# Effect of Chain Length on the Template Activity of Polyribonucleotides

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The effect of chain length on the template activity of synthetic polynucleotides has been studied in a cell-free system for protein synthesis from *E. coli*. Poly-U (polyuridylic acid) was subjected to partial enzymic hydrolysis and fractions were separated on Sephadex columns. Fractions whose average chain length varied from 16 to 42 appeared to have low activity in stimulating polyphenylalanine synthesis. Uridine oligonucleotides up to $(pU)_{11}$ had no such activity. Fractions of short average chain length, obtained from hydrolysates of poly-U and poly-C (polycytidylic acid), proved to be very effective inhibitors of the template activity of highly polymerized poly-U. It is possible that these fractions act by competing for sites on the ribosome. Fractions from poly-A (polyadenylic acid) digests with an average chain length of 11–68 showed significant activity in stimulating the synthesis of polylysine. In contrast to the previous results, such fractions were found not to inhibit the template activity of highly polymerized poly-A.

The discovery by Nirenberg and Matthaei that synthetic ribopolynucleotides can direct the incorporation of $^{14}C$-amino acids into protein in a cell-free system from *E. coli* (Nirenberg and Matthaei, 1961a; 1961b) provided a new experimental approach to protein synthesis. These techniques have led to increased knowledge concerning the general nature of the genetic code and to a better understanding of the characteristics and functions of polyribonucleotides acting as "messenger" RNA.

The initial work with this system, using polyuridylic acid to direct synthesis of polyphenylalanine, demonstrated that highly polymerized poly-U chains[1] were more active than short ones in directing $^{14}C$-phenylalanine incorporation (Matthaei *et al.*, 1962). Subsequently, Martin and Ames (1962) showed that poly-U with an average chain length of 450 nucleotide residues was optimal for template activity.

In the present communication[2] we have undertaken a systematic examination of the template activity of fractions of poly-U and poly-A of short average chain length.[3] Fractions of short average chain length also proved to be effective inhibitors of the template activity of more highly polymerized poly-U.

## Materials and Methods

The lithium salts of UDP, ADP, and CDP were obtained from Schwarz BioResearch, Inc., Mount Vernon, N. Y. All $^{14}C$-amino acids were obtained from Nuclear Chicago Corp., Des Plaines, Ill. Commercial preparations of poly-U, poly-A, and poly-C were obtained from Miles Laboratories, Inc., Clifton, N. J. Polylysine was obtained from Schwarz BioResearch, Inc., Mount Vernon, N. Y.

*Polymers.*—Poly-A, poly-U, and poly-C were synthesized with polynucleotide phosphorylase purified from extracts of *A. vinelandii* by the procedure of Ochoa and Mii (1961), with minor modifications. The turnover number of the enzyme fraction used for polymerization of ADP was 900 moles/minute/$10^5$ g of protein at 37°.

Poly-A was isolated according to Steiner and Beers (1961). Poly-U and poly-C were prepared using the following procedures:[4] The reaction mixture (10 ml) contained 80 mM nucleoside diphosphate, 20 mM $MgCl_2$, 120 mM Tris-HCl buffer, *p*H 8.2, 0.4 mM EDTA, and 53 μg polynucleotide phosphorylase. After 5 hours at 37°, release of $P_i$ corresponded to 50% utilization of nucleoside diphosphate. At this point, 0.7 ml of 1 M ammonium acetate buffer, *p*H 8.6, and 0.4 ml of 1.5 M $MgCl_2$ were added. The sides of the glass container were scratched with a stirring rod and a precipitate of $Mg(NH_4)PO_4$ soon appeared. A few drops of toluene were added. After 11 hours at 37° a second addition of 0.4 ml 1 M ammonium acetate and 0.2 ml of 1.5 M $MgCl_2$ was made. After 24 hours the reaction mixture was chilled and $Mg(NH_4)PO_4$ was removed by centrifugation. The precipitate was washed with 1.5 ml cold water, and the washings were added to the reaction mixture (total volume, 12.2 ml). This was treated with 24.4 ml of 3 M KCl and 9 ml of absolute ethyl alcohol at 3°, with stirring. The precipitated polymer was collected by centrifugation at 18,000 × *g* for 7 minutes. The precipitate was dissolved in 12 ml $H_2O$ and the treatment with KCl and ethyl alcohol was repeated twice. Chromatography on paper of a quantity of polymer equivalent to 1 μmole of phosphorus showed no detectable nucleoside diphosphate. The solution of polymer (10 ml) was vigorously shaken, at 23°, with one-fourth volume of $CHCl_3$ and one-tenth volume of isoamyl alcohol for 5 minutes and centrifuged, and the aqueous layer was collected. This was done three times. The polymer was precipitated with three volumes of alcohol at 3° and collected by centrifugation for 5 minutes at 18,000 × *g*. The precipitate was washed successively with 20-ml portions of 75%, 90%, 95%, and 100% ethanol. Then it was washed with ethyl ether and dried in a vacuum desiccator. From 500 μmoles CDP as much as 400 μmoles poly-C has been obtained (80% of theory). With poly-U the over-all

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[1] Abbreviations used in this work: poly-U, polyuridylic acid; poly-C, polycytidylic acid; poly-A, polyadenylic acid; EDTA, ethylenediaminetetraacetate; DEAE-, diethylaminoethyl-.

[2] Highly polymerized poly-U or "long-chain poly-U" has a sedimentation coefficient of 6.0 and chain length greater than 250 nucleotide units. This material has had some fractionation by ethyl alcohol which removes short polynucleotide material with chain length of 24 or less, but is not completely homogeneous.

[3] "Short-chain poly-U" has a chain length between 14 and 60 and has been fractionated by Sephadex or DEAE-cellulose but *not* separated into defined species.

[4] L. A. Heppel, unpublished observations.

yield has averaged 50%; without the precipitation of $Mg(NH_4)PO_4$, yields of these polymers have ranged from 20–30%.[5]

*Digests of Poly-A, Poly-C, and Poly-U.*—These were made by incubation of polymer and a ribonuclease fraction from pork liver.[6] The reaction mixture contained, in a total volume of 1 ml: 7 μmoles of polymer (as phosphorus), 0.75 mM $MgCl_2$, 0.05 M Tris-HCl buffer, *p*H 7.1, 2 mg of crystalline bovine serum albumin, 0.2 mM β-mercaptoethanol, and 1.5–6.0 μg of the ribonuclease. With poly-C and poly-U it is important to use enzyme fractionated with ammonium sulfate at *p*H 6 and at *p*H 8, and then precipitated by dialysis against dilute Tris-HCl, *p*H 7.2. It should be free of phosphatase and other types of ribonuclease activity. After a suitable period of incubation at 37° (1–3 hours) the ribonuclease was inactivated by adjusting the mixture to *p*H 4.5 with dilute HCl. After 25 minutes at 23°, 1 N $NH_4OH$ was added to restore the *p*H to its initial value. Digests were kept at −15°. Control experiments showed that treatment with acid in this manner does not injure the activity of poly-U fractions in stimulating polyphenylalanine synthesis.

*Micrococcal Nuclease.*—This enzyme was fractionated from *Staphylococcus aureus* cultures (Martin *et al.*, 1961). It hydrolyzes poly-A to form 3′-AMP and small polynucleotides bearing a 3′-phosphomonoester end group. Conditions of the incubation have been described. The enzyme was inactivated by acidifying the reaction mixture to *p*H 2, followed by digestion with crystalline pepsin.

*Estimation of Average Chain Length.*—The number average chain lengths of polymers and of various low molecular weight fractions was determined by measuring the ratio of total organic phosphate to phosphate susceptible to *E. coli* alkaline phosphomonoesterase (Horiuchi, 1959; Torriani, 1960; Garen and Levinthal, 1960). It is important to establish that the preparation of phosphatase is free of nuclease (Harkness and Hilmoe, 1962). Details of the procedure have been published (Lipsett *et al.*, 1961).

*Isolation of Uridine Oligonucleotides.*—Exhaustive digestion with pork liver ribonuclease gave a mixture consisting largely of pUpU, $(pU)_3$, and $(pU)_4$. Lesser amounts of enzyme gave rise to a digest rich in the components $(pU)_4$-$(pU)_7$ or $(pU)_7$-$(pU)_{11}$. Separation of these oligonucleotides[7] was achieved on columns of DEAE-cellulose by elution with a gradient of ammonium bicarbonate–carbonate in the presence of 7 M urea (Tomlinson and Tener, 1962; Lipsett and Heppel, 1963) or by chromatography on Whatman 3MM paper in solvent 1. Rechromatography in solvent 1 was carried out in all cases. An amount of oligonucleotide equivalent to 0.5–1.0 μmole phosphorus, from each band of solvent 1 chromatograms, was subjected to chromatography in solvent 2 and electrophoresis on paper in 0.05 M formate, *p*H 3.5 (Markham and Smith, 1952). This afforded additional evidence of homogeneity and, in particular, demonstrated the absence of oligonucleotides that did not bear a terminal phosphomonoester group.

Characterization of $(pU)_2$, $(pU)_3$, $(pU)_4$, and $(pU)_5$ was carried out by a variety of methods previously described (Lipsett *et al.*, 1961; Markham and Smith, 1952; Volkin and Cohn, 1953; Whitfeld, 1954; Heppel *et al.*, 1955, 1962). They included: (1) quantitative measurement of chain length with *E. coli* alkaline phosphatase, (2) treatment with snake venom phosphodiesterase, (3) stepwise degradation with periodate, and (4) demonstration of resistance to spleen phosphodiesterase. Characterization of the larger members of this homologous series was limited to a measurement of chain length and determination of $R_F$ in solvents 1 and 2 before and after treatment with alkaline phosphatase.

*Solvent Systems.*—The solvent systems used for descending chromatography were as follows: solvent 1, 1-propanol–concentrated $NH_4OH$–water (55:10:35, v/v/v); solvent 2, saturated ammonium sulfate–isopropanol–1 M sodium acetate (80:2:18, v/v/v) (Markham and Smith, 1951).

*Assays.*—All assays were performed in duplicate and contained the following: 0.1 M Tris-HCl buffer, *p*H 7.8; 0.01 M or 0.014 M magnesium acetate; 0.05 M KCl; $6 \times 10^{-3}$ M mercaptoethanol; $1 \times 10^{-3}$ M ATP; $5 \times 10^{-3}$ M potassium phosphoenolpyruvate; 10 μg crystalline phosphoenolpyruvate kinase (California Corp. for Biological Research); 50 mμmoles of $^{14}C$–amino acid of specific radioactivity of 2.0–4.0 mc/mmole; and 0.05 ml of S-30 *E. coli* extracts prepared as described previously (Nirenberg and Matthaei, 1961b), and containing approximately 0.8 mg of protein, in a final volume of 0.250 ml per reaction mixture. For some experiments, a reaction mixture volume of 0.5 ml was used containing 100 mμmoles of $^{14}C$–amino acid. Synthetic polynucleotides were added as indicated in the legends of the appropriate tables and figures. The concentration of polymer is expressed as mμmoles of phosphorus/ml, or, in the case of poly-U, as mμmoles of nucleotide residue based upon absorbancy at 261 mμ. The value of $E_{max}$ at *p*H 2.0 is taken to be 10 for all fractions, and this is justified by the fact that there is less than 5% hypochromicity in poly-U (Fresco, 1959). We have observed that with homopolymers there is no enhancement of $^{14}C$–amino acid incorporation by the addition of $^{12}C$–amino acids to the reaction mixtures, so they were omitted from these experiments. All reaction mixtures were incubated at 37°.

Other workers, as well as ourselves, have observed that the addition of s-RNA to the reaction mixtures may enhance the incorporation of $^{14}C$–amino acids into protein (Lengyel *et al.*, 1961). For certain experiments we have added *E. coli* "stripped" s-RNA obtained from General Biochemicals, Laboratory Park, Chagrin Falls, Ohio.

Reactions involving poly-U were stopped by the addition of 3.0 ml of cold 10% trichloroacetic acid. The samples were heated at 90° for 20 minutes, plated on Millipore filters (pore size, 0.45 μ, disk diameter, 25 mm), and washed many times with cold 5% trichloroacetic acid. At the end of the incubation period reaction mixtures containing poly-A received 0.2 mg of cold carrier polylysine, then 3.0 ml of cold 5% trichloroacetic acid in 0.25% sodium tungstate, *p*H 2.0. The mixtures were heated at 90° for 20 minutes then chilled to 4° for 5 minutes. The precipitates were plated on Millipore filters as described previously (Gardner *et al.*, 1962) and washed with cold 5% trichloroacetic acid in 0.25% sodium tung-

[5] Removal of phosphate during the course of incubation as $Mg(NH_4)PO_4$ may be used to advantage in the removal of terminal phosphate by *E. coli* alkaline phosphatase, an enzyme that is inhibited by $P_i$ in excess of about $10^{-3}$ M. Polynucleotide solutions with as much as 0.015 M monoesterified phosphate are made up in 0.1–0.2 M ammonium bicarbonate buffer, *p*H 8.8, and a small excess of $MgCl_2$. After treatment with the enzyme, the buffer salts may be removed by lyophilization.

[6] M. N. Lipsett, L. A. Heppel, and W. E. Razzell, unpublished data.

[7] Oligonucleotides represent homogeneous, completely separated polynucleotide fractions ranging from $(pU)_2$ to $(pU)_{11}$.

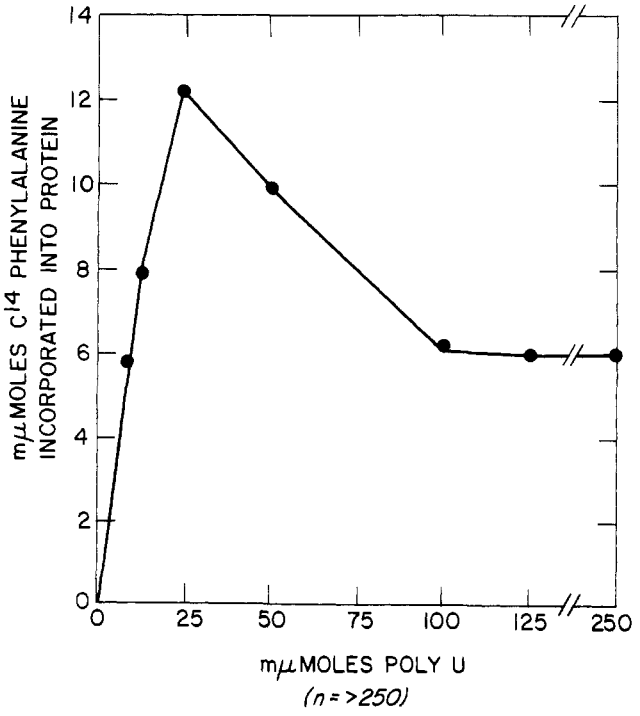


FIG. 1.—$^{14}$C-Phenylalanine incorporation into polyphenylalanine as a function of the concentration of highly polymerized poly-U. Assays were performed as described in Table II.

state. In order to lower background incorporation of $^{14}$C-lysine the following precipitation technique was used for some experiments. After incubation in conical centrifuge tubes, each reaction mixture received exactly 2.0 ml of cold 10% trichloroacetic acid containing 1 mg/ml $^{12}$C-lysine. Tubes were placed in ice for 15 minutes then spun at maximum speed for 10 minutes in an International refrigerated centrifuge. From each supernatant 1.8 ml was aspirated. To this fraction was added 0.2 mg polylysine and 1.8 ml cold 5% trichloroacetic acid in 0.5% sodium tungstate at *p*H 2.0. After 15 minutes at 4° the reaction mixtures were filtered in Millipore filters and washed with cold 5% trichloroacetic acid in 0.25% sodium tungstate, *p*H 2.0. All samples were assayed for radioactivity in a Nuclear Chicago gas-flow counter with a Micromil window and a counting efficiency of 23%.

*Column Chromatography.*—Sephadex G-75 and G-200 were obtained from Pharmacia, Uppsala, Sweden. Sephadex was washed exhaustively with water, placed in a column and washed with water, 1 N NaOH, water to neutral *p*H, absolute ethanol, and water until all the ethanol was removed, and then finally equilibrated with 1 M ammonium carbonate or 1 M NaCl. Pooled fractions eluted from these columns were prepared for assay and chain length determination after desalting and lyophilization. Desalting was performed by diluting the fractions 10-fold in 0.01 M ammonium carbonate and then passing the samples through a Sephadex G-25 column.

## RESULTS

*Effect of Diminishing Chain Length upon Template Activity of Poly-U.*—Since several different preparations of *E. coli* S-30 extracts were used, the activity of the short chain poly-U fractions must be compared only to the untreated poly-U control in the same experiment. For this reason, a portion of the same poly-U preparation which was degraded to shorter chain

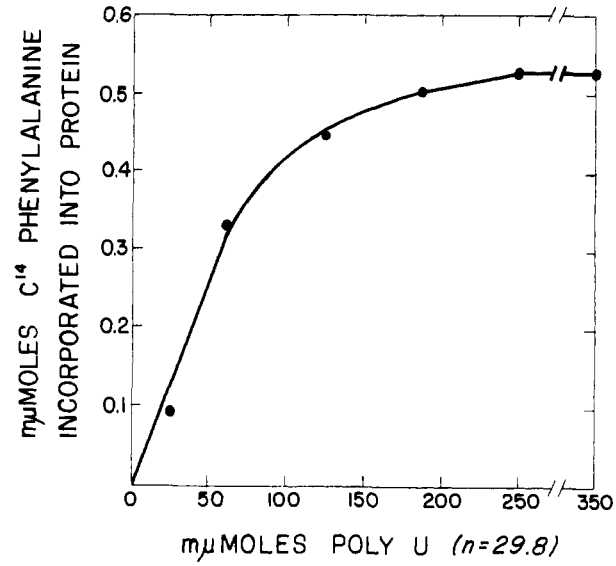


FIG. 2.—$^{14}$C-Phenylalanine incorporation in the presence of an increasing concentration of poly-U ($n$ = 29.8). Assays were performed as described in Table II.

TABLE I

EFFECT OF AVERAGE CHAIN LENGTH OF POLY-U FRACTIONS ON SYNTHESIS OF POLYPHENYLALANINE[a]

| Uridine Polynucleotide Addition | mμMoles $^{14}$C-Phenylalanine Incorporated into Polyphenylalanine |
|---|---|
| None | 0.08 |
| 25 mμmoles poly-U ($n$ > 250) | 2.1 |
| 25 mμmoles poly-U ($n$ = 60) | 1.3 |
| 25 mμmoles poly-U ($n$ = 41) | 0.41 |
| 125 mμmoles poly-U ($n$ = 27.5) | 0.62 |
| 125 mμmoles poly-U ($n$ = 16.0) | 0.40 |

[a] All poly-U fractions were prepared by hydrolysis of the control, long-chain polymer ($n$ > 250), using pork liver nuclease as described under Materials and Methods. Here ($n$) represents average chain length. The reaction mixtures contained 0.1 M Tris-HCl buffer, *p*H 7.8; 0.01 M magnesium acetate; 0.05 M KCl; $6 \times 10^{-3}$ M mercaptoethanol; $1 \times 10^{-3}$ M ATP; $5 \times 10^{-3}$ M potassium phosphoenolpyruvate; 10 μg crystalline phosphoenolpyruvate kinase; $1.6 \times 10^{-4}$ M $^{14}$C-phenylalanine, specific radioactivity 2.0 mc/mmole; polynucleotide in mμmoles of uridylic acid residue as indicated in the table; and dialyzed S-30 *E. coli* extracts, 1.6 mg protein/reaction mixture. Volume for each assay was 0.5 ml. Reaction mixtures were incubated at 37° for 15 minutes.

lengths was also used as the control untreated polymer in most of these experiments. Table I demonstrates the effect of decreasing average length of the polymer upon its ability to direct $^{14}$C-phenylalanine incorporation into protein. It is evident that the ability to direct $^{14}$C-phenylalanine incorporation declines markedly when the average chain length falls below 60, although poly-U with average chain length of 41 still has approximately 20% of the template activity of the untreated polymer. When five times as much polymer is added to the reaction mixture, poly-U fractions with average chain lengths of 27.5 and 16.0 have roughly the same activity as the fraction with chain length of 41. When corrected to equal molar concentration, these shortest fractions, $n$ = 27.5 and $n$ = 16, have template activity of approximately 6% of the untreated polymer. Materials used for these experiments represent unresolved partial digests of poly-U made with a ribonuclease whose attack appears to be random.

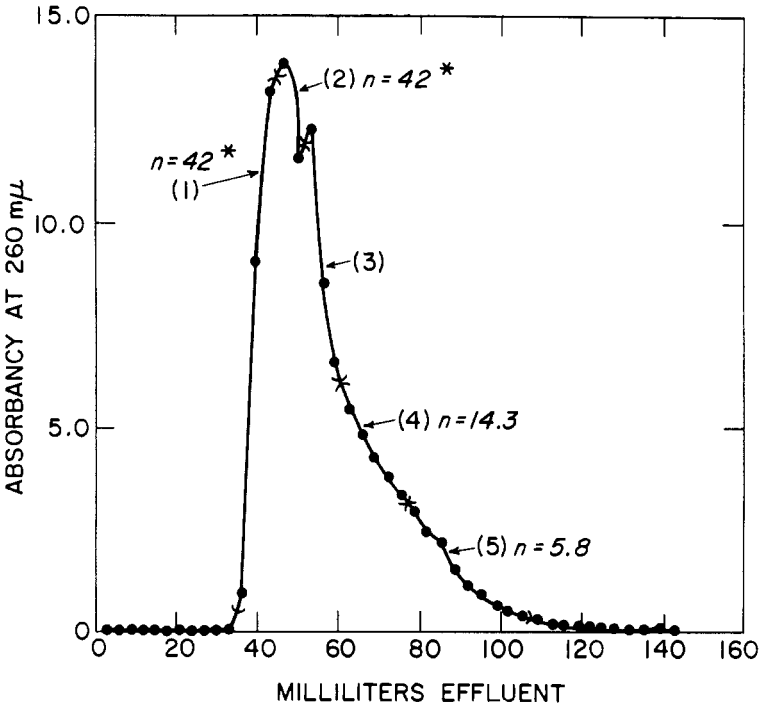


FIG. 3.—Fractionation of poly-U ($n$ = 29.8) in Sephadex G-75. Sephadex G-75 column was prepared as described under Materials and Methods. Size, 1.3 × 101 cm; flow rate, 12.9 ml/hour; fraction cut, 15 minutes; volume/fraction, 3.22 ml; load, 480 OD units at 260 mμ. Column was eluted with 0.01 M ammonium carbonate. Total recovery, 91%. Material in the elution peak was divided into five fractions as indicated by parentheses on the elution diagram. These fractions were desalted and lyophilized for assay and chain-length determination. The asterisk indicates that fractions (1) and (2) were combined for chain-length determination and assay.

Table II shows the results of experiments with homogeneous polynucleotides ranging from $(pU)_2$ to $(pU)_{11}$. It is clear that these oligonucleotides do not have significant template activity (less than 0.5% of the control). This indicates that the minimal chain length for $^{14}$C-phenylalanine incorporation is greater than 11. Addition of excess s-RNA in this experiment failed to enhance the template activity of these oligonucleotides.

*Effect of Concentration of Poly-U on Extent of $^{14}$C-Phenylalanine Incorporation.*—Figure 1 demonstrates that incorporation of $^{14}$C-phenylalanine is approximately linear between 0 and 25 mμmoles of highly polymerized poly-U per 0.250 ml reaction mixture, and that concentrations of 50 mμmoles, or greater, actually inhibit $^{14}$C-phenylalanine incorporation. As shown in Figure 2, incorporation of $^{14}$C-phenylalanine directed by poly-U with an average chain length of 29.8 is linear between 0 and 62.5 mμmoles per 0.250 ml reaction mixture and this increase ceases at 250 mμmoles poly-U. Thus it appears that a greater concentration of short-chain poly-U is required for saturation in this system, and there is no apparent inhibition of $^{14}$C-phenylalanine incorporation at these high concentrations of short-chain polymer. Furthermore, under conditions of saturation the rate of $^{14}$C-phenylalanine incorporation is only 5% of that observed with highly polymerized poly-U.

*Fractionation of Poly-U by Column Chromatography.*—The template activity of poly-U preparations of average chain length as low as 16 might be accounted for by a 5–10% contamination by long poly-U chains. Therefore column chromatographic separation of such poly-U fractions was performed. In Figure 3 is shown the results of a typical experiment in which poly-U ($n$ = 29.8) has been fractionated on Sephadex G-75. Absorbancy at 260 mμ is plotted along the ordinate and milliliters effluent along the abscissa. The peak was divided arbitrarily into several fractions as indicated in the figure. Chain-length determinations and assay for template activity were performed with each fraction. There is clearly no sharp separation of differently sized polymers in this system. In Table III is presented the results of assay and chain-length determinations performed on these fractions. Poly-U fractions with average chain lengths varying from 14.2 to 42 have an activity ranging from 4% to 10% of the

TABLE II

EFFECT OF URIDINE OLIGONUCLEOTIDES ON SYNTHESIS OF POLYPHENYLALANINE[a]

| Uridine Polynucleotide Addition | mμMoles $^{14}$C-Phenylalanine Incorporated into Polyphenylalanine |
|---|---|
| None | 0.09 |
| 12.5 mμmoles poly-U* ($n > 250$) | 4.7 |
| 75 mμmoles $(pU)_2$ | 0.10 |
| 75 mμmoles $(pU)_3$ | 0.10 |
| 75 mμmoles $(pU)_4$ | 0.09 |
| 75 mμmoles $(pU)_6$ | 0.10 |
| 75 mμmoles $(pU)_7$ | 0.08 |
| 75 mμmoles $(pU)_8$ | 0.10 |
| 75 mμmoles $(pU)_{11}$ | 0.08 |

[a] Uridine oligonucleotides were prepared as described under Materials and Methods. Each reaction mixture contained 0.1 M Tris-HCl buffer, $p$H 7.8; 0.014 M magnesium acetate; 0.05 M KCl; $6 \times 10^{-3}$ M ATP, $2 \times 10^{-4}$ M $^{14}$C-phenylalanine, specific radioactivity 4.0 mc/mmole; 75 mμmoles (as base) for each oligonucleotide $(pU)_2$ through $(pU)_{11}$; 1.0 mg *E. coli* s-RNA (General Biochemicals), and dialyzed S-30 *E. coli* extracts, 0.8 mg protein/reaction mixture. Final volume for each assay was 0.250 ml. Reaction mixtures were incubated at 37° for 60 minutes. The asterisk represents undegraded poly-U assayed for comparison.

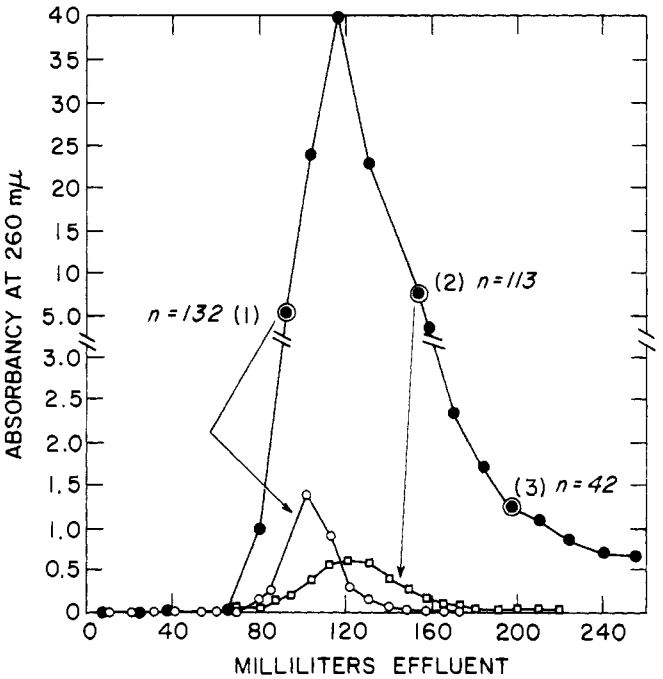


FIG. 4.—Fractionation of poly-U on Sephadex G-200. Sephadex G-200 column was prepared as described under Materials and Methods. Size, 2.1 × 67.4 cm.; flow rate, 14 ml/hour; fraction cut, 60 minutes; fraction volume, 14 ml; load, 2,221 OD units at 260 mμ. Column eluted with 1 M NaCl. Fractions (1), (2), and (3) represent single collections of 14 ml each whose positions on the elution diagram are indicated by the symbol ⊙. Their average chain length is shown on the figure, and template activity is recorded in Table IV. Fractions (1) and (2) were rechromatographed with results shown in the lower part of the figure, —O—O— representing fraction (1) and —□—□— denoting fraction (2).

TABLE III

EFFECT OF CHAIN LENGTH ON TEMPLATE ACTIVITY OF POLY-U FRACTIONATED ON SEPHADEX G-75[a]

| Uridine Polynucleotide Addition | mμMoles $^{14}$C-Phenylalanine Incorporated into Poly-phenylalanine |
|---|---|
| None | 0.06 |
| 37.5 mμmoles poly-U ($n > 250$)+ | 3.2 |
| 225 mμmoles combined fractions 1 and 2 ($n = 42$)* | 0.32 |
| 250 mμmoles fraction 3 | 0.21 |
| 250 mμmoles fraction 4 ($n = 14.3$) | 0.12 |
| 125 mμmoles fraction 5 ($n = 5.8$) | 0.06 |

[a] The poly-U sample was fractionated as described under Materials and Methods. The plus sign represents undegraded poly-U which had not been chromatographed on Sephadex. The asterisk represents fractions 1 and 2 which were pooled for chain-length determination and assay. All fractions were prepared for assay as described under Materials and Methods. Assays were performed as described in Table I. Here ($n$) represents average chain length.

untreated poly-U control, and this activity is achieved by increasing the concentration of polymer 6-fold over that required by highly polymerized poly-U.

A mixture of 2 poly-U fractions (a molar ratio of 2 parts poly-U ($n > 250$) to 1 part poly-U, $n = 29.8$) was also fractionated on Sephadex G-75. This experiment failed to provide satisfactory separations, and accordingly the use of Sephadex G-200 was explored.

TABLE IV

EFFECT OF CHAIN LENGTH ON TEMPLATE ACTIVITY OF POLY-U FRACTIONATED ON SEPHADEX G-200[a]

| Uridine Polynucleotide Addition | mμMoles $^{14}$C-Phenylalanine Incorporated into Poly-phenylalanine |
|---|---|
| None | 0.2 |
| 25 mμmoles poly-U ($n > 250$)* | 8.6 |
| 25 mμmoles fraction 1 ($n = 132$) | 3.0 |
| 25 mμmoles fraction 2 ($n = 113$) | 1.4 |
| 25 mμmoles fraction 3 ($n = 42$) | 0.45 |

[a] All fractions were prepared for assay as described under Materials and Methods. Assays were performed as described in Table I. Final volume of reaction mixture was 0.250 ml. All reaction mixtures were incubated for 15 minutes at 37°. The asterisk represents undegraded poly-U control which had not been chromatographed in Sephadex G-200. Here ($n$) = average chain length.

In Figure 4 is presented the result of poly-U fractionation on Sephadex G-200. This particular preparation of poly-U was a commercial lot obtained from Miles Laboratories, Inc. The encircled numbers in this figure indicate the fractions arbitrarily taken for assay and chain-length determination. It is quite unlikely that fractions representing short-chain material are significantly contaminated with highly polymerized poly-U. This is indicated in part by the results of rechromatography of fractions 1 and 2 (shown in lower part of figure). Material from these fractions is eluted in the same area of the chromatograms on the second run although no resolution into individual polynucleotides is achieved. Fraction 3 was selected as a representative fraction of poly-U with relatively short chain length. Table IV shows considerably more separation in template activity than noted previously. The activity in fraction 3 is 5.2% of the poly-U control and it seems unlikely that this fraction contains a significant quantity of long-chain poly-U.

There is another way to show that fractionation according to size has been achieved. This depends upon evidence presented in the next section showing that polynucleotides of low chain length (but *greater* than 11) effectively inhibit the template activity of highly polymerized poly-U.

Table V shows that fraction 3 (Fig. 4; $n = 42$) inhibits the activity of long-chain poly-U by 47% at a concentration of only 12.5 mμmoles per 0.250 ml assay. Fraction 1 ($n = 132$) and fraction 2 ($n = 113$) do not inhibit $^{14}$C-phenylalanine incorporation. This suggests that the quantity of short chains of poly-U in fractions 1 and 2 is probably minimal, and, with this evidence of resolution, it is unlikely that fraction 3 contains any significant concentration of long-chain poly-U.

*The Study of the Inhibitory Effect of Short Polynucleotide Fractions on Template Activity of Synthetic Polymers.* —As mentioned earlier, concentrations of relatively long-chain polymer in excess of 50 mμmoles/0.250 ml reaction mixture actually gave a reduced incorporation of $^{14}$C-phenylalanine.

An explanation for this is that when large amounts of poly-U are used, significant concentrations of short-chain material are also introduced into the reaction mixture. We have already mentioned the data of Table V showing that material of ($n = 42$) results in good inhibition of $^{14}$C-phenylalanine incorporation, whereas poly-U of chain length above 100 does not inhibit.

TABLE V

INHIBITION OF HIGHLY POLYMERIZED POLY-U BY SHORT-CHAIN POLY-U FRACTIONS[a]

| Uridine Polynucleotide Addition | mμMoles $^{14}$C-Phenylalanine Incorporated into Poly-phenylalanine | Inhibition (%) |
|---|---|---|
| None | 0.3 | |
| 25 mμmoles poly-U ($n > 250$) | 8.9 | |
| 25 mμmoles poly-U ($n > 250$) | 9.7 | 0 |
| + 12.5 mμmoles fraction 1 ($n = 132$) | | |
| 25 mμmoles poly-U ($n > 250$) | 9.9 | 0 |
| + 12.5 mμmoles fraction 2 ($n = 113$) | | |
| 25 mμmoles poly-U ($n > 250$) | 4.7 | 47.1 |
| + 12.5 mμmoles fraction 3 ($n = 42$) | | |

[a] Assays were performed as described in Table I. Final volume of reaction mixture was 0.250 ml. All reaction mixtures were incubated for 15 minutes at 37°. Fractions 1, 2, and 3 represent the same poly-U fractions described in Figure 4 and Table IV.

The inhibition phenomenon was examined in greater detail. In Table VI is presented the effect of increasing the concentration of short-chain poly-U in assays containing a constant quantity of long-chain polymer. The effectiveness of short poly-U as an inhibitor is quite striking; a concentration of the order of 5 mμmoles in a 0.250 ml reaction mixture reduces the template activity of 12.5 mμmoles of long-chain poly-U by 40%.

Only the last two assays in this table contain molar quantities of polymer exceeding 37.5 mμmoles, so that the inhibitory effect demonstrated by these polymers cannot be due merely to an excessive level of uridine polynucleotide in the reaction mixtures (see Fig. 1).

The order of addition of the long and short polymers to each reaction mixture does not alter the inhibitory effect. However, if long-chain poly-U is preincubated at 0° for 3 minutes with the complete system including the S-30 fraction before addition of short-chain poly-U, the inhibitory effect observed in Table VI is abolished. Preincubation with short-chain poly-U before adding the highly polymerized chains does not appear to increase the inhibitory effect.

As noted in Table V, poly-U with an average chain length of 42 was also quite effective as an inhibitor of polyphenylalanine synthesis. The failure of poly-U fractions, with chain lengths greater than 100, to inhibit polyphenylalanine synthesis suggested that only polymers with chain lengths less than 100 were effective as inhibitors. It is also possible that there is a minimum-chain-length requirement for effective inhibition (Table VII). The molar concentration of polymer in these experiments did not exceed the saturating level for long-chain poly-U, and the equivalent ratio of 1 part long poly-U to 1 part short should have resulted in 40–50% inhibition (Table V). Poly-U ($n = 16$) did inhibit $^{14}$C-phenylalanine incorporation by 45%, however homogeneous polynucleotide fractions with chain lengths ranging from $p(U)_2$ to $p(U)_{11}$ showed only slight inhibition or at most 19%. There is no apparent correlation, in these shorter fractions, between chain length and effectiveness of inhibition. These data show that a chain length greater than 11 is required for inhibition and that an average chain length of 16 provided optimal inhibition in this series.

An unexpected inhibitory effect upon highly polymerized poly-U was observed with short-chain poly-C.

TABLE VI

EFFECT OF POLY-U ($n = 29.8$) UPON TEMPLATE ACTIVITY OF POLY-U ($n > 250$)[a]

| Uridine Polynucleotide Additions | | mμMoles $^{14}$C-Phenyl-alanine Incorporated into Poly-phenyl-alanine | Inhibition (%) |
|---|---|---|---|
| Poly-U ($n > 250$) | Poly-U ($n = 29.8$) | | |
| None | None | 0.10 | |
| 12.5 mμmoles | None | 2.70 | |
| 12.5 mμmoles | 1 mμmole | 2.10 | 22.2 |
| 12.5 mμmoles | 5 mμmoles | 1.60 | 40.7 |
| 12.5 mμmoles | 12.5 mμmoles | 1.37 | 49.2 |
| 12.5 mμmoles | 30 mμmoles | 0.62 | 77.0 |
| 12.5 mμmoles | 60 mμmoles | 0.47 | 82.5 |

[a] Assays were performed as described in Table II except that the reaction mixtures were incubated at 37° for 15 minutes and additional s-RNA was omitted.

As shown in Table VIII, long-chain poly-C does not inhibit $^{14}$C-phenylalanine incorporation, however a moderate effect is noted with polymers ranging in size from $n = 9.9$ to $n = 200$.

*Effect of Average Chain Length on Template Activity of Poly-A.*—Gardner *et al.* (1962) reported that poly-A preparations were quite active in directing the synthesis of $^{14}$C-polylysine. They were able to detect the template activity of poly-A by modification of the technique used to precipitate synthesized polypeptides. We have confirmed this observation and have identified the polypeptide products of the reaction.[8] After brief incubation with trypsin all the product was degraded to di- and trilysine.

In Table IX is shown the effect of decreasing the average chain length of poly-A fractions upon the incorporation of $^{14}$C-lysine into polylysine. For these experiments a slight modification of the technique described by Gardner *et al.* (1962) was used to precipitate the polylysine product as described under Materials and Methods. It was established that the tungstic acid procedure recovered more than half the added radioactive tetralysine. The recovery of higher homologs would be expected to be greater. In addition, we have also observed that s-RNA added to the reaction mixtures results in a several-fold enhancement of $^{14}$C-lysine incorporation with only slight increase in incorporation in the absence of polymer. For this reason, these assays all received additional s-RNA. As shown here, poly-A ($n > 250$) directs 3.3 mμmoles $^{14}$C-lysine into protein and a poly-A fraction with average chain length of 68 directs approximately 50% as much $^{14}$C-lysine into protein. Poly-A fractions with average chain length of 16.8 direct $^{14}$C-lysine into protein with over 20% of the efficiency of untreated poly-A. Even smaller poly-A fractions with average chain length of 11.1 and 8.7 have greater than 10% template activity relative to untreated poly-A. The data from Table X shows that short poly-A fractions when added to untreated poly-A do not inhibit $^{14}$C-

[8] In a number of incorporation experiments using a poly-A template of $n = 60$ and higher, the lysine products found in a 10% trichloroacetic acid supernatant fraction of a single run were distributed between oligolysines of 3–15 residues in length with the greatest amount around 8–10 residues. It is by no means clear that these results described the original product adequately, and further work is in progress (H. A. Sober and A. Yaron, unpublished results).

TABLE VII

EFFECT OF URIDINE OLIGONUCLEOTIDES ON POLY-U-DIRECTED POLYPHENYLALANINE SYNTHESIS[a]

| Uridine Polynucleotide Addition | mμMoles $^{14}$C-Phenylalanine Incorporated into Polyphenylalanine | Inhibition (%) |
|---|---|---|
| None | 0.2 | |
| 12.5 mμmoles poly-U ($n > 250$) | 2.0 | |
| 12.5 mμmoles poly-U ($n > 250$) + 12.5 mμmoles poly-U ($n = 16$) | 1.09 | 45 |
| 12.5 mμmoles poly-U ($n > 250$) + 12.5 mμmoles $(pU)_{11}$ | 2.05 | 0 |
| 12.5 mμmoles poly-U ($n > 250$) + 12.5 mμmoles $(pU)_8$ | 1.91 | 4.5 |
| 12.5 mμmoles poly-U ($n > 250$) + 12.5 mμmoles $(pU)_7$ | 1.73 | 13.3 |
| 12.5 mμmoles poly-U ($n > 250$) + 12.5 mμmoles $(pU)_6$ | 1.61 | 19.7 |
| 12.5 mμmoles poly-U ($n > 250$) + 12.5 mμmoles $(pU)_3$ | 1.81 | 9.5 |
| 12.5 mμmoles poly-U ($n > 250$) + 12.5 mμmoles $(pU)_2$ | 2.0 | 0 |

[a] Assays were performed as described in Table II except that the reaction mixtures were incubated at 37° for 15 minutes. The molar concentrations of oligonucleotide represent mμmoles of compound rather than uridylic acid residues. Poly-U ($n = 16$) is an unfractionated partial digest made with pork liver ribonuclease. The other materials are chromatographically pure oligonucleotides.

TABLE VIII

EFFECT OF POLY-C FRACTIONS OF VARYING CHAIN LENGTH ON THE TEMPLATE ACTIVITY OF HIGHLY POLYMERIZED POLY-U[a]

| Polynucleotide Addition | mμMoles $^{14}$C-Phenylalanine Incorporated into Polyphenylalanine | Inhibition (%) |
|---|---|---|
| None | 0.26 | |
| 12.5 mμmoles poly-U ($n > 250$) | 2.6 | |
| 12.5 mμmoles poly-U ($n > 250$) + 12.5 mμmoles poly-C ($n > 250$) | 2.6 | 0 |
| 12.5 mμmoles poly-U ($n > 250$) + 12.5 mμmoles poly-C ($n = 200$) | 2.2 | 15.3 |
| 12.5 mμmoles poly-U ($n > 250$) + 12.5 mμmoles poly-C ($n = 133$) | 2.2 | 15.3 |
| 12.5 mμmoles poly-U ($n > 250$) + 12.5 mμmoles poly-C ($n = 40$) | 2.0 | 23.0 |
| 12.5 mμmoles poly-U ($n > 250$) + 12.5 mμmoles poly-C ($n = 29.8$) | 1.8 | 30.8 |
| 12.5 mμmoles poly-U ($n > 250$) + 12 mμmoles poly-C ($n = 12.1$) | 1.7 | 34.5 |
| 12.5 mμmoles poly-U ($n > 250$) + 12 mμmoles poly-C ($n = 9.9$) | 1.6 | 38.4 |

[a] Assays were performed as described in Table II, except that reaction mixtures were incubated for 15 minutes at 37°.

lysine incorporation. On the contrary, in most cases the short poly-A fractions seem to stimulate the effectiveness of long poly-A strands in directing polylysine synthesis. This is in marked contrast to the

TABLE IX

$^{14}$C-LYSINE INCORPORATION DIRECTED BY POLY-A WITH VARYING AVERAGE CHAIN LENGTH[a]

| Adenine Polynucleotide Addition | mμMoles $^{14}$C-Lysine Incorporated into Polylysine |
|---|---|
| None | 0.32 |
| 75 mμmoles poly-A* ($n > 250$) | 3.3 |
| 75 mμmoles poly-A ($n = 68$) | 1.4 |
| 75 mμmoles poly-A ($n = 28$) | 0.94 |
| 75 mμmoles poly-A ($n = 16.8$) | 0.77 |
| 75 mμmoles poly-A ($n = 11.2$) | 0.69 |
| 75 mμmoles poly-A ($n = 8.7$) | 0.46 |

[a] Assays were performed as described under Materials and Methods in a total volume of 0.250 ml including 1 mg s-RNA/assay mixture. Reaction mixtures contained 50 mμmoles of $^{14}$C-lysine with a specific radioactivity of 4.0 mc/mmole. Reaction mixtures were incubated at 37° for 60 minutes. The asterisk represents undegraded poly-A control.

TABLE X

EFFECT OF SHORT-CHAIN POLY-A UPON TEMPLATE ACTIVITY OF POLY-A ($n > 250$)[a]

| Adenine Polynucleotide Addition | mμMoles $^{14}$C-Lysine Incorporated into Polylysine |
|---|---|
| None | 0.24 |
| 37.5 mμmoles poly-A* ($n > 250$) | 1.6 |
| 37.5 mμmoles poly-A* + 25 mμmoles poly-A ($n = 68$) | 1.6 |
| 37.5 mμmoles poly-A* + 25 mμmoles poly-A ($n = 28$) | 1.8 |
| 37.5 mμmoles poly-A* + 25 mμmoles poly-A ($n = 16.8$) | 1.7 |
| 37.5 mμmoles poly-A* + 25 mμmoles poly-A ($n = 11.2$) | 1.8 |
| 37.5 mμmoles poly-A* + 25 mμmoles poly-A ($n = 8.7$) | 1.9 |
| 37.5 mμmoles poly-A* + 25 mμmoles $(pA)_4$ | 1.7 |
| 37.5 mμmoles poly-A* + 25 mμmoles poly-C ($n = 29$) | 1.6 |

[a] Assays were performed as described under Materials and Methods. All reaction mixtures received 1.0 mg s-RNA. Incubations were performed at 37° for 15 minutes. The asterisk represents undegraded poly-A.

observation of the inhibitory effect of short poly-U fractions. It is also of interest to note that whereas short poly-C fractions do inhibit poly-U activity, they do not appear to inhibit poly-A.

Bretthauer and Bock (1963), as well as Marcus and co-workers (1963) reported on studies with polynucleotide fractions, of different chain lengths, that were recovered following alkaline degradation of long-chain polymers. These workers found that uridine polynucleotide fractions from $U_9$ to $U_{25}$ were quite active in directing $^{14}$C-phenylalanine incorporation. Since alkaline hydrolysis of synthetic polynucleotides results in short chains with 3′- and 2′-phosphomonoester end groups, whereas treatment with pork liver nuclease results in chains with a 5′-phosphomonoester end group, it seemed desirable to examine other types of polynucleotides in the *E. coli* system. For this reason a series of short-chain poly-A fractions was prepared by treating long-chain poly-A with a nuclease prepared from *Staphylococcus aureus* which hydrolyzes poly-

TABLE XI

INCORPORATION OF $^{14}$C-LYSINE INTO PROTEIN DIRECTED BY POLY-A FRACTIONS WITH 3′-PHOSPHOMONOESTER END GROUP[a]

| Adenine Polynucleotide Addition | mμMoles $^{14}$C-Lysine Incorporated into Polylysine |
|---|---|
| *Experiment (1)* | |
| None | 0.28 |
| 75 mμmoles poly-A* ($n > 250$) | 1.3 |
| 75 mμmoles poly-A ($n = 137$) | 0.8 |
| 75 mμmoles poly-A ($n = 9$) | 0.9 |
| 75 mμmoles poly-A ($n = 6.3$) | 0.47 |
| *Experiment (2)* | |
| None | 0.13 |
| 75 mμmoles poly-A* ($n > 250$) | 2.9 |
| 75 mμmoles $(Ap)_6$ | 0.21 |
| 75 mμmoles $(Ap)_4$ | 0.13 |
| 75 mμmoles $(Ap)_3$ | 0.12 |
| 75 mμmoles $(Ap)_2$ | 0.14 |

[a] Assays were performed as described under Materials and Methods. All reaction mixtures received 1.0 mg s-RNA. Incubations were performed at 37° for 60 minutes. The poly-A fractions of experiment (1) ($n = 6.3$ to $n = 137$) were enzymic digests (see text) bearing 3′-phosphomonoester end groups. Experiment (2) was carried out with discrete oligonucleotides that were chromatographically pure. All concentrations are expressed as nucleotide equivalents. The asterisk represents undegraded poly-A with a 3′-OH end group.

nucleotides to products with the 3′-phosphomonoester end groups. In Table XI are presented the chain-length determinations and the results of assays for template activity on such fractions. It does appear that poly-A ($n = 9.0$) has relatively more template activity than comparable fractions ($n = 11.2$ and $n = 8.7$), with a 5′-phosphomonoester end group (Table IX). These fractions also do not inhibit the template activity of long-chain poly-A. Oligonucleotides up to $(Ap)_6$ were found to be inactive.

## DISCUSSION

It is important, for several reasons, to establish whether polynucleotides of relatively short chain length can direct $^{14}$C–amino acid incorporation into protein. Any active materials could be used as primers for end addition of different nucleotides. This would provide model template fractions to be used, for example, in studies of polarity in reading messenger RNA, since the influence of alterations in end groups would probably be more evident in a relatively small molecule. In addition, it may be possible to synthesize chemically relatively short polymers with known sequence in order to determine sequence of letters in different amino acid code words.

In the case of poly-U, our evidence shows that fractions below 100 in chain length have, at best, low activity compared with highly polymerized poly-U. The question arises whether they have any significant activity at all or whether the observed stimulation by fractions of low average chain length is due to the presence of a small amount of long-chain polymer. The experimental conditions happen to be very difficult because of (1) low activity of the fractions, (2) relatively large concentrations required to saturate the system, and (3) the fact that poly-U appears to function catalytically. As little as 1% contamination by long-chain polymer in a fraction of short-chain polymer would be enough to account for the activity found with poly-U of average chain length 16–42. However, this value of 1% may be too low because the activity of presumed long-chain contaminant would be subject to maximal inhibition by the short chains amounting to 80% (see Table VI). Therefore a content of at least 5% of long-chain polymer would have to survive digestion with nuclease and fractionation by column chromatography to account for the activity observed with the short-polymer fractions. The results of fractionation, especially with Sephadex-200, led us to believe that fractions of low chain length probably have intrinsic activity, but one must have reservations until the methods of resolution are improved. It appears that uridine polynucleotides of chain length ($n = 11$) and less have no template activity because these compounds were tested as pure compounds.[9]

The apparent failure to detect template activity in these oligonucleotides, and perhaps even the relatively low degree of activity seen in poly-U fractions with chain lengths varying from 16 to 30, may be due to the inability to detect the presence of very short chains of newly synthesized polyphenylalanine by the method used for precipitation in this study. It is quite possible that polyphenylalanine chains with 5 phenylalanine residues or less might not be precipitated in 10% trichloroacetic acid. Preliminary experiments failed to show the presence of such chains on paper chromatograms, but more work along these lines is contemplated.

The striking inhibition by "short-chain" poly-U fractions of the ability of highly polymerized poly-U to direct polyphenylalanine synthesis was unexpected. Substantial inhibition was obtained with as little as 5 mμmoles/0.250 ml, a considerably lower concentration than that of the long-chain polymer present. It is conceivable that formation of such fragments *in situ*, on the ribosome, helps to account for the decrease in polyphenylalanine synthesis with increased time of incubation. It is also possible that formation of polynucleotide chains of this kind may be part of a regulatory function in protein synthesis. The inhibition appears to be a property of chains less than 100 in length and greater than 11, since only small and variable inhibition was displayed by the polynucleotides $(pU)_2$ to $(pU)_{11}$.

The precise nature of the inhibitory effect of short-chain poly-U fractions on polyphenylalanine synthesis is not readily apparent. It is highly unlikely that U—U base pairing occurs at the temperature used to synthesize polyphenylalanine (Lipsett, 1960). Furthermore, short poly-C fractions also inhibit the activity of poly-U so that the inhibitory effect is apparently more related to the size of the polymer chain than to its base composition. By contrast, neither short poly-C fractions nor short poly-A fractions inhibit the template activity of highly polymerized poly-A. There is evidence that poly-U and ribosomes associate to form rapidly sedimenting aggregates (Barondes and Nirenberg, 1962; Spyrides and Lipmann, 1962; Gilbert, 1963), but this has not been demonstrated with poly-A and ribosomes (Okamoto and Takanami, 1963).[10] Accordingly, short-chain poly-C and poly-U may exert their inhibitory effect upon highly polymerized poly-U by competing for sites on the ribosome, and in the case of short-chain poly-U the "inhibitory" effect may

[9] From the results of Marcus *et al.* (1963) it appears that the cell-free system from yeast utilizes short-chain poly-U more effectively as template material than is true for the *E. coli* system.

[10] Also observed by R. Cukier and M. W. Nirenberg (unpublished data).

simply be a much less efficient template replacing long-chain poly-U in the active ribosomes. Preincubation with long-chain poly-U abolished the inhibition of short-chain poly-U.

Moller and von Ehrenstein (1963) have reported that phage RNA–induced protein synthesis is specifically inhibited by synthetic homopolymers. All polynucleotides tested for inhibitory effect had $s_{20,w}$ values of 4.25 or greater, and poly-U in this size range seemed to be the least effective inhibitor. While their system for protein synthesis is different from the *E. coli* system, it is possible that the site of inhibition is at the ribosome in both cases.

The study of template activity of short poly-A fractions has provided information which could be interpreted as strong evidence that such fractions have significant ability to direct polylysine synthesis. It seems very unlikely that these fractions could have enough long-chain material remaining after nuclease degradation to account for the degree of activity observed. Our experiments indicate that having a 3′-phosphomonoester group rather than a 5′-phosphate end group does not appreciably alter the template activity of these short chains. Also, removal of all terminal phosphate does not reduce the efficiency of such chains.

The data do suggest that a minimal chain length of 6–9 is required for polylysine synthesis. Our major reservations for this interpretation are based on the fact that in the crude 30,000 × *g* extract from *E. coli* the added short-chain poly-A in the presence of ATP may be serving as template or primer for the synthesis of relatively highly polymerized poly-A. Such a product, even though present in small quantities, could account for the polylysine synthesis observed. August *et al.* (1962) have described a system for poly-A synthesis dependent upon the presence of RNA. In addition to *de novo* synthesis, their data indicate that some chains of poly-A utilize RNA fractions as primer. In our system for polylysine synthesis, short poly-A chains bearing a 3′-phosphate end group are as effective as those with a 5′-terminal phosphate or lacking an end group. Polynucleotides bearing a 3′-phosphate end group would not be expected to serve as primers for chain growth unless they were first acted upon by an active phosphomonoesterase. We may also mention that short-chain poly-U and short-chain poly-C in concentrations as high as 400 mμmoles/ml do not stimulate polylysine synthesis. This suggests that if long chains of poly-A are being made during the period of incubation used for these experiments, there is a specific dependence upon short-chain poly-A rather than poly-C or poly-U. Thus, the evidence that short poly-A fractions stimulate polylysine synthesis directly, rather than by first forming long poly-A chains, is fairly strong but not conclusive. Further experiments on this point are contemplated for the future.

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