STUDIES ON THE SPECIFICITY OF PANCREATIC DEOXYRIBONUCLEASE (DN'ase I)

by

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SUMMARY

The kinetic and specificity properties of a highly purified fraction of pancreatic DN'ase (DN'ase A) were re-evaluated in the presence of most divalent metal cations. The enzyme was stabilized by inhibition of proteolytic contaminants with FDP and purified by phosphocellulose chromatography.

A column chromatographic method was developed for the fractionation of oligonucleotides according to chain length up to the pentadecanucleotide level. Methods of fractionation according to base composition were developed for nucleosides and nucleotides, all possible non-isomeric dinucleotides and 14 of the 20 possible non-isomeric trinucleotides.

The specificity of the enzyme was studied by analysis of the 5' or 3' termini of the oligonucleotides released during the digestion. DN'ase A was shown to change its specificity of cleavage during the several stages of native DNA hydrolysis when activated by cations such as Mg^{++}, Mn^{++}, Ni^{++}, Sr^{++} and Zn^{++}. The dpA-pN bond has been found to be highly resistant during the advanced phases of hydrolysis. Mono and dinucleotides produced by extensive hydrolysis of short oligonucleotides do not represent the enzyme's specificity.

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DNA - Deoxyribonucleic acid

RNA - Ribonucleic acid

tRNA - Transfer RNA

DN'ase I - Pancreatic deoxyribonuclease

DN'ase A - A purified fraction of DN'ase I

RN'ase - Ribonuclease

N - Purine or pyrimidine base: adenine (A), cytosine (C), guanine (G), thymine (T), uracil (U)

Py, Pu - Pyrimidine, purine

dN - Deoxyribonucleoside: dA, dC, dG, dT

d-pN - 5' phosphomononucleoside (5' mononucleotide)

d-Np - 3' phosphomononucleoside (3' mononucleotide)

d-pN-pN - 3'-hydroxy-5'-phosphomonoester internucleotide bond

N_xP_z - Oligonucleotide containing x bases and z phosphates

poly dX:dZ - Double stranded homopolymer with poly dX in one strand and poly dZ on the other

poly dXZ - Deoxyribopolymer with the nucleotides X and Z arranged in alternating sequence

Pi, Pt - Inorganic phosphorus, total phosphorus

A_{260} - Absorbance at 260 nm

$E^{0.1\%}_{1\ cm}$ - Absorbance of a 0.1% solution in a 1 cm light path

S.E.M. - Standard error of the mean

rpm - Revolutions per minute

cpm	- Counts per minute
DEAE-cellulose	- 0-(diethylaminoethyl)-cellulose
DEAE-Sephadex	- 0-(diethylaminoethyl)-Sephadex
CM-cellulose	- Carboxymethyl-cellulose
Tm	- Temperature at the midpoint of a thermal hyperchromic transition
TEAB	- Triethylammonium bicarbonate
FDP	- Diisopropylphosphorofluoridate
Tris	- Tris (hydroxymethyl)-aminomethane
Isostich	- Oligonucleotides of identical chain length, disregarding their base composition (246)
EDTA	- Ethylendiaminotetraacetate

CHAPTER 1

INTRODUCTION

1.1 Studies on the primary structure of DNA

The primary sequence of nucleic acids is intensively being investigated today for correlation with their biological properties.

The primary structure of several RNA molecules was elucidated due to the availability of specific RN'ases (1, 4), methods for the chemical modification of certain RNA bases affecting enzyme specificity (3, 101) and the occurrence and purification of relatively small molecules such as tRNA (1, 2) and 5S RNA (3). Furthermore the sequence of oligonucleotides produced by complete digestion with pancreatic RN'ase and T_1 RN'ase (1, 4), the construction of longer oligonucleotide sequences from the overlaps provided by minor bases or unique sequenced oligonucleotides and the availability of new methods of fractionation (5-9) provided the tools for the complete analysis of these molecules.

Studies on the sequence of segments of higher molecular weight RNA have been approached in a similar way. This has led to the sequence of the first 175 nucleotides from the 5' terminus of Qß RNA (10), the sequence of fragments of R 17 and Qß RNA which include the sites for the binding of ribosomes and the initiation of phage protein synthesis (11, 12) and the sequence of 44 nucleotides of R 17 RNA, including the site for the termination of translation of the phage coat protein (13).

The complete sequence of DNA molecules or particular DNA segments is still awaiting the tools which proved so successful in the analysis of RNA.

Progress is being made in the isolation of small functional units of DNA (14-16), in the chemical characterization of DNA (17-21), and in the isolation of enzymes which specifically cleave the DNA molecule (22-24).

The present study was initiated on the basis of suggestive evidence from several laboratories (25-27) that the specificity of DN'ase I could be modified according to the activating ion used. In this case, the enzyme could be used for the preparation of different types of DNA fragments. Methods for the fractionation and analysis of DN'ase I digests were developed during the investigation and applied to studies on the specificity of DN'ase I (28, 29).

The known sequence of DNA segments could facilitate the understanding of certain problems such as the extent of degeneracy of the genetic code, the redundancy of genetic elements, the influence of the primary and secondary structure of sequences specifically recognized by proteins or nucleic acids, and mechanisms related to operation and control of DNA—RNA— PROTEIN synthesis.

1.1.1 Recognition of DNA sequences or structural singularities by proteins

The interaction of two dissimilar macromolecules as DNA and protein requires the presence of specific "recognition sites" in which groups of nucleotides or groups of atoms at the periphery of the heterocyclic ring of the bases play a primary role (30). Sequences which are recognized by given proteins must be specific and unique in order to be distinguishable from other segments (31). This would account for the highly specific DNA interaction with the repressor protein which controls one or at most two operons of thousands present in the genome. It would also explain the low number of these protein molecules in the cell and their rapid kinetics of interaction with the operator regions (32, 33).

The specificity which governs the DNA-protein interaction can be classified as follows:

1) Highly specific recognition sites, as the one for the repressor, the ter system which recognizes the base sequence within the cohesive ends of mature λ phage (34) and the integration and excision protein systems for the λ phage genome (35).

   The combined action of RNA polymerase with the initiator (σ) and terminator (ρ) factors is also highly specific because of their ability to recognize a limited number of promoter and terminator sites (36).

2) Recognition of lower specificity can be attributed to enzymes which detect:
   a) internucleotide bonds: DN'ase I (section 1.4), DN'ase II (38) and other nucleases (39).
   b) base modifications: glucosyl transferases (40), endonuclease IV (22).
   c) single or double stranded DNA: exo or endonucleases (section 1.3), Mung bean endonuclease I (37), Neurospora crassa endonuclease (74).
   d) single strand breaks: DNA polymerase (41) and ligase (42).
   e) free ends of linear DNA: phosphatases, polynucleotide kinase and nucleotidyl transferase (42, 43).

Some of the different binding sites could be closely linked and even share some common recognition elements as proposed for the promoter - operator regions of the genome (44). Interaction models between DNA and protein have been extensively discussed by Yarus (30), Arber and Linn (45), and can be exemplified in the following cases:

1) DNA polymerase. The initiation of DNA replication is believed to be preceded by the production of an endonucleolytic single-strand break which creates a 3' hydroxy-active binding site (template and priming site) for the DNA polymerase (46-48). This hypothesis is supported by experiments done *in vitro* on single, double-stranded and synthetic templates (42, 49) and *in vivo* with the replicative form of bacteriophage øX174 DNA whose nicked form (RF II) is believed to be the active replicative intermediate (50).

2) RNA polymerase. The enzyme together with the appropriate σ factor recognizes the promoter regions or RNA polymerase binding site on the DNA; transcription is then commenced (51). A promoter has been genetically mapped in the lactose operon and it appears to determine the polarity of transcription (52). It has been postulated that recognition of the promoter requires asymmetric sequences at the site such as d Pu-d Py clusters (18, 31). A partial localized denaturation of the double-stranded site would allow the polymerase to intercalate to the specific nucleotide sequences and start transcription (30).

3) Initiator and terminator sites. Fine control of transcription is required in order for RNA polymerase to initiate synthesis of a RNA molecule at the correct site on the template and terminate its synthesis at another specific

site to ensure that RNA molecules produced are discrete units of genetic information. The σ factor of *Escherichia coli* and several bacteriophages has been shown to control the initiator site on the DNA template by binding to RNA polymerase before starting transcription (53, 54). Another protein molecule, the Rho (ρ) factor, has been found by Roberts (55) in *E.coli* systems and causes specific termination and release of RNA being made *in vitro* on DNA templates.

4) *Repressors*. These are specific control elements blocking RNA synthesis at the level of the controlled DNA (56). The purified protein molecules bind to operator sites on the DNA *in vitro* (32, 33). The operator (as well as the promoter) must be non redundant and simple enough to be effectively destroyed by point mutations which create new similar functions (57). The small size of the repressor molecule indicates that it cannot recognize operator sequences longer than 8 – 10 base pairs (58). These findings support its postulated high degree of specific recognition.

5) *Enzymes*. Those involved in other biological processes such as host-controlled modification and restriction of bacteriophages (30, 59, 60), maturation and recombination (42, 61) include nucleases, phosphatases, transferases and ligases. These show specificity towards singularities such as the sugar moiety, certain bases, internucleotide bonds or groups of nucleotides on the DNA.

6) *Polypeptides and histones*. Selectivity for different regions on the DNA molecule has been shown with basic polypeptides and other polycations (30).

Polylysine has greater affinity for AT-rich regions than for GC-rich segments (62). Other polypeptides such as poly L-arginine and poly L-ornithine were observed to complex DNA in a co-operative type of reaction (63). Histones, being very basic chromosomal proteins, seem to act as a major factor in the control of genetic activity in mammalian systems. Chromosomal nucleoproteins, in which the majority of the DNA is repressed, are not available to RNA polymerase (64).

1.1.2 Isolation of specific fragments for sequence analysis

Studies on the primary structure of DNA are hindered by the large size of the molecules which vary in molecular weight between 10^{12} in rat liver cells to 1.5×10^{6} (2,500 nucleotide base pairs) in the E.coli plasmid (65, 66). The analysis of specific functional units in DNA (initiator, terminator, promoter, repressor, enzymes and nucleic acids binding sites) will provide insight into the structure-function relationship as was the case for several RNA molecules (2, 10-13).

Several approaches for the isolation of such specific fragments have been reported as follows:

a) DNA-RNA Hybridization (14, 15, 67, 68).
b) Hybridization or Fractionation after Physical Breakage (69, 70).
c) DNA-protein Binding (71).
d) Deletion Mutants (72).
e) Genetic Transposition (16, 44, 56).
f) Repetitive Sequences (73).
g) Specific Enzymes (22-24, 74).

All of the above approaches can be used for isolating particular enriched DNA units. The most successful for isolation of pure homogeneous fragments has so far proved to be the isolation of DNA sequences complementary to E.coli tRNA by hybridization techniques (14, 15) and the isolation of the E.coli lac-operon from the DNA of transducing phages (16). Both approaches made use of the enzyme N.crassa endonuclease (74) for purifying the complementary hybridized segments.

1.1.3 Methods of fractionation for the analysis of DNA fragments

The determination of DNA sequences depends on several factors including the availability of pure homogeneous DNA or DNA segments, reliable methods for specific degradation, separation and analysis of the fragments produced.

Numerous procedures for analysis of nucleic acid sequences have been reviewed in recent years (6, 75-79). Simultaneous fractionation of deoxyribonucleotides on the basis of both length and base composition was obtained by column chromatography (26, 80-82), two dimensional paper chromatography (78), combination of paper chromatography and electrophoresis (3, 7, 8) or by electrophoresis on thin layer plates (9). However, most of these methods do not resolve long oligonucleotides or many of the short positional isomers, being limited in many cases by capacity and recovery.

The introduction of DEAE-cellulose columns (88) and the use of 7.0 M urea buffers (83) improved the resolution and recovery of oligonucleotides fractionated according to chain length by reducing non-ionic affinities between the oligonucleotides and the column matrix. The residual binding could further be reduced by using DEAE-Sephadex (5, 6, 84). The technique proved to be successful for the fractionation of mixtures of deoxyoligonucleotides up to the octa or nonanucleotide fraction. Sedat and Sinsheimer (85) used DEAE-Sephadex and 7.0 M urea in a heated column in order to fractionate purine oligodeoxynucleotides released by chemical degradation of DNA. They achieved a separation according to chain length up to the dodecanucleotide level.

Subfractionation according to base composition was successful on DEAE-cellulose or DEAE-Sephadex in the presence or absence of 7.0 M urea when pancreatic RN'ase, T_1 RN'ase (6, 75, 77) pyrimidine (17) or purine (E. Junowicz, unpublished results) isostichs were separated under acid or alkaline conditions.

The protonation of A, C and G at pH 2.7 to 3.3 or the partial ionization of U, T and G at pH 8.5 to 9.5, together with the preferential affinities of the purines to the exchangers facilitated the separations. However, isostich mixtures in the hydrolyzates listed above are relatively simple when compared to mixtures of non-isomeric isostichs released by extensive digestion of DNA by pancreatic (or spleen) DN'ase (Table 1). This is due to the lack of base specificity of the enzyme.

Thus, the problem of subfractionating DN'ase I dinucleotides is equivalent, in numeral terms, to the fractionation of pancreatic RN'ase

TABLE 1

Theoretical Number of Oligonucleotides Released by Different Methods of RNA and DNA Degradation

Chain Length	DN'ase I	Py or Pu clusters	Pancreatic RN'ase	T_1 - RN'ase
	$\frac{(n+3)!}{n!\,3!}$	$(n+1)$	$(2n)$	$3(n-1)$
	$n \geq 1$	$n \geq 1$	$n \geq 1$	$n \geq 2$
	$a_1 = 4$	$a_1 = 2$	$a_1 = 2$	$a_1 = 1$
Mono	4	2	2	1
Di	10	3	4	3
Tri	20	4	6	6
Tetra	35	5	8	9
Penta	56	6	10	12
Hexa	84	7	12	15
Undeca	364	12	20	30

a = number of bases in mixture

pentanucleotides. However, the presence of all four bases in the DN'ase I dinucleotide fraction and the variable purine/pyrimidine ratio are important factors in determining the elution position of each non-isomeric component (86). Hence, new methods were required for separation of large amounts of oligonucleotides and quantitative fractionation according to base composition (section 4.2).

1.2 Analysis of DNA sequences

1.2.1 Chemical analysis: pyrimidine and purine clusters

The lack of specific DN'ases and the characteristic chemical reactivity of the purine-N-glycoside bond in DNA initially facilitated the search for chemical degradative methods. Due to the impossibility of formation of 2'-3' cyclic phosphates as in RNA, the internucleotide linkages in DNA are relatively stable to acid. However, the purine-N-glycosidic bonds are more labile than the pyrimidine-N-glycosidic bonds (89). When the purine nucleotide bases have been removed, ß-elimination reactions are facilitated at the 3' and 5' phosphodiester positions by mild degradation with aqueous formic acid in the presence of diphenylamine (90).

The ß-elimination reaction yields uninterrupted pyrimidine clusters with general formula $Py_n \, p_{n+1}$. The method was extensively studied in various laboratories (18, 20, 90, 91, 117) and led, by development of new fractionation techniques, to the total purine-pyrimidine catalogs of bacteriophages S13, λ and T_7 (17, 18, 19). The different DNA's were found to contain certain unique long pyrimidine tracts unevenly distributed on both strands of the molecule.

These could be correlated to asymmetric binding of poly UG and poly U ribopolymers to the strands and by extension to the *in vivo* transcription mechanism (17-19, 31).

The method also has been used to study the distribution pattern of the glucosyl residues attached to hydroxymethylcytosine in the DNA of T-even phages. Sequences of adjacent hydroxymethylcytosine nucleotides were found to be glucosylated only at the 5' end residue (92) suggesting that limited glucosylation may be based on selective recognition of local nucleotide sequences (30).

Alternatively, purine tracts may be analyzed by selective removal of pyrimidine bases with nucleophilic agents such as hydrazine and its derivatives under alkaline conditions (21, 93, 94). This method is not entirely specific due to a partial degradation of the purine clusters during alkaline hydrolysis. Certain aldehyde reagents, such as aniline have been studied recently by Vanyushin and Buryanov (95) and were found to be highly specific for free deoxyribose moieties with no undesirable side reactions. Such procedures may finally prove to be satisfactory for purine cluster analysis. All of these studies consisted so far of fractionations according to chain length, and statistical analysis of distribution of tracts are reported to be similar to the pyrimidine isostichs. Fractionation according to base composition was successfully attempted on hot Sephadex columns up to the pentanucleotide level (E. Junowicz, unpublished results).

1.2.2 Chemical modification of specific nucleotides in DNA

Chemical modification of specific nucleotides in DNA provides transformed structures which may selectively be cleaved. Apurination or apyrimidination of DNA weakens the internucleotide bonds adjacent to the modified units releasing unique types of oligonucleotides (section 1.2.1). Permanganate oxidizes the G, C and T groups of DNA to ureido groups and degradation of the modified molecules in alkali will selectively produce $A_n p_{n+1}$ tracts (98). The lability of cytosine-3 N-oxide in alkali was employed for selective splitting of DNA sequences at the cytosine units (99).

Chemical modification of bases also produces more resistant internucleotide bonds to the action of nucleases. Modification of the uracil moieties by hydroxylamine or soluble carbodiimide allows pancreatic RN'ase to split only the cytidylic acid residues (101). Soluble carbodiimide was also found to bind to G and T residues of denatured DNA (102). The number of bonds cleaved by DN'ase significantly decreased after modification of the DNA and snake venom phosphodiesterase was unable to cleave bonds adjacent to the modified nucleotides.

Total deaminated DNA is completely resistant to the action of DN'ase I, indicating that the amino groups of the base are essential for the catalytic activity of the enzyme (103). Another method which has been used is degradation and quantitative release of thymine from pyrimidine-containing oligodeoxynucleotides by treatment with osmium tetroxide (87). The selective reaction of thymine with osmium tetroxide and selective attachment of other markers to the DNA polynucleotide chain are currently being investigated for the determination of base sequences with the electron microscope by Beer, et al. (110, 111).

None of these methods is completely specific for modifications of a given purine or pyrimidine base and were not applied up to now for quantitative sequential studies.

1.3 Use of enzymes in sequence studies of DNA

Some of the enzymes involved in DNA metabolism (39, 42, 45, 78, 107–109) have a wide variety of applications as instruments in the study of the primary structure of DNA. Those which are relevant to the present topic will be discussed in this section.

1.3.1 Polynucleotide kinase

This enzyme has been used widely for RNA analysis (42, 97) and has been applied to the study of pyrimidine sequences in DNA. The non-radioactive oligonucleotides were phosphorylated with the aid of polynucleotide kinase using (γ-^{32}P) ATP as a phosphate donor (97). The enzyme transfers the γ-phosphate group of ATP to the 5' hydroxyl of each nucleotide in the digest which is then subjected to two-dimensional electrophoresis by the method of Sanger, et al. (7). The oligonucleotides are fractionated by this technique and the fingerprints detected by autoradiography. However, the fractionation is limited up to the octanucleotide level. The method is suitable for studying

DNA's which have low turnover and are difficult to label in vivo. The polynucleotide kinase has also been applied for the identification of the 5' termini of the DNA of bacteriophages λ, ø80, 21 and 186 (114).

1.3.2 DNA polymerase

The physical and chemical properties of DNA polymerase and its involvement in the mechanisms of replication and repair were recently reported (41,42). Information regarding the opposite polarity of the DNA strands was provided by nearest-neighbour analysis (96). The relative frequencies of the sixteen possible nearest-neighbour sequences of a DNA molecule were determined by using the DNA as a template and the four deoxyribonucleoside triphosphates (dNTP) in the presence of DNA polymerase. The DNA was synthesized with a mixture of one α-^{32}P-labeled dNTP and the other three unlabeled dNTP's. Four parallel reactions, each containing a different α-^{32}P-dNTP were prepared. Each of the synthetic DNA's was then hydrolyzed by Micrococcal nuclease and spleen phosphodiesterase producing a mixture of 3'-mononucleotides. Each of the mononucleotides carried the α-^{32}P from the 5' position of the nearest-neighbour nucleotide. Determination of the relative ^{32}P content of the four 3'-mononucleotides isolated from the separate reactions gave the total frequency with which any nucleotide is linked to any other in the newly replicated chain.

In addition, Berg, et al. (112) used the enzyme to incorporate ribonucleotides in the presence of manganous ions. By replacing one of the four deoxynucleotide triphosphates by its corresponding ribonucleotide the replicated chain will be labile to alkali only at the ribonucleotide site. A complete map of the sequences for each ribonucleotide could then be made. The method could be extended by including one α-^{32}P-labeled deoxynucleoside triphosphate to the mixture. This could provide information regarding the nearest-neighbour frequency of each particular oligonucleotide (section 1.2.1). Moreover, a combination of two ribonucleoside triphosphates in a replicating mixture could render information on longer sequences if a RN'ase is used prior to alkaline hydrolysis:

$$\begin{array}{c}
\ldots\ldots \mathrm{NpGpNpNpCpNpNpGpNp}\ldots\ldots \\
\downarrow \mathrm{T_1\ RN'ase} \\
\ldots\ldots \mathrm{NpGp} + \mathrm{NpNpCpNpNpGp} + \mathrm{Np}\ldots\ldots \\
\downarrow \mathrm{HO^-} \\
\mathrm{NpNpCp} + \mathrm{NpNpGp}
\end{array}$$

<u>E.coli</u> B DNA polymerase has been proteolytically cleaved into two separate exonuclease and polymerase units (113). Both activities were previously considered to be part of the same active site of the polymerase (41, 46). The pure polymerase unit combined with the methods described above could replicate the DNA without contamination by the 5' exonuclease.

1.3.3 Exonucleases

By applying exonucleases for sequence determination two valuable approaches are possible:

a) The analysis of mononucleotides released by the enzyme would theoretically allow the determination of 25 nucleotides from one end of an homogeneous substrate to 2% accuracy (115).

b) The analysis of exonucleolytic partial digests (79) proved to be less sensitive to contaminating nucleases.

It has been used with a combination of two exonucleases of opposite polarity and the new fractionation techniques developed (3, 7, 8) for the analysis of RNA nucleotide sequences. This method consists of partial digestion of an oligonucleotide with snake venom diesterase (also spleen diesterase, Exonuclease I, etc.), separation of the components according to chain length, and analysis of the total composition of the successive peaks which will differ only by the terminal nucleotide and will thus indicate the arrangement of the sequence. The sensitivity of the method has been increased by ^{32}P-labeling of the 5' ends of a spleen diesterase partial digest by means of polynucleotide kinase (42,97). The method was applied for the detection of some isomeric pyrimidine nucleotide sequences in fd DNA (97). The analyzed oligonucleotides were never higher than octanucleotides.

Short oligonucleotides from tri to pentanucleotides were analyzed by Micrococcal nuclease (79) or by E.coli exonuclease I (118). The latter is unable to hydrolyze the phosphodiester bond of the 5' terminal dinucleotide released. SP3 DN'ase (116), an exonuclease which sequentially cleaves dinucleotides starting from the 5' end has been applied to analysis of short synthetic oligonucleotides. A procedure was recently developed for the determination of a partial sequence of deoxynucleotides in the cohesive ends of

λ DNA (194). It consisted of:

1) The use of E.coli DNA polymerase for the repair synthesis of the 5' terminated single strands with highly labeled nucleoside triphosphates (210).
2) The isolation of the 3'-OH terminated radioactive segments liberated by partial Micrococcal nuclease hydrolysis.
3) Sequence analysis of the purified segments by the use of partial and complete digestion with snake venom diesterase and spleen diesterase.

By only labeling with dGTP and dCTP it was found that the composition of the right cohesive end was dCCCGCCGC, the complementary sequence, dGGGCGGCG, corresponded to the left cohesive end.

1.3.4 Endonucleases

The classification and properties of most of the known DN'ases as well as their functions in vivo have been reviewed (39, 42, 45, 59, 100, 118-120). Some of the enzymes which have been employed or are potentially useful for DNA analysis are presented.

1) Micrococcal nuclease and Mung bean endonuclease I. Both enzymes are unspecific with respect to the sugar moiety but have activity toward RNA and DNA. Both are activated by divalent cations (37, 39, 121), Ca^{++} being the best ion activator for Micrococcal nuclease. With Sr^{++} the DN'ase activity is still maintained whereas no activity toward RNA is found at 10^{-4} to 10^{-2} M ion concentration. This differential activation suggests that two closely related active sites, or a single modified site, may occur in the enzyme. Micrococcal

nuclease releases 3'-phosphomonoesterified oligonucleotides whereas Mung bean endonuclease releases oligonucleotides carrying a 5'-phosphate. Micrococcal nuclease attacks single-stranded DNA faster than native DNA whereas Mung bean endonuclease is comparable to E.coli exonuclease I (118) and N. crassa endonuclease (74, 118) in being highly specific for the single-stranded structure. Micrococcal nuclease has a pronounced initial specificity towards dNp-Ap and dNp-Tp bonds, a property attributed to the higher instability of the A-T regions of DNA which undergo strand separation (opening $\rightleftharpoons$ closing reactions) at a greater rate than G-C regions and thus, favour recognition by the enzyme (122). Similarly the Mung bean endonuclease shows high preference for dpA-pN and dpT-pN bonds. It has been used to eliminate denatured DNA from a mixture of native and denatured DNA and to cleave double-stranded λ DNA into two halves, probably at an A-T rich region (37).

Another enzyme, venom endonuclease was also found to have an early stage preference for dGp-Gp bonds (39).

2) T_4-induced endonucleases. The presence of hydroxymethylcytosine in the T_4 DNA does not allow endonuclease II (endo II) and endonuclease IV (endo IV), two enzymes induced by the phage upon infection of E.coli, to hydrolyze the phage DNA. Endo IV exhibits its high specificity by breaking the host DNA only at the deoxycytidylic level and liberating 5' P-nucleotides. It does not totally degrade the DNA to short oligonucleotides.

With bacteriophage fd the size of the limit digestion products was about 150 nucleotides. A change in the incubation conditions might reduce the size of the oligonucleotides as observed with poly dC which was degraded to an average chain length of octanucleotides. This has been the only report to date of a highly specific partially purified DN'ase. Endo II is specific for native DNA making single-strand breaks. It was shown to have preferential activity towards dpX-pG and dpX-pC bonds producing oligonucleotides 1,000 to 1,300 nucleotides long from λ DNA (22).

3) DN'ases K_1 and K_2. These were isolated from the mycelia of *Aspergillus oryzae* and both act on native and denatured DNA (123, 124) releasing oligonucleotides of an average chain length of six. None of the enzymes were found to be completely specific. The K_1 enzyme preferentially splits dGp-Gp and dGp-Ap bonds releasing 3'-phosphorylated ends whereas K_2 DN'ase preferentially attacks the same bonds releasing 5'-oligonucleotides. At least 80% of the released termini are purines. DN'ase K_2 also was used for the isolation of thymidylic acid-rich fragments from bacteriophage f_1 (125).

4) Repair, restriction and recombination enzymes. Pyrimidine-pyrimidine dimers are formed when DNA is irradiated with UV light. The presence of the dimers in DNA is lethal but dark repair was observed when ^{3}H-thymine dimers were released from the UV-treated DNA during a recovery period (109). This was shown to be caused by two repair enzymes. The UV-endonuclease (incision enzyme) was shown to break a single phosphodiester bond near each

pyrimidine dimer, creating a nicked region. An exonuclease capable of degrading these regions then releases 5 to 6 nucleotides per incision creating a single-stranded region (126). Together the two enzymes will completely eliminate the distorted regions allowing further repair of the damaged stretch.

Repair enzymes for lesions produced by x-rays, nitrous acid, mitomycin and certain mono and bifunctional alkylating agents have also been described (100). E.coli endonuclease II (127) was found to degrade alkylated glucosylated and non-glucosylated DNA at or near the site of alkylation. The distortion created by the alkylation must be specific since reaction of DNA with other physical and chemical agents did not create a substrate for the enzyme.

The restriction enzymes recognize and introduce double-strand scissions at a limited number of sites (of unique sequential characteristics) on non-homologous DNA molecules. The E.coli B restriction endonuclease (128) was estimated to recognize and break 3 to 5 sites on the λ vir DNA, one per fd replicative form molecule, and about 100 per bacterial molecule. The nucleotide sequence of T_7 DNA recognized by a restriction enzyme from Haemophilus influenzae was recently reported (129). The sequence appears to have a two-fold rotational symmetry conferring on the site a structural distortion specifically identified by the enzyme:

5'......pGpTpPy ———— pPupApCp......3'
3'......pCpApPup ———— PypTpGp......5'.

Modification presumably occurs on the phage DNA molecule at the same site as the restriction enzyme making these sites unavailable for its specific action (45). All of the above mentioned enzymes could eventually be used for the preparation of homogeneous and specific DNA fragments for sequence analysis.

1.4 Pancreatic deoxyribonuclease (DN'ase I)

1.4.1 Properties of the enzyme

Several extensive reviews on the preparation and the physical, chemical, kinetic and in vivo properties of the enzyme have been published (39, 119, 120). Some of the properties relevant to the present topic will be discussed.

Pancreatic DN'ase is an endonuclease which forms oligonucleotides bearing a 5' monophosphate. It attacks the native faster than the denatured form of DNA and will not hydrolyze dinucleotides with 50-fold excess of enzyme (39). The enzyme was crystallized by Kunitz (130) in 1950. Commercial enzyme was fractionated by Potter (120) into five bands by electrophoresis on cellulose acetate strips. One of the five bands isolated was shown to be Chymotrypsinogen B. The crystallized DN'ase was shown also to contain a small contaminant of RN'ase which could mostly be removed on DEAE-cellulose columns. The remaining RN'ase could be specifically abolished by treatment with iodoacetate (206). A purified preparation of DN'ase I was obtained by gel filtration on Sephadex G-100 by Lindberg (141) in 1966 but was unstable in solution. Investigations on stabilization of the enzyme in solution led, in 1969, to the discovery that inactivation of contaminating proteases by FDP treatment produced a stable DN'ase I preparation (131). The enzyme was separated into two active fractions by chromatography on a Sulphoethyl-Sephadex column. Further experiments with the stable DN'ase indicated that an additional peak could be separated by chromatography on phosphocellulose (157). Thus, up to 1969, kinetic and specificity studies were performed with unstable and impure DN'ase fractions. This led to contradictory reports concerning metal ion activation or inhibition, the effect of pH, ionic strength, synergism between ions, etc. (132-136) which had to be re-evaluated before studying the specificity of action of the enzyme.

Studies on the kinetics of DNA hydrolysis showed that at low substrate concentration the process is a first order reaction. At relatively high concentrations the reaction decreases rapidly with increase in DNA concentration, being continuous but not linear with time (39, 130).

DN'ase I is activated by divalent cations and inhibited by chelating agents such as EDTA and citrate (39, 130, 132-134). Different degrees of activation were postulated for several ions activating DN'ase I. Mn^{++} was found to be a better activator than Mg^{++} by several investigators (133, 207). Eichhorn, et al. (174) found Co^{++} and Zn^{++} at concentrations of 10^{-3} to 10^{-2} M to be much better activators than Mn^{++} or Mg^{++}. Shack and Bynum (134) showed that activation depends on pH and ionic strength of the buffer as well as the concentration of DNA in the mixture.

A few other important points have to be stressed.

a) Evidence exists for the involvement of bivalent cations on the active site of the enzyme; the substrate for DN'ase I is a metallo-deoxypolymer and not the polymer alone (43, 130, 133). A shift in the mechanism of activation from double to single hit kinetics was observed by substitution of Mn^{++} for Mg^{++} suggesting that the effect of the metal must be on the site of hydrolysis (137). Co^{++} was found to exert a strong synergistic effect on the Mg^{++}-activated reaction indicating increase in susceptibility of certain DNA-binding sites (133) as well as a change in the mechanism of hydrolysis (137). The phenomena of alkylation of the active site and reduction and reformation of the disulfide bonds of DN'ase I were both shown to be metal-dependent (135, 136). Only one histidine residue out of a total of six is stereospecifically alkylated when iodoacetate is added in the presence of Mn^{++} or Cu^{++}. Similarly, Ca^{++} is essential to protect one of the two disulfide bonds from reduction, or to reform the S-S bond when acting on the totally reduced enzyme. Reactivation of the reduced DN'ase I is also promoted by Mn^{++}, Cu^{++} or Mg^{++}.

b) The rate of reaction slows down during the degradation of native DNA due to the phenomenon of autoretardation (39). This was shown to be due to the higher resistance for cleavage of the shorter oligonucleotides produced during the hydrolysis (138). The continuous transition of the reaction does not allow it to be terminated at precise and predictable stages. However, products of the reaction can be analyzed at initial stages of the reaction (139) and be compared to digests at an apparent equilibrium.

Structural studies on the molecule implicated the presence of the imidazole rings of histidine at the active site, a common property with other pancreatic enzymes (135). Another structural feature is the presence of a carbohydrate moiety forming a N-ß-aspartylglycosylamine bond at a single point on the polypeptide. The only sugars present in the glycoprotein were mannose and glucosamine (140). Natural protein inhibitors of DN'ase I were isolated from several sources (39, 119, 141, 142) amounting in the case of the calf thymus inhibitor to 10% of the total protein in the gland (142).

1.4.2 Specificity of cleavage

Almost all studies performed with DN'ase I for determining its specificity of cleavage were done in the presence of Mg^{++} as an activating ion (39). The analysis of the released oligonucleotides was performed at the mono, di and trinucleotide level and the absence or presence of certain compounds was related to the preferential susceptibility or resistance of certain bonds.

Analysis of sequences of nucleotides in dinucleotide fractions obtained from extensive DNA digests revealed that the sequences d pPu - pPu, d pPy - pPy and d pPy - pPu are present in high amounts whereas the sequence d pPu - pPy is almost absent except for dpG - pC (81). It was then suggested that the d pPu - pPy sequences are preferentially hydrolyzed by DN'ase. However, d - ApCp was shown to be resistant to DN'ase hydrolysis despite its Pu - Py arrangement (119).

Investigations on the action of DN'ase I on short substrates demonstrated a labilizing effect of a proximal 3'-monophosphate on the preceding linkage (82) and the resistance of substrates such as pTpTp and TpTpT to digestion (25). Ralph, et al. (25) also found that certain short homo-oligonucleotides are attacked at different rates when degraded in the presence of Mn^{++}, $d(pA)_{5-6}$ being cleaved the fastest.

Becking and Hurst (26) showed that under various environmental conditions the oligonucleotides obtained vary considerably in composition. The mono and dinucleotides in a Mn^{++}-activated reaction were different from those obtained in Mg^{++}-activated digests (80, 81, 143, 144).

Conclusive results on the specific action of bivalent cations on complementary deoxy-homopolymers were obtained by Bollum (27). With the homopolymer poly dA:dT and in the presence of Mg^{++}, the extent of hydrolysis of poly dA was smaller than that of the poly dT chain. With the poly dI:dC homopolymer only poly dI was digested in the presence of Mg^{++}. When Ca^{++} was added to the reaction mixture poly dC was completely degraded. By changing the synergistic mixture of Ca^{++} plus Mg^{++} to Mn^{++} the same results were obtained with both the poly dI:dC and poly dG:dC fractions. The digestion of single-stranded polydeoxynucleotides was also shown to be non-random. The enzyme produced oligonucleotides of average length 12 to 14 whereas the shorter oligonucleotides appeared to come from the cleaved ends of the very long oligonucleotides (145).

Scheffler, et al. (146) examined the oligonucleotides released from the alternating copolymer d(A-T) by Mg^{++}-activated DN'ase I and showed a specific type of cleavage where T predominated at the 5' end and A at the 3' end.

The data presented above suggests that the susceptibility of certain internucleotide linkages to hydrolysis by DN'ase I can be substantially altered in the presence of specific bivalent cations. This was found to be the case with other enzymes too (212). With carboxypeptidase A the peptidase activity can be abolished while maintaining the esterase activity by replacing the Zn^{++} ion with Co^{++}, Cd^{++}, etc.

No direct evidence exists to show possible differences between initial and advanced scissions produced by the enzyme on native DNA. For this reason, studies on the kinetic properties of the purified DN'ase I activated by divalent cations become important for the re-evaluation of its specificity of action.

1.4.3 Purpose of the present study

The information gained from the above chemical and enzymatic methods was limited to purine or pyrimidine catalogs (17, 18, 19, 21) and short sequences of DNA (97,194).

Thus, the purpose of the work presented in this thesis has been to reassess the specificity of DN'ase I on its natural DNA substrate under a wide range of hydrolytic conditions and, if a strict specificity could be determined, to apply the metal-activated enzyme to studies on the primary structure of DNA.

The investigation led to the development of several fractionation techniques which were applied throughout these studies.

An overall scheme for the experiments carried out during these studies is shown in the following diagram.

The three main subjects investigated were:

a) DN'ase I: purification and properties.

b) Methods of fractionation and analysis.

c) Specificity of DN'ase I.

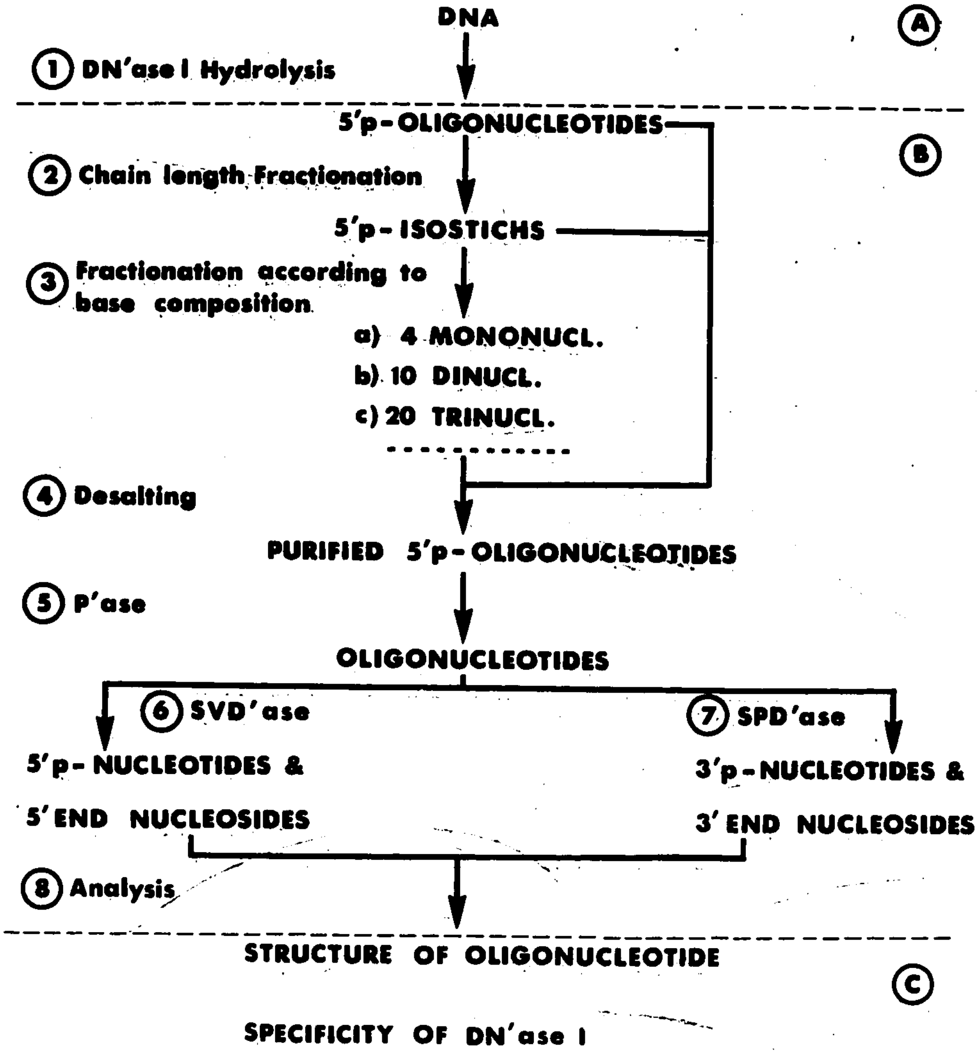

FIGURE A

Schematic Plan for the Analysis of DN'ase I Specificity

P'ase, phosphatase

SVD'ase, snake venom diesterase

SPD'ase, spleen diesterase

CHAPTER 2

MATERIALS AND METHODS

2.1 Materials

Charcoal, Norit A (Matheson, Coleman and Bell) was activated by boiling with 2N HCl followed by washing with ethanol:ammonia:water (50:15:35) and subsequently with water to neutrality.

Reagent grade urea was obtained from Baker and Adamson. Solutions of 7.0 M urea were slurried with 0.25 g activated charcoal/l urea to reduce optical densities.

The charcoal-urea solutions were filtered twice through 3MM Whatman filter paper and then through a 2.5 x 5 cm DEAE-cellulose column. A 7.0 M solution of urea in 0.005 M Tris-HCl buffer, pH 7.6, had an A_{260} of less than 0.005.

Reagent grade phenol (Mallinckrodt) was freshly distilled prior to use.

DEAE-cellulose (Whatman DE-11) was sieved prior to use. The portion passing through a 200 mesh (U.S. standard number) but retained by the 325 mesh size was used. The DEAE-cellulose was washed according to Staehelin (75). Formate, carbonate or bicarbonate forms of DEAE-cellulose were prepared by suspension of the cellulose in 1M formic acid, 1M ammonium carbonate, 1M bicarbonate or 1M TEAB respectively. The ion exchanger was then washed with water and equilibrated in the buffer to be used for elution. TEAB buffer (147) was prepared by mixing triethylamine with water and, keeping the solution at

$0^{\circ}C$ in an ice-salt bath, CO_2 was bubbled through until the desired pH was obtained.

DEAE-Sephadex A-25 (Pharmacia) and Sephadex G-100 were prepared according to the instructions of the manufacturer.

Cellulose phosphate (Whatman P-11) was washed with cycles of 0.2 N HCl and 0.2 N NaOH, then with concentrated buffer, water and finally equilibrated in the starting elution buffer for 12 hours. Carboxymethyl cellulose (Whatman CM-32) was prepared according to the instructions of the manufacturer. Dowex 50-AG50W-X4, minus 400 mesh (Bio-Rad) was cleaned according to Blattner and Erickson (148). Dowex 50W-X8, 200 - 400 mesh (Baker Analytical Reagent) was treated as described by Keller (149).

Nucleotides and nucleosides of the deoxy and ribo series were obtained from Calbiochem.

DN'ase I (EC 3.1.4.5), 1 x crystallized, grade D and purified precrystalline (amorphous), grade DP, E.coli alkaline phosphatase (EC 3.1.3.1), electrophoretically purified, grade BAPF, and chromatographically purified, grade BAPC, snake venom phosphodiesterase I (EC 3.1.4.1), grade VPH, spleen phosphodiesterase II (EC 3.1.4.1), grade SPH, and RNA-core, grade RNAC, were obtained from Worthington Biochemical Corp.

Pancreatic RN'ase (5 x crystallized, grade A) and lysozyme (3 x crystallized) were obtained from Sigma Chemical Co.

p-nitrophenylphosphate, Ca-bis-p-nitrophenylphosphate, and thymine 5'-monophospho-p-nitrophenyl ester were from Sigma Chemical Co.

E.coli K12 cells (late log phase, high peptone medium) were obtained from General Biochemicals.

Diisopropylfluorophosphate was from Aldrich Chemical Co.

The chlorides of Sn^{++}, Sr^{++}, Cd^{++}, Mg^{++}, Hg^{++} and Ca^{++} were spectrographically standardized "Specpure" salts from Johnson Matthey Chemicals Ltd. All other salts and chemicals used were "Reagent" grade.

2.2 Methods

2.2.1 Preparation of calf thymus and E.coli K12 DNA

Calf thymus DNA was prepared from fresh tissue by the method of Kay, et al. (150). It contained 0.4% protein (151), no detectible RNA (152) and the $\epsilon(P)_{260}$ was 6875. The base composition was adenine: 28.1%; thymine: 28.1%; guanine: 21.9%; cytosine: 20.5%; 5-methylcytosine: 1.4%.

E.coli K12 DNA was prepared by the phenol extraction procedure of Saito and Miura (153). Pancreatic RN'ase used in the preparation at a concentration of 2 mg/ml in 0.15 M NaCl, pH 5.0, was heated at 80°C for 10 minutes before use in order to eliminate contaminating DN'ases (154). Following the isopropanol step polysaccharides were eliminated by treatment with 2-methoxyethanol according to Kirby (155). The DNA was precipitated by adjusting the solution to 3.0 M NaCl and addition of one volume of ethanol. The DNA was then redissolved, precipitated in isopropanol and washed with increasing concentrations of ethanol from 70 to 100%. The purified DNA was dissolved in 0.1 M EDTA, pH 7.0, and dialyzed against 5 changes of 0.005 M Tris-HCl buffer, pH 7.6. The yield of DNA was 2.4 mg per gram of wet-packed cells. The DNA contained 0.4% protein (151), 0.7% RNA (152) and the $\epsilon(P)_{260}$ was 6900. The base composition was adenine: 24.5%; thymine: 24.9%; guanine: 25.7%; cytosine: 24.8%.

The temperature-absorbance profile of the DNA was measured in standard saline citrate, pH 7.0, (0.15 M NaCl - 0.015 M sodium citrate) at 260 nm in a Gilford Model 220 spectrophotometer. Temperature was controlled by a Haacke circulating bath and was measured directly in the cuvette by means of a digital read-out thermosensor (156). The Tm was 90.0°C.

The DNA was completely excluded from Sephadex G-100 (2.5 x 100 cm column; buffer, standard saline citrate, pH 7.0).

2.2.2 Purification of DN'ase I

DN'ase I was purified by a modification of the method of Salnikow, *et al*. (157).

Preparative scale purification of DN'ase I from DP grade enzyme consisted of an initial treatment with DFP following the procedure of Price, *et al*. (131). 300 mg protein were dissolved in 6 ml of 0.38 M sodium acetate buffer*, pH 4.7, containing 400 µl of DFP. After 30 minutes at 25°C, the sample was applied to a phosphocellulose column (2.2 x 88 cm) previously equilibrated with the same buffer for 18 hours at room temperature. The column was washed with 0.38 M sodium acetate buffer, pH 4.7, until the first protein peak appeared (190 ml) and then eluted with a linear gradient of 0.38 M to

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* The molarity refers to the acid component of the buffer.

1.0 M sodium acetate buffer, pH 4.7, (total volume 2.0 l). Linear gradients were generated by a two-chamber system (158). The effluent was monitored at 254 and 280 nm with a Gilford Model 2000 spectrophotometer and 8.5 ml fractions collected. The activities of several samples were measured as described in section 2.2.3. The pooled fractions were adjusted to pH 5.2 with 1.0 M NaOH and dialyzed three times against 12 l of distilled water at 4°C for 28 hours. The dialysis tubing was pretreated by boiling in 0.002 M EDTA for 20 minutes. The salt-free protein solutions were lyophilized and stored at -20°C.

Rechromatography of the main DN'ase fraction (DN'ase A) was performed on a P-11 phosphocellulose column (94 x 2.2 cm) equilibrated with 0.6 M sodium acetate, pH 4.7, for 18 hours at 25°C. The lyophilized fraction was dissolved in 10 ml of 0.6 M sodium acetate buffer, pH 4.7, and applied to the column. The column was washed with 160 ml of 0.6 M sodium acetate, pH 4.7, followed by a linear gradient of 0.6 M to 1.0 M sodium acetate, pH 4.7, total volume 1.5 l. The column was monitored at 254 and 280 nm and 10 ml fractions collected. Intermittent gradient elution was used in this case (159). When the major enzyme peak started to elute from the column the connection between the diluted and the concentrated buffers was interrupted and the elution continued stepwise at the concentration of the diluted buffer. When the majority of the enzyme had been eluted the gradient bottles were reconnected and the linear gradient continued.

The major fraction (DN'ase A) was adjusted to pH 5.2 with 1.0 M NaOH, dialyzed twice against 12 l distilled water, then twice against 12 l of 0.002 M

EDTA and finally twice against double-distilled water. The enzyme was lyophilized and stored at -20°C.

Polyacrylamide gel electrophoresis

Samples of crystalline DN'ase I prior to purification and DN'ase A (40 to 80 µg) purified by rechromatography on phosphocellulose were analyzed on a 7% polyacrylamide gel at 4°C according to the method of Davis (168). Both the running and stacking gels were chemically polymerized and sucrose was used instead of the sample gel. The running gel was prepared with 0.037 M Tris-glycine buffer, pH 8.3. The potential drop was 150 volts through the stacking gel and 250 volts through the running gel. The electrophoresis proceeded for 5 hours and bromophenol blue was used as indicator for the advancing front. The gels were stained with 0.13% coomassie brilliant blue (Colab Laboratories Inc.) in 7% acetic acid.

2.2.3 DN'ase I assays

The enzyme was assayed by a modification of the method of Kunitz (130) in two different ways:

Assay A The substrate stock solution contained 0.05 mg/ml DNA dissolved in 0.005 M Tris-HCl buffer, pH 7.6, containing 0.005 M $MgCl_2$ or 0.001 M $MnCl_2$. A 3.0 ml sample of substrate solution in a stoppered cuvette was placed in a Gilford 2000 spectrophotometer fitted with thermospacers and a Haacke constant temperature circulating bath, equilibrated to 25°C. 10 to 100 µl of enzyme solution containing 0.02 mg/ml in 0.005 M Tris-HCl buffer, pH 7.6, were added.

The cuvette contents were mixed by stirring with a plastic rod and the increase in absorbance at 260 nm recorded. The slope of the linear portion of the curve was proportional to the amount of enzyme over this concentration range. One unit of enzyme is defined as the activity which caused an increase in absorbance of 1.0 optical density unit/minute/3 ml assay mixture under the conditions described above.

Assay B The substrate solution contained 0.5 mg/ml DNA dissolved in 0.01 M Tris-HCl, pH 7.6, and 3.3×10^{-3} M $MgCl_2$. A 0.3 ml sample of substrate solution was equilibrated at 37°C in a water bath and 1 to 10 µl of DN'ase solution containing 0.2 mg/ml enzyme in 0.005 M Tris-HCl buffer, pH 7.6, was added. The incubation mixture was shaken and placed in a quartz cuvette with a 0.1 cm optical path. Absorbance at 260 nm was recorded with a Gilford 2000 spectrophotometer at 37°C. One unit of enzyme activity is defined as the activity which caused an increase in absorbance of 1.0 optical density unit/minute/0.3 ml assay mixture under the above set of conditions.

Both assays were found suitable for wide variations of conditions such as pH, concentration of substrate, type of buffer and type of ion used when kinetic properties of the enzyme were investigated.

One unit as measured by assay A is equivalent to 3.2 units when measured by assay B.

Except where otherwise specified the buffers never exceeded 0.01 M ionic strength in the final reaction mixture. Buffers used were Tris-HCl, Tris-maleate and acetate.

During the purification of DN'ase I (section 2.2.2) activities were determined as indicated in assay A, 0.05 M Tris-HCl, pH 7.6. Under these conditions the activity of the enzyme is reduced 8.4 fold. The activity was measured in the presence of the sodium acetate at pH 4.7. The concentration of salt in the assay mixture never exceeded 1.0×10^{-2}M. Addition of sodium acetate, pH 4.7, to the standard mixtures in 0.05 M Tris-HCl, pH 7.6, buffer in the range 1.0×10^{-3} to 1.6×10^{-2} M, using purified DN'ase A, showed the assays to be identical to those performed in the absence of salt.

Protein concentration was calculated from the absorbance at 280 nm in 0.2 M sodium acetate, pH 4.7, where $E^{0.1\%}_{1\ \mathrm{cm}} = 1.20$ (131) or in 0.005 M Tris-HCl buffer, pH 7.6, where $E^{0.1\%}_{1\ \mathrm{cm}} = 1.23$ (141).

Acid Soluble Material Assay

The assay determined the release of acid soluble products from DNA after various times of DN'ase I digestion by measurement of absorbance changes at 260 nm. The assays were performed under identical incubation conditions described in procedure B above with 3.3×10^{-3}M activating ion or varying concentrations as required. To 0.3 ml of cold carrier calf thymus DNA (2.0 mg/ml) 0.2 ml of the incubation mixture were added immediately followed by 0.5 ml ice cold 1.0 N perchloric acid. The mixture was kept at 10°C for 5 minutes followed by centrifugation for 10 minutes at 10,000 r.p.m. at 4°C. The supernatant was collected, equilibrated to room temperature and absorbance measured at 260 nm.

All activities measured were the average of at least two assays. The slope of the linear portion of the curve of acid soluble material released vs time was measured and the activity determined.

One unit is defined as the activity which causes an increase in absorbance of 1.0 optical density unit/minute/ml at 260 nm under the described conditions.

2.2.4 Hydrolysis of DNA by DN'ase I

Calf thymus or E.coli K12 DNA was incubated under various conditions in the presence of Tris-HCl buffer and the corresponding divalent cation and the enzyme added after a 10 minute incubation period at 25°C or 37°C. The hydrolysates were monitored by measurement of the increase in absorbance at 260 nm or by the release of acid soluble material. The reaction was completely stopped by addition of EDTA at a concentration 1.4 to 3.0 times higher than that of the activating ion. The DN'ase was immediately denatured by adjusting the pH of the incubation mixture to 13.0 with concentrated KOH. After 30 minutes at room temperature the solution was titrated back to pH 7.6 with concentrated perchloric acid. The solution was kept at 4°C for 1 hour and the insoluble potassium perchlorate crystals removed using a Büchner funnel. The solution was then diluted and desalted on a DEAE-cellulose column or treated with phosphatase (see section 2.2.5 and 2.2.8).

2.2.5 Fractionation of oligonucleotides according to chain length. Desalting.

DN'ase digests were fractionated according to chain length on DEAE-Sephadex A-25 at 65°C in 7.0 M urea (29). Chromatography columns were jacketed for circulation of water and fitted with internally threaded ends (Ace Glass Inc.), a sintered glass disc, teflon flow control stopcock and Chromatronix teflon tubing adaptors. DEAE-Sephadex A-25 was suspended in 0.005 M Tris-HCl buffer, pH 7.6, 7.0 M in urea and degassed at 80°C under vacuum. The exchanger was packed into the column, containing 10 ml of starting buffer, by gravity flow at 65°C. The DN'ase digest was diluted 1:4 with 0.005 M Tris-HCl, 7.0 M urea buffer, pH 7.6, degassed at room temperature and applied to the hot column at a flow rate of approximately 100 ml/hour. Approximately 5 ml of buffer was maintained on top of the exchanger to allow time for temperature equilibration of the entering solution to 65°C and to avoid bubbles in the column during the chromatographic procedure. The column was eluted with a linear gradient of 0 to 0.4 M LiCl in 0.005 M Tris-HCl, 7.0 M urea buffer, pH 7.6. Column effluents were monitored at 254 nm using a LKB Uvicord or at 260 nm using a Gilford model 2000 spectrophotometer. Flow rate was controlled with a Buchler Polystaltic pump. Column temperature was controlled with a Haacke constant temperature circulating bath.

Preliminary experiments were carried out using 7.0 M urea buffers and DEAE-cellulose (83) or DEAE-Sephadex A-25 (160) at room temperature. The columns were prepared and eluted as described above.

Desalting

This was performed essentially as described by Rushizky and Sober (84). Isostich fractions from the chain length separations were pooled, diluted with four volumes of glass distilled water and applied to DEAE-cellulose columns (2.4 x 15 cm, in formate, carbonate or bicarbonate form). The columns were washed with distilled water and then with 0.02 M buffer. The nucleotides were eluted with 1.3 M to 1.5 M buffer. The volatile buffers used were ammonium formate, carbonate or bicarbonate and TEAB.

The nucleotide fractions were concentrated by flash evaporation at 45°C, redissolved in distilled water and reconcentrated. These steps were repeated several times before removing the remaining salt by sublimation *in vacuo* at 65°C (c.f., 84). The use of TEAB buffer was found to be most advantageous, due to the ease of evaporation with no need for additional sublimation (147).

2.2.6 Fractionation of mononucleotides, dinucleotides and trinucleotides according to base composition

1) Mononucleotides were analyzed according to the chromatographic method of Blattner and Erickson (148) on samples previously desalted.

2) Dinucleotides were not desalted separately. The dinucleotide isostich fractions from DEAE-Sephadex chromatography were pooled, diluted with 5 volumes of distilled water and applied to a DEAE-cellulose column, 0.9 x 25 cm, formate form. The column was washed with 0.05 M formic acid to remove urea and eluted with a linear gradient of 0 to 0.1 M ammonium formate, pH 3.4.

3) Trinucleotides. The trinucleotide isostich fraction, desalted as described, was diluted with 5 volumes of distilled water and applied to a DEAE-cellulose column, 0.9 x 130 cm, formate form. The column was washed with 0.05 M formic acid and eluted with a linear gradient of 0 to 0.3 M ammonium formate, pH 3.6. For the trinucleotide fractionation it is essential to remove fines from the DEAE-cellulose as much as possible. In the present experiments 20 g of DEAE-cellulose was suspended in 2 l of water, allowed to settle for 15 minutes and the solution decanted. This was repeated 6 times.

2.2.7 Separation of mononucleotides and nucleosides by cation exchange chromatography

A new method was developed for the rapid separation of nucleosides and nucleotides (28). The cation exchanger used was AG 50W-X4, minus 400 mesh. The resin was washed twice with 10 volumes of 1.0 N HCl, three times with 10 volumes of concentrated NH_4OH followed by three washes with 10 volumes of the elution buffer used. Fines were removed by decantation.

Chromatographic columns were prepared as described previously (section 2.2.5) with the ends fitted with a polyethylene filter disc, pore size 100 µ, and a nylon bottom drip adapter with a Luer fitting connected to Chromatronix teflon tubing adapters. These fittings reduced the dead volume at the end of the columns and minimized mixing of the eluted material.

Columns were packed with a 50% slurry of the resin and washed with the corresponding elution buffer for 1 hour prior to use. Columns stored for even a few hours were routinely washed 1 hour before use. Flow rate was maintained with the use of a Buchler polystaltic pump at 70 - 100 ml/hour.

Sample preparation. Nucleosides and nucleotides of the ribo and deoxyribose series were dissolved in the appropriate chromatography buffers to give a final solution of 0.5 - 0.8 A_{260} units or 5 - 17 units per nucleotide or nucleoside. Enzyme digestion mixtures were adjusted to the pH of the elution buffer and loaded directly on the column. Sample volumes ranged from 0.2 to 2.2 ml. The sample was allowed to soak completely into the resin bed then the column eluted immediately. The UV absorption of the column effluent was determined with a Gilford model 2000 spectrophotometer at 260 nm, an Isco model UA-2 or an LKB Uvicord (type 4701 A) UV monitoring system at 254 nm. 2 to 5 ml fraction were collected. All separations are complete in one and a half to two hours.

Chromatographic systems

Three different chromatographic elution conditions have been developed.

1) Separation of 3' or 5' deoxynucleotides and deoxynucleosides. Column,

42 cm by 11 mm; elution buffer, ammonium formate prepared by adjustment of 0.3 M NH_4OH to pH 9.2 with concentrated formic acid.

2) Separation of 3' or 5' ribonucleotides and ribonucleosides. Column, 60 cm by 11 mm; buffer, ammonium formate prepared by adjustment of 0.3 M ammonium hydroxide to pH 8.9 with concentrated formic acid.

3) Fractionation of all four deoxynucleosides and 5' deoxynucleotides. Column 42 cm by 11 mm; buffer, ammonium formate prepared by adjustment of 0.015 M ammonium hydroxide to pH 3.2 with formic acid. Following elution of the first five components with this buffer a second ammonium formate buffer, prepared by adjustment of 0.3 M ammonium hydroxide to pH 9.2 with formic acid, is applied.

All buffers must be freshly prepared since being volatile they are susceptible to pH change upon standing. The elution buffers are the same as those used for loading the columns and the first two columns automatically regenerate themselves with elution and no further regeneration is required. Nucleotides and nucleosides were distinguished by phosphate analysis and the separated components identified by spectral analysis.

2.2.8 Characterization of oligonucleotides by enzymatic and chemical methods

Chain length determination was based on the elution position from DEAE-Sephadex, Dowex 50 chromatography for the separation of mononucleotides, DEAE-cellulose chromatography for the separation of dinucleotides and trinucleotides, and ratios of total and monoesterified phosphorus. Monoesterified phosphorus was released by E.coli alkaline phosphatase digestion and the inorganic and total phosphorus assayed by the method of King (161).

Dinucleotides were characterized by hydrolysis with snake venom phosphodiesterase and analysis of the mononucleotides released (148) and by E.coli alkaline phosphatase hydrolysis followed by snake venom phosphodiesterase hydrolysis and analysis of the nucleosides and nucleotides on Dowex 50 (section 2.2.7).

Trinucleotides were characterized by base ratio analysis according to the method of Wyatt (162). Free bases were released by hydrolysis with 90 % formic acid at 175°C for 30 minutes and separated by paper chromatography in the isopropanol-HCl solvent system. UV absorbing spots were cut out, along with adjacent blank areas and eluted with 0.1 N HCl in the case of guanine. The concentration of the bases was calculated from the appropriate extinction coefficients (Calbiochem chart, fifth revision, 1964).

E.coli alkaline phosphatase hydrolysis. The enzyme was treated to remove diesterases as described by Garen and Levinthal (182) and assayed with p-nitrophenylphosphate as substrate. Assay with Ca-bis-p-nitrophenylphosphate showed no diesterase activity to be present. Desalted oligonucleotides were dissolved in 0.02 M Tris-HCl buffer, pH 8.0, 0.01 M in $MgCl_2$. DN'ase I digests were adjusted to 0.02 M Tris-HCl buffer, pH 8.0, 0.01 M in $MgCl_2$ with 0.1 M Trizma base. To the prepared solutions 1.0 unit phosphatase/100 A_{260} units of oligonucleotide were added and the solution incubated for 6 hours at 37°C.

Alternatively 1.0 unit phosphatase/250 A_{260} units of oligonucleotide was added and incubated for 3 - 4 hours at 55°C. Two drops of chloroform were added to inhibit bacterial growth.

Phosphatase was removed by 4 phenol extractions and residual phenol by 4 ethyl ether extractions. Ether was removed by evaporation in a water bath at 50°C for 15 minutes. The enzyme activity was also eliminated by autoclaving at 120°C for 3 minutes (section 5.2.2). This method proved to be quantitative and fast and was used in all studies with DN'ase A.

Snake venom diesterase hydrolysis

Purification. Snake venom diesterase was purified according to Keller (149) in order to eliminate contaminating 5' nucleotidase activity which was reduced 2.3 to 4.2 fold depending on the batch of snake venom diesterase used.

Assay. The enzyme was assayed according to Koerner and Sinsheimer (163) using Ca-*bis*-*p*-nitrophenylphosphate as substrate or alternatively by the method of Razzell and Khorana (164) using thymidine 5' monophospho-*p*-nitrophenyl ester as substrate.

5' nucleotidase activity of snake venom diesterase preparations was assayed by a modification of the method of Felix, *et al*. (167). 2.0 ml of a solution containing 0.005 M 5'-AMP in 0.05 M Tris-HCl buffer, pH 9.2, 0.01 M $MgCl_2$ was incubated at 37°C, 0.05 to 0.2 mg of snake venom diesterase added and the mixture reincubated for 30 minutes. 1 ml of ice cold 72% perchloric acid was then added and the precipitate removed by centrifugation. 2 ml of the supernatant was analyzed for inorganic phosphate according to King (161). A unit is defined as the amount of Pi released under the above conditions in 30 minutes.

Hydrolysis. One half (1-2 ml) of the oligonucleotide solutions previously incubated with E.coli alkaline phosphatase was adjusted to pH 8.8 to 9.2 with Trizma base. 0.003 M $MgCl_2$ and 0.2 mg enzyme (potency 0.23-0.28)/100 A_{260} units of oligonucleotide added. The mixture was incubated for 12-24 hours at 37°C after addition of two drops of chloroform.

For oligonucleotide material not treated with phosphatase previously, the desalted oligonucleotides were dissolved in 0.05 M Tris-HCl, pH 8.8 or 9.2, 0.03 M $MgCl_2$, and treated as described previously.

Spleen diesterase hydrolysis

A second 1-2 ml sample of the phosphatase treated material was adjusted to pH 7.2 by addition of 1.0 N HCl. One unit of enzyme was then added/250 A_{260} units of oligonucleotide material and incubated for 24-36 hours at 37°C. Two drops of chloroform were added to the mixture.

The assay method for the enzyme was as described by Hilmoe (165) and modified by Worthington (166).

3' nucleotidase determination in spleen diesterase. 3' nucleotidase activity was assayed similarly to the 5' nucleotidase but with 2'-3'-AMP as a substrate in 0.05 M Tris-HCl buffer, pH 7.2. No release of Pi was detected by incubating with 0.5 units of spleen diesterase for 120 minutes at 37°C.

2.2.9 Spectral analysis of dinucleotides

Dinucleotides separated according to base composition were desalted as described in section 2.2.5 and dissolved in 0.001M sodium phosphate buffer, pH 7.0, to give a final A_{260} of 1.5 to 2.0/ml. Samples at pH 1 and 12 were prepared by titration of 1.6 ml of dinucleotide solution, pH 7.0, with acid or base using a Radiometer, model TTT 1c pH meter, calibrated with the appropriate standard buffers. Final volumes were 3.2 ml. Spectra were measured in a Gilford 2000 spectrophotometer from 220 to 320 nm at 1 nm intervals. The spectra were compared with those obtained from equimolar mixtures of 5' deoxymononucleotides prepared in the same manner. For measurement of the hyperchromicity, the desalted dinucleotide fractions were dissolved in 1.0 ml of 0.001 M sodium phosphate buffer, pH 7.0, 0.005 M in $MgCl_2$. A 0.4 ml sample of this solution was adjusted to pH 9.2 with 1 µl of 0.28 N NaOH, 1 µl of snake venom phosphodiesterase (3.8 µg enzyme, potency 0.33) added and the solution incubated for 2 hours at 37°C. A sample without enzyme diluted in the same way was used as control. The hydrolyzed sample and control were adjusted to pH 3.4 by addition of 1 µl of 0.56 N HCl, the absorbance at maximum wavelength and 260 nm measured and the hyperchromicity calculated. The extinction coefficients of the dinucleotides were calculated from the percent hyperchromicity at 260 nm, pH 3.4, and the extinction coefficients of the equimolar mixtures of the mononucleotides corresponding to the respective dinucleotide according to the expression

$$\epsilon_{260} \text{ dinucleotides} = \frac{\epsilon_{260} \text{ mononucleotide mixtures} \times 2 \times 100}{100 + \% \text{ hyperchromicity}} .$$

2.2.10 Miscellaneous

a) Pyrimidine heptanucleotides of general formula Py_7p_8 labeled with ^{32}P were a gift from Dr. W. E. Mushynski, obtained by fractionation of a diphenylamine-formic acid hydrolysate of bacteriophage λ DNA. ^{32}P was monitored by Cerenkov radiation (209) in a Beckman Liquid Scintillation Counter, model LS250. Samples were counted for 15 minutes.

b) DNA denaturation. DNA, 0.5 mg/ml in Tris-HCl 0.005 M, pH 7.6, was denatured by boiling the solution in a water bath for 30 minutes and quick cooling in an ice-salt bath.

CHAPTER 3

RESULTS

Purification and properties of pancreatic DN'ase

Commercial crystalline DN'ase I (Worthington) was used in preliminary experiments for the reassessment of some of the enzyme properties, for the development of new fractionation techniques and for studies on the mechanism of action of the nuclease. The instability of the enzyme in solution (130) and the findings by Potter (c.f., Laskowski, 120) who fractionated commercial DN'ase into five bands by electrophoresis on cellulose acetate, necessitated the purification of the Worthington enzyme to provide an homogeneous fraction for specificity studies.

3.1 Purification of DN'ase I

3.1.1 Chromatography on phosphocellulose

In 1969 Price, et al. (131) developed a procedure by which contaminating proteases responsible for the instability of the DN'ase preparation, were inactivated by FDP. They subsequently purified the enzyme on Sulfoethyl-Sephadex at pH 4.7 and obtained two major DN'ase fractions. Fractionation of the same preparation on phosphocellulose produced three DN'ase fractions (157). This last procedure was used for the purification of FDP treated amorphous DN'ase (Worthington, DP grade). The method was modified by extending the length of the column and using a shallower sodium acetate gradient. The results are shown in Fig. 1. The

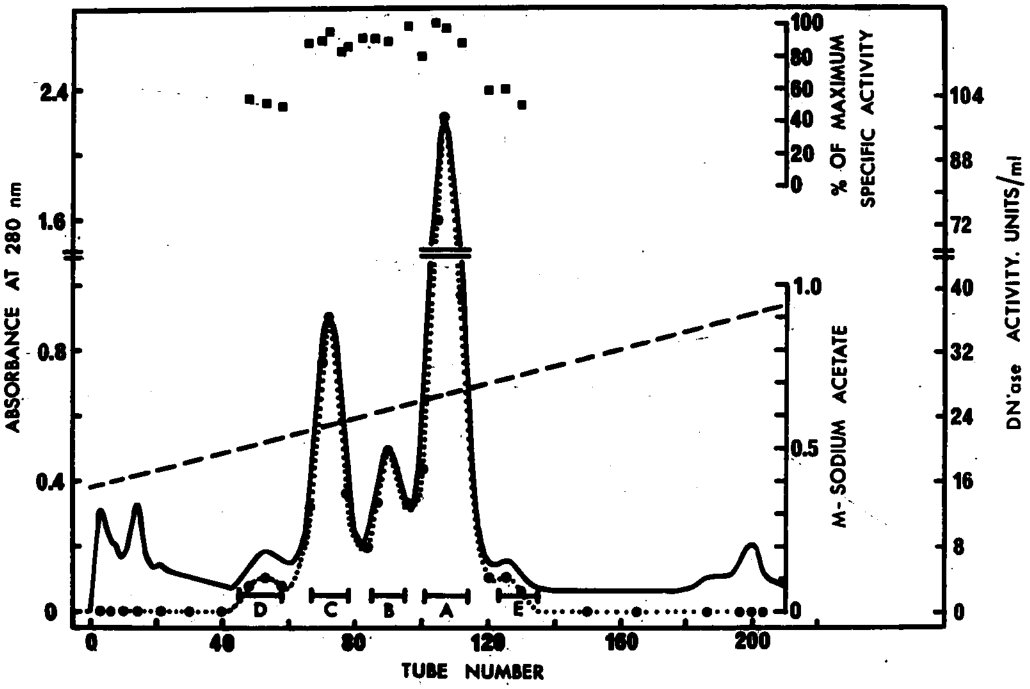


FIGURE 1

Purification of DN'ase I by Phosphocellulose Column Chromatography

FDP-treated amorphous DN'ase I (300 mg enzyme in 6 ml of 0.38 M sodium acetate buffer, pH 4.7, treated with 400 µl FDP for 30 minutes at 25°C) was applied to the phosphocellulose column (88 x 2.2 cm, sodium form). The column was washed with 190 ml of 0.38 M sodium acetate buffer, pH 4.7, and a linear gradient of 0.38 M to 1.0 M sodium acetate buffer, pH 4.7, (total volume: 2.0 l) applied. 8.5 ml fractions were collected at 70 ml/hour. Samples at several stages were analyzed for enzyme activity (section 2.2.3).

—— A_{280}
●--● DN'ase activity
---- molarity of sodium acetate
├──┤ pooled fractions

chromatography results in elution of an initial protein peak devoid of DN'ase activity, followed by five enzymatically active fractions. The major DN'ase band was designated DN'ase A in order to maintain the previous classification by Price, *et al.* (131) and Salnikow, *et al.* (157). When 500 mg enzyme were chromatographed on the same column a sixth peak (F), corresponding to about 0.5% of the total enzyme eluted, appeared on the chromatogram. At the end of the elution of DN'ase activity more inactive protein was obtained.

Fractions A, B and C all had constant specific activities throughout their elution, a positive indication on their degree of purity, whereas fraction D and E had only 50% of the maximal specific activity. The fractions were pooled as indicated in Fig. 1 (├─┤), the intermediate fractions were also pooled and protein concentration and specific activities measured (section 2.2.3). The results obtained in the purification are summarized in Table 2. 78% of the total protein was recovered whereas the specific activity of the main fraction increased during the purification. The three major fractionswere then dialyzed at pH 5.2. No precipitate was formed at this pH and all of the loaded activity was recovered compared to a recovery of 74-90% at pH 4.7 (131), the isoelectric point for the protein, where solubilization is minimal.

The fractions were then lyophilized and stored at $-20^{\circ}C$. No activity was lost by this treatment. DN'ase A was then rechromatographed on phosphocellulose with an intermittent gradient elution method as indicated in Fig. 2a and section 2.2.2.

TABLE 2

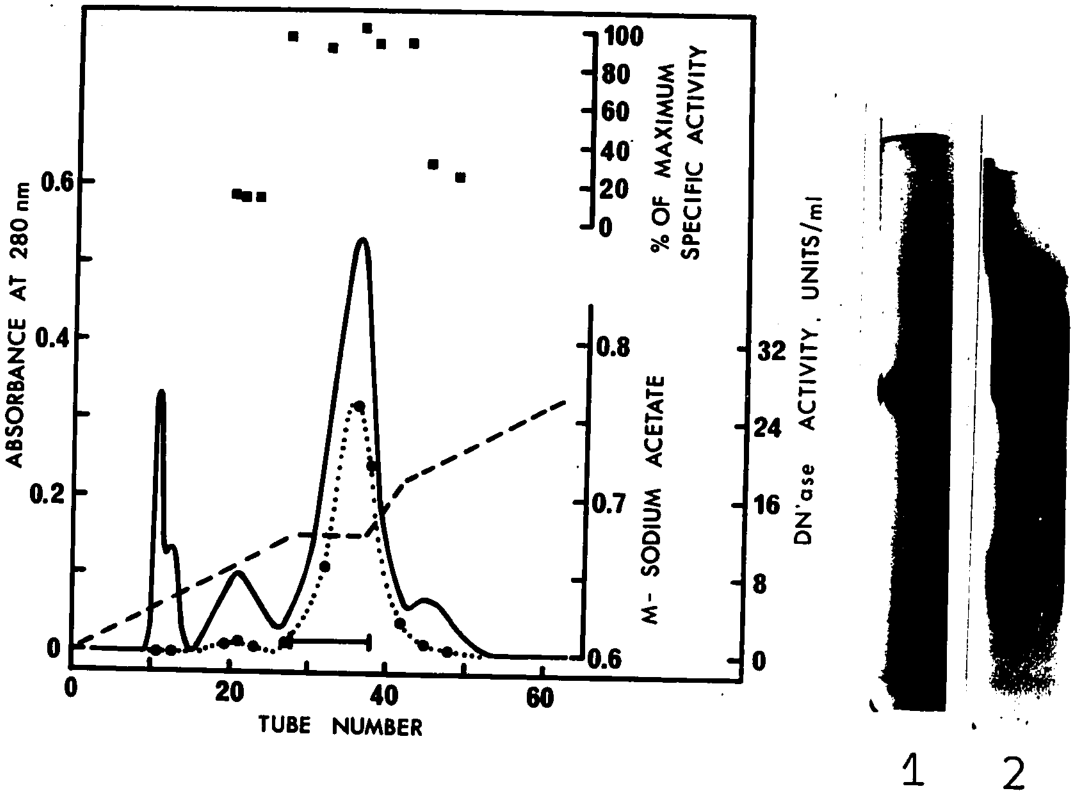
Chromatographic Purification of DN'ase I on Phosphocellulose

DN'ase fraction	Total activity units	% of total activity	Specific activity units/mg protein
DN'ase (DP)	18,144.0	100.0	47.0
A	8,400.0	46.9	61.5
B	2,140.3	12.0	64.8
C	3,460.8	19.0	59.8
D	739.2	4.1	48.0
E	369.6	2.1	30.1
Intermediate	2,772.0	15.5	60.0

Total activity recovered: 98.5%.

Total protein recovered: 78.0%.

Increase in specific activity for DN'ase A: 30.7%.



a

b

FIGURE 2

a) Rechromatography of DN'ase A on a Phosphocellulose Column

A lyophilized portion of DN'ase A (40 mg dissolved in 10 ml of 0.6 M sodium acetate buffer, pH 4.7) was applied to a phosphocellulose column (94 x 2.2 cm, sodium form) and washed with 160 ml of 0.6 M sodium acetate buffer, pH 4.7. An intermittent gradient of 0.6 M to 1.0 M sodium acetate buffer, pH 4.7, was applied (total volume: 1.5 l). 10 ml fractions were collected at 70 ml/hour. Samples at several stages during the elution were analyzed for enzyme activity.

—— A_{280}
●--● DN'ase activity
---- molarity of sodium acetate
├──┤ pooled fractions

b) Polyacrylamide Gel Electrophoresis of DN'ase A

50 μg of rechromatographed DN'ase A (1) and 80 μg of crystalline DN'ase I (2) were analyzed by gel electrophoresis as described in section 2.2.2 in 0.37 M Tris-glycine buffer, pH 8.3. Running gel: 250 volts for 5 hours.

The first fraction eluted was totally devoid of DN'ase activity and is probably a denatured portion of the enzyme. The main DN'ase A peak was flanked by two adjacent fractions having lower specific activities. These probably represent contaminations corresponding to fractions B and E (see Fig.1). By continuing the stepwise elution at 0.675 M sodium acetate buffer, pH 4.7, fraction E was well separated from the main peak, but with a pronounced trailing of the last portion of fraction A. 84.2% of the enzyme activity was recovered in this second chromatography. The DN'ase A had a specific activity of 56.3 units/mg protein. The enzyme was dialyzed as before and stored at -20°C.

3.1.2 Homogeneity and stability of DN'ase A

DN'ase A samples were examined by polyacrylamide gel electrophoresis as described by Davis (168) (section 2.2.2). A single sharp band was observed when 40 to 80 µg enzyme were analyzed, Fig. 2b. The enzyme was devoid of any phosphatase, exonuclease or 5' nucleotidase activity when incubated for 4 hours at 37°C under the assay conditions described in section 2.2.8. Four wide bands could be seen when crystallized and amorphous DN'ase were run on the gels in the same manner, and in some samples the presence of two extra bands could be determined.

The activity of the enzyme, after treatment with FDP, chromatography on phosphocellulose and lyophilization was unchanged following storage at -20°C for at least 14 months.

In 0.005 M Tris-HCl, pH 7.6, and at 0.4 mg/ml the activity was the same over a period of 30 days at room temperature and over a period of 4 months at 4°C. By contrast, crystallized DN'ase I lost its activity at a rate of 30% per hour when stored at 25°C under identical conditions, and at a rate of 8 - 15% per hour in the presence of 3×10^{-3} M $MgCl_2$ or 2×10^{-3} M $MnCl_2$.

3.2 Kinetics of DNA hydrolysis by DN'ase

Although many aspects of the kinetics and specificity of DN'ase I have been established, certain discrepancies remain. This includes the enzymes activation by different divalent cations, ionic strength and pH dependency, protein-activity relationship and amount of enzyme required for different types of hydrolysis. The interdependence of some of these parameters has been studied in various laboratories (130, 133, 134, 137).

The first series of kinetic experiments were performed with commercial crystalline DN'ase I but the major part of the results on the specificity of the enzyme were obtained using the DN'ase A fraction. Optimal conditions for the digestion of DNA at a concentration of 0.5 mg/ml were determined by kinetic studies and were monitored by hyperchromic increase at 260 nm (section 2.2.3, assay B). Only data related to these studies will be presented in this section and, where necessary, will be compared to the earlier results with the crystalline DN'ase I.

All experiments were carried out with DNA and enzyme that had been dialyzed extensively against EDTA (see Methods) and the ions used were spectrometrically pure salt with insignificant contamination.

3.2.1 Proportionality between enzyme activity and protein concentration

Enzyme activity was shown to be proportional to protein concentration over wide ranges depending on the ion analyzed. It can be seen in Fig. 3 that with DN'ase A the linear proportionality is maintained in a 4 fold range for Mg^{++} (0.2 - 0.8 μg protein), 40 fold for Mn^{++} (0.1 - 4.0 μg protein), 4 fold for Sr^{++} (2.0 - 8.0 μg protein), 20 fold for Ni^{++} (0.5 - 10.0 μg protein), 3.2 fold for Zn^{++} (10.0 - 32.0 μg protein). Under the conditions of assay the protein ratio required to obtain the same initial velocity with native DNA (at 10 units of activity) is Mg^{++}: Mn^{++}: Sr^{++}: Ni^{++}: Zn^{++}; 1:3.5: 11.7: 20.4: 51.4.

In the presence of ions such as Cd^{++}, Ca^{++} and Ba^{++}, concentrations of 4-13 μg enzyme/ml were required to obtain linear protein-activity relationships.

Mn^{++} and Ni^{++} showed an initial linear relationship between enzyme concentration and activity while Mg^{++}, Sr^{++} and Zn^{++} showed a lag at low enzyme concentration. The lag was not due to inactivation of the diluted enzyme by the presence of the metal and buffer. Previous incubation of the enzyme with the buffer and the metal did not produce any loss of activity. These three ions also showed a lag when the kinetics of DNA hydrolysis with time were determined. With Mn^{++}, Ni^{++}, Co^{++}, Ca^{++}, Cd^{++} and Ba^{++} no lag was observed. An example of the two different types of kinetics with time

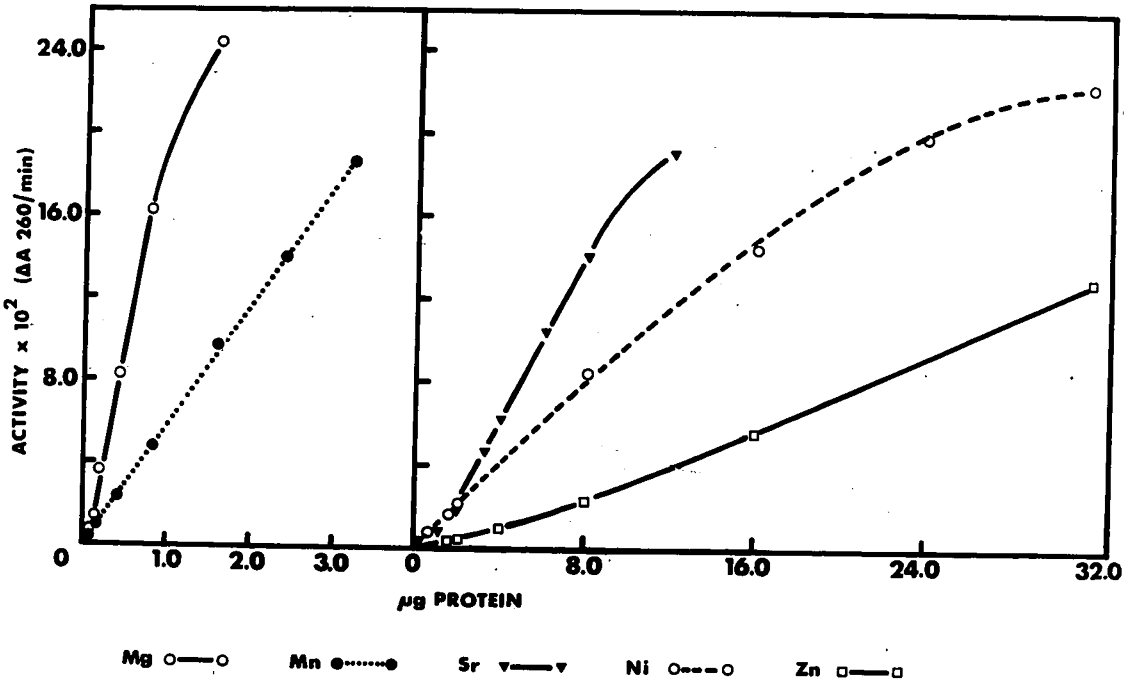


FIGURE 3

Proportionality Between DN'ase A and Protein Concentration in the Presence of Various Metal Cations

The incubation mixture contained 0.5 mg/ml calf thymus DNA in 1.0×10^{-2} M Tris-HCl buffer, pH 7.6. Metal ion: Mg^{++}, 3.3×10^{-3}M; Mn^{++}, Sr^{++} or Ni^{++}, 1.0×10^{-3}M; Zn^{++}, 4.0×10^{-4} M. DN'ase A: as indicated in the abscissa. Total volume of mixture 0.3 ml. The reactions were monitored at 260 nm at 37°C (assay B, section 2.2.3). For reasons of simplicity the metal cations are represented without the valence signs (i.e. Mg^{++} = Mg).

is given in Fig. 4. These observations with DN'ase A can be correlated to the two different mechanisms of DN'ase-metal hydrolysis postulated by Melgar and Goldthwait (137) (see Discussion).

3.2.2 DN'ase A-metal-substrate relationship

The optimal concentration of the activating metal ion has been shown to depend on the DNA concentration and parameters such as the buffer used, pH and ionic strength (130, 134, 169, 170). In agreement with these conclusions it was shown that with DN'ase A the rates reach a limiting value at 0.15 to 0.3 mg DNA/ml depending on the activating cation in the reaction. Optimal metal ion concentrations for two different DNA concentrations in 0.005 M Tris-HCl, pH 7.5, are presented in Table 3.

The rates with heat denatured DNA in the presence of optimal concentrations of Mg^{++} or Mn^{++} at pH 7.5 were always 50 to 70% of the rates with native DNA (39, 119).

3.2.3 Effect of pH on enzyme activity

The pH-activity dependence for all the investigated cations was determined in Tris-maleate buffer which never contributed more than 0.005 M ionic strength to the final reaction mixture. At this concentration the influence of the buffer had no significant effect on the DN'ase A activity. The results for the pH-activity relationship with 0.5 mg/ml DNA at the optimal ion concentration (Table 3) for the various cations tested is given in Fig. 5.

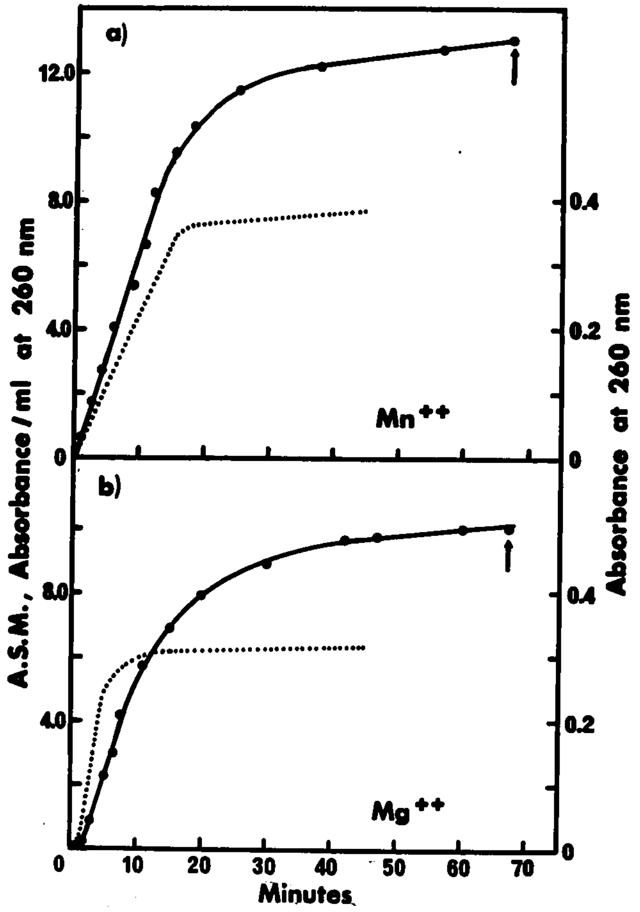


FIGURE 4

Time–activity Record of DNA Hydrolysis by Mn^{++} or Mg^{++}-activated DN'ase A

The hyperchromic increase at 260 nm was monitored in quartz cells with a 0.1 cm optical path in a Gilford 2000 spectrophotometer as described in section 2.2.3, assay B. Acid soluble material (ASM) release was determined at 260 nm in 0.2 ml samples as described in the same section. The incubation mixture contained 0.5 mg/ml calf thymus DNA in 1.0×10^{-2}M Tris-HCl buffer, pH 7.5. Metal ion: 3.3×10^{-3} M. Temperature: 37°C.

a <u>Mn^{++}-activation</u>: 20 mg DNA 0.4 μg/ml enzyme (0.026 units by assay B). Digestion time: 67.5 minutes (digest 13, Table 14).

b <u>Mg^{++}-activation</u>: 100 mg DNA 0.42 μg/ml enzyme (0.054 units by assay B). Digestion time: 67.0 minutes (digest 7, Table 14).

.... A_{260}

●—● acid soluble material at A_{260}

TABLE 3

Dependence of Optimal Ion Concentration on Substrate Concentration at pH 7.5

Ion	Optimal cation concentration, molar 0.05 mg DNA/ml	0.5 mg DNA/ml
Mg^{++}	5.0×10^{-4}	1.0×10^{-3}
Mn^{++}	3.3×10^{-4}	1.0×10^{-3}
Co^{++}	3.3×10^{-4}	1.0×10^{-3}
Sr^{++}	3.3×10^{-4}	1.0×10^{-3}
Ni^{++}	1.0×10^{-4}	3.3×10^{-3}
Zn^{++}	1.0×10^{-4}	3.0×10^{-4}
Cd^{++}	5.0×10^{-4}	1.0×10^{-3}
Ba^{++}	1.0×10^{-3}	1.0×10^{-2}
Ca^{++}	1.0×10^{-3}	1.0×10^{-3}

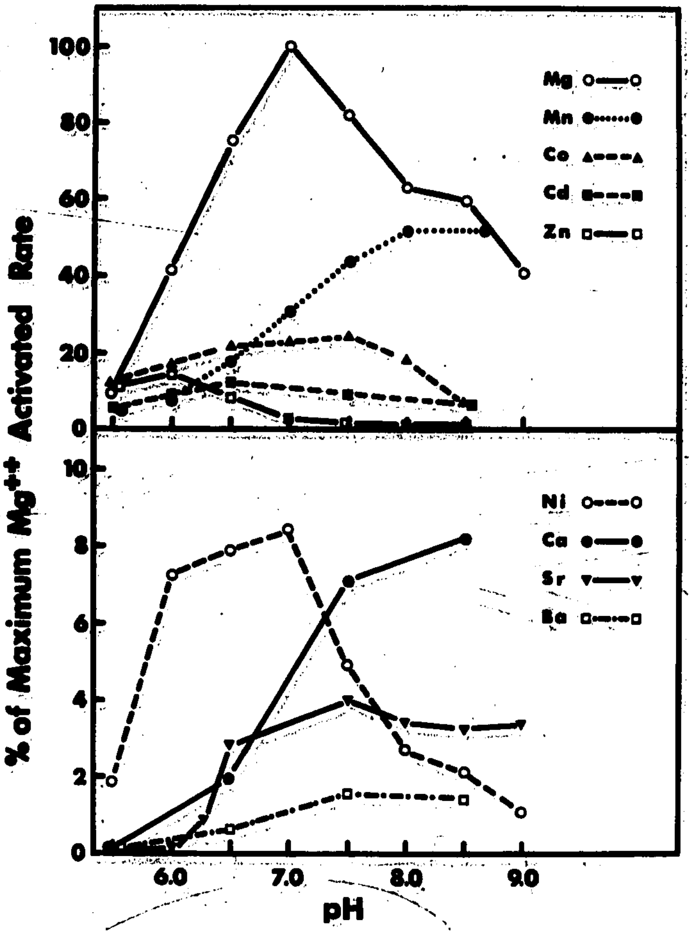


FIGURE 5

Dependence of pH Optimum of DN'ase A on Metal Ion Activation

The incubation mixture contained 0.5 mg/ml calf thymus DNA, in 5×10^{-3} M Tris-sodium maleate buffer at the pH indicated in the abscissa. Metal ion: Mg^{++}, 3.3×10^{-3}M; Mn^{++}, Sr^{++}, Ni^{++}, Co^{++}, Ca^{++}, Ba^{++} and Cd^{++}, 1.0×10^{-3} M; Zn^{++}, 4.0×10^{-4} M. DN'ase A from 0.4 to 4.0 μg per assay as required for optimum conditions for each ion. Total volume of mixture 0.3 ml. The reactions were monitored at 260 nm at 37°C. The ordinate was plotted as % of maximum Mg^{++} activated rate from specific activity data for each ion as measured by assay B, section 2.2.3. The Mg^{++}-activated DN'ase A at pH 7.0 had a specific activity of 210.0 units/mg protein.

Optimum pH generally varies between 6.5 to 8.5 depending on the ions used. For Mg^{++} a sharp optimum was found at pH 7.0 in contrast to a broader range with DN'ase I at 0.05 mg/ml DNA and optimum Mg^{++} concentration (130). For Mn^{++} the optimum pH was between 8.0 and 8.5, similar to Ca^{++} and Ba^{++}.

Broad pH–activity curves were found for Co^{++}, Cd^{++} and Sr^{++}. As demonstrated before (171) Zn^{++} has a maximum at pH 6.0 with lower activity as the pH increases. The rate with Mn^{++} at pH 8.5 was identical to the rate with Mg^{++} at the same pH, but half the maximal rate of Mg^{++} at pH 7.0 where activation was optimal.

3.2.4 Activation of DN'ase A by bivalent cations

The results of the activation of DN'ase A by several divalent cations at various concentrations are presented in Fig. 6.

Mg^{++}, Mn^{++} and Co^{++} were found to be the most effective ions as reported for DN'ase I (119, 132). Mn^{++} and Co^{++} at concentrations above 1.0×10^{-2} M caused the formation of a gel. Mn^{++} always reached 50 to 70% of the maximal Mg^{++} activated rate when 0.5 mg/ml DNA were hydrolyzed. However, when DN'ase I was assayed with 0.05 mg/ml DNA the Mn^{++} activation was 2.2 to 2.5 fold higher than with Mg^{++}. This is in accord with Wiberg (133), Vanecko and Laskowski (138) and Melgar and Goldthwait (137) and contrary to the results of Shack and Bynum (134) who postulated that the high ionic environment in Wiberg's experiments might have produced such an effect.

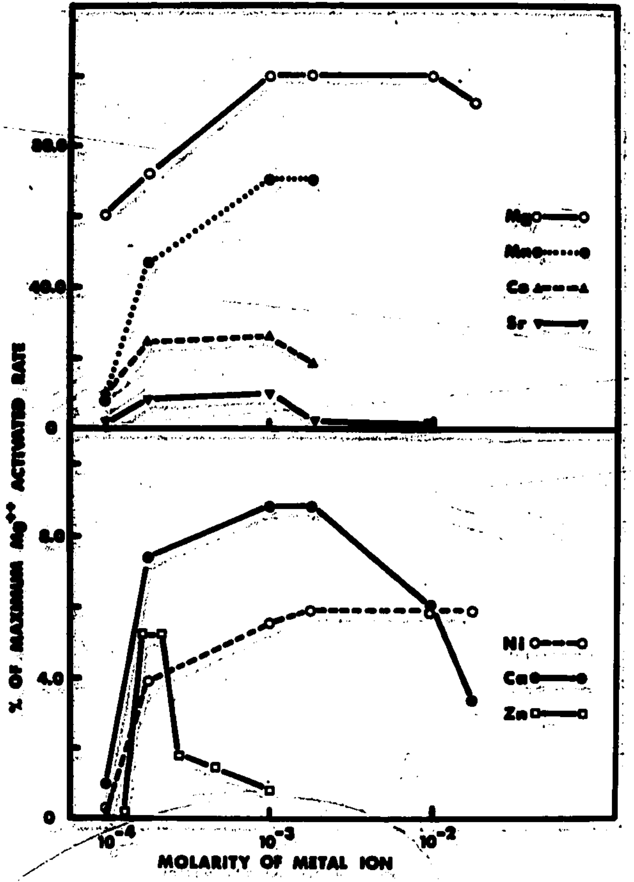


FIGURE 6

Effect of Bivalent Metal Cations on DN'ase A Activity

The incubation mixture contained 0.5 mg/ml calf thymus DNA in 5×10^{-3}M Tris-HCl, pH 7.5. Metal ion as indicated in the abscissa. DN'ase A from 0.4 to 1.6 µg per assay as required for each ion. Total volume of mixture 0.3 ml. The reactions were monitored at 260 nm at 37°C. Specific activity for maximal Mg^{++}-activated rate 157.5 units/mg (see legend to Figure 5).

Contradictory reports have been given on the activating effect (171, 172) or inhibitory effect (134, 173) of Zn^{++}. In our experiments it was found that Zn^{++} has a sharp activating effect at 3.0 to 4.0 x 10^{-4} M whereas below or above this concentration the effect is strongly diminished. Under these conditions of assay no activity could be detected with Zn^{++} at concentrations below 1.0 x 10^{-4} and over 3.0 x 10^{-3} M. At optimal conditions the Zn^{++}-activated DN'ase never accounted for more than 6% of the total Mg^{++}-activated rate when specific activities were compared. The results were similar when acid soluble material determinations were performed under identical conditions. Thus, our data does not support that of Eichhorn, *et al*. (174) in relation to the high activation properties of Zn^{++} and Co^{++}.

Ca^{++} was found to be a good activator with an optimum concentration similar to Mn^{++} (133, 134, 172). Ni^{++} had an optimal activity at 3.3x 10^{-3} M and was maintained constant over a 10 fold concentration. The same results and those with Sr^{++}, with a wide optimum between 3.3 x 10^{-4} and 1.0 x 10^{-3} M have been reported previously (137).

Other ions tested were Cd^{++}, Ba^{++}, Fe^{++}, Cu^{++} and Sn^{++} at concentrations from 10^{-5} to 10^{-1} M. Cd^{++} was found to activate, contrary to other reports (134). Optimal activity was found at 2.0 x 10^{-3} M. Ba^{++} was optimal at a relatively high concentration, 1.0 x 10^{-2} M. No reaction could be detected with Sn^{++} and Fe^{++} at several concentrations and pH's. Cu^{++} produced very slow reactions at high concentrations of enzyme (25 µg/ml), and gels formed, probably due to the formation of a complex between Cu^{++} and Tris which is stable at this pH (175). For these reasons studies with this ion were discontinued.

3.2.5 Ionic strength and synergism

The non-specific inhibition of NaCl on the Mg^{++}, Mn^{++} and Co^{++} activated reactions (134) was shown also with DN'ase A and was partially reversed by addition of an excess of divalent cation. In 0.1 M NaCl the rates at optimal Mg^{++}, Mn^{++} and Co^{++} concentrations were diminished 80, 42 and 75%, respectively. The activity remained higher in the presence of Mn^{++} as previously observed by Melgar and Goldthwait (137).

The influence of increasing ionic strength of Tris-HCl buffer at pH 7.5, was tested with Mg^{++} in 0.01, 0.02 and 0.05 M buffer. The activities, when compared to that at 0.005 M, diminished by 6, 20 and 88%, respectively. The inhibition with Tris-HCl buffer is greater than the non-specific inhibition with NaCl. This could be a reflection of Tris-metal ion interaction at high Tris concentrations (175), where part of the free metal ion is complexed, hence lowering its efficient concentration below the optimum and enhancing the inhibitory effect.

A synergistic effect was demonstrated with DN'ase A when Mg^{++} and Ca^{++} were combined. When a suboptimal concentration of 1.0×10^{-4} M Ca^{++} was added to 3.3×10^{-2} M Mg^{++}, the activity of the enzyme was increased 1.6 to 2.0 fold over the Mg^{++}-activated optimal rate. Shack and Bynum (134) showed that the synergistic effect of Ca^{++} is only demonstrated at suboptimal concentrations of Mg^{++}, and even become inhibitory above 10^{-4} M Ca^{++}. In the present study the activity with Mg^{++} was increased when Ca^{++} was added. This agrees with other reports (133, 137, 176) but the synergistic effect was always to a lesser extent, 1.6 to 2.0 fold compared to 7 to 34 fold in one case (137).

3.2.6 Inhibition of the enzyme activity and irreversible inactivation

It has been reported by several investigators (119) that DN'ase I can be inhibited by low concentrations of anions such as citrate, arsenate, fluoride and particularly EDTA. We choose to work with EDTA for its singular chelating properties with all divalent cations used in this study (177). With EDTA at 3 times the optimal cation concentration of any ion studied, complete and instantaneous inactivation of the enzyme was achieved. Addition of excess cation to the inhibited reaction restored the activity, whereas addition of enzyme alone did not. A typical experiment for a Mn^{++}-activated reaction is shown in Fig. 7.

As shown above, EDTA inhibition is reversible and the necessity for total inactivation in experiments involving subsequent digestion with other enzymes required a procedure for total and irreversible DN'ase denaturation. The following methods were investigated.

a) The removal of DN'ase I on carboxymethyl-cellulose has been reported to be effective for large concentrations of enzyme (0.4 mg/ml) (178).

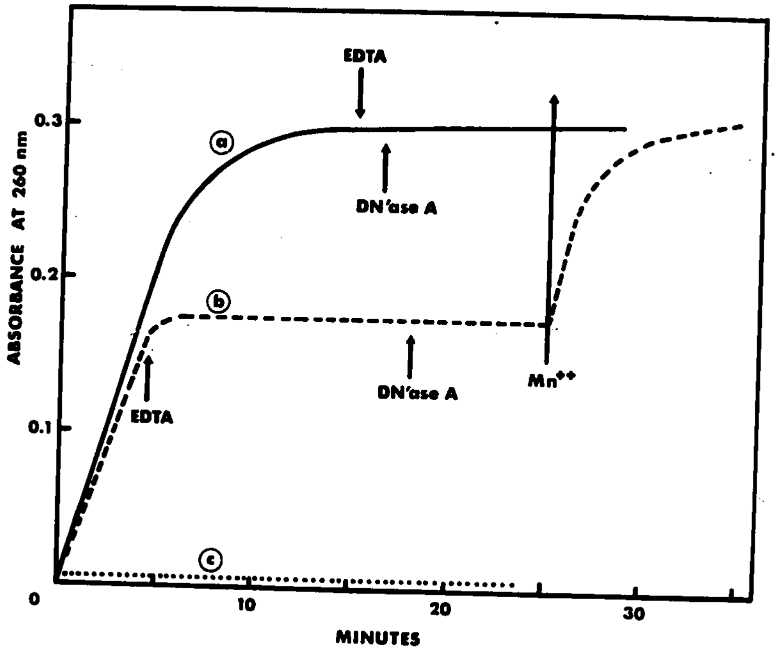


FIGURE 7

Inhibition of DN'ase by EDTA

The incubation mixtures contained 0.5 mg/ml calf thymus DNA in 5 x 10^{-3} M Tris-HCl, pH 7.5. Mn^{++} 1.0 x 10^{-3} M. DN'ase A at zero minute 0.6 μg/0.3 ml (0.045 units by assay B). Total volume of mixture 0.3 ml. The reactions were monitored at 260 nm at 37°C.

——— a) 3.0 x 10^{-3} M EDTA added at 15 minutes. At 16.5 minutes 0.6 μg DN'ase A added. At 25 minutes 6.0 x 10^{-3} M Mn^{++} added.

----- b) 3.0 x 10^{-3} M EDTA added at 4.5 minutes. At 18 minutes 0.6 μg DN'ase A added. At 25 minutes 6.0 x 10^{-3} M Mn^{++} added.

········· c) 3.0 x 10^{-3} M EDTA added at zero minute

Under similar conditions but in the absence of EDTA, total adsorption of the enzyme was achieved when the reaction mixture was shaken for one hour with the exchanger at a weight ratio of 1:60 (enzyme:CM-cellulose) at pH 7.5 and a total ionic strength of 0.01 M. However, after 30 minutes of treatment, 22% of the enzymatic activity was still present. In the presence of EDTA 28% of the enzyme activity remained at 30 minutes. This method was rejected due to the length of time required for complete adsorption of the enzyme.

b) Removal with phosphocellulose. The higher capacity of phosphocellulose (7.4 meq/gr) compared to CM-cellulose (0.6 - 0.7 meq/gr) and the results on the purification of DN'ase on this exchanger suggested the possibility of its use for the removal of the enzyme. Experiments were performed as indicated by Taborsky (179) for the desalting of RN'ase on CM-cellulose. The results of these experiments indicated that in 0.005 M sodium acetate, pH 4.0, and in the presence of 3.0×10^{-3} M to 1.0×10^{-2} M metal ion, 1 mg phosphocellulose can remove a maximum of 0.2 mg DN'ase when shaken at room temperature for 20 minutes (ratio of DN'ase to exchanger 1:5 w/w).

Lower amounts of exchanger could be used to completely remove DN'ase in the experiments cited, but when the same procedure was used after addition of EDTA and in a total volume of 100 ml the enzyme was not completely removed. This is not due to the large volume of the sample since a phosphocellulose column (9 x 2.5 cm) did not remove all contaminating DN'ase and the delay in adsorption time was considerable (see Fig. 8, curve d).

c) Phenol or chloroform: n-amylalcohol deproteinization (180). The enzyme was totally inhibited when treated by either of these methods under optimal conditions of hydrolysis and in the presence of EDTA. However, large volumes of reagent were needed when 30 to 120 mg of DNA were digested and the repeated extractions of the reagents caused 10 - 20% losses of the oligonucleotide material.

d) Alkaline irreversible denaturation. Treatment of the EDTA inhibited digestion mixture with potassium hydroxide (30 minutes, pH 13.0, 25°C) followed by neutralization with perchloric acid (section 2.2.4) produced a totally inactive DN'ase as seen in curve b, Fig. 8. The oligonucleotides were recovered completely after the elimination of the insoluble salt as judged from UV and phosphate (161) determination. The method proved to be reproducible and quantitative. Furthermore, the hydrolyzed material was not affected by the alkaline pH (181). The alkali treatment was used in all further experiments.

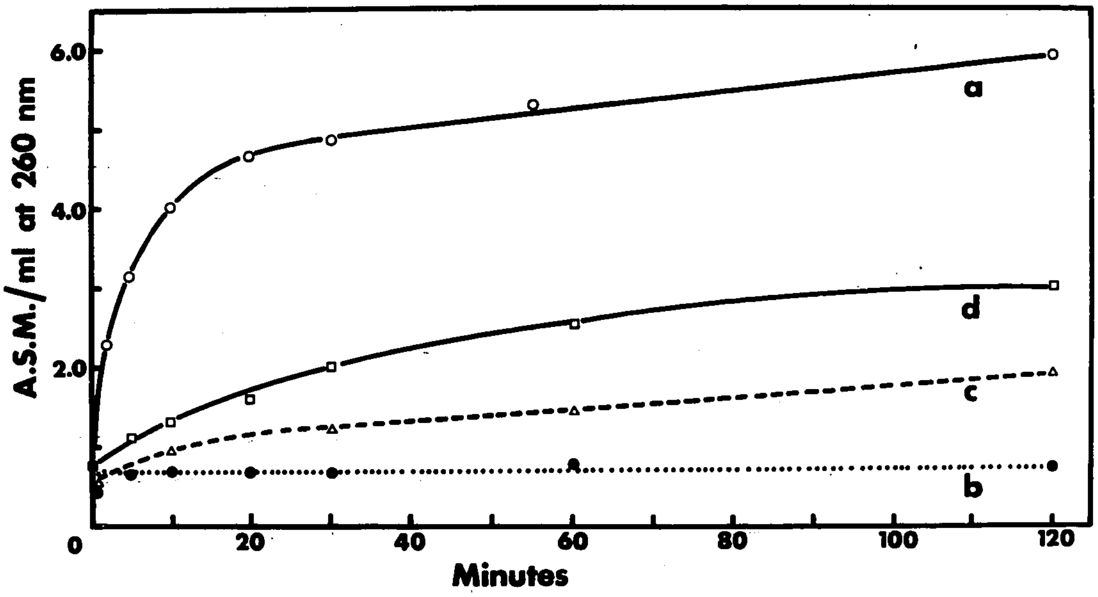


FIGURE 8

Elimination of DN'ase A from the Digestion Mixture

The incubation mixtures contained 0.5 mg/ml calf thymus DNA in 5.0×10^{-3} M Tris-HCl buffer, pH 7.5. Mn^{++}, 6.0×10^{-3} M. DN'ase A, 0.2 μg/ml. Incubation at 55°C. Assay, acid soluble material (ASM) release at 260 nm (section 2.2.3). Total DNA digested: a, b and c, 5 mg; d, 50 mg.

a) 2×10^{-3} M EDTA + enzyme added for pre-incubation. At zero minute Mn^{++} was added and acid soluble material monitored.

b) 2×10^{-3} M EDTA + enzyme added. The mixture was adjusted to pH 13.0 with KOH. At zero minute Mn^{++} was added and the reaction monitored.

c) A partial DN'ase digest performed as in experiment a and inhibited by 2.0×10^{-3} M EDTA was used. The digest produced 3.1 A_{260} units/ml of acid soluble material. It was adjusted to pH 13.0 with KOH to denature the enzyme and incubated at 55°C for 60 minutes with no increase in acid soluble material release. Then, DN'ase A + Mn^{++} were added (zero minute in this graph) and further acid soluble material release monitored.

d) A 100 ml DNA mixture in 0.005 M sodium acetate buffer, pH 4.0, containing 2×10^{-3} M EDTA and enzyme were passed through a 9 x 2.5 cm phosphocellulose column previously equilibrated in the same buffer. The solution was adjusted with 2.0 M Tris to pH 7.5 . Mn^{++} was added, incubated at 55°C and acid soluble material release monitored.

CHAPTER 4

Development of procedures for the separation and analysis of oligonucleotides, nucleotides and nucleosides

4.1 Fractionation of oligonucleotides according to chain length

The object of the work presented in this chapter was to develop the chromatographic procedures necessary for the fractionation of oligonucleotides according to chain length, and the oligonucleotide isostichs, nucleotides and nucleosides according to base composition. Furthermore an investigation of the specificity of hydrolysis of DN'ase A under various conditions was undertaken. All the fractionation experiments were performed on DN'ase I digests of E.coli and calf thymus DNA. Numerous hydrolysates were prepared for this purpose. The conditions for the three hydrolysates whose fractionation will be described were as follows.

Digest A: E.coli K12 DNA (2 mg/ml in 0.005 M $MnCl_2$, 0.005 M Tris-HCl buffer, pH 7.6) was incubated with 0.12 units/ml DN'ase I at 37°C for 6 minutes. Total DNA digested was 100 mg.

Digest B: Calf thymus DNA (2 mg/ml in 0.005 M $MgCl_2$, 0.005 M Tris-HCl buffer, pH 7.6) was incubated with 0.08 units/ml DN'ase I at 25°C for 8 minutes. Total DNA digested was 350 mg.

Digest C: E.coli K12 DNA (0.584 mg/ml in 0.005 M $MnCl_2$, 0.005 M Tris-HCl buffer, pH 7.6) was incubated with 0.176 units/ml DN'ase I at 37°C for 30 hours. Total DNA digested was 400 mg.

The hydrolyses were followed by an increase in absorbance at 260 nm and the reaction stopped completely by addition of EDTA at a concentration 40% higher than that of the activating ion (sections 2.2.3 and 2.2.4).

4.1.1 DEAE-cellulose and DEAE-Sephadex columns

Partial and extensive DNA digests were prepared as indicated above. In preliminary experiments the resulting oligonucleotide mixtures were fractionated according to chain length on DEAE-cellulose (83) or DEAE-Sephadex (160, 183) in the presence of 7.0 M urea buffers. Results of the chromatography of a partial digest of E. coli DNA are shown in Fig. 9.

The incomplete resolution of oligonucleotides on DEAE-cellulose in the presence of 7.0 M urea confirms the previous data presented by Bartos, et al. (86), Carrara and Bernardi (38) and Nestle and Roberts (184).

The poor resolution is attributed to factors other than the net charge of the nucleotide phosphate (86, 160, and section 6.3.1). Changes in the length to width ratio of the column, volume of gradient, eluting salt and pH of the buffers from 5.0 to 8.5 did not improve the separation. Rechromatography of fraction IV from Fig. 9a in a shallower gradient indicated that the main peak was contaminated with the adjacent fractions. The higher the amount of long-chain oligonucleotides the poorer was the separation between the fractions. Above the octanucleotide level resolution disappears.

Peak IX in the chromatogram, a fraction rich in purines (70% of total), was preferentially delayed on the column and addition of higher salt eluted a

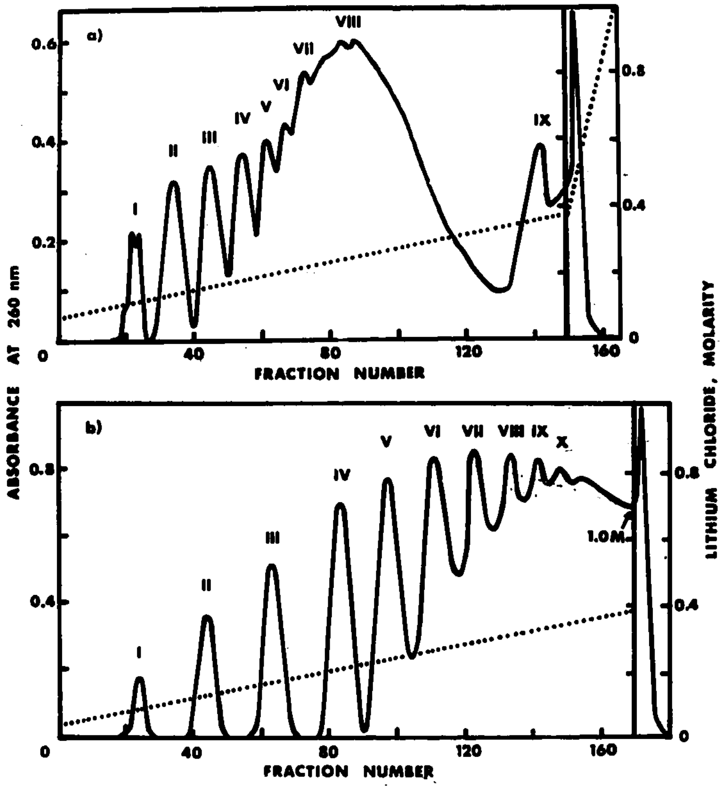


FIGURE 9

Fractionation of a DN'ase I Digest of E.coli K12 DNA on DEAE-cellulose and DEAE-Sephadex at 25°C

The digest (100 mg E.coli K12 DNA, 6.0 units DN'ase I, 37°C, 6 minutes, section 4.1, digest A) was fractionated as follows:

a) DEAE-cellulose: 28 mg of hydrolyzed DNA were chromatographed on a column of DEAE-cellulose (50 x 0.9 cm, chloride form) with a linear gradient of 0.05 to 0.4 M LiCl in 0.01 Tris-HCl, 7.0 M urea buffer, pH 7.6. Total volume of eluent 2.0 l. 12.6 ml fractions were collected at 60 ml/hour. At fraction number 150 a second gradient in the same buffer was applied from 0.36 to 1.0 M LiCl (total volume: 200 ml).

b) DEAE-Sephadex: 50 mg of the hydrolysate were chromatographed on a DEAE-Sephadex column (60 x 0.9 cm, chloride form) with a linear gradient of 0.02 to 0.4 M LiCl in 0.005 M Tris-HCl, 7.0 M urea buffer, pH 7.6. Total volume of eluent 3.0 l. 17.5 ml fractions were collected at 70 ml/hour. The column was eluted with 1.0 M LiCl at the end of the gradient.

—— A_{260}

---- molarity of LiCl.

large fraction of long oligonucleotides.

The use of DEAE-Sephadex improved the separations as demonstrated before by Rushizky, et al. (160) for RN'ase digests. As noticed in Fig. 9b, 10 fractions can be distinguished when part of the same digest was chromatographed. However, above the pentanucleotide peak the separation between the isostichs showed considerable overlap. As with DEAE-cellulose columns, changes in the chromatographic conditions did not improve the fractionations.

4.1.2 DEAE-Sephadex columns at 65°C

The chromatographic fractionation of a DN'ase I digest of calf thymus DNA according to chain length on DEAE-Sephadex at 65°C in 7.0 M urea, pH 7.6, is shown in Fig. 10a. The absorbance profile shows a separation of 14 peaks corresponding to oligonucleotides of chain length 1 to 14. A peak of longer oligonucleotides was eluted from the column by 1 M LiCl. Variations in column length, column diameter, pH of the eluting buffer, and temperature were all investigated. Separation deteriorated below pH 6 and 45°C and with shorter and broader columns.

Removal of the 5' terminal phosphate groups from the oligonucleotides by digestion with E.coli phosphomonoesterase prior to chromatography resulted in an improved resolution of the longer oligonucleotides and separation of 15 peaks as shown in Fig. 10b. The experiments in Figs. 10a and 10b are both from the same initial DN'ase I digest. In Fig. 10b the first peak is nucleosides which are

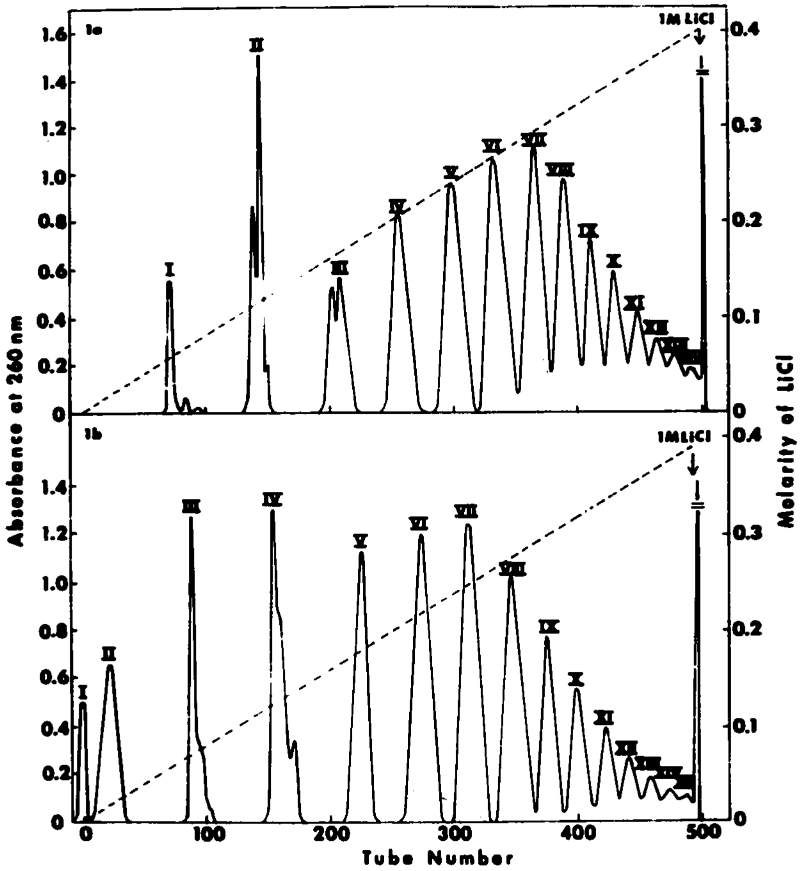


FIGURE 10

Chromatography of a DN'ase I Digest of Calf Thymus DNA on a Column of DEAE-Sephadex (75 cm x 0.9 cm, chloride form) at 65°C.

a) The digest (86.5 mg calf thymus DNA, 3.46 units DN'ase I, 25°C, 8 minutes, section 4.1, digest B) was applied to the column and eluted with a linear gradient of 0 to 0.4 M LiCl in 0.005 M Tris-HCl, 7.0 M urea buffer, pH 7.6, at 65°C. Total volume of eluent 8 l, 16 ml fractions collected. After the gradient elution the column was washed with 1.0 M LiCl.

b) The digest (see Figure 10a) treated with 15 units phosphomonoesterase, 37°C, 6 hours, was applied to the column and eluted as described in Figure 10a.

——— absorbance at 260 nm

- - - - - molarity of LiCl

not adsorbed by DEAE-Sephadex and elute during application of the sample. The corresponding peak in Fig. 10a is the mononucleotide fraction (peak I). To test the efficacy of the separation peak XII from Fig. 10b was rechromatographed on an identical column eluted with a 4 l linear gradient of 0.3 to 0.4 M LiCl in 0.005 M Tris-HCl 7.0 M urea buffer, pH 7.6, at 65°C. No traces of contamination from peaks XI and XIII were observed. The distribution of the oligonucleotides separated according to chain length, based on optical density analyses, and chain length determinations based on phosphate analyses, are shown in Table 4. Total recovery was better than 95%. The very good agreement between the results for oligonucleotides of general formula $N_n p_n$ (Fig. 10a) and $N_n p_{n-1}$ (Fig. 10b) confirms the chain length homogeneity of each peak.

4.1.3 Properties of the DEAE-Sephadex columns at 65°C

To examine the theoretical basis of the fractionation and investigate the possible application of the separation technique to longer oligonucleotides a plot of log chain length versus molarity of the eluting salt at peak maximum for each isostich in Figs. 10a and 10b was made (Fig. 11). Included is data from a DN'ase I digest of calf thymus DNA chromatographed exactly as described in Fig. 10a except that elution was at 25°C. The graph shows the superior separation obtained with the 65°C chromatograms compared to the 25°C chromatogram, particularly of the longer oligonucleotides, and the improved separation at 65°C when 5' terminal phosphates are removed.

TABLE 4

Distribution of Oligonucleotide Isostichs Released from Calf Thymus DNA by DN'ase I Before and After Hydrolysis with *E.coli* Phosphomonoesterase

		Oligonucleotide: general formula N_nP_n			Oligonucleotide: general formula N_nP_{n-1}	
Peak No.	Oligonucleotide isostich	A_{260}*	% of total A_{260}	$\frac{\text{Total P}}{\text{Term P}}$	A_{260}*	% of total A_{260}
I	1	43.34, 39.85	2.1, 2.0		50.81, 50.09	2.5, 2.4
II	2	149.45, 158.20	7.1, 7.8	2.0	139.20, 145.74	6.9, 7.0
III	3	133.15, 132.63	6.3, 6.5		125.65, 136.51	6.2, 6.6
IV	4	197.80, 204.54	9.4, 10.1		202.53, 211.99	10.0, 10.3
V	5	254.28, 245.87	12.1, 12.1	4.9	245.12, 243.95	12.1, 11.8
VI	6	263.13, 268.71	12.5, 13.3		271.83, 266.96	13.4, 12.9
VII	7	246.54, 251.12	11.7, 12.4		249.83, 258.23	12.3, 12.5
VIII	8	181.44, 184.23	8.6, 9.1	7.9	181.64, 186.61	9.0, 9.0
IX	9	144.90, 141.98	6.9, 7.0		143.00, 144.42	7.0, 7.0

TABLE 4.....cont'd.

Peak No.	Oligonucleotide isostich	Oligonucleotide: general formula N_nP_n			Oligonucleotide: general formula N_nP_{n-1}	
		A_{260}*	% of total A_{260}	$\frac{\text{Total P}}{\text{Term P}}$	A_{260}*	% of total A_{260}
X	10	114.28, 107.91	5.4, 5.3		112.50, 105.00	5.6, 5.1
XI	11	79.99, 76.40	3.8, 3.6		72.77, 76.80	3.6, 3.7
XII	12	53.76, 51.80	2.6, 2.6	12.2	50.22, 50.70	2.5, 2.5
XIII	13	44.81, 40.26	2.1, 2.0		33.88, 36.55	1.7, 1.8
XIV	14	26.56, 28.07	1.3, 1.4		23.20, 26.78	1.1, 1.3
XV	15	— —	— —		18.82, 22.27	0.9, 1.1
1.0 M		168.09, 93.47	8.0, 4.6		104.40, 102.87	5.2, 5.0
Total A_{260}		2101.22, 2025.04			2025.40, 2065.47	100.0, 99.8
% Recovery^{t}		99.1, 95.5			96.4, 98.3	

* The values are from two identical separate experiments.

^{t} Determined from the A_{260} of the material applied to the column in 7 M urea.

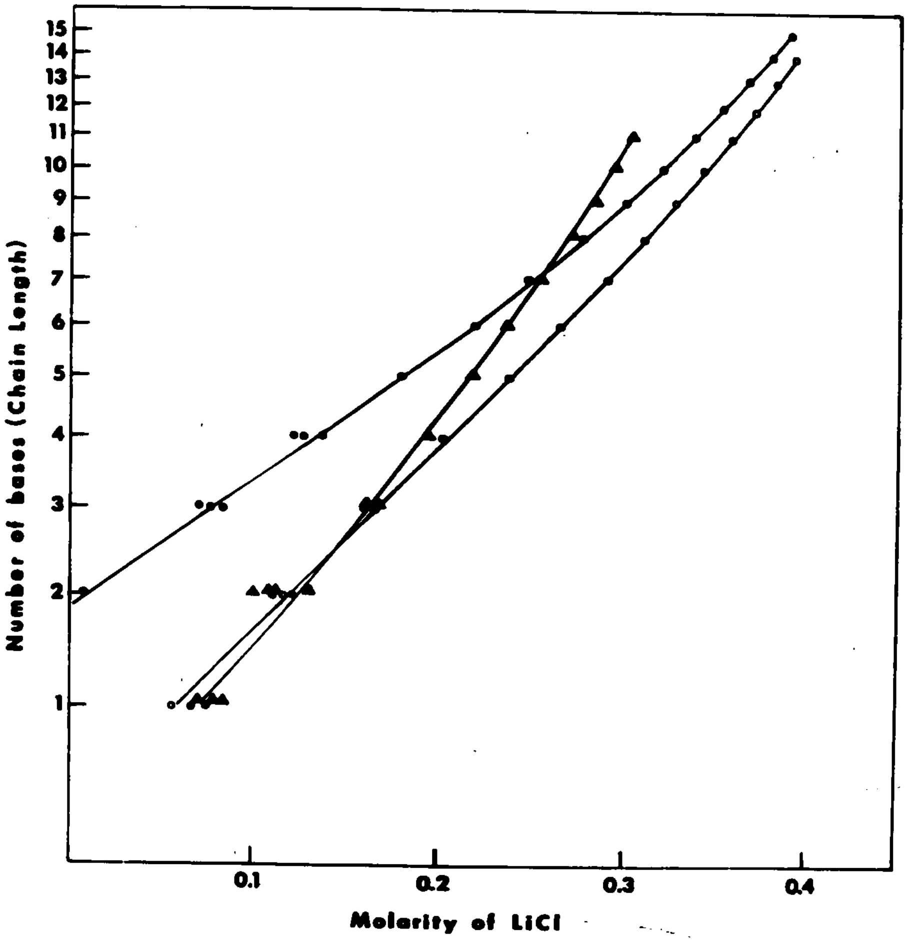


FIGURE 11

Relationship Between Chain Length and Molarity of Eluting Salt of Isostich Fractionations on DEAE-Sephadex

Salt molarities were taken at peak maximum of the isostich elution profiles.

○——○	DN'ase I digest on DEAE-Sephadex at 65°C
●——●	DN'ase I digest dephosphorylated with phosphomonoesterase, on DEAE-Sephadex at 65°C
▲——▲	DN'ase I digest on DEAE-Sephadex at 25°C

The chromatographic fractionation of a 30 hour DN'ase I digest (section 4.1) of E.coli DNA according to chain length is shown in Fig. 12. The absorbance profile shows a separation of 8 peaks. Peaks Ia and Ib were mononucleotides, peaks II to VII corresponding to isostichs 2 to 7. Washing of the column with 1 M LiCl did not elute any ultraviolet absorbing material. Recoveries were better than 98%. The distribution of the oligonucleotides based on optical density and total phosphorus, and chain length analysis data are shown in Table 5. The material from this and other similar chromatograms was used for the fractionation and analysis of mono , di , and trinucleotides.

4.2 Fractionation of isostichs according to base composition

Fractionation of oligonucleotides according to base composition has been attempted by various techniques including ion exchange (5, 6, 26, 82, 185), paper chromatographic (78) and electrophoretic (7, 8, 9, 187) methods. None of the methods provided a satisfactory quantitative separation of all the possible components in a mixture. Recently Satoh and Inoue (186) reported the separation of RNA dinucleotides obtained from an alkaline hydrolysate of RNA on a Dowex exchanger.

4.2.1 Rapid separation of mononucleotides and nucleosides

a) Separation of 3' or 5' deoxynucleotides and deoxynucleosides and 3' or 5' ribonucleotides and ribonucleosides

The chromatographic separation of two mixtures of 5' deoxynucleotides and deoxynucleosides is shown in Fig. 13a and two mixtures of 5' ribonucleotides

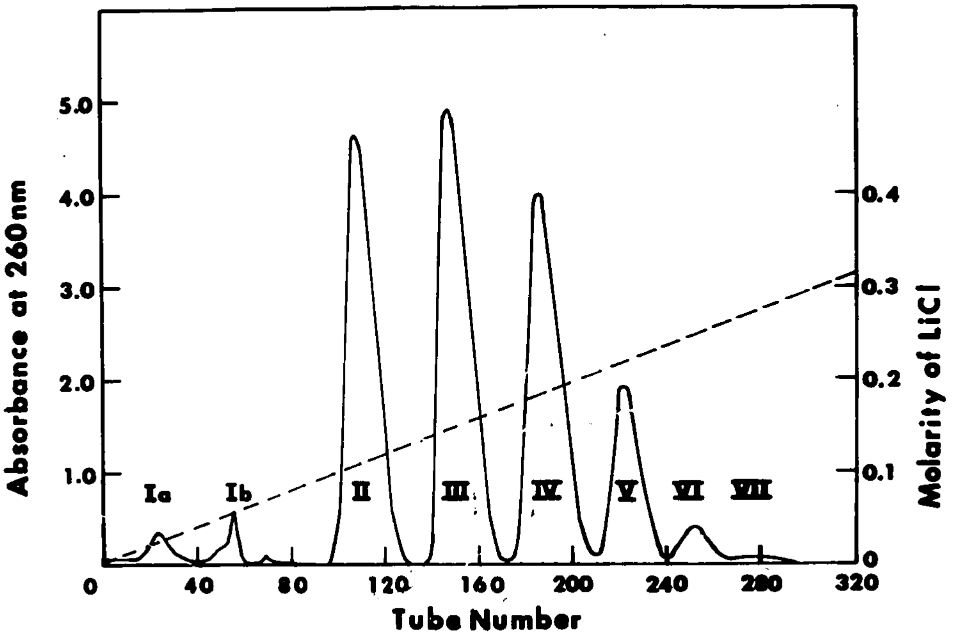


FIGURE 12

Chromatography of a DN'ase I Digest of E.coli K12 DNA on a Column of DEAE-Sephadex (25 cm x 0.9 cm, chloride form) at 65°C.

The digest (133.0 mg E.coli DNA, 40.5 units DN'ase I, 37°C, 30 hours, section 4.1, digest C) was applied to the column and eluted with a linear gradient of 0 to 0.4 M LiCl in 0.005 M Tris-HCl, 7.0 M urea buffer, pH 7.6, at 65°C. Total volume of eluent 6 l, 15 ml fractions collected.

—— absorbance at 260 nm

----- molarity of LiCl

TABLE 5

Distribution of Oligonucleotide Isostichs Released from E.coli K12 DNA by DN'ase I

Peak No.	Oligonucleotide isostich	A_{260}*	% of total A_{260}	µg P	% of total P	$\frac{\text{Total P}}{\text{Term P}}$
Ia	1	75.20 ± 5.16	1.88 ± 0.13	376.2	3.0	1.1
b	1	54.62 ± 2.09	1.35 ± 0.04			
II	2	1173.37 ± 7.08	29.32 ± 0.10	3360.7	26.8	2.0
III	3	1210.20 ± 5.56	30.25 ± 0.12	3686.8	29.4	2.9
IV	4	984.80 ± 5.14	24.62 ± 0.13	3222.8	25.7	4.2
V	5	408.52 ± 1.25	10.20 ± 0.05	1479.7	11.8	5.1
VI	6	89.15 ± 3.26	2.23 ± 0.09	338.6	2.7	6.3
VII	7	15.73†	0.4	62.7	0.5	7.2

* The values are the means of three separate analyses ± S.E.M.

† This fraction was discarded in two experiments.

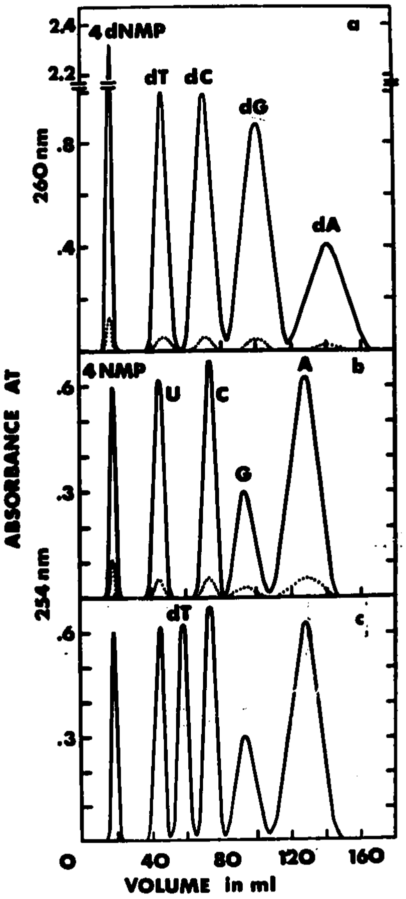


FIGURE 13

a) Chromatography of 5' deoxynucleotides and deoxynucleosides

A mixture containing the four 5' deoxynucleotides and four deoxynucleosides was loaded on a 42 x 1.1 cm column and eluted with a 0.3 M ammonium formate buffer, pH 9.2. ——— , 46.80 absorbance units (260 nm) of mixture in 1.0 ml sample volume. .... , 2.34 absorbance units (260 nm) of mixture in 0.05 ml sample volume.

b) Chromatography of 5' ribonucleotides and ribonucleosides

A mixture containing the four 5' ribonucleotides and four ribonucleosides was loaded on a 60 x 1.1 cm column and eluted with 0.3 M ammonium formate buffer, pH 8.9. ——— , 36.0 absorbance units (260 nm) of mixture in 0.6 ml sample volume. .... , 3.0 absorbance units (260 nm) of mixture in 0.05 ml sample volume.

c) Fractionation of deoxythymidine in the ribonucleoside chromatographic system

To a mixture of 36.0 absorbance units (260 nm) of 5' ribonucleotides and ribonucleosides 7.0 A_{260} units of deoxythymidine were added. The total mixture was chromatographed as described in b). Sample volume: 0.8 ml.

and ribonucleosides in Fig. 13b. Both sets of mixtures contained identical components in equal proportions but differed in concentration by a factor of 20 for the deoxy compounds, and 12 for the ribo compounds. All four nucleotides were eluted together in these systems and the four nucleosides then separated into the individual components in the elution order thymidine or uridine, cytidine, guanosine and adenosine. The separated components were analyzed quantitatively by pooling the peaks, adjustment of the pH and spectral analysis. The results presented in Table 6 list both recoveries and the volume of the fractions eluted from the column for the largest sample. The results shown in Fig. 13 a and b and Table 6 are those obtained by the optimal conditions listed in section 2.2.7. Variations in column length, column diameter, flow rate, molarity and pH of the eluting buffer were all investigated. The separation is extremely pH dependent. Variations of as little as 0.1 pH units will affect the elution position of deoxyguanosine so that it will interfere with either the deoxycytosine or deoxyadenosine separations; similarly for guanosine in the ribo series. Longer columns improve the distance of separation between individual components but also increase the time required for the separation. Oligonucleotides and inorganic phosphate co-chromatograph with the mononucleotide fractions.

Fig. 13c shows an identical pattern to that in Fig. 13b except for the addition of deoxythymidine to the mixture of ribomononucleotides and mononucleosides applied to the column. The deoxythymidine was eluted in

TABLE 6

Quantitative Analysis of the 5' Nucleotides and Nucleosides Separated by Chromatography on Dowex 50

Components	% recovery ± S.E.M.	Volume of fraction
5' deoxynucleotides	99.4 ± 0.06 (4)	8.8
dT	99.0 ± 0.03 (4)	15.8
dC	100.0 ± 0.02 (4)	21.5
dG	101.6 ± 0.03 (4)	37.0
dA	98.4 ± 0.07 (4)	43.8
5' ribonucleotides	99.5 ± 0.06 (3)	7.4
U	101.0 ± 0.09 (4)	12.4
C	100.8 ± 0.06 (4)	20.0
G	100.0 ± 0.03 (4)	27.8
A	98.9 ± 0.11 (4)	37.0

The analyses correspond to the chromatographic separations of the 46.8 A_{260} unit sample shown in Fig. 13a and the 36.0 A_{260} unit sample shown in Fig. 13b. The fractions containing each component were pooled and the total absorbance measured at the pH of the buffer. The figures in parentheses indicate the number of determinations.

a position intermediate between uridine and cytidine and this has an advantage in the sequential analysis of ribo oligonucleotides which may have small DNA oligonucleotide contaminations in that the deoxythymidine will show up as an individual peak. Purified 5' methylcytidine was not available for analysis but when cytosine and 5' methylcytosine were examined on the deoxyribose column (Fig. 13a) they both chromatographed at the same position. It was then assumed that the corresponding nucleosides will chromatograph together.

In studies of the composition of oligodeoxynucleotides or oligoribonucleotides the first deoxynucleotide or ribonucleotide peaks eluted can be conveniently fractionated into their four components by the procedure of Blattner and Erickson (148). This allows a complete nucleotide/nucleoside analysis within a period of 2 to 2.5 hours. The chromatogram shown in Fig. 14a shows results of a phosphomonoesterase and snake venom phosphodiesterase digest of the trinucleotides isolated from a DN'ase I digest (section 4.1.3, Fig. 12). Fig. 14b shows a similar separation of a phosphomonoesterase and spleen phosphodiesterase digest of the same material. Comparing the separations with those obtained in Fig. 13a they are seen to be identical and the salts and enzymes present in the enzyme incubation medium do not interfere with the chromatographic elution.
In these experiments the 5' and 3' termini of the oligonucleotides isolated from the DN'ase I-Mn^{++} digestion of E.coli K12 DNA were analyzed

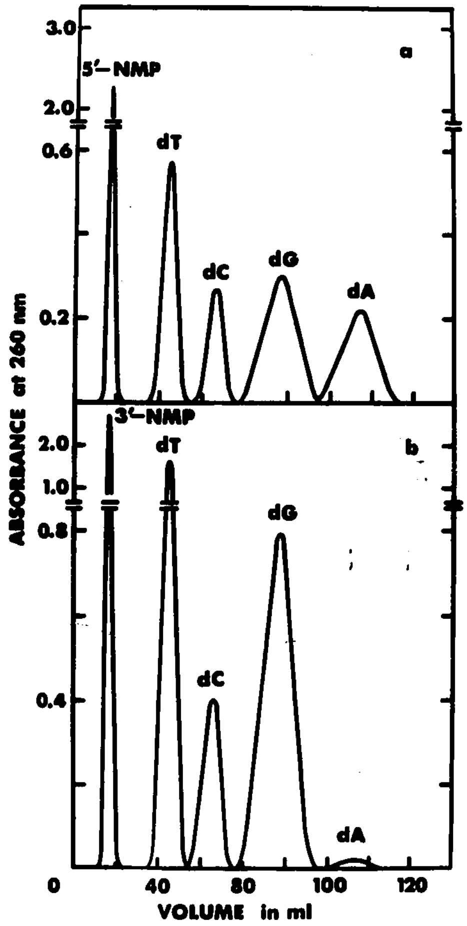


FIGURE 14

a) Chromatography of a snake venom phosphodiesterase digest of the trinucleotides from a DN'ase I digest of *E.coli* K12 DNA. The fractionation was performed as described in Figure 13a. Sample volume: 1.0 ml.

b) Chromatography of a spleen phosphodiesterase digest of the trinucleotides from a DN'ase I digest of *E.coli* K12 DNA. The fractionation was performed as described in Figure 13a. Sample volume: 2.2 ml.

and the results are presented in section 5.3.2, Table 19.

b) Fractionation of all four deoxynucleosides and 5' deoxynucleotides

A second method of approach for a complete nucleotide/nucleoside separation is shown in Fig. 15. The first elution buffer elutes the four deoxynucleotides and deoxythymidine. Following the appearance of adenylic acid the buffer is changed and a peak of absorbance appears immediately following the application of the second buffer due to the change in salt concentration. A similar effect has been noted previously by Khym and Uziel (188). The diesterase enzymes do not interfere with the separation. Recoveries from this column are better than 98%.

The components of the mononucleotide peaks in Fig. 12 (Table 5) representing only 3% of the total DN'ase I digest were analyzed according to Blattner and Erickson (148). The results are shown in Table 7. Neither peak contained cytidilic acid and the total adenylic acid from both peaks was 6.5%.

4.2.2 Separation of dinucleotides on DEAE-cellulose. Analysis and quantitation

Separation of all possible non-isomeric dinucleotides from DN'ase I or DN'ase II digests has been achieved by Laskowski, et al. (78) on paper chromatography. The method is limited to small amounts of material and the recovery of the fractions was not quantitative. Stepwise elution from columns

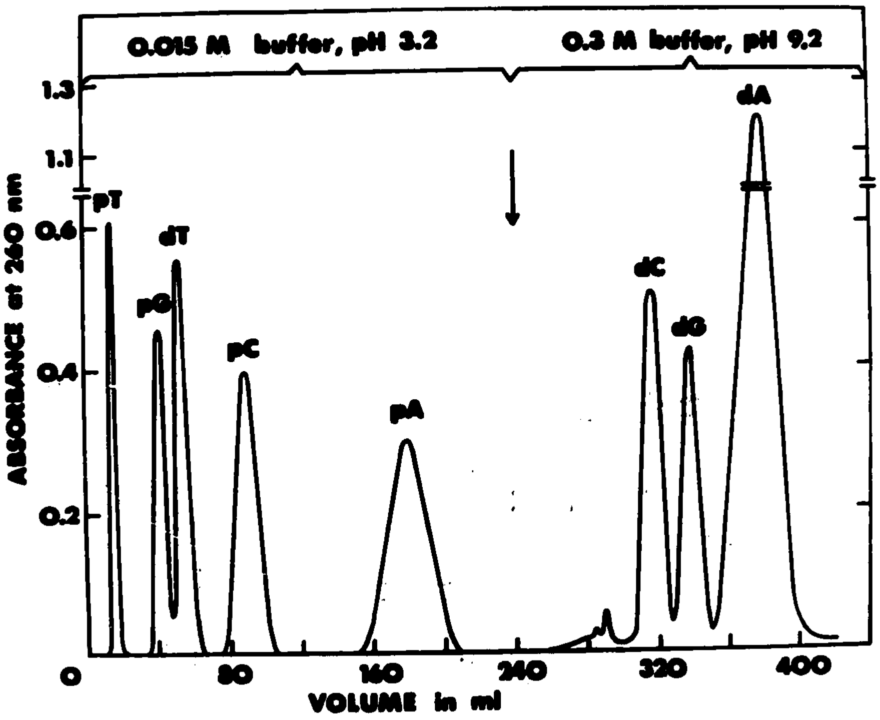


FIGURE 15

Separation of All Four Deoxynucleosides and 5' Deoxynucleotides

A mixture of the eight components totalling 80 absorbance units (260 nm) was loaded on a 42 x 1.1 cm column in 0.015 M ammonium formate buffer, pH 3.2. Sample volume: 1.0 ml. This buffer was then used for elution of the first five components and the second elution buffer, 0.3 M ammonium formate, pH 9.2, applied.

TABLE 7

Distribution of Mononucleotides Released from *E. coli* K12 DNA by DN'ase I

Mononucleotide	Fraction 1a		Fraction 1 b		Total (Fractions 1a and 1b)	
	µmoles	moles %	µmoles	moles %	µmoles	moles %
pT	0.539	54.1	0.050	14.5	0.589	34.3
pG	0.373	37.4	0.280	80.9	0.653	59.2
pA	0.085	8.5	0.016	4.6	0.101	6.5
pC	0	0	0	0	0	0

Fractions 1 a and 1 b refer to Fig. 12 and Table 5 has been attempted also (26, 80, 81, 82, 185) but with strong ionic exchangers and with heterogeneous mixtures of oligonucleotides not fractionated according to chain length. Separation and recoveries have not been satisfactory.

a) Fractionation

To develop a new method for the fractionation of dinucleotides preliminary experiments were attempted on DEAE-cellulose and DEAE-Sephadex columns.

To determine the optimum pH for separation of dinucleotides a plot of net charge versus pH was made (c.f., Cohn, 189). This is shown in Fig. 16. Net charge was calculated from the pK's of the component mononucleotides. In the pH range 1.0 to 4.0 the terminal monoesterified phosphate has only one ionizable group. The largest net charge difference between the various dinucleotides is in the pH range 3.4 to 3.7. Initial attempts to fractionate dinucleotides in this pH range on DEAE-Sephadex at 25°C and 65°C and DEAE-cellulose at 25°C in 7.0 M urea buffers were unsuccessful. Only 6 of the 10 possible dinucleotides were partially separated. The elution positions of the purine containing dinucleotides were not related to their net charge. This indicated preferential adsorption to the column matrix. Bartos, et al. (86) have shown a relationship between the composition of dinucleotides in a mixture and elution position which is also partially dependent on the purine/pyrimidine ratio of the dinucleotides. To delay the elution of the purine rich dinucleotides, fractionation was

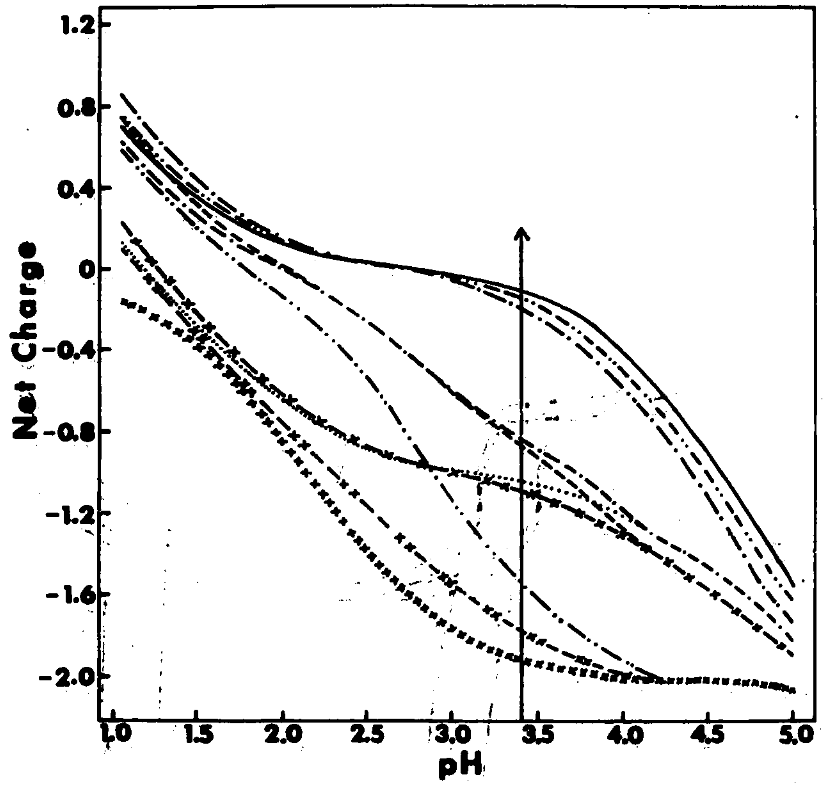


FIGURE 16

Net Charge per Molecule of Dinucleotides as a Function of pH as Calculated from the pK Values of the Mononucleotide Components

——— pCpC
—-·-—-·· pApC
—·—·—·· pApA
—·—·—·-- pCpG
- - - - - pApG
——··—·· pGpG
-x-x- pApT
········· pCpT
--xx-- pGpT
xxxxx pTpT

attempted on DEAE-cellulose with buffers at pH's from 3.2 to 3.7 in the absence of 7.0 M urea. Optimal separation of peak II from Fig. 12 into 11 discrete peaks was obtained at pH 3.4 as shown in Fig. 17. The positional isomers pCpA and pApC were completely resolved by the procedure. pCpA was eluted before pApC similar to the results of Satoh and Inoue (186) for ribodinucleotides. The isomers having a purine at the 3' termini are eluted earlier than their corresponding positional isomers. Larger amounts of dinucleotides can be successfully fractionated provided the volume of the gradient is increased. Variations in pH from 3.2 to 3.7 always produced interference between the various fractions. For example at pH 3.5 CG-AA and AG-TT were well separated but AT and AG were superimposed.

b) Spectral properties

The spectral properties of the dinucleotides at pH's 1, 7 and 12 are presented in Table 8 and are compared to equimolar mixtures of the corresponding mononucleotides. The results show the purity of the isolated dinucleotides and confirms their identity. The properties of the positional isomers pCpA and pApC were identical. Absence of differences in the spectral properties of ribodinucleotide and trinucleotide positional isomers have been reported by Toal, et al. (190).

The hyperchromic effect found by digestion of the dinucleotides with snake venom diesterase and analysis at pH 3.4 is similar to previously reported

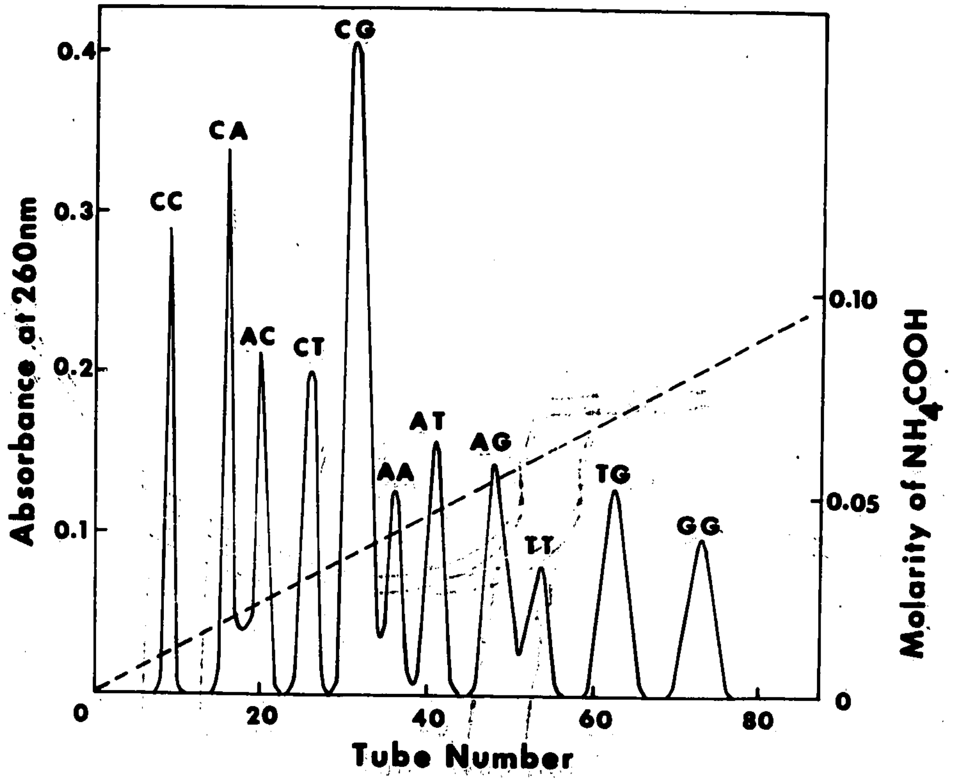


FIGURE 17

Chromatography of Dinucleotides from E.coli K12 DNA on a Column of DEAE cellulose (4.0 g, 325 mesh, 25 cm x 0.9 cm, formate form)

The pooled dinucleotide fractions from Figure 12 (110 A_{260} units), diluted with distilled water, were applied to the column. The exchanger was washed with 0.05 M formic acid and then eluted with a linear gradient of 0 to 0.1 M ammonium formate, pH 3.4. Total volume of eluent 2 l, 21 ml fractions collected.

—— absorbance at 260 nm

----- molarity of ammonium formate

TABLE 8

Spectral Properties of Dinucleotides

Dinucleotide	Source	pH 1.0				pH 7.0				pH 12.0			
		λmax	λmin	250/260	280/260	λmax	λmin	250/260	280/260	λmax	λmin	250/260	280/260
CC	M*	279	241	.44	2.03	271	250	.84	.98	271	250	.84	.98
	D†	279	241	.53	1.93	270	250	.92	.97	270	251	.88	.91
CA	M	266	233	.71	.81	261	227	.79	.42	261	227	.80	.41
	D	266	234	.71	.81	262	228	.84	.51	262	228	.84	.50
AC	M	266	233	.71	.81	261	227	.79	.42	261	227	.80	.41
	D	266	234	.72	.80	262	228	.85	.53	262	228	.85	.51
CT	M	274	237	.58	1.29	269	239	.75	.86	270	247	.79	.83
	D	274	237	.59	1.22	269	238	.78	.83	270	247	.84	.83
CG	M	278	232	.79	1.21	254	224	1.03	.78	268	232	.87	.75
	D	278	233	.82	1.13	254	225	1.04	.79	268	231	.90	.77

TABLE 8.....cont'd.

Dinucleotide	Source	pH 1.0				pH 7.0				pH 12.0			
		λmax	λmin	$\frac{250}{260}$	$\frac{280}{260}$	λmax	λmin	$\frac{250}{260}$	$\frac{280}{260}$	λmax	λmin	$\frac{250}{260}$	$\frac{280}{260}$
AA	M	257	229	.83	.26	260	227	.77	.18	260	227	.78	.14
	D	257	230	.87	.30	260	228	.86	.22	259	228	.82	.21
AT	M	261	232	.77	.45	261	232	.74	.36	261	233	.77	.31
	D	261	232	.79	.45	261	232	.80	.43	261	233	.78	.41
AG	M	257	228	.90	.47	256	225	.94	.38	259	229	.83	.36
	D	257	228	.92	.47	256	226	.97	.46	258	230	.90	.46
TT	M	267	235	.68	.74	267	235	.69	.76	266	244	.74	.68
	D	267	235	.71	.70	267	234	.74	.72	266	244	.84	.69
TG	M	259	231	.85	.74	256	228	.95	.70	263	234	.84	.64
	D	258	231	.85	.67	256	229	.96	.69	261	232	.95	.69
GG	M	256	228	1.00	.74	253	223	1.14	.66	258	231	.89	.62
	D	256	227	.99	.75	253	224	1.14	.67	258	231	.89	.63

* M refers to an equimolar mixture of 5' mononucleotides.

† D refers to dinucleotides isolated as in Fig. 17.

data (191). Thus in calculations of the extinction coefficients and molar proportions of the dinucleotides (Tables 9 and 10) it has been assumed that the spectral properties of the positional isomers are identical.

c) Positional isomers

Each peak from Fig. 17 was identified by spectrophotometric and base analysis. The distribution of the dinucleotides and hyperchromicity and extinction data at 260 nm, pH 3.4, are shown in Table 9. The order of elution showed the purine containing dinucleotides delayed in elution from the column. They were not eluted according to their net charge. The elution order is:

CT (-1.04) > CG (-0.84) > AA (-0.20)
TA (-1.09) > AG (-0.88) and
TT (-1.90) > TG (-1.78) > GG (-1.56).

The elution pattern should be inverted according to their total charge at pH 3.4.

The distribution of each positional isomer, determined by end group analysis (80, 81) is shown in Table 10. The total end group analysis of the dinucleotide fraction is presented in Table 11. There is a preferential hydrolysis of DNA by DN'ase I adjacent to G and C residues indicated by the larger proportion of pGpC, pCpG, pCpT, pCpC and pGpG present in the dinucleotide fraction (Table 10) and the high content of G + C at the 5' and 3' ends of the total dinucleotide fraction (Table 11).

TABLE 9

Distribution of Dinucleotides Released from *E.coli* K12 DNA by DN'ase I and Separated According to Base Composition

Dinucleotide	Net charge	A_{260} pH 3.4	% hyperchromicity at 260 nm, pH 3.4	$\epsilon_{260} \times 10^{-3}$	µmoles	Moles %
CC	0.12	33.8 ± 0.68	5.8	12.5	2.70	8.4
CA + AC	0.16	63.66 ± 2.16	2.2	20.9	3.05	9.5
CT + TC	1.04	64.54 ± 2.06	2.7	14.8	4.36	13.6
CG + GC	0.84	145.36 ± 3.02	2.2	19.0	7.65	23.9
AA	0.20	28.73 ± 0.66	12.7	26.6	1.08	3.4
AT + TA	1.09	61.84 ± 2.28	4.3	22.6	2.74	8.5
AG + GA	0.88	61.41 ± 1.55	3.6	26.8	2.29	7.1
TT	1.90	35.15 ± 1.72	2.4	16.6	2.12	6.6
TG + GT	1.78	79.71 ± 0.78	1.4	21.5	3.71	11.6
GG	1.56	59.36 ± 2.83	3.0	25.2	2.36	7.4

Dinucleotides were from fraction II, Fig. 12 and Table 5.

Net charge is from Fig. 16.

Values for A_{260} are the means of four separate analyses ± S.E.M.

TABLE 10

Distribution of Isomeric Dinucleotides Released from *E.coli* K12 DNA by DN'ase I

Dinucleotide	% of isomer in dinucleotide	Moles %
pCpC	100.0	8.4
pCpA	63.8	6.1
pApC	36.2	3.4
pCpT	67.0	9.1
pTpC	33.0	4.5
pCpG	42.7	10.2
pGpC	57.3	13.7
pApA	100.0	3.4
pTpA	56.6	4.8
pApT	43.4	3.7
pApG	30.4	2.2
pGpA	69.6	4.9
pTpT	100.0	6.6
pTpG	44.8	5.2
pGpT	55.2	6.4
pGpG	100.0	7.4

TABLE 11

Base Composition of the 5' and 3' Termini of Dinucleotides Released from

E. coli K12 DNA by DN'ase I

Base	C	T	A	G
Moles % at 5' end	33.8	21.1	12.7	32.4
Moles % at 3' end	30.0	25.8	19.2	25.0

4.2.3 Separation of trinucleotides on DEAE-cellulose. Analysis and quantitation

Chromatographic separation of the trinucleotide peak III from Fig. 12 on DEAE-cellulose at pH 3.6 is shown in Fig. 18.

Variations in column size and pH of the eluting buffer from 3.3 to 3.7 did not improve the separation. Recoveries were better than 92%. The distribution and base composition of the separated peaks is shown in Table 12. Peaks 1 and 2 did not contain nucleotide material. Sixteen of the 22 separated trinucleotide peaks were identified from their base ratios and accounted for 14 of the 20 possible non-isomeric trinucleotides; 2 pairs of peaks having the same composition. The 4 peaks with base ratios which did not allow easy identification of trinucleotide content, comprise mixtures of trinucleotides not resolved by chromatographic conditions. As in the case of the dinucleotides, purine rich trinucleotides are retained longer on the column.

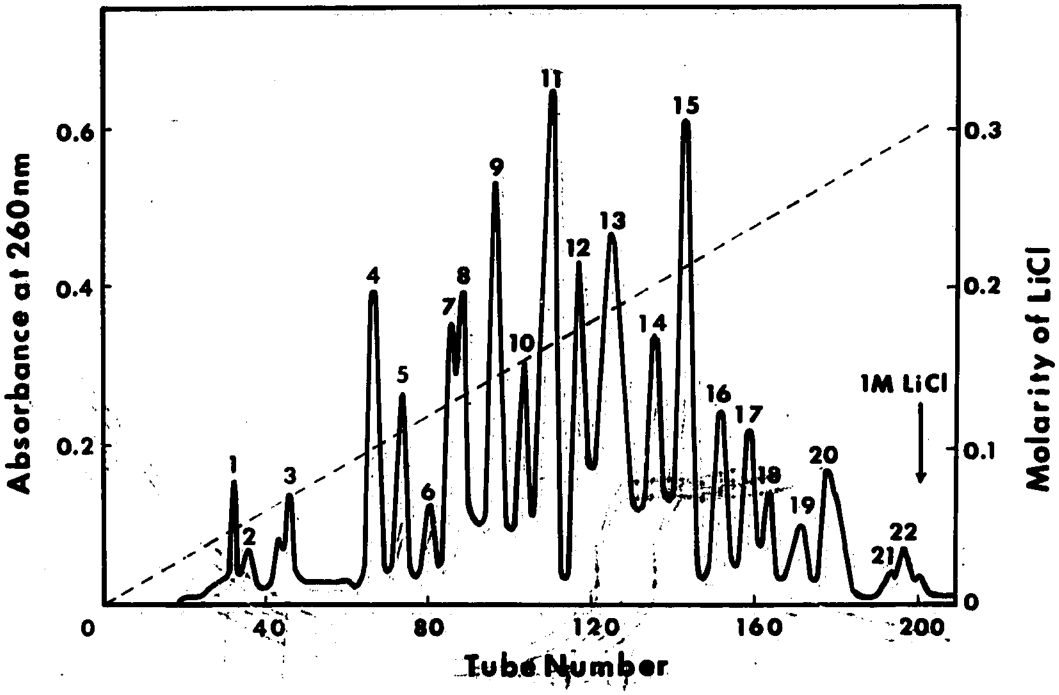


FIGURE 18

Chromatography of Trinucleotides from E.coli K12 DNA on a Column of DEAE cellulose (20 g, 325 mesh, 130 cm x 0.9 cm, formate form)

The pooled trinucleotide fractions from Figure 12 were desalted, dissolved in distilled water and 150 A_{260} units applied to the column. The exchanger was washed with 0.05 M formic acid and then eluted with a linear gradient of 0 to 0.3 M ammonium formate, pH 3.6. Total volume of eluent 2 l, 10 ml fractions collected.

——— absorbance at 260 nm

---- molarity of ammonium formate

TABLE 12

Distribution of Trinucleotides Released from E.coli K12 DNA by DN'ase I and Separated According to Base Composition

Peak No.	Moles %	Base Ratio G	A	C	T	Trinucleotides present
3	0.5	—	—	1.0	—	CCC
4	8.0	—	1.0	2.2	—	CCA
5	6.8	—	—	1.9	1.0	CCT
6	1.5	1.0	—	6.6	2.3	Mix*
7	5.2	1.0	—	2.2	—	CCG
8	4.1	—	1.8	1.0	—	AAC
9	12.3	—	1.0	1.2	1.2	ACT
10	4.1	1.4	1.9	1.0	1.1	Mix
11	14.7	1.0	1.0	1.2	—	GAC
12	3.2	1.0	1.9	1.3	1.3	Mix
13	13.6	1.0	—	1.3	1.1	CTG
14	3.1	—	1.0	—	2.0	TTA
15	6.5	1.2	1.0	—	1.1	GAT
16	4.3	1.0	1.0	—	3.1	Mix
17	4.4	2.0	1.0	—	—	GGA
18	1.5	1.0	—	—	2.3	TTG
19	2.0	1.0	—	—	2.0	TTG
20	3.7	1.8	—	—	1.0	GGT
21	0.2	1.9	—	—	1.0	GGT
22	0.3	1.0	—	—	—	GGG
Total		23.0	22.5	31.3	23.3	

Trinucleotides were from fraction III, Fig. 12 and Table 5.

Values are the means of two separate analyses.

* Probably CCG and CCT.

CHAPTER 5

Studies on the specificity of DN'ase I

The experiments described in this chapter were designed to investigate the influence of the activating cation on the specificity of DN'ase A. The general scheme for the approach to these studies was outlined in section 1.4.3.

5.1 Enzymatic digestion of calf thymus and *E.coli* K12 DNA in the presence of metal ions

5.1.1 DN'ase A digestion: kinetic properties and extent of hydrolysis

Calf thymus and *E.coli* K12 DNA were digested in the presence of various divalent metal ions under specific conditions for each of the ions. The reactions were monitored by measuring the hyperchromic shift at 260 nm or the acid soluble material released at 260 nm as described in section 2.2.3 and shown in Fig. 4. Partial digests (P) were analyzed when one third to one half of the linear hyperchromic shift was reached. Intermediate digests (I) represent reactions terminated at the beginning of the UV plateau, and extensive digests (E) were those which were stopped when the plateau of acid soluble material released reached apparent equilibrium. The main procedure for the hydrolysis and inhibition of the reaction has been described in section 2.2.4.

In all experiments the digestions were performed at a DNA concentration of 0.5 mg/ml in 0.005 M to 0.01 M Tris-HCl buffer, pH7.2 to 8.0 and at 37°C.

In some instances the digestions were not done at the optimal pH for the ion used (see section 3.2.3) because the buffers containing monovalent ions interfered with the action of the specific cations (39, 134, 137). DNA and DN'ase were extensively dialyzed against EDTA before use and metal ions spectrochemically free of contaminants were used in all experiments (section 2.1, 2.2.1 and 2.2.2). The experimental conditions and kinetic properties of the hydrolysis of calf thymus and E.coli K12 DNA used in the specificity studies are given in Tables 13 and 14 respectively.

Different amounts of enzyme were added to the reaction mixtures in order to maintain linear UV kinetics for an interval of 5 to 20 minutes. Specific activity was measured under the conditions of each experiment and calculated from the slopes of the absorbance curves. The reactions were monitored on 0.3 ml samples of the digestion mixtures in 0.1 cm quartz cuvettes. Activities were calculated as described in assay B, section 2.2.3.

Partial, intermediate and extensive degradations amounted to 4.4 to 10%, 12% to 32% and 34% to 94% of the total acid soluble material, respectively. The percent of the total acid soluble material was calculated relative to a total Mg^{++}-activated digest (conditions: identical to digest 8 in Tables 13 and 14, incubation was stopped after 8 hours at 37°C) in which 14.2 A_{260} units/ml of acid soluble material were released. With a longer digestion period no increase in acid soluble material release was observed.

TABLE 13

Digestion Conditions for DN'ase A Hydrolysis of Calf Thymus and *E. coli* K12 DNA

Digest No.	DNA source *	Total DNA digested mg	Ion	Ion concentration M	pH of reaction	Enzyme added µg/ml
1	CT	20	Ba^{++}	3.3×10^{-2}	7.5	4.0
2	CT	30	Ca^{++}	2.0×10^{-3}	7.5	4.0
3	CT	40	Co^{++}	2.0×10^{-3}	7.5	0.6
4	EC	100	Co^{++}	1.0×10^{-3}	7.5	0.5
5	CT	20	Cd^{++}	2.0×10^{-3}	7.5	4.0
6	CT	20	Mg^{++}	3.3×10^{-3}	7.5	0.2
7	CT	100	Mg^{++}	3.3×10^{-3}	7.5	0.4
8	CT	40	Mg^{++}	3.3×10^{-3}	7.5	1.3
9	CT	20	Mg^{++}	3.3×10^{-3}	7.2	1.3
10	EC	40	Mg^{++}	3.3×10^{-3}	7.2	1.3
11	CT	30	Mg^{++} and Ca^{++}	3.3×10^{-2} and 1.0×10^{-4}	7.2	0.4
12	CT	20	Mn^{++}	3.3×10^{-3}	7.5	0.4
13	CT	20	Mn^{++}	3.3×10^{-3}	7.5	0.4

TABLE 13.....cont'd...

Digest No.	DNA source *	Total DNA digested mg	Ion	Ion concentration M	pH of reaction	Enzyme added µg/ml
14	CT	80	Mn^{++}	3.3×10^{-3}	7.5	1.3
15	CT	120	Mn^{++}	3.3×10^{-3}	7.5	0.4
16	EC	20	Mn^{++}	3.3×10^{-3}	8.0	1.3
17	CT	20	Ni^{++}	1.0×10^{-3}	7.5	4.2
18	CT	40	Ni^{++}	2.0×10^{-3}	7.5	4.0
19	CT	20	Ni^{++}	1.0×10^{-3}	7.5	13.3
20	CT	120	Ni^{++}	1.0×10^{-3}	7.5	13.3
21	EC	40	Ni^{++}	1.0×10^{-3}	7.2	6.7
22	EC	120	Sr^{++}	1.0×10^{-3}	7.5	13.3
23	CT	20	Sr^{++}	1.0×10^{-3}	7.5	4.0
24	CT	40	Sr^{++}	1.0×10^{-3}	7.5	4.2
25	EC	20	Sr^{++}	1.0×10^{-3}	7.5	13.3
26	EC	40	Sr^{++}	1.0×10^{-3}	7.5	13.3
27	CT	20	Zn^{++}	1.0×10^{-3}	7.5	4.0
28	CT	40	Zn^{++}	1.0×10^{-3}	7.5	4.2

TABLE 13.....cont'd.

Digest No.	DNA source *	Total DNA digested mg	Ion	Ion concentration M	pH of reaction	Enzyme added µg/ml
29	CT	20	Zn^{++}	3.3×10^{-4}	7.2	6.7
30	EC	20	Zn^{++}	3.3×10^{-4}	7.2	13.3

DNA: 0.5 mg/ml in 5.0×10^{-3} to 1.0×10^{-2} M Tris-HCl buffer. Temperature: 37°C.

* CT = Calf Thymus.
EC = _E.coli_ K12.

TABLE 14

Kinetic Properties and Extent of Hydrolysis of Calf Thymus and E. coli DNA

Digest	Type of digest	Digestion time (min.)	Specific activity $\frac{\text{units enzyme}}{\text{mg protein}}$	% of total ASM* at 260 nm	Chain length Pt/Pi
1	P	80	5.6	6.4	
2	I	42	11.8	18.8	
3	P	3	45.8	7.8	
	I	15	45.8	28.0	28.0
4	E	18	41.7	35.1	8.3
5	I	24	1.6	17.1	
6	P	3	110.0	6.8	38.2
7	E	67	128.0	71.4	
8	P	4.5	98.2	9.9	24.2
	E	123	98.2	62.8	7.4
9	E	90	109.8	48.3	
10	E	120	110.5	72.0	6.3
11	E	30	165.0	62.0	
12	P	2.2	75.0	5.4	32.4
13	E	67.5	64.2	94.0	
14	E	66	69.2	83.0	
15	E	60	70.0	86.0	7.3
16	E	90	72.9	92.6	5.4
17	P	12	4.8	7.6	27.4
18	P	12	4.0	8.2	
	E	122	4.0	56.6	

TABLE 14.....cont'd.

Digest	Type of digest	Digestion time (min.)	Specific activity $\frac{\text{units enzyme}}{\text{mg protein}}$	% of total ASM* at 260 nm	Chain length P_t/P_i
19	E	120	5.3	68.6	
20	I	74	5.6	31.4	14.2
21	E	120	5.4	74.6	7.9
22	E	183	6.0	66.6	
23	P	20	8.0	4.4	46.1
24	I	36	6.7	12.0	
	E	193	6.7	43.5	
25	E	158	6.6	41.6	
26	E	180	5.8	56.4	8.4
27	P	30	1.5	10.0	
28	P	42	1.0	6.8	
	E	280	1.0	50.2	
29	E	240	3.4	34.3	
30	E	240	3.8	41.8	11.0

P = partial, I = intermediate, E = extensive digest.

* ASM, acid soluble material.

The average size of the oligonucleotides at given stages during the course of digestion were determined by measurement of the total to terminal phosphate ratios, Pt/Pi (section 2.2.8). These values were not always proportional to the results expressed as percent of total acid soluble material. This is true especially in partial digests where the change in average size of the oligonucleotides is much faster than in the advanced stages. Similar data was provided by Anai, et al. (192). The average chain length of the extensive digests was between 5.4 and 8.4.

5.1.2 Fractionation of digests according to chain length. Average distribution and size determination of hydrolysates

In order to study the distribution of oligonucleotides in DN'ase A-metal ion activated hydrolyses, digests of calf thymus DNA in the presence of Mg^{++}, Mn^{++} and Ni^{++} (digests 7, 15 and 20, Tables 13 and 14) and E.coli K12 DNA in the presence of Co^{++} and Sr^{++} (digests 4 and 22, Tables 13 and 14) were examined.

The fractionation of the mixtures was carried out as described in section 4.1.2 and separations identical to the ones presented in Fig. 10 were obtained. The fractions were pooled and the total UV content in 7.0 M urea measured at 260 nm. The results are presented in Table 15. From the plot of percent of total A_{260} versus isostich chain length in Fig. 19 it can be seen that the average chain length for the extensive Mg^{++}, Mn^{++} and Sr^{++} degradations vary between 5.5 to 7.0, the patterns being similar, especially on the left side of the bell-

TABLE 15

Distribution of Oligonucleotide Isostichs Released from Calf Thymus DNA and E.coli K12 DNA by DN'ase A in the Presence of Mg^{++}, Mn^{++}, Ni^{++}, Sr^{++} and Co^{++} as Activating Ions

	Calf Thymus DNA						E.coli K12 DNA			
	Mg^{++}		Mn^{++} *		Ni^{++}		Sr^{++}		Co^{++}*	
Oligonucleotide isostich	A_{260}	% of total A_{260}	A_{260}	% of total A_{260}	A_{260}	% of total A_{260}	A_{260}	% of total A_{260}	A_{260}	% of total A_{260}
Non adsorbed	0	0	0	0	0	0	0	0	3017.6	50.2
1	34.3	2.0	15.0	0.8	33.6	1.7	72.3	2.7	†	
2	102.7	5.9	57.2	3.2	19.5	1.0	116.4	4.3	50.5	1.7
3	166.3	9.5	114.4	6.4	34.3	1.8	173.0	6.4	94.0	3.1
4	227.0	13.0	167.1	9.4	51.2	2.7	213.2	7.8	132.8	4.4
5	256.2	14.6	191.7	10.7	60.2	3.1	270.9	10.0	122.9	4.1
6	256.5	14.7	194.3	10.9	62.8	3.3	290.6	10.7	118.5	3.9
7	233.4	13.3	188.0	10.5	65.9	3.4	295.7	10.9	114.2	3.8
8	156.5	8.9	172.1	9.6	70.9	3.7	281.1	10.3	104.0	3.4
9	104.2	6.0	152.9	8.6	77.9	4.0	250.2	9.2	98.7	3.3
10	68.1	3.9	130.2	7.3	79.6	4.1	181.3	6.7	86.5	2.9

TABLE 15.....cont'd.

	Calf Thymus DNA						*E.coli* K12 DNA			
	Mg^{++}		Mn^{++} *		Ni^{++}		Sr^{++}		Co^{++} *	
Oligonucleotide isostich	A_{260}	% of total A_{260}	A_{260}	% of total A_{260}	A_{260}	% of total A_{260}	A_{260}	% of total A_{260}	A_{260}	% of total A_{260}
11	46.9	2.7	108.9	6.1	84.8	4.4	130.3	4.8	86.2	2.9
12	29.7	1.7	86.1	4.8	81.7	4.2	96.8	3.6	73.7	2.4
13	21.8	1.2	64.2	3.6	81.6	4.2	80.0	2.9	70.7	2.3
14	16.2	0.9	48.5	2.7	72.7	3.8	62.1	2.3	48.6	1.6
15	11.0	0.6	32.9	1.8	—	—	58.6	2.1	—	—
16	—	—	22.3	1.2	—	—	44.6	1.6	—	—
17	—	—	10.0	0.6	—	—	—	—	—	—
1.0 M	18.6	1.1	29.7	1.7	1047.1	54.4	104.3	3.8	302.4	10.0
Total A_{260}	1749.4		1785.5		1924.0		2721.4		3017.6	
% Recovery		97.2		99.1		95.9		97.2		96.1

* The fractionations were performed after phosphatase treatment of the digest.

† The dephosphorylated mononucleotide fraction is included in the non adsorbed fraction.

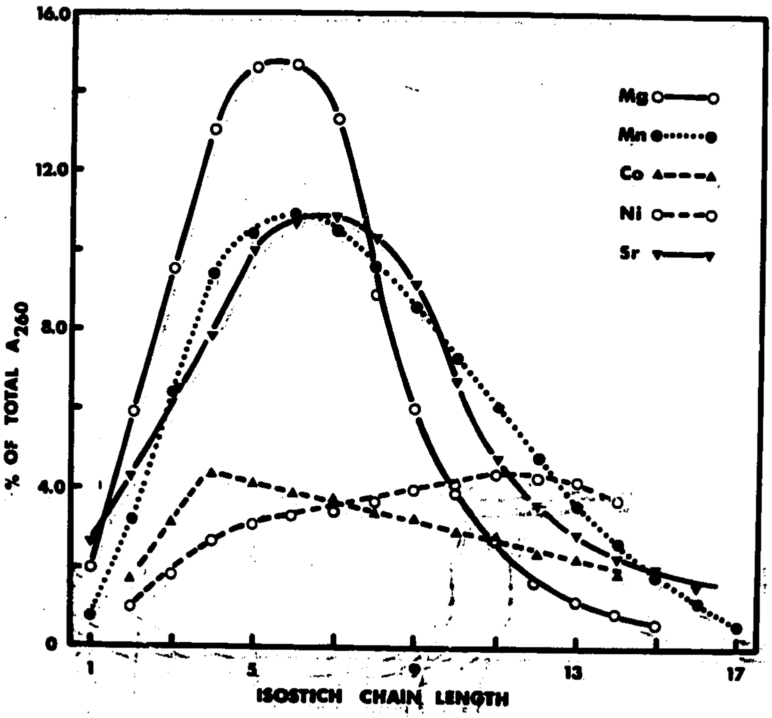


FIGURE 19

Chain Length Distribution of Oligonucleotides from DNA Digests Fractionated on DEAE-Sephadex at 65°C shaped curves. The maximal height of any peak and its sharpness are functions of the total amount of oligonucleotides appearing in the range 1 to 17 nucleotides. The position of the maximum is a function of the average chain length of the mixture and the trailing depends on the amount of long oligonucleotides present.

For the intermediate Ni^{++} digest the average size distribution indicates a maximum oligonucleotide chain length between 11 and 13 but it should be considered that over 50% of the mixture contains oligonucleotides longer than 14. The total P/terminal P determination indicates an average chain length of 14.2.

An interesting result was obtained with the E.coli K12 DNA-Co^{++} digest where 50.2% of the loaded material was not adsorbed to the column. This was probably due to the strong attachment of Co^{++} to certain oligonucleotides during the loading and washing periods. The non-adsorbed fraction had a pink colour typical of the Co^{++} ion. Addition of an excess of a 2.0 M solution of Na_2SO_3 did not result in formation of a precipitate of $CoSO_3$ as occurred in a control experiment with $CoCl_2$ alone in 7.0 M urea. This further suggests formation of a strong complex between the Co^{++} and the oligonucleotides not allowing the latter to attach to the DEAE groups of the ion exchanger under optimal conditions of adsorption. Co^{++} ions have also strong chelating properties with proteins. With glycylglycine it forms a strong red complex which was shown to be resistant to enzymatic action (213). The distribution of oligonucleotides in the case of the Co^{++}-activated digest decreased slowly with increase in isostich chain length, starting from the tetranucleotide fraction. The shallowness of the distribution between the trinucleotide and mononucleotide fractions indicates that the shorter oligonucleotides were preferentially complexed by Co^{++} and consequently eluted in the non-adsorbed fractions.

5.2 Quantitative assessment of the enzymatic methods used for the analysis of the oligonucleotides termini

The oligonucleotides released during the various DN'ase digests were enzymatically analyzed as described in section 2.2.8. The method depends on the purity of the enzymes used. Commercial enzyme preparations were tested for contaminating phosphatase, exo or endonuclease and nucleotidase activity. When possible, the contaminating enzyme was eliminated or its activity reduced in order to minimize artifacts in the determination of end nucleoside composition. Other steps in the analysis, such as total elimination of phosphatase and effect of temperature on the oligonucleotides, also demanded critical evaluation.

5.2.1 Kinetics of phosphomonoesterase digestion

E.coli alkaline phosphatase was treated to remove diesterases (182) and assayed with *p*-nitrophenylphosphate at 37°C and 55°C. Assaying the enzyme with Ca-*bis*-*p*-nitrophenylphosphate showed no diesterase to be present over a period of 4 hours, incubating both at 37°C and 55°C. The use of high temperature was introduced in order to expose internal phosphomonoester~~ase~~s to the phosphatase (196). In analysis of the oligonucleotide ends in partial

DN'ase digests it was possible that some of the internal phosphate might not be available for dephosphorylation at 37°C as shown by Weiss, et al. (196). Experiments testing the efficacy of the phosphatase reaction at 55°C are presented in Fig. 20. 31.5 mg E. coli K12 DNA at a concentration of 0.5 mg/ml in 0.01 M Tris-HCl buffer, pH 7.6, 3.3×10^{-3} M $MgCl_2$ were digested with 1.4 µg/ml (specific activity 108.0 units/mg) of DN'ase A for 2 hours at 37°C. The reaction was terminated with 6.0×10^{-3} M EDTA and the DN'ase was denatured. The mixture was desalted on DEAE-cellulose with TEAB buffer and concentrated to 3 ml in the phosphatase buffer (sections 2.2.4, 2.2.5 and 2.2.8). A solution of 6.5×10^{-3}M dCMP was prepared with the phosphatase buffer and 3 ml samples were taken for hydrolysis and analysis.

The mixtures were incubated at 55°C for 15 minutes and phosphatase was added to each sample as indicated in Fig. 20. Aliquots of 50 to 200 µl were taken at several times during the digestion and the inorganic phosphorus released measured by the method of King (161).

The average chain length of the DN'ase A digest was 5.8 (total P/terminal P) and the phosphatase reaction was essentially complete after 60 minutes of digestion. The dCMP solution was prepared in order to release the amount of Pi expected from an oligonucleotide mixture of average chain length 5.0. Under the same conditions of phosphatase treatment for the DN'ase digest, all phosphate was released from dCMP in 40 minutes. With one third of the amount of phosphatase the reaction was complete after two hours. It should be noticed that the linearity

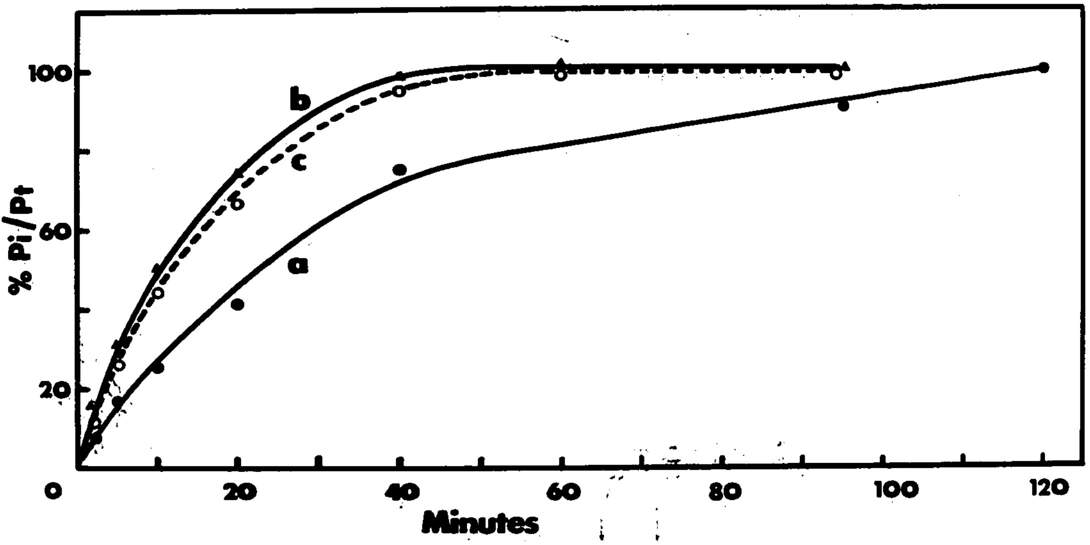


FIGURE 20

Kinetics of E.coli Alkaline Phosphatase Hydrolysis at 55°C

Preparation of the samples for phosphatase hydrolysis was described in the text. Reactions a) and b) were done with 3.0 ml of 6.5 x 10^{-3}M dCMP and reaction c) with an extensive Mg^{++}-DN'ase A digest containing 31.5 mg DNA (3 mg phosphorus) in 3.0 ml solution. Buffer: 2.0 x 10^{-2} Tris-HCl, pH 8.0, 1.0 x 10^{-2} M $MgCl_2$. Temperature of incubation 55°C.

a), 1 unit phosphatase

b) and c), 3 units phosphatase (as measured at 37°C)

Aliquots of 50 to 200 µl were taken for analysis of Pi by the method of King (161).

of the reaction was maintained over a short period of time only, probably due to partial inactivation of the enzyme by prolonged incubation at 55°C (182). Nevertheless the reaction always reached completion. Since the reaction was complete in two hours for oligonucleotides of average size 5.8, it was assumed that all mixtures of higher average chain length (and less terminal phosphates) would be totally dephosphorylated when incubated under identical conditions for periods of 2 to 4 hours.

5.2.2 Phosphatase denaturation. Effect of temperature on deoxyoligonucleotides

In preliminary experiments phosphatase was removed by phenol extraction and the phenol by ethyl ether extractions (29). The procedure proved to be adequate when volumes larger than 3 to 4 ml were extracted, but 20 to 30 percent of the material was lost during the repeated cycles of solvent elimination. Thus it was decided to use high temperature to terminate the reaction. Due to the high stability of phosphatase at high temperature especially in the presence of Mg^{++} ions (182, 193) it was necessary to boil the solution for 10 minutes in order to denature the enzyme completely. However, prolonged treatment at high temperature can produce internucleotide bond cleavage, dephosphorylation and deamination of the purines and cytosine (178, 197). By autoclaving the solution at 120°C for 3 minutes, phosphatase was irreversibly denatured.

To assess the effect of temperature on oligonucleotides and nucleotides two control experiments were performed.

a) A desalted sample of ^{32}P-labeled pyrimidine heptanucleotide (Py_7p_8) was treated with phosphatase and the reaction terminated by autoclaving at 120°C for 3 minutes. Another sample was treated the same way and then mixed with an autoclaved portion of phosphatase. Both samples, and a third one containing untreated pyrimidine heptanucleotide, were analyzed separately on identical DEAE-Sephadex columns at 65°C in the presence of 7.0 M urea. The results are shown in Fig. 21. Recoveries of ^{32}P from the columns were quantitative in all cases. The results showed that:

1) The phosphatase reaction was complete due to the fact that the total P/terminal P ratio, as monitored by ^{32}P counting of the separate peaks in Fig. 21 b, was 4.15 (theoretical ratio: 4.0).

2) No contaminating diesterases were present. They would produce labeled mono and oligonucleotides shorter than Py_7p_8 which would be eluted before Py_7p_8.

3) As seen in Fig. 21 c, phosphatase is totally denatured by autoclaving at 120°C for 3 minutes. Incubation over a period of 24 hours did not produce any inorganic phosphate.

4) The last experiment also demonstrated that the short heat treatment did not produce breakage or dephosphorylation of the oligonucleotides.

b) The second control experiment was done by autoclaving an equimolar mixture of 5' deoxymononucleotides at 120°C for 3 minutes and chromatographing them on a column at pH 9.2 (section 4.2.1) to detect dephosphorylation of any of the nucleotides. The deoxymononucleotides were prepared at a concentration

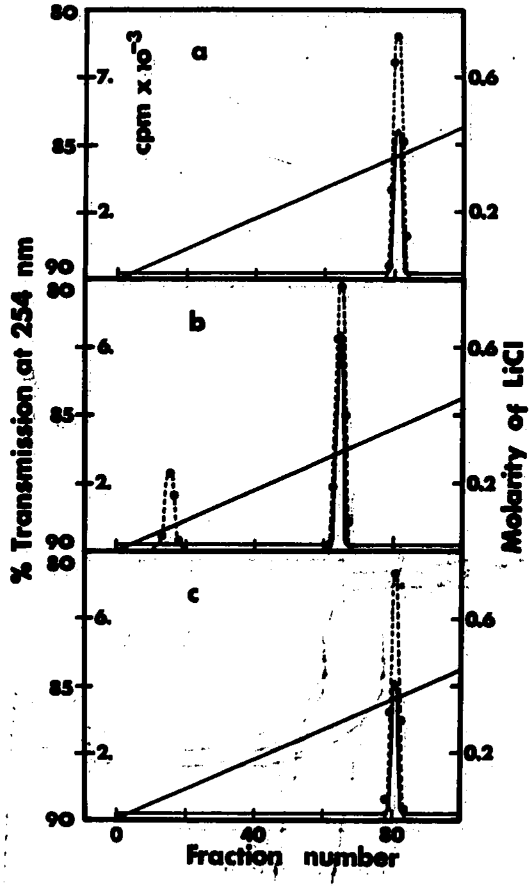


FIGURE 21

Effect of High Temperature on Phosphatase Denaturation and Oligonucleotide Structure

A ^{32}P-labeled Py$_7$p$_8$ sample from bacteriophage λDNA in 1 x 10^{-2} M Tris-HCl buffer, pH 8.0, 2.5 x 10^{-2} M MgCl$_2$ was divided into 3 equal portions of 10 ml. Each sample treated as described in the text was loaded on DEAE-Sephadex (30 x 0.9 cm, chloride form) and eluted at 65°C with a gradient of 0 to 0.4 M LiCl in 5 x 10^{-3} M Tris-HCl, 7.0 M urea buffer, pH 7.6. Total volume of eluent 1.0 l. 10 ml fractions collected at 80 ml/hour. The fractions were monitored at 254 nm on a LKB spectrophotometer, then transferred to scintillation vials and Cerenkov radiation (209) measured directly.

a) untreated Py$_7$p$_8$

b) Py$_7$p$_8$ treated with 0.3 units of phosphatase at 37°C for 24 hours. Reaction ended by autoclaving at 120°C for 3 minutes.

c) Py$_7$p$_8$ and 0.3 units of phosphatase treated separately at 120°C for 3 minutes, mixed and incubated at 37°C for 24 hours.

—— % transmission at 254 ●--● ^{32}P radioactivity of 2 x 10^{-3} M each in 0.05 M Tris-HCl buffer, pH 8.0. The solution was heat treated, the pH readjusted to 9.2 and analyzed on the cation exchanger. No nucleoside material appeared during the fractionation showing that all of the applied mixture was eluted in the first fraction as mononucleotides. It could still be postulated that longer oligonucleotides could have been cleaved during the heat treatment. In such a case they would still not contribute to the original 5' and 3' nucleosides obtained after phosphatase treatment of the DN'ase digest because the 5'-P terminals would not allow their identification when using snake venom diesterase for analysis of the 5' end nucleosides. They would also inhibit the action of spleen diesterase for the analysis of the 3' ends (195).

5.2.3 Snake venom diesterase hydrolysis. Reduction of 5'-nucleotidase contamination

The enzyme was purified as described in the methods section 2.2.8 in order to reduce the 5'-nucleotidase contamination. The impurity was reduced between 2.3 to 4.2 fold depending on the batch of snake venom diesterase used.

Ni^{++} and Zn^{++} have been shown to be strong inhibitors of 5'-nucleotidase at concentrations of 10^{-3} to 10^{-2} M (198, 199) while the same ions at 10^{-3} M have been shown to have no effect on snake venom diesterase (164).

Experiments were done to confirm these observations and to reduce further any possible nucleotidase activity. The results showed that with the enzyme purified by Keller's method (149), 60% of the snake venom diesterase activity was inhibited in the presence of 10^{-3}M $NiCl_2$ and 45% in the presence of 10^{-3} M $ZnCl_2$. The 5'-nucleotidase activity was reduced by 25% in the presence of either ion at a concentration of 10^{-3}M. At a Ni^{++} concentration of 10^{-2} M, 84% of the snake venom diesterase activity was inhibited and only 40% of the 5'-nucleotidase activity whereas at 10^{-4} M Ni^{++} or Zn^{++} only 10% of the 5'-nucleotidase contaminant was inactivated. Thus, much of the activity of the snake venom diesterase was lost when incubated under the above conditions with no great improvement in the reduction of the 5'-nucleotidase contaminant. The Pi released upon incubation of snake venom diesterase with AMP under the conditions of the 5'-nucleotidase assay (section 2.2.8) indicated that after 24 hours at 37°C only 1.6% of the total phosphorus was released. It was finally decided that the purified snake venom diesterase could be used in the absence of any additional ions due to the low amount of interference produced by 5'-nucleotidase with the terminal 5' or 3' nucleoside determinations.

5.2.4 Spleen diesterase hydrolysis

Spleen diesterase was tested for 3'-nucleotidase activity as described in section 2.2.8. No nucleotidase activity could be detected when 2'-3'-AMP was incubated under optimal diesterase conditions for 24 hours at 37°C. A test for phosphatase activity was also done with p-nitrophenylphosphate as substrate and no activity was detected after 12 hours at 37°C. Thus no purification of the commercial enzyme was required.

5.2.5 Evaluation of the end group analysis methods by total analysis of nucleosides and nucleotides in a DN'ase A-Mg^{++} digest of E.coli K12 DNA

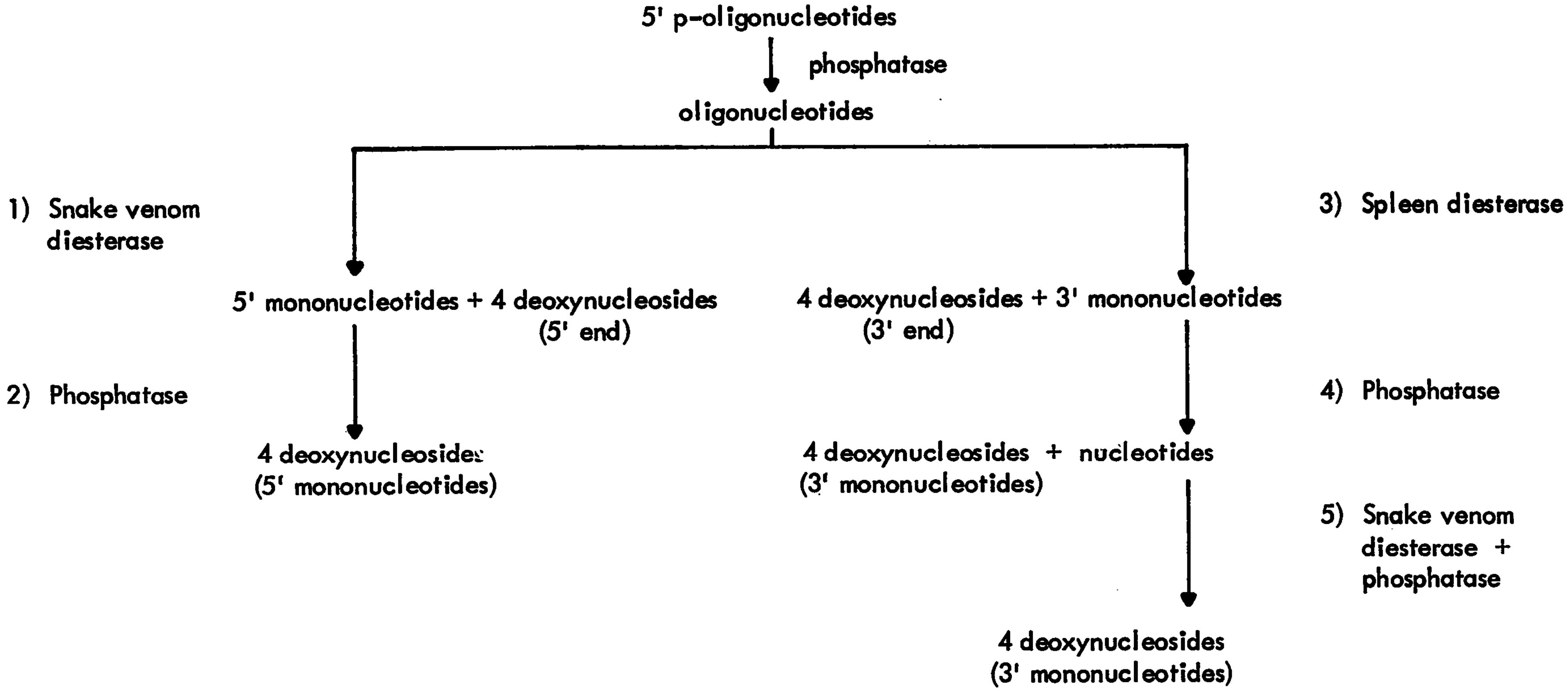
All of the end group analysis methods examined in section 5.2 were collectively evaluated with an extensive DN'ase A-Mg^{++} digest of E.coli K12 DNA. The conditions for the reaction are given in Table 13 (digest 10). All enzymatic treatments, desalting and analysis on the cation exchanger at pH 9.2 were done as outlined in sections 2.2.3, 2.2.4, 2.2.5 and 2.2.7.

Aliquots of the hydrolyzed samples at different stages in the analysis were chromatographed at pH 9.2. The steps involved in the determination of the total nucleoside-nucleotide content are schematically illustrated in Fig. 22.

1) Half of the phosphatase-treated material was hydrolyzed with snake venom diesterase and the products analyzed by Dowex 50 chromatography at pH 9.2 (section 2.2.7). The nucleosides released are the 5' terminals of the oligonucleotides.
2) The first peak from the above fractionation comprising 5' deoxymononucleotides (see Fig. 13) was desalted and treated with phosphatase for total composition analysis by Dowex 50 chromatography at pH 9.2.
3) The second half of the dephosphorylated material was digested with spleen diesterase and analyzed at pH 9.2. The nucleosides released are the 3' terminals of the oligonucleotides.

FIGURE 22

Schematic Outline of a Total Composition Analysis of a DN'ase A - Mg^{++} Digest of E. coli K12 DNA



4) The nucleotide peak from the above fractionation comprising the 3' deoxymononucleotides was desalted and treated with phosphatase for total composition determination at pH 9.2

5) A nucleotide fraction appeared in the above separation which was further treated with snake venom diesterase and phosphatase for total analysis and was assumed to be part of the 3' mononucleotide fraction.

The results are shown in Table 16.

Several points in the total analysis of the DN'ase digest should be emphasized.

a) The difference in total recovery of nucleosides for the two complete analyses of nucleoside content in DNA is probably due to the large amount of nucleotide material involved and calculation factors used (x2 to x6) in step 2) and 4). The recoveries of the terminal 5' and 3' end nucleosides are almost identical. These results lead to an average chain length of 5.9 and 6.9, whereas by phosphate analysis an average of 6.3 was found.

b) Digestion of the 5' nucleotides from step 1) by phosphatase was virtually complete, only 0.6% of the total mononucleotide fraction remaining unhydrolyzed, indicating that the nucleotide peak was composed totally of 5' mononucleotides.

c) Degradation of the 3' nucleotides from step 3) by phosphatase gave an extra nucleotide peak upon analysis, which represented 5.8% of the total nucleotide fraction. The addition of an excess of spleen diesterase did not

TABLE 16

Total Composition Analysis of a DN'ase A – Mg^{++} Activated Digest of E.coli K12 DNA

Nucleosides	5' End				3' End				
	Step 1 Snake venom diesterase	Step 2 Phosphatase	Total DNA nucleosides		Step 3 Spleen diesterase	Step 4 Phosphatase	Step 5 Snake venom diesterase and phosphatase	Total DNA nucleosides	
	µmoles	µmoles	µmoles	Molar %	µmoles	µmoles	µmoles	µmoles	Molar %
1		0.312*							
dT	2.612	13.836	16.448	25.1	3.380	10.504	0.360	14.244	24.7
dC	1.902	14.136	16.038	24.4	1.428	12.608	0.408	14.444	25.1
dG	2.822	13.512	16.334	24.9	4.352	9.368	0.700	14.420	25.0
dA	2.126	14.700	16.826	25.6	0.636	13.040	0.844	14.520	25.2
Total	9.462	56.496	65.646		9.796	45.520	2.312	57.628	
Chain length†			6.9					5.9	

* The value corresponds to the nucleotide fraction which was not further digested and analyzed.

† $\frac{\text{Total nucleotides + nucleosides}}{\text{Nucleosides}}$; by $\frac{\text{Total P}}{\text{Terminal P}}$ = 6.3.

alter the pattern. This peak could represent an oligonucleotide fraction formed during the hydrolysis by spleen diesterase, an enzyme which has a tendency to catalyze transfer reactions leading to higher oligonucleotide species, especially in the presence of high substrate concentration (195, 200, 201). The distribution of nucleosides in this oligonucleotide fraction after total digestion with snake venom diesterase plus phosphatase, shows that 60% of them are purines, but no drastic preferential release of any of the four nucleosides occurred.

d) The molar percent of the total DNA nucleosides by the two separate analyses with snake venom diesterase and spleen diesterase gave a similar distribution of nucleosides in agreement with the distribution of bases in E.coli DNA (section 2.2.1). This indicates that no preferential loss of any of the nucleosides or nucleotides occurred which could impair the use of any of the methods discussed for the quantitative analysis of the oligonucleotide termini.

5.3 Analysis of the 5' and 3' ends of the DN'ase-metal activated hydrolysates of E.coli K12 and calf thymus DNA

All of the methods of fractionation and analysis described in the previous sections were applied to studies on the specificity of DN'ase activated by divalent cations. Partial digests were done in order to determine whether there is any difference in the specificity of early cleavages compared to the extensive degradations as found for enzymes such as Micrococcal nuclease (202, 203), venom endonuclease (204) and DN'ase II (205). The extensive digests were done to detect changes in the specificity of DN'ase at a stage where the rate of the reaction slows down considerably due to autoretardation (39). It must be emphasized that large differences in the type of oligonucleotides obtained by specific cleavage in the presence of certain cations, could eventually be extrapolated to studies on the primary structure of DNA.

5.3.1 Distribution of deoxyribonucleosides at the 5' and 3' termini of the oligonucleotides released from calf thymus DNA

The hydrolysis of the DNA samples with the various metal ions, the conditions of incubation and the kinetic results were described in section 5.1.1.

The total analysis of both termini in total oligonucleotide mixtures indicated the following general pattern.

Two classes of cations could be categorized according to the way they activate DN'ase A:

Group A includes Cd^{++}, Ca^{++}, Ba^{++}, Co^{++} and Mg^{++} plus Ca^{++} and showed no difference in the distribution of terminal nucleosides in partial, intermediate or extensive digests.

Group B, which includes Ni^{++}, Mg^{++}, Mn^{++}, Sr^{++} and Zn^{++}, showed a change in distribution of terminal nucleosides from the partial to the extensive digests.

a) Hydrolysis of calf thymus DNA in the presence of ions of group A

The results from this group of ions are presented in Table 17.

Partial and extensive digests with Co^{++} as activating ion corresponding to 7.8% and 35.0% of the total acid soluble material released showed preferential resistance of the C -C bond (39, 211) as deduced from the low content of dC at the 5' and especially at the 3' end. The data would suggest that under extensive hydrolytic conditions the enzyme will preferentially split bonds of the type Pu-Py (80, 81, 143, 144, 207) and also Pu-Pu. The patterns of degradation are similar for the partial and extensive digests and there are no signs of a change in the preferential cleavage or resistance of certain bonds. A partial digest with Ba^{++} showed similar results to the intermediate hydrolysis with Ca^{++} and slightly lower release of dG at the 3' end probably indicating the partial resistance of sequences of the type dpG-pN.

For the intermediate Cd^{++} digest the 5' end analysis was identical to the Co^{++} hydrolysates whereas the reversal of the dG and dA content at the 3' end occurred probably representing partial resistance of sequences such as dpA-pN.

In all the above cases the dC content at both 5' and 3' ends was lower than the rest, suggesting the resistance of dpC-pC sequences and others rich in dC content such as dpN-pC and dpC-pN.

TABLE 17

Distribution of Deoxyribonucleosides at the 5' and 3' Ends of the Oligonucleotides Released from Calf Thymus DNA by DN'ase Activated by Ions from Group A

Digest No.	Ion	Type of digest	Nucleoside distribution	Molar Proportions									
				5' End					3' End				
				dT	dC	dG	dA	Total	dT	dC	dG	dA	Total
3	Co^{++}	P	µmoles	0.099	0.072	0.108	0.089	0.368	0.098	0.063	0.143	0.094	0.398
			Moles %	26.9	19.6	24.2	24.2		24.6	15.8	35.9	23.6	
		I	µmoles	0.386	0.234	0.393	0.320	1.333	0.324	0.104	0.316	0.752	1.496
			Moles %	29.0	17.5	29.5	24.0		21.7	7.0	21.1	50.2	
1	Ba^{++}	P	µmoles	0.279	0.051	0.182	0.160	0.672	0.194	0.100	0.080	0.312	0.700
			Moles %	41.5	7.6	27.1	23.8		27.7	14.3	13.4	44.6	
2	Ca^{++}	I	µmoles	0.360	0.118	0.332	0.300	1.110	0.304	0.122	0.158	0.480	1.064
			Moles %	32.4	10.6	30.0	27.0		28.6	11.5	14.8	45.1	
5	Cd^{++}	I	µmoles	0.323	0.187	0.321	0.258	1.089	0.343	0.131	0.576	0.234	1.284
			Moles %	29.6	17.2	29.5	23.7		26.1	10.2	45.2	18.4	
11	Mg^{++} and Ca^{++}	E	µmoles	2.378	1.518	1.191	1.739	6.826	1.852	0.572	2.015	2.252	6.691
			Moles %	34.8	22.2	17.4	25.5		27.7	8.5	30.1	33.7	

P = partial, I = intermediate, E = extensive digest.

When the synergistic effect of Mg^{++} plus Ca^{++} was examined the same results were obtained, whereas Mg^{++} alone belonged to the second category.

In these experiments the actual percentages of terminal nucleosides for the various ions showed individual variations but the overall distributions are similar with all four nucleosides represented in both ends and with lower proportions of dC at both termini.

b) Hydrolysis of calf thymus DNA in the presence of ions of group B

The results of the total analysis of the 5' and 3' end nucleosides are given in Table 18.

The common feature for this group of ions in the partial digests was the small amount of pyrimidine nucleosides at the 5' end whereas at the 3' end only dC is lower than the others. Depending on the extent of the partial digest and on the ion used the total pyrimidine content at the 5' end varied between 3 to 12% of the total. The dC content at the 3' end varied between 6.5 to 14% of the total. Only in the Mg^{++} hydrolysate did the dC nucleoside amount to 23% of the total nucleosides at the 3' end.

In the intermediate digest with Sr^{++} as activator (digest 24), the pattern characteristic for the extensive digest became apparent. At the 5' end the pyrimidines were still low but dT was increased substantially. However the total amount of dA and dC at the 3' end remained low and, at this stage, almost similar.

TABLE 18.

Distribution of Deoxyribonucleosides at the 5' and 3' Ends of the Oligonucleotides Released from Calf Thymus DNA by DN'aseA Activated by Cations from Group B

			µmoles nucleosides										Moles % nucleosides							
Digest No.	Ion	Type of digest	5' End					3' End					5' End				3' End			
			dT	dC	dG	dA	Total	dT	dC	dG	dA	Total	dT	dC	dG	dA	dT	dC	dG	dA
6	Mg^{++}	P	0.003	0.003	0.122	0.107	0.235	—	—	—	—	—	1.3	1.3	51.9	45.5	—	—	—	—
8	Mg^{++}	P	0.027	0.023	0.169	0.179	0.390	0.095	0.100	0.122	0.108	0.425	6.9	5.9	43.3	45.9	22.4	23.5	28.7	25.4
	Mg^{++}	E	1.118	1.050	1.346	1.112	4.626	2.585	0.319	1.903	0.155	4.962	24.2	22.7	29.1	24.0	52.1	6.4	38.4	3.1
9	Mg^{++}	E	—	—	—	—	—	1.771	0.460	1.293	0.088	3.612	—	—	—	—	49.0	12.7	35.8	2.4
12	Mn^{++}	P	0.009	0.015	0.222	0.208	0.454	0.122	0.028	0.065	0.160	0.375	2.0	3.3	48.9	45.8	32.5	7.5	17.3	42.7
13	Mn^{++}	E	1.779	1.726	2.544	1.848	7.897	3.419	1.265	2.239	0.151	7.074	22.5	21.8	32.2	23.4	48.3	17.9	31.7	2.1
14	Mn^{++}	E	8.342	5.062	7.310	6.302	11.456	13.180	4.588	9.200	0.360	11.908	26.3	18.5	32.0	23.2	36.2	16.4	44.1	3.2
17	Ni^{++}	P	—	—	—	—	—	0.164	0.038	0.113	0.130	0.445	—	—	—	—	36.8	8.5	25.4	29.2
18	Ni^{++}	P	0.004	0.026	0.134	0.167	0.331	0.104	0.044	0.090	0.087	0.325*	1.2	7.9	40.5	50.4	32.0	13.5	27.7	26.8
	Ni^{++}	E	0.798	0.689	1.196	1.690	4.373*	3.334	1.011	2.024	0.177	6.546	18.2	15.8	27.3	38.6	50.9	15.4	31.0	2.7
19	Ni^{++}	E	1.516	0.802	1.460	1.916	5.694	3.057	0.723	2.150	0.108	6.038	26.9	13.2	25.9	34.0	50.6	12.0	35.6	1.8
23	Sr^{++}	P	0.006	0.014	0.138	0.165	0.323	0.054	0.022	0.134	0.102	0.312	1.8	3.9	41.0	49.4	17.3	7.0	42.9	32.7
24	Sr^{++}	I	0.076	0.032	0.282	0.422	0.812	0.338	0.064	0.293	0.089	0.784	9.4	3.9	34.7	52.1	44.8	8.4	38.9	7.9
	Sr^{++}	E	1.263	1.174	1.383	1.016	4.836	2.511	0.497	1.821	0.090	4.919	26.1	24.3	28.6	21.0	51.0	10.1	37.0	1.8
27	Zn^{++}	P	0.008	0.017	0.115	0.172	0.312	0.078	0.018	0.061	0.144	0.301	2.6	5.5	36.9	55.1	25.9	6.0	20.3	47.8
28	Zn^{++}	P	0.012	0.008	0.078	0.096	0.194	—	—	—	—	—	6.2	4.1	40.2	49.5	—	—	—	—
	Zn^{++}	E	1.573	0.771	1.475	1.415	5.234	2.543	0.696	2.083	0.075	5.397	30.1	14.7	28.2	27.0	47.2	12.9	38.6	1.4
29	Zn^{++}	E	—	—	—	—	—	2.034	0.352	1.703	0.215	4.304	—	—	—	—	47.3	8.2	39.6	5.0

* Part of the samples were lost before analysis.

P = partial, I = intermediate, E = extensive digest.

Examination of the extensive digests with an average chain length of 5.4 to 8.4 showed the four nucleosides at the 5' end with dC always lower than the rest of the nucleosides. As previously shown individual variations in the total amount of each nucleoside were observed. These were due to the impossibility of performing completely identical digests and to the several steps involved in the analysis of the terminal ends which, as shown in section 5.2.5, can lead to methodological inaccuracies. Nevertheless, in all cases, the overall distribution of terminal nucleosides is similar.

At the 3' end the most relevant feature produced by this group of cations was observed. dT and dG varied between 32 to 52% of the total ends, dC between 6.4 to 18%, but dA never increased over 6.5% of the total. These low amounts of dA at the 3' ends corresponded to approximately the same amounts of nucleosides released in the partial digests when concentration in µmoles is considered.

5.3.2 Distribution of deoxyribonucleosides at the 5' and 3' termini of the oligonucleotides released from *E.coli* DNA extensively digested with DN'ase I - Mn^{++}

a) Total DN'ase I - Mn^{++} digestion

Initial experiments on the fractionation and analysis of DN'ase digests were done with the commercial enzyme as mentioned in section 4.1. A total digest of *E.coli* DNA with DN'ase I and Mn^{++} as activating ion was carried out as described in section 4.1, digest C. End analysis of the fractionated mononucleotide and dinucleotide components was performed as outlined in section 5.2. From the results in Tables 5, 7 and 11, the composition of the 5' and 3' ends of the mixtures was determined. Each of the isostich fractions from the trinucleotides to the hexanucleotides was analyzed for total nucleoside distribution at both the 5' and 3' ends of the oligonucleotides. The results are presented in Table 19. It is clearly seen that the general distribution pattern for both the mono and dinucleotides at both ends are completely different from those of the trinucleotides and higher isostich fractions. The dT and dG represented 92.5% of the total mononucleotides whereas dA was very low and dC was not detected at all in the mixture. For the dinucleotides, dT and dA are low at both ends while dC is much higher than was found at both termini in any of the other isostich fractions. The nucleoside distributions for these fractions correspond to the general pattern found for the DN'ase A - Mn^{++} digests with calf thymus and <u>E.coli</u> K12 DNA (sections 5.3.1 and 5.3.2, b), particularly in the low proportions of dA at the 3' ends of the oligonucleotides and diminished amounts of dC at both ends. The seeming preference for hydrolysis of certain internucleotide bonds as deduced from the characterization of dinucleotides does not appear in the distribution of ends in the longer oligonucleotides nor in the total digests. Similar results were found by Antonoglou and Georgatsos (211) when digests of mouse kidney DN'ase

TABLE 19

Distribution of Deoxyribonucleosides at the 5' and 3' Ends of the Oligonucleotides Released from *E.coli* K12 DNA by Mn^{++}DN'ase I Hydrolysis

Isostich	% of total P in digest	5' End				3' End			
		dT	dC	dG	dA	dT	dC	dG	dA
Mono	3.0	54.1	0	37.4	8.5	54.1	0	37.4	8.5
Di	26.8	21.1	33.8	32.4	12.7	25.8	30.0	25.0	19.2
Tri	29.4	25.1	7.9	38.9	28.0	38.0	16.9	43.4	1.7
Tetra	25.7	41.6	6.1	39.8	12.5	38.6	14.7	42.4	4.3
Penta	11.8	42.8	3.9	23.1	30.2	37.5	12.5	44.0	5.9
Hexa	2.7	39.9	9.7	23.5	26.9	39.6	18.1	42.3	0

Heptanucleotides comprising 0.5% of the total phosphorus in one case were not analyzed.

were analyzed. The percent of total base at both ends of the total digest was calculated from the distribution of termini in the various isostich fractions and the isostich proportion (total phosphorus content) in the hydrolysis mixture. The results are presented in Table 20.

In the total mixture, the figures indicate a similar distribution of dT at both ends, low dC at both ends, slightly lower proportions of dA in the 5' end and higher at the 3' end. Taking into account that this was an exhaustive digest with an average chain length of 3.6 and that it was done with the commercial DN'ase I, the results do not appear to differ to a great extent from the DN'ase A-Mn^{++} digests discussed in sections 5.3.1 and 5.3.2, b. It should be mentioned that low dA content at the 3' ends are noticeable in isostichs longer than dinucleotides. The "abnormal" distribution of nucleosides in the dinucleotide fraction when compared to the longer oligonucleotides probably represents the result of "forced cleavage" which might abolish any preferential splitting at advanced stages in the hydrolysis. This was found to be the case for Micrococcal nuclease (39) and DN'ase II (38).

b) Extensive DN'ase A - Metal hydrolysis

The results for the digestion of E.coli K12 DNA with the ions in group B are shown in Table 21.

These results are similar to those obtained with calf thymus DNA in the general distribution of nucleosides at both ends. However, the G + C

TABLE 20

Base Composition of the 5' and 3' Termini of the Total DN'ase I - Mn^{++} Digest of E.coli K12 DNA

	Moles % of total base							
	5' End				3' End			
Isostich	dT	dC	dG	dA	dT	dC	dG	dA
Mono	1.62	0	1.12	0.26	1.62	0	1.12	0.26
Di	5.65	9.05	8.68	3.40	6.91	8.04	6.70	5.14
Tri	7.38	2.32	11.44	8.23	11.17	4.97	12.76	0.49
Tetra	10.69	1.57	10.23	3.21	9.92	3.78	10.90	1.10
Penta	5.05	0.46	2.73	3.56	4.43	1.48	5.19	6.99
Hexa	1.07	0.26	0.63	0.73	1.06	0.49	1.14	0
% of total	31.4	13.7	34.8	19.4	35.1	18.7	37.8	14.0
% of total (adjusted)*	31.7	13.8	35.1	19.5	33.2	17.8	35.8	13.2

* % of total adjusted to 100% in both the 5' and 3' ends.

TABLE 21

Distribution of Deoxyribonucleosides at the 5' and 3' Ends of the Oligonucleotides Released from <u>E.coli</u> K12 DNA Extensively Digested by DN'ase A Activated by Cations from Group B

Digest No.	Ion	Nucleoside distribution	Molar Proportions									
			5' End					3' End				
			dT	dC	dG	dA	Total	dT	dC	dG	dA	Total
10	Mg^{++}	µmoles	2.612	1.902	2.822	2.126	9.462	3.380	1.428	4.352	0.636	9.796
		Moles %	27.6	20.1	29.8	22.5		34.5	14.6	44.4	6.5	
16	Mn^{++}	µmoles	3.012	2.020	3.668	2.556	11.456	4.316	1.952	5.256	0.384	11.908
		Moles %	26.3	18.5	32.0	23.2		36.2	16.4	44.1	3.2	
21	Ni^{++}	µmoles	1.612	0.688	1.424	1.852	5.576	2.244	0.792	2.536	0.256	5.828
		Moles %	28.2	12.0	26.6	32.2		38.5	13.6	43.5	4.4	
25	Sr^{++}	µmoles	0.454	0.423	0.651	0.395	1.923	0.427	0.226	0.604	0.056	1.313
		Moles %	23.6	22.0	33.9	20.5		32.5	17.2	46.0	4.3	
26	Sr^{++}	µmoles	1.020	0.860	1.248	1.110	4.238	1.528	0.586	1.820	0.194	4.128
		Moles %	24.1	20.3	29.6	26.2		37.0	14.2	44.1	4.7	
30	Zn^{++}	µmoles	0.634	0.435	0.595	0.565	2.229	0.758	0.399	1.022	0.086	2.265
		Moles %	28.6	19.6	26.7	25.1		33.5	17.6	45.1	3.8	

content at both ends in the E.coli digests were higher than those in the calf thymus DNA hydrolysates. This trend is more manifest at the 3' end where dG has increased the most. The data can be explained by the higher G + C content in E.coli DNA and its reflection on the distinct frequency characteristics of the molecule when compared to calf thymus DNA which is an A + T type DNA (208). The values for dC and dA were almost identical to those in calf thymus DNA indicating that sequences of the type dpN-pC, dpC-pN and dpA-pN were also preferentially resistant to hydrolysis in this DNA.

The action of the ions described in group B can be summarized as follows:

1) In partial digests very low amounts of pyrimidines were present in the 5' ends being never more than 12% of the total. At the 3' ends the release of nucleosides was more or less similar with slightly lower proportions of dC.

2) In the extensive digests the distribution was similar for dT, dG and dA in the 5' end whereas dC was slightly lower. In the 3' end dC was maintained more or less in the same proportions. dA was not produced in higher amounts than during the partial digests, indicating that all susceptible bonds with a dA in the 3' position were broken during the first stages of the digestion. During the late stages these bonds became resistant.

5.4 Selective 3' end analysis of isostichs from several DN'ase A-Metal digests

Portions of some of the long isostichs obtained in the chain length fractionations described in section 5.1.2, Table 15, were analyzed for total composition of their 3' ends. The results are shown in Table 22.

The data showed a very high distribution of dT and dG in the three separate isostichs amounting to 85% of the total ends. dC is low as before and dA is almost insignificant being never higher than 0.5% of the total.

From these results it can be concluded that DN'ase A will exert its specificity properties in the presence of group B ions especially on long poly or oligonucleotides which are cleaved to shorter ones at certain preferential sequences.

5.5 Specificity of DN'ase A in the presence of cations from group B as activators

The susceptibility of the various DNA sequences to partial or extensive hydrolysis by DN'ase A activated by ions such as Ni^{++}, Mg^{++}, Mn^{++}, Sr^{++} or Zn^{++} is summarized in Table 23. The most interesting features are:

1) Internucleotide bonds containing dA at the 3' end are preferentially split or preferentially resistant at the beginning of the digestion but very resistant at the end. When dA is on the 5' end of the bond it will generally be split.

2) Bonds containing mostly dT and dG at the 3' or 5' ends such as G-G, G-T, T-T and T-G will be preferentially split or preferentially resistant at early stages in the reaction but more readily split at the end.

TABLE 22

Distribution Analysis of the 3' Ends of Selected Isostichs Obtained from Several DN'ase A – Metal Digests

			Molar proportions at 3' end			
Ion	Isostich	Nucleoside distribution	dT	dC	dG	dA
Mg^{++}	Octa	µmoles	0.440	0.112	0.190	0.004
		Moles %	59.0	15.0	25.5	0.5
Ni^{++}	Octa	µmoles	0.223	0.047	0.137	0
		Moles %	54.8	11.5	33.7	0
Sr^{++}	Hexa	µmoles	0.453	0.120	0.273	0.004
		Moles %	53.3	14.1	32.1	0.5

TABLE 23

Specificity of DN'ase A in the Presence of Ni^{++}, Mg^{++}, Mn^{++}, Sr^{++} or Zn^{++} as Activating Ions

Bond	Type of Digest Partial	Extensive
pA — pA	S	VR
pG — pA	S	S
pC — pA	SS	S
pT — pA	S	S
pA — pG	S	VR
pA — pC	R	VR
pA — pT	R	VR
pG — pG	S	HS
pG — pC	R	S
pG — pT	R	HS
pT — pT	R	S
pT — pG	S	HS
pT — pC	R	S
pC — pC	R	R
pC — pG	SS	S
pC — pT	R	SS

S = Split
SS = Slightly Split
HS = Highly Split
R = Resistant
VR = Very Resistant

3) C-C is resistant through all of the hydrolysis as found for digests comprising ions of group A. The low proportions of dC at both ends indicate that linkages which contain this base will be split but not in major proportions.

The results presented show that the divalent metal ions can be classified into two groups according to their activation properties on DN'ase and that ions such as Mg^{++}, Mn^{++}, Ni^{++}, Sr^{++} and Zn^{++} exert a more sophisticated discrimination against certain internucleotide linkages throughout the several stages in the depolymerization of DNA.

CHAPTER 6

DISCUSSION

6.1 Purification of DN'ase I

A purified, homogeneous and stable DN'ase A fraction has been obtained by a combination of FDP treatment and phosphocellulose chromatography of a commercial DN'ase I preparation (sections 3.1.1 and 3.1.2). Five DN'ase I active fractions were obtained and a sixth was observed when larger amounts of the commercial enzyme were chromatographed. The three major fractions (A, B and C) had the same specific activity, each of which was 30% higher than the specific activity of the untreated enzyme. Fractions D and E had 50 to 70% of the maximal specific activity of DN'ase A.

It was shown recently that the amino acid compositions of DN'ase A and B separated on Sulfoethyl-Sephadex are identical (131). However, the carbohydrate moieties of the two preparations were found to contain different amounts of components when totally degraded. Price, et al. (131) found only 2 mannose and 2 glucosamine residues to be present in DN'ase A, while Catley, et al. (140) found a heptasaccharide comprising two N-acetyl-glucosamine and five mannose residues.

The five or six DN'ase fractions obtained during the present study probably differ in the saccharide moiety which may vary in chain length and thus confer to each DN'ase fraction a typical chromatographic behavior. Variations in oligosaccharide chain length might be due to the method of preparation of the commercial DN'ase starting material which uses 0.25 N sulfuric acid in the first step of the purification (130, 131). Similar differences have been shown with the sugar moieties of RN'ase A and B (214) which are extracted from the pancreas by the same acid extraction procedure.

Rechromatography of DN'ase A produced a highly purified enzyme which showed a single sharp band when analyzed by gel electrophoresis at pH 8.3 (Fig. 26). Single bands on gel electrophoresis were observed by Price, et al. (131) when one of the two DN'ase fractions obtained from the Sulfoethyl-Sephadex column was examined by one hour electrophoresis at pH 8.9 (131). Lindberg (141) obtained a single active fraction on G-100 Sephadex with enzyme from a different commercial source (Sigma). Electrophoresis of this fraction at pH 10.3 showed only one band. It is probable that these purified fractions (131, 141) were still contaminated since both commercial preparations used in these studies were initially obtained by the acid extraction method (130).

The rechromatographed DN'ase A did not have any contaminating phosphatase, exonuclease or nucleotidase activity. The enzyme was found to be completely stable when stored at -20°C for 14 months and in solution at 4°C for 4 months. The rate of inactivation of the unpurified crystalline DN'ase I could be diminished in the presence of Mg^{++} or Mn^{++} ions (section 3.1.2). This was also found to be the case with Ca^{++} (131, 136, 140) and it is probably due to the stabilization against proteolytic inactivation as is the case with Ca^{++}-protected Micrococcal nuclease (121).

6.2 Properties of the enzyme

Some of the parameters affecting the activity of DN'ase I had to be re-evaluated with the purified DN'ase fraction before using it for the specificity studies.

The two assay systems, used in parallel, provided complementary information to each other. The spectrophotometric assay provided rapid and accurate measurements of initial velocities which were linear over a wide range of protein concentration depending on the activating ion used (Fig. 3). The other assay, based on the production of acid soluble material released during the hydrolysis of DNA as measured at 260 nm (130, 215), was found to be appropriate for the kinetic determination at advanced stages of the reaction where much slower hydrolysis occurs due to the effect of autoretardation (39, 138, 207).

Varying the concentration of DNA involves a change in the optimal concentration of the activating ions as was shown in Table 3 confirming the observations of Kunitz (130), Shack and Bynum (134) and others. The problem can in part be solved by adding an excess of ions above the concentration of the highest amount of DNA used. However, the relatively high ionic strength of the ions used (section 3.2.5) had a depressing effect on the velocity of the reaction. This is probably the reason why double reciprocal plots for the determination of Vmax or Km with native DNA as a substrate were never linear, similar to the results of Melgar and Goldthwait (137).

Studies on the influence of the pH in the activity of the enzyme (Fig. 5) indicated that metal ion requirement is pH dependent. A wide activation was achieved with the several ions in the range of pH 6.0 to 8.5. Below pH 5.0 the activity was always nil as expected, since protons strongly compete with metal ions in the formation of complexes (216).

For Mn^{++} and Ca^{++} the activities were maximal over pH 8.0. Contrary to the results of Shack and Bynum (134) we found that the requirement for Mg^{++} (also for Ni^{++} and Co^{++}) decreases above pH 7.0 to 7.5. However, the largest increase in activity lies in the region of pH 5.5 to 7.0. As suggested by these authors (134) the titration curve of native DNA does not change over this region of pH (217), at the low ionic strength of these experiments. This indicates that the binding of ions by DNA is pH independent in the range 5.5 to 7.0. Since the activity of the enzyme exhibits pH dependence in the same region it is concluded that the change in bivalent cation requirement with pH is due to the titration of different groups in the enzyme. However, the increase of ion concentration needed for higher concentrations of DNA is in good agreement with data (130, 133, 171, 218) which implicate metal-DNA binding for enzyme activation.

Increase in the concentration of monovalent ions such as Na^{+} or $Tris^{+}$, produced a marked decrease in the rate of digestion as previously demonstrated (39, 130, 134). However, with the activating divalent ions, the decrease in activity at identical ionic strength of the monovalent ions is lower. This could also be an indication of the metallo-substrate interaction: in high salt concentration divalent metal binding to DNA is reduced (218). Partial restoration of activity was obtained by addition of an excess of cation to the NaCl inhibited reaction (section 3.2.5).

The activation of DN'ase A by divalent cations showed that Mg^{++} was the best activator when 0.5 mg/ml DNA was used in the assays. At this DNA concentration the activation with Mn^{++} was never higher than that with Mg^{++} as reported by several investigators (133, 134, 137). However Wiberg's (133) experiments were done with denatured DNA and thus are not truly comparable, while Melgar and Goldthwait (137) performed their experiments at 3.3×10^{-2} M to 5.0×10^{-2}M Tris-HCl at which concentration 88% of the DN'ase A activity was shown to be inactivated (section 3.2.5).

Contradictory reports on activation and inhibition by certain divalent ions were examined. High activation properties of Zn^{++} and Co^{++} between 10^{-4}M to 10^{-2}M reported by Eichhorn, et al. (174) were due to the addition of non-specific ions to the solution (3.3×10^{-3}M $NaNO_3$) and to the high ionic strength and suboptimal pH for all of the ions (0.1 M acetate, pH 5.0). The same applies to results obtained by Melgar and Goldthwait (137b). Calculating from the data of one of their experiments, the activity ratio of Mg^{++}:Mn^{++}:Co^{++} was 1.0:2.0:2.4 whereas in another experiment the ratio was 1.0:33.0:8.0.

Zn^{++} and Cd^{++} were shown to be activators (Fig. 6) contrary to previous reports (134, 173). Larger amounts of enzyme had to be added to the reaction mixtures and in the case of Zn^{++} a narrow activation range was found between 1.0×10^{-4}M and 3.0×10^{-4}M (section 3.2.4).

For most of the ions an increase in concentration above 3.0×10^{-3} M inhibited the reaction. The synergistic effect of Mg^{++} plus Ca^{++} was shown to produce increased rates of hydrolysis over the maximal Mg^{++} activated rate. As mentioned in section 3.2.5, a lower synergistic effect was noticed when compared to other reports (133, 137, 176).

Biphasic or triphasic kinetics were observed depending on the ion used for the activation of DN'ase A. Ions such as Mg^{++}, Sr^{++} and Zn^{++} showed a lag when measured by both the hyperchromic and the acid soluble material assays. This was followed by a rapid linear increase in absorbance and then a much slower hydrolytic step with a shallow plateau for which no final end point could be observed (Fig. 4b). With Mn^{++}, Ni^{++}, Co^{++}, Ca^{++} and Ba^{++} no lag was observed under the conditions of assay (Fig. 4a). Mg^{++}, Sr^{++} and Zn^{++} also showed a lag at low enzyme concentrations when the protein-activity relationship was investigated (Fig. 3) whereas the other ions did not. These results can be compared to the studies of Melgar and Goldthwait (137) who postulated two different mechanisms of activation according to the metal ion used in the reaction of DN'ase I with native DNA. Double-hit kinetics or cleavage of only one of the strands per encounter was attributed to ions such as Mg^{++} Sr^{++} and Zn^{++} which present a lag phase. Single-hit kinetics or simultaneous scission of both strands was described for Mn^{++} and other ions which do not present a lag. The mechanism could be shifted from double to single hit by substituting Mg^{++} for Mn^{++} or Co^{++}, or by adding Ca^{++} to the Mg^{++}-activated hydrolysis. When a monovalent ion such as K^{+} was added to the Mn^{++} or Mg^{++} plus Ca^{++} reactions the lag phase reappeared showing a reversion to double-hit kinetics.

These differences in the mechanism of activation of DN'ase I are supported by the present kinetic data (section 3.2.1). However, the enzyme activated by ions which behaved kinetically differently has been shown to produce the same type of specificity (section 5.3.1).

The important role of divalent cations in the activation of DN'ase A is further concluded from the total inactivation produced by addition of EDTA to the reaction mixture (Fig. 7). The weak and reversible binding of cations to the enzyme (131, 136) allows the rapid chelation of the total metal ions (bound plus free) by EDTA, promptly stopping the reaction. Other factors indicating the existence of an enzyme-metal ion interaction came from the studies of Prince, et al. (131, 135, 136) described in section 1.4.1. Correlating this data with the pH-activity studies of DN'ase A (section 3.2.3, 134, 171) would suggest that the activity of the enzyme depends on the interaction of a metal cation with the unprotonated form of the imidazole group of the histidine (pK 6.0) residue at or near the active site (134).

Peptide-divalent metal ion interactions have been studied with most of the divalent cations. Metal ions like Ni^{++}, Cu^{++} and Zn^{++} have been found to interact strongly with the $-NH_2$ group of histidine and the N of the imidazole ring. For Mn^{++} low chelating values were found whereas Co^{++} and Zn^{++} form chelates through the -SH bond of cysteine and $-NH_2$ groups (219). Since imidazole ordinarily has a very low affinity to Mg^{++} (220) it would mean that if this cation takes place in the binding there must be other contributions to the chelating site.

6.3 Methods of fractionation and analysis of DNA hydrolysates

The availability of methods for discrete chain length fractionation of deoxyoligonucleotides up to the pentadecanucleotide level and quantitative separation of all possible non-isomeric dinucleotides and most of the non-isomeric trinucleotides extends the possibilities for obtaining new information on nucleotide sequences in DNA molecules.

6.3.1 Fractionation according to chain length

The method described for chain length fractionation of deoxyribonucleotides on DEAE-Sephadex at 65°C (section 4.1.2) has the advantage over previous methods (4, 38, 83) of allowing the discrete separation of isostichs up to the pentadecanucleotide level. In one case the separation of heptadecanucleotides was achieved (Table 15, Mn^{++} digest). Recoveries from the columns were better than 95% with no cross contramination of isostich fractions, at least up to the dodecanucleotide level.

Investigation of the possibility of extending the method for separation of even longer oligonucleotides by plotting chain length versus eluting salt concentration in Fig. 11 showed a progressively smaller difference in the salt concentration increase necessary to elute fractions of increasing chain length. Thus with longer oligonucleotides an asymptotic limit will be reached and resolution will disappear. This is contrary to the suggestion of Matthews (221) of a possibly limitless chain length fractionation of long oligonucleotides on DEAE-Sephadex. The non-linearity of the relationships in Fig. 11 obviate linear extrapolation of eluting salt concentration to estimate chain length (222). Since the relationship between eluting salt concentration and chain length is not linear and in addition, the mono, di and trinucleotides are somewhat subfractionated, some secondary binding forces of the oligonucleotides to the column are not overcome by 7.0 M urea at 65°C. Nevertheless, the higher temperature reduces drastically secondary binding forces which interfere in separations at 25°C (Fig. 11), and increases the dependence of the fractionation on the eluting salt concentration, reducing cooperative displacement where the longer oligonucleotides displace the shorter oligonucleotides which have lower affinity for the DEAE groups (8).

The fractionation is also influenced by the length of the oligonucleotides and the 5' terminal phosphate. For example, the tetranucleoside triphosphates (peak IV, Fig. 10b) which have the same charge as the dinucleotides (peak II, Fig. 10a) at pH 7.6 are eluted later. This could only be due to the difference in length between the two oligonucleotides, the larger having a higher non-ionic affinity to the matrix. Removal of the 5' terminal phosphates indicated that they play a role in the interaction with the matrix. Otherwise the two curves in Fig. 11 would be parallel. The dephosphorylation resulted in an improved separation confirming previous observations (83, 223). By increasing the length of the column the procedure could be extended for fractionation of oligonucleotides of chain length 20 to 22 (Fig. 11).

6.3.2 Fractionation of dinucleotides and trinucleotides according to base composition

Assuming random cleavage of DNA by DN'ase I each isostich fraction should contain $\frac{(n+3)!}{n!\,3!}$ oligonucleotide components where n = chain length. Thus 4 mononucleotides, 10 non-isomeric dinucleotides and 20 non-isomeric trinucleotides should be present in isostichs 1, 2 and 3 which were separated according to chain length (Fig. 10). No satisfactory methods were available for fractionation of all the non-isomeric di and trinucleotides according to base composition. The shortcomings of column or paper chromatography and paper or thin-layer plate electrophoresis have been outlined in sections 1.1.3 and 4.2.2.

Two methods, using DEAE-cellulose and ammonium formate as the eluting salt, were devised for the subfractionation of dinucleotides and trinucleotides. The volatile salt is easily removable by flash evaporation and sublimation, allowing the quantitative recovery of oligonucleotides in pure form for further investigation. Recoveries of material from the columns were always better than 92%.

The optimum pH for the separation of dinucleotides was pH 3.4 and was found from a plot of net charge versus pH in the range 1.0 to 5.0 (Fig. 16). Eleven dinucleotide fractions were separated by the procedure, the extra fraction being due to the completely resolved positional isomers dpCpA and dpApC. The spectral properties of the desalted dinucleotides were compared to equimolar mixtures of the corresponding mononucleotides and were found to be identical (Table 8). The results showed that positional isomers such as pCpA and pApC have identical ultraviolet absorption spectra at the three pH values measured. The isomers are thus undistinguishable when determined in a mixture. Identical results were obtained by Toal, et al. (190) for oligoribonucleotide isomers such as ApUp - UpAp, CpGp - GpCp and ApGpUp - ApUpGp - UpApGp. Dinucleotide concentrations were calculated by determining the hyperchromicity of each fraction at pH 3.4 and 260nm after being degraded with snake venom diesterase in a similar way to the method of Staehelin (224) for oligoribonucleotides. The hyperchromic values of the 11 fractions varied significantly between fractions ranging from 1.4% for TG + GT to 12.7% for AA. Similar large differences have been reported previously (190, 224, 225).

Twenty two trinucleotide peaks were fractionated at pH 3.6 (Fig. 18). Sixteen of them were identified and accounted for 14 of the 20 possible non-isomeric trinucleotides. Two sets of positional isomers were subfractionated, TTG and GGT. In both the dinucleotide and trinucleotide fractionations the purine rich trinucleotides were preferentially retained on the column. This indicated that the purine/pyrimidine ratio in the total mixture of isostichs has to be taken into consideration when subfractionating a large mixture of isomers on a cellulose matrix (86). Slight variations of pH, column length and width and volume of the eluant can lead to improper separations (sections 4.2.2 and 4.2.3).

6.3.3 Rapid separation of nucleosides and nucleotides

Recently a number of methods for separation of nucleosides or nucleotides have been reported (104, 148, 226, 227, 228). Two of these methods (104, 148) are rapid, that is the separations can be accomplished in 1 to 1.5 hours. The method described by Blattner and Erickson (148) is applicable only to nucleotides and that of Uziel, *et al*. (104), for nucleosides, has been developed for use with a highly automated chromatographic system and will not separate nucleotides from nucleosides under the conditions described. We therefore designed procedures for both the ribo and deoxyribo series in which a mixture of nucleotides and nucleosides can be separated by column chromatography within 1.5 to 2 hours (section 4.2.1).

The separation of nucleosides at high pH values on a cation exchanger where the total charge of the nucleosides is nil (for A and C) or partially negative (for G, T and U) is due to preferential non-ionic interactions with the resin (229). The partial negative charge of the hydroxyl groups of guanosine, deoxyguanosine, uridine and thymidine at the pH used results in the differential elution of all the components of the mixture. Together with the differences in pK values the additional methyl group of deoxythymidine may explain the increased retention of dT at pH 8.9, a factor used for the separation of this nucleoside from uridine.

The optimum pH was found to be a function of the exchanger and the particular nucleosides analyzed. The concentration of the eluting ion (NH_4^+) was set to give the closest positions of the peaks that still allowed effective separation. A compromise between several other variables affecting the separations had to be found. The volume in which a peak is eluted is a function of the flow rate (fast flow rates permit less equilibration at each level of the column) and the column length and diameter (230). Longer and narrower columns decrease the width (W) of the peak at half its maximum height (H) and hence increases the separation because H and W are inversely proportional to each other. By increasing the length of the columns the resolution of the peaks was much higher but the total analysis took 3 to 4 hours. The effect of other parameters, such as temperature, bead size and cross linking, on the fractionation were not investigated.

The first two systems described, the separation of 3' or 5' deoxynucleotides and deoxynucleosides and the separation of 3' or 5' ribonucleotides and ribonucleosides, were on column systems which did not require any regeneration. This allowed a very fast turn-over of experiments. A complete separation of all four deoxynucleosides and deoxynucleotides on the single column by two buffers at two different pH values required regeneration of the column and equilibration with the first buffer before re-use. An advantage with all methods is that the enzymes and ions in the digestion mixtures do not interfere with the separations and quantitative analyses. Attempts to determine the actual elution position of the enzyme by using ten-fold excess of enzyme and measuring enzyme activity and protein were unsuccessful. However, the columns have been re-used up to 40 times without repacking. The separation of deoxythymidine from the ribonucleosides provides an easy method for identification of DNA contamination in RNA preparations. The pH at which the elutions occur is in the most stable range for the purine glycosidic links. This is compared to the disadvantage of elution of purine nucleosides and nucleotides with acidic solutions (104). However, the main advantages of the procedures described above are the rapid separations achieved in 1.5 to 2 hours, the quantitative recoveries of the material and the accuracy of the method which allows very small quantities of material to be quantitatively measured (Fig. 13).

6.3.4 Applications of the fractionation methods

The availability of the new fractionation methods allows their application to a number of studies.

1) Fractionation according to chain length at 65°C

   a) The procedure can be applied to differentiate between DN'ase (or RN'ase) endo or exonucleolytic activities (39, 59, 118). Only mononucleotides will be produced with an exonuclease whereas a wide range of oligonucleotides will be fractionated after endonucleolytic hydrolysis.

   b) Partial exonuclease digests of long homogeneous oligonucleotides could be fractionated for sequential studies according to the method of Holley (1, 79, section 1.3.3). Combined analysis with two exonucleases of opposite polarity could render information on sequences of 35-40 nucleotides long. In addition, small amounts of nucleic acids could be analyzed by radioactive labeling of the oligonucleotide termini of the partial exonuclease digests. This can be performed by means of polynucleotide kinase (42, 97) for the 5' hydroxy ends, by chemical labeling of the 5' or 3' phosphate ends by formation of a ^{14}C-methylpyrophosphate derivative or by radioactive acetylation of the free 3' hydroxyl groups (77).

   c) The method can be used for the selective analysis of the 5' or 3' termini of DNA or RNA molecules (194, 210, 231, 232). Any complicated oligonucleotide mixture can be separated by this technique regardless of its purine-pyrimidine content. Thus the procedure is suitable for the fractionation of pyrimidine (17, 18, 19) or purine (21, 93) clusters, RN'ase (4, 77) or DN'ase digests containing 5' (DN'ase I) or 3' (DN'ase II) oligonucleotides (38,39).

SP3 DN'ase (116), could be applied to investigate oligonucleotides longer than 15 or 17 which will separate noticeably better on the anion exchanger due to the increase in charge difference between two adjacent fractions at pH 7.5 (section 4.1.2).

2) Fractionation of mononucleotides, nucleosides and bases

a) The columns developed for nucleotides and nucleosides fractionation (section 4.2.1) were used in the present study for composition analysis of the dephosphorylated ends of oligonucleotides. The method can be applied to DNA or RNA oligonucleotide mixtures or homogeneous fractions, which are totally hydrolyzed by 5' or 3' exonucleases. The columns can also be used for detecting contaminating DNA in purified RNA preparations (Fig. 13 c).

b) Stepwise chemical degradation has been achieved on model deoxyoligonucleotides by mild oxidation of the free 3'-OH groups. The labile terminus is reduced to a free 3' end base, sugar fragments and the 3'-phosphomonoester of the shorter oligonucleotide by ß-elimination (105). The newly generated 3'-phosphate is removed by phosphatase, freeing the deoxyribose for a second cycle. The bases liberated at each step can be directly and quantitatively analyzed by our procedure with no interference by the inorganic phosphate or oligonucleotides which fractionate in a different position. A similar approach has been used for sequential analysis of 26 residues from the 3' end of phenylalanine tRNA (233). The 5' end of the oligonucleotides can be attached to an insoluble cellulose support allowing the fast and efficient removal of each base for analysis (106).

3) Fractionation of dinucleotides and trinucleotides

a) The specificity of DN'ases could be detected by analysis of the dinucleotide or trinucleotide isostichs on the two separate columns (sections 4.2.2 and 4.2.3). If by intensive digestion with T4-induced endonuclease IV (22) short oligonucleotide isostichs could be separated, only three dinucleotides would be fractionated on the DEAE-cellulose column at pH 3.4: d-pCpA, d-pCpT and d-pCpG corresponding to peaks 2, 4 and 5, respectively. If another enzyme was found which would release dC and dT at the 5' end only the dinucleotides d-pCpA, d-pCpG, d-pTpA and d-pTpG would be fractionated. Thus, the number of dinucleotides and their position on the chromatogram will indicate the specificity of the enzyme.

b) The sequential release of dinucleotides by SP3 DN'ase (116) could be monitored by the dinucleotide column serving as a tool for sequential analysis from the 5' end of oligonucleotides.

All of the above described methods could be combined with several enzymes for analysis of homogeneous tri to heptanucleotide sequences. Trinucleotides can be analyzed by Micrococcal nuclease (79) or by E.coli exonuclease I (118) which is unable to hydrolyze the phosphodiester bond of the 5' terminal dinucleotide released from the fragment.

SP3 DN'ase (116) could be applied for studying tetra to heptanucleotides. The oligonucleotides have to be dephosphorylated and the hexanucleotide labeled at the 3' terminus.

$$\begin{array}{lcl} & \text{SP3 DN'ase} & \\ \text{CpGpApT} & \longrightarrow & \text{CpG + pApT} \\ \text{CpGpApTpC} & \longrightarrow & \text{CpG + pApTpC} \\ \text{CpGpApTpCpA*} & \longrightarrow & \text{CpG + pApT + pCpA*} \\ \text{CpGpApTpCpApG} & \longrightarrow & \text{CpG + pApT + pCpApG} \end{array}$$

Separation of the digested fractions according to phosphate charge of the dinucleotides according to composition and end analysis of the di and trinucleotides by cation exchange chromatography will render complete information on their structure.

6.4 Assessment of the methodology for quantitative end analysis of oligonucleotides

All of the methods of fractionation and analysis were quantitatively evaluated for studies on the specificity of DN'ase A.

1) The recoveries from the several fractionation columns were generally better than 98%. The percent distribution of oligonucleotides as measured at 260 nm in the presence of 7.0 M urea was in close agreement with the phosphorus analysis (Table 5). Mandeles and Cantor (234) found that at pH 7.0 and in 7.0 M urea a RNA spectrum was almost identical to the spectrum of the sum of the monomers. However, some residual hyperchromicity still remained in the longer deoxyoligonucleotides as was noticed from the lower recovered percentages compared to those obtained by phosphorus determination.

2) No contaminant activity interfered with the action of DN'ase A (section 3.1.2). The inhibition of DN'ase A by EDTA and its denaturation by alkali did not affect the recovery and composition of the hydrolyzed material (section 3.2.6d).

3) Dephosphorylation of the 5'-P-oligonucleotides at 55°C was complete in 1 to 2 hours depending on the chain length of the digest treated with the *E.coli* alkaline phosphatase (section 5.2.1).

4) The phosphatase was irreversibly denatured by autoclaving at 120°C for 3 minutes. The short heat treatment did neither affect the integrity of a ^{32}P-labeled pyrimidine heptanucleotide nor damage any of the components of a mixture of the four 5' mononucleotides (section 5.2.2).

5) Snake venom diesterase was purified for elimination of the 5' nucleotidase activity shown to be present in the commercial enzyme. The contaminant

was reduced 2.3 to 4.2 fold (section 5.2.3). The snake venom diesterase was used directly with the contaminant which contributed a minimal amount of nucleosides to the actual 5' end nucleosides. This is concluded from the different data obtained from the analysis of 5' terminal nucleosides produced by the two groups of activating ions (sections 5.3 and 5.4) and by comparing the total concentration of nucleosides recovered at both ends of the oligonucleotides (Table 16).

6) Spleen diesterase did not present nucleotidase contamination. Total digestion by the enzyme produced a residual fraction which was longer than mononucleotides and amounted to about 6% of the total nucleotide fraction (section 5.2.5). This was postulated to be due to the tendency of the enzyme to catalyze transfer reactions which produce longer oligonucleotides (195, 200, 201). However, no preferential distribution of any of the four bases was found in this fraction, indicating that it represents a random undegraded portion of material.

7) The largest source of error in these experiments came from the repeated manipulation of the different fractions before the final data on the terminal composition of the fractions was assembled. At the level of the analysis by cation exchange chromatography at pH 9.2 an excess of 25 µl for a total sample of 0.5 ml will represent a 5% excess of material analyzed. Most of the experiments were run with an error margin of 5 to 10%. In some of them the error was much larger, as in experiment 25, Table 21, where

the differences between the total concentration of bases at both ends amounted to 32%. This was probably due to an imperceptible loss of material during the preparation of the sample. However, when compared to experiment 26 with the same ion, the molar percentage of end nucleosides at both the 5' and 3' ends were almost identical, indicating the randomness of the losses.

6.5 Analysis of DN'ase I - Mn^{++} digests with E.coli K12 DNA

The intensive digest (30 hours) of E.coli K12 DNA in the presence of Mn^{++} ions produced mononucleotides equivalent to 3% of the total hydrolysate (Table 5). Only 3 mononucleotides were found (Table 7). The absence of pC indicated a non-random degradation by DN'ase I. The chromatographic separation of these mononucleotides into two peaks, Ia and Ib (Fig. 12), each of which contained all 3 mononucleotide components but in different proportions, was unusual but was not investigated further.

Comparison of the present results with those of the only Mn^{++}-activated digest published (26) is not possible. Becking and Hurst used calf thymus DNA at a concentration of 16.7 mg/ml at 2×10^{-2}M Mn^{++} and the pH adjusted to 6.6 during the reaction by titration with NaOH. In the present study this concentration of Mn^{++} produced a gel whereas addition of Na^{+} to the Mn^{++} activated reaction was shown to produce a change in the mechanism of hydrolysis when compared to the kinetics with Mn^{++} alone (Table 5, 137b).

All 16 possible dinucleotides were shown to be present (Table 10) but the non-random distribution supports the previous evidence from the mononucleotide fraction of non-random degradation by DN'ase I. Previous studies of the dinucleotides present in DN'ase digests (26, 80, 81, 144, 235) have failed to observe all possible dinucleotides and positional isomers. The quantitative distribution of the dinucleotide positional isomers pApC, pCpA; pCpT, pTpC; pCpG, pGpC; and pApT, pTpA was similar to the results reported by Becking and Hurst (26). They obtained an extensive Mn^{++} digest where 49% of the mixture corresponded to the dinucleotides (compared to 27% in the present study) but did not detect the dinucleotides pApG, pGpA and pGpG. This was probably due to the utilization of a strong ion exchanger for their fractionation. Sinsheimer (81), Privat de Garilhe, et al. (235) and Lehman (144) found predominantly pPy-pPu sequences in the positional dinucleotide isomers they examined. The present study of all 12 positional isomers verified these observations when A is the purine base but pPu-pPy predominates when G is the purine base.

In the total dinucleotide fraction from the E.coli K12 DNA-manganese digest, G and C predominate at both the 3' and 5' ends (Table 11). However, in those dinucleotides containing A and T, A and T predominate at the 3' end. Becking and Hurst (26) showed that T and C predominate at the 5' end (67.8%) and A and T at the 3' end (70.4%). The composition of the 3' ends of the oligonucleotides in their experiments was similar to the results obtained in the present study.

Sinsheimer (81) using Mg^{++} as co-factor showed T and C predominate at the 5' end and A and G at the 3' end of the dinucleotides. The difference in these three types of results with Mn^{++} (section 4.2.2), Mn^{++} plus Na^{+} (26) and Mg^{++} (80, 81, 144, 235) would indicate a different specificity of degradation of DN'ase I according to the co-factor used in the activation.

Impure trinucleotide peaks (Fig. 18) could contain mixtures of positional isomers of identified trinucleotides (i.e., peak 6) or of any of the six trinucleotides not identified in the analysis. Four of the five trinucleotides identified by Becking and Hurst (26) were not found in our chromatograms (CTT, TTT, AAA and AAG). Presumably some of them were part of the unresolved mixtures. The largest proportion of recovered trinucleotides were those where three of the bases appear in the molecule. GAC, CTG and ACT accounted for 40% of the total trinucleotides produced.

The results from the analysis of the trinucleotide to hexanucleotide termini (Table 19), and from the base composition of the 5' and 3' termini of the total mixture (Table 20) were completely different from those obtained with the mono and dinucleotides and were similar to those with DN'ase A digests using Mn^{++} as a co-factor (Table 21).

From these results it is concluded that mono and dinucleotides do not reflect the specificity of the enzyme and even alter the data obtained from end analysis of the total mixture of oligonucleotides or isostichs longer than trinucleotides. Similar results were obtained by Antonoglou and Georgatsos (211) for the termini of separated dinucleotides or those of the total digest obtained with mouse kidney DN'ase. This is presumably a result of the degradation of larger fragments (8 - 15 nucleotides long) into smaller ones during the slow digestion phase. Thus, mono and dinucleotides are produced by extensive hydrolysis and they should not be correlated with the specificity of the enzyme. Extensive cleavage was shown to exhibit similar effects with DN'ase II (38), venom endonuclease (204) and Micrococcal nuclease (202, 203). A very specific enzyme such as pancreatic RN'ase was found to split Ap-Np bonds under extensive hydrolytic conditions (232). However, it should be emphasized that even under extensive conditions of hydrolysis the ion is still important in determining the manner in which the long oligonucleotides are fragmented to mono and dinucleotides. The difference in composition of the latter when Mg^{++} or Mn^{++} are used in the reaction is significant.

6.6 Correlation between end analysis of oligonucleotides and the specificity of DN'ase A activated by divalent cations

Most of the divalent metal ions were tested for their activation properties on DN'ase A by studying the 5' and 3' ends of the oligonucleotides released during the hydrolysis of calf thymus and E. coli K12 DNA.

Two groups of ions appear to activate the DN'ase A in different ways.

1) Group A, which includes Cd^{++}, Ca^{++}, Ba^{++}, Co^{++} and Mg^{++} plus Ca^{++} did not show large differences in the distribution of terminal nucleosides

when partial, intermediate or extensive digests were analyzed. The differences were smaller at the 5' end, whereas at the 3' end larger variations occurred. For the intermediate Co^{++} digest there was an 8 fold increase in dA content, only a 2 fold increase in the distribution of dG and dC and a 3 fold increase for dT compared to the partial digest (Table 17). This is an indication of the higher susceptibility of dpA-pN bonds. Cd^{++}, which showed identical results to Co^{++} at the 5' end indicated partial resistance of dpA-pN bonds as seen from the data at the 3' end. Ca^{++} alone behaved similarly to Co^{++} and Ba^{++}. However, the synergistic mixture of Ca^{++} plus Mg^{++} presented different results when compared to the separate effects of each ion (Table 18). Mg^{++} plus Ca^{++} also presented a lower dG content at the 5' end in contrast to all other ions of this group which present higher dG:dA ratios. From the results it is clear that Mg^{++} plus Ca^{++} exert the lowest degree of specificity when acting together. In all of these digests the resistance of dpC-pN and dpN-pC bonds is noticeable. The data suggests that under intermediate conditions of digestion the enzyme activated by ions of group A will preferentially split bonds of the type dpPu-pPy and dpPu-pPu. Several investigators have postulated that the dpPu-pPy bonds are preferentially split (80, 81, 143, 144, 207, 236). However, it should be taken into account that their conclusions generally came from analysis of mono and dinucleotides and with ions such as Mg^{++} and Mn^{++} which according to the present study belong to the second group of activators.

In summary, ions of group A present individual variations in the distribution of termini released in the DN'ase A hydrolysis but the general patterns are similar with all of the nucleosides appearing at both ends and always with lower dC proportions.

2) Group B, which includes Ni^{++}, Mg^{++}, Mn^{++}, Sr^{++} and Zn^{++}, showed marked variations in the distribution of the terminal nucleosides between the partial and extensive digests. Examination of the results obtained from calf thymus (Table 18) and E.coli K12 DNA (Table 21) showed an increase in the G + C content at both ends of the E.coli digests. This was attributed to the higher G + C/A + T ratio of E.coli DNA when compared to calf thymus DNA (208).

Since the present DN'ase digests were obtained at different stages of the linear and slow degradation phases, (Tables 13 and 14 and section 6.4) it was expected that some scatter in the 5' or 3' terminal nucleosides would be found. Also differences in the proportions of the terminal nucleosides of the various DN'ase digests activated by ions of this group were found as was discussed in section 5.3.1b, because of difficulties in duplicating the termination of hydrolysis. In spite of these facts it is clear from the results that a typical distribution pattern of terminal nucleosides is produced by this group of ions. In partial digests, low amounts of pyrimidines were released at the 5' ends whereas at the 3' end the four nucleosides were released in approximately equal amounts with dC slightly lower. In extensive digests the four nucleosides were represented at the 5' end. At the 3' end dA was maintained in the same molar concentration as it was during the partial digest. The other nucleosides increased proportionally. At both termini dC was present in lower quantities. The results are schematically summarized in Table 24.

Analysis of the 3' termini of three oligonucleotides obtained in several DN'ase A digests (with Mg^{++}, Ni^{++} and Sr^{++}, Table 22) showed that very minute amounts of dA appear at the 3' end of these isostichs. From the data presented it is concluded that the mechanism of recognition and cleavage of certain internucleotide bonds is changed during the process of DN'ase A hydrolysis in the presence of group B ions. Highly preferential dpN-pPu scission occurs during the very early stages of digestion. The Pu at the 5' side of the split sequences are still predominant at intermediate levels of hydrolysis (digest 24, Table 18) with thymidine increasing in amount faster than dC. On the 3' side dC remained low and dA did not vary at all. Thus, at this stage dpT-pPu and dpG-pPu sequences are preferentially split.

Analysis of the extensive digests indicates that at this stage dpA-pN bonds are completely resistant and sequences containing dG and dT preferentially cleaved. As for ions of group A, the dpC-pC sequences are resistant through all phases of hydrolysis.

TABLE 24

Distribution of Deoxyribonucleosides at the 5' and 3' Ends of the Oligonucleotides Released from DNA by DN'ase A Activated by Group B Cations

	Nucleoside distribution							
	5' End				3' End			
Type of digest	dT	dC	dG	dA	dT	dC	dG	dA
Partial	x	x	xxx	xxx	xxx	xx	xxx	xxx
								↕
Extensive	xxx	xx	xxx	xxx	xxx	xx	xxx	x

6.7 Comparison between the specificity of DN'ase A and other studies

The results obtained in the present investigation are in many respects different from those obtained from several other laboratories when the mechanism of action and the specificity of the enzyme in the presence of different activating cations is correlated.

The interpretation of specificity from the analysis of short oligonucleotides such as mono and dinucleotides is incorrect as was discussed in section 6.5. The mono and dinucleotide fractions represent products of extensive hydrolysis, not characteristic of the normal action of the enzyme. Hoard and Goad (145) postulated that oligonucleotide fragments less than 15 residues long are attacked more slowly than larger ones. The slow rate of accumulation of shorter fragments also indicated to these authors that hydrolysis of the short fragments must be greatly inhibited. They also concluded that very few short oligonucleotides (2 to 5 residues long) come from the very long ones under normal conditions of hydrolysis. But when the total digestion mixture contains only short oligonucleotides the enzyme breaks them down in an increasingly random fashion. The only ions which presented a lag when the kinetics of hydrolysis with time was determined were Mg^{++}, Sr^{++} and Zn^{++} (section 3.2.1). The rest of the ions did not. However, according to the specificity studies described Mn^{++} and Ni^{++}, which behave kinetically like ions which split DNA by a single-hit mechanism, are grouped together with Mg^{++}, Sr^{++} and Zn^{++} (double-hit mechanism) when specificity is compared. All of the other cations or mixtures (Mg^{++} plus Ca^{++})

which split the DNA in a more random-like pattern belong to the single-hit mechanism type of ions. In Melgar and Goldthwait's (137) experiments, all ions of either type to which a monovalent ion was added showed a lag phase. Mg^{++} plus Ca^{++} alone did not produce a lag but by addition of Na^{+} to the mixture it occurred. Thus, evidently the shift in mechanism is primarily at the level of the enzyme. Addition of a non-specific monovalent ion will preferentially bind to the phosphate groups on the DNA (217). This will not cause a change in the mechanism of the reaction unless the ion is involved, directly or indirectly, in the active site of the enzyme.

DN'ase I - Mg^{++} digests of the alternating copolymer poly d AT produced fragments where dT predominated at the 5' end of the oligonucleotides and dA at the 3' end (146). In the present experiments small amounts of dA were produced at the beginning of the hydrolysis in the presence of Mg^{++} (Table 18) but not from dpA-pT cleaved bonds which are resistant through all stages of degradation. The preferential splitting found with poly d AT is probably related to the conformation of the copolymer which, due to its self complementary properties, can form multiple hairpin helices at low ionic strength (0.01 M Na^{+}) (146).

Bollum (27) has also shown differences in the rate of degradation of the double-stranded homopolymer poly dA :dT. Poly dA was digested slowly by Mg^{++} but rapidly by Mg^{++} plus Ca^{++} or Mn^{++}. The rigidity of certain secondary structures may account for the slower hydrolysis of certain polynucleotides by DN'ase I. Poly dA (100 residues long) is attacked three times slower than when a segment of 6 thymidylate residues are substituted at the 3' end of the homopolymer (145). This is in contrast with the results obtained with the dT oligomers alone (25) which are hydrolyzed at half the rate of the corresponding deoxyadenylate. Hoard and Goad (145) proposed that the rate of cleavage by DN'ase I increases with the helical content of the substrate. Thus, if the hexathymidylate is hydrogen bonded to some regions in the polyadenylate portion of the chain they will be preferentially split. This is also the case with native DNA which is degraded faster than denatured DNA (section 3.2.2). However, it has to be considered that the homopolymers of the dPu-dPy type exhibit structural differences from that of DNA and of double-stranded copolymers with alternating sequences as shown by x-ray diffraction studies (237).

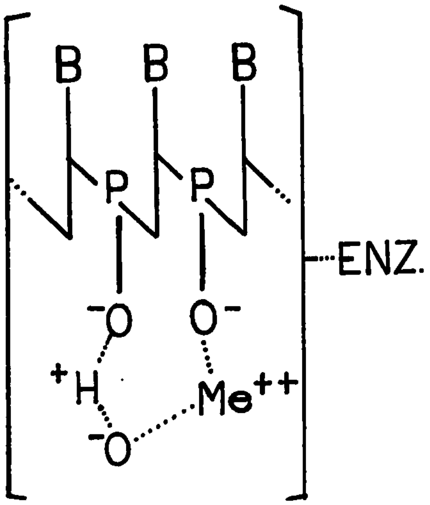
Poly dG:dC was not degraded at all by DN'ase I in the presence of Mg^{++} whereas the addition of Ca^{++} did degrade both strands (27). With poly dI:dC only dI was cleaved with Mg^{++}, dC was not. This reflects the resistance of the dpC-pC bond which was also found to be preferentially resistant through all of the present experiments.

The similar specificity found for ions of group B would indicate that DN'ase A in the presence of ions of the single-hit kinetic type such as Mn^{++} and Ni^{++} cleave both strands of the DNA at almost opposite but separate places. The enzyme could split both DNA strands at the same level only if susceptible complementary sequences such as A-T (or T-A), G-C (or C-G) would be found. From the data obtained in this investigation it would seem that sequences such as dpT-pA and dpC-pG and the complementary pairs dpC-pA and dpT-pG could be cleaved at the same level. When shorter oligonucleotides are produced the mechanism of cleavage shifts, becoming similar to that of Mg^{++}, Sr^{++} and Zn^{++}.

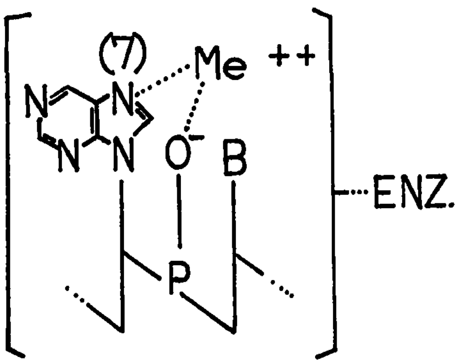
The rate of DNA hydrolysis cannot be correlated with the specificity of the enzyme in the presence of different divalent cations. It was shown (137b) that with single-stranded DNA and in the presence of Mn^{++}, the Vmax is increased several hundred fold when compared to the Mg^{++}-activated hydrolysis. The Vmax ratios were Mg^{++}: Co^{++}: Mg^{++} plus Ca^{++}: Mn^{++}, 1 : 13 : 230 : 430. However Mg^{++} and Mn^{++} showed similar specificities whereas Co^{++} and Mg^{++} plus Ca^{++} belong to the activators of group A. Moreover, when DNA was digested in the presence of Mg^{++} and dAMP single-hit kinetics were found with only a two fold increase in Vmax when compared to Mg^{++} alone. This suggests that the shift from one mechanism to another is not a function of the maximum rate of hydrolysis.

Several models have been proposed to explain the shift in the mechanism of action of DN'ase I in the presence of several bivalent cations (137). Any one of them, the existence of a double-active site on the DN'ase, the formation of enzyme dimers, and the consecutive cleavage of single strands at the same level by one molecule, have to take into account the influence of the ion on both the enzyme and the DNA. Many divalent cations were found to bind to the phosphoryl groups of the DNA at pH 5.0 to 7.0 and at low ionic strength, stabilizing the native DNA structure (238). The alkaline earth metals (Mg^{++}, Ca^{++}, Sr^{++}, Ba^{++}) behave as the strongest stabilizers whereas transition metal ions (Mn^{++}, Ni^{++}, Co^{++}, Zn^{++}) are mostly weaker and probably act in a different manner (239). These authors reported that ions such as Ca^{++}, Mn^{++} and Mg^{++} enhanced the stability of DNA and increased the Tm whereas Cu^{++}, Cd^{++}, Fe^{++} and Pb^{++} decreased it.

An explanation of why the mechanism is shifted for the same ion during the course of the reaction can be visualized from the experiments of Fishman, <u>et al</u>. (240). Mg^{++} added to native or denatured DNA binds to the phosphate groups alone. However, the combination of Mg^{++} with the N of the bases is facilitated when DNA is denatured in the presence of Mg^{++} and hydrogen bonds between the bases are broken. Thus, in the presence of DN'ase I the initial steps of DNA hydrolysis probably involve a phosphate-Mg^{++}-enzyme (I) ternary complex.



I



II

During the course of the reaction when the native structure collapses and denatured poly and oligonucleotides form phosphate and purine (N_7)-Me^{++}-enzyme (II) complexes or other structures (241), and the specificity of the enzyme is changed.

The present study has shown a difference in the characteristics of divalent metal cations as activators of DN'ase A. The results from the specificity studies with ions of group B indicated that following the initial hydrolytic phase sequences of the type dpA-pN are highly resistant. Analysis of certain fractionated isostichs indicated that oligonucleotides almost free of deoxyadenosine at their 3' ends can be isolated. However, DN'ase A does not behave in the presence of these cations as a highly specific enzyme which could be applied directly to the sequence analysis of DNA.

6.8 Outline of proposed future studies with DN'ase A

Several approaches could further be investigated for the analysis of the specificity of DN'ase A.

1) It would be interesting to test if the dA content at the 3' end of the oligonucleotides is chain length dependent and to see if the same characteristic sensitivity or resistance of certain internucleotide bonds is maintained when short oligonucleotides (6 to 17 long) are cleaved. For these studies, purine and pyrimidine clusters or oligodeoxynucleotide isostichs obtained from enzymatic digests could be used.
2) Chemical modification of DNA nucleotides (section 1.2.2) provides structures which could selectively be cleaved by the enzyme. Some evidence exists

about the reduced sensitivity of modified DNA to DN'ase (102, 103).

3) The interaction of DNA with histones was shown to provide protection against the action of DN'ase I (242). By increasing the proportion of histone in the nucleoprotein complete inhibition of the breakdown of the DNA molecules is achieved. In addition, the calf thymus inhibitor of DN'ase I forms a complex with the enzyme in a 1 : 1 ratio (142). Some of the activity of DN'ase I is maintained after complex formation. It should be interesting to investigate if the protection of DNA by the histones or the complexation of DN'ase I by its natural inhibitor changes the specificity of cleavage of the enzyme.

4) The modification of DN'ase A by chemical or enzymatic methods could prove to be the most valuable method for changing its enzymatic properties. The selective alteration of amino acid residues or peptides of enzymes with multiple specificities proved to be successful with many enzymes. Some are involved in nucleic acid metabolism such as phosphatase, pancreatic RN'ase, Staphylococcal nuclease and DNA polymerase (113, 212, 243, 244).

The modified DN'ases should initially be tested for their specificity in the presence of divalent cations, by degrading deoxypolynucleotides containing two or more monomer units (245) and then tested on DNA oligo or polynucleotides for final assessment of its specificity.

CHAPTER 7

CLAIMS TO ORIGINAL RESEARCH

1) Purification of pancreatic deoxyribonuclease (DN'ase I) by phosphocellulose chromatography to a homogeneous and stable fraction (DN'ase A) devoid of any exonuclease, phosphatase or nucleotidase activity.

2) Kinetic re-evaluation of enzyme-activity relationship, the influence of most divalent cations, pH and ionic strength of the incubation mixture with native DNA as substrate.

3) Development of a column chromatographic method for the fractionation of oligonucleotides according to chain length. Up to pentadeca or heptadecaoligonucleotides were separated on DEAE-Sephadex at 65°C in the presence of 7.0 M urea buffers.

4) Development of two column chromatographic methods for the fractionation of the non-isomeric dinucleotides and trinucleotides according to base composition, using DEAE-cellulose at pH 3.4 and 3.6, respectively. Elution was performed in both cases with volatile buffers. All possible non-isomeric dinucleotides and 14 of the 20 possible non-isomeric trinucleotides were quantitatively separated.

5) Development of a cation exchange column chromatographic technique for the rapid separation of nucleosides and nucleotides. Three systems were developed for the fractionation of

   a) 3' or 5' deoxynucleotides and all four deoxynucleosides.

b) 3' or 5' ribonucleotides and all four ribonucleosides.

c) all four 5' deoxyribonucleotides and all four deoxyribonucleosides.

6) Studies on the specificity of DN'ase A in the presence of divalent cations by analysis of the 5' or 3' termini of the oligonucleotides obtained in partial, intermediate and extensive calf thymus and E.coli K12 DNA digests.

7) Analysis of extensive DN'ase I - Mn^{++} digests of E.coli K12 DNA which showed that mono and dinucleotide fractions are the result of extensive cleavage of longer oligonucleotides and do not represent the primary specific products of hydrolysis.

8) Classification of divalent cations into two groups according to the way they activate DN'ase A.

Group A includes Cd^{++}, Ca^{++}, Ba^{++}, Co^{++} and Mg^{++} plus Ca^{++}. These ions do not show significant differences in the distribution of terminal nucleosides at the different stages of digestion.

Group B includes Ni^{++}, Mg^{++}, Mn^{++}, Sr^{++} and Zn^{++} and shows a change in the distribution of terminal nucleosides during the different phases of hydrolysis.

9) With group B ions, dpN-pPu internucleotide bonds were shown to be cleaved preferentially during early stages of digestion whereas dpA-pN bonds were found to be very highly resistant under extensive hydrolysis. The dpC-pC bond was shown to be preferentially resistant through all phases of hydrolysis with all cations.

BIBLIOGRAPHY

1. Holley, R.W., Apgar, J., Everett, G.A., Madison, J.T., Marquisee, M., Merril, S.H., Penswick, J.R., and Zamir, A. Science 147, 1462 (1965).
2. Zachau, H.G., Dütting, D., Feldmann, H., Melchers, F., and Karau, W. Cold Spring Harb. Symp. Quant. Biol. 31, 417 (1966).
3. Brownlee, G.G., Sanger, F., and Barrell, B.G. Nature 215, 735 (1967).
4. Egami, F., and Nakamura, K. "Microbial Ribonucleases", Springler-Verlag, New York Inc. 1969.
5. Rushizky, G.W., Skavensky, I.H., and Sober, H.A. J.Biol.Chem. 240, 3984 (1965).
6. Rushizky, G.W., and Sober, H.A. In Progress in Nucleic Acid Research and Molecular Biology. Eds. Davidson, J.N., and Cohn, W.E. Academic Press, New York. 1968. Vol. 8, p. 171.
7. Sanger, F., Brownlee, G.G., and Barrell, B.G. J.Mol.Biol. 13, 373 (1965).
8. Brownlee, G.G., Sanger, F., and Barrell, B.G. J.Mol.Biol. 34, 379 (1968).
9. Brownlee, G.G., and Sanger, F. European J.Biochem. 11, 395 (1969).
10. Billeter, M.A., Dahlberg, J.E., Goodman, H.M., Hindley, J., and Weissmann, C. Nature 224, 1083 (1969).
11. Steitz, J.A. Nature 224, 957 (1969).
12. Hindley, J., and Staples, D.H. Nature 224, 964 (1969).
13. Nichols, J.L. Nature 225, 147 (1970).
14. Marks, A. Ph.D. Thesis, McGill University, 1969.
15. Marks, A., and Spencer, J.H. J.Mol.Biol. 50 (1970). (in the press).
16. Shapiro, J., MacHattie, L., Eron, L., Ihler, G., Ippen, K., and Beckwith, J. Nature 224, 768 (1969).
17. Černý, R., Černá, E., and Spencer, J.H. J.Mol.Biol. 46, 145 (1969).

18. Mushynski, W.E. Ph.D. Thesis, McGill University, 1970.

19. Mushynski, W.E., and Spencer, J.H. J.Mol.Biol. 51 (1970). (in the press).

20. Burton, K., and Petersen, G.B. Biochem. J. 75, 17 (1960).

21. Cape, R.E., and Spencer, J.H. Can.J.Biochem. 46, 1063 (1968).

22. Sadowski, P.D., and Hurwitz, J. J.Biol.Chem. 244, 6182, 6192 (1969).

23. Ando, T., Takagi, J., Kosawa, T., and Ikeda, Y. J.Biochem. 66, 1 (1969).

24. Fielding, P.E., and Lunt, M.R. Febs Letters 5, 214 (1969).

25. Ralph, R.K., Smith, R.A., and Khorana, H.G. Biochemistry 1, 131 (1962).

26. Becking, G.C., and Hurst, R.O. Can.J.Biochem.Physiol. 41, 1433 (1963).

27. Bollum, F.J. J.Biol.Chem. 240, 2599 (1965).

28. Junowicz, E., and Spencer, J.H. J.Chromatog. 44, 342 (1969).

29. Junowicz, E., and Spencer, J.H. Biochemistry (accepted for publication).

30. Yarus, M. Ann.Rev.Biochem. 38, 841 (1969).

31. Szybalski, W., Bovre, K., Fiandt, M., Guha, A., Hradecna, Z., Kumar, S., Lozeron, H.A., Sr., Maher, V.M., Nijkamp, H.J.J., Summers, W.C., and Taylor, K. J.Cell.Physiol. 74 (Suppl. 1), 33 (1969).

32. Ptashne, M. Nature 214, 232 (1967).

33. Gilbert, W., Muller-Hill, B. Proc.Nat.Acad.Sci. U.S. 58, 2415 (1967).

34. Mousset, S., and Thomas, R. Nature 221, 242 (1969).

35. Gottesman, M.E., and Yarmolinsky, M.B. Cold Spring Harb. Symp. Quant. Biol. 33, 735 (1968).

36. Richardson, J.P. In Progress in Nucleic Acid Research and Molecular Biology. Eds. Davidson, J.N., and Cohn, W.E. Academic Press, New York. 1969. Vol. 9, p. 75.

37. Johnson, P.H., and Laskowski, M., Sr. J.Biol.Chem. 245, 891 (1970).

38. Carrara, M., and Bernardi, G. Biochemistry 7, 1121 (1968).

39. Laskowski, M., Sr. Advanc. Enzymol. 29, 165 (1967).

40. Whittmann, H.G., and Scholtissek, C. Ann.Rev.Biochem. 35, 299 (1966).

41. Kornberg, A. Science 163, 1410 (1969).

42. Richardson, C.C. Ann.Rev.Biochem. 38, 795 (1969).

43. Bollum, F.J. J.Biol.Chem. 235, 2399 (1960).

44. Szybalski, W., Kubinski, H., and Sheldrick, P. Cold Spring Harb. Symp. Quant. Biol. 31, 123 (1966).

45. Arber, W., and Linn, S. Ann.Rev.Biochem. 38, 467 (1969).

46. Englund, P.T., Deutscher, M.P., Jovin, T.M., Kelly, R.B., Cozzarelli, N.R., and Kornberg, A. Cold Spring Harb. Symp. Quant. Biol. 33, 1 (1968).

47. Goulian, M., Lucas, Z.J., and Kornberg, A. J.Biol.Chem. 243, 627 (1968).

48. Goulian, M. Cold Spring Harb. Symp. Quant. Biol. 33, 11 (1968).

49. Wells, R.D., Büchi, H., Kössel, H., Ohtsuka, E., and Khorana, H.G. J.Mol.Biol. 27, 265 (1967).

50. Sinsheimer, R.L., Knippers, R., and Komano, T. Cold Spring Harb. Symp. Quant. Biol. 33, 443 (1968).

51. Epstein, W., and Beckwith, J.R. Ann.Rev.Biochem. 37, 411 (1968).

52. Ippen, K., Miller, J.H., Scaife, J., and Beckwith, J. Nature 217, 825 (1968).

53. Burgess, R.R., Travers, A.A., Dunn, J.J., and Bautz, E.K.F. Nature 221, 43 (1969).

54. Travers, A.A. Nature 223, 1107 (1969).

55. Roberts, J.W. Nature 224, 1168 (1969).

56. Attardi, G., Naono, S., Rouviere, J., Jacob, F., and Gros, F. Cold Spring Harb. Symp. Quant. Biol. 28, 363 (1963).

57. Martin, R.G. Ann. Rev. Genetics 3, 181 (1969).

58. Dove, W.F. Ann. Rev. Genetics 2, 305 (1968).

59. Lehman, I.R. Ann. Rev. Biochem. 36, 645 (1967).

60. Arber, W. J. Mol. Biol. 11, 247 (1965).

61. Wu, R., and Kaiser, A.D. J. Mol. Biol. 35, 523 (1968).

62. Ohba, Y. Biochim.Biophys.Acta 123, 84 (1966).

63. Olins, D.E., Olins, A.L., and von Hippel, P.H. J.Mol.Biol. 24, 157 (1967).

64. Bekhor, I., Kung, G.M., and Bonner, J. J.Mol.Biol. 39, 351 (1969).

65. Nass, M.M.K., and Buck, C.A. Proc.Nat.Acad.Sci. U.S. 62, 506 (1969).

66. Cozzarelli, N.R., Kelly, R.B., and Kornberg, A. Proc. Nat.Acad.Sci. U.S. 60, 992 (1968).

67. Gillespie, D. In Methods in Enzymology. Eds. Grossman, L., and Moldave, K. Academic Press, New York. 1968. Vol. 12, part B, p. 641.

68. Walker, P.M.B. In Progress in Nucleic Acid Research and Molecular Biology. Eds. Davidson, J.N., and Cohn, W.E. Academic Press, New York. 1969. Vol. 9, p.301.

69. Köhne, D.E. Biophys. J. 8, 1104 (1968).

70. Skalka, A., Burgi, E., and Hershey, A.D. J.Mol.Biol. 34, 1 (1968).

71. Matsukage, A., Murakami, S., and Kameyama, T. Biochim.Biophys.Acta 179, 145 (1969).

72. Matsubara, K., and Kaiser, A.D. Cold Spring Harb. Symp. Quant. Biol. 33, 769 (1968).

73. Britten, R.J., and Köhne, D.E. Science 161, 529 (1968).

74. Rabin, E.Z., and Fraser, M.J. Can.J.Biochem. 48, 389 (1970).

75. Staehelin, M. In Progress in Nucleic Acid Research. Eds. Davidson, J.N., and Cohn, W.E. Academic Press, New York. 1963. Vol. 2, p.169.

76. Burton, K. In Essays in Biochemistry. Eds. Campbell, P.N., and Greville, G.D. Academic Press, New York. 1965. Vol. 1, p. 57.

77. RajBhandary, U.L., and Stuart, A. Ann.Rev.Biochem. 35, 759 (1966).

78. Laskowski, M., Sr. In Methods in Enzymology. Eds. Grossman, L., and Moldave, K. Academic Press, New York. 1967. Vol. 12, part A, p. 281.

79. Holley, R.W. In Progress in Nucleic Acid Research and Molecular Biology. Eds. Davidson, J.N., and Cohn, W.E. Academic Press, New York. 1968. Vol. 8, p. 37.

80. Sinsheimer, R.L. J.Biol.Chem. 208, 445 (1954).

81. Sinsheimer, R.L. J.Biol.Chem. 215, 579 (1955).

82. Vanecko, S., and Laskowski, M., Sr. J.Biol.Chem. 236, 1135 (1961).

83. Tomlinson, R.V., and Tener, G.M. Biochemistry 2, 697 (1963).

84. Rushizky, G.W., and Sober, H.A. Biochim.Biophys.Acta 55, 217 (1962).

85. Sedat, J., and Sinsheimer, R.L. J.Mol.Biol. 9, 489 (1964).

86. Bartos, E.M., Rushizky, G.W., and Sober, H.A. Biochemistry 2, 1179 (1963).

87. Burton, K., and Riley, W.T. Biochem. J. 98, 70 (1966).

88. Peterson, E.A., and Sober, H.A. J.Amer.Chem.Soc. 78, 751 (1956).

89. Cohn, W.E., and Volkin, E. Biochim.Biophys.Acta 24, 359 (1957).

90. Burton, K. In Methods in Enzymology. Eds. Grossman, L., and Moldave, K. Academic Press, New York. 1967. Vol. 12, part A, p. 222.

91. Spencer, J.H., Cape, R.E., Marks, A., and Mushynski, W.E. Can.J.Biochem. 47, 329 (1969).

92. Burton, K., Lunt, M.R., Petersen, G.B., and Siebke, J.C. Cold Spring Harb. Symp. Quant. Biol. 28, 27 (1963).

93. Shapiro, H.S. In Methods in Enzymology. Eds. Grossman, L., and Moldave, K. Academic Press, New York. 1967. Vol. 12, part A, p. 212.

94. Habermann, V., Habermannová, S., and Cerhová, M. Biochim.Biophys. Acta 76, 310 (1963).

95. Vanyushin, B.F., and Buryanov, Ya. I. Biokhimiya 34, 718 (1969).

96. Josse, J., Kaiser, A.D., and Kornberg, A. J. Biol. Chem. 236, 864 (1961).

97. Székely, M., and Sanger, F. J. Mol. Biol. 43, 607 (1969).

98. Jones, A.S., and Walker, R.T. Nature 202, 1108 (1964).

99. Seidel, H. Biochim.Biophys.Acta 138, 98 (1967).

100. Howard-Flanders, P. Ann.Rev.Biochem. 37, 175 (1968).

101. Kochetkov, N.K., and Budowsky, E.I. In Progress in Nucleic Acid Research and Molecular Biology. Eds. Davidson, J.N., and Cohn, W.E. Academic Press, New York. 1969. Vol. 9, p. 403.

102. Drevitch, V.F., Salganik, R.I., Knorre, D.G., and Mal'gyn, E.G. Biochim.Biophys.Acta 123, 207 (1966).

103. Matsuda, M., and Ogoshi, H. Biochim.Biophys.Acta 119, 210 (1966).

104. Uziel, M., Koh, C.K., and Cohn, W.E. Anal.Biochem. 25, 77 (1968).

105. Gabriel, T., Chen, W.Y., and Nussbaum, A.L. J.Amer.Chem.Soc. 90, 6833 (1968).

106. Wagner, T.E., Chai, H.G., and Warfield, A.S. J. Amer.Chem.Soc. 91, 2388 (1969).

107. Lehman, I.R. In Progress in Nucleic Acid Research and Molecular Biology. Eds. Davidson, J.N., and Cohn, W.E. Academic Press, New York. 1963. Vol. 2, p.83.

108. Shugar, D., and Sierakowska, H. In Progress in Nucleic Acid Research and Molecular Biology. Eds. Davidson, J.N., and Cohn, W.E. Academic Press, New York. 1967. Vol. 7, p. 369.

109. Setlow, R.B. In Progress in Nucleic Acid Research and Molecular Biology. Eds. Davidson, J.N., and Cohn, W.E. Academic Press, New York. 1968. Vol. 8, p. 257.

110. Beer, M., Stern, S., Carmalt, D., and Mohlhenrich, K.H. Biochemistry 5, 2283 (1966).

111. Gal-or, L., Mellena, J.E., Moundrianakis, E.N., and Beer, M. Biochemistry 6, 1909 (1967).

112. Berg, P., Francher, H., and Chamberlin, M. In Informational Macromolecules. Eds. Vogel, H.J., Bryson, V., and Lampen, J.O. Academic Press, New York. 1963. p. 467.

113. Klenow, H., and Overgaard-Hansen, K. Febs Letters 6, 25 (1970).

114. Wu, R., and Kaiser, D. Proc.Nat.Acad.Sci. U.S. 57, 170 (1967).

115. Cantor, C.R., Tinoco, I., Jr., and Peller, L. Biopolymers 2, 51 (1964).

116. Aposhian, H.V., Friedman, N., Nishihara, M., Heimer, E.P., and Nussbaum, A.L. J.Mol.Biol. 49, 367 (1970).

117. Černý, R., Mushynski, W.E., and Spencer, J.H. Biochim.Biophys.Acta 169, 439 (1968).

118. Lehman, I.R., Linn, S., and Richardson, C.C. Fed. Proc. 24, 1466 (1965).

119. Laskowski, M., Sr. In The Enzymes. Eds. Boyer, P.D., Lardy, H., and Myrbäck, K. Academic Press, New York. 1961. Vol. 5, p. 123.

120. Laskowski, M., Sr. In Procedures in Nucleic Acid Research. Eds. Cantoni, G.L., Davies, D.R. Harper and Row, New York. 1966. p. 85.

121. Cuatrecasas, P., Fuchs, S., and Anfinsen, C.B. J.Biol.Chem. 242, 1541 (1967).

122. Wingert, L., and von Hippel, P.H. Biochim.Biophys.Acta 157, 114 (1968).

123. Kato, M., Ando, T., and Ikeda, Y. J.Biochem. 64, 329 (1968).

124. Kato, M., and Ikeda, Y. J.Biochem. 65, 43 (1969).

125. Shishido, K., Kato, M., and Ikeda, Y. J.Biochem. 65, 49 (1969).

126. Grossman, L., Kaplan, J.C., Kushner, S.R., and Mahler, I. Cold Spring Harb. Symp. Quant. Biol. 33, 229 (1968).

127. Friedberg, E.C., Hadi, S.-M., and Goldthwait, D.A. J.Biol.Chem. 244, 5879 (1969).

128. Roulland-Dussoix, D., and Boyer, H.W. Biochim.Biophys.Acta 195, 219 (1969)..

129. Kelly, T.J., Jr., and Smith, H.O. Fed. Proc. 29, 405 (1970).

130. Kunitz, M. J.Gen.Physiol. 33, 349, 363 (1950).

131. Price, P.A., Liu, T.-Y., Stein, W.H., and Moore, S. J.Biol.Chem. 244, 917 (1969).

132. Greenstein, J.P., Carter, C.E., and Chalkley, H.W. Cold Spring Harb. Symp. Quant. Biol. 12, 64 (1947).

133. Wiberg, J.S. Arch.Biochem.Biophys. 73, 337 (1958).

134. Shack, J., and Bynum, B.S. J.Biol.Chem. 239, 3843 (1964).

135. Price, P.A., Moore, S., and Stein, W.H. J.Biol.Chem. 244, 924 (1969).

136. Price, P.A., Stein, W.H., and Moore, S. J.Biol.Chem. 244, 929 (1969).

137. Melgar, E., and Goldthwait, D.A. J.Biol.Chem. 243, (a) 4401, (b) 4409 (1968).

138. Vanecko, S., and Laskowski, M., Sr. J.Biol.Chem. 236, 3312 (1961).

139. Young, E.T., and Sinsheimer, R.L. J.Biol.Chem. 240, 1274 (1965).

140. Catley, B.J., Moore, S., and Stein, W.H. J.Biol.Chem. 244, 933 (1969).

141. Lindberg, U. Biochemistry 6, 335 (1967).

142. Lindberg, M.U., and Skoog, L. European J.Biochem. 13, 326 (1970).

143. Privat de Garilhe, M., and Laskowski, M. J.Biol.Chem. 215, 269 (1955).

144. Lehman, I. R. J.Biol.Chem. 235, 1479 (1960).

145. Hoard, D.E., and Goad, W. J.Mol.Biol. 31, 595 (1968).

146. Scheffler, I.E., Elson, E.L., and Baldwin, R.L. J. Mol. Biol. 36, 291 (1968).

147. Bachelet, M., and Guibe, M. Bull. soc. chim., France 18, 554 (1951).

148. Blattner, F.R., and Erickson, H.P. Anal.Biochem. 18, 220 (1967).

149. Keller, E.B. Biochem. Biophys. Res. Commun. 17, 412 (1964).

150. Kay, E.R.M., Simmons, N.S., and Dounce, A.L. J.Amer.Chem.Soc. 74, 1724 (1952).

151. Lowry, O.H., Rosebrough, N.J., Farr, A.L., and Randall, R.J. J.Biol. Chem. 193, 265 (1951).

152. Dische, Z. In The Nucleic Acids. Eds. Chargaff, E., and Davidson, J.N. Academic Press, New York. 1955. Vol. 1, p. 285.

153. Saito, H., and Miura, K.I. Biochim.Biophys.Acta 72, 619 (1963).

154. Marmur, J. J.Mol.Biol. 3, 208 (1961).

155. Kirby, K.S. Biochem. J. 66, 495 (1957).

156. Brown, R.H.L., Szer, W., and Spencer, J.H. Lab. Practice 18, 547 (1969).

157. Salnikow, J., Stein, W.H., and Moore, S. Fed. Proc. 28, 344 (1969).

158. Parr, C.W. Biochem. J. 56, xxvii (1954).

159. Rudner, R., Karkas, J.D., and Chargaff, E. Proc.Nat.Acad.Sci. U.S. 60, 630 (1968).

160. Rushizky, G.W., Bartos, E.M., and Sober, H.A. Biochemistry 3, 626 (1964).

161. King, E.J. Biochem. J. 26, 292 (1932).

162. Wyatt, G.R. In The Nucleic Acids. Eds. Chargaff, E., and Davidson, J.N. Academic Press, New York. 1955. Vol. 1, p. 243.

163. Koerner, J.F., and Sinsheimer, R.L. J.Biol.Chem. 228, 1049 (1957).

164. Razzell, W.E., and Khorana, H.G. J.Biol.Chem. 234, 2105 (1959).

165. Hilmoe, R.J. In Biochemical Preparations. Ed. Miester, A. John Wiley and Sons, New York. 1961. Vol. 8, p. 105.

166. Worthington Catalog, Worthington Biochemical Corporation, Freehold, N.J., U.S.A., 1968.

167. Felix, F., Potter, J.L., and Laskowski, M. J.Biol.Chem. 235, 1150 (1960).

168. Davis, B.J. Ann.N.Y.Acad.Sci. 121, 404 (1964).

169. McCarthy, M. J.Gen.Physiol. 29, 123 (1946).

170. Gregoire, J., and Gregoire, J. Bull.Soc.Chim.Biol. 34, 291 (1952).

171. Erkama, J., and Suutarinen, P. Acta Chem. Scand. 13, 323 (1959).

172. Miyaji, T., and Greenstein, J.P. Arch.Biochem. 32, 414 (1951).

173. Gilbert, L.M., Overend, W.G., and Webb, M. Exptl. Cell. Res. 2, 349 (1951).

174. Eichhorn, G.L., Clark, P., and Tarien, E. J.Biol.Chem. 244, 937 (1969).

175. Hanlon, D.P., Watt, D.S., and Westhead, E.W. Anal.Biochem. 16, 225 (1966).

176. Feinstein, R.N. J.Biol.Chem. 235, 733 (1960).

177. Vogel, A.I. In Textbook of Quantitative Inorganic Analysis. John Wiley and Sons, New York. 1961. p. 415.

178. Rosenkranz, H.S., and Bendich, A. Biochim.Biophys.Acta 51, 186 (1961).

179. Taborsky, G. J.Biol.Chem. 234, 2652 (1959).

180. Sevag, M.G., Lackman, D.G., and Smolens, J. J.Biol.Chem. 124, 425 (1938).

181. Ehrlich, P., and Doty, P. J.Amer.Chem.Soc. 80, 4251 (1958).

182. Garen, A., and Levinthal, C. Biochim.Biophys.Acta 38, 470 (1960).

183. Rushizky, G.W., and Sober, H.A. Biochemistry 2, 697 (1963).

184. Nestle, M., and Roberts, W.E. J.Biol.Chem. 244, 5219 (1969).

185. Solymosy, F., Tener, G.M., and Reichmann, M.E. Virology 27, 409 (1965).

186. Satoh, K., and Inoue, Y. Biochem. J. 114, 271 (1969).

187. Smith, J.D. In The Nucleic Acids. Eds. Chargaff, E., and Davidson, J.N. Academic Press, New York. 1955. p. 267.

188. Khym, J.X., and Uziel, M. Biochemistry 7, 422 (1968).

189. Cohn, W.E. In The Nucleic Acids. Eds. Chargaff, E., and Davidson, J.N. Academic Press, New York. 1955. Vol. 1, p. 211.

190. Toal, J.N., Rushizky, G.W., Pratt, A.W., and Sober, H.A. Anal.Biochem. 23, 60 (1968).

191. Michelson, A.M. In Molecular Associations in Biology. Ed. Pullman, B. Academic Press, New York. 1968. p. 93.

192. Anai, M., Hirahashi, T., and Takagi, Y. J.Biol.Chem. 245, 767, 775 (1970).

193. Heppel, L.A., Harkness, D.R., and Hilmoe, R.J. J.Biol.Chem. 237, 841 (1962).

194. Wu, R. Fed. Proc. 29, 465 (1970).

195. Razzell, W.E., and Khorana, H.G. J.Biol.Chem. 236, 1144 (1961).

196. Weiss, B., Live, T.R., and Richardson, C.C. J.Biol.Chem. 243, 4530 (1968).

197. Greer, S., and Zamenhof, S. Fed.Proc. 18, 238 (1959).

198. Ahmed, Z., and Reis, J.L. Biochem. J. 69, 386 (1958).

199. Kaye, M.A.G. Biochim.Biophys.Acta 18, 456 (1955).

200. Heppel, L.A., and Whitfeld, P.R. Biochem. J. 60, 1 (1955).

201. Heppel, L.A., Whitfeld, P.R., and Markham, R. Biochem. J. 60, 8 (1955).

202. Alexander, M., Heppel, L.A., and Hurwitz, J. J.Biol.Chem. 236, 3014 (1961).

203. De Meuron-Landolt, M., and Privat de Garilhe, M. Biochim.Biophys. Acta 91, 433 (1964).

204. Georgatsos, J.G., and Laskowski, M., Sr. Biochemistry 1, 288 (1962).

205. Vanecko, S., and Laskowski, M., Sr. Biochim.Biophys.Acta 61, 547 (1962).

206. Zimmerman, S.B., and Sandeen, G. Anal.Biochem. 14, 269 (1966).

207. Sinsheimer, R.L., and Koerner, J.F. J.Amer.Chem.Soc. 74, 283 (1952).

208. Swartz, M.N., Trautner, T.A., and Kornberg, A. J.Biol.Chem. 237, 1961 (1962).

209. Clausen, T. Anal.Biochem. 22, 70 (1968).

210. Wu, R., and Kaiser, A.D. J. Mol. Biol. 35, 523 (1968).

211. Antonoglou, O., and Georgatsos, J.G. Arch.Biochem.Biophys. 135, 36 (1969).

212. Valee, B.L., and Riordan, J.F. Ann.Rev.Biochem. 38, 733 (1969).

213. Gilbert, J.B., Clyde Otey, M., and Price, V.E. J.Biol.Chem. 190, 377 (1951).

214. Plummer, T.H., Jr., and Hirs, C.H.W., J.Biol.Chem. 238, 1396 (1963). 239, 2530 (1964).

215. Cunningham, L. Ann.N.Y.Acad.Sci. 81, 788 (1959).

216. Gurd, F.R.N., and Goodman, D.S. J.Amer.Chem.Soc. 74, 670 (1952).

217. Jordan, D.O. In The Chemistry of the Nucleic Acids. Butterworth Inc., Washington. 1960.

218. Shack, J., and Bynum, B.S. Nature 184, 635 (1959).

219. Gurd, F.R.N., and Wilcox, P.E. Advanc. Prot. Chem. 11, 311 (1956).

220. Vallee, B. Advanc. Prot. Chem. 10, 317 (1955).

221. Matthews, H.R. J.Gen.Virol. 3, 403 (1968).

222. Petersen, G.B., and Reeves, J.M. Biochim.Biophys.Acta 179, 510 (1969).

223. Ishikura, H., Neelon, F.A., and Cantoni, G.L. Science 153, 300 (1966).

224. Staehelin, M. Biochim.Biophys.Acta 49, 20 (1961).

225. Michelson, A.M. In Molecular Associations in Biology. Ed. Pullman, B. Academic Press, New York. 1968. p. 93.

226. Braun, R. Biochim.Biophys.Acta 149, 601 (1967).

227. Carrara, M., and Bernardi, G. Biochim.Biophys. Acta 155, 1 (1968).

228. Busch, E.W. J. Chromatog. 37, 518 (1968).

229. Cohn, W.E. J.Amer.Chem.Soc. 72, 1474 (1950).

230. Hamilton, P.B. Advanc. Chromatog. 2, 4 (1966).

231. Tomlinson, R.V., and Tener, G.M. Biochemistry 2, 703 (1963).

232. Bishop, D.H.L., Mills, D.R., and Spiegelman, S. Biochemistry 7, 3744 (1968).

233. Uziel, M., and Khym, J.X. Biochemistry 8, 3254 (1969).

234. Mandeles, S., and Cantor, C.R. Biopolymers 4, 759 (1966).

235. Privat de Garilhe, M., Cunningham, L., Laurila, U., and Laskowski, M. J.Biol.Chem. 224, 751 (1957).

236. Matsuda, M., and Ogoshi, H. J.Biochem. 59, 230 (1966).

237. Langridge, R. J. Cell. Physiol. 74 (Suppl. 1), 1 (1969).

238. Banerjee, K.C., and Perkins, D.J. Biochim.Biophys.Acta 61, 1 (1962).

239. Eichhorn, G.L. Nature 194, 474 (1962).

240. Fishman, M.M., Isaac, J., Schwartz, S., and Stein, S. Biochem.Biophys. Res.Commun. 29, 378 (1967).

241. Daune, M., Biopolymers 7, 659 (1969).

242. Shah, V.C., and Hughes, W.L. Biochim.Biophys.Acta 161, 261 (1968).

243. Brutland, D., Atkinson, M.R., Setlow, P., and Kornberg, A. Biochim. Biophys.Res.Commun. 37, 982 (1969).

244. Ward, D.C., Fuller, W., and Reich, E. Proc.Nat.Acad.Sci. U.S. 62, 581 (1969).

245. Ratliff, R.L., Hoard, D.E., Ott, D.G., and Hayes, F.N. Biochemistry 6, 851 (1967).

246. Shapiro, H.S., and Chargaff, E. Biochim.Biophys.Acta 91, 262 (1964).