THE INTERMEDIATE METABOLISM OF PSEUDOMONAS AERUGINOSA: A STUDY OF A NEW PATHWAY OF GLUCOSE OXIDATION

- by -

Flora Christina ~~Norris~~ Stokes

A Thesis Submitted
In Partial Fulfilment of the Requirements
for the Degree of
MASTER OF SCIENCE IN AGRICULTURE
in the
DEPARTMENT OF DAIRYING

Accepted Sept. 7, 1949

THE UNIVERSITY OF BRITISH COLUMBIA

August, 1949.

ABSTRACT

The present study was undertaken in an effort to establish the oxidative pathway of glucose degredation by P. aeruginosa. It was postulated that because this organism had no anaerobic system of metabolism a study of the path by which it utilized glucose would reveal a system of carbohydrate breakdown not previously recognized in bacteria.

A study of the endogenous respiration of the organism as an essential prerequisite to detailed studies of intermediate metabolism shows that the endogenous respiration of P. aeruginosa differs in several important respects from that reported for other organisms. The significance to be attached to the rate of endogenous respiration in interpreting data of the intermediate metabolism of micro-organisms is shown to be of great importance. The endogenous respiration of P. aeruginosa is found to function normally in the presence of substrate. Although the degree of oxidative assimilation is in agreement with figures reported in the literature, oxidative assimilation is not inhibited by usual concentrations of 2:4 dinitrophenol. Aeration of resting cells or storage at low temperatures do not reduce the endogenous respiration. Storage products of the organism as determined by R.Q. values are similar regardless of growth substrate.

Since the oxidation of glucose by P. aeruginosa is governed by an adaptive enzyme system, it was thought that

it would be a simple matter to eliminate or identify compounds as intermediates in glucose oxidation by the use of the technique of simultaneous adaption. Adaption studies with glucose-grown cells indicate that of the forty-three possible intermediates tested only gluconic acid could be an intermediate in glucose oxidation by this organism.

Acetic acid has been isolated as an intermediate compound in the oxidation of glucose or α-ketoglutaric acid by P. aeruginosa. Cells of this organism produced under conditions of intense aeration were found to have lost the ability to oxidize acetic acid and this compound was found in large quantities in the growth medium. When these cells were used in the oxidation of glucose, it was found that the oxygen consumed was the amount needed to convert glucose to acetic acid thus confirming the role of acetate as an intermediate in glucose oxidation. In contrast to the above-mentioned criterion the technique of simultaneous adaptation ruled out acetic acid as a possible intermediate in the oxidation of either glucose or α-ketoglutaric acid. The status of the theory of simultaneous adaption is discussed in relation to the data obtained.

The technique of paper chromatography has been adapted to the identification of gluconic, 2-ketogluconic and α-ketoglutaric acids as intermediates in glucose oxidation. Combinations of methyl and ethyl alcohol were found to be

the most suitable solvents and ammoniacal silver nitrate was found to give the most satisfactory reaction. When grown under normal physiological conditions where glucose was metabolized to carbon dioxide and water P. aeruginosa was shown to have oxidized glucose by way of gluconic and 2-keto-gluconic acids. Since a strong system for oxidizing both gluconic and 2-ketogluconic acids was demonstrated, the presence of these acids over at least an eight hour period of growth is taken as evidence of their importance as intermediates in the oxidation of glucose by bacteria.

This study establishes the presence of an aerobic system of glucose breakdown by P. aeruginosa which has not previously been recognized.

ACKNOWLEDGMENT

I would like to express my sincere thanks to Dr. J. J. R. Campbell for his unfailing help and interest. I wish also to thank the National Research Council for grants to carry out this study.

TABLE OF CONTENTS

Although the mechanism of carbohydrate fermentation by micro-organisms has been elucidated and it is now possible to outline the pathway of carbohydrate dissimilation which yields either lactic acid or ethyl alcohol as a complete series of reactions, very little is known of the mechanism whereby aerobic micro-organisms convert carbohydrate entirely to carbon dioxide and water. The concepts of the oxidative mechanisms which function in bacterial metabolism have arisen chiefly by analogy from scattered information of the aerobic pathway of tissue metabolism. Early theories of Thunberg and Knoop (81)(45), of Toeniessen and Brinkmann (82) and of Szent-Gyorgyi (79) on the oxidations of carbohydrate by tissue have provided the basis for the present accepted theory: the Krebs (47)(48) tricarboxylic acid cycle. According to the Krebs cycle, acetic acid condenses initially with oxalactic acid to yield citric acid which is converted through cis-aconitic acid, isocitric acid, ∝-ketoglutaric acid, succinic acid, fumaric acid, and malic acid to oxalacetic acid and the cycle will be initiated again with the condensation of more acetate. The net result of the cycle is the complete oxidation of pyruvate to carbon dioxide and water.

It is generally assumed that the mechanism of aerobic metabolism by micro-organisms proceeds via the Embden-

Meyerhof (58)(78) anaerobic scheme to the triose stage and thence through the Krebs cycle to produce carbon dioxide and water and incidentally to make available to the cells much greater amounts of energy than are liberated by an anaerobic scheme. Evidence that a portion of the Embden-Meyerhof scheme may function in aerobic metabolism was obtained by Doudoroff, Wiame, and Wolochow (28)working with thephosphorolysis of sucrose by P.putrefaciens. They found that sucrose was split to fructose plus glucose-1-phosphate and that the breakdown of the glucose portion of the molecule proceeded by way of glucose-6-phosphate and fructose-6-phosphate. First evidence of a possible tri-carboxylic acid cycle in micro-organisms was obtained from data indicating carbon dioxide fixation by bacteria (88) (89). More recent data of Ajl and Werkman (1)(2) shows that compounds which are constituents of the Krebs cycle are able to replace carbon dioxide in heterotrophic metabolism of E.coli and A.aerogenes and that these compounds do not function solely as a source of carbon dioxide. Foster (32) expressed the current conception of aerobic metabolism by micro-organisms when he stated that "in common with that of all other living systems, the dissimilation of hexose sugars by molds follows uniformly the well-known mechanism of sugar breakdown through the triose form. The end products depend on how molds

dispose of the intermediate C_3 compounds."

The assumption that the initial breakdown of carbohydrate proceeds by an anaerobic mechanism is probably justifiable in the case of facultative organisms where an anaerobic system is known to be present. However, sufficient data has appeared in the literature to question the assumption that the anaerobic mechanism is the most important means of initiating the breakdown of the carbohydrate molecule by strict aerobes. Moreover, in the case of some organisms at least, an anaerobic mechanism may not exist. Dickens (25) has postulated a system of direct oxidation of hexoses without primary conversion to trioses for yeast and muscle. According to this scheme, the hexose molecule would be phosphorylated and subsequently oxidized to phosphohexonic acid, then by dehydrogenation a phosphoketohexonic acid would be formed, and by decarboxylation a phosphopentonic acid would arise. The triose formed by a continuation of this process could conceivably be oxidized directly or enter a cyclic system of oxidation. Lipmann (50) has also suggested that phosphogluconic acid might be the first product of carbohydrate oxidation by a scheme different from fermentative breakdown. A similar system was indicated when Bernhauer and Gorlich (11) found α-ketoglutaric acid as a product of the oxidation of gluconic acid by bacteria. This

type of system would explain gluconic acid production by a great number of molds. Muller (59) has obtained an enzyme from expressed juice of Aspergillus niger which catalyzed the oxidation of glucose to gluconic acid by molecular oxygen. He found no evidence that co-enzyme was necessary. An enzyme catalyzing the oxidation of glucose to gluconic acid and requiring co-enzyme I was obtained from liver by Harrison (36). Under commercial conditions of submerged fermentation, Lockwood, Tabenkin, and Ward (52) have demonstrated the ability of cultures of Pseudomonas and Phytomonas to produce gluconic and 2-ketogluconic acids from glucose. Using similar conditions, Lockwood and Stodola (51) demonstrated a further possible step in the dissimilation of glucose when they isolated ∝-ketoglutaric acid from the fermentation of glucose, gluconate or 2-ketogluconate by P.fluorescens. Although the conditions employed by these workers were far from normal physiologically, the production of gluconic, 2-ketogluconic, and ∝-ketoglutaric acids by these organisms points to the possibility of the occurrence of a system similar to that proposed by Dickens.

A different type of evidence for the existence of an aerobic system in bacteria which does not involve the Embden-Meyerhof scheme was obtained by Barron and Friedemann (8) in their studies of the oxidation of glucose and hexose phosphates by micro-organisms which cannot ferment

glucose. Evidence of a similar nature was obtained by Barker, Shorr, and Malann (5) who reported continued respiration in tissues after inhibition of glycolysis by iodoacetate.

The present study was undertaken in an effort to establish the oxidative pathway of glucose degradation by P.aeruginosa. It was postulated that because this organism had absolutely no anaerobic system of metabolism (62) a study of the path by which it utilized glucose would reveal a system of breakdown not previously recognized in bacteria.

PART 1

THE STATUS OF THE ENDOGENOUS RESPIRATION

In studies of aerobic micro-organisms the influence of the rate of endogenous respiration is of importance in interpreting data relative to their metabolism. However, little attention has been paid to the problem of the endogenous respiration of these micro-organisms.

The most common approach to the problem of endogenous respiration is to work with cultures whose endogenous activity has been reduced to a point where it is not considered a serious interfering factor. This is usually done by devising a medium which produces cells with good metabolic activity but with practically no storage materials (87). It is not always possible to produce cells with low storage materials and other means such as aeration of the resting cell suspension or ageing at low temperatures have been employed. Quastel and Whetham (67) working with E.coli were the first to suggest the starvation technique now used. This technique consists of vigorously aerating a resting cell suspension in non-nutrient solution for 2 to 4 hours before use, thus forcing the organisms to oxidize their stored products.

Many workers report that endogenous respiration is suppressed in the presence of a readily oxidizable substrate and thus should be disregarded. Stier and Stannard (75)

working with bakers yeast, Clifton (15) with P.calco-acetica, Clifton and Logan (17) with E.coli, Doudoroff (27) with P. saccharophila, Bernstein (12) with P.saccharophila, and Barker (4) with Prototheca Zosfii concluded that endogenous respiration is completely inhibited by substrate. On the other hand, Reiner, Gest, and Kamen (68) by use of radioactive carbon obtained data indicating a stimulation of the endogenous respiration of yeast in the presence of glucose or acetate.

This portion of the investigation was undertaken to clarify the status of the endogenous respiration of the obligate aerobe, P.aeruginosa, particularly with reference to the influence of oxidizable substrate. This information is an essential prerequisite to a detailed study of the metabolism of the organism.

METHODS

Bacteriological: The culture of P.aeruginosa ATC 9027 used throughout was a typical, strongly pigmenting strain. Stock cultures of the organism were maintained in a liver extract gelatin agar of the following composition: 1.0% tryptone, 0.3% K_2HPO_4, 0.1% glucose, 0.3% glycerol, 10.0% liver extract, o.5% agar, 2.0% gelatin, pH 7.2. After growth was initiated at 30°C, cultures were refrigerated. For use in experimental work, the culture was transferred 2 to 3 times at 24 hour intervals in Sullivan's (77) medium. In order to maintain a vigorous undissociated culture a fresh transfer was taken semi-monthly from a refrigerated stock. The medium used for growing cells for Warburg experiments consisted of 0.3% $(NH_4)\ H_2PO_4$, 0.1% $MgSO_4 \cdot 7\ H_2O$, 0.3% carbon source and 0.5 p.p.m. of iron added as ferric chloride. The medium was brought to neutrality and dispensed in 100 ml. quantities in Roux flasks. After sterilization a 10% solution of K_2HPO_4 was added aseptically to a final concentration of 0.3%. The medium used for the growth curve and bacterial nitrogen curve was of similar composition but contained 0.5% glucose as carbon source. One percent of a 24 hr. culture in Sullivan's medium was used as inoculum. The cells for Warburg experiments were harvested after 18 to 24 hours incubation at 30°C, washed with half the growth volume of

0.9% saline and finally made up to the desired concentration. The concentration of cells in the washed suspension as determined by a Fisher Electrophotometer was measured before each experiment and values as bacterial nitrogen were calculated from a standard curve.

A conventional Warburg apparatus (83) was used to follow the oxygen uptake and carbon dioxide production of the cell suspensions. In studies of the influence of inhibiting substances on oxygen uptake, the cells were incubated 20 minutes with the inhibitor prior to the addition of substrate from the side-arm. All Warburg experiments were run at 31°C. The reaction mixture in the cups unless otherwise indicated was pH 7.2.

Cells subjected to the aeration technique were prepared as described above except that aseptic technique was observed throughout. The concentrated cells were aerated vigorously at room temperature while cells which had remained undisturbed at room temperature served as a control. At specified intervals samples of aerated and control cells were removed and their respective respiratory rates determined.

Chemical: Bacterial nitrogen was determined by the micro-Kjeldahl method. One ml. sample of cell suspension, 1 ml. conc. H_2SO_4 and 2 drops 30% H_2O_2 were digested 15 to 30 minutes over a micro-burner. The colorless sample was cooled, and diluted with 6 ml. water. Ten ml. carbon

dioxide-free 10 N NaOH were added and the sample distilled in a micro-Kjeldahl distillation apparatus using steam. Thirty ml. of distillate was collected in 20 ml. N/100 HCl. Excess acid was back titrated with N/100 NaOH using methyl red indicator.

EXPERIMENTAL

Growth Curve: To determine the optimum age of cells for further studies, the growth curve of P.aeruginosa was followed. Two flasks of glucose ammonium phosphate medium were inoculated and incubated at 30°C. Platings using nutrient agar were made of samples removed from the duplicate flasks at 0,2,4,6,8,10,12,15,18,21,24,34, and 40 hours and the plates incubated at 30°C. Average counts of duplicate plates are recorded in Table I.

Table I

Growth Curve of P.aeruginosa.

Time in Hours	Organisms / ml.
0	5×10^6
2	3.6×10^6
4	6×10^6
6	24×10^6
8	64×10^6
10	89×10^6
12	$1,000 \times 10^6$
15	$1,300 \times 10^6$
18	$4,000 \times 10^6$
21	$40,000 \times 10^6$
24	$55,000 \times 10^6$
34	$70,000 \times 10^6$
40	$74,000 \times 10^6$

From Table I it can be seen that P. aeruginosa has a 4 hour lag period and that the peak of the growth curve is obtained at 18 to 24 hours. The maximum number of viable organisms was 74,000,000,000 per ml.

Nitrogen Curve for the Calculation of $QO_2(N)$: Varying volumes of washed 24 hour glucose ammonium phosphate cells re-suspended at 20 times growth concentration were diluted to 10 ml. and the % light transmission determined on duplicate samples using a Fisher Electrophotometer. Nitrogen values on the suspensions were determined by the micro-Kjeldahl method. Figure I shows the % light transmission plotted against the bacterial nitrogen content which has been calculated as mg. N/cup. (If 0.2 ml. cell suspension is diluted to 10 ml. and the % light transmission determined, then the nitrogen value determined from the curve will correspond to the nitrogen content in a Warburg cup containing 0.5 ml. cell suspension.) The $QO_2(N)$ can then be calculated readily.

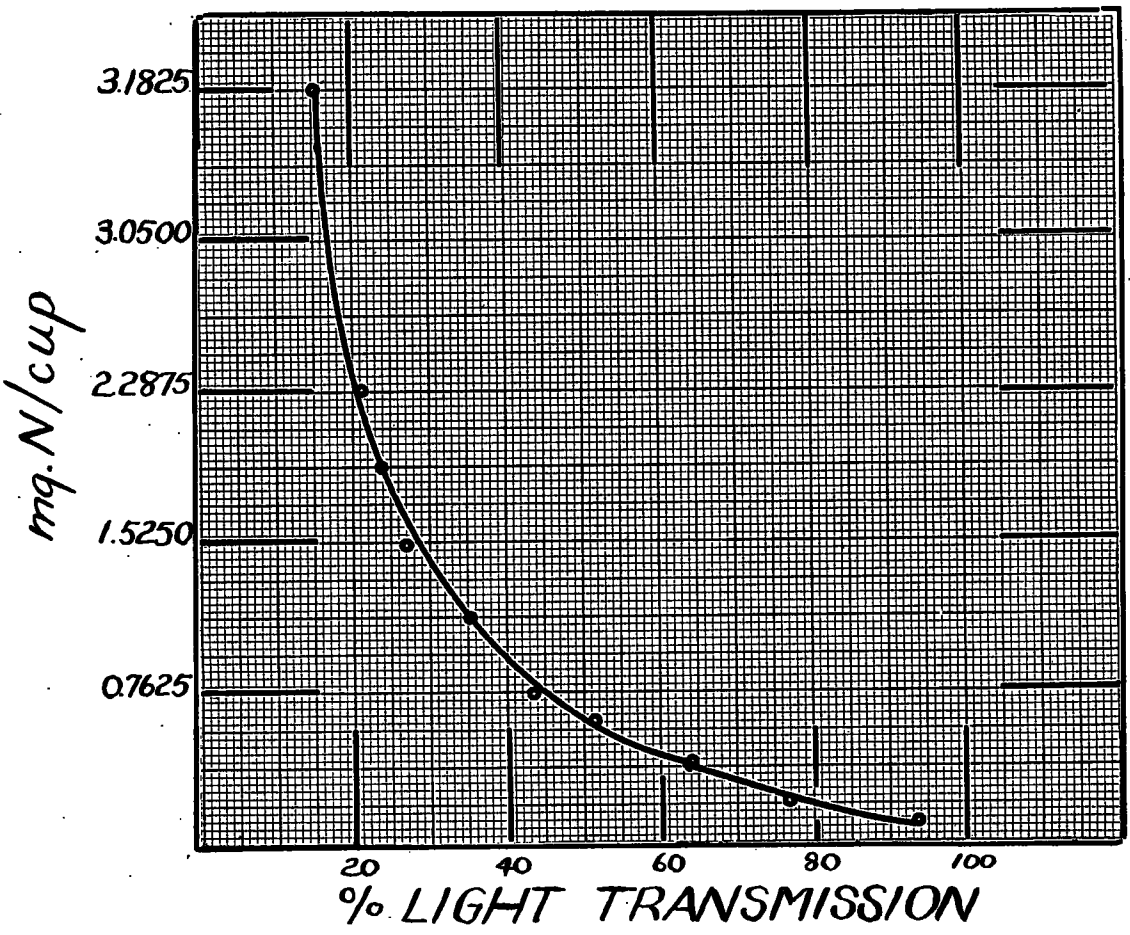

Figure I: Nitrogen curve for the calculation of QO_2 (N).

Nature of Storage Products: In order to determine the nature of the storage products of P. aeruginosa, R.Q. values were obtained using heavy suspensions of cells which had been grown with glucose, ammonium succinate, or sodium acetate as the sole source of carbon. Glucose and succinate cells were harvested at 24 hours and brought to 20 times growth concentration while acetate cells were harvested at 24 and 49 hours and brought to 40 times growth concentration. Cell suspensions of essentially the same turbidity were obtained in all cases. From Table II it can be seen that identical R. Q. values were obtained in all cases, indicating that the storage product of the cells is the same regardless of growth substrate or age and is a compound that is slightly oxidized.

Table II

R.Q. of Cell Suspensions in the Absence of Substrate.

Growth Substrate	Age of cells Hours	R.Q.
glucose	24	1.11
succinate	24	1.12
Acetate	24	1.13
acetate	49	1.11

Influence of Substrate: In order to determine the influence of concentration of substrate on the endogenous respiration, cells were suspended at 20 times growth concentration and allowed to oxidize 2,3,4, and 5 uM of glucose for 70 minutes in the Warburg apparatus. These concentrations require 12,18,24, and 30 uM oxygen respectively for complete oxidation. A similar experiment was set up using 6,9,12, and 15 uM of acetate (equivalent to 12, 18, 24, and 30 uM oxygen). If the endogenous respiration is disregarded, there is a variation of 40% in the degree of oxidation of a given substrate depending upon its concentration. And with the lower concentrations of substrate, the oxygen uptake is greater than is theoretically possible. If, on the other hand, the endogenous respiration is considered to function normally in the presence of substrate and the oxygen uptake due to substrate dissimilation is determined by subtracting the endogenous oxygen uptake from the total uptake, the degree of oxidation is found to be comparable for all substrate concentrations. Moreover, the extent of oxidation is in agreement with the data reported in the literature for related organisms. According to the concept of oxidative assimilation proposed by Barker (4) and Clifton (15), micro-organisms oxidize a portion of the energy source to carbon dioxide and water and assimilate the remainder

Table III

Oxidations of Glucose-Grown Cells on Varying Concentrations of Glucose and Acetate.

Warburg Substrate	conc.in cups uM.	O_2 uptake ul.	theoretical uptake ul.	% theoretical	uptake minus endogenous ul.	% theoretical
	nil	152				
x	2	336	270	124	184	68
glucose	3	425	402	106	273	68
	4	505	535	92	353	66
	5	565	672	84	413	61
	nil	153				
⁂	6	354	270	131	201	74.5
acetate	9	453	402	113	300	75
	12	530	535	99	377	70.5
	15	625	672	93	470	70

x 22 Hour cells.

⁂ 26 Hour cells.

directly into cellular material. The usual reported figures are approximately two-thirds oxidation and one-third assimilation which is the ratio obtained with P.aeruginosa if oxygen uptake due to endogenous respiration is subtracted from the total, Table III.

In order to determine the influence of concentration of cells on amount of oxygen taken up in the presence of a constant amount of substrate, cells harvested from glucose medium were re-suspended at 10,20, and 40 times growth concentration. The activity of the cell suspensions was determined in the Warburg respirometer with 5 uM glucose as substrate. If the endogenous respiration has been repressed, the same total oxygen uptake should be obtained in all cases since the same amount of substrate is being oxidized by each of the cell suspensions. The results recorded in Table IV show, however, that the total oxygen uptake increases proportionately with an increase of endogenous respiration. If, on the other hand, the endogenous respiration is subtracted, the percentage oxidation is found to be relatively constant in all cases, Table IV. The lower value obtained with cells concentrated 10 times may be explained by the fact that the oxygen uptake for these cells had not leveled off at 80 minutes.

Table IV

Oxidation of 5 uM Glucose by

Cell Suspensions of Varying Concentration.

Concentration of Cells	O_2 uptake endogenous ul.	O_2 uptake 5 uM glucose ul.	O_2 uptake glucose minus endogenous ul.	percent of theoretical uptake
10 x	37	420	383	57
20 x	95	540	445	66
40 x	200	645	445	66

The data in Tables III and IV show conclusively that the endogenous respiration of P.aeruginosa is unaffected by the presence of oxidizable substrate. It follows that in determining the oxygen uptake due to the oxidation of a given substrate, the endogenous respiration must be subtracted from the total uptake in order to obtain an accurate value.

Influence of Aeration and Storage: In studies on the influence of aeration and storage on endogenous respiration, 24 hour glucose-grown cells were resuspended in saline at 10 times growth concentration and the suspension divided into two portions. One portion of the suspension was aerated vigorously at room temperature while the other half, serving as control, was allowed to stand undisturbed. Samples of aerated cells were removed at 2, 5, and 8 1/2 hours while samples of control cells were removed at 0, 2, 5, and 8 1/2 hours. The endogenous activity and the ability to oxidize glucose were determined. The purity of suspensions was checked by Gram stain at the conclusion of the experiment.

Since results similar to those obtained after 8 1/2 hours aeration were also obtained at 2 and 5 hours, curves depicting the results for only 0 and 8 1/2 hours are recorded in Figure II.

From these data it can be seen that there is no significant decrease in the endogenous respiration nor is there any appreciable decrease in the ability of the cells to oxidize glucose after 8 1/2 hours aeration. The slight decrease in activity at 8 1/2 hours cannot be attributed to aeration since control cells showed a similar slight decrease.

Since the use of aeration is a recognized procedure for

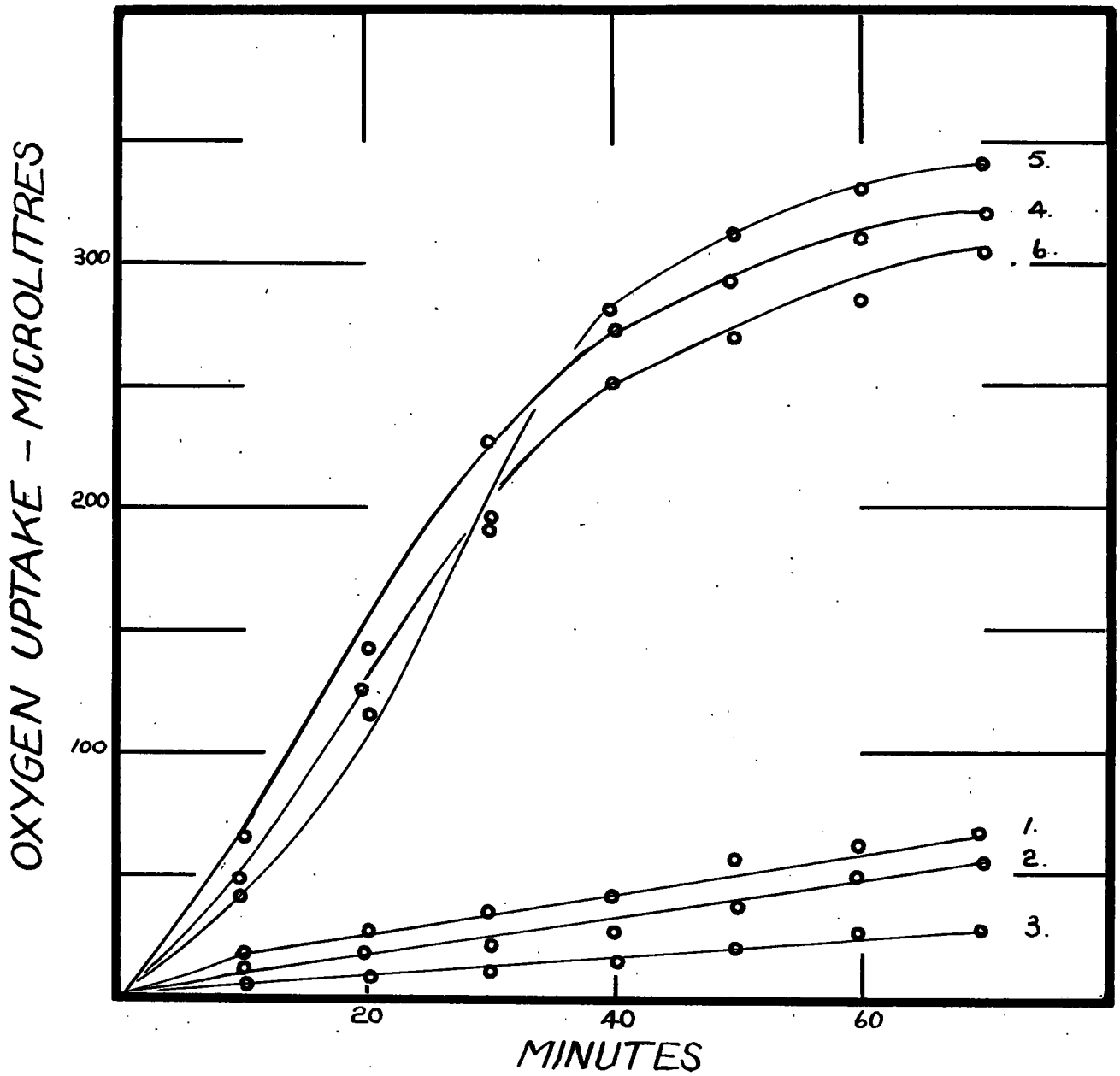

Figure II: Influence of aeration on resting cell suspension of glucose-grown cells.
1. endogenous, 0 hr; 2. endogenous, 8 1/2 hr. aeration; 3. endogenous, 8 1/2 hr. at room temperature; 4. glucose, 0 hr; 5. glucose, 8 1/2 hrs. aeration; 6. glucose, 8 1/2 hr. at room temperature.
Theoretical uptake for substrate = 402 ul.O_2.

reducing the endogenous respiration of micro-organisms, the inability of this technique to affect the autorespiration of P.aeruginosa indicates that there may be something unique about the storage products of the organism.

Additional data on the influence of storage conditions were obtained when a cell suspension was stored in the refrigerator and samples removed daily for study. The endogenous respiration and ability to oxidize glucose were determined. Results recorded in Table V show that after 4 days storage there was no change in the activity of the cells.

Table V

Storage of Cells.

Time of Storage	O_2 uptake by endogenous at 70 minutes ul.	O_2 uptake for 3 uM glucose at 70 minutes ul.
0 days	60	290
1 day	53	310
2 days	57	294
3 days	53	312
4 days	57	285

Inhibition of Synthesis: Since P.aeruginosa appears to be exceptional in its behaviour when aerated and since it has been shown to possess a mechanism for the oxidative assimilation of substrates, it was considered of value to determine the action of compounds which inhibit synthetic processes.

Sodium azide and 2, 4 dinitrophenol have been used extensively as inhibitors of oxidative assimilation. Solutions of sodium azide at final concentrations of M/200, M/400, and M/1000 and 2, 4 dinitrophenol at M/1000, M/1500, M/2000, M/2500, M/4000, and M/8000 were used in Warburg experiments with 3 uM glucose as substrate. The presence of dinitrophenol produced no inhibition of oxidative assimilation in any of the concentrations used. M/1000 azide, however, increased the oxidation from 64% to 90% of the theoretical value. In the calculation of these results, the respective endogenous respiration values were subtracted from those obtained in the presence of glucose. Higher concentrations of azide inhibited both rate and total amount of oxygen uptake. Oxygen uptake in the presence and absence of M/1000 sodium azide is shown in Figure III.

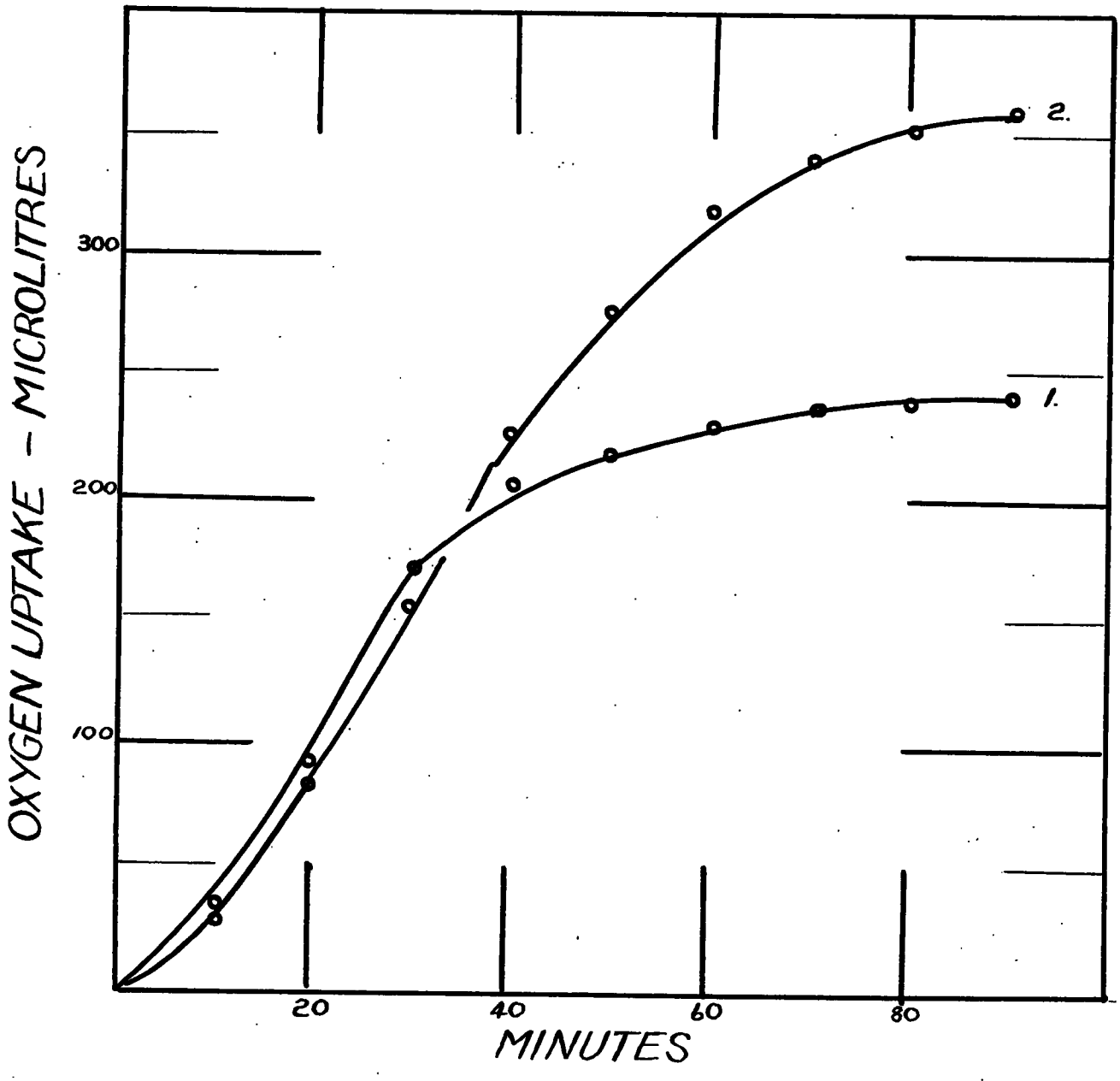

Figure: III — Inhibition of synthesis by Na N_3.
1.glucose; 2.glucose + M/1000 Na N_3.
Theoretical uptake for substrate = 402 ul.O_2.

Influence of Suspending Fluid and pH of Reactions Mixture in Warburg Cups: To determine the influence of suspending fluid used in the preparation of resting cells on the oxidations of P. aerugenosa, washed 26 hour cells were suspended at, 20 times growth concentration in distilled water, 0.85% saline, or M/15 Sorenson's phosphate buffer. These cells suspensions were allowed to oxidize 3 uM glucose for 60 minutes in the Warburg. Results recorded in Table VI show that oxygen uptake is constant regardless of suspending fluid.

Table VI

Influence of Suspending Fluid.

Suspending Fluid	O_2 uptake endogenous ul.	O_2 uptake 3 uM glucose ul.	O_2 uptake endogenous subtracted ul.
dist. water	109	390	281
0.85% saline	112	386	273
M/15 phosphate buffer	124	390	266

To determine if slight variations in the pH of the reaction mixture would materially affect oxygen uptake by the organism, the oxidation of 3 uM glucose by cell suspensions of a 22 hour culture was tested at pH 6.5, 7.2, 8.3, and 9.2. The uptake at 80 minutes, recorded in Table VII shows

that a variation in pH between 6.5 and 9.2 has little effect on total oxygen uptake. However, a slight period of adaptation was required for the oxidation of glucose at pH 8.3 and 9.2 while no adaptation period was required at pH 6.5 and 7.2.

Table VII

Influence of pH in Warburg Reaction Mixture.

pH of cup contents	O_2 uptake endogenous ul.	O_2 uptake 3 uM glucose ul.	O_2 uptake 3 uM glucose endog. subtr. ul.
6.5	116	386	270
7.2	145	440	295
8.3	175	442	267
9.2	152	425	273

DISCUSSION

From this study it is evident that one cannot make generalizations regarding the endogenous respiration of micro-organisms since the endogenous respiration of P. aeruginosa has been shown to differ in several important respects from that reported for other organisms. Although data obtained using azide as an inhibitor of oxidative assimilation agree with those reported in the literature for other organisms, 2, 4 dinitrophenol, the most common reagent employed for this purpose, failed to inhibit the oxidative assimilation of P. aeruginosa. It was also found that the endogenous respiration of this organism could not be reduced by either vigorous aeration or storage of the organism at low temperatures, indicating a possible peculiarity in the nature of the storage products elaborated or the elimination by this technique of certain enzymes associated with the utilization of this storage product. The conclusion that the endogenous respiration functions normally in the presence of added substrate, and thus should be subtracted from the values obtained in the presence of substrate in calculations of the degree of oxidation of the substrate, contradicts the practice followed by most workers.

It is possible that the type of endogenous respiration exhibited by P. aeruginosa is common to organisms which do not

have a constitutive enzyme system for oxidizing glucose (62). The lack of such enzymes indicated that the storage products are not oxidized via glucose or closely related compounds. The enzymes required for the oxidation of stored products thus would not compete with those involved in the oxidation of the substrate.

Much of the confusion and contradictory results reported in the literature may be explained by the fact that there is considerable variation in the nature of the endogenous respiration of various micro-organisms. The significance that is to be attached to the rate of endogenous respiration in interpreting data on the intermediary metabolism of micro-organisms is thus of paramount importance.

SUMMARY

The significance to be attached to the rate of endogenous respiration in interpreting data of the intermediary metabolism of micro-organisms is shown to be of great importance.

The endogenous respiration of P.aeruginosa is shown to function normally in the presence of added substrate.

Although the degree of oxidative assimilation of glucose is in agreement with figures reported in the literature, oxidative assimilation is not inhibited by usual concentrations of 2, 4 dinitrophenol. Sodium azide does inhibit oxidative assimilation, however. Aeration of resting cells or storage at low temperatures do not reduce the endogenous respiration.

Storage products of the organism as determined by R.Q. studies are similar regardless of growth substrate.

Variation in the pH of Warburg reaction mixtures does not influence oxygen uptake, nor does suspending fluid used for resting cell suspension.

The growth curve of P.aeruginosa from 0 to 40 hours on glucose ammonium phosphate is shown.

Annitrogen curve relating density of cell suspensions to bacterial nitrogen content is given to facilitate rapid calculations of $QO_2(N)$ values.

PART II

WARBURG STUDIES ON INTERMEDIATE METABOLISM

The intermediate compounds formed during the oxidation of carbohydrates by an organism such as P.aeruginosa normally do not accumulate during growth but are oxidized to the end products carbon dioxide and water. Thus it is not possible to isolate and identify compounds in the manner employed in anaerobic studies. Moreover, the use of inhibitors to block the oxidation of compounds is not justifiable in studies where the mechanism of dissimilation is completely unknown for the products accumulating in the presence of inhibitors may arise from reactions which do not function in the absence of the inhibitor. The same argument may be used in criticizing the use of vigorous aeration as a technique for causing the accumulation of intermediate compounds since the enzyme systems are damaged by these conditions (63).

The routine procedure employed to avoid unphysiological conditions and interfering growth reactions is the use of manometric techniques (26) with resting cell suspensions (67). Concentrated washed cells, at the peak of their metabolic activity are suspended in non-nutrient solution. These cells, deprived of essential nutrients, are unable to multiply but are endowed with active enzyme systems normally

present in growing cells. By the use of the Warburg respirometer, the ability of the cells to attack the parent substrate and probable intermediate compounds is determined by measuring relative rates and amounts of oxygen uptake and carbon dioxide liberation. A more specific method of determining whether or not a compound is an intermediate in carbohydrate oxidation is suggested by Stanier's (73) simultaneous adaption technique. According to this theory, cells grown on a substrate which is dissimilated by an adaptive enzyme system should be adapted simultaneously to oxidize all the intermediates formed during the oxidation of the parent substrate. Stanier summarized his argument in the following three postulates:

"(1) If the dissimilation of a given substance A proceeds through a series of intermediates B,C,D,E,F,G,---, and if the individual steps in this chain of reactions are under adaptive enzymatic control, then growth on a medium that contains A will produce cells that are simultaneously adapted to B,C,D,E,F,G,---.

(2) If growth on A fails to adapt the cells to a postulated intermediate X, then X cannot be a member of the reaction chain.

(3) Growth on E will adapt the cells for F,G,--- but not necessarily for A,B,C, and D. The probability that the growth on E will adapt the cells to precursors decreases

with the number of intervening steps; i.e., the adaption to D is more probable than adaption to A."

Since P. aeruginosa oxidizes glucose by an adaptive enzyme system (62) it was thought that it would be a simple matter to eliminate or identify compounds as intermediates in glucose oxidation by the use of Stanier's technique.

The following section consists of a study of the oxidation of glucose and probable intermediate compounds by cells adapted by previous growth with glucose or the probable intermediate as the sole carbon source. A survey of the oxidation of 43 possible intermediate compounds by glucose-grown cells was also carried out.

METHODS

Bacteriological: The methods of maintaining stock cultures were similar to those described in Part I. Growth medium for the preparation of resting cell suspensions was similar to that used before but the carbon sources at a final concentration of 0.5% were added aseptically after sterilization through sintered glass. Resting cell suspensions of 18 - 24 hour cultures were prepared as before. A conventional Warburg apparatus was used to measure oxygen uptake. Warburg cups contained 1.5 ml. Sorenson's phosphate buffer pH 7.2 and 0.5 ml. cell suspension in the main compartment. The substrate to be oxidized and any supplements were tipped in from the side-arm after equilibration. The centre well contained 0.15 ml. of 20% KOH for the absorption of carbon dioxide. Total volume of fluid in the cups was 3.15 ml. All substrates were neutralized where necessary and added at such a concentration that 0.2 ml. substrate was equivalent to 18 uM oxygen. In experiments where supplements were added, the final concentrations of Tween 40 (Batch 520B) were 0.01% and 0.001% and the final concentration of adenosine triphosphate was 0.001M.

Chemical: Calcium gluconate was a purified sample prepared from technical gluconic acid. Sodium pyruvate was prepared from pyruvic acid by the method of Robertson (69). A

sample of calcium 2-ketogluconate was obtained from Dr. L. B. Lockwood, Northern Regional Research Laboratories.

EXPERIMENTAL

Adaptation Studies with Glucose-, Gluconate-, 2-ketogluconate-, ∝-ketoglutarate-, Pyruvate-, and Acetate-Grown Cells.

Gluconic acid, 2-ketogluconic acid, ∝-ketoglutaric acid, pyruvic acid, and acetic acid are considered the most probable intermediate compounds in glucose oxidation by P.aeruginosa. Aeration studies by Lockwood, Tabenkin, and Ward (52) indicated that members of the genus Pseudomonas are able to produce gluconic and 2-ketogluconic acid from glucose. The presence of 2-ketogluconic acid has been shown in the supernatant of glucose-grown cells of P.aeruginosa ATC9027 (62). Isolation of ∝-ketoglutaric acid from submerged fermentations of glucose and gluconic acid by P.fluorescens (51) indicated that this acid was an intermediate compound. Since it is difficult to imagine an aerobic system of metabolism which does not involve pyruvic acid and acetic acid, these two compounds were also selected for study..

Adaptive enzymes are formed in response to the presence of small amounts of substrate. Consequently any decomposition of the carbon source during sterilization of the growth medium would produce cells adapted to the decomposition products as well as to intermediate compounds formed in substrate dissimilation. Because carbohydrates

are known to decompose slightly when they are autoclaved in the presence of phosphates, all carbon sources for adaptation studies were sterilized separately by passage through sintered glass.

Cells were grown with glucose, calcium gluconate, calcium 2-ketogluconate, α-ketoglutaric acid, sodium pyruvate or sodium acetate as the sole source of carbon. The resting cell suspensions from the various growth media were allowed to oxidize glucose, calcium gluconate, calcium 2-ketogluconate, α-ketoglutaric acid, sodium succinate, sodium pyruvate, and sodium acetate. The oxidation curves are shown in Figures IV to IX. Endogenous respiration values have been subtracted in all cases.

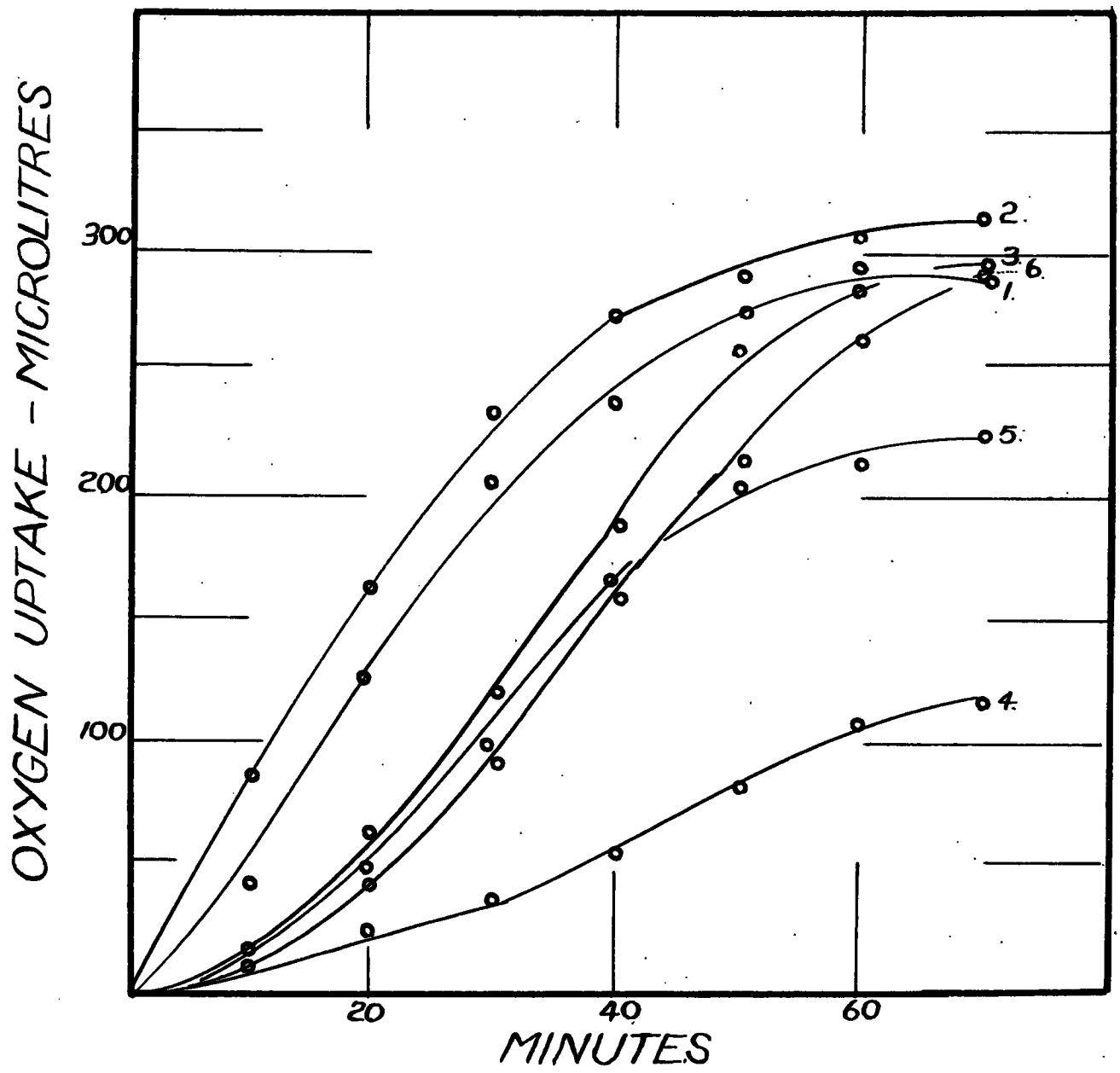

Figure IV: Oxidation of glucose-grown cells.
1. glucose; 2. calcium gluconate; 3. calcium 2-ketogluconate; 4. α-ketoglutaric acid; 5. sodium pyruvate; 6. sodium acetate.
Theoretical uptake for all substrates = 402 ul. O_2.

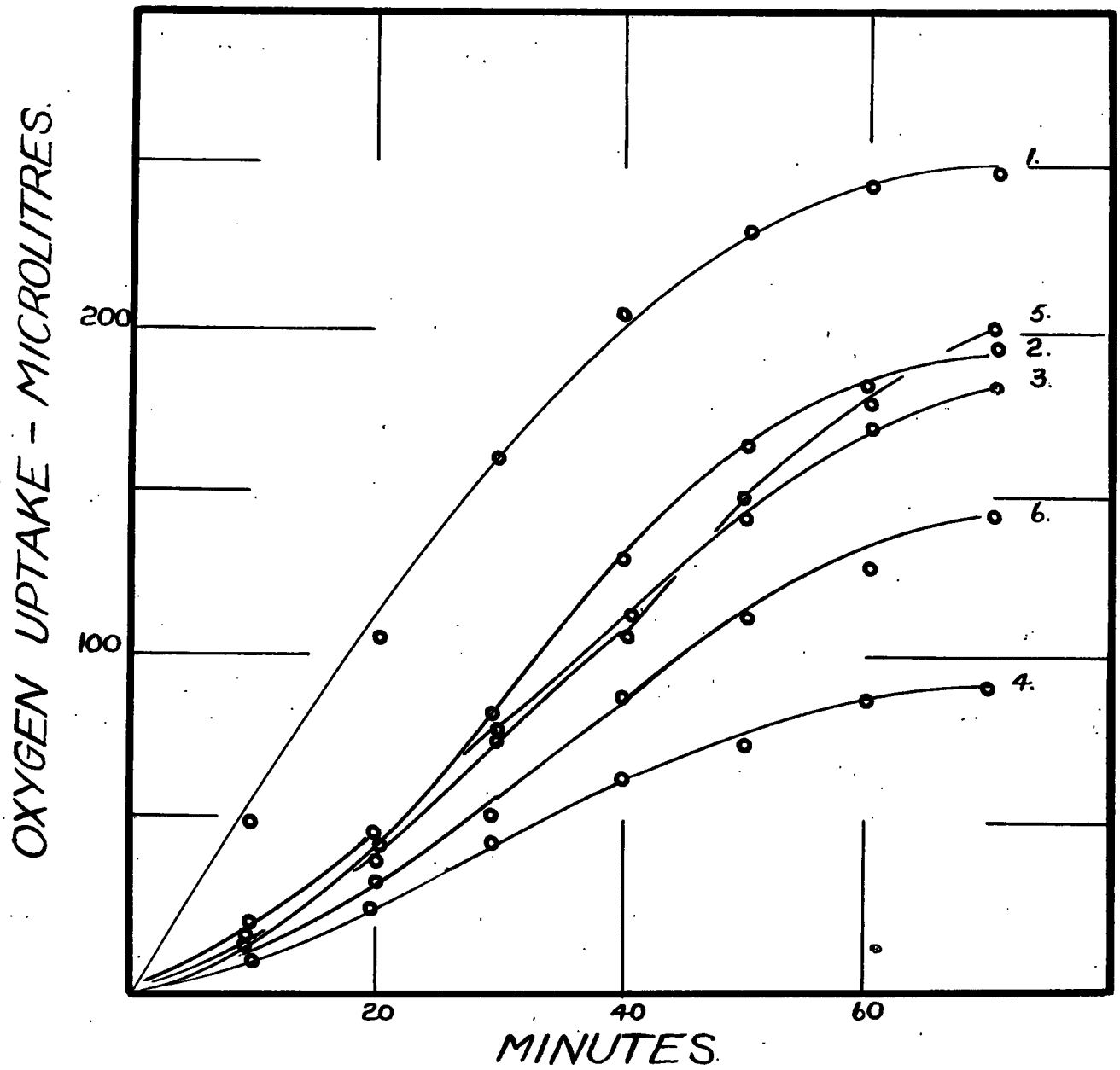

Figure V: Oxidations of calcium gluconate-grown cells. 1. calcium gluconate; 2. glucose; 3. calcium 2-ketogluconate; 4. α-ketoglutaric acid; 5. sodium pyruvate; 6. sodium acetate. Theoretical uptake for all substrates = 402 ul.O_2.

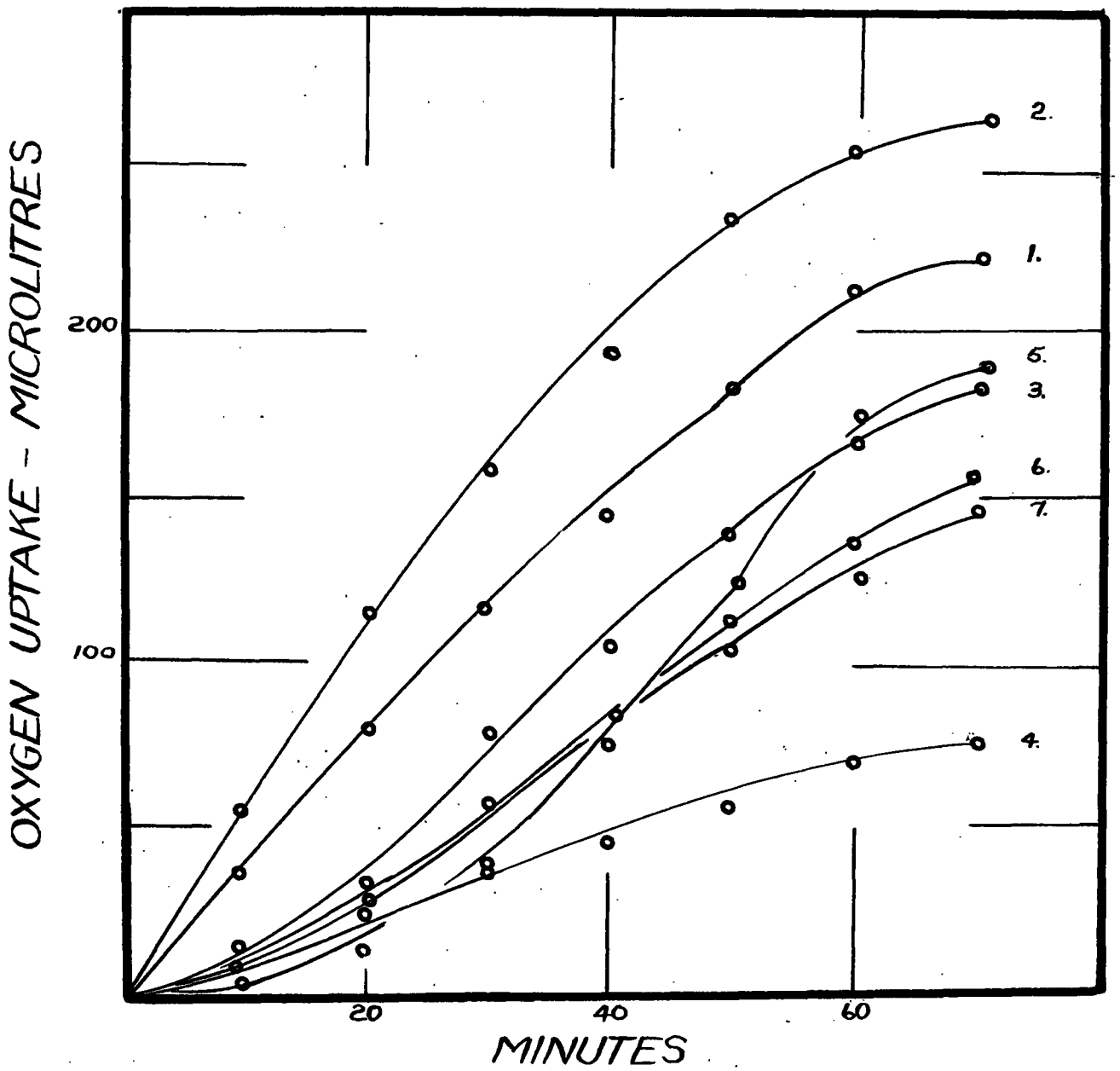

Figure VI: Oxidation of calcium 2-ketogluconate-grown cells. 1.calcium 2-ketogluconate; 2.calcium gluconate; 3.glucose; 4. α-ketoglutaric acid; 5. sodium succinate; 6. sodium pyruvate; 7. sodium acetate. Theoretical uptake for all substrates = 402 ul.O_2.

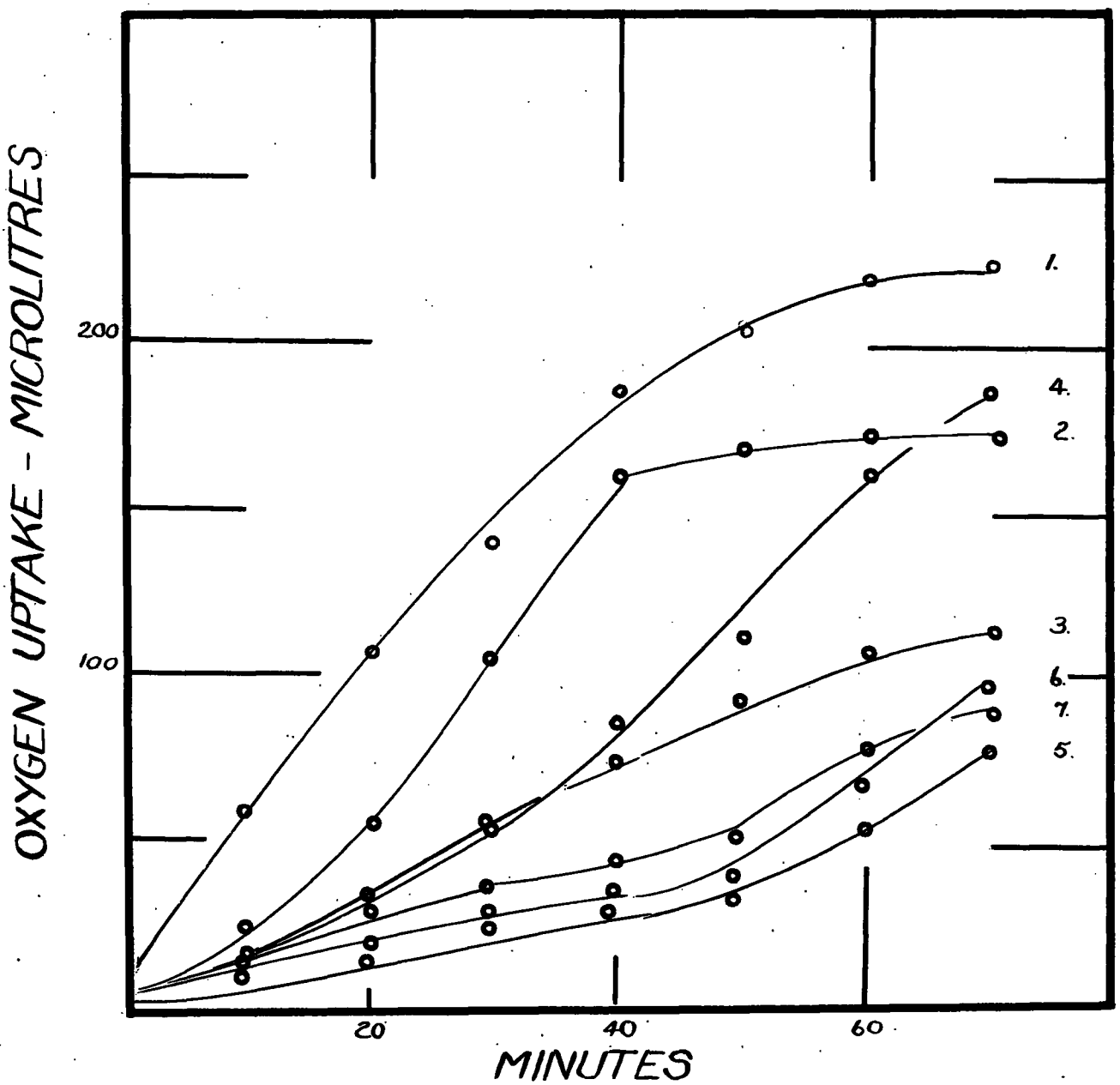

Figure VII: Oxidations of α-ketoglutarate-grown cells.
1. α-ketoglutaric acid; 2. sodium succinate;
3. sodium pyruvate; 4. sodium acetate; 5. glucose;
6. calcium gluconate; 7. calcium 2-ketogluconate.
Theoretical uptake for all substrates = 402 ul. O_2.

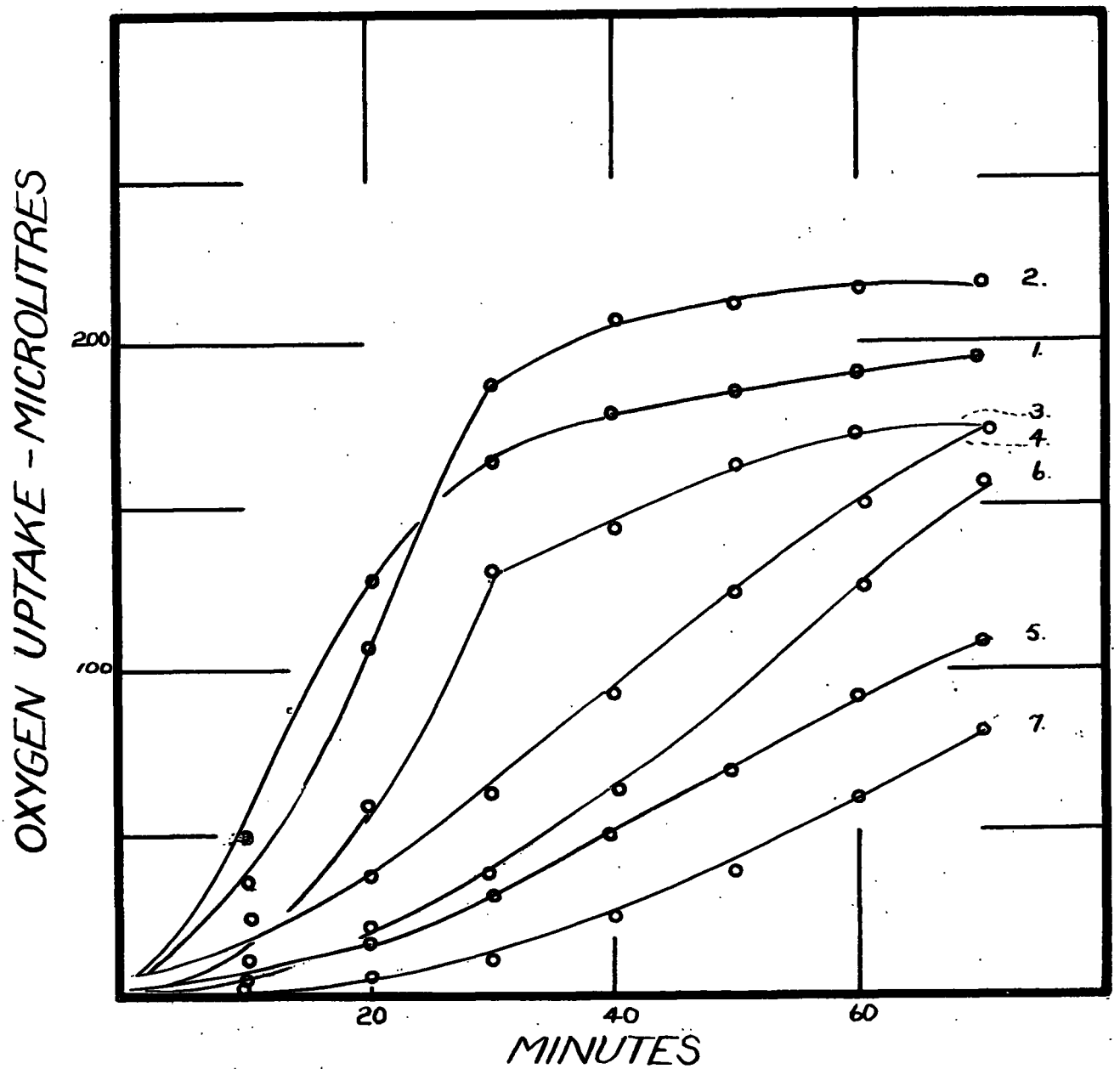

Figure VIII: Oxidations of sodium pyruvate-grown cells. 1.sodium pyruvate;2.sodium acetate; 3. sodium succinate; 4.α-ketoglutaric acid; 5.glucose; 6.calcium gluconate; 7.calcium 2-ketogluconate.
Theoretical uptake for all substrates=402 ul.O_2.

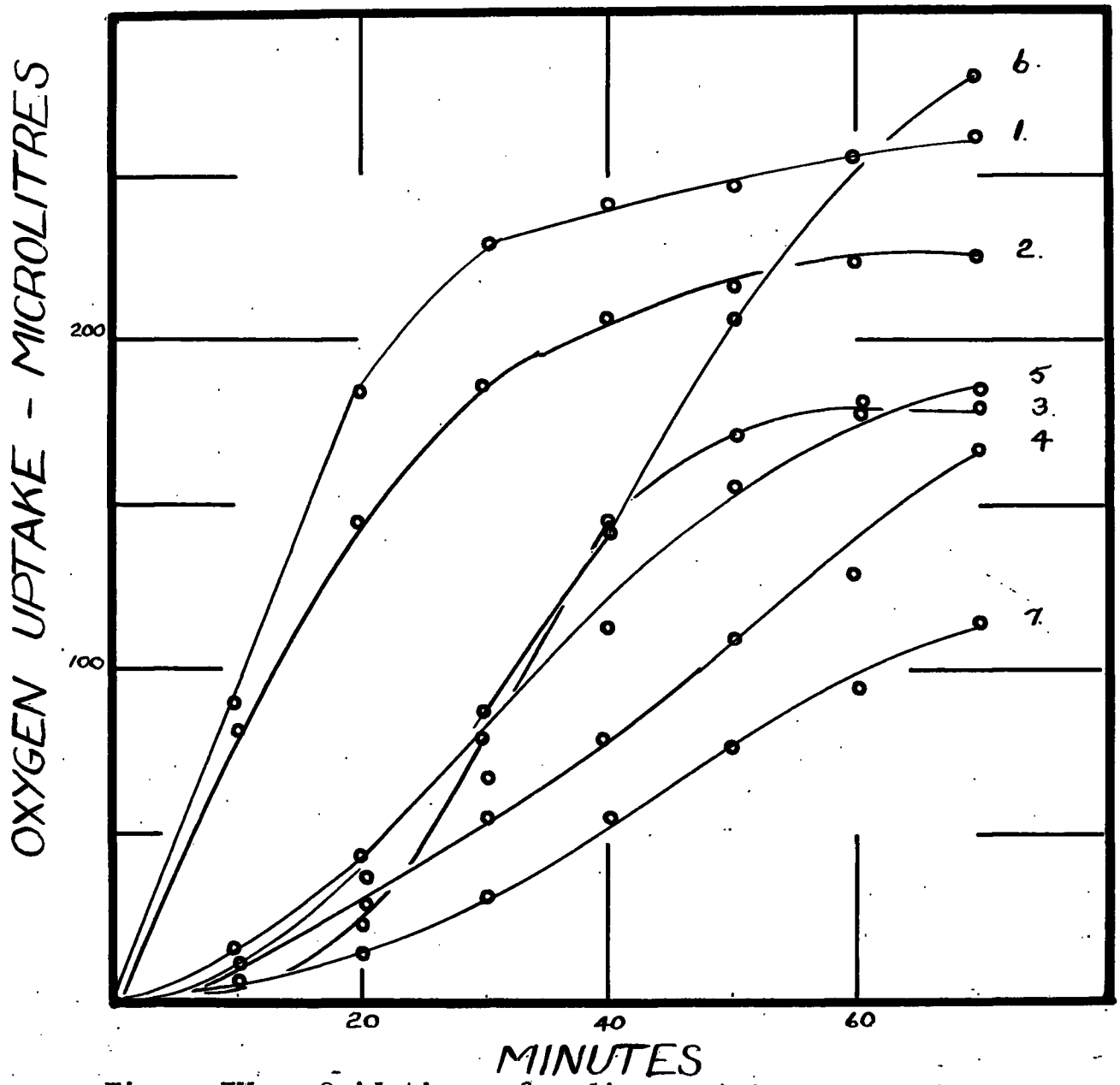

Figure IX: Oxidations of sodium acetate-grown cells. 1.sodium acetate; 2.sodium pyruvate; 3. sodium succinate; 4.α-ketoglutaric acid; 5.glucose;6.calcium gluconate; 7. calcium 2-ketogluconate.
Theoretical uptake for all substrates =402 ul.O_2.

If simultaneous adaptation is the criterion used to determine whether or not a compound is an intermediate in carbohydrate oxidation, then one must conclude that of the postulated intermediate compounds only gluconic acid is an intermediate in glucose oxidation; only gluconic acid is an intermediate in 2-ketogluconic acid oxidation; only acetic acid is an intermediate in pyruvic acid oxidation and only pyruvic acid is an intermediate in acetic acid oxidation. One must also conclude that none of the compounds tested are intermediate in gluconic acid or α-ketoglutaric acid oxidation. Figures IV to IX. In view of these conclusions it would appear that the criterion employed is not applicable to this study possibly because some factor such as permeability is responsible for the period of adaptation required before most compounds are oxidized. For example, one must conclude that the oxidation of α-ketoglutaric acid does not involve succinic acid, pyruvic acid or acetic acid as intermediate compounds since a 20 to 40 minute period of adaptation is required before these compounds are attacked by α-ketoglutarate grown cells. Figure VII. But it does not seem possible that oxidation of this compound does not involve succinic acid, pyruvic acid or acetic acid. Nor does it seem possible that glucose oxidation does not involve pyruvic or acetic acids as intermediate compounds although simultaneous

adaptation excludes these acids as intermediates. Figure IV.

Permeability factors could be responsible for the adaptative nature of the curves of pyruvate and acetate oxidation by glucose-grown cells of P. aeruginosa. If the cell membrane were relatively impermeable to strongly ionized compounds such as these, then a short period of time would be required for the compound to cross. In the case where an adaptation period is due to permeability factors, the presence of a surface active agent to increase cellular permeability or the presence of a small amount of readily available energy to aid the compound in crossing the cell membrane might allow the cells to attack the compounds immediately and at maximum rate.

To test the influence of a surface active agent on the oxidation of sodium pyruvate and sodium acetate by glucose-grown cells, a suspension of organisms grown for 22 hours on glucose ammonium phosphate medium was allowed to oxidize pyruvate and acetate in the presence of 0.01% and 0.001% Tween 40. Similar results were obtained with both concentrations of Tween. Only curves showing oxidations in the presence of 0.001% Tween are recorded in Figure X. In curves depicting the oxidation of glucose, pyruvate, and acetate, the endogenous values have been subtracted and in curves depicting oxidation of pyruvate plus 0.001% Tween and acetate plus 0.001% Tween, the values for oxidation of 0.001% Tween alone have been subtracted.

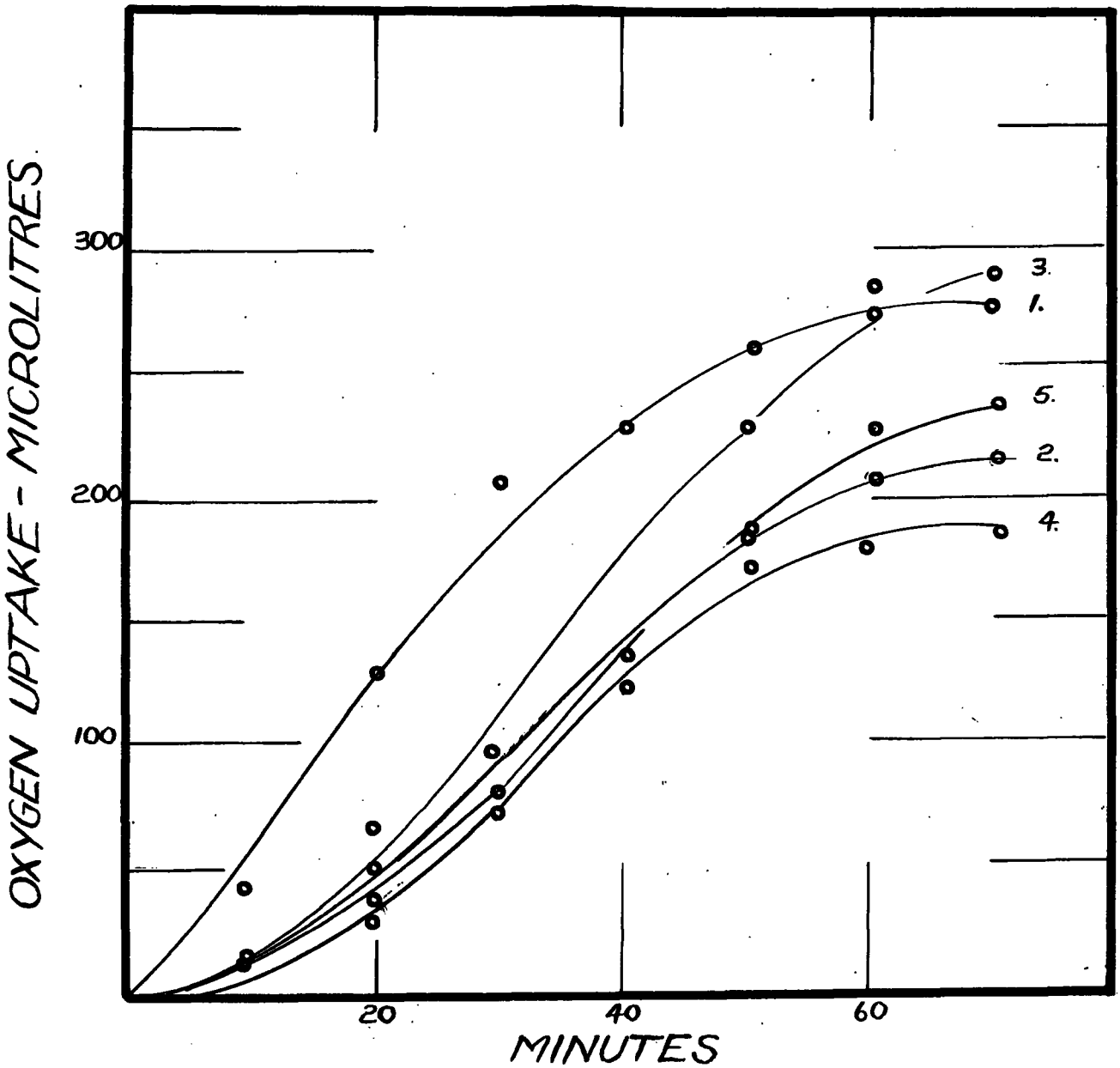

Figure X: Influence of a surface active agent on pyruvate and acetate oxidation by glucose-grown cells.
1.glucose;2.sodium pyruvate;3.sodium acetate;
4.sodium pyruvate + 0.001% Tween 40;5.sodium acetate + 0.001% Tween 40.
Theoretical uptake for all substrates = 402 ul.O_2.

From the results recorded in Figure X it can be seen that the presence of a surface active agent does not cause the immediate oxidation of pyruvate and acetate as would be the case if the lag in oxidation of these compounds were due to permeability effects. Further evidence that permeability was not a factor influencing the adaptive nature of these oxidations was obtained when it was found that the presence of 0.001 M adenosine triphosphate had no influence on the oxidation of pyruvate and acetate by glucose-grown cells. The high energy phosphate in adenosine triphosphate would supply energy for the substrates to cross an impermeable cell membrane. Curves in the presence and absence of adenosine triphosphate were similar to those recorded in the presence and absence of Tween. Figure X.

Warburg Studies of Possible Intermediates in Glucose Oxidation: If the theory of simultaneous adaptation is sound then of the compounds tested only gluconic acid is an intermediate in glucose oxidation by P.aeruginosa. It must therefore be concluded that compounds not usually thought of as intermediates in aerobic metabolism are intermediates with the organism under study. As a result a large number of organic compounds were tested as possible intermediates. For this survey, 18 to 25 hour glucose-grown cells were used. All substrates were neutralized if necessary and made up at a concentration so that 0.2 ml. of solution contained substrate

equivalent to 18 uM oxygen. The following compounds were tested: glucose, calcium gluconate, calcium 2-ketogluconate, α-ketoglutaric acid, sodium succinate, fumaric acid, malic acid, sodium pyruvate, sodium acetate, acetic anhydride, sodium oxalate, sodium formate, glutaric acid, glycolic acid, calcium acetone-dicarboxylate, propionic acid, calcium iso-butyrate, iso-butyric acid, α-hydroxy-iso-butyric acid, butyric acid, iso-valeric acid, iso-caproic acid, formaldehyde, ethyl alcohol, n-butyl alcohol, iso-butyl alcohol, n-propyl alcohol, iso-propyl alcohol, amyl alcohol, allyl alcohol, capryl alcohol, ethylene glycol, propylene glycol, α-methyl glucoside, sodium B-glycerophosphate, ρ-hydroxy-phenylacetic acid, n-amylamine, iso-amylamine, α-amino-methyl-butyric acid, α-amino-iso-butyric acid, methyl glyoxime, choline chloride, alanine and amino-acetic acid. (The nitrogen containing compounds were included in the survey since it is probable that in growing cultures carbohydrate oxidation is closely related to nitrogen metabolism through deamination and transamination.) No intermediates of the Embden-Meyerhof system were tested because it has been shown that under anaerobic conditions P.aeruginosa is unable to produce carbon dioxide or acid from glucose (6), to take up inorganic phosphorous, or to utilize glucose or fructose (62). The oxygen uptake after 20 and 60 minutes incubation with the

various substrates is recorded in Table VIII. The endogenous uptake has been subtracted in all cases.

Of the 44 compounds tested, only glucose and calcium gluconate were attacked without a period of adaptation. Of the other compounds tested, only calcium 2-ketogluconate, sodium pyruvate, and sodium acetate were oxidized to the same extent as glucose (approximately 66% of the theoretical value). A period of adaptation was required before sodium succinate, fumaric acid, malic acid, and acetic anhydride were attacked and the oxygen uptake with these compounds was about 40% of the theoretical value. α-ketoglutaric acid, ethyl alcohol, and n-propyl alcohol were oxidized very slowly and the curves for the oxidation of these compounds were almost linear.

This survey of the oxidation of organic compounds gives no additional evidence as to what compounds might be intermediates in glucose breakdown by P.aeruginosa.

Table VIII -48-

Oxidation of Possible Intermediates by Glucose-Grown Cells.

Compound	* Oxygen uptake ul.	
	at 20 min.	at 60 min.
glucose	130	272
Ca-gluconate	164	314
Ca-2 ketogluconate	60	290
∝-ketoglutaric acid	31	117
Na succinate	15	164
fumaric acid	40	177
malic acid	74	155
Na pyruvate	48	234
Na acetate	45	280
acetic anhydride	73	181
Na oxalate	3	9
Na formate	6	56
glutaric acid	10	48
glycolic acid	6	17
Ca acetone-decarboxylate	6	18
propionic acid	15	59
Ca iso-butyrate	19	41
iso-butyric acid	1	19
butyric acid	1	27
∝-hydroxy-iso-butyric acid	1	4
iso-valeric acid	9	35

Table VIII (cont.)

Compound	* Oxygen uptake ul.	
	at 20 min.	at 60 min.
iso-caproic acid	1	22
formaldehyde	13	28
ethyl alcohol	31	110
n-butyl alcohol	6	15
iso-butyl alcohol	7	20
n-propyl alcohol	17	87
iso-propyl alcohol	7	8
amyl alcohol	0.5	15
allyl alcohol	11	39
capryl alcohol	12	35
ethylene glycol	4	11
propylene glycol	-5 (inhibitory)	-47
α-methyl glucoside	5	24
Na β-glycero phosphate	5	8
p-hydroxy-phenylacetic acid	8	25
n-amylamine	11	28
iso-amylamine	6	23
α-amino-methyl-butyric acid	11	30
α-amino-iso-butyric acid	-1 (inhibitory)	-38
methyl glyoxime	1	2
choline chloride	6	27

Table VIII (cont.)

Compound	* Oxygen uptake ul.	
	at 20 min.	at 60 min.
alanine	17	54
amino acetic acid	7	20

* Theoretical oxygen uptake for all compounds = 402 ul.

DISCUSSION

If the data reported in this section is interpreted in the light of simultaneous adaptation, then of the 43 compounds tested only gluconic acid can qualify as an intermediate in glucose dissimilation since this is the only compound oxidized immediately and at a maximum rate by glucose-grown cells. One might suggest, then, that glucose is oxidized to gluconic acid which is phosphorylated by the organism and all other intermediates occur as phosphorylated derivatives. This interpretation would explain adaptation to compounds such as pyruvate and acetate, for a lag period would be required to phosphorylate the compounds before they could be attacked. However, in view of observations made during this study, a more probable explanation of the results would be that simultaneous adaptation is not an adequate criterion for the identification of intermediate compounds. Previous isolation data (62) indicated that 2-ketogluconic acid is formed by this organism during glucose oxidation under physiological conditions. Yet simultaneous adaptation excludes 2-ketogluconate as an intermediate. The observations recorded in Part I that glucose-grown cells which attack glucose at pH 8.3 and 9.2 require a period of adaptation while the same cells at pH 6.5 and 7.2 oxidize immediately at maximum rate make one question the reliability of adaptation as a tool for identifying intermediate compounds. Cell concentration was also observed

to be a factor in the oxidation of substrates. More concentrated cell suspensions oxidized their parent substrate immediately while light suspensions of the same cells often required a period of adaptation before they could attack at maximum rate. As indicated before, permeability factors could also cause a lag in the oxidation of an intermediate compound. It is considered likely that some of the compounds eliminated on the basis of simultaneous adaptation will actually be shown to be intermediates in further studies.

SUMMARY

If the technique of simultaneous adaptation is accepted as the test for the identification or elimination of intermediate compounds then only gluconic acid has been identified as an intermediate in glucose dissimilation.

A survey has been made of the oxidation of 43 organic compounds by cells of P.aeruginosa harvested from a glucose medium.

The reliability of the simultaneous adaptation technique as a means of identifying intermediate compounds is questioned.

PART III

The Identification of Acetic Acid as an Intermediate in Glucose Oxidation.

Because data (Part II) interpreted on the basis of simultaneous adaptation eliminate all compounds tested but gluconic acid as intermediates in glucose oxidation by P.aeruginosa, the reliability of the technique for identifying compounds intermediate in carbohydrate metabolism has been questioned. By demonstrating the presence of one of the compounds requiring a period of adaptation before it can be oxidized under physiological conditions in a growing culture, one could also show that simultaneous adaptation was not applicable to our studies.

Aubel (3) has isolated alcohol, acetic acid, and formic acid from glucose fermentation by B.pyocyaneus. Acetic acid and traces of succinic acid were reported by Schreder, Brunner, and Hampe (71) and by Neuberg and Kobel (60)(61) working with P.lindneri. Conner, Riker, and Peterson (18) also reported the occurrence of small quantities of pyruvic and acetic acids in glucose cultures of Phytomonas rhizogens. If the assumption that aerobic metabolism of bacteria involves a Krebs cycle is correct, then acetic acid should be intermediate in aerobic carbohydrate metabolism of P.aeruginosa since acetic acid is the compound condensing with oxalacetate to yield citric acid. Acetic acid was one of the compounds

found to be oxidized to the same extent as glucose by glucose-grown cells of P.aeruginosa. Part II. Consequently acetic acid was considered one of the most probable compounds intermediate in glucose degradation. The object of the present investigation was to attempt to identify acetic acid as an intermediate in the oxidation of glucose by P.aeruginosa and to check on the soundness of the technique of simultaneous adaptation.

METHODS

Bacteriological: The growth medium for cells used in Warburg experiments was similar to that described in Part II. The same medium was used when analysis of the growing cultures was carried out. The carbon sources (glucose, ∝-ketoglutarate, or acetate) were added toa final concentration of 0.5% after they had been sterilized by passage through sintered glass. Substrates in Warburg experiments were equivalent to 18 uM oxygen contained in 0.2 ml. of solution. In experiments using large Warburg cups, the reaction mixture was acidified with sulphuric acid and the cup contents subjected to steam distillation in a modified Kjeldahl distillation apparatus. No direct heat was applied to the distillation flask containing the cup contents since it was found that small amounts of volatile acids were formed by the decomposition of substrate in the presence of protein when direct heat was applied. The distillates were titrated with N/100 NaOH.

In order to isolate a quantity of volatile acid adequate for identification purposes the following procedure was carried out for each of the two substrates glucose and ∝-ketoglutarate. Two litres of media were prepared and one litre of this was held as uninoculated control while the other, dispensed in Roux flasks, was inoculated and incubated at 30ºC for 18 hours. At the end of this time the cells were centrifuged off, the supernatant acidified with sulphuric acid and two litres of

distillate collected by steam distillation. The distillate was neutralized, concentrated on a hot plate to about 150 ml., reacidified with sulphuric acid and about 130 ml. collected by slow distillation. The litre of uninoculated medium which contained substrate and phosphate, was treated similarly with the exception that the cells from 100 ml. of the uninoculated medium were added before distillation to ensure the presenre of at least as much protein as was present in the litre of supernatant from the inoculated media. The distillates from these controls were alkaline to brom thymol blue and so the procedure was not carried beyond the initial distillation. Duclaux constants were run on the distillates from the inoculated media.

Chemical: Duclaux constants (40) were determined as follows; 25 ml. of sample was set aside while exactly 100 ml. was placed in a 250 ml. distilling flask equipped in the usual manner for such determinations. After discarding the first 10 ml. of distillate the subsequent three 25 ml. fractions were collected. These three samples plus the original 25 ml. aliquot were then titrated using a Beckman potentiometer. It was found that end-points using an indicator were not reliable for the titration of such dilute solutions. Three end-points were taken for each sample - pH 6.0, 7.5, and 8.5. Essentially the same constants were obtained at

each of the end-points. Titrations required from 3 to 12 ml. of N/400 NaOH. Standards of formic, acetic, and propionic acids of about the same concentrations as the unknowns were also run. Constants were calculated by dividing the titration value for each 25 ml. fraction by the total titration value for the 100 ml. sample distilled.

EXPERIMENTAL

Cells of P.aeruginosa were harvested from media containing glucose, α-ketoglutaric acid or sodium acetate as the sole source of carbon. The cell suspensions were tested in the Warburg respirometer for their ability to oxidize their parent substrate and sodium acetate. The curves recorded in Figure XI show that glucose-grown, α-ketoglutarate-grown or acetate-grown cells are able to oxidize their respective parent substrates immediately and at a maximum rate. However, cells grown on glucose or α-ketoglutarate require a period of adaptation before they are able to oxidize acetate. Therefore on the basis of the theory of simultaneous adaptation, acetic acid would be ruled out as an intermediate in glucose or α-ketoglutarate oxidation. Before eliminating acetic acid as an intermediate it was considered wise to test for its presence or absence by chemical methods.

In order to determine whether or not acetic acid or at least some volatile acid was formed during the oxidation of glucose or α-ketoglutaric acid, cells were harvested from media containing one or the other of these carbon sources and allowed to oxidize their parent substrate in 125 ml. Warburg cups. The reaction mixture which had a total volume of 30 ml. contained 9 mg. substrate. The oxidations were stopped by the addition of H_2SO_4 and the flask contents steam distilled. Since 100% recovery of standard acetic

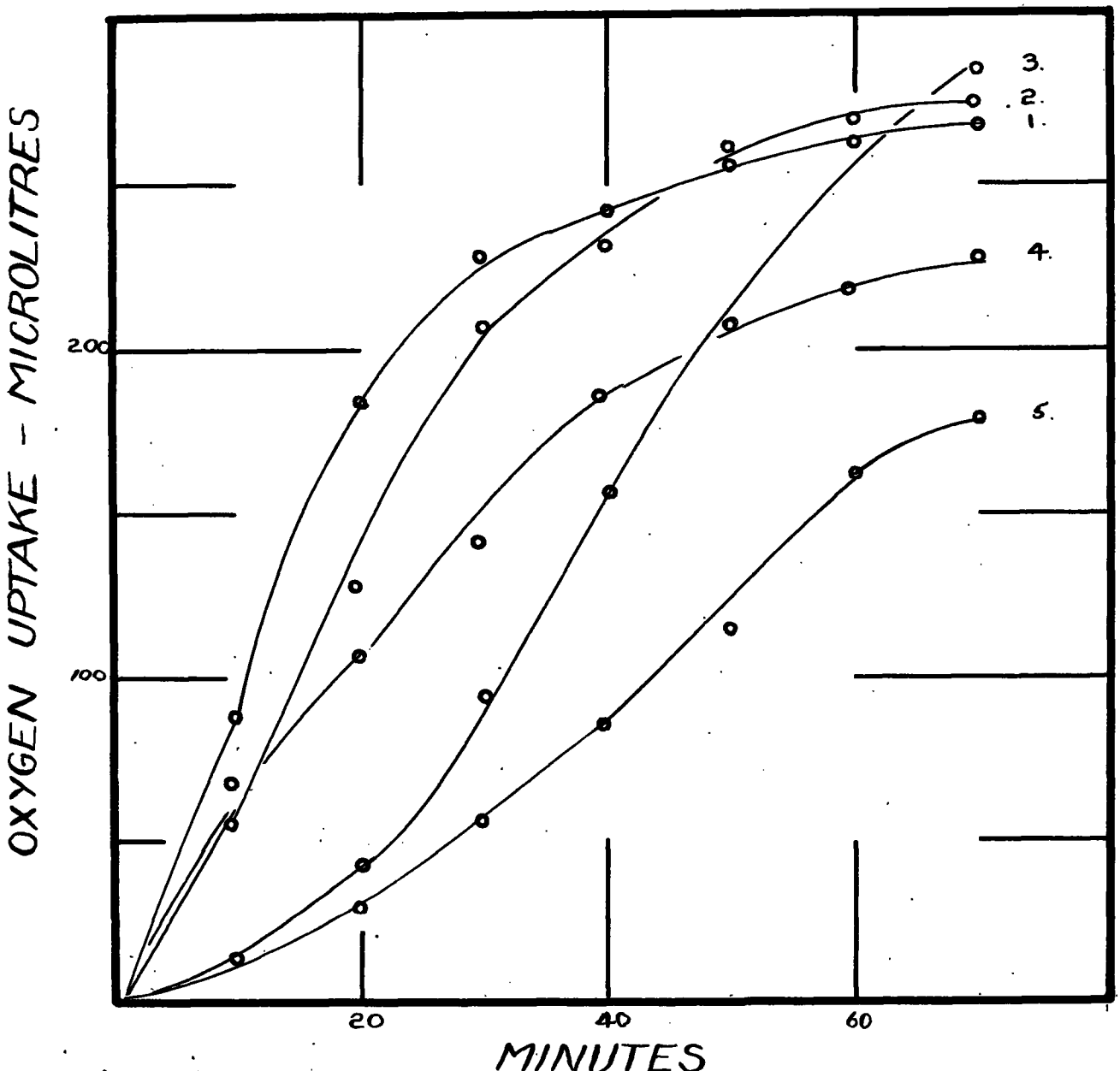

Figure XI: The adaptive nature of sodium acetate oxidation. 1.oxidation of sodium acetate by sodium-acetate-grown cells;2.oxidation of glucose by glucose-grown cells;3.oxidation of sodium acetate by glucose-grown cells;4.oxidation of α-ketoglutaric acid by α-ketoglutarate-grown cells;5.oxidation of sodium acetate by α-ketoglutarate-grown cells. Theoretical uptake for all substrates =402 ul.O_2.

acid was obtained only when the distillate was titrated to an end-point of pH 8.2, the distillates from reaction mixtures were titrated with N/100 NaOH to pH 8.2 using Beckman potentiometer. In experiment 5 an atmosphere of 10% air and 90% nitrogen was used in the hope that intermediate compounds would accumulate because limiting oxygen prevented their further oxidation. However, the results were similar to those of the four experiments run in an atmosphere of air. In every case small amounts of volatile acid were formed during the oxidation of glucose or α-ketoglutarate by resting cells of P.aeruginosa.

Table IX

The Production of Volatile Acid from Glucose or α-ketoglutarate.

Experiment Number	Atm.	Substrate	ml.of N/100 NaOH to neutr. distillate from flask run for		
			10 min.	15 min.	60 min.
1	air	nil(endog)	—	1.25	—
		α-ketoglutarate	1.55	1.40	
2	air	nil(endog)	—	1.10	—
		glucose	1.80	1.85	—
3	air	nil(endog)	—	1.30	—
		glucose	—	1.85	—
4	air	nil(endog)	—	1.25	—
		glucose	—	2.95	
5	10% air 90% N_2	nil(endog)	—	—	1.25
		glucose	—	—	1.50

It was considered probable that the acid formed was acetic acid since this was the only volatile acid oxidized to the same extent as glucose. Part II. However, the amounts of acid formed were too small to permit their identification.

In an effort to identify this volatile acid, the supernatant from a litre of 18 hour culture in which glucose or α-ketoglutarate had been the only source of carbon was acidified and steam distilled. Duclaux constants were determined for the concentrated distillates and compared to the values obtained for comparable concentrations of formic, acetic and propionic acids. Table X. The determinations were repeated 3 to 4 times with similar results each time. The results for the distillates of the growth media are seen to parallel those obtained for acetic acid.

Table X

Duclaux Constants for Supernatant Distillates.

	Standards			Distillate from glucose medium	Distillate from α-ketoglutarate medium
	formic acid	acetic acid	propionic acid		
Fraction A	.1250	.2486	.2860	.2447	.2183
Fraction B	.1526	.2322	.2523	.2380	.1940
Fraction C	.2300	.2612	.2374	.2760	.2360

The constants for acetate differ from those usually reported in the literature but apparently they are an idiosyncrasy of the apparatus or more probably of the dilute solutions used

since the constants usually given are for much more concentrated solutions. Under any circumstances this idiosyncrasy can be used to show definitely that the volatile acid formed during growth is almost solely acetic.

Further evidence that acetate is an intermediate in glucose oxidation by P.aeruginosa was found by Norris (63) and her findings are recorded here since they confirm the role of acetic acid as intermediate in glucose oxidation. Cells harvested after 4 days growth in an aerated liquid glucose medium had almost completely lost their ability to oxidize acetic acid. It follows therefore that if acetate is an intermediate it should accumulate under these conditions of growth. This was found to be the case and about thirty times as great a concentration of acetic acid, as identified by its Duclaux constants, was found in the supernatant from cells which had lost their ability to oxidize acetic acid as in the supernatant from normal cells.

Since one-third of the oxygen consumed during the degradation of glucose is used in converting acetic acid to carbon dioxide and water, if acetate is an intermediate cells which cannot oxidize acetate should use only two-thirds as much oxygen as cells which can oxidize this compound. This was found to be true. Table XI. The value for normal cells is an average value from the data presented in Parts I and II.

Table XI

Oxidation of Glucose by Normal and Aerated Cells.

	Substrate	O_2 uptake ul.
Average value, normal cells	glucose	274
Observed value for cells unable to oxidize acetate (63)	glucose	190
Calculated value for cells unable to oxidize acetate	glucose	183
Observed value for cells unable to oxidize acetate (63)	acetate	7

From this data it can be seen that when glucose was oxidized by cells which had lost the ability to attack acetic acid, the amount of oxygen which was consumed was exactly the amount necessary to convert glucose to acetate. These data in combination with results showing volatile acid production by resting cells oxidizing glucose and the isolation of acetic acid from an 18 hour supernatant of glucose-grown cells furnish convincing evidence of the role of acetic acid as an intermediate in glucose oxidation by P.aeruginosa.

DISCUSSION

In the discussion of the previous section examples of cases where a period of adaptation occurs before resting cell suspensions can oxidize their parent substrate have been shown. The occurrence of such cases raises the question of the reliability of simultaneous adaptation as a tool for the detection of intermediate compounds. On further consideration it becomes evident that the principle of the technique is basically unsound. Yudkin (90) explained enzyme adaptation with the theory that "all examples of enzyme production are cases of increase in enzyme and none are instances of the formation of completely new enzyme" and further that "it is clear that the adaptive enzyme is produced from a precursor or precursors ..... it is assumed that an equilibrium exists between such precursors and the formed enzyme." The combination of the enzyme with substrate would result in a disturbance of the equilibrium and more enzyme would be formed from the precursor in order to restore it. It therefore follows that within the physiological limits of the organism, an increased amount of substrate will result in the production of a greater amount of enzyme. This deduction is supported by the work of Kertesz (42) who found this to be true of invertase production by yeast. Stanier's theory of simultaneous adaptation is in contradiction to this work for it assumes that when a substrate is attacked by an adaptive

enzyme the same amount of enzyme will be produced regardless of the amount of substrate present in the growth medium. That is, when cells are transferred from conditions where the compound in question is a metabolic intermediate and therefore present in only very small amounts to conditions where the same compound is present as parent substrate and in much larger quantities no increase in amount of enzyme will occur.

In his initial demonstration of simultaneous adaptation, Stanier rather than working with compounds of a known system, used aromatic compounds which do not function in any known metabolic pathway. In addition he used no other method to identify a compound as an intermediate and by his technique he recorded only one compound which could be an intermediate in the oxidation he describes.

In our studies it was found that compounds which were probably separated from the parent substrate by only one enzyme reaction could be attacked immediately, for example glucose-grown cells could oxidize only gluconic acid immediately and pyruvate-grown cells could oxidize only acetic acid. This observation can be readily explained. All the reactions involved in the oxidation of glucose will be in equilibrium and consequently the initial breakdown products will be present in relatively large amounts while the products which arise further down in the system will be

present in much smaller amounts. By the same reasoning, the enzyme necessary for the initial step in the oxidation will be present in large amounts while the enzymes necessary for each subsequent step will be present in smaller amounts. Thus sufficient enzyme is present in glucose-grown cells to oxidize glucose to gluconic acid but a period of adaptation is required to produce enough formed enzymes for the oxidation of relatively larger amounts of acetic acid.

SUMMARY

Resting cell suspensions of glucose-grown organisms have been shown to produce small amounts of volatile acid during the oxidation of glucose.

Acetic acid has been identified by Duclaux constants as the volatile acid present in the supernatant of an 18 hour culture of P.aeruginosa in glucose medium.

The identification of acetic acid as an intermediate in glucose oxidation shows that the technique of simultaneous adaptation is not applicable in our studies since glucose-grown cells require a period of adaptation before they are able to attack acetate.

The Identification of Gluconic and 2-ketogluconic Acids as Intermediates in Glucose Oxidation by the Use of Paper Chromatography.

Paper chromatography was introduced in 1944 by Consden, Gordon, and Martin (20) as an analytical technique for the identification of amino acids. Since its introduction, the technique has been found extremely useful and numerous publications have appeared on its varied applications. The aim of the technique is to effect the separation of a mixture of closely related substances present in extremely small quantities. To effect such a separation, the compounds being studied are introduced as small spots near the top of a large sheet of filter paper. The paper is hung vertically from a trough which contains a water-saturated solvent so that the upper edge of the paper is immersed in the solvent. The trough containing the paper is suspended in a closed vessel whose atmosphere is kept saturated with respect to the solvent. After the paper has been irrigated to within an inch or two of the bottom, the sheet is removed, dried, and then sprayed with a solution which will give a general color reaction with the type of compounds being identified. The compounds are identified by their characteristic rates of movement. To identify a compound positively, its movement in at least two solvents must be found to agree with movement of a known compound run as a control under the same conditions. For

the separation of compounds which move similarly, a two-dimensional chromatogram may be employed.

The theory to explain the differences in rate of movement is as follows: partition of the solute takes place between water bound by the cellulose and the solvent which is moving over the surface of the cellulose particles. The distance moved by the solute is a function of (1) the partition coefficient of the solute between water and the solvent, (2) the volume of water bound by a unit area of paper, and (3) the volume of solvent held by a unit area of paper after irrigation. Since (2) and (3) are constant if the filter paper is uniform, the movement of the solute is dependent upon its partition coefficients only. The position of the compounds is expressed as the R_F value which is obtained by dividing the distance moved by the solute by the total distance moved by the advancing front of the liquid.. Rather than a water-saturated solvent, a solvent of two or three liquids in suitable proportions may be used.

Paper chromatography has become a standard technique in the qualitative determination of amino acids with saturated phenol, saturated collidine and butanol-acetic acid as the usual solvents and the ninhydrin reaction the common color reaction for locating the compounds. Consden, Gordon, and Martin (21) have reported a quantitative modification where

the spots are leached out and amounts of amino acids determined colorimetrically. Some sixty amino acids and ninhydrin-reacting substances have been identified by Dent (23) using paper chromatography.

Partridge (64) introduced paper chromatography to the field of sugar chemistry when he used the method to separate reducing sugars. Later he extended the method to include 22 sugars and sugar derivatives (65). The position of the sugars was located using ammoniacal silver nitrate as a spray. An extension to include a quantitative analysis of sugars was suggested by Flood, Hirst, and Jones (30).

Since the original publication of the method for identifying amino acids, the technique has been applied to such different studies as the determination of nitrogenous lipide constituents (14), the separation and quantitative estimation of purines and pyrimidines (29)(84), the analysis of microbial growth factors (86); the separation of organic acids in plant materials (54), the separation of anthocyanin pigments (9), the separation of flavine nucleotides (2), the analysis of inorganic ions (49), the determination of nucleic acid derivatives (55), the quantitative estimation of penicillin (34)(44) and streptomycin (19), the detection of creatine and creatinine (56)(57), the separation vegetable tannins (41), and the analysis of adrenalin (39).

Williams and Kirby (85) reported the use of a simplified

apparatus employing capillary ascent rather then the usual descending method. In their technique the filter paper sheet was shaped into a cylinder (the compounds being tested placed near the bottom of the sheet) and set in a closed earthenware jar containing solvent in the bottom. The solvent irrigated the sheet by capillary ascent. In a further modification, Rockland and Dunn (70) used capillary ascent in test tubes rather than earthenware jars.

Although the only information available on the use of paper chromatography for the study of intermediate carbohydrate metabolism was a preliminary report by Forsyth and Webley (31) on the hydrolysis of sucrose to the constituent hexoses by Lc.mesenteroides, exploratory studies with P.aeruginosa indicated that the technique would be applicable to the compounds and conditions used in the present study. The purpose of the following unit of work was to apply the technique of paper chromatography to the identification of the initial intermediates formed when glucose is dissimilated by P.aeruginosa under normal physiological conditions.

METHODS

Bacteriological: For the preparation of concentrated supernatants, the organism was grown in 200 ml. of glucose ammonium phosphate medium as described in Parts II and III. After growth at 30°C for either 16 or 24 hours, the cells were removed by centrifugation. The clear supernatant was adjusted to pH 7.2 with 4N NaOH and then heated in a boiling water bath for 5 minutes, to aid in suppressing further enzyme action. The supernatant was cooled and immediately vacuum distilled under nitrogen at approximately 30°C until the final volume was 20 ml. This method of concentrating under nitrogen was employed in order to prevent further dissimilation of glucose during the bubbling necessarily accompanying vacuum distillation.

A control flask containing 200 ml. of uninoculated medium was treated in a similar manner.

Chemical: Paper chromatography was carried out both by the more conventional descending method (20) and by the ascending method (85). The latter method was used in exploratory work while the former was used in the subsequent more exact determinations. In the ascending chromatograms the compounds being tested were introduced as drops from a Pasteur pipette; these drops were placed at intervals 1 1/2" from the bottom of a sheet of Whatman No.1 filter paper which had been cut to

measure 13" by 22". The sheet was pinned in the form of a cylinder and set in a large Petri dish containing 25 ml. of solvent. The cylinder and dish were placed in a clean wastebasket, a wooden cover was put in place and the sheet was irrigated at room temperature for 15 - 18 hours. The sheet was then dried and sprayed with 0.1N $AgNO_3$ in 5 NNH_4OH. The sprayed sheet was allowed to dry in the absence of direct light. In the descending chromatograms, drops of the two supernatants, the concentrated medium and known compounds (2 mg/ml.) were introduced near the top of a sheet of Whatman No.1 (18" by 22 1/2") filter paper which was hung vertically from a trough. The trough and filter paper sheets were then suspended inside a closed vessel whose atmosphere was kept saturated with respect to the solvent. The trough was filled with the solvent and the sheets irrigated at room temperature to within 2" of the bottom. The sheets were dried and sprayed with silver nitrate solution. The sprayed sheets were then dried.

The ethyl and methyl alcohols used as solvents were purified prior to use by refluxing with zinc dust in an alkaline solution for 60 minutes before redistillation. Phenylhydrazones were prepared by allowing solutions to stand at room temperature overnight in contact with an equal volume of 10% phenylhydrazine - HCl. Ether extracts were

prepared by adjusting solutions to pH 5.5 and extracting with ether for 6 hours in a continuous extractor.

EXPERIMENTAL

Data reported inthe literature for related organisms (51)(52)(66) and data for P.aeruginosa ATC9027 (43)(62) (63) indicated that gluconic, 2-ketogluconic, and α-keto-glutaric acids are among the probable initial degradation products of glucose. Since the use of paper chromatography for the detection of these compounds had not been previously reported a great deal of background information had to be accumulated. It was first necessary to find a general color reaction for these compounds which could be adapted as a spray for chromatographic analyses. Preliminary investigations were made of reactions indicating the presence of reducing groups, acid groups, and ketones. The suitability of several of these reactions is recorded in Table XII. Although acid-base indicators detected a larger number of compounds than the $AgNO_3$ reagent, the latter reagent was chosen for future work because it was capable of detecting compounds present in much smaller amounts. The alkaline picrate reagent appears promising for future determinations of pyruvic acid.

In searching for a solvent for the chromatographic analysis, one which would not interfere with the silver reduction reaction, would move the compounds being tested, and would be readily volatilized was essential. Saturated phenol, collidine, and butonol-acetic acid, the solvents commonly employed for chromatographic analysis of reducing

Table XII

Suitability of Reactions for

Detection of Compounds.

Type of Reaction	Reagent	Remarks
Reducing group	N/100 $KMnO_4$	filter paper reduces reagent
	2 N NaOH followed by saturated aqueous picric acid	detects pyruvic acid and -ketoglutaric acid
	Copper reagent (40)	detects only glucose and faint reaction for 2-ketogluconic acid.
	Nessler's reagent (37)	detects only glucose and 2-ketogluconic acid and the latter spot fades rapidly.
	Nylander's reagent (37)	gives no reaction.
	0.1N $AgNO_3$ in 5N NH_4OH (65)	suitable for detection of desired compound, sensitivity good.
Acid group	Brom cresol blue (0.2% in 95% ethyl alcohol) (54)	solvent used must be neutral in reaction or volatile, not so sensitive as $AgNO_3$ in NH_4OH but detects any acidic compound
	Brom cresol purple (0.2% in 95% ethyl	spots not so definite as with brom cresol blue.
	Brom cresol green (0.2% in 95% ethyl alcohol)	spots fade rapidly.
	Chlorphenol red (0.2% in 95% ethyl alcohol)	spots spreading.

Spots are

Table XII (cont.)

Type of Reaction	Reagent	Remarks
Ketone group	2:4 dinitro-phenylhydrazine reagent (72)	gives no reaction.
	½% α naphthol in ethyl alcohol equal volume 2% trichloracetic acid (65)	detects fructose but not keto acids.

sugars (65) were found to be unsuitable since they did not move the possible degradation products of glucose through a sufficient distance to allow differentiation. It was found that methyl and ethyl alcohols at various concentrations, alone or in combination served as excellent solvents for the compounds being tested.

The following compounds were found to give a definite spot with the silver nitrate reagent: glucose, glucose - 1 - phosphate, glucose - 6 - phosphate, hexose-diphosphate, gluconic acid, 2-ketogluconic acid, 5-ketogluconic acid, α-ketoglutaric acid, pyruvic acid, lactic acid, malic acid, citric acid, glycerol, glycero phosphate, and fructose. R_F values of these compounds are recorded in Table XIII. No reduction was given by succinic acid, fumaric acid, maleic acid, malonic acid, acetic acid, oxalic acid, formic acid, formaldehyde or acetone - dicarboxylate. The spots obtained with

silver nitrate were not always due to the presence of metallic silver; for instance, lactic acid gave a yellowish spot while citrate gave an orange spot. The gluconic acid spot was initially a yellowish shade turning dark brown with age. Although the silver nitrate reaction was not specific, many of these compounds could, however, be identified by color and R_F values.

Table XIII

R_F Values of Known Compounds in 45% Methyl Alcohol, 45% Ethyl Alcohol, and 10% Water.

Compound (2 mg./ml.)	R_F	Remarks
glucose	0.59	
glucose-1-phosphate	0.72	
glucose-6-phosphate	0.36	
hexose-di-phosphate	0.03	
gluconic acid	0.425	
2-ketogluconic acid	0.45	
5-ketogluconic acid	0.45 0.03	The sample always gave two spots. This compound was not considered a possible intermediate since the organism could not grow with the compound as sole carbon source.
α-ketoglutaric acid	0.46	
pyruvic acid	0.70	
lactic acid	0.65	yellow spot
glycerol	0.76	
malic acid	0.60	orange - brown spot
fructose	0.63	grey-brown spot
citric acid	0.28	orange spot
glycerophosphate	0.51	faint orange spot

* We are indebted to Dr. L.B.Lockwood of the Northern Regional Research Laboratory for samples of 2-ketogluconic and 5-ketogluconic acids; to Dr. C.E.Georgi of the University of Nebraska for a sample of 5-ketogluconic acid; to Dr.D.E.Green of the University of Wisconsin for a sample of α-ketoglutaric acid; to Dr. I.C.Gunsalus of the University of Indiana for samples of glucose-1-phosphate, glucose-6-phosphate, and hexose-diphosphate; and to Dr.H.L.Tarr, Fisheries Exper.Stn., for gluconic acid and glucose-6-phosphate.

Analysis of the culture supernatants always revealed at least two spots. One corresponded to glucose and the second with a smaller R_F value to some glucose degradation product. From a comparison of the R_F values obtained for known compounds, it was found that the value for this lower spot corresponded to that for gluconic, 2-ketogluconic or α-ketoglutaric acid. Since the movement of these three acids was very similar in all of the 55 combinations of solvents used, some supplementary means of separation had to be found.

Advantage was taken of the fact that 2-ketogluconic and α-ketoglutaric acids react with keto fixatives, whereas gluconic acid does not. The influence of sodium bisulfite, phenylhydrazine-HCl, hydroxylamine-HCl, semicarbazide, and 2,4, dinitrophenylhydrazine on the movement of these compounds was determined. Phenylhydrazine-HCl was selected as the fixative to be used in further work, since the R_F values of the derivatives which were formed with it differed most markedly from those of the free acids while the R_F value of gluconic acid remained unchanged. An equal volume of a 10% solution of phenylhydrazine-HCl was allowed to react for 24 hours at room temperature with the solution to be tested. These conditions permitted the complete conversion of any reasonable amount of 2-ketogluconate or α-ketoglutarate to its corresponding hydrazone.

The R_F values obtained, both before and after phenlhydrazine

treatment, for known compounds and for the concentrated supernatant of a 24 hour culture are recorded in Table XIV.

Table XIV

Movement of Intermediate Compounds with a Solvent of 45% Methyl Alcohol, 45% Ethyl Alcohol, and 10% Water.

Compound	R_F Value	
	Before phenylhydrazine treatment	After phenylhydrazine treatment
(1) Glucose	0.55	0.66
(2) Gluconate	0.43	0.43
(3) 2-ketogluconate	0.47	no spot
(4) α-ketoglutarate	0.46	no spot
(5) Concentrated uninoculated medium	0.55	0.66
(6) Concentrated 24 hour culture supernatant	0.45	0.43
(7) 1:1 dilution of (6)	0.44 0.48	0.46 no spot

In Tables XIV and XV, the R_F values of spots corresponding to glucose in the supernatant and also those due to free phenylhydrazine have been omitted in order to minimize confusion. It can be clearly seen from these results (Table XIV) that the supernatant contained gluconic acid since a reducing spot with R_F value corresponding to this compound remained after phenylhydrazine treatment. However, the size of the spot was considerably reduced by phenylhydrazine treatment, indicating that some compound which would react with phenylhydrazine had been removed. On dilution of the concentrated supernatant with an equal volume of water, the larger single spot which had an R_F of 0.45 was seen to actually be composed of two smaller spots one with an R_F of 0.44 and the other with an R_F of 0.48. The spot with an R_F of 0.44 was gluconic acid since it remained undiminished after phenylhydrazine treatment, while the spot with an R_F of 0.48 could be either 2-ketogluconic or α-ketoglutaric acid or a mixture of these compounds since it was eliminated by phenylhydrazine treatment.

In order to confirm the findings recorded in Table XIV, a second solvent containing 90% redistilled ethyl alcohol and 10% water was used (Table XV).

Table XV

Movement of Intermediate Compounds with a Solvent of 90% Ethyl Alcohol and 10% Water.

Compound	R_F Value	
	Before phenylhydrazine treatment	After phenylhydrazine treatment
(1) Glucose	0.56	0.56
(2) Gluconate	0.33	0.33
(3) 2-ketogluconate	0.33	no spot
(4) α-ketoglutarate	0.32	no spot
(5) Concentrated uninoculated medium	0.56	0.54
(6) Concentrated 24 hour culture supernatant	0.36	0.35
(7) Concentrated 16 hour culture supernatant	0.41	0.36

In this experiment, the concentrated supernatant from a 16 hour culture was analysed with a view to demonstrating the presence of the same intermediate compounds in young cultures. The lower reducing spot observed previously was again detected and it remained after treatment with phenylhydrazine but was reduced in size. These findings

indicate the presence of gluconic acid and a keto acid in the 16 hour supernatant as well as in the 24 hour supernatant.

Further separation of the reducing compounds was attempted on the basis of the relative solubilities of α-ketoglutaric, 2-ketogluconic, and gluconic acids in ethyl ether. Gluconic acid, 2-ketogluconic acid, α-ketoglutaric acid, concentrated uninoculated medium, concentrated 24 hour supernatant, and concentrated 16 hour supernatant were ether extracted for 6 hours at pH 5.5. The ether soluble and ether insoluble fractions of all solutions as well as fractions of each solution which had not been extracted were analyzed using a solvent consisting of 95% ethyl alcohol and 5% ammonium hydroxide. Values are recorded in Table XVI. The ether fractions were treated with 10% phenylhydrazine-HCl with results similar to those recorded in Tables XIV and XV. Again the reducing spot representing the intermediate products was reduced but not completely removed by the phenylhydrazine treatment indicating that gluconic acid and either 2-ketogluconic or α-ketoglutaric acids or both were formed by the organism. Since no product could be detected in the ether extract of the culture, it can be concluded that α-ketoglutarate was not present in detectable quantities, and that 2-ketogluconic acid was the keto acid present.

Table XVI

R_F Values of Ether Fraction of

Culture Constituents.

Compound	R_F	R_F ether insoluble residue	R_F ether soluble fraction
(1) Glucose	0.42		
(2) Gluconate	0.15	0.15	no spot
(3) 2-ketogluconate	0.143	0.142	no spot
(4) ∝-ketoglutarate	0.056	no spot	0.06
(5) concentrated uninoculated medium	0.40	0.38	no spot
(6) concentrated 24 hour culture supernatant	0.39 0.135	0.40 0.142	no spot
(7) concentrated 16 hour culture supernatant	0.39 0.135	0.42 0.152	no spot

Further confirmation of the presence of gluconic acid in the culture supernatants was recorded in every experiment. Shortly after the chromatogram was sprayed with $AgNO_3$, gluconic acid came up as a characteristic yellow which later turned brown and a spot with the same characteristics and with the same R_F value always appeared in the culture supernatant.

DISCUSSION

The R_F values obtained were found to vary quite markedly with small differences in the water content of the solvent. It was therefore necessary to run known compounds with each individual chromatogram rather than use values obtained in previous determinations. Such wide variations do not occur inamino acid determinations where solvents are water-saturated and consequently have a constant water content. The free acids or salts of the acids always gave identical R_F values since the compounds moved as ions.

From the data presented it can be concluded that both gluconic and 2-ketogluconic acids were present in detectable amounts in 16 and 24 hour cultures of P.aeruginosa grown in glucose ammonium phosphate liquid medium. It was also found that in a 38 hour culture all the glucose had disappeared and only the faintest trace of gluconic or 2-ketogluconic acid could be detected chromatographically. Curves in Figure IV, (Part II) show that P.aeruginosa has a strong mechanism for oxidizing both gluconic and 2-ketogluconic acids. One must therefore conclude that these compounds are being formed at a continuous and vigorous rate and are being removed in the same manner. This shows that these compounds are not just the end-products of a minor side reaction and can only mean that they are part of the system through which the majority if not all of the glucose is oxidized.

The facts that cells harvested from a glucose medium require a period of adaptation before oxidizing 2-keto-gluconic acid and that 2-ketogluconic acid can be detected chromatographically confirm the evidence in Part III that the technique of simultaneous adaption is not applicable to studies such as these.

Failure to identify a five, four, or three carbon intermediate compound could mean either that the lower products formed do not reduce silver nitrate or that they are present in amounts too small to detect by the methods employed. The second interpretation is probably correct since the initial products will be present in much larger concentrations than the products arising farther down in the dissimilation scheme. (This has been discussed in Part III).

P.aeruginosa seems to be unique in its oxidation of glucose to yield free gluconic and 2-ketogluconic acids as intermediates under physiological conditions. In all reported cases of the direct oxidation of glucose, the glucose molecule is pictured as being phosphorylated and then oxidized to phosphogluconic acid. According to Dickens' concept (24),phosphorous is attached to all compounds intermediate in the scheme of oxidation. But in a consideration of Dickens' scheme, it can be seen that phosphorous attached as he suggests it cannot yield energy to the system. Lipmann (50) postulated phosphogluconic

acid rather than free gluconic acid as an intermediate in glucose oxidation by yeast. In studies of organisms which cannot ferment glucose but are able to oxidize it, Barron and Friedemann (7)(8) consider that the glucose molecule does not become oxidizable until it has been phosphorylated. In the Embden-Meyerhof scheme, too, the hexose is initially phosphorylated and all compounds remain phosphorylated through to the triose stage. Thus there is no known energy-yielding scheme of metabolism which does not rely on the generation of high energy phosphate bonds.

Two possibilities remain, however, and one of these could function in the case of P.aeruginosa. It has been shown by Friedkin and Lehninger (33) that hydrogen transport between dihydrogen-diphosphopyridine nucleotide (Co I) and oxygen can be doupled with phosphorylation of a second system to yield energy. By a system such as this, glucose could be oxidized directly to gluconic acid while a coupled phosphorylation of an entirely different compound yielded energy. On the other hand, it is possible that phosphogluconic and 2-ketophospho-gluconic acids are actually the compounds formed in the dissimilation of glucose but that P.aeruginosa possesses strong phosphorylases which split the compounds to the free acids. In any case, free gluconic and 2-ketogluconic acids do arise under physiological conditions during the dissimil-ation of glucose. The pathway of oxidation is probably

very similar to that functioning in the metabolism of molds.

This study establishes the presence of an oxidative system of glucose breakdown by P.aeruginosa which has not previously been recognized in bacteria.

SUMMARY

Paper chromatography has been adapted to the identification of gluconic, 2-ketogluconic and α-ketoglutaric acids. Combinations of ethyl and methyl alcohols were found to be the most suitable solvents and ammoniacal silver nitrate the most satisfactory reaction to determine the position of the compounds.

Under normal physiological conditions P.aeruginosa was shown to have oxidized glucose by way of gluconic and 2-ketogluconic acids. Since a strong system for oxidizing both of these acids has been demonstrated, the presence of these acids over at least an eight hour period of growth is taken as evidence of their importance as intermediates in the oxidation of glucose by this organism.

BIBLIOGRAPHY

(1) Ajl, S. J., and Werkman, C. H. Replacement of CO_2 in heterotrophic metabolism. Arch. Biochem. 19: 483 - 492. 1948.

(2) Ajl, S. J., and Werkman, C. H. On the mechanism of CO_2 replacement in heterotrophic metabolism. J.Bact.57: 579 - 593. 1949.

(3) Aubel, E. Attaque du glucose et du levulose par le bacille pyocanique. Compt.Rend. 173: 1493 - 1495. 1921.

(4) Barker, H. A. The oxidative metabolism of the colorless algae, Protothecia Zopfii. J. Cell. and Comp. Physiol. 8: 231 - 250. 1936.

(5) Barker, S. B., Shorr, E., and Malam, M. Studies on the Pasteur reaction: effect of iodo acetic acid on the carbohydrate metabolism of isolated mammalian tissues. J. Biol. Chem.129: 33 - 50. 1939.

(6) Barron, E. S. G. Mechanisms for the complete oxidation of carbohydrates by aerobic bacteria. Symp. on Resp. Enz. University of Wisconsin Press. 262 - 263. 1941.

(7) Barron, E. S. G. Mechanisms of carbohydrate metabolism. An essay on comparative biochemistry. Adv. in Enz. 3: 149 - 189. 1943.

(8) Barron, E. S. G., and Friedimann, T. E. Studies on biological oxidations. XIV. Oxidations by microorganisms which do not ferment glucose. J. Biol. Chem. 137: 593 - 610. 1941.

(9) Bate-Smith, E. C. Paper chromatography of anthocyanins and related substances in petal extracts. Nature 161: 835 - 838. 1948.

(10) Bergey, D. H. Manuak of determinative bacteriology. Williams and Wilkins Co. Baltimore. 1948.

(11) Bernhauer, K., and Gorlich, B. Oxydationen mittels Essigbakterien. IV Mitteilung. Uber die Bildung von 2-keto-gluconsaure durch Bact. gluconicum. Biochem. Z. 280: 367. 1935.

(12) Bernstein, D. E. Studies on the assimilation of dicarboxylic acids by Pseudomonas saccharophila. Arch. Biochem. 3: 445 - 458. 1943.

(13) Binkley, W. W., and Wolfrom, M. L. Chromatography of sugars and related substances. Sugar Res.Found. Inc. Sci.Rep.Series 10. 1948.

(14) Chargaff, E., and Levine, C., and Green, C. Techniques for the demonstration by chromatography of nitrogenous lipide constituents, sulfur-containing amino acids and reducing sugars. J. Biol.Chem. 175: 67 - 71. 1948.

(15) Clifton, C. E. On the possibility of preventing assimilation in respiring cells. Enzymologia 4: 246 - 253. 1937.

(16) Clifton, C. E. Microbial assimilations. Adv. in Enz. 6: 269. 1946.

(17) Clifton, C. E., and Logan, W. A. On the relation between assimilation and respiration in suspensions and in cultures of Escherichia coli. J. Bact.37: 523 - 540. 1939.

(18) Conner, H. A., Riker, A. J., and Peterson, W. H. The carbon metabolism of the crown-gall and hairy-root organism. J.Bact. 34: 221 - 236. 1937.

(19) Consden, R. Partition chromatography on paper, its scope and application. Nature 162: 359 - 361. 1948.

(20) Consden, R., Gordon, A. H., and Martin, A. J. P. Qualitative analysis of proteins: a partition chromatographic method using paper. Biochem. J. 38: 224 - 232. 1944.

(21) Consden, R., Gordon, A. H., and Martin, A. J. P. The identification of lower peptides in complex mixtures. Biochem. J. 41: 590 - 596. 1947.

(22) Crammer, J. L. Paper chromatography of flavine nucleodides. Nature 161: 349 - 350. 1948.

(23) Dent, C. E. A study of the behavior of some 60 amino acids and other ninhydrin-reacting substances on phenol-collidine filter paper chromatograms, with notes as to the occurrence of some of them in biological fluids. Biochem. J. 43: 169 - 180. 1948.

(24) Dickens, F. Mechanism of carbohydrate Oxidation. Nature 138: 1057. 1936.

(25) Dickens, F. CCXI Oxidation of phosphohexonate and pentose phosphoric acids by yeast enzymes. Biochem. J. 32: 1626 - 1653. 1938.

(26) Dixon, M. Manometric methods as applied to the measurement of cell respiration and other processes. Cambridge : at the University Press. New York: MacMillan Co. 1943.

(27) Doudoroff, M. The oxidative assimilation of sugars and related compounds by Pseudomonas saccharophila. Enzymologia 9: 59 - 72. 1940.

(28) Doudoroff, M., Wiame, J. M., and Wolochow, H. Phosphorolysis of sucrose by P. putrefaciens. J. Bact.. 57: 423 - 427. 1949.

(29) Edman, P., Hammarsten, E., Low, B., and Reichard, P. Partition chromatographic separation of adenine and guanine. J. Biol. Chem. 178: 395 - 398. 1949.

(30) Flood, A. E., Hirst, E. L., and Jones, J. K. N. Quantitative estimation of mixtures of sugars by the paper chromatogram method. Nature 160: 86 -87/ 1947.

(31) Forsyth, W. G. C., and Webley, D. M. A method for studying the carbohydrate metabolism of microorganisms. Nature 162: 150 - 151. 1948.

(32) Foster, J. W. Introspections on Mold metabolism. Bact. Revs. 11: 167 - 188. 1947.

(33) Friedkin, M., and Lehninger, A. L. Phosphorylation coupled to electron transport between dihydrogen diphosphopyridine nucleotide and oxygen. J. Biol. Chem. 174: 757 - 758. 1948.

(34) Goodall, R. R., and Levi, A. A. A microchromatographic method for the detection and approximate determination of the different penicillins in a mixture. Nature 158: 675 - 676. 1946.

(35) Green, D. E. The mechanisms of biological oxidations. Cambridge : at the University Press. Cambridge Biological Studies. 1941.

(36) Harrison, D. C. CXIII Glucose dehydrogenase: a new oxidising enzyme from animal tissues. Biochem. J. 25: 1016 - 1027. 1931.

(37) Hawk, P. B., Oser, B. L., and Summerson, W. H. Practical physiological chemistry. 12th Ed. The Blakeston Co. 1947.

(38) Hawthorne, J. R. Micro-estimation of sugars separated on the filter paper chromatogram. Nature 160: 714. 1947.

(39) James, W.O. Paper chromatography of adrenalin and related substances. Nature 161: 851 - 1948.

(40) Johnson. Dept. of Biochem. Lab. Manual. Wisconsin. 1947.

(41) Kenten, R. H. Brit. Leather Man. R. A. Lab. Reports 26 (2): 1947. Cited in Consden, R. Nature 162: 359 - 361. 1948.

(42) Kertesz, Z. I. Reiqwirkung - sversuche mit der Saccharase von Penicillium glaucum. Ferment-forschung 9: 300 - 305. 1928.

(43) King, J. V. 2-ketogluconic acid production by P.aeruginosa. Report to the B. C. Research Council. 1948.

(44) Kluener, R. G. Paper chromatography for quantitative estimation of penicillin entities. J. Bact. 57: 101 - 109. 1949.

(45) Knoop, F. Klin. Worschr. 2: 60. 1923. Cited in Krebs, H. A. Adv. in Enz. 3: 191 - 252. 1943.

(46) Krebs, H. A. Intermediate metabolism of carbohydrates. Nature: 138: 288. 1936.

(47) Krebs, H. A. The intermediary stages in the biological oxidation of carbohydrate. Adv. in Enz. 3: 191 - 252. 1943.

(48) Krebs, H. A. and Johnson, W. A., The role of citric acid in intermediate metabolism in animal tissues. Enzymologia 4: 148 - 156. 1937.

(49) Lacourt, A., Sommereyns, G., Degeyndt, E., Baruh, J., and Gillard, J. Quantitative inorganic paper chromatography. Nature 163: 999 - 1000. 1949.

(50) Lipmann, F. Fermentation of phosphogluconic acid. Nature 138: 588. 1936.

(51) Lockwood, L. B., and Stodola, F. H. Fermentation process for production of α-ketoglutaric acid. U.S.Patent 2, 443, 919. 1948.

(52) Lockwood, L. B., Tabenkin, B., and Ward, G. E. The production of gluconic acid and 2-keto-gluconic acid from glucose by species of Pseudomonas and Phytomonas, J. Bact. 42: 51 - 61. 1941.

(53) Lockwood, L. B., Ward, G. E., Stubbs, J. J., Roe, E. T., and Tabenkin, B. Fermentation process for the production of 2-ketogluconic acid. U.S.Patent 2, 227, 716. 1941.

(54) Lugg, J. W. H., and Overell, B. T. Partition chromatography of organic acids on a paper sheet support. Nature 160: 87 - 88. 1947.

(55) Markham, R., and Smith, J. D. Chromatography of nucleic acid derivatives. Nature 163: 250 - 251. 1949.

(56) Maw, G. A. Identification of creatine by partition chromatography. Nature 160: 261. 1947.

(57) Maw, G. A. The detection of creatine and creatinine by partition chromatography. Biochem. J. 43: 139 - 142. 1948.

(58) Meyerhof, O. Intermediate carbohydrate metabolism. Symp. on Resp. Enz. University of Wisconsin Press. 3 - 15. 1941.

(59) Muller, Biochem Z. 213: 211. 1931. Cited in Green, D. E. Mechanisms of biological oxidations.

(60) Neuberg, C., and Kobel, M. Uber den mechanismus des Abbaus von Zucker durch das Thermobakterium mobile, Lindner. Biochem. Z. 243: 451 - 460. 1931.

(61) Neuberg, C., and Kobel, M. Weiteres uber biochemische Leistungen des Thermobakterium mobile, Linder. Biochem Z. 247: 246 - 248. 1932.

(62) Ney, P. W. A study of the intermediate metabolism of P. aeruginosa. Thesis for M.S.A. degree. Dept. of Dairying. U.B.C. 1948.

(63) Norris, M. E. A study of the oxidative activities of aerated cells of P. aeruginosa. Thesis for B.S.A. degree. Dept. of Dairying. U.B.C. 1949.

(64) Partridge, S. M. Application of the paper partition chromatogram to the qualitative analysis of reducing sugars. Nature 158: 270 - 271. 1946.

(65) Partridge, S. M. Filter paper partition chromatography of sugars. 1. General description and application to the quantitative analysis of sugars in apple juice, egg white and foetal blood of sheep. Biochem. J. 42: 238 - 253. 1948.

(66) Pervozvanskii, V. V. Formation of gluconic acid during the oxidation of glucose by bacteria. Microbiology (U. S. S. R.) 8: 149 - 159. 1939.

(67) Quastel, J. H., and Whetham, M. D. The equilibria existing between succinic, fumaric, and malic acids in the presence of resting bacteria. Biochem. J. 18: 519 - 534. 1924.

(68) Reiner, J. M., Gest, H., and Kamen, M. D. The effect of substrates on the endogenous metabolism of living yeast. Arch. Biochem. 20: 175 - 177. 1949.

(69) Robertson, W. B. The preparation of sodium pyruvate. Science 96: 93 - 94. 1942.

(70) Rockland, L. B., and Dunn, M. S. A capillary test tube method for separating amino acids by filter paper chromatography. Science 109: 539 - 540. 1949

(71) Schreder, K. R., Brunner, R., and Hampe, R. Pseudomonas lindneri Kluyver (Termobacterium mobile Lindner)Seine aerobe unde anaerobe Garung mit besonderer Berusksichtigung seiner Alkoholbildung. Wocschr. Brau. 50: 43 - 48; 233 - 237; 243 - 245. 1933. 51: 241 - 245; 249 - 253. 1944

(72) Shriner, R. L., and Fuson, R. C. The systematic identification of organic compounds. John Wiley and Sons. 1940.

(73) Stanier, R. Y. Simultaneous adaptation: a new technique for the study of metabolic pathways. J. Bact. 54: 339 - 348. 1947.

(74) Sternberg, H. E. The use of the two phase chromatogram in columns and on paper. Ferm. J. Club. Dept. of Biochem. University of Wisconsin. Report 13. 1949.

(75) Stier, T. J. B., and Stannard, J.M. A kinetic analysis of the endogenous respiration of baker's yeast. J. Gen. Physiol. 19: 461 - 477. 1936.

(76) Strain, H. H. Chromatographic Adsorption analysis. 1942.

(77) Sullivan, M. Synthetic culture media and the biochemistry of bacterial pigments. J. Med. Res.14: 109 - 160. 1905.

(78) Sumner, J. B., and Somers, G. F. Chemistry and methods of enzymes. 2nd Ed. New York: Academic Press. 1947.

(79) Szent - Gyorgyi, A. Z. Physiol. chem.235: 1. 1936. 242: 1. 1936. Cited in Krebs, H. A. Adv. in Enz. 3: 191 - 252. 1943.

(80) von Szent - Gyorgyi, A. Studies on biological oxidation and some of its catalysts. Leipzig. 1937.

(81) Thunberg, T. Skand. Arch. Physiol. 40: 1. 1920. Cited in Krebs, H. A. Adv. in Enz. 3: 191 - 252. 1943.

(82) Toeniessen, E., and Brinkmann, E. Z. physiol. chem. 187: 137. 1930. Cited in Krebs, H. A. Adv. in Enz. 3: 191 - 252. 1943.

(83) Umbreit, W. W., Burris, R. H., and Stauffer, J. F. Manometric techniques and related methods for the study of tissue metabolism. Minneapolis. Burgess Publishing Co. 1945.

(84) Vischer, E., and Chargaff, E. The separation and quantitative estimation of purines and pyrimidines in minute amounts. J. Biol. Chem. 176: 703 - 714. 1948.

(85) Williams, R. J., and Kirby, H. Paper chromatography using capillary ascent. Science 107: 481 - 483. 1948.

(86) Winsten, W. A., and Eigen, E. Paper partition chromatographic analysis of microbial growth factors. Proc. Soc. Exp. Biol. Med. 67: 513 -517. 1948.

(87) Wood, A. J., and Gunsalus, I. C. The production of active resting cells of streptococci. J. Bact. 44: 333 - 341. 1941.

(88) Wood, H. G., and Werkman, C. H. The utilization of CO_2 by the propionic acid bacteria in the dissimilation of glycerol. J. Bact. 30: 332. 1935.

(89) Wood, H. G., and Werkman, C. H. The utilization of CO_2 in the dissimilation of glycerol by the propionic acid bacteria. Biochem. J. 30: 48 - 53. 1936.

(90) Yudkin, J. Enzyme variation in micro-organisms. Biol. Rev. Cambridge Soc. 13: 93. 1938.