

STUDIES ON THE BIOSYNTHESIS
OF STREPTOMYCES GRISEUS PHAGE

by

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Typed by Margaret Barber

TO MY HUSBAND

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STUDIES ON THE BIOSYNTHESIS
OF STREPTOMYCES GRISEUS PHAGE

INTRODUCTION

Phage biosynthesis is known to take place at the expense of the host cell. This process is initiated by adsorption of the infecting particle on the cell surface and the release of an activating substance (DNA) into the cellular material. The activating substance is then thought to divert the normal cell metabolism toward the biosynthesis of new phage progeny. Many aspects of the nature of this host-phage relationship remain obscure, particularly the sequence of events which occur during the period of phage biosynthesis.

The primary objective of the present investigation was to study the comparative carbohydrate metabolism of the Streptomyces griseus host-phage system. Attention was focused on any alterations which might occur in the pathways of carbohydrate catabolism, and on the detection of any changes in cellular biosynthesis of infected and non-infected cells. In view of the chemical nature of bacteriophage, it seems likely that this approach would elucidate further the mechanisms by which the biosynthesis of bacteriophage occurs. The conventional manometric technique (60, pp.1-16) and the radiorespirometric method (62, pp.1869-1872; 64, in press) were found to be well suited for these studies.

HISTORICAL

Early studies of bacteriophage involved the identification and characterization of phage using the electron microscope. It was observed that variation in size, shape and nutritional status occurred in the types of phage which were isolated (21, p.4). Bacteriophage specific for Escherichia coli have been shown to differ morphologically and physiologically, but they are all related in their specificity of attack on the same host, and for this reason have been the most widely studied. Gilmour and Buthala isolated a phage specific for Streptomyces griseus from the soil, which was shown to be morphologically similar to the tailed coliphages (27, p.17).

The S. griseus phage was further characterized by Noller and shown to be tad-pole shaped, having an overall length of 455 millimicrons. The head was approximately 95 millimicrons and the tail 360 millimicrons in length. Unlike the coliform phages, S. griseus phage has a long latent period (120 to 136 minutes), and most of the adsorption takes place during the first 30 minutes of infection. The percentage of adsorption is usually about 28.6 per cent. It also has been shown to be more active at pH 6.8 and at temperatures ranging from 28° C. to 36° C. Optimum lysis with this phage was shown to occur in one

and one-half hours under conditions of multiple infection (52, pp.22-31).

Adsorption of coliphage, as well as actinophage, has been shown to be enhanced by the addition of calcium (28, p.48; 23, pp.1-35; 41, p.29), yet certain ions such as sodium, potassium and ammonium have been shown to be inhibitory for S. griseus phage (52, p.32; 61, p.521). In addition, phage has been observed to be inactivated at high temperatures and upon prolonged refrigeration (36, p.32; 56, p.315).

Most bacteriophage is recognized to contain protein and DNA (22, p.827), and studies regarding the origin of these constituents have been made using radioactive isotopes of carbon, phosphorus, nitrogen and sulfur. Putnam (57, pp.345-378) used labelled phosphorus and heavy nitrogen and observed that 70 per cent to 80 per cent of both elements contained in coliphage T2, T4 and T6 were drawn from the medium and not from the host cell. Labow, in his studies of T5 coliphage (47, pp.724-725), and Jeener, using the Bacillus megaterium system (37, pp.229-230), arrived at this same conclusion. Klungsøyr observed that if infected S. griseus cells were unwashed, an appreciable amount of P^{32} contained in the phage could be traced to the host cell and not to the medium. Washing the cells decreased the amount of phosphorus derived from the host

by 10 per cent. This suggests that derived phosphorus might be dependent on the amount of endogenous material contained in the host cells at the time of infection (40, pp.1-52).

French, using C^{14} labelled phage protein, noted that less than two per cent of the original activity could be traced to the phage progeny (25, p.45). Other evidence indicates that there is little or no utilization of bacterial proteins or amino acids for viral synthesis, but that the protein of phage progeny is primarily synthesized from new materials in the medium. In a like manner, Kozloff was unable to demonstrate a transfer of bacterial protein to the phage. He observed, however, that new protein was synthesized by the cells after infection (43, p.218). In related studies, Cohen observed that protein synthesis continues at a constant rate during the latent period, and that the ratio of rates of increments in nitrogen and phosphorus content were similar to that found in the normal cells. The rate of protein synthesis was found to decrease during lysis but the synthesis that did occur was shown to originate from constituents in the medium. It also was observed that there was no net RNA synthesis, but that a marked increase in DNA occurred (14, pp.281-293). The possibility that DNA could be synthesized from a turnover of RNA was investigated, and it was shown that RNA remained

inert and that DNA exceeded the amount originally present in the culture. This suggested that RNA was not the precursor of DNA, and that new synthesis of DNA was occurring (15, pp.295-303). Similar results were reported by Manson (50, pp.703-711).

Along somewhat similar lines, Zelle, using chloramphenicol, (an inhibitor of protein synthesis, but not of RNA or DNA synthesis), demonstrated an inhibition of phage maturation if the drug were added during the growth cycle. If the addition were carried out in the latter half of the latent period, lysis of the cells occurred and mature phage particles were released (67, p.61). Pardee demonstrated that RNA and DNA synthesis in E. coli was dependent on the presence of amino acids (53, p.683). Moreover, Amos and Vollmayer using pentamidine, which has the same effect as chloramphenicol (1, pp.172-177), showed that phage was produced in the absence of active protein synthesis. The suggestion was made that a shift in pathway of glutamic acid synthesis occurred (2, pp.178-185).

Further studies relating to RNA and DNA were made by Watson and Maeløe (65, pp.432-442), French (25, p.45) and Kozloff (42, pp.103-108). They observed that only 30 per cent to 50 per cent of the parent nucleic acid is transferred to the phage progeny, and that this transfer does not occur uniformly, but only during the first part of the

latent period. This suggests that a direct transfer of all of the parent DNA to progeny does not occur. Another finding was that reported by Price (55, pp.741-759), who suggested that RNA synthesis appeared to be associated with the rate of growth and not with multiplication.

More recent avenues of investigation of bacteriophage have been directed toward the mode of attack and mechanisms by which phage particles are reproduced. These studies are less conclusive than the ones previously mentioned, but they have stimulated a great deal of interest.

It is generally believed that bacteriophage consists of a head and tail, and that these structures are surrounded by a protein coat, very similar in nature to the host protein. The head is thought to contain DNA and the tail to consist of strands which are attached by disulfide bonds to a tail spike. These disulfide bonds are thought to complex with the metal cations required for adsorption, and the amino group of the tail protein to form bonds with the carboxyl group of the cell wall. The metal is believed to activate a lysing enzyme, which on adsorption causes disruption of the cell wall and a leaking of essential cell metabolites into the medium. Spizizen was able to detect quantities of DPN and CoA in infected cell extracts at the time of phage adsorption (59, p.337). Phage DNA is then thought to be released into the host cell, at which time

phage synthesis begins. This period is known as the latent period (22, pp.827-832; 44, pp.511-527; 45, pp.529-535; 46, pp.537-546). This sequence of events is still obscure, but would tend to explain the effect of ions, pH and temperature on phage activity.

Evidence supporting the release of DNA on infection was shown by Hershey (33, pp.39-56). He used S^{35} and P^{32} to label the phage protein and DNA, and was able to identify viral "ghosts" attached to the cell wall after infection. These "ghosts" contained 75 per cent of the original radioactivity and were lacking in DNA. Upon treatment and removal of the "ghosts", cell lysis was shown to occur, and the "ghosts" alone were shown to be inactive. Only 85 per cent of the original P^{32} was traced to the DNA within the infected host cell. This is in keeping with the findings of Putnam, mentioned earlier.

Other studies on the latent period have been reported. Herdick, using the electron microscope, observed a thickening of the cell followed by the appearance of many uniform globules which then merged to form rings. These rings later thickened and became filled with a "protuberance", which he suggested might be the beginning of the tail (32, pp.1-12). These immature particles are called prophage (9, p.72).

Several workers have reported the absence of active and mature phage during the early stages of the latent period, but approximately 35 minutes after infection, intact and active virus particles were observed (51, p.37). Phage DNA has been reported to appear about seven to 10 minutes after adsorption, and 5-hydroxymethylcytosine has been detected during synthesis of T2 coliphage (22, pp. 827-832).

Pollard and workers were able to extend the latent period of T1 coliphage by X-Ray, deuteron and alpha bombardment. Partial disruption of the phage nucleoprotein was demonstrated, and it was observed that lysis of the cell could still occur, although it was somewhat delayed (54, pp.514-522).

The mechanisms by which the bacterial host is lysed after infection with phage remain largely unknown. It seems evident, however, that lysis is a result of the phage reproductive mechanisms. The metabolic processes by which phage is synthesized seem to be very similar to those used by the normal cell, and the required constituents of the environment do not seem to vary greatly.

The pathways which might be implicated in the synthesis of bacteriophage have been of interest for some time, but until techniques for the study of normal metabolic pathways were developed, a realistic approach to this

problem could not be made. Pathways involved in the metabolism of carbohydrates have received the most attention, primarily because they are thought to be involved in both protein and nucleic acid synthesis.

Gunsalus et al. (31, pp.79-112) have provided an excellent review of the pathways of carbohydrate metabolism in microorganisms. The glycolytic, phosphogluconate, Entner-Doudoroff pathways and the pentose and TCA cycles have all been implicated in microbial metabolism.

Cochrane's group studied the pathways of carbohydrate metabolism found in several Streptomyces species, particularly S. coelicolor. Participation of the direct oxidative pathway, the glycolytic pathway and the TCA cycle were shown to be present in this organism (11, pp.37-44; 12, pp. 308-313). Cohen (16, pp.781-782) has reported evidence for both the direct oxidative and glycolytic pathways in normal E. coli cultures.

Extensive work on the normal metabolic pathways of glucose metabolism in Streptomyces griseus has been carried out in this laboratory. Evidence for the direct oxidative pathway, glycolytic pathway and TCA cycle were shown by Gilmour et al. (29, pp.719-724) and Wang et al. (63, pp.31-37). Butterworth et al. (10, pp.725-727) were able to demonstrate a CO₂ fixation reaction with this same organism. The glycolytic pathway operates in the breakdown of glucose, and although the age of the culture produces changes

in the degree of operation of this pathway, it still seems to be the major route of metabolism (63, pp.31-37). Evidence for these findings have been reported by other workers (26, pp.26-31; 34, pp.353-370).

Bialy conducted degradation studies on the cellular constituents isolated from cultures of S. griseus utilizing C^{14} labelled ribose. He observed that approximately 61 per cent of the sugar was incorporated in young, 12 hour old cultures. Of the 61 per cent, 70 per cent was recovered as amino acids; 15 per cent as RNA and four per cent as fatty material. About 40 per cent of the amino acid activity was traced to aspartic and glutamic acids. Evidence for a ketolase cleavage-type reaction yielding acetate and pyruvate was also observed (6, pp.1-95). An enzyme catalyzing this reaction was described by Racker and workers (58, pp.408-409).

Cohen's studies on the metabolic pathways of carbohydrate metabolism in E. coli showed similarities to the metabolism reported for S. griseus, except that the degree of utilization of the direct oxidative pathway (14 per cent) was slightly more in this organism (16, pp.781-782; 17, pp.746-747). In addition, he observed that under conditions of phage infection, E. coli cells showed little change in their oxygen consumption, or in their respiratory quotients when grown on glucose (13, pp.511-523). Joklik

corroborated these findings (38, pp.368-379). Using C^{14} labelled glucose, however, it was observed that a shift toward the glycolytic pathway occurred in phage infected E. coli cells (17, pp.746-747). This shift in pathway seemed to parallel an increase in the production of DNA, and it was suggested that this might be due to inhibition of an enzyme in the direct oxidative pathway during infection. In an effort to further investigate this possibility, studies using various carbohydrates including glucose, ribose and gluconate were carried out. It was concluded from these studies that lysis could occur and that inhibition of the direct oxidative route was not the immediate cause of decreased RNA synthesis in infected cells (18, pp.490-494).

Spizizen recently reported that T7 completely inhibited the utilization of pyruvate, while T2 coliphage caused only a slight reduction in the rate of utilization. He suggested that this might reflect a difference between carbon and energy requirements between the two types of phage (59, pp.333-341). Amos and Vollmayer observed that T1 coliphage required glucose or pyruvate for maximum adsorption, and suggested that these substrates might be used as energy sources during biosynthesis (3, pp.325-332).

The most recent work relating to the biosynthesis of bacteriophage has been directed toward the pathways of carbohydrate metabolism, which might be implicated in the

synthesis of RNA and DNA. The work of Lanning and Cohen (48, pp.193-199) and that of Bernstein et al. (5, pp.873-878) suggested that although ribose can be synthesized from glucose by way of the glycolytic pathway, most of the synthesis occurred by way of the direct oxidative route. Racker demonstrated that DNA was synthesized by the glycolytic pathway, and was dependent on the presence of triose phosphate, acetaldehyde and triose phosphate isomerase. This reaction was shown to occur in Bacillus cereus in the absence of DPN, and was shown to be affected by variations in pH (35, pp.305-309). Green and Cohen used glucose C¹⁴ and observed that this substrate might be a possible precursor for thymine synthesis (30, pp.387-396). These studies support the findings that an increase in the glycolytic pathway occurs during infection, and the suggestion that this shift is responsible for the increased synthesis of DNA.

EXPERIMENTAL METHODS

Two methods were used in the present study. The first consisted of conventional manometric techniques (60, pp.1-16) to establish a method for obtaining a level of infection that would allow for normal respiration and also for observing the subsequent decreased rate of respiration characteristic of the onset of cell lysis. The second method was the radiorespirometric approach (62, pp.1869-1872; 64, in press) used for the metabolic tracer studies.

Preparation of Cells

Streptomyces griseus (3475, Waksman) was used throughout this study. For preparation of the spore inoculum, soil stocks were streaked on 0.5 per cent glucose nutrient agar in 200 ml. prescription bottle slants. Nine bottle slants were used for each experiment. Sporulation was achieved after two days incubation at 30° C. The spores were washed off the slants with 10 ml. of nutrient broth and each filtered through 30 layers of sterile gauze to remove the clumps. The concentration of the resulting suspension was approximately 1×10^8 spores per milliliter, as predetermined by plate counts and optical density readings.

Prior to each experiment, 12 ml. of the filtered spore suspension were added to each of two flasks containing 100

ml. of 0.1 per cent glucose nutrient broth. The flask contents were allowed to germinate on a rotary shaker at 30° C. for four and one-half hours. After this incubation period, the cells were centrifuged and washed twice in sterile distilled water, then resuspended in the following carbohydrate-free synthetic medium:

NH ₄ Cl	1.0 gram
K ₂ HPO ₄ ·3H ₂ O	0.25 gram
MgSO ₄ ·nH ₂ O	0.10 gram
FeSO ₄ ·7H ₂ O	Trace
CaCl ₂	0.222 gram
Yeast extract	0.050 gram
Distilled water	1000.0 ml.

(pH adjusted to 6.8 with dilute HCl prior to CaCl₂ addition; autoclaved for 15 to 20 minutes at 15 lbs. pressure.)

The phage used was a soil isolate designated as 514-3 (27, p.17). High titer stocks (1×10^{10} per ml.) were prepared by the agar layer method as described by Adams (19, pp.7-8).

The infected cells were prepared by inoculating 12 ml. of the uniform spore suspension into each of two flasks containing 100 ml. of 0.1 per cent nutrient broth, and incubating as described for the host cells. At the end of four hours, 45 ml. of the high titer phage stock was added

to each flask and the contents allowed to incubate for the remaining half hour. This provided a 200:1 phage to cell ratio.

After this preincubation period, the cells were removed by centrifugation, washed twice in sterile distilled water and resuspended in the aforementioned carbohydrate-free synthetic medium.

Streak plates and duplicate flasks were prepared prior to each experiment as a control in estimating the extent of lysis, and as an indication of the presence of any contaminants.

Manometric Studies

Infected and non-infected cells, prepared as outlined, were used for manometric studies (60, pp.1-16). Aliquots containing 0.5 to 1.0 mg. of nitrogen were placed in the respiration flasks in the following sequence: 2.5 ml. of cells; 0.5 ml. of substrate in the sidearm and 0.2 ml. of 20 per cent KOH in the center well. Substrate concentrations ranged from 10 micromoles to 20 micromoles per flask and a bath temperature of 30° C. was used.

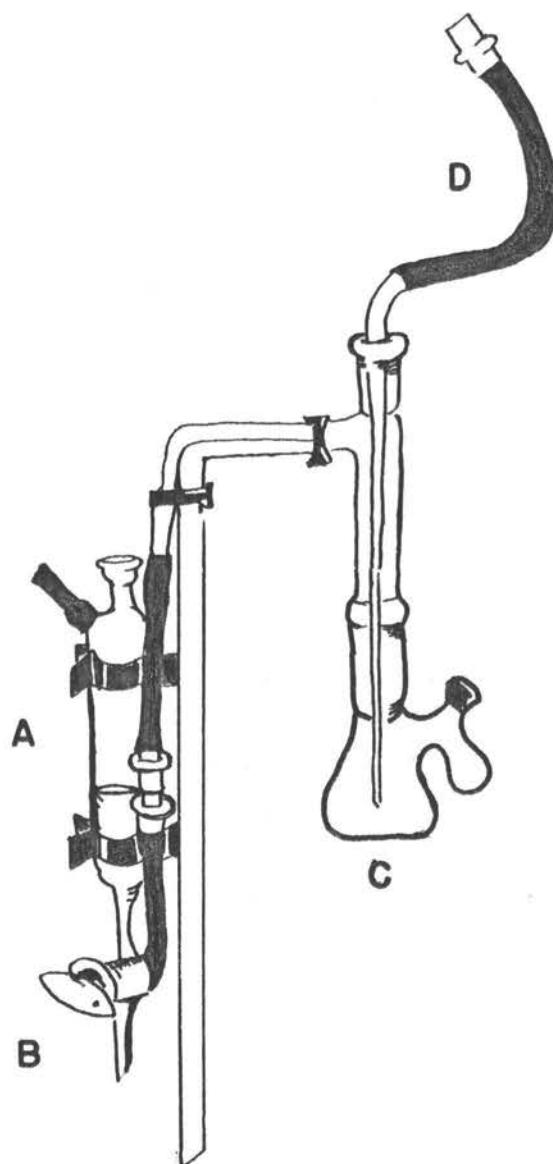
Tracer Studies

Infected and non-infected cells were cultivated and harvested as outlined previously. Ten to 20 milligram cell

aliquots per 10 ml. of the medium were transferred to the respiration flasks, each of which contained a different labelled substrate. The flasks were then connected to the radiorespirometric apparatus (Figure 1), which consisted of a Warburg bath (30° C.), air flow regulator and manifold and CO₂-free NaOH traps. The entire system was connected by latex rubber tubing and operated by allowing CO₂-free air to pass through the respiration flasks and thus flush the respired CO₂ into the NaOH traps. Each trap was equipped with a three-way stopcock which allowed for sampling at desired intervals. Samples were removed each hour and the traps washed and refilled with fresh NaOH. The entire assembly allowed for the simultaneous testing of 12 cell treatments (24, pp.24-25).

Glucose-1,2,6-C¹⁴ and ribose-1-C¹⁴ were obtained from the Bureau of Standards; glucose-U-C¹⁴ and acetate-1-C¹⁴ were procured from Tracerlab, Incorporated. Pyruvate-1-C¹⁴ was obtained from Nuclear-Chicago, and glucose-3,4-C¹⁴ was prepared in Dr. Wang's laboratory according to the method of Wood et al. (66, pp.475-489). All of the labelled substrates were diluted with non-isotopic substrate to a definite specific activity prior to use. The levels of substrates used in these experiments were as follows: glucose 4.5 mg.; ribose 1.8 mg.; pyruvate 1.1 mg. and acetate 0.75 mg. per 10 ml. cell suspension. The radioactivity levels

FIGURE 1.
RADIORESPIROMETRIC APPARATUS



- A = CO₂ TRAP
- B = SAMPLING OUTLET
- C = RESPIRATION FLASK
- D = AIR FLOW

used ranged between 0.5 microcurie and 1.0 microcurie per flask.

Radioactive recovery was determined by precipitation of the sample with $\text{BaCl}_2\text{-NH}_4\text{Cl}$, which was then centrifuged on weighed aluminum planchets. 1N Na_2CO_3 (0.2 ml.) carrier was added to each sample prior to precipitation. The planchets were counted by means of an end-window Geiger-Müller counter, and after corrections for background and self-absorption were made, the results were expressed as per cent interval and cumulative recovery.

The standardization of all substrates and specific activity of the medium following each experiment was determined by the soluble carbon combustion method of Katz et al. (39, pp.1503-1504).

Analysis of Cells and Medium

Radioautograms were prepared by the method outlined in Aronof (4, pp.41-42). The method of preparing cell hydrolyzates for use in paper chromatograms was a modification of that described by Block (7, p.56). An accurately weighed sample of dried cells was treated with 6N HCl and incubated in a sealed tube at 121° F. under 15 pounds pressure for eight hours. The hydrolyzate was then filtered and the supernatant placed in a vacuum desiccator with P_2O_5 and KOH pellets. The resulting residue was diluted with

water so that the specific weight of both the infected and non-infected cells were identical.

Saturated phenol (80 per cent), water; and butanol, acetic acid and water (4:5:1) were used as the solvents in one-dimensional descending chromatograms for analysis of the medium. The solvents and procedure outlined by Block (8, pp.75-80) were used for analysis of the cell hydrolyzates.

Chemical sugar determinations were made by the methods outlined in Umbreit (60, pp.190-191).

EXPERIMENTAL RESULTS

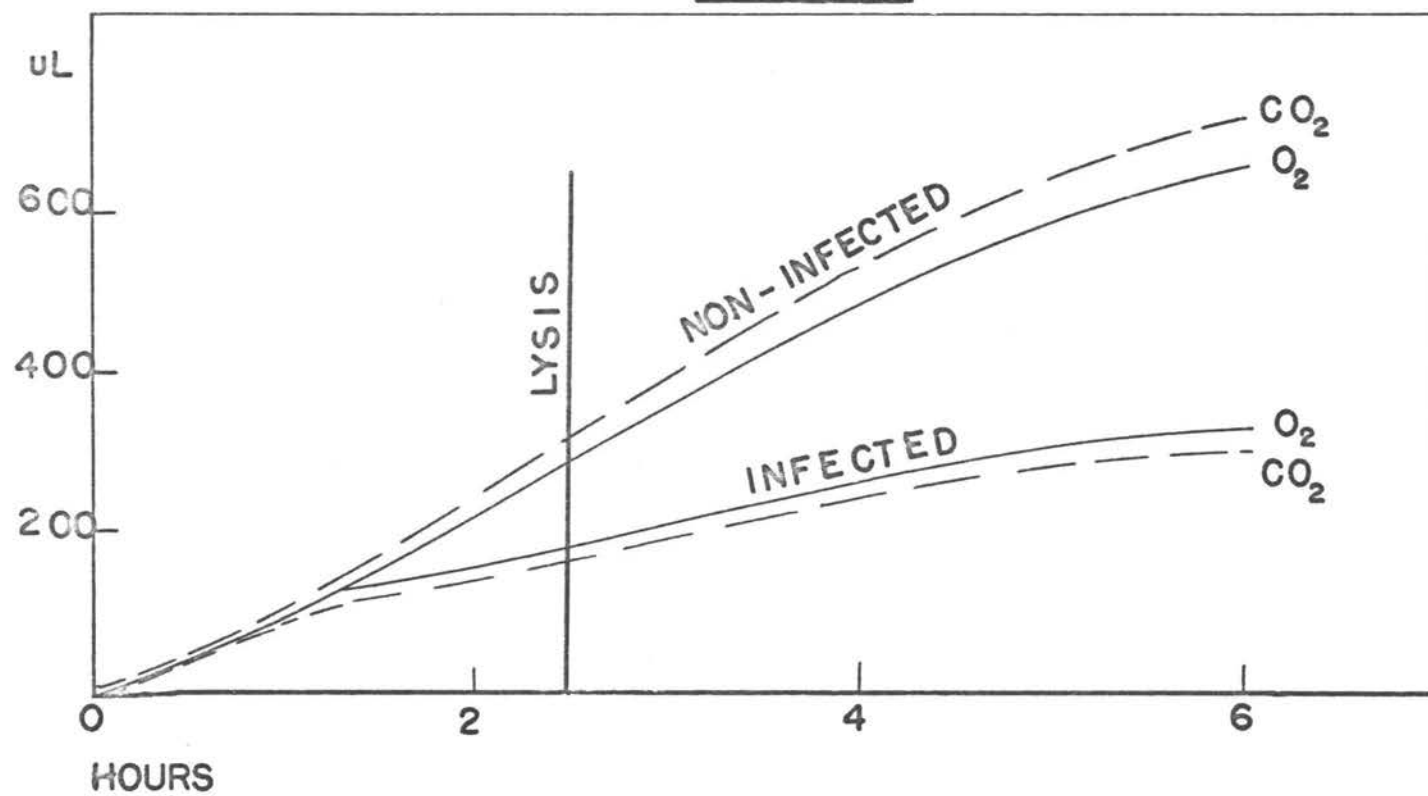
Manometric Studies

Initial metabolic studies were carried out on the Warburg apparatus to test the effectiveness of various synthetic media on phage synthesis, and to observe the rates of respiration and substrate utilization of both the control and infected cells under various experimental conditions. Figures 2, 3 and 4 illustrate the results of these studies using the methods which were outlined earlier. In all studies, lysis could be observed within two to two and one-half hours after the addition of phage.

Lysis was shown to occur when D-ribose was used as a carbon source; however, cell lysis was not as marked, and appeared to occur after a slightly longer period of time than when D-glucose was used. The endogenous metabolism in this system was observed to range between 30 to 40 microliters of oxygen uptake per hour, and before extensive lysis occurred, essentially no differences were noted between the infected and the control cells.

The most outstanding difficulty that was noted during this phase of the study was the establishment of a multiply infected system. Small phage to spore ratios resulted in respiration rates in the infected cells which were equal to or greater than the rates observed for the normal cell,

FIGURE 2.
GLUCOSE UTILIZATION OF NON-INFECTED AND
INFECTED S. GRISEUS CELLS



and no lysis occurred. The infection level is largely determined by the growth rate of the host cell. The variability in sporulation and associated filamentous nature of S. griseus thus makes a high phage to spore ratio (200:1) imperative. The results of the above studies indicated the presence of a multiply infected system and showed that respiration continues at a measurable level for a period of time following phage infection. It was considered that the described respiration period would serve as a basis for the proposed metabolic tracer studies.

Metabolic Tracer Studies

Having established a satisfactory procedure for infection, radioactive tracer studies were started in an attempt to identify and estimate, if possible, the pathways of carbohydrate metabolism in the S. griseus host-phage system.

All of the radioactive data were interpreted by the method of Wang et al. (62, pp.1869-1872). This method provides a means of quantitatively estimating pathway distribution on the basis of comparative respiratory $C^{14}O_2$ recoveries of variously labelled substrates. It has been adapted for the study of growing and intact cell preparations, and allows for detection of respiration rates at any given time interval. This proved of particular advantage

for the study of the metabolism of a phage-infected system.

Expression of tracer data

The calculations were based on known amounts of added substrate utilized. The added substrates in these studies were calculated so that complete utilization would occur in a given time period. The data were expressed according to the following equations:

$$\begin{array}{l} \text{Per cent Interval} \\ \text{Recovery} \end{array} = \frac{\text{Respiratory C}^{14}\text{O}_2 \times 100}{\text{Total Substrate Activity}} \dots\dots\dots (1)$$

$$\begin{array}{l} \text{Per cent Cumulative} \\ \text{Recovery} \end{array} = \text{Summation of the per cent Interval Recoveries} \dots\dots (2)$$

Pathway distribution can be estimated as follows:

1. Let T = Total activity of the administered glucose recovered as C^{14}O_2 (3)
2. Phosphogluconate route (Gp)

When glucose is metabolized via this route, C_1 is preferentially decarboxylated. In the glycolytic route, C_1 and C_6 are equal; therefore, any increase in the recovery of C_1 over C_6 will be due to the

functioning of the direct oxidative route, assuming that these are the only two pathways operating in the organism.

Then:

$$Gp = C_1 - C_6 \dots\dots\dots (4)$$

$$\text{Per cent } Gp = \frac{C_1 - C_6}{T} \times 100 \dots\dots\dots (5)$$

3. Glycolytic pathway

Assuming again that only the phosphogluconate route and the glycolytic pathway are functioning, the remaining fraction of the glucose not metabolized by the direct oxidative route will be directed toward the glycolytic and TCA sequences.

Thus:

$$Ge = 1 - Gp \dots\dots\dots (6)$$

$$\text{Per cent } Ge = \frac{1 - Gp}{T} \times 100 \dots\dots\dots (7)$$

4. TCA cycle and the fate of acetate

The recovery of glucose-3(4)-C¹⁴ and glucose-2-C¹⁴ reflects the decarboxylation of pyruvate and subsequent oxidation of acetate via the TCA cycle. The C-2 of glucose in this case would become the

carboxyl carbon of acetate; thus, the recovery of glucose-2-C¹⁴ would be an indication of pyruvate decarboxylation followed by TCA activity. The fate of acetate is determined as follows:

Let R_c = Per cent of acetate carboxyl
converted to CO_2

$$\text{Per cent } R_c = \frac{G_2}{G_3} \times 100 \dots\dots\dots (8)$$

G_6 represents the methyl carbon of acetate. The per cent of acetate methyl converted to CO_2 can be estimated as follows:

Let R_m = Per cent of acetate methyl
converted to CO_2

$$\text{Per cent } R_m = \frac{G_6}{G_3} \times 100 \dots\dots\dots (9)$$

An estimation of the amount of acetate being used for synthesis can also be calculated.

Let S_c = Per cent of acetate $COOH$
utilized in synthesis

and S_m = Per cent of acetate CH_3
utilized in synthesis

Then:

$$\text{Per cent } S_c = 1 - \text{Per cent } R_c \dots (10)$$

$$\text{Per cent } S_m = 1 - \text{Per cent } R_m \dots (11)$$

Respiration of normal and infected cells

The per cent interval and cumulative recoveries obtained with non-infected and infected cells may be found in Tables I and II, respectively, and a graphic representation is presented in Figures 5 and 6. Several points of primary interest are readily observable from these data. The lower interval and cumulative recoveries observed with infected cells probably reflect the lowered respiration level which occurred as a result of phage infection. For example, with the normal cells, 51 per cent of the administered glucose-U-C¹⁴ was converted to C¹⁴O₂ within four hours, while the total C¹⁴O₂ recovery of this same substrate in the infected cells was no more than 44 per cent over a seven hour period. Radioautograms and glucose determinations of the medium at the termination of the experiment showed that, as far as could be ascertained, all of the glucose was utilized by both normal and infected cells.

A further study of the data in Tables I and II indicated that the C¹⁴O₂ recoveries obtained from the infected cells, although slightly lower, were very similar to those obtained from the control cells, but that the glucose-3, 4-C¹⁴ recoveries were strikingly different. For example, the total cumulative C_{3,4} recovery of the non-infected cells was 80 per cent, while that of the infected cells

Table I

PERCENTAGE INTERVAL AND CUMULATIVE RECOVERY IN RESPIRATORY C¹⁴O₂
FROM NON-INFECTED S. GRISEUS CELLS

Time hrs	G-U-C ¹⁴		G-1-C ¹⁴		G-2-C ¹⁴		G-3,4-C ¹⁴		G-6-C ¹⁴	
	*I	**C	I	C	I	C	I	C	I	C
1	5.0	5.0	5.0	5.0	2.0	2.0	10.0	10.0	1.0	1.0
2	21.0	26.0	14.0	19.0	10.0	12.0	30.0	40.0	7.0	8.0
3	20.0	46.0	18.0	37.0	15.0	27.0	20.0	60.0	12.0	20.0
4	5.0	51.0	5.0	42.0	7.0	34.0	16.0	76.0	6.0	26.0
5	3.0	54.0	4.0	46.0	5.0	39.0	2.0	78.0	5.0	31.0
6	2.0	56.0	2.0	48.0	3.0	42.0	1.0	79.0	4.0	35.0
7	2.0	58.0	2.0	50.0	2.0	44.0	1.0	80.0	3.0	38.0

*I = Per cent interval recovery calculated by equation (1).

**C = Per cent cumulative recovery calculated by equation (2).

Table II

PERCENTAGE INTERVAL AND CUMULATIVE RECOVERY IN RESPIRATORY C¹⁴O₂
FROM INFECTED S. GRISEUS CELLS

Time hrs	G-U-C ¹⁴		G-1-C ¹⁴		G-2-C ¹⁴		G-3,4-C ¹⁴		G-6-C ¹⁴	
	*I	**C	I	C	I	C	I	C	I	C
1	3.0	3.0	3.0	3.0	2.0	2.0	6.0	6.0	0.6	0.6
2	8.0	11.0	8.0	11.0	6.8	8.0	16.0	22.0	4.0	4.6
3	11.0	22.0	9.0	20.0	7.0	15.0	18.0	40.0	8.0	12.6
4	7.0	29.0	9.0	29.0	8.0	23.0	13.0	53.0	7.0	19.6
5	9.0	38.0	8.0	37.0	6.0	29.0	2.0	55.0	6.0	25.6
6	4.0	42.0	6.0	43.0	6.0	35.0	1.0	56.0	6.0	31.6
7	2.0	44.0	2.0	45.0	3.0	38.0	1.0	57.0	3.0	34.6

* = Per cent interval recovery calculated by equation (1).

** = Per cent cumulative recovery calculated by equation (2).

FIGURE 5.
PERCENTAGE INTERVAL AND CUMULATIVE RECOVERY
IN RESPIRATORY $C^{14}O_2$ FROM NON-INFECTED S. GRISEUS
CELLS

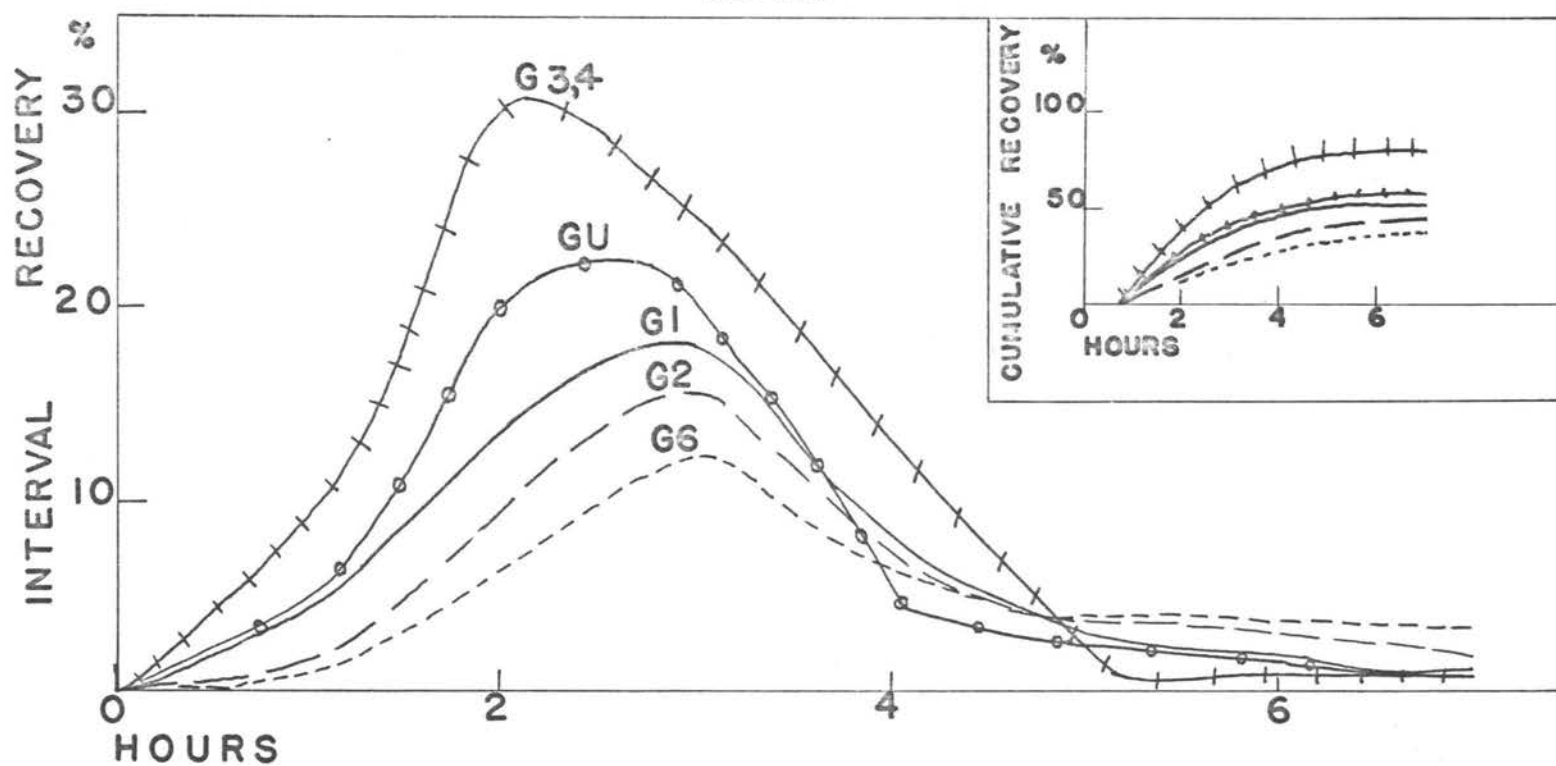
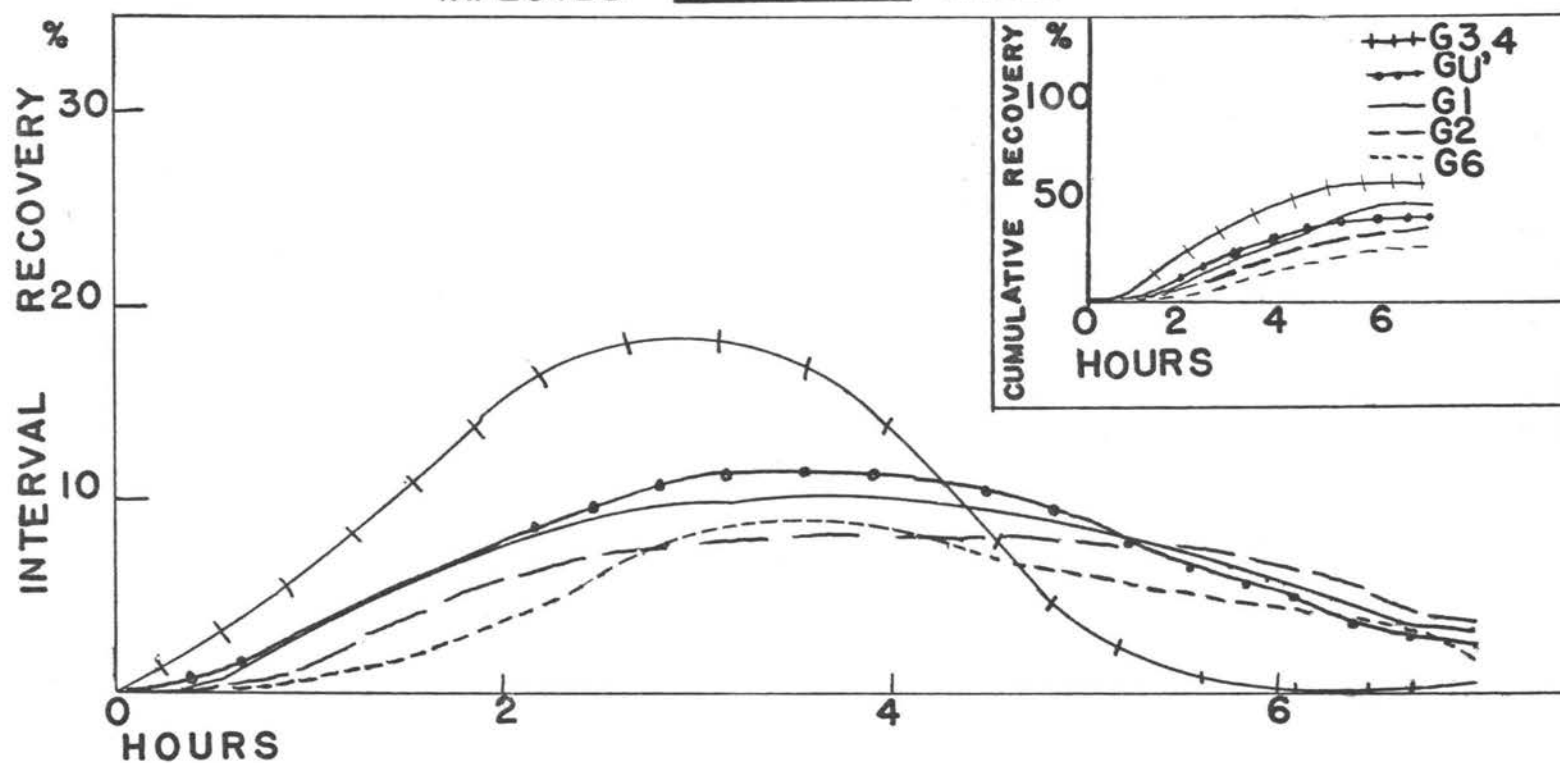


FIGURE 6.
PERCENTAGE INTERVAL AND CUMULATIVE
RECOVERY IN RESPIRATORY $C^{14}O_2$ FROM
INFECTED S. GRISEUS CELLS



was only 57 per cent. Since the total amount of administered glucose was utilized, this observation suggested an interference in acetate formation. The possibility of decreased TCA activity was also considered. Reference to Table III shows the $C^{14}O_2$ recoveries obtained when pyruvate-1- C^{14} and acetate-1- C^{14} were used as substrates instead of glucose. There appeared to be no difference in the utilization of pyruvate or acetate with the infected and control cells. Presumably there was no impairment of pyruvate decarboxylation after infection. These data also suggested that no interference of TCA activity occurred. Using glucose as a substrate, however, marked differences in the fate of acetate were shown. The results in Table IV (calculated by equations 8 and 9) illustrate a marked increase in the combustion of acetate in the infected cells as compared to those calculated for the control cells. Further, the calculations in Table V (derived by equations 10 and 11), show a marked decrease in the utilization of acetate for biosynthesis in contrast to the control cells.

The differences in the recoveries of C_1 and C_6 of both the infected and non-infected cells are indicative of the operation of the direct oxidative route (Table VI). Estimation as to the extent of operation of this pathway was made by using equations 4 and 5. The high recoveries of glucose-3,4- C^{14} and glucose-2- C^{14} for both cell types

Table III

PER CENT CUMULATIVE RECOVERY IN RESPIRATORY $C^{14}O_2$
 FROM ACETATE-1- C^{14} AND PYRUVATE-1- C^{14} WITH
 PHAGE-INFECTED AND NON-INFECTED
S. GRISEUS CELLS

Time hrs	<u>Pyruvate-1-C^{14}</u>		<u>Acetate-1-C^{14}</u>	
	Non-inf	Infect	Non-inf	Infect
1	15.2	13.7	7.1	7.2
2	34.2	14.3	24.1	21.2
3	46.3	30.0	40.6	35.0
4	50.0	45.6	49.2	44.7
5	52.0	51.2	53.8	50.3
6	53.1	53.5	56.3	53.2
7	53.8	54.9	57.6	55.5
8	54.5	56.0	58.6	56.0

Table IV

PERCENTAGE OF ACETATE-CARBON (Carboxyl and Methyl)
 CONVERTED TO CO₂ FROM C¹⁴-LABELLED GLUCOSE

Experi- ment	Non-infected		Infected	
	CH ₃ (COOH)	(CH ₃)COOH	CH ₃ (COOH)	(CH ₃)COOH
	G-2/G-3	G-6/G-3	G-2/G-3	G-6/G-3
1	39.0	31.0	66.6	60.7
2	44.7	34.2	64.1	49.2
3	<u>52.1</u>	<u>33.5</u>	<u>69.0</u>	<u>54.0</u>
Average	45.2	32.9	66.5	54.6

Table V

OBSERVED PER CENT CONTRIBUTION OF G-2
(Acetate Carboxyl) AND G-6 (Acetate
Methyl) IN CELLULAR BIOSYNTHESIS

Experiment	<u>Non-infected</u>		<u>Infected</u>	
	<u>G-2</u>	<u>G-6</u>	<u>G-2</u>	<u>G-6</u>
1	61.0	69.0	33.4	39.3
2	55.3	65.8	35.9	50.8
3	<u>47.9</u>	<u>66.5</u>	<u>31.0</u>	<u>46.0</u>
Average	54.7	67.1	33.4	45.4

Table VI

RELATIVE CONTRIBUTION OF GLYCOLYSIS (Ge) AND
 PHOSPHOGLUCONATE DECARBOXYLATION (Gp) IN
 NON-INFECTED AND PHAGE-INFECTED S. GRISEUS CELLS

Hour	<u>Non-infected</u>		<u>Infected</u>	
	Gp	Ge	Gp	Ge
1	4.0	96.0	2.4	97.6
2	11.0	89.0	6.4	93.6
3	17.0	83.0	7.4	92.6
4	16.0	84.0	9.4	90.6
5	* <u>15.0</u>	<u>85.0</u>	* <u>11.4</u>	<u>88.6</u>
6	13.0	87.0	11.4	88.6
7	12.0	88.0	10.4	89.6

*Hour selected for comparison.

indicated that the glycolytic pathway and TCA cycle were also operative.

Estimations of the proportion of administered glucose catabolized via each pathway were made using the values obtained at the time of glucose depletion. This point is represented by a leveling off of cumulative radiochemical recoveries from glucose-3,4- C^{14} (Tables 1 and 2). With both the control and infected cells, the exhaustion of intact glucose occurred at five hours. Thus it is evident that although $C_{3,4}$ of glucose appeared as CO_2 at a slower rate in the infected cells, the point of glucose exhaustion occurred at the same time.

As was mentioned above, the estimation of pathways distribution is made on the basis of the total amount of glucose utilization in both infected and non-infected cultures. The relative contribution of the glycolytic pathway, of course, must be based on the assumption that, of the glucose utilized at a certain hour, any substrate not catabolized by way of the direct oxidative pathway is necessarily directed toward the glycolytic sequence. Values which were calculated for each hour interval are summarized in Table VI. It will be observed that only a small difference in pathway distribution exists between the infected and non-infected cells, (Gp = 15.0 per cent

for the non-infected cells and $G_p = 11.4$ per cent for the infected cells). Yet for all calculations shown, the trend appeared to favor a slight decrease in the direct oxidative pathway. With a lysing system however, typical of any phage infected cell suspension, it is difficult to differentiate between decreases in $C^{14}O_2$ recoveries attributable to glucose depletion and progressive lysis of the cells. Thus, the degree of change in the actual pathway distribution might remain uncertain. It is felt that the data pertaining to pathway distribution does support the conclusion that in infected cultures of S. griseus, more of the glucose utilized is metabolized via the glycolytic pathway.

Since there does not appear to be any interference in the TCA cycle, and increased oxidation of acetate in the infected cells was indicated, the alteration in pathway distribution might reflect an increased demand for some intermediate of the glycolytic pathway, possibly a three carbon fragment prior to the formation of pyruvate.

Participation of a ketolase-type cleavage (6, p.85) reaction with formation of two and three carbon compounds which could undergo oxidation via the TCA cycle might be mentioned at this point; however, the contribution of the

direct oxidative pathway in this system is small, and hence it is not expected that this reaction contributes greatly to the phage biosynthetic sequence.

In an attempt to evaluate further the contribution of the direct oxidative pathway or preferential appearance of C_1 of glucose, experiments were performed using ribose 1- C^{14} as substrate. The results from these experiments indicated that a marked decrease in the conversion of C_1 of ribose to CO_2 occurred in infected cells. The same results were suggested by the data shown in Figure 3, page 22. As far as could be determined, complete utilization of administered ribose had occurred in both the control and infected cells. This area was not investigated further, however, and no definite conclusions were made.

In addition to the analysis of $C^{14}O_2$ liberation with labelled glucose substrates, chromatographic analyses of HCl hydrolyzates of the cells were carried out. On the basis of the methods used, no marked differences could be observed in the qualitative amino acid content of the infected cells, although there appeared to be a lower concentration of amino acids in the infected as compared to the control cells. It was also apparent that glutamic acid and alanine contained the largest

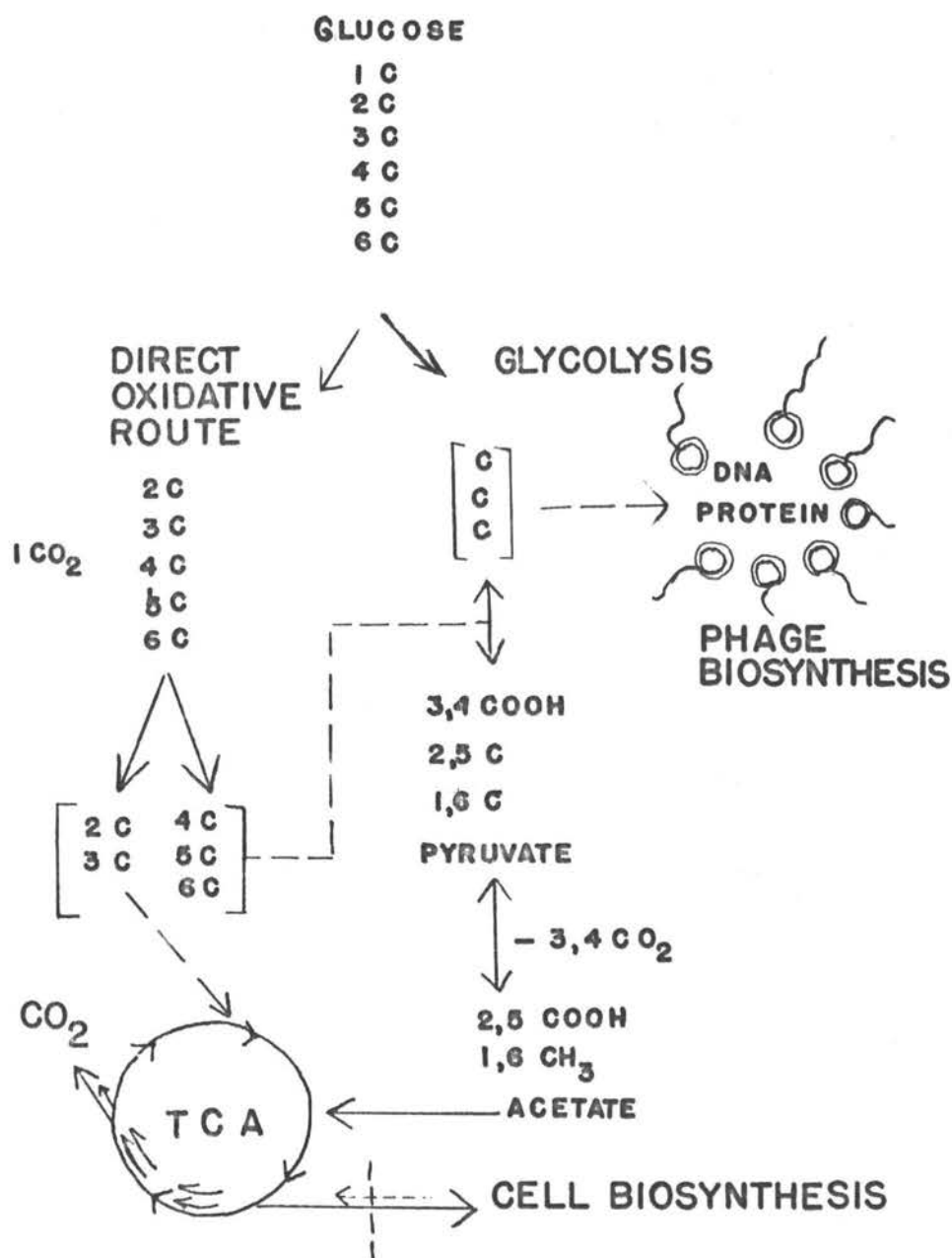
portion of the labelling in both cultures. Cultures supplied with ribose showed results similar to those obtained for cells grown on glucose.

In view of the above data, it would appear that there may be essentially little difference in the cellular protein of infected and non-infected cells. The methods employed were not sensitive enough to detect very small differences, however, and because of this, no definite conclusions were drawn.

DISCUSSION

The nature of this problem necessitates that this discussion be confined to some degree of conjecture based, of course, on what is felt to be sound experimental evidence. The data suggest that there is no marked alteration in the overall pathways of carbohydrate metabolism, but that changes do occur at the level of the formation of a three carbon fragment. Based on these observations, a proposed alteration in the pathway of carbohydrate metabolism of infected cells during bacteriophage synthesis is presented (Figure 7). An attractive idea concerns a possible interference of glucose catabolism at some site prior to or at the pyruvate level. The three carbon moiety, formed either by the direct oxidative route or via glycolysis, might well be drained off in the direction of pentose formation and thereby result in a net decrease in the per cent combustion of C_2 and C_6 of glucose. This in effect would stand as a stimulation in glycolysis, perhaps for DNA production during phage synthesis. This conclusion is further corroborated by the previous observation of the lower cumulative recoveries observed with glucose-3,4- C^{14} . However, since the glucose was completely utilized by both the infected and non-infected cells, it appears probable that in view of the higher combustion efficiency of C_2 and C_6 and the lower cumulative recoveries with $C_{3,4}$, that a

FIGURE 7.
PROPOSED SEQUENCE OF CARBOHYDRATE
UTILIZATION IN THE BIOSYNTHESIS OF
BACTERIOPHAGE



specific interference has occurred between triose phosphate and pyruvate with the infected cells.

The increased combustion of acetate in the infected cells is thought also to reflect the decreased need for biosynthesis of cellular materials in the dying system. The possibility that this increased combustion stands as an energy source in phage synthesis, however, cannot be overlooked.

It is proposed that an impairment in the glycolytic scheme occurs, and that this impairment is at a level intermediate to the formation of pyruvate. The possibility of a decrease in some biocatalyst upon adsorption of the infecting particle, as suggested by Spizizen (59, pp. 333-341), would support this proposal. However, the suggestion that this biocatalyst might be CoA and/or DPN would not fully explain the apparent normal rate of decarboxylation when pyruvate is used as the substrate.

Another interesting possibility might be that upon infection the activating substance derived from the parent phage reacts in such a way that it directs slightly different enzyme-substrate reactions, and thus diverts the normal cell metabolism to one of phage biosynthesis. These and other concepts remain as a working basis for future investigations.

SUMMARY

1. Prior to the onset of lysis, multiply infected S. griseus cells are shown to be metabolically active. Normal biosynthesis directed toward cell proliferation is markedly reduced, however. The reduction in biosynthesis appears to be accompanied by an increase in terminal oxidative activity of the infected cells.
2. Glucose catabolism of normal and infected cells appears to follow the same metabolic pathways. The direct oxidative route, TCA cycle and primarily the glycolytic pathway operate in this system. A slight decrease in the direct oxidative activity and a shift toward the glycolytic pathway are suggested after phage infection.
3. An accumulation or drainage of a carbon moiety may occur with the infected cells, but this is not apparent with the control cells. This point suggests either a re-routing of a portion of the administered carbohydrate to phage synthesis or infers a net accumulation of a product of glycolysis.

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