References and Notes

1. P. B. Price and R. M. Walker, *Nature* **196**, 4856 (1962).
2. ———, *J. Geophys. Res.* **68**, 4847 (1963).
3. R. L. Fleischer and P. B. Price, *ibid.* **69**, 331 (1964).
4. ———, *Geochim. Cosmochim. Acta* **28**, 1705 (1964).
5. R. L. Fleischer, P. B. Price, R. M. Walker, *J. Geophys. Res.* **69**, 4885 (1964).
6. M. Maurette, P. Pellas, R. M. Walker, *Nature* **204**, 821 (1964).
7. R. L. Fleischer, P. B. Price, R. M. Walker, E. L. Hubbard, *Phys. Rev.* **133**, A1443 (1964).
8. R. L. Fleischer, P. B. Price, E. M. Symes, D. S. Miller, *Science* **143**, 349 (1964); M. Maurette, P. Pellas, R. M. Walker, *Bull. Soc. Franc. Mineral. Crist.* **87**, 6 (1964); R. L. Fleischer and P. B. Price, *Geochim. Cosmochim. Acta* **28**, 755 (1964).
9. P. B. Price and R. M. Walker, *Phys. Rev. Letters* **8**, 217 (1962).
10. For a recent review, see K. W. Ford, *Sci. Am.* **209**, 122 (1963).
11. U. B. Marvin and C. Klein, *Science* **146**, 919 (1964).
12. H. Hamaguchi, G. W. Reed, A. Turkevich, *Geochim. Cosmochim. Acta* **12**, 337 (1957).
13. R. A. Schmitt, R. H. Smith, D. A. Olehy, *General Atomic Rept. GA-4221* (General Atomic Div. of General Dynamics Corp., San Diego, Calif., 1963).
14. For a review, see E. Anders, in *The Moon, Meteorites and Comets*, B. M. Middlehurst and G. P. Kuiper, Eds. (Univ. of Chicago Press, Chicago, 1963), p. 402.
15. M. Maurette and R. M. Walker, *J. Phys. Radium* **25**, 43 (1964).
16. R. L. Fleischer, P. B. Price, R. M. Walker, *J. Geophys. Res.*, in press.
17. P. B. Price and R. M. Walker, *Appl. Phys. Letters* **2**, 23 (1963).
18. For recent measurements and a summary of previous work, see J. F. Lovering and J. W. Morgan, *J. Geophys. Res.* **69**, 1979 (1964).
19. G. W. Reed, K. Kigoshi, A. Turkevich, *Geochim. Cosmochim. Acta* **20**, 122 (1960).
20. H. D. Holland and D. Gottfried, *Acta Cryst.* **8**, 291 (1955).

21. Part of this work was carried on at the Dept. of Geological Sciences, Harvard Univ., under a program of meteorite research administered by Dr. C. Frondel, NASA grant NSG 282-63.

* Present address: Southern Methodist University, Dallas, Texas.

15 February 1965

Enzymatic Synthesis of Tri- and Tetranucleotides of Defined Sequence

Abstract. *Conditions have been found under which polynucleotide phosphorylase adds only a few nucleotide residues to the 3′ end of a dinucleotide primer. Pure tri- and tetranucleotides can be isolated from reaction mixtures in high yield.*

Block oligonucleotides containing nearly any desired trinucleotide sequence at the point of union of the two blocks can be prepared in pure form (*1*). The polymerization of a nucleoside diphosphate (*2*) was primed with an appropriate oligonucleotide by means of polynucleotide phosphorylase from *Micrococcus lysodeikticus*. The successful synthesis of the desired product was due first to the enzyme having an essentially absolute primer requirement for initiation of polymerization (*3*), and second to our finding that high NaCl concentrations terminated chain growth at the desired length (*1*). Typical products were CpCpCpCpCpApApA and CpCpCpCpCpApUpUpU.

One remaining limitation of the preparative procedure arose from the failure of very short oligonucleotides such as the dimer, NpN, to prime polymerization at the high NaCl concentrations required: consequently, it was not possible to prepare trimers and tetramers of specified sequence. We now report how this may be done with the same system.

This new procedure has its origin in the observation that, in the absence of NaCl, reaction products were unutilized dinucleotide primer and long polynucleotide chains with primer at the 5′ end. Evidently these two species of polymer did not represent the product that should be obtained at thermodynamic equilibrium. Such a product should instead have a single peaked distribution of the type known as the "most probable" distribution with its mean value being determined by the mole ratio of NDP polymerized to primer. In principle, the continuation of the incubation for a sufficiently long time should bring about a redistribution of monomeric units to this state. We have now found conditions that make this redistribution possible in a practical time span and that result in a very low molecular weight product consisting of only a few species, such as ApUpA, ApUpApA and ApUpApApA, which can then readily be fractionated.

Polynucleotide phosphorylase from *M. lysodeikticus* was purified 90-fold (*3*). The purified enzyme was shown to be free of nuclease and phosphatase. It polymerized NDP at a very slow rate in the absence of added primer, but this endogenous activity was completely inhibited by the addition of 0.4*M* NaCl to the reaction mixture (*1*). In studies of reaction kinetics, the polymerization of NDP and the incorporation of radioactive primer into polymer was followed by removing samples from a reaction mixture at various time intervals and applying them to Whatman 3MM chromatography paper. Chromatograms were developed in a solvent in which primer and NDP migrated away from the origin, while oligonucleotides longer than the tetramer remained at the origin. Spots at the origin were eluted, and the amount of NDP polymerized was determined by absorbance measurement, whereas the amount of primer incorporated was determined by ra-

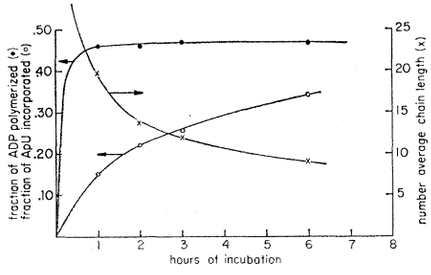

Fig. 1. Prolonged incubation of a polymerization reaction mixture. The ADP is polymerized in the presence of ApU-C^{14} primer. Samples of the reaction mixture (text), withdrawn at the indicated times, were applied to Whatman 3MM paper, and the chromatogram was developed in a solvent composed of 60 parts 95 percent ethanol to 40 parts 1*M* ammonium acetate. Origin spots were cut out and eluted with buffer (0.01*M* tris, *p*H 8.2, 1m*M* EDTA). The fraction of ADP polymerized (•) was determined by absorbance measurement at 260 mμ; the fraction of ApU-C^{14} incorporated into polymer (○) was determined by radioactivity measurement (*1*). The average chain length of polymer at the origin (×) is the mole ratio of polymer residues to incorporated primer residues.

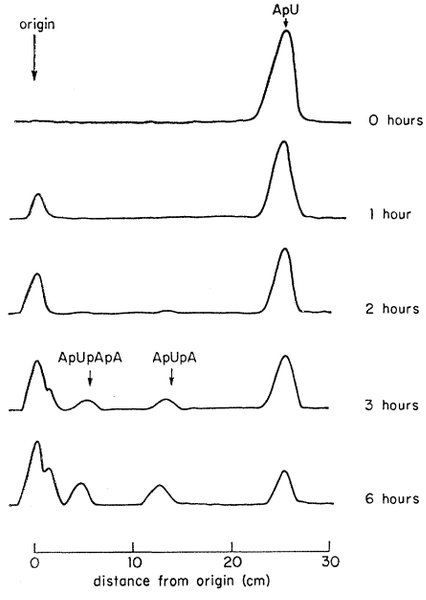

Fig. 2. Distribution of ApU-C^{14} primer as a function of time. The chromatogram from Fig. 1 was cut into strips (before elution of origin spots) and scanned for radioactivity in a Vanguard automatic chromatogram scanner. Radioactivity peaks corresponding to ApUpA and ApUpApA appear in the samples taken at 3 and 6 hours.

dioactivity measurement. The ratio (nucleotide residues) : (primer residues) provides the number average chain length of the polymer product.

Reaction conditions which are optimum for the synthesis of long polymer chains (*5*) cannot be used for the preparation of tri- and tetranucleotides. For example, when a dinucleotide is used to prime the polymerization of ADP (*6*) under the usual reaction conditions (*5*), oligonucleotides [NpNpA, NpN(pA)$_2$, and others] are not present in significant amounts at any time during the course of the reaction (*4, 7*); instead only long polymer chains (more than 300 residues) and unincorporated primer molecules are found.

In a study of the polymerization kinetics of this system we have shown (*4*) that the incomplete utilization of primer, and hence the bimodal distribution of the product, is due to the fact that the addition of the first nucleotide residue on to the 3′ end of the primer (initiation step) is extremely slow as compared to the subsequent chain-growth steps. The very skewed distribution of chain lengths which results from this kinetic barrier is not in a state of maximum entropy (*8*). Hence a redistribution of chain lengths can be expected to occur long after the net polymerization has ceased, and it results ultimately in the "most probable" chain length distribution in which entropy is at a maximum (*8*).

Such a redistribution process would be characterized by continued incorporation of primer into polymer; moreover, since the overall NDP concentration remains constant during this process, there must also be a simultaneous phosphorolysis of the long polymer chains already formed. The polymer product formed as a result of this action would be of intermediate size, the actual length being proportional to the mole ratio of NDP to primer originally present in the reaction mixture. Thus it should be possible to obtain a preparation rich in tri- and tetranucleotides if the initial ratio of NDP to primer is sufficiently low and if the reaction mixture is then incubated for a very long time until the true state of chemical equilibrium is attained.

To test this hypothesis a reaction mixture whose NDP-primer ratio was 6.5 was incubated for several hours after the net polymerization had ceased. Reaction conditions were: 3.4 m*M* ADP, 2 m*M* magnesium acetate, 0.1 m*M* $CuSO_4$, 0.2*M* tris buffer, *p*H 8.2, 0.52 m*M* ApU (labeled with C^{14}) and 0.6 mg of enzyme per milliliter; incubation at 37°C. Samples were withdrawn at regular intervals and analyzed chromatographically as already described (Fig. 1). Evidently, though polymerization has leveled off at 47 percent completion after 1 hour, the incorporation of primer does in fact continue for 6 hours more. Also, there is a corresponding decrease in the average chain length of the product, since the average chain length is defined as the ratio of polymer residues to incorporated primer residues. These results are in complete accord with the expectations based on theoretical considerations.

When the chromatograms from which the data in Fig. 1 were obtained are analyzed for the distribution of radioactivity above the origin (Vanguard strip scanner), peaks corresponding to ApUpA, ApU(pA)$_2$, and ApU(pA)$_3$ are observed in the samples taken at 3 and 6 hours (Fig. 2). This result not only further confirms our expectation that the average chain

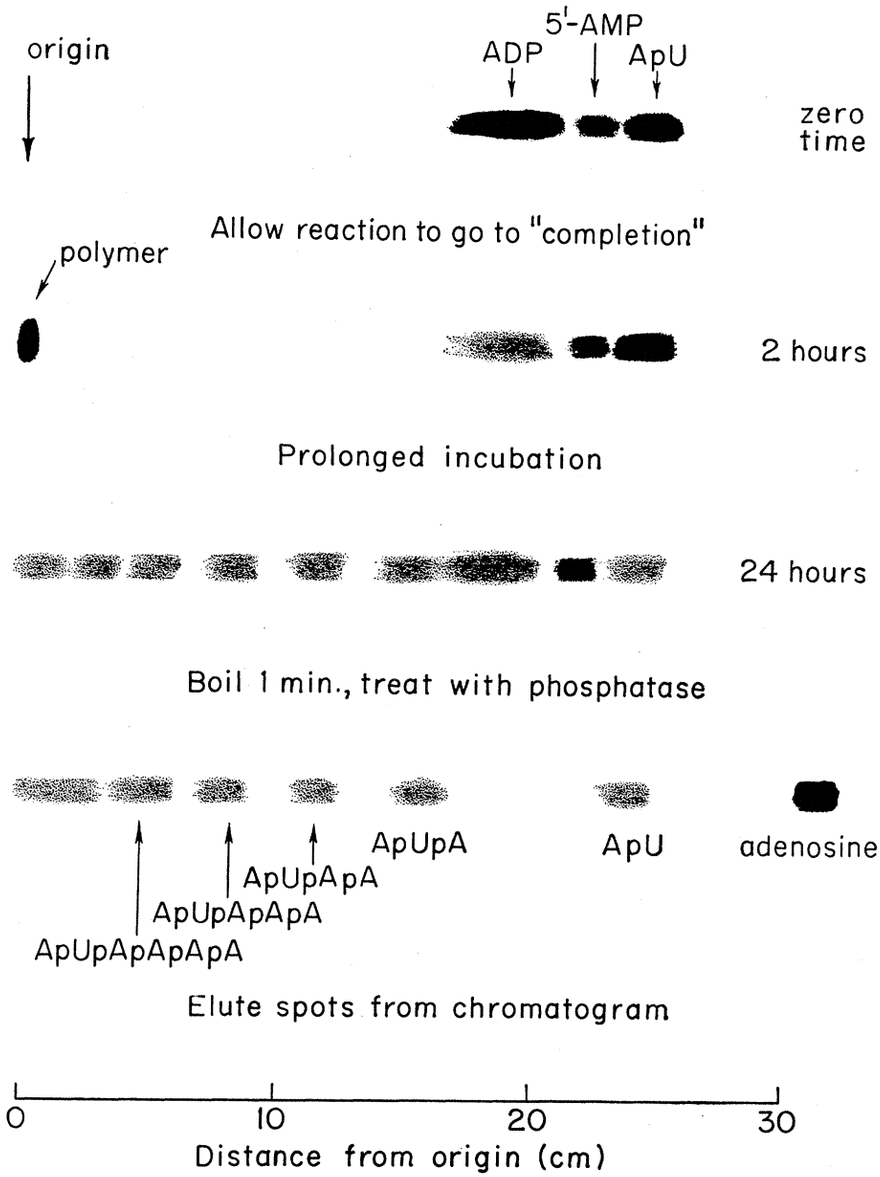

Fig. 3. Sequence of events in a reaction mixture most suitable for the synthesis of oligonucleotides. Samples were withdrawn from a reaction mixture at various times and applied to a chromatogram, which was developed in a solvent composed of equal parts of 95 percent ethanol and 1*M* ammonium acetate. The distribution of ultraviolet-absorbing material in the various strips is reproduced here.

length should diminish with prolonged incubation, but also demonstrates that this technique can be used to prepare tri-, tetra- and pentanucleotides of defined sequence.

After studying reaction conditions and chromatography techniques we developed the following procedure for routine preparations of large amounts (about 100 μg) of oligonucleotides. In a 0.1-ml reaction mixture were 10 to 30 m*M* NDP; 10 m*M* magnesium acetate, 0.2*M* glycine buffer at *p*H 9.3, 0.1 m*M* $CuSO_4$, 0.40*M* NaCl, 7.5 m*M* dinucleotide primer, and 0.12 mg of enzyme per milliliter (*9*). The reaction mixture was incubated at 33°C for 14 to 24 hours, the longer time being necessary for the polymerization of purine nucleoside diphosphates. The course of a typical reaction is shown in Fig. 3, where ApU is used to prime the polymerization of ADP. Samples were removed from the reaction at the indicated times and applied to chromatography paper. The chromatograms were developed overnight with a solvent composed of equal parts of 95 percent ethanol and 1*M* ammonium acetate. The appearance of the dried chromatograms under a source of ultraviolet light is reproduced in Fig. 3. At zero time the only visible components are ADP, ApU and a trace of 5′-AMP which usually contaminates commercial preparations of ADP. After 2 hours of incubation the polymerization of ADP has leveled off at about 50-percent incorporation, the commonly misnamed "equilibrium" state. A subsequent 22 hours of incubation results in a redistribution of chain lengths, and during this process the true equilibrium state is approached. In order to remove excess ADP, which partially overlaps the ApUpA region, the reaction mixture is heated for 1 minute at 100°C to denature polynucleotide phosphorylase, and it is then treated with 20 μg of purified alkaline phosphatase for 2½ hours at 37°C. In large-scale preparations of oligonucleotides, the entire reaction mixture may now be applied to paper for chromatography in the usual way (*1*). Dried chromatograms are soaked for 1 hour in absolute ethanol in order to remove ammonium acetate, and the soaking is followed by a 10-minute wash in ether. The various oligonucleotide components are then cut out, eluted with H_2O and lyophilized.

We tested purity of oligonucleotide fractions by hydrolyzing a portion in alkali and determining the reaction products. In one experiment, alkaline hydrolysis of ApApU gave only AMP-2′, 3′ and uridine in a ratio of 2.05:1.00; in a second experiment, hydrolysis of ApApUpU gave only AMP-2′, 3′; UMP-2′, 3′; and uridine in a ratio of 2.16 : 1.05 : 1.00. These results not only show the high degree of purity of the oligonucleotide fractions but also demonstrate the reliability of the determination of chain length on the basis of chromatographic mobility.

This technique is exceedingly useful for the preparation of large (0.1 to 1 mg) amounts of many tri-, tetra- and pentanucleotides of defined base sequence. Of the NDP's that we have studied in this reaction (ADP, UDP, CDP, IDP, XDP), all give excellent results. Poor yields obtained with GDP are due to the very slow phosphorolysis of the polyguanylic acid chains originally synthesized, and this in turn results in low yields of the shorter chains.

Oligonucleotides synthesized in this fashion have been valuable in studies of coding specificity, in both the specific binding of aminoacyl-sRNA to ribosomes (*10*) and in polypeptide synthesis (*11*).

R. E. THACH
PAUL DOTY
Department of Chemistry, Harvard University, Cambridge, Massachusetts

References and Notes

1. R. E. Thach and P. Doty, *Science* **147**, 1310 (1965).
2. Conventions used: N, (pN) and NDP represent respectively the nucleoside, nucleotide residue, and nuculeoside diphosphate of any base. Specific bases are abbreviated in the usual way: adenosine, A; uridine, U; cytidine, C; guanosine, G; inosine, I; and xanthosine, X. sRNA, soluble RNA; AMP, adenylic acid; UMP, uridylic acid.
3. M. F. Singer and B. M. O'Brien, *J. Biol. Chem.* **238**, 328 (1963).
4. R. E. Thach, thesis, Harvard University (1964).
5. Normal reaction conditions are: 40 m*M* NDP, 10 m*M* magnesium acetate, 0.1 m*M* $CuSO_4$, 0.2*M* tris buffer, *p*H 8.2, 0.1 to 1.0 m*M* dinucleotide primer and enzyme. Incubation at 37°C.
6. Only the polymerization kinetics of purine nucleoside diphosphate have been studied in detail. Preliminary studies on the kinetics of pyrimidine nucleoside diphosphate polymerizations have indicated similar behavior, but specific exceptions to the general rule may occur (*8*). In any case, the methods developed here for the synthesis of tri-, tetra-, and pentanucleotides apply equally well to both purines and pyrimidines.
7. L. A. Heppel, *J. Biol. Chem.* **238**, 357 (1963).
8. L. Peller and L. Barnett, *J. Phys. Chem.* **66**, 680 (1962).
9. NaCl is included specifically for the purpose of depressing the rate of the polymerization reaction which proceeds in the absence of primer (*1*, *4*). The extent to which this concentration of salt inhibits the formation of long polymer chains (*4*) under the conditions employed is not significant.
10. R. E. Thach and T. A. Sundararajan, *Proc. Natl. Acad. Sci. U.S.*, in press; E. Akinrimisi, R. E. Thach, P. Doty, in preparation.
11. T. A. Sundararajan, R. E. Thach, P. Doty, in preparation.
12. This work, as well as that previously reported (*1*), was supported by the National Institutes of Health grant HD-01229.

23 March 1965

Glossopteris Discovered in West Antarctica

Abstract. *Leaf impressions from the Polarstar Formation in the northern Ellsworth Mountains are the first* Glossopteris *and the oldest identifiable plant fossils reported from West Antarctica. Their occurrence in a thick, probably marine, geosynclinal sequence in close association with coals and probable glacial deposits increases the similarity between the late Paleozoic history of Antarctica and the other southern continents.*

Three geologic expeditions, sponsored by the University of Minnesota, to the Ellsworth Mountains have made discoveries in the Polarstar Formation which now demonstrate the existence of *Glossopteris* in West Antarctica. In 1961 to 1962 the party collected several argillite specimens with bedding plane markings within the lower 15 m of the formation on the east side of Mt. Wyatt Earp (Fig. 1) in the northern Sentinel Range. The markings are mainly smooth, structureless, linear impressions or low ridges 1 to 2 mm wide and up to 9 cm long. They might be interpreted as animal trails, ice crystal impressions, or plant stems, but their existence indicated the possible presence of fossil plants in the formation. The following season definite plant stems with cell structure and carbonaceous matter were found in slates and argillites higher in the formation on Mt. Weems and on the ridge to the north. Continued searching in the area with helicopter support during 1963 to 1964 culminated in the discovery of abundant *Glossopteris* leaves in the upper part of the exposed Polarstar Formation at 77°33′15″S, 85°54′W, on a ridge 2.4 km northeast of Polarstar Peak. This *Glossopteris* site lies near Mt. Ulmer, considered the highest peak in the Sentinel Range by Ellsworth when he first viewed the mountains from the north during his 1935 flight across West Antarctica.

The general geology of the Ellsworth Mountains has been described (*1*); most exposed bedrock consists of intensely folded metasedimentary