5.66; acetyl, 26.0. Found: C, 50.85; H, 7.48; N, 5.65; acetyl, 23.2.

Vigorous Acid Hydrolysis: β-Thioethyl Salicylate (7) and Hygric Acid (8).—Celesticetin salicylate (15 g., 0.022 mole) was added to 250 ml. of constant boiling hydrochloric acid which was being heated under reflux. After 5 min. of continued boiling, the mixture was chilled rapidly, diluted with 200 ml. of water, and extracted with 0.5 volume of ether. The boiling and extraction procedure was repeated twice. The combined extracts were shaken with 200 ml. of water containing 6 g. of mercuric chloride. The resulting white precipitate, when washed and dried, weighed 4.35 g. This was dissolved in 80 ml. of purified dioxane and treated with hydrogen sulfide. The 2.1 g. of precipitated mercuric sulfide was removed and the filtrate diluted with water, then extracted with 125 ml. of ether. After several water washings and drying over magnesium sulfate, the ether was evaporated, leaving an oily residue. This was distilled and a fraction boiling *ca.* 110°, 50 μ, was collected (1.1 g.).

Anal. Calcd. for $C_9H_{10}O_3S$: C, 54.53; H, 5.09; S, 16.17; CH_3–C, 0. Found: C, 55.09; H, 5.20; S, 16.77; CH_3–C, 0.

The aqueous residue from the initial extraction above was evaporated *in vacuo* to dryness. A 4-g. aliquot of the residue was dissolved in 40 ml. of water and washed with two 20-ml. portions of chloroform, then diluted to 160 ml. with water and washed with two 40-ml. portions of butanol. The water was next removed by evaporation *in vacuo* and the residue was dissolved in 40 ml. of ethanol and diluted with 200 ml. of ether. After the first gummy precipitate was discarded, 160 ml. of slower-growing white crystals were recovered. The 90 mg. of recrystallized product melted at 183–184°, had an acidic function (pK_a' 1.8) and a basic one (pK_a' 10.4 in water), and was optically active, $[\alpha]_D$ −70° (*c* 1%, water) lit. −81°. The equivalent weight was 165.5 (calcd. 165.6). Analyses for this compound were rather unsatisfactory, owing perhaps to loss of hydrogen chloride in drying.

Anal. Calcd. for $C_6H_{12}ClNO_2$: C, 43.50; H, 7.30; N, 8.46; Cl, 21.40; N-methyl, 17.6. Found: C, 44.40 to 45.50 (4 analyses); H, 7.02; N, 8.52, 9.63; Cl, 20.95; N-methyl, 6.4.

This material was converted to its copper salt by evaporation to dryness with copper hydroxide. The blue salt, then crystallized from chloroform, melted 208° dec. (lit. 200–208° dec.). Its infrared spectrum was identical with that of L-N-methylproline which was synthesized from L-proline *via* its betaine and methyl hydrate.

Milder Acid Hydrolysis of Celesticetin: Celestose Pentacetate (9).—A solution of 5 g. (0.0094 mole) of celesticetin in 100 ml. of 4 *N* sulfuric acid was heated under reflux for 15 hr. At this time a Van Slyke amino-nitrogen determination indicated 80% hydrolysis. The reaction mixture was cooled and filtered. The filtrate was extracted twice with 100 ml. of ether, then adjusted to pH 4.3 with barium hydroxide. The barium sulfate was removed and the filtrate was concentrated *in vacuo* to 70 ml. A 20-ml. aliquot of this was evaporated to dryness (*in vacuo*) and the residue was heated under reflux for 3 hr. with 25 ml. of pyridine and 5 ml. of acetic anhydride. After this solution was treated with water and neutralized with sodium bicarbonate, it was evaporated *in vacuo* to dryness. The residue was suspended in water and extracted with 50-ml. portions of ether. The pooled extracts, dehydrated over magnesium sulfate, were evaporated to dryness, yielding 0.39 g. of dark gum. From this, using ether and Skellysolve B, 100 mg. of tan crystals was obtained. A solution of these in ethanol, after treatment with carbon to decolorize, yielded 28 mg. of colorless needles after addition of ether and Skellysolve B. These exhibited a transition point at 215–216° and melted at 234–234.5°.

Anal. Calcd. for $C_{19}H_{29}NO_{11}$: C, 51.00; H, 6.53; N, 3.13; acetyl, 48.3; methoxyl, 6.9. Found: C, 51.26; H, 6.45; N, 3.06; acetyl, 48.7; methoxyl, 6.1.

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[CONTRIBUTION FROM THE DEPARTMENT OF BIOLOGICAL SCIENCES, PURDUE UNIVERSITY, LAFAYETTE, INDIANA]

The Synthesis of Polynucleotide–Celluloses and Their Use in the Fractionation of Polynucleotides[1]

BY P. T. GILHAM

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A method for the incorporation of chemically synthesized polynucleotides onto cellulose has been developed. By the polymerization of the appropriate mononucleotides, celluloses have been obtained to which thymidine, deoxyadenosine, or deoxycytidine polymers are attached at one of their ends by covalent linkages. Series of oligonucleotides can be bound to columns of these substituted celluloses in base-pairing complexes of different stabilities and subsequently can be fractionally eluted by using a temperature-gradient technique. The application of this method to separation of complex polynucleotides and to the sequence analysis of nucleic acids is discussed.

One of the approaches to the analysis of the fine structure of nucleic acids involves their specific degradation to smaller polynucleotides, the separation of these fragments, and the determination of their individual base sequences. Some success has been achieved in specific degradation by the use of enzymes and by the use of chemically modified nucleic acids while the fractionation of polynucleotides with ion-exchange resins[2] and DEAE–cellulose,[3] together with chromatography and electrophoresis,[4] has permitted the isolation of components up to the tetranucleotide level. However, the separation of mixtures of larger polynucleotides is expected to prove a difficult problem especially in cases where the mixtures contain polynucleotides of approximately the same size with approximately the same base ratio content. In the case of larger polynucleotides it is obvious that the distinctive property of their base sequences must be exploited in any method aimed at their separation and purification. We have briefly described[5] such a method, one that involves the use of celluloses to which chemically synthesized polynucleotides have been permanently attached. A mixture of polynucleotides can then be fractionated on these celluloses according to the ability of the individual components to form base-pairing complexes at various temperatures with the incorporated polynucleotides. This approach, involving base-pairing complexes, has also been used for the fractionation of nucleic acids by Bautz and Hall[6] and for the fractionation of large homologous ribo-

(1) This work has been supported by the National Institutes of Health, U. S. Public Health Service, and the National Science Foundation.

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polynucleotides by Rich and Adler.[7] This report describes further work on the synthesis of polynucleotide–celluloses and their use in the fractionation of polynucleotides by a technique involving temperature-gradient elution.

We have observed that oligonucleotides are capable of base-pairing complex formation with themselves in salt solutions and that the amount of complexing is temperature dependent.[8] In order to make use of this observation for the purpose of a separation technique it was considered that the most efficient polynucleotide–cellulose would be one in which the polynucleotides were attached at their terminals by covalent linkages to the cellulose. Thus, the chain of the attached polynucleotide would be available for maximum interaction with the mixture to be fractionated while the whole polynucleotide–cellulose macromolecule would be reasonably chemically stable.

The polynucleotide–celluloses containing a single nucleotide base are the easiest to prepare. Polythymidylic acid is prepared chemically from the reaction of thymidine 5′-phosphate with dicyclohexylcarbodiimide in pyridine by a modification of the procedure reported by Tener, *et al.*[9] The reaction is allowed to proceed to completion and the mixture then is added to dry cellulose powder. After a further reaction of 5 days the cellulose is isolated and the amount of thymidine polymers incorporated is determined from the amount left in solution. A control experiment, in which no cellulose was added, showed that, under the conditions used, thymidine 5′-phosphate was polymerized to chains up to at least 12 nucleotides long. From the method of preparation and the observed subsequent stability of the product, the structure of the polynucleotide–cellulose can be represented as a mixture (in size) of polythymidylic acid chains attached at their terminal 5′-phosphoryl ends by phosphodiester linkages to the sterically favored hydroxyl groups of the cellulose. The unincorporated material (about 40%) probably arises from products which do not have a terminal (or potentially terminal) 5′-phosphate group and are thus unavailable for reaction with the cellulose. Such products are known to occur in considerable quantities in chemical polymerizations of nucleotides and include macrocyclic polynucleotides, polynucleotide pyrophosphates, and nucleoside 3′,5′-cyclic phosphates. Thus, the polymers attached to the cellulose are considered to have a homogeneous linear structure while the postulated diester linkage is consistent with the observed stability of the substituted cellulose. During the use of these celluloses for separations, the background optical density is rarely above 0.03 and one particular column has been used for some 30 fractionation runs over a period of 6 months without loss of activity.

Deoxycytidine and deoxyadenosine polynucleotide–celluloses can be prepared in a similar way although, in these cases, as with the chemical synthesis of the polynucleotides themselves, it is necessary to use protecting groups for the ring nitrogens. The protecting groups are then removed after the attachment of the polynucleotides to the cellulose. The N^6-anisyl derivative[10] of deoxycytidine 5′-phosphate was polymerized and reacted with cellulose. The anisyl groups were subsequently removed with strong ammonia solution and the incorporation of polymers was estimated at 60%. For the preparation of the deoxyadenosine polynucleotide–cellulose the diacetyl derivative[11] of deoxyadenosine 5′-phosphate was partially deacetylated to N-acetyldeoxyadenosine 5′-phosphate with sodium hydroxide by an analogous method to that used for the partial deacetylation[12] of diacetyldeoxyguanosine 5′-phosphate. Polymerization of this N-acetyl derivative, reaction with cellulose, and the subsequent removal of the acetyl protecting groups gave a cellulose on which 55% of the deoxyadenosine polymers had been incorporated.

The ability of these celluloses to function as predicted was first tested with the thymidine and deoxyadenosine hexanucleotides. When a mixture of these polymers in 1 *M* sodium chloride solution was run through a temperature-controlled column of the thymidine polynucleotide–cellulose the thymidine polymer was eluted at the solvent front at 4° while the deoxyadenosine polymer was retained. The latter was eluted when the temperature of the column was raised to 35°. The recovery was quantitative and it was shown by paper electrophoresis that the separation was complete. The dependency of the temperature of elution on the number of consecutive deoxyadenosine residues in a polynucleotide was then investigated with a series of deoxyadenosine polymers. These were prepared by the chemical polymerization of N-acetyldeoxyadenosine 5′-phosphate followed by the removal of the acetyl groups with ammonia. The products were purified and characterized by the methods used for the polynucleotides obtained from the polymerization of N-benzoyldeoxyadenosine 5′-phosphate.[13] A mixture of the deoxyadenosine tri-, tetra-, penta-, hexa-, and heptanucleotides dissolved in the standard salt solution was applied to the thymidine polynucleotide–cellulose column and elution effected by a stepwise change in the temperature of the column (Fig. 1). The peak fractions were combined, dialysed to remove salt, and the products identified by paper chromatography. Thus, it could be shown that the peaks represented the series of polymers of chain length 3–7 as numbered in Fig. 1. The small unidentified peaks are presumed to be the impurities known to contaminate the chemically synthesized polymers.[13]

With these results it is suggested that the elution temperature for a particular oligonucleotide depends on the dissociation temperature of the complex it forms with the oligonucleotides attached to the cellulose. In other experiments[8] in this laboratory the dissociation curves for the complexes between various deoxyadenosine oligonucleotides with thymidine deca- and dodecanucleotides in solution have been determined and the dissociation temperatures correspond roughly with the elution temperatures of the same deoxyadenosine polymers on the column. For example, the complex between deoxyadenosine hexanucleotide and thy-

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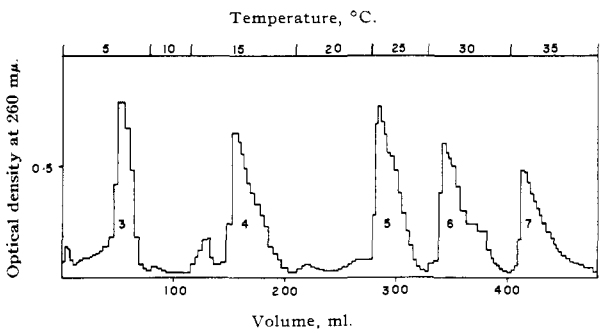

Fig. 1.—Separation of the deoxyadenosine tri- through hepta-nucleotides on the temperature-controlled column of thymidine polynucleotide–cellulose, 24 × 0.9 cm. The elution solvent was 1 *M* sodium chloride–0.01 *M* sodium phosphate, pH 7, and the flow rate *ca.* 2 ml. hr.$^{-1}$

midine dodecanucleotide in solution is completely dissociated at 30°,[5] the temperature at which that deoxyadenosine polymer is eluted from the column. Differences in dissociation temperatures have also been observed for the complexes formed between adenosine oligonucleotides and large molecular weight polyuridylic acid.[14]

The deoxyadenosine polynucleotide–cellulose was tested with a series of uridine oligonucleotides[15] prepared by the partial hydrolysis of polyuridylic acid with a pork liver nuclease.[14] The mixture (100 optical density units at 262 mμ) in the standard salt solution was fractionated using the stepwise temperature gradient as before and the separated peaks were analysed by paper chromatography (Table I). The tetra- and pentanucleotides were eluted together at −2° although this mixture was well separated from the following hexanucleotide.

TABLE I

Elution temp, °C.	Polymer	R_f[a]	O.D. units at 262 mμ recovered
−2	$(pU)_4$, $(pU)_5$	0.215, 0.165	17.3
−2	$(pU)_6$	0.135	11.8
5	$(pU)_7$	0.100	12.5
10	$(pU)_8$	0.083	12.0
15	$(pU)_9$	0.054	12.5
20	$(pU)_{10}$	0.040	10.5
25	$(pU)_{11}$	0.035	7.6
25	$(pU)_{12}$	0.026	4.5
30	$(pU)_{13}$	0.021	3.6
35	$(pU)_{14}$	0.017	2
35	$(pU)_{15}$	0.011	2

[a] Solvent system: *n*-propyl alcohol–concentrated ammonia–water (55:10:35).

The experiments reported above show a difference in the complexing ability of the two columns for a particular size oligonucleotide in that the deoxyadenosine polymers are eluted at higher temperatures from the thymidine column than the corresponding uridine polymers from the deoxyadenosine column. This may be simply owing to a difference in the stability of the complexes formed between deoxyribo- and ribooligonucleotides or to a difference between the complex-forming ability of adenine oligonucleotides with thymine and uracil oligonucleotides. In this regard it has been shown that RNA–DNA complexes are less stable than DNA–DNA complexes in the fractionation of nucleic acids on nucleic acid–agar columns,[16] and Shugar and Szer[17] have reported that the complex between the homologous adenine and uracil ribopolynucleotides has a lower temperature stability than that between the homologous adenine and thymine ribopolynucleotides. However, a more important factor in the present case may be the differences in the average lengths of the oligonucleotides attached to the cellulose. In the chemical polymerization of deoxyadenosine 5′-phosphate reasonable quantities of oligonucleotides up to a chain length of eight are produced, whereas equivalent quantities up to a chain length of twelve are formed from thymidine 5′-phosphate.

Although the fractionations reported above can be achieved by much simpler means such as partition chromatography on paper or by DEAE–cellulose chromatography in the presence of 7 *M* urea,[18] they do serve to demonstrate the potential of the base-pairing method. Theoretically, there is no limit to the type and complexity of polynucleotide sequence which can be chemically incorporated onto cellulose powder and thus provide unlimited selectivity in the fractionation of complex polynucleotides. The present method has been used successfully in a preliminary sequence study of the ribonucleic acid from a plant virus, the results of which are documented in the following paper.

Experimental

Thymidine Polynucleotide–Cellulose.—Anhydrous thymidine 5′-phosphate (pyridine salt, 2 mmoles) was dissolved in dry pyridine (3 ml.) and dicyclohexylcarbodiimide (4 mmoles) and some glass beads were added. A thick gum formed and the flask was shaken vigorously for 5 days. The total product was transferred in the absence of moisture to another flask containing dicyclohexylcarbodiimide (2 g.) and Whatman standard grade cellulose powder (5 g.) which had been dried *in vacuo* at 100° overnight. Dry pyridine (50 ml.) was added and the mixture was shaken for 5 days. The cellulose was collected by filtration, washed with pyridine, and allowed to stand overnight in 50% aqueous pyridine. The product was collected by centrifugation, washed extensively with warm ethanol to remove dicyclohexylurea, and finally washed with water. The combined washings and filtrates were diluted with water and extracted with light petroleum to remove excess carbodiimide, and the aqueous solution was concentrated in the presence of ammonia to remove pyridine. The unincorporated thymidine compounds obtained in this way measured 7600 optical density units at 267 mμ (a recovered yield of 40%). The cellulose was placed in a column and washed with 1 *M* sodium chloride–0.01 *M* sodium phosphate solution, pH 7, until the optical density (260 mμ) of the eluent was less than 0.03.

Deoxycytidine Polynucleotide–Cellulose.—Deoxycytidine 5′-phosphate (2 mmoles) was converted to N^6-anisoyldeoxycytidine 5′-phosphate with anisoyl chloride.[10] The product, in the form of the anhydrous pyridine salt, was allowed to react with dicyclohexylcarbodiimide and then with cellulose (7 g.) as in the preparation of the thymidine polynucleotide–cellulose. The product was washed as before with pyridine, aqueous pyridine, and finally with ethanol to remove dicyclohexylurea. The cellulose was then mixed with concentrated ammonia solution and allowed to stand for 2 days with occasional shaking. The product was then filtered and washed with water until the washings were free of ultraviolet-absorbing materials. The combined washings were extracted with light petroleum and concentrated to dryness to remove pyridine. Spectrophotometric analysis showed that these washings contained 40% of the initial cytidine, indicating an incorporation of 60% on the cellulose.

Deoxyadenosine Polynucleotide–Cellulose.—Deoxyadenosine 5′-phosphate (ammonium salt, 2 mmoles) was shaken with a mixture of dry pyridine (40 ml.) and acetic anhydride (40 ml.). The material dissolved in 5 hr. and the solution was allowed to stand for 20 hr. The solution was evaporated *in vacuo* to dryness,

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water (5 ml.) and pyridine (5 ml.) were added, and the solution was allowed to stand for 3 hr. Paper chromatography[19] showed the presence of only the diacetyl derivative (R_f 0.63). The product was concentrated from aqueous pyridine solution *in vacuo* a number of times to remove acetic acid and finally taken up in water (20 ml.) and the pH adjusted to 7 with sodium hydroxide. The solution was cooled to 0° and 2 *N* sodium hydroxide (20 ml., previously cooled to 0°) was added. After 3 min. at 0° the solution was quickly neutralized by the addition of an excess of Dowex 50 (pyridinium) ion-exchange resin. The mixture was placed on top of a short column of Dowex 50 (pyridinium) and the product was washed through with water. The total eluate was concentrated *in vacuo* in the presence of pyridine. Paper chromatography[19] showed the presence of the N-acetyldeoxyadenosine 5′-phosphate (R_f 0.53) with a trace amount of deoxyadenosine 5′-phosphate (R_f 0.37). The N-acetyl derivative was rendered anhydrous by repeated evaporation *in vacuo* of its pyridine solution and finally was dissolved in 10 ml. of dry pyridine. Some glass beads were added and dicyclohexylcarbodiimide (4 mmoles) and after the mixture was shaken for 5 min. half of the pyridine was removed by evaporation *in vacuo*. The mixture was shaken for 1 week, added to a mixture of dry cellulose (6 g.), dicyclohexylcarbodiimide (2 g.), and pyridine (50 ml.), and then shaken for a further 6 days. The cellulose was isolated by centrifugation and washed with pyridine. It was then mixed with aqueous pyridine and allowed to stand overnight. The dicyclohexylurea was removed by exhaustively washing the product with warm ethanol, chloroform, and then ethanol. Finally, the N-acetyl groups were removed by mixing the product with 7 *N* ammonia and allowing it to stand for 24 hr. After washing with water the cellulose was ready for use. Spectrophotometric analysis of the combined washings as described for the thymidine polynucleotide–cellulose showed that 55% of the original deoxyadenylic acid had been incorporated onto the cellulose.

Separation of Thymidine and Deoxyadenosine Hexanucleotides.—The thymidine polynucleotide–cellulose prepared as above was mixed with 1 *M* sodium chloride–0.01 *M* sodium phosphate solution, pH 7, and packed as a slurry into a water-jacketed column. The final dimensions of the cellulose were 24 × 0.9 cm. and the column was brought to 4° by a circulating coolant. Ten optical density units (260 mμ) each of thymidine and deoxyadenosine hexanucleotides were applied to the thymidine polynucleotide column in 1 *M* sodium chloride–0.01 *M* sodium phosphate solution, pH 7. Elution was carried out at 4° and the thymidine polymer (λ_{max} 266 mμ) emerged quantitatively as a sharp peak at the solvent front. Elution at 24° produced no further material and at 35° all of the deoxyadenosine polymer (λ_{max} 258 mμ) emerged. The products were dialysed to remove salt and submitted to paper electrophoresis in ammonium citrate buffer (0.03 *M*, pH 2.7). The thymidine polymer migrated at a rate 2.6 times that of the deoxyadenosine polymer, and no contamination of one polymer by the other was apparent.

Fractionation of Deoxyadenosine Polymers.—A mixture of deoxyadenosine oligonucleotides was prepared from the reaction of N-acetyldeoxyadenosine 5′-phosphate with dicyclohexylcarbodiimide as described above in the preparation of the deoxyadenosine polynucleotide–cellulose. The product was treated with 7 *N* ammonium hydroxide for 12 hr. and the mixture of oligonucleotides was fractionated on a DEAE–cellulose column.[13] A mixture of approximately 20 optical density units (260 mμ) each of the deoxyadenosine tri-, tetra-, penta-, hexa-, and heptanucleotides in 1 *M* sodium chloride–0.01 *M* sodium phosphate, pH 7 (1 ml.), was applied to the top of the thymidine polynucleotide–cellulose column and elution was carried out at 0° with the salt solution at *ca.* 2 ml. hr.$^{-1}$. The temperature of the column was then raised in a stepwise manner (Fig. 1) during the elution and the peak fractions were combined. The fractions were then dialysed for 5 hr. against water to remove salt, a procedure which caused only small losses of the shorter oligonucleotides. The products were identified by paper chromatography[20] alongside samples of standard oligonucleotides as prepared above (see Table II).

TABLE II

Fraction		R_f
3	Deoxyadenosine trinucleotide	0.33
4	Deoxyadenosine tetranucleotide	0.27
5	Deoxyadenosine pentanucleotide	0.21
6	Deoxyadenosine hexanucleotide	0.15
7	Deoxyadenosine heptanucleotide	0.11
	Deoxyadenosine-5′ phosphate	0.49

Fractionation of Uridine Oligonucleotides.—The deoxyadenosine polynucleotide column was prepared in a similar way to the thymidine column above and a mixture of uridine oligonucleotides [99 optical density units (262 mμ)] was applied in 1 *M* sodium chloride–0.01 *M* sodium phosphate solution, pH 7.0, at −2°. Elution rate was then maintained at 2–4 ml. hr.$^{-1}$ and at appropriate points the temperature was raised in a stepwise fashion. The optical density peaks were collected and dialysed against water and the solutions were concentrated. Identification was effected on Whatman No. 1 paper by partition chromatography,[20] extended over some days, of samples of the products applied in small spots alongside standard samples of uridine oligonucleotides.[15] The R_f values recorded in Table I were obtained from a single descending chromatogram in which the solvent front was not allowed to reach the bottom of the paper.

(19) Solvent system: ethanol–1 *M* ammonium acetate (7:3), pH 7.5.

(20) *Cf.* Table I, ref. *a*.

[CONTRIBUTION FROM THE DEPARTMENT OF BIOLOGICAL SCIENCES, PURDUE UNIVERSITY, LAFAYETTE, INDIANA]

The Use of Polynucleotide–Celluloses in Sequence Studies of Nucleic Acids[1]

BY P. T. GILHAM AND W. E. ROBINSON

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Thymidine polynucleotide–cellulose has been used to fractionate some of the larger polynucleotides derived from the ribonuclease digestion of the ribonucleic acid from the bromegrass mosaic virus. By the use of specific nucleases partial sequence determinations have been carried out on the fractions obtained and thus the relationship between the base sequences of various polynucleotides and their positions of elution from the polynucleotide–cellulose column has been defined. The use of this method of analysis in studies on the structure of nucleic acids is discussed.

As an approach to the determination of the fine structure of nucleic acids we have considered the need for a method which will separate fragments of nucleic acids by making use of the property of their base sequences. Thus, we have described a procedure[2] by which chemically synthesized polynucleotides may be permanently attached to cellulose, and it was shown that mixtures of simple polynucleotides may be fractionated on columns of these substituted celluloses, the fractionation depending on the relative stabilities of the complexes formed between the components of the mixture and the polynucleotides attached to the cellulose. While a series of homologous polynucleotides could be separated in this way it remained to be seen whether

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(2) P. T. Gilham, *J. Am. Chem. Soc.*, **86**, 4982 (1964).