

ROLE OF THE CITRIC ACID CYCLE  
IN THE METABOLISM OF STREPTOMYCES SPECIES

by

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ROLE OF THE CITRIC ACID CYCLE  
IN THE METABOLISM OF STREPTOMYCES SPECIES

INTRODUCTION

The tricarboxylic acid cycle has received general acceptance as the principal pathway for the oxidation of acetic acid by animal tissues. However, data obtained in studies with bacteria do not always satisfy the criteria on which the cycle was based in animal tissues (20, p.166). Factors such as limiting cell permeability and the existence of other known metabolic pathways are mainly responsible for the difficulties encountered.

Until quite recently research on the physiology of the actinomycetes dealt largely with general nutritional requirements of the group. Few studies of a fundamental nature have been conducted on the metabolism of these organisms.

This investigation was designed to study the mechanism(s) involved in the oxidative dissimilation of various substrates by intact cells of S. griseus with special reference to the citric acid cycle. Techniques adapted to the Warburg constant volume respirometer and to



radioactive tracer experiments were used.

## HISTORICAL

Since the announcement of the isolation of the antibiotic, streptomycin (34, pp.66-69), an extensive literature has accumulated on the general nutrition of Streptomyces griseus. For the most part these studies dealt with streptomycin titers and not with the accompanying physiology of the organism. However, some aspects of the physiology of this organism have been elucidated by fragmentary data accumulated during the studies on streptomycin production.

Studies conducted by Dulaney and Perlman (12, pp. 504-511) indicated that there were two phases of metabolic activity. During the growth phase the production of mycelium was accompanied by a reduction in the soluble constituents of the medium (N-C-P), fermentation of the available carbohydrate, a high oxygen demand and little production of streptomycin. During the autolytic phase the mycelium weight decreased markedly, inorganic phosphorus and soluble nitrogen were released into the medium, the oxygen demand dropped, and considerable quantities of streptomycin were produced. The pH rose gradually throughout the period of the fermentation.

S. griseus has been reported to utilize various organic acids, some of which are directly or indirectly

associated with the tricarboxylic acid cycle. Pridham and Gottlieb (33, p.109) found that acetate, succinate, and citrate were utilized for growth by various strains of S. griseus. Dulaney (13, p.10) observed that acetate and malonate would not support growth. However, citrate, fumarate, gluconate, lactate, malate, pyruvate and succinate were utilized to varying degrees. In a related study, Hubbard and Thornberry (19, pp.61-74) noted that acetate, citrate, lactate, glutarate, malonate, malate and succinate were utilized by S. griseus. It should be mentioned, however, that in the aforementioned investigation glucose was a constituent of the basal medium. The investigations of Perlman and Wagman (31, p.257) revealed that cells grown on lard oil appeared to be more active metabolically than cells grown on a medium containing glucose ( $QO_2$  was 50 as compared with 27 on glucose). The addition of inorganic phosphate speeded the metabolism of glucose and glycerol by both types of cells (as measured by oxygen demand) but did not affect the rate of metabolism of stearate, acetate or lard oil by the cells.

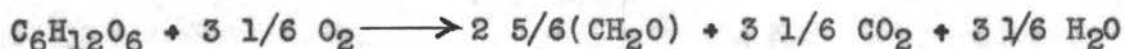
The utilization of amino acids such as glutamate, aspartate and alanine commonly associated with Krebs cycle intermediates was observed by Dulaney (14, p.313). The data obtained by Eiser and McFarlane (15, pp.164-173) suggested that the utilization of aspartic acid, involving

the aspartase-transamination reaction, brought about increased growth at the cost of streptomycin production. These workers were able to replace aspartic acid with glutamic acid, or even with succinate and nitrate, and obtain similar results.

Several investigations have indicated that carbon dioxide is the main product of carbohydrate utilization. Detailed analysis of the various growth media used has revealed no large accumulation of volatile or non-volatile acids. The minor accumulation of lactic acid has been reported by several research groups (12, pp.510-511; 7, p.729; 31, pp.254-260). Cochrane and Dimmick (7, p.729) also reported that small amounts of succinate were produced but did not appear until a full day after the glucose had essentially disappeared from the medium. The accumulation of acetate during the utilization of both glucose and lipids was observed by Perlman and Wagman (31, pp.254-260). While investigating the biosynthesis of streptomycin, Numerof, et al. (27, p.1342) noted the presence of free alanine and glutamic acid in the fermentation medium.

Until recently little emphasis was placed on the actual mechanisms by which S. griseus derived from organic substrates the energy required by the cell for growth and other cell processes. The incomplete oxidation

of glucose was reported by Sevcik (35, p.303) according to the following equation:



Lactic and acetic acids were isolated and identified as intermediates in the above oxidation. Scevola and Valcurone (36, p.624), also working on the intermediates of carbohydrate metabolism by S. griseus, were able to intercept triose phosphate and pyruvic acid in the respiration of living cells of this organism. As acetaldehyde was found in only one instance it did not seem probable that pyruvate was oxidized by simple decarboxylation.

Preliminary evidence against the existence of a tricarboxylic acid cycle in S. griseus was reported by Garner and Koffler (16, p.139). The respiration of cells grown in liquid media containing single carbon sources led to the conclusion that only the enzymes responsible for the oxidation of succinate and pyruvate were constitutive. Enzymes enabling cells to use other intermediates of the cycle were thought to be adaptive, and hence did not appear to be components of a main terminal oxidative pathway. However, a subsequent investigation by Coty, et al. (11, p.74) demonstrated that cell free extracts of S. griseus prepared from alumina-ground cells had the ability to perform nearly all the critical reactions of the tricarboxylic acid cycle as shown by the following

1. Oxalacetate + acetylphosphate  $\longrightarrow$  citrate.

Scevola and Zorzoli (37, p.552) have reported the formation (and isolation as the acetyl phenylhydrazine) of an active acetyl radical during the respiration of S. griseus.

2. Citrate or cisaconitate  $\longrightarrow$  alpha ketoglutarate.

This reaction was reversible as evidenced by the reoxidation of reduced triphosphopyridine nucleotide (T.P.N.) if sodium bicarbonate was added to the reaction.

3. Malate oxidation was reversed by the addition of oxalacetate. Manganous ions were found to be essential. Reactions 2 and 3 were T.P.N. specific.

4. Succinate  $\longrightarrow$  fumarate  $\longrightarrow$  malate.

Labeled fumarate and malate were formed from  $C^{14}$  labeled succinate. Succinic dehydrogenase activity was demonstrated by the Thunberg technique.

Preliminary work carried out in this laboratory (6, p.94) indicated that several members of the tricarboxylic acid cycle were oxidized by non-proliferating cells. No oxygen uptake was observed with alpha ketoglutarate, succinate, fumarate, and malate unless substrate concentrations approaching 0.16M were employed. Citrate was not oxidized even in the presence of high concentrations of substrate.



Oxidative mechanisms have been postulated for other members of the genus Streptomyces. Cochrane, et al. (10, pp.17-23) concluded that the hexose monophosphate shunt was present in S. scabies and S. coelicolor. That the shunt sequence might be mainly or exclusively responsible for the initial steps in the metabolism of glucose was suggested by the results with labeled glucose, by the failure to locate the early steps of the Embden-Meyerhof-Parnas system, and by the observation that glucose-6-phosphate was 80 to 90 per cent metabolized in a system containing only T.P.N. as coenzyme.

Cochrane and Peck demonstrated that S. scabies (8, p.5) and S. coelicolor (9, pp.37-44) were able to carry out the reactions of the tricarboxylic acid cycle. The use of cell free extracts was essential with both organisms, primarily because of the low permeability of the cells to certain key substrates. Matsuoka, et al. (26, p.169) have suggested that a tricarboxylic acid cycle is operative in S. venezuelae. They were able to detect most of the intermediates free in the medium. They also proposed that glutamic acid was the most important amino acid, probably linking nitrogen metabolism with carbohydrate metabolism in S. venezuelae. Glutamic acid was readily detected free in the medium.

## EXPERIMENTAL METHODS

Two approaches were used in studying the metabolism of S. griseus. The first phase of the work centered on the investigation of terminal oxidations as evidenced by manometric techniques. The second phase entailed the use of radioactive acetate and radioactive carbon dioxide with intact cells. Each of these approaches is treated separately in the following section on experimental methods.

### Manometric Studies

#### Cultures.

All the cultures used were obtained from the American Type Culture Collection. S. griseus (3475) was used throughout all manometric studies. S. albus (3004), S. viridochromogenus (3356) and S. willmorii (6867) were used in preliminary comparative studies. Stocks were maintained in sterile soil and successive transfers on laboratory media were limited to insure physiological constancy of the organisms.

#### Preparation of cells.

Stock cultures were prepared by adding spore suspensions to tubes of sterile soil. Cultures for inocula were obtained by spreading spores from a soil tube over



Blake bottle slants of modified nutrient agar (2.0% agar, 1.0% glucose, 0.5% sodium chloride). After 4 to 5 days incubation at 28°C the spores were harvested by adding 10 ml. of sterile broth to each Blake bottle. The spores were teased off the agar with a sterile wire loop. One ml. of this spore preparation served as inoculum. Fifty ml. of nutrient broth supplemented with 0.1 per cent glucose and 0.5 per cent sodium chloride contained in 250 ml. Erlenmeyer flasks were inoculated and mounted on a rotary shaker set at a speed of 230 R.P.M. The temperature of incubation was 28°C for a period of 24 hours. The resulting cell crop was harvested by centrifugation, washed four times in phosphate buffer (0.06 M, pH 6.9) and then resuspended at growth volume. The colonial aggregates of S. viridochromogenus were subjected to 15 seconds in a Waring blender. The final growth of the other organisms was finely dispersed and did not require the blending procedure. In some instances the cells were subjected to a starvation procedure. Usually 50 ml. of a washed cell suspension contained in sterile 250 ml. Erlenmeyer flasks were returned to the rotary shaker. Samples were taken at various time intervals for manometric determinations.

#### Manometry.

Measurements of the gaseous exchange of resting cell

suspensions were made by the use of conventional manometric techniques as detailed by Umbreit, Burris and Stauffer (40, pp.1-16) and will not be repeated except where modifications in method were made. A standard Warburg constant volume microrespirometer was used throughout the work. The water bath was maintained at 30.1°C. Flasks were shaken at the rate of 60 complete strokes per minute through a distance of 3.2 cm. In a typical experiment to determine the rate of oxygen consumption, each flask contained 2.5 ml. of cell suspension (0.6 to 0.8 mgms of N.), the sidearm contained 0.5 ml. of substrate in 0.067M buffer and the center well contained 0.2 ml. of 20 per cent potassium hydroxide to give a total flask content of 3.2 ml. All experiments were carried out in an air atmosphere. Substrate concentrations were 0.003 M unless otherwise stated. Carbon dioxide evolution was determined by replacing the potassium hydroxide with buffer in one pair of flasks and calculating carbon dioxide by difference (40, pp.17-20). Allowance was made for the retention of carbon dioxide in the buffer at the pH of the reaction.

#### Chemical.

Total cell nitrogen was determined by the micro-Kjeldahl method (25, pp.280-282). The one dimensional paper chromatographic method of Lugg and Overall (23,

pp.98-104) using a water:butanol:formic acid (5:4:1) solvent was employed for the detection of non-volatile carboxylic acids. The method is sensitive to from 2 to 8 gamma of acid. In some instances the phosphate buffer used for suspending the cells was replaced with distilled water as phosphate salts proved troublesome during chromatographic analysis.

### Radioactive Tracer Studies

#### Acetate incorporation.

Studies on the incorporation of labeled acetate by intact cells were made using both growing and non-proliferating cells of S. griseus (3475). The non-proliferating cells were suspended in phosphate buffer (0.067M, pH 6.9). Growing cells were suspended in a synthetic medium. This medium was prepared as follows:

NaCl	-- 5 grams	MgSO <sub>4</sub> .7H <sub>2</sub> O	-- 0.5 grams
K <sub>2</sub> HPO <sub>4</sub>	-- 2 grams	FeSO <sub>4</sub> .7H <sub>2</sub> O	-- 0.002 grams
(NH <sub>4</sub> ) <sub>2</sub> HPO <sub>4</sub>	-- 4 grams	ZnSO <sub>4</sub> .7H <sub>2</sub> O	-- 0.001 grams

Distilled water was added to 1000 ml. The pH of the medium after autoclaving was 6.8.

#### Treatment of cells and apparatus.

The apparatus consisted of a 250 ml. three necked

flask with attachments as follows: entering through one side neck was an aeration tube with a sintered tip extending to the bottom of the flask. Air was passed through a soda-lime tower, a sulfuric acid trap, and finally through a sterilized cotton-filled tube before entering the culture flask.

Carbon dioxide leaving the flask was dispersed through a sintered glass tube into 25 ml. of 3 N. NaOH. Provision was made to permit sampling through the center neck of the flask. The temperature for all experiments was 28°C. Forty-five mgs. of acetate-1- $\text{Cl}^{14}$  with a total activity of  $7.28 \times 10^6$  counts per minute were mixed thoroughly with the 100 ml. of cells in the culture flask. Samples were taken periodically. Activity of the medium, the cells, and the carbon dioxide was counted and expressed as counts per minute. These were deposited and counted directly in cupped metal planchets after evaporation. Appropriate aliquots were used. Although the content of inorganic salts of the samples of media was relatively high and the resulting activities were thereby reduced owing to increased self-absorption, preliminary experiments indicated that the method was suitable for control and comparative purposes. The experiments were terminated by adding 6 N HCl. The cells, separated from the medium by centrifugation and repeated washing (twice

with distilled water, once with alcohol), were hydrolysed in 6 N HCl for 20 hours under reflux. The hydrolysate was freed of HCl by vacuum desiccation then resuspended in a minimum of water for analysis. The final medium and the alcohol extract were chromatographed. The descending method was employed in preparing both one and two dimensional paper chromatograms (22, pp.396-399). One dimensional chromatographs were developed in a water:butanol:acetic acid (5:4:1) solvent system. The second solvent system was water saturated phenol containing a trace of 8 hydroxy quinoline. Amino acids were detected by spraying dried chromatograms with a 0.2 per cent solution of ninhydrin in butanol saturated with water. The method of Lugg and Overall (23, pp.98-104) was used to detect other non-volatile organic acids. Radioautographs were prepared after the method of Benson, et al. (4, p.1716).

For kinetic studies 5 ml. of boiling alcohol were added to 5 ml. aliquots from the reaction vessel. The cells were washed twice with distilled water, once with ether, then were dried over  $P_2O_5$ . Weighed amounts of the dried cells were placed in vials to which were added 1 ml. of 6 N HCl. The vials were sealed, then autoclaved at  $121^{\circ}C$  for 6 hours. The hydrolysates were dried and freed of HCl by vacuum dessication over  $P_2O_5$  and KOH. After resuspension in a small amount of distilled water

appropriate aliquots were taken for chromatographic analysis.

Glutamic acid was isolated from the alcohol extract of the non-proliferating cells in the form of the free acid after dilution with non-isotopic L-glutamic acid. The purity of the isolated glutamic acid was checked with paper chromatography. It was then subjected to the following degradation procedures:

- (a) Combustion provided a measure of the total activity,
- (b) Ninhydrin decarboxylation (42, p.253) removed the alpha carboxyl,
- (c) Schmidt's reaction (2, pp.1564-1568) yielded alpha, gamma diaminobutyric acid and the gamma carboxyl as carbon dioxide.

Radioactivities were determined as barium carbonate in the conventional manner; counting data were corrected for background and self-absorption.

#### Carbon dioxide fixation.

##### Treatment of cells and apparatus.

The apparatus consisted of a three neck flask with attachments as follows. One neck was equipped with a rubber hood. The carbon dioxide, oxygen and pyruvate were admitted into the vessel with the appropriate

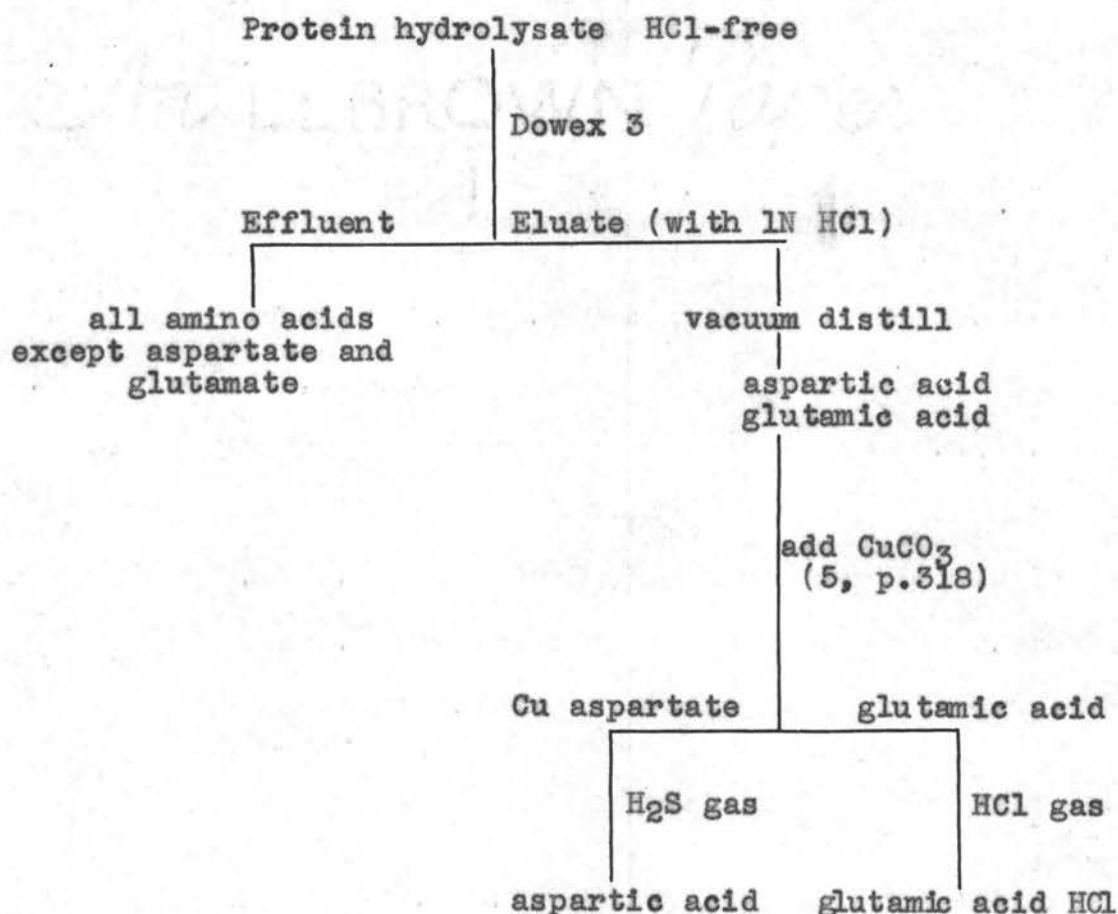


attachments on a hypodermic needle. Provision was made on one neck of the flask for attachment to a water aspirator. The third neck was connected to a mercury manometer.

100 ml. of S. griseus cells suspended in the inorganic basal medium were put in the three neck flask. The system was evacuated and checked for leaks. Two millimoles of sodium pyruvate in aqueous solution was added. Then labeled carbon dioxide generated from sodium carbonate with a total activity of  $5.4 \times 10^6$  counts per minute was passed in. Finally oxygen was introduced. The experiment was conducted under a negative pressure. The culture flask was agitated in a specially constructed water bath at a temperature of 28°C for a period of 8 hours. At the end of the experiment 10 ml. of 6 N HCl were added. The cells were separated from the growth medium by centrifugation, washed twice with water, once with alcohol, and finally, once with ether. The resulting cells were dried over  $P_2O_5$ , weighed, then acid hydrolysed. Aliquots of the resulting hydrolysate were analysed with two dimensional paper chromatography. Subsequent preparation of radioautographs revealed the extent and degree of incorporation of radioactivity in the amino acids of the hydrolysate.

Chemical.

The following scheme outlines the procedures employed for the separation of glutamic acid and aspartic acid from the protein hydrolysate.



Controls were maintained throughout the separation operations with ninhydrin spot tests. Microbiological assays (18, pp.1-12) were used to determine the amount of glutamate and aspartate present.

The aspartic acid was subjected to the following



degradation procedures:

- (a) Combustion provided a measure of the total activity,
- (b) Ninhydrin decarboxylation (42, p.253) removed both carboxyls,
- (c) Deamination with silver nitrite produced malic acid which was subjected to decarbonylation with concentrated sulfuric acid (41, p.581). This was followed by oxidation of the carbon monoxide to carbon dioxide to provide a measure of the alpha carboxyl.

The glutamic hydrochloride was degraded by the combustion and ninhydrin reactions used in a previous section for glutamic acid.

## EXPERIMENTAL RESULTS

I. Manometric StudiesOxidative patterns of selected Streptomyces species.

The cultures used in the initial stages of this study were selected on the basis of species differences observed during 'phage typing' work. An investigation of the oxidative behavior of these cultures (Table 1) showed considerable variation in respect to the number and kind of substrates oxidized. It should be noted that glucose was the only substrate commonly utilized and that alpha ketoglutarate was the only substrate not oxidized by any of the species. It is interesting that citrate was not used by any of the test cultures except S. albus. This culture also oxidized all of the other compounds employed with the exception of the alpha keto acids. S. viridochromogenus oxidized malate, succinate and fumarate but, oddly enough, would not utilize acetate. S. griseus differed from the other species in oxidizing pyruvate and oxalacetate and in showing no oxygen uptake on the remaining Krebs cycle intermediates. The oxidation of pyruvate and oxalacetate by intact cells of S. griseus has been reported by Oginsky, et al. (29, p.640). In the case of S. willmorii only acetate and glucose were utilized.

TABLE 1

The oxidation of selected substrates by various  
Streptomyces species

Substrate	<u>S. virido-</u> <u>chromogenus</u>	<u>S. albus</u> *	<u>S. will-</u> <u>morei</u>	<u>S. griseus</u>
Glucose	213(87) <sup>†</sup>	256(132)	145(53)	260(82)
Acetate	No	190(132)	100(53)	166(82)
Oxalacetate	No	No	No	180(82)
Alpha keto- glutarate	No	No	No	No
Malate	150(87)	190(132)	No	No
Fumarate	135(87)	190(132)	No	No
Succinate	130(87)	172(132)	No	No
Citrate	No	164(132)	No	No
Pyruvate	No	No	No	160(82)

\* S. albus--oxygen consumption, in microlitres, for 220 minutes. All others are based on a 60 minute period.

† Endogenous respiration values are in parenthesis.

### Oxidative behavior of *S. griseus*.

Since *S. griseus* is presently being used as the host organism in the study of phage multiplication, it was decided to use this culture in all subsequent physiological studies.

### Endogenous respiration.

It may be noted in Table 1 that the endogenous respiration of *S. griseus* and of the other species is very high. This high endogenous respiration is characteristic of actinomycetes and fungi (45, p.315; 39, p.267). Since marginal rates of respiration are difficult to ascertain in the presence of a high endogenous, experiments designed to reduce the endogenous were carried out. The influence of age of cells on the endogenous respiration of *S. griseus* is shown in Figure 1.

No significant differences were observed in the endogenous rates of respiration between 24 and 48 hour old cells. However, the rate of oxygen uptake of 24 hour cells in the presence of glucose was twice that of the 48 hour old cells. Gottlieb and Anderson (17, pp.172-173) have reported a similar decrease in *S. griseus* respiration during the growth period.

The procedure of incubating cells in the absence of substrate for a three hour period served to reduce the

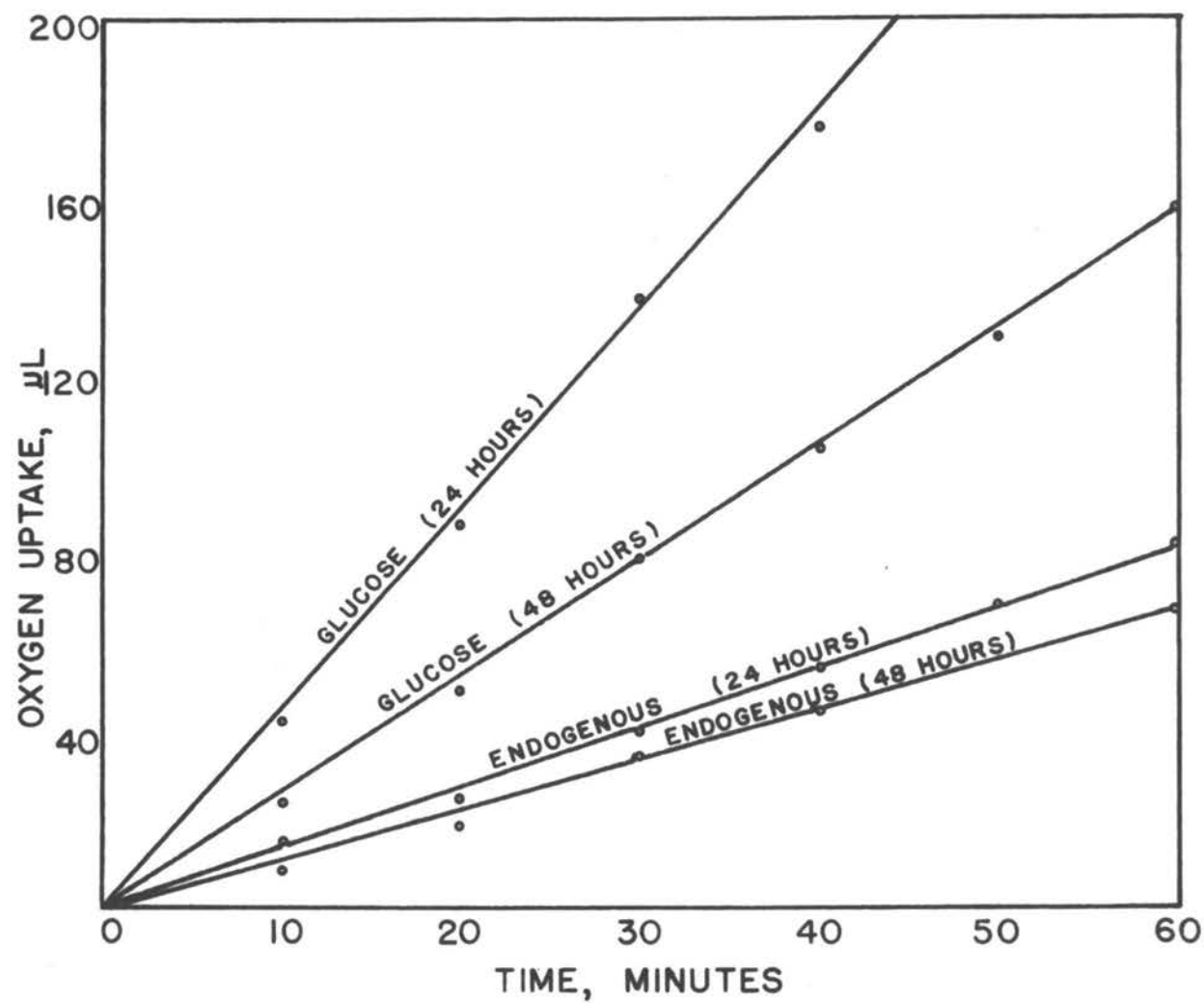


FIGURE 1

The effect of age of cells on respiration of S. griseus.

endogenous (Figure 2) but subsequent substrate oxidation was proportionally reduced. Similar effects were observed with starved 48 hour cells. Periods of starvation longer than 3 hours decreased the rates of substrate and endogenous respirations but these reductions were not so definite as were those of the 3 hour starvation periods. Since no reduction in endogenous respiration was obtained without a concomitant reduction in substrate respiration, 24 hour unstarved cells were used for all subsequent manometric determinations.

#### Mole ratio studies.

The amount of oxygen used by cells in the presence of a known amount of a specific substrate may be used to determine the extent of oxidation of the substrate by the cells. The oxygen consumption per mole of substrate metabolized is compared with the theoretical values for various stages of oxidation. Such information can often be used to identify the product(s) of oxidation of the substrate by the cells. Manometric determinations showed (Table 2) that resting cells of S. griseus consumed less than one third of the theoretical oxygen required for the complete oxidation of these substrates. Apparently glucose and glutamate were oxidized to the level of succinate. Similarly acetate, if oxidized via the Krebs cycle,

The effect of starvation on respiration  
of S. Erisens.

FIGURE 2

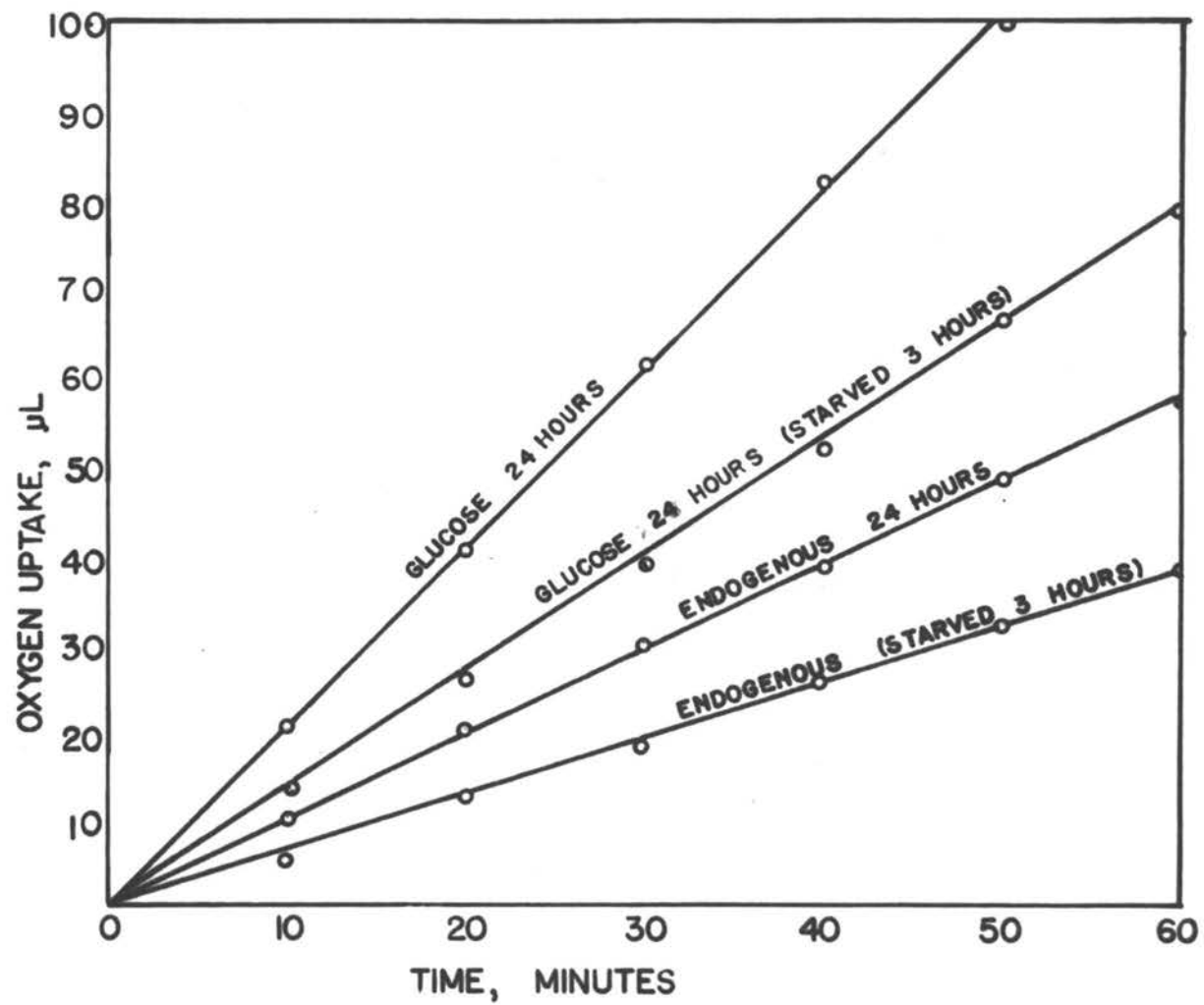


TABLE 2

Stoichiometric ratios for the dissimilation of various compounds

Substrate	Moles of oxygen consumed per mole of substrate		
	Actual	Theoretical	% of Theoretical
Glucose	2.08	6.0	35
Acetate	0.62	2.0	31
Glutamic	1.20	4.5	27



was oxidized to the level of succinate. It was recognized, however, that oxidative assimilation could account for the observed low oxygen consumption. Although it has been reported that S. griseus does not form succinate until late in the growth period (7, p.725) an analysis of the acetate medium was carried out using paper chromatography. No succinate was detected. However, a slow moving spot with an Rf lower than any of the acid controls employed was shown to give a positive ninhydrin test. Subsequent investigations proved the compound to be glutamic acid. The relation between acetate oxidation and the production of glutamic acid is discussed in more detail in a following section.

#### Respiratory quotient.

The respiratory quotient can often be used advantageously to interpret results and to buttress results obtained by other methods of investigation. The results of the mole ratio studies (Table 2) indicated incomplete oxidation of glucose. If succinate were the product of oxidation, for example, 2 moles of carbon dioxide would be evolved for every 2 1/2 moles of oxygen consumed. The R. Q. of this theoretical system would be 0.80. The observed R. Q. for the oxidation of glucose by S. griseus was very close to 1.0. In a typical experiment the following R. Q. values were obtained: endogenous 1.00,

glucose 1.07, endogenous plus substrate 1.03.

The results of mole ratio and R. Q. studies, using glucose as the substrate, attests to the combined effects of incomplete oxidation combined with considerable assimilation.

#### Substrate oxidation.

The results of experiments already tabulated in Table 1 have been represented in graphical form in Figure 3. The data illustrated in Figure 3 discloses that glucose, acetate, pyruvate, lactate and oxalacetate were oxidized at significant rates by S. griseus at a final substrate concentration of 0.003M. No significant increase in the respiration of S. griseus was noted with succinate, citrate, alpha ketoglutarate, fumarate and malate. However, as shown in Figure 4, the amino analogues of pyruvate, oxalacetate, and alpha ketoglutarate were rapidly oxidized. It is apparent from a comparison of these data that cells of this organism show some inconsistency between the relative rates of oxidation of amino acids and related organic acids. Compounds such as alpha ketoglutarate and succinate that can be predicted as intermediates in the dissimilation of glutamic acid showed no oxygen uptake. However, glutamic acid was oxidized at a rate closely approaching that of glucose. In the same sense, aspartate (after a short lag) and

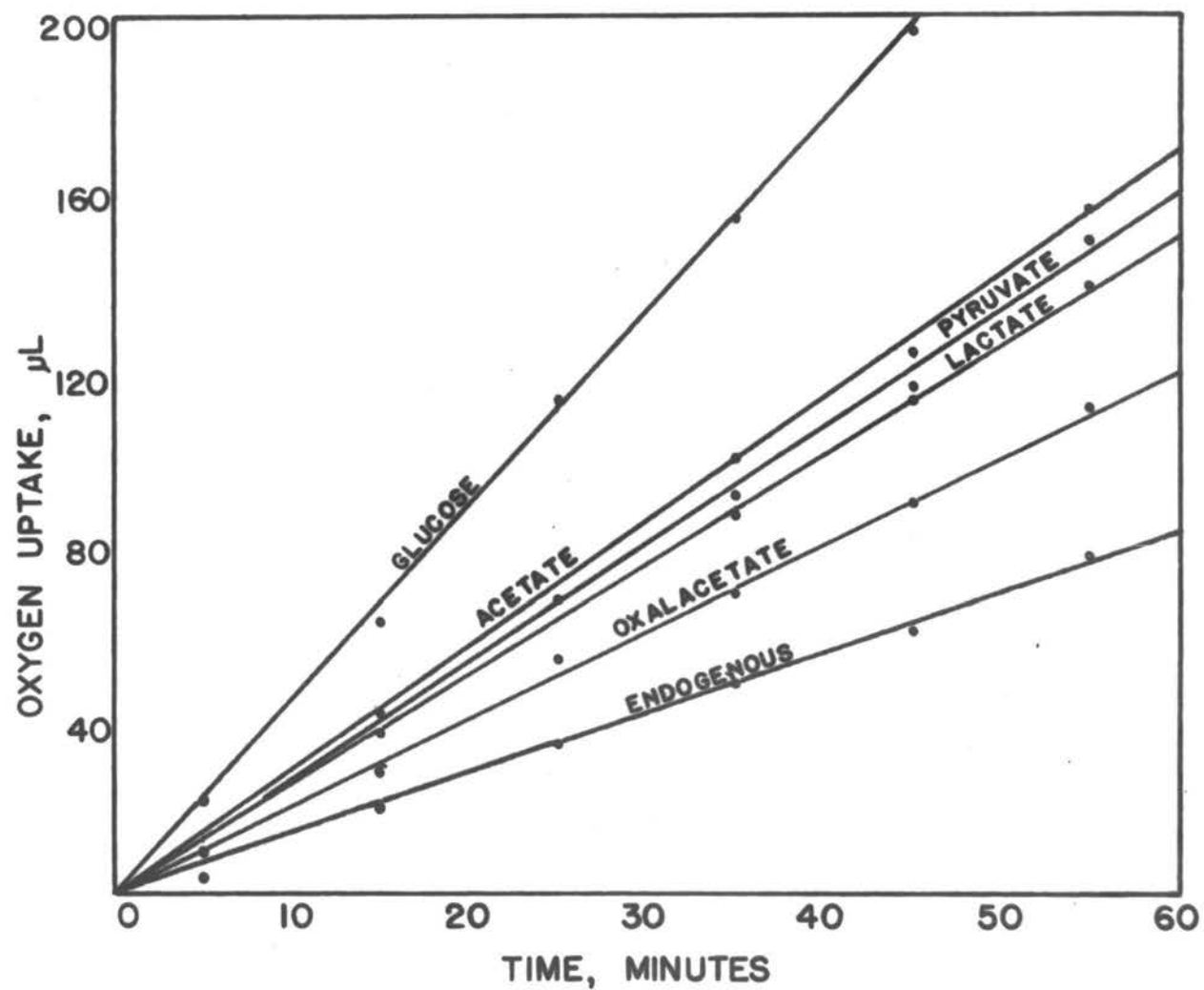


FIGURE 3

Observed respiration of intact cells of *S. griseus*.

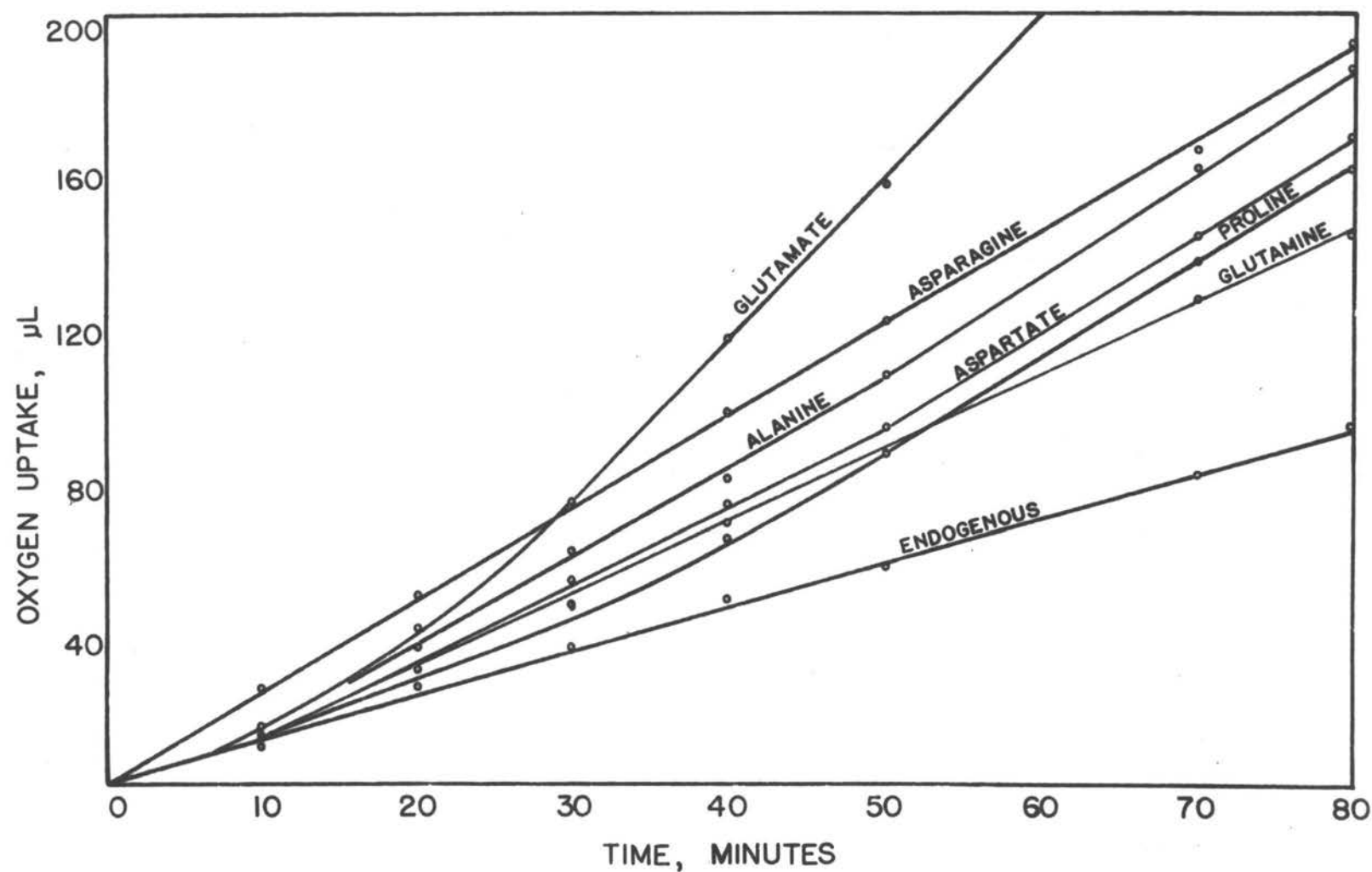


FIGURE 4  
The oxidation of selected amino acids by cells of *S. griseus*.

asparagine were oxidized more rapidly than oxalacetate. The lag in oxidation shown by glutamate and proline (Figure 4) is worthy of note. A lag in proline utilization has been reported by Woodruff and Ruger (46, pp.315-321). These workers showed that cells could not be adapted to proline which indicated that the lag was not one of adaptation but one of permeability.

#### Permeability effects.

During the course of investigations on the oxidation of Krebs cycle intermediates by species of the genus Streptomyces (8, p.5; 9, pp.37-44) several key substrates were not oxidized by intact cells. These workers resorted to cell free extracts to overcome the inability of some substrates to penetrate the cell. The data to be presented indicates that high substrate concentrations can be used to overcome similar permeability barriers with S. griseus cells.

In early experiments glutamic acid was rapidly oxidized by intact cells of S. griseus. Subsequent mole ratio studies indicated that the product of glutamate oxidation went beyond the alpha ketoglutarate stage. This observation suggested that permeability might be responsible for the lack of oxidation of 0.003M alpha ketoglutarate and other key intermediates. This premise was tested by a number of indirect methods having the

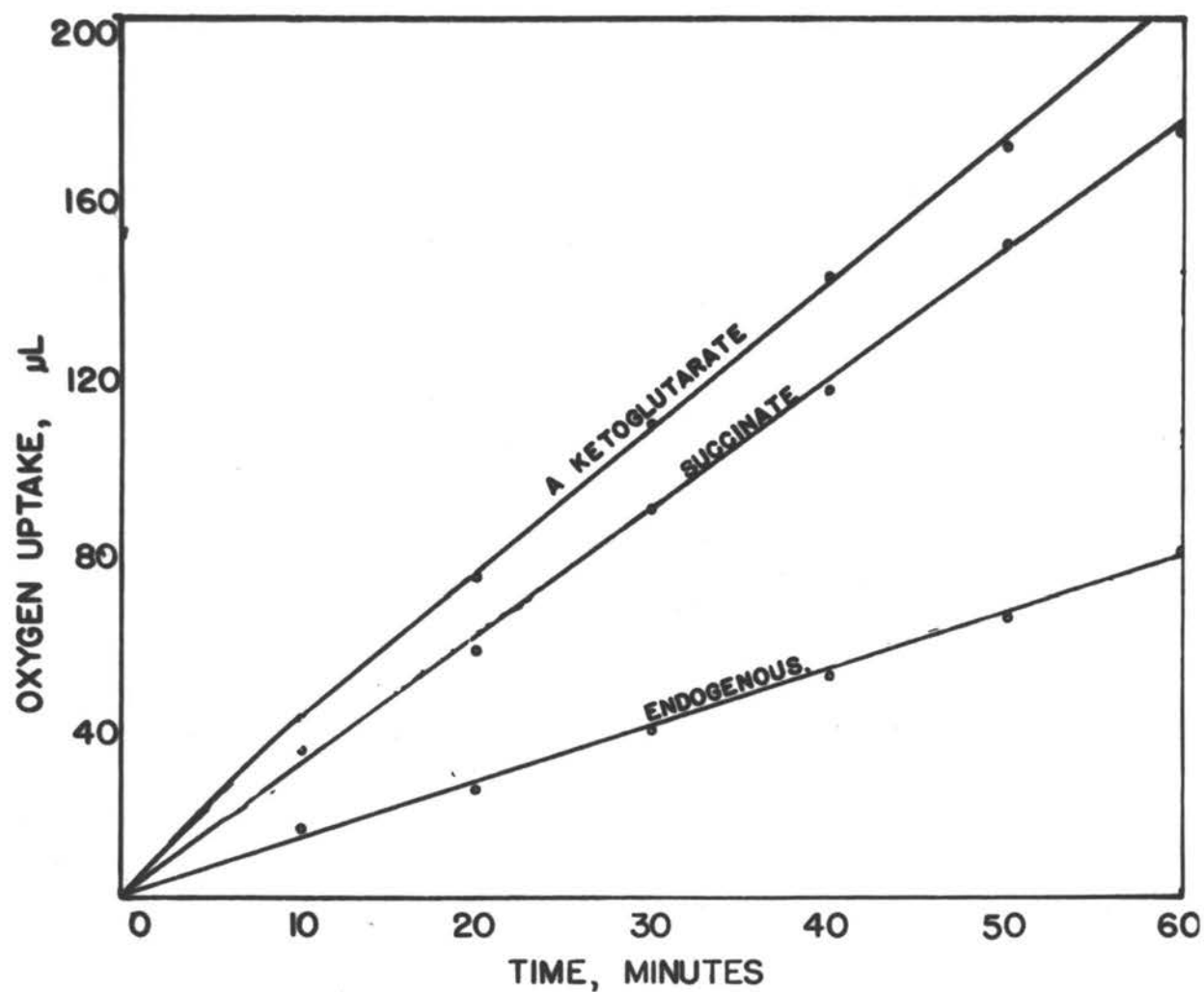


FIGURE 5

The oxidation of 0.167 M succinate and alpha ketoglutarate by intact cells of S. griseus.

common effect of influencing cellular permeability.

In the present study when the concentration of substrate in the reaction flasks was increased considerably in excess of that normally used, two compounds that were not metabolized were now oxidized rapidly. In both cases optimal substrate oxidation was attained at the 0.167 M level (Figure 5). As shown in Figure 6 successive increases in oxygen consumption occurred with concomitant increases in the concentration of alpha ketoglutarate. The results with succinate, though not as pronounced, proved to be quite conclusive. Succinate was oxidized at concentrations ranging from 0.03M ( $QO_2N = 113$ ) to 0.167M ( $QO_2N = 201$ ).

#### pH effects.

If the permeability of the cell wall to various substrates is a function of the unionized acid concentration then an increase in the hydrogen ion concentration should have the same effect as an increase in the substrate concentration. Lowering the pH to 6 in the presence of 0.003M substrate slightly but significantly increased the rate of oxygen consumption of succinate and alpha ketoglutarate over endogenous. The activity of the organism was reduced at pH 5.5 as was evidenced by lower rates of oxidation of substrates. The presence of malate, fumarate or citrate at 0.003M concentration inhibited the

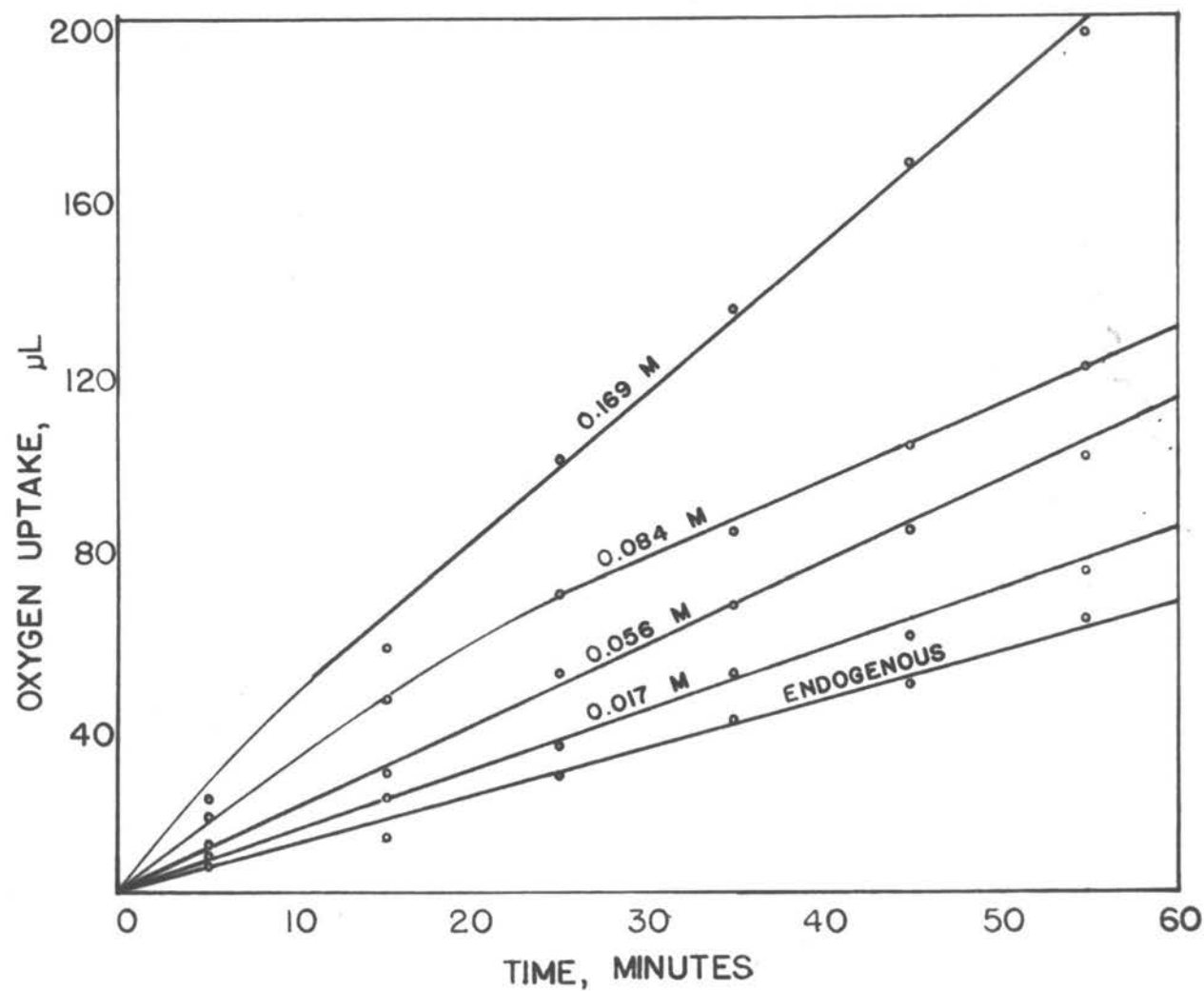


FIGURE 6

The oxidation of alpha ketoglutarate at various substrate concentrations by S. griseus.



endogenous respiration between pH 5.5 and 6.5. Although the oxidation of citrate, fumarate and malate were not observed with these procedures, it can be assumed that permeability barriers inhibited the oxidation of 0.003M succinate and alpha ketoglutarate.

Recent publications by Stone and Wilson (38, pp.605-617), Williams and Wilson (44, pp.353-360), and particularly Barrett and Kallio (3, pp.517-524) showed that factors other than enzyme compliment may be responsible for a slow initial rate of utilization or a negative utilization of substrate. These workers and others have demonstrated conclusively that apparent adaptive lags or negative utilization can often be attributed to the inability of a substrate to cross the cell wall.

It has been shown in the present study that the permeability barriers imposed by cells of S. griseus may be overcome by increasing the concentration of substrate in the case of succinate and alpha ketoglutarate. Although the oxidation of malate, fumarate and citrate were not obtained even with high concentrations of substrate it was possible to obtain oxidation of these acids by preincubating the cells for several hours with a specific substrate. Reference to Figure 7 discloses that malate and fumarate were oxidized after 4 hours in contact with the cells. The oxidation of citrate was observed after

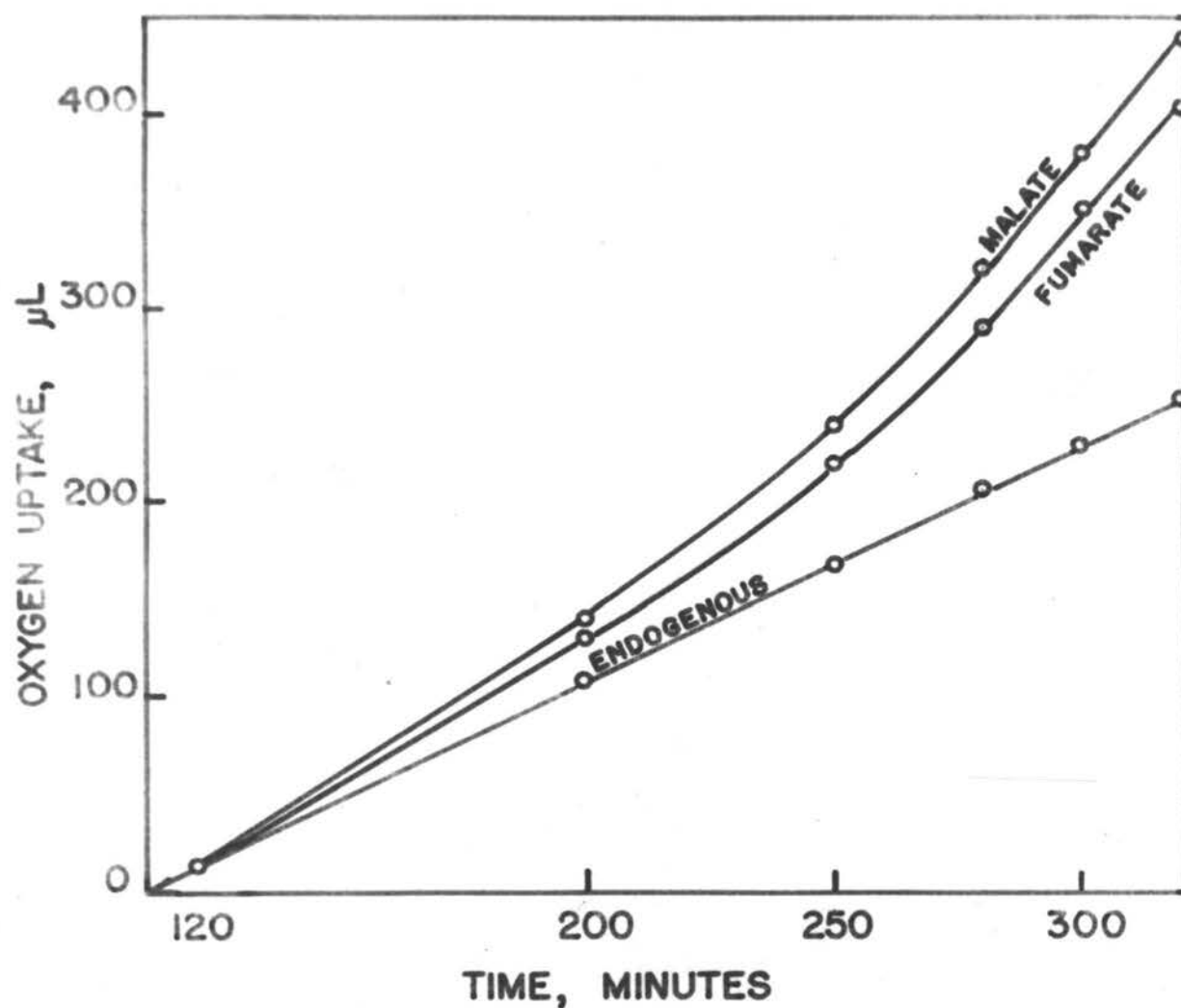


FIGURE 7

The oxidation of 0.167 M malate and fumarate by *S. griseus* cells after a 120 minute preincubation period.

a 10 hour preincubation period (Figure 8). This acid slightly inhibited the endogenous respiration until oxidation occurred. The possibility of bacterial contamination being responsible for the observed oxygen uptake was negated by repeating the experiments under aseptic conditions.

#### Inhibition studies.

Malonic acid. Garner and Koffler (16, p.139) found that the respiration of S. griseus on succinate was inhibited by malonate. In the present investigation it was not possible to demonstrate inhibition unless the malonate was used at concentrations much higher than those recommended by Pardee and Potter (30, pp.241-250). It was presumed that effective diffusion barriers reduced the amount of malonate penetrating the cells.

Pyrophosphate. This inhibitor has also been reported to competitively inhibit the oxidation of succinate (21, pp.81-88). In the present work pyrophosphate stimulated the endogenous respiration of S. griseus. The degree of stimulation increased with concentration of pyrophosphate from 0.01M to 0.04M. As shown in Figure 9 the stimulation of endogenous by 0.04 pyrophosphate was very definite. The rates of glucose oxidation with and without added pyrophosphate were identical. Similarly,

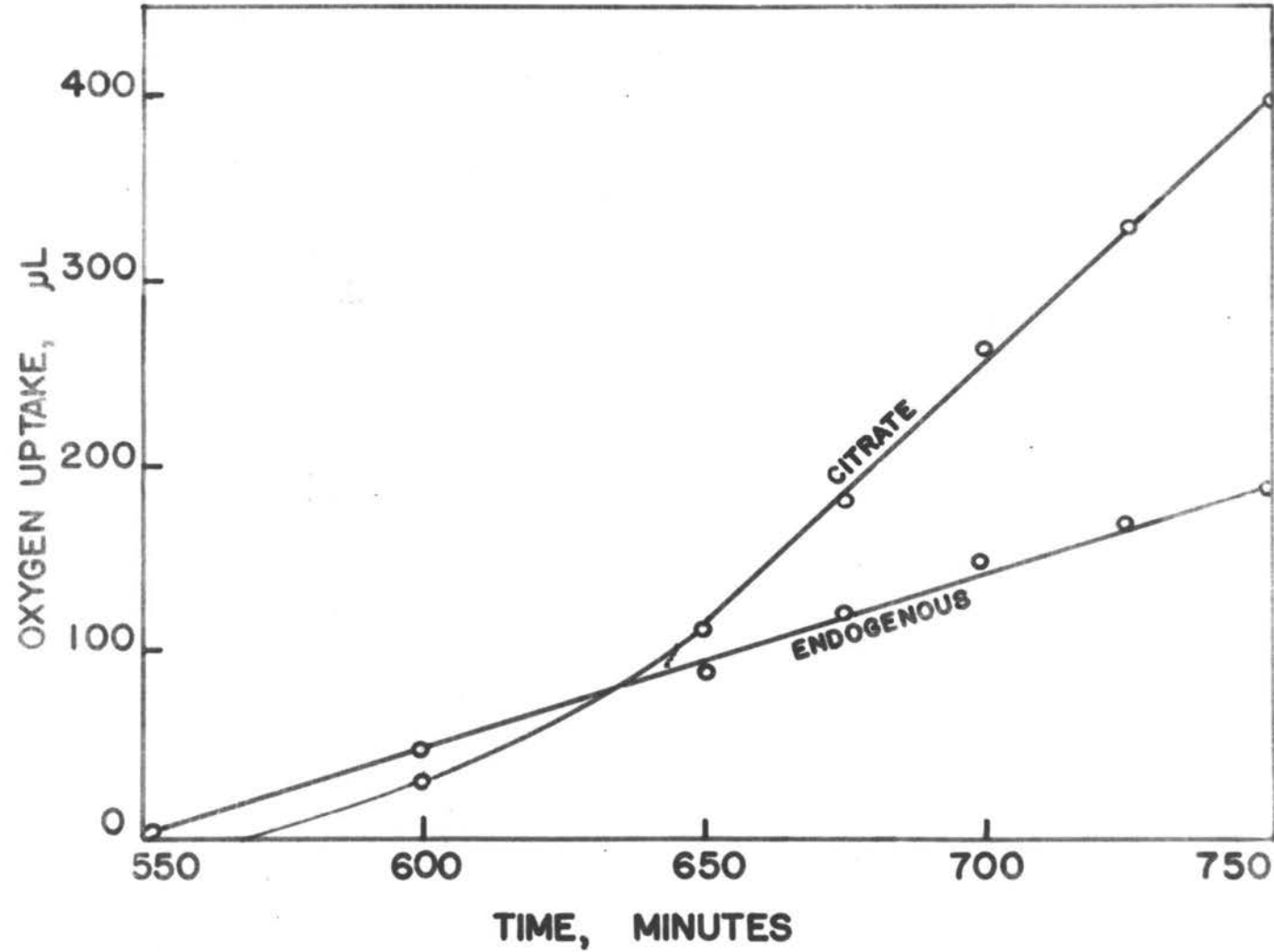


FIGURE 8

The oxidation of 0.167 M citrate by S. griseus cells after a 550 minute preincubation period.

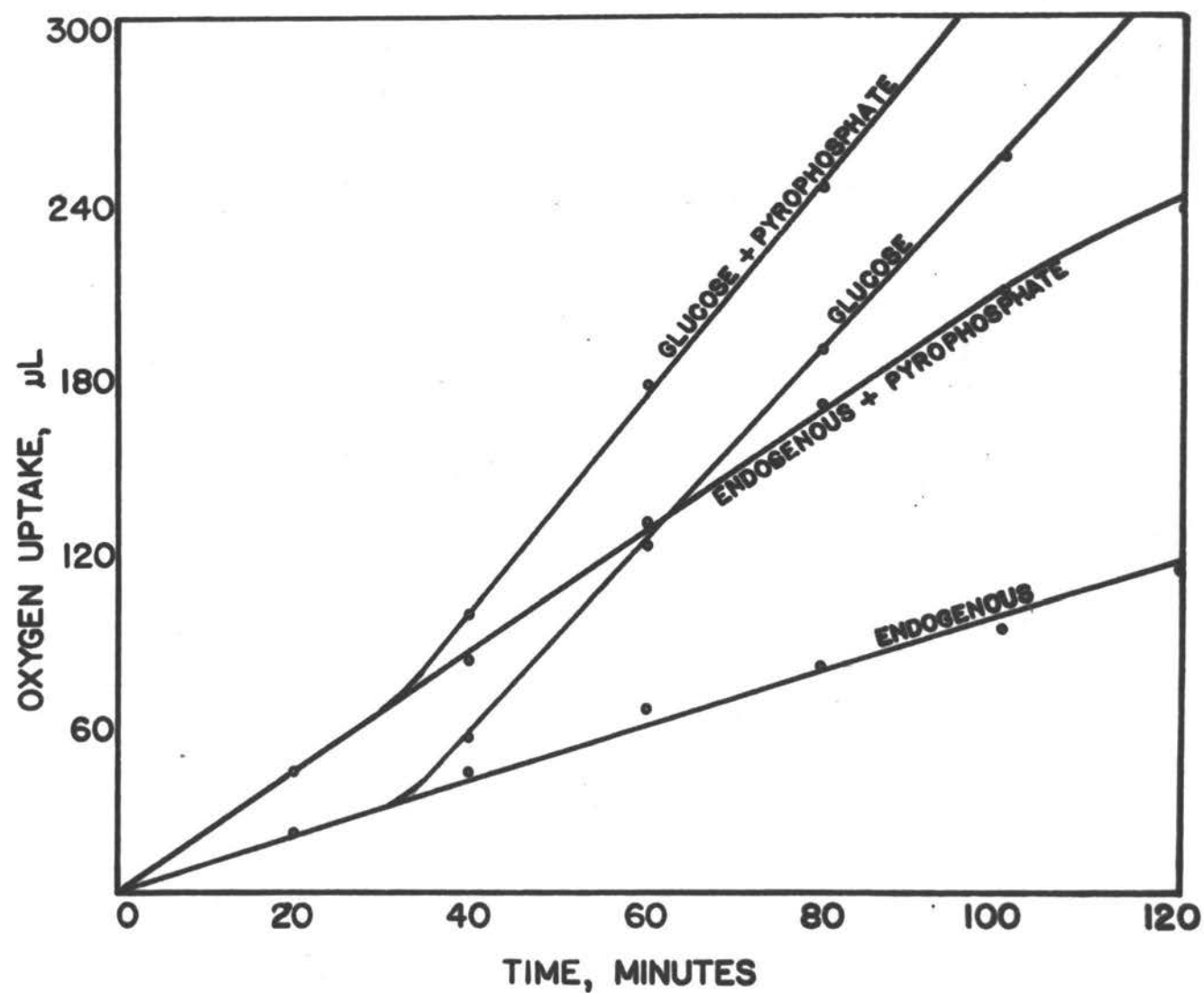


FIGURE 9

The stimulation of the endogenous respiration of *S. griseus* by 0.04 M pyrophosphate.

the rate of 0.003M glutamate oxidation was not inhibited by pyrophosphate. Complete inhibition was observed with 0.0006 M (2  $\mu$ M) glutamate. However, the evaluation of pyrophosphate inhibition was complicated by the marked stimulation of the endogenous. It appeared that the stimulation was responsible to a reaction peculiar to the endogenous since the rates of 0.003M glucose or glutamate oxidation were identical with and without the addition of pyrophosphate.

Fluoroacetate. It was shown (Figure 8) that citrate is oxidized by intact cells of S. griseus. Further proof of this oxidation was obtained by indirectly inhibiting the oxidation of citrate with fluoroacetate. Fluoroacetate has been shown to inhibit aconitase (32, pp.310-315) through the formation of a fluorotricarboxylic acid which competes with citric acid for the enzyme site. The inhibition by 0.003M fluoroacetate of cells oxidizing 0.003M oxalacetate is shown in Figure 10. Attempts to detect citrate in the fermentation medium were not successful. This might be expected, however, since the cell wall was not freely permeable to citrate. Glutamic acid was detected free in the medium after the inhibition of the oxidation of either pyruvate or oxalacetate with fluoroacetate.

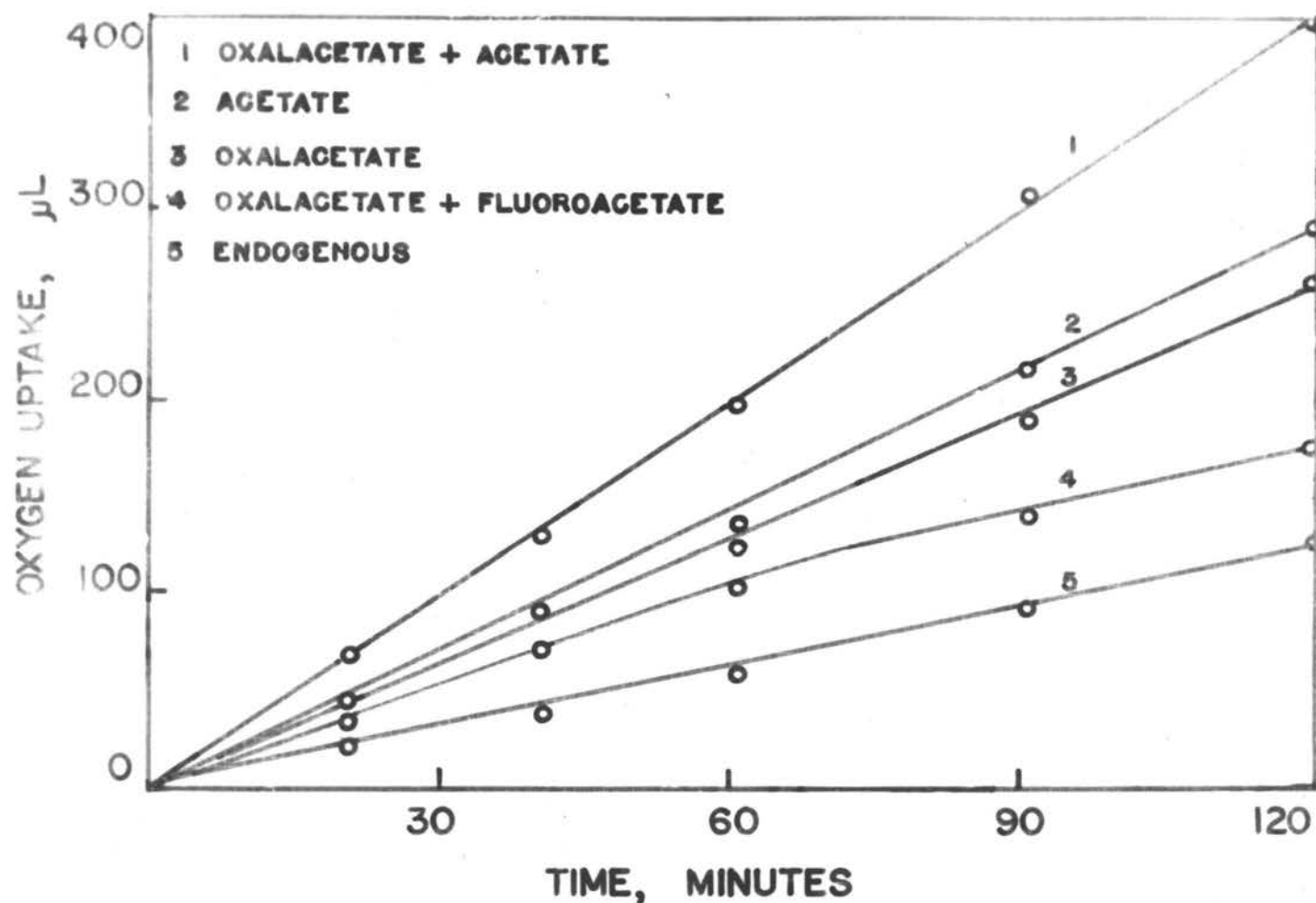


FIGURE 10

The inhibition of 0.003 M oxalacetate oxidation with 0.003 M fluoroacetate and the stimulation of acetate oxidation by oxalacetate.



The stimulation in acetate oxidation noted with oxalacetate was not regarded as a sparking effect. This observation was included in Figure 10 for comparison with the fluoroacetate data.

## II. Radioactive Tracer Studies

The initial experiment was designed to demonstrate any existing relationship between the oxidation of acetate by non-proliferating cells and the presence of free glutamic acid in the medium. Since acetate carbon was incorporated into cell protein by non-proliferating cells it appeared logical that a more extensive incorporation would result if a growth supporting medium were used. The extent of this incorporation and the sequence of the synthesis of amino acids from labeled acetate was determined with growing cells. The probability of carbon dioxide fixation by growing cells was also investigated using  $C^{14}O_2$ .

### Incorporation of acetate-1- $C^{14}$ by non-proliferating cells.

Radioactive glutamic acid was detected in the medium throughout the experiment. No other non-volatile compounds were observed. The incorporation of  $C^{14}$  into cellular protein was demonstrated with radioautographs. The main activity on the radioautographs of the protein

hydrolysate was found in glutamic and aspartic acids. These acids were identified by their respective Rf values. No other amino acids present showed significant activity.

It is interesting to note that the alcohol extract of the cells contained free glutamic acid as well as lipid material. Glutamic acid was isolated as the free acid and its purity was established by paper chromatography. The intramolecular distribution of  $C^{14}$  in the glutamic acid is given in Table 3. The conversion of acetate to glutamic acid resulted in isotopic labeling exclusively in the carboxyl groups. The ratio of activities in the gamma and alpha carboxyls approached 2 to 1, respectively.

#### Incorporation of acetate-1- $C^{14}$ by growing cells.

The salient qualitative results were the extensive incorporation and the apparent sequence of incorporation of  $C^{14}$  into the amino acids. The number of amino acids derived from acetate carbon was much greater in the protein of growing cells. As with non-proliferating cells, glutamic and aspartic acids became radioactive early in the experiment. Whereas no other amino acids were labeled in the case of non-proliferating cells, growing cells were shown to incorporate activity into other amino acids in the following sequence: glycine, serine and the

TABLE 3

Distribution of  $C^{14}$  in glutamic acid from non-proliferating cells of S. griseus utilizing  $CH_3C^{14}OOH$

Carbon atom	Radioactivity	
	c.p.m. x $10^7$ *	Per cent of total
Total	2.51	100
COOH	0.77	30.7
CHNH <sub>2</sub> } CH <sub>2</sub> } CH <sub>2</sub> }	0.02	0.8
COOH	1.72	68.5

\* Specific activity (total) is expressed as counts per minute per millimole of glutamic acid; the activities of individual carbon atoms are counts per minute per millimole of carbon.

threonine-alanine group followed by valine, proline and the leucine-isoleucine-phenylalanine group.

A radioautograph of the protein hydrolysate, further resolved with two dimensional chromatography, demonstrated that glutamic acid, aspartic acid and serine contained the highest activity at the end of 5 hours. These were followed, in order, by alanine, glycine and threonine. The faster moving group of amino acids was not further resolved. Although the sequence of synthesis of amino acids was apparent, the separation was not complete enough to permit the formulation of definite conclusions.

#### Incorporation of $C^{14}O_2$ by growing cells.

The demonstration of  $C^{14}$  in the protein fraction of S. griseus indicated the participation of carbon dioxide in protein synthesis. The protein hydrolysate was found to have a total activity of  $4.32 \times 10^5$  counts per minute. Since the total activity of the initial radioactive carbon dioxide was  $5.7 \times 10^6$ , counts per minute, this figure represented a carbon dioxide fixation of 8 per cent.

A radioautograph prepared from a two dimensional chromatogram (Figure 11) served to demonstrate the relative activities of some of the amino acids. Aspartic acid, glutamic acid, serine, glycine and threonine were radioactive.

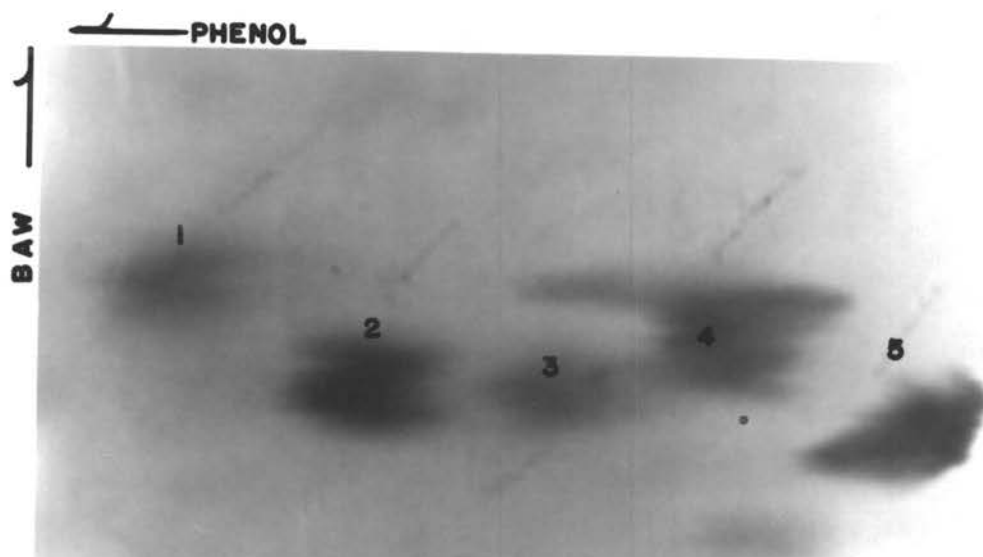


FIGURE 11

Radioautograph of a two dimensional chromatogram demonstrating the incorporation of radioactivity into amino acids during fixation of  $C^{14}O_2$ . The spots were identified as follows: (1) threonine, (2) glycine, (3) serine, (4) glutamate and (5) aspartate.

Glutamic acid and aspartic acid were separated from the protein hydrolysate. Subsequent degradation procedures gave the intramolecular distribution of radioactivity. Only the carboxyl carbons of aspartic acid were labeled (Table 4). As shown in Table 5, the alpha carboxyl carbon of glutamic acid contained all of the radioactivity.

TABLE 4

Distribution of  $C^{14}$  in aspartic acid from growing cells of S. griseus utilizing  $C^{14}O_2$  and non-radioactive pyruvate

Carbon atom	Radioactivity	
	c.p.m. x $10^5$ *	Per cent of total
Total	6.10	100
COOH	1.30	21.3
CHNH <sub>2</sub>	0.0	0.0
CH <sub>2</sub>	0.0	0.0
COOH	4.87	79.7

\* Specific activity (total) is expressed as counts per minute per millimole of glutamic acid; the activities of individual carbon atoms are counts per minute per millimole of carbon.

TABLE 5

Distribution of  $C^{14}$  in glutamic acid from growing cells of S. griseus utilizing  $C^{14}O_2$  and non-radioactive pyruvate

Carbon atom	Radioactivity	
	c.p.m. $\times 10^5$ *	Per cent of total
Total	2.79	100
Alpha carboxyl	2.68	96
Gamma carboxyl	0.11	4

\* Specific activity (total) is expressed as counts per minute per millimole of glutamic acid; the activities of individual carbon atoms are counts per minute per millimole of carbon.



## DISCUSSION

The citric acid cycle as finally proposed by Krebs (20, pp.165-199) for animal tissue has been the subject of intensive study and of considerable controversy in the field of microbial physiology. The question of the role of the aforementioned cycle in the metabolism of Streptomyces cultures has been investigated in the present study. Lines of evidence in favor of the citric acid cycle representing the principal oxidative pathway have been presented.

The reliability of the first method of proof is dependent upon the validity of the concept that a demonstration of substrate oxidation by intact cells is indicative of the organization of the enzymic complement of the cells. If this concept be valid there can be little doubt that S. griseus cells possess constitutive enzymes enabling them to utilize acetate, pyruvate, oxalacetate, alpha ketoglutarate and succinate. Only one known oxidative pathway requires the active participation of the five acids mentioned above, the citric acid cycle. It should be noted that in the case of succinate and alpha ketoglutarate recourse was made to the accepted method (24, p.31) of using high substrate concentration to overcome a limiting cellular permeability. The positive results obtained through the use of this method were

substantiated by the observation that glutamic acid is oxidized beyond the stage of alpha ketoglutarate. The slight inhibition of succinate oxidation observed with malonate requires further study.

The constitutive nature of the enzymes necessary for the oxidation of malate, fumarate and citrate is, however, not beyond question even though it has been shown that the presence of any one of these acids will cause an increase in oxygen consumption by cells of S. griseus. The question of the nature and the effects of the processes causing a limiting permeability becomes a subject of definite importance. In the case of all three of the acids, oxidation was observed only after long periods of complete inactivity. Such data are commonly interpreted as a definite indication of adaptive processes within the cell and not as manifestations of permeability. Whether or not critical significance can be attributed to the observation of adaptive lags in oxidation has been the subject of several recent publications. Barret and Kallio (3, pp.517-524) demonstrated conclusively that Krebs cycle intermediates were oxidized by way of the conventional Krebs cycle despite the fact that intact cells showed lags in oxidative activity towards the same intermediates. In the present investigation inhibition studies were employed to demonstrate that at least the

enzymes required for the synthesis and the oxidation of citric acid are part of the normal enzyme complement of S. griseus cells. Fluoroacetate itself is not toxic to oxidative processes. The inhibitory action of this acid is manifested through the fluorotricarboxylic acid formed by the condensation of oxalacetate with fluoroacetate (32, pp.310-315). The condensation product inhibits the oxidation of citric acid by competing for the enzyme, aconitase. The observed inhibition of oxidation by fluoroacetate infers that citric acid is formed and that citric acid is further oxidized. The stimulatory effect of oxalacetate on acetate oxidation, though open to several explanations, is assumed by the writer to be an expression of the requirement for a 4 carbon intermediate to overcome a rate limiting reaction.

Although the large part of the data presented thus far is strongly suggestive of the participation of the citric acid cycle in some phase of the oxidative processes, one observation in particular detracts from the finality of such a conclusion. The demonstration of glutamic acid accumulation is not a point in favor of a complete cyclic oxidation. Such an accumulation could indicate that there is a block in the cycle in the sense of a rate limiting reaction.

On the other hand, since no quantitative recovery

of glutamate was attempted, no definite relationship between acetate oxidation and glutamate accumulation can be inferred. The latter point was clarified when it was shown that radioactive glutamate accumulated during the oxidation of acetate-1-C<sup>14</sup>.

Since the exchange reactions resulting in the incorporation of radioactive carbon into cellular protein were localized in glutamic and aspartic acids the involvement of oxalacetate and alpha ketoglutarate in the oxidation of acetate is apparent. Further, from the intramolecular distribution of C<sup>14</sup> in the isolated glutamic acid (Table 3) it can be seen that the conversion of acetate to glutamate results in isotopic labeling exclusively in the carboxyl groups. Pertinent to this observation is the report of Wang, et al. (43a, pp.683-688). These workers showed that bakers yeast, grown in the presence of carboxyl labeled acetate, yielded glutamic acid with activity in the carboxyl carbons, but with twice as much activity in the gamma as in the alpha carboxyl. It was made clear that this was the ratio to be expected if glutamate was derived from alpha ketoglutarate formed from extensive operation of the tricarboxylic acid cycle. If the cycle stopped at alpha ketoglutarate the glutamate would have been labeled only in the gamma carboxyl. The extent of labeling in the alpha carboxyl indicated that

essentially all of the acetate reacted with 'recycled' oxalacetate; such extensive recycling showed that the cycle represented a major oxidative pathway rather than just a means of synthesis of glutamic acid. In the present work the gamma carboxyl contained 2.2 times the activity of the alpha carboxyl. Such distribution is almost in accord with expectations if the citric acid cycle is assumed to operate extensively. The glutamic acid used for the degradation procedures accumulated in the cell during the oxidation of acetate. Therefore, not all of the labeled acetate would react with 'recycled' oxalacetate. The cyclic activity of some of the endogenous oxalacetate would be reduced to less than one complete turn of the cycle. This is reflected in the difference between the  $C^{14}$  content of the alpha and gamma carboxyl groups of the glutamic acid. Thus the observation that the gamma carboxyl contained 2.2 times the activity of the alpha carboxyl is in accord with the operation of the tricarboxylic acid cycle.

It is significant that the tricarboxylic acid cycle participates extensively in the synthesis of the carbon skeletons of some of the amino acids during the growth of S. griseus on acetate. Most of the amino acids detected in the protein hydrolysate were radioactive. It appears significant that glutamate and aspartate should

be the first amino acids to become radioactive during the growth of S. griseus on labeled acetate. Abelson (1, pp. 335-343) has postulated that many of the other aliphatic amino acids are synthesized through glutamic and aspartic acids during the growth of E. coli. Although it appears possible that this could also be true in the case of S. griseus, further work of a more quantitative nature is necessary to establish these relationships.

The incorporation of radioactivity into alanine from carboxyl labeled acetate suggests that reactions similar to the "malic" enzyme (28a, p.979) or the Wood Werkman reaction (43b, p.135) could be operative. The removal of the beta carboxyl group from alpha, beta carboxyl labeled oxalacetate would result in carboxyl labeled pyruvate. Since the source of the four carbon condensing partner necessary for the oxidation of acetate is not known the possibility of a  $C_3-C_1$  condensation was investigated. The demonstration of the incorporation of  $Cl^{14}O_2$  into the protein fraction of S. griseus leaves no doubt that this organism possesses a reaction for the fixation of carbon dioxide. As shown in Figure 11, aspartic acid, glutamic acid, serine, glycine and threonine were radioactive. Alanine was not radioactive. The intramolecular distribution of  $Cl^{14}$  in aspartic acid (Table 4) and in glutamic acid (Table 5) was in accord



with a  $C_3-C_1$  condensation. The total activity of aspartic acid was found in the carboxyl carbons. The higher activity in the beta carboxyl carbon is in agreement with the occurrence of a  $C_3-C_1$  condensation. A partial equilibrium with a symmetrical four carbon acid such as fumaric would account for the activity on the alpha carboxyl of oxalacetate or aspartic acid (47, p.205).

The activity of glutamic acid was located almost exclusively in the alpha carboxyl carbon. This labeling is in agreement with the existence of an extensive cycling through an asymmetrical  $C_6$  compound (43a, p.687). The low activity on the gamma carboxyl carbon indicates that the reversal of a Thunberg type reaction is not occurring to any extent. This data shows that carbon dioxide enters the carbon skeletons of glutamic and aspartic acids via carbon dioxide fixation and the tricarboxylic acid cycle. The carboxylation of pyruvate leading to malate or oxalacetate (28, pp.56-106) appears to represent a major pathway of carbon dioxide utilization in S. griseus.

## SUMMARY

The oxidation of most of the tricarboxylic acid intermediates was demonstrated with intact cells of S. griseus. Limiting cellular permeability proved responsible for the lack of oxygen consumption encountered during the investigations with several intermediates of the tricarboxylic acid cycle. The constitutive nature of the enzymes required for the formation and the oxidation of citric acid was demonstrated by the inhibition of oxalacetate oxidation with fluoroacetate.

The sequence of the tricarboxylic acid cycle reactions was shown by determining the intramolecular distribution of  $C^{14}$  in glutamic acid formed during the oxidation of acetate- $1-C^{14}$ .

It has been shown that a  $C_3-C_1$  condensation coupled with tricarboxylic acid cycle activity is the major pathway of carbon dioxide fixation by S. griseus.

The intramolecular distribution of  $C^{14}$  in glutamic acid arising from the incorporation of labeled carbon dioxide further confirmed that the tricarboxylic acid cycle operates extensively in the terminal respiration of S. griseus.



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