A MAPPING PROCEDURE FOR NUCLEOTIDES AND OLIGONUCLEOTIDES*

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It is now often assumed that the sequence of nucleotides in nucleic acids is of paramount genetic significance. However, there is at present no stepwise degradation procedure for determining the sequence of nucleotides in a polynucleotide chain. Fragmentary information concerning sequential arrangements can be obtained by degradation of nucleic acids with specific enzymes followed by separation and analysis of the nucleotide and oligonucleotide products. Separation of digestion products can be accomplished by use of columns employing ion exchange resins (Volkin and Cohn, 1953), or cellulose derivatives (Staehelin, Peterson, and Sober, 1959), and also by use of paper chromatography and paper electrophoresis (Reddi, 1959). The column methods provide numerous discrete fractions but tend to require larger amounts of material than separations on paper. However, no simple, integrated procedure had apparently been developed for separations on paper. The successful combination of paper electrophoresis and paper chromatography to give a mapping procedure for peptides (Ingram, 1956; Woody and Knight, 1959) suggested that a similar technique might be useful for separating oligonucleotides. We have developed such a method using as test material digests obtained by the action of pancreatic ribonuclease on tobacco mosaic virus nucleic acid (TMV-RNA).

TMV-RNA was obtained from centrifugally purified preparations of tobacco mosaic virus (TMV) by treatment with phenol (Gierer and Schramm, 1956).

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About 5 mg. of the viral RNA in 0.04 M phosphate buffer at pH 7.1 were treated with 0.25 mg. of ribonuclease (Worthington Biochemical Corporation, Freehold, New Jersey) and the mixture was held for twenty-four hours at 37°.

Fractionation of the digest was started by a paper electrophoretic step. A sheet of Whatman 3MM paper (46 x 57 cm) was inserted into a Durrum-type electrophoresis cell(Block, Durrum, and Zweig, 1955) equipped with a special rack facilitating removal of the paper when wet. After wetting the paper, a half-hour run was made before the sample was applied at the voltage to be used subsequently and in a buffer prepared by adding 7.5 ml. of formic acid (98%) to 2.5 liters of water and adjusting the pH to 2.7 with concentrated ammonium hydroxide. The paper was then removed and after 5-10 minutes of drying in air, the digest (equivalent to 2-7 mg of RNA) was applied in a corner near the cathode edge of the paper. A picric acid marker was applied to the same edge but near the opposite corner. The paper was returned to the electrophoresis cell and a run made at 6 volts per cm and a current of about 12 mA. In 17-20 hours the picrate marker had reached the anode reservoir and the run was finished. (The marker moves faster than the RNA digestion-product front.) The support with the paper was then removed and the paper dried in air at room temperature. After turning the paper 90° from the electrophoretic direction and serrating the lower edge for run-off, the paper was developed in a mixture of equal parts of the electrophoresis buffer adjusted to pH 3.8 and tertiary butanol, the pH of the final mixture being 4.8. It is important to saturate the atmosphere of the chromatography tank with the developing solvent. This is done by having an extra sheet of Whatman 3MM paper wet with the solvent and suspended in the tank. Solvent is also placed in a tray in the bottom of the tank. The chromatography run requires about 36 hours at 19-22° at which time the fastest running components are approaching the bottom of the paper. Higher temperature decreases the running time of the chromatographic step, but results in less satisfactory resolution of components.

After chromatography, the paper was dried in air at room temperature

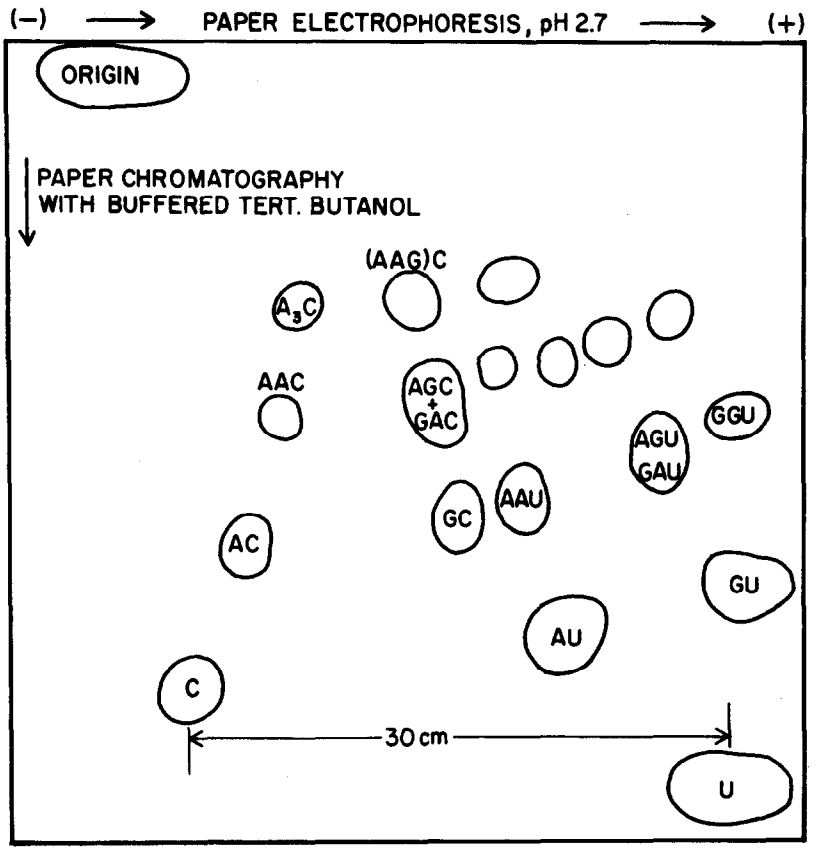

Fig. 1. Fractionation of ribonuclease digests of phenol TMV-RNA.

and the spots were located by examination with an ultraviolet light and marked. The spots were also recorded by the printing method of Smith and Allen (1953), thus providing a map. A tracing of such a map is presented in Figure 1.

The two-dimensional mapping procedure just outlined yielded with digests of TMV-RNA 19 discrete spots. The material in these spots was eluted either with water or dilute acid and subjected to a variety of analytical procedures. By readings in the Beckman DU spectrophotometer and summing up the optical densities (OD) it was found that about 90% of the applied OD_{260} could be accounted for as characteristic fractions. The reproducibility of the method was tested by spectrophotometric analysis of the eluted major spots from different runs. The reproducibility was found to be $\pm$ 3% for aliquots of the

same digest and ± 4% with digests of different preparations, all of them TMV-RNA.

The compounds eluted from the various spots of a map were analyzed by a standard procedure (Markham and Smith, 1952) to determine the purines and pyrimidines present and their proportions. The sequences of bases in the oligonucleotide fractions were deduced partly on the basis of the known specificity of pancreatic ribonuclease, partly by comparison of properties with those of known substances, and in some cases by application of techniques available for determining the sequences of smaller oligonucleotides (see Reddi, 1959). Using the method of abbreviation introduced by Markham and Smith (1952) in which each individual nucleotide, or each nucleotide residue in an oligonucleotide chain is indicated by the first letter of the nucleotide's name, the following compounds can be quantitatively eluted from our maps of ribonuclease digests of TMV-RNA: U, C, AU, GU, GC, AAU, AGU, GAU, GGU, AAC, AGC, GAC, AAAC, and (AAG)C. The sequences of (AAG)C have not been determined yet. The locations of the above substances are shown in Figure 1. The unlabeled spots are due to single or mixed compounds which are not present in amounts sufficient for direct quantitative determination.

The full potentiality of the mapping procedure has not yet been evaluated. However, preliminary runs indicate that the method will quickly provide data on the nature and frequency of nucleotide sequences in the nucleic acids of mutant strains of viruses, and permit similar comparisons to be conveniently made of RNA from different viruses, soluble RNAs, host cell RNAs, etc. Furthermore, the method can be diversified by use of nucleases of different specificities, by examination of the products of incomplete digestion, and various other modifications. The procedure can also be applied to the survey of products obtained by the action of specific nucleases on deoxyribonucleic acid.

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