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*LINEAR BIOSYNTHESIS OF TOBACCO MOSAIC VIRUS: EVIDENCE THAT SHORT VIRUS RODS ARE NATURAL PRODUCTS OF TMV BIOSYNTHESIS**

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This paper considers whether the ribonucleoprotein rods that are the distinctive product of the tobacco mosaic virus (TMV) infection process are inherently variable in length. The problem is of interest because it influences conclusions regarding the mechanisms of TMV biosynthesis and replication.

In previous studies of the incorporation of C^{14} into TMV in the infected tobacco leaf we have shown that the virus protein and RNA components are synthesized concurrently from the leaf's pools of free amino acids and ribonucleotides.[1] This observation, which has now been confirmed in two other RNA-bearing viruses, poliovirus[2] and fowl plague virus,[3] mitigates against the possible occurrence of free RNA and protein precursors of TMV, and suggests instead that incomplete or alternative forms of the virus are more likely to consist of ribonucleoprotein. Since the virus' protein subunits and RNA fiber form coaxial structures in the TMV rod, any ribonucleoprotein alternative to the predominant 3,000 Å rod is likely to take the form of a rod of some different length.

The problem of TMV rod length has been the subject of considerable study, but there is little agreement as to the significance of the results. While it is now clear that rods longer than 3,000 Å are preparative artefacts,[4, 5] there is wide disagreement regarding the origin of the rods shorter than 3,000 Å that are always observed in TMV preparations. Two recent reviews of this question reach opposite conclusions. Pirie[6] supports the view that short rods are natural products of TMV bio-

synthesis, while Schachman[7] states that "the most reasonable supposition to be made at this time is that the particles of TMV are essentially monodisperse and are 3,000 Å long."

Accordingly, as a prerequisite to analysis of the role of short rods in TMV biosynthesis (to be considered in succeeding papers), we report in the present paper experiments designed to determine whether such rods are artificially derived from breakage of 3,000 Å rods, or whether they represent alternative forms of the virus which are synthesized as such in the infected leaf.

Methods.—In these experiments TMV was isolated from blade tissue of leaves of greenhouse-grown plants of *N. tabacum* 6–7 days after these leaves had been inoculated with 200 mg/ml of a solution of purified TMV. The inoculated plants, which were nearly mature, were maintained at a temperature of 24°C under constant illumination for the period between inoculation and isolation of the virus. Except where indicated in the text, purification of virus was achieved by standard procedures which are described elsewhere.[1]

The distribution of rod lengths in TMV preparations was determined by the following procedures: To each 0.5 ml sample of TMV is added 0.5 ml of a 1 mg/ml solution of bovine serum albumin, 0.25 ml of polystyrene spheres, and 0.25 ml of distilled water. Albumin facilitates uniform spreading of the rods on the electron microscope sample grid; polystyrene spheres provide a standard both for magnification and for determination of microdroplet size. The sample is then atomized onto 200-mesh carbonized collodion sample grids. The preparations are shadowed with uranium at a 30° angle, and examined in an RCA EMU-2 electron microscope. The atomized microdroplets form characteristic patterns on the sample grid, which are readily recognized from the circular array of the polystyrene spheres. An example of such a microdroplet is shown in Figure 1. For each sample 10 random microdroplet patterns are photographed at a magnification

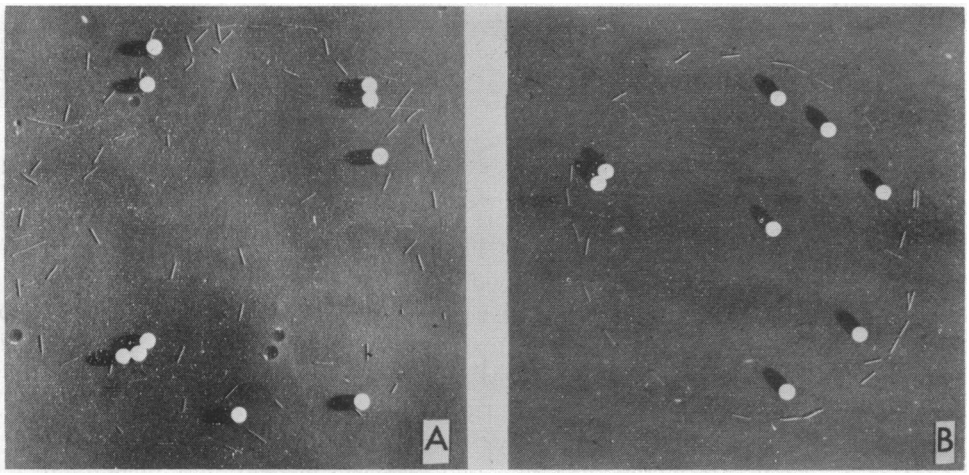

FIG. 1—Examples of electron micrographs of TMV microdroplets containing standard polystyrene spheres (mean diameter, 2,640 Å). *A:* Standard TMV preparation; *B:* Fraction of nearly homogeneous 3,000 Å rods obtained by ultracentrifugal fractionation of a standard TMV preparation on a sucrose gradient.

of approximately 3,600. To determine the frequency distribution of rod lengths in a sample, *all* the TMV rods visible in the electron micrographs of 10 microdroplets are measured, a minimum of several hundred rods being measured for any given sample.

Rod lengths were measured from the electron micrograph plates at a magnification of 25×, using a variable-magnification low power microscope. Two different techniques were employed. In most experiments rod lengths, and for comparison purposes the diameters of the polystyrene spheres, were determined with an ocular micrometer. However, certain samples (as indicated in the text) were measured by an automatic device. This consists of a filar ocular micrometer

geared to a potentiometer which actuates a servomechanism that drives an index device across the top of 100 channels uniformly spaced in a sheet of clear plastic. The index device is attached to a reservoir of steel ball bearings ($^1/_{32}$ in. in diameter) by means of a flexible tube. The size of each rod measured by the ocular micrometer is automatically recorded by pressing a switch which releases a steel ball into the channel that lies beneath the index device, after the latter has been positioned by the adjustment of the micrometer index wire to the length of a given rod. The accumulated steel balls form a histogram of the rod lengths observed in the population. This automatic apparatus has the advantage of eliminating possible personal bias in recording rod lengths that approximate whole numbers on the ocular scale. It is also more rapid and more accurate than the ocular scale technique. Results obtained by both methods are comparable, except that the automatic apparatus resolves the rod lengths about 3–4 times more accurately than the scale method.

Experimental.—1. *Rod lengths in purified TMV preparations:* The problem that this paper seeks to analyze is exemplified by Figure 2 which shows the frequency distribution of rod lengths in a standard purified TMV preparation. The preparation is obviously inhomogeneous with respect to rod lengths. Two classes centered at 3,000 Å and 6,000 Å predominate, but a significant proportion of the population falls into a wide range of intermediate lengths. The experiments which follow describe the origins of this heterogeneity in rod length.

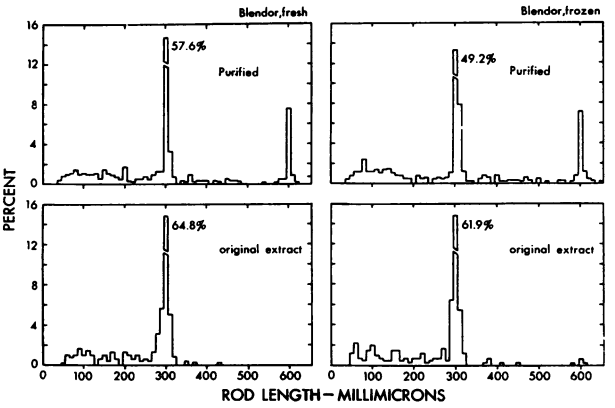

FIG. 2—Frequency distributions of rod lengths in TMV isolated from fresh and frozen infected leaf after grinding in a blendor. The lower frequency distributions represent the rod lengths observed in the original extract obtained after grinding; the upper figures represent the rod lengths observed after the respective original extracts were purified by the standard procedure described in the text. Number of rods measured: original extract fresh, 478; original extract frozen, 407; purified fresh, 587; purified frozen, 666. Length measurements by ocular micrometer.

It is apparent from the lower part of Figure 2, which shows the rod lengths observed in the original unpurified extract of infected leaf, that part of the observed variation in rod length is a result of purification. Almost all of the rods in the original extract are 3,000 Å or shorter. Rods longer than this dominant length are therefore artefacts resulting from the effects of preparative procedures. It has been demonstrated by Welsh[4] that TMV preparations readily form dimers of the 3,000 Å rods, and Boedtker *et al.*[5] have reported similar effects. Since rods of lengths intermediate between the 3,000 Å and 6,000 Å classes are also absent in the original extract, but appear after purification, these must arise from dimerization of 3,000 Å rods with shorter ones. It may be concluded, therefore, that all rods longer than the 3,000 Å class are not naturally present in the infected leaf. Figure 2 also shows that freezing the leaf previous to TMV extraction has no significant effect on the distribution of rod lengths in either the original extract or in the purified preparation.

There remains the question of determining the origin of the rods shorter than the

predominant 3,000 Å class, for these are observed in both purified and unpurified TMV preparations. Several possible modes of origin of these rods must be considered: (*a*) Breakage of 3,000 Å rods due to grinding or other extraction processes. (*b*) Breakage of 3,000 Å rods during evaporation of microdroplets on electron microscope sample grids. (*c*) Breakage of previously synthesized 3,000 Å rods within the infected cell prior to extraction. (*d*) Short rods are actually synthesized as such in the infected leaf.

2. *The effect of extraction procedures:* Infected leaf cells are ordinarily crushed by some method as an initial step in the preparation of purified TMV and the resultant mechanical effects might conceivably cause some virus rods to break. Accordingly, we have examined the rod length in TMV preparations isolated by a method which does not involve crushing or grinding. By means of a bundle of ordinary sewing needles, the blade of an infected leaf is gently perforated, with a density of several hundred holes per square centimeter. Several segments of perforated leaf tissue, each about 2 × 5 cm, are placed in a beaker containing about 50 ml of pH 7 phosphate buffer, which is then gently stirred (by means of a magnetic stirrer) for about 15 minutes. At the end of that time, the leaf tissue is removed and the TMV dissolved in the buffer examined in the electron microscope immediately, and also after the usual purification procedures. The results of such an experiment are shown in Figure 3.

As in ordinary TMV extracts, the virus prepared from perforated tissue contains almost no rods longer than the 3,000 Å class, except after purification. Short rods are present in both the original and purified preparations, although the relative concentration is somewhat lower than that found in TMV prepared by grinding methods. Since the amount of TMV isolated from perforated tissue is only 5% of that obtained from the same tissue by grinding, this difference may have two alternative explanations: (*a*) Grinding causes some proportion of the 3,000 Å rods to break into shorter rods, or (*b*) long rods are preferentially extracted from perforated leaf. The distribution of TMV in infected cells suggests that the second of these explanations is the correct one. Infected cells contain inclusion bodies, such as crystals, many of which are suspended in the extensive vacuole of the cell. It has been observed that the crystalline inclusion bodies break up when a cell is perforated by a needle;[8] moreover, electron micrographs of crystals found in the cell vacuole show that they consist entirely of 3,000 Å rods.[9] It is likely, therefore, that the small amount of TMV released from perforated leaf is chiefly virus that was originally present in the cell vacuole in which 3,000 Å rods tend to predominate. Table 1 shows that TMV exposed to the action of a blendor for 20 minutes exhibits a decrease of 12% in the proportion of 3,000 Å rods. Grinding in a motor-driven glass homogenizer for 4–15 minutes has little or no effect on rod length. These procedures are considerably more severe than the grinding actually used in preparing TMV which involves only a 75-second period of treatment.

TABLE 1

EFFECT OF ADDITIONAL GRINDING ON ROD LENGTH

Preparation	Grinding method	Additional grinding (min)	Per cent 3,000 A Rods	
			Original preparation	After additional grinding
1	Blendor	20	83.8	73.8
2	Glass homogenizer	4	82.4	78.4
3	Glass homogenizer	15	81.2	85.0

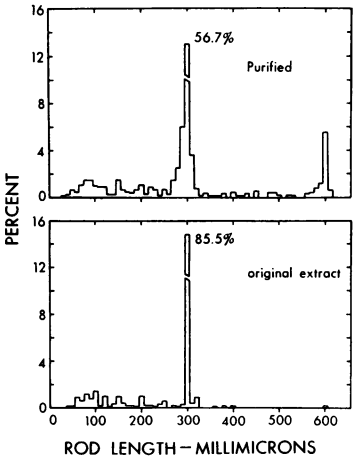

FIG. 3—Frequency distribution of rod lengths in TMV isolated from infected leaf by diffusion through needle holes (without grinding). Lower figure represents TMV in the original diffusate from the leaf; the upper figure represents TMV prepared from the latter by the standard purification procedure. Number of rods measured: original diffusate, 975; purified preparation, 751. Length measurements by ocular micrometer.

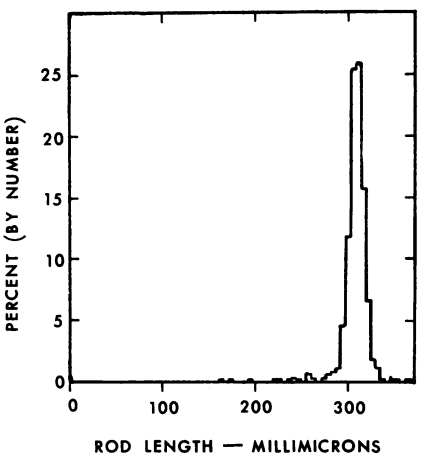

FIG. 4.—Frequency distribution of rod lengths in a highly homogeneous fraction isolated from a standard TMV preparation by means of ultracentrifugation on a sucrose gradient. Total number of rods measured, 698. Length measurements by automatic recording micrometer.

3. *Possible breakage during electron microscope grid preparations:* By means of ultracentrifugation on a sucrose gradient it is possible to isolate the 3,000 Å class of TMV rods from ordinary inhomogeneous preparations (see Table 2 for details). An electron micrograph of a typical microdroplet observed in such a homogeneous preparation is shown in Figure 1, and a typical frequency distribution of rod lengths is shown in Figure 4. About 95% of the rods fall into the 3,000 Å class, and nearly all of the remaining rods are between 2,000 Å and 2,500 Å long. The rather few short rods present in this preparation cannot arise from fragmentation of 3,000 Å rods, for if this occurred we would expect to find a comparable number of rods in the 500–1,000 Å class, and these are absent. Since the preparation described in Figure 4 was subject to the standard preparative steps that are required to obtain an electron micrograph (isoelectric precipitation, ultracentrifugation, dialysis, evaporation of microdroplets on the sample grid) these procedures apparently do not cause an incidence of rod breakage in excess of 2–3%.

A certain number of rods in most electron micrographs show very fine breaks between adjoining fragments that together comprise a length of 3,000 Å. Such breaks obviously originate *in situ*, on the grid. A count of these *in situ* breaks in a number of preparations totaling several thousand (2,793) 3,000 Å rods showed that such breaks occur with an average frequency of 2.7%. In the measurements reported in this paper, rods exhibiting breaks of this type are classified in the 3,000 Å group.

4. *Possible in vivo breakage of 3,000 Å rods:* There remains the possibility that

the short rods, while actually present in the infected tobacco cell, are produced within it by breakage of previously synthesized 3,000 Å rods. Evidence on this problem is provided by the experiment reported in Table 2. TMV is labeled by brief exposure

TABLE 2

SPECIFIC ACTIVITIES OF FRACTIONS OF C^{14}-LABELED TMV ISOLATED FROM SUCCESSIVE ZONES OF A SUCROSE GRADIENT

Fraction	Composition: Per cent by weight, Rods 3,000 ± 300 Å long	Composition: Per cent by weight, Rods less than 2,700 Å long	Mean length of rods less than 2700 Å long	Specific activity (cpm/mg)
Original TMV preparation	77	23	1,230	123
1	91	9	990	74
2	86	14	1,830	90
3	39	61	1,590	121
4	41	59	810	176

The original TMV preparation was purified by the standard method from infected tobacco leaf (7 days after inoculation) after a 15-minute period of photosynthetic $C^{14}O_2$ incorporation. Fractions of the purified C^{14}-labeled TMV were prepared by ultracentrifuging 150 μg of the original TMV preparation on sucrose gradient tubes (according to the technique described by Brakke[16]) at 46,000 *g* for 150 minutes. Fraction 1 represents the lowermost zone retained, the succeeding fractions following in order upward in the tube. Parallel fractions from 6 tubes were pooled to obtain sufficient material in each fraction. Specific activities were determined from C^{14} counts of 18–31 μg samples on a low-background counter (1.5 cpm background). Rod lengths were determined by the methods described in the text.

of an infected leaf to $C^{14}O_2$, and fractions which vary with respect to rod length are isolated from the purified virus by ultracentrifugation on a sucrose gradient. The specific activities of the various fractions are clearly dependent on the relative proportions of rods of different lengths which they contain. If the short rods represented broken fragments of virus originally synthesized as 3,000 Å rods, the specific activities of these fractions should be identical. The isotope data show that short rods cannot arise from broken 3,000 Å rods at any time subsequent to the synthesis of the latter. This rules out the possibility that the short rods are due to breakage which occurs either in the infected cell, or at any stage during subsequent treatment of the isolated virus.

Discussion.—As noted earlier there has been considerable disagreement in the literature regarding the source of the short rods that are universally observed in TMV preparations. Some of the foregoing data help to resolve this difficulty.

Our observation that short rods are present in crude unpurified extracts of infected leaf confirms similar observations by Oster[10] and by Watanabe and Kawade.[11] This result cannot be ascribed to the extraction process, for we also find short rods in virus which simply diffuses out of infected leaf through needle holes. Furthermore, since we also find considerable differences in the C^{14} specific activities of 3,000 Å rods and short rods the latter cannot arise from *in vivo* breakage. These observations, and the experimental demonstration that preparative procedures do not cause more than a few per cent of the rods in nearly homogeneous preparations of 3,000 Å rods to break, establish that short rods are natural products of TMV biosynthesis.

Frampton[12] measured the lengths of the rods shown in electron micrographs published by Stanley and Anderson,[13, 14] and from the resultant frequency distribution found that only 30% were 3,000 Å ± 400 Å long and that the rod lengths tended to fall into classes with mean lengths of about 400, 1,000, 1,500, and 2,000 Å. Figure 5 shows that length-classes quite similar to those found by Frampton in Stanley's electron micrographs occur in certain of our preparations. Although short rods found in the large amount of TMV isolated from infected leaf after grind-

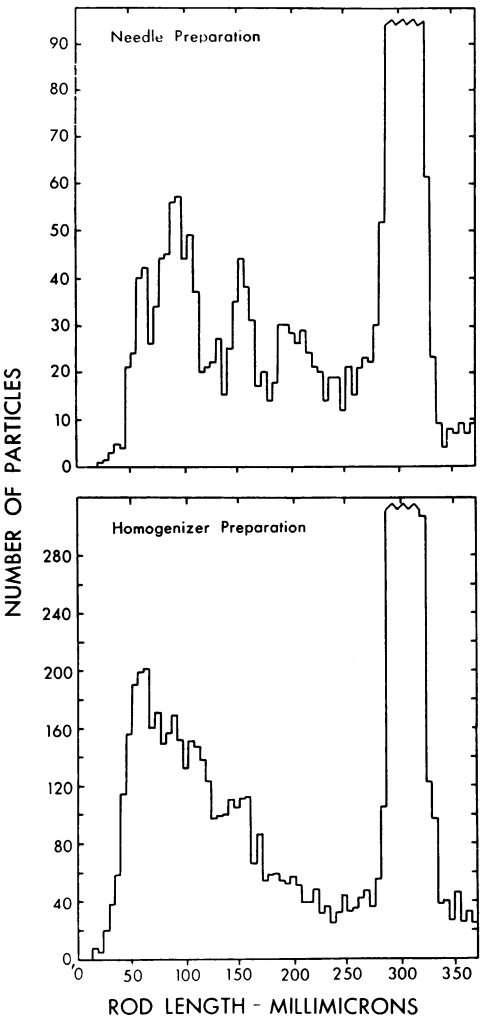

FIG. 5—Partial frequency distributions to show rod lengths in the short particles observed in a series of purified TMV preparations isolated by the needle-hole technique (without grinding) and by grinding in a glass homogenizer. Number of rods (less than 2,700 Å long) measured: needle preparation, 1,256; homogenizer preparation, 4,397. Length measurements by automatic recording micrometer.

ing in a glass homogenizer form a continuous distribution, preparations isolated from perforated leaf show a similar over-all nongaussian distribution, on which is superimposed certain distinct classes. These have lengths of 500, 1,000, 1,500, and 2,000 Å. Considering the relatively small number of measurements (159 rods) involved in Frampton's tabulation of Stanley and Anderson's data, the agreement between our results and his is remarkable.

These observations suggest an explanation of Williams' and Steere's conclusion that short rods arise from breakage of 3,000 Å rods during preparation of electron micrographs.[15] These investigators measured the rods in a number of microdroplets and found that the sum of the lengths of the short rods present in a single microdroplet often approximated some multiple of the predominant length of 3,000 Å. This appeared to support the conclusion that the short rods represented broken fragments of 3,000 Å rods. However, it is apparent that populations of short rods that fall into classes that approximate a simple fraction of the 3,000 Å length will more readily follow this rule than do populations composed of short rods of random length. Hence, the tendency of short rods to yield total lengths that are multiples of 3,000 Å does not necessarily constitute evidence that they arise from breakage of 3,000 Å rods.

The conclusion that TMV rods shorter than 3,000 Å are synthesized as such in the infected tobacco leaf emphasizes the importance of considering the heterogeneity of ordinary TMV preparations in interpreting analytical results based on them. The evidence reported in Figure 4 that rather homogeneous populations of 3,000 Å rods can be prepared by ultracentrifugal fractionation provides an experimental basis for more rigorous analysis of the composition and properties of the TMV rod. This technique routinely yields preparations consisting of 90–95% 3,000 Å rods. The best preparations reported previously by Williams and Steere[15] and by Boedtker

et al.[5] contained 70% 3,000 Å rods.

Figure 5 illustrates the value of detailed consideration of the lengths of TMV rods. This shows that TMV rods isolated from the cell vacuole tend to fall into length classes which are multiples of $^1/_6$ of 3,000 Å (except that relatively few $^5/_6$ rods are observed). It would appear that the rod's structure is governed in part by forces that vary in a periodic fashion along its length. Such periodicity might arise from two possible sources: (*a*) periodicity in the nucleotide sequence of the RNA fiber, or (*b*) variations in the structure of successive protein subunits. Either alternative suggests important restrictions on proposals regarding the biochemical locus of the virus' genetic specificity. Experimental analysis of this problem is in progress.

The basic outcome of the observations summarized above, that rods of various lengths are natural products of TMV biosynthesis, shows that the product of TMV biosynthesis is a heterogeneous population of ribonucleoprotein rods. This conclusion places certain restrictions on possible explanations of the mechanisms of TMV biosynthesis and replication, and leads to new experimental opportunities to elucidate these processes. In particular it enables investigations of the relationship between the biochemical properties of TMV rods of different lengths and the mechanism of TMV biosynthesis. These are to be described in forthcoming papers.

Summary.—(1) In agreement with previous evidence, it is apparent that TMV rods longer than 3,000 Å do not exist as such in the infected cell, but represent the effects of polymerization induced by preparative procedures. Rods shorter than 3,000 Å are present in untreated extracts of infected leaf and in material which diffuses out of needle holes made in such leaf. (2) The effects of preparative procedures cannot account for more than a few per cent of the short TMV rods observed in purified preparations. This can be directly demonstrated by preparing an ultracentrifugal fraction from a standard TMV preparation, which yields electron micrographs in which 95% of the rods are 3,000 Å ± 300 Å long. (3) The observation that short rods and 3,000 Å rods isolated from C^{14}-labeled infected leaf exhibit considerably different specific activities shows that short rods do not arise from *in vivo* breakage of 3,000 Å rods. (4) The foregoing results establish that rods shorter than 3,000 Å long are natural products of the process of TMV biosynthesis, and help to resolve some of the discrepancies in the literature on this subject. (5) The conclusion that the product of TMV biosynthesis is a heterogeneous population of ribonucleoprotein rods places certain restrictions on possible mechanisms of TMV biosynthesis and replication leads to new experimental opportunities to elucidate these processes.

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*SYNTHETIC POLYNUCLEOTIDES AND THE AMINO ACID CODE, VI**

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Previous experiments with synthetic polynucleotides of random nucleotide distribution have provided information about the base composition of the triplet code letters for 19 amino acids.[1] These results were confirmed by other investigators.[2] However, the sequence of bases in the code triplets is known only in the case of phenylalanine (UUU).[3] Through the use of nonrandom copolymers it should be possible to establish the base sequence of some code letters, the direction in which the code is read, and the colinearity of the synthesized polypeptides with the template polynucleotide.

The simplest approach to the problem is to place one or more triplets of known sequence at one end of a poly U chain. This is made possible by the fact that short oligonucleotides prime polynucleotide phosphorylase by acting as nuclei for growth of the polynucleotide chains.[4] By priming with a mixture of ApU and ApApU[5] we have obtained polymers which promoted the incorporation of small amounts of tyrosine besides phenylalanine, but not that of isoleucine, asparagine, or lysine. In contrast, random poly UA stimulated the incorporation of tyrosine and isoleucine to the same extent. This suggested that tyrosine incorporation may be caused by a beginning AUU sequence in these polymers.

Preparation of oligonucleotide primers of polynucleotide phosphorylase:—Poly UA(5:1), 16 mg, from a previously prepared batch[6] (actually determined U:A ratio, 4.8:1) was dissolved in 1.6 ml of 0.02 *M* Tris-HCl buffer, pH 7.4, and digested for 15 hr at room temperature with 0.32 mg of crystalline pancreatic ribonuclease (Worthington) after adding a few drops of toluene. The solution was then adjusted to pH 5.6 with acetic acid and made 0.01 *M* with sodium acetate. Ribonuclease digestion of the above polymer yields Up, ApUp, ApApUp, and traces of higher oligonucleotides (ApApApUp, etc.). In order to remove the terminal phosphate, monoesterified at ribose carbon 3′, the solution was incubated with prostatic phosphomonoesterase[7] until no more orthophosphate was released. Following this treatment the solution was adjusted to pH 7.9 with NaOH and digested with 0.1 mg of crystalline trypsin (Armour) for 15 min to destroy the ribonuclease and phosphomonoesterase. The phosphomonoesterase and trypsin digestions were conducted at 37°. The solution was shaken with phenol for 1 hr at room temperature. The aqueous layer was washed with ether and acidified to pH 3.0 with HCl. The oligonucleotides (mostly ApU and ApApU) together with the uridine were then adsorbed on 100 mg of charcoal,