Nucleotide 'Maps' of Digests of Deoxyribonucleic Acid

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Various digests of ^{32}P -labelled DNA were examined by two-dimensional ionophoresis on cellulose acetate and DEAE-cellulose paper. The products from digestion with pancreatic deoxyribonuclease and *Neurospora crassa* endonuclease were qualitatively closely similar, but very complex, and were used to investigate the mapping behaviour of nucleotides in various ionophoretic systems. Ionophoresis on DEAE-cellulose paper in triethylamine carbonate, pH 9.7, followed by ionophoresis in the second dimension at pH 1.9 gave high resolution of nucleotides in very complex mixtures and permitted the fractionation of larger quantities than is possible on cellulose acetate. High resolution of nucleotides in compact spots was obtained with two-dimensional ionophoresis on cellulose acetate and AE-cellulose paper, a system that is a useful supplement to those based on DEAE-cellulose paper.

Although the determination of the complete nucleotide sequence of a DNA molecule is likely to remain an imposing problem for some considerable time, recent developments in nucleic acid biochemistry and enzymology have led to new micro methods that are eminently suitable for the determination of limited sequences of deoxyribonucleotides. In particular, the application of ionophoresis on ion-exchange papers to the rapid separation and identification of small quantities of (labelled) nucleotides (Sanger, Brownlee & Barrell, 1965), the use of polynucleotide kinase to label the 5'-terminus of oligo- and poly-nucleotides (Richardson, 1965; Novogrodsky & Hurwitz, 1965, 1966) and subsequent extension of a terminally labelled polydeoxynucleotide in appropriate circumstances by polynucleotide ligase (Gellert, 1967; Weiss & Richardson, 1967; Olivera & Lehman, 1967; Geftter, Becker & Hurwitz, 1967) provide, in combination, an entirely practicable approach to the study of nucleotide sequences of DNA molecules in regions of particular biological interest. The recent determination of partial sequences at the cohesive ends of DNA from bacteriophage λ (Wu & Kaiser, 1968) is an excellent example of the application of repair reactions with DNA polymerase (Richardson, Inman & Kornberg, 1964) to nucleotide sequence studies.

To facilitate the determination or comparison of sequences in specific fragments or regions of DNA molecules, a bank of information on the fractiona-

tion of complex mixtures of oligonucleotides is necessary. There are several methods for the degradation of DNA and fractionation of the degradation products. Examples are the breakage of apurinic acid by aromatic amines (Burton & Petersen, 1960) and the hydrolysis of DNA by endonucleases such as pancreatic deoxyribonuclease (Smith & Markham, 1952; Sinsheimer, 1954). Column chromatography, often with modifications of the Tomlinson & Tener (1963) system, has been used widely in the examination of products from these reactions, but resolution of complex mixtures of nucleotides often requires combinations of several methods and so becomes quite tedious and time-consuming. In contrast, a nucleotide 'mapping' analysis of one or more digests of DNA could provide a relatively simple, quick and highly discriminating means for the characterization or 'fingerprinting' of DNA or large fragments of it. This paper describes some methods for the production of nucleotide 'maps' from DNA preparations, and the identification of many of the smaller oligonucleotides resolved by these 'mapping' techniques.

MATERIALS AND METHODS

Materials. The following enzymes were purchased from Worthington Biochemical Corp., Freehold, N.J., U.S.A.: Pronase, pancreatic deoxyribonuclease, spleen acid deoxyribonuclease (deoxyribonuclease II), micrococcal nuclease, snake-venom phosphodiesterase, spleen phosphodiesterase, bacterial alkaline phosphomonoesterase and pancreatic ribonuclease. Pig liver nuclease (prepared

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by the method of Dr L. Heppel, Cornell University; unpublished work) was kindly provided by Dr B. F. C. Clarke. *Neurospora crassa* endonuclease was kindly given by Dr I. R. Lehman. Preparations of highly purified spleen acid deoxyribonuclease (Bernardi & Griffe, 1964) were kindly provided by Dr G. Bernardi. Semen phosphomonoesterase was prepared as described by Wittenberg & Kornberg (1953).

Cellulose acetate (Oxoid) electrophoresis strips were obtained from Oxo Ltd., London E.C.4, U.K. Ion-exchange papers used were Whatman Chromedia DE 81, AE 81 and ET 81. The dyes used for electrophoretic markers were Xylene Cyanol FF, Orange G, and Acid Fuchsin, and were obtained from G. T. Gurr Ltd., London S.W.6, U.K. CsCl used was the AnalaR grade from British Drug Houses Ltd., Poole, Dorset, U.K. The scintillator 2,5-bis-(5-*tert*-butylbenzoxazol-2-yl)thiophen (BBOT) was obtained from Ciba Ltd., Duxford, Cambs., U.K.

Preparation of ^{32}P -labelled DNA. (i) *Escherichia coli* (strain M.R.E. 600) was grown overnight in limiting phosphate medium (Garen & Levinthal, 1960) and 5 ml was subcultured into 100 ml of the medium containing 2–25 mCi of ^{32}P P_i. After 7 h growth with vigorous aeration at 37°C the cells were harvested by centrifugation (3000g for 10 min) and DNA was extracted from them by the procedure of Lehman (1960). In subsequent preparations the method was modified as follows. The cells were suspended in 0.14 M-NaCl–0.01 M-sodium citrate (3 ml) and shaken at room temperature for at least 2 h (and often overnight) with sodium dodecyl sulphate (210 mg). Ethanol (2 vol.) was added to the translucent solution. The gelatinous precipitate was collected by centrifugation after removal of most of the supernatant with a Pasteur pipette and extracted three times with 1.4 M-NaCl–0.01 M-sodium citrate (3 ml). (In some preparations about 0.5 mg of calf thymus DNA was added to the combined extracts as carrier, but this was discontinued in later preparations.) Ethanol (2 vol.) was added to the combined extracts and the fibrous precipitate was collected by spooling and the solution was centrifuged. The pellet, which was hardly visible, was dispersed in 0.14 M-NaCl (3 ml) and the spooled precipitate was added to this solution. Pronase (4 mg) was added and the mixture was stirred and then shaken at room temperature for several hours during which time the precipitate dissolved. The solution was stored overnight in the refrigerator and was then extracted twice with water-saturated phenol (1.5 ml). The phenol layers were combined and extracted with water (1 ml) and the aqueous phase was then mixed with the DNA solution, which was then extracted about 12 times with ether. The ether layers were rejected and ether was removed from the aqueous phase by bubbling air (gently) through the solution for several minutes. Ribonuclease (2 mg/ml; 50 μ l) was added to the solution and after 2 h at room temperature the solution was passed through a column (37 cm $\times$ 1.5 cm diam.) of Sephadex G-200. In some cases an ethanol precipitation step was introduced before ribonuclease treatment; the precipitate was then dissolved in 0.5 mM-NaCl (1 ml) and stored overnight at 4°C before treatment with ribonuclease (50 μ g) as before. The Sephadex column was eluted with 0.5 mM-NaCl and radioactivity in the effluent was monitored. [To minimize possible loss of DNA by adsorption, the column

was previously 'saturated' with DNA by application of calf thymus DNA solution (1 mg/ml) followed by a large wash (200 ml) with 0.5 mM-NaCl. This procedure was followed only when particularly small quantities of labelled DNA were being processed.] When radioactivity began to appear in the effluent, fractions (1.5 ml) were collected and counted for radioactivity. Two well-resolved peaks of radioactivity were obtained and were coincident with extinction at 259 nm. The material in the first peak was stable to alkali but was hydrolysed by pancreatic deoxyribonuclease; that in the second peak was hydrolysed by alkali to mononucleotides. Fractions in the first peak of radioactivity were therefore used as solutions of DNA, and were stored frozen.

(ii) In some cases, it was possible to recover DNA from preparations of radioactive cells (*E. coli*) that had been treated with phenol for extraction of tRNA. In these instances, the debris from the interface of the phenol and aqueous phases was washed with ether and dissolved in 5% (w/v) sodium dodecyl sulphate (3 ml). After centrifugation (1000g for 15 min) the solution was treated with ethanol (2 vol.) and precipitated fibres were collected, dissolved in 0.5 mM-NaCl (1 ml), then digested with ribonuclease and the preparation completed as described above.

(iii) Bacteriophage DNA. Labelled bacteriophage λ DNA was prepared from the heat-inducible lysogen, C600 (λ_{C1857}), grown at 31°C in supplemented M9 medium (Levine & Borthwick, 1963) but at one-tenth the normal phosphate concentration. ^{32}P P_i (25 mCi) was added to the culture (83 ml), at a cell density of about 3×10^8 cells/ml and 30 min later the temperature was raised to 42°C for 30 min. Aeration was then continued at 37°C and the culture was lysed about 20 min later. After a further 30 min (phosphoglycerol monolaurate was used to minimize foaming) the lysate was clarified by centrifugation (10000g for 10 min) and the number of plaque-forming units/ml was determined with *E. coli* strain W1485 as the indicator bacteria (about 10^{10} bacteriophage/ml). The bacteriophages were collected by $(\text{NH}_4)_2\text{SO}_4$ precipitation (21.3 g) and the precipitate was dissolved in λ -dil medium (3 ml) (Kaiser & Hogness, 1960) and CsCl was added to a concentration of 41.5% (w/w). After centrifugation at 130000g for 24 h, the fractions containing bacteriophage were diluted with water (2 ml), dialysed, extracted twice with water-saturated phenol and dialysed exhaustively against 1 mM-NaCl. The dialysed solution of bacteriophage λ DNA was stored frozen. ^{32}P -labelled DNA from bacteriophage T4 was kindly provided by Dr A. A. Travers and ^{32}P -labelled DNA from bacteriophage fd was kindly provided by Dr J. D. Smith.

Degradation of DNA. (i) Burton degradation (Burton & Petersen, 1960). DNA solution (10 μ l) in mM-NaCl was mixed with 3% (w/v) diphenylamine (recrystallized from aqueous ethanol) in formic acid (20 μ l) and incubated at 30°C in a sealed capillary for 18 h. After digestion, reaction mixtures were evaporated to dryness on a sheet of polythene in a vacuum desiccator (water pump) and their residues were extracted with pyridine-acetate buffer solution, pH 3.5 (5 μ l), for ionophoresis. Details of these and other micro procedures were as described by Sanger *et al.* (1965).

(ii) Digestion with micrococcal deoxyribonuclease (Cunningham, Catlin & Privat de Garilhe, 1956). DNA

solution (10–100 μ l) in 1mM-NaCl was evaporated to dryness on polythene and dissolved in 0.05M-borate buffer, pH 8.8, 0.01M with respect to CaCl_2 (20 μ l). Micrococcal nuclease solution (1mg/ml in water; 10 μ l) was added and the mixture was incubated at 37°C for 12 h.

(iii) Digestion with pancreatic deoxyribonuclease (Kunitz, 1950). DNA solution (10–100 μ l) in 1mM-NaCl was evaporated to dryness on polythene and dissolved in a solution (10 μ l) of pancreatic deoxyribonuclease (1mg/ml) in 0.1M-sodium acetate, pH 5.0, containing MgCl_2 (5mM). After digestion for 18 h at 37°C (or in some cases 30°C) the reaction mixture was dried for ionophoresis as described above, or sometimes applied directly to cellulose acetate strips or to DEAE-cellulose paper. In one series of experiments the effect of time of digestion on the reaction products was studied at room temperature (20°C) over a range of enzyme concentrations.

(iv) Digestion with pig liver nuclease (L. Heppel, unpublished work). DNA in 1mM-NaCl (100 μ l) was dried on polythene and dissolved in water (50 μ l) containing 0.67M-tris, pH 7.1 (2 μ l), 0.04M- MgCl_2 (1 μ l), 14mM-2-mercaptoethanol (1 μ l) and the enzyme preparation (6 μ g). The mixture was digested at 37°C for 4 h.

(v) Digestion with *N. crassa* endonuclease (Linn & Lehman, 1965a). DNA in 1mM-NaCl (50–200 μ l) was denatured by heating in sealed tubes in boiling water for 20 min followed by rapid cooling in ice. The solution was then either dried on polythene or mixed with 0.5M-tris, pH 8.5 (40 μ l), 1M- MgCl_2 (2 μ l) and 0.2 unit of enzyme (5 μ l): dried samples were dissolved in the following reaction mixture: 0.1M- NH_4HCO_3 , pH 8 (20 μ l), 0.1M- MgCl_2 (2 μ l), about 0.05 units of enzyme (2 μ l). Reaction mixtures were incubated at 50°C for 2 h or at 37°C for 14 h.

(vi) Digestion with spleen endonuclease (spleen acid deoxyribonuclease) (Bernardi & Griffe, 1964). A solution of the Worthington enzyme preparation (1mg/ml) in 0.05M-sodium acetate, pH 4.6, 1mM- MgCl_2 , or a purified preparation (E_{280} 240) from Dr G. Bernardi was used. With the former preparation, DNA in 1mM-NaCl was digested at an enzyme concentration of 0.1mg/ml in 0.01M-sodium acetate, pH 4.6, 0.01M- MgCl_2 at 30°C for 18 h. With Bernardi's preparation of the enzyme, DNA was digested in 0.01M-sodium acetate, pH 5.0, with one-tenth the volume of the enzyme solution (E_{280} 240) at 37°C for 3½ h. In some cases lower enzyme concentrations and shorter digestion times were used.

Ionophoresis. Details of basic procedures and general conditions for ionophoresis on cellulose acetate strips or DEAE-cellulose paper were as described by Sanger *et al.* (1965). When two-dimensional ionophoresis was carried out on DEAE-cellulose paper, the first ionophoresis was usually done in triethylamine carbonate buffer at pH 9.7. When dry, the piece, or strip, of DEAE-cellulose paper was stitched without difficulty to another sheet of DEAE-cellulose paper for ionophoresis in the second direction. [Sheets of DEAE-cellulose paper that had been sewn together, or to sheets of Whatman no. 1, no. 52, or AE-cellulose (aminoethyl-cellulose) paper, required particularly careful handling when wet.] Sheets of AE-cellulose paper had much better wet strength than DEAE-cellulose paper, and although some batchwise variation in this respect was found it was usually possible to conduct experiments with AE-cellulose paper in Michl

tanks in the usual way (Michl, 1951) at any pH, and support racks were not normally necessary. Sheets of ET-cellulose (epichlorhydrin triethanolamine-cellulose) paper, on the other hand, were particularly fragile when wet. Both AE-cellulose paper and ET-cellulose paper tended to distort appreciably more than DEAE-cellulose paper on drying. When ionophoresis was carried out in triethylamine carbonate buffer, a 5% solution at pH 9.7 was used in the buffer tanks, but it was necessary to add triethylamine to the 'white spirit' used as coolant in the tanks. The behaviour of nucleotides in this system was markedly dependent on the triethylamine content of the 'white spirit': increasing concentrations of triethylamine increased the movement of even quite large nucleotides. In general, a triethylamine concentration of 0.5–1.0% (v/v) proved the most useful. With the triethylamine carbonate system it was especially important to avoid overheating because the papers then developed a dry band very easily: voltages were restricted to a maximum of 25 V/cm.

Radioautography. Radioautographs were made on Ilford Industrial G X-ray film and developed in Phenisol developer, or on Kodak Blue Brand or Autoprocess Kodirex X-ray film which was developed in DX 80 developer (20°C for 4 min) or machine-processed.

Analyses of nucleotides. Details of the micro techniques used for these analyses, and for the elution of nucleotides from the ion-exchange papers with 30% (v/v) triethylamine carbonate solution were as described by Sanger *et al.* (1965).

(i) Digestion with snake venom phosphodiesterase (Razzell & Khorana, 1959). Complete digests for the determination of the composition of 5'-oligonucleotides were made by incubation of the nucleotide in a solution (10 μ l) of the enzyme (0.1mg/ml) in 0.02M-tris-HCl buffer, pH 8.5, at 37°C for at least 2 h and sometimes overnight. Some batches of the enzyme were contaminated with phosphatase, in which case it was preferable to use a low concentration of tris and a shorter digestion time. Studies of the effect of time, temperature and enzyme concentration showed that optimum conditions for partial digestion were an enzyme concentration of 0.01mg/ml and a digestion time of 20–40 min at room temperature (19°C). The enzyme solutions were diluted immediately before use from a stock solution (1mg/ml) in water.

(ii) Digestion with venom phosphodiesterase on paper. It was frequently desirable to determine the composition of the series of products from partial digestion of an oligonucleotide. Often the amount of radioactivity in each product was small, which meant that an exposure of nearly 2 weeks was necessary to detect the products, and it was then virtually impossible to elute and digest them further for compositional analysis. In such cases an analysis was obtained on some occasions by cutting from the DEAE-cellulose paper a strip on which the products of partial digestion had been separated electrophoretically and moistening this strip with a solution of venom phosphodiesterase (0.2mg/ml in 0.02M-tris, pH 8.5). The moist strip was then placed on a glass spiral rack and kept in a humid atmosphere at 37°C for several hours. It was dried and stitched to a sheet of Whatman no. 52 paper for ionophoresis (pH 3.5) in a direction at right-angles to that used for the separation of the partial digest.

(iii) Digestion with spleen phosphodiesterase (Bernardi & Bernardi, 1966). Complete digests for the determination

of the composition of 3'-oligonucleotides were made by incubation of the nucleotide in a solution (10 μ l) of the enzyme (0.5–1.0 mg/ml) in 0.1M-sodium acetate buffer, pH 5.0, at 37°C for 2 h. For partial digests, an enzyme concentration of 0.1 mg/ml was used at room temperature (23°C) for 30–60 min.

(iv) Digestion with phosphomonoesterase. For the determination of terminal nucleotides, the oligonucleotides were dissolved in 10 μ l of bacterial alkaline phosphatase (0.5 mg/ml) in 0.1M-NH₄HCO₃ and incubated at 37°C for about 1 h. The nucleotides were separated from the phosphatase by ionophoresis on Whatman no. 52 paper at pH 3.5, but a far preferable procedure is the inactivation of the phosphatase by treatment with EDTA at pH 2 (Dahlberg, 1968). Complete digestion of the nucleotides with venom or spleen phosphodiesterase then gave the identity of the terminal nucleotide by difference. In some cases semen phosphomonoesterase was used; the nucleotides were incubated with 10 μ l of the preparation in 0.1M-sodium acetate buffer, pH 5 (Wittenberg & Kornberg, 1953) at 37°C for 1 h and the enzyme was then inactivated by heating in boiling water for 5 min (when this is done, it is essential to seal the drawn-out end of the capillary tube first).

RESULTS AND DISCUSSION

Several types of digest of ³²P-labelled DNA have been fractionated by two-dimensional ionophoretic methods. Nucleotide 'maps' of the various digests are included here for reference, although analysis of digestion products was restricted to some of the endonuclease reactions. Of these, pancreatic deoxyribonuclease and *N. crassa* endonuclease gave very complex mixtures of oligonucleotides (and the products released by the two enzymes were very similar) and therefore these digests were used for the construction of nucleotide reference 'maps'.

[Abbreviation of nucleotides: since only deoxynucleotides are discussed in this paper, the usual prefix d has been omitted throughout. All the nucleotides referred to in the text, Figures and Tables have a 5'-phosphate (except for those in Fig. 4 and the relevant discussion, which have a 3'-phosphate) which has been omitted throughout.]

Problems in the analysis of nucleotide 'maps' of deoxyribonuclease digests, and the analysis of the nucleotides themselves, differ from those arising in equivalent studies with RNA (Sanger *et al.* 1965; Brownlee & Sanger, 1967) in the following respects. (a) The endonucleases for DNA generally lack base specificity so that a more complex mixture of nucleotides is produced (quite apart from the far greater complexity of the nucleic acid being digested). (b) One cannot deduce a terminus of an oligonucleotide from the base specificity of the nuclease used. (c) Another consequence of the lack of base specificity is that many, or all, of the possible isomers of a given oligonucleotide may be present in a nuclease digest with the result that many

of the nucleotides separated will still be heterogeneous (particularly with the larger nucleotides); this makes interpretation of analyses of partial digests of separated nucleotides difficult if not impossible in many cases. (d) The change in mobility on DEAE-cellulose paper of an oligonucleotide after removal of a T residue is similar to that resulting from the removal of a G residue, so that the position of these residues in a nucleotide cannot always be assigned simply on the basis of difference in mobility of two overlapping nucleotides on DEAE-cellulose paper at pH 2. (e) Analysis of oligodeoxynucleotides can only be effected enzymically (in work on this scale), with snake venom or spleen phosphodiesterase; the presence of even very small quantities of phosphatase in these enzyme preparations makes quantitative determination of mononucleotides in the phosphodiesterase digest less precise than in the case of an alkali digest of an oligoribonucleotide.

Nucleotide 'maps' of a Burton digest, a micrococcal nuclease digest, and a pig liver nuclease digest of DNA from *E. coli* are shown in Plate 1(ai), (a ii) and (a iii). These were made from small quantities of DNA (about 10–50 μ g) by two-dimensional ionophoresis on cellulose acetate (pH 3.5) and DEAE-cellulose paper (pH 1.9). Some fast-running nucleotides present in the digests may have been lost off the end of the paper. The deoxyribonuclease activity in the pig liver nuclease appeared to have an absolute requirement for Mg²⁺ ions, but the preparation hydrolysed RNA whether Mg²⁺ ions were present or not. The nucleotide 'map' (Plate 1a iii) showed that a complex range of products was released from DNA by the pig liver nuclease, suggesting that this enzyme is an endonuclease with little or no obvious base specificity (cf. results obtained with pancreatic deoxyribonuclease; Plate 3). Burton digests have been studied in more detail with these and similar techniques by Szekeley & Sanger (1969) and by E. M. Southern (unpublished work). None of the nucleotides separated in these three experiments was analysed but several of them may be identified by comparison with the results given in Plate 3 and Table 1. The results of experiments with spleen endonuclease are shown in Plate 1(b). Two of the 'maps' (Plate 1bi and 1b ii) were obtained with a digest at a low concentration of the highly purified enzyme (Bernardi & Griffe, 1964), and the third (Plate 1b iii, a more complex 'map'), with a higher concentration of the commercial enzyme preparation. It is apparent from Plate 1(bi) and 1(b ii) that a considerable proportion of the radioactivity remained as large oligonucleotides, in accord with the findings of Bernardi & Griffe (1964), but the presence of several small nucleotides was unexpected and the composition of these nucleotides (Plate 1bi) indicates that the enzyme

lacks an absolute specificity for G residues (although this could apply to the large fragments that were not analysed).

Digests of *E. coli* DNA with pancreatic deoxyribonuclease (deoxyribonuclease I) or *N. crassa* endonuclease gave very similar nucleotide 'maps'. Some of the nucleotides separated from a *N. crassa* endonuclease digest by two-dimensional ionophoresis on cellulose acetate (pH 3.5) and DEAE-cellulose paper (pH 1.9) were analysed and the composition of these nucleotides is shown in Plate 2(b). This type of nucleotide 'map', and these analyses, were used to compare DNA from *E. coli* with that from calf thymus nuclei with the aid of neutron activation of the paper after ionophoresis (Murray & Offord, 1966). Sequence analysis was not undertaken on nucleotides from the *N. crassa* endonuclease digest. The composition of the nucleotides in the *N. crassa* endonuclease digest show that here also the enzyme does not have an absolute specificity, for G residues (of the nucleotides analysed, 56% are devoid of G), but this certainly does not argue against a preference, or early specificity for G residues (Linn & Lehman, 1965b) and indeed the proportion of guanylic acid in the digest [nucleotide no. 4 (Plate 2bii) is a mixture of (CT) and G] is very much higher than in the case of a pancreatic deoxyribonuclease digest [where (CT) accounts for nearly all of the nucleotide in the corresponding 'map' position].

The qualitative similarity of pancreatic deoxyribonuclease and *N. crassa* endonuclease digests of *E. coli* DNA is apparent in the nucleotide 'maps' shown in Plate 2(a), but some quantitative differences are apparent. Nucleotide 'maps' obtained with a given DNA species and a given nuclease proved to be highly reproducible with several independent DNA preparations and different batches of enzymes, not only in the number and relative positions of spots on the 'maps', but also in the relative intensities of the spots. Pancreatic deoxyribonuclease digests were used in preference to *N. crassa* endonuclease digests for more detailed work on nucleotide maps because the former enzyme is more readily available. Resolution of the more complex area of the slower-moving nucleotides can obviously be improved by extending the duration of ionophoresis, as shown in Plate 3. The three samples analysed were taken from a single 12 h digest that had been stored frozen; the apparent loss of some of the very slow-moving nucleotides is not due to depurination during ionophoresis (which may be sometimes manifest as pronounced streaking if the ionophoresis tanks are allowed to become overheated), but simply to storage of the digest, i.e. to further, slower, action of the nuclease. Use of these 'maps' of pancreatic deoxyribonuclease digests as 'fingerprints' of DNA was explored with DNA from *E. coli* and its bacteriophage λ and

fd, for encouraging differences had been demonstrated between DNA from *E. coli* and calf thymus nuclei (Murray & Offord, 1966) and, similarly, ribonuclease digests had revealed a difference between 16S and 23S rRNA from *E. coli* (Sanger *et al.* 1965). The results, however, showed that the nucleotide 'maps' made from the three DNA preparations were very similar (Plate 4). At first sight, the 'map' of the digest of fd DNA appears different from the other two, but this is more apparent than real and results partly from relatively prolonged ionophoresis in the second dimension (i.e. on DEAE-cellulose paper; also the pH of the buffer solution probably differed slightly from the other two experiments), and partly because the proportion of larger digestion products (those in the heavy arc near the top of the maps of the λ and *E. coli* digests) is much smaller, probably due to a more efficient digestion of the simpler, single-stranded DNA molecule by the nuclease. Different extents of digestion may well result in differing proportions of smaller nucleotides, so that differences in relative abundance of certain nucleotides (and such differences can be emphasized in photographs when compared with the original radioautographs) should be treated with care. Thus, the relatively large proportion of AAA and AAAA (identified by reference to Plate 3, and Table 1) in the digest of fd DNA, for example, may or may not be of special significance. The nucleotides indicated by the arrow in the lower right of the 'map' of the digest of λ DNA have been found in corresponding digests of λ DNA and *E. coli* DNA (see the boxed area in the lower right of Plate 3bi), but that area of the paper was not included when the other radioautographs in Plate 4 were made. When comparing these DNA digests, one might note that the nearest-neighbour nucleotide frequencies of DNA from *E. coli* and bacteriophage λ are essentially the same (Josse, Kaiser & Kornberg, 1961).

Pancreatic deoxyribonuclease degrades DNA to 5'-nucleotides (Sinsheimer & Koerner, 1952), so the composition of nucleotides from these digests was determined by hydrolysis to 5'-mononucleotides with snake venom phosphodiesterase. In most cases, the composition of an oligonucleotide could be assessed from the relative intensities of its mononucleotide spots on radioautographs, but usually this was confirmed by cutting out appropriate areas of paper and counting them for radioactivity. The sequence of bases in an oligonucleotide was deduced as far as possible from the products of a partial digest with venom phosphodiesterase. This procedure for sequence determination, which was exploited by Holley, Madison & Zamir (1964) in the determination of the nucleotide sequence of alanine tRNA from yeast, rests upon the fractionation and identification of the components

of an overlapping series of nucleotides; for example, a partial digest of the 5'-tetranucleotide WXYZ with venom phosphodiesterase would contain WXYZ, WXY, WX, and the four mononucleotides. The basis of the method used for identification of the overlapping nucleotides (Sanger *et al.* 1965) is that the degree of ionophoretic separation (on DEAE-cellulose paper) between individual nucleotides in an overlapping series depends on the base in the mononucleotide by which two immediately overlapping nucleotides differ. Sanger *et al.* (1965) expressed this difference in mobility by the ratio, M , of the difference between the distance migrated by two overlapping oligonucleotides to the distance moved from the origin by the larger (i.e. slower) of the two. The M values found for deoxynucleotides (on DEAE-cellulose paper at pH 2) are similar to those observed by Sanger *et al.* (1965) with ribonucleotides: loss of a 3'-deoxycytidylate residue gives a value between 0.08 and about 0.25, loss of a 3'-deoxyadenylate residue gives a value in the range 0.4 to 1.0 (but usually about 0.5), and loss of a 3'-deoxyguanylate or 3'-thymidylate residue gives a much more pronounced effect with M values in the range 1.4–3.0 (but usually about 2, and G and T are indistinguishable). Some examples of these experiments are shown in Plate 5(a). Interpretation of patterns is frequently complicated by heterogeneity of the nucleotide under analysis; it may be slightly contaminated with a neighbouring nucleotide from the 'map', or it may contain more than one isomer. Nevertheless, the identity of at least the principal component of a mixture may often be deduced with reasonable confidence from consideration of mobility shifts (i.e. M values), mobilities of the new nucleotides produced by partial digestion, and the composition of the original nucleotide (and, sometimes, the mononucleotides released, although identification of these on DEAE-cellulose paper is often equivocal). Nucleotide 114 (Plate 5a and Table 1), for example, has the composition CAGT and gives two successive products with M values of 0.44 and 0.13 (corresponding to the loss of A and C residues) and a nucleotide whose mobility equals that of the dinucleotide (GT). Its principal constituent is thus (GT)CA, but the trinucleotide (AT)C and the dinucleotides (AT) and (CG) also occur in the partial digest [(CT) cannot be distinguished here from G] so that other isomers are undoubtedly present as well. Apart from the rather infrequent case where the two bases nearest the 5'-terminus are identical, the complete base sequence of an oligonucleotide cannot be obtained unambiguously by this method, but the identity of the 5'-terminus was established for a number of the nucleotides from the change in base composition of the nucleotide after treatment with phosphatase. Although it is very desirable that sequences deduced

in this way be confirmed by determination of the composition of each of the products in a partial digest, this was possible in rather few cases, because in these complex mixtures each original nucleotide contained only a very small proportion of the radioactivity present in the DNA and this was then divided further between the products of digestion. If a given nucleotide in a partial digest was visible on radioautography only after a few days' exposure, it was usually futile to elute this nucleotide for further analysis since its radioactivity would then have been divided into even smaller units. The capacity of the cellulose acetate strips used for the first ionophoresis limits the amount of a nuclease digest that may be fractionated in a given experiment. In some instances, it proved possible to determine the composition of each product in a partial digest by separating the products ionophoretically on DEAE-cellulose paper in the usual way and by then digesting them completely to mononucleotides by treatment of the whole strip of paper with venom phosphodiesterase. The strip of paper was then sewn to another sheet of paper for ionophoresis at right-angles to the first direction to separate the mononucleotides formed by the second treatment with venom phosphodiesterase. The procedure was not used widely, however, for it is expensive and if used on many samples it becomes time-consuming and monopolizes ionophoresis and radioautography facilities.

Table 1 contains the composition and, as far as possible, the sequence of nucleotides eluted from nucleotide 'maps' (Plate 3b) made with pancreatic deoxyribonuclease digests of *E. coli* DNA. The number of nucleotides unequivocally identified is disappointingly small. This is because a non-specific endonuclease digest of a large nucleic acid will usually contain several if not all of the possible isomers of small oligonucleotides. A series of nucleotide 'maps' of the type shown in Plate 3(bi) was made with solutions of *E. coli* DNA that had been digested at various concentrations of pancreatic deoxyribonuclease for various periods of time at 20°C before addition of excess of EDTA to stop the reaction. Digests made with 1 or 0.1 mg of enzyme/ml (0.35 mg of DNA/ml) for periods varying from 2 min to 12 h gave virtually identical nucleotide 'maps', although the proportion of the larger oligonucleotides (i.e. those remaining as a streak at the origin on ionophoresis on DEAE-cellulose paper) was somewhat greater in 2 min and 10 min digests at the lower enzyme concentration than in the other digests. Only traces of the oligonucleotides resolved on nucleotide 'maps' (as in Plate 3b) were found in 2 min and 10 min digests at an enzyme concentration of 0.01 mg/ml, but all of the oligonucleotides were detected in both digests. None of the small oligonucleotides was found in a 60 min

Table 1. *Composition and sequence of some of the nucleotides (from a pancreatic deoxyribonuclease digest of E. coli DNA) shown in the nucleotide 'map' in Plate 3(b)*

Nucleotide	Composition	Sequence	Nucleotide	Composition	Sequence
4	AT ₁	(AT)TT	123	A ₂ G	(AG)A
5	AGT ₂		124	CA ₂ T	(AT)CA
10	GT ₂		126	AG ₂	GGA
16	A ₂ GT	(GT)AA	127	A ₂ G	(AG)A
17	CAT ₂	(AT)CT	128	CA ₂ T	(AT)AC
21	AT ₂	TTA	135	A	AAAA
24	C ₂ GT	(GT)CC	137	CA ₂	
25	CT ₂	TTC	138	CA ₂	
26	CGT	(GT)C	139	C ₂ A	
27	CG ₂	GGC	141	C ₂ A	
28	G	GG	142	CAG	(AG)C
29	CGT	(GT)C	144	CAG	(AG)C
30	T	TT	145	CAG	(CG)A
31	GT	TG	146	CAT	(CT)A
32	GT	GT	147	AG	(AG)
34	CA ₂ G	(AG)CA	148	AG	(AG)
37	A ₂ T	(AT)A	150	CAT	(CT)A
39	A ₂ T	(TA)A	151	A	AAA
40	A ₂ G	(AG)A	152	C ₂ AT	
41	A ₂ T	(AT)A	153	C ₂ T	
54	CA ₂ T	(CAT)A	154	C ₂ G	(CG)C
61	CA ₂ T	(AT)AC	155	C ₂ G	(CG)C
66	AT ₂	(AT)T	156	AT	(AT)
69	CA ₂ T	(AT)AC	157	AT	(AT)
84	A ₂ G	(AG)AA	158	C ₂ T	TCC
90	AGT	(GT)A	159	C ₂ G	GCC
		(AT)G	160	CG	(CG)
96	CGT	(GT)C	161	CG	(CG)
102	CT ₂	TTCT	163	CA ₂	
109	A ₂ GT	(AT)GA	165	CT	TC
112	CAT ₂		166	A	AA
113	CAGT	(CAG)T	167	T	T
114	CAGT	(GT)CA	168	CTG	CT
115	AGT	(AT)G			G
117	A ₂ T	(AT)AA	205	C ₂ A	ACC
120	C ₂ GT		206	C ₂ A	
121	CG ₂	(CG)G	207	C ₂ A	CAC

digest with 0.001mg of enzyme/ml. No conclusions could be drawn about base specificity of pancreatic deoxyribonuclease in either early or limit reactions, but some oligonucleotides appear more resistant than others to the enzyme, for example, the tetranucleotides AAAA and TTTT (see Table 2) occur in the reaction products, but the tetranucleotides GGGG and CCCC were not found. The ionophoretic behaviour of the deoxynucleotides in this two-dimensional system is similar to that of corresponding ribonucleotides (Sanger *et al.* 1965), and shows the same dependence on base composition. Since these ionophoretic properties are pH-dependent, a pancreatic deoxyribonuclease digest of *E. coli* DNA was analysed by two-dimensional ionophoresis on cellulose acetate (pH 3.5) followed by DEAE-cellulose paper in 7% (w/v) formic acid, which has proved so valuable for the separation of many

ribonucleotides. This produced some changes in the nucleotide 'map', and although resolution was improved in the central area to the immediate and upper right of the dinucleotides TT and GT (i.e. nucleotides 30 and 32 in Plate 3b), resolution in other regions of the 'map' was distinctly worse than at pH 1.9. Two-dimensional ionophoresis on cellulose acetate (pH 3.5) and DEAE-cellulose paper in triethylamine carbonate buffer (pH 9.7) was also used, but here the behaviour of nucleotides was strongly dependent on the triethylamine content of the 'white spirit' in the ionophoresis tank; Plate 5(b) shows a nucleotide 'map' of a pancreatic deoxyribonuclease digest of *E. coli* DNA made with this system.

One factor limiting the use of two-dimensional ionophoresis on cellulose acetate and DEAE-cellulose paper is the low capacity of cellulose

Table 2. *Composition and sequence of nucleotides (from a pancreatic deoxyribonuclease digest of E. coli DNA) from the nucleotide 'map' shown in Plate 6(aii)*

Nucleotide no.	Composition	Sequence	Nucleotide no.	Composition	Sequence
1	GT ₃	(GT)TT	76	GT	(GT)
2	T	TTTT	78	CG ₂	(CG)G
3	CT		79	CG ₂	CGG
4	CAGT				GCG
5	AGT ₂		81	GT	(GT)
6	AT ₃	(AT)TT	82	CA	
		TTTA	84	CA	
		TTAT	85	CA ₂ T	(AT)AC
7	T	TTT	86	CA ₂ T	
8	C ₂ AG ₃ T		87	CA ₂ T	
9	CAGT		90	CA ₂ G	
10	CAGT		91	A ₂ G	(GA)A
11	CA ₂ G ₂ T		92	A ₂ T	AAT
15	CG ₂ T ₂				(AT)A
16	CT ₃	TTCT	94	CA ₂ G	
		(CT)TT	95	CA ₂ T	(AT)CA
17	GT ₂	TTG	96	A ₂ G	
18	GT ₂	(GT)T	97	A ₂ T	AAT
19	GT ₂	(GT)T	100	CA ₂ T	
21	CA		101	A ₂ G	
22	CA ₂ GT		102	C ₂ AT	
23	CA ₂ GT		103	A ₂ T	(AT)A
24	CA ₂ GT		104	CA	
25	A ₂ GT		105	C ₂ A	
26	AT	(AT)TA	106	C ₂ AG	(CAG)C
		TTAA	107	C ₂ AT	(CT)AC
27	AT	TTAA	108	C ₂ AT	(AT)CC
28	AT		109	CAG	(AG)C
29	AT		114	C ₂ AT	
30	CAGT		115	C ₂ AT	
34	CAGT		116	A	
36	CAT ₂	(AT)TC	117	CAT	
38	CAGT	(AT)CG	118	CAG	(CA)G
39	CAGT	(GT)CA	119	CA ₂ T ₂	
40	AG ₂	GGA	120	CA	
41	AGT	(AT)G	121	CAG	(CG)A
42	AGT	(AG)T	122	CAG	
43	AGT		123	CAG	(CG)A
44	AT ₂	(AT)T	124	CAT	
45	AT ₂	(AT)T	125	CAT	
51	A ₂ GT		126	AG	(AG)
52	CAT		128	AG	(AG)
53	CT		129	C	
54	CT		130	C ₃ T	(CT)CC
55	A ₃ T	(AT)AA	131	C ₂ G	
56	CT ₂		133	CA ₂	
61	CAGT		134	C ₂ G	
62	C ₂ GT	(GT)CC	135	A	AAA
63	CG	(CG)GC	136	C ₃ GT	(CG)C
64	CT	(CT)TC			(CT)C
	CGT	(CG)T	138	AT	(AT)
65	CGT	(GC)T	139	A	AA
66	CGT	(GT)C	140	A	AA
		(CT)G	141	A	
67	CT ₂	(CT)T	142	G	
68	CT ₂	CTT	143	CT	(CT)
		TCT	144	CT	(CT)
69	C ₃ G ₂		145	T	TT
71	C ₂ GT	(CG)CT	146	CT ₂	TTT
		(CT)CG	147	C ₂ T	
72	CG	(CG)CG	148	C ₂ T	(TC)C
73	CG ₂	(CG)G	151	C ₂ G	(CG)C
74	CGT	(CT)G	152	C ₂ G	(CG)C
75	G	GG			

Table 3. *Composition and sequence of nucleotides (from pancreatic deoxyribonuclease digests of E. coli DNA) from the nucleotide 'maps' shown in Plate 7(b)*

Nucleotides 1–50 are from 'map' (bii), nucleotides 51–110 are from 'map' (bi), and nucleotides 111–124 are from 'map' (biii).

Nucleotide no.	Composition	Sequence	Nucleotide no.	Composition	Sequence
1	T	TTTT	62	T	TTT
2	GT ₃		63	CAGT	(AT)CG
3	AT ₃	(AT)TT			(GT)CA
4	GT	(G ₂ T ₂)			(AG)CT
5	CGT ₂		66	AGT	(GT)A
6	CAGT	(GT)CA	72	AT ₂	(AT)T
9	C ₂ AG		73	G	GG
10	GT ₂		74	CA ₂ T	(AA)C
11	GT ₂		77	CGT	(GT)C
12	CT ₃	(CT)TT	78	CGT	(GT)C
		TTCT	80	A ₂ G	
16	C ₂ AG		84	GT	(GT)
17	C ₂ AG		85	CT ₂	TTC
18	T	TTT	86	A ₂ T	(AT)A
19	AGT	(GT)A	87	A ₂ T	
20	AGT		89	CAG	
21	AT ₂	(AT)T	90	GT	(GT)
22	AT ₂	TTA	91	T	TT
		(AT)T	92	A ₂ G	AA + (AG)
23	G	GG	93	CAT	(AT)C
24	CGT	(GT)C			(CT)A
26	A ₂ T	(AT)A	94	C ₃ G	(CG)CC
28	CAG		95	C ₂ G	(CG)C
29	CAG		96	AT	(AT)
31	GT	(GT)	97	CA	(C ₂ A ₂)
32	CT ₂	(CT)T	98	C ₂ T	(CT)C
33	CAT	(CT)A	99	CG	(CG)
35	C ₂ G	(CG)C	100	CA ₂	
36	CA ₂		101	A	AA
37	C ₃ A		102	CT	(CT)
38	GT	(GT)	103	C ₃ A	
39	A ₂ G	(AG)A	104	C ₃ A	
40	C ₂ A		105	C ₂ A	
41	C ₂ A		106	C ₂ A	
42	C ₄ GT	(CG)C	107	CA	(CA)
		(CT)C	108	CA	(CA)
43	A	AA	109	CA	(CA)
44	AT	(AT)	110	C	
45	CG	(CG)	111	CA	(CA)
46	T	TT	112	CG	(CG)
47	AT	(AT)	113	CAT	(AT)C
48	CA	(CA)			(CT)A
49	CA	(CA)	114	AG	(AG)
50	CT	(CT)	115	CT ₂	(CT)T
51	T	TTTT			TTC
52	GT ₃	(GT)TT	116	GT ₂	(GT)T
54	AT ₃	(AT)TT	117	T	TTT
55	AGT ₂	(AG)TT	118	CT	(CT)
		TTGA	119	CT	(CT)
		(AT)GT	120	AT	(AT)
		(AT)TG	121	GT	(GT)
57	G ₂ T	GGT	122	T	TT
		(GT)G	123	T	T
58	CGT ₂	(GT)CT	124	T	T
59	GT ₂	(GT)T	125	P ₁	
60	CT ₃	(CT)TT			

acetate. The capacity of DEAE-cellulose paper for nucleotides is very much higher, indicating that a two-dimensional system on DEAE-cellulose paper only (triethylamine carbonate buffer, pH 9.7, in the first dimension, followed by formic acid-acetic acid buffer, pH 1.9, in the second dimension) would be useful, particularly for the analysis of nucleotides in complex reaction mixtures. The nucleotide 'map' illustrated in Plate 6(ai) shows that very high resolution of nucleotides is attainable by this method and in one experiment 1 mg of a digest of DNA was used without appreciable loss of resolution. Analyses of nucleotides from this 'map' (Plate 6a(ii)) are given in Table 2 and show a rather different spatial distribution of the nucleotides from that obtained on 'maps' made with the cellulose acetate-DEAE-cellulose paper method, where nucleotides tend to be aligned in a manner predictable on the basis of the 'graticule rules' of Sanger *et al.* (1965). In triethylamine carbonate buffer, pH 9.7, the ionophoretic mobility of nucleotides on DEAE-cellulose paper tends to be inversely proportional to their size (as at low pH). It is also affected by the nature of the bases present in the oligonucleotides, but less dramatically than at low pH. A marked difference from the behaviour at low pH is that mobility often appears to be more sensitive to base sequence in the nucleotide, thus giving particularly good resolution of isomers in some cases, for example (Plate 6a(ii)), nucleotides 17, 18 and 19, which are isomers of GT₂, nucleotides 41, 42 and 43, which are isomers of AGT, and nucleotides 107, 108, 114 and 115, which are isomers of C₂AT. [It should be noted that the numbers of nucleotides given in Plate 3(b) and Table 1 do not correspond to those in Plate 6(a(ii)) and Table 2. The numbers in Plate 6(a(ii)) and Table 2 have been italicized to emphasize this difference.] This degree of separation of isomers was quite unexpected and although there is some variation between experiments, it is generally highly reproducible. Resolution in some areas of the map may be improved further by carrying out the second ionophoresis in 7% (w/v) formic acid.

The various two-dimensional ionophoretic methods with DEAE-cellulose paper supplement each other quite usefully, but many of the faster moving nucleotides are not readily separable on DEAE-cellulose paper at pH 1.9 (e.g. CT and G move very close together), and nucleotides rich in C are often poorly resolved. This prompted an examination of other modified papers of which ECTEOLA-cellulose (ET-cellulose paper) and aminoethyl-cellulose (AE-cellulose paper) appeared the most promising for two-dimensional ionophoresis in conjunction with cellulose acetate. Migration on ET-cellulose paper was in all cases very slow (Plate 6b) and the poor handling properties of this paper (the batch used was more delicate than DEAE-cellulose paper) did

not encourage perseverance with it. AE-cellulose paper, however, was much more useful, for a direct comparison with DEAE-cellulose paper produced very compact spots, a high degree of resolution (Plate 7a) and a lower mobility for nucleotides containing C. Two-dimensional ionophoreses of pancreatic deoxyribonuclease digests of *E. coli* DNA on cellulose acetate and AE-cellulose paper were then made at various pH values. Three of the nucleotide 'maps' are shown in Plate 7(b) and the identity or composition of several of the nucleotides is included in this Plate and in Table 3. [It should be noted that the numbers of nucleotides given in Plate 7(b) and Table 3 do not correspond with those in Plates 3(b) and 6(a(ii)) and Tables 1 and 2. Similarly, a given

Table 4. *Mobilities with respect to the blue dye (Xylene Cyanol FF) of some 5'-nucleotides on AE-cellulose and DEAE-cellulose papers*

Nucleotide	AE-cellulose paper		DEAE-cellulose paper
	pH 2.0	pH 3.5	pH 2.0
C	1.38	1.28	2.16*
A		1.07	2.08
G		0.87	1.98
T	1.53	1.51	2.54
CC		0.92	
(CA)	1.28	0.75	
(CG)	0.93	0.61	1.78
(CT)	1.22	0.90	1.98
AA	1.04	0.58	
(AG)	0.67	0.49	1.47
(AT)	0.87	0.65	1.59
GG	0.34	0.24	0.79
(GT)	0.49	0.50	0.93
TT	0.62	0.82	0.90
(CCA)	1.15	0.48	
(CAA)	0.93	0.32	
(CTT)	0.47	0.38	0.78
(CGT)	0.39	0.30	0.88
(AAT)	0.48	0.27	1.05
(ATT)		0.29	0.50
(GGT)	0.14	0.11	0.24
(GTT)	0.19	0.17	0.27
TTT	0.24	0.32	0.27

* The values for the mononucleotides, particularly C, are not reliable. It is often difficult to resolve and identify C and A with certainty, and occasionally C is found just ahead (i.e. faster-moving) of T. Temperature changes or small fluctuations in pH are likely causes of these problems.

EXPLANATION OF PLATE I

(a) Nucleotide 'maps' of (i) Burton digest, (ii) micrococcal digest and (iii) pig liver nuclease digest of *E. coli* DNA. The digests were fractionated by ionophoresis on cellulose acetate, pH 3.5 (3kV, 1h) followed by ionophoresis on a standard size sheet (57 cm) of DEAE cellulose paper, pH 2 (2kV, 2.5h). The spots drawn in ink show the position of the blue (Xylene Cyanol FF) marker. (b) Nucleotide 'maps' of spleen endonuclease digests of *E. coli* DNA. (bi) The digest was fractionated by ionophoresis as in (a), but with a longer sheet (85 cm) of DEAE-cellulose paper. Composition of the 3'-nucleotides was determined by digestion with spleen phosphodiesterase. (bii) The same digest as in (bi), but fractionated by two-dimensional ionophoresis on DEAE-cellulose paper at pH 2 and pH 9.7 (as in Plate 6a). (biii) A later experiment with a high concentration of a commercial enzyme preparation. The digest was analysed in the same way as that shown in (bi).

EXPLANATION OF PLATE 2

Nucleotide 'maps' of digests of *E. coli* DNA, with *N. crassa* endonuclease (ai and bi) or pancreatic-deoxyribonuclease (aai) fractionated as described for Plate 1(a); (bii) is a line drawing of the radioautograph (bi). The numbers given to the nucleotides do not correspond to those in any of the other Plates. The compositions shown were obtained by digestion of the nucleotides, after elution from the DEAE-cellulose paper, with venom phosphodiesterase and ionophoretic analysis of the 5'-mononucleotides.

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EXPLANATION OF PLATE 4

Nucleotide 'maps' of pancreatic deoxyribonuclease digests of DNA from: (a) *E. coli*; (b) bacteriophage λ ; (c) bacteriophage fd. Ionophoresis of the digests on cellulose acetate, pH 3.5, was followed by ionophoresis in the second dimension on DEAE-cellulose paper, pH 2.

EXPLANATION OF PLATE 5

(a) Partial digests with snake-venom phosphodiesterase of some nucleotides eluted from DEAE-cellulose paper after two-dimensional ionophoretic fractionation of a pancreatic deoxyribonuclease digest of *E. coli* DNA shown in Plate 3. The digests were examined, in parallel with a reference sample of the undigested nucleotide, by ionophoresis on DEAE-cellulose paper at pH 2. The zones ringed by a dotted line denote the position of the blue dye; (a_{ii}) is a line drawing of the radioautograph (a_i). (b) Two-dimensional separation of a pancreatic deoxyribonuclease digest of *E. coli* DNA. Ionophoresis on cellulose acetate, pH 3.5, was followed by ionophoresis on DEAE-cellulose paper in 5% triethylamine carbonate buffer at pH 9.7.

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EXPLANATION OF PLATE 6

Nucleotide 'map' of a pancreatic deoxyribonuclease digest of *E. coli* DNA after two-dimensional ionophoresis on (a) DEAE-cellulose paper, first in 5% triethylamine carbonate buffer, pH 9.7, second in formic acid-acetic acid solution, pH 2, (b) cellulose acetate, pH 3.5, followed by ET-cellulose paper, pH 2. The dotted zones denote the positions of blue dye markers; (aii) is a line drawing of the radioautograph (ai). Analyses of some of these nucleotides are given in Table 3.

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EXPLANATION OF PLATE 7

Comparative nucleotide 'maps' of the same pancreatic deoxyribonuclease digest of *E. coli* DNA. In each case cellulose acetate, pH 3.5, was used for ionophoresis in the first dimension but was followed by ionophoresis in the second dimension on: (ai) DEAE-cellulose paper, pH 1.9; (aii and bi) AE-cellulose paper, pH 1.9; (aiii and bii) AE-cellulose paper, pH 3.5; (biii) AE-cellulose paper, pH 6.5; (b) shows tracings of radioautographs. Analyses of some of these nucleotides are given in Table 3.

nucleotide carries a different number in Plate 7(bi), 7(bii) and 7(biii).] In contrast with DEAE-cellulose paper, the results obtained with AE-cellulose paper in triethylamine carbonate buffer at pH 9.7 were extremely poor, for all the nucleotides appeared to move very fast indeed and were located only as diffuse streaks, hence two-dimensional separations on AE-cellulose paper only were not pursued. The change in mobility of nucleotides on AE-cellulose paper attending a relatively small change in pH (from 2 to 3.5) is quite large and is particularly dramatic in the dinucleotides. Normally at pH 3.5 only mononucleotides move ahead of the blue marker (mobilities of some nucleotides with respect to the blue dye are given in Table 4), but at pH 2 some di- and tri-nucleotides move just ahead of this dye. The retarding effect on mobility of size of nucleotides is greater on AE-cellulose paper than on DEAE-cellulose paper. This is particularly apparent, and useful, with C residues, for the retardation attending progressive addition of residues is very large at pH 3.5. Another noteworthy difference between the papers is that nucleotides containing G residues move appreciably more slowly at either pH on AE-cellulose paper than the corresponding nucleotide with T substituted for G. These features of AE-cellulose paper, together with its high resolving power, good handling properties, and relatively low cost, make it a useful complement to DEAE-cellulose paper. One does, however, find some variation between batches in both handling properties and ionophoretic mobility of nucleotides with this paper.

The results presented in this paper show that the nucleotide 'mapping' procedure developed for ribonucleotides (Sanger *et al.* 1965) is, as one would expect (Murray & Offord, 1966), generally applicable to deoxyoligonucleotides. There seems to be little or no tendency to depurination of deoxynucleotides during ionophoresis on ion-exchange paper at the low pH values (about 2) normally used, provided that excessive heating is avoided. Two-dimensional ionophoresis systems involving cellulose acetate and AE-cellulose paper, or DEAE-cellulose paper at two different pH values proved useful additions to the cellulose acetate-DEAE-cellulose paper methods. These systems have been used to fractionate complex mixtures of nucleotides produced by the action of pancreatic deoxyribonuclease on *E. coli* DNA and several of the nucleotides have been identified. Nucleotide 'maps' based on these results should be useful for the analysis of complex nucleotide mixtures for comparative work, for sequence studies on larger oligodeoxynucleotides, and for studies of the base specificity of deoxyribonucleases. The 'maps' are of particular value in sequence analysis of specific regions of DNA molecules when applied to terminally labelled preparations (K. Murray, unpublished work).

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