The Synthesis of Celluloses Containing Covalently Bound Nucleotides, Polynucleotides, and Nucleic Acids*

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ABSTRACT: A new and versatile method for the incorporation of nucleotides, polynucleotides, and nucleic acids onto cellulose has been developed. The method involves specific activation of the terminal monosubstituted phosphate or polyphosphate group of the nucleotide or polynucleotide (in aqueous solution at pH 6) in the presence of cellulose chromatographic paper. The activation is brought about by *N*-cyclohexyl-*N'*-β-(4-methylmorpholinium)ethyl carbodiimide *p*-toluenesulfonate and the condensation with the cellulose is achieved by a novel technique in which the reaction mixture is concentrated within the fibers of the cellulose. The products of the reaction are considered to consist of nucleotides or polynucleotides connected to the cellulose by ester linkages between the terminal phosphate or polyphosphate groups of the nucleotide chains and the sterically favored hydroxyl groups of the cellulose. The reaction has been applied to a variety of nucleotides, polynucleotides, and nucleic acids, containing terminal phosphate, diphosphate, or triphosphate groups, and the yields of incorporation have varied from 15 to 71%. Polynucleotide celluloses prepared by this method are expected to be of some value in the study of those enzymes which are concerned with the synthesis or degradation of nucleic acids.

In addition, the capacity of the method to readily incorporate nucleotides and polynucleotides onto an insoluble support should permit the development of new methods for the chemical and enzymatic synthesis of polynucleotides as well as new techniques for the fractionation and sequence analysis of polynucleotides from natural sources.

The exposure of cellulose powder to anhydrous solutions of chemically polymerized mononucleotides allows the formation of cellulose products which contain simple polynucleotides covalently bound through phosphodiester linkages at their 5'-phosphate terminals (Gilham, 1962, 1964). Polynucleotide celluloses formed in this way have been exploited in a number of studies concerned with nucleic acid research. For example, insoluble polymers prepared by this method have been used in new fractionation methods in which the components of mixtures of polynucleotides can be separated by virtue of the variation in the stabilities of base-paired complexes formed between the components of the mixtures and the cellulose-bound polynucleotides (Gilham, 1964; Gilham and Robinson, 1964). More recently, a consideration of these findings has led to the use of these polynucleotide celluloses as insoluble primers and templates for the nucleotide-polymerizing enzymes. Thymidine polynucleotide cellulose has been found to serve as a primer and template for DNA polymerase, as a template for RNA polymerase, and as an initiator for the terminal deoxynucleotidyltransferase (Jovin and Kornberg, 1968), while Cozzarelli *et al.* (1967) have used the same insoluble polymer together with a complementary polynucleotide to assay for the enzymatic joining of polynucleotide strands. Although the anhydrous method of preparation of polynucleotide celluloses is an efficient one, the products formed contain only homopolymers of relatively short chain length. In fact, the polynucleotides which may be incorporated in this way are limited in base sequence and chain length to those which can be prepared chemically in anhydrous solution. We now wish to describe a new and more versatile method for the preparation of polynucleotide celluloses. The new technique involves the covalent binding of nucleotides and polynucleotides through the specific activation, in aqueous solution, of their terminal phosphate or polyphosphate groups, and the method is applicable equally well to polymers of both natural and synthetic origin. In addition, there is an extra practical advantage in the method, in that, polynucleotides may be readily incorporated onto cellulose paper strips, a technique which facilitates the handling of these insoluble polymers.

N-Cyclohexyl-*N'*-β-(4-methylmorpholinium)ethyl carbodiimide *p*-toluenesulfonate in aqueous solution has been shown to act as a specific activating agent for the monoesterified phosphate groups of nucleotides or polynucleotides, providing the pH of the solution is maintained at about 6.0 (Naylor and Gilham, 1966). It has also been shown that this activation of the phosphate groups can result in the formation of phosphodiesters if there is a sufficiently high concentration of hydroxylic component also present in the reaction mixture, or if the molecules themselves contain hydroxyl groups in favorable steric relationships to their activated phosphate groups. Thus, the reagent can convert, in aqueous solution, nucleoside 2'(3')-phosphates into the corresponding cyclic phosphates, and nucleoside 5'-phosphates into nucleoside 5'-alkyl phosphates. In addition, it has been demonstrated that this reagent is capable of

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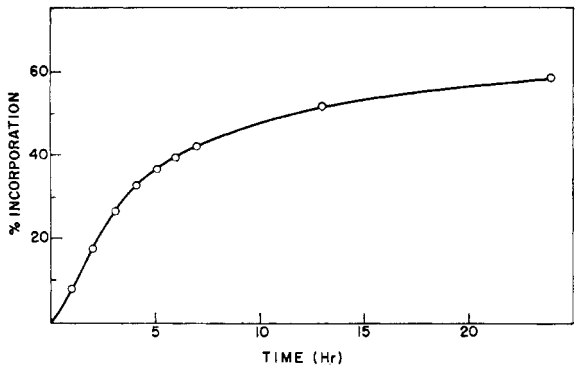

FIGURE 1: Rate of incorporation of thymidine 3′-phosphate onto Whatman No. 3MM chromatographic paper. The reaction mixture contained 0.3 μmole of the nucleotide and 4.5 mg of *N*-cyclohexyl-*N*′-β-(4-methylmorpholinium)ethyl carbodiimide *p*-toluenesulfonate in 0.04 M sodium 2-(*N*-morpholino)ethanesulfonate buffer (pH 6.0) (50 μl). The mixture was applied to a 2 × 3 cm paper strip and allowed to dry in air. The percentage yields of incorporation were determined at various times measured from the point of application of the mixture to the cellulose.

effecting the joining of two oligonucleotide chains by normal phosphodiester linkages providing that, during the activation step, the 5′-phosphate terminals of one oligonucleotide are held in a conformation contiguous to that of the 3′-hydroxyl ends of the other. The required conformations can be achieved by complex formation between the oligonucleotides and larger polynucleotides containing complementary bases (Naylor and Gilham, 1966).

In the extension of these findings to the study of the formation of phosphodiesters between terminal phosphate groups of polynucleotides and the hydroxyl groups of cellulose, two new experimental problems soon became apparent. While the homogeneous nature of the condensation reactions cited above allowed the use of a pH meter together with the addition of strong acids or bases for the control of pH, it was considered necessary to employ a buffer to control the pH of heterogeneous reactions involving cellulose. The choice of buffer is important since it is desirable to avoid the use of weak acid or weak base buffers which could react rapidly with the activating reagent. For this reason 2-(*N*-morpholino)ethanesulfonic acid (pK = 6.15) and its sodium salt have been used as the buffer in the present experiments. A second problem arose from the observation that the exposure of cellulose to solutions of nucleotides in the presence of the buffer and the activating agent resulted in essentially no incorporation of the nucleotide, a result which undoubtedly reflected the requirement, mentioned above, that there should be a high effective concentration of the hydroxylic component for the efficient synthesis of phosphodiesters. It was considered, however, that the effective concentration of the hydroxyl groups could be increased by allowing the aqueous solution of the reactants to slowly concentrate itself within the cellulose fibers. Thus, it was found that, when this concentration was brought about by the slow evaporation of water from the reaction solution–cellulose mixture, good yields of incorporation of nucleotides and polynucleotides could be obtained.

Figure 1 shows a graph of the rate of incorporation of thymidine 3′-phosphate obtained by applying a solution of the nucleotide together with the buffer and the activating agent to a cellulose paper strip and allowing the solution to dry in air at room temperature for various periods of time. The yield of incorporation was 59% 24 hr after the application of the solution to the paper. It was found subsequently that slightly higher yields of incorporation could be obtained if the drying procedure was occasionally interrupted by short exposures of the paper strip to an atmosphere saturated with water vapor. The yields of various substituted celluloses obtained in this way are given in Table I. The reaction method appears to be quite versatile in that, not only molecules with terminal phosphate groups can be incorporated with high efficiency but also those containing di- and triphosphate groups. However, it should be pointed out that, in the special case of ribonucleotides or polyribonucleotides containing phosphate groups at their 2′- or 3′-terminal positions, it is necessary that, during the incorporation reaction, the vicinal 2′- or 3′-hydroxyl groups should be maintained in a protected condition (*e.g.*, with an acetyl group as in 2′(3′),5′-diacetyluridine 3′(2′)-phosphate, Table I). In the absence of this protection the activation of the phosphate group results instead in the sterically favored cyclization reaction yielding the corresponding 2′,3′-cyclic phosphate.

The results shown in Table I indicate that there is a decrease in yield with an increase in the size of the polynucleotide incorporated. This effect could be due to a deficiency of phosphorylated ends in the large polymers or, alternatively, the effect may be the result of the secondary structure of the larger molecules causing some steric restriction of the ends and thus preventing some of them from approaching the hydroxyl groups of the cellulose. However, the amount of material that can be incorporated onto a particular area of cellulose paper is not limited by the yields shown in Table I. It has been found that, subsequent to one incorporation reaction, the paper strip may be washed, dried, and subjected to a second reaction with essentially the same yield of incorporation as obtained in the first. For example, a strip of cellulose paper was subjected to five consecutive incorporation reactions each of which contained the same quantity of deoxyadenosine 5′-phosphate. After the fifth reaction the total amount of incorporated material was measured and found to be five times the amount incorporated during the first reaction.

A number of methods have been developed for the quantitative analysis of incorporated nucleotides. The yields shown in Table I were obtained by a general method of analysis which involves the spectrophotometric determination of the unreacted material eluted from the cellulose after the reaction, allowance being made for the absorption due to the activating agent itself. In some cases, a direct estimate of the amount of incorporated material can also be made. For example, bound polyribonucleotides containing pyrimidines may be released from the cellulose by treatment with pancreatic ribonuclease, and polyribonucleotides in general

TABLE I: Incorporation Yields of Nucleotides, Polynucleotides, and Nucleic Acids.

Nucleotide Compound Incorporated	Amt Added to Cellulose (μmoles of nucleotide base)	Amt Bound to Cellulose (μmoles of nucleotide base)	Yield (%)
Deoxyguanosine 5′-phosphate	4.5	3.2	71
Cytidine 5′-phosphate	4.5	3.0	67
Uridine 5′-diphosphate	4.7	3.0	64
Thymidine 3′-phosphate	3.8	2.4	63
Uridine 5′-phosphate	4.5	2.7	60
Deoxyadenosine 5′-phosphate	5.1	3.0	59
2′(3′),5′-Diacetyluridine 3′(2′)-phosphate	3.9	2.1	54
Thymidine tetranucleotide (pTpTpTpT)	3.6	1.7	47
Adenosine 5′-triphosphate	4.0	1.8	45
Transfer ribonucleic acid	7.0	2.1	30
Polyadenylic acid	1.3	0.3	23
Polyuridylic acid	4.0	0.6	15

may be hydrolyzed and released by treatment with 0.25 N sodium hydroxide at 20° for 20 hr. In the case of substituted celluloses containing purine deoxyribonucleotides, the purine bases may be released by treatment of the cellulose with 1 N hydrochloric acid at 20° for 10 hr.

The type of linkage which joins the nucleotides and polynucleotides to the cellulose in these products displays considerable chemical stability. For example, uridine 5′-phosphorylcellulose is stable at neutral pH and remains undegraded on treatment with either 0.25 N sodium hydroxide or 1 N hydrochloric acid at 20°, and these observations are consistent with the proposed phosphodiester structure of the linkage. However, in the special case of uridine 2′(3′)-phosphorylcellulose (obtained from the acetylated derivative by treatment with dilute ammonia) the linkage is unstable toward strong acid or alkali, a property which is undoubtedly due to the presence of the contiguous 2′(3′)-hydroxyl groups. For example, treatment of this substituted cellulose with 0.25 N sodium hydroxide results in the quantitative release of the uridine phosphate. These observations are also consistent with the proposed phosphodiester linkage since the observed lability of the uridine 2′(3′)-phosphorylcellulose toward acid and alkali is analogous to that of ribonucleic acid, the degradation of which results also from the direct participation of the 2′-hydroxyl groups in the mechanism of the acid- and base-catalyzed hydrolysis.

In conclusion, it seems probable that the present method will permit the covalent binding to cellulose of any polynucleotide or nucleic acid providing the molecules to be incorporated contain phosphate or polyphosphate groups at one or the other of their terminals and providing these groups do not suffer excessive steric hindrance by the nonterminal sections of the polynucleotide chains. The availability of a general method for connecting polynucleotides to an insoluble support is expected to be of value in a number of studies in the nucleic acid field. For example, the method should permit wider applications in the use of polynucleotide celluloses as matrices for the separation of polynucleotides and as substrates for the nucleotide-polymerizing enzymes mentioned above. In addition, the present finding that incorporated ribonucleic acids are recognized by pancreatic ribonuclease indicates that these substituted celluloses may be useful in studying many other enzymes concerned with the degradation or synthesis of polynucleotides and nucleic acids. Polynucleotide celluloses synthesized by the present method should be of particular value in studying those enzymes whose synthetic or degradative activities are confined to the terminals of polynucleotide chains since the method of synthesis of these insoluble substrates clearly indicates the polarity of the incorporated polynucleotides. The present findings may also aid in the development of new methods involving the use of insoluble supports for the specific chemical or enzymatic synthesis of polynucleotides. Finally, the observation of the ease with which oligonucleotides may be covalently bound to cellulose is expected to lead to the development of new approaches for the sequence analysis of such polymers derived from natural sources. The condensation of oligonucleotides onto insoluble supports should have a special application to those methods in which nucleotide sequences are derived by the stepwise removal of nucleotides from one of the polynucleotide terminals (Weith and Gilham, 1967).

Experimental Section

Materials. *N*-Cyclohexyl-*N*′-β-(4-methylmorpholinium)ethyl carbodiimide *p*-toluenesulfonate was purchased from Aldrich Chemical Co., Milwaukee, Wis., and pancreatic ribonuclease was the product of Worthington Biochemical Corporation, Freehold, N. J. Yeast tRNA and polyadenylic acid (potassium salt) were obtained from Calbiochem, Los Angeles, Calif., and poly-

uridylic acid (potassium salt) was purchased from Schwarz BioResearch Inc., Orangeburg, N. Y. All incorporation experiments were carried out on Whatman No. 3MM chromatographic paper which had been washed with 0.005 M EDTA, pH 7 for 1 hr, then washed with distilled water for 3 hr and dried in air at room temperature.

Incorporation of Nucleotides, Polynucleotides, and Nucleic Acids onto Cellulose. The substrate solution was prepared by dissolving the alkali metal salt of the nucleotide, polynucleotide, or nucleic acid in water and adjusting the pH of the solution to 6.0 by the addition of either dilute hydrochloric acid or dilute sodium hydroxide. This solution (0.4 ml, containing 1–10 μmoles of nucleotide or nucleotide component, in the case of the polymers) was added to the buffer, 0.2 M sodium 2-(*N*-morpholino)ethanesulfonate (pH 6.0) (0.1 ml), in a small tube and this solution was mixed with the reagent, *N*-cyclohexyl-*N'*-β-(4-methylmorpholinium)ethyl carbodiimide *p*-toluenesulfonate (50 mg). The mixture was applied with a Pasteur pipet to a 5 × 12 cm area of prepared chromatographic paper such that the solution was spread evenly across the area. The tube was washed out with water (0.2 ml) and the washing was also applied to the rectangle of paper. The paper strip was hung up to dry in the laboratory atmosphere (temperature, 20°; relative humidity, 30–40%). At 2, 5, 8, and 14 hr after the application of the reaction mixture, the paper strip was suspended in a tank containing air saturated with water vapor for 1 hr and then returned to the laboratory atmosphere. After a total of 24 hr from the start of the reaction the paper strip was washed by gentle agitation in a large volume of 0.05 M sodium phosphate solution (pH 7) in order to remove the reagent, buffer, and unincorporated substrate. The paper strip was finally washed with water and hung up to dry at room temperature.

Determination of Yields of Incorporation Reactions. A. GENERAL METHOD. In order to obtain an accurate estimate of the amount of incorporation, the reaction of each substrate was accompanied by three controls. For each substrate, four areas (5 × 12 cm each) were marked out, adjacent to one another, on a single sheet of prepared chromatographic paper. The substrate solution prepared in a similar way to that described above (50 μl, containing 1–10 μmoles of nucleotide or nucleotide component, in the case of polymers) was placed in a small tube (tube 1) and mixed with 0.2 M sodium 2-(*N*-morpholino)ethanesulfonate (pH 6.0) (0.1 ml) together with 0.35 ml of a solution of *N*-cyclohexyl-*N'*-β-(4-methylmorpholinium)ethyl carbodiimide *p*-toluenesulfonate (freshly prepared by dissolving 570 mg of the reagent in water and making the volume of the solution up to 4 ml). Tube 2 contained the same quantity of substrate and buffer together with 0.35 ml of water, while tube 3 was prepared by mixing 0.1 ml of the buffer with 50 μl of water and adding 0.35 ml of the reagent solution. In a fourth tube (tube 4) was placed the same quantity of buffer together with 0.4 ml of water. The contents of each tube were transferred quantitatively to one of the rectangular areas of paper using a water wash of 0.2 ml as described above. The paper strip was allowed to dry in air with intermittent exposures to air saturated with water as described in the previous experiment. After 24 hr the four rectangles were separated and each area was cut into small strips and extracted with 0.05 M sodium phosphate (pH 7) (80 ml) for 2 hr. Ultraviolet spectra were recorded for the extracts corresponding to tubes 1–3 with the extract corresponding to tube 4 placed in the reference cell. Optical density measurements taken at appropriate wavelengths were used to estimate the yield of incorporation. The optical density of the extract from tube 2 provided a check on the amount of nucleotide or polynucleotide originally present in the reaction mixtures. The amount of incorporation was calculated by adding the optical density (at an appropriate wavelength) of the extract corresponding to tube 2 to that corresponding to tube 3 and substracting that corresponding to tube 1. The results of these experiments are shown in Table I.

B. ALKALINE HYDROLYSIS METHOD. In the case of celluloses containing polyribonucleotides, tRNA, and 2'(3'),-5'-diacetyluridine 3'(2')-phosphate the amount of incorporation was measured directly by alkaline hydrolysis. The incorporation reactions were carried out as described in the preceding experiment except that after the 24-hr drying period the paper strip was washed with 0.05 M sodium phosphate (pH 7) to remove unreacted substrate together with the buffer and reagent. The paper was then dried in air and the four rectangles were separated. Each area was cut into small strips and immersed in 0.25 N sodium hydroxide (50 ml). After 20 hr at 20° ultraviolet spectra were recorded for each extract with the extract corresponding to tube 4 occupying the reference cell. The amount of incorporation was obtained by adding the optical density (at an appropriate wavelength) of the extract corresponding to tube 2 to that corresponding to tube 3 and subtracting this value from the optical density of the extract corresponding to tube 1. The theoretical value for 100% incorporation was obtained from the optical density of a solution prepared by treating a quantity of the substrate (equal to that originally added to the reaction mixtures) with 50 ml of the sodium hydroxide solution for 24 hr. The amounts of incorporation obtained by this method were essentially identical with those obtained by the general method.

C. ACID HYDROLYSIS METHOD. This method was used for those celluloses containing purine deoxyribonucleotides. The assay was carried out as described for the alkaline hydrolysis method except that, in place of the extraction with sodium hydroxide, the paper rectangles were each treated with 1 N hydrochloric acid (50 ml) for 10 hr at 20°. The theoretical optical density values to be expected for 100% incorporation were obtained by exposing the same amount of substrate to the same volume of acid for the same time. The amount of incorporation was then calculated as described in the alkaline hydrolysis method, yielding values which agreed with those obtained using the general method.

D. RIBONUCLEASE HYDROLYSIS METHOD. This method was used for substituted celluloses prepared from nucleic acids and polyribonucleotides containing pyrimidines. The procedure was identical with that used in the alka-

line hydrolysis method except that, subsequent to the washing of the paper with the sodium phosphate solution the paper rectangles were dried in air, separated, and then soaked with a solution of pancreatic ribonuclease (0.25 mg/ml). The wet paper strips were then suspended in a tank saturated with water vapor for 10 hr at 20°. The strips were then removed and dried in air. Each rectangle was then cut into small strips and extracted with 0.05 M sodium phosphate (pH 7) (50 ml) for 2 hr. The amount of incorporation was then calculated as described in the alkaline hydrolysis method, yielding values which agreed with those obtained using the general method.

Multiple Incorporation of Deoxyadenosine 5'-Phosphate. A 5 × 12 cm area of chromatographic paper was treated with a reaction mixture containing deoxyadenosine 5'-phosphate (5.24 μmoles) as described above. After the 24-hr drying period the strip of paper was washed in water for 0.5 hr to remove unreacted substrate, buffer, and reagent. The paper strip was dried in air and subjected to four more reaction cycles each one identical with the first. After the 24-hr drying period and the water washing of the fifth reaction cycle the paper strip was cut up and analyzed according to the acid hydrolysis method described above. The amount of incorporation measured in this way was 15.7 μmoles (indicating an over-all yield of 60% based on the total amount of substrate (26.2 μmoles) added to the reaction mixtures).

Kinetic Analysis of the Incorporation of Thymidine 3'-Phosphate. A solution of thymidine 3'-phosphate (sodium salt, pH 6.0, 0.4 ml containing 35 $ODU_{266\,m\mu}$) was mixed with the pH 6 buffer (0.1 ml) and the activating reagent (50 mg) as described above. A number of 2 × 3 cm areas were marked out, adjacent to one another, on a single sheet of the chromatographic paper and ten of these areas were treated with the same volume of the reaction mixture (50 μl, containing 3.1 $ODU_{266\,m\mu}$, 0.3 μmole of the thymidine phosphate). One of the rectangles containing the reaction mixture was immediately cut out together with a blank rectangle and each was extracted with 0.05 M sodium phosphate (pH 7) (10 ml). The ultraviolet spectrum of this sample measured with the extract of the blank in the reference cell gave the zero-time reading. The rest of the rectangles were hung up to dry in the laboratory atmosphere and at various times rectangles were removed and analyzed as described for the zero-time sample. The amount of incorporation at time, *t*, was taken to equal the change in optical density at 266 mμ between the zero-time spectrum and that corresponding to time, *t*. The results of this analysis are shown in graphical form in Figure 1.

Stability of Uridine 5'-Phosphorylcellulose. Uridine 5'-phosphorylcellulose (5 × 6 cm, containing 1.3 μmoles of uridine 5'-phosphate) and a 5 × 6 cm area of blank chromatographic paper were each treated with 0.25 N sodium hydroxide (50 ml) at 20° for 10 hr. Spectrophotometric analysis of the extract of the sample *vs.* that of the blank showed no release of the uridine phosphate. The same result was obtained when a similar sample of the uridine 5'-phosphorylcellulose was treated with 1 N hydrochloric acid at 20° for 10 hr.

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