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A General Method for Fractionation of Nucleic Acids on the Basis of Sequence Homology*

W. Stuart Riggsby†

ABSTRACT: Techniques used for detection of homologous nucleic acid duplexes on nitrocellulose disks were adapted for use in preparative fractionation of genetically specific nucleic acids. The technique used consists of preparation of a deoxyribonucleic acid–nitrocellulose complex, drying the complex at high temperature, preincubation in a polymer solution, batch incubation of the deoxyribonucleic acid–nitrocellulose with the preparation to be purified, and subsequent elution of the nucleic acids of interest. Mixtures of labeled deoxyribonucleic acid from bacteria and bacteriophage were purified about 50-fold; recovery was about 70%. The same technique was applied to the preparation of messenger ribonucleic acid specific for T2 bacteriophage, yielding milligram quantities of T2 messenger ribonucleic acid. These techniques are applicable to a variety of experimental situations in which a relatively large amount of genetically specific nucleic acid is desired.

Methods of separation of nucleic acids on solid-phase nitrocellulose fall into two classes. The first depends only upon the interactions of the nucleic acids with the nitrocellulose; the second depends, in addition, upon interactions between complementary[1] sequences in the nucleic acids. Although both classes of methods may, in special circumstances, be used for preparative fractionation of genetically specific nucleic acids, the second class is applicable to a greater variety of problems in biochemistry and genetics.

Nygaard and Hall (1963) discovered that nitrocellulose membrane filter disks bind DNA–RNA hybrids

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[1] The vocabulary of this paper was chosen to conform mainly with the usage suggested by Britten (1968). I have, however, retained the terms "homologous" and "heterologous" on the grounds that Britten's suggested term "identical" is almost certain to be understood in its ordinary sense, and is more likely to cause confusion in certain types of experiments (*e.g.*, strand separation) than is "homologous."

and dissociated DNA but do not bind either native DNA or free RNA. They took advantage of this binding specificity to study the RNA formed in response to bacteriophage infection (Nygaard and Hall, 1964). Preparative column techniques based on this discovery were quickly developed (Armstrong and Boezi, 1965; Klamerth, 1965; Boezi and Armstrong, 1967). The original technique of Nygaard and Hall was refined by Wohlhieter *et al.* (1966) to permit detection of genetically distinct DNA in cases where an episomal gene from *Escherichia coli* can be transferred to *Proteus mirabilis*, an organsim whose DNA base composition is quite different from that of *E. coli*. Their technique is based on the differential thermal stability of the two DNA species.

An elegant extension of the Nygaard–Hall technique was introduced by Gillespie and Spiegelman (1965). They bound dissociated DNA to nitrocellulose disks, stabilized the complex by drying, and then exposed the complex to RNA, which they found to be capable of forming stable hybrids with the bound DNA. The flexibility of this technique as an analytical tool has been widely exploited (Bautz *et al.*, 1966; Skalka, 1966; Oda and Joklik, 1967; Cohen and Hurwitz, 1967; Shearer and McCarthy, 1967; Church and McCarthy, 1967a,b; Scott and Smillie, 1967; Niyogi and Thomas, 1967; Bonner *et al.*, 1968), and a small-scale preparative procedure based on this technique has been reported (Taylor *et al.*, 1967). Bautz and Reilly (1966) combined the technique of Gillespie and Spiegelman (1965) with the earlier cellulose column technique of Bautz and Hall (1962) to obtain a method for detecting RNA specific for certain genes of T4 bacteriophage. The specific RNA obtained by the Bautz–Reilly method has also been used in an attempt to purify, by sedimentation techniques, DNA complementary to the specific RNA (Mazaitis and Bautz, 1967).

Another extension of the Gillespie–Spiegelman technique is due to Denhardt (1966). By exposing prepared DNA–nitrocellulose filters to a complex organic preincubation medium, Denhardt was able to suppress further DNA–nitrocellulose complex formation and to detect specific DNA–DNA duplex formation.

The techniques described in this paper were developed for separating genetically specific DNA and RNA in much larger quantities than has been possible with the hitherto available techniques. Two types of separation are of particular interest: (a) separation of mRNA complementary to bacteriophage DNA from the host cell RNA, and (b) separation of bacteriophage DNA from the DNA of infected bacteria. No attempt has been made to apply the procedures detailed here to isolation of specific messengers by deletion techniques such as those of Bautz and coworkers (Bautz and Reilly, 1966; Mazaitis and Bautz, 1967). The improved capacity and stability of these columns should, however, be equally applicable to experiments involving deletion techniques.

Experimental Section

Preparation of Nitrocellulose. Nitrocellulose (Hercules Type RS, 5–6 sec)[2] was ground with an approximately equal volume of 2 × SSC in an electrically driven mortar and pestle for 1 hr, and then pressed through a no. 40 mesh wire sieve (Bautz and Reilly, 1966). The resulting paste was washed ten times with four volumes of 2 × SSC; each time, the nitrocellulose was allowed to settle for 10 min, and the fine material was decanted. The nitrocellulose was poured into a large fritted glass Büchner funnel and washed with four volumes of 2 × SSC. The nitrocellulose was then suspended in four volumes of 6 × SSC, allowed to stand for 10 min, and decanted. Finally, the nitrocellulose was suspended in three volumes of 6 × SSC and stored at 4° until used. Such preparations have been kept for as long as 1 year. The concentration of the nitrocellulose suspensions was determined by filtering a 10-ml portion of a vigorously shaken suspension, drying overnight *in vacuo*, and weighing. Since dry nitrocellulose is an explosive, care should be taken not to subject the dried material to physical shock or to expose it to an open flame.

Binding of DNA to Nitrocellulose. DNA was dissociated by exposure to 0.15 N NaOH for 10 min, followed by neutralization with HCl. The ionic strength of the solution was then raised by the addition of four-tenths volume of 20 × SSC. Usually 1–4 mg of DNA/g of nitrocellulose was used; this DNA is referred to as DNA-I.[3] The solution was added to the appropriate amount of a nitrocellulose slurry in 6 × SSC and stirred for 30–60 min at room temperature. The slurry was then filtered through Whatman No. 3MM paper. The powder was dispersed in a Petri dish, dried *in vacuo* at room temperature overnight, and then dried *in vacuo* at 80° for 3 hr.

Preincubation of the DNA–Nitrocellulose. The dried DNA–nitrocellulose powder was resuspended with vigorous shaking in a preincubation medium (PM6) containing 0.02% each Ficoll (Pharmacia, mol wt ~400,000), polyvinylpyrrolidone (Sigma, mol wt ~360,000), and bovine serum albumin (Pentex fraction V) in 6 × SSC. This medium is identical with that used by Denhardt (1966) except for the ionic strength. The suspension was dispersed in 50-ml ehrlenmeyer flasks and shaken for 6 hr at 66°, and the slurry was filtered through Whatman No. 3MM paper. The preincubation step was usually omitted in the experiments where RNA was to be analyzed but must be included when DNA is to be analyzed.

Incubation with Nucleic Acid to Be Analyzed. The prepared DNA–nitrocellulose was resuspended in 6 × SSC and the nucleic acid to be analyzed was added. In the DNA experiments, dissociation was done as described just before use. The added DNA is referred to as DNA-II. In most, but not all, cases the DNA-II was fragmented by sonication before dissociation to obtain

[2] The term "5–6 sec" is the manufacturer's designation for a particular type of nitrocellulose, and specifies the time for a standard object to fall a standard distance in a standard alcoholic solution of a given batch of nitrocellulose. It is related to the viscosity of the solution and, through the viscosity, to the polymer length of the nitrocellulose.

[3] Abbreviations used: SSC, 0.15 M NaCl–0.015 M sodium citrate (pH 7.2); *n* × SSC, SSC concentrated by a factor *n*; DNA-I, reference DNA (see methods); DNA-II, DNA to be analyzed (see methods); PM6, preincubation medium (see methods).

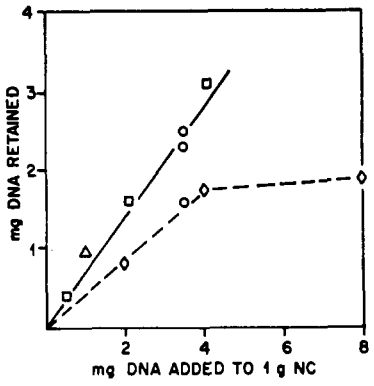

FIGURE 1: Retention of DNA by nitrocellulose. Aliquots of denatured DNA were applied to a slurry of nitrocellulose as described in the Experimental Section. Several DNA preparations and two types of nitrocellulose were used. Stirring was for the length of time indicated. (◇) *E. coli* DNA, 5–6 sec nitrocellulose, 30-min stirring; (○) *E. coli* DNA, 5–6 sec nitrocellulose, 30-min stirring, variable flow rate as explained in text; (△) T2 DNA, 5–6 sec nitrocellulose, 60-min stirring; (□) *E. coli* DNA, 0.5 sec nitrocellulose, 30-min stirring.

TABLE I: Retention of DNA-I by Nitrocellulose after Incubation and Elution.[a]

Column	DNA-I Input (mg)	DNA Lost in Binding Step (mg)	DNA Lost in Incubation and Elution (mg)	% Retained
1	5.0	0.3	0.2	90
2	15.0	1.9	1.2	79

[a] The complex contained 10 g of nitrocellulose, and each was incubated and eluted four times. The bulk of the DNA lost in incubation and elution appears in the effluent and in the 6 × SSC elution of the first cycle. It is probable that this DNA was never bound to nitrocellulose, since the prebaking wash commonly used in filter methods was not employed here.

more efficient hybridization (Denhardt, 1966).[4] The suspension was placed in 50-ml flasks and shaken at 66° for 16–24 hr, then transferred to a jacketed chromatography column at the desired temperature.

Elution. Effluent from the column was collected as a single fraction. The column was then eluted with successive portions of 6 × SSC to recover the material (DNA-II or RNA) not in the form of duplexes. The column was then eluted with successive portions of water to recover the material that had formed duplexes with the DNA-I.

Hybridization on Nitrocellulose Disks. DNA–DNA complementarity analysis on nitrocellulose disks was carried out by the method of Denhardt (1966) except that the preincubation medium was made up in 6 × SSC. After the preincubation period, disks were removed to fresh 6 × SSC for incubation with DNA. RNA was hybridized to DNA on nitrocellulose disks by the method of Gillespie and Spiegelman (1965), with modifications described elsewhere (Riggsby and Merriam, 1968).

Preparation of Nucleic Acids. E. coli DNA was prepared by the method of Marmur (1961). RNA from *E. coli* cultures was prepared, except where otherwise noted, by repeated extraction with hot phenol (Scherrer and Darnell, 1962). DNA from phages T2 and T5, also prepared by hot phenol extraction (Massie and Zimm, 1965), were gifts respectively of Dr. Elliot Volkin and Dr. Robert Fujimura.

Centrifugation. Radioactively labeled nucleic acid samples recovered from nitrocellulose columns were subjected to isopycnic centrifugal analysis in CsCl (Meselson *et al.*, 1957). Centrifugation was in 56% CsCl at 37,000 rpm in the SW-39 rotor. Fractions of about 0.05 ml were collected dropwise from the bottom of the tube onto Whatman No. 3MM paper disks, which were treated by the method of Bollum (1966) before being counted.

Assay of Radioactivity. Most radioactivity measurements were made in a Packard Tri-Carb liquid scintillation counter with a toluene, 2,5-bis[2-(5-*t*-butylbenzoxazolyl)]thiophene (Packard's BBOT) counting solution.

In some of the experiments advantage was taken of the fact that the energy of the β particle emitted by the unstable ^{32}P nucleus is sufficient to produce Cerenkov radiation in aqueous solutions, and that this radiation may be detected in a conventional liquid-scintillation counter (Clausen, 1968). Samples to be counted in this manner were sometimes diluted to a standard volume of 10 ml with water; otherwise a small, empirically determined volume correction was applied. With Packard Tri-Carb counters in this laboratory, maximum counting efficiency is obtained with a window setting of 40–1000 and a gain of 39%, although rather large departures from these values produce only small changes in counting efficiency of ^{32}P. Under these conditions, the counting efficiency of ^{32}P is 31% with respect to the value obtained in the toluene counting solution.

Results

Capacity of Nitrocellulose Powder to Bind and Retain DNA. Figure 1 and Table I show the amount of input DNA-I retained by nitrocellulose throughout the entire process of incubation in 6 × SSC and elution with 6 × SSC and with water. In the experiments shown in Figure 1, a small (1 × 20 cm) column was used to contain 1 g of nitrocellulose. The flow rates on this size column tend to be variable, and the amount of DNA-I retained is to some extent related to the flow rate. Points on the solid line of Figure 1 were obtained in experiments where the flow rate was moderately fast (about 10 min/10 ml).

[4] Sonication was carried out for 3 min at maximum output in a Raytheon Model DF101 oscillator.

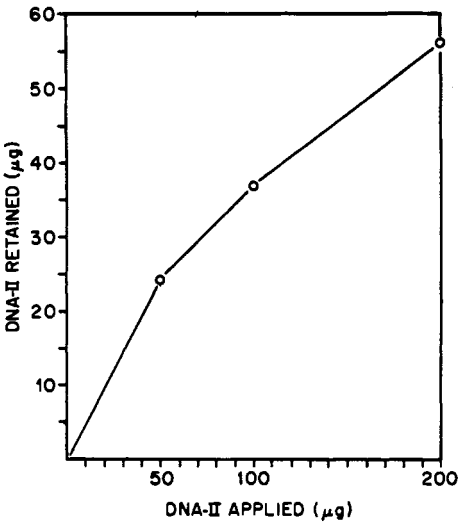

FIGURE 2: Binding of DNA-II to DNA–nitrocellulose complex. DNA-I (1.0 mg) was bound to each of three aliquots of nitrocellulose slurry, each containing 1 g, 5–6 sec nitrocellulose. These samples were incubated with various/amounts of ^{32}P-labeled *E. coli* DNA after preincubation. (It will be noted that DNA-I is in excess.) The fraction of the DNA bound was determined from the amount of unbound label.

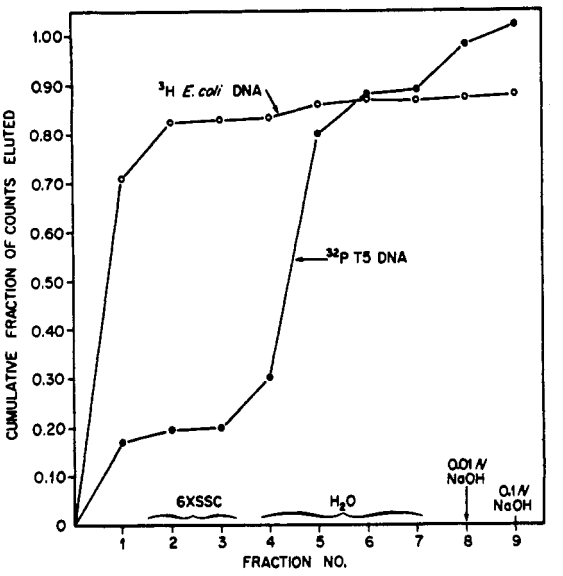

FIGURE 3: Elution profile of an artificial mixture of ^{32}P-labeled T5 DNA and ^{3}H-labeled *E. coli* DNA from a column containing 2 mg of T5 DNA bound to 1 g of nitrocellulose. Each fraction represents elution with 10 ml of the solution described at the bottom of the figure. Aliquots (0.1 ml) of each fraction were pipetted onto 25-mm Schleicher & Schuell B6 filter disks, dried and counted as described in the Experimental Section.

Points on the broken line represent experiments in which the flow rate was slower (30–45 min/10 ml). Circles represent aliquots of the same slurry placed on identical columns and eluted at different flow rates; the most rapid flow rate gave the highest retention, and the slowest flow rate gave the poorest. Addition of Celite (Johns-Manville), up to 1 g/g of nitrocellulose, improved flow rates to some extent; but the improvement was largely offset by the inconvenience of the much larger bed volumes resulting from use of this very low density material.

Table I presents the results of experiments with a large (2 × 20 cm) column containing 10 g of nitrocellulose. The DNA-I used in these experiments did not contain radioactive label; values given are therefore subject to an error in conversion of optical density units into DNA concentration, as will be discussed. Nevertheless, retention of these columns is considerably better than that of the smaller columns. It should be noted that the flow rates of these columns were also faster than those of the small columns. Flow time of column 2 was initially 4 min/10 ml and increased to about 7.5 min/10 ml after collection of eight 10-ml fractions. After four cycles of incubation and elution, the initial and final flow rates were 5 and 12 min 10 per ml, respectively.

Ability of Nitrocellulose–DNA Complex to Bind DNA-II or RNA. The stabilized nitrocellulose–DNA complex is capable of binding an amount of homologous DNA or RNA equal to about 10% of the amount of DNA in the complex. Varying amounts of sonicated, dissociated *E. coli* DNA (DNA-II) were incubated in slurries, each of which contained 1.0 mg of *E. coli* DNA bound to 1.0 g of nitrocellulose. The amount of DNA-II bound (*i.e.*, eluted by water but not by 6 × SSC) is shown in Figure 2. The fraction of DNA-II retained decreases with increasing concentration, but even at a ratio DNA-II/DNA-I = 0.2, no plateau has been reached. Although in some applications it may be desirable to use saturating conditions, selective recovery of specific nucleic acids is favored when lower inputs are used. Moreover, at higher inputs, flow rates decrease rapidly. For these reasons, no attempt was made to determine the upper limit of such saturation curves. In the RNA separation experiments, carried out considerably below the saturation level, as much as 1.0 mg of RNA was bound to complex containing 15 mg of DNA-I. These results show that the capacity of the complex to bind homologous sequences is probably about the same for RNA and DNA.

Fractionation of DNA. The specificity of the DNA–nitrocellulose column technique with respect to fractionation of DNA was tested with mixtures of DNA from *E. coli* and T5 bacteriophage. A complex of 2 mg of unlabeled T5 DNA with 1 g of nitrocellulose was prepared, preincubated, and exposed for 18 hr to a mixture of 160 μg of ^{3}H-labeled *E. coli* DNA and 180 μg of ^{32}P-labeled T5 DNA. The slurry was eluted from the column at 66° as described, using two 10-ml portions of 6 × SSC and four 10-ml portions of water, followed by NaOH. Results of this elution are shown in Figure 3. More than 80% of the heterologous *E. coli* DNA was eluted in the 6 × SSC fractions, and less than an additional 1% was eluted in the water fractions. The ^{3}H label not accounted for is probably in the 6 × SSC eluate, which quenches ^{3}H counts slightly in the counting technique used. More than 50% of all the ^{32}P label is in fraction 5; 70% of it is in the combined water fractions. The ratio of ^{32}P to ^{3}H in these combined fractions is increased by a factor of about 50 over that of the starting material. The ^{3}H *E. coli* DNA, although enriched in the 6 × SSC

TABLE II: Recovery of RNA from a Column Containing 5 mg of T2 DNA Bound to 15 g of Nitrocellulose.[a]

Sample	RNA (mg)	Sp Act. (cpm/μg)
Starting RNA	1.0	3.9×10^3
6 × SSC effluent	0.77	2.1×10^3
First water fraction	0.02	4.5×10^4

[a] The RNA was extracted from *E. coli* infected with T2, and pulse-labeled 4–9 min after infection. Four 6 × SSC fractions were collected between the 6 × SSC effluent and the first water fraction. These fractions contained rapidly diminishing amounts of RNA with a specific activity similar to that of the 6 × SSC effluent. Total recovery of radioactivity was 85%. Since the column was to be recycled, no attempt was made at complete recovery, which would have required the use of NaOH and concomitant destruction of the DNA–nitrocellulose complex.

TABLE III: Successive Elution of RNA Samples from the Same DNA–Nitrocellulose Complex.[a]

Elution	Sample	RNA (mg)	Sp Act. (cpm/μg)
I	Starting material	11.4	2.3×10^3
I	Effluent	4.3	8.0×10^2
I	Water-1	0.24	2.2×10^4
II	Effluent	9.0	7.1×10^2
II	Water-1	0.26	2.8×10^4
III	Effluent	7.2	6.0×10^2
III	Water-1	0.21	3.6×10^3
IV	Effluent	6.9	5.5×10^2
IV	Water-1	0.37	7.6×10^2
	Starting material	16.8	9.2×10^2
V	Effluent	10.3	4.2×10^2
V	Water-1	0.75	7.0×10^3

[a] Elutions I and II followed incubation with identical samples of $^{32}PO_4$ pulse-labeled T2 RNA. Elutions III and IV followed incubation with the effluent fraction from elutions II and III, respectively. Elution V followed incubation with a different RNA sample. One-half of the original 11.4 mg of starting material was added in the first incubation, the other half, along with the effluent from the first elution, was added in the second incubation.

fractions, is not so extensively purified as is the T5 DNA The ratio of ^3H to ^{32}P in the combined 6 × SSC fractions is only about four times that of the starting material.

Fractionation of RNA. The specificity of the nitrocellulose column technique with respect to fractionation of RNA was tested with RNA extracted from *E. coli* cells infected with T2 bacteriophage. Cells grown in a phosphate-depleted medium and infected with T2 at a multiplicity of about 5 were pulsed with $^{32}PO_4$ 4–9 min after infection. Under the conditions used, fewer than 1% of the cells were uninfected. At 9 min, the cells were poured over frozen buffer, and the RNA was extracted. RNA so extracted should contain, in addition to possible minor components, unlabeled *E. coli* rRNA and ^{32}P-labeled T2 mRNA; fractionation of these two major species should be detected in the different specific activities of the fractions obtained.

A slurry of 5 mg of T2 DNA bound to 15 g of nitrocellulose was incubated as described with 1.0 mg of the RNA preparation. Elution was at 60°; results are shown in Table II. The 6 × SSC fraction was recycled through the same nitrocellulose–DNA complex with recovery of additional labeled RNA in the water fractions; however, the concentration (A_{260}) of these samples was too low to permit an estimation of the specific activity (see Discussion).

Reuse of the Nitrocellulose–DNA Complex. To obtain large amounts of specific nucleic acids by the technique described, it is necessary either to have a very large amount of DNA-I bound to the nitrocellulose or to be able to reuse the same material in consecutive runs. The baking of the DNA–nitrocellulose complex permits such reuse. Table III shows a four-step fractionation procedure. The complex of nitrocellulose and T2 DNA was incubated with 5.7 mg of pulse-labeled RNA from *E. coli* infected with T2, and eluted (elution I). The total amount of RNA and the specific activity of the effluent and of the first water fraction are shown. By "first water fraction" is meant that fraction in which the water front appears in the eluate; it is easily detected by the convective disturbances on mixing with the 6 × SSC. The 6 × SSC effluent and those 6 × SSC eluate fractions showing appreciable activity were combined, added to an additional 5.7 mg of RNA, reincubated with the same DNA–nitrocellulose complex, and eluted (elution II). Recoveries and specific activities here were comparable with those of the first elution. The 6 × SSC effluent was incubated and eluted twice more, without addition of new starting material. At the relative concentrations used here, nearly all the material with high specific activity was removed in the first elution after addition of new starting material. By elution IV, no appreciable fractionation is achieved. When a new sample (of a different preparation) was applied and eluted (elution V) the customary separation was observed. This particular DNA–nitrocellulose complex was used eight times without noticeable decrease in efficiency. In all cases, most of the T2-specific RNA is removed in the first cycle. On subsequent elutions, there appears to be a considerable amount of non-T2 RNA bound. If such nonspecific binding occurs in the first cycle, it must be small, since the optical density recovered as "specific" RNA (4.2%) agrees closely with other estimates (5%) of the fraction of T2 specific RNA in infected *E. coli* (Nygaard and Hall, 1964).

Sedimentation Behavior of Nucleic Acids Eluted from

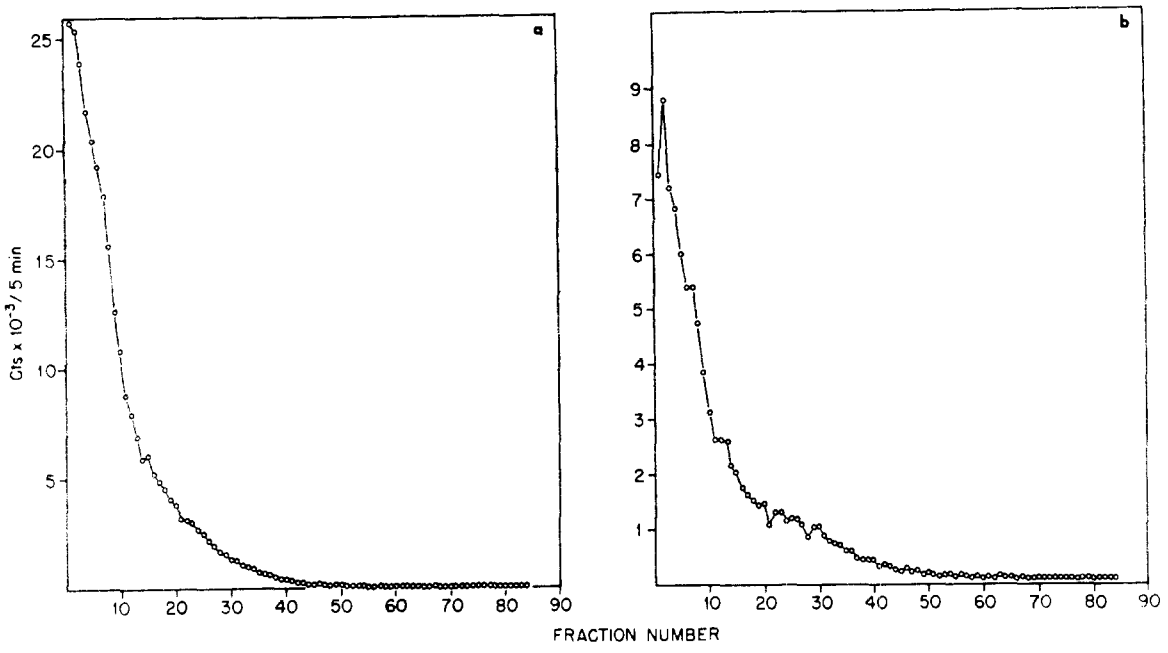

FIGURE 4: Isopycnic sedimentation of eluates from a DNA–nitrocellulose column used to fractionate RNA from T2-infected *E coli*. Conditions for both experiments were the same (56% CsCl, 25°, 37,000 rpm, 70 hr). (a) Sedimentation of the first water fraction (RNA homologous to the T2 DNA on the column). (b) Sedimentation of the 6 × SSC effluent (RNA not homologous to the DNA on the column).

Columns. Sedimentation studies were done primarily to determine whether the RNA and DNA-II eluted from nitrocellulose columns were present as single-stranded chains or as hybrids with DNA-I. Isopycnic sedimentation in 56% CsCl as described by Hall and Spiegelman (1961) was carried out on both effluent and water fractions from an elution of T2 RNA; Figure 4 shows the results. In both cases the RNA is sedimented well to the bottom of the tube, and no significant amount of RNA label is found in fractions 30–40, where DNA–RNA hybrid would be expected. Similarly, DNA-II eluted from the nitrocellulose column bands in CsCl at the density of single-stranded T2 DNA (Figure 5).

Hybridization Behavior of Fractionated RNA. Hybridization studies on RNA preparations, before and after fractionation on DNA–nitrocellulose, were carried out by a modification of the exhaustive technique of Landy and Spiegelman (1968). Successive filters loaded with 100 μg each of T2 DNA were incubated with small amounts (usually less than 1 μg) of various RNA fractions. Departing from the Landy–Spiegelman procedure, I employed the ribonuclease treatment with each filter used, in order to determine both the RNase-resistant and the RNase-sensitive fractions of the RNA bound. Before treatment with RNase, each filter was rinsed at room temperature with 1 ml of 6 × SSC; this solution was then added to the original RNA solution for subsequent analysis. Each preparation was carried through three rounds of hybridization. In each case the last filter bound less than 1% of the RNA present. The sum of the counts bound to the three filters was taken to represent the RNase-resistant hybrid. The radioactivity remaining in the solution after the three rounds of hybridization was determined by Cerenkov counting, and the difference was taken to represent the RNase-sensitive hybrid lost in the ribonuclease treatment. An independent assay of the radioactivity in the wash solutions, greatly concentrated by boiling, showed that no detectable counts were lost in this step.

The results of these experiments are shown in Table IV. The sums of the RNase-resistant and RNase-sensitive fractions in unfractionated RNA are consistent with the values obtained by Landy and Spiegelman (1968) in a similar system. The values obtained with fractionated material confirm that a considerable enrichment of RNA sequences homologous to T2 DNA has been obtained.

TABLE IV: Exhaustive Hybridization with T2 DNA.[a]

	RNase-Resistant Hybrid	RNase-Sensitive Hybrid	Unhybridized
Unfractionated RNA	0.54	0.20	0.26
Effluent	0.05	0.09	0.86
First water fraction	0.86	0.09	0.05

[a] Exhaustive hybridization was carried out as described in the text. *E. coli* cells were infected with T2 bacteriophage and pulse labeled with [^{32}P]- and [^{14}C]-nucleosides from 1.5 to 5 min after infection. The RNA was a 2 M NaCl precipitate prepared by the method of Midgley (1965).

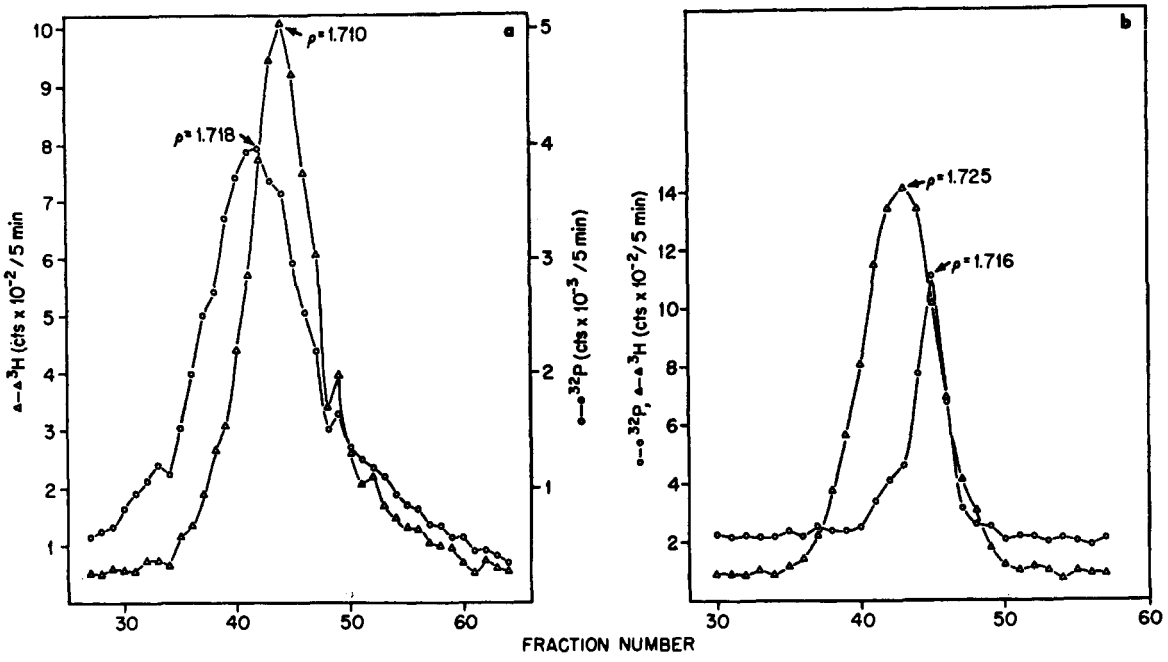

FIGURE 5: Isopycnic sedimentation of the first water fraction from a column used to analyze a preparation of ^{32}P-labeled T2 DNA. The column was prepared with a complex of nitrocellulose and unlabeled T2 DNA. The position of the ^{32}P-labeled DNA is indicated by the circles; triangles represent the position of the markers used: (a) ^{3}H-labeled native *E. coli* DNA; (b) ^{3}H-labeled denatured *E. coli* DNA. Using the density values tabulated by Schildkraut *et al.* (1962) and the difference in density between native and denatured DNA reported by Meselson and Stahl (1958), T2-denatured DNA should have a density of 1.716. In all, 85 fractions were collected from each of these gradients.

Discussion

Characteristics and Application of the Method Described. The enrichment technique described here is essentially a modification of the RNA isolation technique of Bautz and Reilly (1966), and incorporates refinements suggested by Gillespie and Spiegelman (1965) and by Denhardt (1966) for nitrocellulose disk assays. With respect to RNA fractionation, it is similar except for capacity to the small-scale disk fractionation procedure described by Taylor and coworkers (1967). Such a technique is useful for preparative as well as for analytical purposes. The results seem to meet requirements of a specific separatory system. (a) Separation of both DNA and RNA on the basis of genetic specificity is possible. (b) Relatively large amounts of the nucleic acids of interest can be separated. (c) The methods were tested with a bacterial system, two bacteriophage systems, and various combinations of these; they seem to be applicable to microbial systems in general. Application to higher organisms will be discussed. (d) Methods described allow an estimation of the (radioactive) specific activity of the separated DNA or RNA. In hybridization experiments, and especially in hybridization competition and in experiments designed to determine what fraction of a given nucleic acid preparation is homologous to another preparation, this parameter is of critical importance. As will be discussed in the succeeding section, both the absolute concentration and the concentration of label in fractions eluted from these columns may be obtained.

Separation of nucleci acids on the basis of sequence homology has numerous applications. In genetic and biochemical investigations at several levels of complexity, isolation of large amounts of mRNA unique to a specific organism or gene would be useful. The first of these aims, isolation of organism-specific RNA, was achieved directly, and extension to gene-specific RNA requires only that the appropriate deletion mutations be available. Of perhaps even greater interest is the isolation of DNA of comparable specificity, particularly in view of the greater stability of DNA under the conditions used. Use of isolated specific nucleic acids for *in vitro* synthesis of specific proteins may be possible (Salser *et al.*, 1967; DeVries and Zubay, 1967). In this connection, however, it should be pointed out that some degradation of hybridized RNA is observed in sucrose density gradient centrifugation (W. S. Riggsby, unpublished). The degradation does not interfere with subsequent hybridization of the RNA, but may be sufficient to destroy biological activity.

In view of recent studies on the nature of the base sequence distribution of DNA and RNA in higher organisms (Waring and Britten, 1966; Britten, 1968, Britten and Kohne, 1967, 1968a,b; Britten and Chamberlin, 1968; Church and McCarthy, 1968), it is clear that meaningful results in the area of separation of genetically distinct sequences require the preliminary separation of the unique and the imperfectly complementary fractions of such DNA (Britten and Kohne, 1968a). The DNA–nitrocellulose column may prove to be a useful tool for separating RNA transcribed from these two fractions. Under conditions favoring the formation of imprecisely complementary duplexes (Church and Mc-

Carthy, 1968), it would be predicted that RNA bound to DNA–nitrocellulose complex after a relatively short incubation should be mostly redundant, with the unique fraction left in solution. An alternate procedure would be to prepare the DNA–nitrocellulose complex from the prefractionated DNA. Experiments along these lines are in progress in this laboratory.

Estimation of Nucleic Acid Concentrations in Eluates. Aqueous solutions in which nitrocellulose powder has been allowed to stand for some time develop a nonfilterable absorbancy in the ultraviolet. This is particularly true of nitrocellulose incubated at high temperatures. Consequently, eluates from nitrocellulose columns contain ultraviolet-absorbing nitrocellulose products as well as nucleic acids, and the precise amount of such material is not known. Corrections for this material, however, are usually possible when determining nucleic acid concentrations in eluates, provided that the optical density of the solution is measured at two wavelengths. This correction may be carried out in the following manner.

Let ϵ'_{260} and ϵ'_{280} be the mass extinction coefficients at 260 and 280 mμ, respectively, of the nucleic acid being studied; let ϵ''_{260} and ϵ''_{280} be the corresponding extinction coefficients of the nitrocellulose product. Then the concentration, c_1, of nucleic acid in a solution whose optical densities at 260 and 280 mμ are A_{260} and A_{280} is given by

$$c_1 = \frac{A_{260} - R(A_{280})}{\epsilon'_{260} - R(\epsilon'_{280})}$$

where $R = \epsilon''_{260}/\epsilon''_{280}$. Only the ratio, and not the absolute values, of the extinction coefficients of the nitrocellulose product need be known.

In the experiments reported, R varies between 1.25 and 1.40, depending on the solvent, temperature, and time of incubation. Even when these three conditions are controlled, the values of R are 1.30–1.33; in all experiments in which the appropriate value of R could not be determined directly, the value 1.3 was chosen to compute c_1. The error introduced by use of an improper value of R is illustrated in Table V. Consider a series of samples, all having unit optical density at 280 mμ and having the A_{260}/A_{280} ratios given. Values of c_1 were computed for both $R = 1.3$ and $R = 1.4$. Errors resulting from the wrong choice of R at low optical density ratios are rather large, whereas the error is very small at high ratios. Specific activities reported in this paper were calculated on the basis of solutions whose optical density ratios were 1.75–1.80 (water elutions) and 1.9–2.0 (6 × SSC elutions). Errors that would be introduced by incorrect choice of R would thus be in the neighborhood of 10% for the water elutions and less than 5% for the 6 × SSC elutions. Preliminary results indicate that the interference by ultraviolet absorbing materials may be decreased in the 6 × SSC fractions by incubating them at room temperature in aqueous formamide solutions instead of at elevated temperatures, as suggested by Bonner *et al.* (1967) for hybridization on nitrocellulose disks. This advantage has however been offset by a lowering of the amount of DNA-II bound to the DNA–nitrocellulose complex.

TABLE V: Calculated Nucleic Acid Concentrations in Hypothetical Eluates from Nitrocellulose Columns.[a]

	c_1 Calculated		
A_{260}/A_{280}	$R = 1.3$	$R = 1.4$	% Error
1.31	0.001	×	×
1.41	0.013	0.0001	1200
1.51	0.024	0.015	60
1.6	0.034	0.027	26
1.7	0.046	0.040	15
1.8	0.057	0.053	8
1.9	0.069	0.067	3
2.0	0.080	0.080	0

[a] All samples have unit optical density at 280 mμ, and the optical density at 260 mμ is the same as the ratio given in the first column of the table. The method of computation is described in the text. The concentration c_1 is in milligrams per milliliter; per cent error is defined as $(a - b) \times 100/b$, where a and b are the values of c_1 calculated on the respective hypotheses that $R = 1.3$ and $R = 1.4$.

Subsequent Hybridization of Eluted DNA. R. Fujimura (personal communication) used the method described to fractionate DNA isolated from phage-infected bacteria. In subsequent complementarity analyses using filter methods, he found that the water fraction, when exposed to blank nitrocellulose disks, gave extremely high background values. Further investigation showed that this high background was caused by a trichloroacetic acid soluble component of high specific radioactivity. This component is eluted from the column as a sharp peak when the original 6 × SSC elution is carried out exhaustively. This phenomenon is present, but is not so serious when *E. coli* DNA alone is eluted from a column, and is not observed at all in RNA preparations as judged either by hydridization behavior or by sedimentation analysis.

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