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The presented study was undertaken to determine the operational catabolic carbohydrate pathway(s) contributing to the synthesis of the DNA of the actinophage for *Streptomyces griseus*. The incorporation of glucose-6-^{14}C versus glucose-1-^{14}C was determined for the deoxyribose and purine and pyrimidine bases of the phage DNA. The specific activities of the pentose and base moieties were calculated. Methods for the purification of the phage and isolation of the phage DNA were used. Development of deoxyribose isolation and quantitation procedures were of major importance in the investigation. The purine and pyrimidine bases were determined by standard methods. Radiotracer experiments were performed by liquid scintillation technique.

It was concluded that the *S. griseus* phage DNA deoxyribose was synthesized primarily by the

non-oxidative transketolase-transaldolase series of reactions. A minor deoxyribose synthesizing pathway was determined to be the oxidative hexosemonophosphate scheme. The phage DNA bases were not synthesized via a carbohydrate mechanism.

BIOSYNTHESIS OF *STREPTOMYCES GRISEUS* PHAGE DEOXYRIBONUCLEIC ACID

by

JUDITH ANN SHEARER

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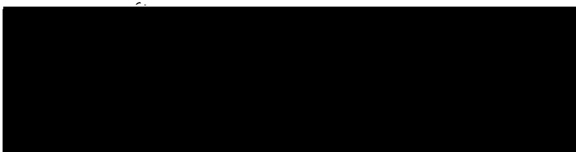

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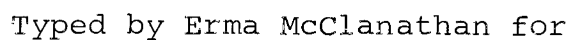
Typed by Erma McClanathan for 

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TABLE OF CONTENTS

INTRODUCTION.......... 1

HISTORICAL REVIEW.......... 2

Early Investigators.......... 2
Animal Virus Studies.......... 4
Ribose, Precursor of Deoxyribose.......... 5
Biosynthesis of Deoxyribose.......... 9
Streptomyces Host-Phage System.......... 15

MATERIALS AND METHODS.......... 17

Spore and Phage Stocks.......... 17
Preparation of High Titer Lysates.......... 18
Phage Purification.......... 19
Isolation of Phage DNA.......... 21
Hydrolysis of DNA to Release Deoxyribose.......... 22
Quantitation of Deoxyribose.......... 24
Paper Chromatography of Deoxyribose.......... 25
Hydrolysis of Phage DNA to Release Bases.......... 25
Paper Chromatography of DNA Bases.......... 26
Quantitation of DNA Bases.......... 26
Radiotracer Experiments.......... 27

RESULTS AND DISCUSSION.......... 30

Input Ratio of Labelled Glucose.......... 30
$^{14}CO_2$ Evolution During Lysis.......... 31
Specific Activity of Deoxyribose.......... 34
Quantitation of Deoxyribose.......... 34
Comparative Radioactivities of Deoxyribose Samples.......... 36
Comparative Radioactivity of the DNA Hydrolysates.......... 37
Specific Activity of the Bases.......... 39
Quantitation of the Bases.......... 39
Radioactivity of the Bases.......... 41
Theoretical Synthesis of Deoxyribose.......... 43

SUMMARY.......... 47

BIBLIOGRAPHY.......... 48

LIST OF TABLES

Table

I. Comparative Input Values for Radiotracer Experiments.................... 30

II. Quantitation of Deoxyribose Derived from ^{14}C Labelled S. Griseus Phage DNA.......... 36

III. Observed ^{14}C Specific Activity of Isolated Phage Deoxyribose................. 37

IV. Comparative Labelling of Phage DNA Enzyme Hydrolysates........................ 38

V. Comparative Incorporation of Glucose-1-^{14}C and Glucose-6-^{14}C by the Phage Purine and Pyrimidine Bases........................... 40

VI. Specific Activity of the Carbon for Bases and Deoxyribose...................... 42

LIST OF FIGURES

Figure

1. Radiorespirometric Pattern for the Utilization of Glucose-1-^{14}C During Host Lysis.......................... 32

2. Radiorespirometric Pattern for the Utilization of Glucose-6-^{14}C During Host Lysis.......................... 33

3. Standard Curves for the Determination of Deoxyribose.............................. 35

4. Theoretical Pathways for Deoxyribose Synthesis................................ 44

BIOSYNTHESIS OF STREPTOMYCES GRISEUS PHAGE DEOXYRIBONUCLEIC ACID

INTRODUCTION

The nucleic acid of viral particles is capable of entering specific host cells and subsequently directing the cell mechanism to produce viral components rather than cell components. This parasitic dependence of viruses for host metabolites and enzymes is eventually fatal to the host cell. While the virus must depend on the host cell for its metabolic equipment, it is feasible that the major routes of cell biosynthesis may be altered and that a minor pathway may become more important in virus production. Such an alteration in the metabolism of an infected cell may be important in the chemotherapy of viral diseases.

The primary objective of the present investigation was to study the pathway(s) of carbohydrate catabolism operative during *Streptomyces griseus* bacteriophage synthesis. The comparative incorporation of glucose-1-^{14}C and glucose-6-^{14}C into the phage particle was considered to be indicative of the operational metabolic pathway(s). However, the primary emphasis was placed on extent of the labelling of the deoxyribonucleic acid, especially the pentose moeity since its formation from glucose has been well studied. The ^{14}C labelling pattern of the purine and pyrimidine bases was also considered.

HISTORICAL REVIEW

Early Investigators

For many years, virology was primarily concerned with the chemical characterization of virus particles. However, during the past two decades greater emphasis has been placed on the metabolic behavior of virus-infected cells.

A principal investigator in the virus biosynthesis field has been Dr. Seymour Cohen. He chose the bacteriophage which infects *Escherichia coli* for his virus studies because this host-phage complex offered a well-controlled biological system. In 1946, Cohen and Anderson reported that the addition of T2 phage or T4 phage to *E. coli* grown on glucose did not significantly alter the oxygen consumption or respiratory quotient from that of the uninfected cells (16). A study by Joklick of the oxidation of certain carbohydrate substrates utilized by T1 and T2 infected *E. coli* supported Cohen's findings (29). However, in later studies, Cohen reported that the pattern of synthesis with respect to nucleic acid changed markedly after infection with phage. In infected cells only the DNA fraction increased in contrast to normal cells which showed three times as much RNA synthesized as DNA (13).

A further question related to the source of viral components in terms of uptake from the medium versus direct transfer from *in situ* host components. Using ^{32}P, Cohen (14) demonstrated that phosphorylated virus constituents were synthesized in the main from P assimilated in the medium after infection. In the same experiment it was shown that RNA had a very low turnover rate and was not a precursor of virus DNA.

Cohen (15) further showed that when gluconate served as the sole source of carbon for *E. coli* growth, more RNA was produced than DNA. However, this carbohydrate was found to be a poor carbon substrate for virus synthesis during which time an increased demand for DNA appeared. Therefore, Cohen concluded that during glucose utilization by *E. coli* ribose was derived mainly from the oxidative, pentose phosphate (PP) pathway and deoxyribose may come from constituents generated most readily from the glycolytic Embden-Meyerhoff-Parnas (EMP) pathway.

Cohen and Roth (19) also showed that virus infection does not inhibit the enzymes of the phosphogluconate pathway and that the PP pathway was induced to function by the appropriate choice of substrates; however, if glucose was offered, these enzymes tended not to function (11).

Animal Virus Studies

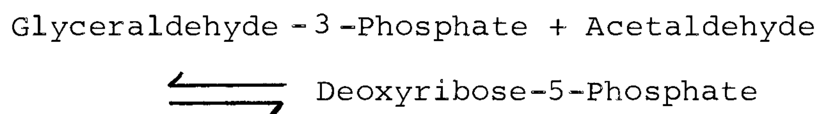
With monkey kidney tissue, Levy and Baron (33) found that infection with poliomyelitus virus type III very quickly led to increased glycolysis under both aerobic and anaerobic conditions. Becker, Grosswics and Bernkopf (5) found a higher glucose uptake with poliovirus type II in human amnion cell cultures than in control cultures. Inhibitors of oxidative pathways (PP) of glucose metabolism did not alter cell maintenance or virus reproduction. It was therefore assumed that glycolytic rather than oxidative PP processes were important in the synthesis of this virus-host system. Tissue culture studies by Planterose (43) on foot and mouth disease virus showed a progressive decrease in PP reactions and an increase in glycolysis. Apparently, the oxidative reactions supplied the energy for virus synthesis, but these reactions could be replaced completely by glycolysis.

Zelma (60) also carried out inhibitor studies of virus synthesis. Using either Hela cells, with normal high EMP pathway activity, or chick embryo cells, with a primary PP pathway, it was found that tick-borne encephalitis virus infection required glycolytic cleavage of glucose for further virus synthesis. In both host tissues, glycolysis appeared essential for the virus production and could not be circumvented by any

alternative metabolic pathway.

Ribose, Precursor of Deoxyribose

Before 1952, little progress had been made in investigating the biosynthetic pathways involved in the synthesis of the sugar moeity of DNA. Racker (44) isolated an enzyme from *E. coli* extracts which would catalyze the reversible reaction:



The enzyme, deoxyribose-phosphate aldolase, was found to be widely distributed in microorganisms and animal tissues. The deoxyribose-phosphate aldolase was distinct from the aldolases which split fructose 1,6-diphosphate and ribose-5-phosphate. Deoxyribose-5-phosphate synthesized by Racker's enzyme system could be utilized for nucleoside formation. In consequence, the $C_2 + C_3$ condensation was theorized to be the normal pathway of nucleic acid synthesis. The finding that deoxyribose aldolase had a low affinity for acetaldehyde suggested that acetaldehyde may not be the normal substrate. It was considered possible that an aldehyde linked to a purine or pyrimidine precursor could condense with glyceraldehyde to directly synthesize nucleotides.

Manson (36) argued that polymeric RNA could not be

the precursor of DNA in T4r phage infected *E. coli*. Using the incorporation of glycine-2-^{14}C into the purines as an indicator of RNA metabolism, he observed no degradation of endogenous RNA, no incorporation of isotopic precursors into the existing RNA, and no loss of isotope from RNA labelled before phage infection. The inertness of RNA during phage infection was explained by the fact that *de novo* synthesis of RNA had been prevented. Evidence was presented which suggested that a conversion of ribose to deoxyribose occurred at the nucleoside or nucleotide level. Somatic cells of rats were not found to incorporate ^{15}N-labelled uracil or cytosine but administration of ^{15}N-labelled uridine and cytidine resulted in the incorporation into the nucleosides of both RNA and DNA as reported by Hammarsten, Reichard and Saluste (24).

An experiment by Rose and Schwigert (51) in which rats were injected with ^{14}C-labelled cytidine showed that the ribose of cytidine was converted to deoxyribose in the pyrimidine nucleosides of DNA. The counts on the pyrimidine base and sugar of RNA and DNA nucleosides were essentially identical, indicating that the base and sugar moeities were incorporated as a unit and that the intact ribonucleoside had served as a precursor of deoxycytidine units. Reichard (47) reported similar findings with chick embryos.

Using ^{32}P labelled ribonucleotides, Roll (49,50) found extensive dephosphorylation to occur before the pyrine and pyrimidine moeities were incorporated into RNA or converted to a deoxyribose derivative and incorporated into DNA of the viscera of the rat. Thus, ribose in nucleosidic linkage had been implicated as a precursor of the deoxyribose portion of thymidine in animal tissues.

Amos and Magasanik (2) used the _E. coli_ T1 phage system to determine whether the conversion of ribose to deoxyribose was of major importance for DNA synthesis. Radioactive uridine was fed to _E. coli_ which in turn were infected with phage. It was found that a significant portion of phage thymidine was derived from exogenous uridine, and that deoxyribose originated largely from ribose without dilution by unlabelled metabolites. Presumably, this conversion occurred without cleavage of the bond between pyrimidine base and pentose. McNutt has also shown the latter route to be quantitatively important in the biogenesis of DNA of _Neurospora_ (38,39).

With cell free extracts of _Salmonella typhimurium_, Grossman and Hawkins (23) demonstrated the formation of deoxyribonucleosides from corresponding pyrimidine ribonucleosides. Reichard and Ruthberg (48) reported that dCMP could be produced directly from CMP by particulate

free *E. coli* extracts; the reaction depended upon the presence of ATP, Mg^{++} and TPNH. In addition, Reichard (46) partially purified two protein fractions from *E. coli* which: (1) catalyzed the phosphorylation of CMP to CDP, (2) catalyzed the reduction of the ribonucleoside diphosphate to deoxyribonucleoside diphosphate. While studying the very active deoxyribosyl-synthesizing system induced in *E. coli* during phage infection Cohen and Barner (17) discovered that extraneous enzymes and inhibitors complicated isolation of the enzyme system. Extraction in spermidine stabilized the enzymes and eliminated DNA and significant amounts of phage induced RNA.

Reichard (45), Pizer and Cohen (42) theorized that in the reduction of ribonucleotides to deoxyribonucleotides that an intermediate, cyclonucleotide was involved. This compound, which was bound between position two of the pyrimidine ring and position 2' of the ribose by an addition reaction, could be cleaved to give the deoxyribosyl derivative. However, these cyclonucleosides have been metabolically inert. As pointed out by Pizer (42) this observation may only indicate a requirement for phosphorylation prior to cyclonucleoside formation.

Biosynthesis of Deoxyribose

Several possible routes for the biosynthesis of deoxyribose have been proposed. In the Entner-Douderoff (ED) pathway the conversion of 6-phosphogluconate to pyruvate and 3-phosphoglycerate has been shown.

An intermediate in the ED reaction sequence has been identified as 2-keto-3-deoxy-6-phosphogluconate. A decarboxylation of this intermediate compound should yield deoxyribose-5-phosphate (32). However, no evidence has been obtained for such a biological mechanism.

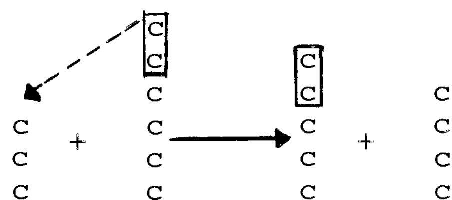
Many investigators have reported experiments which suggested that the pentose moeity of the deoxyribonucleotides was a product of ribose. Therefore, the problem became essentially that of determining the balance of operative pathways which contribute to ribose synthesis. According to Cohen's recent review (12), the balance of pathways varied according to the carbon source used and other pacterial growth conditions.

Lanning and Cohen (31) isolated the ribose from the RNA of *E. coli* grown on glucose-1-^{14}C. The ribose was found to be 20 to 30 per cent as active per mole as the initial glucose. These results suggested that the majority of the ribose was formed via the phosphogluconate pathway. However, a part of the ^{14}C labelled ribose may have been derived from the Embden-Meyerhoff pathway

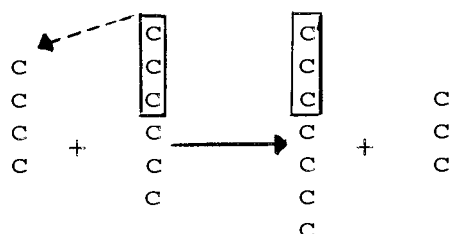
by a condensation of glyceraldehyde-3-phosphate and acetaldehyde. Alternatively, the ^{14}C labelled triose formed via the Embden-Meyerhoff pathway may have been equilibrated with ribulose-5-phosphate and subsequently with ribose from the phosphogluconate pathway.

In Horecker and Mehler's review of carbohydrate metabolism (27) it was proposed that a reversal of the transketolate-transaldolase sequence would result in ribose phosphate synthesis by the following mechanism:

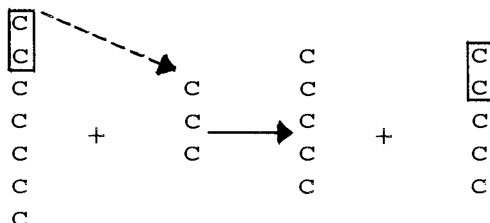
I. Transketolase catalyzed reaction of triose phosphate and hexose phosphate to yield pentose-phosphate and tetrose phosphate.



II. Transaldolase catalyzed reaction of tetrose phosphate with a second mole of hexose phosphate to yield heptulose phosphate and triose phosphate.



III. Transketolase catalyzed reaction of heptulose phosphate and triose phosphate to yield two pentose phosphate molecules.



The net result of the described mechanism was: two and one-half hexose molecules to yield three pentose molecules.

In another set of experiments, Lanning and Cohen (32) exposed normal E. coli and T6r^{+} infected E. coli to glucose-1-^{14}C as the sole carbon source. The deoxyribose of the bacterial DNA contained 20 to 30 per cent of the molar ^{14}C specific activity. Again, such results suggested that a loss of C_1, via the phosphogluconate route was of major importance in bacterial deoxyribose synthesis. That deoxyribose from the phage DNA contained 41 to 59 per cent of the ^{14}C molar specific activity of the medium suggested that the metabolism of the infected cell was altered by C_1 conservation, giving glycolysis a greater importance in the phage infected system than in the non-infected cells. The data also showed that deoxyribose had a molar ^{14}C specific activity of the same

order as found for ribose, suggesting the probable conversion of ribose to deoxyribose. This view was further supported by Horecker, Domagk and Hiatt who demonstrated similar ^{14}C labelling of ribose and deoxyribose in mammalian cells (26).

Bernstein, Fossit and Sweet designed a procedure to degrade deoxyribose from the nucleic acid of _E. coli_ grown on ^{14}C labelled glucose (6). Analysis of the bacteriological fermentation products revealed the relative specific activity of each of the component carbons of the deoxyribose. Bernstein and Sweet concluded that the labelling pattern indicated that a considerable portion of deoxyribose from _E. coli_ nucleic acid arose from hexose by loss of the C_1 carbon, although other pathways appeared to be involved (7,8). The discrepancies in the labelling patterns were partially explained by a transketolase-transaldolase (TK-TA) type of reaction. Bernstein and Sweet further reported the similarity in ^{14}C labelling patterns of ribose and deoxyribose derived from the same labelled carbon source. Purine and pyrimidine bound ribose was shown to have the same ^{14}C distribution pattern.

Loeb and Cohen (34,35) confirmed that the deoxyribose of all the deoxyribonucleotides of the DNA of _E. coli_ grown on glucose-1-^{14}C have essentially the same

amount of isotope, which, in turn, was the same amount of isotope as that incorporated into the ribose from each ribonucleotide of the RNA. Furthermore, it was found that the deoxyribose of the purine and pyrimidine bases of the DNA of phage T5 were also derived from a common pathway. Again, the phage deoxyribose showed an increased conservation of the C_1 of glucose over that of the non-infected cell deoxyribose.

The work of Bagatell, Wright, and Sable (4) indicated that a non-oxidative pathway was of even greater importance in the biosynthesis of ribose by E. coli than previous reports had deduced. Their ^{14}C labelling patterns were best explained by a combination of the transketolase-transaldolase and hexose monophosphate pathways. However, unlike previous investigations, acetate instead of glucose was used as carbon source. The data again confirmed that ribose and deoxyribose have the same precursor, or that deoxyribose is derived from ribose.

Cohen, Barner and Lichtenstein (18) incorporated uracil-2-^{14}C into T6r^+-infected E. coli. Ribonucleic acid comprising four per cent of the total RNA and resembling the base composition of the phage DNA was identified. The latter phage induced RNA selectively disappeared from the extracts and a large portion of its ^{14}C label appeared in deoxynucleotides. These

investigators concluded that the phage-specific RNA was degraded to 5-ribonucleotides which in the presence of reduced TPNH were rapidly converted to deoxyribonucleotides. This mechanism has been discussed in a review article by Champe (9).

More recently, Szynkiewics, Sable and Pflueger (54) have investigated pathways involved in _E. coli_ deoxyribose biosynthesis. They reaffirmed that the TK-TA pathway was responsible for the majority of nucleic acid pentoses when _E. coli_ was grown on acetate. Glucose, however, induced four-fifths of the pentose synthesis to take place by the direct oxidative (hexose monophosphate) pathway. Using a resting cell culture, different ^{14}C labelling patterns in the ribose and deoxyribose of the bacterial nucleic acids were obtained. It was suggested that two ribose pools were present in the cell, one which supplied the intermediates for RNA synthesis, the other for the synthesis of DNA. These pools may be separated either by subcellular organization or by time. The synthesis of RNA and DNA showed evidence of being asynchronous.

Wright, Sable and Bailey (57) observed no change in the pathways involved with a T2H _E. coli_ phage infected system over a non-infected _E. coli_ system. About sixty per cent of the pentose appeared to have been synthesized

via the oxidative pathway, the remainder by the TK-TA pathway. There was no evidence for a condensation of 2-carbon and 3-carbon precursors to yield deoxyribose. Again, it should be mentioned that these workers adapted their systems to acetate before exposure to glucose. As determined in a previous paper (54) age, carbon source and other conditions of bacterial growth and infection manifested a determinative effect on the metabolic pathway utilized for biosynthesis of phage and cellular components.

Streptomyces Host-Phage System

Time course experiments on the utilization of ^{14}C specifically labelled glucose and acetate by *Streptomyces griseus* have been carried out by Wang *et al.* (55). The results point to the operation of the Embden-Meyerhoff-Parnas glycolytic scheme in conjunction with the tricarboxylic acid cycle as the major pathways of glucose catabolism in this organism. A minor portion of glucose was metabolized by a direct oxidative pathway involving the C-1 decarboxylation of phosphogluconate. Supporting evidence for these pathways were shown with pathway inhibition studies by Hockenhill *et al.* (25).

Gilmour and Buthala (22) isolated a phage specific for *S. griseus* which was shown to be morphologically

similar to the tailed coliphages. Recently, Nishikawa and Gilmour (41) have demonstrated that the amino acids of the *S. griseus* protein coat are synthesized primarily by glycolysis although evidence for an operational pentose pathway is shown.

MATERIALS AND METHODS

Spore and Phage Stocks

Streptomyces griseus strain 3475 (Waksman collection) was the host cell used in all experiments. Stock cultures were maintained in sterile soil. Soil inocula was streaked on a medium containing: 1.0% glycerol, 0.1% asparagine, 0.1% glucose, 0.1% K_2HPO_4, and 1.7% agar. After incubation for two days at 30°C and five days at room temperature, the spore growth was scraped from the surface and transferred to a 0.25% peptone solution. This suspension was filtered through 30 layers of gauze. Spore plate counts were made and the volume adjusted to 7×10^8 spores/ml.

Actinophage strain 514-3, isolated by Gilmour and Buthala (22), was suspended in 0.25% peptone. The soft agar overlay technique of Adams (1) was used to obtain confluently lysed plates and high titered phage stocks. Glucose nutrient agar containing 0.5% glucose, 0.5% peptone, 0.3% beef extract and 0.01% yeast extract with 0.65% Bacto-agar for the overlay soft agar and 1.7% Bacto-agar for the base was used. Three ml of soft agar maintained at 45°C, 0.2 ml spore stock (7×10^8 spores/ml), and 0.1 ml phage suspension (about 1×10^{10} phage/ml) were mixed and poured over a plate of base agar. The

plates were incubated at 30°C for 15 hours by which time confluent lysis had occurred. The soft agar layer was then scraped into 0.25% peptone, shaken on a rotary shaker for ten minutes and centrifuged to remove the agar. The phage suspension was sterilized by filtration through a Seitz filter. Assay of the resulting phage stock was also performed by the soft agar overlay technique. However, several plates were prepared, each containing 0.1 ml of one of the various dilutions of the serially diluted phage stock in place of the 1×10^{10} phage/ml suspension. The number of individual lytic areas on the spore lawns generally showed the phage stock titer to be $1\text{-}5 \times 10^{10}$ plaque forming units/ml (pfu/ml).

Preparation of High Titer Lysates

One ml of spore stock (7×10^8 spores/ml) was added to 100 ml of germination media containing 0.20% glucose and 0.25% yeast extract, pH 7.0, and shaken on a rotary shaker at 30°C for 14 hours. The 100 ml of germinated culture was then added to a 2500 ml Fernbach flask containing about 900 ml of semi-synthetic media. The semi-synthetic media consisted of 800 ml distilled water, 8 ml of 5% yeast extract, 5 ml of 2% $CaCl_2$ and 100 ml of synthetic base medium adjusted to pH 7.0. The synthetic base medium contained 0.2% K_2HPO_4, 0.2% $(NH_4)_2HPO_4$,

0.007% $MgSO_4 \cdot nH_2O$ and a trace of $FeCL_3$, pH 7.0. A supply of air, passed through sterilized cotton filters, was bubbled into the transferred 14 hour cultures and then shaken on a reciprocal shaker at 30°C for two hours. At the end of this two hour adaptation period about one half of the initial glucose (or about 100 mg) had been removed from the media. Three ml of phage stock (6×10^{10} pfu/ml) was then added to the culture and aeration and shaking at 30°C was continued until complete lysis occurred, about 20-22 hours after the phage addition.

About 60% of the glucose present at the time of phage addition was utilized during the lysis period. Glucose was determined by modifying the method of Dubowski (20). To 6% o-toluidine in glacial acetic acid, 100 lambda of the sample was added. The mixture was boiled for ten minutes and the resulting optical density was read spectrophotometrically at 630 mμ. The optical density was referred to a standard curve to determine glucose concentration.

Phage Purification

The resulting lysates, with titers of $4\text{-}5 \times 10^{10}$ pfu/ml as determined by the soft agar overlay assay, were purified by differential and density gradient centrifugation according to the method of South (52). The

raw lysates were centrifuged in a Servall GSA rotor at 5000 G for one hour. The supernatant was filtered through Whatman #3 filter paper and the pellet of cell debris discarded. The filtered phage suspension was centrifuged in a Beckman L-2 ultracentrifuge #21 rotor at 16,000 rpm for 90 min at 10°C. The supernatant was discarded and each pellet allowed to resuspend overnight in two ml of glycerol solution at 4°C. The glycerol solution consisted of: 1.5% glycerol, 0.01 M $CaCl_2$, and 0.01 M $MgCl_2 \cdot 6H_2O$, pH 7-8.

The resuspended phage was centrifuged in a Servall SS-34 rotor at 3000 G for ten minutes to remove extraneous material. The supernatant was again centrifuged in the Beckman L-2 ultracentrifuge. The #30 rotor was used at 19,000 rpm for 90 minutes at 10°C. Again, the phage pellet was resuspended in the glycerol solution. A fresh solution of DNAse and RNAse in 0.1 M Tris buffer, pH 7.0, was added to the phage suspension so that the final concentration of each enzyme was 2 ug/ml. The mixture stood for 60 minutes at room temperature to allow destruction of host nucleic acids. The suspension was again centrifuged in the Servall SS-34 rotor at 3000 G for ten minutes. Three ml of the glycerol suspension was placed in a small cellulose nitrate tube with 2.5 ml of saturated cesium chloride solution and centrifuged in the

SW-39 swinging bucket rotor of the Beckman L-2 ultracentrifuge at 29,000 rpm for 18 hours at 10°C.

A phage band formed in the center of the tube was isolated by using a knife tube slicer. The phage band was removed into a small volume of 0.01 M tris buffer with 0.01 M $CaCl_2$ and 0.01 M $MgCl_2 \cdot 6H_2O$ at pH 8.0. The phage suspension was dialyzed against this same buffer for 36 hours.

Isolation of Phage DNA

A combination of detergent, salt, and phenol techniques with subsequent ethanol precipitation according to the modified method of Foldes and Trautner (21) was used to isolate the DNA from the *S. griseus* phage. The dialyzed phage suspension was added to an equal volume of 0.2 M NaCl in 0.01 M Tris buffer at pH 7.2, containing 20 mg/ml of sodium lauryl sulfate. The mixture was allowed to digest at room temperature for 30 minutes, after which time an equal volume of phenol, recently distilled and saturated with 0.01 M Tris buffer at pH 8.0, was added. This phenol mixture was gently shaken for 15 minutes at 60°C. The layers were separated by centrifugation in the Servall SS-34 rotor at 5000 G for five minutes. The viscous aqueous layer was pipetted off and extracted twice with phenol; the lower phenol

layer and interface containing protein was discarded. To a tube containing the final extracted aqueous layer, 100% ethanol was slowly added by carefully pouring it down the side of the tube. The DNA fibers precipitated and were wound around a stirring rod. The isolated phage DNA was air dried.

Hydrolysis of DNA to Release Deoxyribose

Several methods of isolating the free deoxyribose or deoxyribose-5-phosphate from the phage DNA were attempted. Bromination, to release pyrimidine bound deoxyribose after the method of Itzhaki (28) was found inconvenient for the milligram amounts of phage DNA available. Degradation of DNA with 0.1N HCl and heat followed by treatment with Darco G Charcoal, according to the method of Coffey, Morse and Newburgh (10), did not result in a compound which could be isolated by paper chromatography. However, modification of the enzymatic method of Laland and Overend (30) proved useful for degradation of the phage DNA.

The DNA isolated from one liter of high titer phage lysate was dissolved in a two ml aqueous mixture containing 0.15 mg DNAse and 12.0 mg of $MgSO_4 \cdot nH_2O$. The pH was adjusted to 7.4 with 1% NH_4OH. This mixture was stoppered and incubated at 37°C for 24 hours. After the

first incubation period, one ml containing 0.6 mg of alkaline phosphatase and one ml containing 10 mg NH_4SO_4 was added. One per cent NH_4OH was used again to adjust the pH to 10.4. The phosphatase treated DNA was incubated at 37°C for 48 hours.

After the enzymes had been allowed to act, 25 mg of $Ba(OH)_3$ in 1.0 ml of hot H_2O was added to the reaction mixture. the precipitated sulfates were filtered from the mixture by passing it through Whatman #5 filter paper. To remove the organic bases the filtrate was shaken for 30 minutes with one ml of treated 1R-120 Amberlite resin. The resin was filtered and washed on Whatman #5 filter paper. The filtrate was then added to treated 1R-4B Amberlite resin and shaken for 30 minutes. The resin also was filtered and washed on Whatman #5 filter paper.

Ten grams of Amberlite 1R-120 resin were prepared by adding 52 ml of 10% HCl and then rinsing on a Buchner funnel with three liters of distilled water. The treatment of ten grams of Amberlite 1R-4B consisted of adding 360 ml of 2% $NaCO_3$ and then, in a Buchner funnel, washing with distilled water until the wash water was free of carbonate ions.

The filtrate resulting after the two resin treatments was concentrated to dryness at 40°C under reduced

pressure. Three ml of acetone was added to the residue and maintained at 0°C overnight. The acetone solution was then filtered through Whatman #5 filter paper and evaporated to dryness. The residue was resuspended in one ml of distilled water and reserved for further analysis.

Quantitation of Deoxyribose

Deoxyribose was estimated by using a modified version of the Waravdekar and Saslaw method (56). It was necessary to change the amount and concentration of some of the reagents in the reported method in order to obtain repeatable results. This test depends on periodate oxidation and the estimation of the ensuing malonaldehyde with 2-thiobarbituric acid. A standard curve, prepared using solutions of pure 2-deoxyribose, was obtained by using the identical method of analysis by which the phage deoxyribose was quantitated.

To three ml of distilled water in a tube, 100 lambda of sample was added. One-tenth ml of 0.025 N periodic acid in 0.125 N H_2SO_4 was mixed with the diluted sample and allowed to remain at room temperature for 20 minutes. One-half ml of 2% sodium arsenite in 0.5 N HCl was added and mixed well to stop the oxidation. An aliquot of one ml was removed to a tube containing 2.0 ml of 0.15%

thiobarbituric acid (pH 2.0). Tubes were fitted with tear drop condensors and heated in a boiling water bath for 20 minutes. The tubes were cooled at room temperature and the optical density measured at 532 mμ. The optical density was directly related to deoxyribose concentration by comparison with a standard curve.

Paper Chromatography of Deoxyribose

Whatman #1 paper previously washed with distilled water was spotted with the deoxyribose stock solution; a volume which contained at least 5 ug of the pentose was applied. One-way, descending chromatography was developed for 12 hours in a solvent of isopropanol, ammonia and water (7:1:2), according to the method of McDonald and Fletcher (37).

The spot, with an R_f of 0.65, was detected by spraying the developed chromatograph with a solution of 0.5 ml alanine, 0.5 g diphenylamine, 5.0 ml phosphoric acid and 50 ml of acetone. The sprayed chromatograph was placed in a 90°C oven for one minute to create a red-brown spot for visual detection.

Hydrolysis of Phage DNA to Release Bases

The phage DNA was hydrolyzed in 88% formic acid by the method of Wyatt (58). To the DNA from one liter of

high titered purified phage, 1.0 ml of 88% formic acid was added, and the tube sealed. Maintenance at 120°C for 15 hours was sufficient for complete hydrolysis. The tube was carefully opened and the acid evaporated at 75°C under reduced pressure. The residue was dissolved in 100 lambda of 1 N HCl.

Paper Chromatography of DNA Bases

Ten lambda of the hydrolyzed DNA was spotted on one corner of Whatman #1 paper. The two-way chromatographs were developed as described by Wyatt (59). The first solvent, isopropanol, concentrated HCl, and water (68:16.7:15.3) developed the chromatograph by the descending method for 24 hours. The chromatographs were then air dried in a hood for one hour. The second solvent, n-butanol, water and concentrated NH_4OH (100:18:3) developed the chromatographs for about 15 hours in the direction perpendicular to the first solvent. The chromatographs were again dried and then viewed with an ultraviolet lamp. The bases appeared as dark spots against a light colored background.

Quantitation of DNA Bases

Circles with a 4.5 cm diameter, containing the chromatographed bases, were marked and cut from the paper.

Similar circles from appropriate locations on the paper were cut to serve as blanks. The paper circles were shredded into screw cap tubes; five ml of 0.1 N HCl was added to the tubes and they were shaken for 48 hours. The acid was decanted from the paper and centrifuged at 10,000 G for 45 minutes to remove the particulate material. The optical densities of the solutions were measured in a Beckman DU-2 model spectrophotometer, using the appropriate blank to set the base line. Quantity of bases present could be calculated from the extinction coefficients using the formula:

$$\text{molar concentration} = \frac{\text{O.D. at maximum}}{\text{extinction coefficient}}$$

The extinction coefficients used were 13.1×10^3 at 262 mμ for adenine, 10.4×10^3 at 275 mμ for cytosine, 11.4×10^3 at 248 mμ for guanine, and 7.9×10^3 at 265 mμ for thymine.

Radiotracer Experiments

High titer lysates were prepared as previously described. However, at the point of phage addition, 50 μc of glucose-1-^{14}C was also added to one flask and 50 μc of glucose-6-^{14}C was added to another. As the Fernbach flasks were sparged with air at a constant flow rate, evolved $^{14}CO_2$ was trapped in 15 ml of

ethanol-ethanolamine (2:1). Each hour, fresh ethanol-ethanolamine was placed in the traps. One hundred lambda of each hourly $^{14}CO_2$ sample was counted in a Packard Tri-Carb Liquid Scintillation Spectrophotometer. The fluor consisted of 0.6% 2,5-diphenyloxazole (PPO) and 0.01% 1,4-bis-2-(5-phenyloxazolyl)-benzene (POPOP) in toluene. Five ml of ethanol-ethanolamine (2:1) and ten ml of fluor solution were placed in counting vials to which 100 lambda of sample were added. The counting was done at the balance point of the scintillation counter.

DNA was extracted from the labelled phage by the techniques previously described. One set of glucose-1-^{14}C and glucose-6-^{14}C labelled DNA was hydrolyzed to give free deoxyribose; another identical set was hydrolyzed to give free bases. Ten lambda of the enzyme hydrolysates were counted in the same manner as the $^{14}CO_2$ samples.

The labelled deoxyribose samples were suspended in one ml of water. Three hundred lambda were used to quantitate the samples in triplicate. Then, the volume of the suspension which contained 0.50 µg of deoxyribose was spotted for chromatography along with ten µg of pure deoxyribose. Several identical spots were applied, some were sprayed with the indicator, and the location of the other spots detected by using the sprayed spots as a standard. A lab monitor also helped indicate the area in

which the labelled deoxyribose could be located. Squares, four cm by four cm, containing the labelled deoxyribose, were marked and cut from the chromatographs. These squares were folded in half and placed in a counting vial with 20 ml of fluor solution. The fluor solution for all paper counting consisted of 0.4% PPO and 0.005% POPOP in toluene. These paper squares were counted by liquid scintillation at the balance point.

Bases labelled by incorporation of ^{14}C-glucose were quantitated as previously described. Identical chromatographs of the bases were made. Circles, 4.5 cm in diameter, containing the bases were cut out, folded in half, and placed in counting vials with 20 ml of the paper fluor solution and counted by liquid scintillation at the balance point.

RESULTS AND DISCUSSION

Input Ratio of Labelled Glucose

Since the data to be presented have been based on a comparison of the incorporation of ^{14}C from glucose-1-^{14}C and glucose-6-^{14}C, it was first considered critical to have identical input levels for the two glucose isotopes (50.0 μc). After addition of the labelled glucose, the flasks were well mixed, and an aliquot removed and centrifuged. Twenty lambda of the supernatant was counted by liquid scintillation as previously described. As shown in Table I, the input for glucose-6-^{14}C experiments was only slightly higher than the input value for the glucose-1-^{14}C experiments. While this difference in input values was probably insignificant, all data for glucose-1-^{14}C experiments were corrected by a factor of 1.010.

Table I. Comparative Input Values for Radiotracer Experiments.

Labelled substrate	Input cpm	Ratio of $\frac{\text{glucose-6-}^{14}C}{\text{glucose-1-}^{14}C}$
Glucose-1-^{14}C	1498	1.010
Glucose-6-^{14}C	1514	

$^{14}CO_2$ Evolution During Lysis

With a bacteriophage infected system the infected cells continue to metabolize phage components at a detectable rate for some time after infection. However, after several hours, the cells unable to metabolize their own substrates become inactive. This phenomenon was observed by evaluating the $^{14}CO_2$ evolved during the lysis period in the radiotracer experiments. As the lysing cultures were sparged with air, the evolved $^{14}CO_2$ was trapped in an ethanol-ethanolamine solution (2:1). Each hour the traps were replenished with a fresh ethanol-ethanolamine solution. Aliquots of the trapped $^{14}CO_2$ samples were counted as previously described. The comparative hourly and cumulative $^{14}CO_2$ activity may be observed for glucose-1-^{14}C, and glucose-6-^{14}C, in figures 1 and 2, respectively. During glycolysis the C-1 and C-6 carbons of glucose become the methyl group of pyruvic acid. Decarboxylation gives rise to acetate which is further oxidized via the Tricarboxylic Acid Cycle. If glycolysis was the only pathway functioning during phage synthesis, identical interval $^{14}CO_2$ recoveries should be obtained with both glucose-1-^{14}C and glucose-6-^{14}C. Clearly, this was not the case. Over one-third more $^{14}CO_2$ was evolved with glucose-1-^{14}C as compared to glucose-6-^{14}C substrate.

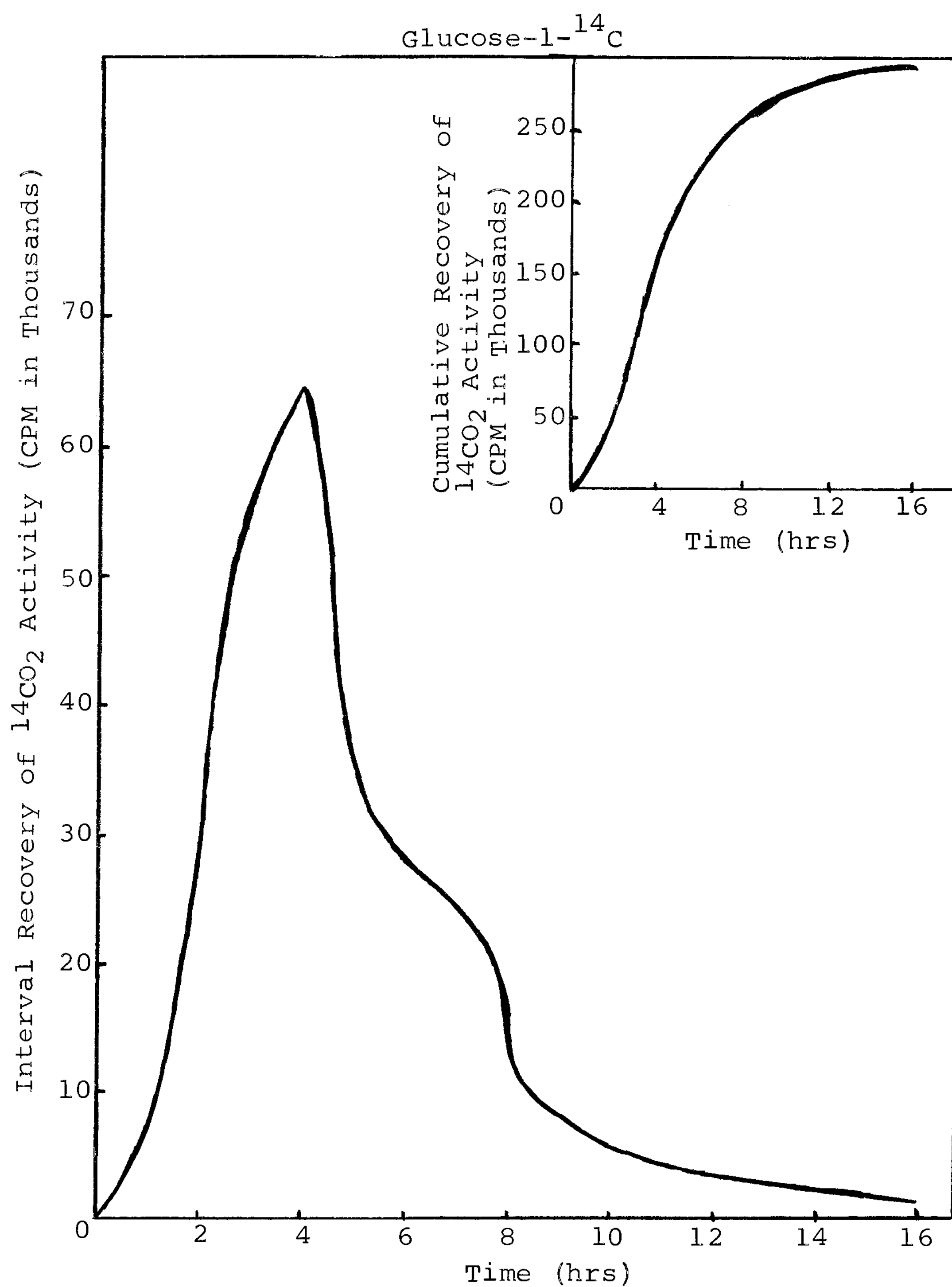


Figure 1. Radiorespirometric pattern for the utilization of glucose-1-^{14}C during host lysis.

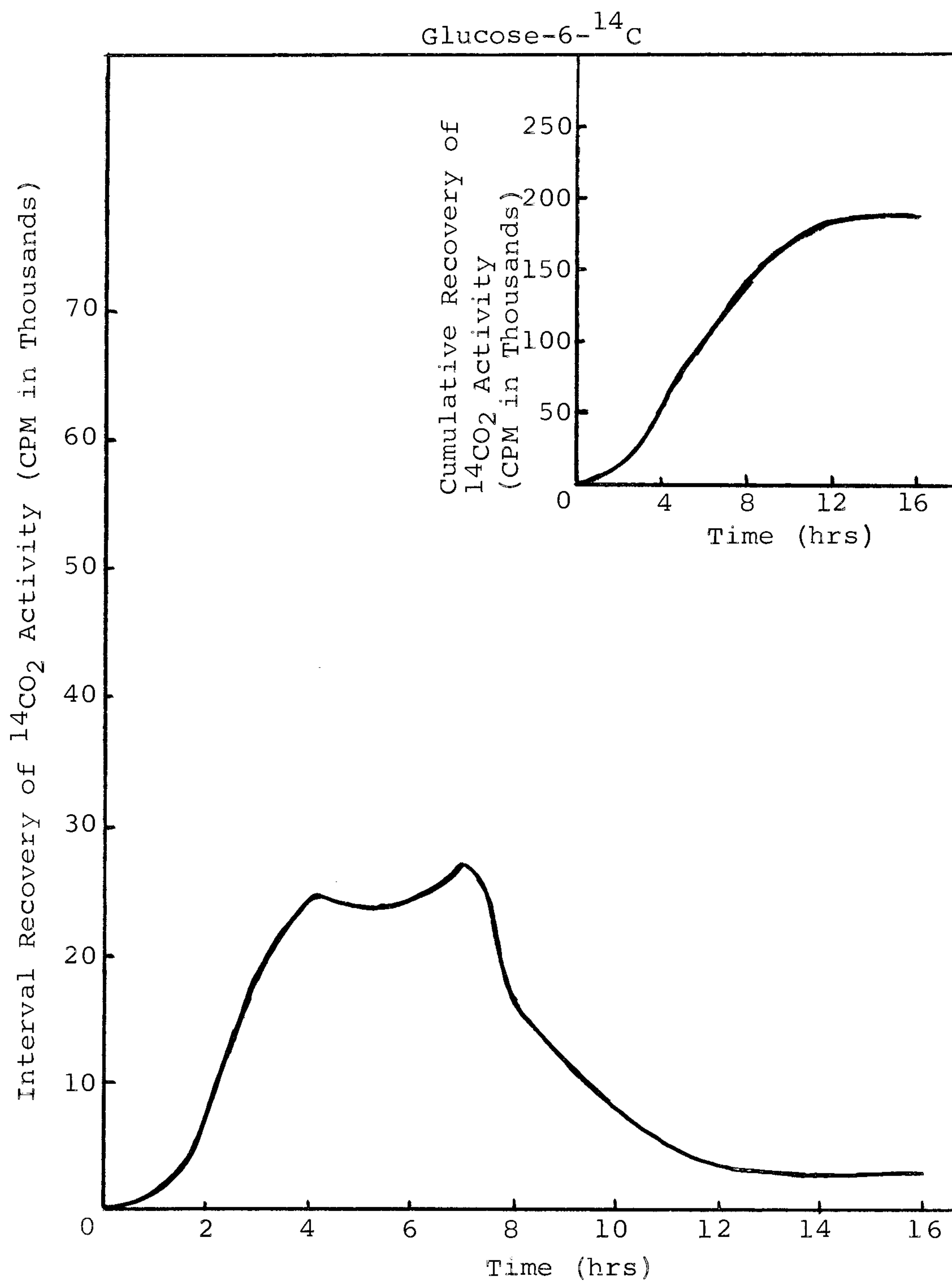


Figure 2. Radiorespirometric pattern for the utilization of glucose-6-^{14}C during host lysis.

Specific Activity of Deoxyribose

Quantitation of Deoxyribose

The method for the microquantitation of deoxyribose as described by Waravdekar and Saslaw (56) did not prove satisfactory; the results were erratic and not repeatable. Turbidity interfered with spectrophotometric readings. However, decreasing the concentration and amount of several reagents resulted in solutions which were not turbid. This modified method which was used in the quantitation of the deoxyribose for the radiotracer experiments was previously described (page 24). In figure 3 a sample standard curve from the original quantitation procedure is shown along with the standard curve for the modified method. The final standard curve used was a straight line graph based on averaged points.

Using the modified deoxyribose quantitation procedure, the samples of deoxyribose isolated from the ^{14}C labelled *S. griseus* phage DNA were analyzed. In this regard, 14.8 ug of deoxyribose were obtained for the glucose-1-^{14}C label and 13.0 µg of deoxyribose were derived for the glucose-6-^{14}C label. The pertinent yield data are shown in Table II.

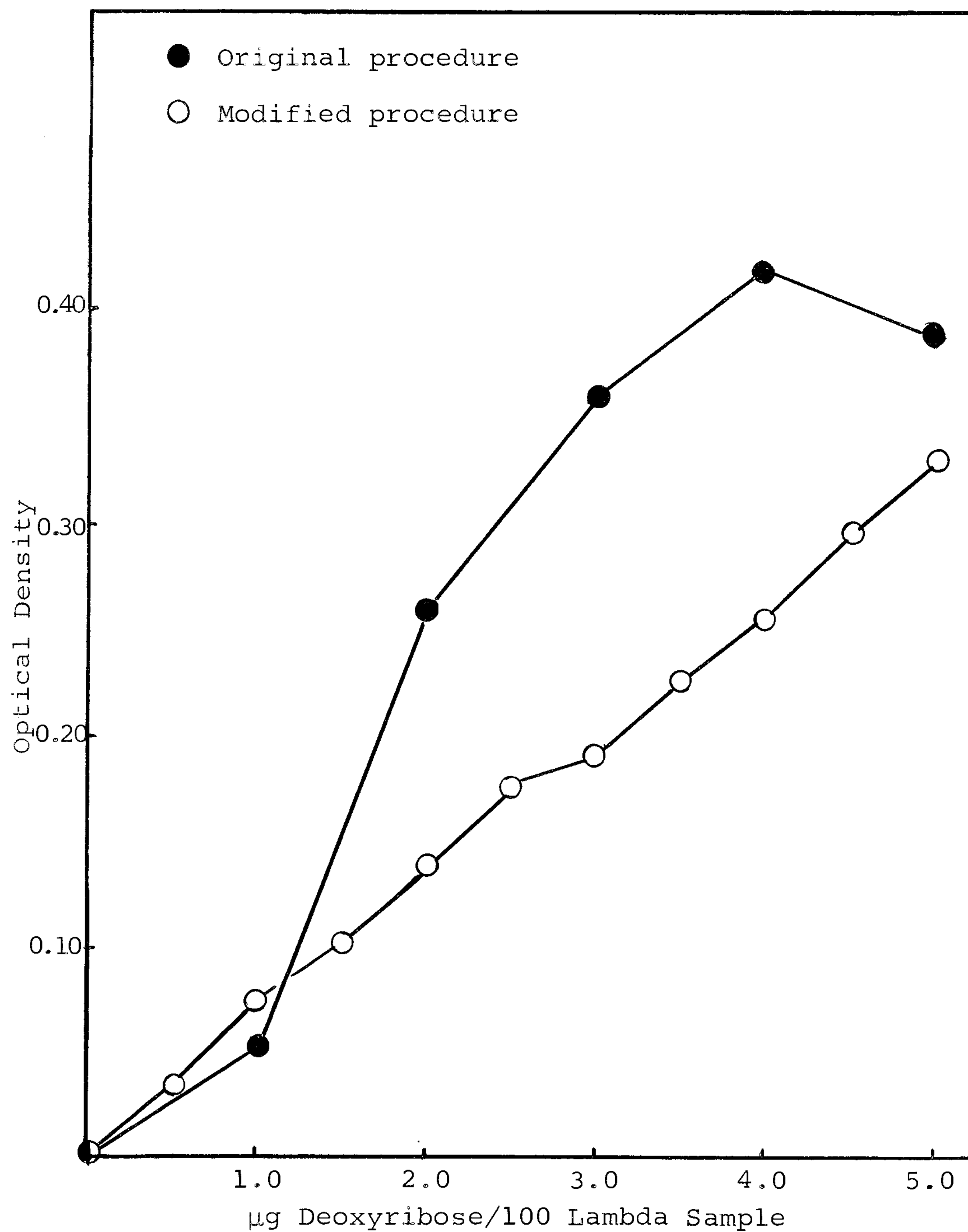


Figure 3. Standard curves for the determination of deoxyribose.

Table II. Quantitation of Deoxyribose Derived from ^{14}C-labelled S. Griseus Phage DNA.

Source of ^{14}C Label (Triplicate determination)	Optical Density	Average Optical Density	μg Deoxyribose per 100 λ
Glucose-1-^{14}C (a)	0.098		
Glucose-1-^{14}C (b)	0.098	0.097	1.48
Glucose-1-^{14}C (c)	0.095		
Glucose-6-^{14}C (a)	0.085		
Glucose-6-^{14}C (b)	0.087	0.086	1.30
Glucose-6-^{14}C (c)	0.090		

Comparative Radioactivities of Deoxyribose Samples

Using aliquots of the same deoxyribose samples which had previously been quantitated a volume equal to 0.50 μg was spotted on Whatman #1 filter paper and the deoxyribose isolated by paper chromatography as previously described. Sections of the paper containing the deoxyribose were counted by liquid scintillation as described previously. The counts per minute (cpm) for 0.50 μg of deoxyribose were obtained. In Table III the specific activities for the deoxyribose labelled via glucose-1-^{14}C and the deoxyribose labelled via glucose-6-^{14}C are depicted. Specific activity was determined in units of cmp/μg deoxyribose.

Table III. Observed ^{14}C Specific Activity of Isolated Phage Deoxyribose.

Source of ^{14}C Label in Deoxyribose	cpm/0.50 µg sample	Specific Activity cpm/µg Deoxyribose
Glucose-1-^{14}C	6402	12804
Glucose-6-^{14}C	8421	16842

It is evident that the incorporation of glucose-6-^{14}C into deoxyribose as compared to glucose-1-^{14}C was in the ratio of 16842/12802 or 1.30. A ratio of 1.30 implies that conservation of the C_6 and loss of the C-1 of glucose occurred during formation of the deoxyribose of the phage DNA.

Comparative Radioactivity of the DNA Hydrolysates

Aliquots of the enzymatically hydrolyzed DNA were counted by the liquid scintillation method. It was considered probable that aliquots of the hydrolysates which contained equal amounts of deoxyribose originally contained equal amounts of DNA. From the previous quantitation of deoxyribose for the radiotracer experiments it was found that the deoxyribose labelled via glucose-1-^{14}C had a DNA concentration of 14.8 µg/ml and that the deoxyribose labelled by glucose-6-^{14}C had a DNA concentration of 13.0 µg/ml. Since volumes were kept equal, the hydrolysates must originally have had 14.8/13.0 or

1.14 times greater DNA extracted with the glucose-1-^{14}C experiment as was obtained for the glucose-6-^{14}C experiment. Since equal volumes of the hydrolysates were counted the values obtained for the glucose-6-^{14}C hydrolysate were corrected by the value of 1.14 to account for the differences in DNA content.

The data given in Table IV show the comparative radioactivity of 20 lambda of the DNA hydrolysates. The correction factor of 1.14 for the glucose-6-^{14}C hydrolysate was considered. It was noted that the ratio of comparative incorporation by the glucose-6-^{14}C labelled hydrolysate as compared to the glucose-1-^{14}C labelled hydrolysate was 1.35. The comparative ratio for the labelling of the deoxyribose and the total hydrolysate were of the same order. Allowing for experimental variation, the ratios for the deoxyribose and the hydrolysates were virtually identical.

Table IV. Comparative Labelling of Phage DNA Enzyme Hydrolysates.

Source of ^{14}C Label in Hydrolysate	cpm	cpm corrected for DNA content	$\frac{\text{Glucose-6-}^{14}C}{\text{Glucose-1-}^{14}C}$
Glucose-1-^{14}C	15,533	15,688	1.35
Glucose-6-^{14}C	18,519	21,112	

Knowing the comparative incorporation ratio of 1.35 for the total DNA hydrolysates as compared to the 1.30 ratio obtained for the deoxyribose moeity suggested that both the entire DNA and deoxyribose moeities of *Streptomyces griseus* phage utilized identical pathways of incorporation for the ^{14}C-labelled glucose.

Specific Activity of the Bases

Quantitation of the Bases

Portions of the DNA derived from the radiotracer experiments as used for deoxyribose determinations were hydrolyzed in formic acid to release free purine and pyrimidine base by the method of Wyatt (58) as previously described. Aliquots of the acid hydrolysate were spotted and developed by paper chromatography. The separated bases were eluted from the paper and the optical densities were read, allowing calculation of the concentration of each individual base by the method outlined previously. In Table V the values obtained for the molar concentration of each base have been listed. Guanine proved to be highly insoluble and could not be eluted from the paper.

Table V. Comparative Incorporation of Glucose-1-^{14}C and Glucose-6-^{14}C by the Phage Purine and Pyrimidine Bases.

Bases	cpm	Molar Concentration	Specific Activity	Ratio: Glucose-6-^{14}C Bases / Glucose-1-^{14}C Bases
Adenine-1*	66	1.36×10^{-5}	49 cpm/10^{-5} moles	2.22
Adenine-6+	71	0.65×10^{-5}	109 cpm/10^{-5} moles	
Cytosine-1	207	3.46×10^{-5}	60 cpm/10^{-5} moles	2.48
Cytosine-6	308	2.07×10^{-5}	149 cpm/10^{-5} moles	
Thymine-1	843	6.92×10^{-5}	122 cpm/10^{-5} moles	3.01
Thymine-6	1381	3.75×10^{-5}	368 cpm/10^{-5} moles	
Guanine-1	134	1.87 (estimated	72 cpm/10^{-5} moles	2.19
Guanine-6	158	1.00 ratio)#	158 cpm/10^{-5} moles	

*1 denotes that the base was labelled via glucose-1-^{14}C

+6 denotes that the base was labelled via glucose-6-^{14}C

#Since molar concentration values were not obtained for guanine, the estimated yield of guanine-1 as compared to guanine-6 was calculated by averaging the yield of the three other base-1 as compared to the yield for the three base-6. In other words, the glucose-1-^{14}C experiment yielded 1.87 greater amount of base than the glucose-6-^{14}C experiment.

Radioactivity of the Bases

Another set of identical paper chromatographs as used for quantitation of the purine and pyrimidine bases were used for radioactivity measurements. Rather than eluting the bases from the chromatograph paper squares, the individual bases were cut out and counted by liquid scintillation techniques. The specific activity of each base was calculated in terms of cpm/10^{-5} moles of base as shown in Table V. Also in Table V the comparative incorporation of glucose-1-^{14}C versus glucose-6-^{14}C has been expressed as a ratio: $\frac{\text{specific activity of base-6}}{\text{specific activity of base-1}}$.

The comparative incorporation ratios for the bases had values of about 2.20 to 3.00, significantly higher than the ratio of 1.30 found for deoxyribose. Therefore, the biosynthetic pathway which was responsible for the loss of C_1 of glucose must have been more active in the formation of the organic bases than in the synthesis of deoxyribose.

The specific activity of the carbon of the bases and deoxyribose were calculated and expressed in terms of cpm/10^{-5} moles of carbon. These specific activities are shown in Table VI.

Table VI. Specific Activity of the Carbon for the Bases and Deoxyribose.

DNA moeity	cpm/10^{-5} moles of carbon
Adenine-1*	10
Adenine-6+	22
Cytosine-1	12
Cytosine-6	30
Thymine-1	24
Thymine-6	74
Guanine-1	--
Guanine-6	--
Deoxyribose-1	3,427,470
Deoxyribose-6	4,513,660

*1 denotes that the moeity was labelled via glucose-1-^{14}C

+6 denotes that the moeity was labelled via glucose-6-^{14}C

Table VI quite decisively showed that the carbon atoms of the deoxyribose were labelled with ^{14}C to a far greater extent than the carbon atoms of the bases. This phenomenon suggested that there was little active synthesis of the purine and pyrimidine bases of the S. griseus phage DNA as compared to a very pronounced synthesis of deoxyribose. Perhaps the bases for the phage DNA were derived from the breakdown products of the host cell nucleic acid or were derived from components of the media other than glucose, i.e., yeast extract.

Theoretical Synthesis of Deoxyribose

Through the various studies on biosynthesis of deoxyribose, four possible pathways have been suggested. These pathways and their theoretical relationship to deoxyribose are depicted in figure 4. It was first thought that the Embden-Meyerhof-Parnas end-products, glyceraldehyde-3-phosphate and acetaldehyde, condensed under the direction of an aldolase to form deoxyribose-5-phosphate. However, the aldolase reaction appeared to be more important in the breakdown of deoxyribose than in its synthesis. Thus, the glycolytic pathway as such was rarely considered important in the formation of deoxyribose.

Another pathway which theoretically could be involved in the biosynthesis of deoxyribose is the Entner-Douderoff pathway. An intermediate of the ED pathway, 2-keto-3-deoxy-6-phosphogluconate, theoretically may be decarboxylated to deoxyribose-5-phosphate. No occurrence of such a decarboxylation has been reported in the literature. Thus, in the light of reports which implicated ribose as a necessary precursor to deoxyribose it was not difficult to postulate that the ED pathway was not involved in deoxyribose synthesis.

A pathway which has been known to form ribose, was the Hexosemonophosphate pathway. The decarboxylation of

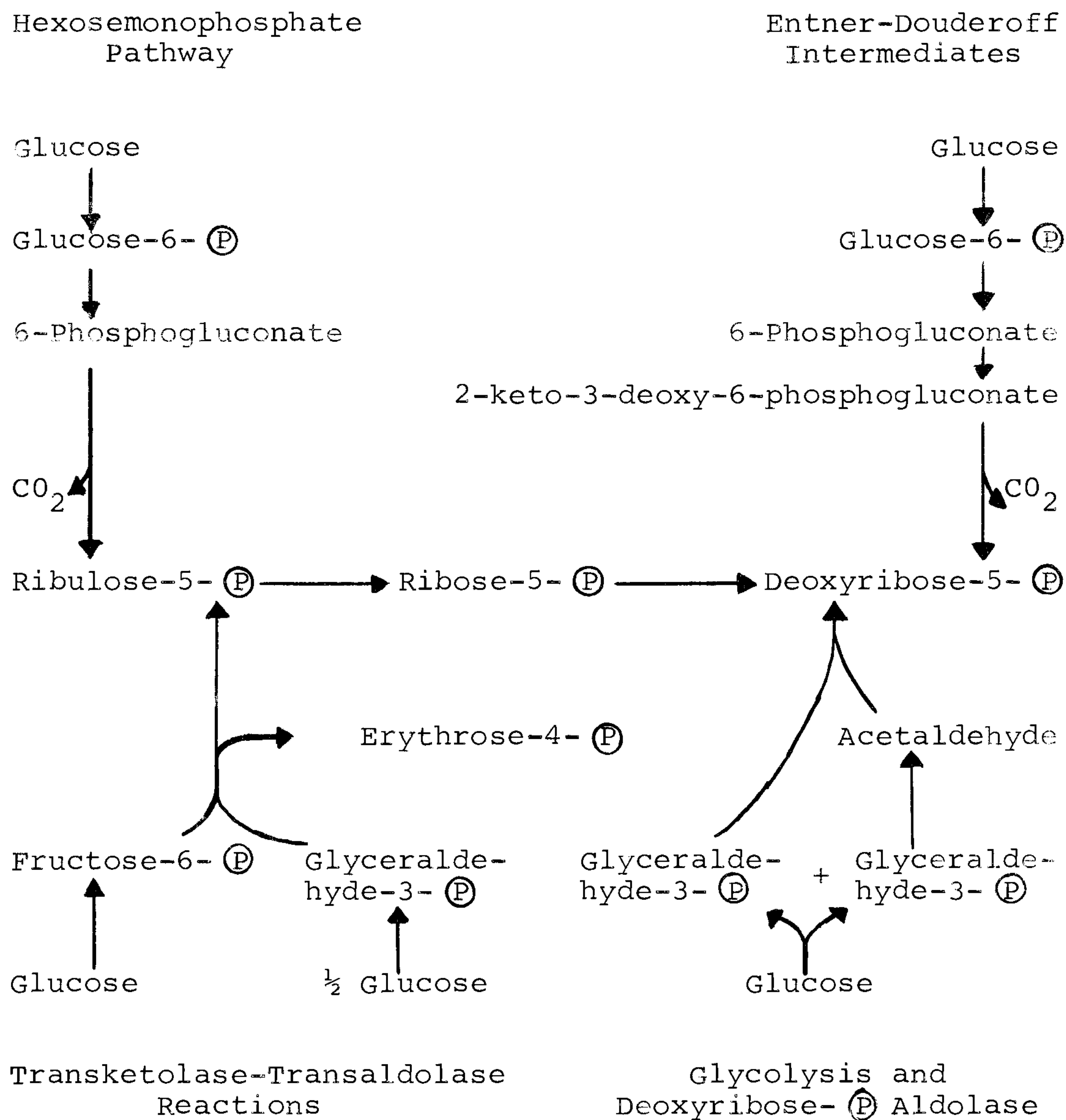


Figure 4. Theoretical pathways for deoxyribose synthesis.

6-phosphogluconate resulted in ribulose-5-phosphate formation. Ribulose-5-phosphate in turn was converted to ribose-5-phosphate by action of a phosphoriboisomerase. Such ribose synthesized via the oxidative HMP pathway may be reduced to form deoxyribose. It should be noted that the ribose formed by the HMP scheme would be in equilibrium with a glyceraldehyde-3-phosphate. It was possible for such a triose to have its origin in the EMP pathway and thereby influenced the labelling of ribose when radiotracer experiments were performed.

Another series of reactions resulting in ribose synthesis is called the transketolase-transaldolase pathway. The sequence of TK-TA reactions, which were described under HISTORICAL, result in 2½ glucose molecules giving rise to 3 ribose molecules. This non-oxidative pathway forms ribose which may enzymatically be converted to deoxyribose.

The comparative incorporation data for deoxyribose of the _S. griseus_ phage DNA showed that a mechanism which utilized both the C_1 and C_6 of glucose was involved. If C_1 and C_6 had both been incorporated to the same extent a ratio of 1.0 would have been expected. Since the ratio was of a slightly higher order, 1.3, some other pathway which was responsible for a C_1 loss was also operating. It has been shown that the comparative incorporation

ratio for the amino acids of an organism using exclusively an oxidative HMP metabolism is on the order of 20.0 (41).

The study presented suggested that the deoxyribose of *S. griseus* phage DNA is formed primarily by a non-oxidative pathway, although there was evidence for a slightly active oxidative pathway. In view of the literature, it is further suggested that the TK-TA was the non-oxidative major pathway and that the HMP was the oxidative minor pathway.

It is doubtful that any major change in metabolic pathways has occurred in the phage infected *S. griseus* as compared to the non-infected cells. The comparative incorporation of 1.3 is most likely a reflection of a major non-oxidative pathway and a minor HMP oxidative pathway which was reported previously for *S. griseus* (55). It would be interesting to compare *S. griseus* metabolism to the infected cell metabolism utilizing the method of comparative radiotracer incorporation data.

SUMMARY

The results and conclusions obtained from the study of the biosynthesis of *Streptomyces griseus* phage DNA may be summarized as follows:

1. Methods for the isolation and quantitation of deoxyribose were developed.

2. The specific activity of the phage DNA deoxyribose labelled with glucose-6-^{14}C versus glucose-1-^{14}C gave a comparative incorporation ratio of 1.30.

3. The specific activity of the phage DNA pyrimidine and purine bases labelled with glucose-6-^{14}C versus glucose-1-^{14}C gave comparative incorporation ratio of 2.20 to 3.00.

4. Deoxyribose was formed primarily by a non-oxidative pathway, probably the transketolase-transaldolase sequence, and to a lesser extent by an oxidative pathway, probably the hexosemonophosphate pathway.

5. The bases are synthesized by a slightly more active oxidative pathway than the deoxyribose but at an extremely lower rate. The bases are either synthesized from other components in the media or from breakdown products of the host cell nucleic acids.

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