DEGRADATION STUDIES ON CELLULAR CONSTITUENTS ISOLATED FROM STREPTOMYCES GRISEUS UTILIZING C^{14} LABELED CARBOHYDRATES

by

JERZY JOZEF BIALY

A THESIS

submitted to

OREGON STATE COLLEGE

in partial fulfillment of
the requirements for the
degree of

DOCTOR OF PHILOSOPHY

June 1957

TO MY NEW COUNTRY

APPROVED:

Redacted for Privacy

Associate Professor of Chemistry

In Charge of Major

Redacted for Privacy

Chairman of Department of Chemistry

Redacted for Privacy

Chairman of School Graduate Committee

Redacted for Privacy

Dean of Graduate School

Date thesis is presented NOV. 15, 1956

Typed by LaVon Peek

ACKNOWLEDGMENT

I would like to express my appreciation to Dr. C. H. Wang for his inspiring direction of this portion of my education and for the many hours which he gave to guide this research.

I would like to thank Dr. B. E. Christensen whose friendly interest and encouragement has been deeply appreciated.

Grateful thanks are given to Dr. C. M. Gilmour for his spirit of cooperation and for the use of his laboratory during the bacteriological phase of this work.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

DEGRADATION STUDIES ON CELLULAR CONSTITUENTS ISOLATED FROM *STREPTOMYCES GRISEUS* UTILIZING C^{14} LABELED CARBOHYDRATES

INTRODUCTION

Prior to the discovery of the antibiotic, streptomycin, the organism responsible for its production, *S. griseus* was not extensively studied in any respect. The lack of interest, or even reluctance to undertake such an investigation, stemmed mainly from the fact that, at the time of the discovery of the antibiotics, the whole order of *Actinomycetales*, to which *S. griseus* belongs, was rather chaotic in respect to its taxonomy and the inherent characteristics of this order. The latter can be exemplified by the loss of pigmentation (29), the loss of ability to liquefy agar, and the ready formation of stable variants from the degenerate ones. The task is further complicated by the ability of *Streptomyces* to vary morphologically. Consequently, while other organisms, such as *E. coli*, had most of their metabolic processes known, or at least under investigation, the members of the order *Actinomycetales* were left to remain in obscurity. Various attempts have also been made to classify the organisms of this order, sometimes even resorting to desperate and unorthodox methods. Conn (18), and Cochrane and Conn (13) attempted to distinguish between *Streptomyces* species by employing spectrophotometric

measurements of specific absorption curves from pigments, while discovery of specific actinophages capable of lysing various species of Streptomyces have advanced ideas regarding the possibility of their use in species differentiation (21). Considering that the most recent work by Waksman (73) still relies on the mode of reproduction as the fundamental basis for separation of genera, it is apparent that taxonomy in the order of Actinomycetales has yet to be clarified.

On the other hand, the spark of the discovery of compounds which are antibiotic in nature (11, 19, 24, 27,) has initiated feverish activity in the study of the physiology of Actinomycetes and may soon lead to better understanding of biochemical processes of each species which should provide not only an insight into a puzzle of long standing, but also a solution to the taxonomical problem by giving new means of classification based on a more reliable criterion than morphological observations.

Most of the early physiological data, as might be expected, were obtained as by-product of experiments designed for the study on production of streptomycin. The earlier work, although concerned mainly with either the development of cultural conditions for improvement of the yield of known antibiotics (38, 67, 70, 71, 72), or search for new ones (1, 25, 55, 58), has stimulated the

search for deeper understanding of basic metabolic processes in the organism itself. Similarly, as augmented by the discovery that S. griseus is a vitamin B_{12} producer, a great deal of work was done on nutritional requirements of this organism (11), although one cannot help noticing the shift of emphasis to the metabolism studies as a primary object in recent years (15,27). Some of the early investigators (24) noted that the growth characteristics of the S. griseus culture consists of two phases. The early phase corresponds to the classical bacterial growth phase, which involves a steady increase of cell mass and simultaneous disappearance of substrate from the medium, as well as a high oxygen demand and copious CO_2 evolution. The second phase, corresponding to the death phase, is characteristic of the autolytic stage accompanied by a relative decrease in cell mass, release of inorganic phosphate and nitrogenous compounds into the medium, decrease in oxygen requirement, decrease in CO_2 evolution, and gradual increase of pH. It was found that streptomycin was produced in significant amounts only during this second phase of growth.

Considerable advances in identification of compounds released into the medium during this phase have been made since the appearance of the earlier work. Cochrane and Dimmick (14) identified several organic acids in the growth medium of Streptomyces coelicolor, the most

important ones being succinic, lactic, and fumaric acids. The above workers as well as Bialy et al. (8) also reported the presence of succinic acid in the cultural media of S. griseus. Accumulation of lactic acid was reported by several workers (14,24,8 ,53) while acetate was reported by Perlman and Wagman (53) as a fermentation product of both glucose and lipids. Perlman and Wagman (53) found that cells grown on lard oil exhibited superior activity as compared to that grown on glucose, and there was no reduction of antibiotic production while using lard oil as substrate. Nitrogenous compounds released into the cultural medium were identified as amino acids, some of them were characterized as glutamic acid, aspartic acid, methionine, threonine, and lysine. There were also indications of the presence of arginine, cysteine, and histidine (19). Alanine and glutamic acid were also reported in the fermentation medium by Numerof (51).

A wide variety of carbohydrates have been used as a sole carbon source for growth of S. griseus and it was found that the ability to utilize certain sugars or their derivatives varies from strain to strain. Perlman and O'Brien (52) report that among the carbohydrates utilized by all strains under investigation were: glucose, fructose, mannose, galactose, mannitol, alpha-methyl

glucoside, maltose, and inulin. Some of the strains varied in their ability to grow on galactose, lactose, starch, rhamnose, and xylose, while none of the strains were able to utilize arabinose, raffinose, sucrose, and sorbose.

It is noteworthy that with such a wide spectrum of carbohydrates used as substrates in growth of Streptomyces the metabolic studies were, up to date, confined exclusively to the use of glucose. This lack of diversity was felt most acutely in the case of pentoses which play important role as intermediates in metabolic processes. Several modes of breakdown processes of pentose have been established in various microbial systems. In the case of Streptomyces, although it was reported that all strains under investigation were unable to utilize arabinose, and only a few of them demonstrated the ability to grow on xylose, it is conspicuous that the utilization of ribose, an important intermediate carbohydrate, by Streptomyces is not registered in the literature.

Utilization of amino acids by S. griseus has been thoroughly studied by Ganguly and Ganguli (31) who observed preferential utilization of cystine, aspartic acid, and arginine. In contrast to their work, which indicates that even after 120 hours of fermentation glutamic acid remained in the medium, other workers

(22,27) have found that glutamic acid and alanine, in addition to aspartate, are readily utilized by S. griseus.

Extensive studies have also been carried out by several investigators on the utilization of various organic acids by S. griseus with some divergence in findings. The ultimate goal of this type of work was evidently the detection and estimation of the operation of the tricarboxylic acid cycle, since all of the compounds used were either a member of the cycle or related to the latter by only a few metabolic steps. According to Dulaney (23), citrate, pyruvate, fumarate, succinate, malate, lactate, and gluconate were utilized by this organism while acetate and malonate were not metabolized. A similar study by Hubbard and Thornberry (44) indicates that acetate, citrate, malonate, succinate, glutarate, and lactate can be utilized by S. griseus. The latter findings were subsequently confirmed by Pridham (54). Butterworth and Gilmour (9) have shown that S. griseus can oxidize acetate, lactate, pyruvate, and oxalacetate under resting conditions. Succinate, fumarate, and malate were metabolized by this organism only at higher concentrations, while citrate remained unoxidizable. More recent work by these authors (10,35) indicates that alpha-ketoglutarate and succinate were oxidized at higher concentrations than normally employed and a preincubation of the cells was necessary

in the oxidation of citrate, fumarate, and malate. Cochrane and Peck (16), in a similar study, have been able to show oxidation of certain compounds of the tricarboxylic acid cycle by Streptomyces coelicolor with the exception of alpha-ketoglutarate and citrate. By using the cell-free extracts they were able to demonstrate the presence of most of the specific enzymes responsible for the operation of the tricarboxylic acid cycle.

The next obvious step in gathering and interpreting the information pertaining to the metabolic activity of S. griseus was the isolation of intermediates of the oxidative pathways and several workers in the field have obtained data of this general nature. Sevcik (63) isolated acetic and lactic acids, and postulated incomplete oxidation of glucose to those compounds in addition to CO_2 and water. Pyruvate and triose phosphate were isolated by Scavola and Valcurone (59) in similar studies, and Scavola and Zorzoli (60) have actually been able to isolate acetyl phenylhydrazine and thus prove the existence of the active acetyl radical.

The advent of radioactive tracers as a tool in biochemical studies has recently made important advances on this problem. Whereas a minor portion of

tracer studies on S. griseus was concerned with the mechanism of synthetic pathways of antibiotics formation (45, 46), the main emphasis was placed by some workers on the study of the metabolism of the organism.

Despite the fact that there is some early work doubting the operation of the Krebs cycle (33), the work of Coty et al. (20) has not only demonstrated in cell-free extracts of S. griseus, that most of the enzymes responsible for the critical steps of this terminal oxidative pathway are present, but also was successful in showing the operation of the following reactions:

Oxalacetate + acetylphosphate → citrate;

citrate → alpha-ketoglutarate:

Addition of oxalacetate reversed malate oxidation. It was further shown employing C^{14} labeled succinate, that labeled fumarate and malate were obtained, hence suggesting very strongly that the tricarboxylic acid cycle was operating in this organism. Gilmour and coworkers (35) in studies on the utilization of CH_3-$C^{14}OOH$ by S. griseus have shown that the isotopic distribution patterns of aspartic and glutamic acids, isolated from the labeled cells, are in good agreement with the operation of tricarboxylic acid cycle in this organism. Cochrane and Peck (16) have come to the same conclusion based on experimental findings in both Warburg manometric studies

and tracer studies. It has also been demonstrated by Butterwoth *et al.* (10) by using $C^{14}O_2$ and degradation of metabolic intermediates that a C_3-C_1 condensation of probably the Wood-Werkman type is also a major biosynthetic pathway in *S. griseus*.

The catabolic pathways of glucose operative in this organism are not yet clearly understood. The results obtained by a series of inhibition studies by Hockenhull and coworkers (43) point to the operation of the classical Embden-Meyerhof scheme, yet the findings could not clearly rule out the presence of some alternative mechanism, such as the direct oxidative pathway. It should, however, be noted that work upon related organisms, *Streptomyces scabies* and *Streptomyces coelicolor* (17), indicates that the direct oxidative pathway is indeed one of the means by which glucose is broken down by these organisms. The preferential liberation of $C^{14}O_2$ from glucose-1-C^{14} over that from glucose-3,4-C^{14} was observed in these experiments.

In addition to the above evidence the presence of necessary enzyme systems for glucose-6-phosphate and ribose-5-phosphate oxidation was also demonstrated. Inhibition studies seem also to corroborate the conclusion that the direct oxidative pathway is present and operative in *Streptomyces coelicolor* and

Streptomyces scabies.

Little is known about the nature of the relatively high endogenous respiration of Streptomyces which is characteristic of actinomycetes and fungi (69 ,81). Some attempts have been made in the past (50) to study various methods of reducing this endogenous respiration. Although some reduction could be affected by starvation, it was found that subsequent substrate oxidation was proportionally reduced. The effect of age of the culture on endogenous respiration was quite insignificant although the rate of oxygen uptake in the presence of glucose was higher with younger cells, thus making it more difficult to ascertain the marginal rates of respiration in older cultures.

EXPERIMENTAL AND RESULTS

General Procedures

Cultural methods

The organism used throughout these studies was a strain of Streptomyces griseus (3475 Waksman). Spore stock cultures were kept in sterile soil, from which they were transferred to bottle slants of modified agar[1] and incubated for several days, until maximum sporulation was obtained. Inocula were obtained by introducing nutrient broth[2] into the slant bottle, scraping the spores off the surface agar with a loop and pipetting aliquots of the resulting suspension into 300 ml. Erlenmeyer flasks containing 200 ml. of nutrient broth, supplemented with 0.1 percent glucose and 0.5 percent NaCl.

Cultures were incubated on a rotary type shaker for prescribed time interval at 28-32°C, harvested by centrifugation, washed twice with buffer (0.067 M, pH 6.9), and suspended in a chemically defined growth medium to a desirable concentration. The growth medium used

---

[1] 1.5 percent agar, 0.5 percent glucose, 0.5 percent peptone, 0.3 percent beef extract and a trace of yeast extract.

[2] 0.3 percent beef extract, 0.5 percent peptone, 0.1 percent glucose and a trace of yeast extract

throughout these experiments consisted of NaCl, 5.0g.; K_2HPO_4, 2.0g.; $(NH_4)_2HPO_4$, 2.0g.; $MgSO_4.7H_2O$, 0.1g.; $FeSO_4.7H_2O$, 0.001g. in 1000 ml. of distilled water. The medium was autoclaved for 20 minutes and its acidity adjusted to pH 6.8 prior to the addition of cells.

Radioactivity measurement.

Radioactivity of respiratory CO_2 was counted as $BaCO_3$ on aluminum plates prepared by the centrifugation method. Radioactivity in the medium was measured by conversion of the carbonaceous compounds to $BaCO_3$ by persulphate wet combustion method, that in the cells by the Van Slyke-Folch wet combustion method with subsequent counting the mounted barium carbonate planchets. All radioactivity measurements were carried out to a standard deviation of 3% and corrected for background and self-absorption.

All radioactivity determinations in the degradation work were done as $BaCO_3$ in the conventional manner using stainless steel planchets. Samples were counted with 1% accuracy and corrected for background and self-absorption.

C^{14} labeled substrates.

Glucose-1,2,6-C^{14}, fructose-1,6-C^{14}, arabinose-1-C^{14}, and ribose-1-C^{14} were obtained from the Bureau of Standards through the kind cooperation of Dr. Isbell. Glucose-u-C^{14} and acetate-1 and 2-C^{14} were procured

from Tracerlab Inc. Glucose-3,4-C^{14} was prepared in this laboratory according to the method of Wood and coworkers (80). All labeled substrates were diluted with nonisotopic substrate to a defined specific activity before they were used.

Time Course Studies

Apparatus.

A special apparatus was designed to carry out time course studies of the utilization of labeled carbon source in respect to the interval radiochemical recovery in the respiratory CO_2, the fermentation products in the medium and cellular constituents at any specific time.

The assembly illustrated in Figure 1 permits the study of the utilization of six substrates by microorganisms under identical conditions. The flow rate of the gas sweeping through each system is controlled by the master flowmeter (F) which can be connected to any single incubation flask (I) by way of the manifold-stopcock (S) manipulation. Each growth flask is also equipped with a sampling device (D) for the collection of cell and medium samples at desirable intervals. Testing of the absorption efficiency or the CO_2 trap (T) indicated that leakage is less than 2% at a gas-flow rate of 200 ml. per minute, using 25 ml. of 0.5 N

CO_2-free sodium hydroxide solution and a few drops of octanol as antifoaming agent in the trap.

Figure 2 shows a flow diagram of the entire assembly consisting of six incubation flasks.

Experimental procedure.

Procedures used in the time course experiments on the utilization of different substrates were essentially the same as described by Wang et al. (76). Usually 200 ml. of cell suspension containing from 350 to 500 mgs of cells were used in each growth flask. Air was passed through the medium at a rate of 200 or 400 ml. per minute. Samples of the medium were assayed at regular intervals for glucose content according to the method of Shaffer and Somogy (64,66). The NaOH solutions for trapping respiratory CO_2 were replaced every half an hour in the early stage of the experiment and every hour thereafter. Usually a battery of 6 to 12 flasks was used in each series of experiments to permit a comparison of factors in question with cells grown under identical conditions.

At the end of each experiment , cells were separated from the medium by centrifugation, thoroughly washed and dried in vacuo over P_2O_5. Products of fermentation in the medium were examined by means of paper-chromatography, followed by radioautographic detection of activity for individual compounds. Colorimetric

determinations were used for the detection and assay of different components of the incubation media.

Nature of endogenous respiration.

In order to study the contribution of the respiratory CO_2 from the endogenous carbon sources, the nutrient broth was modified to contain 0.5 percent glucose with appropriate level of glucose-u-C^{14} to render cells radioactive. Uniformly labeled cells so obtained were washed and suspended in a carbon-source-free proliferating medium and transferred into two time course study flasks. While one flask contained no extraneous carbon source, into the other flask was introduced 0.7 mM of unlabeled glucose as sole carbon substrate. This arrangement therefore made it possible to obtain information on the effect of external substrate on endogenous respiration.

The results obtained in this experiment are summarized graphically in Figure 3.

Utilization of labeled substrates.

The initial phase of the present work involves a survey of utilization of various substrates by S. griseus with respect to the relative distribution of activity among the respiratory CO_2, cells, and media, using tracer techniques. Procedures outlined previously were followed in all of these experiments, with amounts of incubation media and cell weight kept within the

limits specified. At the end of each experiment incubation media were extracted with ether in a liquid-liquid extractor to determine the nature of the labeled fermentation products. By using cells incubated for a different time interval prior to the time course study, the effect of age of culture on the pattern of utilization of glucose and ribose was also studied.

The level of radioactivity used in this series of experiments was 0.5 uc per incubation flask, while the total amount of substrate employed in each experiment was not the same due to the fact that the rate of utilization for different substrates by S. griseus was far from constant. The level for glucose and fructose employed was 126 mg. per flask, that for acetate was 45 mg. per flask, while in the case of experiments with arabinose or ribose 30 mg. per flask was used.

The data obtained in the above experiment are given in Table 1.

With labeled glucose as substrate, paper-chromatographic examination of the labeled compounds in the incubation medium revealed that the ether-soluble fraction consisted mainly of succinic acid, and some ten amino acids were identified in the ether-insoluble fraction, although glutamic acid was the only compound labeled with detectable level of radioactivity.

Identification of pathways of glucose catabolism.

In view of the fact that little is known about the metabolic pathways and the possible variation in the pathway distribution for cultures of this organism, incubated for different lengths of time, time course experiments on the utilization of glucose-1,2,6 and u-C^{14} by cultures of S. griseus, 12, 36 and 72 hours old were carried out. Radiochemical recovery of substrate activity in respiratory CO_2 observed in these experiments are given in Table 2. The interval specific activities of respiratory CO_2 as derived from glucose-1,2, or 6-C^{14} were given in Figures 4,5, and 6, as time course graphs.

Furthermore, additional information was also obtained by studying the utilization of some of the key breakdown products of glucose by this organism, namely ribose and acetate, in a series of time course experiments. The interval specific activities and cumulative radiochemical recovery of respiratory CO_2 as derived from ribose-1-C^{14}, acetate-1-C^{14} and acetate-2-C^{14} are given in Figures 7, 8, and 9.

Estimation of pathways of glucose catabolism.

With the information obtained in the foregoing experiment on hand, efforts were then directed to the estimation of individual catabolic pathways of glucose in S. griseus culture incubated for 36 hours. Time course experiments were again carried out on the utilization of

glucose-1,2 and 6-C^{14}(along with glucose-3,(4)-C^{14}), by this organism, in the presence and absence of sodium arsenite in the incubation medium. The experimental conditions employed in this series of experiments were otherwise identical in every respect.

The interval and cumulative radiochemical recovery in respiratory CO_2 as well as chemical recovery of metabolic CO_2 from the respective labeled substrate are given in Figures 10, 11, 12, 13, and Table 3. Distribution of activity derived from the labeled substrate among respiratory CO_2, incubation media and cellular constituents are given in Tables 4 and 5. The incubation media were further assayed for the amount of keto acids and lactic acid according to the method of Friedman (30) and that of Baker and Summerson (5), respectively. With normal incubation medium no significant amount of keto acids and only a trace of lactic acid (.5 mg per flask) were detected. On the other hand, as one would expect, the presence of arsenite in the incubation medium resulted in formation of 5 mgs of total keto acids and 16 mgs of lactic acid, evidently from administered substrate.

TABLE 1

DISTRIBUTION OF RADIOACTIVITY IN *STREPTOMYCES GRISEUS* UTILIZING VARIOUS C^{14} LABELED SUBSTRATES

Substrate	Age of cultures in hours	Percent incorporated				Medium content	
		Cells	Medium	CO_2		Ether extract	Ether insoluble
				%	mg		
Glucose-u-C^{14}	12	28	7	41	114	4%	4%
Glucose-u-C^{14}	36	17	25	32	106	10%	11%
Glucose-u-C^{14}	72	13	31	33	74	21%	8%
Acetate-1-C^{14}	36	7	--	71	--	--	--
Acetate-2-C^{14}	36	18	--	52	--	--	--
Ribose-1-C^{14}	36	24	22	30	--	--	--
Ribose-1-C^{14}	12	61	4	23	--	--	--
Fructose-1,6-C^{14}	36	2	82	11	--	--	--
Arabinose-1-C^{14}	36	not utilized				--	--

Time of incubation with labeled substrate: 12, 36 and 72 hour cultures were incubated with glucose-u-C^{14} for $5\frac{1}{4}$, 6 and $4\frac{1}{2}$ hours, respectively: 12 hour culture was incubated with ribose-1-C^{14} for 6 hours. All other experiments were run for 12 hours.

TABLE 2

DISTRIBUTION OF RADIOACTIVITY IN RESPIRATORY CO_2 FROM 12, 36 AND 72 HOURS OLD CULTURE OF STREPTOMYCES GRISEUS UTILIZING C^{14} LABELED SUBSTRATE

Substrate	Percent activity recovery in respiratory CO_2					
	12 hours culture		36 hours culture		72 hours culture	
	At 5 hours	At 11 hours	At 3 hours	At 11 hours	At 7 hours	At 11 hours
Glucose-1-C^{14}	22	31	9	29	14	20
Glucose-2-C^{14}	24	35	11	42	22	29
Glucose-3,4-C^{14}	61*	65*	25	54	39*	44*
Glucose-6-C^{14}	19	30	6	27	14	20
Glucose-u-C^{14}	35	43	17	39	21	26

Weight of cells per flask: 350 mg, 450 mg, and 350 mg for cultures 12, 36, and 72 hours old, respectively.

Air-flow rate per flask: 200 ml per minute.

Level of substrate per flask: 126 mg.

Level of radioactivity per flask: .5 uc.

Temperature: 30°C.

Time of incubation: 11 hours.

* Calculated value (76)

TABLE 3

CUMULATIVE WEIGHT OF RESPIRATORY CO_2 FROM 36 HOURS OLD CULTURE OF STREPTOMYCES GRISEUS UTILIZING GLUCOSE

Time in hours	Weight of CO_2	
	Normal medium	.01M sodium arsenite in medium
0	33.6	15.0
1	60.0	24.7
2	86.4	36.2
3	104.7	47.3
4	119.0	57.3
5	131.8	64.4
6	143.0	70.2
7	153.8	75.9
8	165.2	81.6
10	189.8	91.9
12	214.3	100.7

TABLE 4

DISTRIBUTION OF RADIOACTIVITY IN 36 HOURS OLD CULTURE OF STREPTOMYCES GRISEUS UTILIZING C^{14} LABELED GLUCOSE

Substrate	Percent radiochemical recovery			
	Respiratory CO_2	Medium	Cells	Total
Glucose-1-C^{14}	29	16	46	91
Glucose-2-C^{14}	42	15	38	95
Glucose-3,4-C^{14}	54	14	24	92
Glucose-6-C^{14}	27	19	50	96
Glucose-u-C^{14}	39	16	37	92

Cell weight per flask: 500 mg.

Air-flow rate per flask: 400 ml per minute.

Level of substrate per flask: 126 mg.

Level of radioactivity per flask: .5 uc.

Temperature: 30°C.

Time of incubation: 12 hours.

TABLE 5

DISTRIBUTION OF RADIOACTIVITY IN 36 HOURS OLD CULTURE OF STREPTOMYCES GRISEUS UTILIZING GLUCOSE C^{14} IN THE PRESENCE OF .01 M SODIUM ARSENITE

Substrate	Percent radiochemical recovery			
	Respiratory CO_2	Medium	Cells	Total
Glucose-1-C^{14}	47	43	4	94
Glucose-2-C^{14}	15	65	7	87
Glucose-3,4-C^{14}	16	72	5	93
Glucose-6-C^{14}	2	91	5	98
Glucose-u-C^{14}	19	61	8	88

Cell weight per flask: 450 mg.

Air-flow rate per flask: 400 ml per minute.

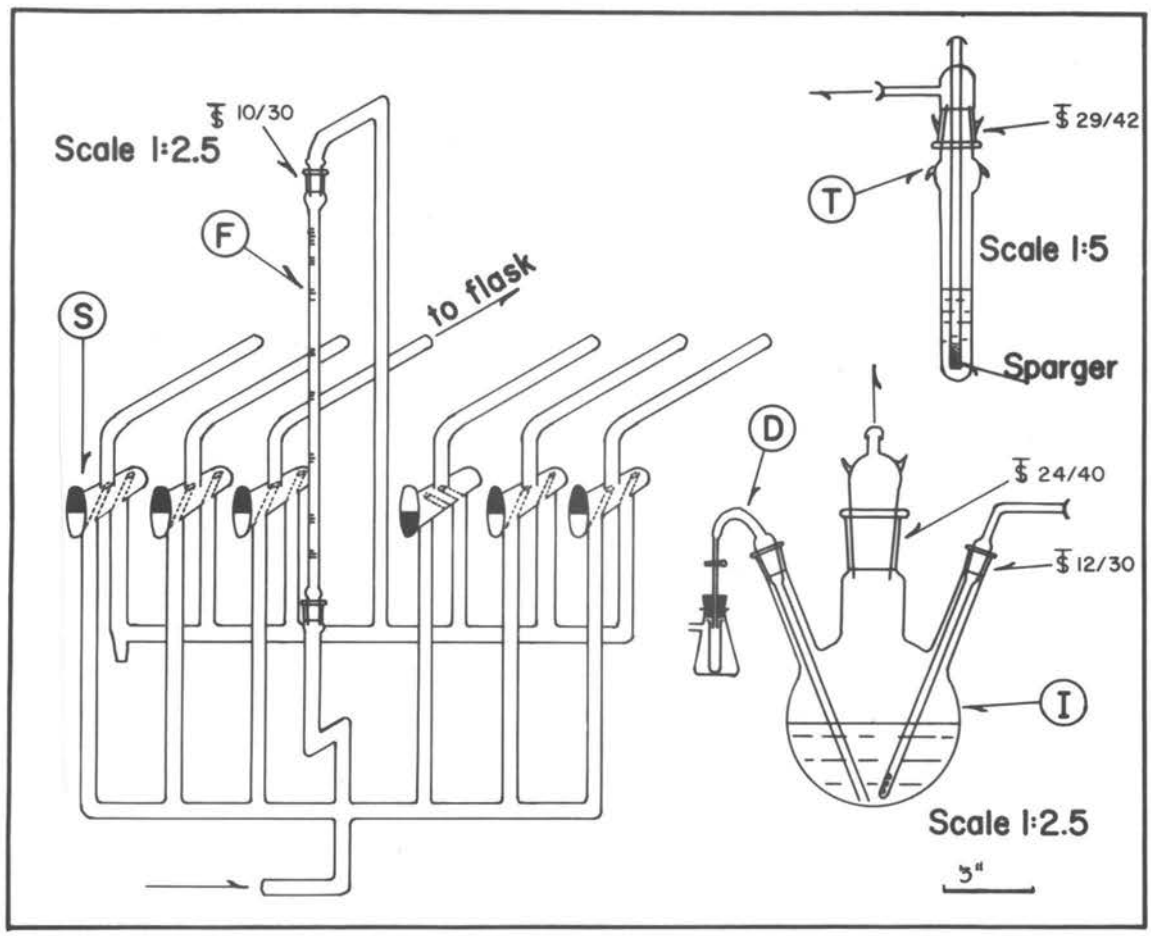
Level of substrate per flask: 126 mg.

Level of radioactivity per flask: .5 uc.

Temperature: 30°C.

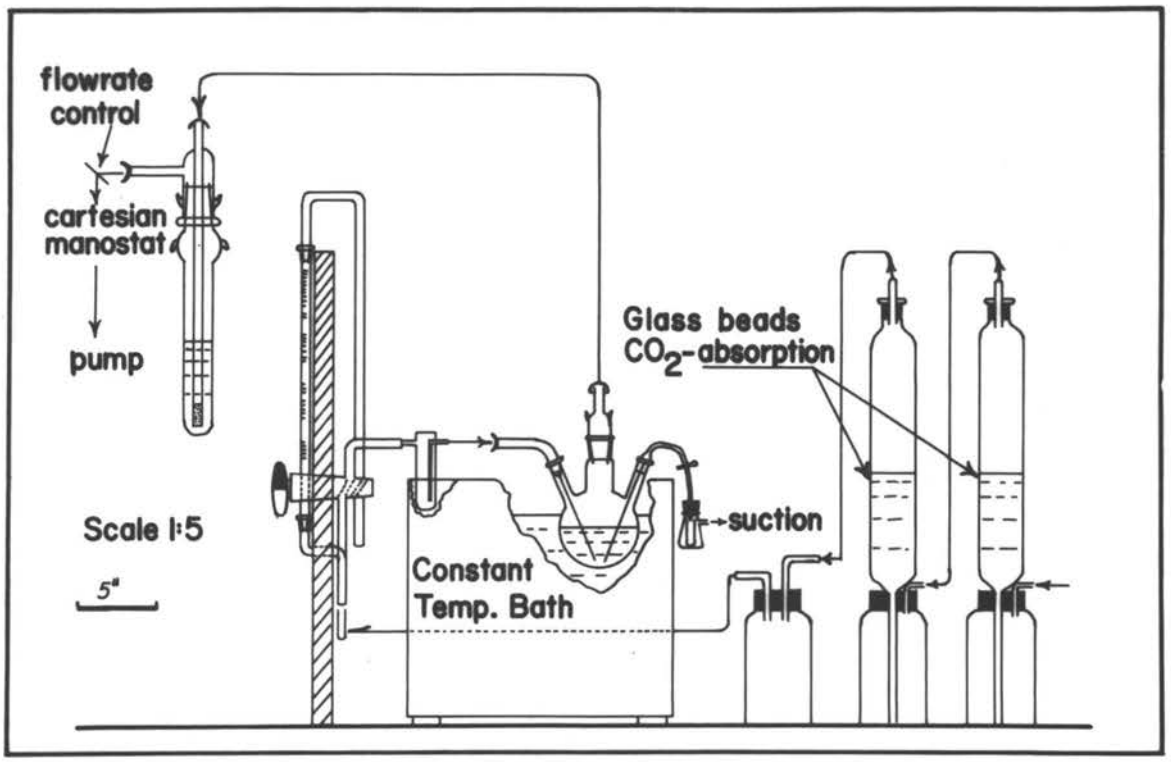
Time of incubation: 12 hours.

FIGURE 1



APPARATUS FOR THE TIME COURSE STUDY OF CARBOHYDRATE METABOLISM IN MICROORGANISMS

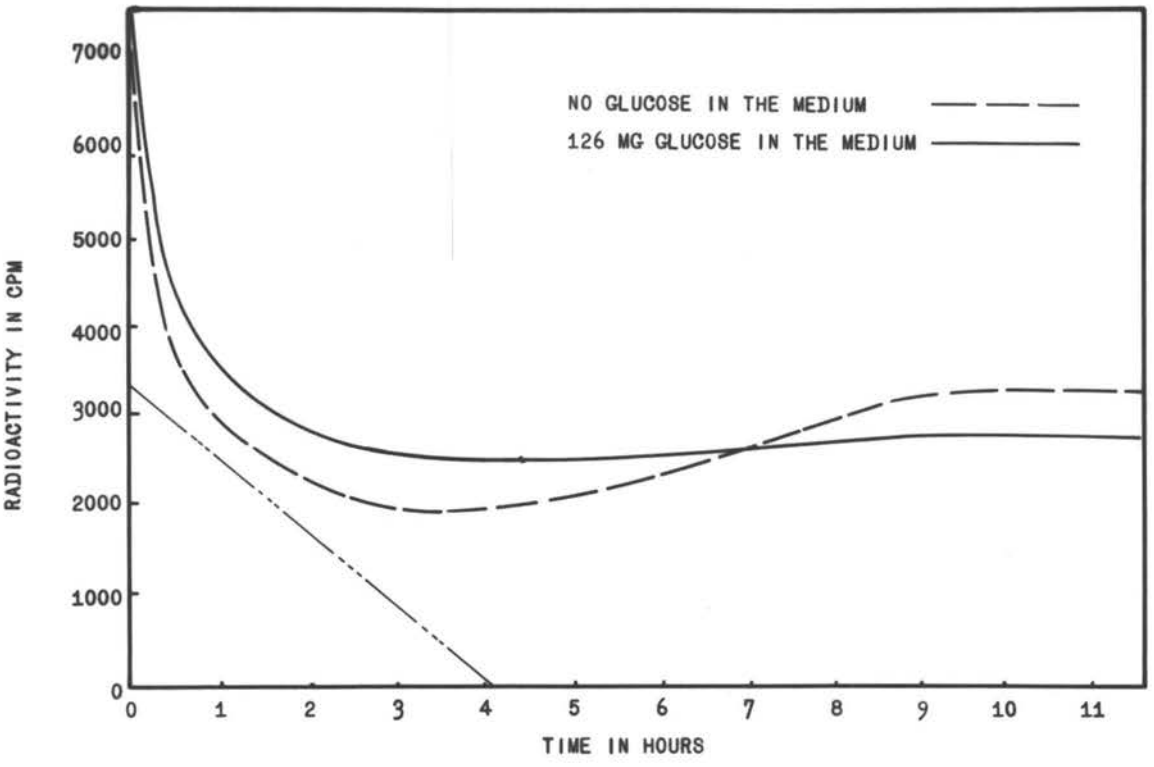
FIGURE 2



ASSEMBLY FOR THE TIME COURSE STUDY OF CARBOHYDRATE METABOLISM IN MICROORGANISMS

FIGURE 3

EFFECT OF SUBSTRATE ON ENDOGENOUS RESPIRATION IN 36 HOURS OLD CULTURE OF S. GRISEUS



——··· —— medium glucose disappearance.

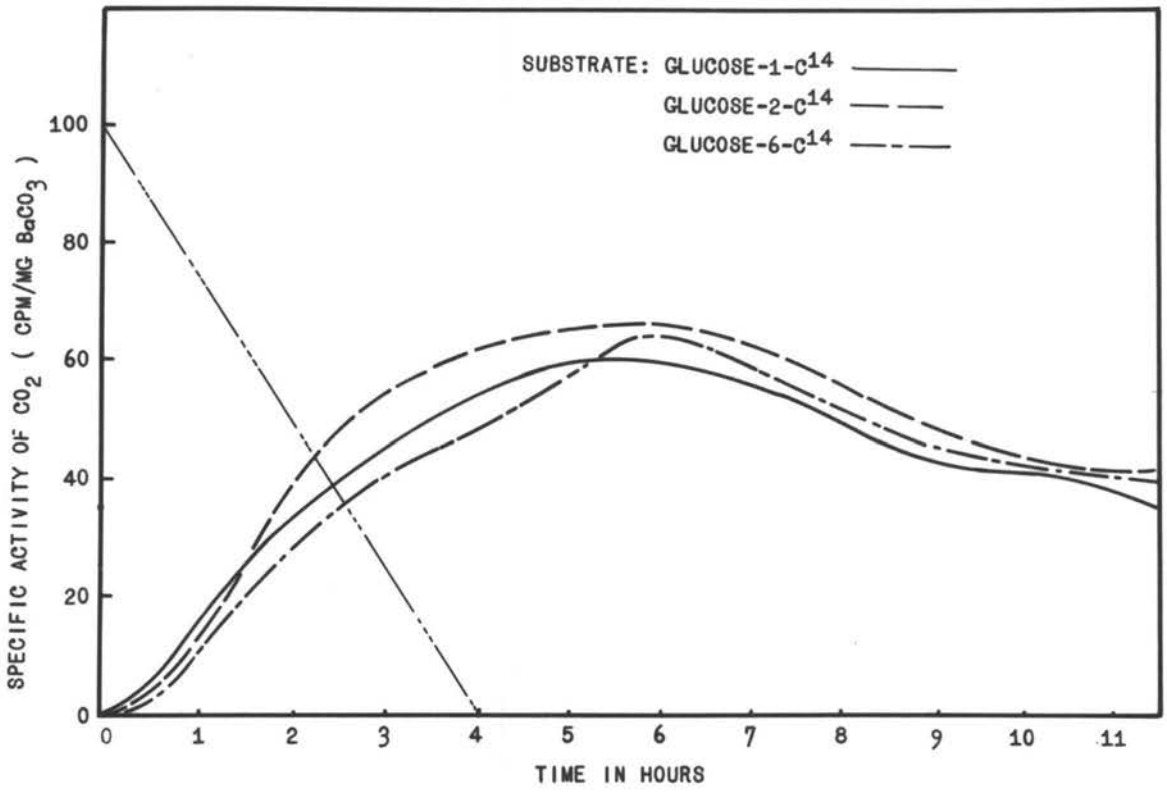
Weight of cells per flask: 500 mg.

Air-flow rate per flask: 400 ml per minute.

Temperature: 30°C.

FIGURE 4

SPECIFIC ACTIVITY OF RESPIRATORY CO_2
FROM 12 HOURS OLD CULTURE OF S. GRISEUS
GROWN ON SPECIFICALLY C^{14} LABELED GLUCOSE



——·····—— medium glucose disappearance.

Weight of cells per flask: 350 mg.

Air-flow rate per flask: 200 ml per minute.

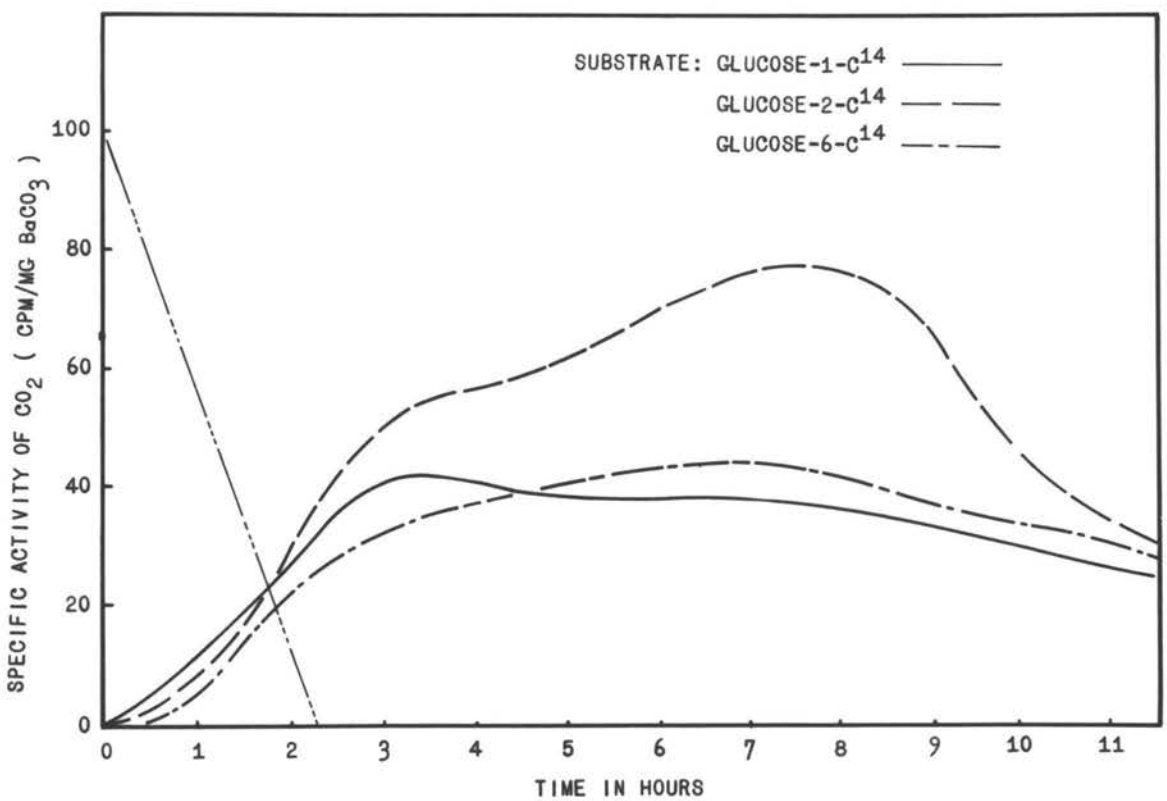
Level of substrate per flask: 126 mg.

Level of radioactivity per flask: .5 uc.

Temperature: 30°C.

FIGURE 5

SPECIFIC ACTIVITY OF RESPIRATORY CO_2
FROM 36 HOURS OLD CULTURE OF S. GRISEUS
GROWN ON SPECIFICALLY C^{14} LABELED GLUCOSE



——·-·—— medium glucose disappearance.

Weight of cells per flask: 450 mg.

Air-flow rate per flask: 200 ml per minute.

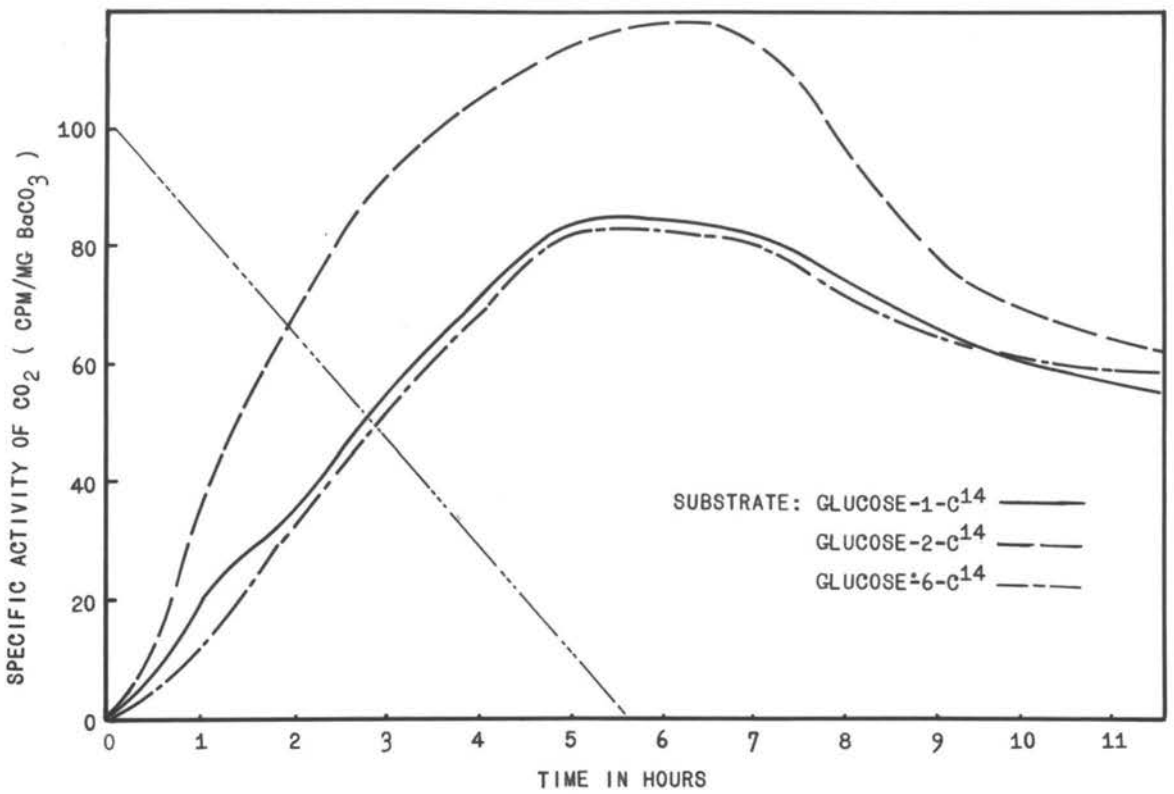
Level of substrate per flask: 126 mg.

Level of radioactivity per flask: .5 uc.

Temperature: 30°C.

FIGURE 6

SPECIFIC ACTIVITY OF RESPIRATORY CO_2
FROM 72 HOURS OLD CULTURE OF S. GRISEUS
GROWN ON SPECIFICALLY C^{14} LABELED GLUCOSE



—·····— medium glucose disappearance.

Weight of cells per flask: 350 mg.

Air-flow rate per flask: 200 ml per minute.

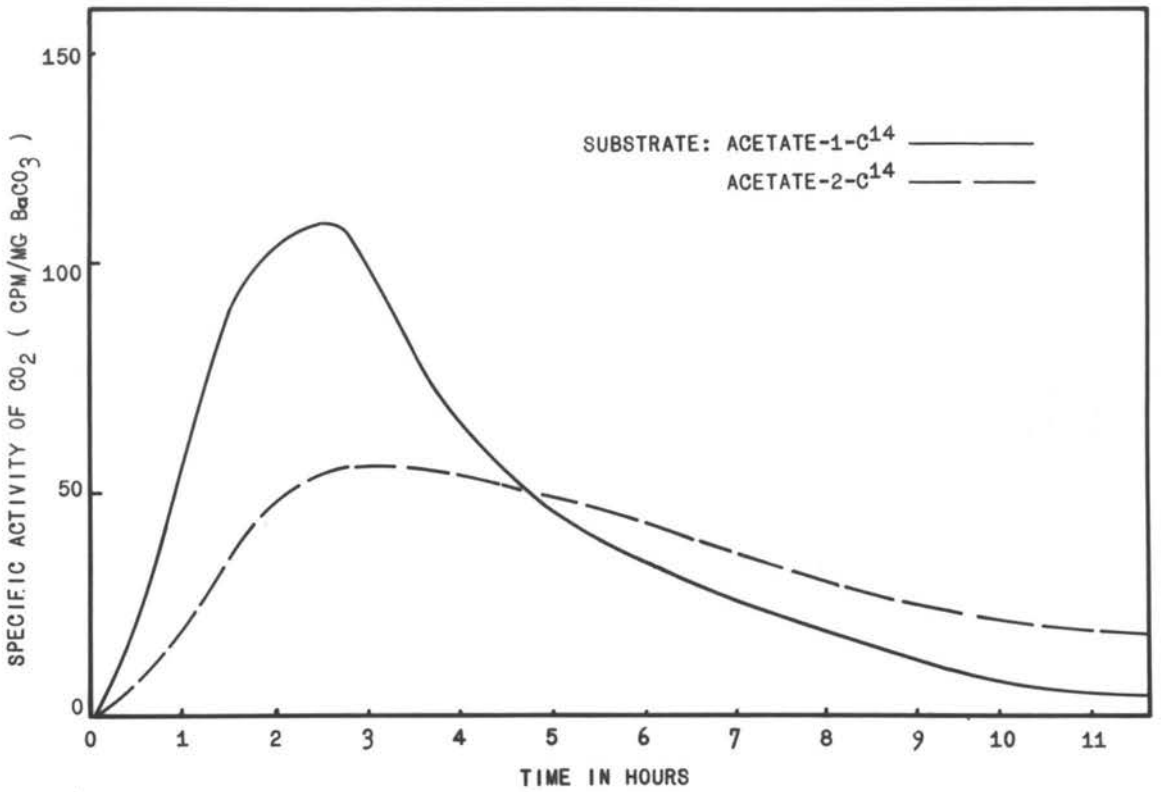
Level of substrate per flask: 126 mg.

Level of radioactivity per flask: .5 uc.

Temperature: 30°C.

FIGURE 7

SPECIFIC ACTIVITY OF RESPIRATORY CO_2
FROM 36 HOURS OLD CULTURE OF S. GRISEUS
GROWN ON ACETATE-1 OR 2-C^{14}



Weight of cells per flask: 400 mg.

Air-flow rate per flask: 200 ml per minute.

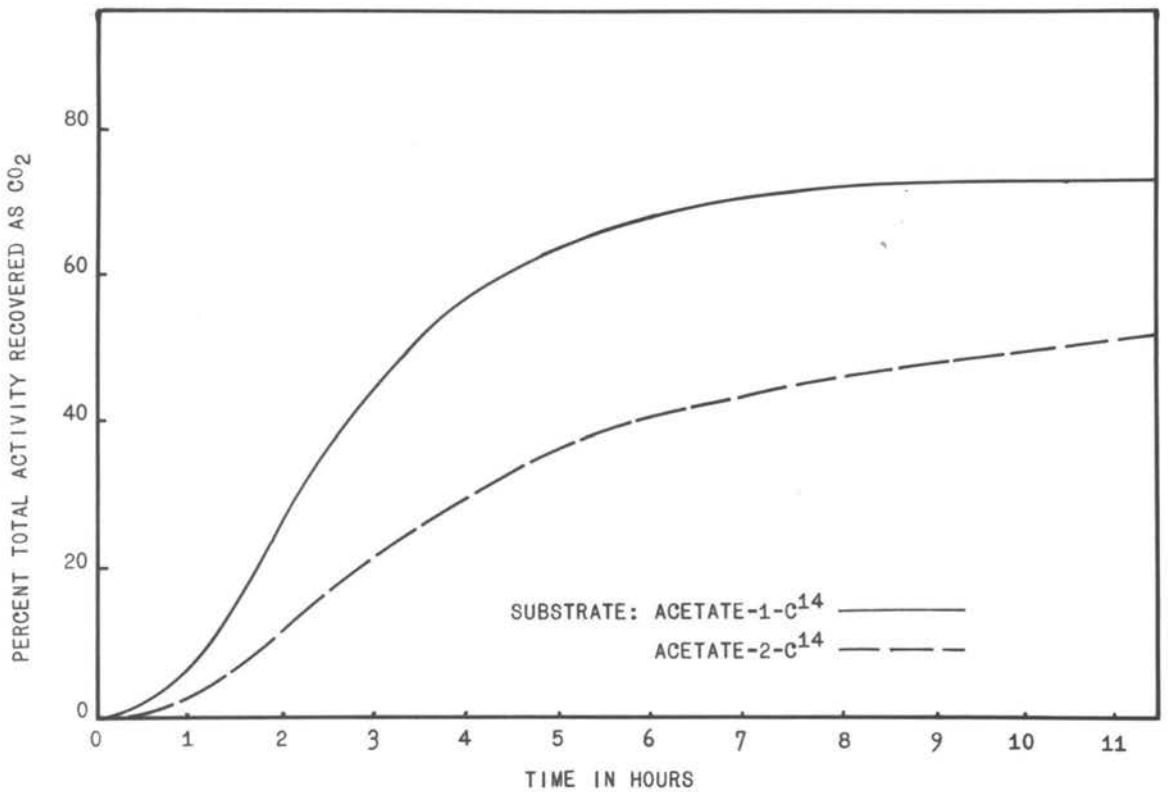
Level of substrate per flask: 45 mg.

Level of radioactivity per flask: .5 uc.

Temperature: 30°C.

FIGURE 8

CUMULATIVE RADIOCHEMICAL RECOVERY IN RESPIRATORY CO_2 FROM 36 HOURS OLD CULTURE OF S. GRISEUS GROWN ON ACETATE-1 OR 2-C^{14}



Weight of cells per flask: 400 mg.

Air-flow rate per flask: 200 ml per minute.

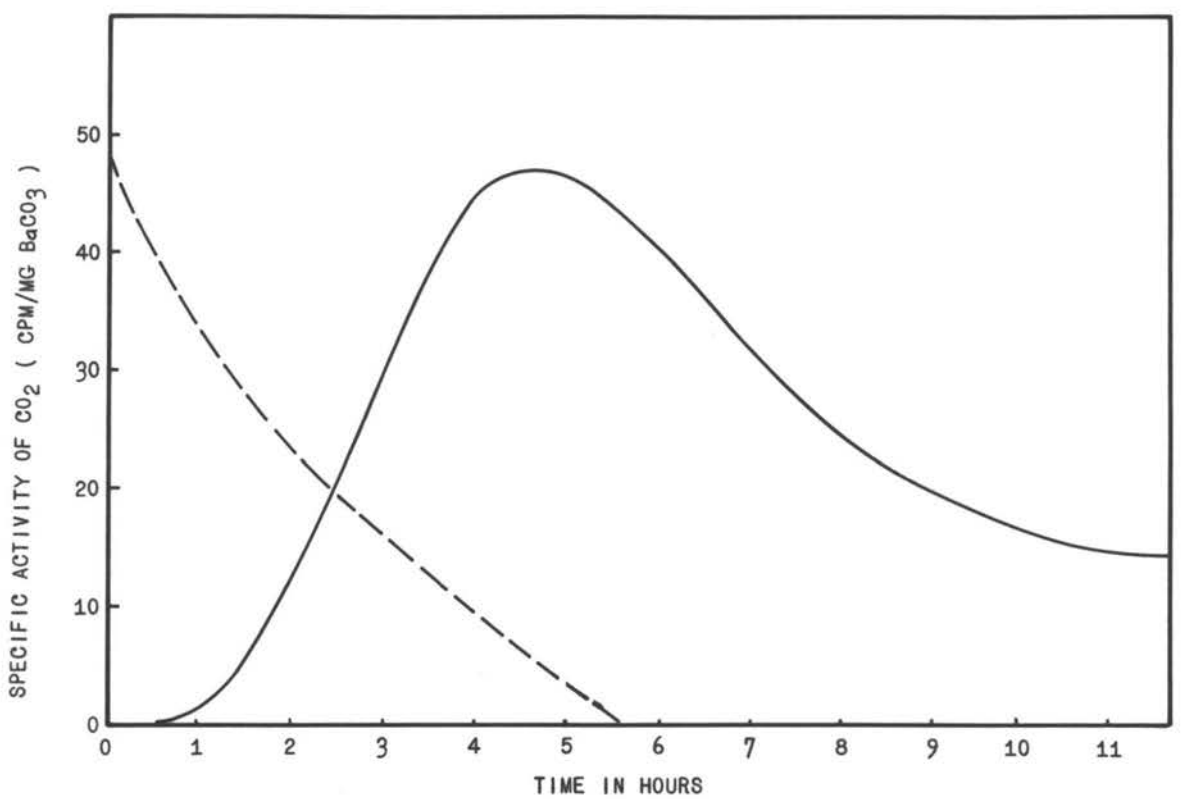
Level of substrate per flask: 45 mg.

Level of radioactivity per flask: .5 uc.

Temperature: 30°C.

FIGURE 9

SPECIFIC ACTIVITY OF RESPIRATORY CO_2
FROM 36 HOURS OLD CULTURE OF S. GRISEUS
GROWN ON RIBOSE-1-C^{14}



— — — medium ribose disappearance.

Weight of cells per flask: 400 mg.

Air-flow rate per flask: 200 ml per minute.

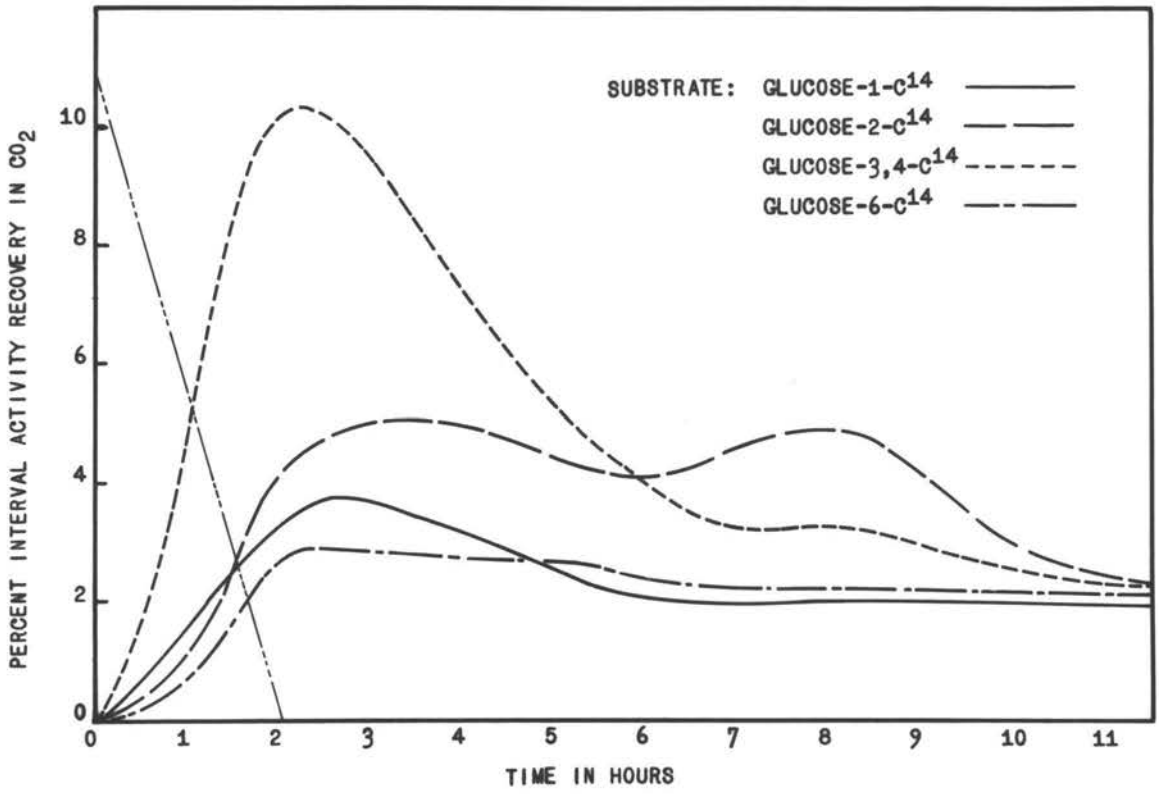
Level of substrate per flask: 30 mg.

Level of radioactivity per flask: .5 uc.

Temperature: 30°C.

FIGURE 10

INTERVAL RADIOCHEMICAL RECOVERY IN RESPIRATORY CO_2
FROM 36 HOURS OLD CULTURE OF S. GRISEUS
GROWN ON SPECIFICALLY C^{14} LABELED GLUCOSE



——·····—— medium glucose disappearance.

Weight of cells per flask: 500 mg.

Air-flow rate per flask: 400 ml per minute.

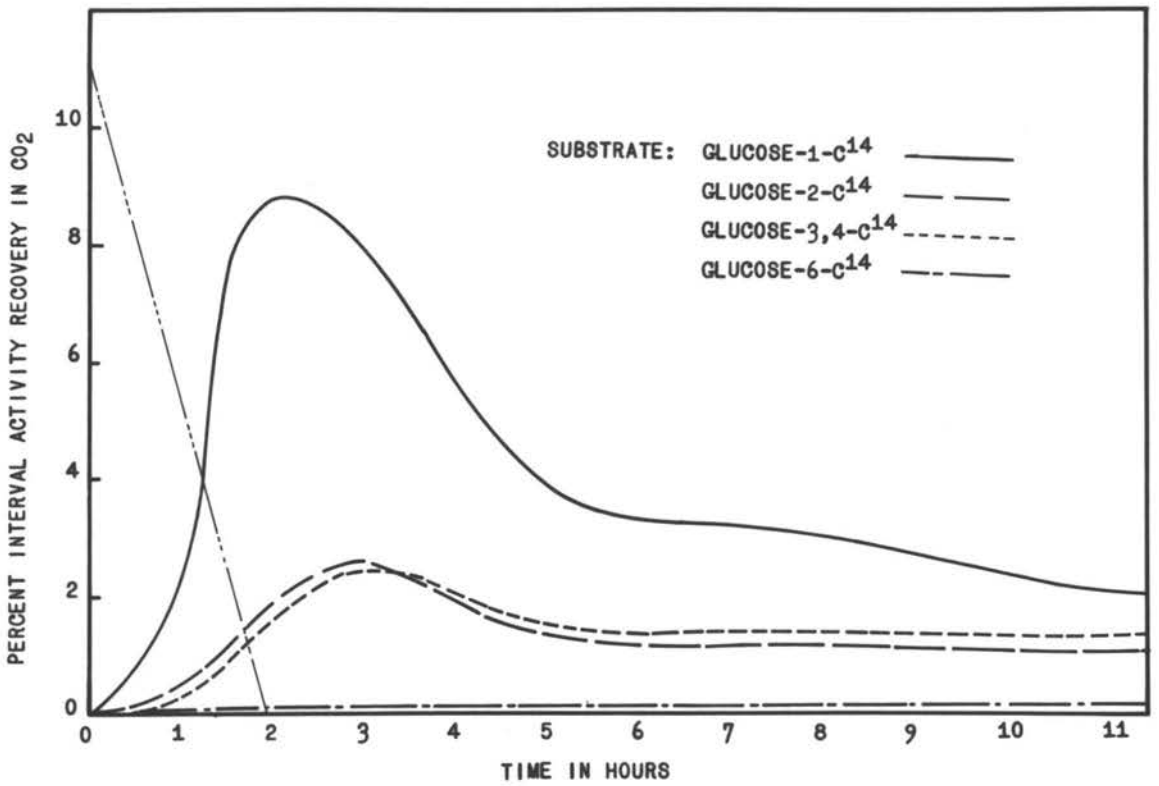
Level of substrate per flask: 126 mg.

Level of radioactivity per flask: .5 uc.

Temperature: 30°C.

FIGURE 11

INTERVAL RADIOCHEMICAL RECOVERY IN RESPIRATORY CO_2 FROM 36 HOURS OLD CULTURE OF S. GRISEUS GROWN ON SPECIFICALLY C^{14} LABELED GLUCOSE, IN THE PRESENCE OF SODIUM ARSENITE



——·····—— medium glucose disappearance.

Weight of cells per flask: 450 mg.

Air-flow rate per flask: 400 ml per minute.

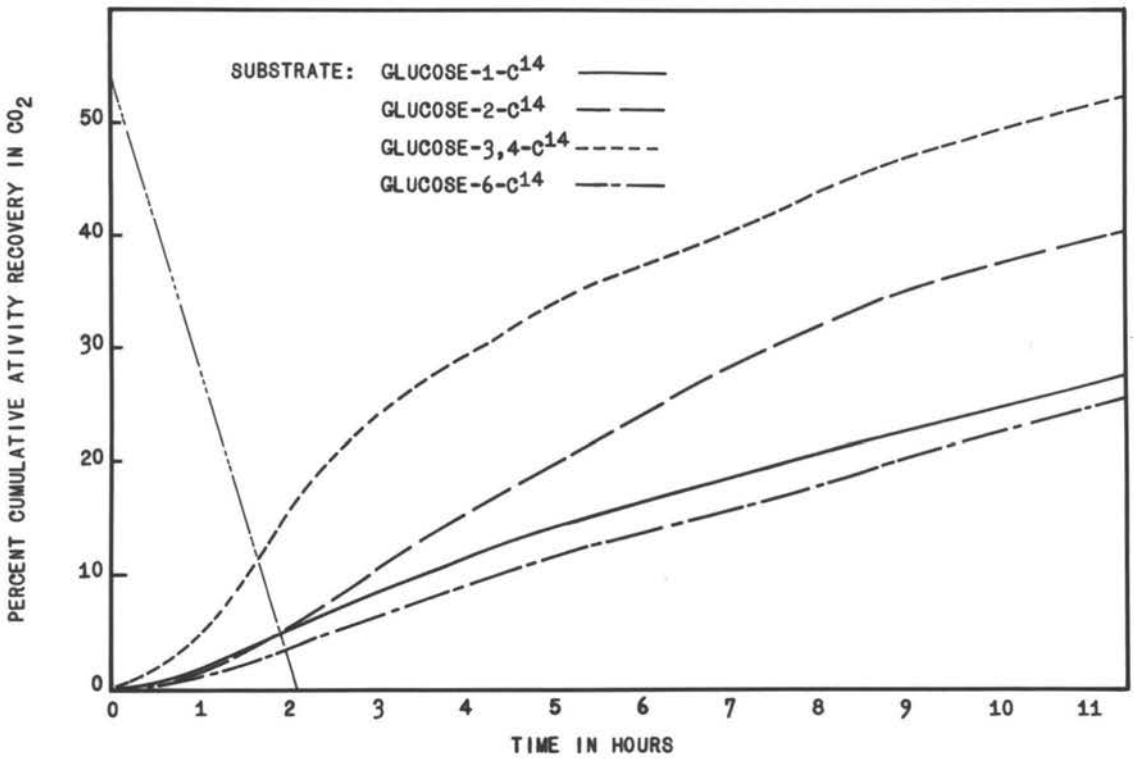
Level of substrate per flask: 126 mg.

Level of radioactivity per flask: .5 uc.

Temperature: 30°C.

FIGURE 12

CUMULATIVE RADIOCHEMICAL RECOVERY IN RESPIRATORY CO_2
FROM 36 HOURS OLD CULTURE OF S. GRISEUS
GROWN ON SPECIFICALLY C^{14} LABELED GLUCOSE



—— ··· —— medium glucose disappearance.

Weight of cells per flask: 500 mg.

Air-flow rate per flask: 400 ml per minute.

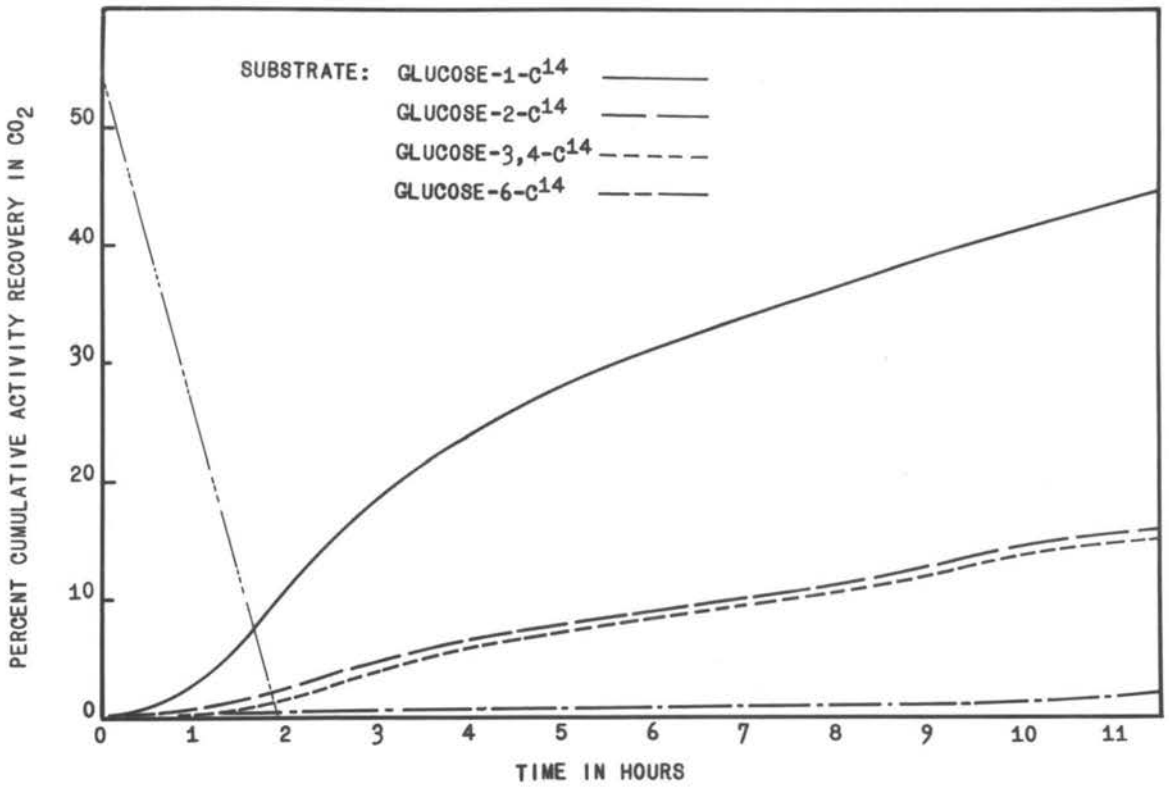
Level of substrate per flask: 126 mg.

Level of radioactivity per flask: .5 uc.

Temperature: 30°C.

FIGURE 13

CUMULATIVE RADIOCHEMICAL RECOVERY IN RESPIRATORY CO_2 FROM 36 HOURS OLD CULTURE OF S. GRISEUS GROWN ON SPECIFICALLY C^{14} LABELED GLUCOSE, IN THE PRESENCE OF SODIUM ARSENITE



——·····—— medium glucose disappearance.

Weight of cells per flask: 450 mg.

Air-flow rate per flask: 400 ml per minute.

Level of substrate per flask: 126 mg.

Level of radioactivity per flask: .5 uc.

Temperature: 30°C.

Utilization of Ribose-1-C^{14} by Streptomyces griseus

Incorporation experiment.

Preliminary experiments designed to test the utilization of ribose by S. griseus have indicated that a fair amount of activity from labeled ribose was incorporated into this organism, and moreover, that younger culture (12 hours) utilizes ribose to a greater extent than older culture (36 hours).

To insure a sizeable crop of cells in a reasonably short duration the amount of spores employed for inocula was increased over the dose used in the time course experiments described previously. Six spore slants (6 days old) were used to inoculate medium contained in 24 Erlenmeyer flasks and the cultures were incubated on the rotary type shaker for 12 hours at 28-30°C. Cells were harvested as described previously and were transferred into a battery of 12 growth flasks. After an hour-long depletion under vigorous aerobic conditions, 30 mg. of ordinary ribose were introduced into each of ten flasks and 30 mg. of ribose-1-C^{14} (specific activity of 125 uc per mM) were added to each of the remaining two flasks. The flow rate of air was maintained at 200 ml per minute throughout the duration of the experiment.

Ribose disappearance from the medium was followed by

the colorimetric method of Mejbaum (49) and exhaustion of substrate from medium was realized after 4 hours. The experiment was terminated one hour after ribose disappearance from the media. Cells in each flask were harvested in identical manner by centrifugation and were washed twice with distilled water and dried over P_2O_5 in a vacuum desiccator. The average weight of dry cells from each flask was 350 mgs.

The distribution pattern of radioactivity among the respiratory CO_2, medium, and cells is summarized in Table 8.

Isolation of cellular constituents from S. griseus utilizing ribose-1-C^{14}.

Small portions of labeled cells were taken for hydrolysis and subsequent determination of activity distribution among the cellular constituents.

One portion (6.5 mg) was extracted with hot trichloroacetic acid for 15 minutes according to the method of Schneider (62). Removal of trichloroacetic acid was accomplished by ether extraction, after which the water phase containing nucleic acids was taken to dryness by lyophylization and subsequently hydrolyzed with 1N HCl for one hour at 100°C (65). The resulting hydrolysate was taken to dryness repeatedly under vacuum to remove HCl and the residue was then paper-chromatographed in

TABLE 8

DISTRIBUTION OF C^{14} ACTIVITY IN *STREPTOMYCES GRISEUS* UTILIZING RIBOSE-1-C^{14}

Fractions	Percent
Cells	61
Respiratory CO_2	23
Medium	5
Total recovery	89

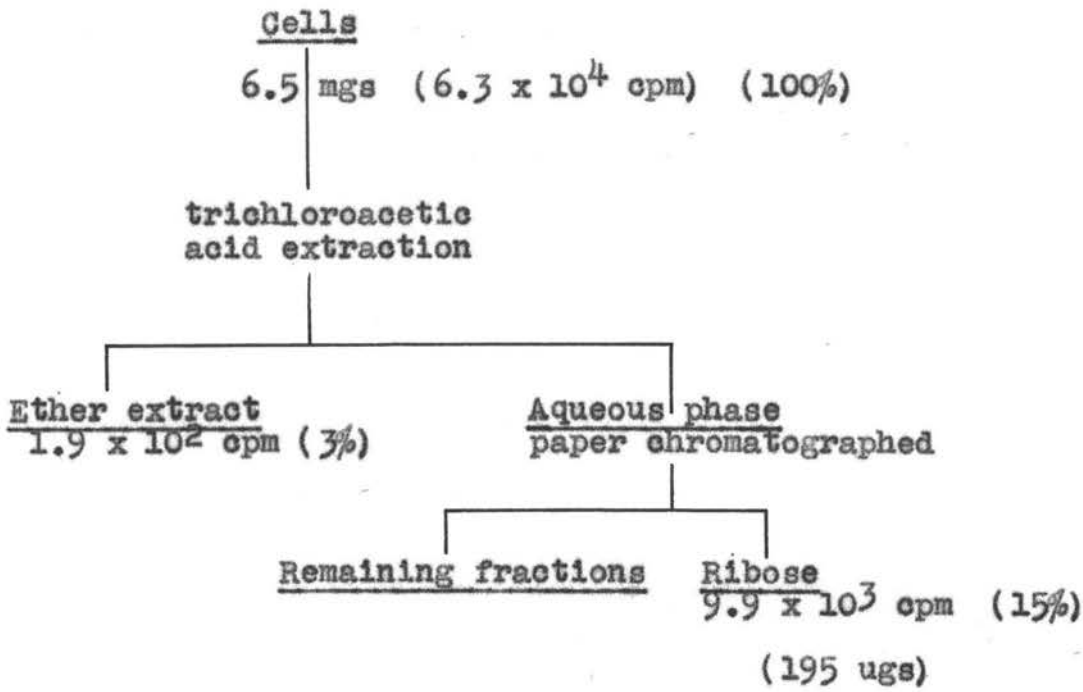
Weight of cells per flask: 350 mg.
Air-flow rate per flask: 200 ml per minute.
Level of substrate per flask: 30 mg.
Level of radioactivity per flask: 25 uc.
Temperature: 30°C.

duplicate in phenol-water system. The areas corresponding to ribose were located and eluted with water. The eluate from one spot was assayed for ribose by means of colorimetric method (49). The amount of radioactivity corresponding to this weight of ribose was estimated by wet combustion method using the eluate from the second spot. Distribution of radioactivity among cellular constituents resulting from this fractionation is represented in Diagram 1.

The next portion (100 mg) of cells was processed according to the method of Gilvarg (36) for the determination of the amount of activity incorporated into protein and carbohydrate fractions. The cells were defatted and extracted with .2N NaOH. The protein fraction was precipitated from the alkali extract with trichloroacetic acid. The residue from the extraction, consisting mainly of polysaccharide material, was hydrolyzed with 1N HCl at 100°C for one hour. Bulk of the HCl was removed by taking the hydrolysate to dryness, and the residue was then taken up in water and passed through Dowex-50 and Dowex-3 columns consecutively. Aliquots of the purified carbohydrate solution so obtained were paper-chromatographed in phenol-water systems. Spots corresponding to ribose and glucose were eluted respectively from the paper and their specific activities determined.

DIAGRAM 1

DISTRIBUTION OF C^{14}-ACTIVITY IN VARIOUS FRACTIONS OF *STREPTOMYCES GRISEUS* CELLS GROWN ON RIBOSE-1-C^{14}



Quantitative distribution of activity following the above scheme is summarized in Diagram 2.

Protein fraction in the above scheme was hydrolyzed in the conventional manner and analyzed by two-dimensional paper-chromatography and radioactivity scanning was then carried out for activity distribution among the various amino acids. It was found that the aspartic and glutamic acids were very heavily labeled, with some activity distributed among alanine, glycine, serine, threonine, proline, valine, histidine, lysine, leucine and/or isoleucine, arginine, phenylalanine and tyrosine.

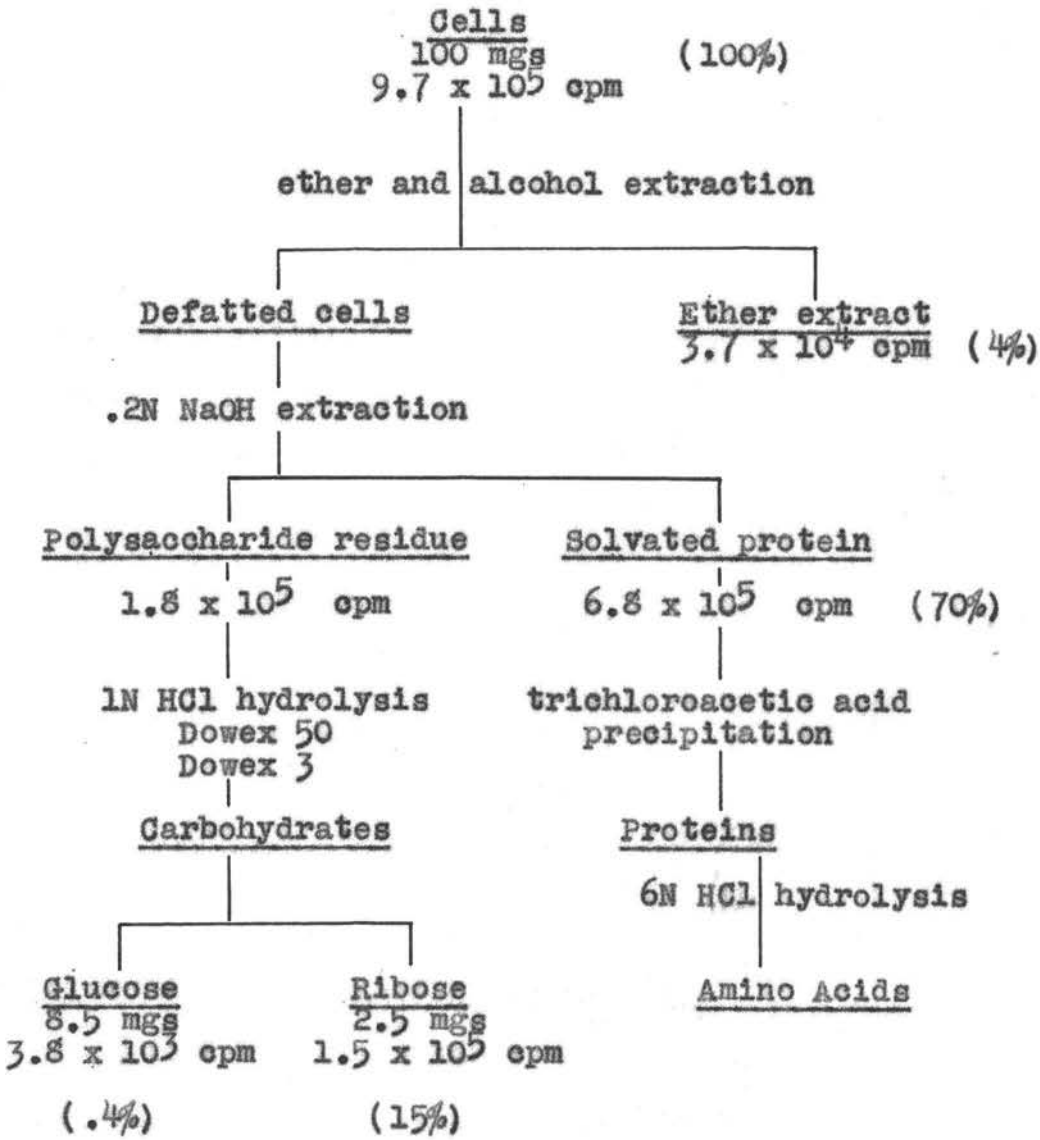
Isolation of aspartic acid and glutamic acid.

Three hundred milligrams of the labeled cells were mixed with 2.70 grams of unlabeled cells obtained in the incorporation experiment. The cells were hydrolyzed with constant boiling HCl under reflux conditions for 24 hours. After removal of HCl by repeated evaporation to dryness under vacuum, humin was removed by filtration and the hydrolysate was extracted exhaustively with ether in a liquid-liquid extractor.

The hydrolysate was then passed through a Dowex-1-X8 column according to the procedure of Hirs et al. (42). Glutamic and aspartic acids isolated by this method were diluted to appropriate levels with the respective non-labeled amino acid and subjected to further

DIAGRAM 2

DISTRIBUTION OF C^{14}-ACTIVITY IN VARIOUS FRACTIONS OF STREPTOMYCES GRISEUS GROWN ON RIBOSE-1-C^{14}



purification. Glutamic acid was recrystallized as a hydrochloride while aspartic acid was purified by converting it to its copper salt. The purity of both amino acids was established by paper-chromatography.

The scheme of the above separation procedure is given in Diagram 3.

Degradation of aspartic acid and glutamic acid isolated from S. griseus utilizing ribose-1-C^{14}.

The methods of Wang et al. (75 ,77) were followed in the degradation studies of both amino acids, with minor modifications.

The series of reactions carried out in the process of aspartic acid degradation were as follows:

(a) Total combustion provided a measure of total activity.

(b) The sum of alpha- and beta-carboxyl groups were obtained by ninhydrin reactions.

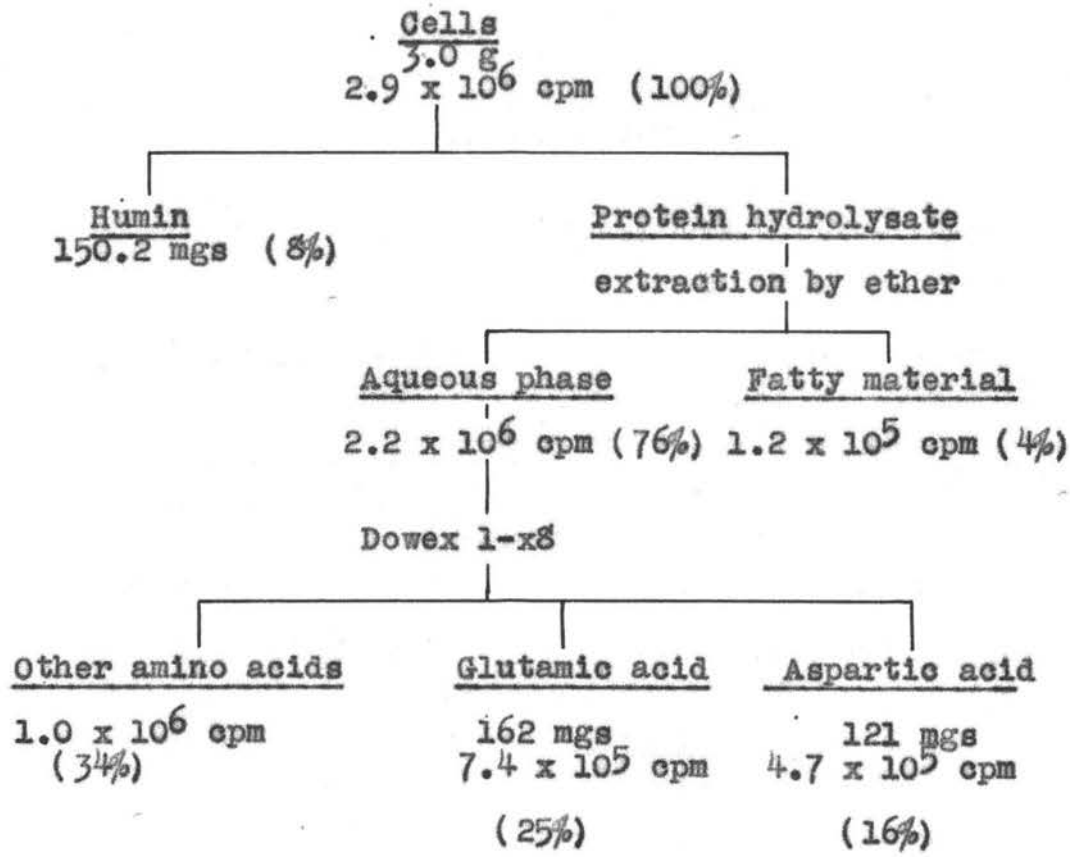
(c) Conversion of aspartic acid to malic acid and subsequent decarboxylation according to the method of Von Pechmann 56) gave activity of alpha-carboxyl group.

(d) Direct oxidation of aspartic acid by means of sodium hypoiodate and combustion of the resulting iodoform gave activity on methylene carbon.

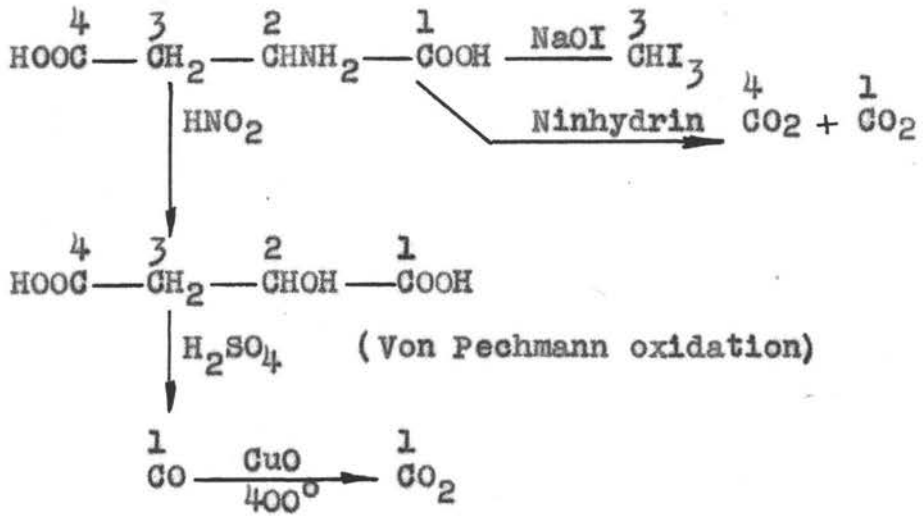
(e) The activity of amino carbon atom was obtained by difference.

DIAGRAM 3

THE ISOLATION OF ASPARTIC ACID AND GLUTAMIC ACID FROM STREPTOMYCES GRISEUS GROWN ON RIBOSE-1-C^{14}



Degradation Scheme for Aspartic Acid



Glutamic acid was degraded according to the following scheme:

(a) Total combustion gave total activity of the molecule.

(b) Ninhydrin decarboxylation provided a measure of activity of alpha-carboxyl carbon.

(c) Schmidt (26, 75) degradation on glutamic acid revealed the activity of C-5 and the resulting byproduct alpha, gamma-diaminobutyric acid was obtained in chromatographically pure state by passing the reaction mixture through a Dowex-3 column. Combustion of the dipicrate derivative (m.p. 182-4°C) prepared therefrom gave average activity of carbons 1, 2, 3, and 4.

TABLE 9

DISTRIBUTION OF C^{14} IN ASPARTIC ACID ISOLATED FROM *STREPTOMYCES GRISEUS* UTILIZING RIBOSE-1-C^{14}

Carbon atom	cpm X 10^4*	per cent of total
Total	3.23	100
1 COOH	.46	14
2 $CHNH_2$	1.12	35
3 CH_2	1.16	36
4 COOH	.49	15

* Total activity is expressed as counts per minute per millimole of aspartic acid; the specific activity of individual carbon atoms are counts per minute per millimole of carbon.

(d) The bulk of the alpha, gamma-diaminobutyric acid was oxidized with silver oxide (41) to beta-alanine and isolated as beta-alanine 2-nitro-1,3-indandionate. Subsequent liberation of the free amino acid with HCl and ether extraction yielded chromatographically pure beta-alanine. The combustion of a portion of the latter provided the average value of activity in C-2, 3, and 4.

(e) KOH-NaOH fusion of beta-alanine gave rise to acetic acid which was isolated as its Ba salt; combustion of the latter gave average activity of C-2 and 3;

(f) Schmidt reaction on acetic acid resulted in the conversion of acetate carboxyl to CO_2 and hence gave the activity of C-2 directly and C-3 by difference.

Degradation of Glutamic Acid

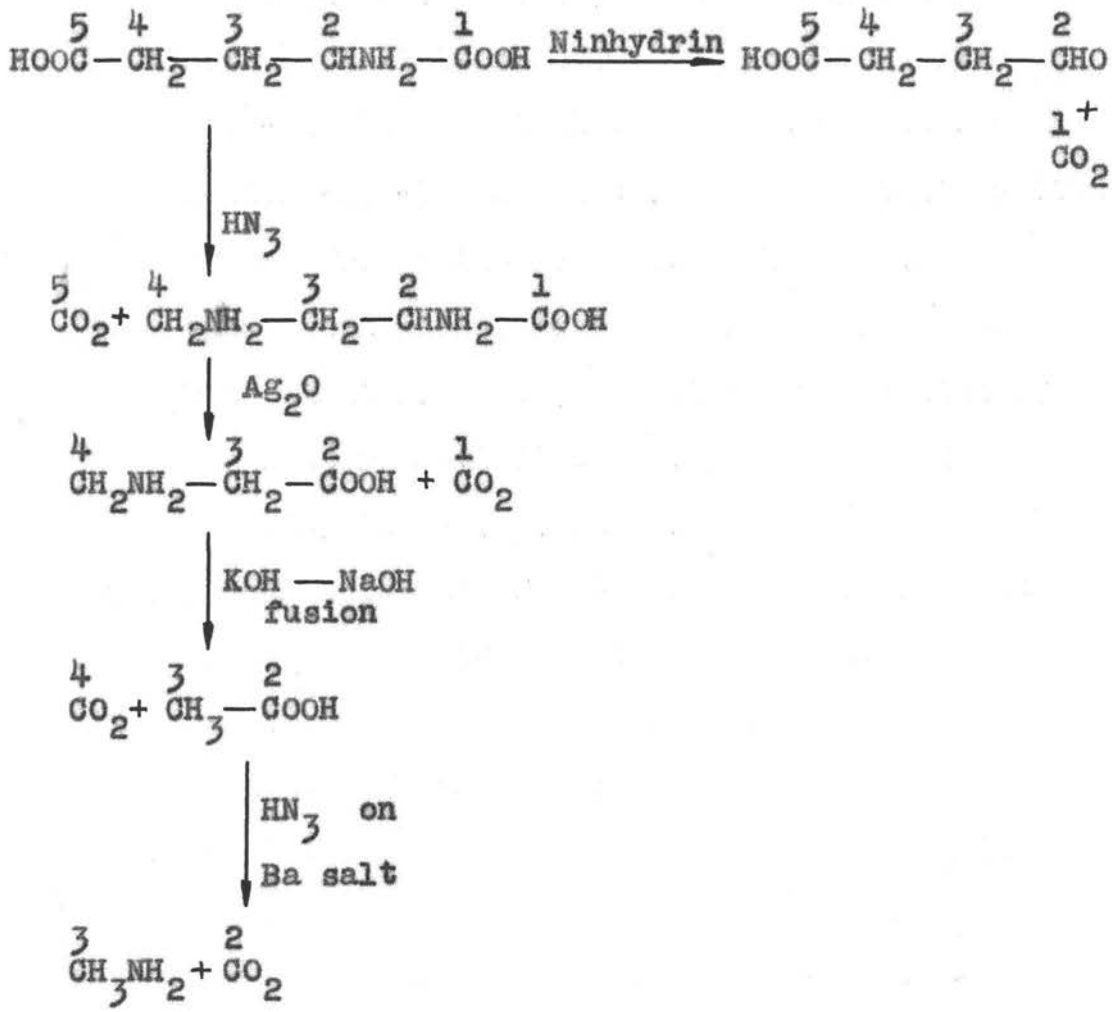


TABLE 10

DISTRIBUTION OF C^{14} IN GLUTAMIC ACID ISOLATED FROM *STREPTOMYCES GRISEUS* UTILIZING RIBOSE-1-C^{14}

Carbon atom	cpm X 10^4*	per cent of total
Total	1.48	100
1 COOH	.20	13
2 $CHNH_2$	.46	31
3 CH_2	.35	24
4 CH_2	.42	29
5 COOH	.05	3

* Total activity is expressed as counts per minute per millimole of glutamic acid; the specific activity of individual carbon atoms are counts per minute per millimole of carbon.

DISCUSSION

Time Course Studies

Nature of endogenous respiration.

In the studies on the physiology of microorganisms the nature of the endogenous respiration is of great importance in interpreting the data of respiration relative to substrate metabolism, particularly when experiments are conducted under proliferating conditions. At present, the status of the microbial respiration of endogenous source is still in a rather chaotic state. Contradictory results reported by various workers who venture into the realm of this problem seem only to add to the general confusion. Many workers reported that endogenous respiration of microorganisms is suppressed in the presence of a readily oxidizable substrate and suggested that the rate of endogenous respiration observed in concurrent experiments should not be used directly as basis for obtaining net respiratory rate of substrate. Thus Steir and Stannard (68) working with baker's yeast and Clifton and Logan (12) with *E. coli* concluded that endogenous respiration is completely inhibited by substrate. On

the other hand, Reiner and coworkers (57), by the use of radioactive carbon, obtained data indicating that the endogenous respiration of yeast is actually stimulated by the presence of glucose or acetate. Norris and coworkers (50) have demonstrated that in the case of Pseudomonas aeruginosa the endogenous respiration functions independently in the presence of added substrate.

The data obtained in the present study of the nature of endogenous respiration in C^{14} labeled S. griseus cells are illustrated by Figure 3, which indicates that there is considerable stimulation of endogenous respiration when substrate is present in the media. It should be noted however, that irrespective of absence or presence of substrate in the medium, an initial precipitous drop of $C^{14}O_2$ production from the labeled cells occurs within the first hour of the experiment; this is followed with an almost constant level of radioactivity turnover during the rest of the study. These facts suggest that the endogenous carbon source of S. griseus could be classified into two different types. First, it appears that a small labile pool is existing within the cell, comprising of readily available intermediates, which is subject to rapid turnover. One might surmise that the compounds in this pool consist of fragments resulting from the breakdown of glucose-u-C^{14} in which the cells

were originally incubated, and that those fragments are intimately related to the respiratory system of the organism. One should therefore expect that soon after the administration of external inactive substrate the newly formed unlabeled intermediates would enhance the operation of respiratory activity, thus directing some of the internal labeled depletable products into terminal respiratory pathway and result in noticeable stimulation in C^{14} turnover.

Meanwhile there is probably also existing another class of endogenous respiration in the organism which is the result of exchange reactions between the newly formed anabolic intermediates and existing cellular constituents. It is reasonable to believe that the turnover rate of this type of exchange reactions is slow, relatively constant, and is consequently not subject to rapid depletion. The relatively flat portion of the depletion curve as shown in Figure 3 is probably the result of this type of exchange reactions. It is also interesting to note that in the depletion experiment greater amount of cellular activity was later released into respiratory CO_2. This possibly reflects a partial lysis of the cells on extensive starvation.

Contrary to this, in the case where external

substrate was present in the medium for the first four hours, one finds that the later part of the $C^{14}O_2$ production curve is essentially flat in nature. This fact supports the speculated partial lysis in the later phase of the depletion experiment.

In light of the above discussion, it would appear that in a study of the respiration of substrate by S. griseus, using Warburg manometric method, caution should be exercised in deducting endogenous respiration from the observed substrate respiration data.

In the subsequent experiments on the utilization of labeled glucose, cells were depleted for one hour prior to the addition of labeled glucose to minimize the effect of the readily depletable pool upon the catabolism of the labeled substrate under investigation.

Utilization of labeled substrates.

In order to evaluate the overall glucose metabolism of S. griseus, under proliferating conditions, with respect to the detection and quantitation of individual pathways, first of all it is necessary to acquire fundamental information on the possible variation on the pattern of utilization of glucose by this organism, in cultures of different age. For this purpose uniformly C^{14} labeled glucose was employed as a sole carbon source to cultures of 12, 36 and 72 hours respectively.

The information observed in these experiments (Table 1) can be summarized as follows.

(1) The incorporation of glucose activity into cellular constituents decreases with the increasing age of culture.

(2) Considerably more fermentation products were detected in the medium of older cells.

(3) Whereas, in the case of 12- and 36-hour cultures the excreted labeled compounds in the medium consist of equal portions of the ether-soluble and ether-insoluble fractions, the bulk of that in the 72-hour experiment was ether-soluble in nature. The ether-insoluble fractions contain manly amino acids, while succinic acid accounted for most of the activity in the ether phase.

(4) With respiratory CO_2, as expected, one finds that the chemical recovery decreases drastically with the increasing age of cultures. However, it is noteworthy that, although the radiochemical yield of glucose activity in CO_2 is high with young cells, no significant difference was observed in comparing 36- and 72-hour cultures. The results clearly show that at the exponential phase of cell growth the activity of cellular biosynthesis and respiratory function is at its highest peak.

Identification of pathways of glucose catabolism.

As indicated in the foregoing section, although the overall picture of utilization of glucose by cultures of this organism of different age, are not exactly the same, it remained to be elucidated whether there are variations with respect to the distribution of catabolic pathways of glucose in cultures of different age under proliferating conditions. The detection of individual pathways was accomplished by the use of specifically labeled glucose.

It is evident from the fact that preferential utilization of C-1 as compared to C-6 of glucose is observed in cultures of all three different ages, (Figures 4,5 and 6) that a certain amount of glucose is catabolized by way of the phosphogluconate decarboxylation in cultures of all ages. That the bulk of the glucose is utilized by this organism via the conventional Embden-Meyerhof-Parnas (EMP) pathway in conjunction with the TCA cyclic processes is clearly indicated by the much greater radiochemical recovery of C-3 (4) of glucose, given in Table 2, as calculated according to the method of Wang et al. (76). It is understood that the assumptions made for the original method might not be exactly adequate in the present case. However, it is believed that a similar treatment

on the present data is justifiable in seeking a first approximation of radiochemical yield of C-3 (4) of glucose in the respiratory CO_2. Later experiment using glucose-3(4)-C^{14} has further supported the validity of this theoretical treatment.

It is noteworthy that the specific activities of the respiratory CO_2 from labeled glucose samples are much higher in 72-hour culture as compared to the younger cultures and moreover a striking resemblance is observed (Figure 6) between ratio of

$$\frac{CO_2 \text{ from C-2 of glucose}}{CO_2 \text{ from C-6 of glucose}}$$

(According to the conventional EMP-TCA pathway one would expect that C-1 and C-2 of acetate are derived from C-2, 5 or C-1,6 of glucose respectively) and that of

$$\frac{CO_2 \text{ from C-1 of acetate}}{CO_2 \text{ from C-2 of acetate}}$$

(Figure 7).

This fact, together with the experimental evidence for operation of TCA cyclic processes in this organism (35) reveals that in the 72-hour old culture, extensive operation of TCA cycle is predominant, with little side-tracking of TCA intermediates for other functions. This is not surprising in view of the earlier discussion that cellular biosynthesis is greatly reduced when a culture is aged.

It is also interesting to note that in all of the

glucose experiments, the observed peaks of the specific activities of respiratory CO_2 appeared after glucose has been exhausted from the media. This fact, particularly in the case where glucose-1-C^{14} or glucose-3 (4)-C^{14} were concerned, is not in line with the observed rapid conversion of C-1 or C-3 (4) of glucose to CO_2 by way of EMP and phosphogluconate decarboxylation pathway respectively, in yeast and E. coli (74). On the other hand, similar findings were noted in experiments with molds, such as Penicillium digitatum, Penicillium chrysogenum and Aspergillus niger (74). This anomaly probably can be explained by the relatively higher endogenous respiration of mold and actinomyces as compared to that of yeast and true bacteria, together with the information concerning the readily depletable endogenous pool in S. griseus described previously. It is conceivable that the observed delay in the appearance of the CO_2-specific-activity peak in the glucose experiments is the result of the preferential utilization of the endogenous intermediates over that of external substrate.

The pentose formed in the phosphogluconate decarboxylation process is probably not metabolized extensively by the pentose cyclic pathway in this organism. This is concluded from the following facts:

(1) The nonresemblance observed in the slopes of

the time course curves (Figure 5) for CO_2 from glucose-1-C^{14} and -2-C^{14} respectively, particularly in the early phase of incubation. Theoretically, the recovery in CO_2 from C-2 should equal to that from C-1 of glucose when pentose cycle is operating extensively.

(2) The similarity in maximal values of $C^{14}O_2$-specific activity curves, derived from ribose-1-C^{14} (Figure 9) and acetate-2-C^{14} (Figure 7) (after being adjusted on the basis of uc/mM and assuming that each ribose molecule may yield two acetate molecules), which indicates that C-1 of ribose resembles C-2 of acetate in catabolism. This conclusion is further supported by the experimental evidence on incorporation of ribose into cellular matter.

Estimation of pathways of glucose catabolism.

In estimating the contribution of individual pathway to the overall glucose catabolism of microorganisms, it is essential to establish not only the time when glucose is exhausted from the medium but also the time when substrate glucose is consumed within the cell. While disappearance of glucose from media can be easily followed by chemical means the point at which cell has attacked last traces of substrate glucose within the cell can only be surmised by some indirect method. In view of the rapid conversion of C-3 (4) of glucose to CO_2 in organisms capable to metabolize glucose

glycolytically, it appears that the point at which bulk of C-3 (4) of glucose has been converted to respiratory CO_2 can be considered as a point at which all substrate glucose is consumed within the cell. In the early part of this work glucose-3 (4)-C^{14} was not available, nevertheless reasonably close values for C-3 (4) could be obtained by the use of the recovery data for glucose-u-C^{14} along with glucose-1,2 and 6-C^{14} and calculation could then be made according to the following equation (76):

$$G_3 = \frac{6Gu-(G_1 + 2G_2 + G_6)}{2}$$

Where: G_1, G_2, G_6, and Gu represent the recovered radioactivity in respiratory CO_2 from glucose-1,2,6 and u-C^{14}, respectively, when identical levels of substrate are used.

This equation is derived from the following assumptions:

(1) Under prevailing experimental conditions, substrate glucose is utilized either by direct decarboxylation of phosphogluconate or the classical Embden-Meyerhof pathway, followed by the tricarboxylic acid cycle (10, 32, 35, 43). This is true in light of the discussion in the preceding section.

(2) Polysaccharide fraction of cells incorporates only limited amounts of substrate glucose.

Later work on ribose incorporation studies support this assumption to some extent.

(3) Direct decarboxylation of phosphogluconate is a rapid process. Examination of curves in Figures 4, 5, and 6 bear evidence to this point.

(4) The pentose formed from direct decarboxylation of phosphogluconate is limited in amount and is metabolized through the C_2-C_3 cleavage. Work involving degradative studies of cellular constituents under the ribose incorporation experiments supports this assumption.

(5) Except for the portion taking part in CO_2 fixation, the pyruvate formed by glycolysis is decarboxylated rapidly. Early appearance of C-3 activity in respiratory CO_2 (Figure 12) portrays this fact.

(6) Trioses formed in the glycolytic processes are identical in respect to further metabolic reactions.

(7) The aldolase coupling of trioses to hexose is not operative. Failure of C-6 activity to appear in respiratory CO_2, in the presence of sodium arsenite (Figure 11) supports this assumption.

Using equation (1) the calculated values for C-3 (4) of glucose are given in Table 2. The values given in the left side of the column for each age of culture correspond to the time when $C^{14}O_2$ evolution from C-3 (4) of glucose has leveled off.

Subsequent experiments employing glucose-3 (4)-C^{14} (Figure 12) revealed the following conclusions:

(1) There are indications that glucose -u-C^{14} samples obtained from commercial suppliers are more heavily labeled in C-3 (4) as compared to C-1,2,5 and 6 of glucose as a result of incomplete randomization of carbon atoms of glucose in the original synthesis. This nonhomogeneity in labeling, although not extensive, has been estimated to be 10-15 percent in favor of C-3 (4). This is also illustrated by the fact that, whereas the calculated value of C-3 (4) activity decreased to a negligible amount a few hours after the exhaustion of glucose from medium, there is continuous evolution of CO_2 from glucose-3 (4)-C^{14} in a comparable experiment.

The nonhomogeneity of labeling in glucose -u-C^{14}, although introduces inaccuracy into the estimated values for the extent of catabolic pathways, should not alter qualitatively the conclusion drawn from experiments involving the use of this compound.

(2) It appears that the $C_3 + C_1 = C_4$ type reaction is playing an important role in overall carbohydrate anabolism in this organism. The operation of the latter type of reaction will result in C_4 acid with one of its carboxyl carbon atoms corresponding to C-3 (4) of glucose. The interaction of C_4 acid with other biosynthetic

intermediates such as aspartic acid would undoubtedly reduce the rate of conversion of C-3(4) of glucose to CO_2. This is clearly shown by the slow rise of the curve for the cumulative radiochemical recovery of glucose-3 (4)-C^{14} in respiratory CO_2 (Figure 12).

(3) However, the estimation on the time of exhaustion of substrate glucose in cells can still be ascertained by examining the above mentioned curve, since a noticeable change in slope of the curve is traceable. In the present case, an abrupt change in slope is observed at 3 hours, i. e., one hour of time is required to consume all the substrate glucose after being transported into the cells. This factor is used throughout the subsequent section in the estimation of pathways in cultures of different ages.

Knowing the exact time of exhaustion of glucose substrate within the cells and following the assumptions made previously, it is then possible to calculate the distribution of the administered glucose between the individual pathways. Let G_1', G_2', $G_3'(4)$ and G_6' be the percent radiochemical recovery of respective labeled glucose in respiratory CO_2 at the time of substrate exhaustion within the cells, one finds that fraction of glucose catabolized via phosphogluconate decarboxylation, G_p:

$$G_p = G_1 - G_6 \qquad (2)$$

Since it has been assumed that there are only two catabolic pathways functioning in this organism, consequently, the fraction of glucose catabolized glycolytically, G_e:

$$G_e = 1 - G_p \qquad (3)$$

Fate of pyruvate

In order to trace the fate of pyruvate as derived from external glucose glycolytically, one has to consider two factors:

(1) the extent of labeling in C-1,2,5 and 6 inherent to the biosynthetic method employed in the preparation of glucose-3(4)-C^{14}. Available data from work of Wood (79), and Marks and Horecker (48) indicated that on the average, randomization occurred to the extent of 3 percent.

(2) Considerable amount of glucose-3(4)-C^{14} activity has been detected in the medium. For example, in the experiment involving culture of 36 hours, 14 percent of the substrate activity (Table 4) was detected in the incubation medium. In view of the nature of the excreted compounds in the medium, (lactic acid, succinic acid, amino acids) it is then reasonable to consider that this fraction is derived from glucose by the glycolytic pathway, with or without the subsequent condensation of the $C_3 + C_1$ type. In either case C-3(4) of glucose is retained.

Theoretically, for each mole of glucose catabolized glycolytically one should expect the formation of one mole of lactic acid from C-1,2 and 3 of glucose and likewise for C-4,5 and 6, therefore the overall amount of pyruvic acid, in moles should equal then to twice the number of moles of glucose catabolized glycolytically (G_e). Similarly, for each mole of pyruvic acid decarboxylated oxidatively to acetate one should expect one mole of CO_2 from C-3 or C-4 accompanying the decarboxylation process. Consequently, fraction of pyruvate converted to acetate should then equal to fraction of pyruvate decarboxylated, P_d:

$$P_d = \frac{2G'_3}{2G_e \times .97} = \frac{G'_3}{.97\, G_e} \qquad (4)$$

The factor 0.97 is inherited from the partial randomization in glucose-3(4)-C^{14}.

Fraction of pyruvate remained intact, including the fraction of pyruvate metabolized by way of CO_2 fixation, P_i should then be:

$$P_i = G_e - m - P_d \qquad (5)$$

The term m represents the amount of pyruvate or its equivalents accumulated in the incubation medium. In the case of 36 hour old culture, m is equal to 0.14 (Table 4).

The amount of activity found in cells should also serve as a lower margin of the value of P_i, since most, if not all, of the glucose-3(4)-C^{14} activity incorporated into

the cells can be visualized as by way of $C_3 + C_1 = C_4$ type mechanism. Naturally, some of the activity originally incorporated in such a fashion could subsequently be converted to respiratory CO_2 by extensive TCA processes, however the amount left in cells even at the end of the experiment should portray the lower limit of the value of P_f, while the value calculated from equation (5) should provide the upper limit.

Fate of acetate.

The efficiency of the conversion of carboxyl carbon atom of acetate to respiratory CO_2 is calculated according to the equation:

$$R_c = \frac{G'_2}{G_3} \qquad (6)$$

The efficiency of utilization of carboxyl carbon atom of acetate (Sc) in biosynthesis is given by:

$$Sc = 1 - Rc \qquad (7)$$

The efficiency of combustion of methyl carbon atom of acetate (Rm) is obtained from:

$$Rm = \frac{G'_6}{G'_3} \qquad (8)$$

The efficiency of utilization of methyl carbon atom of acetate (Sm) in biosynthesis is calculated from:

$$Sm = 1 - Rm \qquad (9)$$

Naturally, in each case acetate is assumed to be formed exclusively from glycolytic pathways.

The estimation of various catabolic pathways as calculated according to the foregoing discussion for the culture 36 hours old are given as follows. In all cases, experimental data at 3 hours were selected for the calculation.

Extent of glycolysis	97%
Extent of phosphogluconate decarboxylation	3%
Fraction of pyruvate decarboxylated oxidatively	27%
Fraction of pyruvate remained intact at the specified time	59% (lower limit 24%)
Fraction of acetate-carboxyl oxidized to CO_2	44%
Extent of utilization of carboxyl carbon atom of acetate in biosynthesis	56%
Extent of combustion of methyl carbon atom of acetate	24%
Extent of utilization of methyl carbon atom of acetate in biosynthesis	76%

The important role played by the EMP glycolysis and $C_3 + C_1$ condensation, of either Wood-Werkman or malic type, in this organism is self-evident from examining the foregoing figures. The preferential utilization of the methyl carbon atom of acetate moiety in biosynthesis is in line with the established TCA process in S. griseus.

Korkes (47), in a recent review paper, written just before his untimely death, has devised a method for the

calculation of pathway participation in respiratory functions of biological systems. Specific activity data of CO_2 as derived from glucose-1-C^{14} and glucose-6-C^{14} were used as a basis for calculations. The method is derived on the theoretical consideration that all carbon atoms of glucose are converted to CO_2 at equal rates in the glycolytic process. In the case of the direct oxidative pathway, the author suggested assumptions be made that either C-1 of glucose is converted to CO_2 exclusively (phosphogluconate decarboxylation) or C-1,2 and 3 are converted to CO_2 at equal rate if pentose cyclic process is prevailing.

It is noteworthy that the method does not rely on the radiochemical yield data in any respect, although the author did point out the necessity and the possible difficulties one might encounter in selecting a proper set of specific activities for the calculation.

In the present work it is clearly shown by the data given in Figure 12 that in the EMP-TCA sequence, rates of converting carbon atoms of glucose to CO_2 are far from uniform. Consequently using specific activity data of CO_2 from glucose-1,2,3(4) and 6-C^{14}, the distribution of catabolic pathways can be derived in a manner similar to that of Korkes, as follows:

Let: A equal specific activity of glucose (cpm/mM)

C_p equal the fraction of CO_2 via the phosphogluconate decarboxylation.

C_e equal the fraction of CO_2 via glycolysis and TCA processes.

X_1 equal the specific activity of CO_2 from C-1 of glucose, in cpm/mM.

X_6 equal the specific activity of CO_2 from C-6 of glucose, in cpm/mM.

n equals the combustion efficiency of C-1 or C-6 of glucose to CO_2 by way of EMP-TCA pathway.

Then:

$$C_e = 1 - C_p \qquad (10)$$

$$X_1 = AC_p + \frac{AC_e}{n} = \frac{nAC_p + AC_e}{n} = \frac{nAC_p + A(1 - C_p)}{n} =$$

$$= \frac{nAC_p + A - AC_p}{n} = \frac{(n-1)AC_p + A}{n} = \frac{A}{n} \quad (n-1)C_p \; 1; \qquad (11)$$

$$X_6 = \frac{AC_e}{n} = \frac{A}{n}(1 - C_p) \; ; \qquad (12)$$

$$R = \frac{X_6}{X_1} = \frac{\frac{A}{n}(1 - C_p)}{\frac{A}{n}(n - 1)C_p + 1} = \frac{1 - C_p}{(n - 1)C_p + 1}$$

$$(n - 1)RC_p + R = 1 - C_p$$

$$(n - 1)RC_p + C_p = 1 - R$$

$$C_p = \frac{1 - R}{(n - 1)R + 1} \quad ; \qquad (13)$$

Fundamental assumptions underlying the derivation of the foregoing relationship are:

(1) The total catabolism of glucose is channeled through only two pathways, namely the glycolytic pathway and the direct oxidation of phosphogluconate.

(2) The isomerase and aldolase reactions are ignored.

(3) Decarboxylation of phosphogluconate is prompt.

(4) Pentose formed by the decarboxylation of phosphogluconate is not catabolized further by way of the pentose cycle. Although it is demonstrated in the present work that pentose formed in direct

decarboxylation of phosphogluconate does undergo extensive cleavage similar to that reported by Heath (39), yet in view of small amount of glucose metabolized through the phosphogluconate decarboxylation, the contribution of pentose to CO_2 is probably insignificant.

The term n designates the combustion-efficiency of C-6 of glucose to CO_2. In Korkes' work n is assigned a value of 6. In the present work it is defined in the following manner: At any given time,

$$n = \frac{2G'_2 + 2G'_{3,(4)} + 2G'_6}{G'_6} \qquad (14)$$

Using experimental findings given in Table 4 for the culture of 36 hours the n values for different time intervals are shown in the following Table.

Time in hours	n value
1	19.7
2	14.1
3	13.3
4	11.8
5	11.5
6	10.9
7	10.6
8	10.4
10	9.7
12	9.3

It is obvious, due to the much greater rate of conversion of C-3(4), and to a less extent C-2,5 of glucose CO_2, as compared to that of C-1 and 6 in the EMP-TCA sequence, the value of n tends to be high in the early phase of experiment and should be eventually reduced to the theoretical value of 6. However, it is recalled that the specific activities of CO_2 from glucose-1 or 6-C^{14} reach their respective peaks, i.e. turnover equilibrium, at 8 hours (Figure 5). For this reason, in the present work calculations are made on a basis of a n value of 10.4 chosen according to the foregoing criteria. Furthermore, since Equation 13 concerns with the fraction of CO_2 originated by way of phosphogluconate decarboxylation, to convert value of C_p to the corresponsing fraction on the basis of glucose, designated hereafter as G'_p, the following equation is derived:

$$G'_p = \frac{CO_2 \text{ via PGD pathway} \times 1}{CO_2 \text{ via EMP-TCA} \times P + CO_2 \text{ via PGD} \times 1}$$

$$= \frac{C_p}{C_e \times P + C_p} \qquad (15)$$

Where P represents the average radiochemical yield of CO_2 from glucose via EMP-TCA pathway, or:

$$P = \frac{2G6 + 2G2 + 2G3(4)}{6} \qquad (16)$$

In the following Table is given a comparison of pathway participation in glucose catabolism of proliferating S. griseus cultures, 12, 36 and 72 hours in age.

Age of culture	12 hour	36 hour	72 hour
Turnover equilibrium at	5 hours	8 hours	5 hours
G_1	22.3%	20.9%	9.5%
G_2	23.9%	32.7%	16.2%
$G_{3,4}$	60.8%	44.0%	31.6%
G_6	19.1%	18.1%	9.0%
R (X_6/X_1)	.86	.87	.95
n (Eq. 14)	11.5	10.4	11.5
P (Eq. 16)	.346	.316	.189
G_p (Eq. 2)	3%	3%	0.5%
G_e ($1-G_p$)	97%	97%	99.5%
G_p (Eq. 13)	1.4%	1.4%	0.5%
G'_p (Eq. 15)	4%	4%	0.5%
G'_e ($1-G'_p$)	96%	96%	99.5%

The agreement between the values of G_p and G'_p in each set thus reflects the validity of the theoretical considerations involved in the respective types of calculation (Equations 2 and 14).

In the course of estimating the catabolic pathways of glucose in microorganisms the research workers are often haunted by two fundamental problems. They are:

(1) Identification of the trail followed by C-2 of glucose to the eventual conversion to CO_2, since the distinction between EMP-TCA processes and the extensive

extensive pentose cyclic processes insofar as C-2 of glucose is concerned is far from clear. This is mainly because of the relatively rapid reaction rates involved in either sequence.

(2) Although the excellent reproducibility observed in the present series of experiments on the estimation of catabolic pathways of glucose implies that the distribution of these pathways in microorganisms are by and large constant in nature under normal experimental conditions, yet it remains to be investigated whether the introduction of external factors in incubation media will result in shuffling of glucose among individual pathways. It is for this reason that the experiments employing specifically labeled glucose were repeated under otherwise identical conditions with the addition of appropriate level of sodium arsenite as respiration inhibitor. The experimental findings in this series are given in Tables 3 and 5 as well as in Figures 11 and 13. In evaluating these data once again, one has to make some assumptions based on existing information in literature. Thus, in the present work the inhibitory effect of arsenite is assumed to be functioning principally at the stages concerning decarboxylation of alpha-keto acids (6, p. 419 and 43). It is further assumed that arsenite ion is not interfering in any

respect with the reactions of the pentose cycle, although no experimental proof is available at present to substantiate this point. Bearing these assumptions in mind, one should then expect that the addition of arsenite to the incubation media of this organism would result in partial or complete blocking on the conversion of all carbon atoms of glucose to CO_2 by the EMP-TCA pathway. On the other hand, the conversion of C-1,2,3 of glucose to CO_2 via the pentose cyclic pathway should not be impaired by the inhibitor. Likewise, if one can assume that the isomerase-aldolase reaction sequence is not affected by arsenite, oxidation of C-4,5,6 of glucose to CO_2, if it is operative under normal conditions, should remain operative.

A comparison of the data presented in Tables 3,4 and 5 as well as Figures 10, 11, 12 and 13 reveals some very interesting information:

(1) The overall recovery of respiratory CO_2 in experiment using sodium arsenite amounts to about one half of that collected in the control experiment. This is the indication of the effectiveness of the inhibitor upon the respiratory function.

(2) The role of the EMP-TCA processes played in biosynthesis of this organism cannot be over stressed. This is evidenced by the negligible amount of glucose

activity incorporated into cellular constituents.

(3) The great amount of glucose activity accumulated in the incubation media of the nature of C_3 acids substantiates the previously assumed inhibitory action of arsenite upon the decarboxylation of alpha keto acids in this organism. It is interesting to note that practically all of the glucose-6-C^{14} activity was detected in medium as compared to 43 percent accumulation observed in the glucose-1-C^{14} experiment. This is understandable in view of the fact that the C-4,5,6 of glucose, irrespective of its derivation, is accumulated as a result of arsenite inhibition. Moreover, the failure of the conversion of C-6 of glucose to CO_2 in arsenite experiment also indicates that under normal conditions, the combustion of glucose does not involve the operation of pentose cyclic pathway.

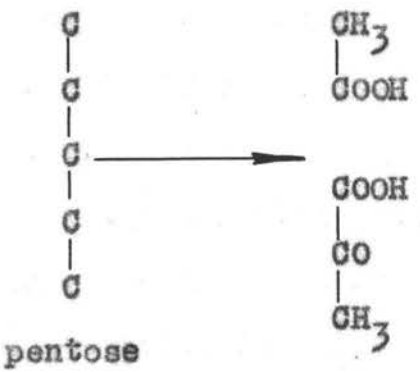
(4) In the case of arsenite experiment using glucose-2-C^{14} and glucose-3 (4)-C^{14}, one finds that the radiochemical recoveries in CO_2 are also substantially reduced as compared to the control experiment.

It is recalled that 96 percent of glucose-6-C^{14} activity was recovered in the incubation medium as C_3 acids, it is therefore reasonable to assume that the bulk of the $C^{14}O_2$ from glucose-3 (4)-C^{14} is actually derived from C-3 of glucose. The corrected $C^{14}O_2$ recovery is

then 32 percent and 15 percent from C-3 and C-2 of glucose respectively. Significance of these values will be discussed later.

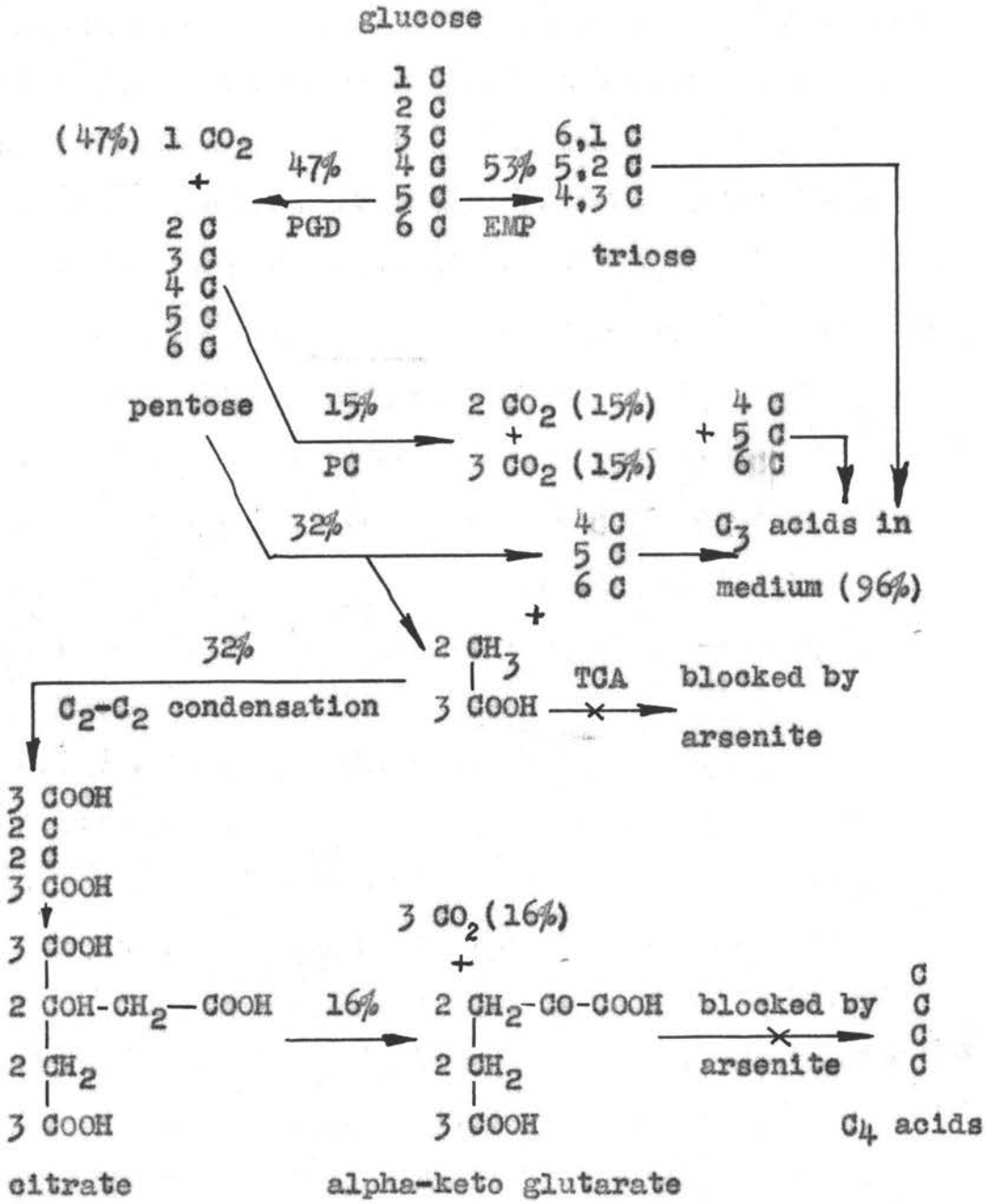
(5) The most interesting finding in the arsenite experiment is the extraordinary high recovery of glucose-1-C^{14} activity in CO_2. At the time of termination of the experiment, 47 percent of C-1 of glucose was recovered as CO_2. This fact points to the inertness of phosphogluconate decarboxylation toward arseinte and the shuffling of glucose from one to the other pathway in the event of a major road block along one of the major pathways.

(6) That pentose cycle pathway is operating only to a limited extent is indicated by considerably smaller recovery of $C^{14}O_2$ from C-2 of glucose as compared to that from C-1 (15 and 47 percent respectively). From the experimental findings concerning the utilization of ribose by this organism, it is conceivable that the bulk of the pentose resulting from the phosphogluconate decarboxylation reaction is further catabolized by the following cleavage-reactions:



pentose

(7) Indirect evidence of the operation of a $C_2 - C_2$ condensation of acetate units is also provided by the present work. The condensation could be of the nature of Thunberg type or malate-synthetase type, reported recently (2). An inventory of pathway distribution in the glucose-arsenite experiment as based on percentage radiochemical recoveries at the conclusion of the experiment (Table 5) can be summarized as follows:



Legend: EMP=Embden-Meyerhof-Parnas pathway; PGD = phosphogluconate decarboxylation process; PC = pentose cyclic process, TCA = tricarboxylic acid cycle. All percentages are based on glucose administered to the incubation medium.

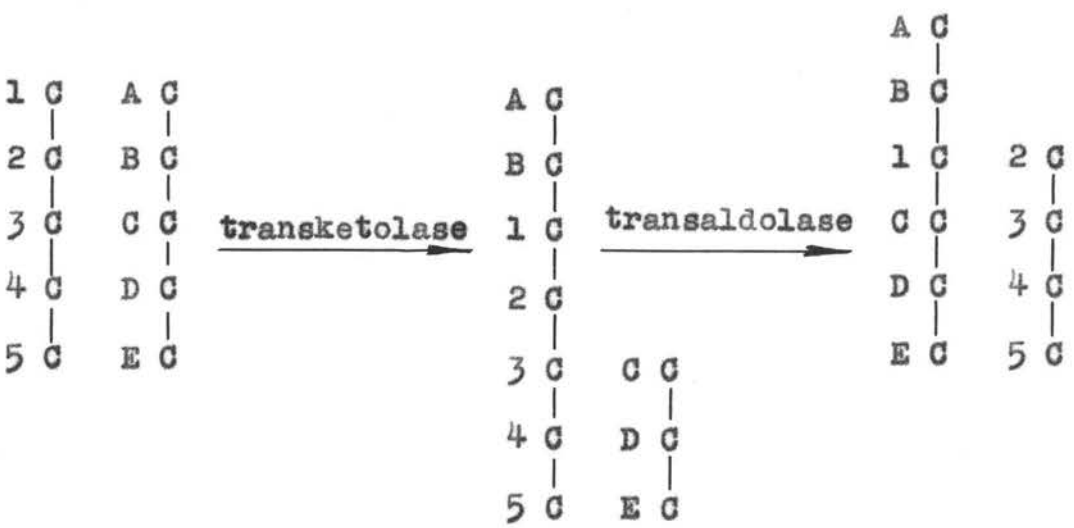
The incorporation of ribose-1-C^{14} into cellular constituents.

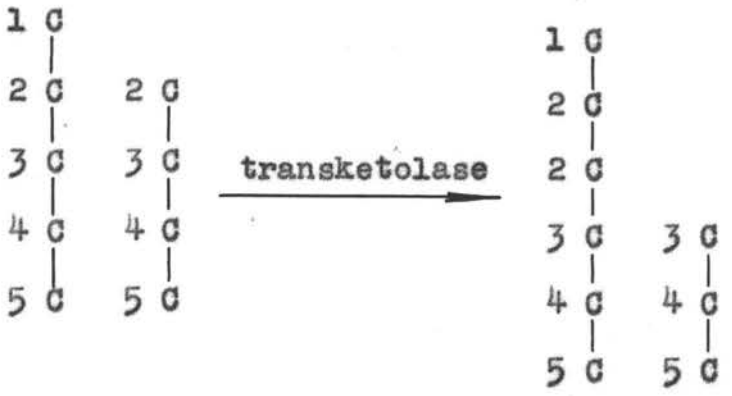
The knowledge of the operation of direct oxidative pathway in microorganisms has stimulated a great deal of interest concerning the fate of pentose, a key intermediate in pentose cyclic pathway, in the overall picture of the carbohydrate metabolism.

Six catabolic pathways of pentose have so far been reported in various biological systems. They can be summarized schematically as follows.

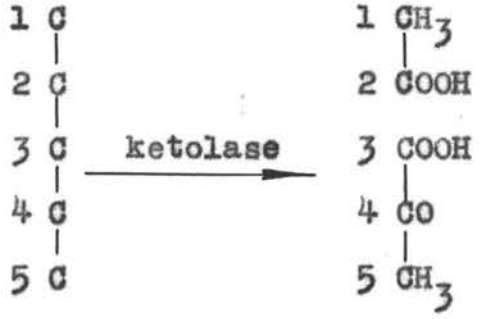
(1) Ribose ⟶ Nucleic acids.

(2) C_2—C_3 cleavage of the transaldolase-transketolase type (34).

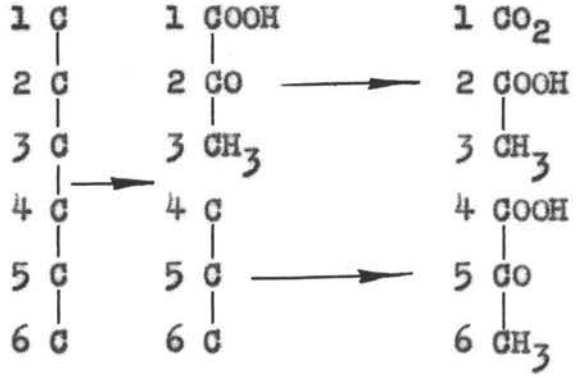




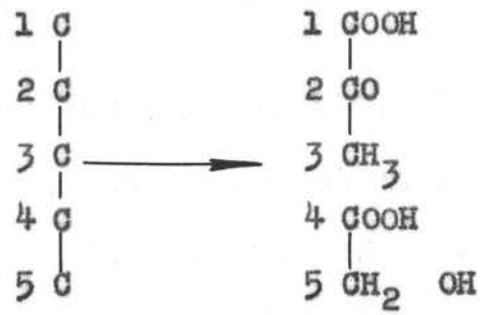
(3) C_2-C_3 cleavage of the ketolase type (7,39).



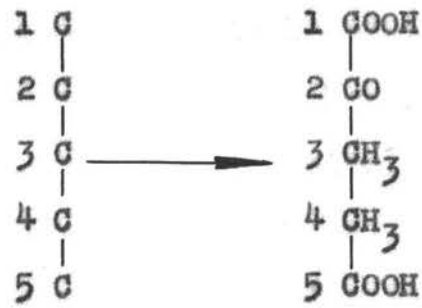
(4) C_2-C_3 cleavage of the Entner-Doudoroff type (28).



(5) C_3—C_2 cleavage (21).



(6) Direct conversion of pentose to alpha-keto-glutarate (78).



In the present work, it is demonstrated that ribose, but not arabinose, is utilized as the sole carbon source by S. griseus, particularly in the case of cultures of younger age. Consequently, efforts were focused on the incorporation of ribose-1-C^{14} into cellular constituents of this organism to shed some light on the metabolic pathways followed by ribose in both respiratory and biosynthetic functions.

Preliminary time course studies on radiochemical recovery of ribose-1-C^{14} activity in CO_2 revealed a striking resemblance between the latter and that of

acetate-2-C^{14}. In order to secure a complete inventory of radioactivity of ribose-1-C^{14}, incubation media and cells were examined. These results are given in Table 6. The active role played by C-1 of ribose in cellular synthesis is clearly indicated by the heavy incorporation of ribose-1-C^{14} activity into cellular constituents.

In view of the many possible pathways ribose could have used in its catabolism, the cells were therefore analyzed for labeling distribution. As indicated in Diagram 1, very little of the labeled ribose remained intact in the cells. This fact therefore minimizes the importance of the direct incorporation of ribose into ribonucleic acids in the overall ribose catabolism.

In Diagram 2, radiochemical inventory among cellular constituents is given in detail. Insignificant amount of ribose activity detected in glucose fraction reflects the fact that extensive pentose cycle (transketolase-transaldolase type) is not operative in this organism, at least insofar as ribose is the starting material.

As indicated also in Diagrams 2 and 3, the bulk of the ribose activity in the cells was incorporated into various amino acids, among which glutamic acid and aspartic acid are by far the heavier labeled ones. It

is therefore mandatory to carry out degradation studies on the latter two amino acids in order to pinpoint the catabolic mechanism responsible for this observation.

The isotopic distribution patterns of glutamic acid and aspartic acid are given in Tables 7 and 8. Extensive labeling was observed on practically all carbon atoms in glutamic acid which rules out the operation of direct conversion of ribose to alpha-keto-glutarate. Since the operation of the latter pathway would give rise to practically exclusive labeling in C-1 of glutamic acid. The observation further implies that ribose is probably catabolized by way of a cleavage reaction, followed by the subsequent utilization of the breakdown products by the TCA processes.

Of the three types of known cleavage reactions described previously, two of them (4 and 5) would have resulted exclusively in carboxyl labeled acetate or pyruvate from ribose-1-C^{14}. The detection of heavy labeling in the non-carboxyl carbon atoms in amino acid and glutamic acid hence eliminates the possible involvement of these two types of cleavage reactions in the catabolism of ribose in this organism. This is deduced from the fact that in classical TCA process one would expect acetate-1-C^{14} and pyruvate-1-C^{14}

would give rise to labeling in glutamic acid and aspartic acid in a manner confined exclusively to the carboxyl carbon atoms (9).

The foregoing discussion therefore led to the belief that the cleavage reaction of the ketolase type is probably the predominant, if not exclusive pathway, responsible for catabolism of ribose in this organism. The conclusion is further supported by an examination of isotopic patterns in aspartic acid and glutamic acid. In the case of the former, the incorporation of labeling from acetate-2-C^{14} into aspartic acid by way of oxalacetate can be visualized as via either one or both of the following pathways:

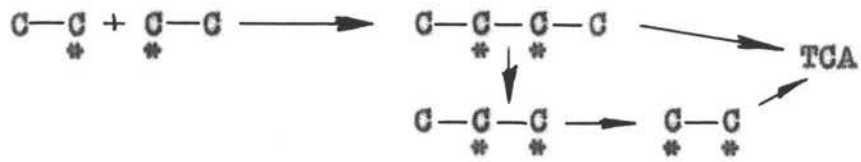
(1) Classical TCA processes would give rise to C_4 acids labeled as follows (3, p. 91):

	HOOC—	—C—	—C—	—COOH
First cycle	0	50	50	0
Second cycle	25	75	75	25
Third cycle	37.5	87.5	87.5	37.5
Fourth cycle	43.8	93.4	93.4	43.8
Steady state value	50	100	100	50

(2) $C_2—C_2$ condensation of the Thunberg or malate-synthetase type should result in exclusive labeling in the middle carbon atoms of aspartic acid.

In the present case the ratio of the extent of labeling in the middle carbon atoms to that of carboxyl carbon atoms is slightly greater than 2. This fact could have reflected either that the operation of TCA cycle with respect to the labeled substrate is not extensive, or that the C_2-C_2 type condensation plays a significant role in the utilization of acetate units.

Similar theoretical treatment can be applied in the case of glutamic acid. However, detection of a small amount of activity in C-5 of glutamic acid would have to be explained by the partial randomization of the two carbon atoms in acetate, since no activity can be introduced into this position by way of the classical TCA process. It is possible that this finding also reflects the operation of C_2 C_2 condensation as shown in the following scheme:



In conclusion, it is hoped that the present work on the metabolism of glucose and ribose in S. griseus may have provided the bacteriologist and biochemist with some information in securing a better understanding of the overall metabolism in actinomyces.

BIBLIOGRAPHY

1. Acker, F. B. Some nutritional requirements of Streptomyces griseus 3570 for growth and cancidin production. Applied microbiology 2:152-157. 1954.

2. Ajl, Samuel J. Conversion of acetate and glyoxylate to malate. Journal of the American chemical society 78:3230. 1956.

3. Aronoff, Sam. Techniques of radiobiochemistry. Ames, Iowa, The Iowa state college press, 1956. 1st ed. 228 p.

4. Baddiley, G. et al. Acetic acid metabolism in Torulopsis utilis. II. Metabolic connection between acetic acid and tyrosine and a method of degradation of the phenolic ring structure in tyrosine. Journal of biological chemistry 183:777-788. 1950.

5. Baker, S.B. and William H. Summerson. The colorimetric determination of lactic acid in biological material. Journal of biological chemistry 138:535-554. 1941.

6. Baldwin, Ernest. Dynamic aspects of biochemistry. Cambridge, University press, 1952. 2nd ed. 544 p.

7. Bernstein, A. I. Fermentation of ribose-C^{14} by Lactobacillus pentosus. Journal of biological chemistry 205:309-329. 1953.

8. Bialy, J. J., S. Klungsoyr, C. M. Gilmour and C. H. Wang. Studies of the biochemistry of Streptomyces. III. Glucose catabolism in Streptomyces griseus. In press.

9. Butterworth, E. M. and C. M. Gilmour. Oxidation of members of the tricarboxylic acid cycle by Streptomyces griseus. Proceedings of the society of American bacteriologists 53:94. 1953.

10. Butterworth, E. M., C. M. Gilmour and C. H. Wang. Studies on the biochemistry of the Streptomyces. II. Fixation of $C^{14}O_2$ by intact cells of Streptomyces griseus. Journal of bacteriology 69:725-727. 1955.

11. Chesters, C. G. C., and G. N. Rolinson. Trace elements and streptomycin production. Journal of general microbiology 5:559-565. 1951.

12. Clifton, C. E. and W. A. Logan. On the relation between assimilation and respiration in suspensions and in cultures of Escherichia coli. Journal of bacteriology 37:523-540. 1939.

13. Cochrane, V. W. and Conn, J. E. The growth and pigmentation of Actinomyces coelicolor as affected by cultural conditions. Journal of bacteriology 54:213-218. 1947.

14. Cochrane, V. W. and I Dimmick. The metabolism of species of Streptomyces. I. The formation of succinic and other acids. Journal of bacteriology 58:723-730. 1949.

15. Cochrane, V. W. and H. D. Peck, Jr. The respiratory metabolism of Streptomyces scabies. Phytopathology 42:5. 1952.

16. Cochrane, V. W. and H. D. Peck, Jr. The metabolism of Streptomyces. VI. Tricarboxylic acid cycle reactions in Streptomyces coelicolor. Journal of bacteriology 65:37-44. 1952.

17. Cochrane, V. W., H. D. Peck, Jr. and A. Harrison. The metabolism of Streptomyces. VII. The hexose monophosphate shunt and associated reaction. Journal of bacteriology 66:17-23. 1953.

18. Conn, J. E. The pigment production of Actinomyces coelicolor and A violaceus-ruber. Journal of bacteriology 46:133-150. 1943.

19. Corum, C. J., W. M. Stark, G. M. Wild and H. L. Bird, Jr. Biochemical changes in a chemically defined medium by submerged cultures of Streptomyces erythreus. Applied microbiology 2:326-329. 1954.

20. Coty, V. F., H. R. Garner and H. Koffler. Tricarboxylic acid cycle reactions in Streptomyces griseus. Proceedings of the society of American bacteriologists 53:74. 1953.

21. Doudoroff, M., J. De Ley, N. J. Palleroni and R. Weimberg. New pathways in oxidation of D-galactose and of D-arabinose. Federation proceedings 15:244. 1956.

22. Dulaney, E. L. Observations of Streptomyces griseus. II. Nitrogen sources for growth and streptomycin production. Journal of bacteriology 56:305-315. 1948.

23. Dulaney, E. L. Observations of Streptomyces griseus. III. Carbon sources for growth and streptomycin production. Mycologia 41:10. 1949.

24. Dulaney, E. L. and D. Perlman. Observations on Streptomyces griseus. I. Chemical changes occurring during submerged streptomycin production. Bulletin Torrey botanical club 74:504-511. 1947.

25. Dulmage, H. T. A synthetic medium for neomycin production. Applied microbiology 1:103-106. 1953.

26. Ehrensvard, G., L. Reio, E. Saluste and R. Stjernholm. Acetic acid metabolism in Torulopsis utilis. III. Metabolic connection between acetic acid and various amino acids. Journal of biological chemistry 189:93-108. 1951.

27. Eiser, H. M. and W. D. McFarlane. Studies on the metabolism of Streptomyces griseus. In relation to the production of streptomycin. Canadian journal of research 26:164-173. 1947.

28. Entner, N. and M. Doudoroff. Glucose and gluconic acid oxidation of Pseudomonas saccharophila. Journal of biological chemistry 196:853-862. 1952.

29. Erickson, D. Differentiation of vegetative and sporogenous phases of Actinomycetes. III. Variations in the Actinomyces coelicolor species group. Journal of general microbiology 2:252-259. 1948.

30. Friedeman, Theodore E. and Gladys E. Haugen. Pyruvic acid. II. The determination of keto acids in blood and urine. Journal of biological chemistry 147:415-442. 1943.

31. Ganguly, S., N. C. Ganguli and S. C. Roy. Utilization of amino acids by Streptomyces griseus during the production of vitamin B_{12}. Journal of scientific and industrial research 13B:847-849. 1954.

32. Ganguly, S. and S. C. Roy. Utilization of phosphorus by Streptomyces griseus during its aerobic and and anaerobic growth. Archives of biochemistry and biophysics 63:26-31. 1956.

33. Garner, H. R. and H. Koffler. Preliminary evidence against the existence of a Krebs cycle in Streptomyces griseus. Proceedings of the society of American bacteriologists 51:139. 1951.

34. Gibbs, Martin, Joan M. Earl and Jean L. Ritchie. Metabolism of ribose-1-C^{14} by cell-free extracts of yeast. Journal of biological chemistry 217:161-168. 1955.

35. Gilmour, C. M., E. M. Butterworth, E. P. Noble and C. H. Wang. Studies on the biochemistry of the Streptomyces. I. Terminal oxidative metabolism in Streptomyces griseus. Journal of bacteriology 69:719-724. 1955.

36. Gilvarg, Charles and Konrad Bloch. The utilization of acetic acid for amino acid synthesis in yeast. Journal of biological chemistry 193:339-346. 1951.

37. Gottlieb, D. and H. W. Anderson. The respiration of Streptomyces griseus. Science 107:172-173. 1948.

38. Gottlieb, D. and L. Diamond. A synthetic medium for chloromycetin. Bulletin Torrey botanical club 78:56-60. 1951.

39. Heath, E. C., J. Hurwitz and B. L. Horecker. Acetyl phosphate formation in the phosphorolytic cleavage of pentose phosphate. Journal of the American chemical society 78:5449. 1956.

40. Herbert, D., H. Gordon, V. Subralimanyan and D. E. Green. Zymohexase. Biochemical journal 34: 1108-1123. 1940.

41. Herbst, R. M., and H. T. Clarke. Oxidation of amino acids by silver oxide. Journal of biological chemistry 104:769-788. 1934.

42. Hirs, C. H. W., Stanford Moore and William H. Stein. The chromatography of amino acids on ion exchange resins. Use of volitile acids for elution. Journal of the American chemical society 76:6063-6065. 1954.

43. Hockenhull, D. J. D., K. H. Fantes, M. Herbert and B. Whitehead. Glucose utilization by Streptomyces griseus. The journal of general microbiology 10:353-370. 1954.

44. Hubbard, C. V. and H. H. Thornberry. Utilization of some organic acids by Streptomyces griseus for streptomycin production and growth. Illinois academy of science transactions 43:61-74. 1950.

45. Hunter, G. D., M. Herbert and D. J. D. Hockenhull. Actinomycete metabolism: origin of the guanidine groups in streptomycin. The biochemical journal 58:249-254. 1954.

46. Hunter, G. D. and D. J. D. Hockenhull. Incorporation of C^{14}-labeled compounds into streptomycin. The biochemical journal 59:268-272. 1955.

47. Korkes, Seymour. Carbohydrate metabolism. III. The shunt pathway. Annual review of biochemistry 25:703-713. 1956.

48. Marks, Paul A. and B. L. Horecker. Distribution of radioactive carbon dioxide incorporated into rat liver glycogen. Journal of biological chemistry 218:327-333. 1956.

49. Mejbaum, W. Uber die Bestimmung Kleiner Pentosemengen, insbesondere in Derivaten der Adenylsaure. Zeitung physiolgische cheme 258: 117-120. 1939.

50. Norris, Flora C., Jack J. R. Campbell and Phyllis W. Ney. The intermediate metabolism of Pseudomonas aeruginosa. I. The status of the endogenous respiration. Canadian journal of research 27C: 157-164. 1949.

51. Numeroff, P., et al. Biosynthesis of streptomycin. I. Studies with C^{14} labeled glycine and acetate. Journal of American chemical society 76:1341-1344. 1954.

52. Perlman, D. and E. O'Brien. Utilization of carbohydrates by strains of Streptomyces griseus. Journal of bacteriology 72:214-218. 1956.

53. Perlman, D. and G. H. Wagman. Studies on the utilization of lipids by Streptomyces griseus. Journal of bacteriology 63:253-262. 1952.

54. Pridham, T. G. and D. Gottlieb. The utilization of carbon compounds by some actinomyces as an aid for species determination. Journal of bacteriology 56:107-114. 1948.

55. Principe, P. A. and H. H. Thornberry. Effect of cobalt on production of streptomycin and vitamin B_{12} by Streptomyces griseus. Phytopathology 42:123-126.

56. Racusen, D. W. and S. Aronoff. Metabolism of soy bean leaves. V. The dark reactions following photosynthesis. Archives of biochemistry and biophysics 42:25-40. 1953.

57. Reiner, J. M., N. Gest and M. D. Kamen. The effect of substrates on the endogenous metabolism of living yeast. Archives of biochemistry 20:175-177. 1949.

58. Reynolds, D. M. and S. A. Waksman. Grisein, an antibiotic produced by certain strains of Streptomyces griseus. Journal of bacteriology 55:739-752. 1948.

59. Scavola, M. E. and G. Valcurone. Intermediates of carbohydrate metabolism of Streptomyces griseus. Pavia. Farm science and technology 7:622-624. 1952. (Abstracted in Chemical Abstracts 47:4422. 1953).

60. Scavola, M. E. and P. Zorzoli. The formation of an active acetyl radical during the respiration with acetate by Streptomyces griseus. Pavia. Il farmaco ed. science 8:552. 1953. (Abstracted in Chemical abstracts 48:799a. 1954).

61. Schatz, A., E. Bugie, and S. A. Waksman. Streptomycin, a substance exhibiting antibiotic activity against gram positive and gram negative bacteria. Proceedings of the society of experimental biological medicine 55:66-69. 1944.

62. Schneider, Walter C. Phosphorus compounds in animal tissues. I. Extraction and estimation of desoxypentose nucleic acid and of pentose nucleic acid. Journal of biological chemistry 161:293-303. 1945.

63. Sevcik, V. Metabolism of Streptomyces griseus. Ceskasloveska biologia 297-303. 1952. (Abstracted in Chemical abstracts 47:7596h. 1953.

64. Shaffer, P. A. and A. F. Hartmann. The iodometric determination of copper and its use in sugar analysis. Journal of biological chemistry 45:365-390. 1921.

65. Smith, D. J. and R. Markham. Chromatographic studies on nucleic acids. II. The quantitative analysis of ribonucleic acids. Biochemical journal 46:509-513. 1950.

66. Somogyi, M. Notes on sugar determination. Journal of biological chemistry 70:599-612. 1926.

67. Spilsbury, J. F. Observations on the nutritional requirements of Streptomyces griseus (Krainsky) Waksman and Schatz. Transactions of British mycological society 31:210-228. 1948.

68. Stier, T. J. B. and J. M. Stannard. A kinetic analysis of the endogenous respiration of baker's yeast. Journal of general physiology 19:461-477. 1936.

69. Stout, H. A. and H. Koffler. Biochemistry of the filamentous fungi. I. Oxidative metabolism of glucose by Penicillium chrysogenum. Journal of bacteriology 62:253-268. 1951.

70. Thornberry, H. H. Nutrient requirements of an antibiotic soil fungus, Streptomyces griseus (Krainsky) Waksman and Henrici. Phytopathology 36:412. 1946.

71. Thornberry, H. H. The role of minerals in production of streptomycin by Streptomyces griseus. Phytopathology 38:26. 1948.

72. Thornberry, H. H. and H. W. Anderson. Synthetic medium for Streptomyces griseus and the production of streptomycin. Archives of biochemistry 16:389-398. 1948.

73. Waksman, Selman A. and Hubert A. Lechevalier. Guide to the classification and identification of the actinomycetes and their antibiotics. Baltimore, Williams and Wilkins, 1953. 243 p.

74. Wang, Chih H. Associate Professor of Chemistry, Oregon state college. Personal communication.

75. Wang, Chih H., Bert E. Christensen and Vernon H. Cheldelin. Conversion of acetate and pyruvate to glutamic acid in yeast. Journal of biological chemistry 201:683-688. 1953.

76. Wang, C. H. et al. Carbohydrate metabolism in baker's yeast. I. Time course study of glucose utilization. Journal of American chemical society 78:1869-1872. 1956.

77. Wang, Chih H., Richard C. Thomas, Vernon H. Cheldelin and Bert E. Christensen. Conversion of acetate and pyruvate to aspartic acid in yeast. Journal of biological chemistry 197:663-667. 1952.

78. Weimberg, R. and Michael Doudoroff. The oxidation of L-arabinose by Pseudomonas saccharophila. Journal of biological chemistry 217:607-624.

79. Wood, Harland G. Fermentation of 3,4-C^{14}- and 1-C^{14} labeled glucose by Clostridium thermoaceticum. Journal of biological chemistry 199:579-583. 1952.

80. Wood, H. G., N. Lifson and V. Lorber. The position of fixed carbon in glucose from rat liver glycogen. Journal of biological chemistry 159:475. 1945.

81. Woodruff, H. B. and J. W. Foster. Microbiological aspects of streptomycin. I. Metabolism and streptomycin formation in stationary and submerged cultures of Actinomyces lavendulae. Archives of biochemistry 2:301-315. 1943.