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THE PYRIMIDINE TRACTS OF

BACTERIOPHAGE ϕX174

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DEDICATION

To my loving parents, who set me on the way, my long-suffering roommates who bore me through the middle and my wonderful husband, who made the end possible.

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To the National Institutes of Health, U.S. Public Health Service, for financial support during the period 1964-1968.

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ABSTRACT

The bacteriophage ϕX174, containing single stranded, circular DNA as its genetic material, has been studied in some detail with respect to its chemistry and biological function. The frequency of the pyrimidine tracts in the wild type bacteriophage has been determined, and the preponderance of the amount of longer tracts of seven to ten pyrimidine nucleotides, over that which would be expected from a random distribution noted.

Further studies of the longer pyrimidine tracts were carried out using the ρ^- mutant bacteriophage, which is lysis defective, showing delayed lysis in dilute cultures of the host, E. coli, and lysing only about ten per cent of the cells in dense culture. The DNA of the phage contains a discontinuity, a small region which is not single stranded, and therefore not susceptible to the action of E. coli exonuclease I. This enzyme digests only single stranded DNA and works only on free 5' OH ends. Therefore, digesting ϕX174 chromosomes with pancreatic DNA'ase, an endonuclease, to a limited extent, followed by digestion with E. coli exonuclease I, leaves a population of DNA of varying lengths, all having the same 5' end at the discontinuity. Within this population, then, the frequency of an individual tract's survival, depends on its distance from the discontinuity, and can be used to determine its position on the ϕX174 chromosome.

Methods for preparation of pyrimidine tracts, their separation by length into isopliths, and their subfractionation according to their relative cytosine and thymine content have already been developed. When more than one tract of the same length and base composition existed, further separations were not possible. Only the unique tracts could be mapped. This, in the ρ^- mutant, consisted of one decanucleotide, two each of nona and octanucleotides and one each of hepta and hexanucleotides. All of these tracts were located in the region of about 30 to 80 per cent of the distance around the chromosome from the discontinuity.

The pyrimidine tracts of the ρ^- mutant and wild type bacteriophage were compared. There is a decanucleotide of composition C_3T_7 present in the wild type phage, but not in the ρ^- mutant. Some evidence indicates that this mutation, that of a pyrimidine to a purine, occurs at a cytosine residue, leaving an octanucleotide, C_1T_7 present in the ρ^- mutant, but not in the wild type phage.

The nature of the ρ^- mutation is discussed, along with the possibility that the longer pyrimidine tracts may have a role in initiation of the DNA to messenger RNA transcription process.

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INTRODUCTION

A quarter of a century has passed since the momentous discovery of the genetic properties of DNA. Before that time, DNA was considered not nearly as interesting a study as proteins; it appeared to be merely a repetitive structure, containing its four nucleotides in equal amounts. There were hints as to its genetic nature; gently prepared DNA was found to be of higher molecular weight than most proteins (1), and it had been postulated that all the viruses, known carriers of genetic information, contained nucleic acids (2,3). The final proof, however, was left to Avery, MacLeod and McCarty, who demonstrated that high molecular weight DNA could be used to change the genetic properties of pneumonia bacteria (4).

With this finding, and the subsequent one that, for at least some of the viruses, all the genetic material is contained in the DNA (5), the serious study was begun. Molecular biology took on the task of finding out the detailed structure and mode of replication of genetic material, as well as its method of translation into cellular composition and function.

Further work has progressed rapidly. The elucidation of the double helical structure of DNA by Watson and Crick (6), based on their fitting of a molecular model of the DNA to the available x-ray data (7), while making use of Chargaff's base ratio laws (8) took place in 1953, and furnished an immediate explanation for some of the replicative properties of the genetic material. In addition, the details of DNA transcription to RNA along with the translation of RNA into protein have been subject to close scrutiny. Thus, at least the general outline of the primary and secondary functions of DNA has been clarified.

The final goal in the study of the action of DNA, however, is yet to be realized: a knowledge comparable to that of the exact function of every nucleotide within the DNA chain. Much progress has been made in the interpretation of the information available within the DNA chromosome. The realization of the triplet nature of the genetic code (9) was quickly followed by the introduction of synthetic messenger RNA into an *in vitro* system (10), allowing assignation of amino acid identities corresponding to many of the base triplets. Binding studies on s-RNA (11) elucidated many of the remaining triplet-amino acid correspondences, so that assignations of meaning could be definitively made for most of the possible nucleic acid triplets. The triplet codes not definitely assigned were at least given tentative identifications. Meanwhile, studies on mutants were revealing the possible single base changes which could lead to corresponding single amino acid substitutions.

Furthermore, it had been realized that not all the information carried in the DNA can be directly translated into amino acid sequences. In particular, the amber and ochre triplets appear to be "stop" signals, leading to polypeptide chain termination. Similarly, at least in many instances, amino acid chain initiation appears to be dependent upon a special triplet coding for N-formyl methionine. Since messenger RNA has been found to be multicistronic in many instances, different "stop" and/or "start" signals are needed for DNA transcription into RNA as compared with RNA translation into protein. And, because proteins specified by the same multicistronic messenger RNA can be manufactured at different rates, there must be some means for this rate differential to be accomplished; perhaps the rates are regulated by the abundance of the different soluble RNAs corresponding to the various alternate coding triplets for the same amino acid.

Areas of DNA entirely devoted to control functions are also found, these being either repressor or operator regions. The gene product of the repressor region can by itself, or in combination with an inhibitor, which is generally a small molecule, block the formation of all the proteins coded for by a single polycistronic messenger (12). The operator region, which controls whether or not a repressor can be effective, appears to be the DNA site on which the repressor must sit in order to prevent genetic expression. These are controls that are known; others may as yet be undiscovered.

In the attempts at elucidating the modes of action of DNA, RNA and their assisting molecules, many organisms have come under study. The tendency is toward the use of smaller, less complicated systems. Fathered by the sweet pea, genetic study has passed through the fruit fly and on down to the bacteria and viruses. The last two make exceptionally good subjects; both have short generation times, and culture methods are such that very rare genetic events can easily be picked out from millions of non-events.

Bacterial viruses make a particularly good subject. Aside from their coat proteins, which do not enter the host cell to participate in reproduction, they are composed almost solely of nucleic acids. And their very small size assures that they represent only a few genetic functions. These functions are not necessarily simple ones. Indeed, there is certainly an element of temporal control in that coat proteins are not produced until quite late in the infective process. However, viral functions are certainly less complicated than those of higher organisms. Certainly not before they are understood can we expect to progress to an understanding of even the bacterial cell. *Escherichia coli*, the best understood of bacteria, contains approximately 2000 genes as compared with the 100 of T4 bacteriophage and the 6 or 7 of ϕX174.

The bacteriophage ϕX174, because of its small size, even among bacteriophages, and its relative ease of culture, is a particularly good subject for study. First classified by Sertic and Bulgakov in 1935 (13), this phage is one of a group of serologically similar ϕX viruses which attacks sensitive strains of the host bacterium *E. coli*.

For a long time after the initial identification of ϕX174, its existence was essentially ignored, until 1959 when Sinsheimer developed a relatively simple procedure for isolation of the phage in pure form (14). Studies on the virus at this time showed it to have a molecular weight of 6.2×10^6, with a diameter of 30 mμ as viewed in the electron microscope. The genetic material of the phage consists of one single stranded chromosome of DNA, of molecular weight 1.7×10^6, which is approximately equivalent to 5500 nucleotides (14,15).

Subsequent experiments showed that all the genetic material of the virus is contained in the DNA, as demonstrated by the ability of *E. coli* protoplasts infected with ϕX174 DNA to produce whole phage which are in turn normally infectious (16).

To date, much of the research carried out on ϕX174 has been concerned with its biological functions within the host bacterium.

Although few in number, studies of the chemical structure of the DNA have been carried out as well. In the course of these chemical studies, ϕX174 gained distinction as the first recorded example of a circular viral chromosome (17-19). Additional work has also been carried out to determine the frequency of runs of purine and pyrimidine oligonucleotides (20,21). In these studies each individual run of uninterrupted purines or pyrimidines is known as a tract and tracts of identical length are termed isopliths.

The primary purpose of the present work was to add to the small fund of information about the DNA of bacteriophage ϕX174 through a study of its pyrimidine tracts. There were two facets to this study: the first a mapping of the unique pyrimidine isopliths of the ρ^- mutant of ϕX174, the second a comparison of the isopliths of the ρ^- mutant with the wild type phage to see if any differences could be observed.

The ρ^- mutant of the bacteriophage was originally chosen as a subject for study because it could be obtained in higher yields from the E. coli host than could the wild type phage. The ρ^- phage are designated lysis defective: they show delayed lysis of dilute cultures of host bacteria. In dense cultures they only lyse about 10 per cent of the E. coli cells. Their longer stay within the host cell allows them to produce between 100 and 500 phage per cell, compared with about 150 - 200 for the wild type.

The mapping of pyrimidine tracts could be accomplished by taking advantage of a special property of ϕX174, first observed by Fiers and Sinsheimer while obtaining ultracentrifugal evidence for a ring structure of the phage DNA. They were digesting ϕX174 chains with E.

coli exonuclease I, a phosphodiesterase which attacks single stranded DNA stepwise from the 3' hydroxyl end, liberating 5' mononucleotides (22). Since the DNA of the phage is circular, intact rings having no free 3' hydroxyl ends were not digested. However, even when the rings had first been cleaved with pancreatic deoxyribonuclease, digestion by the exonuclease proceeded only until the enzyme reached a discontinuity in the single stranded structure of the DNA, at which point the enzyme activity ceased.

It was possible, therefore, to break the chromosome by using pancreatic DNA'ase, which, they found, cleaves the chain at random points leaving free 3' hydroxyl ends (17). The broken DNA could then be digested with exonuclease I. This treatment would yield a set of DNA chains of all lengths ranging from a complete chromosome to very short tracts (see Figure 1). In all of the DNA strands the 3' end would originate at the same point in the original chromosome, this point being the discontinuity. Thus, the frequency of occurrence of a given tract in the set of digested DNA molecules would be a function of its distance from the discontinuity. This distance would always be measured in the $3' \rightarrow 5'$ direction of the DNA chain.

By enzymatically treating ^{14}C labeled ϕX174 DNA in this manner, and then combining it with untreated ^{3}H labeled ϕX174 DNA, it was possible to determine the position of each of the unique tracts from its $^{3}H/^{14}C$ ratio. This use of two labels eliminates any errors due to unequal loss of tracts during the various manipulative procedures.

Methods for the formation of pyrimidine tracts (23), and for their separation into isopliths (24,25,21) have already been perfected by other

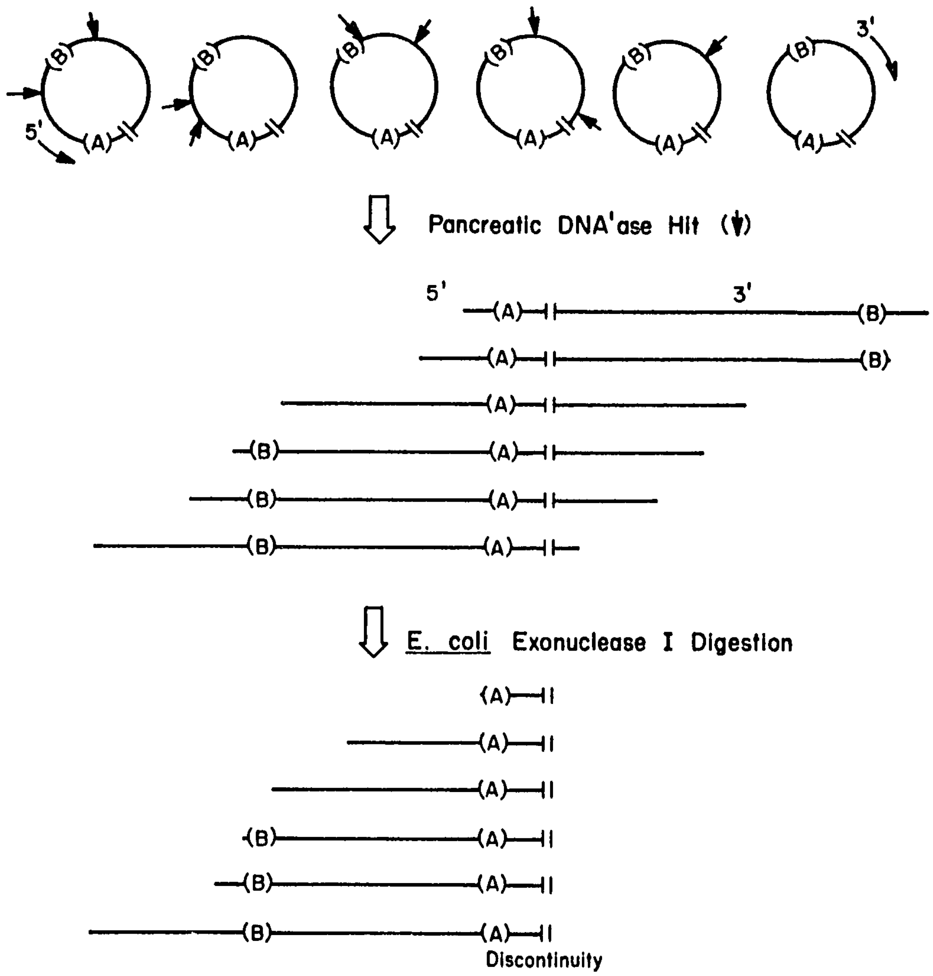


FIGURE 1. LIMITED DIGESTION OF ϕX174 CHROMOSOMES

The double lines on the DNA ring indicate the discontinuity. Small arrows indicate random points of attack by pancreatic DNA'ase. A and B are arbitrary tracts, located at positions f_1 and f_2 respectively. Since A is closer to the discontinuity in the 5' sense, it occurs more frequently in the DNA after exonuclease digestion.

workers. Electrophoresis and paper chromatography have yielded excellent results for the separation of some of the isopliths according to their cytosine thymidine composition. It was hoped that these methods could be extended to all of the isopliths, or, if that were not possible, that the method of Petersen and Reeves (26) for separation of phosphorylated pyrimidine isopliths by chromatography on DEAE cellulose could be adapted to the needs of these experiments.

It was also possible to prepare a mixture of ^{14}C labeled wild type and ^{3}H labeled ρ^- mutant ϕX174 DNA which could be degraded to pyrimidine tracts and separated into isoplith subfractions of identical composition by the same methods. By so doing, any differences between the longer, less common isopliths of the two phages could be detected.

Not only would the information concerning the pyrimidine tracts of ϕX174 that could be obtained by these methods add to the fund of data on the chemical structure of DNA, but also some important indications as to its functions may be made available. The spacings of the larger pyrimidine tracts could indicate either that these tracts are random in nature, or, perhaps, perform some special genetic function. Certainly any mutational changes observable at the DNA level as well as the functional level of the ρ^- mutation would be of interest. And were any of the differences between the pyrimidine tracts of the ρ^- mutant and the wild type bacteriophage to occur in one of the unique tracts of the ρ^- mutant, it would even be possible to map the location of this mutation on the physical ϕX174 chromosome and correlate it with eventual genetic mapping data on the ρ^- mutation.

MATERIALS AND METHODS

A. Materials

1. Materials Obtained Commercially: Uracil-2-^{14}C, uracil-^{3}H and uridine-^{3}H were products of the New England Nuclear Corporation, Boston, Massachusetts. Both 2,5-diphenyloxazole (PPO) and 1,4-bis-(4-methyl-5-phenyloxazolyl)-benzene (dimethyl POPOP) were purchased from Packard Instrument Company, Le Grange, Illinois. Allied Chemicals, Morristown, New Jersey, supplied resublimed naphthalene.

The compressed gases used were purchased from Gaspro, Limited, Honolulu, Hawaii, as was dry ice.

Carbowax 6000 was obtained from Union Carbide Chemicals, New York, New York. California Corporation for Biochemical Research, Los Angeles, California, was the source of bis-(p-nitrophenyl) phosphoric acid and glycine.

Cesium chloride was obtained from two sources, the first being Matheson Company, Incorporated, Norwood, Ohio, and the second Fischer Scientific Company, Fair Lawn, New Jersey. The latter company was also the source of diphenylamine.

Mallinckrodt Chemical Works, New York, New York was the source of analytical grade phenol which was redistilled in the laboratory before use. Trichloroacetic acid and gelatin were purchased from the J. T. Baker Chemical Company, Morristown, New Jersey.

Ethylenediaminetetra-acetic acid (EDTA) was purchased from Eastman Organic Chemicals, Rochester, New York. Tris (2-amino-2-hydroxymethyl-propane-1;3-diol) (Tris) was purchased as reagent grade Trizma Base from

Sigma Chemical Company, St. Louis, Missouri.

Agar was obtained from Case Laboratories, Chicago, Illinois. Nutritional Biochemical Corporation, Cleveland, Ohio supplied L-amino acids and ribonucleic acid. Tryptone, Casamino acids and Nutrient Broth were products of Difco Laboratories, Detroit, Michigan.

Sigma Chemical Company was the source of lysozyme which had been crystallized from egg whites, bovine pancreatic ribonuclease, bacterial alkaline phosphatase and the Dowex 50w resins. The Worthington Biochemical Corporation, Freehold, New Jersey supplied snake venom phosphodiesterase, beef pancreatic deoxyribonuclease and calf thymus DNA. Armour Pharmaceutical Company, Kankakee, Illinois was the supplier of sterile 30 per cent bovine serum albumin (BSA).

DEAE Sephadex A-25 and sodium dextran sulfate were obtained from Pharmacia Fine Chemicals, New York, New York. Whatman DEAE ion exchange cellulose in microgranular form (Chromedia) was purchased from W. & R. Ralston, Limited, England.

Deionized water was used in making all aqueous solutions for these experiments.

All other chemicals used were standard laboratory reagents.

2. Materials Obtained as Gifts: Cultures of *E. coli C* and *C1*, derived from British Type Culture Collection 122, and *E. coli W6* were gifts of Dr. R. L. Sinsheimer, California Institute of Technology, Pasadena, California. Dr. Sinsheimer also supplied stocks of ρ^- mutant and wild type strains of the bacteriophage ϕX174 in addition to the DNA of a γH, temperature sensitive mutant of the same phage to be used as a positional marker in velocity sedimentation experiments. In addition,

Dr. Sinsheimer provided a standard solution of wild type ϕX174 DNA for use in obtaining standard curves of DNA in the protoplast assay system.

Dr. I. R. Lehnam, Stanford University, Palo Alto, California kindly supplied purified exonuclease I from *E. coli*.

B. Methods

1. Preparation of ϕX174 DNA

a. *Titer of ρ^- Mutant and Wild Type ϕX174*: Plaque assays were performed using the agar layer technique of Adams (27). Two ml of top agar and 0.2 ml of plating bacteria were used per plate. The plating bacteria, *E. coli C*, were grown in TKB broth (10 g tryptone and 5 g NaCl/liter H_2O) with aeration to a concentration of about 5 x 10^8/ml and then stored in the cold until used. The initial phage dilution was through saturated borate solution in order to kill any contaminating bacteria. Subsequent dilutions were in TKB broth.

b. *Growth and Purification of ρ^- Mutant ϕX174*: The method of Sinsheimer for the preparation of pure ϕX174 of the ρ^- strain was followed (28). The *E. coli* culture to be infected was transferred from an actively growing culture in Fraser's 3XD medium to 2 liters of the same medium (29). Infection was with a multiplicity of 3 phage/bacteria, with the host cells at a concentration of between 2 and 3 x 10^9cells/ml. After three hours of aeration, the cells were spun down, resuspended in 0.25 M Tris, pH 8.1, and treated with lysozyme (10 mg/ml) and 8 per cent disodium EDTA to remove the cell walls. This treatment was followed by two successive freezings and thawings in order to lyse the cells.

The phage suspension was dialyzed against D medium and concentrated in the polyethylene glycol-sodium dextran sulfate phase system of Wesseln, et al. (30). The interface, containing the phage, was sedimented, resuspended in 0.01 M Tris acetate, pH 8.0, and once again sedimented. The pellet was then extracted twice with saturated borate solution, and the borate fractions pooled.

To the pooled phage was added 1/10th volume of 5X tryptone followed by CsCl to bring the density of the solution to 1.41. Ultracentrifugation followed and the band of phage was collected by dropwise collection. For this process the lusteriod tube was pierced and the pressure controlled either by a simple hydraulic apparatus or by use of a piercing needle attached to thin Nalgene tubing that was run through a Buchler polystaltic pump. In the region of the visible band fractions of two drops each were collected.

The ultracentrifugation process serves to separate whole ϕX174 bacteriophage from less dense particles containing host DNA fragments surrounded by ϕX174 coat proteins.

c. Growth and Purification of Wild Type ϕX174: The method of Sinsheimer for the preparation of pure wild type ϕX174 was followed (28). The *E. coli C* culture to be infected was transferred from an actively growing culture of TPG3A medium to 1 liter of the same medium. Infection was with a multiplicity of 3 phage/bacterium with the host cells at a concentration of between 4 and 5 x 10^8 cells/ml. Aeration was continued until the culture cleared, after which the broth was added to polyethylene glycol and sodium dextran sulfate to concentrate the phage.

The procedure subsequently followed that for collection of the ρ^- mutant of the phage.

For use in the experiments described in this paper, some modifications of Sinsheimer's methods were necessary. Acid washed glassware was used in order to obtain the highest possible phage yield per infection. Also, due to the difficulty in preventing culture contamination with air-borne ϕX174 during the overnight growth of the E. coli C for infection, it was necessary to test the ϕX174 sensitivity of the bacteria with some stock phage at the same time as the transfer from the overnight growth to fresh medium. In this way, plaques were visible on the trial plate of sensitive cultures at the same time that the large flask of bacteria was ready for infection.

It was also necessary to ascertain that the bacteria were in healthy log phase from the time of transfer until the time of infection. A halt in the bacterial growth during this time period could be indicative of the death of the susceptible bacteria due to contamination by the phage, followed by normal growth of ϕX174 resistant bacteria. Determinations of cell density were taken at regular intervals in a Petroff-Hauser counter. The Petroff-Hauser counter was used for all cell density determinations during the course of these experiments.

Infections were made with phage stocks which were found to be more stable when stored in the interface material of the polyethylene glycol-sodium dextran sulfate mixture than in saturated borate solution. When the ϕX174 pyrimidines were to be labeled, labeled uridine or uracil was added immediately after the phage. The labeled material was dissolved in sterile water prior to its aseptic addition to the culture.

All fractions discarded during these procedures were first titered to ascertain that they contained no more than 10 per cent of the phage as compared with the fraction retained.

d. Hot Phenol Extraction of ϕX174 DNA: The DNA was extracted from the phage according to the method of Sinsheimer (28). After purification through ultracentrifugation, the phage were dialyzed against saturated sodium borate. Extraction was performed at 70°C with redistilled phenol which had been preequilibrated with saturated borate. Phenol was removed from the aqueous layer by extraction with ether followed by removal of the ether by bubbling N_2.

An aliquot of the DNA was tested with DNA'ase and RNA'ase to ascertain that the material recovered was, indeed, DNA.

2. Limited Pancreatic DNA'ase Digestion of ^{14}C Labeled ρ^- Mutant ϕX174 DNA

a. Velocity Sedimentation of ϕX174 DNA in Alkaline Sucrose: The method of Burton and Sinsheimer was used for the preparation of alkaline sucrose gradients (31). Four ml of a 5 $\rightarrow$ 20 per cent sucrose solution in 0.04 M trisodium phosphate was used in each tube.

Centrifugation of the solution was performed in the 65 head of a Beckman model L-2 ultracentrifuge at 368,000 x g for 2-1/2 hours.

After piercing the tube, drops were collected as described by Burton and Sinsheimer, alternately into vials of scintillation fluid for radioactivity determination and into prechilled tubes for bioassay.

b. Bioassay of ϕX174 DNA: The assay procedure of Guthrie and Sinsheimer for biologically active, circular, wild type ϕX174 was used for ρ^- mutant's DNA in these experiments (16,32). An actively growing culture of *E. coli* W6 from an overnight innoculation into modified Fraser's 3XD medium was transferred into more of the same medium. When the cell density reached between 3 and 5 x 10^8 cells/ml the cells were spun down.

The cells were resuspended in 1.5 M sucrose, stabilized with 30 per cent BSA, then treated with lysozyme (2 mg/ml in sterile 0.25 M Tris, pH 8.1) and 4 per cent EDTA in order to form protoplasts. Nutrient broth was then added, followed by 10 per cent $MgSO_4$. The cells were then examined for protoplast formation.

For each assay of the biological activity of the ϕX174 DNA a standard curve was necessary. Ten fourfold dilutions of the standard ϕX174 DNA solution were made in 0.05 M Tris, pH 8.1.

Each unknown ϕX174 DNA sample, and each dilution of the standard ϕX174 was incubated individually with an equal volume of protoplast suspension for ten minutes. Nutrient broth, containing 0.5 per cent $MgSO_4$ was then added to the mixture. The tubes were then incubated for an additional 90 minutes before being frozen and thawed three times in succession to release the whole phage which had been produced.

Figure 2 shows a typical standard curve in which an efficiency of approximately 15 per cent was obtained (viable phage obtained per DNA molecule put in). Efficiencies ranged between 15 and 30 per cent in the course of those experiments involving the bioassay procedure.

FIGURE 2. STANDARD CURVE FOR PROTOPLASTS

A typical function of ϕX174/ml in growth tube versus DNA/ml in growth tube. In this curve the efficiency (viable phage produced/DNA put into protoplasts) is approximately 15 per cent. During the course of these experiments the efficiency ranged from 15 to 30 per cent.

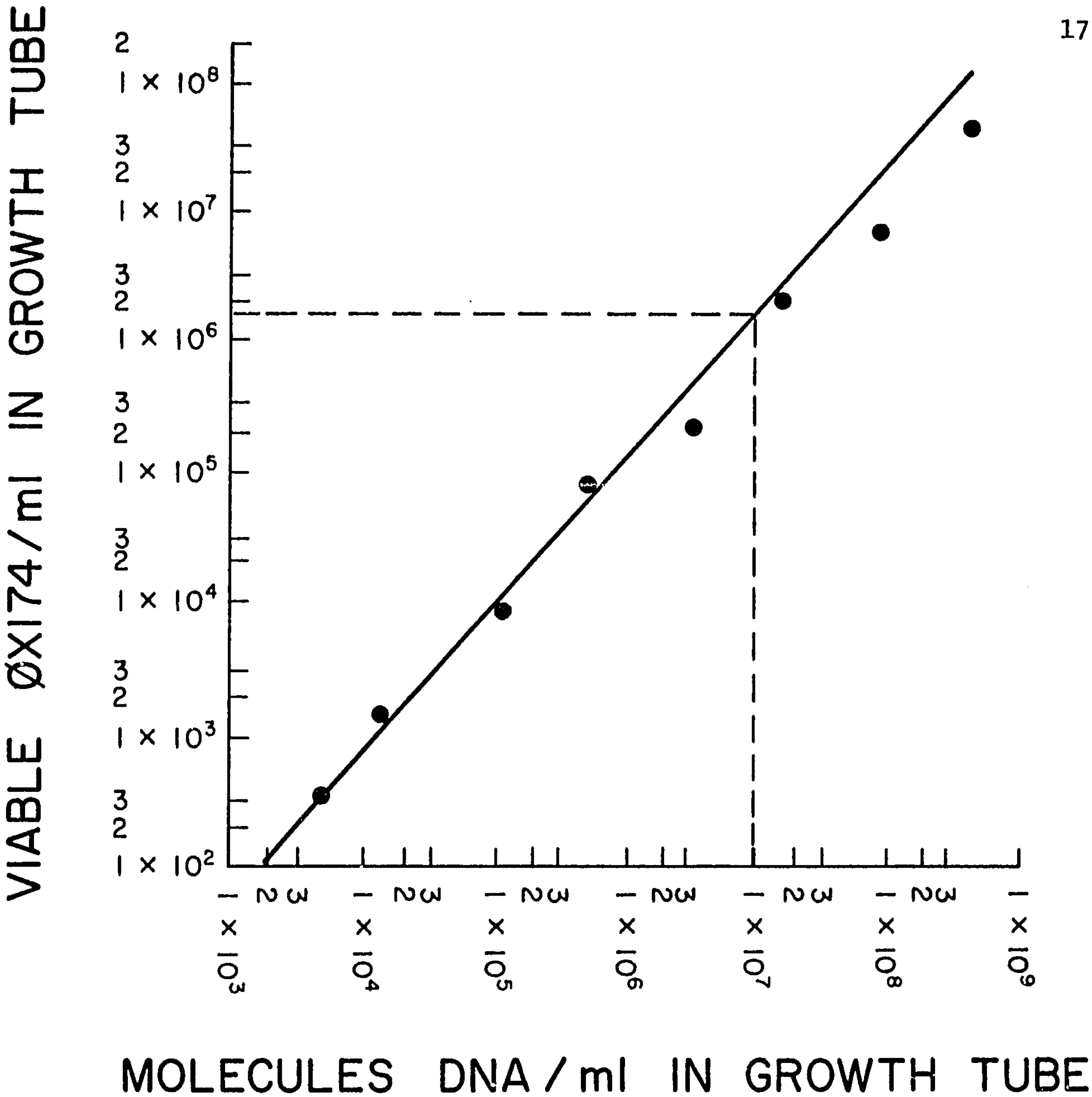

VIABLE ØX174/ml IN GROWTH TUBE
MOLECULES DNA / ml IN GROWTH TUBE
1 × 10²
1 × 10³
1 × 10⁴
1 × 10⁵
1 × 10⁶
1 × 10⁷
1 × 10⁸
1 × 10⁹

FIGURE 2

c. <u>Assay of Pancreatic DNA'ase Activity by Bioassay Method</u>: In order to avoid extraneous nuclease contamination, sterile plastic tubes were used for all dilutions and incubations. Pipets were of sterile plastic and were discarded after use. All micropipets were washed in 1:3 concentrated H_2SO_4:HNO_3 prior to use.

The incubation mixture for assay consisted of varying amounts of ϕX174 DNA, DNA'ase and BSA buffer solutions in a total volume of 250 μl. An aliquot of ^{14}C labeled ϕX174 DNA was diluted 2.5 fold with buffer B so that the final DNA solution was in 0.1 M NaCl, 0.02 M Tris, pH 7.5, and 0.008 M $MgCl_2$. Buffer B consisted of 0.1 M NaCl, 0.03 M Tris, pH 7.5, and 0.01 M $MgCl_2$. Further dilutions of DNA were in buffer C, consisting of 0.1 M NaCl, 0.02 M Tris, pH 7.5, and 0.008 M $MgCl_2$. The second component, 0.1 per cent bovine serum albumin, was made up in buffer C. Crystalline bovine pancreatic DNA'ase, diluted to 1.5 x 10^{-8} g/ml in a buffer containing 0.02 per cent BSA, 0.05 M Tris, pH 8.1 and 0.002 M $MgCl_2$, was the third component. All solutions were sterile.

Incubation of solutions containing 100 μl DNA, DNA'ase (between 1 and 100 μl) and sufficient BSA solution to bring the total volume to 250 μl were carried out at 37°C. At times ranging from 15 to 120 minutes, 50 μl aliquots of the incubation mixture were removed from each tube and mixed with 100 μl of 0.5 M Na_2HCO_3, pH 11.0, in order to stop the DNA'ase activity. The solutions in bicarbonate were allowed to remain at 30°C for two hours and then were heated to 65°C to insure denaturation as well as inactivation of the DNA'ase.

After heating, each of the solutions was diluted with 0.05 M Tris, pH 8.1, and aliquots were taken for bioassay.

d. Assay of DNA'ase Activity by Velocity Sedimentation in Alkaline Sucrose: Five μl of the stock ϕX174 DNA solution, which was the product of phenol treatment of whole ^{14}C labeled phage, was allowed to react with 5 μl of the DNA'ase solution for 30 minutes (for concentrations and buffers involved see section 2c of Methods). Twenty μl of bicarbonate buffer was added, and the solution incubated at 30°C for 1 hour, then heated to 65°C for 5 minutes. Seventy μl of Tris buffer was then added and the solution was frozen for storage.

After thawing, the digested DNA solution was centrifuged on an alkaline sucrose gradient and drops were collected for bioassay and determination of radioactivity. At the same time, a small aliquot of digested ϕX174 DNA that had been reserved was also subjected to bioassay.

e. Pancreatic DNA'ase Treatment of ^{14}C ϕX174 DNA: The DNA which had been extracted by phenol treatment of ^{14}C labeled ϕX174 was separated into two portions, one of 280 μl (portion I), and one of 500 μl (portion II). Each portion was digested with an equal volume of pancreatic DNA'ase for 107 minutes (these conditions were determined from the results of the experiment described in section 2d of Methods). After the DNA'ase digestion was completed to the predetermined extent, twofold volumes of 0.5 M NaH_2CO_3 buffer, pH 11.0, were added to each of the aliquots; they were then incubated at 30°C for two hours after which they were heated to 65°C for five minutes.

Aliquots from each portion were taken for bioassay and for alkaline sucrose gradient centrifugation in order to determine the extent of digestion.

f. E. coli Exonuclease I Treatment of ^{14}C ϕX174 DNA: After destruction of the pancreatic DNA'ase activity, an equal volume of 0.13 M HCl-glycine buffer, pH 9.5, was added to each portion of the digested ϕX174 DNA. Then 240 μl of E. coli exonuclease I (33,000 units/ml diluted 1:50 in sterile 0.1 per cent gelatin, pH 8.0, and stored frozen in aliquots) was added to portion I. The sample was next incubated at 30°C for 24 hours. Approximately 425 μl of the exonuclease was added to portion II, and the solution incubated at 30°C for 48 hours.

After incubation with exonuclease, each portion of DNA was dialyzed individually against 200 ml of buffer D, consisting of 0.005 M Tris, pH 7.5, and 0.001 M EDTA. Dialysis was overnight at 4°C.

3. Preparation of Dephosphorylated Pyrimidine Tracts

a. Preparation of Pyrimidine Tracts via Apurinic Acid: The ϕX174 DNA was added to 1 gm of carrier calf thymus DNA in order to minimize loss of the labeled phage DNA due to such phenomena as absorption to glass tubes and pipets. Apurinic acid was prepared from the mixed DNA solution and degraded to pyrimidine tracts according to the method of Burton and Petersen (23). The DNA was dissolved overnight in 0.01 M Tris formate, pH 7.5. Once completely dissolved, the DNA was treated with 15 g of diphenylamine in 88 per cent formic acid for about 18 hours. Water was added to the solution, followed by 5 successive extractions with equal volumes of ether to remove the formic acid and various of the nonnucleotide degradation products.

The purines were removed from the aqueous phase by passage through a Dowex 50w column which had been prepared in the H^+ form. The pyrimidine tracts were eluted with H_2O, then lyophilized to dryness.

b. Assay of Bacterial Alkaline Phosphatase Activity: Three ml cuvettes were used for the mixing of 1.5 ml each of solutions E and F. Solution E contained 0.004 M disodium p-nitrophenyl phosphate in 0.01 M ammonium acetate, pH 8.1. Solution F consisted of 0.1 M Tris, pH 8.2, and 0.01 M $MgCl_2$. One cuvette served as reference, while to the other was added 1 μl of the bacterial alkaline phosphatase (0.05 mg in 5 ml of 0.01 M Tris acetate, 0.01 M $MgCl_2$, pH 8.2, previously heated to 90°C for 10 minutes and then cooled to room temperature over a two hour period).

Absorbance readings at 410 mμ were taken at timed intervals in a Beckman model DU spectrophotometer. The absorptivity of the reaction product, p-nitrophenol, at 410 mμ is 1.62×10^4. The activity of the bacterial alkaline phosphatase can be determined from a plot of the absorbance versus time (33).

c. Dephosphorylation of Pyrimidine Tracts: The lyophilized powder, containing the phosphorylated pyrimidine tracts, was redissolved in 20 ml of a buffer containing 0.2 M Tris, 0.2 M $MgCl_2$ and 0.2 M NH_4HCO_3, pH 7.6. To this solution was added 0.5 ml of bacterial alkaline phosphatase with a specific activity of about 10^{-6} moles/μl/min. The phosphatase was dissolved in 0.01 M Tris acetate, 0.01 M $MgCl_2$, pH 8.2. The phosphatase solution had been heated to 90°C for 10 minutes and then cooled to room temperature over a two hour period before use.

The solution of pyrimidine tracts and phosphatase enzyme was incubated at 37°C until crystals of ammonium magnesium phosphate no longer appeared. At approximately 12 hour intervals, the crystals were spun down, washed with 1 ml of the Tris, $MgCl_2$, NH_4HCO_3 buffer, resedimented and discarded. The buffer wash was added to the incubation mixture along with an additional 0.1 ml of the bacterial alkaline phosphatase. Due to the long total incubation periods involved it was necessary to add a drop of chloroform before incubation in order to prevent bacterial contamination.

The dephosphorylated pyrimidine tracts were passed through a Dowex 50w column which had been prepared in the NH_4^+ form; they were then eluted with H_2O and lyophilized to dryness.

4. Separation of Pyrimidine Tracts by Chain Length

The oligonucleotides were chromatographed by the method of Knorana and Vizsolyi (24) as modified by Hall and Sinsheimer (21). This method, which required that the pyrimidine tracts be dephosphorylated, was found to give good resolution of the longer pyrimidine tracts, and had the additional advantage of using a completely volatile eluant solution.

The pyrimidine oligonucleotides, after being dissolved in a buffer of 0.001 M triethylammonium carbonate (TAC), were absorbed onto a DEAE Sephadex A-25 column which had been preequilibrated with the same buffer. Elution from the column was with a linear gradient of 0.001 to 1.3 M TAC, the total volume of the gradient being at least 4 liters.

The oligonucleotide isopliths were eluted in order of increasing chain length, the elution being dependent not only on molecular size, but also on total charge.

5. Subfractionation of Pyrimidine Isopliths

a. Paper Chromatography of Isopliths: Nucleosides and free bases were spotted on Whatman no. 1 paper and separated by ascending chromatography in the butanol: H_2O (86:14, v:v) solution of Markham and Smith (34). Chromatography required between 20 and 26 hours for optimum resolution of spots.

Oligonucleotides were spotted on Whatman no. 1 or no. 3 paper and separated by descending chromatography for approximately 30 hours in the solvent system of Wyatt, consisting of 170 ml isopropanol, 40 ml concentrated HCl and H_2O to a total volume of 250 ml (35).

In all cases spots were detected by photography in ultraviolet light. The ultraviolet absorbing spots were cut out and eluted in 2 to 3 ml aliquots of 0.1 N HCl.

b. Column Chromatography of Nucleosides: Nucleosides were chromatographed on Dowex 50w NH_4^+ columns according to the method of Crampton, et al. (36). The nucleosides were dissolved in 0.2 M Na Citrate, pH 4.0, before application to the Dowex column which had been preequilibrated with the same buffer. Thymidine was eluted with the same buffer; deoxycytosine elution was carried out in 1 M Na citrate buffer, pH 4.0.

c. Column Chromatography of Isopliths: The isopliths of length six and longer were separated according to their relative deoxycytosine and thymidine contents. Each isoplith was chromatographed individually after having first been alternately lyophilized to dryness and redissolved in deionized H_2O until the remaining powder no longer smelled of triethyl ammonium carbonate. The isopliths were dissolved in 0.1 M formic acid prior to chromatography.

Whatman DEAE ion-exchange cellulose in microgranular form (Chromedia) was prepared according to the directions of the manufacturer. The resin was washed with HCl, H_2O, NaOH, H_2O, NaOH, and, finally, H_2O, until the effluent was near neutrality.

The method of Petersen and Reeves (26) for the chromatography of phosphorylated pyrimidine tracts was modified for use in these experiments.

The resin was equilibrated with 0.1 M formic acid prior to use by stirring a volume of the resin into at least one equivalent of the acid and allowing the resin to settle for a minimum of ten minutes before decanting. This process was repeated eight times. After the final decantation, the resin was poured into the column and settled by passing another equivalent of the 0.1 M formic acid through it.

The pyrimidine isopliths were dissolved in 0.1 M formic acid and absorbed onto a preequilibrated column of DEAE Chromedia. The columns were 1 cm in diameter and varied in length from 7 to 15 cm; the shorter columns were used for the longer oligonucleotides which were present in smaller quantities. In all instances the oligonucleotide subfractions were reasonably well resolved.

After absorption of the isopliths, the column was washed with the formic acid until a small 270 mμ absorbance peak of unidentified material was eluted. The column was then eluted with a linear gradient of increasing NaCl concentration in 0.1 M formic acid. Each chamber contained 350 ml of the formic acid solution; the mixing chamber initially contained no NaCl. The reservoir contained 0.2 M NaCl in the formic acid. In some instances, the volumes in the chambers were increased to as much as 1 liter and the final NaCl concentration to 1.0 M. These longer gradients were used for the elution of the longer isopliths.

Fractions of 3 ml were collected at a flow rate of 12 ml/hour. The relative thymidine content of the subfractions eluted increased with increasing NaCl concentration, as had been noted by Petersen and Reeves. This was confirmed by ultraviolet spectra and, later, base analysis of the peaks.

d. Column Desalting of Isoplith Subfractions: The method of Petersen and Reeves (26) for column desalting of oligonucleotides was modified as follows: The resin used was DEAE Chromedia. Columns of 1 x 10 cm were preequilibrated with 0.001 M triethylammonium carbonate, pH 8.3. The oligonucleotides were absorbed onto these columns and then eluted with 1.3 M TAC, pH 8.6. The fractions were then lyophilized to dryness and stored at 4°C.

e. Dialysis Desalting of Isoplith Subfractions: It was possible to desalt the larger pyrimidine isopliths (length>6) by dialysis against deionized H_2O. Two or more six hour dialyses at 4°C were carried out for each sample.

6. Identification of Isoplith Subfractions

a. Assay of Snake Venom Phosphodiesterase Activity: Snake venom phosphodiesterase was assayed by the method of Koerner and Sinsheimer (37). The optical density of the enzyme at 278 mμ was taken. An aliquot of enzyme was allowed to react with the substrate, Ca bis (p-nitrophenyl) phosphate in Tris acetate buffer with magnesium and ammonium acetate added. Readings were taken at 440 mμ. The potency was then obtained from the formula:

$$\text{Potency} = \Delta A_{440}/\text{min}/\text{ml}/A_{278} \times 0.66$$

b. Base Composition of Isoplith Subfractions: Subfractions were degraded to nucleosides according to the method of Hall and Sinsheimer (21). The oligonucleotides were digested with snake venom phosphodiesterase for 48 hours at 37°C, then with bacterial alkaline phosphatase for 24 hours. Both digestions took place in 0.02 M Tris acetate, pH 8.8, 0.02 M $MgCl_2$, 0.02 M NaCl.

After digestion, between 5 and 10 ml of 0.2 N Na citrate, pH 4.0, was added to the 0.6 ml of digestion solution and the nucleosides were separated by elution from a Dowex 50w column as described in section 5b of Methods. The 270 mμ absorbance of each peak, the first being thymidine and the second deoxycytosine, was taken on a Beckman model DU spectrophotometer.

7. Determination of Radioactivity

Radioactivity was usually measured in a Packard Tri-Carb liquid scintillation spectrophotometer, model 3003. Polyethylene vials were used for counting. In most instances, 0.1 ml of sample was dissolved in 10 ml of Hunt's solution (38), which is a modification of the solution described by Werbin, et al. (39). In addition to the sample, 0.3 ml of H_2O was added in order to prevent freezing.

For the determination of the $^{3}H/^{14}C$ ratio in the concentrated isoplith subfractions, 12 ml of Hunt's solution was used as scintillation fluid. To this was added 0.1 ml of oligonucleotides and

0.36 ml of H_2O. The larger volume of scintillation fluid yielded greater efficiency of counting, due to a higher volume to surface ratio. At this volume the total number of counts obtained was not dependent upon small fluctuations in the volume of the scintillation fluid. These measurements were used for the localization of the individual pyrimidine tracts in the ϕX174 chromosome.

For these determinations of $^3H/^{14}C$ ratios, quenching estimates were made using an external standard (see Fig. 3). Optimum window and gain settings for all channels were those recommended by the manufacturer for double labeling with ^3H and ^{14}C. Standardization of the external standard for these studies was made on a series of increasingly quenched solutions of uridine ^{14}C and uracil ^3H; each isotope was determined separately. Corrections to dpm for cpm obtained were thereafter made from calculations from figure 3 which plots external standard cpm against per cent efficiency for each isotope in the channels in which it registers. Channel 1 contained primarily ^3H counts as well as a small percentage of ^{14}C counts. Channel 2 contained solely ^{14}C counts.

Radioactivity measurements in connection with alkaline sucrose gradients and pancreatic DNA'ase and E. coli exonuclease I digestion of ^{14}C labeled ϕX174 DNA, were performed in a Nuclear Chicago liquid scintillation spectrophotometer, using Bray's solution (40) as scintillation fluid. Samples of 0.1 ml were counted in 10 ml of scintillation fluid.

FIGURE 3. QUENCHING STANDARDIZATION

This figure is for standardization of quenching of doubly labeled pyrimidine isopliths by the use of an external standard. Per cent efficiency is plotted against cpm of external standard for each isotope in the channels in which it registers.

Samples of 0.1 ml are placed in 12 ml of Hunt's solution in which is dissolved 0.36 ml of H_2O in order to prevent freezing. Polyethylene vials are used. Settings: Channel 1 (primarily ^3H) windows 50→550, gain 100%; Channel 2 (entirely ^{14}C) windows 200→1000; gain 11.5%; Channel 3 (external standard) windows 300→∞, gain 1%.

Curve A (X-X-X) = ^{14}C in channel 2

Curve B (●-●-●) = ^3H in channel 1

Curve C (O-O-O) = ^{14}C in channel 1

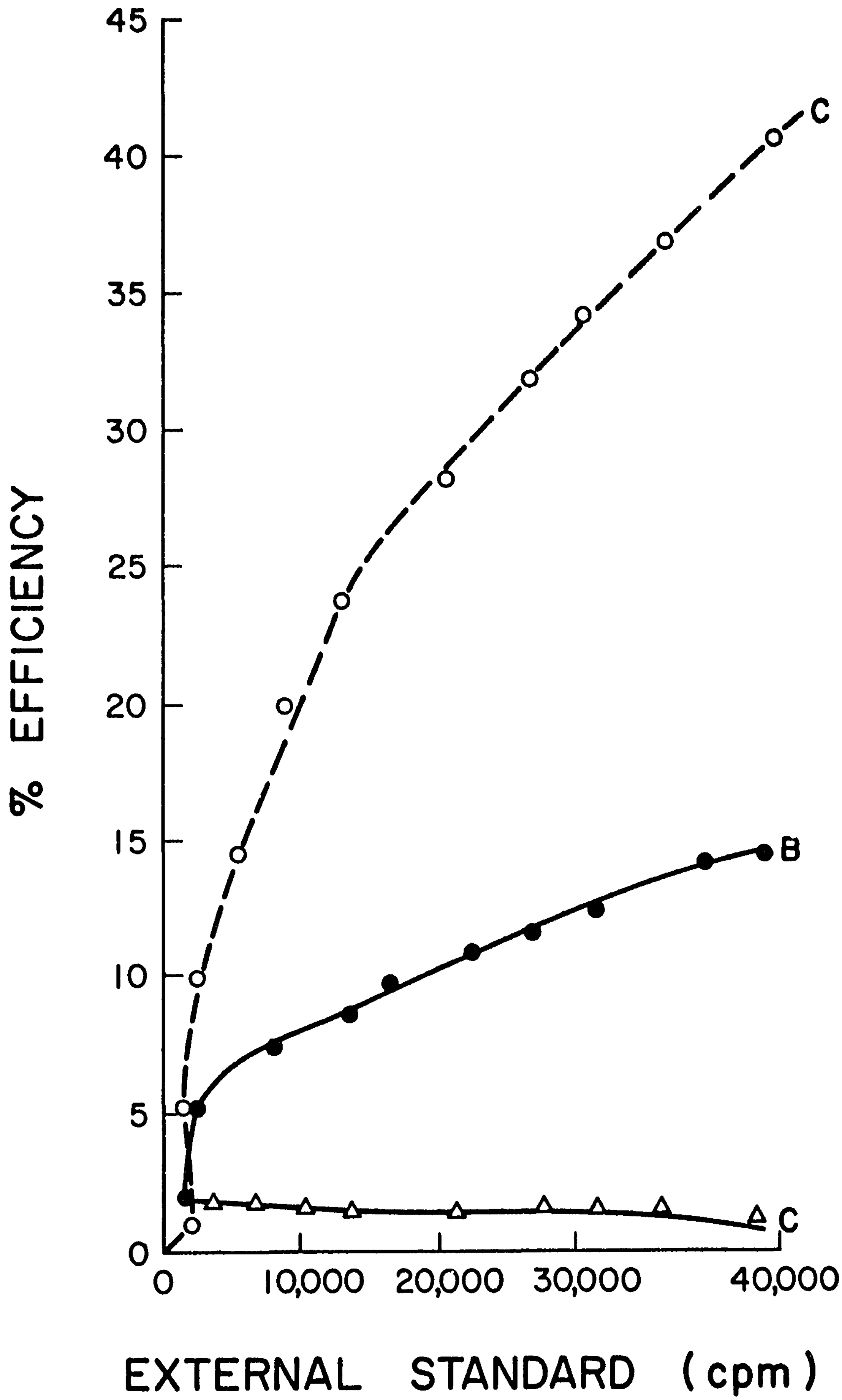

% EFFICIENCY
45
40
35
30
25
20
15
10
5
0
C
B
C
0
10,000
20,000
30,000
40,000
EXTERNAL STANDARD (cpm)

FIGURE 3

RESULTS

1. Preparation of Labeled DNA for Tract Localization

a. Preparation of ^{14}C Pyrimidine Labeled DNA: Figure 4 shows a typical growth curve for the *E. coli C* bacteria immediately prior to their infection with ρ^- bacteriophage. Along with the ϕX174 was added 0.20 mc (0.75 mg) of uracil ^{14}C in 2.0 ml of sterile H_2O. Table I indicated the recoveries of ϕX174 titer and ^{14}C activity during the purification of the phage. The initial recovery from the infection of 2 l of bacteria was 6.5×10^{14} viable phage containing a total of 6.1×10^7 cpm ^{14}C.

After the hot phenol extraction, the ^{14}C labeled DNA was found to contain 7.5×10^{15} molecules of ϕX174 DNA with a total radioactivity of 2.8×10^7 cpm at a counting efficiency of approximately 73 per cent. Prior to phenol extraction, about 2 per cent of the phage present were found to be viable.

Of the total ^{14}C counts used for infection, about 19 per cent was recovered.

b. Preparation of ^3H Pyrimidine Labeled DNA: Tritiated DNA was obtained by the same method as ^{14}C labeled DNA. A number of separate infections were carried out using either uracil ^3H or uridine ^3H, the latter being used in almost all instances as its labeling efficiency was found to be much higher. The initial purification procedure yielded only 2.5×10^6 cpm of phage with a viable count of 4.3×10^{14}. More infections were carried out. The product of the first purification was added to the new phage before the cesium chloride centrifugation step of the second purification.

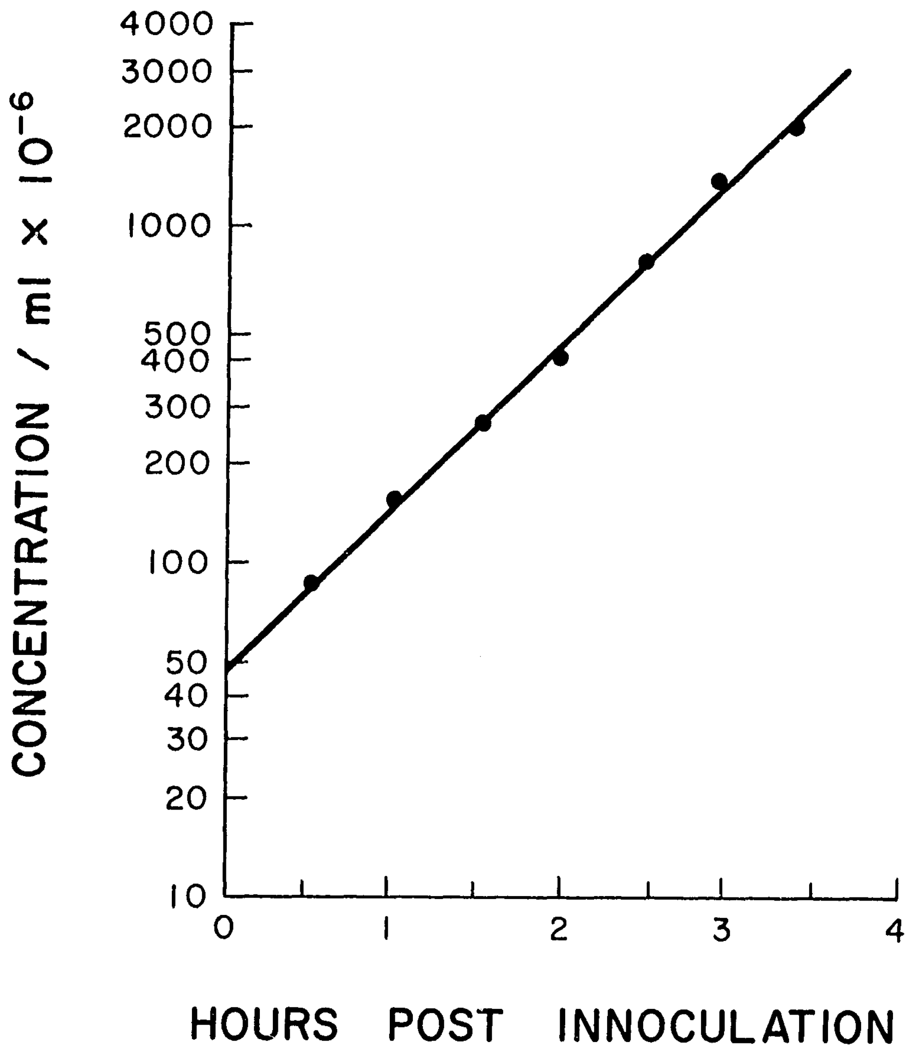


FIGURE 4. GROWTH OF E. COLI C FOR ρ^- ϕX174 INFECTION

E. coli C are grown for infection by ρ^- mutant of bacteriophage ϕX174. The figure plots the concentration of the bacteria versus time after innoculation into fresh Fraser's 3XD medium. Concentration was determined by cell count in a Petroff-Hauser counter.

TABLE I. RECOVERIES IN PURIFICATION OF ^{14}C AND ^{3}H ρ^{-} MUTANT ϕX174

Location of Activity	Titer of ^{14}C ϕX174 ($x10^{-13}$)	Cpm ^{14}C ($x10^{-6}$)	Titer of ^{3}H ϕX174 ($x10^{-13}$)	Cpm ^{3}H ($x10^{-6}$)
Lysed Cell Debris	65	61[a]	340	1500
PEG-Dextran Interface	80	160	46	80
Tris Suspension	34	24	33	37
First Borate Supernatant	10	24	47	b
Second Borate Supernatant	6	6	2.3	b
Purified Phage (Post CsCl)	3	30	7.3	2.3

[a]There was much quenching of the samples until the cell debris was removed. Quenching corrections were not made at this time as the machine was not yet calibrated.

[b]These samples were not counted.

The final yield of tritiated ϕX174 DNA was 9.7×10^{15} molecules of phage DNA with a total radioactivity of 1.3×10^{7} cpm at a counting efficiency of approximately 17 per cent. Table I shows the recovery of counts and viable titer during the purification of the phage. Of the total ^{3}H counts used during infection, about 0.1 per cent were recovered.

2. Preparation of Labeled DNA for Wild Type-ρ^{-} Tract Comparison

Figure 5 shows a typical growth curve for the *E. coli C* bacteria prior to their infection with wild type bacteriophage. An aliquot of wild type ϕX174 and 0.10 mc (0.37 mg) of uracil ^{14}C in 1.0 ml of sterile H_2O was added to the bacteria for each infection.

The ρ^{-} bacteriophage which contained ^{3}H labeled pyrimidines was prepared as usual. The wild type and ρ^{-} phages were pooled together in the polyethylene glycol-sodium dextran sulfate interface; titers and counts of the pooled phage were taken during the rest of the purification procedure. Table II indicates the recoveries of counts and titers during these procedures.

After hot phenol extraction, 8.6×10^{5} cpm of ^{14}C and 3.6×10^{6} cpm of ^{3}H were recovered.

3. Limited Digestion of ^{14}C Labeled ϕX174 ρ^{-} DNA

a. Velocity Sedimentation in Alkaline Sucrose: The amount of breakage of the ^{14}C labeled ϕX174 chromosome rings during the DNA extraction procedure and subsequent handling was determined by ultracentrifugation. The DNA, which had been stored in saturated borate solution, was first dialyzed against two changes of a buffer of 0.1 M NaCl, 0.001 M Tris, pH 8.1, at 4°C. A 1 µl aliquot of the ϕX174

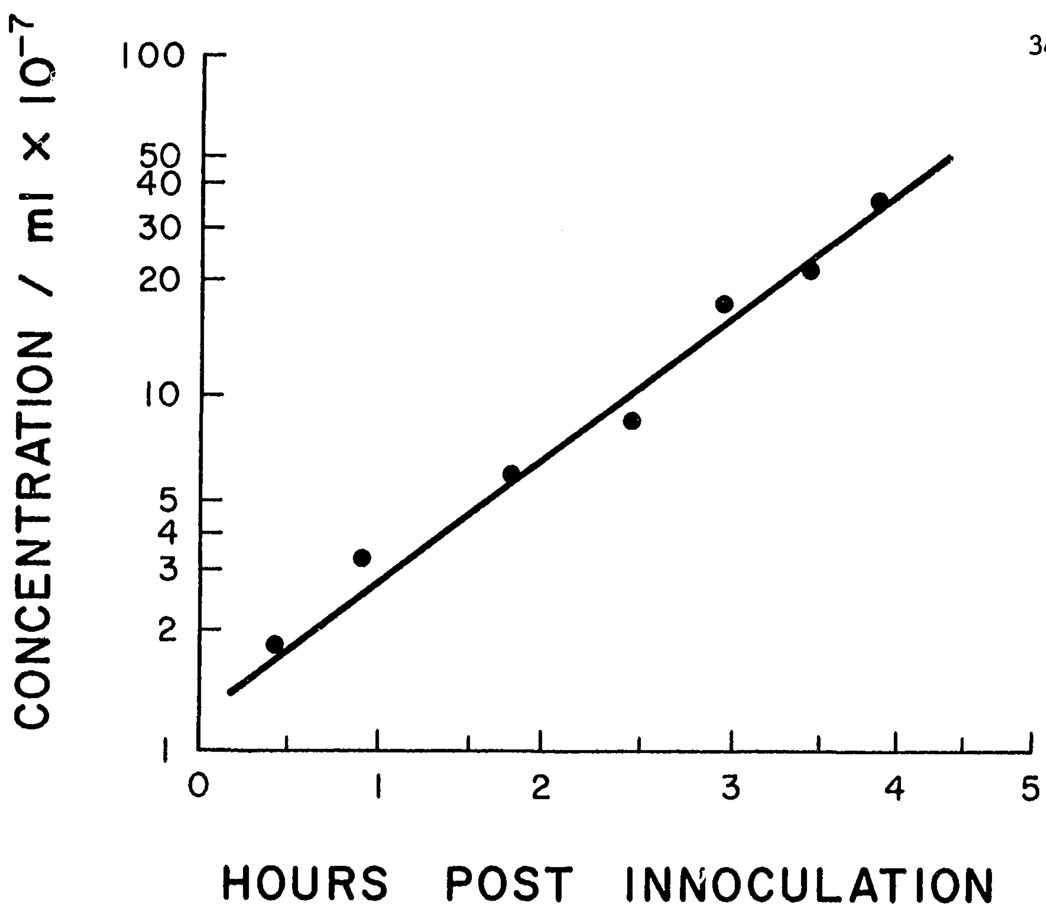


FIGURE 5. GROWTH OF E. COLI C FOR WILD TYPE ϕX174 INFECTION

E. coli C are grown for infection by the wild type bacteriophage ϕX174. The figure plots the concentration of the bacteria versus time after innoculation into fresh TPG3A medium. Concentration was determined by cell count in a Petroff-Hauser counter.

TABLE II. RECOVERIES DURING PURIFICATION OF ρ^--Wild Type ϕX174

Location of Activity	ϕX174 Titer ($x10^{-11}$)	Cpm ^{14}C (Wild Type) ($x10^{-6}$)	Cpm ^{3}H (ρ^-) ($x10^{-6}$)
Lysed Cell Debris	2600		610
PEG-Dextran Interface	300		
Tris Suspension	100	40,000	330
First Borate Supernatant	96	150	380
Second Borate Supernatant	34	21	40
Purified Phage (Post CsCl)	1.4	1.6	3.9

DNA was diluted to 100 μl in the same buffer, and to it was added 1 μl of marker phage, equivalent to approximately 1 x 10^9 phage chromosomes. The γH mutant, used as marker, is a temperature sensitive mutant which can be assayed selectively in a mixture with the ρ^- mutant. The γH, but not the ρ^- mutant, will grow on E. coli C_1, while the ρ^-, but not the γH mutant, will grow at 20°C on E. coli C. For optimal efficiency in plating, 10 per cent of E. coli C is added to the E. coli C_1 which is used for titering (41). The DNA from the two phage mutants was routinely layered on an alkaline sucrose gradient and spun for two hours.

The first run gave unexpected results. The biological activity was correlated with the slower moving component, although it should be the faster moving, unbroken chromosomes which are biologically active (19).

One possible cause for this phenomenon would be incomplete removal of the low density protein during the phenol treatment of the phage. Phenol extraction of the phage was therefore repeated, yielding 1.1 x 10^7 cpm of ^{14}C labeled φX174 DNA. An aliquot of this DNA was placed on an alkaline sucrose gradient along with γH marker and spun for two hours. Two main peaks were observed (see Figure 6). The faster moving one, contained in tubes 41-56, yielded biologically active, circular phage, making up 63 per cent of the radioactive label. The second peak, contained in tubes 57 through 68, was of the slower moving, broken DNA, and contained most of the remaining radioactivity.

b. Assay of DNA'ase Activity by Bioassay Method: Because of the random nature of φX174 DNA breakage by pancreatic DNA'ase, it had been expected that plots of the log of circular DNA remaining versus time of incubation with pancreatic DNA'ase, would yield a series of straight

FIGURE 6. ALKALINE SUCROSE CENTRIFUGATION OF ^{14}C LABELED ϕX174 DNA

Drops were collected from the bottom of the gradient in alternating tubes and scintillation vials. Centrifugation was for 2 hours at 65,000 rpm. The counting was performed in Bray's solution.

Curve A (———) = cpm ^{14}C

Curve B (●-●-●) = yield of ρ^- DNA x 10^{-5}

The biological activity of the γH marker was located primarily in tubes 42 through 52. Approximately 1/50th of that activity was present in tubes 58 through 64 and, again, in tubes 66 through 70.

The faster moving peak contained the biologically active ring form of the ϕX174 and accounted for 63 per cent of the total radioactivity.

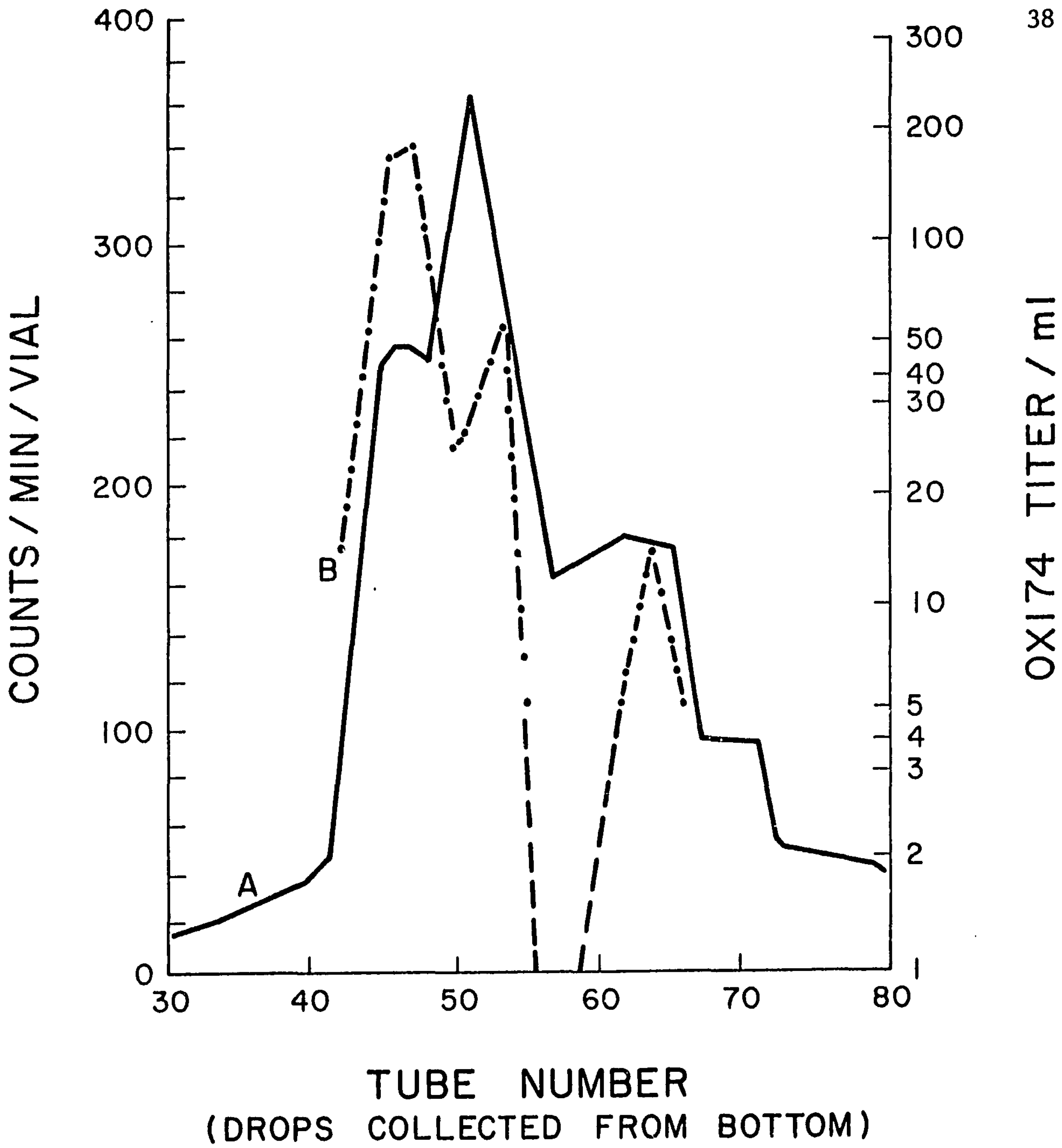

400
300
200
100
0
COUNTS / MIN / VIAL
300
200
100
50
40
30
20
10
5
4
3
2
1
OX174 TITER / ml
A
B
30
40
50
60
70
80
TUBE NUMBER
(DROPS COLLECTED FROM BOTTOM)

FIGURE 6

lines with negative slopes, originating at 100 per cent initial activity and with the slopes becoming more negative with increasing DNA'ase concentration.

Such plots, however, showed widely scattered points, so that only a rough estimate of DNA'ase activity could be made. Extra care taken in acid washing micropipets and in using sterile, disposable pipets and test tubes to prevent possible contamination by extraneous nucleases did not noticeably improve the results.

A final assay was performed, using a single DNA to DNA'ase ratio, with samples removed at 0, 15, 30, 45, 60 and 90 minutes of incubation, and diluted to 2.0 ml after destruction of DNA'ase. Samples of 0.4 ml from each tube were then bioassayed in quadruplicate. The values obtained for each point were found to vary by as much as a factor of two, reflecting on the lack of precision of the assay. Since this method, then, was not precise enough for the needs of the experiment, an alternate assay method was instituted.

c. <u>Assay of DNA'ase Activity by Velocity Sedimentation in Alkaline Sucrose</u>: From the results of the pancreatic DNA'ase digestion of ϕX174 DNA which were obtained by the bioassay method, as discussed in section 3b, it was possible to estimate to some extent the proper conditions for the pancreatic DNA'ase digestion of ^{14}C labeled ϕX174 to an average of 2 hits/chromosome. A small aliquot of DNA was digested for an arbitrary time period under conditions which appeared to lead to anywhere from 10 to 95 per cent digestion of the DNA in about that time period under the conditions of concentration, pH, and buffer strength described in section 2d of Methods.

Since alkaline sucrose gradient centrifugation yields a very exact determination of the number of rings present, it was argued that two very good points on a graph of time of digestion versus per cent of rings remaining would be better than a number of very inexact points, in order to determine the proper time of digestion.

Alkaline sucrose gradient centrifugation of the DNA digested in this manner showed that 41 per cent of the DNA remained in ring form. Direct bioassay of the digested DNA showed that 50 per cent of the original rings were still intact.

In figure 7 these data are plotted as per cent rings versus time of digestion. The results of the centrifugation assay showed that under the conditions used for DNA digestion, a 107 minute incubation of a 1:1 DNA:DNA'ase solution should yield 13.5 per cent rings, as desired.

d. <u>Pancreatic DNA'ase Treatment of ^{14}C ϕX174 DNA</u>: Portion I (280 μl ^{14}C ϕX174 DNA) was shown by alkaline sucrose gradient centrifugation and bioassay in quadruplicate to consist of 12.9 per cent intact circular DNA after treatment with DNA'ase as described in section 2e of Methods.

Portion II (500 μl) was shown to have 7 per cent of its DNA remaining as rings after digestion with pancreatic DNA'ase.

e. <u>E. coli Exonuclease I Treatment of ^{14}C ϕX174 DNA</u>: After treatment with DNA'ase and exonuclease I, 18 per cent of the total counts of portion I of the ϕX174 DNA were dialyzed out as mononucleotides, as were 17 per cent of the total counts of portion II. Dialysis of mononucleotides is very slow from a solution of high salt concentration into a solution of low salt concentration.

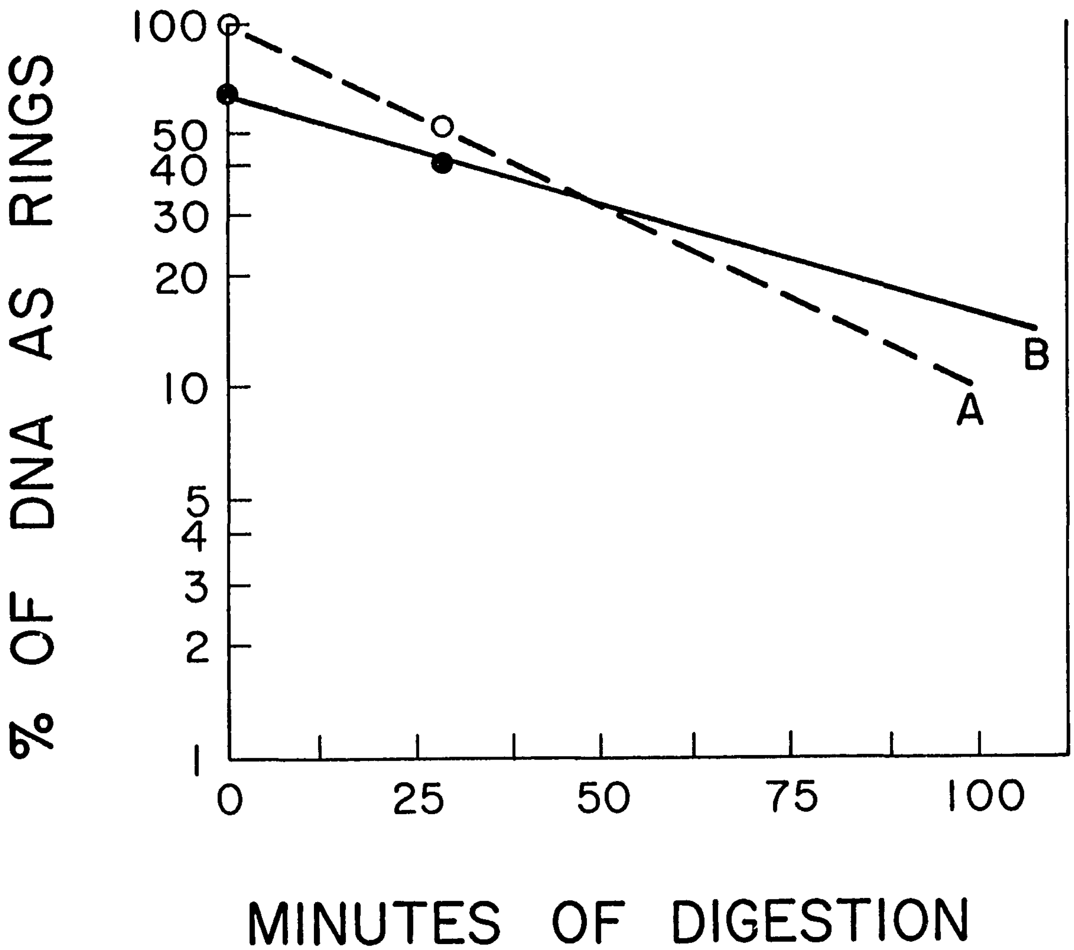


FIGURE 7. PER CENT RINGS VERSUS TIME OF DIGESTION

Curve A (0-0-0) represents the bioassay results where the initial rings are taken as 100 per cent. The actual value was 63 per cent so that 21.3 per cent of the initial rings is equivalent to 13.5 per cent of the total DNA. Curve B (●-●-●) represents the data from the alkaline sucrose sedimentation of the digested DNA, and was used to determine the final time of incubation.

To determine if the digestion with *E. coli* exonuclease I was complete, small aliquots of each of the digested DNA portions was added to enough carrier calf thymus DNA to show a visible precipitate when denatured. The DNA mixture was then precipitated with 1 ml of 10 per cent trichloroacetic acid for each 5 ml of DNA solution.

Portion II yielded 44 per cent mononucleotides, indicating the presence of 55 per cent mononucleotides prior to dialysis, and showing that digestion with the exonuclease had been complete.

Portion I required an additional 24 hours of digestion with 2 μl of undiluted *E. coli* exonuclease I which was added to the DNA after that solution was adjusted to pH 9.4 with 0.041 g of glycine. After this second incubation, precipitation with ten per cent TCA showed 50 per cent of the counts in the supernatant. Thus, this portion of ϕX174 DNA contained 64 per cent mononucleotides before dialysis, indicating complete digestion up to the discontinuity by the *E. coli* exonuclease.

4. Preparation of ϕX174 ρ^- Pyrimidine Isopliths

a. Preparation of ϕX174 ρ^- DNA: One half of the pancreatic and exonuclease digested ^{14}C labeled ϕX174 DNA was mixed together with half of the whole ^{3}H labeled ϕX174 DNA so that the $^{3}H/^{14}C$ ratio of the DNAs could be followed and any unequal loss of material from the different tracts during the following procedures would not effect the results obtained for the location of those tracts. Carrier calf thymus DNA was added to the labeled ϕX174 DNAs in order to minimize losses of the labeled material due to absorption. After production of phosphorylated pyrimidine tracts by formic acid-diphenylamine treatment as described in section 3a of Methods, the oligonucleotides were treated with bacterial alkaline phosphatase whose specific activity was found to be 1.2 x 10^{-2} mole/min/mg. Treatment was under the conditions described in section 3c of Methods. The dephosphorylated pyrimidine oligonucleotides obtained contained 8,420 absorbance units at 270 mμ, 1.28 x 10^6 cpm of ^{14}C and 3.11 x 10^6 cpm of ^3H, which was equivalent to 5.81 x 10^6 dpm of ^{14}C and 2.53 x 10^7 dpm of ^3H.

b. Separation of ρ^- Pyrimidine Oligonucleotides by Length: Preliminary practice separations were performed with calf thymus DNA, and showed that oligonucleotides of length 6 and longer were not completely resolved, as demonstrated by the fact that optical absorbance was far above background between the peak tubes.

During the separation of the labeled ϕX174 derived pyrimidine oligonucleotides, the gradient was made more shallow after the collection of the sixth peak. This was accomplished by addition of 587 ml of 1.3 M triethylammonium carbonate buffer, pH 8.6, to the reservoir chamber, and the same amount of an 0.54 M solution of the TAC buffer to the mixing chamber.

Figure 8 shows the elution pattern of the labeled pyrimidine isopliths from the DEAE Sephadex A-25 column. Eleven distinct peaks were observed, with what appeared to be five additional peaks following. Some loss of peaks 15 and 16 occurred during collection, so that their actual heights could not be determined.

The fractions under each absorbance peak were pooled, and the number of absorbancy units and disintegrations per minute determined for each

FIGURE 8. SEPARATION OF DOUBLY LABELED ϕX174 PYRIMIDINE TRACTS BY LENGTH

The oligonucleotides, dissolved in 200 ml of 0.001 M triethylammonium carbonate, pH 8.3, were absorbed onto a 2 x 40 cm column of DEAE Sephadex A-25 which had been preequilibrated with the TAC buffer. Elution was with a gradient of 0.001 to 1.3 M solution of the TAC buffer. The gradient is indicated on the figure, which also plots absorbance at 270 mμ versus tube number. Fractions of 15 ml/tube were taken at a flow rate of 1.6 ml/min.

The first 270 mμ absorbance peak, containing primarily nucleosides, is not absorbed on the column. The number of each successive peak represents the number of nucleotides in the isoplith which was eluted.

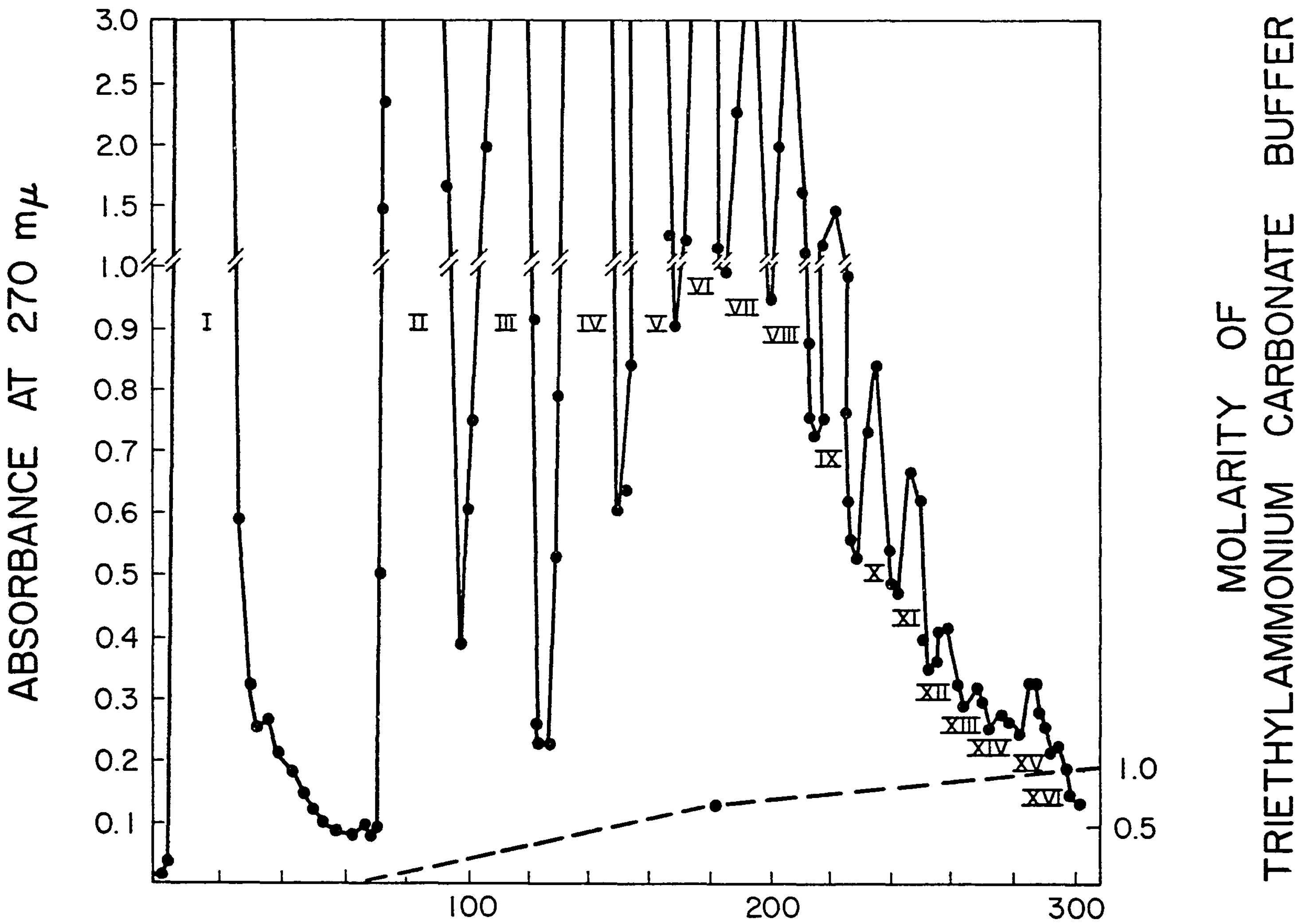

ABSORBANCE AT 270 mμ
3.0
2.5
2.0
1.5
1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
I
II
III
IV
V
VI
VII
VIII
IX
X
XI
XII
XIII
XIV
XV
XVI
100
200
300
TUBE NUMBER (APPROXIMATELY 15 ml/TUBE)
1.0
0.5
MOLARITY OF
TRIETHYLAMMONIUM CARBONATE BUFFER

FIGURE 8

peak. Table III lists the per cent absorbance, the actual and per cent dpm ^{14}C and dpm ^{3}H, the $^{3}H/^{14}C$ ratio, and the apparent number of tracts present in each pyrimidine isoplith. The per cent absorbance indicates the isoplith distribution in calf thymus DNA, and the per cent dpm ^{3}H the isoplith distribution in whole ϕX174 DNA.

Table IV lists the percentages of pyrimidines in each isoplith reported in the literature for the same elution system (21) as well as the results obtained during the preliminary separations performed with calf thymus DNA during the present experiments. The results obtained from Table III are also listed. It can be seen that the chromatographic separations are fairly reproducible. There does seem to be some tendency, however, for the first two or three peaks to contain a varying amount of material. Paper chromatography confirmed that in some instances, portions of the di or trinucleotides were eluted in the first peak.

c. Labeling Determination on ρ^{-} Nucleotides: In order to ascertain that the radioactive counts in the ϕX174 were located in the pyrimidines, rather than the purines, the purines were eluted from the Dowex H^{+} column with 0.1 N HCl. Approximately 1/500th of the total ^{14}C and ^{3}H counts present in the DNA were located in the purine fraction.

The pyrimidine nucleosides were chromatographed in butanol: H_2O to determine what percentage of each of the radioactive labels was present in the deoxycytosine, and what percentage in the thymidine. Photography through ultraviolet light showed two spots whose Rf values were 0.30 and 0.63 (Fig. 9a). Ultraviolet absorption spectra taken on a Cary 14 recording spectrophotometer identified these spots as

TABLE III. PYRIMIDINE ISOPLITHS

Tract[a] no.	% A_{270}[b]	dpm ^{14}C (10^{-3})	dpm ^{3}H ($x10^{-3}$)	% dpm ^{14}C	% dpm ^{3}H	$^{3}H/^{14}C$	No. of[c] Tracts
1	13.1	2570	7060	44.4	28.0	2.74	272
2	19.0	573	5950	9.86	23.5	10.4	229
3	18.2	442	3680	7.60	14.5	8.33	142
4	12.7	210	1840	3.62	7.29	8.23	71
5	8.60	166	1280	2.86	5.06	7.68	49
6	7.19	80.7	485	1.39	1.92	6.01	18.7
7	3.66	53.7	418	0.93	1.65	7.81	16.1
8	3.20	27.6	170	0.48	0.67	6.16	6.6
9	2.09	16.1	104	0.28	0.41	6.10	4.0
10	1.20	11.6	54.3	0.20	0.21	4.67	2.1
11	0.85	9.31	5.65	0.16	0.22	0.61	2.2
12	0.59	6.08	3.54	0.10	0.14	0.58	1.4
13	0.39	1.23	9.39	0.02	0.37	7.61	3.6
14	0.30	1.59	3.02	0.04	0.12	1.82	1.2

[a]The tract number is the same as the number of nucleotides in the isoplith.

[b]The percentages are of the total absorbance units put on the column.

[c]The number of tracts are the maximum possible number. Many counts were also present in non-pyrimidine material, so that the actual number of tracts present was less than the number given.

TABLE IV. PER CENT OF PYRIMIDINES PER ISOPLITH

Tract Length	Calf Thymus[a]		Calf[b] Thymus	Calf[c] Thymus	Calf[d] Thymus	ϕX174[a]	ϕX174[d]
1	20.6	21.6	21.8	53.7	13.1	19.0	28.0
2	20.0	20.0	23.0	16.0	19.0	27.3	23.5
3	15.5	15.2	19.0	12.0	18.2	17.6	14.5
4	12.8	12.7	13.9	10.1	12.7	10.9	7.29
5	8.7	7.7	8.8	7.4	8.6	10.6	5.06
6	6.8	6.6	6.8	6.8	7.2	5.2	1.92
7	4.9	4.5	4.4	4.3	3.7	4.2	1.65
8	3.4	3.1	3.5	3.5	3.2	2.3	0.671
9	2.4	2.2	2.2	2.8	2.1	1.5	0.410
10	1.5	1.3	1.2	1.9	1.2	0.7	0.214
11	1.4	1.4	1.2	1.5	0.85	0.3	0.224
12	1.1	0.8	0.92	0.75	0.59	-	0.140
13			0.77		0.39		0.371
14					0.30		0.119

[a]Hall and Sinsheimer (21).

[b]Experiment 1.

[c]Experiment 2.

[d]From Table III.

FIGURE 9. CHROMATOGRAPHY OF PYRIMIDINE NUCLEOSIDES

Figure 9a is a drawing of the chromatogram which was run for 24 hours in butanol:H_2O (Scale = 1/2).

Figure 9b is the ultraviolet absorption spectrum from 310 to 240 mμ of spot 1, identified as thymidine.

Figure 9c is the spectrum of spot 2, identified as deoxycytosine.

A blank, eluted from the chromatogram as indicated in Figure 9a, was used in the reference cell of the Cary 14 Recording Spectrophotometer. Both nucleosides are in 0.1 N HCl.

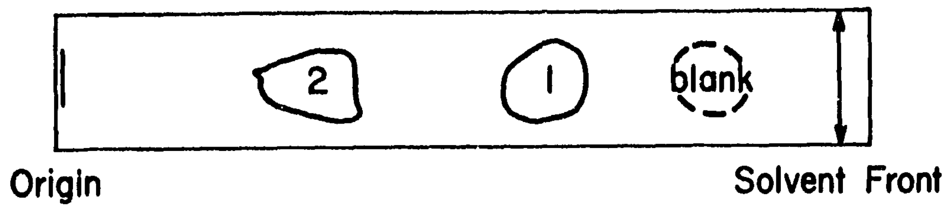


FIGURE 9A

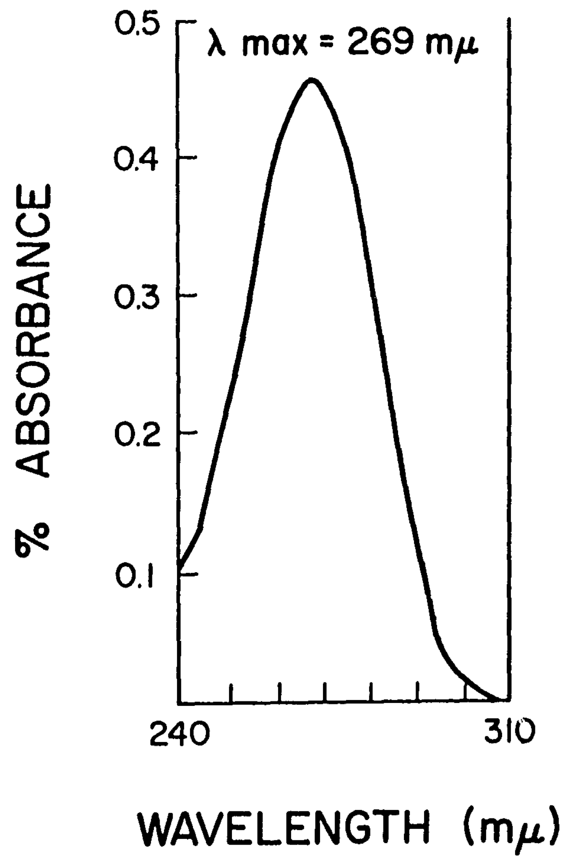


FIGURE 9B

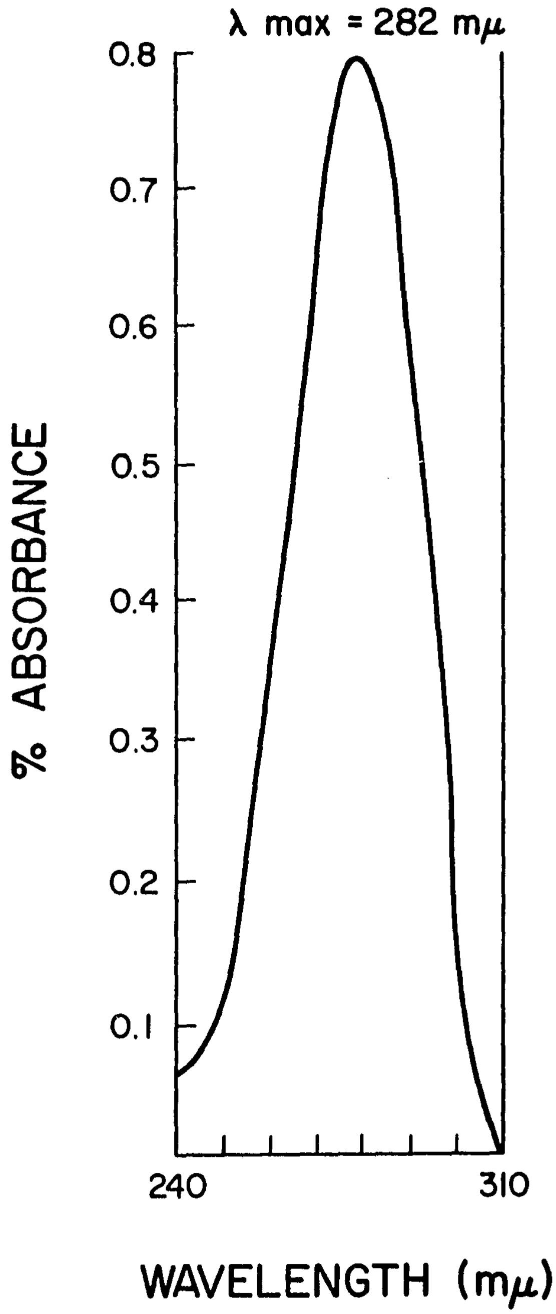


FIGURE 9C

deoxycytosine and thymidine respectively (Figs. 9b, 9c).

The radioactivity of the deoxycytosine and thymidine spots, together with their absorptivity at 270 mμ in acid was determined. The thymidine recovered represented 62 per cent, 44 per cent and 64 per cent of the total 270 mμ absorbance, dpm of ^3H and dpm of ^{14}C respectively. The deoxycytosine totaled 38 per cent, 56 per cent and 36 per cent respectively of these activities.

The molar absorptivity of thymidine in acid is 7.4×10^{-3} at 270 mμ, while for deoxycytosine it is 9.1×10^{-3}, so that the molar fractions of thymidine and deoxycytosine are 0.66 and 0.34 respectively. The molar fractions of these two nucleosides have been found to be $T = 0.59$ and $C = 0.41$ for the first peak (nucleosides) of calf thymus DNA's pyrimidine oligonucleotides, and $T = 0.56$, $C = 0.44$ for the first peak of ϕX174 DNA's pyrimidine oligonucleotides (21).

Assuming proportionate loss of deoxycytosine from both the ϕX174 and the calf thymus DNA, the molar fraction of the ϕX174 thymidine present in the eluted spot would be 0.64, while the deoxycytosine would be 0.36. This, however, would only be true of the ^3H labeled ϕX174 nucleosides. The ^{14}C labeled nucleosides that are present are composed of both ordinary peak I nucleosides of pyrimidine isoplith separation and of the products of E. coli exonuclease I digestion of pancreatic DNA'ase treated ϕX174 chromosomes. The original ^{14}C labeled DNA contained 280 μl of which 50 per cent of the DNA was in the form of mononucleotides, and 500 μl of which 44 per cent was mononucleotides - an average of 46 per cent mononucleotides prior to formic acid treatment.

One must assume that 19 per cent of the remaining DNA material became pyrimidine nucleosides upon production of the phosphorylated pyrimidine tracts (21). It is also necessary to assume that the mononucleotides produced by E. coli exonuclease I digestion reflected the base ratio of the entire ϕX174 chromosome, i.e. 51 per cent of pyrimidine nucleotides with the molar fraction of thymidine being 0.61 and that of deoxycytosine 0.39. Assuming, as before, proportionate loss of deoxycytosine from ϕX174 and calf thymus DNA, the molar fraction of thymidine and deoxycytosine in 82 per cent of the nucleosides would become 0.58 and 0.42 respectively, while the remaining 18 per cent would be the 0.64 and 0.36 of peak I. The overall molar fraction of thymidine is thus 0.59 and of deoxycytosine 0.41 within the ^{14}C labeled ϕX174 DNA.

The E. coli exonuclease treated DNA, then, has 64 per cent of the ^{14}C counts in the thymidine with a molar fraction of 0.59, and 36 per cent in the deoxycytosine with a molar fraction of 0.41. Thus, 55 per cent of the ^{14}C label is in each average thymidine residue and 45 per cent in each average deoxycytosine residue.

The untreated DNA contains 44 per cent of the ^{3}H counts in the thymidine with a molar fraction of 0.64, and 56 per cent in the deoxycytosine with a molar fraction of 0.36. The tritium label, then, is distributed between the thymidine and the deoxycytosine in the ratio of 31:69.

5. Subfractionation of Pyrimidine Isopliths by Composition for Tract Localization

a. Paper Chromatography of Isopliths: Dinucleotides obtained as a result of chromatographic separation of the pyrimidine isopliths were chromatographed in isopropanol:HCl:H_2O. Three spots resulted, with Rf values of 0.97, 0.78 and 0.42. They were identified as T_2, CT and C_2 respectively from their spectra.

b. <u>DEAE Chromedia Chromatography of Hexanucleotides</u>: To the doubly labeled pyrimidine isopliths of length 6 was added more carrier calf thymus DNA of the same length, resulting in a total absorbance of 1.16×10^4 at 270 mμ.

Elution from the chromedia column was with a linear gradient of 0 to 0.15 M NaCl in 0.1 M formic acid. Alternate tubes were analyzed for absorbance at 270 mμ and activity of ^3H and ^{14}C (Fig. 10). The 270 mμ absorbing peaks were each pooled, desalted on short Chromedia columns, and concentrated. Aliquots of the concentrated peaks were taken in order to determine the $^3H/^{14}C$ ratio.

Aliquots of each of the subfractions represented by the 270 mμ absorbance peaks were enzymatically digested to nucleosides and chromatographed on a Dowex 50w column as described in section 6b of Methods. The eluted peaks were subjected to spectral analysis to determine the quantity of each nucleoside present, and thus the identity of each peak.

Properties of the 11 subfractions of the length 6 pyrimidine isopliths are listed in Table V. The first four and the last one of the subfractions contain unidentified nonpyrimidine material. Subfractions 2 through 6 contain the hexanucleotide series C_4T_2 through T_6, as determined by hydrolysis and column chromatography. The hexanucleotide C_3T_3 is present in two peaks, one large and one small

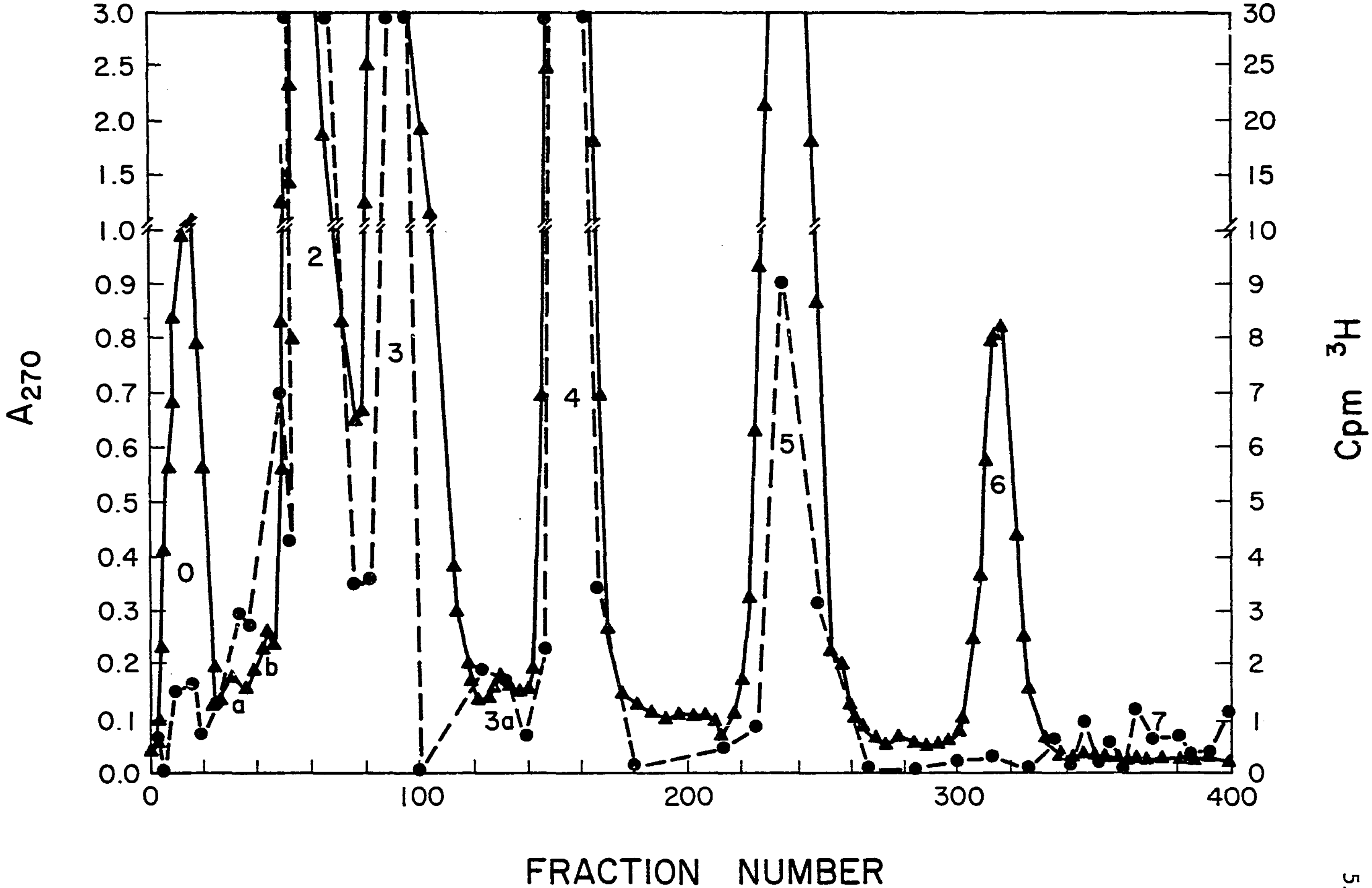
FIGURE 10. HEXANUCLEOTIDE CHROMATOGRAPHY FOR TRACT LOCATION

The figure represents the elution pattern of the ^3H and ^{14}C labeled pyrimidine hexanucleotides from a DEAE Chromedia column. Fractions of 3.5 ml were eluted with a linear gradient of 0 to 0.15 M NaCl in 0.1 N formic acid. The flow rate was 15 ml/hour.

1. (X-X-X) = Absorbance at 270 mμ versus tube number.
2. (●-●-●) = cpm ^3H versus tube number.

The cpm of ^{14}C followed that of ^3H, but was omitted from the figure for the sake of clarity.

Elution is in order of increasing relative thymidine content.



3.0
2.5
2.0
1.5
1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
A_{270}
30
25
20
15
10
9
8
7
6
5
4
3
2
1
0
Cpm ^3H
0
100
200
300
400
0
a
b
2
3
3a
4
5
6
7
FRACTION NUMBER

FIGURE 10

TABLE V. HEXANUCLEOTIDE SUBFRACTIONS

Peak no.	% A_{270}	dpm ^{14}C ($x10^{-3}$)	dpm ^{3}H ($x10^{-3}$)	% dpm ^{14}C	% dpm ^{3}H	$^{3}H/^{14}C$ (dpm)	A_{270}[a] T	A_{270}[a] C	C:T[b]	Identity	Tract[c] no.
0	3.31	1.83	6.80	1.44	1.18	56.0					
a	0.36	0.62	2.58	0.49	0.43	>106					
b	0.48	2.06	1.52	1.62	0.25	>50					
1	0.93	1.37	1.00	1.07	0.18	40.6					
2	6.97	20.6	201	16.3	32.5	14.8	13.2	27.9	3.4:2	C_4T_2	6.4
3	33.1	29.9	129	23.5	22.0	9.05	67.1	95.5	3.5:3	C_3T_3	4.9
3a	0.54	1.87	8.83	1.47	1.52	13.1					
4	31.2	21.0	143	16.5	24.6	104	29.8	16.3	1.8:4	C_2T_4	5.7
5	17.3	38.4	69.0	30.2	11.7	>206	80.5	20.0	1.0:5	C_1T_5	3.2
6	5.41	2.86	14.4	2.25	2.48	4.33	56.5	1.8	0.1:6	T_6	0.8
7	0.39	6.55	17.2	5.16	2.96	>82					

[a]Identities are determined by enzymatic hydrolysis followed by chromatography on Dowex 50w. Peaks are pooled and the absorbance at 270 mμ for C (deoxycytosine) and T (thymidine) is taken.

[b]The C:T ratio contains the correction factor of 0.813 x C/T to account for the differences in molar absorptivity at 270 mμ of the two nucleosides.

[c]The number of tracts are calculated from the dpm ^{3}H, using nona and decanucleotides as reference. See explanation later in text.

(3 and 3a). Subfractions 2 through 6 contain most of the 270 mμ absorbance, and subfractions 2 through 5 most of the ^3H activity.

c. DEAE Chromedia Chromatography of Heptanucleotides: To the doubly labeled pyrimidine isopliths of length 7 was added more carrier calf thymus DNA of the same length. The total 270 mμ absorbance of this fraction was thereby brought to 905.

These heptanucleotides were fractionated and treated in the same manner as the hexanucleotides (Figure 11). Table VI, analogous to Table V, shows the presence of nine subfractions. The first two and the last one contain primarily unidentified material. Subfractions 2 through 6 were identified as the heptanucleotide series C_6T_1 through C_1T_6. These subfractions contained most of the 270 mμ absorbing material as well as most of the tritium counts.

d. DEAE Chromedia Chromatography of Octanucleotides: Calf thymus carrier DNA of length 8 was added to the doubly labeled pyrimidine octanucleotides. Total 270 mμ absorbance for this fraction was 485.

The octanucleotides were subfractionated and treated in the same manner as the hexanucleotides except that the subfractions were eluted from the DEAE Chromedia column with a gradient of 0 to 0.2 M NaCl in 0.1 M formic acid (Fig. 12). Table VII, analogous to Table V, lists the data for the 10 subfractions collected. The first two and the last three subfractions consisted primarily of unidentified material. Subfractions 2 through 7 were identified as the octanucleotide series C_6T_2 through C_1T_7. Most of the 270 mμ absorbing material was present in subfractions 4 through 7; most of the ^3H counts were found in the same subfractions.

FIGURE 11. HEPTANUCLEOTIDE CHROMATOGRAPHY FOR TRACT LOCATION

The figure represents the elution pattern of the ^{3}H and ^{14}C labeled pyrimidine heptanucleotides from a DEAE Chromedia column. Fractions of 3.5 ml were eluted with a linear gradient of 0 to 0.15 M NaCl in 0.1 M formic acid. The flow rate was 15 ml/hour.

1. (X-X-X) = Absorbance at 270 mμ versus tube number.
2. (●-●-●) = cpm ^{3}H versus tube number.

All of peak 8, extending through tube number 410, was collected.

The cpm of ^{14}C followed those of ^{3}H, but were omitted from the figure for the sake of clarity.

Elution is in the order of increasing relative thymidine content.

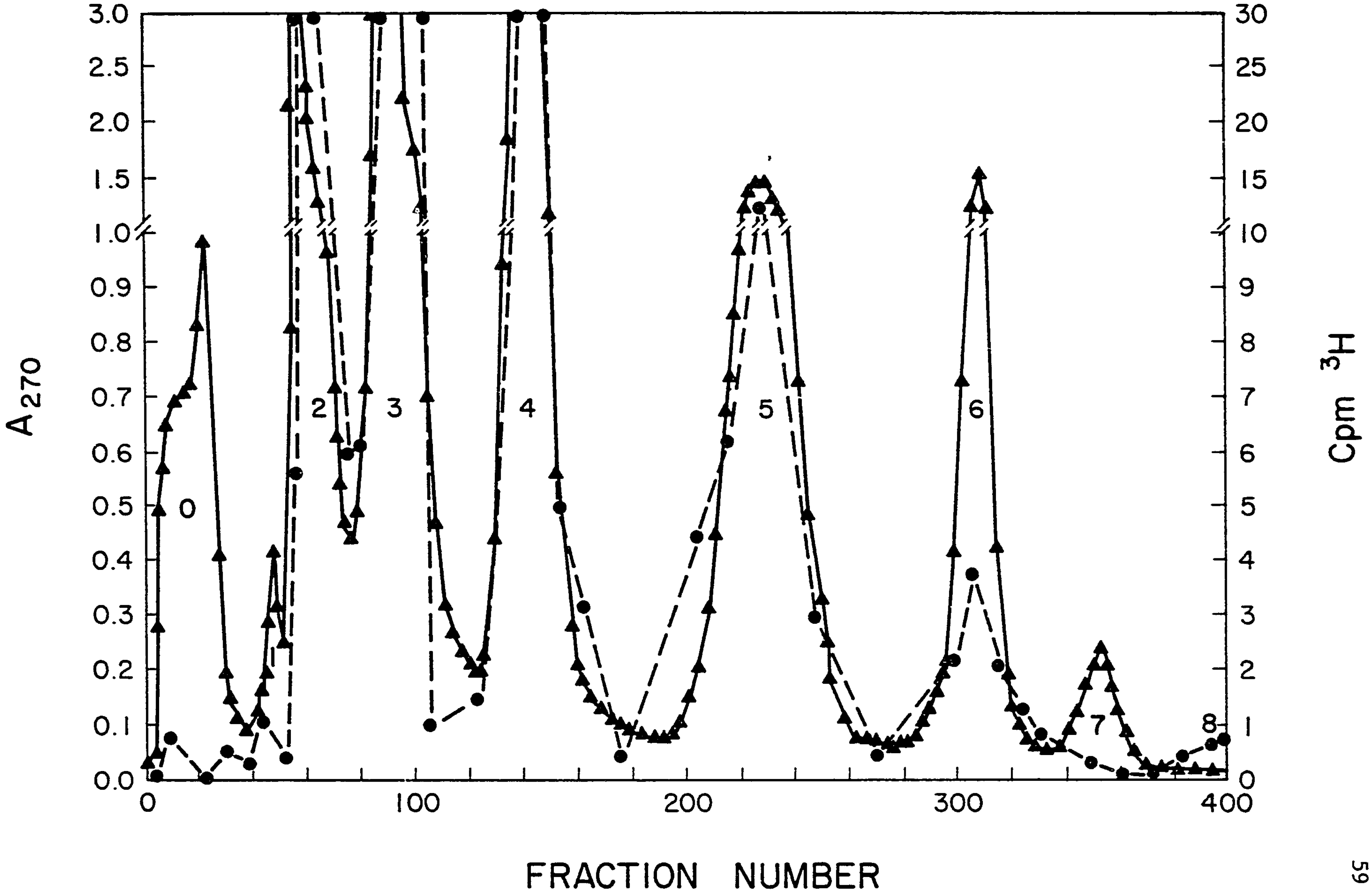

A270
Cpm 3H
FRACTION NUMBER
0
2
3
4
5
6
7
8
0.0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
0.8
0.9
1.0
1.5
2.0
2.5
3.0
0
1
2
3
4
5
6
7
8
9
10
15
20
25
30
0
100
200
300
400

FIGURE 11

TABLE VI. HEPTANUCLEOTIDE SUBFRACTIONS

Peak no.	% A_{270}	dpm ^{14}C ($x10^{-2}$)	dpm ^{3}H ($x10^{-4}$)	% dpm ^{14}C	% dpm ^{3}H	$^{3}H/^{14}C$ (dpm)	A_{270}[a] T	A_{270}[a] C	C:T[b]	Identity	Tract[c] no.
0	8.16					>128					
1	1.16					7.67					
2	14.1	43.7	7.60	10.0	21.2	19.8	5.06	34.2	5.5:1	C_6T_1	1.9
3	25.9	106	10.9	24.4	27.8	12.7	8.71	24.6	4.6:2	C_5T_2	2.9
4	21.4	75.0	12.3	17.4	31.4	16.0	11.5	18.2	3.9:3	C_4T_3	3.6
5	18.3	142	5.18	32.8	13.0	24.3	37.7	31.5	2.7:4	C_3T_4	1.7
6	8.82	46.8	2.59	10.8	6.60	5.1	32.8	14.0	1.7:5	C_2T_5	0.9
7	1.80	6.75		1.55		>74	11.7	2.6	1.1:6	C_1T_6	
8	0.36	11.9		2.75		2.21					

[a]Identities are determined by enzymatic hydrolysis followed by chromatography on Dowex 50w. Peaks are pooled and the absorbancy at 270 mμ for each of C (deoxycytosine) and T (thymidine) is taken.

[b]The C:T ratio contains the correction factor of 0.813 x C/T to account for the differences in molar absorptivity at 270 mμ of the two nucleosides.

[c]The number of tracts are calculated from the dpm ^{3}H, using nona and decanucleotides as reference. See explanation later in text.

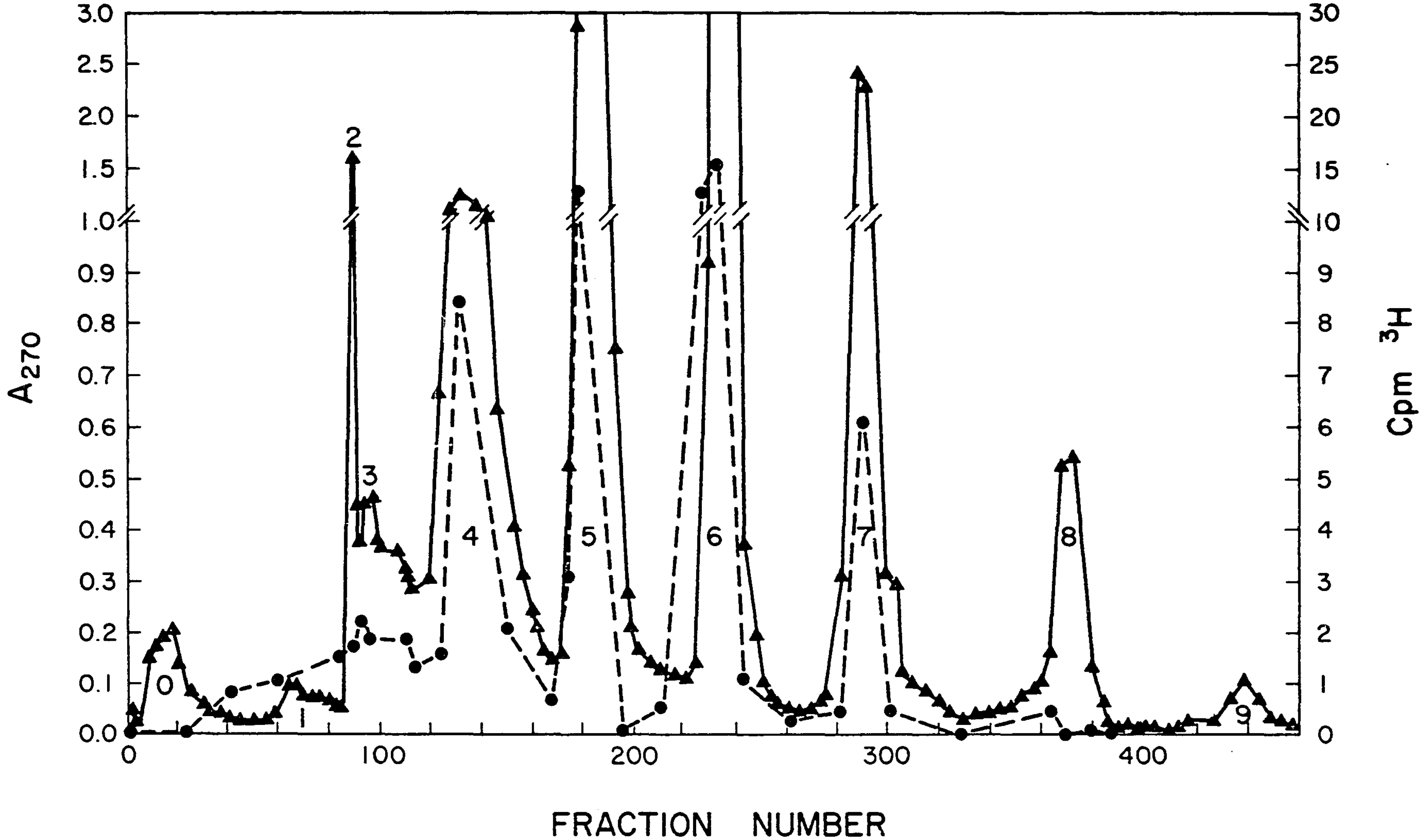
FIGURE 12. OCTANUCLEOTIDE CHROMATOGRAPHY FOR TRACT LOCATION

The figure represents the elution pattern of the ^{3}H and ^{14}C labeled pyrimidine octanubleotides from a DEAE Chromedia column. Fractions of 3.5 ml were eluted with a linear gradient of 0 to 0.2 M NaCl in 0.1 M formic acid. The flow rate was 15 ml/hour.

1. (X-X-X) = Absorbance at 270 mμ versus tube number.
2. (●-●-●) = cpm ^{3}H versus tube number.

The cpm of ^{14}C followed that of ^{3}H, but was omitted from the figure for the sake of clarity. To conserve material only enough of the tubes were counted to ascertain that the radioactivity followed the optical activity.

Elution is in the order of increasing relative thymidine content.



3.0
2.5
2.0
1.5
1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
A_{270}
30
25
20
15
10
9
8
7
6
5
4
3
2
1
0
Cpm ^3H
0
100
200
300
400
0
1
2
3
4
5
6
7
8
9
FRACTION NUMBER

FIGURE 12

TABLE VII. OCTANUCLEOTIDE SUBFRACTIONS

Peak no.	% A_{270}	dpm ^{14}C ($x10^{-2}$)	dpm ^{3}H ($x10^{-3}$)	% dpm ^{14}C	% dpm ^{3}H	$^{3}H/^{14}C$ (dpm)	A_{270} T [a]	A_{270} C [a]	C:T [b]	Identity	Tract [c] no.
0	2.05	8.15	8.35	2.63	4.19	1.10					
1	1.33		5.14		2.55	30					
2	3.22	2.37	3.36	7.63	1.62	7.32	1.93	6.79	5.7:2	C_6T_2	0.1
3	5.06		3.14		1.55	3.48	2.95	5.82	4.8:3	C_5T_3	0.1
4	19.6		19.9		9.86	10.1	19.2	23.8	4.0:4	C_4T_4	0.5
5	24.8	99.6	72.5	32.1	37.5	12.9	6.85	4.13	2.5:5	C_3T_5	2.1
6	21.9	68.6	47.0	21.9	23.3	8.09	7.55	3.64	2.3:6	C_2T_6	1.5
7	14.2	71.2	25.4	22.8	12.6	6.83	11.6	2.05	1.0:7	C_1T_7	0.9
8	5.88	28.5	5.80	9.12	2.90	5.57					
9	1.13	11.9	3.84	3.83	1.90	64.5					
10	0.83		4.11		2.05	67.1					

[a] Identities are determined by enzymatic hydrolysis followed by chromatography on Dowex 50w. Peaks are pooled and the absorbancy at 270 mμ for each of C (deoxycytosine) and T (thymidine) is taken.

[b] The C:T ratio contains the correction factor of 0.813 x C/T to account for the differences in molar absorptivity at 270 mμ of the two nucleosides.

[c] The number of tracts are calculated from the dpm ^{3}H, using nona and decanucleotides as reference. See explanation later in text.

e. <u>DEAE Chromedia Chromatography of Nonanucleotides</u>: Addition of carrier calf thymus DNA of length 9 to the labeled pyrimidine nonanucleotides brought the total absorbance at 270 mμ to 129.

The nonanucleotides were fractionated and treated in the same manner as the octanucleotides (Fig. 13). Table VIII, analogous to Table V, reveals the existence of 8 nonanucleotide subfractions. The first one and last two were of unidentified material. Fractions 1 through 5 were identified as the nonanucleotide series C_7T_2 through C_3T_6. Most of the ^3H activity was located in subfractions 2 through 4, with most of the absorbance at 270 mμ being located in subfractions 2 through 5.

f. <u>DEAE Chromedia Chromatography of Decanucleotides</u>: To the doubly labeled decanucleotides was added calf thymus length 10 oligonucleotides so that the total 270 mμ absorbance was brought to 157.

The decanucleotides were fractionated and treated in the same manner as the hexanucleotides, except that the subfractions were eluted from the DEAE Chromedia column with a gradient of 0 to 0.3 M NaCl in 0.1 M formic acid (Fig. 14). Table IX, analogous to Table V, lists data for the 10 subfractions collected. The first one and the last three subfractions contain unidentified material. Subfractions 1 through 6 were identified as the decanucleotide series C_8T_2 through C_3T_7. Most of the absorbance at 270 mμ was located in subfractions 2 through 6, and most of the ^3H activity in subfraction 2.

FIGURE 13. NONANUCLEOTIDE CHROMATOGRAPHY FOR TRACT LOCATION

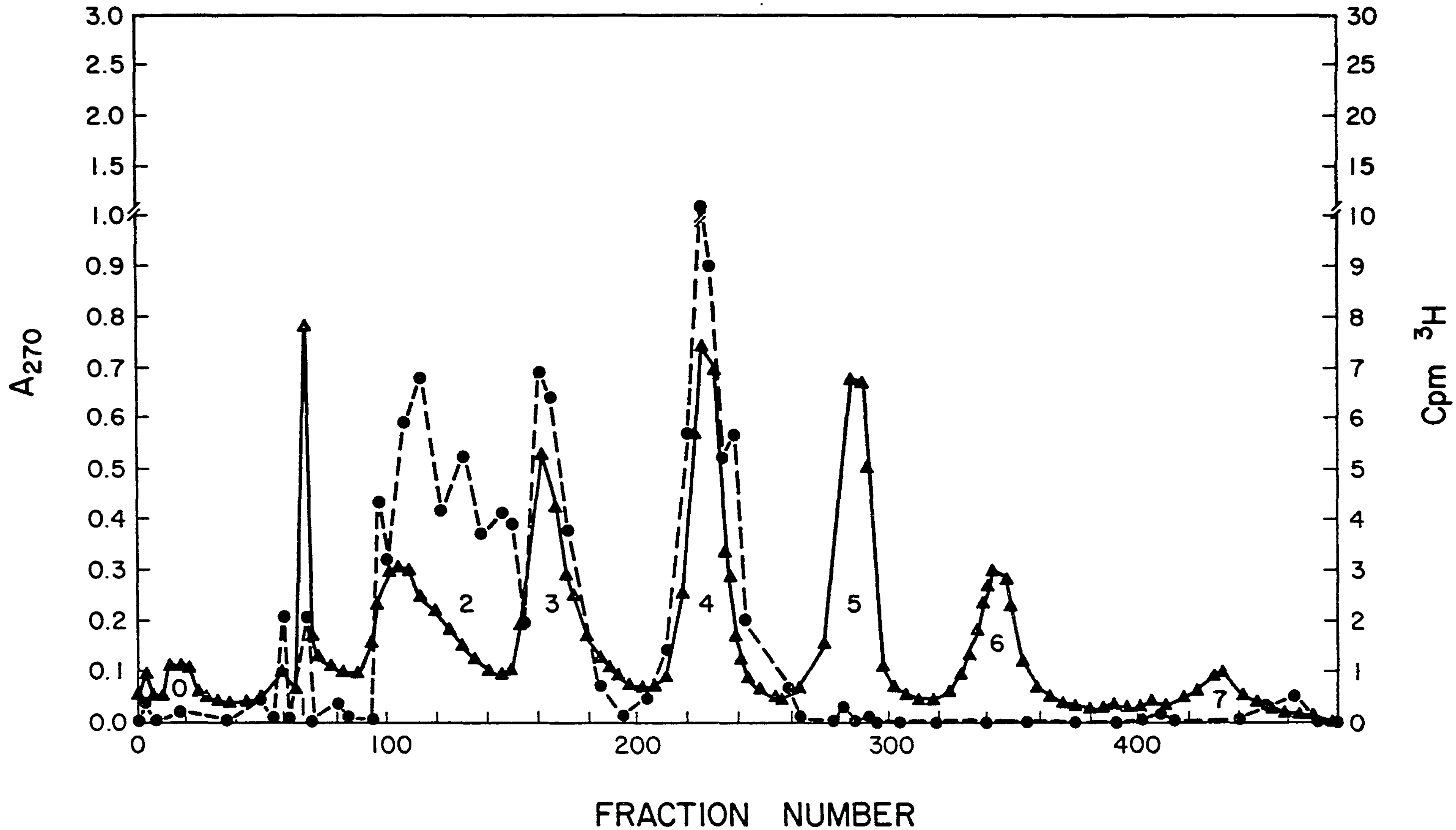
The figure represents the elution pattern of the ^{3}H and ^{14}C labeled pyrimidine nonanucleotides from a DEAE Chromedia column. Fractions of 3.5 ml were eluted with a linear gradient of 0 to 0.2 M NaCl in 0.1 M formic acid. The flow rate was 15 ml/hour.

1. (X-X-X) = Absorbance at 270 mμ versus tube number.

2. (●-●-●) = cpm ^{3}H versus tube number.

Peaks of cpm ^{14}C followed that of ^{3}H, but were omitted from the figure for the sake of clarity. Counts were so low that within a given peak they were quite irregular. Subfraction 2 was contained in tubes 94 through 150.

Elution is in the order of increasing relative thymidine content.



3.0
2.5
2.0
1.5
1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
A_{270}
30
25
20
15
10
9
8
7
6
5
4
3
2
1
0
Cpm ^3H
0
100
200
300
400
0
2
3
4
5
6
7
FRACTION NUMBER

FIGURE 13

TABLE VIII. NONANUCLEOTIDE SUBFRACTIONS

Peak no.	% A_{270}	dpm ^{14}C ($x10^{-2}$)	dpm ^{3}H ($x10^{-3}$)	% dpm ^{14}C	% dpm ^{3}H	$^{3}H/^{14}C$ (dpm)	A_{270}[a] T	A_{270}[a] C	C:T[b]	Identity	Tract[c] no.
0	5.48	4.42		3.40		>44					
1	5.06	37.7	1.56	30.9	0.75	>1260	0.22	0.98	7.2:2	C_7T_2	
2	20.2	46.5	46.5	38.0	16.4	9.72	0.41	1.02	6.1:3	C_6T_3	1
3	13.4	20.2	51.5	16.6	21.5	17.7	0.77	1.34	5.7:4	C_5T_4	1
4	23.4		89.5		37.2	11.6	1.48	1.15	3.2:5	C_4T_5	2
5	19.3	13.8	19.2	11.3	8.00	7.94	2.64	1.32	2.5:6	C_3T_6	
6	9.0		14.7		6.15	7.26					
7	4.18		17.1		7.05	>82					

[a]Identities are determined by enzymatic hydrolysis followed by chromatography on Dowex 50w. Peaks are pooled and the absorbancy at 270 mμ for each of C (deoxycytosine) and T (thymidine) is taken.

[b]The C:T ratio contains the correction factor of 0.813 x C/T to account for the differences in molar absorptivity at 270 mμ of the two nucleosides.

[c]The number of tracts are obtained by comparison of the ^{3}H counts in each peak with the data of Hall and Sinsheimer (21) on the nona and decanucleotides. Adjacent peaks containing 1, 1 and 2 tracts in order of increasing thymidine were found in the nonanucleotides and a single, high cytosine content tract in the decanucleotides. Due to the high amount of unidentified, labeled material present, it was necessary to use these data rather than that of initial tritium label over the whole chromosome.

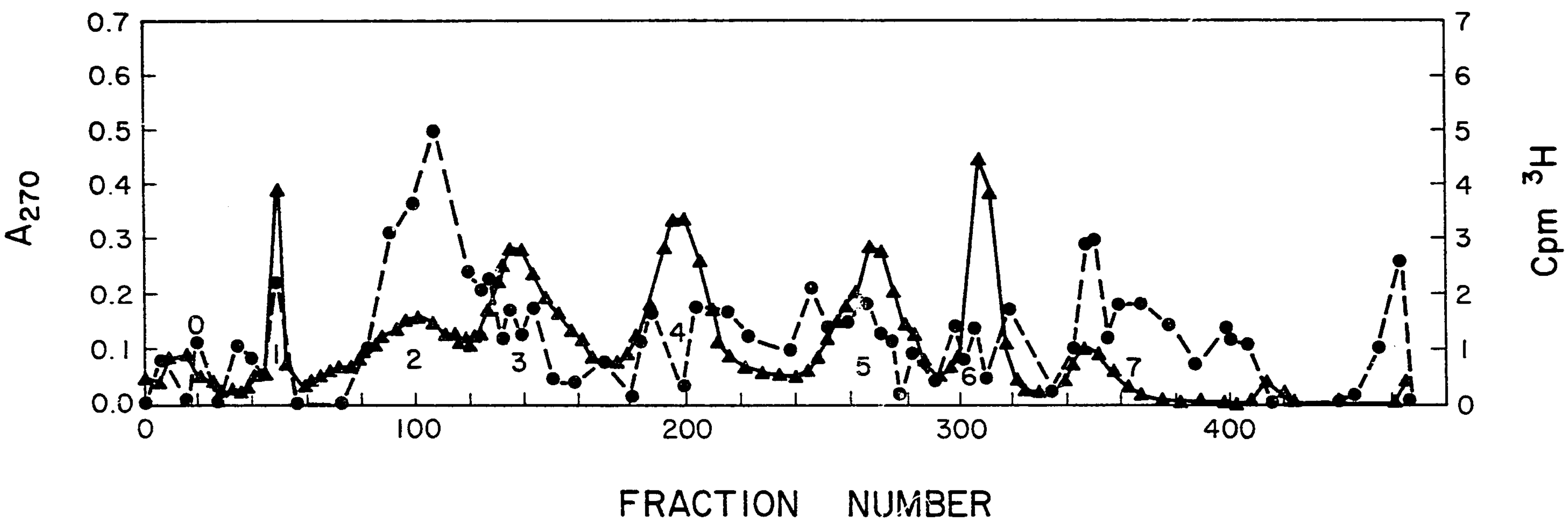
FIGURE 14. DECANUCLEOTIDE CHROMATOGRAPHY FOR TRACT LOCATION

The figure represents the elution pattern of the ^{3}H and ^{14}C labeled pyrimidine decanucleotides from a DEAE Chromedia column. Fractions of 3.5 ml were eluted with a linear gradient of 0 to 0.3 M NaCl in 0.1 M formic acid. The flow rate was 15 ml/hour.

1. (X-X-X) = Absorbance at 270 mμ versus tube number.
2. (●-●-●) = cpm ^{3}H versus tube number.

Peaks of cpm ^{14}C followed those of ^{3}H, but were omitted from the figure for the sake of clarity. Counts were so low that within a given peak they were quite irregular.

Elution is in the order of increasing relative thymidine content.



A270
Cpm 3H
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
7
6
5
4
3
2
1
0
0
100
200
300
400
0
1
2
3
4
5
6
7
FRACTION NUMBER

FIGURE 14

TABLX IX. DECANUCLEOTIDE SUBFRACTIONS

Peak no.	% A_{270}	dpm ^{14}C ($x10^{-2}$)	dpm ^{3}H ($x10^{-3}$)	% dpm ^{14}C	% dpm ^{3}H	$^{3}H/^{14}C$ (dpm)	A_{270}[a] T	A_{270}[a] C	C:T[b]	Identity	Tract[c] no.
0	5.91					131					
1	4.07					58.7	0.17	0.82	7.9:2	C_8T_2	
2	18.0	31.7	41.7	51.7	84.0	14.3	1.36	3.50	6.4:3	C_7T_3	1
3	10.2		3.64		7.33	29.4	3.33	5.84	5.7:4	C_6T_4	
4	19.2					9.26	1.89	2.40	5.3:3	C_5T_5	
5	16.6	3.71	3.17	6.03	6.36	4.18	1.07	0.86	3.9:6	C_4T_6	
6	14.8		1.05		2.31	>70	3.20	0.97	2.8:7	C_3T_7	
7	7.76	24.0		38.9		9.45					
8	1.30					>97					
9	2.16	2.06		3.37		10.1					

[a] Identities are determined from enzymatic hydrolysis followed by chromatography on Dowex 50w. Peaks are pooled and the absorbancy at 270 mμ for each of C (deoxycytosine) and T (thymidine) is taken.

[b] The C:T ratio contains the correction factor of 0.813 x C/T to account for the differences in molar absorptivity at 270 mμ of the two nucleosides.

[c] The number of tracts are obtained by comparison of the ^{3}H counts in each peak with the data of Hall and Sinsheimer (21) on the nona and decanucleotides. Adjacent peaks containing 1,1 and 2 tracts in order of increasing thymidine were found in the nonanucleotides and a single, high cytosine content tract in the decanucleotides. Due to the high amount of unidentified, labeled material present, it was necessary to use these data rather than that of initial tritium label over the whole chromosome.

g. DEAE Chromedia Chromatography of Unadecanucleotides: An absorbance of 192 at 270 mμ was obtained for the length 11 pyrimidine isopliths by adding extra calf thymus carrier oligonucleotides of the same length to the labeled material.

The unadecanucleotides were fractionated and treated in the same manner as the decanucleotides (Fig. 15). Table X, analogous to Table V, reveals the existence of 14 unadecanucleotide subfractions. Most of the absorbance at 270 mμ was located in subfractions 7, 8 and 10. All of the ^3H activity recovered was located in subfraction 2. This accounted for only 1 per cent of the activity placed on the chromedia column. The rest of the counts were so spread out that they did not register in any of the peaks prior to their concentration.

h. DEAE Chromedia Chromatography of Pyrimidine Isopliths of Lengths 12, 13 and 14: Each of the pyrimidine isopliths of length greater than 11 was subfractionated individually, and the subfractions treated in the same manner as the hexanucleotides, except that the gradient of NaCl was extended to 0.5 M for the duodecanucleotides and tridecanucleotides and to 1.0 M for the tetradecanucleotides. In no case was there enough tritium activity in any peak to account for the presence of as much as a single ϕX174 pyrimidine tract, even though the total counts present in each of the isopliths could have represented at least one peak.

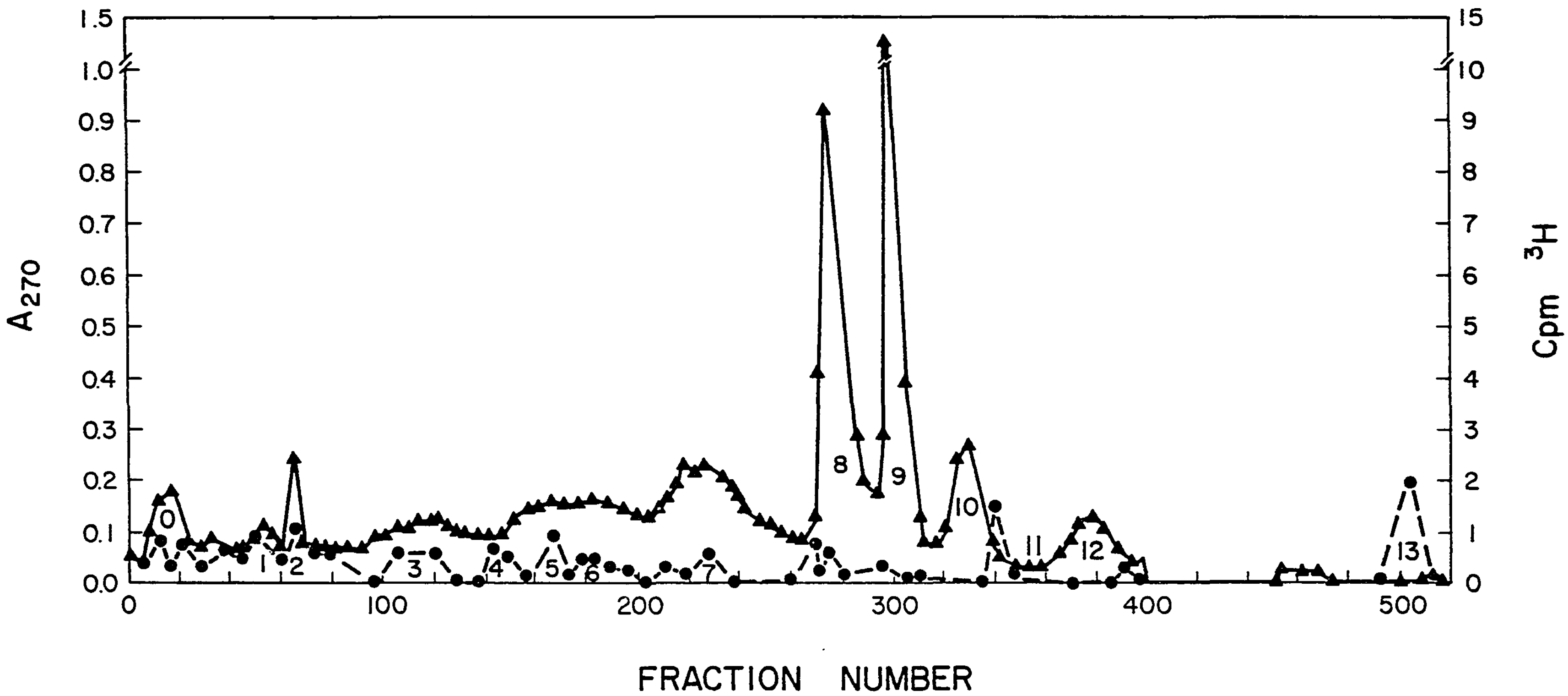
i. Determination of Quantity and Location of ρ^- Pyrimidine Tracts: Tables V through X show that a sizable percentage of both tritium activity and absorbance at 270 mμ that is present in the fractions recovered from the DEAE Sephadex A-25 column exists as unidentified

FIGURE 15. UNADECANUCLEOTIDE CHROMATOGRAPHY FOR TRACT LOCATION

The figure represents the elution pattern of the ^{3}H and ^{14}C labeled pyrimidine unadecanucleotides from a DEAE Chromedia column. Fractions of 3.5 ml were eluted with a linear gradient of 0 to 0.3 M formic acid. The flow rate was 15 ml/hour.

1. (X-X-X) = Absorbance at 270 mμ versus tube number.
2. (●-●-●) = cpm ^{3}H versus tube number.

Counts were so low that they formed no really distinct peaks of either ^{3}H or ^{14}C.



A_{270}
Cpm ^3H
FRACTION NUMBER
0 100 200 300 400 500
0.0 0.1 0.2 0.3 0.4 0.5 0.6 0.7 0.8 0.9 1.0 1.5
0 1 2 3 4 5 6 7 8 9 10 15
0 1 2 3 4 5 6 7 8 9 10 11 12 13

FIGURE 15

TABLE X. UNADECANUCLEOTIDE SUBFRACTIONS

Peak no.	% A_{270}	dpm ^{14}C	dpm ^{3}H	% dpm ^{14}C	% dpm ^{3}H	$^{3}H/^{14}C$ (dpm)
0	4.77					10.2
1	2.62	50.1		15.6		
2	4.17		187		100[a]	1.95
3	5.99					
4	3.06	33.2		10.1		
5	1.46	1.02		2.6		0.71
6	10.9					13.8
7	12.7					>36
8	28.4					2.14
9	6.85					0.26<
10	11.3					0.47<
11	1.63					0.93<
12	4.58					0.47<
13	1.58	23.5		71.7		

[a]Although this amounts to only about 1 per cent of the ^{3}H counts put on the column, the rest of the peaks were too dilute for any of the counts to register.

nonpyrimidine material. Much of this material comes off the column as an initial peak before starting the NaCl gradient, and, in the longer isopliths, much of it is spread throughout the gradient. Due to this, calculations of tritium activity per pyrimidine nucleotide that are derived from the initial activity and absorbance of the oligonucleotide mixture cannot be accurately used to determine the amount of tritium activity expected per tract.

It is known from the results of Hall and Sinsheimer (21), that there are four nonanucleotide tracts and two decanucleotide tracts in the wild type ϕX174 DNA. Of the nonanucleotide tracts, two appear to have identical deoxycytosine-thymidine compositions, although, of course, their sequences may not be identical. Subfractionation of the nonanucleotides in these experiments show that peaks 2 and 3 contain approximately the same amount of tritium activity (see Table VIII). Peak 4 contains approximately double the tritium activity of peaks 2 and 3. These peaks are in the same order and proximity as the ones noted by Hall and Sinsheimer (21). The decanucleotides show only one peak with tritium activity sufficient to account for a single tract (see Table IX). Upon chromatography of a duplicate portion of decanucleotides, extending the NaCl gradient to 1.0 M, the same results were obtained. Further studies showed that although the second decanucleotide tract is not present in the ρ^- mutant DNA, the first tract has the same composition as the one found by Hall and Sinsheimer (21).

Assuming that each of the subfractions, 10-2, 9-2 and 9-3 is equivalent to a single tract, and that the subfraction 9-4 is equivalent to two tracts of identical composition, the average tritium activity for each thymidine and deoxycytosine residue can be calculated. Subfractions 10-2, 9-2 and 9-3 contain 4.17×10^4, 4.65×10^4 and 5.15×10^4 dpm respectively, while subfraction 9-4 contains 8.95×10^4 dpm of tritium. Their respective compositions are C_7T_3, C_6T_3, C_5T_4 and C_4T_5. The ^3H label, as determined from the nucleoside analysis of the first peak from the Sephadex A-25 column, is in the proportion of 31:69, C:T. A weighted average, then, of the tritium activity of each base residue in the ϕX174 chromosome is 3.0×10^3 dpm for each thymidine residue and 6.7×10^3 dpm for each deoxycytosine residue.

From these data it is possible to determine which of the isoplith subfractions of lengths 6, 7 and 8 contain one or more pyrimidine tracts in whole ϕX174 (Table XI). The octanucleotides contain one single tract, one double tract, a half tract which may be a single one, and a subfraction which may consist of either a single or a double tract. The heptanucleotides contain one single and four multiple subfractions for a total of approximately 11 tracts, and the hexanucleotides one single and four multiple subfractions for a total of approximately 20 tracts.

In Table XI is also listed the per cent of each of the ^{14}C, nuclease treated tracts still remaining. This percentage is calculated from the $^3H/^{14}C$ ratio determined for each of the individual tracts. The $^3H/^{14}C$ ratio of the pyrimidine tracts prior to column chromatography was 4.35/1. However, not all of the original ^{14}C

TABLE XI. THE PYRIMIDINE TRACTS IN EACH ISOPLITH SUBFRACTION

Identity[a]	dpm ^3H ($x10^{-4}$)	dpm ^3H[b] per 1 tract calc. ($x10^{-4}$)	Tract no.	$^3H/^{14}C$ Observed	$^3H/^{14}C$[c] Normal	% Tracts Remaining (Psx100)	% Distance[d] from Disc. (fx100)
C_7T_3	4.17	5.59	0.7	14.3	12.1	35.1	56.7
C_6T_3	4.65	4.92	0.9	9.7	8.4	50.6	71.7
C_5T_4	5.15	4.55	1.1	17.7	16.8	25.3	38.7
C_4T_5	8.95	4.18	2.1				
C_4T_4	1.99	3.88	0.5	10.1	10.1	42.1	64.2
C_3T_5	7.25	3.51	2.1				
C_2T_6	4.70	3.14	1.5				
C_1T_7	2.54	2.77	0.9	6.8	10.3	41.2	62.9
C_6T_1	7.60	4.32	1.8				
C_5T_2	10.5	3.95	2.7				
C_4T_3	12.3	3.58	3.4				
C_3T_4	5.18	3.21	1.6				
C_2T_5	2.58	2.84	0.9	5.1	6.3	67.5	83.9
C_4T_2	20.1	3.28	6.1				
C_3T_3	13.8	2.91	4.8				
C_2T_4	14.3	2.54	5.6				
C_1T_5	6.9	2.17	3.2				
T_6	1.4	1.80	0.8	4.3	6.4	66.4	82.9

[a]The determination of identities has already been described in the text and the results listed in Tables V through IX.

[b]The tritium disintegrations per minute are 3.0×10^3 per thymidine residue per position and 6.7×10^3 per deoxycytosine residue per position.

[c]Values are normalized to number of C residues = number of T residues for both the overall chromosome and each tract. See text.

[d]$Ps = e^{-m(1-f)}$. Here m is 2.4 hits/chromosome.

labeled material was present at this time, since an average of 17.2 per cent of it had been removed as mononucleotides by dialysis before the two labeled DNAs were mixed. The $^{3}H/^{14}C$ ratio of the original material, therefore, would have been 3.60/1.

Before the initial $^{3}H/^{14}C$ ratio can be compared with that of an individual tract to determine the percentage of that tract remaining, however, an additional correction is necessary. This is to account for the fact that neither the ^{3}H nor the ^{14}C label is distributed equally between the two pyrimidine bases. Since this unevenness in labeling would have no effect on base ratio comparison if both the initial material and the individual tract under consideration had molar fractions of T = 0.5, C = 0.5, it is only necessary to normalize the $^{3}H/^{14}C$ ratios for each of the tracts considered and for the initial starting material to what it would be for these values of C and T.

The starting material, i.e. intact ϕX174 chromosomes, has a C/T ratio of 0.39/0.61 (13). But the ^{3}H label is distributed so that each C residue contains 69 per cent and each T residue 31 per cent. The ^{14}C label is distributed as T = 0.55 and C = 0.45.

The normal $^{3}H/^{14}C$ ratio, then, can be determined from the following equation:

$$(H/C)_N = (H/C)_O \times (H)_N/(C)_N$$

where $(H/C)_N$ is the normal $^{3}H/^{14}C$ ratio, $(H/C)_O$ the observed $^{3}H/^{14}C$ ratio, and $(H)_N$ and $(C)_N$ the normalized values of ^{3}H and ^{14}C respectively, whether it be for the starting material or for the tract of interest.

For each isotope, the normalized value can be determined from the equation:

$$(I)_N = (1/2)(M_C+M_T)(I_C+I_T)/(M_CI_C+M_TI_T)$$

where M_C and M_T are the molar fractions of each base present (or, in the case of a single tract, one could say the number of residues of that base in the tract) and I_C and I_T are the fractions of the radioactive label present in C and T respectively.

Applying these equations to the intact chromosome, the $^3H/^{14}C$ normalized ratio becomes 4.24/1. The single tracts are calculated individually and listed in Table XI.

From the percentage of each ^{14}C labeled tract remaining after nuclease digestion, it is possible to determine the position of that tract on the ϕX174 chromosome by using the formula:

$$Ps = e^{-m(1-f)}$$

where Ps is the probability of the tract surviving digestion with *E. coli* exonuclease I. This is equivalent to the fraction of the original tracts present after digestion. The quantity m is the average number of hits per chromosome. In order to calculate the average number of hits per chromosome, the above equation is applied to the total of chromosomes present so that it is reduced to:

$$Ps = e^{-m}$$

where Ps is the fraction of rings receiving no hits. Since the 280 µl fraction contained 12.9 per cent remaining rings, and the 500 µl fraction 7 per cent remaining rings, the average value of $m = 2.4$.

Finally, f is the fraction of the distance around the chromosome at which the tract is located. In Table XI, f is designated as the per cent of the distance around the chromosome, with the discontinuity as both 0 and 100 per cent.

Figure 1 indicates two separate tracts, A and B, located at f_1 and f_2 per cent of the distance around the chromosome respectively. The fate of these two tracts upon treatment of the DNA chromosome with pancreatic DNA'ase followed by E. coli exonuclease I is shown. It can be seen that the probability of a given tract's survival is dependent upon its distance from the discontinuity.

6. Comparison of ϕX174 Wild Type and ρ^- Pyrimidine Tracts

a. Separation of Pyrimidine Tracts by Length: The mixture of pyrimidine tracts of wild type and ρ^- ϕX174 DNA was separated routinely by length using DEAE Sephadex A-25. Fifteen peaks were collected, representing isopliths ranging in length from 1 to 15 nucleotides. Table XII lists the per cent absorbance, cpm ^3H and cpm ^{14}C as well as the $^3H/^{14}C$ ratio for each isoplith. Here the ^3H counts represent the ρ^- phage and the ^{14}C counts the wild type phage. The activity of the two labels is presented in terms of cpm rather than dpm as the external standard used for quenching determination was no longer operable.

b. Labeling Determination on Nucleosides of Wild Type and ρ^- ϕX174 DNA: In order to ascertain that the radioactive counts in both the ϕX174 DNAs were located in the pyrimidines rather than the purines, the purines were eluted from the Dowex H^+ column with 0.1 N HCl. Approximately 0.5 per cent of the total ^3H and ^{14}C counts present were

TABLE XII. WILD TYPE-ρ^- PYRIMIDINE ISOPLITHS SEPARATED

Tract[a] no.	% A_{270}	cpm ^3H $x10^{-3}$	cpm ^{14}C $x10^{-2}$	$^3H/^{14}C$	% cpm ^3H	% cpm ^{14}C
1	22.2	140	546	2.56	50.3	69.4
2	22.4	66	94[b]	7.00	23.8	11.9
3	17.0	46	26	1.76	16.5	3.3
4	12.1	11	59	1.82	3.8	7.5
5	7.6	7.2[b]	27	2.66	2.6	3.4
6	7.6	3.1	8.9	3.57	1.1	1.1
7	4.3	4.0	19.5	2.05	1.4	2.5
8	2.8	1.3	7.8	1.67	0.5	1.0
9	1.5	1>	1>			
10	0.86	"	"			
11	0.48	"	"			
12	0.43	"	"			
13	0.31	"	"			
14	0.21	"	"			
15	0.09	"	"			

[a]The tract number is the same as the number of pyrimidines in the isoplith.

[b]The counts in this and the longer isopliths are quite low and contained in large volumes of effluent, making activity totals inaccurate.

located in the purine fraction.

A small aliquot of the pyrimidine tracts was put aside. It was digested with snake venom phosphodiesterase and bacterial alkaline phosphatase, then chromatographed on column of Dowex 50w resin following the procedure used for determination of tract composition. Not enough counts, however, were present to make a good determination of labeling. The nucleosides (peak 1) from the Sephadex A-25 column were therefore used, as before, when the labeling was determined for location of the unique tracts of the ρ^- ϕX174.

The thymidine from tract 1, eluted from the Dowex 50w column, was found to contain 40.8 per cent of the total absorbance at 270 mμ, 5.5 per cent of the ^3H cpm, and 48.4 per cent of the ^{14}C cpm respectively. Calculations analogous to those of section 4c were carried out, with the exception that the corrections were not needed for ^{14}C counts lost on dialysis as there had been no pancreatic DNA'ase-E. coli exonuclease I treatment. The calculations showed the ^3H counts divided between individual thymidine and deoxycytosine residues in a ratio of 7:93 and the ^{14}C counts in a ratio of 55:45.

As in the previous experiment, it was impossible to determine the exact counts of each isotope from the total amount of material present, since much of the 270 mμ absorbing and radioactive material was not identifiable as pyrimidine nucleosides or oligonucleotides.

c. Subfractionation of Decanucleotides: The decanucleotides were subfractionated routinely.

Since it was within this isoplith that the discrepancy between the tracts of the wild type and the ρ^- mutant ϕX174 was first noted, it was these tracts that were subfractionated first. This was to ascertain that the differences observed were actual rather than procedural; Hall and Sinsheimer had subfractionated the wild type decanucleotides by paper chromatography in isopropanol-HCl while column chromatography was used in these experiments (21).

Table XIII lists the results of this subfractionation. It can be seen that fraction 10-4, previously identified as the decanucleotide C_3T_7 (see Table IX) contains one tract each of the wild type and ρ^- phage. Fraction 10-8, however, which is the decanucleotide C_3T_7, contains one wild type tract, but not enough ^3H labeled material for a ρ^- tract.

d. <u>Subfractionation of Heptanucleotides and Octanucleotides</u>: The heptanucleotides and octanucleotides were subfractionated routinely on DEAE Chromedia in order to determine if there was any difference between the ^{14}C labeled wild type tracts and the ^3H labeled ρ^- tracts. The nonanucleotides were not subfractionated as the values for these tracts obtained in the earlier ρ^- experiment were the same as those obtained by Hall and Sinsheimer for the wild type phage (21).

The results of these two subfractionations are listed in Table XIV. Subfractions known to contain 1 or more tracts of ρ^- pyrimidines are listed according to composition. Counts of ^3H and ^{14}C for each of these subfractions are listed, along with the known number of that tract present in ρ^- bacteriophage (see Table XI), and the ^3H cpm and ^{14}C cpm for each deoxycytosine residue as calculated from the total cpm of the tract and the known ^3H:^{14}C label distribution between the pyrimidine residues for each of these isotopes. The calculations are analogous to those described in section 4i. Also listed is a ratio of

TABLE XIII. SUBFRACTIONS OF DECANUCLEOTIDES

Tract no.	% A_{270}	Identity[a]	cpm ^{3}H[b] (Obs.)	cpm ^{14}C[b] (Obs.)	$\frac{\text{cpm } ^{3}H}{\text{Tract}}$[c] (Calc.)	$\frac{\text{cpm } ^{14}C}{\text{Tract}}$[c] (Calc.)	No. w.t. Tracts	No. ρ^- Tracts
0	2.4							
1	0.8							
2	1.0		3	0.8				
3	2.2		7	1.2				
4	7.2	C_7T_3	500	21.8	492	22	1	1
5	14.0	C_6T_4	70	9.5	429	23		
6	21.0	C_5T_5	14	8.9	367	23		
7	19.6	C_4T_6	18	2.5	304	24		
8	15.9	C_3T_7	70	26.1	241	24	1	
9	2.9	C_2T_8	1	3.4	178	25		
10	2.6		13	9.2				
11	2.5		2	8.6				
12	3.8		12	2.4				
13	5.4		7	1.7				

[a]Identities were determined in earlier experiments as described in the text. See Table IX.

[b]The peaks were pooled, concentrated by lyophilization, dialyzed against deionized water and lyophilized to dryness. Half of the total material was counted. Due to the small number of counts counting times of at least 500 minutes were used for each subfraction.

[c]The theoretical cpm per tract for both isotopes was calculated from the data on the hepta and octanucleotides as described in the text.

TABLE XIV. PYRIMIDINE HEPTANUCLEOTIDES AND OCTANUCLEOTIDES

Tract[a]	cpm ^3H	cpm ^{14}C	No. of Tracts[b]	^3H/C res.[c]	^{14}C/C res.[c]	$^3H/^{14}C$[d] for C
C_6T_1	645	25	2	53	1.7	31
C_5T_2	884	47	3	57	2.1	27
C_4T_3	1100	81	4	65	2.6	25
C_3T_4	419	42	2	64	2.6	25
C_2T_5	112	22	1	47	1.8	26
C_4T_4	325	25	1	75	2.8	27
C_3T_5	540	31	2	80	1.8	44
C_2T_6	364	31	2	75	1.7	44
C_1T_7	105	11	1	70	1.1	64

[a]See Tables VI and VII for identification of subfractions.

[b]The tract numbers are determined from Table XI. Decimal values of 0.5 or more are rounded off to the higher number.

[c]The values are calculated from the known counts and compositions of the tracts as well as the labeling ratio for both isotopes in deoxycytidine and thymidine residues. See section 6b for labeling ratios and section 4i for the type of calculations used.

[d]The $^3H/^{14}C$ ratios indicate the ratio of the number of cpm in the deoxycytidines of each pyrimidine tract present in the ρ^- phage to the number of cpm in deoxycytidines of the tract present in the wild type phage. The ratio given is for each deoxycytidine residue. The ratio for each thymidine residue is proportionate to this value.

$^3H/^{14}C$ for each deoxycytosine residue counted. The ratio for each thymidine residue is directly proportionate to this number. This ratio should be constant in all isoplith residues where the number of tracts of wild type pyrimidines is equal to the number of tracts of ρ^- pyrimidines.

DISCUSSION

1. <u>Location of Pyrimidine Tracts in ρ^- ϕX174</u>: The pyrimidine tracts of length 6 and longer were isolated from the ρ^- mutant of bacteriophage ϕX174 and subfractionated according to their relative content of deoxycytosine and thymidine. The location of each of the unique pyrimidine tracts was determined whenever an isoplith subfraction consisted of only that one tract. This was accomplished through a determination of the $^3H/^{14}C$ ratio for each such tract. The double label was used to eliminate any errors in tract localization due to uneven loss of the various pyrimidine tracts during processing. The ^3H label was derived from whole ϕX174 DNA, and the ^{14}C label from ϕX174 DNA which had been digested with pancreatic DNA'ase and <u>E. coli</u> exonuclease I so that the amount of each tract remaining indicated its distance from a discontinuity along the ϕX174 chromosome (see Figure 1). This discontinuity is a small, probably hairpin structured region of DNA, whose integrity seems to be necessary for biological function (42).

Referring once again to Figure 1, it is possible to derive an equation relating the distance of a given tract from the discontinuity to its degree of survival after exonuclease digestion. If the average number of breaks with pancreatic DNA'ase per chromosome is designated as m, and the total length of the chromosome as c, then the average number of hits per unit length of chromosome is m/c. Referring to the arbitrary tract, A, located in position f in the figure, the longest distance between f and the discontinuity can be designated c_1. The shortest distance between these same two points is then $c-c_1$. The average number of hits in this shorter region is then $(m/c)(c-c_1)$, which is the same as $m(1-c_1/c)$. Since the function relating the average number of hits per chromosome to the actual percentage of the total chromosomes present receiving any actual number of hits between zero and infinity, fits an exponential distribution, the chance for no hits occurring in the region $c-c_1$, designated Ps, is equal to $e^{-m(1-c_1/c)}$. The term c_1/c can also be designated as f, the fraction of the distance around the chromosome at which the tract of interest is located, making the relevant equation:

$$Ps = e^{-m(1-f)}$$

where Ps, as the probability of a tract surviving exonuclease digestion, is also equivalent to the fraction of the original tract present after digestion.

This same equation is used to determine the number of hits per chromosome required to obtain an optimal spread of values for the function relating the frequency of occurrence of each tract after digestion to its position. Table XV indicates the values of Ps for different values of f as determined by the number assigned to m. The value of m=2 appears to give the best spread of values for Ps with respect to f, so that the differences in the value of f reflect maximally in the differences in the values of Ps.

Due to the uneven distribution of labels, especially the ^3H label, between the two pyrimidine bases, the actual composition of each of the single tracts is important in determining its location. A possible reason for the large loss of the ^3H label from thymidine as compared with deoxycytosine becomes apparent upon studying the pathways of incorporation of the uridine-^3H into both these nucleosides (Fig. 16).

TABLE XV. DETERMINATION OF OPTIMAL NUMBER OF HITS PER CHROMOSOME

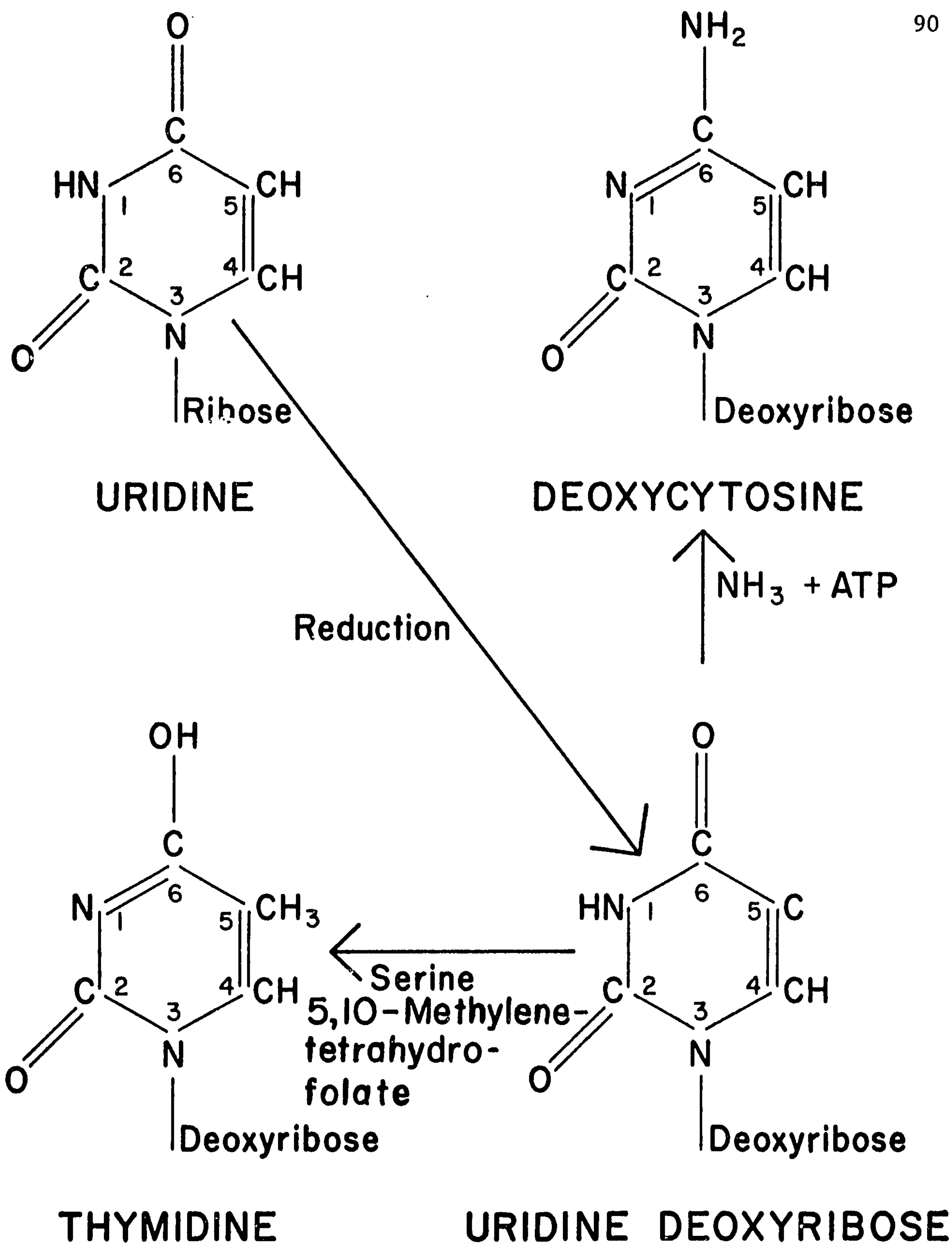
f[a]	Ps[b] m=0.2	Ps m=0.5	Ps m=0.7	Ps m=1.0	Ps m=1.5	Ps m=2.0	Ps m=2.5
0.1	0.835	0.64	0.535	0.405	0.26	0.166	0.067
0.2	0.852	0.67	0.57	0.45	0.30	0.205	0.091
0.3	0.869	0.705	0.613	0.495	0.35	0.248	0.122
0.4	0.885	0.74	0.66	0.55	0.405	0.30	0.165
0.5	0.905	0.775	0.705	0.608	0.472	0.37	0.223
0.6	0.923	0.82	0.75	0.67	0.55	0.45	0.30
0.7	0.942	0.86	0.81	0.74	0.64	0.55	0.405
0.8	0.961	0.90	0.87	0.82	0.74	0.67	0.55
0.9	0.980	0.95	0.93	0.90	0.86	0.82	0.74
1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0

[a] f is the fraction of the distance around the chromosome at which a given tract is located.

[b] The values of Ps are calculated for different values of m for each f in the first column from the formula:

$$Ps = e^{-m(1-f)}$$

where m is the average number of hits per chromosome and Ps is the probability of a tract surviving digestion with exonuclease.



PATHWAY OF INCORPORATION OF URIDINE-^{3}H

FIGURE 16

Only in the formation of thymidine is there loss of nontautomeric hydrogen upon introduction of the $-CH_3$ group into the 5 position of the pyrimidine ring. This is one of only two such hydrogens on the pyrimidine ring, the other being at position 4.

If the ^3H label were evenly distributed between the 4 and 5 position of the uridine, this would perfectly explain the labeling differences of the two nucleosides that was found experimentally in labeling the ρ^- mutant phage. The ^3H label distribution in the wild type phage was much more assymetric, however, A different batch of uridine-^3H had been used for the latter infections and, perhaps, contained uridine labeled primarily in the 5 position. No information as to the position of the labeled ^3H, however, was enclosed by the supplier of the uridine.

Table XVI lists the per cent error in the determinations of location of the unique pyrimidine tracts. These errors range from 3 to 8 per cent and are determined from the counting error for each isotope. The countings errors were determined from the number of counts per minute above background and the total counting time as shown in Figure 17. The effect of the isotope counting errors are applied to the $^3H/^{14}C$ ratio and from there to the error in determining Ps, the per cent of each tract remaining after exonuclease I digestion. The per cent error in Ps is then translated into the per cent error in distance, f, through the formula used to determine f, i.e.

$$Ps = e^{-m(1-f)}$$

TABLE XVI. PER CENT ERROR IN TRACT LOCATION

Tract	% Distance Discont.	% Error[a] ^3H	% Error[a] ^{14}C	% Error in[b] Counting	% Error in[c] Distance
C_7T_3	50.5	4	10	14.2	7.8
C_6T_3	67.6	4	8	11.8	5.6
C_5T_4	34.8	3.8	10	13.8	6.4
C_4T_4	59.2	4	8	11.7	5.8
C_1T_7	57.6	2.7	4	6.5	2.8
C_2T_5	81.5	2.7	3.5	6.0	2.8
T_6	80.4	2.9	3.7	6.4	3.1

[a]Numbers are taken from Figure 17 relating counts per minute and total counting time to per cent error.

[b]The per cent error in counting is taken from the effect of the error in each of ^3H and ^{14}C on the $^3H/^{14}C$ ratio.

[c]Per cent error in distance is taken by using the counting error to determine limits for the per cent of each tract remaining, Ps, and calculating from that the maximum and minimum values of the distance from the discontinuity, i. Errors are expressed in terms of per cent of the entire distance around the chromosome.

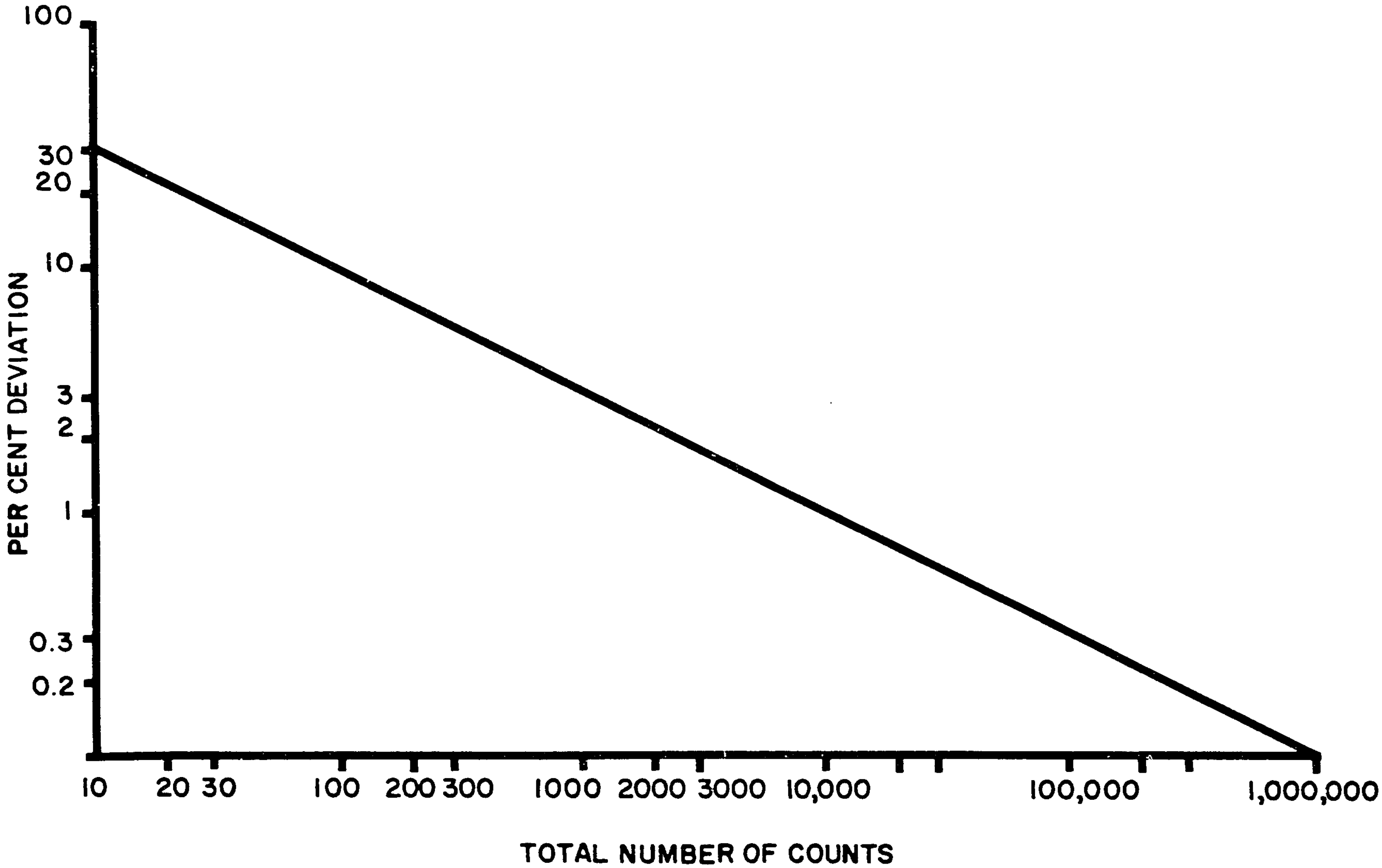


FIGURE 17. STATISTICAL ERRORS

The figure represents the per cent deviation for each total number of counts at a standard deviation of 1.

Figure 18 shows the location of each of the unique pyrimidine tracts on the chromosome of the ρ^- mutant of ϕX174. The figure clearly shows the curious tendency of these longer runs of pyrimidines to cluster in the central section of the chromosome. Their possible locations reach from 35 to 85 per cent of distance from the discontinuity, with two tracts being located at approximately the 83 per cent position and 4 tracts being between 55 and 75 per cent.

It is interesting to speculate upon the question of what special function, if any, the longer pyrimidine tracts might have. Hall and Sinsheimer have already noted the slight deviation from randomness of the pyrimidine tract distribution within both ϕX174 and calf thymus DNA (21). The shorter isopliths, with the exception of the dinucleotides of ϕX174, tend to contain fewer of the pyrimidines than would be expected were their distribution completely random. The ϕX174 isopliths of length 7 and longer contain more of the pyrimidines; this is true to even a greater extent for the calf thymus DNA.

Other workers have also noted a tendency toward an asymmetric grouping together of pyrimidines. Nonrandomness has been noted in the distribution of pyrimidines from the DNA of herring testis and Micrococcus lysodeikticus (43) as well as calf thymus (23). Asymmetries have also been noted in the nearest neighbor frequencies studied for these systems (44). In addition, moderate to large asymmetries have been noted in the distribution of the pyrimidine residues between the two strands of DNA in bacteriophages of Bacillus megaterium (45,46,47), Bacillus subtilis (48,49), Bacillus stearothermophilus (50), satellite DNA from mouse and guinea pig (51,52) as well as T4 phage and 7

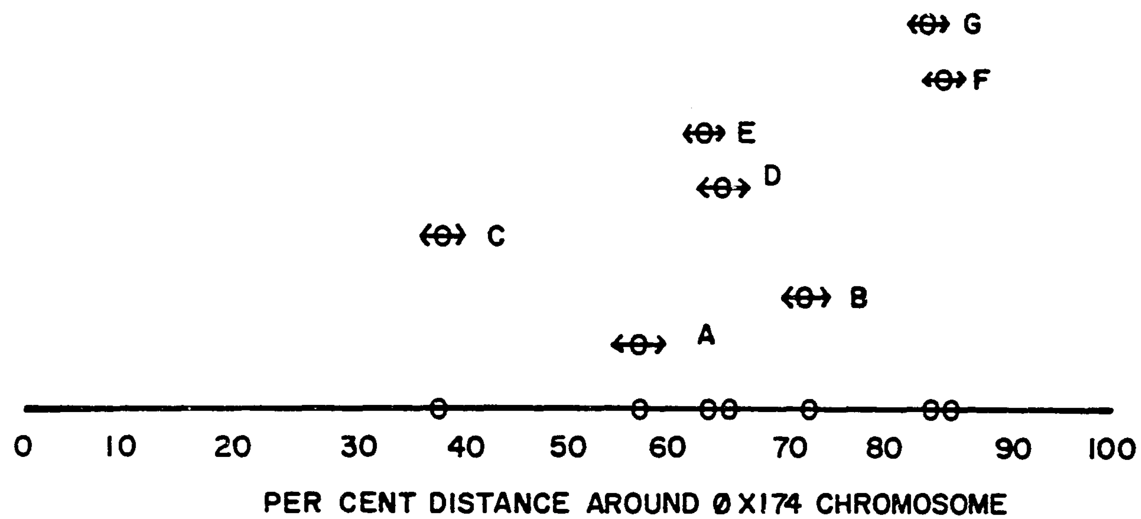


FIGURE 18. LOCATION OF UNIQUE ρ^- MUTANT PYRIMIDINE TRACTS ON ϕX174 CHROMOSOME

This figure shows the location of the pyrimidine tracts which can be uniquely determined on the ϕX174 chromosome. The discontinuity is located both at the 0 and 100 per cent ends of the circular chromosome, here depicted as linear. The open circles are the points that were determined and the arrows represent the limits defined by errors in counting of the double label.

A: C_7T_3 B: C_6T_3 C: C_5T_4 D: C_4T_4 E: C_1T_7 F: C_2T_5 G: T_6

different bacteria including *E. coli* (53).

Findings such as these have lead to speculation about a possible special function for at least some of the longer pyrimidine tracts. The presence of some special purpose long pyrimidine tracts in addition to those already present as adjacent three-pyrimidine code words for amino acids could serve to explain both the excess of these tracts in calf thymus and ϕX174 DNA and the grouping tendencies of pyrimidines in many other systems.

If there are long pyrimidine runs functioning other than as the triplet code for amino acids, the next question would be: what is their function? The small size of the ϕX174 chromosome limits the possible complexity of control functions; the amber and ochre codons, containing purines, seem to adequately account for message termination. There does remain one simple possibility for the function of the longer pyrimidine tracts: chain initiation.

There is another line of evidence which leads to thoughts in these terms. As early as 1958, Stent postulated that a triple stranded DNA-RNA structure serves as an intermediate in DNA directed RNA synthesis (54). Zubay advanced a similar hypothesis in 1962 (55). Reports of the possibility of naturally occurring three stranded DNA-RNA (56) and RNA-RNA complexes have been made since that time although no really good evidence for the existence of an *in vivo* three stranded, base paired, nucleic acid helix has yet been produced.

A number of triple stranded helices consisting of synthetic poly and oligonucleotides have been studied since Felsenfeld and Rich first showed the formation of the three stranded complex $rA:rU_2$ in 1957 (57).

Under the numerously varied conditions used in these experiments, the systems dI_2:dC (58,59) poly rA:rI_2 (60), poly G_2:oligo C (61), oligo rG_2:poly rC and oligo rG:poly rC_2 (62), poly rA:oligo dT_2 (63), and the series of poly dA:rU_2, dA:dT_2, (dA:dT)rU and (rA:dT)rU (64) have been examined. It is the greater stability of the triple helical structures which contain two strands of pyrimidines (65) which indicate their possible role in the initiation of the DNA to RNA transcription process, since it might be expected that messenger RNA production would begin where the three stranded helix is most stable.

It is known that this initiation process takes place at more than one site on the DNA chromosome of small viruses other than ϕX174. Electron microscopy of polyoma and papilloma viruses show that each binds RNA polymerase to a number of sites along the chromosome (66). The polyoma virus, whose DNA is of about the same length as that of the ϕX174 bacteriophage, shows between 6 and 9 binding sites for the RNA polymerase, indicating the existence of approximately one initiation point for each virus genetic function.

Since the initiation of the present study, it has been suggested by Szybalski, et al. (67), perhaps thinking along similar lines, that pyrimidine rich clusters on a number of DNA strands isolated from a variety of bacteria, bacteriophages and higher organisms (68-70) were likely to be specific initiation sites for the single or multi-cistronic messages of these chromosomes. The pyrimidine tracts which he studied, however, were located on the transcribing strand of the DNA. The corresponding areas on the ϕX174 chromosome would consist of purine rich clusters, as it is the complementary strand rather than the parent strand of the ϕX174 which is transcribed into messenger RNA (71).

A most striking aspect of Szybalski's theoretical basis for pyrimidine cluster involvement in messenger RNA initiation, however, is that most of the arguments presented depend merely upon the existence of long runs of either purines or pyrimidines (72). In fact, Szybalski's experimental findings bear this out. On the one hand, for many of the organisms studied, when one of the DNA strands is transcribed either early or exclusively, the same strand was also found to contain almost all the C rich and T rich clusters as determined by preferential binding of that strand with poly G or the opposite strand with poly U (69, 72-74). In fact, studies of coliphage λ and some of its mutants after fragmentation showed C rich clusters in two separated sections of one of its strands and in a medial section on the other strand. Annealing with pulse labeled messenger RNA showed binding of each of the three C rich areas with separate RNA fractions consisting of early and two kinds of late messenger RNA. There were two switchover sites on the λ phage for the change to transcription of the opposite strand (75,76).

On the other hand, it was often the case that neither DNA strand from an organism showed any special affinity for poly G or poly U (69, 73). Or, as was found for both E. coli and ϕX174, both strands of some organisms bind to the synthetic polynucleotides to a similar extent (69,76). In other words, pyrimidine rich regions such as Szybalski describes need not exist on only one strand of the DNA in order for it to be the only strand transcribed, in fact these regions need not exist on either strand at all.

The fact concerning ϕX174 is of special interest here. Szybalski's studies are on broken runs of between 10 and 30 nucleotides in length, consisting primarily of one kind of pyrimidine interspaced with any of the other bases (67). Studies on ϕX174 have shown that there are approximately the same number of unbroken pyrimidine tracts, containing both the pyrimidines, on each of the strands. The present studies have shown six tracts of length 9 or longer on the parent strand of wild type ϕX174; Sedat and Sinsheimer studied the purines on the parent strand, equivalent to the pyrimidines on the transcribing strand, and found sufficient radioactivity to account for 12 tracts of length 9 or longer (19). Three of these tracts seemed to consist of more than 13 adjacent pyrimidine nucleotides on the transcribing strand. No effort was made to subfractionate each of the purine isopliths, however. The work on the pyrimidine isopliths has shown that radioactivity sufficient to account for more than a single tract within an isoplith is often dispersed upon subfractionation. In fact, initial radioactivity data on the longer pyrimidine isopliths prior to subfractionation shown approximately the same number of pyrimidine tracts as purine tracts.

If Figure 18 is examined, it becomes quite obvious that the unique pyrimidine tracts alone cannot account for messenger RNA initiation. At least six complementation groups have been found for ϕX174 (77) which would place the initiation sites about 17 units apart on the scale used in the figure. Perhaps the decanucleotide C_7T_3 and the nonanucleotides C_6T_3 and C_5T_4 are initiation sites. However they only cover the central portion of the chromosome. Initiation sites for genes close to the discontinuity have not been mapped. There are two unmapped nonanucleotides of identical composition whose average position is about the same as that of the two octanucleotides mapped, but they might in actuality be at opposite ends of the chromosome. And perhaps the decanucleotide, missing in the ρ^- mutant of ϕX174 is the sixth initiation site.

However, perhaps Szybalski's criterion of broken runs containing 10 or more nucleotides with a preponderance of one or the other pyrimidines needs to be met. Then, perhaps C_7T_3 is one initiation site and another consists of the oligonucleotides C_2T_5 and T_6 which map so closely together. The other initiator sites could then consist of runs of primarily the same purine on the parent strand. On the other hand, in the absence of any information on the number of cistrons transcribed into a single m-RNA, it may be rash to assume that six initiation sites are required. The cluster of pyrimidine tracts near the center of our map might represent the sole initiation site for the entire genome, and possibilities between these two extremes can be readily imagined.

To be able to eliminate one or both of the suppositions, it would be necessary to obtain additional data: mapping of the missing decanucleotide and all of the unique purine tracts is presently possible. Perhaps at some future time it would also be possible to further subfractionate the multiple tracts on the basis of their actual sequences, which probably do differ, and thus map those tracts as well.

In the meantime, however, both suppositions must be admitted, along with the possibilities that either only pyrimidine clusters of the type described by Szybalski act as initiation sites, or that perhaps initiation depends upon neither runs of purines nor pyrimidines.

2. <u>Decanucleotides and the ρ^- Mutation</u>: The first indication of the existence of a difference between the pyrimidine tracts of the wild type and ρ^- mutant of ϕX174 was noted during the localization of the ρ^- pyrimidine tracts. It became apparent that one of the decanucleotides found in the wild type phage (21) was not present in the ρ^- mutant. Further work with a mixture of phage in which the wild type carried a ^{14}C label and the ρ^- mutant an ^{3}H label confirmed the earlier finding as not being the result of an artifact.

The values for the number of ρ^- mutant pyrimidine nonanucleotides agreed with those of Hall and Sinsheimer for the wild type (21). A satisfactory tract number determination of the hepta and octanucleotides had not been performed, however, so column separations were carried out on the doubly labeled material. Upon inspection of the results in Table XIV, it is interesting to note that the $^{3}H/^{14}C$ ratio for the cytosine residues is unusually high in the octanucleotide C_1T_7. (It should be noted that due to the unevenness in distribution of the ^{3}H label, as discussed in section 1, the overall ratio is not used, because it varies with the amounts of C and T in each tract.) The mean value for this ratio, including the decanucleotide and the unusual octanucleotide, is 35. The value for the tract C_1T_7 differs from the mean by more than two standard deviations. If the value for that octanucleotide is omitted from calculations of the mean and standard deviation, it differs by almost 4 standard deviations. Due to the small sample size, it is impossible to draw any definite conclusions from these values, but they do suggest that there may be one more C_1T_7 tract in the ρ^- mutant than in the wild type ϕX174 bacteriophage. If that is, indeed, the case, then it should be further noted that there is but one such tract in the ρ^- mutant phage, and, therefore, no such tracts in the wild type bacteriophage. This tract had already been mapped in Figure 18, and is very close to the decanucleotide C_7T_3.

The ρ^- mutant of ϕX174 was originally isolated from a preparation of tritium labeled phage by its different plaque appearance from the wild type phage (78). It is likely to be a point mutation. If, then, the C_1T_7 tract is, indeed, unique to the ρ^- mutant of ϕX174 and is not present in the wild type bacteriophage, then it is quite likely that this tract is the product of mutation of the decanucleotide C_3T_7 with one of the cytosines substituted by a purine. This mutation would have to have occurred at a position between a terminal cytosine and the remainder of the pyrimidines. Hall and Sinsheimer had some evidence that the 5' OH terminal end of the decanucleotide tracts consisted of "TC" with unknown sequence (21). If this is so, then the 3' end must contain the CC sequence. The entire sequence of this decanucleotide in the wild type phage, then, would be pCCTTTTTTT(TC) where the order of the final two bases is not known.

If it is the case that the heptanucleotide C_1T_7 was originally the decanucleotide C_3T_7 in the wild type phage, then the hypothesis that long unbroken runs of pyrimidines are necessary for messenger RNA initiation would be greatly weakened. The two decanucleotides do not map far enough apart to consist of two different initiation sites. If they are, instead, part of the same initiation site, it is obvious that such sites need not consist primarily of the same base.

Again, it must be remembered that Szybalski's "C rich" and "T rich" areas are of unknown composition, and, in fact, may not even contain any long pyrimidine tracts. Their identification as being C or T "rich" is merely dependent upon an increased binding affinity to synthetic polynucleotides of complementary bases and need not at all be dependent upon the existence of long runs of a single pyrimidine base.

At least six complementation groups of ϕX174 have been identified (77). No doubt they will be eventually mapped. It will be of interest to see if the location of the C_1T_7 tract of the ρ^- mutant corresponds with the position of the host lysis function of the phage.

In any case, the nature of the mutation involved is of interest. Were mutations to be completely random, the chance of a point mutation occurring in one of the two longest pyrimidine tracts out of 5500 bases is only 0.004. Mutations seem not to be entirely random, however. Fitch, working back to probable code mutations from amino acid changes has found that the transition G→A is the most frequent (79). Interestingly enough, this was not correlated with an increased C→U mutation rate, as reflected in amino acid changes, showing that there is a susceptibility difference between the transcribing and the non-transcribing strand of the DNA. Earlier, however, Freese had proposed that transversions would be more common than transitions (80) and Fitch confirmed this for the overall situation in the case of naturally occurring mutations.

In addition, the bases under consideration do not stand alone. There is an increased susceptibility to mutation of long pyrimidine runs; thymine runs tend to dimerize under ultraviolet light. This being the case, however, it becomes even more striking that longer pyrimidine tracts occur more frequently than would be expected from a merely random distribution. It becomes more likely that they do, after all, have some special function which makes their mutation to shorter tracts unfavorable to the organism. And, unless all the tracts do, indeed, cluster in the center due to some protective function of that area which prevents pyrimidine to purine transversions, thoughts again turn to the possibility of the longer pyrimidine tracts being necessary because they are, after all, sites for RNA initiation.

If the mutation in the pyrimidine decanucleotide is the only difference between the wild type and the ρ^- forms of ϕX174, then one can speculate as to how this change might affect the mutant if the mutation is, indeed, located at a site of chain initiation. The replacement of a pyrimidine base by a purine would weaken the site's effectiveness at chain initiation, perhaps by weakening its binding to RNA polymerase. There would still be sufficient attraction for some binding to take place, however. Transcription and thus translation of the lytic factor would proceed at a slower rate than usual and lysis would be delayed. In a dense _E. coli_ culture, nutrients are limited, and toxic and inhibitory products of metabolism build up. If the cells do not lyse soon enough, these or other factors assure, in most cases, that they do not lyse at all. Perhaps some cofactor needed for lysis is used up, or perhaps some lysis inhibitor reaches sufficiently high levels to be effective. This, of course, is highly speculative, but it is, at least, consistent with what is known about the effects of the ρ^- mutation.

SUMMARY

The location of the unique pyrimidine tracts of the ρ^- mutant of bacteriophage ϕX174 of length six and longer were mapped on the circular viral chromosome. The seven mappable tracts were clustered in the central portion of the chromosome; that is the portion opposite the discontinuity.

The pyrimidine tracts of the wild type and the ρ^- mutant of bacteriophage ϕX174 were compared. A decanucleotide that is present in the wild type phage is absent in the ρ^- mutant. This may have been replaced with an octanucleotide present in the ρ^- mutant which appears not to be present in the wild type phage.

The possible nature of the ρ^- mutation and the possibility of a role for the longer pyrimidine tracts in initiation of RNA transcription are discussed.

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