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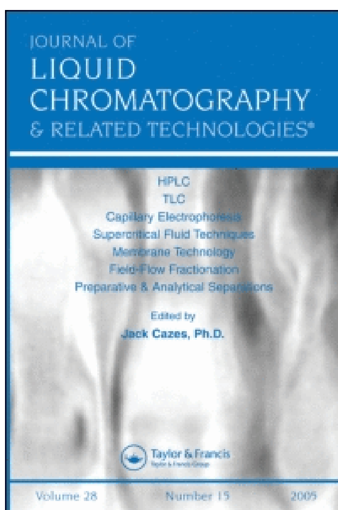


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# Bio-Macromolecular Liquid Chromatography Yesterday and Today: Advancements in the Separation and Study of Nucleic Acids and Their Related Proteins

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JOURNAL OF LIQUID CHROMATOGRAPHY, 12(8), 1347-1366 (1989)

# BIO-MACROMOLECULAR LIQUID CHROMATOGRAPHY YESTERDAY AND TODAY: ADVANCEMENTS IN THE SEPARATION AND STUDY OF NUCLEIC ACIDS AND THEIR RELATED PROTEINS

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Rapid, repeatable, and resolved chromatographic separations of molecules and analyses of their components have finally arrived in the commercial marketplace. This arrival has been long awaited, particularly with regard to nucleic acids (DNA and RNA). Biotechnological advances of protein and nucleic acid sequencing and synthesis have ushered in not only the instrumentation, but also the requirements of pre- and post-chromatographic column separations. A variety of high performance column supports had to be developed and have resulted in highly resolved separations of nmole quantities, as well as quality resolutions of mmole quantities of macromolecules. In addition, analysis of purified protein and nucleic acid components required development of reason-



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ably timed, high resolution separations of the more than 20 amino acids in one case, and 60 nucleosides in the other. This review juxtaposes the past with the present, and finally, gives a peek into the future of macromolecule separations.

Nucleic Acids

An understanding of nucleic acids began with an analysis of their component nucleotides. This had been first accomplished by acid or alkaline hydrolysis, and later by enzyme hydrolysis of the nucleic acids. Bases were separated by Dowex-50 chromatography into the four major varieties and then further analyzed by paper or thin layer chromatography. Some 60 modified nucleosides, mostly ribonucleosides, were discovered and their characterizations begun (1). Nucleosides were applied directly to paper or thin layer chromatography for separation (Figure 1). Visualization was obtained by U.V. absorbance or by autoradiography. Resolution, sensitivity, and quantitation were all poor. The methods were labor intensive, error prone, and time consuming. Today, more than 60 nucleosides in RNA, even in the presence of the additional four major deoxy-ribonucleosides, can be separated, identified, and quantitated in nmole amounts, in less than one hour (Figure 2)(1). Visualization by photodiode array spectrometry in real time permits rapid identification by ultraviolet spectral characteristics and by retention time. Standardization of the chromatography allows one to program the data acquisition for automatic peak identification analogous to that routinely accomplished in amino acid composition analysis.

The separation of nucleic acid polymers, at first limited to short, homopolymers (poly A), and co-polymers (poly dA:dT), was expanded to anion exchange separations of

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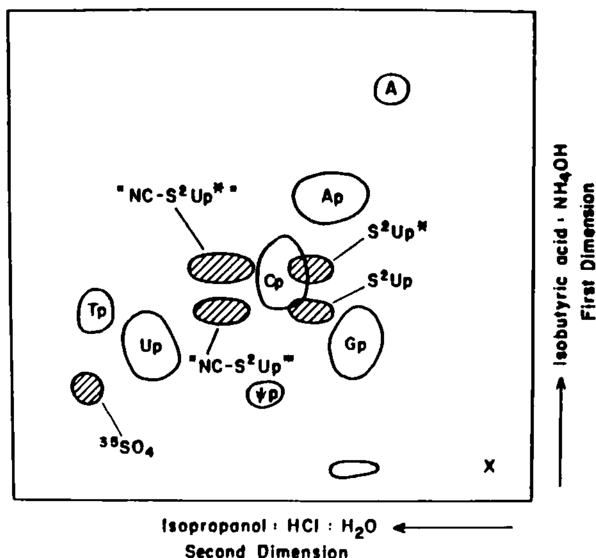


FIGURE 1. Thin layer chromatographic separation of modified nucleotides from E. coli $tRNA^{Glu}$. The tRNA was first treated with CNBr which reacts with the thiolated nucleotides; it was then enzymatically digested and the resulting digest placed on the TLC plate. The first dimension was developed using isobutyric acid: $NH_4OH$ and the second dimension was developed using isopropanol: HCl : $H_2O$.

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short bio-polymers (2 - 20 mers) derived from endonuclease digestions. The separation technique most often chosen was diethylaminoethyl-(DEAE)-cellulose column chromatography under denaturing conditions (Figure 3). In theory, as well as in practice, the polynucleotides are separated on the basis of chain length, solely due to the one negative charge associated with each nucleotide. Positively charged bases (such as 1-methyladenine) reduced the total negative charge for that polymer and its retention as well. Elution was effected by a shallow salt gradient. Resolution of oligomers was within the range of monomers to approximately decamers,

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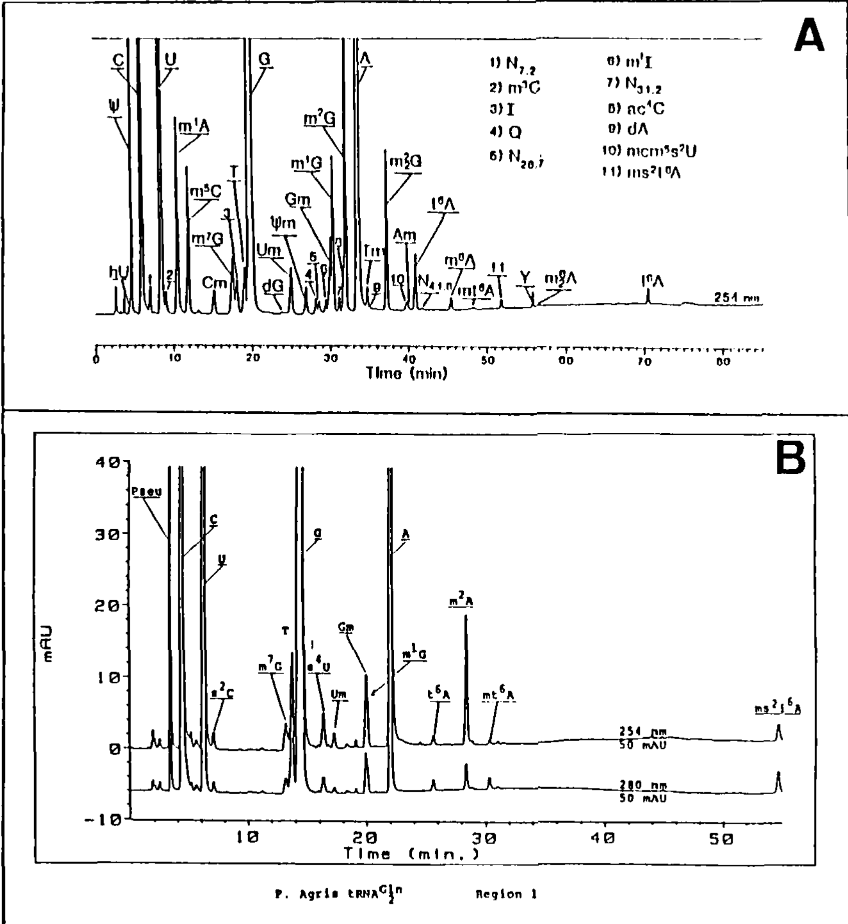


FIGURE 2. HPLC gradient separation of major and modified nucleosides. Panel A is the separation of nucleosides from unfractionated calf liver tRNA. Panel B is the separation of nucleosides from E. coli $tRNA_2^{GLN}$ . The tRNA was enzymatically hydrolyzed with P1 nuclease and dephosphorylated with bacterial alkaline phosphatase (11). The column used was a Supelcosil LC-18-DB 25cm x 4.6mm (Supelco). The solvents used were: A. pH 5.3, 0.01 M $NH_4H_2PO_4$, 2.5% $CH_3OH$; B. pH 5.1, 0.01 M $NH_4H_2PO_4$. 20 % $CH_3OH$: C. pH 4.9, 0.01 M $NH_4H_2PO_4$, 35% $CH_3OH$. Flow was 1 ml/min at 26 °C. In panel A the gradient was A to B 0-45 min., B to C 45-80 min., 100% C for 7 min. before returning to the initial conditions. The gradient for the separation for panel B was: A to B 0-18 min., B to C 18-32 min., 100% C 5 min. before returning to initial conditions.

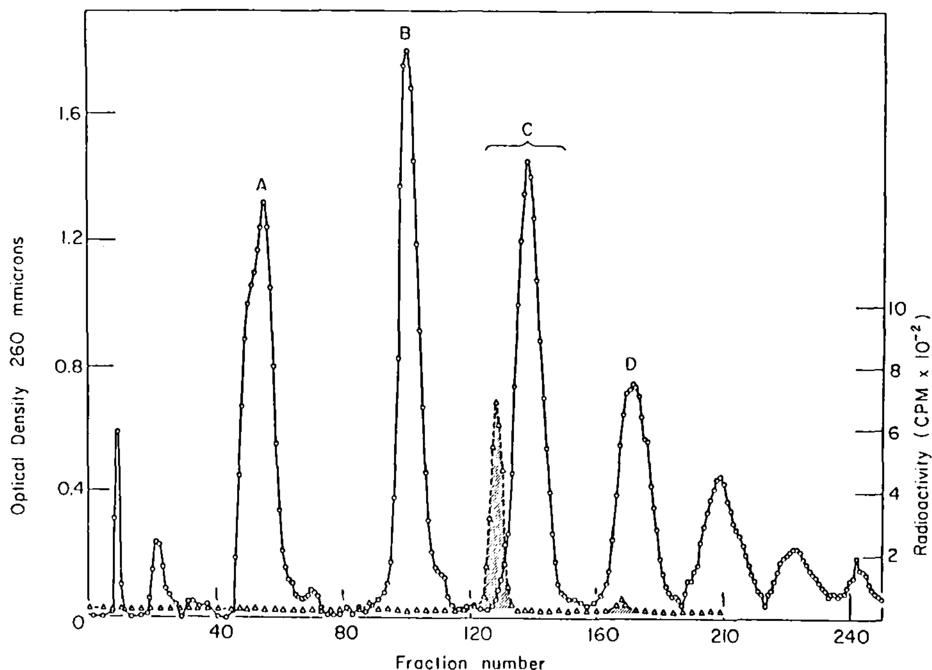


FIGURE 3. Chromatography on DEAE cellulose of a $T_1$ RNase digestion of $tRNA_f^{Met}$ . A 0.5 x 75 cm DEAE cellulose column was equilibrated with 7 M urea in 0.02 M Tris, pH 7.4. Nucleotides loaded onto the column were eluted with an 800 ml linear salt gradient of 0 to 0.4 M NaCl in 7 M urea, 0.02 M Tris, pH 7.4. Fractions of 2 ml were collected every 20 minutes. The absorbance at 260 nm and the radioactivity ($^{14}$C-methyl) of the fractions were determined. The absorbance is plotted with circles, the radioactivity peaks are shaded, and the activity denoted with triangles.

and was complicated by positively charged bases. Equal length oligomers of different base composition were rarely resolved. Separations were time consuming; retention times (salt concentrations) were not all acceptably reproduceable quantitation was difficult. In addition, the solvent (7M urea) made handling the chromatography system, and sample handeling afterwards troublesome.

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Today's polynucleotide separations are based on anion exchange, size exclusion and reversed phase HPLC (2-5). Denaturants are, for the most part, eliminated, or at least concentrations therein reduced significantly. They are not always required for resolution, and their absence is important to the ease of HPLC system operation and to product handling. We have used reversed phase HPLC (Fig. 4) to separate chemically synthesized oligomers. Figure 4 demonstrates the first step in the purification of a manually synthesized oligoribonucleotide pentamer. Reversed phase HPLC sufficiently separated the pentamer (1mg) from starting compounds and incorrect sequences during the first chromatography so that a repeat of the procedure with the eluted fraction yielded pure product (5). Of particular interest is the separation of instrument-synthesized DNA oligomers from aborted sequences; this is especially important for the longer syntheses that produce more aborted sequences. Figure 5 demonstrates the high resolution, reversed phase separation of successful trityl-protected DNA syntheses products (4 mer and 24 mer). This rapid purification of synthesized DNA of 30 mers and smaller from incomplete products proves better than gel electrophoresis. The reverse is true for larger oligomers.

Purification of whole nucleic acid molecules with complete biological functions have always proven difficult. One of the most interesting, but also most difficult, separation problems has been the purification of transfer RNA (tRNA) species. These 70-85 nucleotide length RNAs exist in all cells and are expressed in 60-100 different species. Their relatively common lengths have made size exclusion and traditional ion exchange chromatographies almost ineffective. Gel electrophoresis in the presence of denaturants was similarly ineffectual even at high polyacrylamide concentra-

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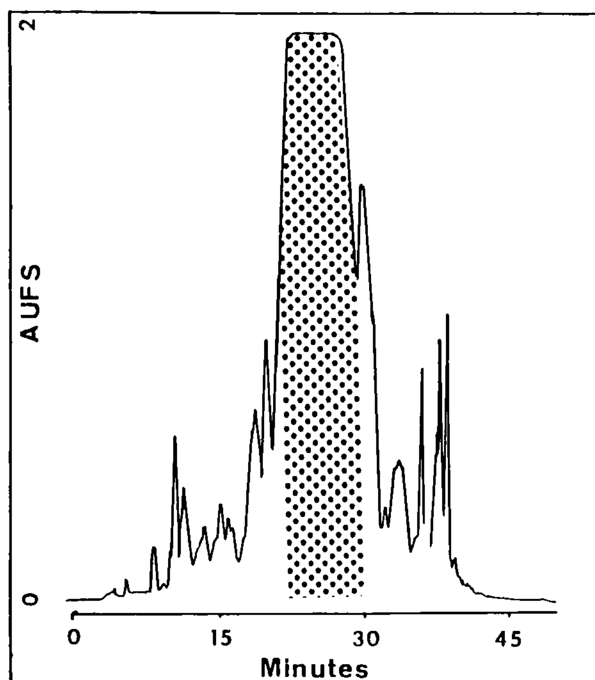


FIGURE 4. Reversed phase HPLC separation of a manually synthesized ribose pentameric oligonucleotide. Here the oligomer UCUUC was purified from the synthesis reagents and failed sequences using a Synchrom RP-P (4.1 x 250 mm) column. The shaded region marks the elution of the purified product. The HPLC system used included two Waters 510 pumps with a Waters 680 gradient controller and a Waters 441 detector with a fixed wavelength of 254 nm. Solvents were: A. pH 5.3, 2.5% $CH_3OH$, 0.001M $KH_2PO_4$; B. pH 5.3, 50% $CH_3OH$, 0.001M $K_2HPO_4$. The gradient was 100% A to 75% A, 25% B in 40 minutes followed by a 10 minute wash at 100% B before returning to the initial conditions. Flow rate was 0.5 ml/min, temperature was ambient, and the single run load limit was 1 mg total RNA.

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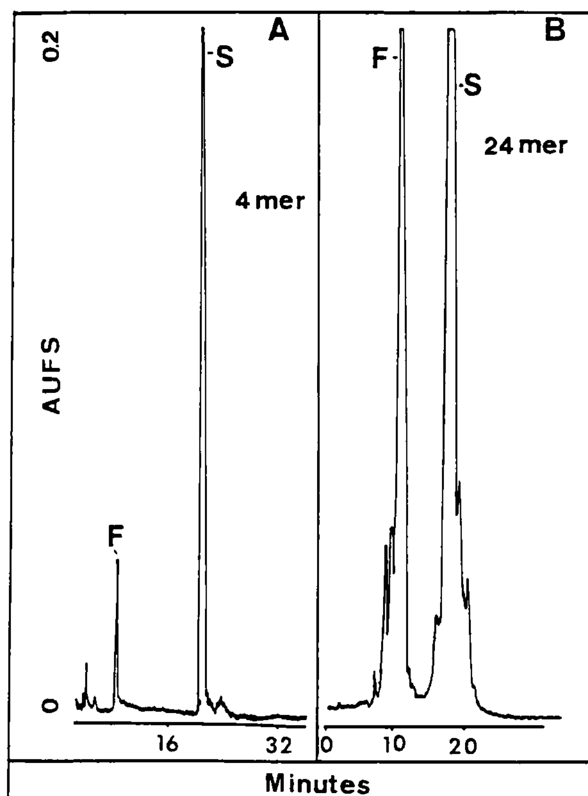


FIGURE 5. HPLC Purification of deoxy-oligo- nucleotides from an automated synthesizer. With the deoxy-trityl blocking group still attached to the completed synthesis product, failed and incomplete sequences are easily resolved from the full length product. Complete sequence samples are denoted with an S; failed sequences are denoted with an F. The system employed for this separation was the same as in Figure 4. Solvents were: A. 0.1M Tri-ethyl amine : acetic acid, pH 7.0; B. acetonitrile. The initial gradient conditions were 90% A, 10% B and were linearly changed to 50% A and B at time 25 minutes. The composition was maintained for 5 minutes and then returned to the initial conditions.

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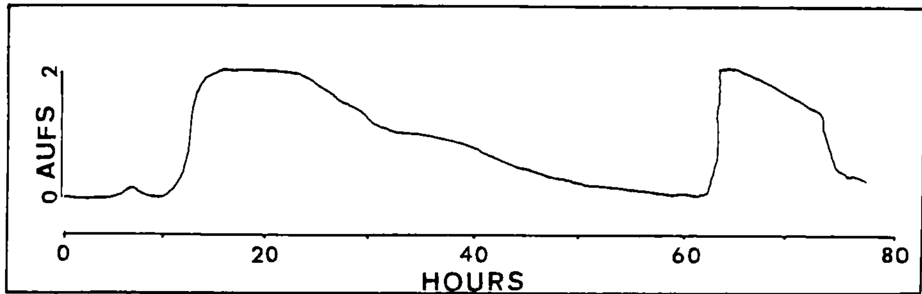


FIGURE 6. Large-scale LC separation of E. coli tRNA using benzolylated DEAE cellulose. The separation shown is of 48,000 $A_{260}$ units of RNA (approximately 2 g) on an LC column with a bed volume of 1850 ml. Elution regions of several tRNA species were: a. valine and f-methionine, b. leucine, c. serine, d. phenylalanine, e. tyrosine and tryptophan. The gradient was 0 to 1.5M NaCl, 0 - 50 hours, then to 1.5M NaCl with 20% ethanol, 50 - 80 hours. Flow was at 3.3 mL/min.

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tions. However, the presence in vivo of post-transcription, hydrophobic, base modifications of some tRNA species permitted separations with mixed-function columns. Benzoylated DEAE cellulose chromatography of unfractioned tRNA (Figure 6) resulted in separations on the basis of the strength of ion exchange coupled with hydrophobic interaction. In the absence of any large hydrophobic interaction, most E. coli tRNA species eluted from the column early and unresolved (Fig. 6) before 1.0 M NaCl was reached in the elution gradient. However, $tRNA^{Phe}$, having the hydrophobic group isopentenyladenosine at nucleotide position 37, was retained until 1.4M NaCl. Tryptophan and tyrosine tRNAs which also contain the same hypermodified nucleoside at position 37 only eluted after application of 1.5M NaCl containing 20% ethanol. This particular column required 3.5 days of continuous running with two days set-up.

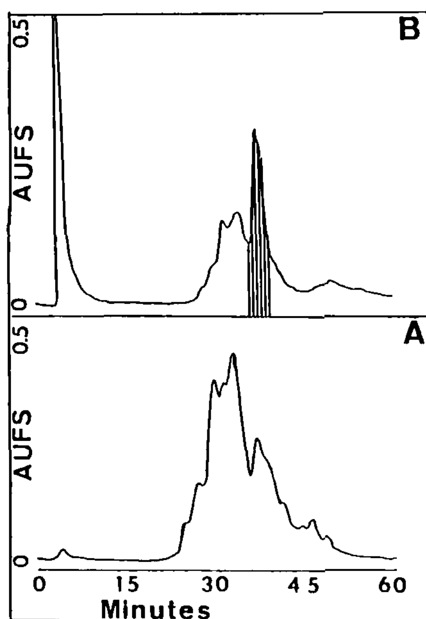


FIGURE 7. HPLC separation of E. coli tRNA using ion exchange DEAE functionality. Panel A is the separation of 2.5 $A_{260}$ units of unfractionated tRNA from a genetically engineered E. coli $tRNA_f^{Met}$ overproducer. The large peak near the injection results from unresolved smaller nucleotides that co-extracted with the RNA, and the shaded region marks the elution of the $tRNA_f^{Met}$. The separation was achieved with a Waters Protein Pak 5PW DEAE 7.5 x 75 mm column. Solvents were: A. 0.25 M NaCl, 0.1 M Tris, pH 7.6; B 0.5 M NaCl, 0.1 M Tris, pH 7.6 . The gradient was linear from solvent A to B over 55 minutes. The HPLC system was a Waters 600 Multisolvent Delivery System with a Milton Roy fixed wavelength (254 nm) detector.

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In contrast, today tRNA species can be readily purified by anion exchange high performance chromatography (6). In fact, with the cloning of tRNA genes and their excessive transcription (due to strong promotors), one chromatographic separation will typically yield an 80 plus percent pure tRNA species (Figure 7). We have cloned two wild type E. coli

tRNA species, $tRNA^{Gln}$ and $tRNA_f^{Met}$ behind the pL promotor (Nickerson, et al., manuscript in preparation). The excessive overproduction of the single tRNA makes identification of this tRNA after DEAE HPLC or gel electrophoresis easy through comparison to standard E. coli tRNA mixtures. Unlike previous LC separations of tRNA, or oligomers by anion exchange, this HPLC separation is accomplished in a minimum of time with analytical and preparative columns and without denaturants. We have found that the high performance weak anion exchangers are sensitive to nucleic acid structural differences that probably expose different numbers of phosphates. In contrast, the weak anion cellulose supports, or Sephadex, were not. Denaturants weakened the resolving power of HPLC supports for 70-85 mers; denaturants strengthened the resolving power of LC supports for oligomers from 2 to 25 nucleotides in length.

The interest in DNA gene cloning has not only placed pressure on the development of restriction endonuclease fragment isolation (probably still best by agarose gel electrophoresis for fragments larger than 100 mers), but also on plasmid isolation (7). Previous methods of plasmid isolation have been tedious, time consuming, and yielded incomplete purifications. Standard of these methods is the isopynic (cesium salt) centrifugation. The DNA is isolated from cells by phenol extraction and alcohol precipitation, then treated with RNase and proteases and subjected to the centrifugation for 6 to 18 hours. Bands of DNA detected by ultraviolet absorbance (enhanced by ethidium bromide intercalation and fluorescence) are collected through a needle. The material is then dialysed to remove the cesium chloride. In comparison, a forty five minute purification is possible by DEAE HPLC (Fig. 8)(5). Figure 8 demonstrates separation of plasmid under one set of conditions and separation of plasmid

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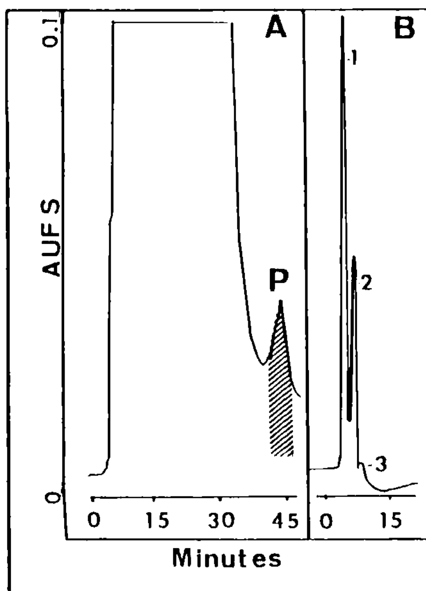


FIGURE 8. Isolation of plasmid DNA and resolution of conformational forms using HPLC. Panel A is the isolation of a derivative of the commonly used PBR 322 plasmid. The plasmid elution region is shaded and denoted with a P. The gradient used was 80% A , 20% B
initially, to 60% A, 40% B at time 50 minutes. The composition was then changed to 50 % B for 10 minutes and then returned to the initial conditions. Panel B is the resolution of a purified plasmid into its conformational forms: 1. relaxed, 2. supercoiled, 3. linear. The gradient used for this separation was 25% B initially to 40% B at time 40 minutes followed by a return to the initial conditions. The solvents employed were: A. 5M urea, 20 mM $KH_2PO_4$, pH 6.5; B
5M urea, 20 mM $KH_2PO_4$, pH 6.5, 1.5 M KCl. The HPLC system was a Waters 600 Multisolvent Delivery System with a Milton Roy fixed wavelength (254 nm) detector. The flow rate in both separations was 0.75 ml/min, and the temperature was ambient.

forms (supercoiled, relaxed) under different chromatography conditions. Although we have purified plasmid from only phenol-extracted, alcohol-precipitated nucleic acid, others have simply applied whole cell extracts (cleared of insoluble material) to the anion exchange column. The plasmids we have purified are completely free of contaminating DNA and RNA, and are cut by restriction enzymes into recognizable restriction fragments.

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Proteins and Peptides

The same weak anion exchanger, DEAE cellulose, used for nucleic acids, was also a favorite method for the separation of proteins. Other LC column supports routinely used for protein purification included the Sephadex ion exchangers and the Sephadex size exclusion gels. Columns were almost always large and many times run in cold rooms. Although ion exchange was not based on size, the poor resolution of these columns meant that multiple chromatographies with reduced yields were necessary. Thus, larger sample amounts were required. Gel filtration chromatography of proteins sometimes required the extremes of two-story columns (run in stairwells). A DEAE cellulose fractionation of HeLa cell extract is shown in Fig 9. The chromatography was completed in 21 hours. The fractions were assayed for tRNA methylase activity and demonstrated the presence of five peaks of activity. Peaks 1 and 2, when assayed for particular methylase activities, were pure, at least in terms of the resulting single methylated nucleotides.

Our research interests in nucleic acid structures, and the proteins that interact with and modify those structures, has led us to the study of superhelical DNA and its relaxation catalyzed by the enzyme DNA topoisomerase I. We have purified wheat germ DNA topoisomerase I activity by size

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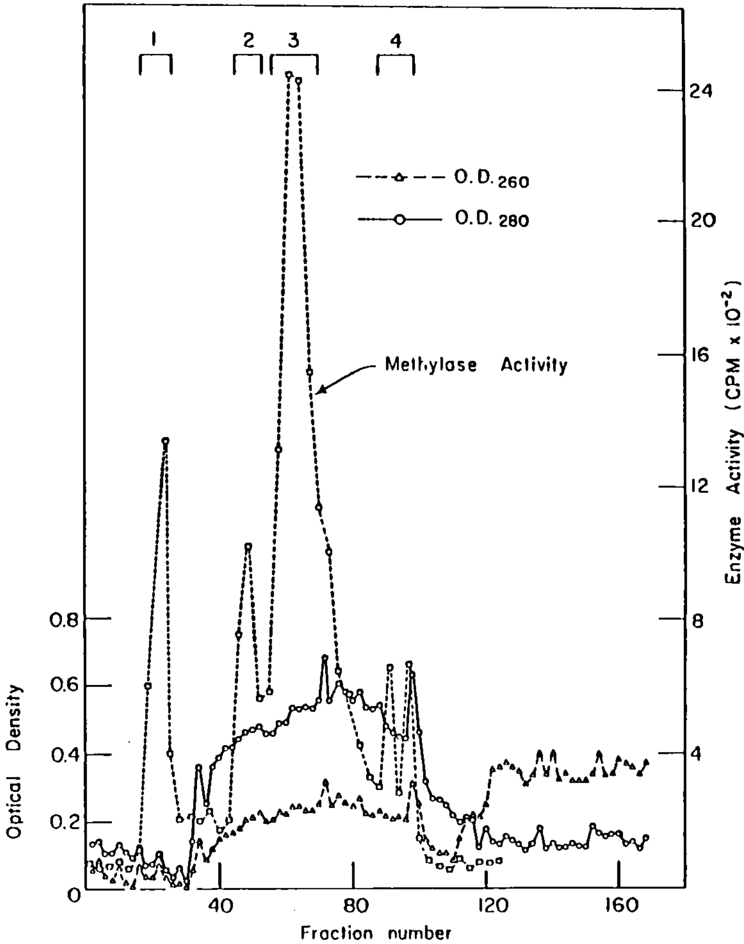


FIGURE 9. LC DEAE cellulose separation of tRNA methylase proteins. A 2 x 24cm column was equilibrated with TSHEG buffer (50 mM Tris, 3 mM DTT, 5 mM EDTA, 10% glycerol, pH 7.8) and loaded with fractions containing methylase activity from a DEAE-sephadex separation of HeLa cell extract. Activity was eluted first with the addition of 120 ml TSHEG. Then successive elutants contained 0.15 M KCl, 0.25 M KCl, and finally 0.5 M KCl all in TSHEG. Fractions of 4 ml were collected at 0.5 ml/min.

exclusion HPLC in 25 minutes (Figure 10) in contrast to the 21 hour fractionation mentioned above. In addition, photodiode array spectrometry during chromatography exhibited one protein peak with significant nucleic acid (260 nm) absorbance. This peak was the topoisomerase with nucleic acid still on the enzyme as demonstrated in agarose gel electrophoretic assays (8) of its DNA unwinding activity (Figure 11).

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Future

HPLC column improvements, especially in mixed-function and affinity supports, will provide the basis for future techniques of macromolecular separation. However, the latest advancements in membrane technology for purifications should not be overlooked. Membrane separations are particularly well-suited for large samples, or for large sample volumes of dilute concentration. They may be useful for pre-treatment before HPLC, or in the case of batch affinity purifications, as the one and only step. An example of the latter is presented here. A protein A disk was used for purification of human IgG. A sample of 15 ml of human serum was applied to the disk which was then eluted with pH 3, 0.1 M glycine. We found that elution was completed in 40 minutes, and the disk was ready for re-use in another 20 minutes (Figure 12). The eluted antibody was checked for purity by polyacrylamide gel electrophoresis and Western blot (with a goat anti-human IgG). Our human IgG preparation was pure with one passage of the membrane (Figure 13).

Conclusion

Liquid chromatography separations of macromolecules have been utilized for decades. Only recently have high performance chromatography methods been developed and exploited for

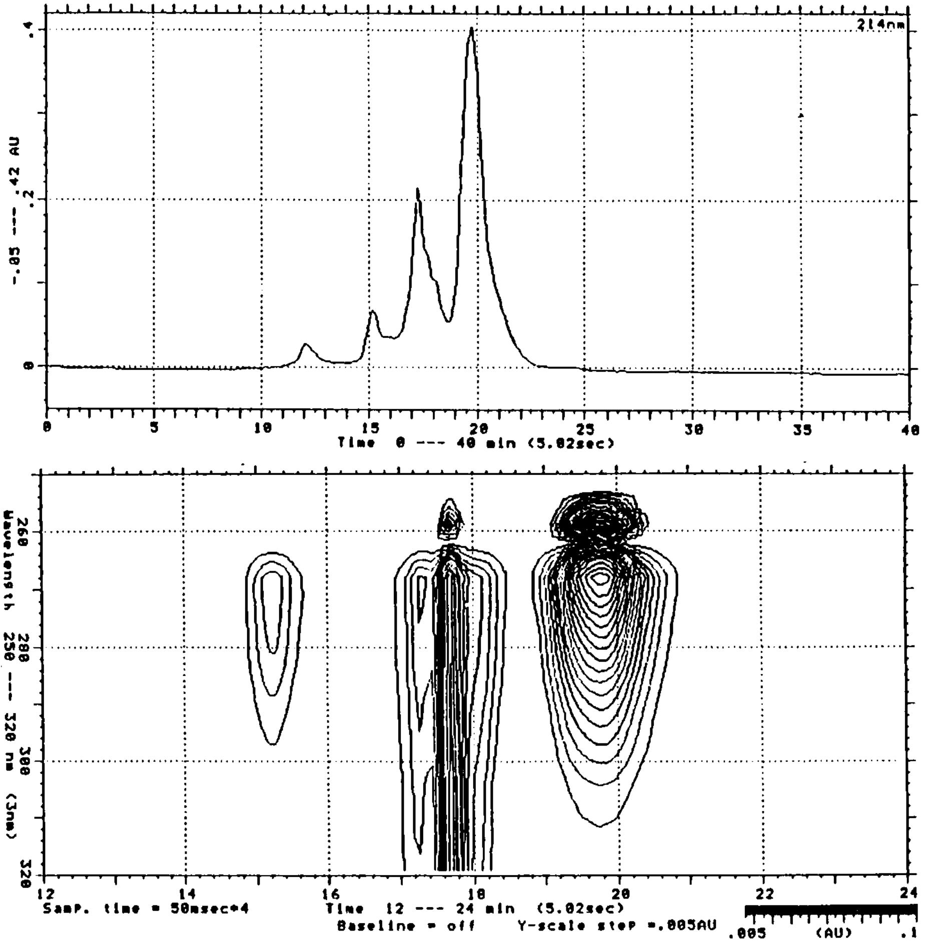


FIGURE 10. HPLC size exclusion separation of a semi-purified sample of wheat germ DNA topoisomerase I. In panel A, a 5 ul sample was injected onto a Synchropak GPC 300 column, 25 cm x 4.6 mm I.D. (Synchrom). Mobile phase was 10 mM $KH_2PO_4$, pH 7.5, with a flow rate of 0.2 ml/min. A Waters U6K injector and 510 pump were used. Detection of the peaks was with a Waters 990 Photodiode Array Detector. Panel B is the visualization of a co-migrating nucleic acid absorbance associated with the protein wheat germ topoisomerase I by use of the contour plot of the profile displayed in panel A.

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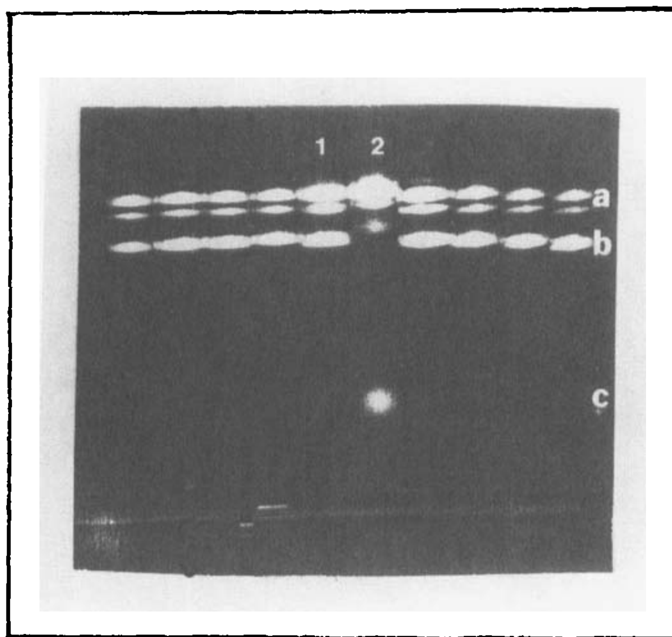


FIGURE 11. Agarose gel of HPLC fractions showing topoisomerase I activity and the co-migration of nucleic acid. A 10 ul sample of wheat germ extract (200 mg wheat germ per ml of a 10 mM NaH2PO4, 150 mM NaCl, pH 7.5 extraction buffer) was injected onto a Synchropak GPC 300 column. Fractions containing enzymatic activity were combined, lyophilized, and reinjected onto a Synchropak GPC 1000 column. Fractions of 200 ul were collected; 100 ul of each sample was then concentrated down to 10 ul and assayed for enzymatic activity by incubation with 0.74 ug OX174 RF DNA (Bethesda Research Laboratories), and 13 ul assay Buffer (50mM Tris-HCl, pH 7.9, 1 mM EDTA, 50 mM NaCl, 20% v/v glycerol) at 37 °C for 45 minutes. DNA topoisomerase I activity was assayed by electrophoretic separation of the reaction products in a 0.8% agarose gel. Lane 1 is representative of a enzymatically inactive fraction in which supercoiled DNA (b) remains after the incubation period. In Lane 2 the DNA topoisomerase I has converted supercoiled DNA to the nicked relaxed form (a). Small nucleic acids associated with topoisomerase are visible at (c).

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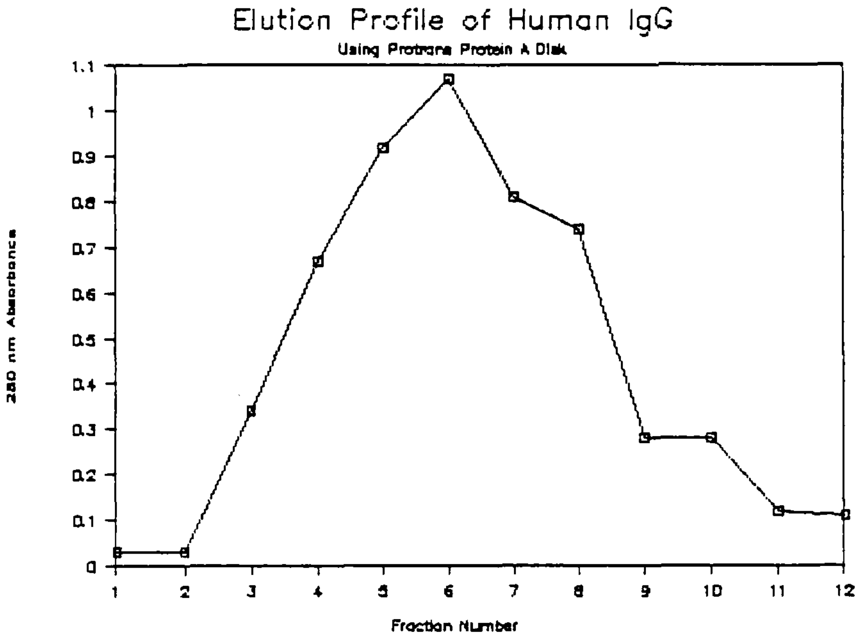


FIGURE 12. Elution profile of human IgG from a Protein A affinity disk. Fifteen ml of human serum was diluted to a 45 ml volume with 10 mM $NaH_2PO_4$, pH 7.2. The diluted serum was then recirculated one hour over a ProTran disk (ICN) at a flow rate of 6ml/min. The disk was then washed with 100 mM glycine, pH 7.6 for 20 minutes while maintaining the same flow rate. IgG was eluted with 100 mM glycine, pH 3.0, at a flow rate of 3 ml/min. Ten ml fractions were collected and the absorbance at 280 nm was plotted.

these large biomolecules and their constituent monomers. Progress has been rapid, and separations of even closely sized nucleic acids are now possible. Various supports are now employed that give equal or better separations than electrophoresis. Even molecules the size of plasmids can be isolated, separated into different forms and easily collected. Undoubtedly, the future will yield higher resolution columns, and specific affinity membranes.

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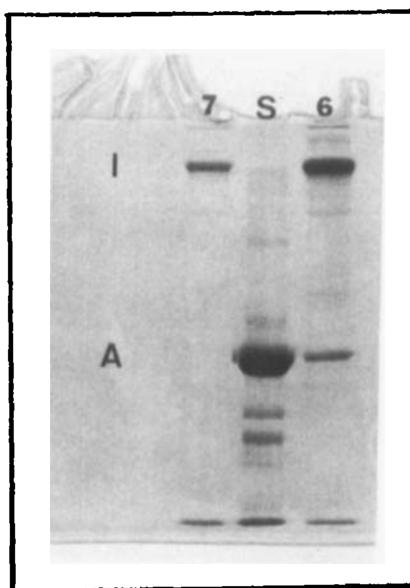


FIGURE 13. PAGE separation of fractions eluted from a ProTran Protein A affinity disk. A 10 ul sample of each fraction was loaded onto a 12.5% SDS gel. The original serum was run in lane S. Lanes 6 and 7 are samples from fractions corresponding to those collected on the purification plotted in FIGURE 12. The IgG band which has been enriched many fold migrates to position I. The large band at A in lane S is serum albumin, which has been virtually eliminated by the affinity disk purification.

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