STUDIES ON NUCLEIC ACIDS OF PLANTS

I. DEOXYRIBONUCLEIC ACID OF PLASTIDS

II. THE USE OF NUCLEOSIDE PHOSPHOTRANSFERASE AND (^{32}P) p-NITROPHENYL PHOSPHATE FOR THE DETERMINATION OF 5'-LINKED TERMINI OF RIBOSOMAL RIBONUCLEIC ACID

Thesis for the Degree of Ph. D.

MICHIGAN STATE UNIVERSITY

VERYL EDWIN BECKER

1967

THESIS

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STUDIES ON NUCLEIC ACIDS OF PLANTS
I. Deoxyribonucleic Acid of Plastids
II. The Use of Nucleoside Phosphotransferase
And (^{32}P)p-Nitrophenyl Phosphate for the Determination
of 5'-Linked Termini of Ribosomal Ribonucleic Acid.

presented by

Veryl Edwin Becker

has been accepted towards fulfillment
of the requirements for

Ph. D. degree in Botany & Plant Pathology

Clifford J. Ballard
Major professor

Date December 7, 1967

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ABSTRACT

STUDIES ON NUCLEIC ACIDS OF PLANTS

I. DEOXYRIBONUCLEIC ACID OF PLASTIDS

II. THE USE OF NUCLEOSIDE PHOSPHOTRANSFERASE AND (^{32}P)p-NITROPHENYL PHOSPHATE FOR THE DETERMINATION OF 5'-LINKED TERMINI OF RIBOSOMAL RIBONUCLEIC ACID

Veryl Edwin Becker

The first part of this thesis documents an investigation aimed at determining whether plastids originate from a common proplastid progenitor. The objective was to compare the properties of the deoxyribonucleic acid (DNA) contained in chloroplasts with that of chromoplasts of a given plant species. Analogous DNA species would indicate an analogous proplastid progenitor. The success of an investigation of this type depends upon the ability to separate different DNA molecules by cesium chloride density gradient centrifugation. Reported are techniques for the isolation of DNA from nuclei, chloroplasts, and chromoplasts from a number of plants and some profiles of DNA as obtained by cesium chloride density gradient centrifugation. Suggestions of two DNA peaks from the plastid preparations are demonstrated, one presumably nuclear and

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the other plastid. Inasmuch as the densities of the DNA components were very similar, separation was not possible and further research on the problem was discontinued.

The second part of the thesis gives the development of a new technique for the detection and identification of minute quantities of nucleosides. The basis of the technique is the transfer of the phosphate moiety from (^{32}P)p-nitrophenyl phosphate to the nucleosides mediated by nucleoside phosphotransferase. (^{32}P)Nucleotides are formed, thereby increasing the sensitivity of detecting the product. A procedure for the partial purification of nucleoside phosphotransferase from carrot leaves was devised whereby the majority of the phosphatase activity was separated from the transferase activity. Purification of the enzyme involved fractionation by acetone, ammonium sulfate and diethylaminoethyl-cellulose column chromatography.

(^{32}P)p-Nitrophenyl phosphate, with high specific activity was synthesized by refluxing (^{32}P)phosphoric acid and phosphorus pentachloride. The (^{32}P)phosphorus oxychloride formed was reacted with p-nitrophenol with the concomitant formation of (^{32}P)p-nitrophenyl phos-

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phoric acid after the hydrolysis of the phosphoryl halides. Incubation of an equimolar mixture of adenosine, cytosine, guanosine, and uridine with nucleoside phosphotransferase and (^{32}P)p-nitrophenyl phosphate resulted in the incorporation of equimolar amounts of radioactivity into the synthesized 5'-nucleotides.

Phosphorylation of the nucleosides obtained from the 5'-linked termini of unfractionated cauliflower ribosomal RNA yielded primarily adenylic and uridylic acid, indicating adenosine and uridine as the terminal nucleosides, with the amount of adenosine being approximately twice that of uridine. Results on the termini of the RNA subunits indicate that the larger subunit is terminated preferentially by adenosine and that adenosine and uridine both terminate the smaller subunit.

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by

Veryl Edwin Becker

A THESIS

Submitted to
Michigan State University
in partial fulfillment of the requirements

DOCTOR OF PHILOSOPHY

Department of Botany and Plant Pathology

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The author wishes to express his sincere appreciation to Dr. C. J. Pollard, his major professor, for his valuable suggestions, encouragement, and assistance, throughout the course of this study. Appreciation is also extended to Drs. J. A. Boezi, N. E. Good, L. W. Mericle, and S. H. Wittwer, members of the guidance committee, and to Dr. A. A. DeHertogh who served as a substitute, for their interest and suggestions. Special consideration is given to Drs. Sipra Guha and E. R. Fowlks for proofreading the manuscript.

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Sincere gratitude is extended to the author's wife, Carol, for her willing assistance, understanding, and sacrifice during this investigation.

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INTRODUCTION

It is generally accepted that chloroplasts contain DNA and that the DNA plays an important role in the replication and development of the plastid. Evidence is accumulating that chloroplasts are autonomous in that the DNA is replicated, ribosomes are present, and protein synthesis occurs in the organelles (see reviews by Kirk, 1966; Granick and Gibor, 1967). Furthermore, from the occurrence of chloroplast to chromoplast transitions, maternal inheritance of plastids, and the restriction of starch synthesis to plastids, it is possible to deduce that in some cases plastids are ontogenetically related (Frey-Wyssling and Mühlethaler, 1965).

The primary objective of the first part of this thesis was to determine the origin of plastid types. Specifically, it was proposed to test the hypothesis that chloroplasts and chromoplasts from a given plant species have their origin in a common proplastid pro-

genitor. It was conceived that this could be accomplished by 1) a comparison of the properties of DNA isolated from chloroplasts to that isolated from chromoplasts and 2) a comparison of the complement of enzymes contained in these organelles. Since chloroplastic preparations generally contain nuclear DNA, however, the success of the comparison of the respective DNA species depended upon the ability to separate contaminating nuclear DNA from plastid DNA. Since Sager and Ishida (1963) had reported definite separation of plastid and nuclear DNA on a cesium chloride density gradient, this experimental approach appeared to be justified. Although this is the best method currently available, it was not possible to distinctly separate the two DNA moieties, as will be demonstrated later. The second approach to the problem involved an investigation of the enzymes restricted to chloroplasts (Smillie, 1963). Alkaline fructose-1,6-diphosphate-6-phosphatase was investigated but since very little enzymatic activity of any kind could be detected in chromoplast preparations this approach was abandoned.

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the basic problem proposed, the writer feels that the work should be documented because useful techniques for the isolation of DNA were devised and it represented an important part of his training.

The second part of the thesis concerns the use of nucleoside phosphotransferase and (^{32}P)p-nitrophenyl phosphate for the detection of minute quantities of nucleosides. Nucleoside phosphotransferase will transfer the phosphate constituent from phosphate donors such as phenyl phosphate and p-nitrophenyl phosphate to a nucleoside; thus yielding a nucleotide (Brawerman and Chargaff, 1954; Katagiri, Yamada, Mitsugi, and Tsunoda, 1964).

Since the objective was to detect minute quantities of nucleosides, it was necessary to use (^{32}P)p-nitrophenyl phosphate of high specific activity as the phosphate donor. Consequently, a method for its synthesis had to be devised since all previous syntheses involved large quantities of reactants (Axelrod, 1948; Desjobert, 1963). As a further ramification of the technique the efficacy of the use of the phosphate ester in the determination of the 5'-linked nucleosides

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of ribosomal RNA released by alkaline hydrolysis was explored. Since alkaline hydrolysis of an RNA molecule yields a mixture of 3'-nucleotides from the center of the chain (Brown and Todd, 1952), a nucleoside-2'(3'), 5'-diphosphate from the 3'-linked end, and a nucleoside from the 5'-linked end (Lane and Tamaoki, 1967), this part of the problem involves the separation of the nucleosides from the hydrolysis mixture followed by the phosphorylation of the nucleosides. Essentially then, the second section gives the isolation and partial purification of nucleoside phosphotransferase, an improved method for the synthesis of (^{32}P)p-nitrophenyl phosphate, and the utilization of these reagents in identifying the 5'-linked terminal groups of ribosomal RNA.

PART I

DEOXYRIBONUCLEIC ACID OF PLASTIDS

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LITERATURE REVIEW

Morphology, Ontogeny, and Genetics of Plastids

Plastids, when mature, may be divided into three general categories: chloroplasts, chromoplasts, and leucoplasts (Frey-Wyssling and Mühlethaler, 1965). Those plastids which possess chlorophyll and carry on photosynthesis are chloroplasts, while those which vary in color from yellow to red and contain carotenoids but little chlorophyll are chromoplasts. Leucoplasts are colorless plastids whose main function appears to be the storage of starch, oil, or protein.

Several proposals are in vogue for the origin of plastids. The oldest and still the most popular is that of Schimper's (1885), who simply stated that all plastids arise from pre-existing plastids. An example of chloroplast division is shown by the unicellular alga, Chromulina pulsilla where the chloroplast undergoes division prior to the division of the cell

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(Manton, 1959). Also, Green (1964) has published elegant time-lapse photographs of the dividing chloroplasts of Nitella.

It has been suggested that plastids may arise de novo since from electron micrographs of very thin sections it is possible to detect what appears to be various stages of plastid development, the earliest being merely a group of macromolecules. As a further extension of this proposal it was suggested that these small bodies may give rise to both mitochondria and plastids (Badenhuizen, 1962; Weier, 1963; Vesk, Mercer, and Possingham, 1965).

Mühlethaler and Bell (1962) proposed that plastids arise from the nucleus. They presented evidence which indicates that in the ovum of the eagle fern (Pteridium aquilinum) the entire cytoplasmic organelle system breaks down just prior to fertilization and at a later time structures that appear to be new plastid and mitochondrial initials are formed around the nucleus by evaginations of the nuclear membrane. It should be noted that on electron micrographs it is difficult to determine whether organelles are completely disinte-

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grated, especially when their external membrane is still present. Also, questions can be raised as to whether evaginations of an organelle membrane really give rise to more organelles or if they are distortions of the same organelle.

Although there are definite disagreements among investigators with regard to the ultimate origin of plastids, it is conceded that an entity called a proplastid does divide and gives rise to all plastid types and that mature chloroplasts can undergo division (Granick, 1961; Frey-Wyssling and Mühlethaler, 1965; Ben-Shaul and Klein, 1965).

Cytological evidence, obtained by both the light and electron microscope, reveals that interdifferentiation among plastid types exists (Granick, 1961; Frey-Wyssling and Mühlethaler, 1965). Several workers have presented evidence that chloroplasts differentiate into chromoplasts by the gradual disappearance of chlorophyll and internal structure with the concurrent replacement by lipids either in the form of droplets or a fibrillar network (Zurzycki, 1954; Frey-Wyssling and Kreutzer, 1958; Lance-Nougarède, 1960; Camefort,

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1964; and Thomson, 1966). Not only is the chloroplast to chromoplast transition plausible, but it is conceivable that a transition can also occur by which leucoplasts can change to either chloroplasts or chromoplasts (Granick, 1961). Although interdifferentiation among plastids may exist, the chromoplast appears to be the final state of differentiation.

The area of plastid genetics has recently been reviewed (Gibor and Granick, 1964; Hageman, 1965; Wettstein and Eriksson, 1965). From the information documented in the reviews it is apparent that plastids are primarily inherited maternally, that a large number of genes are present in the chloroplasts, and that definite nuclear-plastid genome interrelations exist. Studies on the chloroplasts of Euglena indicate that two general types of plastid genes are present, those that function in the differentiation of the proplastid to the mature plastid and those that function in the multiplication of the plastid (Granick and Gibor, 1967). Both the synthesis of chlorophylls and carotenoids appear to be under the control of the nucleus since some of the steps in their biosynthesis are affected

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by a mutation in the nuclear genome (Kirk, 1966). It is realized that there are other aspects of the inheritance system of plastids but a complete review of the area is beyond the scope of this thesis.

Cytochemical and Biochemical Investigations of Nucleic Acids in Plastids

The early cytochemical investigations on the detection of nucleic acids in plastids relied on the use of direct staining, direct staining in combination with ribonuclease, and acid extractions of thin sections (Chiba, 1951; Metzner, 1952; Spiekermann, 1957). These results indicated that both RNA and DNA were present but that RNA was present in much larger quantities.

Further research utilizing the above technique plus deoxyribonuclease, the electron microscope, and the incorporation of radioactive nucleic acid precursors again confirmed the presence of RNA, this time in the form of ribosomes (Jacobson, Swift, and Bogorad, 1963; Brown and Gunning, 1966) and the presence of long DNA filaments (Ris and Plaut, 1962; Kislev, Swift, and Bogorad, 1965, 1966; and Brown and Gunning, 1966).

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Another approach to the problem of the presence of DNA in chloroplasts involves the biochemical analysis of chloroplastic preparations prepared by aqueous or non-aqueous techniques (Granick and Gibor, 1967). The chloroplastic preparations can either be analyzed directly (Biswas and Biswas, 1963; Kirk, 1963; Pollard, 1964a; Ruppel and Wyk, 1965) or DNA can be centrifuged in a cesium chloride gradient, thus allowing for the detection of chloroplastic DNA in the presence of nuclear DNA (Chun, Vaughan, and Rich, 1963; Sager and Ishida, 1963; Brawerman and Eisenstadt, 1964; Ray and Hanawalt, 1964; Edelman, Schiff, and Epstein, 1965; Kislev, Swift, and Bogorad, 1965; Shipp, Kieras, and Haselkorn, 1965; Suyama and Bonner, 1966; Green and Gordon, 1967). In cesium chloride density gradient centrifugation an equilibrium density gradient is established by the centrifugal field. Concurrent with the establishment of the gradient, the DNA present will migrate to a position in the tube where the density of the cesium chloride is equal to its buoyant density. Once complete equilibrium is acheived, the gradient is photographed in the case of the analytical centrifuge

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or in the case of the preparative centrifuge the tube is punctured, fractions are collected dropwise, and the absorbance at 260 mμ for each fraction is determined. The results of the density gradient centrifugation of DNA extracted from chloroplastic preparations usually show a major nuclear band due to nuclear contamination plus a satellite band which is attributed to chloroplastic DNA. Since a correlation exists between the purity of the chloroplast preparation and the size of the satellite band the attribution seems justified.

The most convincing evidence regarding the presence of DNA in chloroplasts stems from studies with a unicellular alga, Acetabularia. The cell is unique in that the nucleus is located in the rhizoid and thus can easily be removed from the chloroplastic part of the plant. Gibor and Izawa (1963) have purified chloroplasts from enucleated, bacteria free, Acetabularia cells and have found them to contain DNA. Schweiger and Berger (1964) indicated that RNA synthesis which appears to be DNA dependent takes place in chloroplast preparations from enucleated Acetabularia cells.

Although numerous reports are present in the literature concerning the presence of DNA in chloroplasts, the presence of DNA in other types of plastids has not been investigated either by genetical, cytochemical, or biochemical techniques. Suyama and Bonner (1966) have detected a satellite band on a densitometer tracing from a photograph of a cesium chloride gradient of DNA isolated from turnip roots, which was not equal in density to either the nuclear, chloroplastic or nitochondrial DNA. Swift (1965) has published a densitometer tracing of DNA extracted from cytoplasmic particles of Swiss chard roots, indicating that a satellite band is present with density equal to that of the DNA obtained from the chloroplasts. Presumably, this DNA had its origin in proplastids or leucoplasts. Swift's results are questionable since he attributed the largest peak represented on the tracing to a contaminating microorganism.

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MATERIALS AND METHODS

Experimental Materials

Cesium chloride (99.9 percent purity) was purchased from Gallard-Schlesinger Chemical Manufacturing Corporation, Carle Place, New York; *Clostridium perfringens* DNA from Worthington Biochemical Corporation, Freehold, New Jersey; trypticase from Baltimore Biological Laboratory, Baltimore, Maryland; and salmon sperm DNA and ribonuclease from Sigma Chemical Company, St. Louis, Missouri. *Pseudomonas putida* DNA was a gift from Dr. John A. Boezi of this University. All other chemicals were reagent grade and are commonly available at chemical supply houses.

Watermelon (*Citrullus vulgaris*, Schrad.), tomato *Lycopersicon esculentum*, Mill., var. *grandifolium*, Bailey), and pepper (*Capsicum frutescens*, L., var. *grossum*, Bailey) plants were grown to maturity in the field. Spinach (*Spinacia oleracea*, L.) was purchased from local commercial sources. Leaves and fruits were

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Escherichia coli K12 was kindly provided as a slant culture inoculum by Dr. Harold L. Sadoff of this University. A portion of the E. coli from the slant culture was transferred asceptically to 100 ml of sterile 2 percent trypticase medium and incubated for 5 hours at 37° on a mechanical shaker. The entire 100 ml inoculum was transferred to 11 liters of sterile 2 percent trypticase medium and incubated with aeration for 11 hours at 37°. The E. coli cells were collected by centrifugation.

Preparation of Chloroplasts and Chromoplasts

All chloroplasts and pepper chromoplasts were prepared according to the method of Chun, Vaughan, and Rich (1963) with some modification. All plant preparations were kept in the cold (0 to 5°) or as close to this temperature as possible unless otherwise designated. The washed and chilled leaves were shreded by hand and ground in a large mortar and pestle with sand. Sufficient homogenizing medium was added to obtain a slurry. The homogenizing medium for pepper chloroplastic prepa-

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ration was 0.4 M sucrose, 0.5 M tris (hydroxymethyl) aminomethane (tris), 0.01 M NaCl, 0.005 M ethylenediaminetetraacetic acid (EDTA), pH 8.0 while the medium for watermelon, spinach, and tomato preparations was 0.4 M sucrose, 0.02 M tris, 0.01 M NaCl, 0.005 M EDTA, pH 8.0. Each preparation was then filtered through four layers of cheesecloth and two facial tissues. The nuclear pellet was obtained by centrifugation of homogenates from pepper leaves for 15 minutes at 160 x g, from watermelon leaves for 10 minutes at 250 x g, and from spinach and tomato leaves for 10 minutes at 400 x g. Further centrifugation for 10 minutes at 1000 x g sedimented the chloroplastic pellet.

Pepper chromoplasts were prepared from mature red fruit pieces by homogenization in a Waring blendor at 1/2 line voltage for 90 seconds using 0.3 M sucrose, 0.5 M tris, 0.005 M EDTA, pH 8.0, as the homogenizing medium. The homogenate was filtered through four layers of cheesecloth and two facial tissues. Successive centrifugations at 400 x g and 1000 x g resulted in nuclear and chromoplastic pellets, respectively.

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they sedimented at greatly reduced centrifugal forces. Consequently, these chromoplasts were prepared by a modification of the method proposed by Strauss (1956). Essentially, the red portion of fruits was removed and crushed by hand with a potato masher. The suspension obtained was filtered through four layers of cheesecloth and two facial tissues. Centrifugation at 4,000 x g for 10 minutes resulted in a mixed nuclear and chromoplastic pellet. The pellet was resuspended in a 30 percent sucrose solution and centrifuged at 15,000 x g for 20 minutes. The chromoplasts floating on top of the solution were carefully removed with a spatula. Nuclei and nuclear fragments along with some chromoplasts were pelleted in the bottom of the centrifuge tube.

In all plastid preparation contamination by nuclei or nuclear fragments, as assessed staining with aceto-carmine, was restricted to a few small nuclear fragments. The majority of the chloroplasts and practically all of the chromoplasts, as viewed under the light microscope, remained intact, even in the case of pepper preparations where it was necessary to raise the concen-

tration of tris buffer to 0.5 M in order to prevent the coagulation of the homogenate caused by the highly acid pepper tissue. Chromoplasts as isolated above due to their crystalline structure, tended to be stable in a wide range of osmotic concentrations.

DNA Preparation

Escherichia coli DNA was prepared according to the method proposed by Marmur (1961), while plastid DNA was prepared by a modification of the same method. Essentially, chloroplast and chromoplast preparations were suspended in 0.15 M NaCl, 0.1 M EDTA, pH 8.0 (approximately 30 ml). To the suspension was added sodium dodecyl sulfate to a final concentration of 1 percent from a stock solution of 25 percent and $NaClO_4$ to 1 M from a stock solution of 5 M, followed by an equal volume of chloroform-isoamyl alcohol (24:1). The mixture was placed on a mechanical shaker for 30 minutes. Following separation by centrifugation, the upper aqueous phase was removed with a pipet, taking care not to obtain any of the denatured protein at the interphase or any of the lower non-aqueous phase. The aqueous phase was again

combined with an equal volume of chloroform-isoamyl alcohol and the preparation deproteinized by shaking, this time by hand for 10 minutes. Upon separation of the two phases and removal of the upper aqueous phase, the deproteinization procedure was repeated until denatured protein no longer collected at the interphase. The aqueous phase was then dialyzed overnight against 0.15 M NaCl, 0.015 M citrate, pH 7.0 (saline citrate) to reduce the high perchlorate concentration (see Chun, Vaughan, and Rich, 1963). Two volumes of ethanol were added to the dialysate and after thorough cooling of the preparation the nucleic acids were collected by centriguation. The nucleic acid pellet was resuspended in 5 to 10 ml of 0.1 M sodium acetate, pH 6.0, ribonuclease (aqueous solution previously heated at 85^{o} for 10 minutes to destroy deoxyribonuclease activity) was added to a concentration of 50 μg/ml, and the mixture was incubated for 1 hour at 37^{o}. Solid NaCl was added to a concentration of 3 M, the preparation was centrifuged to remove the small amount of precipitate. This was followed by the addition of 0.4 volume of isopropanol (Kirby, 1964). After cooling, DNA was collected by centrifugation. If all the polyphenolic brown material

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was not removed, the NaCl-isopropanol step was repeated. The DNA thus obtained was dissolved in saline citrate and again deproteinized with chloroform-isoamyl alcohol until no protein was visible at the interphase. The DNA was collected from the upper aqueous phase by precipitation with 2 volumes of ethanol followed by centrifugation. In order to test for DNA purity the absorbance at 260 mμ and 280 mμ was taken routinely. DNA yield was determined on the basis of 24 optical density units at 260 mμ being equivalent to 1 mg of DNA. In some experiments, DNA content was also determined by the indole test (Ceriotti, 1952).

The greatest difficulty encountered in the isolation of DNA was the presence of a dark brown color in the preparations, apparently caused by the presence of polyphenolic compounds. These polyphenolic compounds exhibited solubility properties similar to nucleic acids and gave extraneous results when analyzing for DNA content and purity. A modification of the procedure first developed by Kirby (1964) for the removal of carbohydrates from E. coli nucleic acid preparations was utilized, as mentioned above, to remove the polyphenolic

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compounds. The procedure involved dissolving the nucleic acids in 0.1 M sodium acetate, pH 6.0 followed by the addition of solid NaCl to a concentration of 3 M. Centrifugation at 9,000 x g resulted in a pellet which was determined by UV absorption not to contain a significant amount of DNA. With the addition of 0.4 volumes of isopropanol and thorough cooling of the solution on ice, the DNA precipitated while the brown color remained in solution. If the pellet was brown in color the NaCl-isopropanol step was repeated.

DNA preparations routinely gave a ratio of absorbance at 260 mμ to 280 mμ of 1.7 to 2.0. Preparations with a ratio less than 1.7 were either discarded or purified further until this ratio was achieved. Yields of DNA varied greatly depending upon the plastid preparation with an average of approximately 200 μg/kg of leaf tissue while fruit tissue yielded lower values, particularly in the case of watermelons.

Cesium Chloride Gradient Preparation, Centrifugation, and Collection

Prior to centrifugation a gradient of CsCl was prepared by means of a double chamber mixing device

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(Martin and Ames, 1961). The density of the CsCl solution in the first chamber was 1.84 g/cm^3 and in the second chamber, 1.60 g/cm^3. To 4.1 ml of prepared gradient was added 0.3 ml of CsCl saline citrate (ρ = 1.57 g/cm^3) solution containing the appropriate DNA preparation. Finally, 0.3 ml of paraffin oil was layered on top of the CsCl solution.

The gradients were centrifuged at 31,000, 35,000, and 37,000 rpm for periods of 52 to 72 hours at 20° in a SW 39L rotor. The centrifugation was stopped without braking, and the gradient was collected through a number 21 syringe needle mounted through a rubber stopper and sealed with four Parafilm disks. To each fraction was added 1.0 ml of saline citrate and the absorbance at 260 mμ was determined.

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RESULTS AND DISCUSSION

DNA preparations, dissolved in 0.3 ml of buffered CsCl solution, were layered on performed CsCl gradients and centrifuged at 31,000 to 37,000 rpm for periods ranging from 52 to 72 hours. In order to test the feasibility of the density gradient technique it was necessary to determine whether 1) a linear CsCl density gradient was established by centrifugation, 2) the DNA was distributed according to a gaussian curve, and 3) DNA species of different densities could be separated. The establishment of a linear density gradient and the symmetrical distribution of the DNA after centrifugation is illustrated by Figure 1. The CsCl solution containing 50 μg of *E. coli* DNA was centrifuged for 52 hours at 31,000 rpm. The density of the CsCl gradient as determined by a hand refractometer is given in arbitrary units since the measurements were taken in percent sucrose and were not converted to density or concentration of CsCl. Partial separation

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Absorbance 260 mμ
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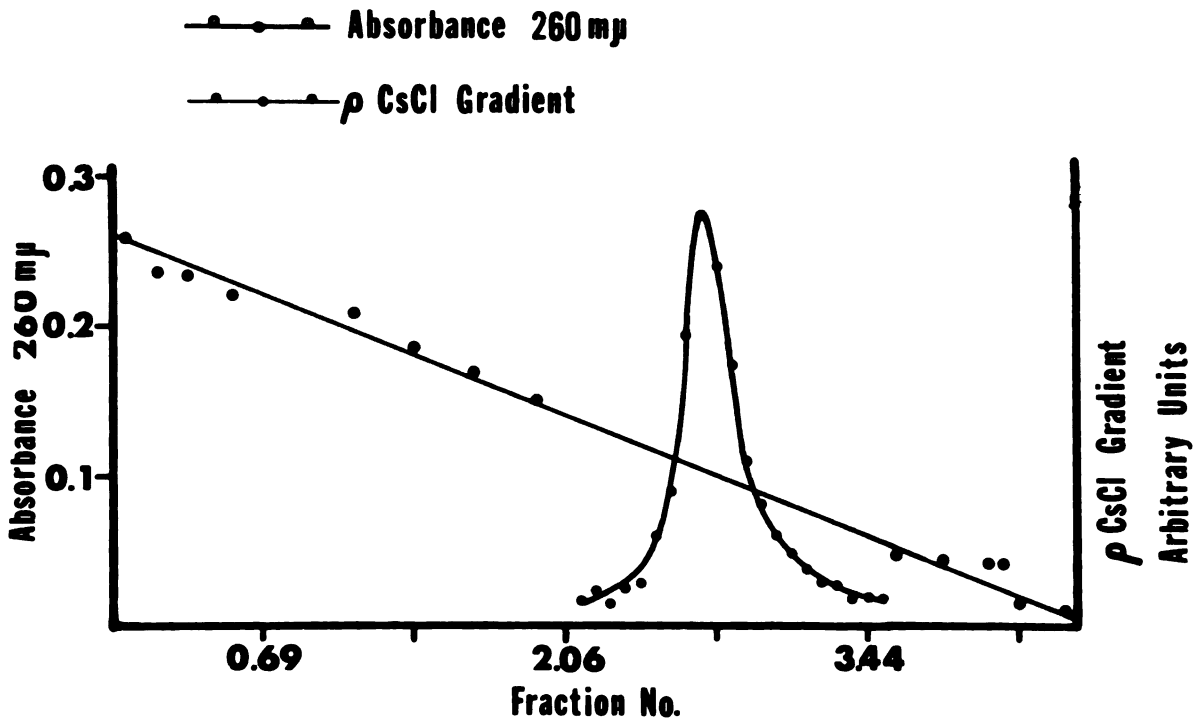

Figure 1: CsCl density gradient profile of E. coli DNA. Centrifugation of the DNA (50 μg) was at 31,000 rpm for 52 hours at 20°. The DNA profile (open circles) and the gradient established (solid circles) are illustrated.

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of a mixture of DNA species from Pseudomonas putida and salmon sperm (50 μg each) was achieved (Figure 2) under identical conditions to those mentioned above for the centrifugation of E. coli DNA. The buoyant densities reported for Pseudomonas putida and salmon sperm DNA are 1.721 and 1.703 g/cm^3, respectively (Schildkraut, Marmur, and Doty, 1962). In order to determine the degree of separation achieved between DNA species of different densities at 35,000 rpm, a mixture of Clostridium perfringens DNA and E. coli DNA (150 μg each) with buoyant densities of 1.691 and 1.710 g/cm^3, respectively (Schildkraut, Marmur, and Doty, 1962) was centrifuged. As illustrated in Figure 3, partial separation of the DNA species was attained.

Although not indicated in Figures 1 and 2, centrifugation at 31,000 rpm tended to give irregular and unpredictable gradient profiles both with plant and bacterial DNA. This effect could conceivably be attributed to an increased effect of diffusion at reduced centrifugal forces. The DNA profiles obtained by centrifugation of tomato (300 μg) and spinach chloroplastic DNA (120 μg) at 37,000 rpm for 72 hours are shown in

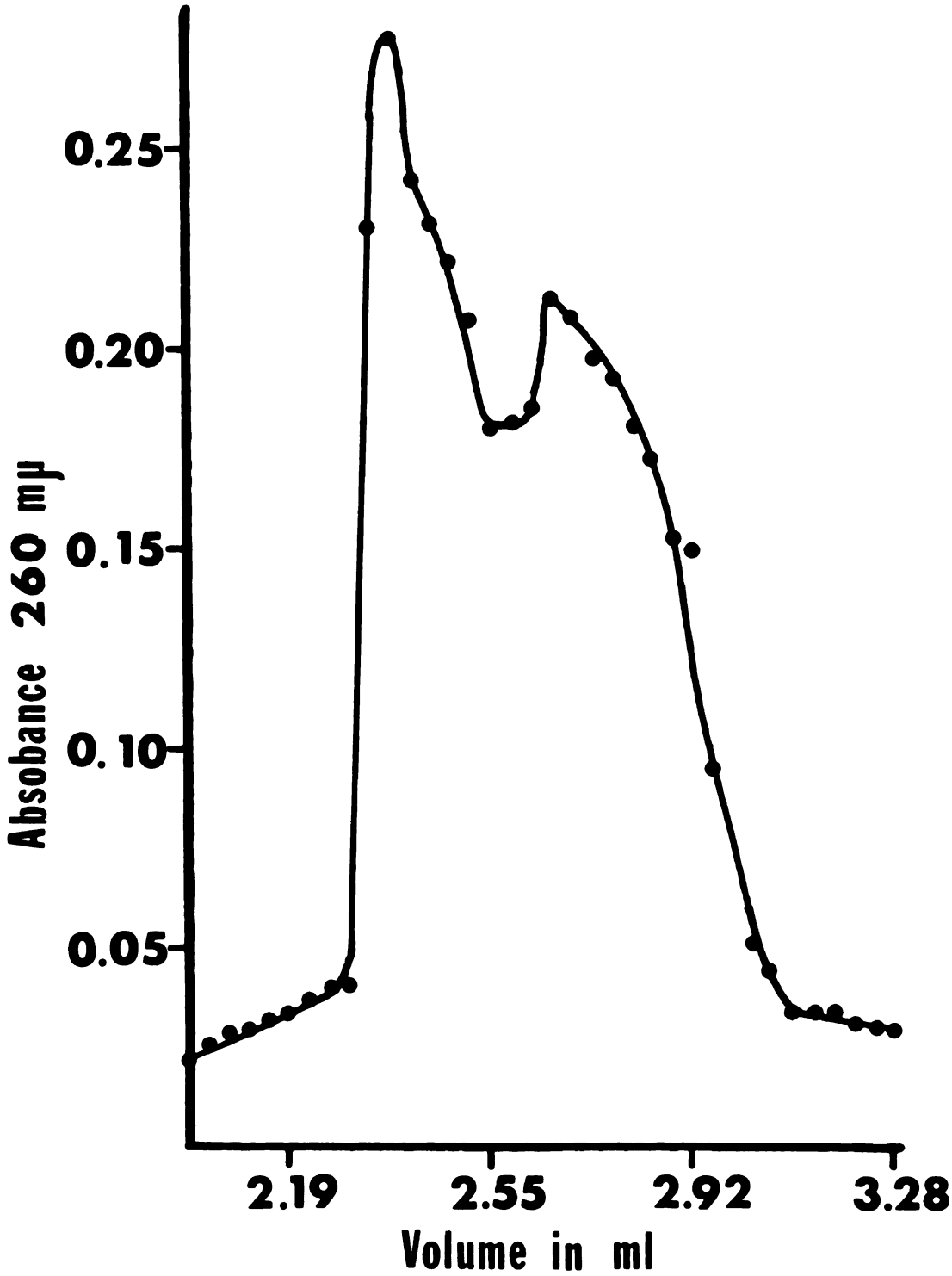

Figure 2: CsCl density gradient profile of a mixture of Pseudomonas putida and salmon sperm DNA. Centrifugation of the DNA species (50 μg each) was at 31,000 rpm for 52 hours at 20°.

Absorbance 260 mμ

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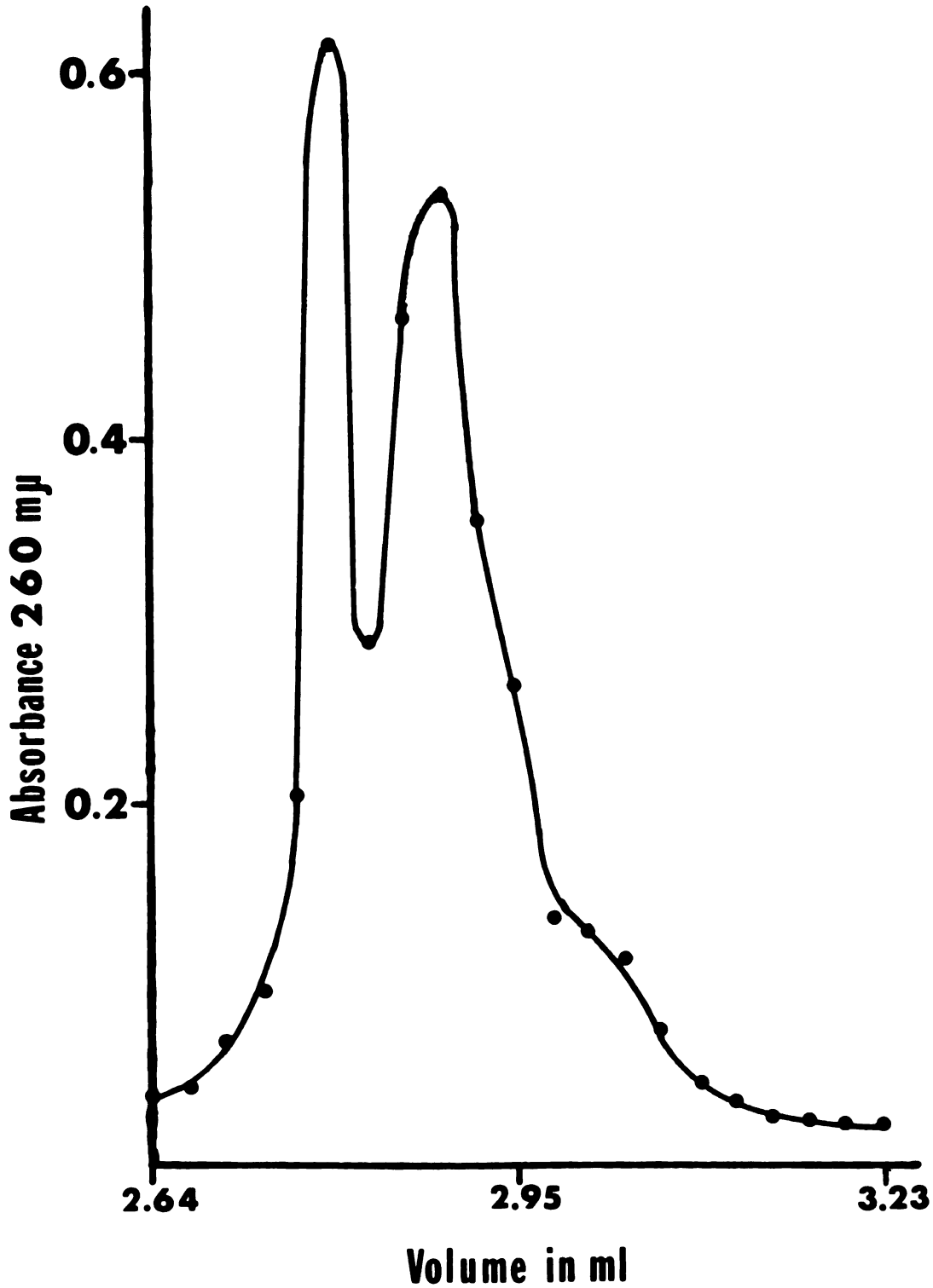

Figure 3: CsCl density gradient profile of a mixture of Clostridium perfringens and E. coli DNA. Centrifugation of the DNA species (150 μg each) was at 35,000 rpm for 72 hours at 20°.

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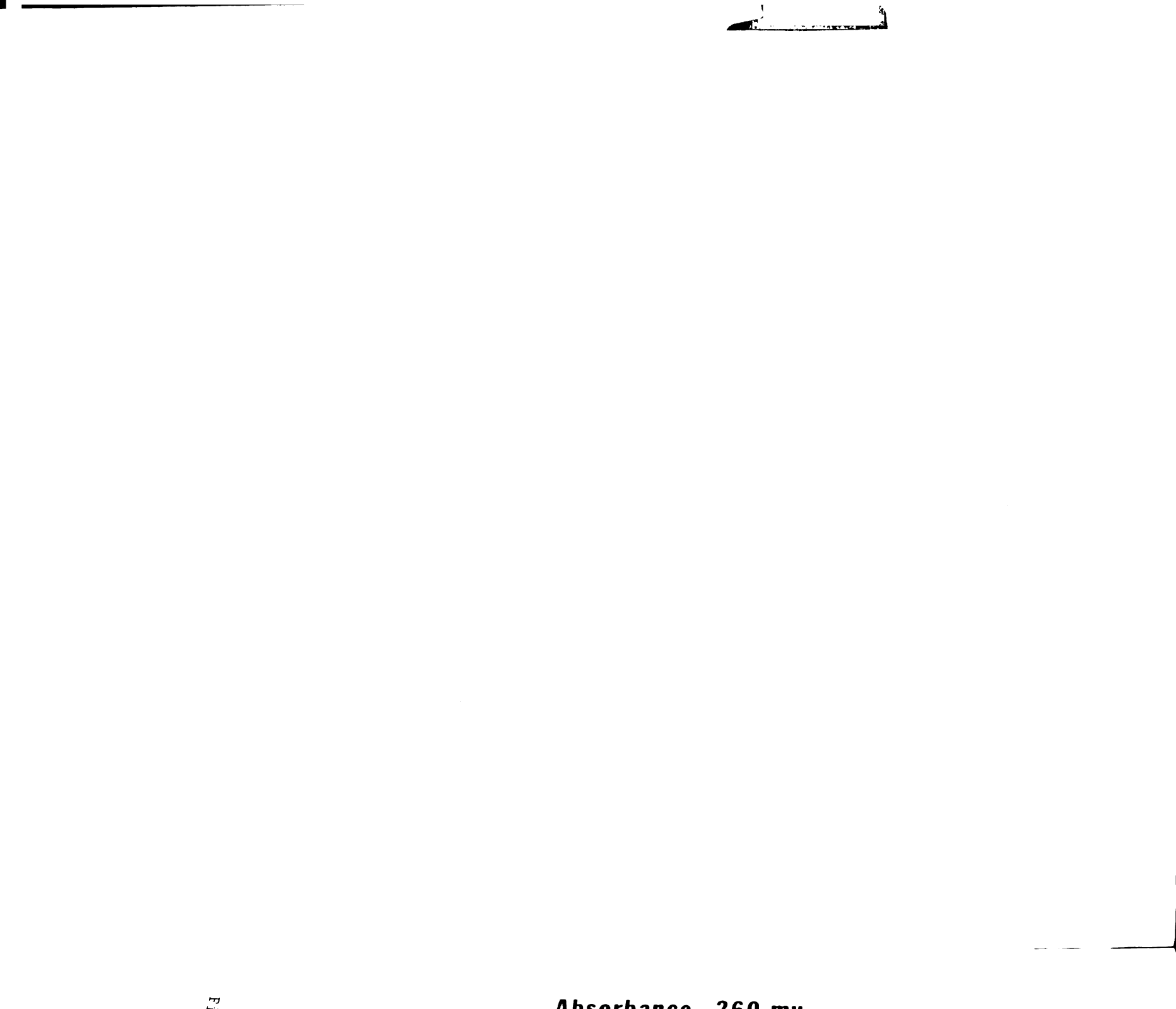
Figures 4 and 5, respectively. Two peaks are evident for the tomato chloroplastic DNA preparation; one, presumably representing nuclear DNA and the other chloroplastic DNA. In the case of the spinach chloroplast DNA preparation, only a single peak is evident.

Centrifugation at 35,000 rpm tended to yield more consistent results than at 31,000 rpm and better separation than at 37,000 rpm. Results of the gradient centrifugations of DNA extracted from preparations of pepper chloroplasts, pepper chromoplasts, watermelon chloroplasts and watermelon chromplasts are shown in Figures 6, 7, 8, and 9, respectively. For pepper chloroplastic DNA (Figure 6), two peaks are observed, one presumably due to nuclear DNA and the other chloroplastic DNA. In the case of watermelon chloroplastic DNA (Figure 8) only a shoulder on a large DNA peak is apparent. Both chromoplastic DNA centrifugations (Figures 7 and 9) show a large DNA peak plus a smaller one of lesser buoyant density. The smaller peak constitutes only one tube of the gradient but since the larger peak tends to slope toward and falls off sharply after this point, it is probably not an artifact. It is obvious


Absorbance 260 mμ

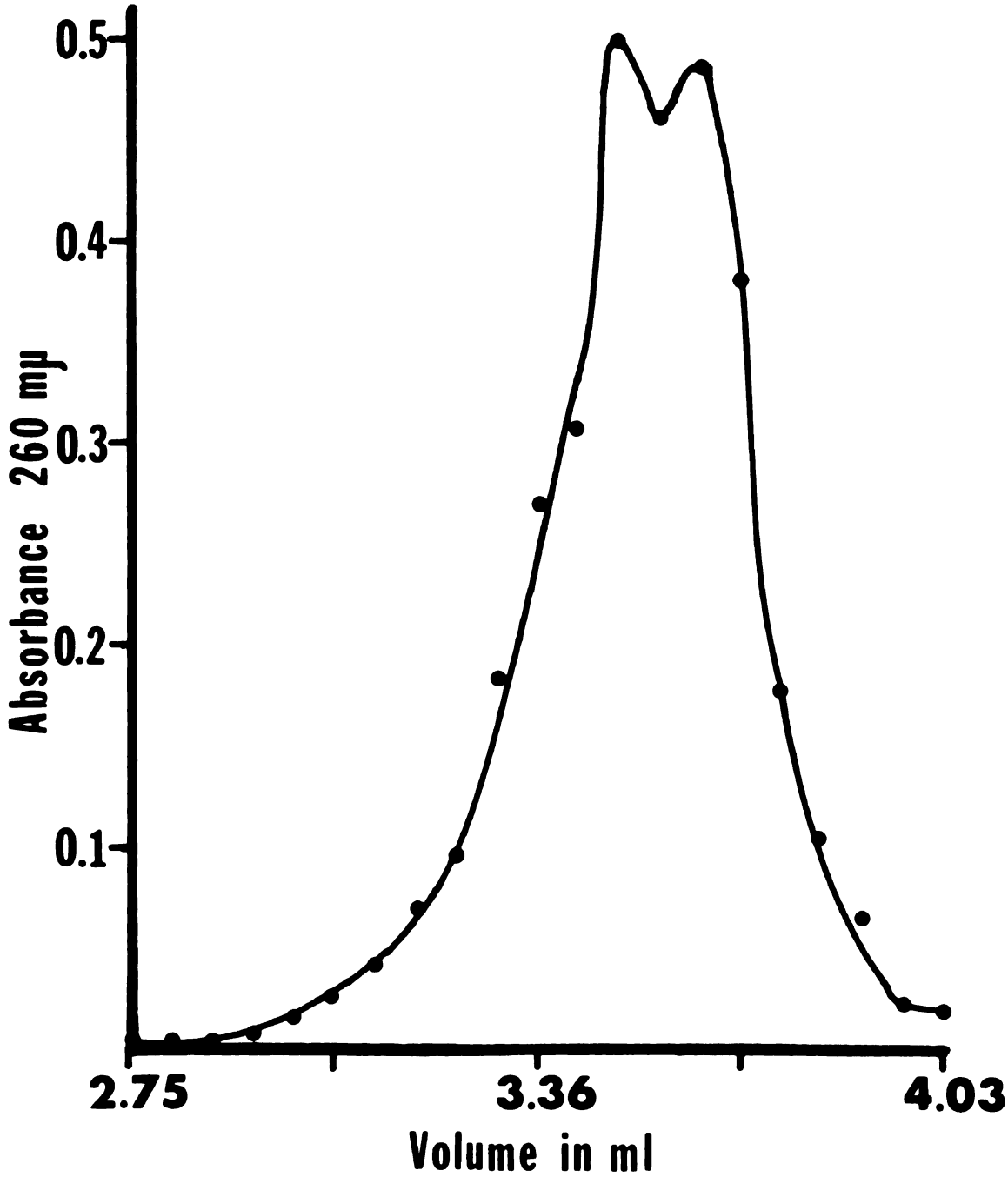


Figure 4: CsCl density gradient profile of DNA from a tomato chloroplast preparation. Centrifugation of the DNA (300 μg) was at 37,000 rpm for 72 hours at 20°.

Absorbance 260 mμ

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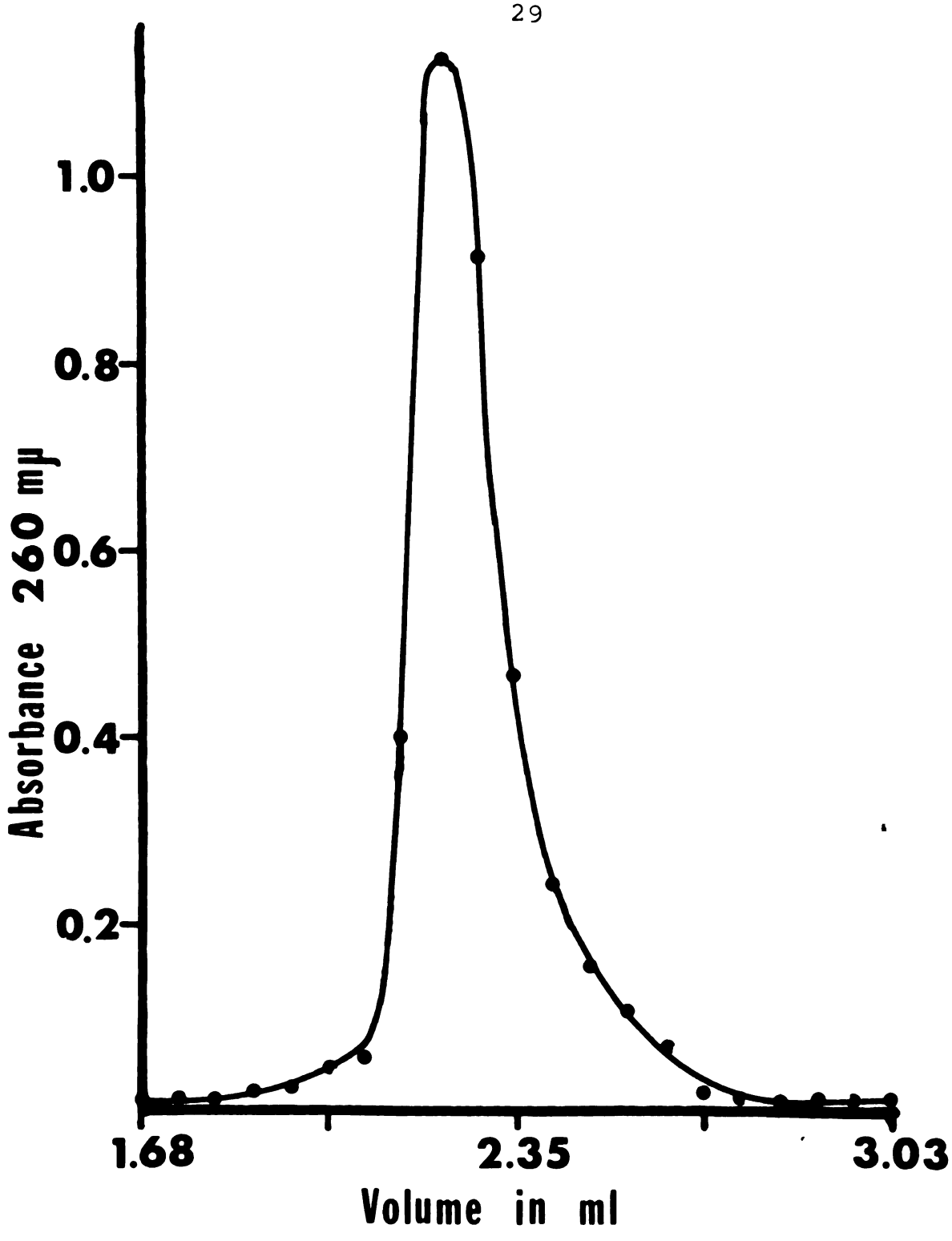

Figure 5: CsCl density gradient profile of DNA from a spinach chloroplast preparation. Centrifugation of the DNA (120 μg) was at 37,000 rpm for 72 hours at 20°.

Absorbance 260 mμ

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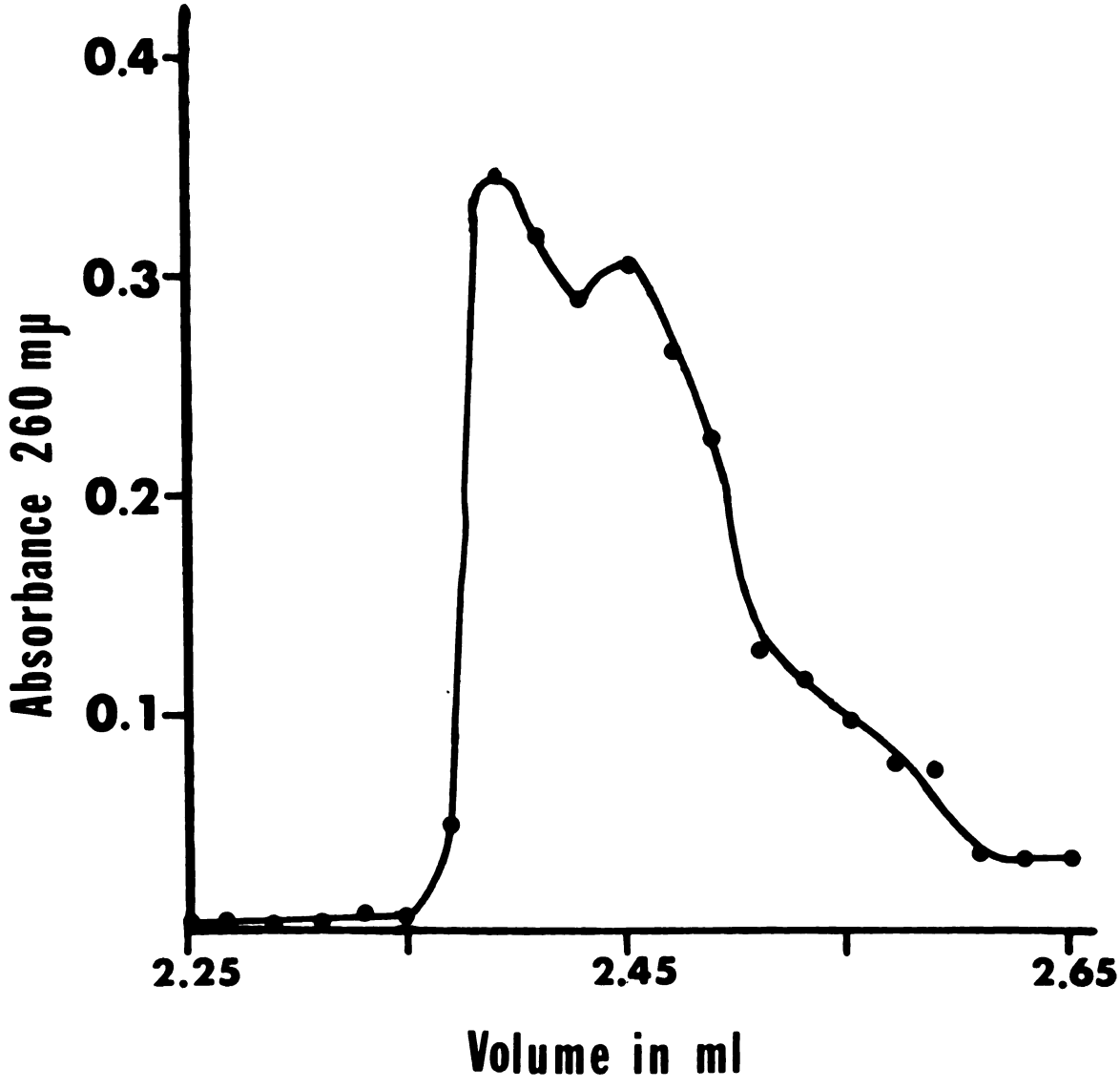

Figure 6: CsCl density gradient profile of DNA from a pepper chloroplast preparation. Centrifugation of the DNA (262 μg) was at 35,000 rpm for 72 hours at 20°.

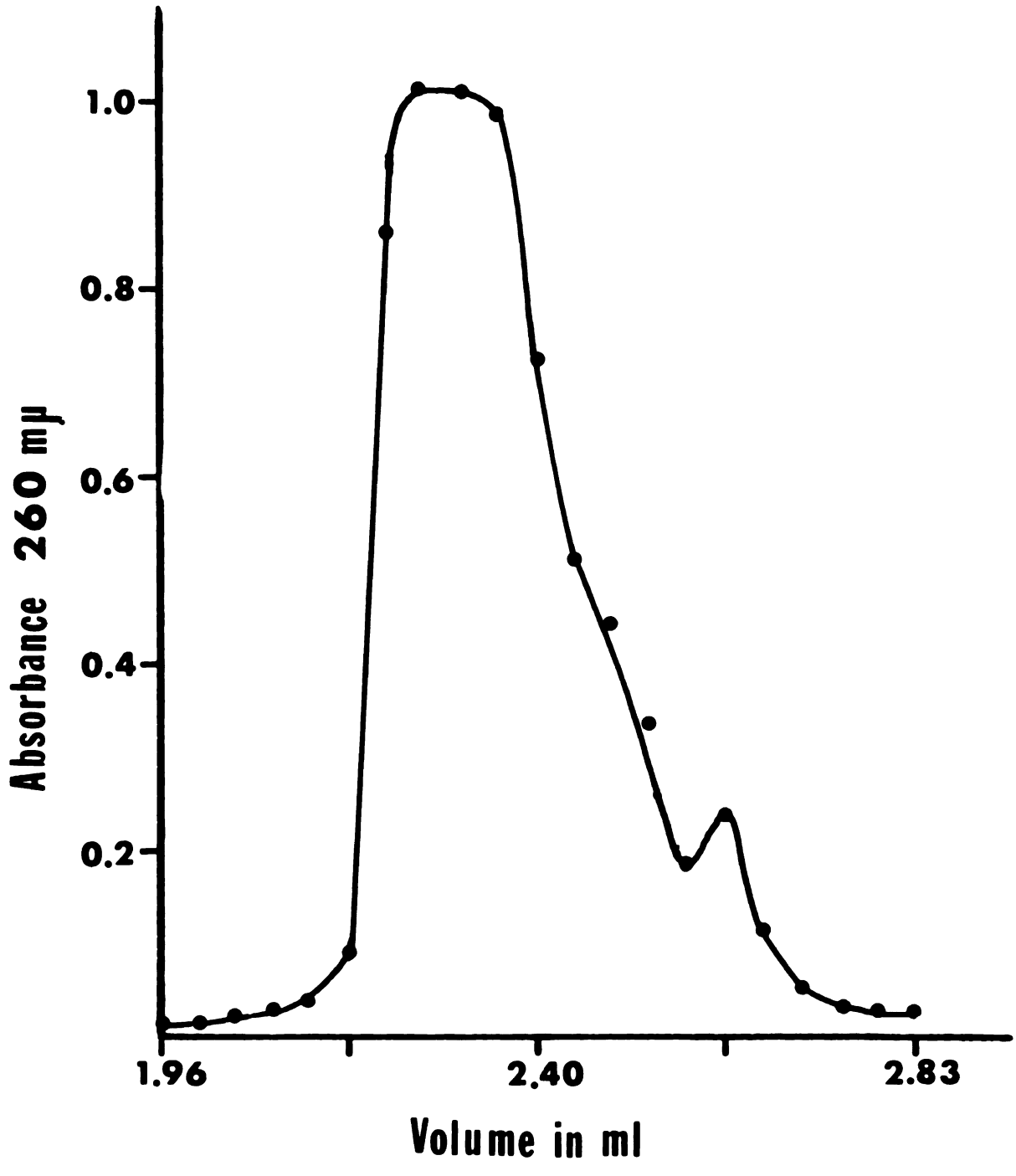

Figure 7: CsCl density gradient profile of DNA from a pepper chromoplast preparation. Centrifugation of the DNA (380 μg) was at 35,000 rpm for 72 hours at 20°.

Absorbance 260 mμ

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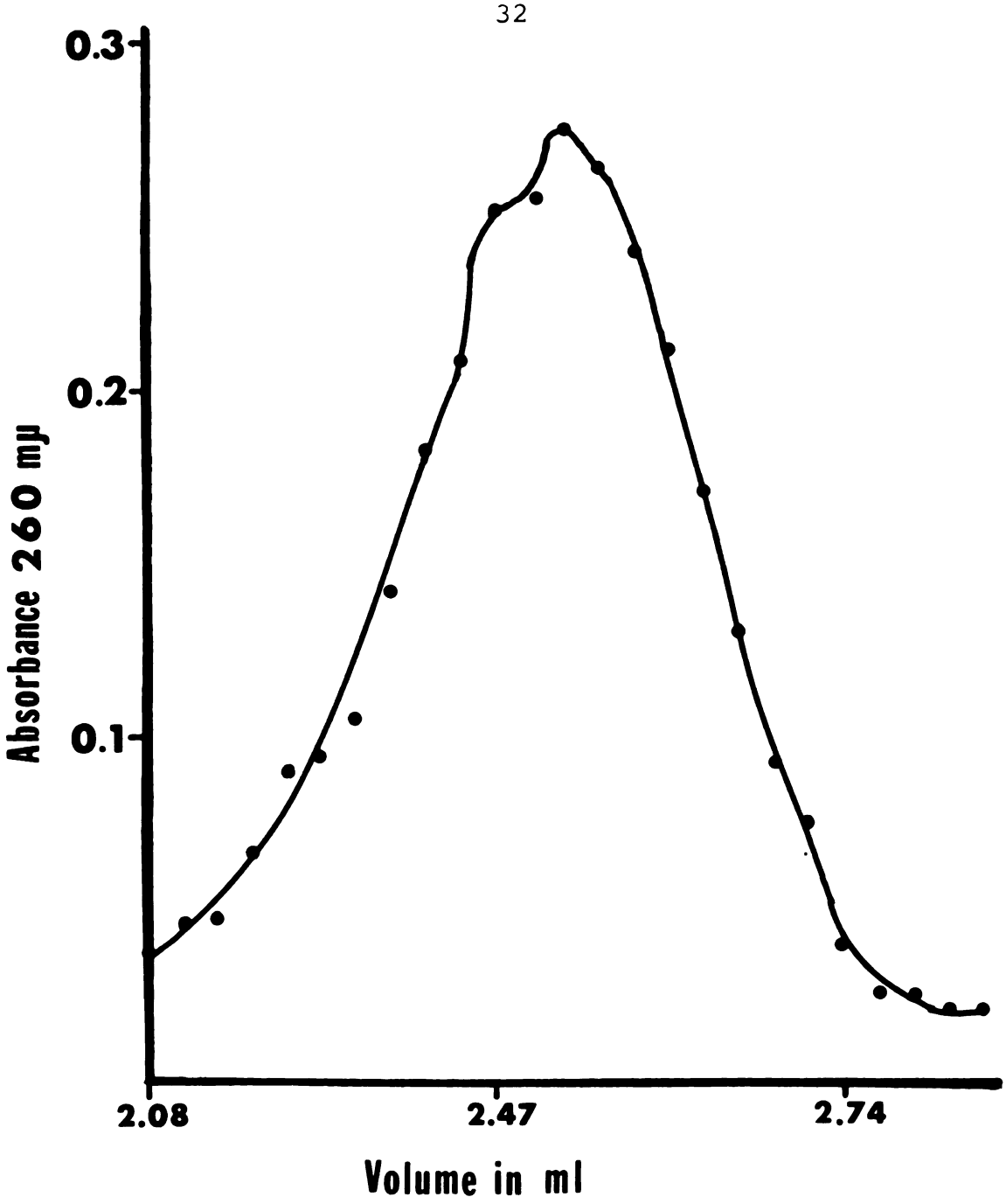
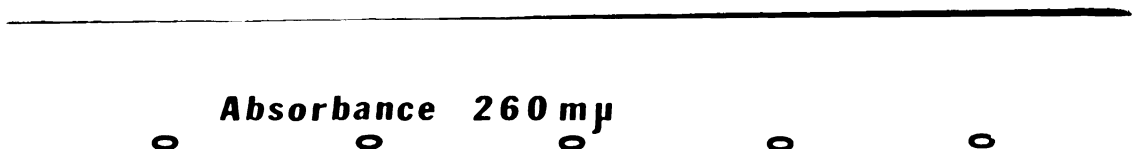


Figure 8: CsCl density gradient profile of DNA from a watermelon chloroplast preparation. Centrifugation of the DNA (238 μg) was at 35,000 rpm for 72 hours at 20^{o}.



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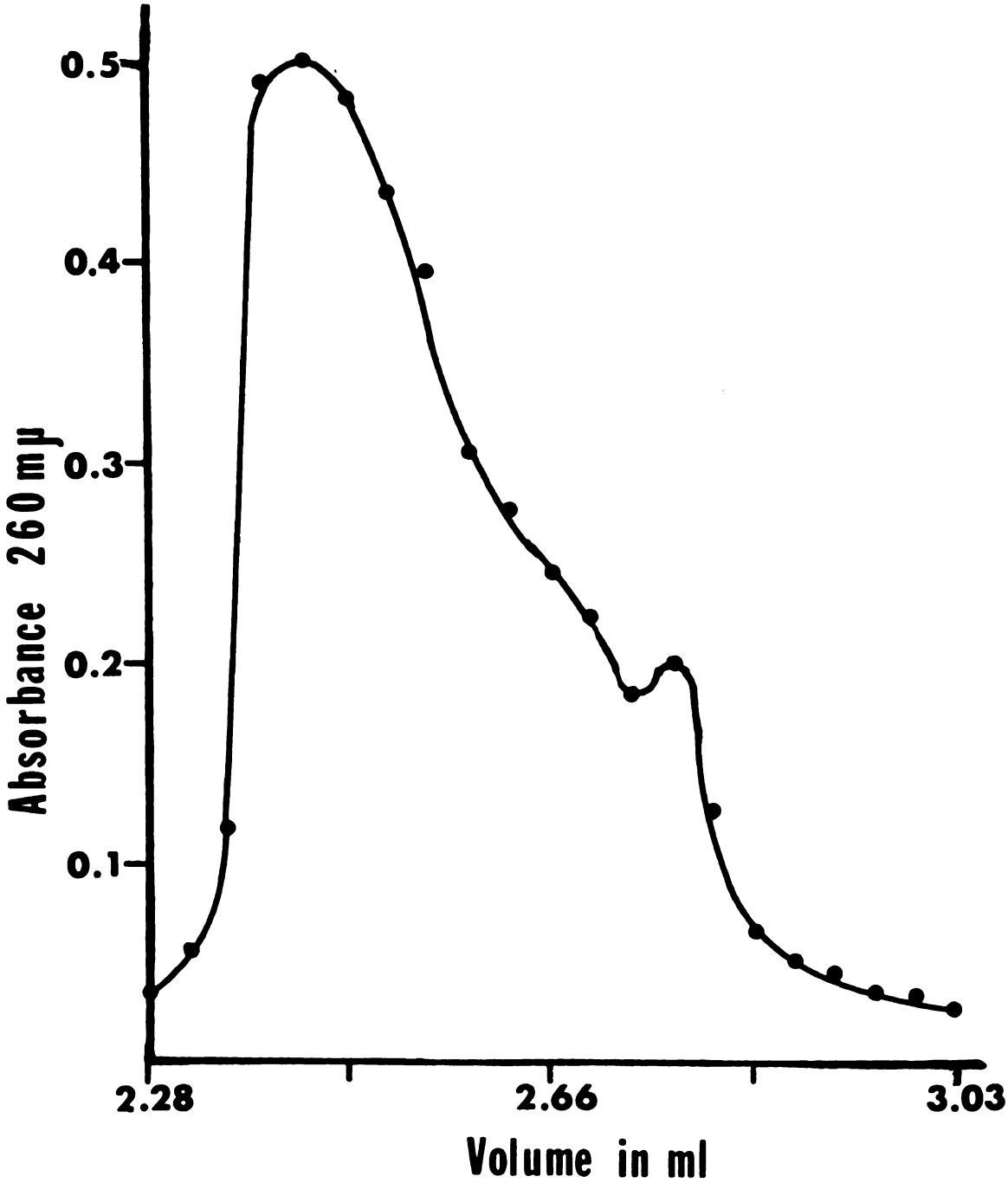

Figure 9: CsCl density gradient profile of DNA from a watermelon chromoplast preparation. Centrifugation of the DNA (396 μg) was at 35,000 rpm for 72 hours at 20^{o}.

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that only partial or suggestive separations were attained between the plastid and nuclear DNA species, due to their similar densities.

A comparison of the results from gradient centrifugations in this thesis to that published by other investigators (Suyama and Bonner, 1966; Green and Gordon, 1967) indicates that the separations at 35,000 rpm as well as the quality of DNA preparations are indeed equivalent. It is noted that extraneous absorbance at 260 mμ did not exceed a reading of 0.050 on any part of the gradient.

It is realized that all aspects of this problem were not pursued to their ultimate conclusion but with the use of experimental procedures and techniques described, it is doubtful that much more information would have been obtained. It is possible that additional information may be gained through the use of the analytical centrifuge, by the recent advent of cesium acetate gradient centrifugation (Zolotor and Engler, 1967) and by centrifugation in a fixed angle rotor (Flamm, Bond, and Burr, 1966). Even with these techniques it is doubtful that sufficient quantities of plastid DNA could be isolated in order to chemically analyze the DNA.

PART II

THE USE OF NUCLEOSIDE PHOSPHOTRANSFERASE AND (^{32}P)p-NITROPHENYL PHOSPHATE FOR THE DETERMINATION OF 5'-LINKED TERMINI OF RIBOSOMAL RIBONUCLEIC ACID

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LITERATURE REVIEW

Since the research for this part of the thesis involved three general areas; namely, the preparation of the enzyme nucleoside phosphotransferase, the synthesis of (^{32}P)p-nitrophenyl phosphate, and experiments on ribosomal RNA termini, each will be reviewed separately.

Nucleoside Phosphotransferase

The existence of enzymes capable of transferring the phosphate moiety of phenyl phosphate to various nucleosides was first reported by Brawerman and Chargaff (1953a, 1953b). Later (Brawerman and Chargaff, 1954, 1955; Tunis and Chargaff, 1956, 1957, 1960a, 1960b) some characteristics of the enzyme were reported. They found, for example, that the enzymes could be isolated from a variety of organisms; enzymes from animal sources yielded both 3'- and 5'-nucleoside monophosphates whereas enzymes from bacteria and plant sources formed only 5'-monophosphates. Phenyl phos-

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phate was the donor used in all of these studies. Phosphate transfer could also be effected from nucleotide donors to other nucleosides or 3'-nucleotides. In other studies it was observed that the pH optimum was 5.2. The phosphatase activity, either inherent or a result of contamination, appeared to be inhibited by cupric or zinc ions while the transferase activity was not affected. Finally, a purification scheme was given in which they reported the separation of phosphatase and transferase activities. It should be noted that even their best preparation exhibited phosphatase activity.

A number of years later a series of papers on nucleoside phosphotransferase was published by a group of Japanese researchers (Katagiri, Yamada, Mitsugi, and Tsunoda, 1964; Mitsugi, 1964a, 1964b; Mitsugi, Kamimura, Nakazawa, and Okumura, 1964; Mitsugi, Kamimura, Okumura, and Katsuya, 1964; Mitsugi, Komagata, Takahashi, Iizuka, and Katagiri, 1964; Mitsugi, Nakazawa, and Okumura, 1964; Mitsugi, Nakazawa, Takahasi, and Yamada, 1964a, 1964b; Mitsugi, Nakazawa, Okumura, Takahashi, and Yamada, 1965; Mitsugi, Okumura, Shiro, and Takahashi, 1965). They

isolated the enzyme from a large number of bacterial species and found that the 5'-nucleotides along with p-nitrophenyl phosphate, phenyl phosphate, and benzyl phosphate served as phosphate donors. The most efficient donor was p-nitrophenyl phosphate. Cupric and zinc ions stimulated both transferase and phosphatase activities with a shifting of the pH optimum from 5.0 to 4.0. Nucleoside-2'(3'), 5'-diphosphates were also synthesized starting with the 2'(3')-monophosphate as acceptor and p-nitrophenyl phosphate as the donor. In all of their experiments a cell-free extract served as the enzyme preparation with the exception of one instance where an extract of E. coli was partially purified by ammonium sulfate fractionation and acid precipitation. In their final publication they reported that phosphate transfer from one nucleotide to another also occurred at an alkaline pH and that cupric and arsenate ions stimulated this synthesis. Maley and Maley (1963), utilizing an homogenate of 4 day old chick embryos, also observed that ribonucleotides, deoxyribonucleotides and phenyl phosphate served as donors for the enzymatic transfer of phosphate to other

ribonucleotides and deoxyribonucleotides at a pH optimum of 9.0.

Synthesis of (^{32}P)p-Nitrophenyl Phosphate

The only method present in the literature for the synthesis of (^{32}P)p-nitrophenyl phosphate is the one reported by Axelrod (1948), who used a modification of the method proposed by Lindberg (1946) for the synthesis of propanediol phosphate. The synthesis involves refluxing anhydrous (^{32}P)H_3PO_4 with PCl_5 to yield (^{32}P)$POCl_3$ which is then vacuum distilled to a reservoir in a dry ice-acetone bath. To the distilled (^{32}P)$POCl_3$ is added p-nitrophenol dissolved in anhydrous chloroform. This is followed by the addition of a small amount of pyridine. After allowing the reaction to go to completion, water is added in the form of ice chunks to hydrolyze the phosphoryl halides. The p-nitrophenyl phosphoric acid is extracted with chloroform and the solvent removed by aeration, leaving a sticky tar. Water is added, the solution made alkaline to phenolphthalein and the disodium p-nitrophenyl phosphate crystallized by the addition of a large excess

ether-acetone (1:1). Further purification involved the formation of a barium salt and its crystallization by the addition of alcohol. The yield was approximately four percent of the theoretical amount and no specific activity data was given.

Variations on the basic synthetic scheme to improve the yield of (^{32}P)$POCl_3$ (Kalinsky and Weinstein, 1954) and to increase the purity of unlabeled p-nitrophenyl phosphate (Bessey and Love, 1952; Aschaffenburg, 1953) have been reported. More recently another method for the synthesis of p-nitrophenyl phosphate has been published (Desjobert, 1963). This method involves the direct reaction of anhydrous H_3PO_4 with P_2O_5 and p-nitrophenol for 48 hours at 116^{o} followed by the extraction of the reaction mixture with distilled water. After the addition of barium carbonate until the pH was 10.0, $Ba_3(PO_4)_2$ removed by filtration. The addition of an equal volume of 90 percent ethanol and cooling to 2^{o} results in the crystallization of the barium salt of p-nitrophenyl phosphate. The crystals obtained by filtration were washed with 60 percent ethanol, 90 percent ethanol, and ether. Yield was

approximately 40 percent of the theoretical amount based on the amount of p-nitrophenol added.

Ribosomal RNA Termini

Present within all living organisms is a class of subcellular particles referred to as ribosomes that are characterized by having a sedimentation coefficient between 70 and 80 S and consisting of approximately 50 percent protein and 50 percent RNA. They can be dissociated into two unequal subunits, a larger one varying between 50 and 60 S and a smaller one ranging from 30 to 40 S, simply by lowering the magnesium ion concentration in the surrounding medium. The ribosomal RNA subunit derived from the 50 to 60 S particle has a sedimentation coefficient varying between 23 and 34 S while the 30 to 40 S subunit yields RNA varying between 13 and 19 S (Spirin, 1963, 1964; Petermann, 1964; Ebel, 1966). The molecular weights of the subunits vary from 1.0 to 1.5 x 10^6 for the larger and 0.5 to 0.7 x 10^6 for the smaller, corresponding to approximately 3000 to 4500 and 1400 to 2000 nucleotides, respectively. Recently another smaller

ribosomal RNA component has been reported (Rosset and Monier, 1963; Rosset, Monier, and Julien, 1964; Virmaux, Mandel, and Urban, 1964) which appears to be closely associated with the 50 to 60 S subunit. It has a sedimentation coefficient of 5 S or slightly larger than transfer RNA (4 S) and a base ratio similar to transfer RNA. It is distinctly different, however, from transfer RNA in that it does not have methylated bases and does not bind amino acids.

Although conflicting evidence exists regarding the continuity of the RNA components (see later discussion in Literature Review) it appears on the basis of end group studies (Midgley, 1965a; Lane and Tamaoki, 1967; Takanami, 1967), physiochemical studies (Midgley, 1965a; Stanley and Bock, 1965; Beck, Duval, Aubel-Sadron, and Ebel, 1966), base ratio differences between the subunits (Petermann, 1964; Pollard, 1964b; Beck, Duval, Aubel-Sadron, and Ebel, 1966), electron microscope observations (Spirin, 1963), and the existence of two cistrons for the biosynthesis of RNA subunits (Yankofsky and Spiegelman, 1963); Attardi, Huang, and Kabat, 1965), that both subunits are composed of one continuous sepa-

rate and distinct polynucleotide chain. Furthermore, from end group studies on viruses (Sugiyama and Fraenkel-Conrat, 1961, 1963; Whitfeld, 1962; Sugiyama, 1965) and end group studies of ribosomal RNA (Midgley, 1965b; Lane and Tamaoki, 1967; Nichols and Lane, 1967; Takanami, 1967) the nucleoside terminus linked to the RNA chain by a phosphate ester at the 5'-position of the ribose sugar is not phosphorylated, while at least in the case of ribosomal RNA, the nucleoside at the other terminus linked via the 3'-position is phosphorylated at the 5'-position.

Several investigators have presented evidence regarding the discontinuity of the polynucleotide chain of the large ribosomal RNA subunit. Hunt (1965) while investigating the 30 S subunit of rabbit reticulocyte RNA, McIlreavy and Midgley (1967) while determining the terminal nucleotide sequences of E. coli ribosomal RNA, and Lane (1962, 1965) investigating whole wheat germ and E. coli ribosomal RNA found approximately one terminal group for each 1200 to 1500 nucleotides or approximately the size as the 13 to 18 S subunit.

Midgley (1965c) determined that when the 23 S component of E. coli RNA was stored under slightly alkaline conditions or if certain RNA isolation procedures were employed, a break in the 23 S chain occurred, resulting in two smaller polynucleotide chains. Beck, Duval, Aubel-Sadron, and Ebel (1966) found that it was necessary to add polyvinyl sulfate, a ribonuclease inhibitor, to their RNA isolation medium to prevent scissions in the RNA chains. It is apparent, therefore, that if ribosomal RNA is isolated by conditions where nucleases are inhibited and hydrolysis by physical and chemical means is eliminated, a continuous polynucleotide chain is obtained.

Thus, alkaline hydrolysis of most RNA molecules yields a mixture of 2'(3')-nucleoside monophosphates from the center of the chain, a nucleoside from the 5'-linked terminus, and a 2'(3'), 5'-nucleoside diphosphate from the 3'-linked terminus (Brown and Todd, 1952; Sugiyama and Fraenkel-Conrat, 1961, 1963; Lane and Tamaoki, 1967). Specific hydrolysis of the chain occurs at the phosphate ester linkage to the 5'-position of the ribose sugar.

Both qualitative and quantitative evidence has been presented concerning the identity of the 5'-linked and the 3'-linked termini of all three classes of ribosomal RNA. Investigations on the termini of the 5 S RNA fraction were conducted by Rosset, Monier, and Julien (1964) and by Brownlee and Sanger (1967). They found that the 3'-linked end was terminated by uridine-3',5'-diphosphate and that 5'-linked end was terminated by uridine.

Lane and co-workers have investigated the terminal groups of E. coli, wheat germ, (^{3}H) L cells, and (^{14}C) E. coli ribosomal RNA released by alkaline hydrolysis (Lane, 1962, 1965; Lane and Tamaoki, 1967; Nichols and Lane, 1967). Their results are listed in Table 1. Lee and Gilham (1965) also working with unfractionated wheat germ found the adenosine, guanosine, cytidine, and uridine released by alkali to be 20, 24, 31, and 25 mole percent, respectively. Hunt (1965) has presented qualitative evidence, based on the reaction of the rabbit reticulocyte ribosomal RNA 5'-linked terminal nucleoside with periodate and (^{3}H) isonicotinic acid hydrazide. Subsequent hydrolysis by pancreatic ribonu-

Table 1. Ribosomal RNA terminal group studies reported by Lane and co-workers.

Source	RNA Component	Terminus	Mole percent A	G	C	U
Wheat germ	unfractionated	5'-linked	18	31	25.5	25.5
(Lane, 1965)	unfractionated	3'-linked	9	40	23	27
L cells	16 S	5'-linked	75	6	4	15
(Lane and	28 S	5'-linked	12	24	6	58
Tamaoki, 1967)	16 S	3'-linked	9	28	52	11
	28 S	3'-linked	5	24	11	60
E. coli (Lane,	unfractionated	5'-linked	47	9	13	30
1962; Nichols	16 S	5'-linked	60	10	10	19
and Lane, 1967)	23 S	5'-linked	19	6	4	70
	16 S	3'-linked	41	25	10	23
	23 S	3'-linked	20	59	9	12

clease or ribonuclease T_1 revealed that unfractionated RNA terminates primarily in adenosine and uridine, the 28 S terminus being mostly uridine and the 18 S terminus being predominantly adenosine. Investigations have been conducted by Midgley and McIlreavy (1966) and McIlreavy and Midgley (1967) on the 5'-linked terminal groups of E. coli RNA by methods similar to those of Hunt (1965) and by alkaline hydrolysis of (^{14}C)RNA. Their results are tabulated in Table 2.

Table 2: Composition of the 5'-linked termini of E. coli ribosomal RNA.

Source	RNA component	Mole percent A	G	C	U
Alkaline hydrolysis of (^{14}C)RNA (Midgley and McIlreavy, 1967)	16 S	70	14	4	11
	23 S	21	27	6	46
Periodate oxidation and reaction with (^{14}C) isonicotinic acid hydrazide (Midgley and McIlreavy, 1966; McIlreavy and Midgley, 1967)	16 S	71	(16 G+C)		16
	23 S	26	(26 G+C)		48
	unfractionated	43	11	8	38

Takanami (1967) investigated the 3'-linked termini of E. coli ribosomal RNA by first removing the phosphate group with alkaline phosphatase, then phosphory-

lating this terminus through the use of polynucleotide kinase and (^{32}P)orthophosphate, next hydrolyzing the RNA chain with alkali, and finally determining the yield of (^{32}P)nucleoside diposphates released. From the 16 S subunit, the mole percent nucleoside diphosphates released were 74.5 A, 7.0 G, 8.7 C, and 9.7 U while the 23 S subunit yielded 16.5 A, 68.2 G, 6.8 C, and 8.4 U.

In a very recent paper by Sagiura and Takanami (1967) utilizing the methods reported by Takanami (1967), it was found that uridine-2'(3'),5'-diphosphate was the predominant terminus of both the larger and smaller ribosomal RNA subunits of Bacillus cercus, Bacillus subtilis, Bacillus stearothermophilus, and Saccharomyces cerevisiae. RNA isolated from Sarcina lutea yielded primarily adenosine-2'(3'),5'-diphosphate from the larger subunit and uridine-2'(3'),5'-diphosphate from the smaller subunit. A composite of their results is listed in Table 3.

Adenosine and uridine were the only nucleosides detected by Pollard (1967) after alkaline hydrolysis of unfractionated cauliflower, spinach, parsnip, cabbage, and mushroom RNA. Further studies on cauliflower RNA

Table 3: The 3'-linked termini of bacterial and yeast ribosomal RNA as reported by Sugiura and Takanami (1967).

Source	RNA Component	Mole percent A	C	G	U
Bacillus cereus	small subunit	13.2	4.4	1.6	81.0
	large subunit	9.6	3.0	2.5	85.0
Bacillus subtilis	small subunit	22.0	9.8	2.2	66.0
	large subunit	23.0	2.7	3.4	71.0
Bacillus stearothermophilus	16 S	10.7	2.2	1.1	85.7
	23 S	21.8	3.8	3.5	70.9
Sarcina lutea	small subunit	10.8	3.1	2.5	83.6
	large subunit	82.0	2.4	2.8	12.7
Saccharomyces cerevisiae	small subunit	11.1	6.0	4.0	84.5
	large subunit	31.6	1.9	9.6	57.0

utilizing the periodate oxidation and amine cleavage scheme of Neu and Heppel (1964) revealed that adenine and uracil were the only free bases released. For the 28 S RNA of cauliflower, it was shown by alkaline hydrolysis, by labeling the nucleoside fraction with $(^{32}P)H_3PO_4$ in conjunction with polyphosphate, and by periodate oxidation with the subsequent reduction of the dialdehydes to alcohols with tritiated borohydride that both adenosine and uridine terminated the subunit. Utilizing similar experimental procedures as for the 18 S fraction, adenosine and uridine were again revealed to be the terminal nucleosides.

Recently Midgley and McIlreavy (1967) found that by altering the growth medium of E. coli cultures they could alter the 5'-linked terminal groups. Following the methods previously described (Midgley, 1965b) it was determined that in glucose or succinate grown cultures, equal amounts of adenosine and uridine were present while cultures grown in richer media such as broth or casamino acids, yielded RNA with a great amount of uridine. The increase of uridine was attributed to an increased amount in the 23 S ribosomal RNA fraction but not in the 16 S fraction.

MATERIALS AND METHODS

Experimental Materials

Diethylaminoethyl-(DEAE) cellulose, carboxymethyl-(CM) cellulose, and Dowex 50X2-200 (H^+-sulfonic acid resin) were purchased from Sigma Chemical Company, St. Louis, Missouri. Rexyn 201 (Cl^--quaternary amine resin) was purchased from Fisher Scientific Company, Detroit, Michigan and (^{32}P)H_3PO_4 (carrier free) from New England Nuclear, Boston, Massachusetts. All other chemicals were reagent grade and are commonly available at chemical supply houses.

Carrot (Daucus carota, L. var. sativa, DC.) plants were grown in the field from which leaves were harvested, washed carefully and cut into smaller pieces. Cauliflower (Brassica oleracea, L. var. botrytis, L.) was obtained from local commercial sources.

The preparation of DEAE-cellulose for column chromatography involved removing the fine particles by repeated decantings of supernatant solutions and thor-

ough washing with successive portions of 1 N HCl, water, 1 N NaOH, and water. Following the washings it was equilibrated with the appropriate buffer. CM-cellulose was prepared similarly except the acid and base washings were reversed. The columns were packed under nitrogen pressure of 10 to 16 lbs/in^2 with several additions of buffered cellulose. The sulfonic acid and quaternary amine resins were prepared by thoroughly washing the resins with water, 1 N NaOH, water, acetone, water, 1 N HCl, and water.

Acid phosphatase was prepared from hypochlorite treated lettuce seeds (Lactuca sativa, var. capitata L.) that had imbibed water for 36 hours. The imbibed seeds (27.7 g) were homogenized in 25 ml of cold 0.1 N NaCl in a stainless steel Waring blendor for three minutes at top speed. Filtering the homogenate through two facial tissues and centrifuging at 15,000 x g for 20 minutes resulted in a crude preparation of acid phosphatase in the supernatant solution. Alkaline phosphatase from calf mucosa (Sigma Chemical Company) was further pruified by eluting a preparation from a DEAE-cellulose column with a linear NaCl gradient

(0 to 1 M) buffered by 0.01 M tris, 0.005 M magnesium acetate, pH 7.5. A fraction exhibiting only monoesterase activity was utilized.

Paper Chromatographic and Electrophoretic Solvent Systems

Unless otherwise indicated all paper chromatography was descending with the paper developed in the following solvent systems:: A) isopropanol (7): concentrated NH_4OH (1): 0.1 M boric acid (2); B) isopropanol (7): concentrated NH_4OH (1): water (2); C) 75 percent ethanol-10 percent saturated $(NH_4)_2SO_4$ impregnated paper (Lane, 1963); D) t-butanol (70): 88 percent formic acid (15): water (15); E) isobutyric acid (66): 2.3 N NH_4OH (34); F) isopropanol (7): water (2): concentrated NH_4OH (1); and G) 0.1 M sodium phosphate, pH 6.8 (1000 ml): $(NH_4)_2SO_4$ (600 g): n-propanol (20 ml). Solvent systems D, E, F, and G along with the accompanying R_F data for the nucleic acid derivatives are listed in the 1967 catalog from Schwartz BioResearch Inc., Orangeburg, New York. The electrophoretic procedure was according to Chandra and Varner (1965) with minor modifications. The electrophoretic solvent system was pre-

pared by dissolving 0.01 M EDTA (tetrasodium salt) and 0.34 ml pyridine in 600 ml water, adjusting the pH to 3.5 with acetic acid, and diluting the solution to 1 liter. Separation was achieved by applying a constant potential of 400 volts across the paper.

Preparation of Nucleoside Phosphotransferase

Preliminary purification of nucleoside phosphotransferase was done according to the methods given by Tunis and Chargaff (1960a), with minor modifications. Four pounds of carrot leaves were homogenized in a Waring blendor with 1 liter of 0.1 M sodium acetate, pH 5.1. After dividing the leaf material into three parts, each part was homogenized with approximately 330 ml of buffer by repeated homogenizations and filtrations through cheesecloth. The fraction precipitating between 30 and 70 percent by volume of cold acetone (-20^{o}) was collected by centrifugation. The pellet was dissolved in a minimum amount of distilled water and the fraction precipitating between 40 and 80 percent of saturation by $(NH_4)_2SO_4$ was collected by centrifugation. The resulting pellet was dissolved in the minimum amount of

distilled water, dialyzed, lyaphilized to dryness and stored at -20° until further use. At this stage the preparation was designated crude nucleoside phospho-transferase.

Further purification resulted by DEAE-cellulose column chromatography. The preparation (180 mg protein) was eluted from a 21 x 1.5 cm column with a linear NaCl gradient from 0 to 1 M (500 ml) in 0.01 M tris and 0.005 M magnesium acetate, pH 7.5. The linear NaCl gradient was prepared with an apparatus constructed according to the principles of the double chamber gradient preparing device reported by Martin and Ames (1961). Essentially, two 500 ml plastic bottles were attached in series to the column via a small diameter rubber hose. The mixing chamber or the bottle leading to the column contained a magnetic stirring bar. The major fractions exhibiting transferase activity were dialyzed against distilled water, lyophilized to dryness, reconstituted by adding 10 ml of distilled water, and dialyzed against distilled water. The enzyme preparation thus obtained after the addition of the appropriate amount of concentrated buffer, was added to a second DEAE-cellulose column

(23 x 1.5 cm). The column was eluted with a 1 liter pH gradient buffered by 0.05 M malic acid, 0.025 M acetic acid and 0.05 M maleic acid from pH 5.75 to 2.4. The mixing chamber contained 500 ml of the buffer adjusted with NaOH to pH 5.75 while present in the adjacent bottle was 500 ml of buffer at pH 1.8. Fractions possessing high transferase activity but low phosphatase activity were combined, dialyzed against distilled water, lyophilized to dryness, reconstituted with distilled water, and dialyzed against distilled water. The final solution (20 ml) was divided into smaller portions and frozen at -20° until further use. At this stage the enzyme preparation was designated purified nucleoside phosphotransferase. A NaCl gradient elution of a CM-cellulose column and another pH gradient elution of a DEAE-cellulose column were completed but these were not included as part of the purification scheme. The procedures employed and the results obtained, thereof, will be described briefly in the Results.

Protein concentration of the preparations was determined on the basis of absorbance at 260 and 280 mμ

(Layne, 1957). Proteinaceous material eluted from the DEAE-cellulose and the CM-cellulose columns by NaCl gradients was determined on the basis of absorbance at 280 mμ.

Enzyme Assays

Assays of crude nucleoside phosphotransferase involved incubating 0.2 ml p-nitrophenyl phosphate (18 μmoles/0.1 ml), 0.5 ml nucleoside (2 μmoles/0.1 ml), 1 ml enzyme preparation and 0.3 ml 0.1 M sodium acetate, pH 5.1 for various lengths of time. Fractions eluting from the DEAE-cellulose and CM-cellulose columns were assayed by incubating 0.3 ml p-nitrophenyl phosphate (18 μmoles/0.1 ml), 0.5 ml uridine (2 μmoles/0.1 ml), 0.5 ml column eluate, and 0.8 ml of 1 M sodium acetate, pH 5.1 for 15 minutes. All phosphotransferase assays were incubated at 37°. The reaction was stopped by the addition of an equal volume of 10 percent trichloracetic acid, neutralized, and diluted with water until the final salt concentration was less than 0.02 M. The solution was then added to a 4 x 1 cm quaternary amine resin column with water to remove the nucleosides, the nucleotides synthesized were eluted with 0.005 N HCl

in the following order: cytidine-5'-monophosphate (CMP), adenosine-5'-monophosphate (AMP), uridine-5'-monophosphate (UMP), and guanosine-5'-monophosphate (GMP). p-Nitrophenyl phosphate and p-nitrophenol eluted after the nucleotides. Fractions were collected and yield was determined on the basis of total absorbance at 260 mμ. Further separation of the nucleotides for the determination of the isomeric product formed involved neutralization of the acid eluate, evaporation under reduced pressure, the addition of a small volume of water, and chromatographing a portion of the sample in either solvent system A or C. In both solvent systems the 3'-monophosphates travel ahead of the 5'-monophosphates.

Phosphatase activity eluting from the cellulose columns was determined by incubating 1 ml of 0.001 M p-nitrophenyl phosphate in 0.1 M sodium acetate, pH 5.1, and 0.5 ml of the column eluate for 10 minutes at room temperature. The reaction was stopped by the addition of 1 ml of one percent NaOH. The amount of p-nitrophenol formed was determined by the absorbance at 410 mμ.

Synthesis of (^{32}P)p-Nitrophenyl Phosphate

p-Nitrophenyl phosphate was synthesized according

to a modification of the method proposed by Axelrod (1948). 0.102 mmoles (6.3 μl) of 85 percent H_3PO_4 and 10 mCi of $(^{32}P)H_3PO_4$ were added to a 14/20 standard taper joint 16 x 150 mm test tube, evaporated to dryness under reduced pressure and placed in an oven at 150 to 160° for 48 hours. After removal from the oven the tube was stoppered immediately with a cork stopper. After cooling to room temperature, 0.208 mmoles (58 mg) of PCl_5 were added and the reaction mixture refluxed by placing the tube at an angle (approximately 20 degrees from the horizontal) with just the very bottom of the tube immersed in an oil bath at 107 to 110°. When all the PCl_5 had reacted with the $(^{32}P)H_3PO_4$ as evidenced by the disappearance of the solid PCl_5, the tube was removed from the bath, allowed to cool to room temperature, and placed in a dry ice-acetone bath. At this time the cork stopper was removed and the tube was evacuated by means of a vacuum pump for 10 minutes to remove any residual traces of HCl. Immediately following the evacuation, 0.414 mmoles (57.6 mg) of p-nitrophenol dissolved in 0.26 ml of dry $HCCl_3$ and 0.062 ml of dry pyridine were added to the reaction mixture. After

sealing the tube, this time with a glass stopper, the reaction was allowed to go to completion at room temperature with occasional mixing. At the end of 30 minutes crushed ice (approximately 1.5 g) was added and the mixture was allowed to stand for another 30 minutes with occasional mixing to decompose the phosphoryl halides. After extracting the aqueous phase with three 8 ml portions of chloroform, it was banded on two large chromatographic sheets and chromatographed for 23 hours in solvent system B. The UV absorbing band (R_F = 0.58) was eluted from the paper with water and evaporated under reduced pressure at 42°. The residue remaining after evacuation was (^{32}P)p-nitrophenyl phosphate. Yield of product formed was determined by absorbance at 285 mμ in 0.1 N HCl.

Radioactivity of samples spotted on filter paper or of paper chromatogram sections was determined by counting the paper in a Packard 3003 Tri-Carb scintillation spectrometer with 15 ml of toluene containing 1,4-bis-2-(4-methyl-5-phenyloxazolyl)-benzene (0.3 g/liter) and 2,5-diphenyloxazole (5 g/liter).

Preparation of Ribosomal RNA and 5'-Linked Terminal Nucleosides

Ribosomal RNA and the 5'-terminal nucleosides were prepared according to a method proposed by Pollard (1967). Essentially, portions of a total of 450 g of cauliflower florets were successively homogenized with 300 ml of 0.01 M $MgCl_2$, 0.01 M $CaCl_2$, 0.025 M sucrose and 0.05 M tris, pH 7.8, and filtered through four layers of cheesecloth. The final filtrate was centrifuged at 15,000 x g for 20 minutes followed by further centrifugation of the supernatant solution at 104,000 x g to pellet the microsomes. The pellet was suspended in 45 ml of a solution containing the following: 0.001 M $MgCl_2$, 5 percent sodium dodecyl sulfate, 0.28 M lithium sulfate, and 0.1 M sodium acetate, pH 6.0. Following the addition of an equal volume of phenol it was homogenized in a stainless steel Waring blendor for 1 minute at 3/4 line voltage. After removal of the upper aqueous layer which separated by centrifugation, the phenol deproteinization was repeated. Following the addition of an equal volume of ethanol, the precipitated nucleic acids were collected by centrifugation and suspended in 20 ml of cold 3 M

sodium acetate, pH 6.0, with a Tenbroeck all glass homogenizer. After thorough cooling of the acetate solution on ice, ribosomal RNA was collected from the pellet by centrifugation while the soluble RNA remained in solution. The acetate treatment was repeated three additional times. Ribosomal RNA (40 mg) obtained in this manner had a ratio of absorbance at 260 mμ to 280 mμ of 2.07. Sucrose density gradient centrifugation in a linear gradient (4 to 20 percent sucrose in 0.05 M NaCl, 0.0001 M $MgCl_2$, 0.05 M sodium acetate, pH 5.3) for 16 hours at 0^{o} in a 25.1 rotor gave the typical bimodal distribution of ribosomal RNA with the area under the curve of the larger subunit being twice that of the smaller subunit and with no obvious contamination by soluble RNA.

The RNA was hydrolyzed in 0.3 N KOH at 37^{o} for 24 hours. The ratio of RNA : KOH did not exceed 1 mg of RNA per ml of KOH solution. After hydrolysis, the solution was neutralized with the sulfonic acid resin and the resin removed by filtration through a sintered glass funnel. The resin was then washed with distilled water. The filtrate and the resin washings were passed through a 3 x 1 cm column of the quaternary amine resin

and the nucleoside fraction eluting before CMP with 0.002 N HCl collected. The HCl eluate was neutralized, combined with the water eluate and evaporated to dryness under reduced pressure. The residue remaining was extracted with boiling pyridine followed by evaporation of the pyridine at reduced pressure. Small volumes of concentrated NH_4OH were added to the flask and evaporated to remove the remaining traces of pyridine. The residue was dissolved in three 0.5 ml portions of water, followed by evaporation of the water in a 16 x 150 mm 14/20 standard taper joint test tube. The nucleosides obtained were present as a residue in the tube.

Identification of 5'-Linked Terminal Nucleosides

Phosphorylation of the nucleosides involved the incubation of the nucleoside residue, 0.1 ml (^{32}P)p-nitrophenyl phosphate (0.53 μmoles/0.01 ml), 0.1 ml enzyme solution, and 0.05 ml of 0.1 M sodium acetate, pH 5.2, for a period of 6 hours. On some occasions, the components were in proportions different than those mentioned previously. Their exact composition will be described in the Results. After incubation, assay mix-

tures were spotted directly on chromatographic papers along with unlabeled carrier nucleotides and developed with the appropriate solvents. The unlabeled carrier nucleotides were spotted on the chromatogram only after the incubation mixture had been completely dried with a heat gun. With high activity (^{32}P)p-nitrophenyl phosphate papers were first developed in solvent system D for 3 days during which the solvent front ran off the chromatographic paper. In solvent system D, (^{32}P)p-nitrophenyl phosphate and (^{32}P)H_3PO_4 travel ahead of the (^{32}P)nucleotides and UMP separates from the other nucleotides since it travels faster. The UV light absorbing nucleotide areas were circled, cut from the paper and eluted with 10 ml portions of water at room temperature 10 times and 10 ml portions of water at 100^{o} five times. The radioactivity of the final eluate was essentially background. After evaporation of the solution at reduced pressure at 42^{o} they were spotted on chromatographic paper and further separated by chromatography in solvent system C, solvent system G, and electrophoresis. For low activity (^{32}P)p-nitrophenyl phosphate, direct electrophoresis afforded sufficient

separations. The radioactivity of the chromatographic and electrophoretic sections was counted as mentioned previously. Separation of extraneous salts from nucleotides eluted from chromatograms where salt containing solvent systems were utilized was accomplished by absorbing the nucleotides on Norit A (0.05 or 0.1 g) in the presence of 10 ml of 0.01 N HCl. After washing the Norit with three 10 ml portions of water, the nucleotides were removed from the Norit by successive exposures to two 10 ml portions of 80 percent ethanol containing one percent concentrated NH_4OH followed by two more 10 ml portions of 50 percent ethanol containing one percent concentrated NH_4OH. In each instance the Norit was removed by centrifugation. Nucleotides were present as a residue after evaporation of the alcoholic solution at reduced pressure.

RESULTS

Preparation of Nucleoside Phosphotransferase

Nucleoside phosphotransferase was obtained from homogenates of carrot leaves since carrot roots, from whence it was previously isolated (Tunis and Chargaff, 1960a), failed to afford a significant amount of enzymatic activity. The crude preparation obtained by acetone and $(NH_4)_2SO_4$ fractionation of leaf homogenates exhibited a considerable amount of phosphatase activity. A time course of the amount of UMP formed reveals that net synthesis occurs for 45 minutes and that periods of incubation longer than this time yield decreasing amount of product (Figure 10). The maximum amount of UMP synthesized was 2.2 μmoles, corresponding to 22 percent of the theoretical yield since 10 μmoles of uridine was present in the incubation mixture. In another experiment acceptor specificity was determined using identical incubation media except for the substitution of adenosine, cytosine, or guanosine for the uridine acceptor. The

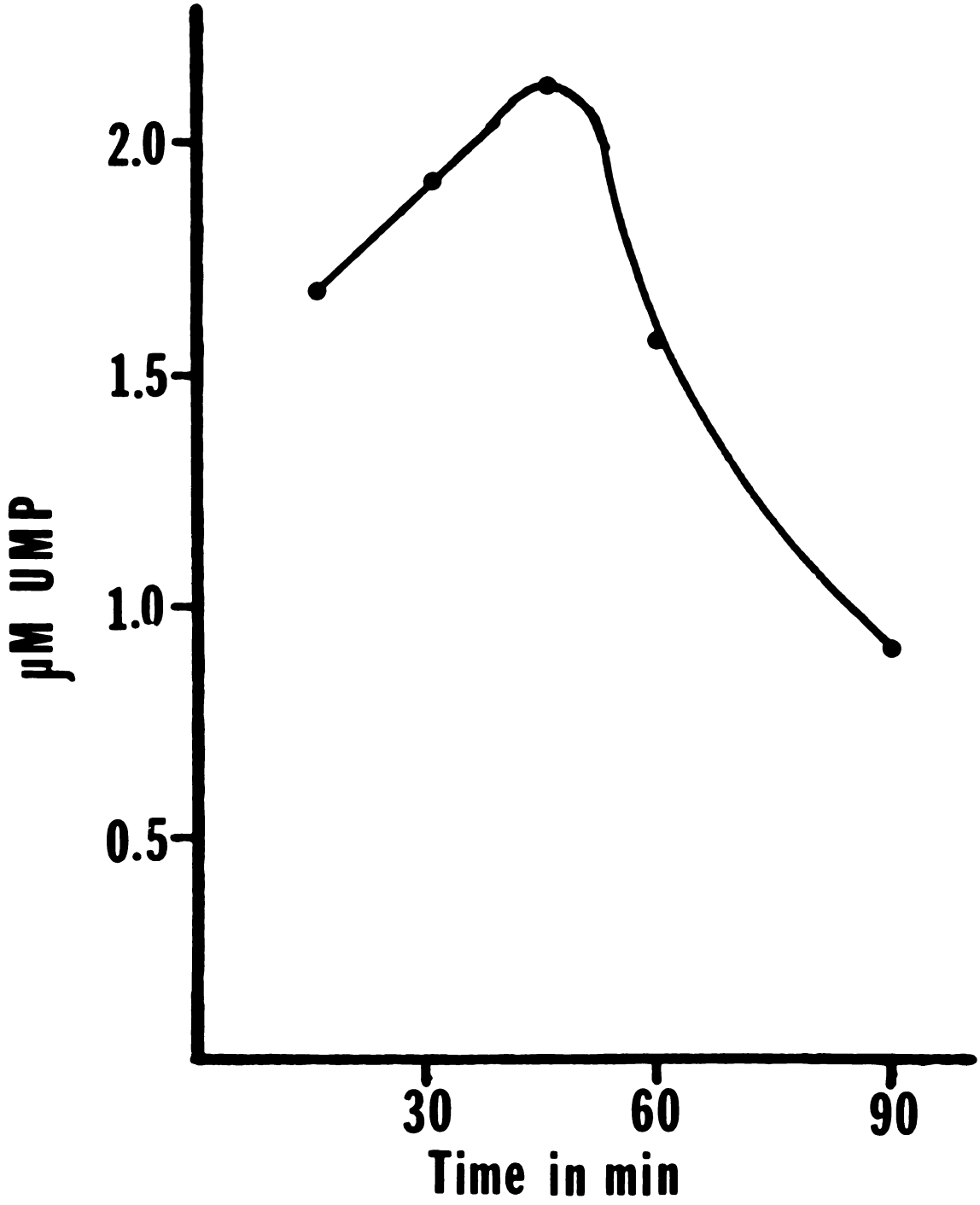

Figure 10: Time course of 5'-UMP synthesis with crude nucleoside phosphotransferase.

amounts of nucleotides synthesized in 30 minutes are shown in Table 4. It is noted that CMP and UMP are synthesized in the much larger quantities than are GMP and AMP.

Table 4: Quantities of nucleotides synthesized by crude nucleoside phosphotransferase.

Nucleotide	μmoles synthesized
AMP	0.5
CMP	2.7
GMP	1.1
UMP	1.9

In order to determine which nucleotide isomer was formed it was necessary to analyze the products by paper chromatography. After neutralization of the 0.005 N HCl eluate and the removal of the water by evaporation under reduced pressure, a portion of the residue dissolved in a small amount of water was spotted on chromatographic paper previously impregnated with 10 percent saturated $(NH_4)_2SO_4$. Reference nucleotides were spotted on adjacent areas of the paper and the chromatogram was developed in solvent system C. Since the 2'(3')- isomers travel faster and are separated from the 5'- isomer, it

was possible to determine that the 5'-isomer was the major product formed in all cases. The only exception was a small amount of 2'(3')-AMP in addition to the 5'-derivative. The UV absorbing AMP, CMP, and UMP and the fluorescent GMP areas of the paper were eluted with 0.1 N HCl and the spectra obtained were identical to those of commercially prepared nucleotides, thus indicating that other transformations of the bases did not occur.

The results in Figure 10 suggested the presence of phosphatase activity. Since it would be very desirable to separate the phosphatase and transferase activities, it was proposed that a new purification scheme be devised to accomplish this goal. After reviewing some of the methods employed by other authors it was decided that a linear gradient elution of a DEAE-cellulose column might be fruitful. It was proposed that a DEAE-cellulose column be eluted with a linear gradient ranging from buffer to a buffered 1 M NaCl solution. The elution profile obtained from such a gradient elution (Figure 11) illustrates that the transferase activity is eluted in three areas but with the major portion corresponding to the major portion of the phosphatase

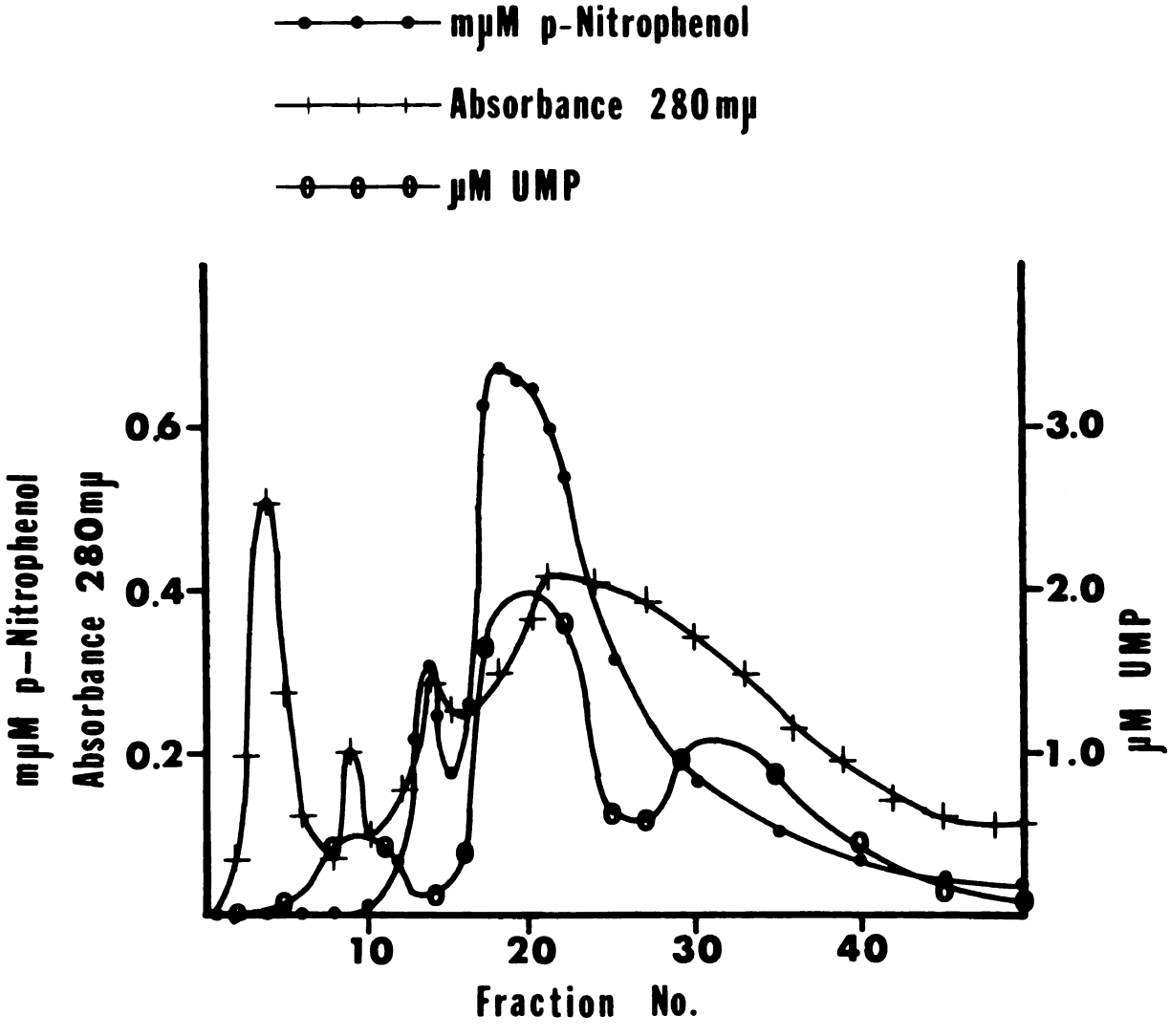

Figure 11: NaCl gradient elution of a DEAE cellulose column. Linear gradient (500 ml) elution from buffer to buffered 1 M NaCl. Buffer consists of 0.01 M tris and 0.005 M magnesium acetate, pH 7.5.

activity. Protein as determined by absorbance at 280 mμ, eluted in four areas, a large fraction eluting directly from the column, two smaller intermediate bands, and another large fraction eluting slightly behind the major portion of the phosphatase activity. The phosphatase activity was distrubted into two areas, a smaller band eluting first and a larger area eluting with the major transferase activity. Although this method failed to separate the majority of the phosphatase and transferase activity, it did remove the greater majority of the brown polyphenolic material since this was not eluted from the column.

The second procedure which was tried was another linear NaCl gradient elution but this time from a CM-cellulose column. In this case the column was eluted with a 1000 ml gradient ranging from buffer to buffered 3 M NaCl with the buffer being 0.005 M sodium acetate, pH 5.5. The result obtained was that practically all the enzymatic activity added was eluted directly, including the phosphatase and transferase enzymes. Since chloride ions displaced the transferase and phosphatase enzymes about equally from the DEAE-cellulose column and

these proteins failed to be attached to the CM-cellulose column it was obvious that a different approach had to be employed in order to separate the activities. For this reason it was proposed to elute a DEAE-cellulose column with a pH gradient. With a pH gradient the proteins are not eluted from the column by simple displacement but are eluted due to a change in net charge of the protein itself. Because of the change in charge it is no longer attracted to the positively charged column. Also at low to neutral pH the charge of the DEAE-cellulose column does not change and thus, the effective result is the titration of the protein from the column.

Utilizing the same pH gradient preparing device as was used with the NaCl gradient elutions, a DEAE-cellulose column was eluted with 0.02 M N-2-hydroxyethylpiperazine-N-2-ethanesulfonic acid (HEPES), 0.005 M 2-(N-morpholino)ethanesulfonic acid (MES), and 0.1 M sodium acetate from pH 7.55 to pH 3.9. Although a linear gradient elution was achieved, the experiment was unsuccessful since the desired enzymes were just beginning to elute from the column at the lowest pH. At this time a second pH gradient was devised. A buffer

system composed of 0.05 M malic acid, 0.05 M maleic acid, and 0.025 M acetic acid was utilized with the higher pH (5.75) buffer mixture in the mixing chamber and the lower pH (1.8) buffer mixture in the adjacent bottle. The proteinaceous material obtained from the major phosphatase and transferase peak eluted from the first DEAE-cellulose column by the NaCl gradient was eluted from this second DEAE-cellulose column by the pH gradient. The actual pH range of the eluate was from 5.75 to 2.4. The elution profile (Figure 12) illustrates that most of the phosphatase and part of the transferase activities are eluted directly from the column. At a lower pH a larger amount of transferase along with a smaller amount of phosphatase activity is eluted. Evident from the eluate obtained at the lowest pH collected is either the appearance of another transferase peak or a more rapid elution of trailing transferase activity from the central transferase area. Fractions including the small phosphatase area and the lowest pH eluate were combined, dialyzed, lyophilized, and dialyzed again to remove the salts and water. The final solution was frozen until further use. This prepa-

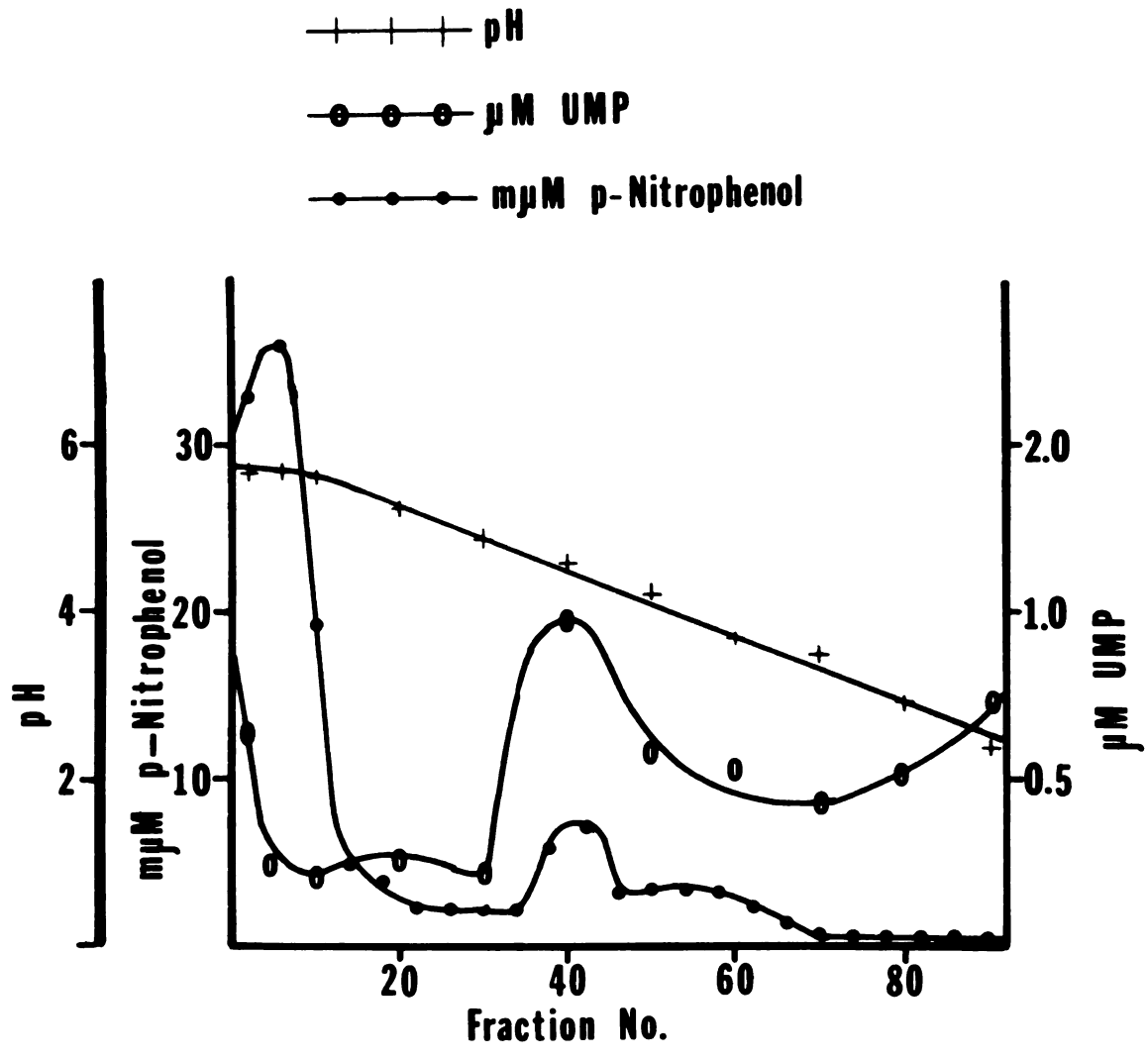

Figure 12: The pH gradient elution profile of a DEAE-cellulose column. Linear gradient (1000 ml) elution from pH 5.75 to pH 2.4 buffered by 0.05 M malic acid, 0.05 M maleic acid, and 0.025 M sodium acetate.

ration designated as purified nucleoside phosphotransferase was used in all of the following transferase assay and incubation mixtures.

Utilization of this partially purified transferase preparation in the synthesis of nucleotides yielded results shown in Table 5. The incubation mixture consisted of 0.4 ml enzyme preparation. 0.2 ml p-nitrophenyl phosphate (18 μmoles/0.1 ml) and 0.5 ml nucleoside (2 μmoles/0.1 ml). Both the nucleosides and the p-nitrophenyl phosphate were dissolved in 0.1 M sodium acetate, pH 5.1. The mixture was incubated for 4 hours. In each case only the 5'-phosphate isomer was synthesized as determined by chromatography in solvent systems A and C.

Table 5: Synthesis of nucleotides with the purified nucleoside phosphotransferase preparation.

Nucleotide	μmoles synthesized
AMP	0.4
CMP	1.2
GMP	0.5
UMP	1.0
TMP	0.4

The pH optimum of the enzyme was investigated using a citrate buffer with the pH ranging from 4.8 to

5.6. The incubation mixture consisted of 0.1 ml uridine (5 mμmoles/0.1 ml), 0.05 ml 0.1 M citrate buffer, 0.02 ml p-nitrophenyl phosphate (0.53 mμmoles/0.01 ml), and 0.04 ml enzyme preparation. The mixture was incubated for 6 hours. Separation of the UMP, after spotting the incubation mixture on chromatographic paper and adding unlabeled carrier, was achieved by development with solvent system C. The results as given in Table 6 indicate that the pH optimum is around 5.2.

Table 6: pH optimum of nucleoside phosphotransferase.

pH	cpm incorporated into UMP
4.8	5975
5.0	5995
5.2	6257
5.4	6012
5.6	4515

Synthesis of (^{32}P)p-Nitrophenyl Phosphate

In 1948 Axelrod proposed a method for the synthesis of (^{32}P)p-nitrophenyl phosphate starting with (^{32}P) H_3PO_4. When this method was tried in the present studies, not only were very small amounts of (^{32}P)p-nitrophenyl phosphate synthesized but a compound of unknown identity

(compound X) was the major product. The compound accounted for most of the product and since it also was highly radioactive, a partial characterization of the compound was performed in order to determine whether the synthetic scheme could be altered to eliminate this product, or conceivably the product itself might be altered to obtain the desired (^{32}P) p-nitrophenyl phosphate. An absorption spectrum of the compound revealed that it appeared to be quite similar to p-nitrophenyl phosphate (Figure 13) but that its maximum absorbance at 280 mμ is slightly displaced from that of commercially prepared p-nitrophenyl phosphate whose maximum absorbance is 285 mμ. Also it was noted that it does not exhibit the characteristic spectrum of a 2,4-dinitrophenyl derivative. By ascending chromatography it can be shown that in solvent system A it has an R_F much greater than p-nitrophenyl phosphate (Figure 14) but considerably less than bis-p-nitrophenyl phosphate which travels ahead of p-nitrophenol. It was determined that it was stable to alkaline hydrolysis (pH 11.5 for 20 minutes at 100°) but was cleaved by acid into p-nitrophenol and an impurity found in small quantities in commercial p-nitrophenyl

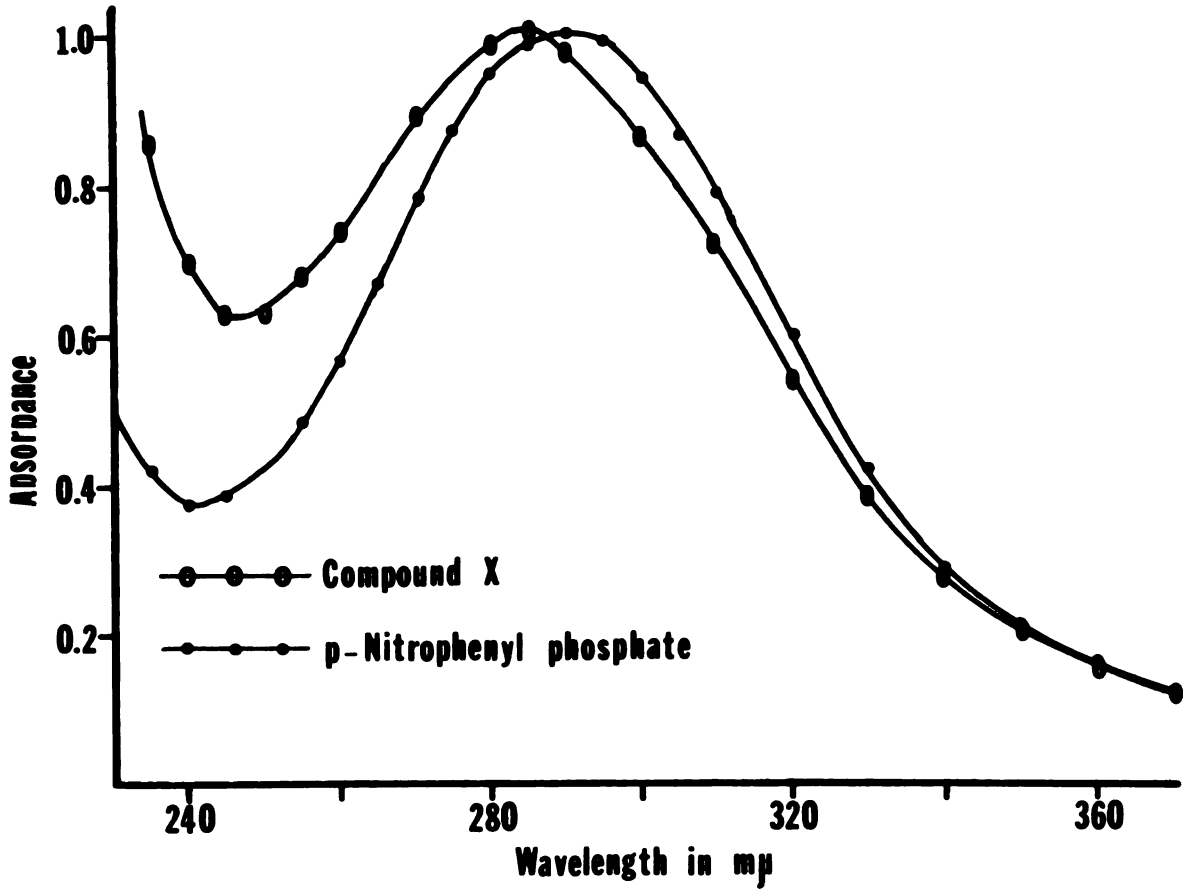

Figure 13: Absorption spectra of compound X and commercial p-nitrophenyl phosphate in 0.1 N HCl.

Figure 14: Acid hydrolysis of compound X. Cleavage by 1 N HCl at 100° for 30 minutes followed by separation of the components by ascending chromatography in solvent system A. Illustrated in lane A of the chromatogram is compound X and a small amount of synthesized p-nitrophenyl phosphate (p-NPP). In lane B is represented the acid hydrolysis product of compound X and p-nitrophenyl phosphate. Products of the hydrolysis are p-nitrophenol (p-NP) and an impurity (I) present in commercial p-nitrophenyl phosphate systems.

phosphate (Figure 14). Cleavage by alkaline phosphatase followed by the addition of one percent NaOH yields a compound whose spectrum resembles p-nitrophenol. Also, compound X did not serve as a donor for the transferase enzymes. The addition of compound X to an incubation mixture containing unlabeled p-nitrophenyl phosphate did not inhibit the transferase enzyme. At this stage the characterization of the compound was abandoned because it appeared that further investigation, although interesting, would not be in line with the original intent.

For obvious reasons it was decided that either a new method of synthesis must be devised or Axelrod's method must be altered in order to obtain the desired product. The decision was reached that the latter alternative was to be taken and that the entire reaction be performed in a single container in order to eliminate the tremendous losses taken during refluxing and distillation. After several futile attempts at the synthesis of $(^{32}P)POCl_3$ in a sealed tube and by a water jacketed tube, a method was devised which proved to be more fruitful. This method involved preparing anhydrous

(^{32}P)H_3PO_4 by heating at 150 to 160° for 48 hours and reacting the anhydrous (^{32}P)H_3PO_4 with added PCl_5. The reaction was heated with an oil bath at 107 to 110° with just the bottom part of the cork stoppered test tube touching the hot oil. The tube was set at an angle of approximately 20° from the horizontal such that the (^{32}P)$POCl_3$ formed would collect on the cool upper side of the tube. After all the PCl_5 had reacted and the tube had cooled to room temperature, it was placed in a dry ice-acetone bath and evacuated with a vacuum pump for 10 minutes. Presumably, this evacuation removed some of the remaining HCl still present in the reaction mixture. Following the evacuation, p-nitrophenol dissolved in pyridine and chloroform was added. After 30 minutes at room temperature ice chunks were added to decompose the phosphoryl halides. At this stage chloroform extractions removed most of the p-nitrophenol leaving primarily (^{32}P)H_3PO_4 and (^{32}P)p-nitrophenyl phosphate in the aqueous phase. Following the chromatography of the aqueous phase in solvent system B, the UV light absorbing, highly radioactive area identical in R_F to commercial p-nitrophenyl phosphate (Figure 15) was eluted from

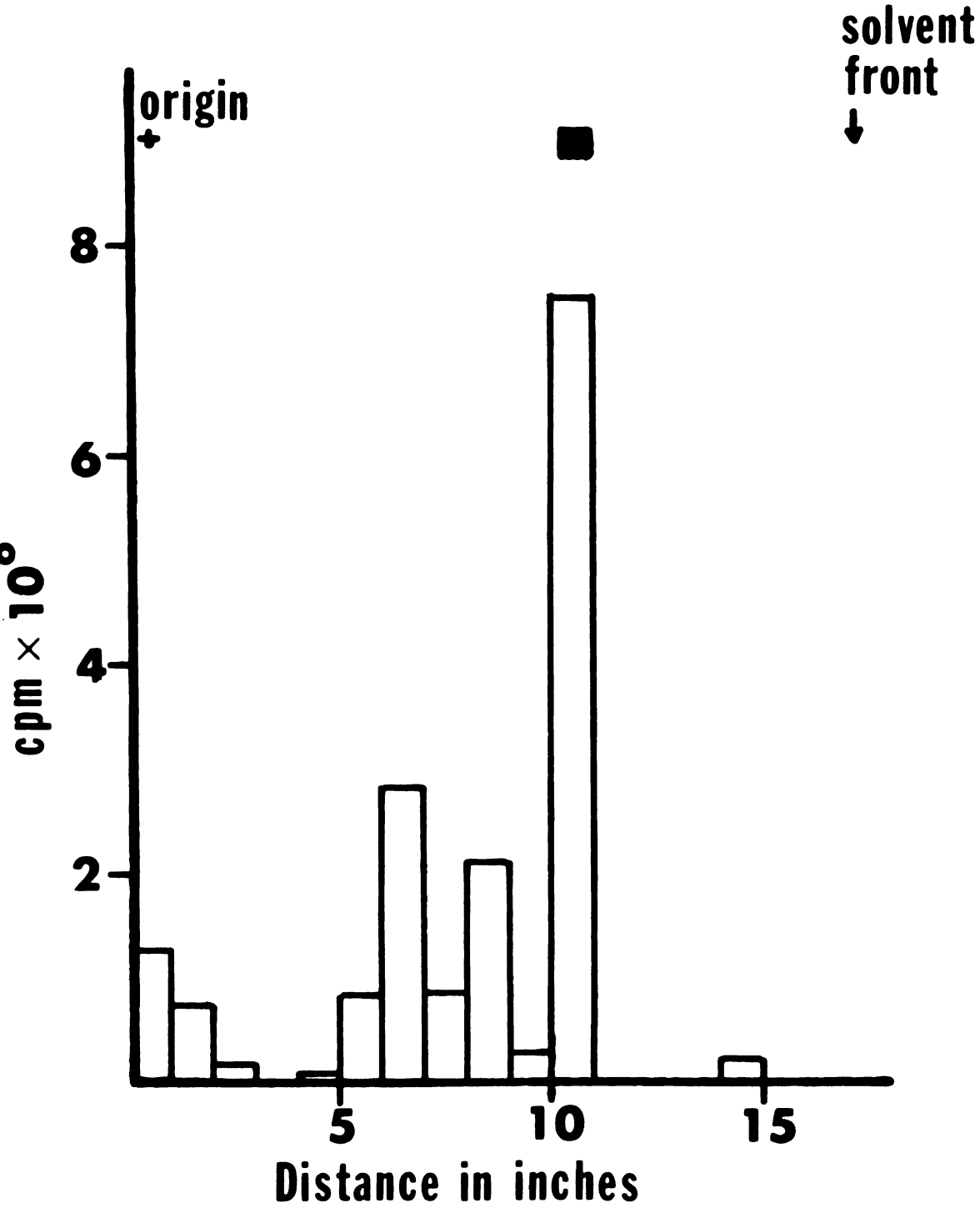

Figure 15: Chromatography of the aqueous phase of the (^{32}P)p-nitrophenyl phosphate reaction mixture in solvent system B. The UV light absorbing area of p-nitrophenyl phosphate on the paper and the distribution of the radioactivity are illustrated.

the paper.

Identification of the product formed was accomplished by co-chromatography with unlabeled p-nitrophenyl phosphate in solvents A, C, E, F, and G, comparing the absorption spectrum to commercial p-nitrophenyl phosphate, hydrolysis by acid, and incubation with both the acid and alkaline phosphatase preparations. Chromatography of the product gave a single peak of radioactivity and a single area of UV light absorbance (Figure 16) and chromatographic R_F similar to commercial p-nitrophenyl phosphate (Table 7).

Table 7: Chromatography of the synthesized (^{32}P)p-nitrophenyl phosphate.

Solvent system	R_F of commercial p-nitrophenyl phosphate	R_F of synthesized (^{32}P)p-nitrophenyl phosphate
A	0.42	0.42
B	0.58	0.58
C	0.82	0.83
E	0.81	0.80
G	0.50	0.48

Partial hydrolysis of the synthesized product by 6 N HCl followed by chromatography in solvent system C yields a new area of radioactivity corresponding to (^{32}P) H_3PO_4 and a new area of UV light absorbance corresponding

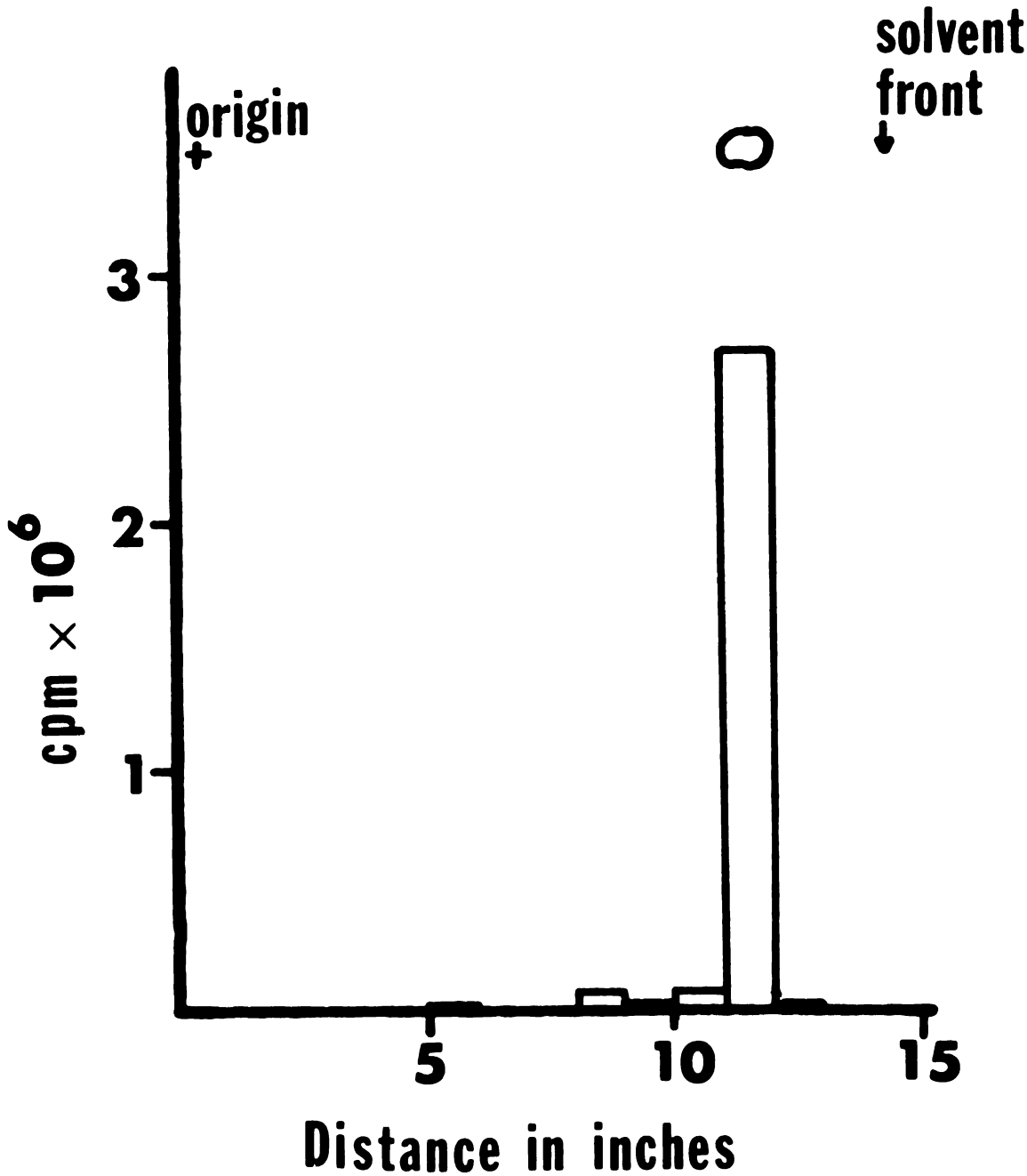

Figure 16: Chromatography of purified (^{32}P)p-nitrophenyl phosphate in solvent system C. The UV absorbing area of p-nitrophenyl phosphate on the paper and the distribution of the radioactivity are illustrated.

to p-nitrophenol (Figure 17).

Hydrolysis by acid phosphatase prepared from lettuce seeds and alkaline phosphatase from calf mucosa yield equivalent amounts of p-nitrophenol from both commercial and synthesized p-nitrophenyl phosphate (Table 8). The incubation mixture for the hydrolysis of the product by alkaline phosphatase consisted of 0.2 ml of 0.1 M glycine, pH 9.5, 0.1 ml enzyme preparation, and 0.02 ml of p-nitrophenyl phosphate (0.53 μmoles/ 0.01 ml). The mixture for the acid phosphatase preparation was 0.1 ml sodium citrate, pH 5.5, 0.1 ml enzyme preparation, and 0.05 ml p-nitrophenyl phosphate (0.53 μmoles/0.01 ml). Both mixtures were incubated for 10 minutes at 37^{o}. In the case of the acid phosphatase the reaction was stopped by adding 0.5 ml of one percent NaOH, a 0.1 ml aliquot was taken from this mixture and diluted to 3.0 ml with additional one percent NaOH. For the alkaline phosphatase preparation the reaction was stopped by adding 2.75 ml of one percent NaOH directly to the incubation mixture. p-Nitrophenol was determined on the basis of absorbance at 410 mμ.

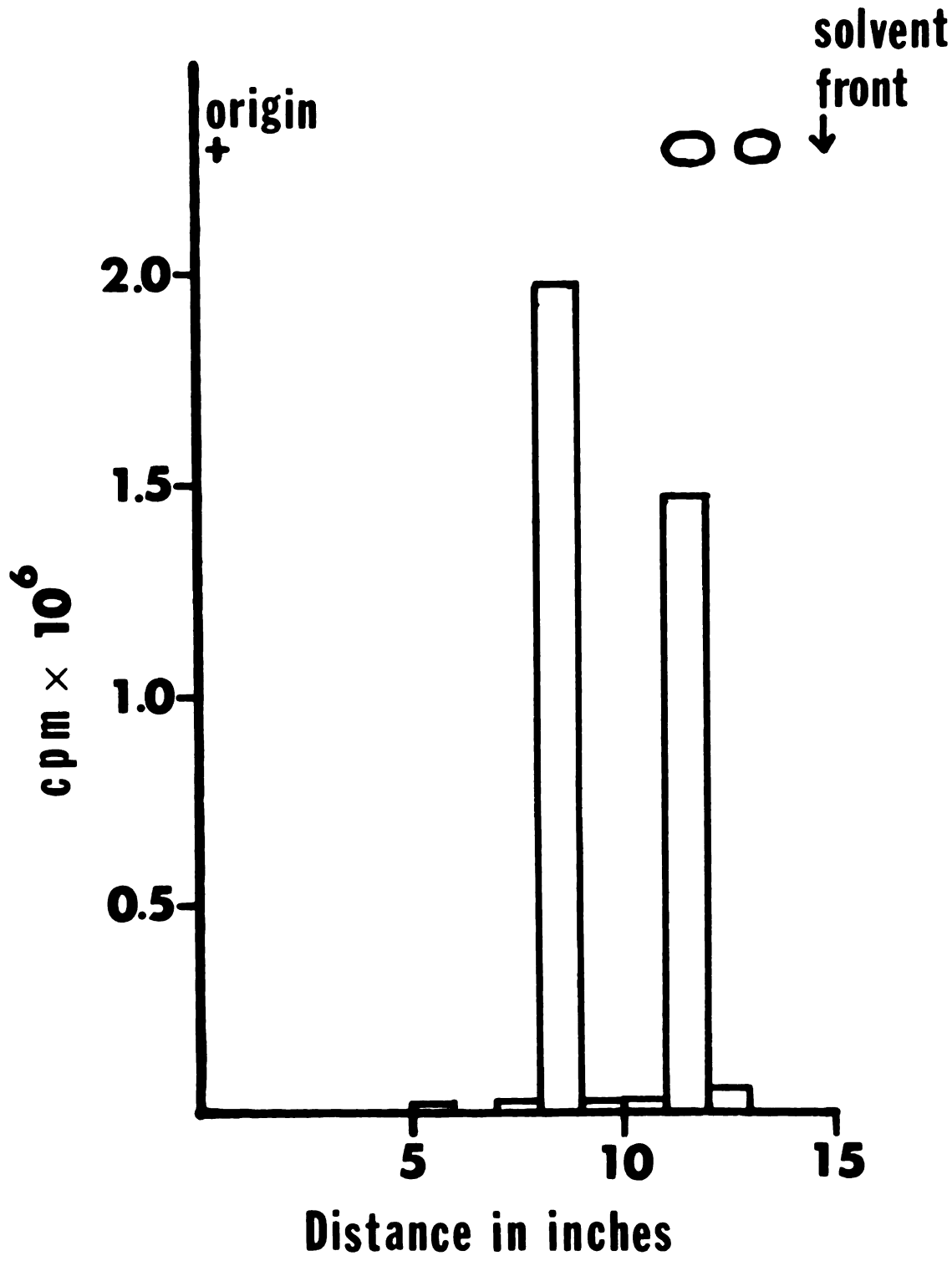

Figure 17: Partial hydrolysis of (^{32}P)p-nitrophenyl phosphate by acid. Cleavage by 6 N HCl for 10 minutes at 100° followed by separation of the components by chromatography in solvent system C. The UV absorbing areas of the paper and the distribution of radioactivity are shown.

Table 8: Hydrolysis of the synthesized (^{32}P)p-nitrophenyl phosphate and commercial p-nitrophenyl phosphate by alkaline and acid phosphatases.

Enzyme	p-Nitrophenyl phosphate	p-Nitrophenol released, absorbance at 410 mμ
Acid phosphatase	unlabeled	0.572
Acid phosphatase	labeled	0.570
Alkaline phosphatase	unlabeled	1.395
Alkaline phosphatase	labeled	1.360

A comparison of the absorption spectra obtained from the radioactive and the commercial p-nitrophenyl phosphate shows that the spectra obtained are practically identical (Figure 18). Maxima at 285 mμ and minima at 230 mμ were obtained in both cases.

The amount of (^{32}P)p-nitrophenyl phosphate synthesized was 0.11 mmoles (33.7 mg) as determined by absorbance at 285 mμ, which is 40.5 percent of the theoretical yield based on the quantity of PCl_5 added. Radioactivity of the sample as determined by placing a small aliquot of the sample on a filter paper, evaporating the water, and counting the sample as mentioned previously was 1.23×10^7 cpm/μmole.

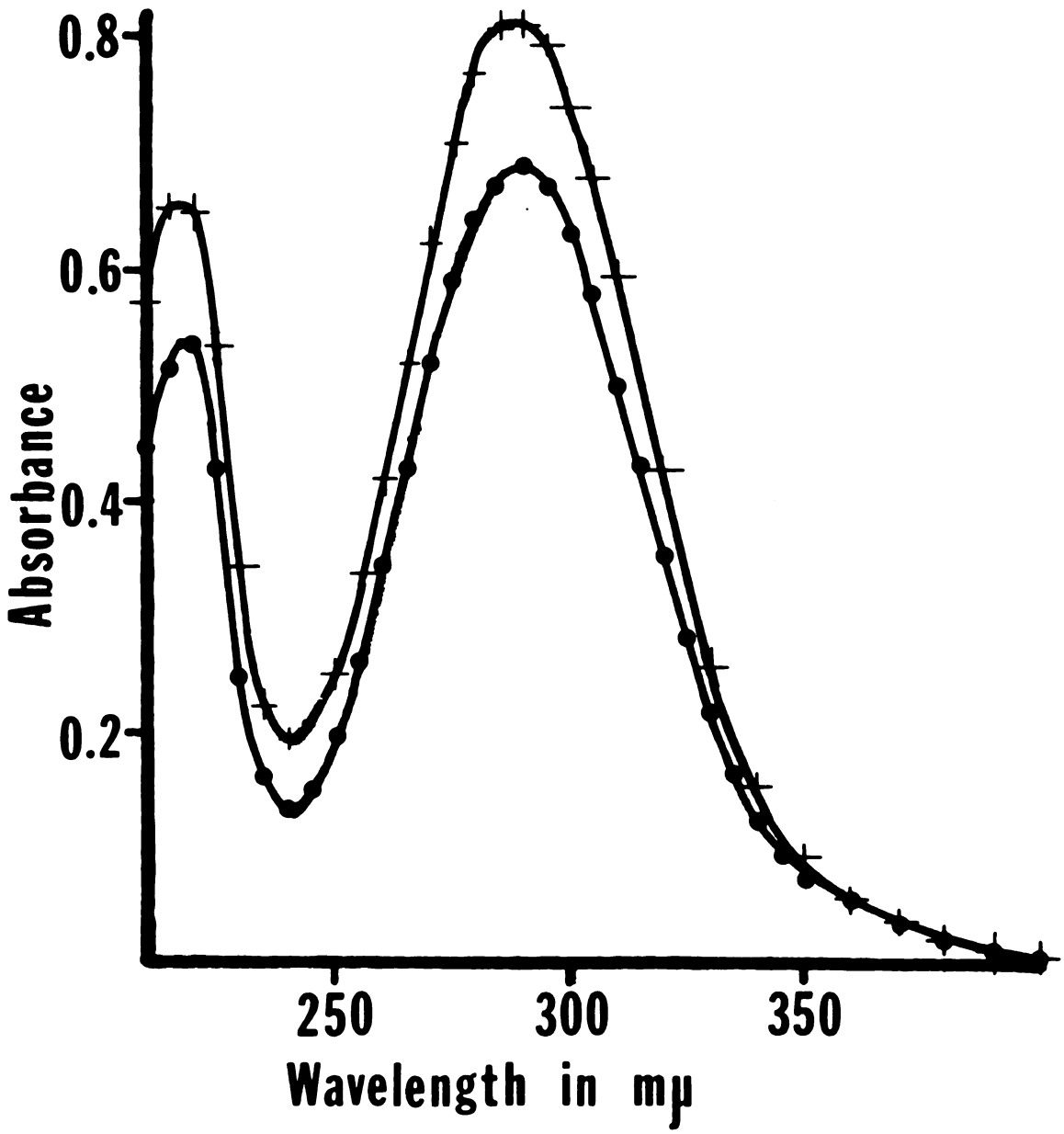

Figure 18: Absorption spectra of synthesized (^{32}P)p-nitrophenyl phosphate (upper curve) and commercial p-nitrophenyl phosphate (lower curve) in 0.1 N HCl.

Detection of Minute Quantities of Nucleosides

In order to test the efficacy of detecting minute quantities of nucleosides, a time course of the incorporation of radioactivity from (^{32}P)p-nitrophenyl phosphate mediated by nucleoside phosphotransferase was performed. The incubation mixture consisted of 0.1 ml enzyme preparation, 0.025 ml (^{32}P)p-nitrophenyl phosphate (0.53 μmoles/0.1 ml) and 0.05 ml uridine (5 mμmoles /0.01 ml). At the end of 1, 2, 4, and 6 hours 0.02 ml was withdrawn and spotted on a chromatogram followed by the addition of unlabeled carrier UMP. The chromatogram was developed with solvent system D for 30 hours. After drying and the circling of the UV absorbing UMP areas, they were cut from the paper and the radioactivity in the area was determined as previously mentioned. The results (Table 9) indicated that radioactivity can be incorporated into quantities of nucleosides as small as 2.9 mμmoles and that the maximum incorporation occurred during the 6 hour incubation period.

Table 9: Time course of incorporation of radioactivity into 2.9 mμmoles of uridine.

Time (hrs)	cpm	Percent incorporation
1	1318	9
2	1808	12
4	2296	15
6	2507	17

To determine the effect of increasing substrate concentrations, a mixture of nucleosides (5 mμmoles each) was incubated with (^{32}P)p-nitrophenyl phosphate ranging in concentration from 0.53 to 2.11 μmoles. The incubation mixture consisted of 0.1 ml enzyme preparation, 0.01 ml nucleoside mixture (5 mμmoles each) dissolved in 0.1 M sodium acetate, pH 5.2 and the appropriate volume of (^{32}P)p-nitrophenyl phosphate (0.53 μmoles/0.01 ml). From the results obtained (Table 10) after separation of the radioactive mixture by electrophoresis for 6.5 hours it is evident that the incorporation of radioactivity into the nucleosides was approximately equimolar. Even at a ratio of p-nitrophenyl phosphate to nucleosides of approximately 100 : 1 saturating substrate concentrations were not achieved.

Table 10: The effect of increasing substrate concentration on the incorporation of radioactivity into a nucleoside mixture (5 mμmoles each).

Concentration of (^{32}P) p-nitrophenyl phosphate	cpm	AMP % Inc	Mole %	cpm	CMP % Inc	Mole %	cpm	GMP % Inc	Mole %	cpm	UMP % Inc	Mole %
0.01 ml (0.53 μmoles)	304	11	23	372	14	28	290	11	22	361	13	27
0.02 ml (1.06 μmoles)	630	23	23	873	32	32	574	21	21	672	24	24
0.04 ml (2.11 μmoles)	1002	37	30	737	27	22	740	27	23	820	30	26

Since the separation of the radioactive components in the incubation mixture by electrophoresis was only feasible when low activity (^{32}P)p-nitrophenyl phosphate was used, another method of separation was devised. This method involved the paper chromatography of the mixture in solvent system D for 3 days so that (^{32}P);-nitrophenyl phosphate and (^{32}P)H_3PO_4 would travel far ahead of the nucleotides. Following the elution of the nucleosides from the paper and evaporation of the water eluate under reduced pressure at 42^{o}, the residue, after being taken up in a small amount of water, was spotted on a second chromatogram and separated by the appropriate chromatographic system or by electrophoresis. UMP was generally eluted separately since in the first system it traveled faster than the AMP, CMP, and GMP mixture. In order to determine whether specific losses of nucleotides occurred in the process of chromatography in solvent system D and the elution from the paper, an equimolar mixture of nucleosides (mμmoles each) was incubated, the incubation mixture banded on a chromatogram along with the unlabled carrier nucleotides, and development of the

paper was effected in solvent system D. Following the elution from the paper AMP, CMP, and GMP were separated by chromatography in solvent system G for 13.5 hours while UMP was chromatographed in solvent system C for 21 hours. The results agree well with those obtained by electrophoresis (Table 10) in that proportional amounts of radioactivity were incorporated into the nucleosides (Table 11).

Table 11: Incorporation of radioactivity into a mixture of nucleosides. Separation of the radioactive components was effected by chromatography first in solvent system D followed by chromatography in solvent systems C and G.

Nucleotide formed	cpm	Percent incorporation	Mole percent of total
AMP	2188	28	28
CMP	1963	25	26
GMP	1570	20	21
UMP	1887	24	25

Ribosomal RNA Terminal Group Studies

Cauliflower ribosomal RNA prepared by the method proposed by Pollard (1967) gave the typical bimodal distribution with the area under the curve for the larger subunit being approximately twice that of the smaller

subunit (Figure 19). No obvious peak of soluble RNA was noted which in turn, establishes the purity of the RNA.

Three experiments were done on the determination of the terminal groups of unfractionated ribosomal RNA. Since the conditions for separating the labeled nucleotides were different, the experiments will be discussed individually. The phosphorylation of the nucleosides obtained from 15 mg of unfractionated RNA yielded a larger proportion of radioactivity incorporated into AMP and UMP than into CMP and GMP (Table 12). The incubation mixture consisted of 0.05 ml (^{32}P)p-nitrophenyl phosphate (0.53 μmoles/0.01 ml), 0.1 ml enzyme preparation, 0.05 ml of 0.1 M sodium acetate, and the nucleoside residue. After incubation for 6 hours the radioactive mixture was separated by electrophoresis.

Table 12: Phosphorylation of the nucleosides obtained by alkaline hydrolysis of 15 mg of ribosomal RNA followed by electrophoretic separation of the radioactive mixture.

Nucleotide	cpm	Mole percent of total
AMP	1040	50
CMP	294	10
GMP	300	11
UMP	485	22

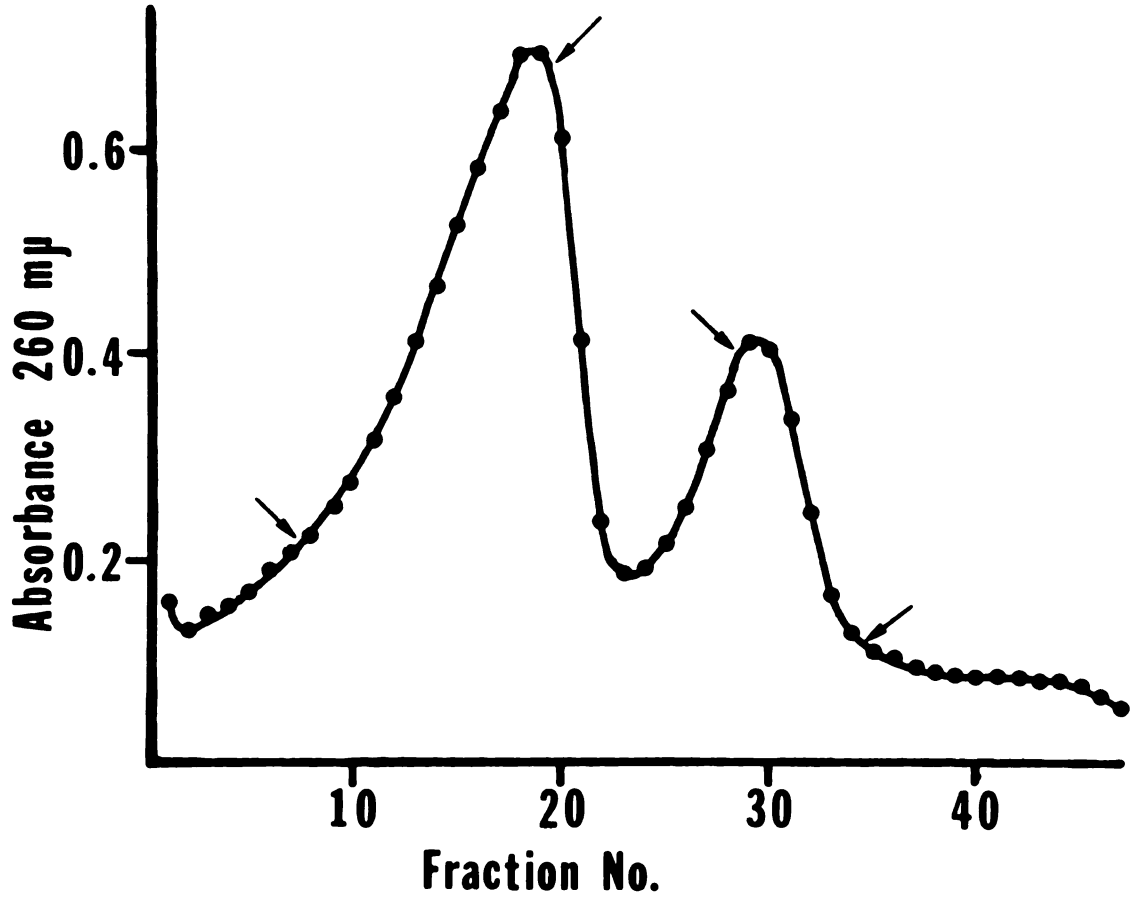

Figure 19: Sucrose density gradient profile of cauliflower ribosomal RNA. Centrifugation of the RNA (2 mg) was at 23,000 rpm for 16 hours in a linear sucrose gradient (4 to 20 percent) in 0.05 M NaCl, 1 x 10^{-4} M $MgCl_2$, 0.05 M sodium acetate, pH 5.3 at 0^{o}.

Using an identical incubation mixture as the one mentioned above except that the nucleosides were obtained from 10 mg of RNA, the proportion of radioactivity incorporated into the respective nucleosides shown in Figure 20 was obtained. Again both AMP and UMP appear to be the most highly labeled. Separation of the radioactive mixture was achieved by chromatography first in solvent system D for 3 days followed by elution from the paper and a second chromatography in solvent system C for 23 hours. A tabulation of the results illustrated in Figure 20 is given in Table 13.

Table 13: Incorporation of radioactivity into the nucleosides obtained from alkaline hydrolysis of 10 mg of cauliflower ribosomal RNA.

Nucleotide	cpm	Mole percent of total
AMP	6160	40
CMP	2670	17
GMP	2090	14
UMP	4450	29

In another instance 21 mg of RNA were hydrolyzed and the nucleoside residue obtained was incubated with 0.1 ml (^{32}P)p-nitrophenyl phosphate (0.53 μmoles/0.01 ml), 0.1 ml enzyme preparation, and 0.05 ml of 0.1 M sodium

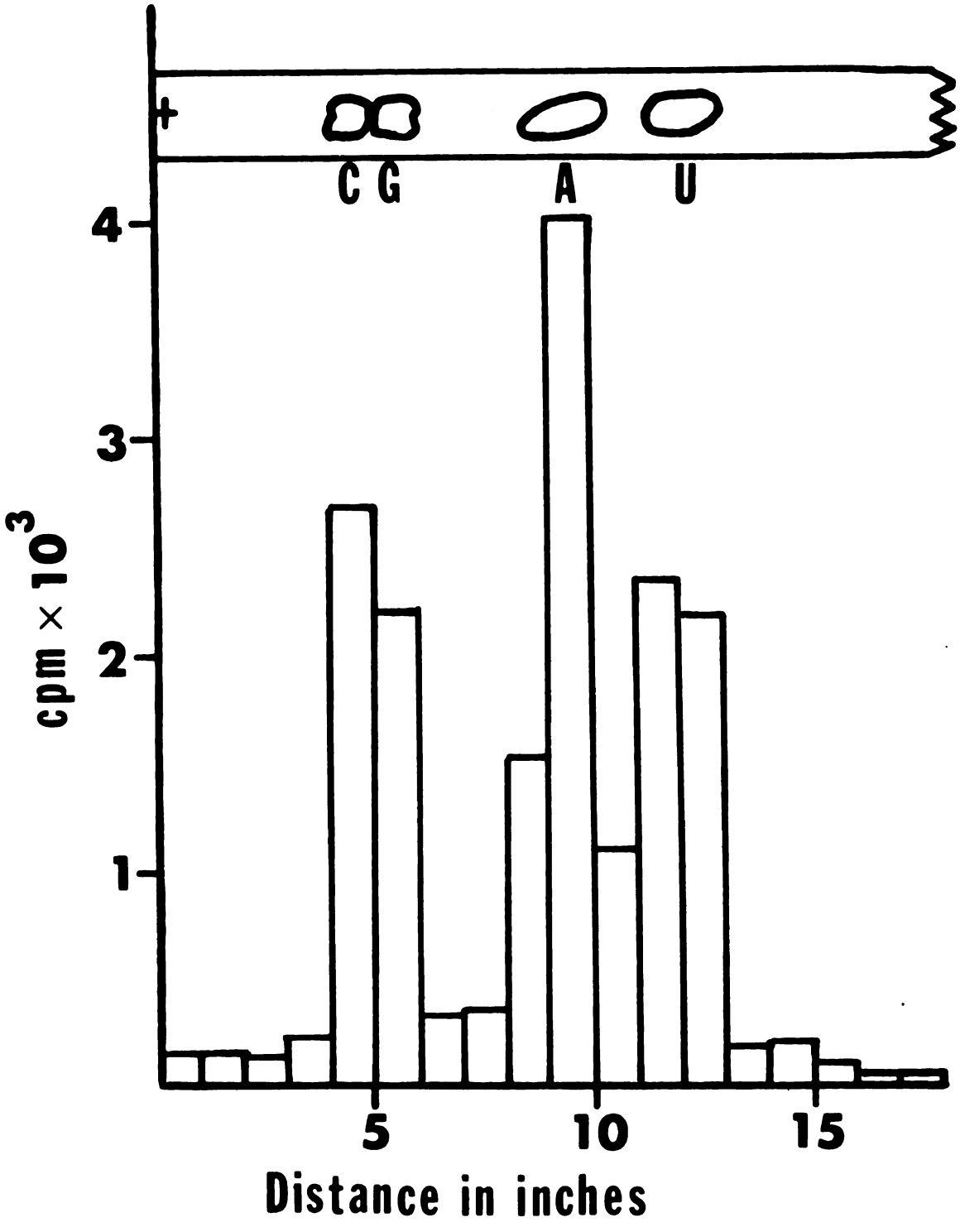

Figure 20: (^{32}P) Phosphate transfer to nucleosides isolated from the 5'-terminus of cauliflower ribosomal RNA. Chromatography in solvent system C after prior chromatography in solvent system D. The UV absorbing areas on the paper and the distribution of radioactivity are illustrated.

acetate buffer, pH 5.2 for 6 hours. Separation of the radioactive components was effected by chromatography in solvent system D, elution of the UMP area and the AMP, CMP, and GMP area from the paper. A second chromatography in solvent system G resulted in the separation of the nucleotides. The relative amounts of radioactivity incorporated are shown in Table 14. Since an area of radioactivity was located slightly behind and over-lapping the last part of the UMP area, presumably due to traces of (^{32}P)p-nitrophenyl phosphate, it was assumed that the radioactivity of the faster traveling half of the UMP spot was equal to that of the slower moving half. Calculations were made on this basis. In order to test the assumption, each of the respective UV absorbing nucleotide areas were eluted from the paper, absorbed and eluted from charcoal, and chromatographed in solvent system C. In this solvent system complete correspondence was noted between radioactivity and UV light absorbance areas of the paper. The relative amounts of radioactivity associated with each nucleotide following the charcoal treatment are also shown in Table 14.

Although losses of radioactivity did occur it should be noted that the assumption appeared to be correct.

Table 14: Radioactivity incorporated into the nucleosides obtained from 21 mg of RNA.

Nucleotide	First separation		Charcoal treated	
	cpm	mole percent	cpm	mole percent
AMP	26076	53	11925	61
CMP	8824	18	2062	11
GMP	1053	2	616	3
UMP	13300	27	4818	25

Assuming an average of 2250 nucleotide residues per RNA molecule, the incorporation as calculated from the first separation was 24 percent of the theoretical amount.

Ribosomal RNA subunits were obtained after separation by sucross density gradient centrifugation. Fractions from both subunits were collected as indicated by the arrows in Figure 19. Only the first half of the larger and the second half of the smaller subunits were collected. The nucleosides obtained from the hydrolysis of 7.0 mg of RNA from the larger subunit and 3.5 mg from the smaller subunit were incubated with 0.1 ml enzyme preparation, 0.1 ml p-nitrophenyl phosphate (0.53 μmoles/0.01 ml) and 0.05 ml of sodium acetate buffer, pH 5.2.

The mixture was incubated for 6 hours. Following chromatography in solvent system D and elution of the nucleotides from the paper, AMP, CMP, and GMP were separated by chromatography in solvent system G while UMP was further purified by electrophoresis. The results obtained are given in Table 15. Although these results are quite preliminary, they indicate that adenosine terminates both subunits and that uridine terminates the smaller subunit. A relatively large amount of radioactivity was found to be present in the CMP area. Although the amount of cytidine detected may be real, it is also conceivable that the radioactivity in the CMP area could be due to the elution of trace amounts of CMP from the quaternary amine resin column by the 0.002 N HCL. Phosphatase activity present in the enzyme preparation could have hydrolyzed this CMP to cytidine which in turn could have been phosphorylated.

Table 15: Radioactivity incorporated into the nucleosides obtained from 7.0 mg of large subunit ribosomal RNA and 3.5 mg of small subunit ribosomal RNA.

Nucleotide	Large Subunit		Small Subunit	
	cpm	Mole Percent	cpm	Mole Percent
AMP	14250	52	5310	34.8
CMP	9470	34	6375	41.7
GMP	2467	9	*	
UMP	1467	5	3580	23.5

*Not significant from background.

DISCUSSION

Nucleoside Phosphotransferase

Although an extensive characterization of nucleoside phosphotransferase was not performed, certain comparisons can be made between the preparation isolated from carrot leaves and the preparation isolated by Tunis and Chargaff (1956, 1957, 1960a, 1960b) from carrot roots. Evident in both instances is the specific transfer to the 5'-position of nucleosides as opposed to the formation of both 3'- and 5'-monophosphates by bacterial and animal phosphotransferases (Brawerman and Chargaff, 1954, 1955; Maley and Maley, 1963; Mitsugi, Komagata, Takahashi, Iizuka, and Katagiri, 1964), a pH optimum of 5.2, and the general presence of phosphatase activity in the transferase preparations. Also it should be noted that similar proportions of nucleotides were synthesized when they were incubated individually as with their preparation. Differences in the two systems were noted in the effect of cupric ions on the synthesis of nucleotides

with the purified enzyme. With the enzyme prepared by Tunis and Chargaff (1960a) a net increase in nucleotides synthesized was observed in the presence of cupric ions. This result conceivably was due to either a decrease in the phosphatase activity, an increase in the transferase activity, or both. In direct contrast, in this study a net decrease in UMP was synthesized. This difference in the effect of cupric ions could be due to a preferential inhibition of a contaminating phosphatase in their preparation. It remains to be determined whether the enzymes are identical.

An intriguing problem is the question of the physiological function of the enzyme in the plant. It is conceivable that the enzyme may actually possess both phosphatase and transferase activity since Georgatos (1967) has reported phosphate transfer from nucleotide donors to nucleoside acceptors by placental alkaline phosphatase. In this preparation, ratio of donor to acceptor to obtain maximum phosphate transfer of 5.8 percent was 1000 : 1. At the present an assessment of the dual role of the enzyme studied here cannot be made since only partially purified preparations were employed

containing both phophatase and transferase activities. Even though a comparison of phosphatase and transferase activities eluting from the DEAE-cellulose columns is not completely valid, since different concentrations of substrate were used for each assay, it appears that phosphatase and transferase activity do not completely coincide and thus separate enzymatic activities are indicated. If they indeed were separate enzymes, it is conceivable that the transferase enzyme could function in a biochemical pathway for the synthesis of nucleotides different from the conventionally described schemes.

(^{32}P)p-Nitrophenyl Phosphate

Some criteria for the synthesis of a radioactive derivative of a compound proposed to have further experimental use have been met in that only inexpensive and readily available chemicals and equipment were utilized in the synthesis, the yield of product was substantial, and the synthesis procedure is easy to perform. The most expensive starting material was (^{32}P)H_3PO_4; cheap in terms of radioactive derivatives.

Although (^{32}P)p-nitrophenyl phosphate itself could be useful in the detection of minute quantities

of phosphatase activity, transferase assays, and for investigating the mechanisms of enzymatic reactions, it is conceivable that the intermediate in the reaction scheme; namely, $(^{32}P)POCl_3$, might have a much greater applicability since it reacts readily with hydroxyl groups and thus could be used for the formation of phosphate esters. In combination with nucleoside phosphotransferase, radioactive nucleotides and other phosphate acceptors could be synthesized which by themselves could have substantial value in scientific research.

5'-Linked Terminal Groups

On the basis of the findings of this study and the results obtained by Pollard (1967) it is evident that adenosine and uridine are the primary 5'-linked nucleoside termini of unfractionated cauliflower ribosomal RNA. In terms of relative concentrations, it appears that adenosine is the terminal nucleoside almost twice as much as uridine. These results agree with those obtained from ribosomal RNA of E. coli (Lane, 1962; Midgley and McIlreavy, 1966; and Nichols and Lane, 1967), L cells (Lane and Tamaoki, 1967), and rabbit reticulocytes (Hunt, 1965). Obvious differences

are noted in the results obtained from wheat germ ribosomal RNA where all four nucleosides were found to terminate the 5'-linked end (Lane, 1965; Lee and Gilham, 1965).

An assessment of the terminal group specificity cannot be made at present since very little is known about the biosynthesis, structure, and function of ribosomal RNA. In comparison to transfer RNA where the -C-C-A sequence is obligatory for the acceptance of amino acids, it may be that specific terminal groups are necessary for "active" ribosomes. Enzymes which transfer specific nucleotides to the 5'-linked terminal end of transfer RNA have been reported (Daniel and Littauer, 1963; Klemperer and Canellakis, 1966). Similar enzymes may be existent which add specific nucleotides to ribosomal RNA. The finding by Midgley and McIlreavy (1967) that the 5'-linked terminal nucleosides of E. coli RNA can be enriched for uridine by culturing them on broth or casamino acid media instead of glucose or succinate media would tend to indicate that there is some variability in terminal groups, possibly due to turnover of the termini.

Evaluation and Future Use of the Technique

The technique proposed for the detection and identification of minute quantities of nucleosides, definitely is feasible but at present will only yield semi-quantitative results, although it is not known whether 100 percent transfer would occur if all the phosphatase activity were eliminated. In any case, before completely quantitative phosphorylations can be achieved, a complete purification of the enzyme and the removal of phosphatase activity may be required, which in turn may alleviate the requirement of adding large quantities of substrate in order to obtain adequate phosphate transfer.

A second aspect of the technique which should be investigated is the development of a better method for the separation of (^{32}P)p-nitrophenyl phosphate, and the four labeled nucleotides. The separation technique now utilized is lengthy and quite laborious since the first chromatography in solvent system D requires 3 to 4 days for developing and the labeled nucleotides must then be eluted from the paper. A two-dimensional chromatographic procedure or a combination of electrophoresis and chro-

matography might prove to be useful.

Additional experiments should also be performed dealing with the 5'-linked termini of the individual subunits of cauliflower ribosomal RNA. These experiments would give more conclusive results regarding the relative proportions of adenosine and uridine terminating these subunits.

Several suggestions for future investigations can be proposed. The first and the one previously mentioned is to determine whether the phosphatase and transferase activity can be separated. It is also conceivable that an investigation of the 3'-linked terminus of RNA could be conducted with the enzyme at the present stage of purity since it does not possess phosphodiesterase activity. The experiment would first involve a pre-incubation of the ribosomal RNA with the enzyme to remove the 5'-phosphate constituent from the end of the chain. After the pre-incubation period, (^{32}P)p-nitrophenyl phosphate would be added and hopefully, during another incubation period, the terminus would again be phosphorylated. The terminal nucleoside diphosphate obtained after alkaline hydrolysis could then be identi-

fied. It is not known whether the enzyme will remove the phosphate from the end of a polynucleotide chain or whether it will phosphorylate a polynucleotide chain but theoretically, it appears to be a worth-while investigation. Other aspects of the problem which deserve investigation include 1) donor specificity, 2) acceptor specificity, 3) the physiological significance of the enzyme, and 4) a determination of the mechanism of the reaction.

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ERRATUM

The correct designations of the ordinates of Figures 10, 11, and 12 should be μmoles or mμmoles of the respective product instead of μM or mμM.