PREPARATION OF SEPHAROSE-BOUND POLY(rI:rC)

by

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SUMMARY

Poly rI was covalently bound through its terminal 5'-phosphate moiety to Sepharose and then annealed with poly rC to yield insoluble matrix bound poly(rI:rC). The method has no serious deleterious effects on the integrity or in vitro biological activity of the double stranded complexes.

INTRODUCTION

Our interest in the synthesis of a stable, insoluble, matrix bound form of poly(rI:rC) stemmed from its potential as a reagent to study the mechanism of induction of host resistance to viral infection at the cellular level and its usefulness as a tool for the isolation of membrane or intracellular receptor sites involved in the process of induction of host resistance to viral infection. Procedures used to bind nucleic acids to insoluble supports include irradiation induced immobilization of double stranded DNA to cellulose (1), carbodiimide mediated esterification of single stranded ribonucleic acids on paper (2), and multi-point attachment of amino group containing ribonucleic acids with some single stranded character to cyanogen bromide activated Sepharose (3). For our study, we devised a two step method to covalently bind double stranded ribonucleic acids to Sepharose. The first step, which is applicable for all single

stranded ribonucleic acids, involves binding the nucleic acid to Sepharose by coupling the terminal 5'-phosphate moiety of the ribonucleic acid to an hydroxyl group of the Sepharose. In the second stage, the Sepharose bound ribonucleic acid is annealed with an appropriate ribonucleic acid to yield a covalently bound, double stranded complex.

MATERIALS AND METHODS

Polyinosinic-8 ^{14}C acid (0.17 μc per μmole of phosphate) was obtained from Miles Laboratories, Inc., Elkhart, Indiana. Polyinosinic acid (λ_{max} 248 nm, E% 280 (pH 7.0); $S_{20,w}$ 12.9) and polycytidylic acid (λ_{max} 268 nm, E% 183 (pH 7.0); $S_{20,w}$ 6.4) were kindly provided by Dr. E. E. Harris of our laboratories.

Prior to activation, Sepharose 4B, a beaded form of agarose, was washed with 0.1N NaCl and then with deionized water. In a typical activation reaction, 15 ml of washed Sepharose 4B was suspended in 30 ml of deionized water and cooled to about 10°. After the addition of 4.5 g of CNBr to the stirred suspension, the pH was raised to 11 and maintained by the addition of 8N NaOH. After 15 min, the temperature had reached 20°. The activated matrix material was isolated by filtration, washed with 600 ml of cold deionized water, and usually used promptly in the condensation reaction. Contrary to published reports, however, the activated matrix material retains its reactivity for several weeks on storage in deionized water at 4°.

Single stranded ribonucleic acids were attached to activated Sepharose by adding three volumes of a solution (3-4 mg/ml) of the ribonucleic acid in pH 6.0, 0.2M 4-morpholine-

ethanesulfonic acid buffer to one volume of activated Sepharose. The suspension was stirred gently overnight at 4°, and the product was isolated by filtration. The matrix bound ribonucleic acid was packed in a column and washed continuously with pH 7.0, 0.15M NaCl - 0.006M sodium phosphate buffer at the rate of 60 ml/hr for about 12 hr. The extent of ribonucleic acid binding to activated Sepharose was established by hydrolysis of an aliquot of the product in 0.25N NaOH (1 ml per 0.1 ml matrix material) overnight. The amount of liberated nucleotide was established by ultraviolet absorption of the filtrate at pH 7.0 (inosinic acid - λ_{max} 248.5 nm, E% 353; cytidylic acid - λ_{max} 270 mu, E% 284).

For the preparation of matrix bound, double stranded ribonucleic acid, Sepharose bound, single stranded ribonucleic acid was suspended in two volumes of a solution (3-4 mg/ml) of the second ribonucleic acid in pH 7.0, 0.15M NaCl - 0.006M sodium phosphate buffer and stirred overnight at 4°. The product was isolated by filtration and exhaustively washed with the pH 7 buffer prior to assay by alkaline hydrolysis or testing for the induction of host resistance to viral infection *in vitro*.*

RESULTS AND DISCUSSION

At the outset, a method for binding ribonucleic acids to Sepharose was sought that would be applicable for all single stranded nucleic acids and result in a stable, single point attachment of the macromolecule to the matrix. This left little

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choice but to bind the macromolecule to the matrix through a phosphodiester moiety forged by condensing the terminal 5'-phosphate group of the nucleic acid with an hydroxyl function of Sepharose. Sepharose was treated with cyanogen bromide to yield cyanic ester moieties or cyclic iminocarbonate functions (4,5), which in turn were condensed with the ribonucleic acid. The latter reaction may be regarded as proceeding by addition of the 5'-phosphate function of the nucleic acid to a cyanic ester moiety of the activated Sepharose to yield an activated 5'-phosphate group (6), which, in turn, reacts with an adjacent hydroxyl group of the Sepharose yielding a phosphodiester. To keep the phosphate moiety in a suitable form for condensation and prevent participation of amino groups, the reaction was carried out at pH 6.0.

A 7.5-ml portion of cyanogen bromide activated Sepharose was stirred overnight in a solution of 70 mg of poly rI in 20 ml of the pH 6.0 buffer. The product was isolated by filtration and washed with four volumes of deionized water. One 2-ml aliquot of the product contained 4.1 mg of poly rI per ml of settled Sepharose after exhaustive washing with 500 ml of the pH 7.0 buffer. Other 2-ml aliquots stirred with 75 ml of 0.1M pH 9.5 carbonate buffer, or 75 ml of 0.1M pH 10.6 carbonate buffer contained 3.3 and 2.6 mg of poly rI per ml of matrix, respectively. These data suggest that poly rI is covalently bound to the Sepharose largely by single point attachment through the terminal 5'-phosphate moiety rather than multi-point attachment through those phosphodiester moieties along the length of the nucleic acid chain, since reaction of the latter groups would yield phosphotriesters that should hydrolyze readily at pH 9.5 - 10.6.

Sepharose bound poly(rI:rC) was prepared by treating 6 ml of Sepharose bound poly rI (7.3 mg poly rI per ml of matrix) with a solution of 68 mg of poly rC in 15 ml of the pH 7.0 buffer overnight, followed by filtration and exhaustive washing with the pH 7 buffer. The product contained 7.3 mg of poly rI and 6.7 mg of poly rC per ml of settled Sepharose and was active at high dilution for the induction of host resistance to viral infection in cell culture.

The stability of the covalently bound poly(rI:rC) at pH 7.0 was demonstrated conclusively by testing washings in the in vitro host resistance induction assay. Stability in cell culture medium, however, could only be determined by labeling techniques. Accordingly, Sepharose bound poly(rI[8 ^{14}C]:rC) containing 0.29 uc of ^{14}C (1.7 μmoles of poly rI) per ml of Sepharose was prepared in the manner described above. There was essentially no leakage of the covalently bound, double stranded complex from the matrix in pH 7.0 buffer at 35°. In cell culture medium, however, about 20% of the poly(rI[^{14}C]:rC) was stripped from the matrix in the course of 18 hr at 35°.

In a comparable study, poly rC was covalently attached to activated Sepharose and then annealed with poly rI. This matrix bound poly(rI:rC) contained 2.7 mg of poly rI and 1.4 mg of poly rC per ml of Sepharose and was active at high dilution for the induction of host resistance to viral infection in vitro.

In conclusion, a general two-step method has been developed for the single point attachment of double stranded ribonucleic acids to insoluble hydroxyl containing matrices. The method, which causes no serious alterations in the structure

or *in vitro* biological activity of the double stranded nucleic acid, is now being extended to the preparation of soluble, bound polynucleotides on macromolecules such as polyglucose.

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