CHROMATOGRAPHIC RESOLUTION OF COMPLEMENTARY STRANDS OF DENATURED PNEUMOCOCCAL DNA

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It has been shown that MAK columns are capable of resolving the complementary strands of pneumococcal DNA. Using stepwise elution of denatured DNA, two fractions were obtained at different eluting salt molarities, which regained high biological activity on annealing only when previously mixed.[1] Unfortunately, resolution could not clearly be demonstrated by means of chromatographic profiles themselves, since denatured DNA could be recovered only very poorly with continuous gradient elution. This difference from the behavior of native DNA had previously been described by others.[2, 3]

Accomplishment of complete strand resolution even by means of stepwise elution from MAK has been hindered by two factors: (1) incomplete recovery of the DNA and (2) variations in the precise salt molarities at which the two fractions appeared, although the same increment in concentration sufficed to resolve them. The uncertainty in the molarity of the eluting salt solution would be of only trivial importance were it possible to recover the denatured DNA with a continuous gradient. The experiments to be described demonstrate that when the columns are maintained at low temperatures, recovery with gradient elution is complete and the denatured pneumococcal DNA is eluted in two peaks. Since these peaks are shown to reflect complementary strand resolution, the chromatographic profiles may now be used as an independent indication of when this is occurring.

Materials and Methods.—Preparation of pneumococcal DNA: DNA was prepared by the method usually employed in this laboratory.[4] Deproteinization was effected by the chloroform emulsion technique.

Alkali denaturation of DNA: Denaturation was accomplished by diluting a concentrated stock DNA solution into 0.10 *N* NaOH at room temperature. After 5 min at the alkaline pH, the solution was neutralized to pH 6.8 by addition of the appropriate quantity of 1 *M* NaH_2PO_4 (two equivs. per equiv. of NaOH). Before adsorption to a MAK column the salt molarity was increased by addition of 5.0 *M* NaCl. The final concentration of DNA was about 50 μg/ml.

MAK chromatography: The preparation of MAK (methylated albumin adsorbed to kieselguhr) was as previously described.[3, 4] Gradient elution was accomplished by dropwise addition of a concentrated salt solution to the initial salt solution in a closed mixing chamber attached to the column. All salt solutions contained 0.02 *M* potassium phosphate buffer at pH 6.8. The molarities and volumes used resulted in nearly linear salt gradients. The usual gradient was achieved by adding 1.50 *M* buffered NaCl to 500 ml of 0.60 *M* buffered NaCl. Both the column and mixing chamber were jacketed for temperature control. The gradients indicated by the dashed lines in Figures 1 and 2 refer to the molarity of eluting salt solution entering the column at the time the frac-

tions were collected. Elution rates varied between 25 ml/hr and 50 ml/hr, depending upon the size of the column.

Treatment with T_1 RNase: T_1 RNase was obtained from Calbiochem. A concentrated stock solution of DNA was incubated for 5 hr at 37°C with 50 units of enzyme per mg of DNA in 0.15 *M* NaCl buffered with 0.01 *M* Na citrate at pH 6.0. The DNA was then precipitated with winding in 50 per cent ethanol and redissolved in 0.15 *M* NaCl. Evidence that the RNase was active came from the fact that the few per cent (at most 5%) low-molecular-weight material contained in the DNA preparation prior to treatment was reduced to a negligible amount. This was demonstrated by analytical boundary sedimentation and by loss of UV-absorbing material which would not adsorb to MAK.

Results.—Temperature dependence of DNA elution from MAK columns: It seemed likely that variability in the column temperature was responsible for the unpredictable salt molarity required to elute denatured DNA from MAK. In previous experiments the DNA was eluted at prevailing room temperatures. This can account for temperature differences as large as ten degrees, even though fluctuations may be quite small over short periods of time. When temperature was first controlled, the DNA was eluted stepwise in a cold room maintained at about 5°C. It was immediately observed that both for native and for denatured DNA recovery was complete, whereas previously, at room temperature, it seldom exceeded 50 per cent. It was then noted that at controlled temperatures considerably higher than ambient, denatured DNA is so tightly bound to MAK that elution cannot be accomplished at neutral pH, even at very high salt molarities. By varying column temperature between 10°C and 40°C, both total recovery and the salt molarity at which the DNA is first eluted were influenced. The lower the temperature used for elution the higher was the amount of DNA recovered and the lower the salt molarity required for elution. These experiments were carried out in collaboration with Dr. Paul Milvy in this laboratory.

Salt gradient elution of denatured DNA at low temperature: When eluted at low temperatures with an increasing salt gradient, denatured DNA is recovered

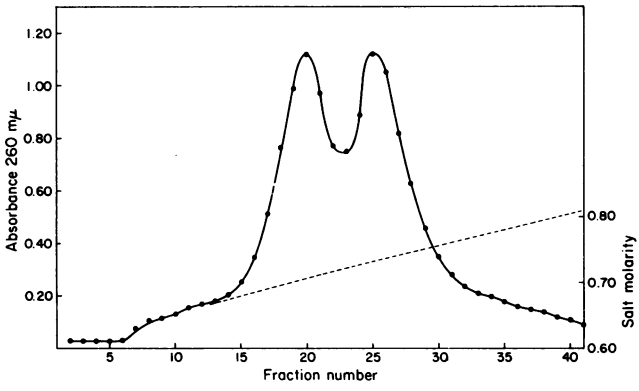

FIG. 1.—Elution of denatured pneumococcal DNA from MAK with a cold linear salt gradient. A total of 50 OD units of alkali-denatured DNA was applied to a 14 × 1.5-cm MAK column in 0.60 *M* saline phosphate. Both the column and gradient mixing reservoir were jacketed and thermostated at 5–6°C throughout. Optical densities are indicated by the solid points. The eluting salt gradient was nearly linear between 0.60 *M* and 0.80 *M* phosphate-buffered NaCl and is indicated by the dashed line. Fractions were collected in 3.25-cc vol. Recovery of optical density was 100%.

completely and, at least with pneumococcal DNA, two peaks are clearly resolved. Figure 1 shows an elution profile obtained at 5–6°C with a nearly linear salt gradient. In this instance recovery of the adsorbed DNA was 100 per cent and is to be compared with the recovery of 20–30 per cent obtained with room temperature gradient elution.

That the resolved peaks are the result of differential recognition of complementary strands by MAK is indicated by biological assay of the individual fractions. The DNA was tested for ability to self-anneal at 65°C, under conditions which are optimal for recovery of transforming activity with unfractionated denatured DNA. Data such as those of Table 1 have been obtained with a number of

TABLE 1. *Renaturability of column fractions with and without mixing before annealing.*

	Self-annealed (%)	First peak mixed with second peak before annealing (%)	Average of first and second peaks annealed separately (%)
Unfractionated denatured DNA	50		
Column fractions			
At first peak	5		
Second peak			
At peak	40	50–60	23
Halfway down peak	22	50–60	14

All activities are for the streptomycin resistance marker and are expressed as percentage of specific native activity. They represent the average of values obtained with six different gradients. Residual activities before renaturation were all between 2 and 7%.

columns whose elution profiles were similar to that shown. In each instance the DNA which was eluted in the first peak did not regain activity when annealed by itself. The DNA which was eluted in the region between the two peaks did renature with rather high efficiency, attaining 40–50 per cent the activity of native DNA, as does the unresolved denatured DNA. The material which was eluted at the height of the second peak also regained considerable activity, but this capability decreased steadily as fractions farther down the trailing edge were assayed. In a typical experiment, the recovery of transforming activity after self-annealing was 40 per cent at the height of the second peak, diminishing to about 20 per cent half way down the peak. Insofar as these renaturation potentials reflect the relative proportion of the two complementary strands, a considerable amount of overlap or trailing of the material of the first peak into the second is indicated; a common occurrence with MAK columns.[1–3]

As observed with fractionation by stepwise elution, the crucial test for strand resolution resides in the enhancement of ability to recover transforming activity when two resolved fractions are remixed. When these experiments were carried out with DNA obtained from the peaks resolved by gradient elution, the specific transforming activities attained after mixing far exceeded the average for the same fractions annealed separately. Values as high as 60 per cent of native activity have been observed after such mixing, and are to be compared with an average value of about 14 per cent for the same fractions when self-annealed. These results are summarized in Table 1.

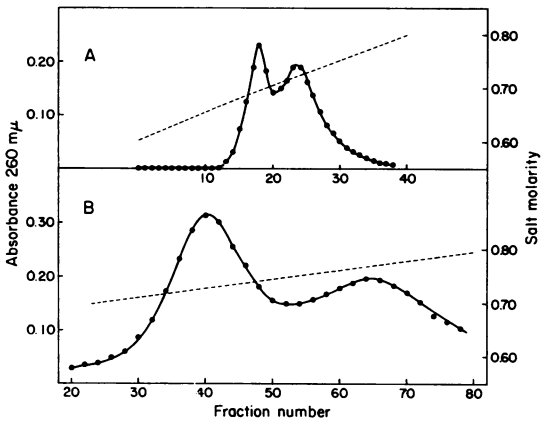

FIG. 2.—Effect of steepness of eluting salt gradient on chromatographic profile. In (*A*), 11.6 OD units of alkali-denatured DNA were applied in 0.60 *M* saline-phosphate to a 5 × 1.5-cm MAK column and eluted with the same salt gradient as in Fig. 1. Temperature was maintained at 18°C. Collection was in 3.25-cc vol. Recovery was 75%. In (*B*), 55 OD units of alkali-denatured DNA were applied in 0.60 *M* saline-phosphate to a 15.5 × 1.5-cm MAK column and eluted with a shallower gradient. Temperature was maintained at 15°C. Collection was in 3.5-cc vol. Recovery was 80%. The dashed lines indicate the eluting salt gradients.

Some factors influencing elution profiles: In attempting to increase the resolution of complementary strands by the MAK columns, a number of factors involved in gradient elution were studied. These experiments did not lead to significantly better resolution. This was finally obtained in the recycling experiments described below. However, some of the results are of interest and will be discussed briefly.

(a) *Steepness of the gradient:* A shallower salt gradient would be expected to decrease both the overlap of the two peaks and the trailing of the material of the first peak into the second. In Figure 2, the upper curve was obtained with the usual salt gradient (similar to that used in the experiment of Fig. 1) and the lower curve with a gradient approximately one third as steep. The shallower gradient has obviously broadened the peaks sufficiently that, despite the greater separation, the overlap is not much decreased, if at all. This broadening (which has been observed several times) is probably caused by heterogeneity of the binding sites on the MAK columns, although heterogeneity in the denatured DNA would certainly contribute as well. The biological assays of the fractions confirmed the fact that resolution was not improved with the shallower gradient.

For a proper comparison of Figure 2*A* with Figure 2*B*, it should be noted that in the former experiment only one fifth the amount of DNA was applied to an appropriately smaller column. This accounts for the low optical densities and early elution from the column. However, in the experiment of Figure 2*B*, the low optical densities arise almost entirely from broadening of the peaks, as the same quantities of DNA and MAK were used as for the column shown in Figure 1. The incomplete recoveries obtained in both these experiments reflect the higher temperature (15°C and 18°C) at which both columns were run.

(b) *Ratio of DNA to MAK:* The usual amount of DNA applied to a given amount of MAK was chosen on the basis of past experience. Under these particular conditions the MAK is approximately half saturated. In Figure 3, three different elution profiles are shown obtained by maintaining a constant column size and varying the quantity of denatured DNA over a fourfold range.

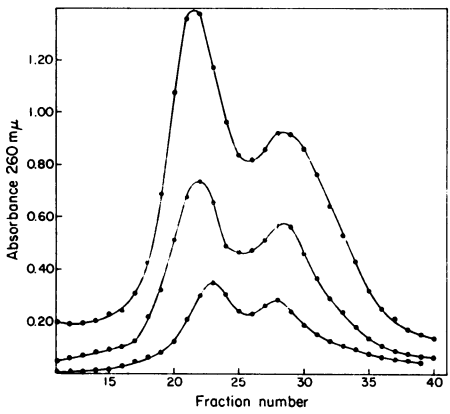

FIG. 3.—Influence of ratio of DNA to MAK on chromatographic profile. The size of the MAK column was kept constant and the amount of DNA applied was varied. In each case the alkali-denatured DNA was applied in 0.60 *M* saline-phosphate to a 14.5 × 1.5-cm MAK column maintained at 5–6°C, and the eluting gradient was as shown in Figs. 1 and 2*A*. In the order of decreasing quantity, 65, 33, and 14.5 OD units, respectively, were applied to the three columns. Recovery was 90–100%.

The highest ratio of DNA to MAK corresponds to the usual proportions employed. Biological assays of the fractions indicated that decreasing the ratio of DNA to MAK leads to only slightly improved resolution. The similarity of the three elution profiles indicated that neither saturation of adsorption sites nor the flow rate was a limiting factor for resolution. The optical densities of the profiles are proportional throughout to the total amounts applied.

(c) *Elimination of traces of guanine-rich polyribonucleotides with T_1 RNase:* RNA has been removed from the pneumococcal DNA's used in these studies by exhaustive treatment with pancreatic RNase and subsequent removal of low-molecular-weight fragments by successive winding of the DNA in 50 per cent ethanol (a minimum of five times). One of the purified pneumococcal DNA preparations was further freed of RNA fragments by incubation with an excess of T_1 RNase, an enzyme which preferentially attacks guanine-containing polyribonucleotides. When this DNA was alkali-denatured and eluted from an MAK column at low temperature, the chromatographic profile shown in Figure 4 was obtained. This profile is similar to those obtained without T_1 RNase digestion, with the trailing edge of the second peak remaining as broad as usual.

The removal of possible traces of guanine-rich ribonucleotides was desirable in connection with the finding that such ribonucleotide sequences can react preferentially with one of the complementary strands of denatured DNA.[5, 6] In the studies of Szybalski and co-workers an excess of ribosomal RNA, or guanine-rich

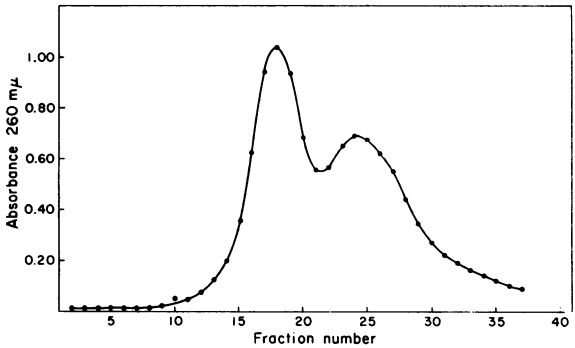

FIG. 4.—Chromatographic profile obtained with DNA treated with T_1 RNase. Purified native DNA which was digested with T_1 RNase, as described in the text, was alkali-denatured, applied to a MAK column, and eluted as in Figs. 1 and 3. A total of 50 OD units were applied to a 15 × 1.5-cm column maintained at 5–6°C.

polyribonucleotides, added to denatured DNA before sedimenting in a CsCl gradient resulted, in some cases, in the appearance of a new band of increased buoyant density. When a denatured pneumococcal DNA preparation, or either of the two fractions resolved from it, were banded in CsCl, single, fairly symmetrical bands were observed, in each case having the same increment in buoyant density with respect to the native DNA.[7]

The observation of two peaks eluting from an MAK column with a preparation of denatured B. *stearothermophilis* DNA from which RNA had not been removed, has recently come to our attention.[8] Perhaps, as with the pneumococcal DNA, the same two peaks would have been observed with a purified DNA preparation from which the RNA had been removed.

Rechromatography of the two components resolved by MAK columns: It was suspected that clean resolution of the more tightly bound DNA strand was hindered by trailing of the first chromatographic peak into the second, due to heterogeneity of binding sites on the MAK columns. Such trailing has been observed during elution of homogeneous native DNA's as well as mixtures of native and denatured DNA.[2-4] Recycling of the fractions obtained under each of the two peaks should show to what extent this is true for the resolution of complementary strands and could also provide fractions with improved resolution. Both objectives have been accomplished.

Appropriate fractions were combined from three separate columns, each of which was similar in elution profile to that shown in the upper curve of Figure 5. The regions within peaks *A* and *B* which were rechromatographed are indicated by the brackets. The elution profiles for each of the two recycled peaks are shown below. It is at once obvious that the DNA in each of the resolved peaks rechromatographs according to its original pattern. The combined fractions from peak *A* re-eluted in one peak at about the same salt molarity in which it was originally obtained. The combined fractions from peak *B* re-eluted at higher salt concentrations in a main peak with an early shoulder near the position of peak *A*. Thus, the first passage through the MAK columns gave material elut-

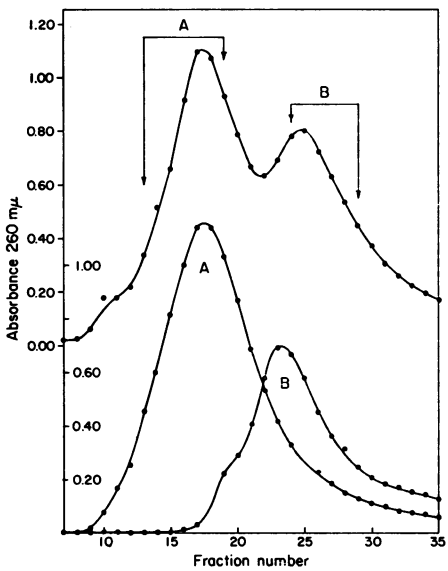

FIG. 5.—Recycling of the two components obtained from MAK columns. The upper curve shows a chromatographic profile obtained under the same conditions as described for Figs. 1, 3, and 4. Selected fractions from the two peaks obtained with three such columns were combined as indicated within the brackets (*A*) and (*B*), and separately applied to two fresh MAK columns. Elution was with the same salt gradient in each case. The recycled fractions from the *A* peaks were eluted as shown in curve *A* and the recycled fractions from the *B* peaks were eluted as shown in curve *B*. All temperatures were maintained at 5–6°C throughout. The eluting salt gradients were as shown in Figs. 1 and 2*A*. Recovery was 90–95%.

ing in peak *B* which should elute with peak *A*. The peaks of the recycled DNA fractions appear to be as wide as those obtained from the first column passage, even though only a limited portion of each of the original peaks was utilized. This observation tends to support the view that it is primarily the heterogeneity of the MAK column binding sites which determines the spread and overlap of the peaks.

The results of the biological assays are given in Table 2. The DNA in the early shoulder of the recycled second peak does not regain activity when self-annealed. Although the concentration of this fraction during annealing was about half that of the others, a recent larger-scale experiment provided a shoulder of optical density 0.65. Again, no renaturation was obtained, the activity remaining at its residual value of 5 per cent. Thus, the shoulder represents *A* strands removed from the original *B* peak, and the region between the two

TABLE 2. *Renaturability of fractions from recycled B peak of Fig. 5.*

	Residual activities before annealing (%)	Self-annealed (%)	Mixed with *A* peak before annealing (%)	Average of *A* and *B* peaks annealed separately (%)
Combined *B* peaks before recycle		32		
Fractions from recycled *B* peaks				
No. 19	5	6		
No. 21	9	30		
No. 24	7	16	52	11
No. 27	5	12	55	9

All activities are for the streptomycin resistance marker and are expressed as percentage specific native activity. Renaturation was carried out at optical densities of 0.4 in each instance except for Fraction no. 19 which was at an optical density of 0.22.

MAK peaks, which always regains high biological activity, does not result from the presence of a third highly renaturable component. Just after the shoulder of the recycled *B* peak the renaturability of the DNA increases to 30 per cent, indicating mixed *A* and *B* strands, and then falls to 16 per cent at the peak and 12 per cent half way down the trailing edge. When these latter poorly renaturable fractions are mixed with purified *A* fraction before annealing, the recovery of activity is very pronounced, reaching values of 52–55 per cent that of native DNA.

The strand resolution which can be effected by two passages of the denatured pneumococcal DNA through MAK columns is almost complete and is remarkably reproducible. Since the residual activity of the resolved strands never decreases below 3–6 per cent, the activity of 12–16 per cent achieved by the fractions of the recycled *B* peak after self-annealing amounts to an increment of only 8 per cent of native activity. From these figures it is roughly estimated that the recycled *B* strands are 85 per cent pure near the top of the peak. The resolved *A* strands obtained after one passage through MAK are about 90 per cent pure.

Conclusions and Discussion.—Two distinct chromatographic peaks are obtained in high yield when denatured pneumococcal DNA is eluted from MAK columns at low temperatures with a salt gradient. The assays for ability to

regain transforming activity after annealing indicate that the peaks correspond to resolved complementary strands, as was previously demonstrated for eight genetic markers with stepwise elution from MAK.[4, 9] Since it now becomes clear that all of the denatured DNA is resolved into two distinct classes by MAK, there must be some chemical or physical distinction between all complementary strands of these bacterial DNA preparations.

The experiments of Szybalski and co-workers[5, 6] indicate that some DNA's possess local sequences of nucleotides which are rich in cytosine and that these regions usually occur on only one of the complementary strands. Those DNA's which have exhibited this behavior are from several bacteriophages and from some, but not all bacteria. Pneumococcal DNA has not been mentioned in these reports, but it is now known that it does interact with guanine-rich polyribonucleotides and, presumably, contains cytosine-rich regions on one strand.[7] Thus, MAK may be responding to these sequences without requiring the addition of ribopolymers for detection. A study of the MAK elution profiles of a variety of DNA's has been begun, but it is not yet known whether a positive correlation exists between two peak MAK profiles and interaction of one specific DNA strand with guanine-rich polymers.

It seems most unlikely that the traces of ribonucleotides remaining in the DNA preparations after both pancreatic and T_1 RNase digestion can account for strand resolution by the MAK columns. Szybalski reports that T_1 RNase completely abolishes the ability of ribosomal RNA to interact with DNA strands.[6] If, nevertheless, RNA is implicated, the recognition by MAK would have to be sensitive to amounts of bound ribonucleotides which cannot be detected by an increase in buoyant density. The denatured pneumococcal DNA's and their resolved strands all show single bands in CsCl at the same buoyant density. In this connection, it will be particularly interesting to observe the MAK elution profiles with denatured bacteriophage DNA's, where contamination by trace quantities of RNA fragments is unlikely.

If it turns out that the resolution of complementary strands by MAK is not caused by regions rich in specific purine or pyrimidine bases, other explanations will have to be sought. In any case, the clean resolution of complementary strands by the MAK columns provides useful material for studies involving the integration and transcription of transforming DNA, and it will now be possible to judge from chromatographic profiles whether MAK is capable of resolving the complementary strands of various other DNA preparations.

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