

hours) incubation at 37°C. The material employed in the experiments described showed negligible activity. *E. coli* RNA was labeled in log phase for 90 seconds with H^3-uridine (5 μc/10μg/ml) and purified according to the method of Hayashi and Spiegleman (1961). T2-specific RNA, labeled with H^3-uridine between 10 and 13 minutes after infection, was prepared according to Kano-Sueoka and Spiegelman (1962). The purity of the RF-DNA was monitored by constant specific radioactivity on repeated chromatography, banding in cesium chloride gradients, and analysis of sedimentation patterns in the analytical ultracentrifuge. Fragmentation of DNA by sonic oscillation was accomplished with a Raytheon oscillator at 4°C, (Raytheon Co., Waltham, Massachusetts). The DNA was dissolved (50 μg/ml) in SSC and treated for the desired period. The S_w^{20} of the DNA preparation was measured with a Spinco model E analytical ultracentrifuge (Beckman Instruments, Inc., Palo Alto, California) using ultraviolet optics in SSC (0.15 Na Cl, 0.015 M Na citrate, pH 7.4) at 20°C.

Enzyme Preparation. Purification of the DNA-RNA polymerase followed the procedure of Chamberlin and Berg (1962). It was isolated from *E. coli* (C122) cells harvested in log phase and stored at −15°C. The final purification step involves elution of the enzyme from the DEAE (diethylaminoethyl) column with 0.23 M KCl. Regions of peak specific activity were employed in the experiments described.

Preparation of Columns of Methylated Albumin Coated on Kieselguhr (MAK). The protocol of Mandell and Hershey (1960) was followed with one modification in the preparation of the methylated albumin. The mixture of albumin, methanol, and HCl was incubated for at least 5 days at 37°C. In our hands this resulted in a preparation which yielded consistently superior separations. The suspension of protein-coated kieselguhr (MAK) is prepared as follows: A suspension of 20 gm of kieselguhr in 100 ml of 0.1 M NaCl buffered with 0.05 M $Na\text{-}PO_4$ buffer at pH 6.5 is heated to boiling and cooled. Add 5 ml of 1 per cent methylated albumin solution and stir. It is then washed with 300 ml of 0.1 M NaCl–0.5 M $Na\text{-}PO_4$ buffer and then made up to 100 ml with the same buffer.

The details of our "standard" column are as follows; bottom layer, 0.5 gm of powdered paper; first layer, 4 gm of kieselguhr plus 1 ml of 1 per cent methylated albumin solution; second layer, 3 gm of kieselguhr plus 5.0 ml of suspension of methylated albumin coated on kieselguhr (MAK); top layer, 0.5 gm of kieselguhr. Excess buffer is driven down to slightly above the level of the packed column by applying air pressure at about 2 lb/in.^{2} All other column sizes are denoted by x, $1/2x$, $4x$, *etc.* The dimensions are in millimeters.

Size	Diameter	Height (excluding top and bottom layers)
$1/4x$	28	13
$1/2x$	28	26
Standard (x)	28	51
$4x$	42	92

Preparation of P^{32}-riboside Triphosphate. UTP (uridine triphosphate) labeled with P^{32} in the alpha phosphorus was synthesized according to the detailed procedure given by Haruna *et al.* (1963). The initial specific activity was about 3.3×10^8 CPM/μmole. All counting was done in a Packard (Packard Co., Des Plaines, Illinois) liquid

scintillation spectrometer. Acid-precipitable material was washed with TCA (trichlorecetic acid) and dried on coarse membrane filters (Schleicher and Schuell, Keene, New Hampshire).

The Enzymatic Synthesis of DNA-RNA Hybrids. The reaction mixture (1 ml) contains 24 μg of template DNA, 40 μmoles of Tris buffer at pH 7.9, 1 μmole of $MnCl_2$, 4 μmoles of $MgCl_2$, 46 μmoles of KCl, 12 μmoles of β mercaptoethanol, 40 to 100 μg of enzyme, 500 mμM of ATP, CTP (cytosine triphosphate), GTP (guanosine triphosphate), and UTP^{32}. The extent of hybrid synthesized was varied by the time and temperature of incubation.

Hybridization by Annealing. The details of formation and detection of DNA-RNA hybrids are as described previously by Hayashi, Hayashi, and Spiegelman (1963*b*). Denatured DNA and RNA are mixed in 2 × SSC[1] buffer and incubated at 42.5°C for 16 hours. The reaction mixtures are treated with pancreatic ribonuclease under the standard conditions of the following section or as detailed in the text.

Standard RNAase Treatment of DNA-RNA Hybrids. Pancreatic RNAase (Sigma Chemical Company, St. Louis, 5 × crystallized, 49 Kunitz unit/mg., Lot R92B-89) is dissolved in pH 5 SSC (0.15 M NaCl, 0.015 M Na citrate adjusted to pH 5 by adding 1 M citric acid) at the concentration of 2 mg/ml. The solution is heated to 80°C and kept for 10 minutes, followed by a quick cooling at 0°C. RNAase, thus treated, is added at 30 μg/ml to the DNA-RNA mixtures dissolved in 2 × SSC[1] and incubated at 26°C for 30 minutes. The reaction mixture is then chilled, diluted 50 times with the starting buffer, and loaded on an MAK column. When estimates of the proportion of RNA hybridized are required, an aliquot of the reaction mixture is precipitated with cold 10 per cent TCA, filtered onto membrane filters, washed, dried, and then inserted into liquid scintillation vials.

RESULTS

The Detection of DNA-RNA Hybrids on Columns of Methylated Albumin Coated on Kieselguhr (MAK). To illustrate the use of MAK columns for detecting hybrids, experiments were carried out hybridizing P^{32}-DNA (*E. coli*) with H^3-RNA (*E. coli*) labeled for 90 seconds in log phase. Subsequent to the hybridization period, the reaction mixture was exposed to ribonuclease at 26°C and then loaded on the column. The resulting elution pattern is shown in Fig. 1A. We see here the appearance of a ribonuclease-resistant structure indicated by coincident peaks of H^3-RNA and P^{32}-labeled DNA. Fig. 1B shows the result of a control experiment in which the same mixture of DNA and RNA was not exposed to the annealing treatment. There is clearly no evidence of ribonuclease-resistant RNA in the region of the P^{32}-DNA.

To insure that the peak of H^3 and P^{32} (Fig. 1A) possesses the density characteristic of DNA-RNA hybrid, samples of the tubes indicated (numbers 35, 38, and 41) were subjected to centrifugation in cesium chloride gradients, as shown in Fig. 2. The absence of free RNA at the bottom of Fig. 2A shows the effectiveness of the ribonuclease pretreatment. Virtually all the resistant H^3-RNA is complexed

[1] SSC, 0.15 Na Cl, 0.015 M Na citrate, pH 7.4.

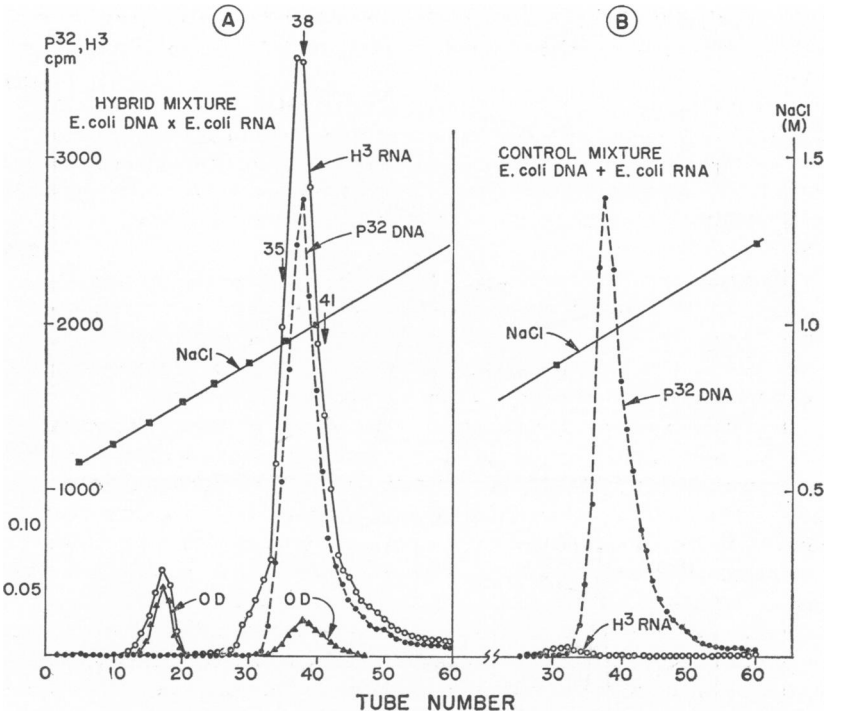

FIGURE 1 Chromatography of hybridized and control mixtures. A. Experimental: 40 μg of P^{32} heat-denatured *E. coli* DNA was mixed with 1 mg of H^3-RNA of *E. coli* in 3 ml of 2 $\times$ SSC (Marmur, 1961). The reaction mixture was incubated at 42.5°C for 16 hours, the mixture was chilled to 26°C, and 30 μg/ml of pancreatic RNAase added. After 30 minutes' incubation at 26°C, the reaction mixture was poured into 50 ml of 0.5 M NaCl, 0.05 M phosphate buffer, pH 6.5 (starting buffer), containing 0.5 g kieselguhr. The mixture was loaded on a 1/4 size MAK column as described in Methods. The column was washed with 200 ml of starting buffer and the NaCl gradient then instituted. Acid precipitable P^{32} and H^3 were assayed on fractions collected. B. Control: The same amounts of heat-denatured P^{32}-DNA and H^3-RNA were incubated separately under conditions identical with A. After 16 hours, the two were mixed, loaded onto the column, and eluted as in A.

with the P^{32}-DNA and occupies a density position very close to that of the marker single-stranded DNA (OD profile). The other tubes (Figs. 2B and 2C) show that all the samples contain RNA-DNA complexes of about the same density. In these experiments, the proportion of RNA to DNA in the hybrid is comparatively low so that the density is very similar to that of single-stranded DNA.

It was of interest to examine the column method for its ability to differentiate between homologous and heterologous hybridizing mixtures. Hybridizations were carried out between H^3-labeled T2-RNA and *E. coli* DNA in one case, and T2-DNA in the other. In this experiment, care was exercised to ensure that all the cells of the

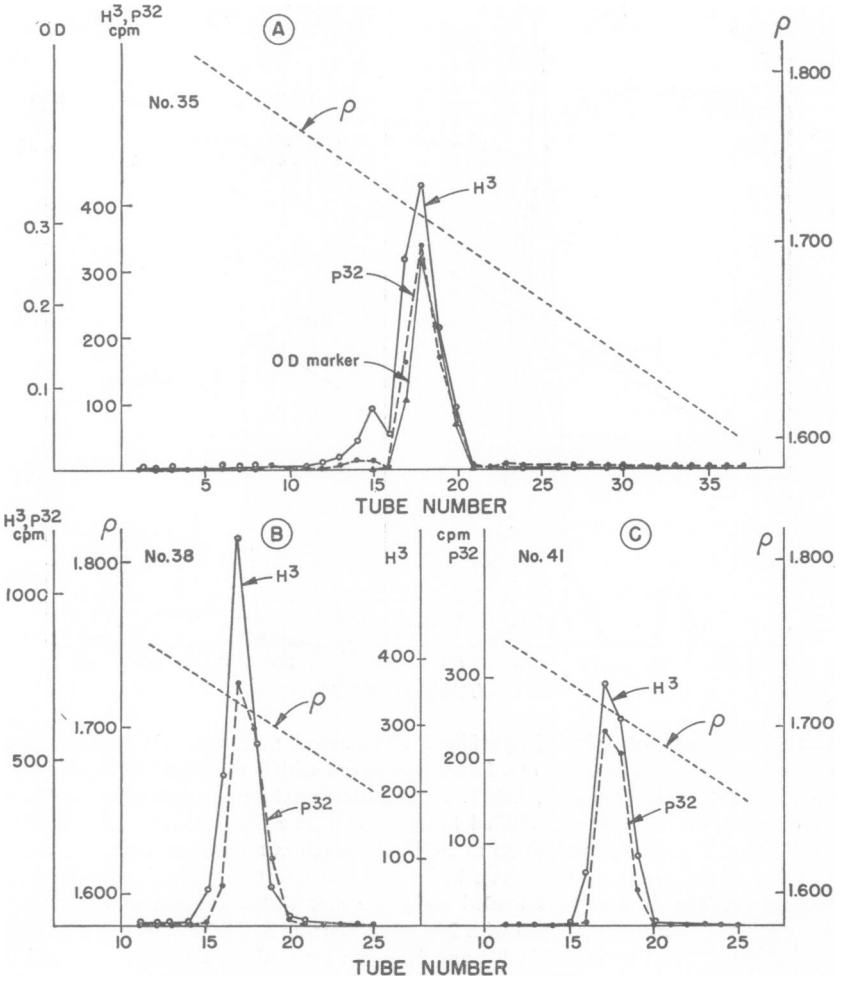

FIGURE 2 Equilibrium density gradient centrifugations of chromatographic samples. Aliquots from tubes 35, 38, and 41 (Fig. 1A) were centrifuged in CsCl (input ρ of 1.720). After 70 hours, the contents were dripped from the bottom of the centrifuge tube and the acid-precipitable counts measured. Heat-denatured *E. coli* DNA was added as an OD marker (not shown in B and C).

culture were infected. No ribonuclease-resistant RNA eluted with the P^{32}-DNA when T2-RNA was challenged with *E. coli* DNA (Fig. 3A). On the other hand, an excellent hybrid structure formed between the RNA synthesized after T2 infection and the T2-DNA (Fig. 3B). These results clearly establish that no detectable messages are produced from the host genome subsequent to infection with the virulent DNA virus.

Similar specificity tests were carried out with other heterologous hybridizations

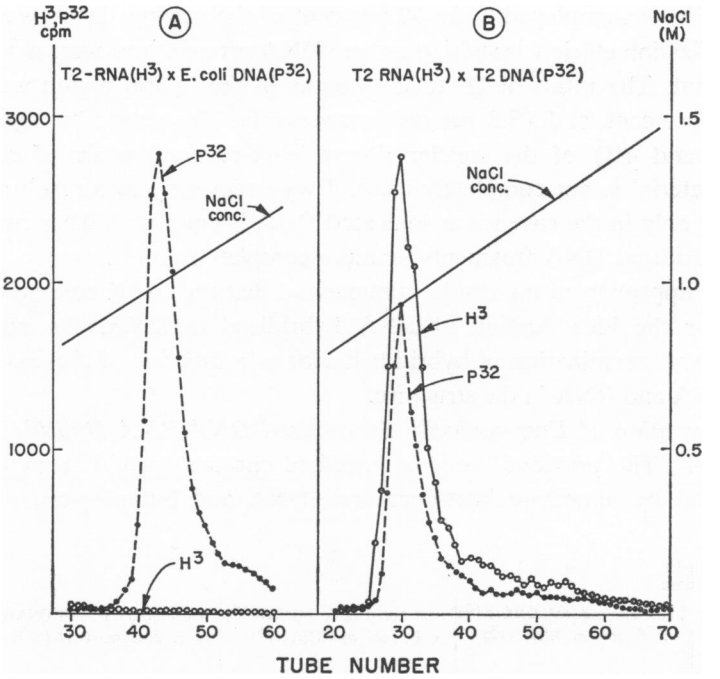

FIGURE 3 Chromatography of homologous and heterologous hybridizing mixtures. T2 infected *E. coli* BB was pulse-labeled for 3 minutes at 37°C, 10 minutes after infection with H^3-uridine. Total RNA was isolated as in Methods. 1 mg of this H^3-labeled RNA was incubated with heat-denatured P^{32} *E. coli* BB DNA (A) or heat-denatured P^{32}-T2 DNA (B) and the mixtures chromatographed. Details of hybridization and subsequent RNAase treatment are as in Fig. 1.

(*e.g., Pseudomonas aeruginosa* DNA × *E. coli* RNA; *Bacillus subtilis* DNA × T2-RNA; *etc.*). In none of these were hybrid structures observed in the elution profile.

Size of DNA and Elution Efficiency. Sueoka and Cheng (1962) had already recognized that MAK columns gave rather poor recovery of the input denatured DNA. Our own experience was in agreement since 40 to 50 per cent of the added denatured DNA was recovered in three bacterial cases and only 20 per cent of the T2-DNA was finally eluted. We had, however, observed that more than 70 per cent of single-stranded ϕX174-DNA was routinely recovered from these columns with the same elution program.

The possibility that the higher recovery was related to the lower molecular weight, was tested by degrading C^{14}-DNA of *E. coli* from 30S to 7.8 and 6.7S by exposure to ultrasonic oscillation. Each of the DNA preparations was then heat-denatured and used in hybridization tests with H^3-labeled *E. coli* message fraction. The results obtained are shown in Figs. 4A and 4B where 80 to 90 per cent of the sonicated DNA eluted from the column. In Fig. 4C where unsonicated

E. coli DNA was employed, only 40 per cent of the original DNA was recovered. The hybridization efficiencies with the three DNA preparations were not distinguishably different. The ratios of H^3 to C^{14} counts in the hybrid region were 15.7 per cent, 15.8 per cent, and 15.3 per cent respectively. The reason for the appearance (Figs. 4A and 4B) of the smaller ribonuclease-resistant peaks of apparent hybridized material is not completely clear. They occur only as a result of annealing and appear only in the case when sonicated DNA is employed. They may represent smaller hybridized DNA fragments or more complete hybrids.

It would appear from the results summarized that the MAK column can, indeed, be used for the identification of RNA hybridized to DNA. We now turn our attention to an examination of hybrid behavior as a function of the relative proportions of DNA and RNA in the structure.

Separation of Enzymatically Synthesized DNA-RNA Hybrids of Differing Proportions. The previous sections described chromatographic separation of hybrids formed by annealing heat-denatured DNA and homologous, naturally oc-

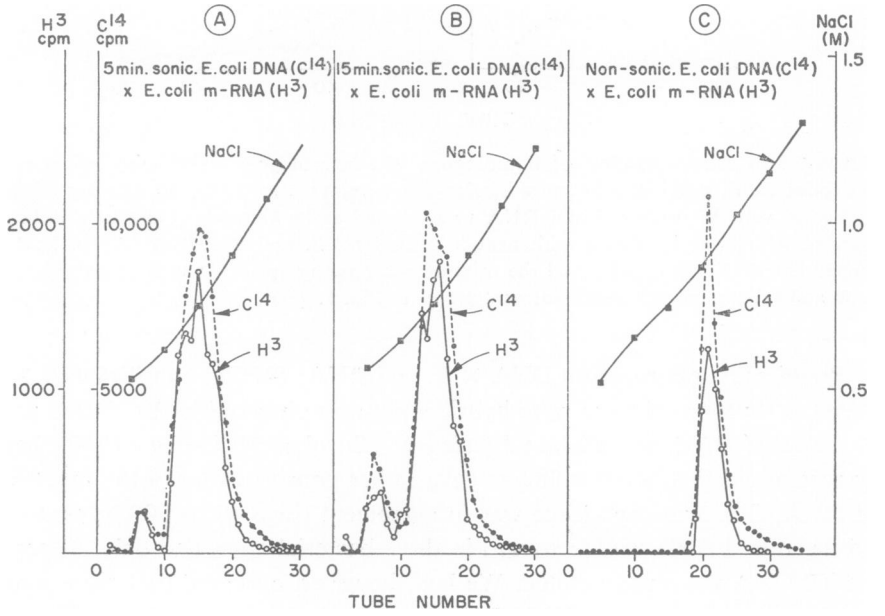

FIGURE 4 Chromatography of hybrids involving DNA of different sizes. Samples of C^{14}-*E. coli* C DNA (30S) were sonicated with a Raytheon sonic oscillator for 5 minutes and 15 minutes in SSC buffer. Sedimentation coefficients, measured in the analytical ultracentrifuge with ultraviolet optics, were 7.8S and 6.7S, respectively, in SSC. The original and sonicated DNA were heat-denatured and hybridizations were carried out with pulse-labeled RNA. The mixtures were then RNAase-treated and chromatographed as detailed in Methods and Fig. 1. The ratio of RNA to DNA in the reaction mixtures were kept constant. (A) 5 minute sonic DNA × H^3-RNA. (B) 15 minute sonic DNA × H^3-RNA. (C) Original DNA × H^3-RNA.

curring RNA messages. The proportion of RNA in the hybrid complexes, in these cases, is relatively small and not readily varied. Consequently, the behavior of the hybrid structure on the column was very similar to single-stranded DNA.

To examine the effect of varying the ratios of RNA to DNA in the hybrid, we turned our attention to the small DNA bacteriophage ϕX174. This material had the obvious advantage provided by a homogeneous population of identical single-stranded DNA molecules. Further, the extensive study of Chamberlin and Berg (1964) and Sinsheimer and Lawrence (1964) had demonstrated that when ϕX174 was used as a template for the transcribing enzyme, the newly formed RNA is initially hybridized to the DNA. Their data indicate that eventually a hybrid is formed which contains approximately a 1:1 ratio of RNA to DNA as evidenced by the position it assumes in a gradient of cesium sulfate. It appears that the density of the hybrid is related to the extent of RNA synthesis permitted.

We wanted to see whether a similar distinction was attainable with MAK columns. RNA-DNA hybrids were enzymatically synthesized as described under Methods using single-stranded DNA isolated from the bacteriophage ϕX174 as the template. The course of the synthesis was monitored by the incorporation of UTP^{32}, in the presence of the other three riboside triphosphates. The extent of synthesis was controlled by variation of both temperature and time of incubation.

At the termination of the reaction, the mixture was chilled and the nucleic acids were isolated by the phenol method and dialyzed against 2 × SSC. Since the amount of the template DNA in the reaction mixture and the specific activity of the UTP^{32} were both known, the ratio of DNA to RNA in the final product could be readily estimated.

An examination was made of the ribonuclease-resistant proportion found in the final products isolated after varying amounts of RNA were synthesized. This was carried out under our standard conditions (Methods) and in every case included an internal control of H^3-23S ribosomal RNA to monitor the effectiveness of the digestion on unhybridized RNA. The results obtained with products which ranged in DNA:RNA from 1:0.3 to 1:1.3 are given in Fig. 5. When the synthesis is limited to 0.3 or 0.5 of the DNA, the proportion of RNA resistant to enzymatic digestion is high (about 80 per cent). As soon, however, as the synthesis approaches the 1:1 ratio, the per cent resistance drops to 60 per cent, and if allowed to go to a ratio of 1:1.3, it falls to 45 per cent. It appears that as the synthesis progresses, more and more of the RNA synthesized is freed from the hybrid structure and becomes, therefore, susceptible to RNAase treatment. This conclusion is in agreement with that of Chamberlin and Berg (1964) and is further supported by the density gradient and chromatographic analysis detailed below.

The RNAase resistance of the hybrid structure is a particularly useful property and it is of some interest to specify the conditions under which it can be usefully employed. Chamberlin and Berg (1964) found that the enzymatically synthesized

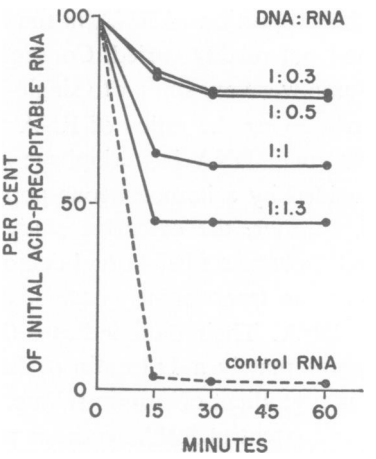

FIGURE 5 RNAase resistance of RNA synthesized on single-stranded DNA. The reaction mixtures (1.0 ml) contain the components listed in Methods with the following changes; 10 μg of ϕX174 DNA; UTP^{32}-labeled in the α-phosphorous. Incubation was carried out at 25°C for 7 minutes (1:03 hybrid) at 36.5°C for 10 minutes (1:0.5 hybrid), for 20 minutes (1:1 hybrid) and for 90 minutes (1:1.3 hybrid). The reaction was terminated by adding water-saturated phenol. The nucleic acid was then extracted from the water phase, and dialyzed against 2 × SSC. RNAase treatment of the final product was performed under the standard conditions. As an internal control, H^3-23S RNA of *E. coli* was added to each reaction mixture. Aliquots were taken from the reaction mixture at the indicated times, precipitated with 10 per cent TCA and acid-precipitable counts determined and expressed as per cent of 0 time.

hybrid appeared to be less stable towards pancreatic ribonuclease than had been reported for other annealed hybrids (Yankofsky and Spiegelman, 1962*b;* Giacomoni and Spiegelman, 1962; Goodman and Rich, 1962; Chipchase and Birnstiel, 1963). We, therefore, subjected this question to some further investigation which we briefly summarize.

The 1:1 DNA to RNA synthesized product was chosen and Fig. 6 summarizes its resistance with variation in temperature, concentration of ribonuclease, and molarity of sodium chloride. Internal controls of H^3-labeled free RNA were included and in all cases the control RNA was virtually completely destroyed. Comparison shows that the hybrid is not stable to 5μg/ml of RNAase at 0.1 M NaCl and 37°C. At 0.3 M NaCl it is stable to even 30μg/ml of enzyme at 37°C. This may explain the results of Chamberlin and Berg (1964), who ran their reaction at 37°C at low ionic strength. The comparative instability of enzymatically synthesized hybrids may be related to the small fragments generated when the RNA polymerase is employing single-stranded DNA as a template. The detailed conditions required for resistance must be examined for each type of hybrid studied. Our experience has been that with pure, DNAase-free, ribonuclease, our standard condi-

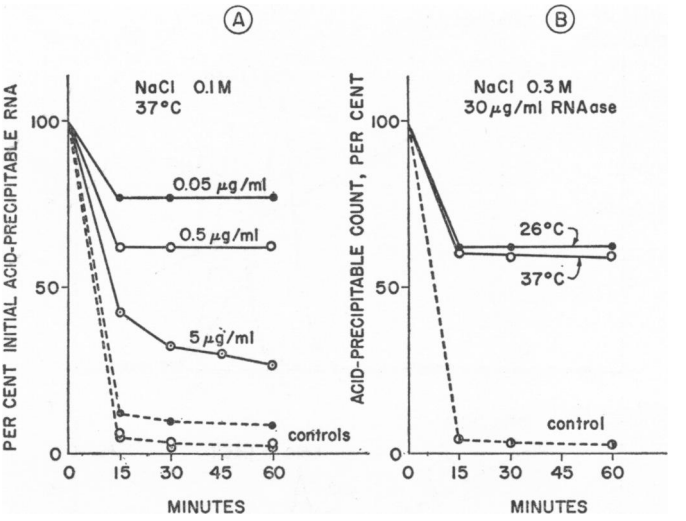

FIGURE 6 RNAase sensitivity of synthesized RNA. The product corresponding to a synthesis of RNA to DNA of 1:1 of Fig. 5 was exposed to RNAase under the conditions specified in the figure. The buffer used in experiments of 6A was Tris-HCl (0.01 M, pH 7.4) and for that of 6B was 2 × SSC.

tions (0.3 M NaCl; 26°C, 30μg/ml of RNAase) yields consistent and reproducible plateaus of resistance which are stable for more than an hour.

The results of equilibrium density gradient centrifugations in Cs_2SO_4 of the various products are shown in Fig. 7. It will be noted that when the extent of synthesis was 1:0.3, there is virtually no free RNA to be found in the corresponding density region. When, however, the reaction is allowed to proceed to a 1:1 ratio of denatured RNA, approximately 20 per cent of the synthesized RNA bands in the density region characteristic of free RNA. The amount of unhybridized RNA increases to approximately 50 per cent when the synthesis proceeds to a ratio of RNA to DNA of 1.3.

Note should be taken of the small shoulder observed in Fig. 7, in all cases on the right side of the hybrid peak and coinciding with the marker single-stranded DNA. These shoulders are ribonuclease-sensitive (see Fig. 8) and may, therefore, represent incomplete synthesis involving hybrids of very short regions of the DNA. Their presence in even limited synthesis where no RNA is observed in the density region corresponding to free RNA (Fig. 7A) may explain the presence of 20 per cent RNAase sensitive material observed in such products (Fig. 5). This would mean that the true hybrid peak is completely resistant. We have further examined the nature of the RNAase-resistant structures formed in such synthetic reactions by means of electron photomicrographs and found them to be circular structures (Bassel *et al.*, 1964).

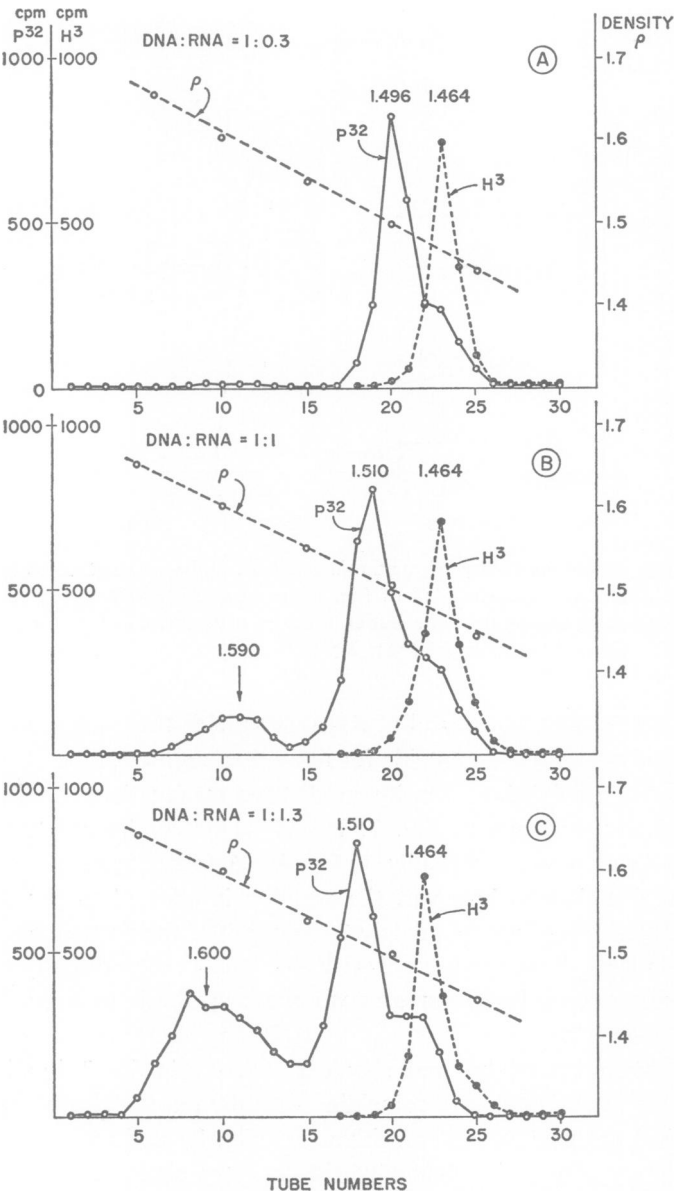

FIGURE 7 Equilibrium density gradient centrifugations. Cs_2SO_4 was added to the products synthesized in the experiments of Fig. 5. The input density was 1.52 in a total volume of 3.0 ml. H^3-ϕX174 DNA was added as a density marker. The mixture was centrifuged in a Spinco model L centrifuge at 21°C for 3 days, then the contents were dripped through a syringe needle from the bottom of the centrifuge tube. Radioactivity was measured on the acid-precipitable material.

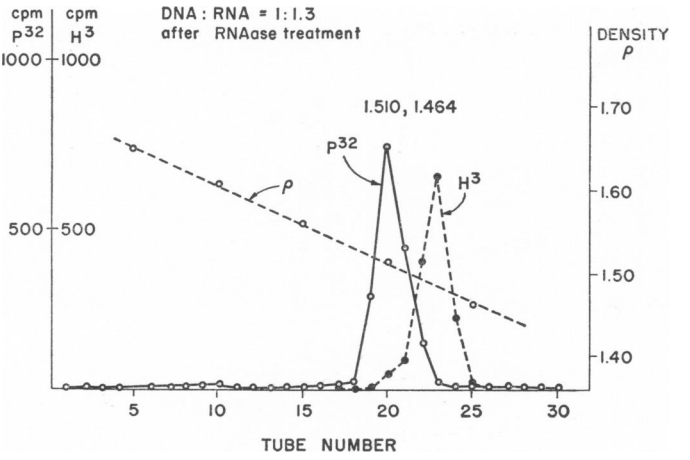

FIGURE 8 Effect of RNAase pretreatment on the P^{32}-RNA profile in a Cs_2SO_4 gradient. The 1:1.3 product of experiments of Fig. 5 was treated with RNAase under standard conditions and the enzyme eliminated by phenol method. After dialysis, the nucleic acids were centrifuged in Cs_2SO_4 as described in Fig. 7.

The ribonuclease resistance of the hybridized material and the sensitivity of the free RNA is shown in Fig. 8. Here, the same reaction mixture used in the experiment of Fig. 7C was treated with ribonuclease under standard conditions prior to centrifugation. We note the complete absence of free RNA in the heavy density region, with retention, however, of the hybridized structure.

In agreement with previous workers (Chamberlin and Berg, 1964; Sinsheimer and Lawrence, 1964), we find that the density of the hybrid is increased as the synthesis progresses to a RNA to DNA ratio of 1.0. Synthesis beyond this ratio does not change the hybrid density, liberating increasing amounts of free RNA.

We now consider what happens to the hybrid structures when chromatographed on an MAK column. In these cases, single-strand and double-strand ϕX RF-DNA, both labeled with H^3, are put in as markers. In all cases, the reaction mixtures were pretreated with ribonuclease before being put on the column. It is clear from a comparison of the three sets of data in Fig. 9 that with the progress of the RNA synthesis, the hybrid structure moves away from the chromatographic position of the single-stranded DNA toward the double-stranded DNA. When the ratio of DNA to RNA becomes 1.0, the hybrid reaches its limiting position and synthesis beyond this ratio does not change its relative position with respect to the two marker materials.

It is evident that the MAK column can be used to identify, separate, and distinguish DNA-RNA hybrids of varying composition.

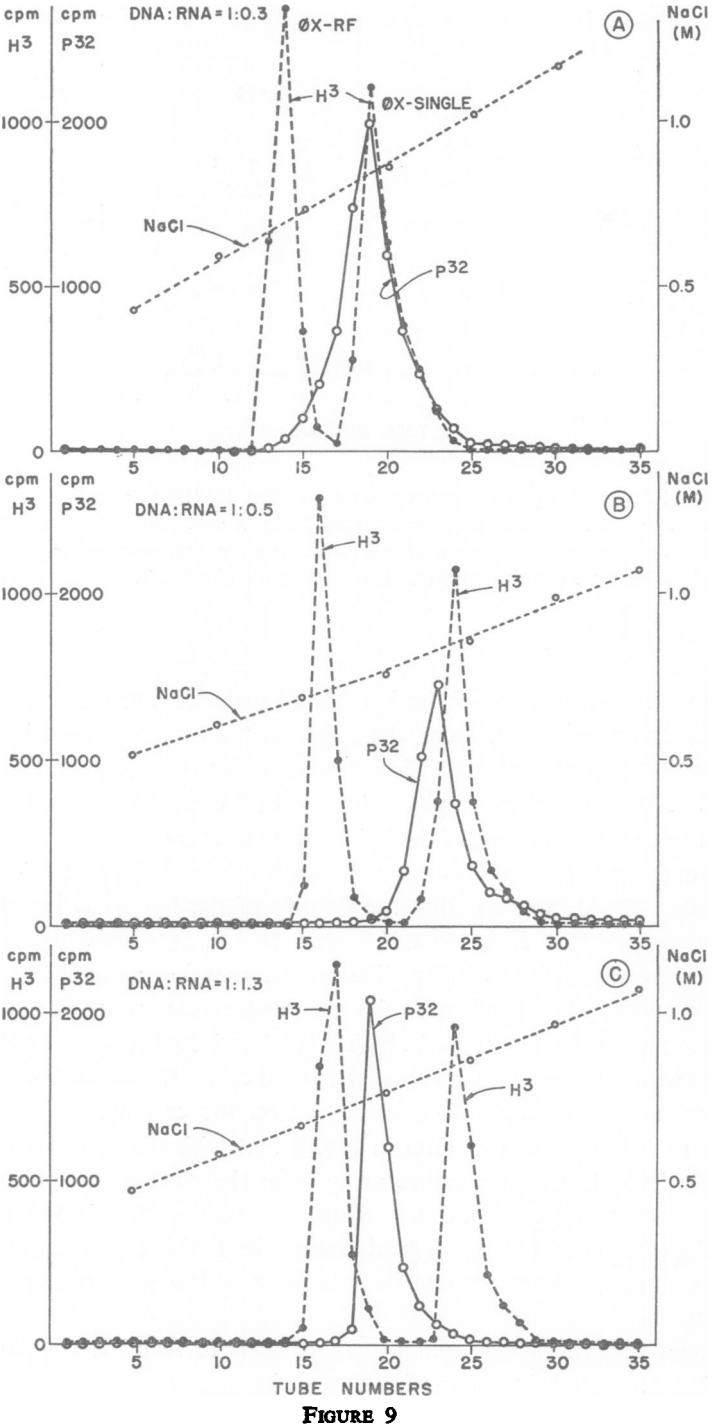
cpm H3
cpm P32
DNA: RNA = 1:0.3
ØX-RF
H3
ØX-SINGLE
NaCl
P32
NaCl (M)
A
B
DNA: RNA = 1:0.5
C
DNA: RNA = 1:1.3
TUBE NUMBERS

FIGURE 9

DISCUSSION

The use of the MAK column for DNA-RNA hybrid detection has two useful properties in common with the swinging bucket density gradient method. Both permit actual isolation of the hybrid structure and an estimation of the extent of DNA involvement. However, the chromatographic procedure is simpler and less time-consuming. In addition, the columns are more flexible with respect to capacity and resolution. Both of these characteristics can be changed over a wide range by variation in column size and type of NaCl gradient employed.

A unique advantage possessed by the chromatographic method is its ability to handle hybrid isolation involving small DNA fragments. Hybridizations with DNA in the size range of 1 to 2 million are clearly useful in any attempts at isolating particular cistrons. Also, by employing DNA of this size, one overcomes a previously observed disadvantage of MAK columns of poor recovery with DNA of high molecular weight.

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FIGURE 9 Chromatographic behavior of hybrids of varying proportions of RNA to DNA. The products synthesized in experiments of Fig. 5 were subjected to standard RNAase treatment. The reaction mixture was chromatographed on MAK column using H^3-ϕX-RF-DNA and H^3-ϕX174 DNA as markers. One-half size of the standard column was used. NaCl concentrated gradient was applied from 0.5 to 1.3 M. Aliquots of the fractions collected were precipitated with TCA and radioactivity was measured with a scintillation counter.

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