IMMOBILIZED BIOCHEMICALS AND AFFINITY CHROMATOGRAPHY

Edited by
R. Bruce Dunlap
Department of Chemistry
University of South Carolina
Columbia, South Carolina

PLENUM PRESS • NEW YORK AND LONDON

Library of Congress Cataloging in Publication Data

Symposium on Affinity Chromatography and Immobilized Biochemicals, Charleston, S. C., 1973.
Immobilized biochemicals and affinity chromatography.

(Advances in experimental medicine and biology, v. 42)
Contains most of the papers presented at the symposium held Nov. 7-9 in conjunction with the Southeastern Regional American Chemical Society meeting.
Includes bibliographical references.
1. Biological chemistry — Technique — Congresses. 2. Affinity chromatography — Congresses. I. Dunlap, Robert Bruce, 1942- ed. II. Series. [DNLM: 1. Biochemistry — Congresses. 2. Chromatography, Affinity — Congresses W1 AD559 v. 42 1974 / QD271 S986i 1973.]
QP519.7.S9 1973 574.1'92'028 74-7471
ISBN 0-306-39042-6

Proceedings of the symposium on Affinity Chromatography and Immobilized Biochemicals held in Charleston, South Carolina, November 7 - 9, 1973

A Division of Plenum Publishing Corporation
227 West 17th Street, New York, N.Y. 10011

United Kingdom edition published by Plenum Press, London
A Division of Plenum Publishing Company, Ltd.
4a Lower John Street, London W1R 3PD, England

Printed in the United States of America

Contents

IMMOBILIZED POLYNUCLEOTIDES AND NUCLEIC ACIDS

P. T. Gilham

Department of Biological Sciences

Purdue University, West Lafayette, Indiana 47907

The use of immobilized nucleic acids and polynucleotides in the study of nucleic acids and their associated enzymes has become widespread in recent years. There are two main areas of application: the fractionation of nucleic acids and polynucleotides through base-paired complex formation and the isolation and purification of nucleic acid-associated enzymes by affinity chromatography. There are now a number of methods for the preparation of immobilized polynucleotides and the potential uses of these materials depend to some extent on the means by which the polymers are attached to the insoluble supports.

METHODS OF IMMOBILIZATION

As indicated in the following table, the methods for the preparation of immobilized polynucleotides can be broadly divided into two categories: those involving covalent linkages between the polymer and the support, and those employing physical entrapment of the polymer within the support matrix.

In those cases where covalent binding of the molecule is desirable, terminal attachment can be readily achieved by activation of the phosphomonoester group at the polynucleotide terminal in the presence of an insoluble polysaccharide. This reaction yields a product in which the polynucleotide is connected to the support by a stable phosphodiester linkage. A second approach exploits the reactivity of the terminal dialdehyde group resulting from the periodate oxidation of ribopolynucleotides. Condensation of these dialdehyde functions with supports containing primary amine or hydrazide

Table I

1. Covalent Binding at the Polynucleotide Terminals.

   (a) Activation of terminal phosphate group.
   (b) Periodate oxidation of 3'-terminals in RNA species.
   (c) Condensation of RNA 3'-terminals with supports containing dihydroxyboryl groups.

2. Covalent Binding at Multiple Points.

   (a) Condensation with phosphocellulose.
   (b) Reaction with CNBr-activated supports.
   (c) UV-irradiation.

3. Physical Entrapment.

groups allows covalent attachment at the polynucleotide 3'-terminals. A third method arises from the discovery that the terminal cis diol groups in ribopolynucleotides are capable of forming specific cyclic complexes with supports containing covalently-bound dihydroxyboryl groups. The use of phosphocellulose and CNBr-activated supports permits the multiple point attachment of polynucleotides and, in the case of CNBr activation, the reaction mechanism appears to be similar to that operating in the immobilization of enzymes by this method. Immobilization induced by ultraviolet irradiation of polynucleotides probably results in multiple point attachment also, although the mechanism of the reaction is obscure.

Covalent Attachment at Polynucleotide Terminals

Insoluble supports containing small homopolynucleotides can be prepared by the chemical polymerization of the appropriate mononucleotide (1,2). For example, the treatment of thymidine 5'-phosphate with dicyclohexylcarbodiimide in anhydrous solution produces thymidine oligonucleotides of the form, pdT-dT_n-dT ($n < 20$). In the reaction mixture, the terminal phosphate groups are present in an activated form and the addition of dry cellulose to the mixture results in the condensation of these groups with the hydroxyl functions of the support. In this case the oligonucleotides are bound to the support through stable phosphodiester linkages at their 5'-terminals. In an analogous way the polymerization of a nucleoside 3'-phosphate yields immobilized oligonucleotides connected to the cellulose at their 3'-terminals.

Preformed polynucleotides from either synthetic or natural sources may also be bound to cellulose at one of their terminals (3). In this case, the reaction is carried out in aqueous solution and a water-soluble activating agent, N-cyclohexyl-N'-β-(4-methylmorpholinium)ethylcarbodiimide, is used. The carbodiimide activates the terminal phosphomonoester group resulting in, again, a phosphodiester linkage to the support. The method was initially tested with mononucleotides, polynucleotides and tRNA (3), and it has recently been shown that, in the case of immobilized tRNA, practically all of the bound nucleotide material can be released by exposure to pancreatic ribonuclease (4), an observation that confirms the proposed terminal linkage to the support.

The reactivity of the 3'-terminal cis diol group in polyribonucleotides and RNA can be exploited in two ways for the terminal binding of these molecules. Periodate oxidation of the diol moiety to the corresponding dialdehyde yields a product that can undergo condensation with aminoethylcellulose, and the resulting linkage can then be stabilized by reduction with sodium borohydride (5). The binding in these products is thought to arise from the formation of a substituted morpholine ring structure that includes the nitrogen of the aminoethylcellulose and the five atoms that originally constituted the ribose ring of the terminal nucleoside. The assignment of this linkage is based on the structure of the product obtained from the borohydride reduction of the complex formed between periodate-oxidized adenosine 5'-phosphate and methylamine (6). Supports containing hydrazide groups will react in a similar manner with periodate-oxidized polyribonucleotides to form reasonably stable linkages. For example, a mixture of agar and polyacrylic acid hydrazide has been used to immobilize tRNA in this way (7), and reduction with sodium borohydride has also been used to stabilize the linkage in a similar complex obtained from periodate-oxidized tRNA and hydrazinyl-Sepharose (8).

Another method for the terminal binding of polyribonucleotides arises out of the observation that polymers that possess terminal cis diol groups are capable of forming specific complexes with supports containing covalently-bound dihydroxyboryl groups (9-11). N-[N'-(m-Dihydroxyborylphenyl)succinamyl]aminoethylcellulose (9) prepared from the condensation of N-(m-dihydroxyborylphenyl)-succinamic acid with aminoethylcellulose forms cyclic boronate structures at pH 8-9 with molecules containing the ribonucleoside diol group. In certain applications immobilized polynucleotides prepared by this method may have some advantages over those mentioned above in that, at pH 6, the complex breaks down, allowing the recovery of the bound polynucleotide from the support and the substituted cellulose is then available for the binding of another polymer.

Covalent Attachment at Multiple Points

Some of the early attempts to immobilize nucleic acids and polynucleotides exploited an activated form of acetylated phosphocellulose (12). Activation was achieved by treating the derivatized cellulose with dicyclohexylcarbodiimide in a non-aqueous solvent and it is presumed that the subsequent binding of the polynucleotide resulted from the formation of phosphodiester linkages between the support and the various hydroxyl groups on the polynucleotide chain. Thus, it was possible to bind the phosphocellulose to the glucosyl hydroxyl groups of phage T4 DNA (12) and to the 2'-hydroxyl groups of various polynucleotides and RNA molecules (13).

More recently, the immobilization technique that has been used so successfully for protein molecules and other ligands has been applied to polynucleotides. Single stranded RNA and DNA may be efficiently bound at pH 8 to cyanogen bromide-activated agarose (14) and, while the mechanism of the immobilization is not known, it seems likely that binding results from the formation of covalent linkages consisting of isourea ether groups connecting the oxygen atoms of the polysaccharide hydroxyl groups to the nitrogen atoms of some of the nucleotide bases within the polynucleotide chains. It has been suggested that, if the condensation reaction with the activated polysaccharide is carried out at pH 6, polynucleotides may be immobilized by single point attachment at the terminal phosphate groups of the polynucleotide strands (15). However, in this study, control experiments involving polynucleotides that do not possess terminal phosphate groups were not reported, and a more recent investigation of the reaction with cyanogen bromide-activated agarose indicates that multi-point attachment of polynucleotides occurs also at pH 6 (16).

Denatured DNA, RNA and polynucleotides are immobilized when exposed to UV-irradiation in the presence of inert supports such as polyvinyl beads, cellulose, and nylon threads (17). In this procedure it is possible that no covalent linkages are formed between the support and the polynucleotide strands since, in the absence of the support, these polymers are capable of forming insoluble gels upon irradiation. These gels are presumably a consequence of the production of intermolecular cross-links resulting from pyrimidine-pyrimidine dimer formation and the supports may serve only to immobilize the insoluble products so formed. Alternatively, the induction of both inter- and intramolecular cross-links of this type may be responsible for the formation of macrocyclic structures resulting in the physical entrapment of the polynucleotide strands within the support matrix. Binding could also result from UV-catalyzed addition reactions involving functional groups on the supports and the 5,6 double bonds in the pyrimidine moieties of the polynucleotide chains. Nevertheless, the method seems to have some general applicability

in that, by exposure to UV-irradiation, calf thymus DNA (18), viral RNA (19), and ribosomal RNA (20) have been bound to cellulose, and poly(U) has been immobilized on fiberglass filters (21).

Physical Entrapment

A number of methods for the physical immobilization of nucleic acids have been devised. With supports such as cellulose acetate (22), agar (22), and polyacrylamide (23) the nucleic acid is mixed with the support in a soluble state and this is followed by a treatment in which the support is rendered insoluble, thereby physically trapping the polynucleotide strands within the support matrix. For example, the DNA-cellulose acetate complex is formed by dissolving the two materials in an anhydrous solvent and then adding water to co-precipitate the two polymers, while immobilization in polyacrylamide gels is effected by the polymerization of acrylamide in the presence of the polynucleotide. Cellulose-DNA is prepared by simply drying a slurry of DNA and cellulose (24). Although these methods of immobilization have the advantage of simplicity in preparation the complexes formed are subject to dissociation and are useful only for the immobilization of nucleic acids of large molecular weight where the rate of diffusion from the support matrix is relatively slow compared with the time taken to complete a particular chromatographic experiment.

APPLICATIONS

There are three main research areas in which immobilized polynucleotides have been exploited:

1. Fractionation of polynucleotides and nucleic acids through base-paired complex formation.

2. Study of the mechanism of enzymes involved in the synthesis or degradation of nucleic acids.

3. Isolation of enzymes involved in the synthesis or degradation of nucleic acids by affinity chromatography.

Fractionation of Polynucleotides and Nucleic Acids

The base-pairing properties of cellulose containing terminally-linked thymidine oligonucleotides was first demonstrated with a mixture of deoxyadenosine oligonucleotides of chain lengths, 3-7 (1,2). The mixture was fractionated on a column of the oligo(dT)-cellulose using an elution procedure involving a stepwise temperature gradient. The temperature at which each oligo-

nucleotide could be eluted corresponded roughly to the dissociation temperature of the complex formed in solution between the oligonucleotide and thymidine dodecanucleotide. Oligoribonucleotide fragments obtained from viral RNA could also be fractionated on the basis of their content of contiguous adenosine sequences by chromatography on oligo(dT)-cellulose using temperature gradient elution (25).

The initial observation that oligo(dT)-cellulose could be used to specifically isolate A-rich polynucleotides from the total mammalian cellular RNA (26) has led to the widespread use of the material for the isolation and study of messenger RNA species that contain covalently-linked poly(A) segments. Examples of the application of the method include the isolation of poly(A)-containing nuclear RNA of HeLa cells (27), the purification of rabbit globin mRNA (28), the study of the in vivo and in vitro synthesis of poly(A)-rich RNA by rat brain (29), the purification of the mRNA coding for a mouse immunoglobin L-chain (30), the purification of the 14s mRNA coding for the A_2 chain of α-crystallin of calf lens (31), and the assay of the poly(A) content of yeast mRNA (32).

It should be pointed out that, in the preparation of oligonucleotide-celluloses for the isolation of nucleic acids by the specific base-pairing mechanism, the commercial source of the cellulose used is of some importance. Oligo(dT)-cellulose synthesized from a cellulose preparation that differed from the type originally specified (2) has been shown to be no more efficient in the binding of poly(A) than the unsubstituted cellulose itself (33). A more recent study (34) of the binding of polynucleotides to various celluloses has shown that some cellulose preparations are capable of binding considerable amounts of poly(A) and poly(I) in the absence of derivatization with oligo(dT) or oligo(dC) and it was suggested that this non-specific binding probably results from a relatively high lignin content in these preparations.

Celluloses containing polynucleotides that have been terminally linked by the water-soluble carbodiimide method have also been investigated for their ability to selectively bind polynucleotides containing complementary sequences. A comprehensive study of the preparation and the binding properties of immobilized oligodeoxyribonucleotides of defined chain length and sequence has been carried out and the results indicate that such materials should be useful in the isolation of mRNA species containing particular oligonucleotide sequences (35,36). In another application, the RNA prepared from the DNA of the simian virus, SV40, has been attached to cellulose by the carbodiimide procedure and the product was shown to be capable of specifically absorbing that portion of fragmented SV40 DNA that contained sequences complementary to the

immobilized RNA (37). The technique has some potential as a general method for gene isolation since the specificity of the absorption could be maintained even in the presence of a large excess of bacterial DNA.

Covalent immobilization via the periodate oxidation of the 3'-terminals of polynucleotides has also been used for the isolation of complementary polynucleotides. Periodate oxidized E. coli 16S rRNA bound to an agarose derivative containing hydrazide groups was found to be effective in purifying complementary DNA chains from a mixture of fragments prepared from sheared E. coli DNA (38). A recent novel application involves the use of the complex formed between a periodate-oxidized tRNA species and a polyacrylamide gel containing hydrazide groups to specifically bind another tRNA species possessing a complementary anticodon sequence (39). The technique constitutes a new approach to the study of complementary anticodons as well as a method for the purification of certain tRNA species.

The use of polynucleotides immobilized by multiple covalent linkages for the isolation of nucleic acids containing complementary sequences was first reported by Bautz and Hall (12). Bacteriophage T4 DNA was attached to acetylated phosphocellulose and the resulting material was used to chromatographically separate T4-specific RNA from E. coli RNA. More recent applications of this separation technique have made use of supports containing polynucleotides that have been attached by the cyanogen bromide or UV-irradiation methods. Poly(U) attached to cyanogen bromide-activated agarose has been employed in the isolation of mRNA from KB-cells (40), and in the study of the poly(A) segments in HeLa mRNA (41,43).

Myeloma cell mRNA containing poly(A) sequences has been isolated by chromatography on polyvinyl beads containing UV-irradiated poly(U) (44), and the same absorbent has been used to effect a partial purification of $tRNA^{ser}$ from crude E. coli tRNA (45). In addition, poly(U)-cellulose prepared by the UV-irradiation technique has found use in a number of studies concerned with the poly(A) sequences in viral and eukaryotic RNA species (21,46,47), while ribosomal RNA-cellulose prepared by the same method has served as an absorbent in the partial purification of ribosomal RNA genes from B. subtilis (20). Merriam et al. (48) have prepared, by the UV-irradiation method, a cellulose complex containing DNA from the bacteriophage, ØX174, and have used the material for the analysis of complementary strands in DNA preparations obtained from cells infected with the phage.

The immobilization of single-stranded DNA by physical entrapment in gels of cellulose acetate or agar produces materials that are capable of forming specific complexes with RNA molecules containing complementary sequences, and this property forms the basis

of a general method for the isolation of RNA species that possess sequences that are complementary to DNA from a particular source. The immobilization of DNA in agar has been subsequently applied to a number of studies on nucleic acids and the techniques of the preparation and uses of these materials have been reviewed (49).

Mechanism of Enzyme Action

The methods used for the binding of polynucleotides by terminal covalent attachment to a support are such that the orientation of the bound molecules is known, and this property allows the use of these immobilized polymers in the study of the action of those enzymes that are associated with the synthesis or degradation of nucleic acids. Oligo(dT)-cellulose can be used as a substrate primer for terminal deoxynucleotidyl transferase to extend the immobilized oligo(dT) chain at its 3'-terminus with a covalently-linked poly(dC) chain (50). This product together with soluble poly(dC) is capable of forming a bihelical structure with a common template molecule, poly(dI), and the resulting complex may be used to assay and study the enzyme, polynucleotide ligase. Further studies along these lines have shown that oligo(dT)-cellulose serves also as a primer and template for E. coli DNA polymerase and as a template for RNA polymerase (51). For example, the synthesis of poly(dT)-poly(dA) by DNA polymerase in the presence of a complex formed between oligo(dT)-cellulose and oligo(dA) yields a product in which the poly(dT) chain is covalently bound to the cellulose and the poly(dA) chain is hydrogen-bonded to the poly(dT) chain.

Polynucleotides with terminal phosphate groups have been linked to soluble polysaccharides such as Ficoll and dextran using the water-soluble carbodiimide technique, and the resulting macromolecules have been shown to possess some novel properties in their use in the study of the action of the enzymes, deoxyribonuclease, polynucleotide kinase, and DNA polymerase (52). The water-soluble carbodiimide technique can also be applied to the attachment of fragmented DNA to an insoluble cross-linked dextran and the resulting complex serves as a substrate for pancreatic deoxyribonuclease and as a template for RNA polymerase (53). Polynucleotides that have been linked at multiple points to cyanogen bromide-activated agarose also act as useful substrates in certain biochemical applications. RNA-agarose prepared in this way forms the basis of an assay procedure for an endonuclease isolated from sea urchin embryos (16). This nuclease degrades RNA to large polynucleotides and is somewhat difficult to assay by the classical methods. Poly(I)-agarose and the base-paired complex that it forms with poly(C) have been prepared as potential reagents for the study of the mechanism of induction of host resistance to viral infection (15).

Affinity Chromatography

The method of purification of proteins that exploits their capacity to specifically bind to immobilized polynucleotides has found extensive application in the study of those enzymes that bind to DNA. Initial applications of the affinity chromatographic method in nucleic acid research have employed DNA physically immobilized in agarose (54) or cellulose (24), and DNA immobilized on cellulose by UV-irradiation (18). These chromatographic materials have been used for the purification of endonuclease I and exonucleases I and II (54), DNA polymerase (18,24), RNA polymerase (24), and the gene 32-protein of bacteriophage T4 (24). The methods for the preparation and use of DNA-cellulose together with other applications of the chromatographic method have been reviewed by Alberts and Herrick (55). DNA immobilized in polyacrylamide gel has also been used for the purification of DNA polymerase (56). Many of the enzymes that are associated with the synthesis of nucleic acids possess the capacity to bind to single-stranded DNA and the use of single stranded DNA-agarose for the preparative purification of E. coli DNA polymerases I and II, RNA polymerase, exonuclease III, and bacteriophage T4 polynucleotide kinase has recently been described (57).

The covalent attachment of DNA to agarose by the cyanogen bromide procedure also produces materials that are suitable for affinity chromatography. DNA polymerase (14) and deoxyribonucleases from pancreas and spleen (58) have been purified by this method. In two other applications, the complex formed by the UV-irradiation immobilization of bacteriophage f2 RNA on cellulose has served in the chromatographic purification of the poly(G) polymerase obtained from f2-infected E. coli cells (19), and oligo(dT)-cellulose, prepared by the carbodiimide procedure, has provided a purification method for the RNA-dependent DNA polymerase from RNA tumor viruses (59).

Immobilized tRNA has been employed in a number of studies on the isolation of specific aminoacyl-tRNA synthetases. The chromatographic absorbent used in two of these studies was prepared by the periodate oxidation of a particular tRNA species and the subsequent covalent binding of the product to a support containing hydrazide groups. Affinity chromatographic purifications have been effected with $tRNA^{val}$ or $tRNA^{lys}$ bound to a polyacrylhydrazide-agar mixture (60), and with $tRNA^{phe}$ attached to hydrazinyl-agarose (8). In the latter case, the chromatographic method permitted the isolation of yeast phenylalanyl-tRNA synthetase in a completely pure state. Aminoacyl-tRNA may be immobilized by the covalent binding of the amino group of the aminoacyl moiety to an activated support. E. coli isoleucyl-tRNA can be bound to bromoacetamidobutyl-agarose and the resulting complex serves as a chromatographic system for the purification of the corresponding tRNA synthetase (61).

CONCLUSION

It is apparent from the foregoing discussion that, for a particular study, the choice of the method of immobilization depends, to a large extent, on the nature of the subsequent experimental application of the immobilized polymer. As with the use of other immobilized substances some consideration should be given to the stability of the linkage to the support and the steric availability of the attached polynucleotide for specific interactions with molecules in solution. In certain applications involving the use of covalently immobilized polynucleotides the methods that produce terminal attachment would seem to offer some advantages over those employing multi-point attachment. In studies concerned with the assay and the mechanism of action of nucleic acid-associated enzymes the methods employing single point attachment at one of the polynucleotide terminals are desirable since they yield products in which the polarity of the attached polymer is known and in which there is likely to be the least steric interference from the support matrix. In the isolation, by the base-pairing mechanism, of nucleic acids possessing homopolymer sequences, supports containing complementary homopolymers immobilized by single or multi-point attachment seem equally effective. In the future however, more sophisticated separations of nucleic acids based on the base-pairing of more complex complementary sequences will be attempted and, in these cases, the use of synthetic oligonucleotides of defined sequence, attached to a support at their terminals, may be preferred. In this regard, it is of interest that a study of the binding capacity of synthetic oligonucleotides bound to cellulose at their 5'-terminals by the water-soluble carbodiimide method indicates that the entire oligonucleotide, in each case, is apparently available for base-paired complex formation with its complementary nucleotide sequence (36).

Future applications of supports containing covalently-linked polynucleotides are likely to add more emphasis to the importance of the nature of the covalent linkage itself. In the case of multi-point immobilized polymers some applications may require a consideration of the number of attachment points per polynucleotide chain that are formed during the immobilization procedure. For example, RNA-agarose prepared by the cyanogen bromide procedure has been used to study an endonuclease that degrades RNA to large oligonucleotides, and the capacity of the enzyme to release RNA fragments from the support was shown to be markedly dependent on the concentration of the cyanogen bromide used to prepare the RNA-agarose (16). For affinity chromatographic applications physical entrapment is a satisfactory method for the immobilization of those nucleic acids that, by virtue of their size, do not undergo rapid diffusion from the support matrix.

ACKNOWLEDGEMENT

This work was supported by grants GM11518 and GM19395 from the National Institutes of Health.

REFERENCES

1. Gilham, P. T., J. Amer. Chem. Soc. 84, 1311 (1962).
2. Gilham, P. T., J. Amer. Chem. Soc. 86, 4982 (1964).
3. Gilham, P. T., Biochemistry 7, 2809 (1968).
4. Jeffers, J. S. and Gilham, P. T., unpublished experiments.
5. Gilham, P. T., Methods Enzymol. 21, 191 (1971).
6. Brown, D. M. and Read, A. P., J. Chem. Soc., 5072 (1965).
7. Knorre, D. G., Myzina, S. D., and Sandakhchiev, L. S., Izv. Sibirsk. Otd. Akad. Nauk SSSR, Ser. Khim. Nauk, 135 (1964).
8. Remy, P., Birmelé, C., and Ebel, J. P., FEBS (Fed. Eur. Biochem. Soc.) Lett. 27, 134 (1972).
9. Weith, H. L., Wiebers, J. L., and Gilham, P. T., Biochemistry 9, 4396 (1970).
10. Rosenberg, M., Wiebers, J. L., and Gilham, P. T., Biochemistry 11, 3623 (1972).
11. Rosenberg, M. and Gilham, P. T., Biochim. Biophys. Acta 246, 337 (1971).
12. Bautz, E. K. F. and Hall, B. D., Proc. Natl. Acad. Sci. U.S. 48, 400 (1962).
13. Adler, A. J. and Rich, A., J. Amer. Chem. Soc. 84, 3977 (1962).
14. Poonian, M. S., Schlabach, A. J., and Weissbach, A., Biochemistry 10, 424 (1971).
15. Wagner, A. F., Bugianesi, R. L., and Shen, T. Y., Biochem. Biophys. Res. Commun. 45, 184 (1971).
16. Berridge, M. V. and Aronson, A. I., Anal. Biochem. 53, 603 (1973).
17. Britten, R. J., Science 142, 963 (1963).
18. Litman, R. M., J. Biol. Chem. 243, 6222 (1968).
19. Fedoroff, N. V. and Zinder, N. D., Proc. Natl. Acad. Sci. U.S. 68, 1838 (1971).
20. Smith, I., Smith, H., and Pifko, S., Anal. Biochem. 48, 27 (1972).
21. Sheldon, R., Jurale, C., and Kates, J., Proc. Natl. Acad. Sci. U.S. 69, 417 (1972).
22. Bolton, E. T. and McCarthy, B. J., Proc. Natl. Acad. Sci. U.S. 48, 1390 (1962).
23. Wada, A. and Kishizaki, A., Biochim. Biophys. Acta 166, 29, (1968).
24. Alberts, B. M., Amodio, F. J., Jenkins, M., Gutmann, E. D., and Ferris, F. L., Cold Spring Harbor Symp. Quant. Biol. 33, 289 (1968).

25. Gilham, P. T. and Robinson, W. E., J. Amer. Chem. Soc. 86, 4985 (1964).
26. Edmonds, M. and Caramela, M. G., J. Biol. Chem. 244, 1314 (1969).
27. Edmonds, M., Vaughan, M. H., and Nakazato, H., Proc. Natl. Acad. Sci. U.S. 68, 1336 (1971).
28. Aviv, H. and Leder, P., Proc. Natl. Acad. Sci. U.S. 69, 1408 (1972).
29. DeLarco, J. and Guroff, G., Biochem. Biophys. Res. Commun. 49, 1233 (1972).
30. Schechter, I., Proc. Natl. Acad. Sci. U.S. 70, 2256 (1973).
31. Berns, A. J. M., Bloemendal, H., Kaufman, S., and Verma, I. M., Biochem. Biophys. Res. Commun. 52, 1013 (1973).
32. McLaughlin, C. S., Warner, J. R., Edmonds, M., Nakazato, H., and Vaughan, M. H., J. Biol. Chem. 248, 1466 (1973).
33. Kitos, P. A., Saxon, G., and Amos, H., Biochem. Biophys. Res. Commun. 47, 1426 (1972).
34. DeLarco, J. and Guroff, G., Biochem. Biophys. Res. Commun. 50, 486 (1973).
35. Astell, C. and Smith, M., J. Biol. Chem. 246, 1944 (1971).
36. Astell, C. R. and Smith, M., Biochemistry 11, 4114 (1972).
37. Shih, T. Y. and Martin, M. A., Proc. Natl. Acad. Sci. U.S. 70, 1697 (1973).
38. Robberson, D. L. and Davidson, N., Biochemistry 11, 533 (1972).
39. Grosjean, H., Takada, C., and Petre, J., Biochem. Biophys. Res. Commun. 53, 882 (1973).
40. Lindberg, U. and Persson, T., Eur. J. Biochem. 31, 246 (1972).
41. Adesnik, M., Salditt, M., Thomas, W., and Darnell, J. E., J. Mol. Biol. 71, 21 (1972).
42. Jelinek, W., Adesnik, M., Salditt, M., Sheiness, D., Wall, R., Molloy, G., Philipson, L., and Darnell, J. E., J. Mol. Biol. 75, 515 (1973).
43. Molloy, G. R. and Darnell, J. E., Biochemistry 12, 2324 (1973).
44. Stevens, R. H. and Williamson, A. R. Nature (London) 239, 143 (1972).
45. Hung, P. P., Science 149, 639 (1965).
46. Kates, J., Cold Spring Harbor Symp. Quant. Biol. 35, 743 (1970).
47. Philipson, L., Wall, R., Glickman, G., and Darnell, J. E., Proc. Natl. Acad. Sci. U.S. 68, 2806 (1971).
48. Merriam, E. V., Dumas, L. B., and Sinsheimer, R. L., Anal. Biochem. 36, 389 (1970).
49. Bendich, A. J. and Bolton, E. T., Methods Enzymol. 12 B, 635 (1968).
50. Cozzarelli, N. R., Melechen, N. E., Jovin, T. M., and Kornberg, A., Biochem. Biophys. Res. Commun. 28, 578 (1967).

51. Jovin, T. M. and Kornberg, A., J. Biol. Chem. 243, 250 (1968).
52. Scheffler, I. E. and Richardson, C. C., J. Biol. Chem. 247, 5736 (1972).
53. Rickwood, D., Biochim. Biophys. Acta 269, 47 (1972).
54. Naber, J. E., Schepman, A. M. J., and Rorsch, A., Biochim. Biophys. Acta 114, 326 (1966).
55. Alberts, B. M. and Herrick, G., Methods Enzymol. 21, 198 (1971).
56. Cavalieri, L. F. and Carroll, E., Proc. Natl. Acad. Sci. U.S. 67, 807 (1970).
57. Schaller, H., Nusslein, C., Bonhoeffer, F. J., Kurz, C., and Nietzschmann, I., Eur. J. Biochem. 26, 474 (1972).
58. Schabort, J. C., J. Chromatogr. 73, 253 (1972).
59. Gerwin, B. I. and Milstein, J. B., Proc. Natl. Acad. Sci. U.S. 69, 2599 (1972).
60. Nelidova, O. D. and Kiselev, L. L, Molekul. Biol. 2, 60 (1968).
61. Bartkowiak, S. and Pawelkiewicz, J., Biochim. Biophys. Acta 272, 137 (1972).