STUDIES ON THE BACTERIAL UTILIZATION OF PHENOL AND RELATED COMPOUNDS

By

Norman Kramer

Thesis submitted to the Faculty of the Graduate School of the University of Maryland in partial fulfillment of the requirements for the degree of Doctor of Philosophy

1950

UMI Number: DP70438

All rights reserved

INFORMATION TO ALL USERS
The quality of this reproduction is dependent upon the quality of the copy submitted.

In the unlikely event that the author did not send a complete manuscript
and there are missing pages, these will be noted. Also, if material had to be removed,
a note will indicate the deletion.

UMI DP70438

Published by ProQuest LLC (2015). Copyright in the Dissertation held by the Author.

Microform Edition © ProQuest LLC.
All rights reserved. This work is protected against
unauthorized copying under Title 17, United States Code

ProQuest LLC.
789 East Eisenhower Parkway
P.O. Box 1346
Ann Arbor, MI 48106 - 1346

TABLE OF CONTENTS

155869

TABLE OF CONTENTS (continued)

LIST OF TABLES

LIST OF FIGURES

CHAPTER I

INTRODUCTION

It is an undisputed fact that no compound, however complex, has ever accumulated to any great extent on the surface of this planet. Had this not been valid, one must certainly admit that over the millions of years of this earth's existence some compounds would have undoubtedly accumulated to such magnitude as to disturb the natural balance that now exists.

The advance of microbiology provided man with techniques which were to prove useful in helping solve some of the problems that underlie the degradation of wastes and thus perpetuate the natural turnover of matter. The process of mineralization was not generally appreciated by early workers in the field of microbiology since the relation of bacteria to disease was of such importance that more research work was given to the process of destroying bacteria than in controlling them for any useful purposes. Thus arose the use of various chemical microbicidal agents. It is a well known fact that phenol and its derivatives are effective agents in the destruction of bacteria, however, phenols are also decomposed by some microorganisms.

It is the purpose of this thesis to re-evaluate the extent of utilization of various ring compounds in the hope of making some correlation between the chemical configuration and utilization of these compounds. An attempt will also be made to investigate the possible metabolic pathway of phenol oxidation.

CHAPTER II

HISTORICAL

This investigation was pursued with two distinct objectives in view; thus for the purpose of clarity and simplicity, the presentation of the historical background is divided into two sections. The first deals with the utilization of various compounds and the second is devoted to those investigations concerned with explaining the possible mechanism of phenol oxidation.

The degradation of ring compounds through biological means was first suggested by the observations that phenol, toluene, xylol and the cresols disappeared when applied to a variety of soils (Russell and Hutchinson, 1909, and Störmer, 1908). This phenomenon was also noted by Fowler, Ardern and Lockett (1911) when they observed that phenol failed to accumulate in sewage. These workers may be considered to be the first to have actually isolated an organism capable of attacking phenol. This organism resembled Flavobacterium helvolum but at that time was called Bacillus fluorescens. Wagner (1914) isolated seven other organisms, which were not clearly described but which were capable of decomposing ring compounds, and also observed the ubiquitous distribution of these organisms in nature.

This work was followed by quantitative studies of phenol disappearance by other investigators. Buddin (1914) observed that the presence in soil of a number of volatile and non-volatile substances significantly influenced the bacterial populations. He noted an initial decrease in the population which was followed by a greater sustained increase. The phenomenon of phenol disappearance was subsequently

confirmed by Sen Gupta (1921) who noted that 99 per cent of added phenol disappeared from the soil in approximately five days. He also observed when successive equal quantities of phenol were added to the same sample of soil, each succeeding quantity disappeared more rapidly than the preceding one. Furthermore, when the soil was either autoclaved or treated with mercuric chloride prior to the phenol application, there resulted no appreciable decrease in the amount of phenol. This evidence indicated that the degradation of phenol and other ring compounds was accomplished by the presence of viable microorganisms in the soil.

As a consequence of these findings, den Dooren de Jong (1926) investigated a wide variety of compounds which were capable of supporting the growth of Pseudomonas fluorescens. He employed the method of incorporating a single known carbon source in a simple synthetic salt agar medium. This work, despite its all-inclusive aspects, was noted to include only a very few strains which were able to attack phenol.

The practice of applying to the soil insecticides containing phenols, toluene and other aromatic compounds prompted many investigators to study more fully the effect of these compounds on the microflora of the soil and to isolate, for metabolic studies, pure cultures of these organisms.

The first investigations on the utilization of these types of insecticides was undertaken by Gray and Thornton (1928) who isolated a variety of organisms from the soil with the ability to attack phenol, cresols, naptholene, phloroglucinol, resorcinol and toluene. These organisms, though not completely studied, were tentatively classified into five families: Coccaceae, Mycobacteriaceae, Bacteriaceae, Spirillaceae, and Bacillaceae. A new genus, Mycoplana, was recognized which included organisms characterized as small, gram negative, motile branching cells.

The outstanding characteristic, however, was the ability of these bacteria to utilize phenol as the sole source of carbon.

After Gray and Thornton's work, there appeared periodically papers which dealt with utilization of various compounds by a number of different microorganisms. Most of the papers which are considered pertinent to this thesis are here briefly reviewed.

Happold and Key (1932) isolated a vibrio from gaswork liquor which utilized 95 per cent of 0.04 per cent phenol in 10 days. The first thermophilic phenol-oxidizing organism was reported by a Russian worker, Egorova (1946). The microbial utilization of petroleum and its by-products was known for a long time (Söhngen, 1913), but in recent years greater significance has been attached to this problem. Grant and Zobell (1942) claimed to have isolated organisms from the sea which tolerated solutions of tricresol and phenol and grew in the presence of gasoline, toluene and kerosene. These organisms belonged to the genera Proactinomyces, Pseudomonas and Mycobacterium. Stone, Fenske and White (1942) isolated gram negative rods classified as Pseudomonas, Achromobacter and Alcaligenes which had the ability to attack hydrocarbons like paraffin and various oil fractions. These investigators noted that continued cultivation of these organisms on nutrient agar resulted in a marked decrease in ability to attack hydrocarbons.

A study of the literature prior to 1942 indicated that most of the workers were concerned primarily with determining the number of compounds supporting the growth of specific organisms. In recent years, however, utilization experiments were conducted with the aim of revealing some of the effects of positional substitution and to indicate the general correlation that existed among the types of compounds attacked.

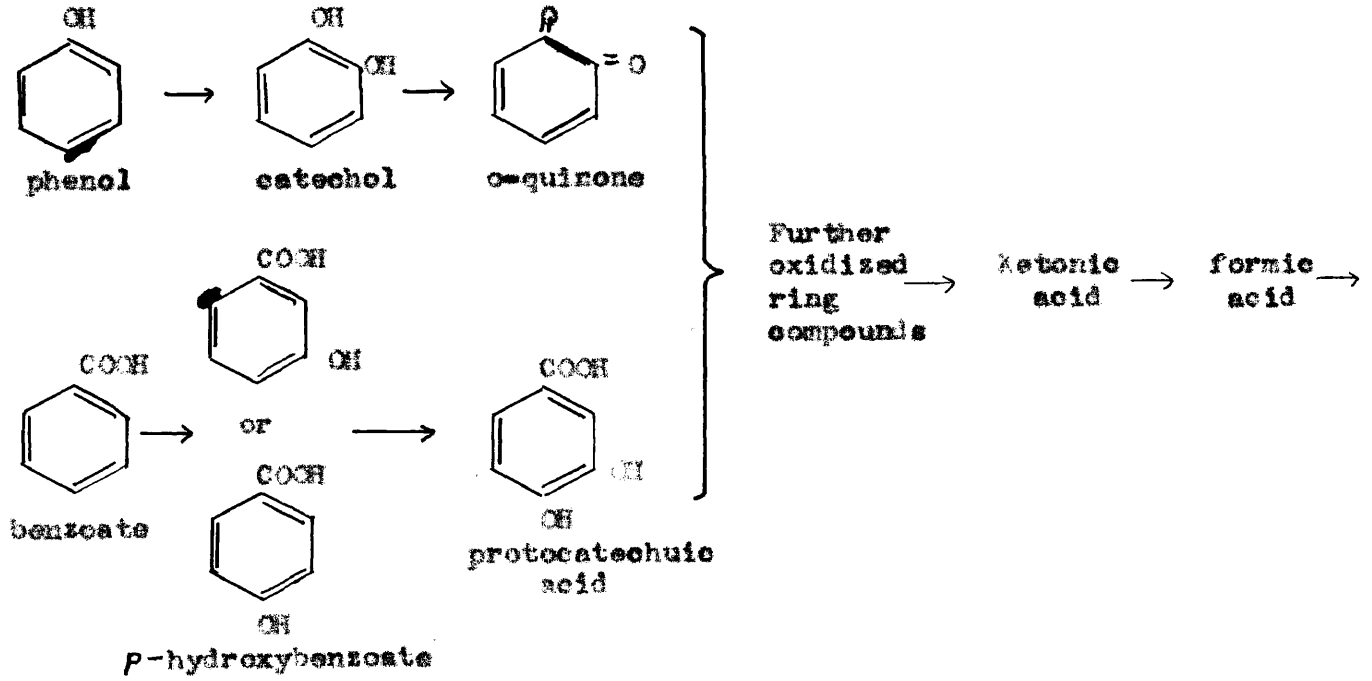
For instance, Bernheim (1942) working with Mycobacterium on the oxidation of benzoic acid and related compounds contended that the position of the hydroxyl group was most important in the oxidation of benzoic acid derivatives. He also showed that pathogenic strains differed from non-pathogenic strains in their ability to attack certain compounds.

Two important papers devoted to the relationship between structure and utilization appeared in the last three years (Evans, 1947; Czekalowski and Skarzynski, 1948). The significance of these papers was greatly weakened by the fact that only one strain or species was employed in the utilization experiments on the various compounds, thus inclusive generalizations could not be justified. These investigators concluded that unsubstituted aromatic compounds, phenolic ethers and introduction to the benzene ring of sulfonic, chloro, nitro and amino groups made the compounds unavailable for attack.

In an attempt to reveal the possible mechanism of the degradation of phenol, various laboratory techniques have been employed each of which involved the use of all types of aromatic compounds. For instance, Tausson (1928) who used growth experiments, speculated that saliginin, salicylic acid and catechol were possible intermediates in the degradation of the triphenyl compound, phenanthrene. This was deduced by the crude method of noting the ability of the organism to grow on these particular substances and ruling out all other compounds by either non-utilization or lack of logical chemical relationship between the substrate and intermediate. Wappold (1930) also showed that catechol in addition to o-benzoquinone could be detected as early oxidative products of phenol. However, the failure to produce colored substances in the oxidation of benzoic acid prompted Bernheim (1942) to suggest

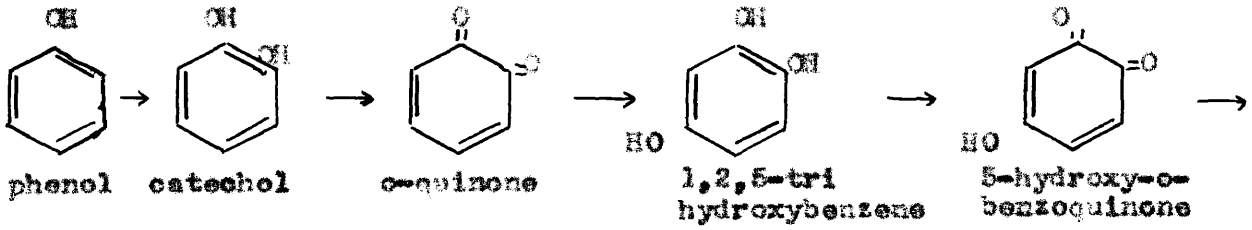
that quinones were not formed as intermediates. Using manometric studies he concluded that salicylic acid was a possible intermediate in the breakdown of benzoic acid.

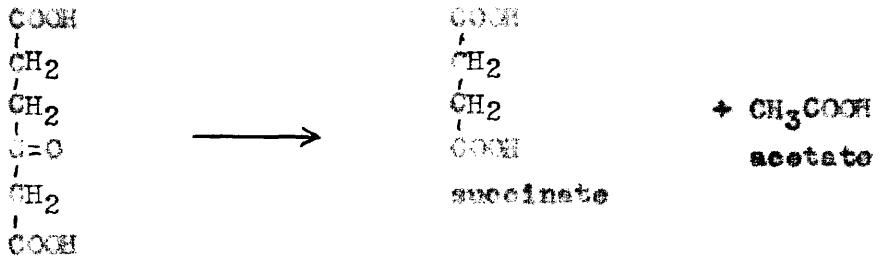
Employing manometric methods and chemical isolation techniques Nelson and Dawson (1944) proposed that phenol was oxidized via catechol and quinone. Evans (1947) used somewhat the same methods as Nelson and Dawson plus isolation of the intermediates and at first proposed the following breakdown scheme:



Later, however, Evans (1950) deleted protocatechuic acid as a possible intermediate in benzoate oxidation and offered no other substitute. This scheme was not in agreement with that of Bernheim (1942) who maintained that salicylic acid (o-hydroxybenzoate) was an intermediate instead of the meta or para forms. This discrepancy may have been due to the difference in the organisms used by the two workers.

Further elucidation on the oxidation of phenol was provided by Kilby (1948) who isolated an unstable keto-acid which he identified as β-ketoadipic acid. With this additional knowledge Kilby prepared his own scheme for the oxidation of phenol.





β-ketoadipate

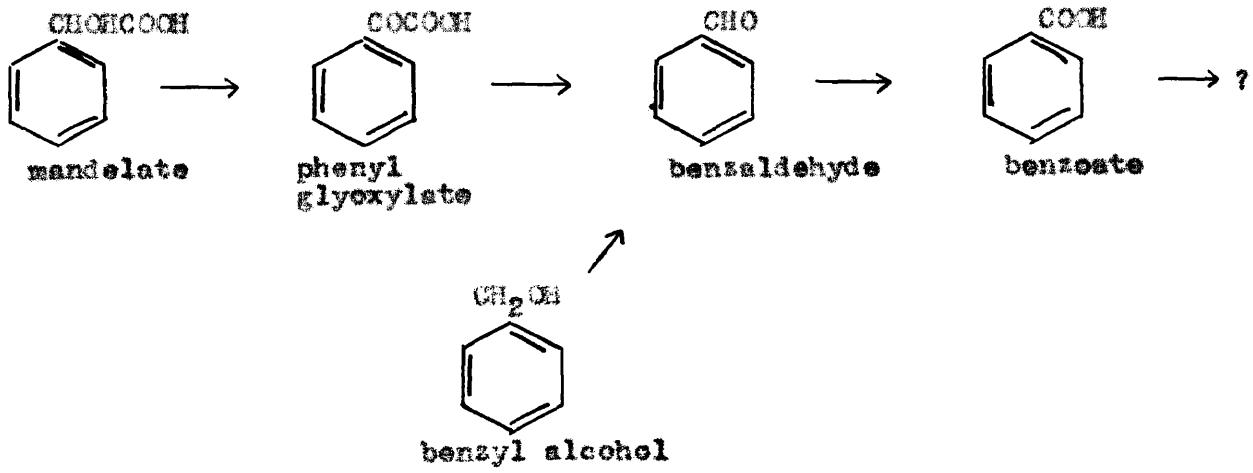
In the last few years with the use of adaptation experiments additional evidence has been accumulated on the metabolic pathway of phenol oxidation. Adaptation in this paper implies that a specific enzyme is manifested only when the cells are present in the homologous substrate. This phenomenon was first observed by Fortman (1882) who noted that the enzyme, invertase, was produced only when starch was present in the medium. This is in contrast to the condition where invertase was produced by cells regardless of whether or not sucrose was present in the medium.

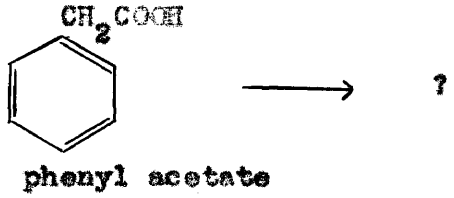
After this period a group of workers re-examined these observations and attempted to study the effect of pH, resting cells, dried cells and various inhibitors on the adaptive mechanism (Karström, 1937; Stephenson and Stickland, 1932, 1933; Yudkin, 1938; Evans and Handley, 1940). A summation of the significance of these investigations was made by Dubos (1940) in a review of production of adaptive enzymes by bacteria.

Stanier (1947) used the adaptation mechanism in a technique for the study of metabolic pathways. This technique was employed as the basis of a portion of this thesis, and as such it was deemed important to list the basic principles that underlie this technique.

1. Every dissimilation is the result of a series of simple, chemically intelligible step-wise reactions.
2. Enzymatic specificity must be accepted.
3. Lack of adaptation is not due to a permeability effect.
4. The degradation of the substrate must take place by oxidation.
5. Cells adapted to attack the primary substrate should be adapted simultaneously to attack all the intermediates formed during the oxidation of that substrate.
6. Each step in a reaction chain is under adaptive enzymatic control.
7. Postulated chemical intermediates must be made available for study.

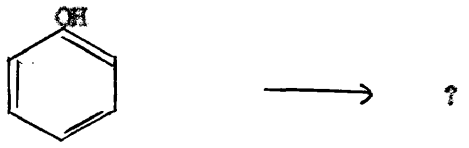
When this technique was applied by Stanier (1947, 1946) it was found that Pseudomonas fluorescens possessed four separate oxidative mechanisms involving a number of very similar aromatic compounds. This scheme is presented diagrammatically as follows:





phenyl acetate

p-hydroxybenzoate

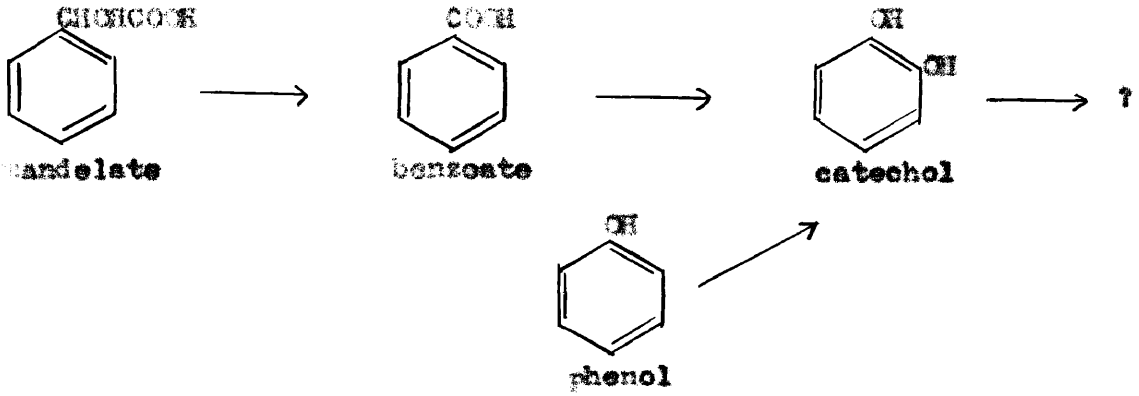


phenol

It was of interest to note that of 20 strains of Pseudomonas that utilized benzoate only 5 strains had the ability to grow in the presence of phenol. It should be noted that Stanier failed to consider hydroxyl compounds as possible intermediates in the breakdown of ring compounds as has been suggested by other previous workers.

In view of the findings of Evans, and Nelson and Dawson who suggested that catechol was a possible intermediate in the oxidation of phenol, Sleeper and Stanier (1950) again examined, by the simultaneous adaptation technique, the role of catechol as an intermediate. The latter observed that cells adapted to mandelate, benzoate and phenol were simultaneously adapted to catechol and thus suggested that catechol

was a common intermediate of all three substrates. Similar evidence indicated that protocatechuic acid was an intermediate in the oxidation of p-hydroxybenzoic acid and not an intermediate in benzoate oxidation. These later findings were not in agreement with those of Evans in which he stated that both p-hydroxybenzoate and protocatechuic acid were intermediates in benzoate oxidation. Cleeper and Stanier's concepts were formulated in the following scheme:



The historical aspects so far were primarily concerned only with the possible changes that were involved within the ring nucleus with very little emphasis on what occurred after the ring had been broken. It was the hope that this gap in the metabolic pathway could be filled using the recent advances in the knowledge of the mechanism of the cyclophorase system. This cycle was known to be operative in the breakdown of carbohydrates, fats and proteins by enzymatic action of many animal tissues (Green, Loomis and Auerbach, 1948; and Baldwin, 1949). Thus it was thought that the cycle may be operative in the bacterial breakdown of aromatic compounds.

Lenti (1948) claimed to have evidence supporting the existence of this cycle in Escherichia coli. He demonstrated inhibition of succinate oxidation while the oxidation of pyruvate was not affected. On the other hand, Karlson and Barker (1948), using the technique of adaptation, submitted evidence against the occurrence of this cycle in Azotobacter agilis. These results have shown the existence of a reaction chain from α-ketoglutarate through succinate, fumarate, malate and pyruvate to acetate. This reaction would support the theory that oxidation of acetate and pyruvate was via part of the cyclophorase cycle. But since the more oxidized members did not cause adaptation to all the higher ones, as the cyclophorase cycle theory required, the cycle appeared to be non-operative in this oxidation scheme. This conclusion was further substantiated by the fact that they could not detect in the medium formation of succinate and oxalacetate from acetate when radioactive tracers were employed.

The ability of an organism to utilize the various components of this cycle was frequently used in the past to indicate the possibility of the organisms oxidizing its substrate via the cyclophorase cycle. For instance, Lewis (1948) working with Neurospora mutants noted that succinic acid or a closely related acid was required for growth. This mutant utilized the following components of the cycle: glutamate, aspartate, succinate, fumurate, malate, α-ketoglutarate, oxalsuccinate, acetate and acetoacetate. It failed to utilize pyruvate, oxalacetate, citrate, isocitrate, cis-aconitate, lactate and formate. From these data he contended that the genetic block occurred somewhere in the cyclophorase cycle.

From this brief review of the literature, it is apparent that there are conflicting opinions and insufficient data on many of the aspects of the bacterial utilization of phenol. In the following pages are presented data supplementing and extending the above work.

CHAPTER III

EXPERIMENTAL METHODS

Media. A chemically defined basic medium described by Gray and Thornton (1928) was used in the initial stages of this work. It was of the following composition:

	per cent
Dipotassium phosphate	0.1
Magnesium sulfate	0.02
Sodium chloride	0.01
Calcium chloride	0.01
Ferric chloride	0.002
Ammonium sulfate	0.1

pH 7.2 to 7.6

Each of these chemicals was prepared in such a concentration that when one ml of each was added to 93 ml of sterile distilled water the desired concentration was obtained. The salts were autoclaved separately, except the ferric chloride which was prepared by adding 0.2 mg to 100 ml of sterile distilled water. In the later stages of this work, ferric chloride and calcium chloride were observed not to be required for growth and were deleted from the basic medium. Solid medium was made by incorporating the basic salts in 2.0 per cent sterile agar. The carbon source compounds were added in concentrations varying from 0.001 M to 0.01 M without sterilization and when necessary were adjusted, prior to incorporation in the basic medium, to pH 7.2 to 7.6 with 0.1 N sodium hydroxide. All carbohydrates and stock vitamin solutions were sterilized by filtration through sintered glass filters.

Cultures. Two known species of Mycoplana were obtained from Dr. R.W.G. Gray of McGill University, one of the investigators who originally described these organisms; and two from the American Type

Culture Collection, Washington, D. C. The phenol-utilizing strains described in this thesis were isolated from arable soils located in the near vicinity of the University of Maryland. Approximately one gram portions of soil were added to 250 ml Erlenmeyer flasks containing the basic medium plus 0.007 M phenol as the sole source of carbon. After three days at 30 C, 0.1 ml was subcultured to a flask of fresh sterile medium. This procedure was repeated three times. From the third flask, one loopful was streaked on basic medium containing 2.0 per cent agar and 0.007 M phenol. Representative colonies were picked and inoculated into tubes containing the basic salts plus 0.007 M phenol. All strains that grew well were maintained as stock cultures on 0.007 M phenol agar slants and stored at 4 C.

Determination of Phenol. The quantitative estimation of phenol when present in the medium was determined by the colorimetric method used by Gomori (1949) with modifications. The two basic reagents required for this test were an anisole reagent and a borax solution which were prepared as follows:

Anisole Reagent: Into 50 ml of ice cold water was added approximately 0.5 gram of 5-nitro-2-aminoanisole (Eastman). The solution was filtered through paper. One ml of 5.0 per cent sulfuric acid and 1 ml of 1.0 per cent sodium nitrite were added to the filtrate. This was kept cold prior to its use.

Borax Solution: Thirty-five grams of sodium borate were added to 1000 ml of 15 per cent ethyl alcohol.

Procedure: A standard linear graph as shown in figure 1 was obtained by preparing known concentrations of phenol in which 3 ml contained 0 to 30 micrograms.

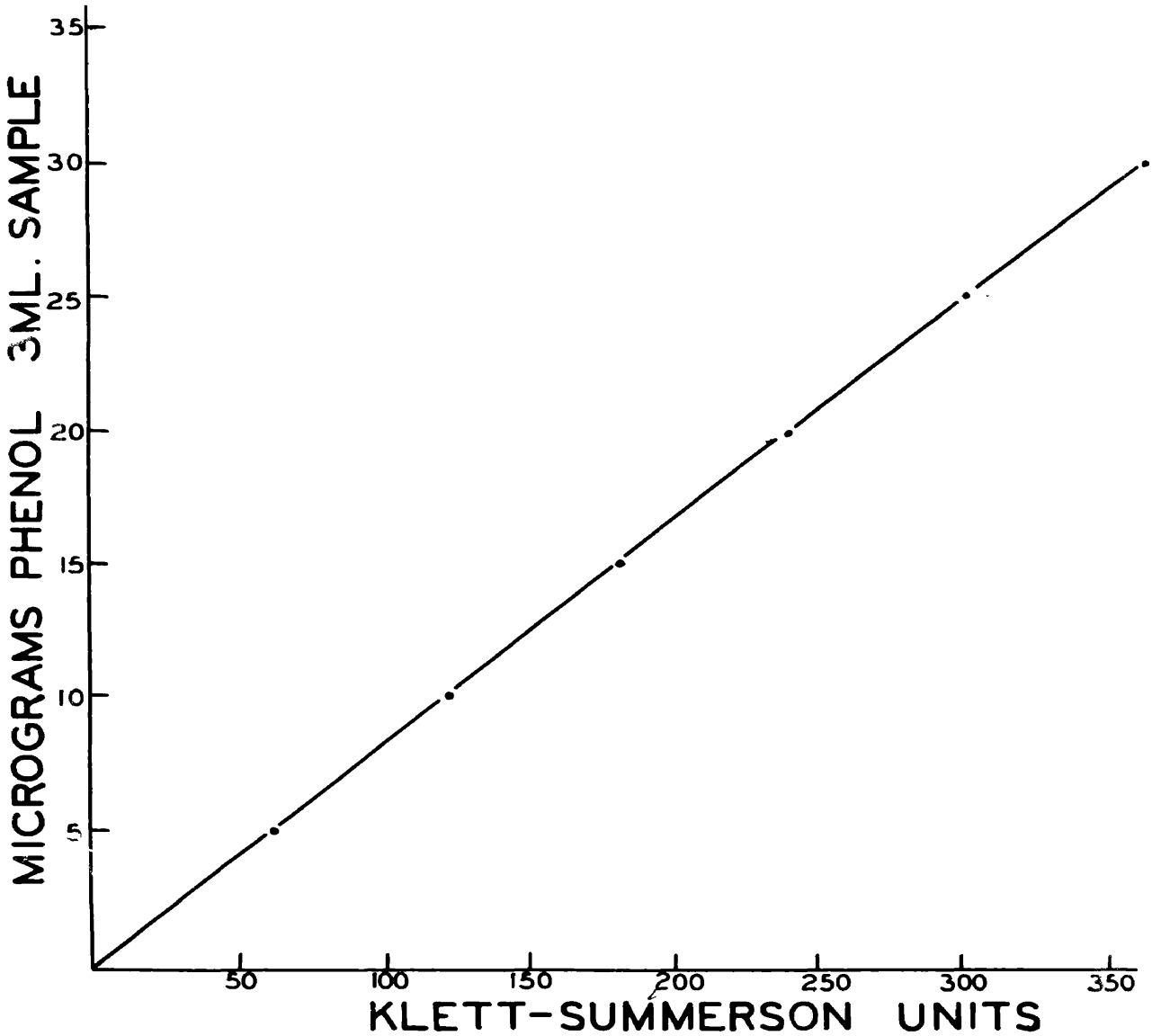


Figure 1. Relation between intensity of color developed and amount of phenol present in a 3 ml sample.

To this amount of standard phenol solution, 2.5 ml of borax solution and 2 ml of water were added and the contents mixed. Then 1.5 ml of aniaole reagent were added and mixed. The intensity of red color which developed was measured in the Klett-Summerson photoelectric colorimeter using a green filter (500-570 mμ). When culture medium was used, the medium was cleared of its turbidity by centrifugation and the supernatant fluid tested in the same manner as above.

Estimation of Growth on Test Compounds. Utilization of the compounds was determined by the method of den Dooren de Jong (1926). Agar plates were prepared by the addition of salts and the specific carbon compound. These plates were streaked with the various organisms. The amount of growth observed on agar plates containing the specific carbon compound was compared with the growth observed on the control plates not containing the compound. In most cases no macroscopic growth was observed on the control plates; however, if growth did appear then the compound under test was considered positive only when there was distinctly a much greater amount on the agar containing the compound. All plates were incubated at 30 C for 5 days.

When the organism was grown in liquid medium, growth was estimated turbidimetrically and measured with the Klett-Summerson photoelectric colorimeter using a blue filter (400-465 mμ).

Morphology and Physiology of Cultures. Morphological studies were performed using a 24 hour culture grown in the basic medium plus 0.007 M phenol. Wet preparations were examined for motility and general structure using the phase microscope. Most of the staining techniques and biochemical tests employed were those as described in the Manual of

Methods for Pure Culture Study (1948). The arrangement of the flagella was determined by the method of Vaneval (1931) and ammonia production was detected by the methods of Hansen (1930).

Fermentation of carbohydrates, alcohols, and glucosides were tested by the addition of these substances, previously filtered, to sterile 1.0 per cent trypticase broth. These determinations were made after 15 days at 30 C.

Optimum temperature requirements were determined by inoculating one loopful of organisms into a tube containing trypticase soy broth in such a manner as to avoid any culture material adhering to the sides of the tube. These were incubated at 5 C, 20 C, 30 C, 37 C, 45 C, 55 C, 60 C and examined for growth as indicated by turbidity every 24 hours for six days. Those cultures tested at 45, 55, and 60 C were carefully removed and placed at 30 C for further incubation. This was done for the purpose of determining whether the failure to grow was the result of an inhibitory effect or an actual destruction of the bacteria at these temperatures.

pH Determination. In most instances when only an approximate pH was required the colorimetar method was employed using brom thymol blue as an indicator. Exact determinations were made with the glass electrode.

Manometric Experiments. The cells necessary for manometric studies were grown in 500 ml of liquid medium containing the basic salts, excluding ferric chloride and calcium chloride, plus the specific carbon compound. The inoculated medium was placed on a shaking machine (3 inch stroke, 120 oscillations per minute) for 24 to 48 hours at 30 C. The cells were harvested by centrifugation, washed twice in Sörensen's 2/15 phosphate buffer (pH 7.0) and resuspended in the same buffer. To the

M/15 phosphate buffer was added magnesium sulfate, 0.02 per cent and sodium chloride, 0.01 per cent. One ml of this cell suspension, usually having a turbidity of 600 Klett units, was used in each Warburg vessel. In most cases one ml of a 0.002 M substrate was added from the side arm and 0.2 ml of 10 per cent potassium hydroxide was placed in the center well. Oxygen consumption of the cell suspension was measured in the usual way at 30 C in an atmosphere of air.

The compounds which were considered stable were prepared in 100 ml quantities (0.002 M) and stored at 4 C for further use. The unstable compounds were prepared each time just prior to its need and placed immediately into Warburg flask side arms. All substrates were added to the side arm in 1 ml quantities containing 2 μ M except oxalacetate which was added in approximately 2 μ M per ml.

All compounds of the cytochrome cycle were stored under vacuum in sealed glass bell jars containing calcium chloride.

CHAPTER IV

RESULTS

A. Observations on the growth of *Mycoplana* on phenol

All of the four known cultures of *Mycoplana*, two of *M. bullata* and two of *M. dimorpha* failed to grow in 0.007 M phenol medium. Concentrations of phenol in the basic synthetic medium varied from 0.01 M to 0.001 M. The possibility that phenol was toxic to the organisms was ruled out when growth occurred in a medium containing 1 per cent trypticase and 0.007 M phenol. Concentrations of phenol higher than 0.007 M were toxic to all strains. These strains conformed to the cultural and biochemical characteristics as reported by Gray and Thornton (1928), but the characteristic branching of cells was rarely observed in any of the strains. When branching did occur it was not uniformly present in all subcultures, and was not consistent with regard to the age of the culture.

In replacing phenol in the basic synthetic medium with the following carbon sources: arabinose, glucose, lactose, maltose, cellobiose, sucrose, raffinose, mannitol, glycerol, inositol and salicin, only glucose and mannitol supported the growth of *M. dimorpha*. *M. bullata* failed to grow in the presence of any of these carbon sources. When these substances were incorporated into a medium containing 1 per cent trypticase, with or without phenol, all four strains grew well. The growth of these strains was always accompanied by a slight rise in the pH.

Addition of thiamine, pyridoxine, pantothenate, niacin and para-amino benzoic acid in concentrations of 1 µg per ml and riboflavin, folic acid, and biotin in concentrations of 0.1 µg per ml failed to support the growth of any of the Mycoplana. Supplements of trypticase and yeast extract in concentrations of 5 mg per cent did not alleviate this situation.

B. Observations on phenol utilizing organisms isolated from soil

1. Quantitative estimation of phenol utilization.

In view of the failure to show any phenol utilization by Mycoplana, 14 soil organisms which grew in phenol were selected for further study. The amount of phenol utilized in the synthetic salt medium and its relationship to pH and growth of these organisms are shown in table 1. Flasks were incubated without shaking. It is seen from this table that as the phenol disappeared the amount of growth increased (higher turbidity measurements) and the pH decreased slightly from 7.45 to about 6.7.

2. Ability of specific chemical compounds to support the growth of 14 organisms.

The ability of 14 test strains to utilize phenol when present as the sole source of carbon was not uniformly observed to hold true when other aromatic compounds were substituted for phenol. When these strains were tested against 87 specific chemical compounds for ability to produce macroscopic growth, they were found to fall into three distinct groups. In tables 2 and 3 it may be seen that the groups are arranged depending on the number of strains that were able to attack any specific compound. There were 16 compounds which supported the

TABLE 1

Correlation of phenol utilization with changes in pH and growth

Organism	mg % Phenol Remaining *			Growth Response **			pH ***		
	Hours Incubation			Hours Incubation			Hours Incubation		
	24	48	72	24	48	72	24	48	72
Control	48.3	48.3	48.3	0	0	0	7.5	7.5	7.45
5	30.0	17.5	0	6	31	52	7.2	6.7	6.65
10	25.0	2.9	0	9	45	53	7.2	6.55	6.50
29	44.0	35.0	0	5	23	38	7.4	7.0	6.90
56	31.3	16.5	0	4	30	51	7.25	6.6	6.65
58	46.6	46.3	42.0	0	4	10	7.6	7.45	7.30
60	44.6	39.5	33.0	0	2	14	7.45	7.25	7.0
69	22.6	7.6	0	9	31	48	6.95	6.55	6.5
73	38.6	15.3	0	3	26	44	7.25	6.7	6.5
77	13.3	6.4	0	16	34	47	6.8	6.5	6.5
81	32.6	6.3	0	3	33	49	7.05	6.65	6.55
85	18.0	7.6	2	10	39	50	7.15	6.75	6.55
87	42.3	24.6	1	5	44	51	7.3	6.8	6.55
90	38.0	18.3	1	4	31	47	7.25	6.7	6.5
94	39.6	33.0	4	6	30	47	7.3	6.60	6.5

* Initial amount of phenol, 50 mg per cent.

** Figures represent turbidity as per cent of light absorption as measured with Klett-Summerson Electrophotometer using blue filter.

*** Initial pH 7.4.

TABLE 2

Ability of 14 phenol utilising bacteria to produce visible growth on compounds after 5 days at 30 C.

Compounds Supporting Visible Growth of All 14 Species	Compounds Supporting Visible Growth of Some of the 14 Species
Benzaldehyde	Benzoic acid (13)
Phenol acetate	Benzyl alcohol (12)
l-Tyrosine	Glycerol (12)
Ethyl alcohol	Mannitol (12)
Zinc acetate	Sucrose (12)
Sodium acetate	Glucose (12)
Potassium acetate	Benzene sulfonate (12)
dl-Alanine	Phenylalanine (12)
Lactate	α-Ketoglutarate (12)
Pyruvate	p-Hydroxybenzoate (11)
Oxalacetate	Maltose (11)
Citrate	Salicylate (10)
Succinate	Catechol (10)
Fumurate	cis-Aconitate
Malate	Cellobiose (11)
Salicin	p-Cresol (9)
	Raffinose (11)
	Pyrogallol triacetate (9)
	Lactose (9)
	Fructose (6)
	Potassium d-isocitrate (6)
	Uranium acetate (6)
	Benzamide (4)
	Inositol (4)
	o-Cresol (3)
	m-Cresol (3)
	Resorcinol (3)
	Sulfosalicylate (3)

TABLE 3

Compounds which do not support the visible growth of any of the 14 species

Compounds Not Allowing Visible Growth of 14 Species	
Saturated ring compounds:	**Miscellaneous compounds:**
Cyclohexane	Phenyl ether
Cyclohexanol	Benzil
	Diphenylamine
Benzene Derivatives:	Benzidine hydrochloride
Cl -- Chlorobenzene	o-Tolidine
-- o-Dichlorobenzene	Tannic acid
NH_2-- Aniline	p-Toluene sulfonic acid
-- p-Phenylenediamine	p-Quinone
-- p-Aminodimethylaniline	Urea
CH_3-- 2-Hydroxy-1,4-dimethylbenzene	Lignin
	Furfural
Phenol Derivatives:	Copper acetate, phenyl benzoate, arabinose, xylose
NO_2-- o-Nitrophenol	
m- "	
p- "	
2,4-Dinitrophenol	
2,6-Dinitrothymol	
3,5-Dinitro-o-cresol	
3,5-Dinitrosalicylic acid	
2,4-Dinitro-6-phenylphenol	
Picric acid	
CH_3-- 2,6-Dimethylphenol	
Orcinol	
Thymol	
C_3H_7- o-Isopropylphenol	
C_5H_{11} Diamylphenol	
OH -- Hydroquinone	
1,3,5-Phloroglucinol	
1,2,3-Pyrogallic acid	
Gallic acid	
4,6-Dimethyl resorcinol	
C_6H_5- o-Phenylphenol	

growth of all 14 strains, while none of the strains grew in 43 compounds and still another group of compounds which varied in the number of strains capable of growing in the presence of the compound. Those compounds which supported the growth of only some of the strains are shown in table 4. Examination of this table revealed no significant correlation in regard to the genera of organism and growth on any particular compound. Other correlations will be reviewed in the discussion of this paper.

3. Effect of deletion of salts from medium on growth.

In orientation experiments with phenol utilizing organisms it was necessary to determine the medium and other factors most suitable for their growth. In pursuance of this end it was observed that the amount of growth varied when media of different compositions were employed. The basic medium of Gray and Thornton (1928) was selected as the medium of choice. However, in view of the discrepancy that existed as to which salts were essential for growth and the fact that this medium had the tendency to precipitate slightly upon standing, it was deemed necessary to determine the indispensibility of each constituent. In table 5 the amount of growth (turbidity) is tabulated in relation to the complete medium and the medium with deletion of various salts. Examination of this table reveals that all species required dipotassium phosphate for growth. As the medium was adjusted to pH 7.5 with 0.1 N sodium hydroxide, it appeared that phosphate in the form of dipotassium phosphate was an essential constituent. It also was observed that the presence of magnesium sulfate was essential for the growth of at least five of these strains and provided for better growth in the remaining

strains with the exception of strain number 94. Although ferric chloride was not noted to be essential it did provide for better growth in 9 strains. Calcium chloride was not only found to be non-essential but actually inhibited the growth in the majority of cases. With the exception of the Vibrio strain number 29, sodium chloride was also noted to be non-essential. Aside from this exception no other significant correlation was noted in regard to species and its requirement for any particular salt.

4. Identification of cultures.

On the basis of morphological, cultural and biochemical characteristics the 14 organisms were classified according to Bergey's Manual (Breed et al, 1948) as belonging to three genera. Eight cultures were classified as members of the genus Achromobacter; five cultures, in the genus Micrococcus; one culture, in the genus Vibrio.

All species were gram negative, non-acid fast, non-spore forming organisms. All were unable to hydrolyse starch, liquefy gelatin and produce hydrogen sulphide. Indole was not produced and the Voges-Proskauer and methyl red tests were negative. In 1 per cent trypticase, growth of the organisms was abundant and the pH changed from the initial 7.5 to a pH of about 8.4. The following carbohydrates, alcohols, and glucosides were inoculated: arabinose, fructose, glucose, lactose, maltose, cellobiose, sucrose, raffinose, mannitol, glycerol, inositol and salicin. Of these compounds only arabinose and glucose were fermented by organisms 3, 10 and 56 when present in 0.5 per cent concentrations in trypticase broth. All other compounds were found to have a terminal alkalinity of pH 7.5. The significant differential characteristics are shown in table 6.

TABLE 4

Compounds supporting the visible growth of some of the 14 species

Compound	Achromobacter spp								Micrococci spp					Vibrio sp
	3	10	56	58	60	85	87	90	59	73	77	91	94	29
Benzoate	+	+	+	+	+	+	+	+	+	+	+	+	+	
Benzyl alcohol	(+)	(+)	+			+	+	+	+	+	+	+	+	
Benzene sulfonate	(+)		(+)	(+)		(+)	(+)	(+)	(+)	(+)	(+)	(+)	(+)	(+)
dl-Phenylalanine			+	+	+	+	+	+	+	+	+	+	+	+
∝-Ketoglutarate				+	+	+	+							+
p-Hydroxybenzoate	+	+	+			+	+	+	+	+	+	+	+	
Salicylate	+	+	+			+		+	+	+	+	+	+	
Catechol		(+)	(+)				+	+	+	+	+	+	+	+
cis-Aconitate	+	(+)	+	+	+	+	+		(+)	(+)				+
p-Cresol						+	+	+	+	+	+	+	+	+
Pyrogallol triacetate		+	+			+			+	+	(+)	(+)	(+)	(+)
D d-isocitrate				+	+	+	+							+
Benzamide		+		+	+			+						
o-Cresol						+	+							+
m-Cresol						+	+							+
Resorcinol						(+)	(+)							(+)
Sulfosalicylate						+					+	+		
Uranium acetate									+	+	+	+		+
Fructose						+	+	(+)				+	(+)	+
Glucose	+	+	(+)			+	+	(+)	(+)	(+)	(+)	(+)	(+)	+
Lactose		(+)	(+)			(+)		+	+	+	(+)	(+)	+	
Maltose		(+)	(+)			(+)	(+)	+	(+)	(+)	(+)	(+)	+	+
Cellobiose	+	+	+			+	(+)	+	+	+	+	+	+	
Sucrose	+	+	+			(+)	(+)	+	+	+	+	+	+	+
Raffinose	(+)	(+)	(+)						+	+				
Mannitol	(+)	(+)	(+)			(+)	(+)	+	+	+	+	+	+	+
Glycerol	(+)	(+)	(+)			+	+	+	+	+	+	+	+	+
Inositol		+							+	+			+	

\+ Good Growth
(+) Moderate Growth
Blank spaces indicate no growth

TABLE 5

Effect of deleting constituents of medium on growth of organisms*

Organisms	Composition of Media***							
	K_2HPO_4 $MgSO_4$ NaCl $CaCl_2$ $FeCl_3$ $(NH_4)_2SO_4$ phenol	----** $MgSO_4$ NaCl $CaCl_2$ $FeCl_3$ $(NH_4)_2SO_4$ phenol	K_2HPO_4 ---- NaCl $CaCl_2$ $FeCl_3$ $(NH_4)_2SO_4$ phenol	K_2HPO_4 $MgSO_4$ ---- $CaCl_2$ $FeCl_3$ $(NH_4)_2SO_4$ phenol	K_2HPO_4 $MgSO_4$ NaCl ---- $FeCl_3$ $(NH_4)_2SO_4$ phenol	K_2HPO_4 $MgSO_4$ NaCl $CaCl_2$ ---- $(NH_4)_2SO_4$ phenol	---- ---- ---- ---- ---- $(NH_4)_2SO_4$ phenol	K_2HPO_4 ---- ---- ---- ---- $(NH_4)_2SO_4$ phenol
3	38	0	14	40	47	11	0	0
10	45	0	8	34	34	12	0	4
23	33	0	0	9	38	16	0	0
56	21	0	2	17	30	11	0	0
58	21	0	0	10	29	4	0	0
60	21	0	0	10	20	7	0	0
69	30	0	5	23	34	19	0	7
73	31	0	7	31	41	14	0	0
77	32	0	7	34	40	22	0	0
81	32	0	8	31	40	22	0	0
85	63	0	25	45	46	41	0	16
87	58	0	20	50	58	58	0	20
90	30	0	9	29	31	15	0	0
94	26	0	23	28	38	20	0	0

* Growth was measured with Klett-Summerson Electrophotometer. Figures represent turbidity as per cent of light absorption using blue filter. Readings made at 60 hours incubation.

** Medium adjusted to pH 7.6 with 0.1 N NaOH.

*** Percentage of each ingredient the same as stated under Materials and Methods.

TABLE 6

Differential morphological and biochemical characteristics of the 14 organisms studied

Genera	Strain No.	Morphology	Motility	Nitrate Reduction	NH_3 Produced	Oxygen Requirements	Litmus Milk	Temperature Requirements 5	20,30 37	35,55 60
Achromobacter	3	rods (pairs)	-	-	-	a	acid ropy	-	+	-
	10	rods (pairs)	-	-	-	a	acid	-	+	-
	56	rods (pairs)	-	+	+	a	acid	-	+	-
	58	rods	+	+	-	a	alk	+	+	-
	60	rods	+	+	-	a	alk	+	+	-
	85	rods	+	-	+	a	alk	+	+	-
	87	rods	+	-	+	f	alk	+	+	-
	90	rods	-	-	-	a	sl. acid	+	+	-
Micrococci	69	diplococcus	-	+	-	a	alk	+	+	-
	73	diplococcus	-	+	-	a	alk	+	+	-
	77	diplococcus	-	-	-	a	alk	+	+	-
	81	diplococcus	-	-	-	a	alk	+	+	-
	94	diplococcus	-	-	-	a	alk	+	+	-
Vibrio	29	vibrio	+	+	+	a	alk	+	+	-

a = aerobic
f = facultative

The organisms were observed periodically for over a period of 96 hours for possible morphological changes and they never showed branched forms. The Achromobacter species were distinctly elongated rods and gram negative; the polar portions stained more intensely than the rest of the cell. The motile organisms, when stained by the method of Maneval (1931) were found to possess 1 to 10 flagella. Peritrichous arrangement was the usual form noted.

The cell shape remained relatively uniform throughout this period except for Achromobacter number 10 which had the tendency to form extremely elongated cells in 24 hour cultures.

All fourteen organisms when grown on trypticase soy agar slants for a period of 11 months retained the ability to utilize phenol as the sole source of carbon when present in the synthetic salt medium. Furthermore, organism number 86 was subcultured on trypticase soy agar slants on the average of every 2 to 3 days for approximately 120 transfers without the loss of its ability to utilize phenol.

C. Studies on phenol oxidation by the adaptive mechanism

1. Influence on the oxygen uptake with different phenol concentrations and when different salts were employed in washing the cells.

In order to determine whether a compound is oxidized by the adaptive mechanism, the cells when grown on a specific compound must show an immediate maximum oxygen uptake when manometrically tested against this compound. In preliminary experiments it was noted that this immediate maximum uptake varied when only approximate concentrations of phenol were employed as a substrate. Thus it was necessary at the outset to obtain conditions, carefully controlled, which consistently

produced an immediate oxygen uptake. When the concentration of phenol (as a substrate in Warburg flask) was increased from 2 µM per ml to 8 µM it had a marked effect on decreasing the rate of oxygen uptake as shown in figure 2. This same effect, although not as well marked in the lower concentrations, was noted when the cells were washed with certain salts present in the phosphate buffer solution. When the cells were washed and resuspended in only the M/15 phosphate buffer, there occurred a lower rate of oxygen uptake in contrast to that when the cells were washed with a salt-phosphate mixture. The salts used were ferric chloride, sodium chloride, magnesium sulfate and calcium chloride in concentrations as that used in the culture medium. Later work indicated that only sodium chloride and magnesium sulfate were required in the phosphate buffer solution when phenol was used as the substrate.

2. Determination of the adaptive oxidation patterns of the 14 organisms.

One of the principal aims of this work was to establish the possibility that phenol oxidation was via the cyclophorase system. In order for this to be realized it was apparent that an organism had to be selected which oxidized phenol by the adaptative mechanism. The 14 organisms were first grown in the basic salt medium containing 0.005 M ethyl alcohol to obtain so-called "unadapted cells". These cells were harvested and tested manometrically against 2 µM phenol. As shown in figure 3, all organisms showed a lag and this indicated that all 14 organisms had to adapt themselves to the phenol in order for the phenol to be attacked. The length of time that elapsed before a maximum rate of oxidation occurred varied among the organisms. It can be seen from

the graph that 8 of the 14 organisms (Nos. 10, 69, 73, 77, 81, 85, 87, 94) show a typical lag ranging from 80 to 100 minutes then the characteristic exponential rise in oxygen uptake. The other six organisms show a much longer lag. Similar unadapted cells were produced when the organisms were grown in 0.05 per cent trypticase.

When, however, the organisms were grown in the basic medium containing 0.005 M phenol as the sole source of carbon, and tested manometrically against phenol a different pattern was observed. In this case as shown in figure 4, 9 of the 14 organisms (Nos. 3, 10, 56, 58, 60, 85, 87, 90, 94) showed an immediate and maximum rate of oxygen uptake after the addition of 2 µM of phenol. In regard to these nine organisms, 8 of which are Achromobacter and one a Micrococcus, the findings at this point indicated that the oxidation of phenol was brought about by adaptive enzymes. On the other hand, the five other organisms (Nos. 29, 69, 73, 77, 81) behaved like the previous unadapted cells. Here they displayed the same initial lag followed by an exponential increase of oxygen uptake. In regard to these five organisms, the findings indicate that other factors, such as permeability, prevent their use as tools for the analysis of metabolic intermediates of phenol.

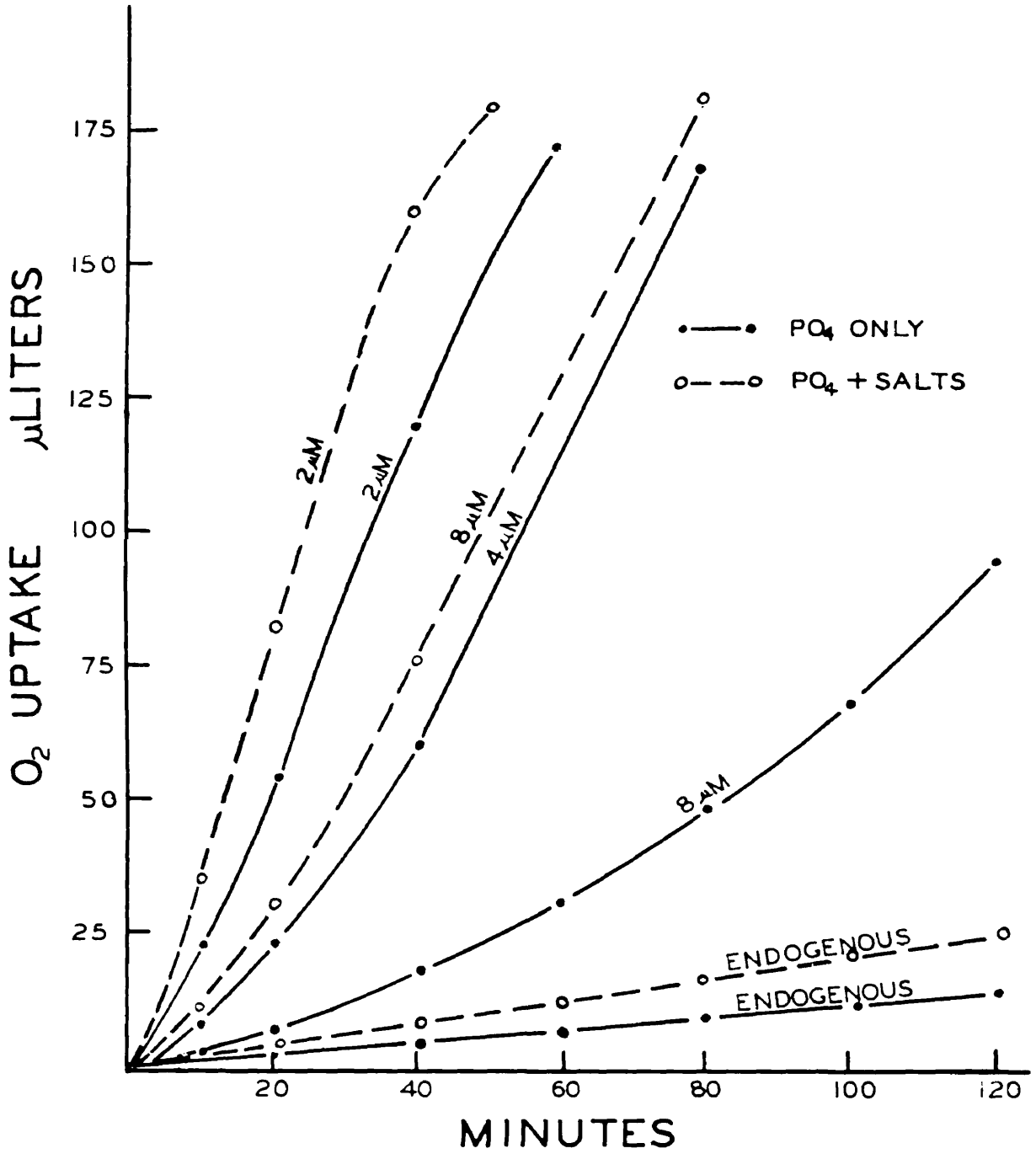


Figure 2. Influence on oxygen uptake with different phenol concentrations with different salts employed in washing cells.

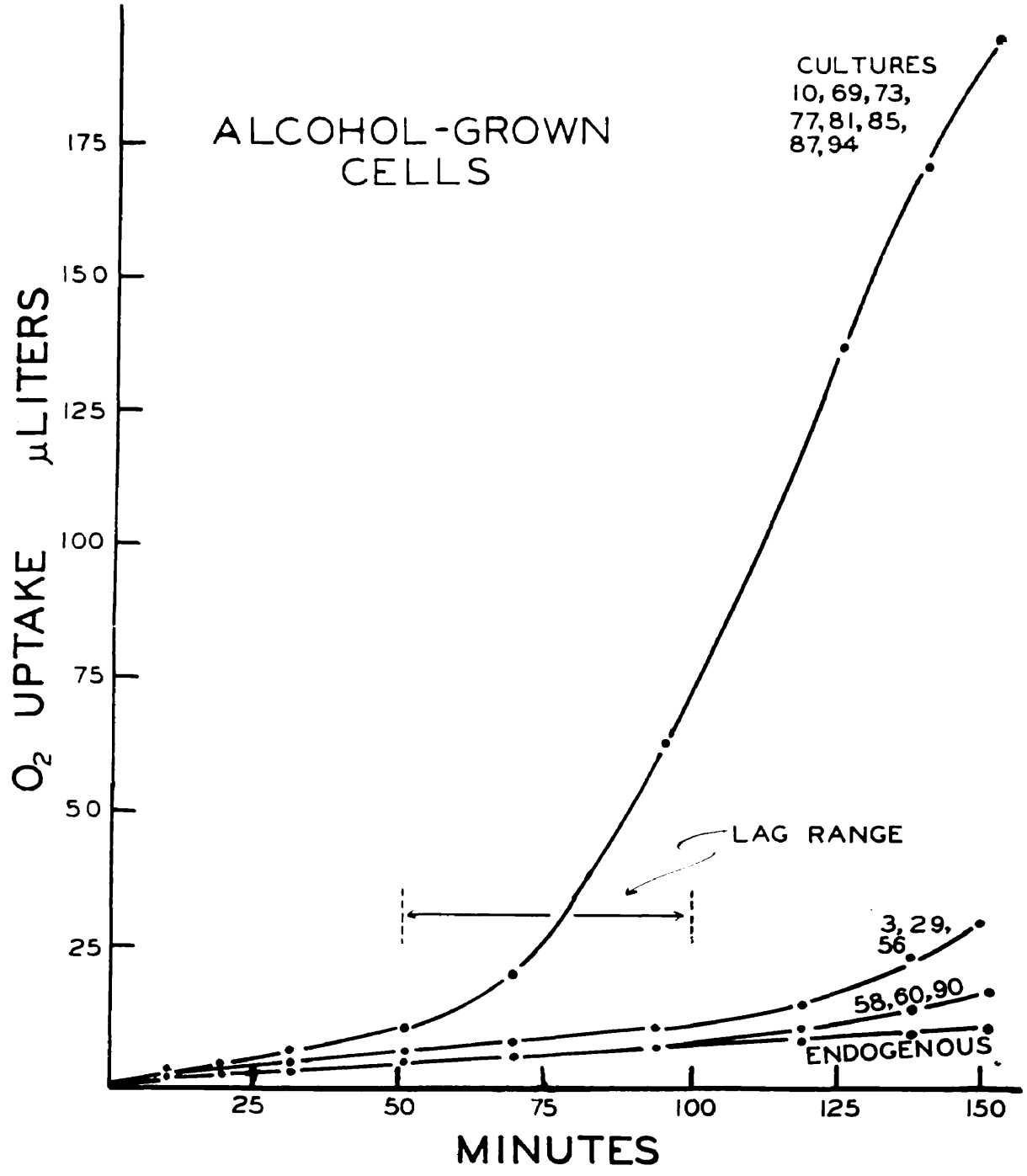


Figure 3. The oxidation of 2 micromoles phenol by 14 organisms grown on ethyl alcohol.

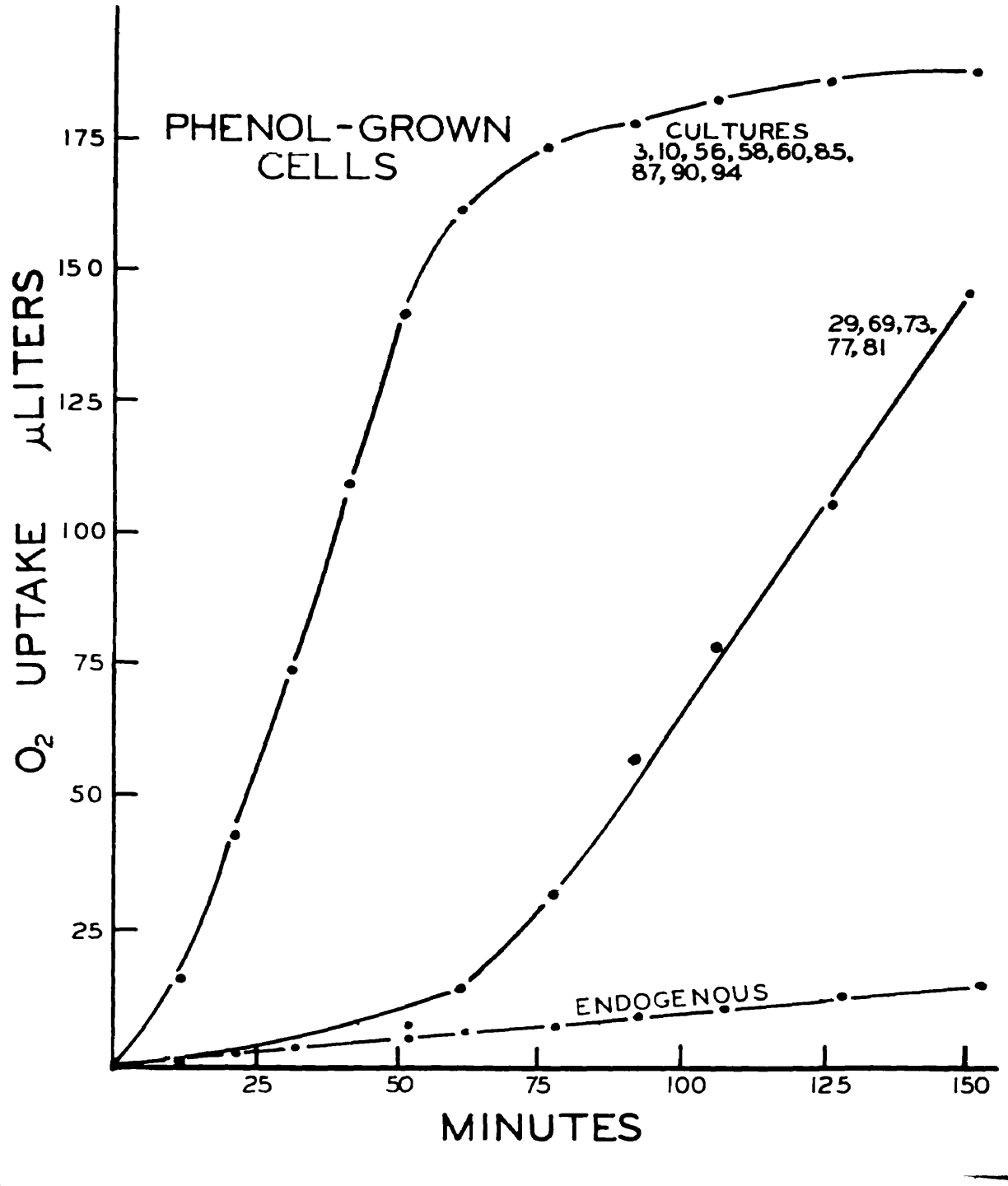


Figure 4. The oxidation of 2 micromoles phenol by 14 organisms grown in phenol.

D. The role of the cyclophorase system in phenol oxidation

1. Oxygen uptake of the cyclophorase cycle compounds by phenol-grown cells.

It has been established that the oxidation of phenol by a majority of the organisms under study was under adaptive control. In addition five of these organisms were able to utilize all of the compounds which are accepted as components of the cyclophorase cycle. On the basis of this evidence one organism, Achromobacter sp. number 85, was selected for the purpose of determining by simultaneous adaptation whether the oxidation of phenol occurred via the cyclophorase cycle.

As previously stated in the historical portion of the thesis (page 8), a postulated compound may be considered a metabolic intermediate in the oxidation of phenol if the phenol grown cells are simultaneously adapted for the oxidation of the postulated compound. When the compounds of the cyclophorase cycle were tested for the presence of this mechanism it was observed that pyruvate, succinate, fumarate, malate, and oxalacetate were all oxidized at an immediate maximum rate of oxygen uptake as shown in figure 5. In contrast to this, α-ketoglutarate, citrate, cis-aconitate, isocitrate and formate displayed a distinct initial lag which was followed by an exponential increase in oxygen uptake as shown in figure 6.

2. Oxygen uptake of the cyclophorase cycle compounds by acetate, succinate, and citrate-grown cells.

The rates of oxygen uptake of the various compounds of the cyclophorase system by cells grown on phenol are illustrated in figures 5

and 6. This information supplemented with the rates of oxygen uptake of these compounds by unadapted cells indicated which of these compounds were simultaneously adapted according to the original premises. In this work unadapted cells were obtained by growing the organism in medium in which sodium acetate was the only source of carbon. It can be seen from figure 7 that citrate, cis-aconitate, isocitrate, ∝-ketoglutarate and phenol all showed an initial slow rate of oxidation followed by an exponential increase to a maximum rate of oxidation. The possibility that these slow rates of oxidation were not due to a permeability effect was ruled out when an intermediate maximum rate of oxidation was observed when the compound was manometrically tested by cells previously grown on the specific compound. This is noted in figure 8. Because of the marked similarity of all the compounds in this type of reaction, only two compounds are shown.

In contrast to this, pyruvate, succinate, fumurate, malate, oxalacetate and acetate were all oxidised with initial rapid maximum rates by cells grown on acetate medium. This is illustrated in figure 9.

For the purpose of establishing whether citrate, isocitrate, cis-aconitate and ∝-ketoglutarate were a distinct group completely divorced from the other adapted compounds, the following experiments were undertaken.

The cells, previously grown on citrate, were tested manometrically against representative compounds from both groups of compounds; and in like manner, the compounds were tested by cells grown on succinate. The results of these experiments are illustrated in figures 10 and 11. It can be seen that citrate, isocitrate and cis-aconitate were all simultaneously adapted and succinate, ∝-ketoglutarate and phenol showed

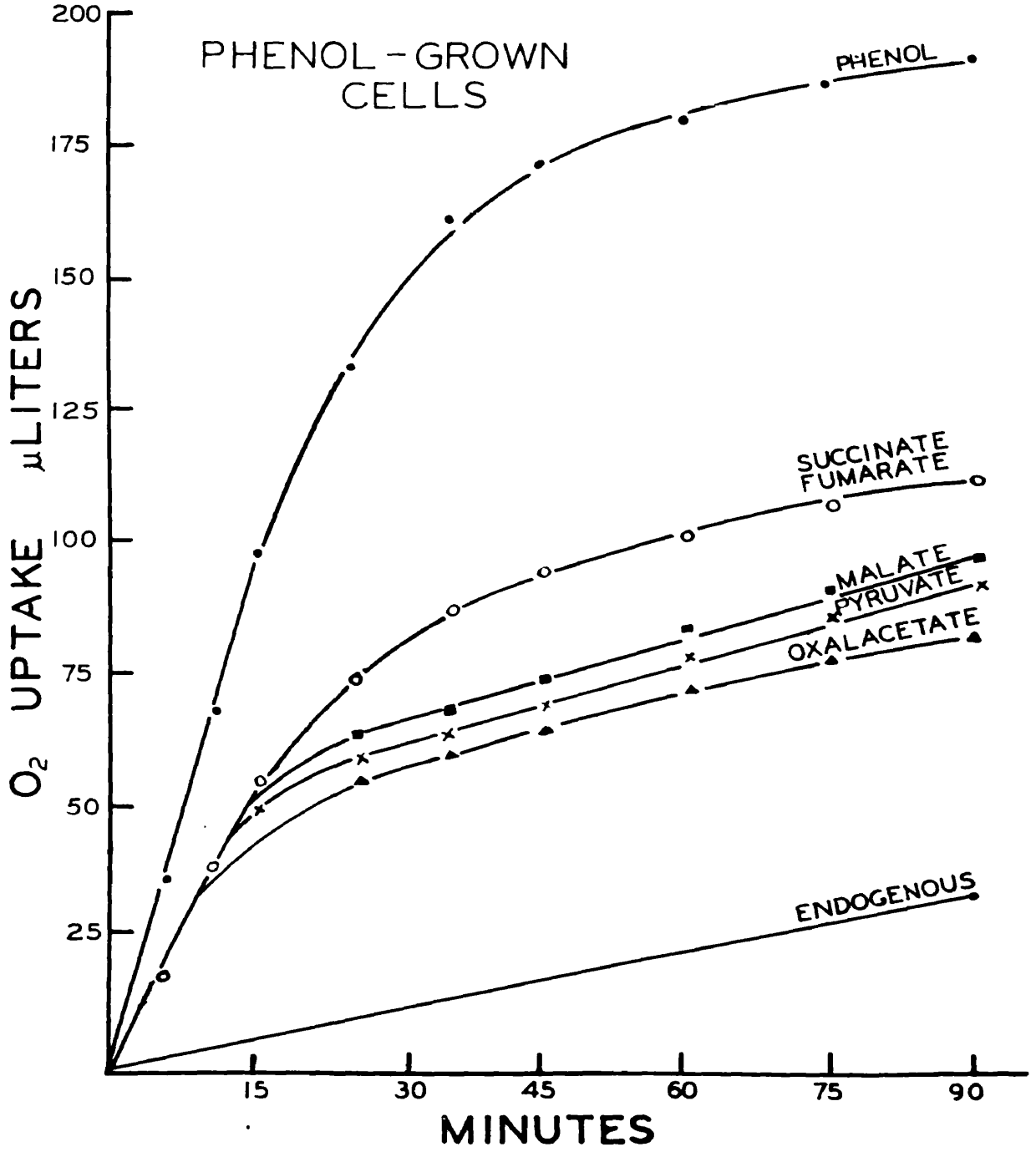


Figure 5. The oxidation of 2 micromoles of phenol, succinate, fumarate, malate, oxalacetate and 10 micromoles of pyruvate by Achromobacter sp. 85 grown on phenol.

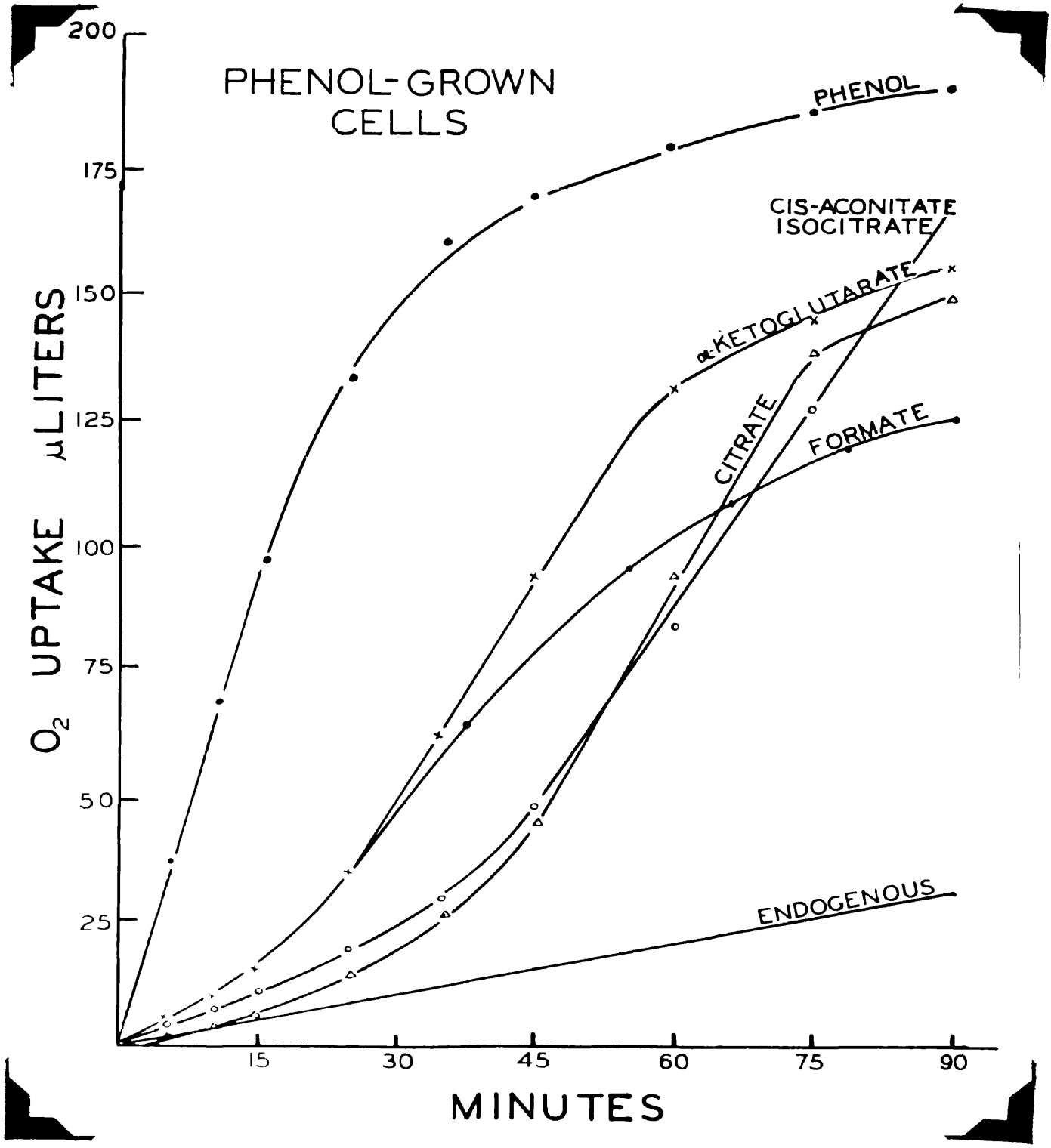


Figure 6. The oxidation of 2 micromoles of phenol, cis-aconitate, isocitrate, citrate, α-ketoglutarate and 10 micromoles formate by Aerobacter sp. 55 grown on phenol.

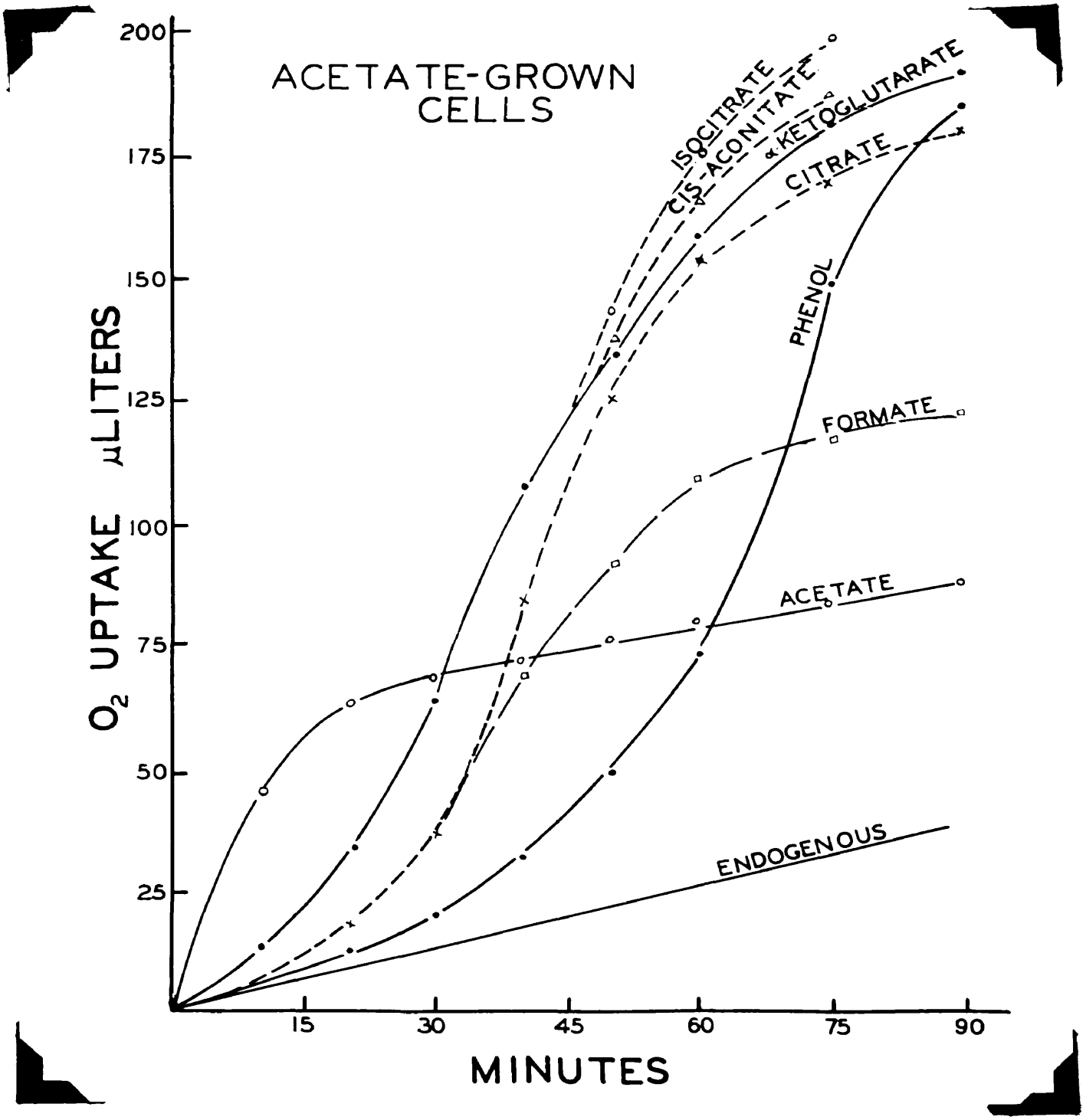


Figure 7. The oxidation of 2 micromoles of acetate, citrate, isocitrate, cis-aconitate, α-ketoglutarate, phenol and 10 micromoles of formate by Achromobacter sp. 65 grown on acetate.

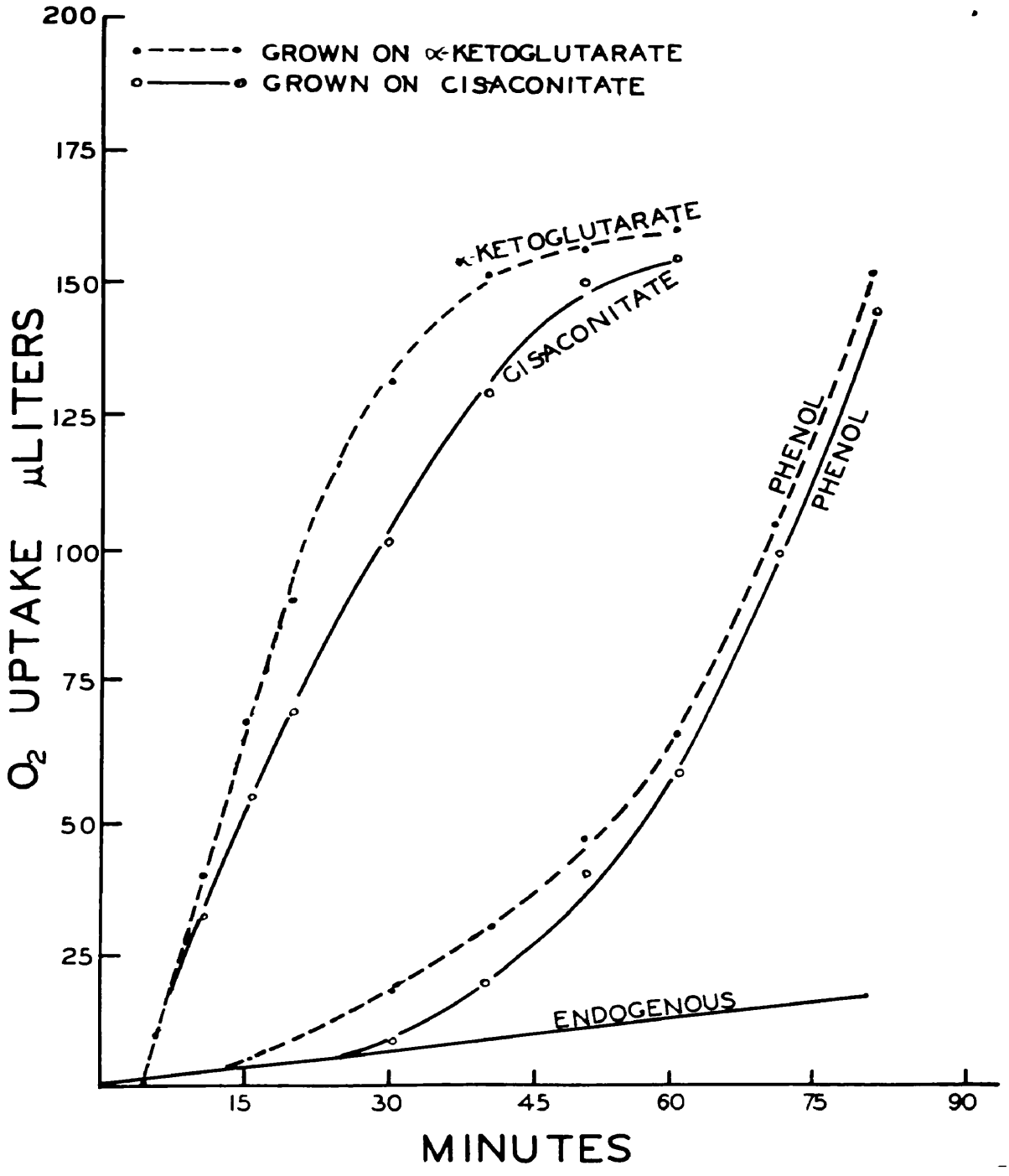


Figure 8. The oxidation of 2 micromoles of ∝-ketoglutarate and phenol by Achromobacter sp. 85 grown on ketoglutarate. The oxidation of 2 micromoles of cis-aconitate and phenol by Achromobacter sp. 85 grown on cis-aconitate.

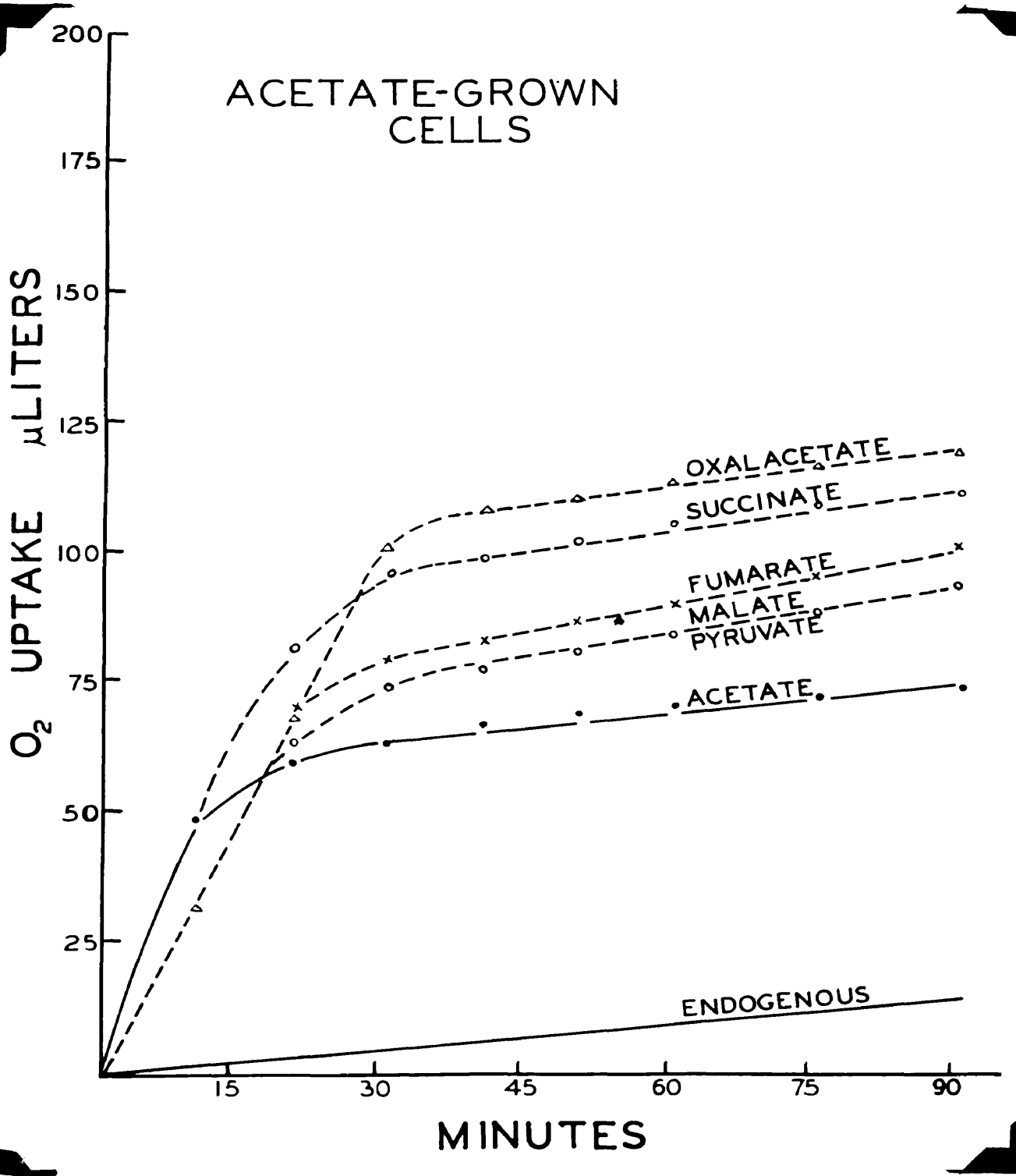


Figure 9. The oxidation of 2 micromoles of acetate, oxalacetate, succinate, fumarate, malate and 10 micromoles of pyruvate by Achromobacter sp. 65 grown on acetate.

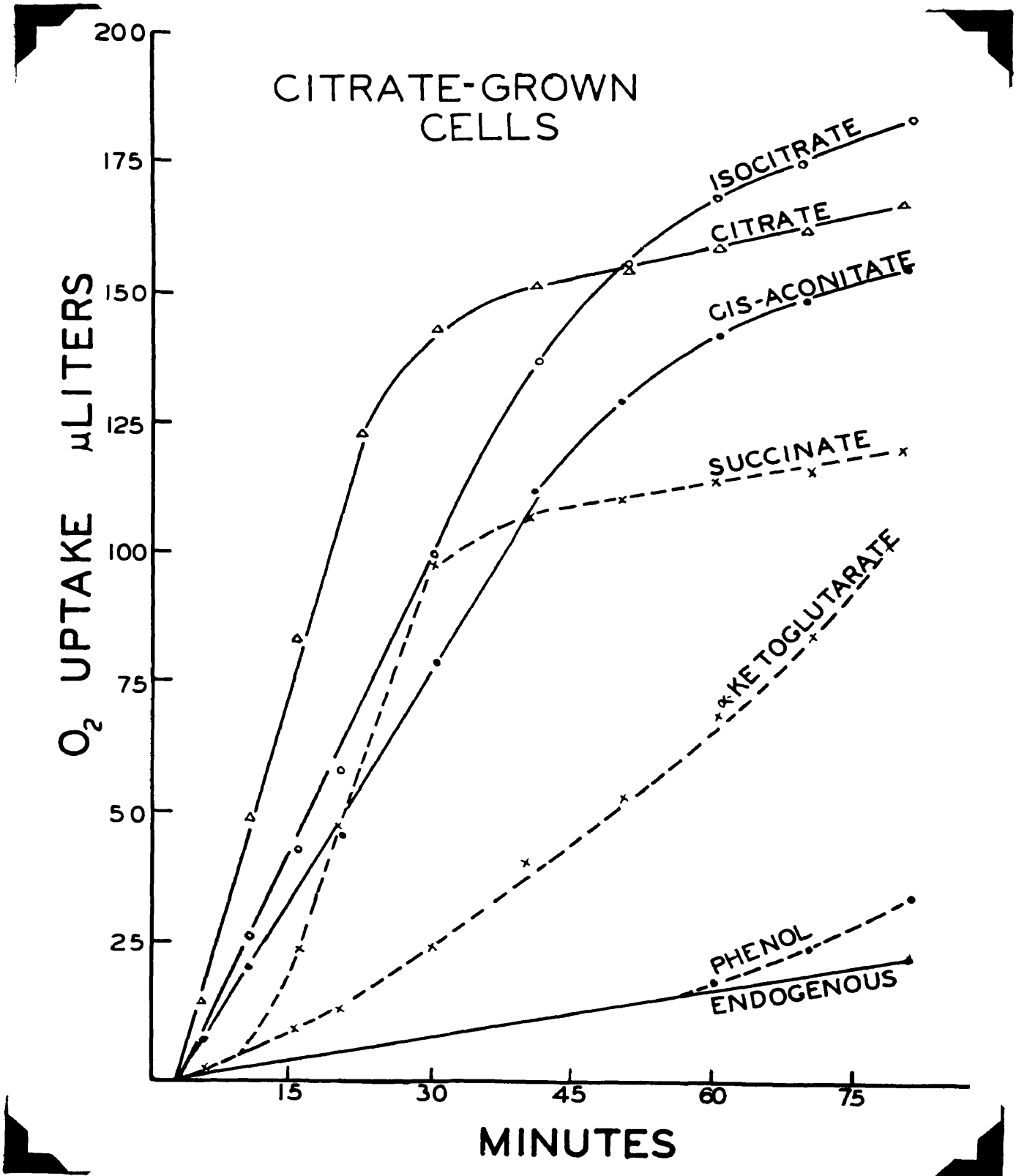


Figure 10. The oxidation of 2 micromoles of isocitrate, citrate, cis-aconitate, succinate, α-ketoglutarate and phenol by Achromobacter s. # 65 grown on citrate.

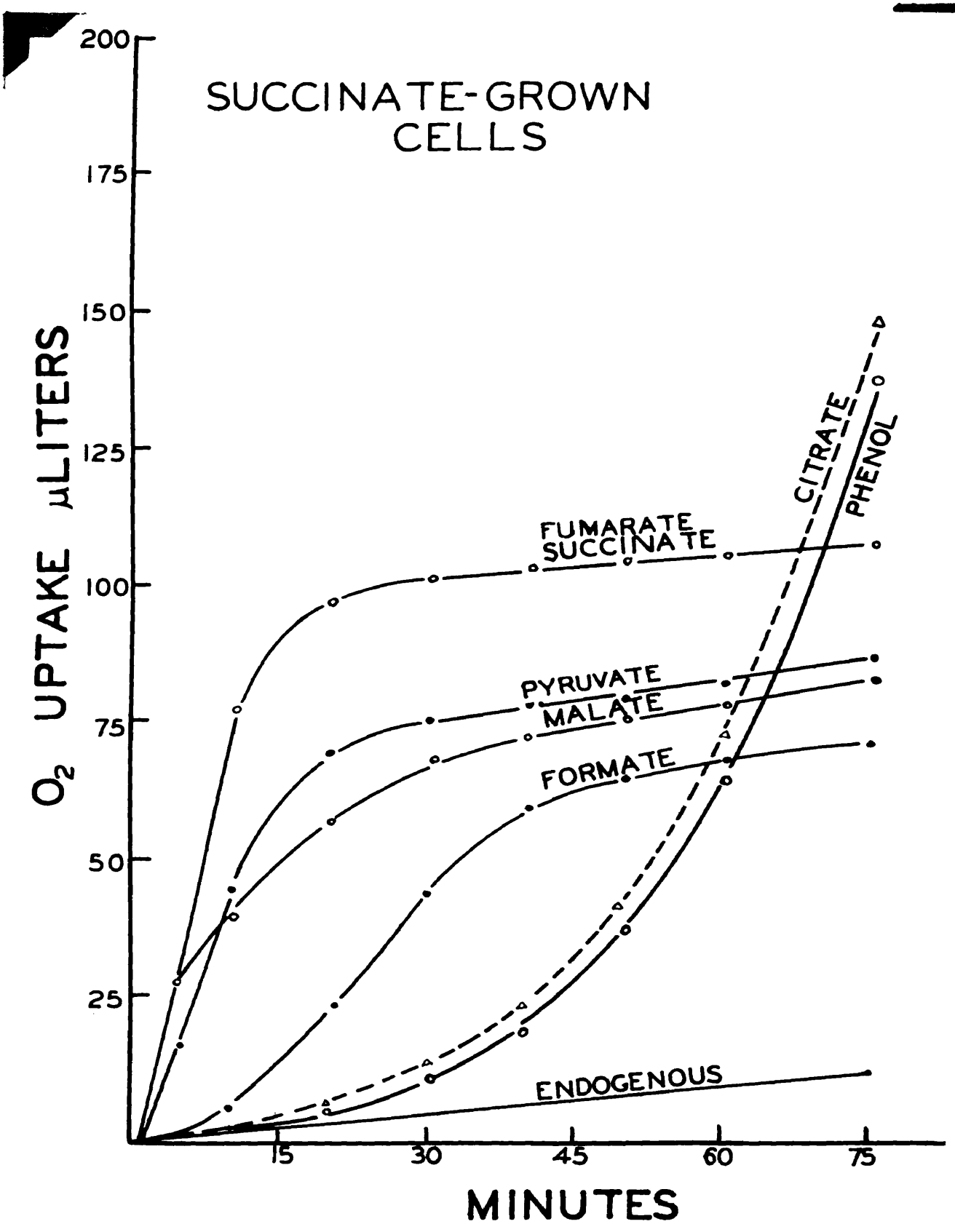


Figure 11. The oxidation of 2 micromoles of citrate, phenol, succinate, fumarate, malate, and 10 micromoles of pyruvate and formate by *Lobrarobacter* sp. 88 grown on succinate.

distinct initial lag phases. With succinate grown cells, succinate, fumarate, malate and pyruvate were simultaneously adapted and the citrate group showed a lack of adaptation.

3. Observations of phenol-grown cells on the adaptation of other closely related aromatic compounds

In accord with the premises that when phenol-grown cells were simultaneously adapted to other compounds it was assumed that these compounds were metabolic intermediates in phenol oxidation. With this in view experiments were conducted to determine how many of the aromatic compounds which supported the growth of the organism Achromobacter sp. (85) were simultaneously adapted.

It was of some interest to note that o-cresol, m-cresol, p-cresol, phenyl acetate, resorcinol, hydroquinone and catechol were all oxidised by phenol-grown cells with an immediate maximum rise in oxygen uptake and thus considered to be simultaneously oxidized. Whether this oxidation was due to constitutive or adaptive enzymes was not determined but the investigations of Stanier (1948, 1950) would indicate that these compounds in all probability were under adaptive control. Other compounds, all manifesting some degree of an initial lag are recorded in table 7.

Those compounds which were considered chemically unstable (resorcinol, catechol, hydroquinone) were tested manometrically for oxygen uptake by boiled cells and viable cells. Those containing the boiled cells gave no indication of an oxygen uptake and thus the possibility of spontaneous oxidation was ruled out.

TABLE 7

Observations of phenol-grown cells on the adaptation of closely related aromatic compounds

Simultaneously adapted	Not simultaneously adapted
o-Cresol	Tyrosine
m-Cresol	Phenylalanine
p-Cresol	Benzyl alcohol
Phenyl acetate	Phenyl benzoate
Resorcinol	Salicylate
Hydroquinone	Sulfo-salicylate
Catechol	Benzaldehyde
	Benzoate
	p-Hydroxybenzoate
	Pyrogallol triacetate
	Adipate

CHAPTER V

DISCUSSION

The failure to obtain any indication of growth on 0.007 M phenol by four known strains of the genus Mycoplana is in direct contrast with the findings of the original investigators (Gray and Thornton, 1928). It is assumed that at one time these strains possessed the ability of growing on phenol, but cultivation of the organisms in the absence of this compound over a long period of time apparently caused them to lose this ability. Similar observations were reported in the utilization of higher hydrocarbons by bacteria (Stone, Fenske, and White, 1942).

The phenomenon of permanently "losing" a function that the cell previously possessed is not disputed, but it definitely raises this important question: If a genus is based primarily on a biochemical function which is not particularly stable, does the characterization of the genus remain valid?

The contention that the description of Mycoplana is not based on a firm foundation is further strengthened by the following: (1) The ability to grow on phenol is a common function of many other bacteria and is not confined to any specific genus. Some of the genera which possess this property of growing on phenol and which are not Mycoplana are Pseudomonas, Micrococcus, Vibrio, Mycobacterium, Bacterium, and Bacillus (Gray and Thornton, 1928; Stanier, 1946; Fowler, Ardern, and Lockett, 1910; Egorova, 1946; Happold and Key, 1932). (2) In general, branched cells with the ability to grow in phenol have not been isolated

with frequency if at all in this study or any other study reported in the literature. (3) Growth on phenol was not correlated with other characteristics which would serve to differentiate these organisms from those of other genera.

In the quantitative estimation of the utilization of phenol, it was noted that all 14 organisms, with the exception of cultures 58 and 60, utilized most of the phenol within three days. Organism 55 was the most rapid in this aspect and utilized most of the phenol in 24 hours. These organisms, however, did utilize phenol at a much more rapid rate than observed by other investigators. For instance, Gray and Thornton (1928) reported that the average rate of utilization of phenol was from 7 to 14 days, and Czekalowski and Skerzynski (1948) reported an average of 5 days.

Again with the exception of cultures 58 and 60, all organisms produced some acid in the degradation of phenol to change the pH from 7.4 to 6.6.

In the utilization of the various related aromatic compounds, there are certain correlations and results that deserve mention. It is observed that these organisms are not merely confined to the oxidation of a single substance like phenol but in fact, when all of the organisms are considered as a group, they are capable of growing in the presence of at least 50 per cent of the other closely related aromatic compounds tested. In general, the organisms were unable to produce visible growth under the conditions tested on saturated ring compounds, diphenyl compounds, or compounds in which the hydroxyl group was substituted by a chloro, amino, or nitro group. The addition of nitro or alkyl groups also tended to prevent growth. Illustrative of this point is the observation that nitrophenols, 2,6-dimethyl phenol, diamyl phenyl, and orcinol

do not support growth. The presence of more than one hydroxyl group on the ring made the compound less readily available as is shown in the case of catechol, resorcinol, phloroglucinol and pyrogallol. These results are in general agreement with the findings of investigators working with species of Achromobacter and Vibrio (Happold and Key, 1932; Evans, 1947; Czekalowski and Skarzynski, 1948).

Observations on the ability of any bacterium to grow in the presence of other chemically related compounds serves to screen the number of compounds which may be considered as possible intermediates in any oxidation process. However, it is emphasized that there are certain definite limitations to this type of experimentation and growth alone should never be considered as conclusive positive evidence for their existence as an intermediate. It might be stated that utilization of a compound indicates that the compound can be an intermediate but lack of utilization by no means implies that the compound cannot be an intermediate. This is based on the assumption that certain unstable compounds on spontaneous oxidation produce toxic substances before the organism is able to attack the original compound but under quick enzymatic control these toxic substances do not accumulate to any great extent. Another explanation is the possible tendency of certain compounds to sufficiently evaporate producing a subminimal quantity in the medium. In this study for instance, it was observed that catechol did not always support growth of the organism, yet when oxygen uptake was determined by the adaptive manometric technique, catechol was definitely utilized. In the former case when growth studies were considered catechol could not be considered a possible intermediate but the fallacy of this was shown by the latter technique. The same behavior was observed with resorcinol and hydroquinone.

The presence of a single hydroxyl group or an aliphatic side chain appeared to aid in allowing the ring compound to support good growth; however, it must be emphasized that this property is definitely limited by the number of these groups and by the position in which they are arranged on the ring. Increasing the number of side groups had the tendency to decrease the possibility of growing on the compound. For example, phenyl acetate, which possesses only one acetate radical on the ring allowed good growth of all 14 organisms whereas the addition of two or more acetate radicals, as in pyrogallol triacetate, resulted in growth of only 9 organisms. The point is also shown when the ring contained only one sulfonic group as in benzene sulfonic acid. Here, 12 organisms grew well on the compound; however, when a carboxyl and a hydroxyl group are added to this compound, making it sulfosalicylic acid, only 3 organisms were able to grow well on it. The ability of these organisms to grow on compounds containing a sulfonic group was considered significant in view of the apparent toxicity of this group and the reported failure to support growth of bacteria.

The influence of positional substitution in the ring was best illustrated with the cresols and dihydroxyl compounds. With the cresols it was observed that in the para position the compound gave better growth than in either the ortho or meta positions. This same preference for para positional compounds was not shown in the use of the dihydroxyl compounds. In the dihydroxyl compounds the ortho position (catechol) gave better growth than either the meta (resorcinol) or the para (hydroquinone) compounds.

These results would indicate the higher specific nature of the mechanism involved in the degradation of aromatic compounds by bacteria.

This conclusion is supported by the observation that minor additions, substitutions or deletions of simple radicals to the ring greatly alter the ability of the compound to support good growth of any individual bacterium tested.

The necessity of certain ions for the growth of these organisms was previously tabulated and it will be noted that dipotassium phosphate and magnesium sulfate were generally required by all organisms. The observation that the medium devoid of dipotassium phosphate will not support the growth of any of the organisms, even when the pH of the medium was adjusted with sodium hydroxide, would indicate the need of either phosphates or potassium. Green, Herbert and Subrahmanyan (1940) reported that magnesium or manganese ions were required for carboxylase activity; thus for the same reason, it is assumed that magnesium ions are required by the organisms in this study. The marked influence that some of these salts had on the oxygen uptake of phenol further substantiates the need of the specified salts in the growth of these organisms.

It can be observed from figure 1 that cells which are washed and resuspended in phosphate buffer plus the salts magnesium sulfate and sodium chloride had a more rapid rate of oxygen uptake than those cells which are washed and resuspended only in phosphate buffer. A similar increase in rate can be observed when the concentration of phenol is decreased from 8 μM to 2 μM.

High concentrations of phenol (8 μM) and washing the cells only in phosphate buffer solution result in rates of oxygen uptake which make it appear that phenol is not oxidized by the adaptive process. This is of extreme importance when this technique is employed to determine possible metabolic pathways. In this particular case when 8 μM of

phenol was used the initial slow rate of oxidation would immediately indicate that phenol degradation could not be investigated by the process of simultaneous adaptation. Another case in point can be cited from the data presented by Evans (1947) in which he attempted to compare the rates of oxygen uptake of phenol and catechol. He stated that a definite latent period was evident with phenol as a substrate but was absent in catechol. The phenol concentration employed by Evans was 0.5 mg per ml but as shown in this study, lower concentrations of phenol causes a more rapid rate of oxidation; thus Evans may have shown erroneously a latent phase in phenol oxidation.

One of the main objectives of this thesis is to indicate the possible intermediate role of the cytophorase cycle compounds. It is shown that the organism used in this study oxidized phenol, citrate, cis-aconitate, isocitrate, α-ketoglutarate and formate adaptively. On the other hand, the oxidation of succinate, fumurate, malate, pyruvate and oxalacetate could not be shown to be under adaptive control. According to the original theory only those compounds under adaptive control can be used to obtain evidence of the possibility that the postulated compound actually is a metabolic intermediate. Thus it clearly can be seen that one of the basic difficulties in this work is to obtain unadapted cells for the purpose of determining the adaptation pattern.

On the basis of these results citrate, cis-aconitate, isocitrate, α-ketoglutarate and formate cannot possibly be members of the reaction chain in the breakdown of phenol. However, the possibility still exists that succinate, fumarate, malate, pyruvate and oxalacetate can be members of this reaction chain.

These are the only conclusions that can be reached concerning the compounds of the cycle on the basis of the accumulated evidence. It is, however, important to realize that the failure to demonstrate that the compounds were possible intermediates could have been due to experimental procedure. For example, it is known that under certain conditions various hydrogen acceptors must be present or possibly some coenzyme or ion must be restored after the washing procedure for the reaction to be initiated.

The observation that o-cresol, m-cresol, p-cresol, phenyl acetate, resorcinol, hydroquinone, and catechol were all simultaneously oxidized from phenol grown cells raises the question of whether these compounds should be considered metabolic intermediates in phenol degradation. By inspection of the chemical structure, the cresols and phenyl acetate cannot logically be considered as intermediates; whereas resorcinol, hydroquinone, and catechol can be so considered. The immediate oxygen uptake in the cresols and phenyl acetate can best be explained on the basis of two concepts. One is that a common intermediate exists in a reversible reaction between the oxidation of phenol and the oxidation of cresols and phenyl acetate. The other explanation is that phenol is an intermediate in the oxidation of cresols and phenyl acetate, and the close similarity in structure causes the phenol grown cells to oxidize cresols and phenyl acetate simultaneously.

CHAPTER VI

CONCLUSIONS

A standard linear graph was obtained for the colorimetric estimation of phenol in culture media. Studies with four known species of Mycoplana failed to indicate any growth on phenol. Because of this and other reasons the emphasis of the characterization of the genus Mycoplana was questioned.

Fourteen organisms capable of utilizing phenol were studied for their ability to grow on 87 chemical compounds. Sixteen compounds supported the growth of all 14 species, while 45 compounds did not support the growth of any of the organisms.

Phenol utilization by the 14 organisms, as determined colorimetrically, was correlated with an increase of growth (turbidity) and a decrease of pH from 7.4 to 6.6. All organisms retained the ability to utilize phenol after 120 transfers on a non-phenol containing medium.

On the basis of growth on the 87 compounds, the following generalizations may be made: Saturated ring compounds, diphenyl compounds or phenolic ring compounds having a chloro, nitro or amino group attached do not support visible growth under test conditions; likewise, increasing the number of side groups tended to decrease the possibility of growth on the compound. The influence of positional substitution on the ring was observed. With the cresols, the para position allowed better growth than either the ortho or meta position. However, with the dihydroxyl compounds the ortho position supported better growth than either of the other two positions. Five of the 14 organisms were

capable of growing in all of the components of the cyclophorase cycle.

The essential salt requirements were determined by observing the growth response in media which varied in composition. It was noted that phosphates were essential for all organisms and magnesium was essential for the growth of all but one species. Ferric chloride was not essential but provided for better growth of 9 strains whereas calcium chloride was noted to be partially inhibitory in most cases.

Of the 14 organisms employed in this study, 8 were classified in the genus Achromobacter; 5 in the genus Micrococcus; and one in the genus Vibrio.

Studies on phenol oxidation with regard to the adaptive mechanism revealed the following: Increasing the concentration of phenol (substrate in Warburg flask) from 2 to 8 micromoles produced a significant decrease in the rate of oxygen uptake. The higher concentrations of phenol made it appear that phenol was not oxidized by the adaptive process. In addition, cells which were washed and resuspended in phosphate buffer plus the salts (magnesium sulfate, sodium chloride, ferric chloride, calcium chloride) caused a greater rate of oxygen uptake than those cells which were washed and resuspended only in phosphate buffer.

Oxidation of phenol by the 14 organisms revealed that 9 were under adaptive control and 5 were not under this control.

With the technique of simultaneous adaptation, the components of the cyclophorase system were tested for their role as intermediates in the degradation of phenol. Of the compounds tested, α-ketoglutarate, citrate, cis-aconitate, isocitrate and formate were oxidized under adaptive control. These same compounds also were not simultaneously oxidized by phenol grown cells. With this evidence it would conclusively

indicate that these compounds were not possible intermediates in phenol oxidation.

In contrast to the above, the adaptive process could not be demonstrated in the oxidation of pyruvate, succinate, fumarate, malate and oxalacetate. These compounds, however, were simultaneously oxidized by phenol-grown cells. Whether this oxidation could be attributed to the presence of constitutive or adaptive enzyme activities remained unknown. Until a definite mechanism is uncovered, the evidence, though not conclusive, indicated that these compounds were possible metabolic intermediates in the oxidation of phenol.

It is also noted that closely related aromatic compounds differed in their rates of oxidation by phenol-grown cells. Phenyl acetate, o-cresol, m-cresol, p-cresol, resorcinol, hydroquinone and catechol were all simultaneously oxidized by phenol-grown cells.

The explanation of this adaptation to so many aromatic compounds which are not logically considered intermediates remains unanswered.

Previously it was suggested, on the basis of growth studies, that the mechanism in the oxidation of phenol was highly specific. In the light of adaptation studies, this concept does not seem to be entirely correct. Simultaneous adaptation to catechol by phenol grown cells can well be explained on the basis of an intermediate, but adaptation to its isomers, resorcinol and hydroquinone, cannot be explained on the theory that they are possible intermediates. It does, however, suggest the inability of the organism to differentiate compounds possessing dihydroxyl groups in the various positions on the ring.

CHAPTER VII

LITERATURE CITED

Baldwin, E. 1949 Dynamic Aspects of Biochemistry. Macmillan Co., New York, N. Y.

Bernheim, F. 1942 The oxidation of benzoic acid and related substances by certain mycobacteria. J. Biol. Chem. 143: 383-389.

Breed, R.S., Murray, E.G.D., and Hitchens, A.P. 1948 Bergey's Manual of Determinative Bacteriology. 6th ed., Williams & Wilkins, Baltimore.

Buddin, W. 1914 Partial sterilization of soil by volatile and non-volatile antiseptics. J. Agr. Sci. 6: 417-461.

Czekalowski, J. W. and Skarzynski, B. 1948 The breakdown of phenols and related compounds by bacteria. J. Gen. Microbiol. 2: 231-238.

Dooren de Jong, L.E. van. 1926 Bijdrage tot de kennis van het mineralisatieproces, Mijgh and Van Ditmar's Uitgevers-Mij. Rotterdam.

Dubos, R. J. 1940 The adaptive production of enzymes by bacteria. Bact. Rev. 4: 1-16.

Egorova, A.A. 1946 Physiological data on phenol-oxidizing thermophilic bacteria. Mikrobiologiia 15: 467-477. Abst. Chem. Abst. 43 (1): 276 (1949).

Evans, E. C. 1947 Oxidation of phenol and benzoic acid by some soil bacteria. Biochem. J. 41: 372-382.

Evans, E.C., Handley, W.R.C., and Happold, F.C. 1940 The tryptophanase-indole reaction. Biochem. J. 35: 207-211.

Fowler, G.T., Ardern, E., and Lockett, W.T. 1911 The oxidation of phenol by certain bacteria in pure culture. Proc. Roy. Soc. (London) B 83: 149-156.

Gomori, G. 1949 Determination of phenol in biologic material. J. Lab. Clin. Med. 34: 275-282.

Grant, C. W. and Zobell, C. E. 1942 Oxidation of hydrocarbons by marine bacteria. Proc. Soc. Exp. Biol. Med. 51: 266-267.

Gray, P.H.H. and Thornton, H.G. 1928 Soil bacteria that decompose certain aromatic compounds. Centr. f. Bakt. Abt. II, 73: 74-96.

Green, D. E., Herbert, D., and Subrahmanyan, V. 1940 On the isolation and properties of carboxylase. J. Biol. Chem. 135: 795-796.

Green, D. E., Loomis, W. F., and Auerbach, V. H. 1948 Studies on the cytophorase system. J. Biol. Chem. 172: 389-403.

Hansen, P.A. 1930 The detection of ammonia production by bacteria in agar slants. J. Bact. 19: 223-229.

Happold, F. C. 1930 The correlation of the oxidation of certain phenols and of dimethyl-p-phenylenediamine by bacterial suspensions. Biochem. J. 24: 1737-1748.

Happold, F.C. and Key, A. 1932 The bacterial purification of gasworks' liquors. The action of liquors on the bacterial flora of sewage. J. Hyg. (Cambridge) 32: 573-580.

Karlsson, J. L. and Barker, H. A. 1948 Evidence against the occurrence of a tricarboxylic acid cycle in Azotobacter agilis. J. Biol. Chem. 175: 913-921.

Karström, H. 1937 Enzymatische Adaptation bei Mikroorganismen. Ergeb. Enzymforsch., 7: 380-378.

Kilby, B.A. 1948 The bacterial oxidation of phenol to β-ketoadipic acid. Biochem. J. 43: V.

Lonti, C. 1946 Ossidazione Dell-Ac Piruvico Prodotta Dal Bacterium coli Communis Boll. Soc. ital. biol. sper., 22: 332-333.

Lewis, Ralph W. 1948 Mutants of Neurospora requiring succinic acid or a biochemically related acid for growth. Am. J. Bot. 33: 292-295.

Maneval, W. E. 1931 The staining of bacteria, with special reference to mordants. J. Bact. 21: 313-321.

Manual of Methods for Pure Culture Study of Bacteria. 1948. Society of American Bacteriologists. Biotech Publications, Geneva, New York.

Nelson, J. M. and Dawson, C. R. 1944 Tyrosinase. Adv. in Enzymology IV: 99-150.

Russell, E. J. and Hutchinson, H.B. 1909 The effect of partial sterilisation of soil on the production of plant food. J. Agr. Sci. 3: 111-144.

Sen-Gupta, N. 1921 Dephenolization in soil. J. Agr. Sci. 11: 136-158.

Sleeper, B.P. and Stanier, R.Y. 1950 The bacterial oxidations of aromatic compounds. J. Bact. 59: 117-127.

Söhngen, N.L. 1913 Benzin, Petroleum, Paraffinöl und Paraffin als Kohlenstoff und Energie - quelle für Mikroben. Centr. f. Bakt. Abt. II, 37: 595-609.

Stanier, R. Y. 1947 Simultaneous adaptation: A new technic for the study of metabolic pathways. J. Bact. 54: 339-348.

Stanier, R. Y. 1948 The oxidation of aromatic compounds by fluorescent pseudomonads. J. Bact. 55: 477-494.

Stephenson, M. and Stickland, L. H. 1932 Hydrogenlyases - Bacterial enzymes liberating molecular hydrogen. Biochem. J. 26: 712-724.

Stone, R. W., Fenske, M. R., and White, A.G.C. 1942 Bacteria attacking petroleum and oil fractions. J. Bact. 44: 169-178.

Störmer, K. 1906 Über die Wirkung des Schwefelkohlenstoffs und ähnlicher Stoffe auf den Boden. Centr. f. Bakt. Abt. II 20: 282-286.

Tausson, W. O. 1928 Die Oxydation des Phenanthrens durch Bakterien. Planta 5: 259-273.

Wagner, P. 1914 Über Benzolbakterien. Z. f. Garungphysiologie 4: 289-319.

Wortmann, J. 1882 Untersuchungen über das diastatische Ferment der Bakterien. Z. physiol. chem. 6: 287-329.

Yudkin, J. 1938 Enzyme variations in microorganisms. Biol. Rev. (Cambridge) 13: 93-106.

ACKNOWLEDGMENT

The author wishes to express his sincere appreciation to Dr. John E. Faber, Jr., for affording him the opportunity to carry on graduate studies and to Dr. Raymond E. Doetsch for the guidance and assistance received in the preparation of this thesis. He also acknowledges his indebtedness to Dr. Norman C. Laffer and fellow colleagues of the Department of Bacteriology for the encouragement and many valuable suggestions they have given. Gratitude is also expressed to Dr. Arthur Kornberg and Dr. Alton Meister of the National Institutes of Health, Bethesda, Maryland, for some of the compounds used in this work.