STUDIES ON THE BACTERIAL METABOLISM OF BENZYLAMINE

A Thesis

submitted by

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to the University of Manitoba

in partial fulfilment of the requirements

for the Degree of

Master of Science in Pharmacy

March 1973

Abstract

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A strain of Pseudomonas aeruginosa isolated from soil was examined for its ability to utilize benzylamine as sole source of carbon and energy. By the use of gas-liquid chromatography, a series of metabolites was identified and their probable sequence of production determined. The results indicated the following sequence: benzylamine to benzyl alcohol, to benzaldehyde, to benzoic acid, and then to catechol.

ACKNOWLEDGEMENTS

Sincere thanks to all the members of the Faculty of Pharmacy, most especially to Dr. J. A. M. Shaw, my thesis supervisor, for his advice, suggestions and most of all, his patience.

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1.1 INTRODUCTION

Many common soil bacteria and fungi possess the ability to utilize aromatic compounds as the sole source of carbon and energy. Of these microorganisms, the members of the bacterial family Pseudomonadaceae are the most common and generally these have been used for metabolic studies with aromatic compounds (54, 11, 14, 43). Pseudomonas, Vibrio (9), Azotobacter (24) and Mycobacterium (37) are the bacterial genera best known for their ability to degrade the benzene nucleus while Aspergillus, Penicillum, and Neurospora are the fungi best known for this ability. The enzymes catalyzing the reactions of aromatic degradation are produced only when the microorganism is growing in the presence of the aromatic compound and are induced rather than constitutive enzymes (10,41). This will be discussed in some detail in a later section.

The key intermediate in the catabolic pathway of the simple aromatic compounds is catechol (11). There are two known pathways for the cleavage of the aromatic ring in catechol. In the first pathway, known as the "ortho" pathway, catechol is oxidized by pyrocatechase, using atmospheric oxygen to produce cis-muconic acid. One mole of water is lost to give the lactone, muconolactone, which is then acted upon by muconolactone isomerase to give the product β-oxoadipate enol lactone. This compound is then acted upon by β-oxoadipate enol lactone hydrolase

to form β-oxoadipate which is readily metabolized to succinyl and acetyl CoA.

In the second pathway, known as the pathway of "meta" cleavage, catechol is oxidized by metapyrocatechase to form 2-hydroxymuconic semi aldehyde. This is in turn acted upon by a hydrolase enzyme to give formate and 4-hydroxy-2-ketovalerate which in turn is degraded by an aldolase to give acetaldehyde and pyruvate (18). These pathways are shown in Figure 1.

Many of the bacterial strains capable of decomposing catechol via the meta pathway also possess the genetic capability to decompose catechol via the ortho pathway (10, 41). This phenotypic expression of the alternate pathways appears to be determined by the chemical nature of the precursor with which the organisms are grown (10, 41). This was shown using Azotobacter sp. where it was demonstrated that catechol was cleaved by the ortho pathway when growth was initiated on p-hydroxybenzoate and yet when growth was initiated on benzoate, the catechol produced was cleared via the meta pathway (24). Thus, although catechol is a common intermediate to both pathways, the pathways operate as a result of the manner in which enzyme synthesis is regulated.

Catechol itself is not an inducer of either the ortho or meta pathway and the enzymes catalyzing the first four steps in the meta pathway are all induced by phenol or the cresols. Benzoate oxidase, the first enzyme of the ortho pathway is induced benzoate. Catechol-1,2-oxygenase is induced only after the

catechol first produced is converted by constitutive catechol oxygenase to cis-muconic acid which then serves as inducer of catechol oxygenase, muconate lactonizing enzyme and muconolactone isomerase. These three enzymes must be induced before extensive substrate conversion can occur.

1.2 ENZYME INDUCTION

Enzyme induction may be defined in very general terms as a specific increase in the amount of an enzyme in response to the presence of a nutrient or a metabolite. In order for an enzyme system to be considered inducible, two criteria must be met:

(a) Induction is a phenotypic change in all of the cells of a given population rather than a genotypic change in part of the population. It should also be noted that the enzyme concentration will increase greatly without a corresponding change in the cell concentration whereas if the increased enzyme levels were due to a genotypic change, the enzyme concentration would be dependent on the concentration of the cells.

The fact that all of the cells are affected was demonstrated by Benzer in 1953 with a rather ingenious experiment. In this experiment, E. coli were induced with lactose to produce β-galactosidase and then infected with a bacteriophage. By infecting at varying times, and comparing the amount of enzyme released with

the number of cells lysing, it could be seen that each bacterium lysed released the same amount of enzyme and thus was equally induced (4).

(b) Induction represents a formation de novo of enzyme molecules rather than the activation of pre-existing precursors. This was shown by inducing cells to produce β-galactosidase in the presence of radioactively labeled amino acids. The isolated β-galactosidase was found to be labeled and thus was formed de novo and did not exist as a preformed precursor, which was activated in some way (44).

The mechanism by which low molecular weight compounds specifically stimulate the synthesis of enzymes is largely unknown. Only a few specific examples have been studied and it is too soon to postulate a general mechanism if indeed only one general mechanism even exists.

An inducible enzyme is generally present even when bacteria are grown in the absence of inducer although the level of this "basal enzyme" is very low. There are two possible explanations for the presence of these enzymes.

(a) Traces of inducer may be present in the medium or be synthesized by the bacteria.

(b) Constitutive mutants (producers of high enzyme levels in the absence of inducer) may be present. These mutants

seem to be present in every population of inducible cells.

A constitutive enzyme, as opposed to an inducible enzyme, is ideally one which represents a constant fraction of the bacterial protein irrespective of growth conditions. The difference between a constitutive enzyme and an inducible enzyme is essentially quantitative with the difference not really being in the enzymes themselves but rather in their production. For example a constitutive mutant can produce β-galactosidase in the absence of inducer and thus can utilize neo-lactose which is a substrate for but not an inducer of β-galactosidase while a wild-type cell (inducible) cannot utilize neo-lactose.

Inducibility provides a selective advantage over constitutibility in the economics of the cell. The level of the enzyme is governed by the amount of available substrate thus excessive amounts of enzyme (which cost in terms of energy to produce) are avoided. Another advantage of inducibility is that the bacterial cell is not physically large enough to carry the full complement of enzymes they are capable of producing were they all to be produced at once. Thus a cell with inducible enzyme systems is able to handle a much wider range of substrates as in effect its limitations of size are greatly reduced. In this light, a microorganism with inducible enzyme systems is much more versatile than one with constitutive enzyme systems.

The advantages of inducibility can thus be summarized as:

1. Excessive levels are not produced when they are not required.
2. The physical and spacial limitations of the cells are greatly reduced.
3. The cells show a high level of metabolic versatility.

1.3 BENZENE DEGRADATION

Marr and Stone investigated the bacterial degradation of benzene in 1960 (37). They isolated microorganisms capable of utilizing benzene as sole source of carbon and energy by the use of soil enrichment culture on benzene. Of the organisms isolated, a Pseudomonas sp. and a Mycobacterium sp. were chosen for their study since members of these genera are widespread in soil. From their work, they concluded that the ability to degrade benzene was due to induced enzymes, probably those postulated by Stanier (49), rather than to a mutational process. From a comparison of mammalian systems and bacterial systems for benzene metabolism, Marr and Stone concluded that several pathways were available to animals as phenol, catechol, and trans-muconic acid could all be recovered in the urine while both bacterial species they studied showed only one general pathway and that that pathway provided complete degradation of the benzene to carbon dioxide and water.

1.4 PHENOL DEGRADATION

Evans, in 1946 (16) examined the utilization of phenol as sole source of carbon and energy by several bacteria he isolated from soil. The microorganism he used for his preliminary work, he designated as "Vibrio 01" and on characterization, he found it to be very similar to Vibrio cuneata (5). Other organisms he isolated which could utilize phenol were from the families Coccaceae, Mycobacteriaceae, Bacteriaceae, Pseudomonadaceae, Spirillaceae, and Bacillaceae. From his work he predicted an oxidative pathway whereby the ring was first oxidized then ring fission occurred giving rise to ketonic and aldehydo-acids which in turn could be further catabolized. He also implicated catechol as an intermediate in the pathway.

More recently Feist and Hegeman (18) studied the metabolism of phenol by Pseudomonas putida. They found that the catechol produced in this sequence was cleared via the "meta" pathway and thus that phenol acted as an inducer to the meta pathway. They concluded that the meta pathway serves as a general mechanism for the catabolism of various alkyl derivatives of catechol derived from substituted phenolic compounds since induction of an entire pathway by the primary substrate (in this case phenol) would indicate a low degree of catabolic specificity. This low specificity of induction also suggests that during evolution, this pathway functioned in the catabolism of a wide

range of aromatic derivatives which would arise from the breakdown of various natural products such as some of the phenolic constituents of various plants.

1.5 BENZOATE DEGRADATION

The degradation of benzoic acid by soil pseudomonads was investigated by Feist and Hegeman in 1969 (18) and earlier by Ornston and Stanier in 1966 (40, 41). The first step of the degradation was found to be the conversion of benzoate to catechol. The reaction is catalysed by benzoate oxidase which is induced by benzoate. The catechol formed from this reaction is cleared via the ortho pathway where the following occurs: The catechol is converted to cis,cis-muconate by catechol-1,2-oxygenase; the cis,cis-muconate is converted to muconolactone by cis,cis-muconate lactonizing enzyme; the muconolactone is converted to β-oxoadipate enol lactone by muconolactone isomerase. These last three enzymes are induced by cis,cis-muconate: β-oxoadipate enol lactone hydrolase then converts the β-oxoadipate enol lactone to β-oxoadipic acid which is in turn converted to succinate and acetyl CoA.

It can be seen that the ortho pathway has a higher degree of specific regulation than the meta pathway where the primary substrate induces all of the enzymes in the pathway. The regulation of induction of the pathways is shown in Figure 1.

The literature shows many more aromatic compounds have

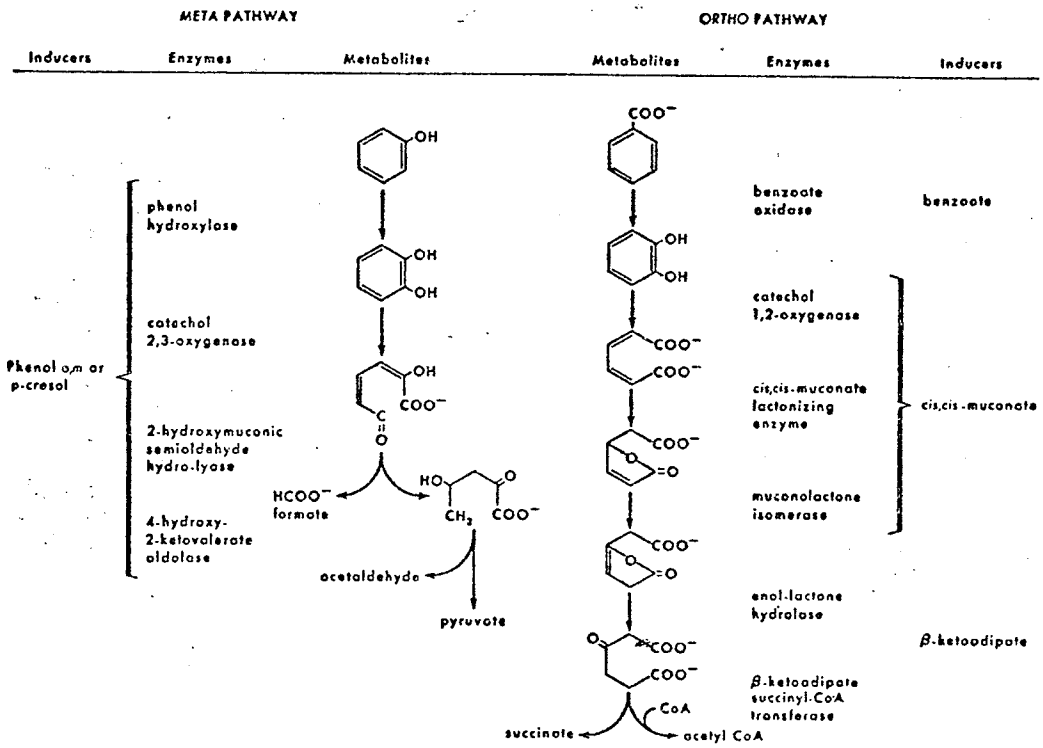


FIG. 1. *Metabolites, enzymes, and inducers of the ortho and meta pathways in P. putida. The regulation of the ortho pathway was described by Ornston* (44) *Enzymes, the names of which are enclosed by brackets, share a common inducer.*

been investigated to determine how they are degraded by various soil organisms--mandelic acid was investigated by Gunsalus in 1953 (22), anthracene (17), naphthalene (12), aniline (54), and arylsulphonates (6, 43).

1.6 OXYGENASES--GENERAL CONSIDERATIONS

In all of the examples cited above, it can be seen that dihydroxylation of the aromatic ring occurs before the aromatic ring undergoes cleavage. The enzymes catalysing the hydroxylation of the ring have been termed mixed-function oxidases (26) as in their reaction one atom of oxygen is incorporated into the substrate molecule and the other is reduced to water in the presence of a suitable electron donor. In other words, oxygen serves as both an oxidizing and oxygenating agent (25, 52).

In the bacterial metabolism of aromatic compounds, oxygenases must be specifically induced. Since the activities of these enzymes are usually of a low order, the hydroxylations which they carry out are usually the rate-determining step of the metabolic pathway. With aromatic molecules, a double hydroxylation is carried out by the bacterial enzymes and molecular oxygen appears to be incorporated into the substrate molecule to form organic peroxide which is then reduced to two hydroxyl groups. Epoxide has been suggested as an intermediate in some reactions of this type (26, 52) yet Gibson in his studies (20, 21) of the enzymatic formation of catechol from benzene using a crude

pseudomonad extract, indicated a cyclic peroxide rather than an epoxide.

Many investigators (42, 50) believe that heavy metals such as iron or copper are involved in the enzymatic activation of oxygen and it has been assumed that the oxygenases contain heavy metal ions probably at their active sites. Little is actually known regarding the function of these ions in the overall reaction mechanism. In general the role of the heavy metal ions has been considered in terms of three categories (26):

1. Iron, as a component of non-heme iron protein participates in an electron transport system associated with hydroxylation reactions.
2. Metal ions may be involved in the aggregation of subunits to form a native enzyme or to maintain the aggregation.
3. The ion is directly involved in the oxygenation reaction and facilitates the activation of substrate and/or molecular oxygen.

Which of these, if any, is generally correct is unknown largely because only a very few of the enzymes hydroxylating the aromatic nucleus have been purified to the point where they can be studied.

1.7 REASONS FOR STUDY

There are several reasons for the study of aromatic catabolism by microorganisms:

(a) Classification

At the present time there is no satisfactory place for many microorganisms in our present taxonomic system. With the introduction of systems of numerical taxonomy, a large number of parameters must be used and although it is not likely, it is still possible that the ability to produce enzymes capable of degrading aromatic molecules may be used as one of these parameters.

(b) Evolutionary aspects

Studies of evolutionary sequences with bacteria are extremely difficult to carry out for several reasons--the most obvious being that unlike plants and animals, bacteria have no fossil record to speak of. Fossil records have been invaluable in determining the progression of both plants and animals but for bacteria another basis must be found. It can be argued that the more exacting an organism is in its needs for metabolites and growth factors the more advanced its evolution, while on the other hand it can be argued that the less exacting it is the more advanced its evolution because it is much more independent and thus more complex. In other words

at which end of the evolutionary scale is the ultimate parasite? In any case the utilization of rather unappetizing aromatic molecules may be significant as it would seem to show a very high degree of versatility. As has already been pointed out, the mode in which these molecules are attacked may also have a bearing on the evolutionary stage as one route of catabolism seems much less specific than the other induction-wise, thus one is further towards the end of the scale in an evolutionary frame of reference. The entire area of bacterial evolution is one of great speculation lacking in facts and proofs so any avenue of exploration which may shed some light on the problem must be investigated and recorded so that at some time in the future it may be re-examined in the light of new ideas and findings and be evaluated in relation to them.

(c) Ecological aspects

At the present time there is an acute concern regarding the effect man is having on his environment and the resulting problems of pollution. Potential pollutants may contain an aromatic nucleus which along with various substituents may persist in our environment for long periods of time thus resulting in increasing levels with time and usage. For peace of mind, if nothing else, these components should be investigated in an ecological

frame of reference. Part of these investigations should involve the microbial flora of soils and water since these materials will ultimately end up pooled in the soil and water. Some materials may be unaffected by microorganisms and persist; others may have an effect on the flora and thus upset an otherwise stable ecological balance; some may be attacked by microorganisms to yield harmless products while some may be biologically altered so that compounds of enhanced toxicity are produced. The study and evaluation of these materials cannot be properly undertaken unless some of the underlying principles regarding the metabolism of simpler derivatives are examined. In other words, if the pathways of simple compounds are established, the pathways of the more complex derivatives may be predicted with some degree of confidence.

(d) Pharmaceutical aspects

Some of the older pharmaceutical preservatives are relatively simple aromatic compounds. If these materials are susceptible to microbiological degradation their efficiency as preservatives is of course abolished. The very real possibility also exists that drugs containing an aromatic moiety could be degraded or altered either in storage or in the human body to produce materials with toxic or potential antigenic activity.

(e) Scientific curiosity

Finally and possibly most importantly is the aspect of scientific curiosity. All practical research which is essentially goal-orientated must be based on the knowledge accumulated from rather abstract or "academic" research. The bits and pieces of information acquired from abstract research may at some later date be integrated with other information and from this integration various practical problems may be solved. Both types of research are essential, the one providing a basis for the other. Perhaps it is unwise to attempt to distinguish practical from abstract research as in any research work the whole object is to attempt to answer the questions, "Why?" and "How?".

With all of this in mind we will now examine the bacterial catabolism of benzylamine.

2.0 EXPERIMENTAL

2.1 OBJECTIVES

The objectives of this study may be summarized as:

(i) to determine whether benzylamine is able to be degraded by soil microorganisms so that benzylamine may be used as sole source of carbon and energy;

(ii) to identify or describe the organism capable of this;

(iii) to suggest a metabolic pathway for the degradation of benzylamine;

(iv) to indicate whether the enzymes needed for this are constitutive or induced.

2.2 PROCEDURES

(a) Medium

A simple salts medium based on Koser's citrate broth (33) was used. The composition was:

NH_4Cl	1 gm
NaCl	5 gm
$MgSO_4 \cdot 7H_2O$	0.2 gm
$NH_4H_2PO_4$	1 gm
K_2HPO_4	1 gm
Water to	1000 ml

The substrate materials were added to the medium and the pH adjusted to 7.0 with either N/1 HCl or N/1 NaOH. With the exception of catechol, all substrates used were stable to autoclaving for 15 minutes at 121° C. The catechol was dissolved in cold medium and sterile-filtered through a millipore filter into a sterile flask. Undue exposure to light was avoided with this medium as at pH 7.0 the catechol was decomposed by light. For a procedure where solid medium was needed as in the viable counts of the cultures, 2% w/v of agar (Difco) was added to the simple salt medium.

(b) Isolation

In order to perform this study, a bacterial species capable of utilizing benzylamine as the sole source of carbon and energy had to be found. To achieve this end about 20 laboratory strains of bacteria were examined, most of which had been isolated from soil during previous investigations (2). Most of these organisms were members of the family Pseudomonadaceae. The cells had been maintained on nutrient agar slants so they were first subcultured into nutrient broth and then onto fresh nutrient agar slants (Oxoid) to ensure that the cells were viable. Incubation at this time was arbitrarily carried out at 35° C. After 24 hours, growth was apparent on the slants and cells were removed from each slant and inoculated into about 10 ml of simple salt medium containing 0.1% benzylamine v/v as sole source of carbon

and energy with the pH adjusted to 7.0. The tubes were incubated at 35° C. for a period of three weeks and from the tubes showing turbidity at the end of this time cells were subcultured into fresh simple salt-benzylamine medium and again incubated at 35° C. At the end of two weeks material from these tubes was streaked onto plates of simple salt-benzylamine medium solidified with 2% agar (Difco) and incubated. Minute colonies began to appear after about 10 days of incubation and the largest of these colonies were picked and subcultured into liquid medium. After five days of incubation turbidity was apparent in one of the tubes and this was the culture selected for this study.

The growth parameters for the cells were roughly determined in the following ways:

(i) pH

Simple salts medium with 0.1% v/v benzylamine was prepared and divided into three tubes. The pH of the media were adjusted so that one tube had a final pH of 6.5, one at 7.0 and one at 7.5. The tubes were inoculated and then incubated at 35° C. for five days. At the end of this time the tubes were examined and the one showing the greatest turbidity was used as an indicator of the pH approaching optimal for growth.

(ii) Temperature

The temperature approaching optimal for growth was determined in a manner similar to that used for pH. The

medium was prepared and the pH adjusted to 7.0. Tubes were inoculated and then incubated at the following temperatures--25° C., 30° C., 35° C. and 40° C. At the end of five days incubation the tubes were examined and the one showing the greatest turbidity was used as an indicator of the temperature approaching optimal for growth.

(iii) Substrate concentration

Since one of the objectives of this study was to identify the probable metabolites of benzylamine, it was desirable that the highest possible concentration of benzylamine be used as substrate. As previously done, six tubes were set up so they contained benzylamine in the following concentration (w/v)--0.1%, 0.2%, 0.3%, 0.4%, 0.5% and 1%. The pH of each was 7.0 and incubation was at 35° C. The tube with the highest benzylamine concentration showing growth was selected as the working substrate concentration.

(c) Identification

The microorganism was next evaluated in the hope of being able to give it a name or a series of characteristics which would allow periodical checks during the various experimental procedures to ensure that the same organism was being used in all the work.

The microorganism was first examined on physical parameters--colony morphology, pigment production, cell shape and size, Gram stain, motility, and growth at 42° C. The biochemical activities were next examined--carbohydrate fermentation, glucose metabolism by fermentative or oxidative means (Hugh-Leifson medium), (32), oxidase test (34), catalase test (1), indole production (35), citrate utilization (33, 46), urease production (8), glycerol (35) and mannitol (35) utilization and H_2S production (23).

(d) Metabolite extractions

Four liters of simple salt medium were prepared containing 0.5% benzylamine buffered to pH 7.0. The complete medium was sterilized by autoclaving at 121° C. for 30 minutes. The cooled medium was inoculated with 180 mgm dry weight of bacterial cells which had been washed in 0.02 M phosphate buffer at pH 7.0. Sterile air was passed through the medium at a flow rate of 500 ml/min. and incubation was carried out at 35° C. in a water bath. At time intervals of 12 hours, 100 ml samples of culture were removed. The sample was passed through a sintered glass filter (course); the filtrate was then passed through a glass-fiber pad (Millipore Co.); then finally the filtrate was passed through a millipore membrane filter (pore diameter 0.45μ). The cell-free solution was then adjusted to pH 4.0 with INHCl and extracted with anhydrous ether (Fischer). The solution was extracted with

100 ml ether then three more times with 50 ml volumes of ether. The ether extracts were combined and dried over anhydrous magnesium sulphate. The anhydrous magnesium sulphate had previously been washed with anhydrous ether, allowed to air-dry then had been dried on glass sheets at 80° C. for two days prior to use as a drying agent. The magnesium sulphate was removed from the ether extract by filtration through a sintered glass filter. The volume of the extract was then reduced to about 3 ml, labeled as "acid extract" and the sample set aside for gas-liquid chromatographic analysis.

The acidified solution was then adjusted to pH 10.0 with 1NNaOH and the solution extracted and treated as before. The product of this procedure was labeled "alkali extract" and the sample set aside for gas-liquid chromatography.

The volume reductions of the ether extracts was done at reduced pressure both to avoid heating which may have altered some components and to reduce the time required so that GC analysis could be done quite soon after the extraction process.

(e) Oxygen Uptake studies

The Warburg constant volume manometer was calibrated by the ferricyanide-hydrazine method. By this method a calculated volume of nitrogen was generated in the system as follows: (53)

$$4Fe^{+++}(CN)_6 + N_2H_4 \longrightarrow 4Fe^{++}(CN)_6 + 4H^+ + N_2$$

From this, the flask constant (k) was determined for each

flask. Since the Warburg manometer has one arm of the manometer tube open, the values obtained will be affected by fluctuations in both atmospheric pressure and temperature during the course of an experiment. To eliminate these variables which would lead to errors, a thermobarometer was set up. This was essentially a manometer containing water instead of substrate and inoculum. When readings were taken, the thermobarometer was read and its value was subtracted from the other manometers thus compensating for any changes in the external conditions of the experiment (13).

Cells, at about the middle of their log phase of growth were harvested from 1000 ml of culture which had been incubated at 35° C. and aerated, during incubation, with sterile air at a flow rate of 500 ml/minute. The suspension was centrifuged at 1000 rpm for 60 minutes using an International centrifuge model U.V. The cells were then resuspended in simple salt medium without substrate at pH 7.0, and again packed. This was repeated three times to wash away the substrate and any debris present in the culture. The packed cells were then finally resuspended in 20 ml of sterile simple salt medium without substrate and sterile air was passed through the suspension at a flow rate of 50 ml/min. for a period of 30 minutes as previous experience had shown that traces of substrate remained in the suspension at high enough levels to affect oxygen uptake experiments. Aeration seemed to increase the rate of consumption so that the levels dropped to a point where they had a negligible effect on the

oxygen uptake studies. The suspension was then checked to determine the dry weight of the cells which was shown to be 19 mgm/ml.

The substrate solutions were prepared so that 1.0 ml contained about 10^{-4} M of substrate. An accurately weighed amount of substrate was weighed out at approximately the calculated weight for a solution containing 1mM/100 ml. The material was dissolved in about 40 ml distilled water and the pH adjusted to 7.0 with either N/2 NaOH or N/2 HCl and then made to a volume of 100 ml with distilled water. Due to the instability of some substances, only freshly prepared solutions were used. The benzoic acid and the catechol were purified before use by the technique of microsublimation under reduced pressure.

The buffer used was in fact the simple salt medium without substrate added and at pH 7.0. 10% w/v KOH was used in the center well to absorb any CO_2 generated in the system. To ensure that ammonia was not being evolved and affecting the readings, a trial was done with 0.2 ml of saturated boric acid solution. No changes in readings compared to the ones where KOH was used were observed indicating (a) no ammonia was evolved; (b) evolved ammonia remained in solution, (c) ammonia evolved was assimilated by the cells to synthesize nitrogenous cell components.

The system was run at 35° C. and shaken at a rate of 70 strokes/minute with a stroke length of 5.0 cm.

In one trial, a blank trial was set up where the inoculum consisted of 19 mgm of heat-killed cells (dry weight) to observe

if any cell components showed oxygen uptake. No change in readings was observed thus buffer was used for inoculum in the blanks.

To check for non-biological oxygen uptake, a trial was done for each substrate using buffer instead of cell suspension as inoculum. With all the substrates tested--benzylamine, benzyl alcohol, benzoic acid, catechol, phenol, and aniline--no oxygen uptake was observed after a two hour period.

(f) Gas-Liquid Chromatography

Qualitative and semi-quantitative analysis was carried out on the "acid and alkaline" ether extracts obtained from the cultures. The instrument used was a Beckman GC-5 with six-foot teflon columns. The oven temperature was programed to start at 100° C. and remain isothermal for sixty seconds and then to rise to 170° C. in three minutes. The run conditions were established experimentally and are summarized in Table I.

The semi-quantitative determinations were calculated by triangulation whereby the area under each peak is geometrically estimated and the value obtained is directly proportional to the amount of material present in the sample. Standards of known concentration were prepared using anhydrous ether as solvent. The instrument was calibrated by estimating the area of these peaks and relating that value back to the concentration of the standard. Eight samples of each standard were run and the

TABLE I

Instrument: Beckman GC-5

Conditions of Run:

(a) Column: Degs 1%
Chromasorb W
AW/DMCS 80/100
six foot teflon column

(b) Injection type - glass inlet

(c) Detector type - H_2 flame

(d) Temperature

(i) column oven linear program:
100° C. to 170° C. in 4'
with a 1' delay

(e) Gases - He - 50 ppsi - 30 ml/min
H_2 - 50 ppsi - 30 ml/min
Air - 40 ppsi - 250 ml/min

(f) Inlet temp - 180° C.

(g) Detector line temp - 206° C.

(h) Detector temp - 260° C.

average value of these runs were used for calibration purposes.

The qualitative determinations were performed by measurement of retention times relative to the solvent peak. Confirmation of peak identity was carried out by co-chromatography with authentic standards both on a Degs 1% column and an Amine-220 column. The Degs column was used for all the semi-quantitative determinations as peak resolution was found to be most satisfactory.

Each "acid extract" obtained as previously described under the heading "Metabolite extraction" was made up to 10.0 ml with anhydrous ether. 1.0 µl of this solution was injected into the instrument and run at attenuations of $2x10^4$, $1x10^4$ and $5x10^3$ on the Degs 1% with three runs being done at each attenuation. 1.0 µl of the sample was then co-chromatogramed with 1.0 µl of each standard--benzylamine, benzyl alcohol, benzaldehyde, benzoic acid and catechol. The remainder of the sample was then stored under refrigeration in a tightly sealed volumetric flask until analysis (qualitative) could be done on the Amine-220 column. The identical procedure was followed with each of the "alkali extract" samples.

When all of the samples had been run and co-chromatogramed with standards using the Degs 1% column, the Amine-220 column was installed in the instrument and the conditions altered as shown in Table I. Each sample was then run and co-chromatogramed with the standards as had been done on the Degs column.

As a check on the quantitative analytical procedure, solutions of the standards were prepared dissolved in the simple salt medium so that the final concentrations were known. 100 ml samples of these solutions were extracted with ether in exactly the same way as the bacterial cultures. The "acid and alkaline" extracts of this procedure were then run on the Degs 1% column and the amount detected was calculated as a percentage of the original concentration of the solution.

For all the standards used in the GLC analysis, the materials were previously purified--the benzylamine, benzyl alcohol and benzaldehyde by re-distillation and the benzoic acid and catechol by micro-sublimation at reduced pressure using a cold finger.

(g) Growth curve analysis

From the four liters of medium used for the extraction of metabolites (see under "Metabolite extractions"), samples were withdrawn at various time intervals for growth analysis. This was done concurrently with the metabolite analysis so that any co-relations between medium composition and growth could be examined. The basic parameters for growth evaluation were dry weight of the cells, optical density and viable counts.

(i) Dry weight

A 20.0 ml sample of the cell suspension was passed through a millipore membrane filter of pore size 0.45μ. The

cells retained on the filter were washed twice with 20 ml volumes of membrane filtered distilled water to remove any medium components from the surface of the cells and the filter. Prior to the filtration, the membrane filters were washed with filtered distilled water and dried to constant weight at room temperature in a desiccator. The weight of the filter was determined using an automatic balance. After the filtration, and washing, the membrane filter was carefully removed from the holder and dried at room temperature in a desiccatoruntil its weight was constant. Twenty-four hours was normally found to be a satisfactory length of time. This weight less the initial weight of the filter was used as the dry weight of the bacterial cells.

(ii) Optical density

For optical density measurements, a Bausch and Lomb Spectronic 20 was used. The same curvettes were consistently used for sample and blank to eliminate the variation inherent from one curvette to another. The sample consisted of 10 ml of the cell suspension and the blank was 10 ml of membrane filtered suspension. Because of pigment production and changes in the composition of the medium which would occur, a blank of the cell-free suspension was always paired with the unfiltered suspension. The measurements were all taken at a wave length of 600 nm.

(iii) Viable counts

Doubling dilutions of the suspension were made into sterile, physiological saline. 10 ml of these dilutions were

assayed for viable cells by spreading the sample over the surface of plates prepared from simple salts medium containing 0.5% benzylamine, and 2% agar. The plates were incubated at 35° C. for 48 hours and the colonies formed were counted. Counts less than 30 or over 300 were not considered to be valid.

Since several of the procedures involved the use of membrane filters, a check was performed to ensure that the possible metabolites did not have a detrimental effect on the membrane filters. Solutions of benzylamine, benzyl alcohol, benzaldehyde, benzoic acid and catechol, each at 1% concentration were prepared in the simple-salt medium and 100 ml of each were passed through a membrane filter which had previously been washed with distilled water and dried to constant weight. After the filtration of the solutions the filter was again dried to constant weight and any changes in the weight of the membrane filter were noted.

(h) Comparative growth curves

About 100 ml of simple-salt medium containing 0.5% of benzylamine were prepared and inoculated with cells. At about the middle of the log phase, the suspension was centrifuged on a Sorvell centrifuge model GLC-1 at about 3000 rpm for 30 minutes. The super natent was removed and the cells resuspended in simple-salt medium only to a volume of about 10 ml. About 5 ml of this suspension was inoculated into 100 ml of simple-salt medium with 0.5% benzylamine and about 5 ml into nutrient broth (Oxoid), also

about 100 ml. Both flasks were incubated at 35° C. and aerated with about 50 ml of sterile air per/minute. After 3 days of growth the cells were removed from suspension by centrifuging and resuspended in fresh medium and again incubated with aeration. After two more days, the cells were again packed and washed twice with sterile physiological saline and finally resuspended in about 20 ml of the sterile saline. In this way, two lots of cells were obtained--one of which had been subcultured and allowed to grow in the absence of benzylamine for several generations and one which had been maintained on benzylamine, yet both sets of cells were derived from the same source of benzylamine-utilizing cells. The optical density reading of the suspensions were adjusted so that a 1:10 dilution gave a reading of 50% absorbance and then 2.0 ml of the nutrient broth grown culture was inoculated into 200 ml of each of the following at pH 7.0 and 0.5% w/v concentration in simple-salt medium--benzylamine, benzyl alcohol, benzoic acid and succinic acid. The identical procedure was followed with the benzylamine-maintained cells. The eight flasks were incubated at 35° C. in a water bath and sterile air was passed through each solution at a rate of about 50 ml per minute. Growth in each flask was followed turbidometrically by withdrawing 10 ml samples at set time intervals, passing 5 ml of the sample through a millipore filter to provide a blank and using the remainder as sample.

3.0 RESULTS

(a) Medium and Isolation

The simple salt medium described under "Procedures" was found to be satisfactory. The results of the growth parameters were as follows:

(i) pH

Tube	pH	Growth
1	6.5	+ +
2	7.0	+ +
3	7.5	+ +

(ii) Temperature

Tube	Temp (^{o}C)	Growth
1	25	+
2	30	+ +
3	35	+ +
4	40	+ +

(iii) Substrate concentration

Tube	Concentration (%)	Growth
1	0.1	+ +
2	0.2	+ +
3	0.3	+ + +
4	0.4	+ + +
5	0.5	+ +
6	1.0	-

From this data, it was decided that growth for the study would be on benzylamine 0.5% w/v at pH 7.0 and 35° C.

(b) Identification of the bacterium

The bacterium was found to be a Gram-negative, aerobic, rod-shaped bacterium which showed motility at 35° C. probably by means of polar flagella by the nature of its movement. It produced pigments in many of the media tested and the color varied from blue-green to a fluorescent yellow-green. Neither acid nor gas were produced from glucose, galactose, lactose or sucrose and no acid was produced from glycerol or mannitol. Citrate was utilized as sole carbon source. The organism was catalase positive and oxidase positive and did not produce indole from tryptophane. It showed oxidative metabolism of glucose on Hugh-Leifson medium and in most media used, extracellular slime was produced. The colonies on nutrient agar were irregular, flat with an irregular margin, transparent and had a smooth glossy surface. Large amounts of blue-green pigment which was water soluble were produced on nutrient agar plates. Growth in nutrient broth occurred at 42° C. but not at 45° C. Neither urease nor H_2S were produced by the organism.

(c) Oxygen uptake studies

The data shown in Table II and Figure 2 have been adjusted to allow for endogenous respiration, thermobarometer readings and also adjusted to give oxygen consumption for exactly

5.0 uM of substrate. The inoculum of cells in each flask was 19 mgm dry weight of cells. The data are the average of three trials for each substrate.

Substrate	Benzylamine	Benzyl Alcohol	Benzoic Acid	Catechol	Phenol	Aniline
Time (min.)						
0	0	0	0	0	0	0
5	6	5	4	6	0	0
10	20	19	21	24	0	0
15	38	36	40	43	0	0
20	56	50	50	61	1	0
25	78	74	72	81	3	2
30	90	87	84	100	5	3
35	110	100	100	120	10	7
40	126	120	118	137	10	8
45	148	140	132	159	11	10
50	162	157	150	176	14	13
55	182	175	166	195	14	14
60	196	193	180	211	15	14
65	216	210	198	233	16	18
70	225	215	213	249	20	18
75	250	230	223	270	20	19
80	260	240	225	291	22	20
85	264	250	226	300	24	20
90	268	250	230	301	23	19
95	270	251	231	300	23	20
100	270	250	230	300	22	20

TABLE II

Metabolite Concentrations as a Function of Time

Oxygen Uptake Curves

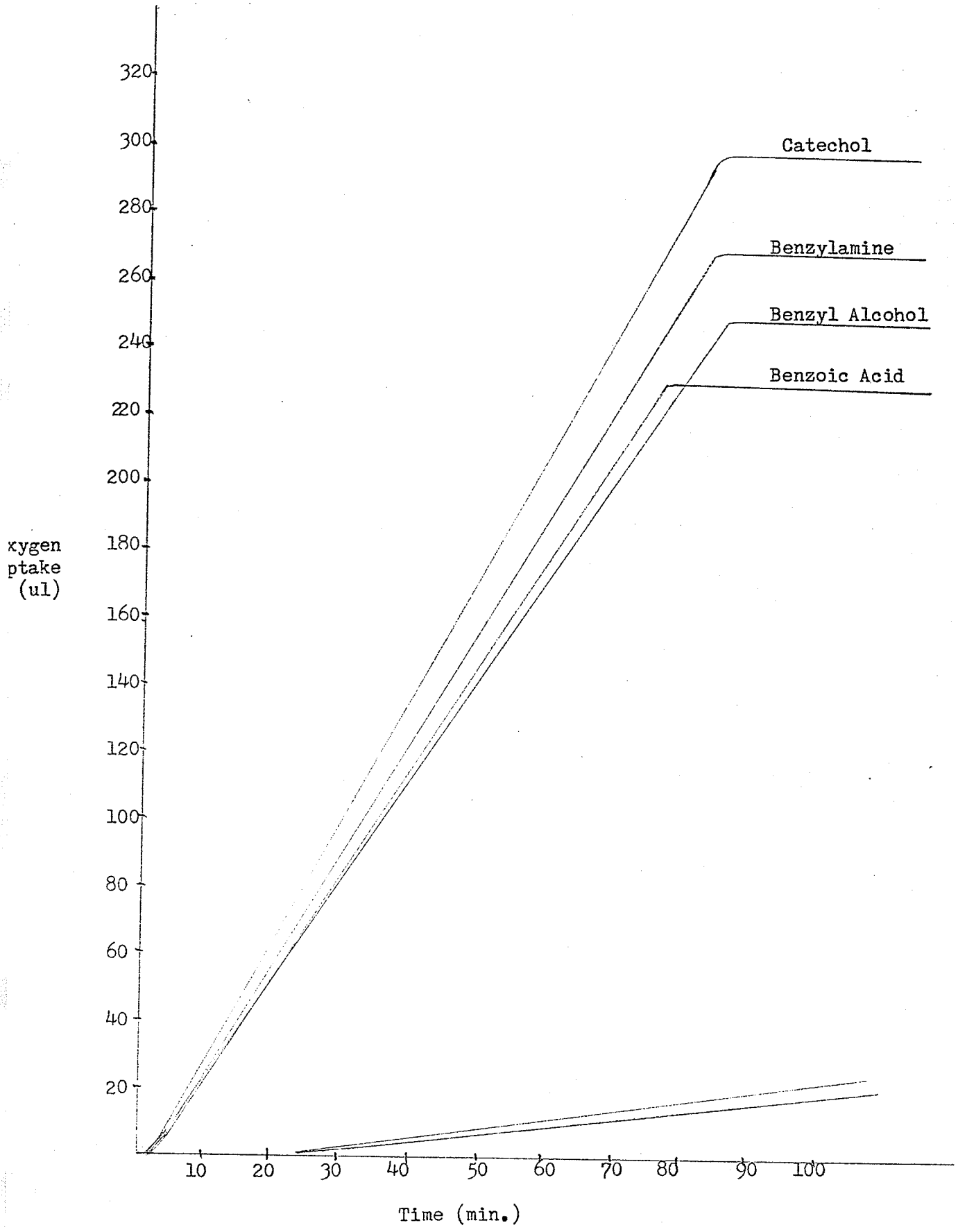


Fig. 2

(d) Gas-liquid chromatography

SEMI-QUANTITATIVE RESULTS

Substrate	Benzylamine	Benzyl Alcohol	Benzaldehyde	Benzoic Acid	Catechol
Day					
0	5.00				
0.5	5.00				
1.0	4.90	0.36			
1.5	4.85	0.40			
2.0	4.75	0.61			
2.5	3.50	1.61			
3.0	1.01	2.78		0.23	
3.5	0.65	2.31	0.10	1.25	
4.0	0.56	1.74		2.21	
4.5	0.40	1.46		2.46	
5.0	0.25	1.00		2.50	
5.5	0.10	0.20		2.48	
6.0				2.41	0.90
6.5				1.91	
7.0				1.23	
7.5				0.30	
8.0					
8.5					
9.0					

TABLE III

Assay Effeciency

Values Obtained	Actual Value
447 mgm	500 mgm
470	
460	
475	
473	
450	
460	
455	
450	
460	
Total 4600	

Average = 460

% recovery = $\frac{460}{500}$ x 100 = 92%

TABLE IV

Metabolite Concentration As A Function Of Time

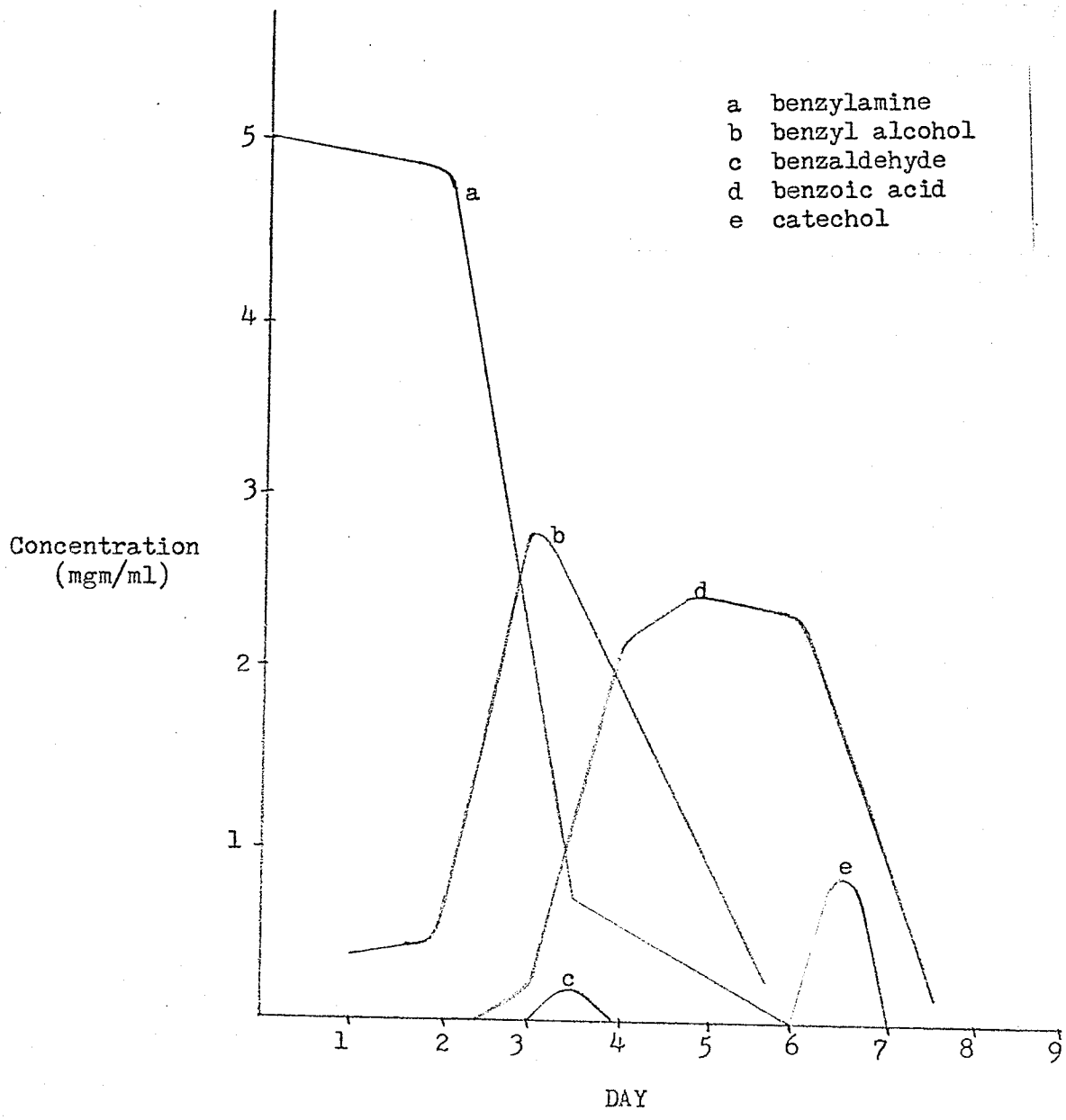
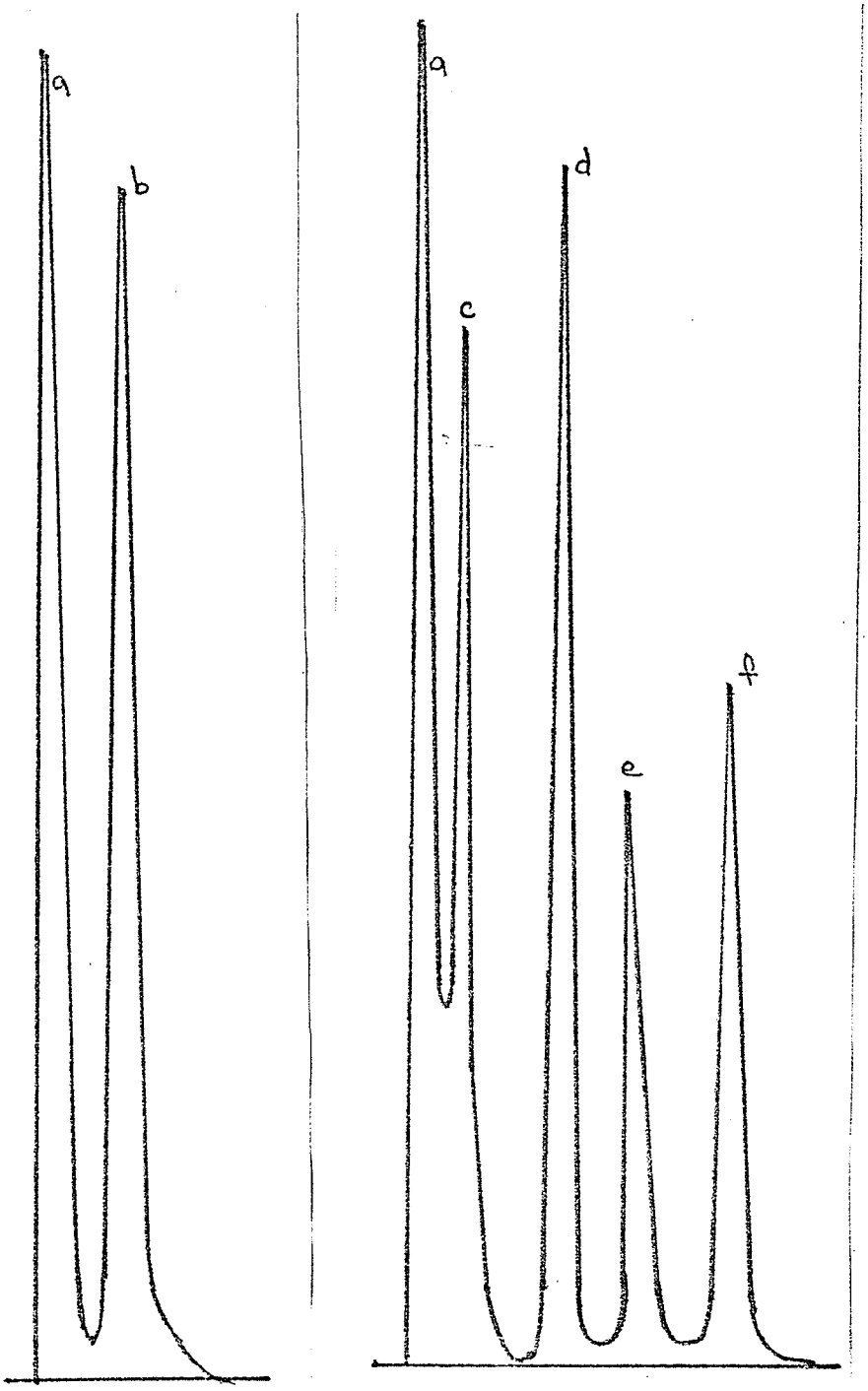


Fig. 3



Alkali Extract

Acid Extract

a solvent
b benzylamine
c benzaldehyde
d benzyl alcohol
e benzoic acid
f catechol

Fig. 4

QUALITATIVE-RETENTION TIMES RELATIVE TO SOLVENT PEAK

	Degs 1%		Amine-220	
Substance	Authentic	Extract	Authentic	Extract
Benzylamine	85 sec.	85 sec.	90 sec.	90 sec.
Benzyl alcohol	210 sec.	210 sec.	14 sec.	14 sec.
Benzaldehyde	60 sec.	60 sec.	445 sec.	445 sec.
Benzoic acid	332 sec.	332 sec.	455 sec.	455 sec.
Catechol	480 sec.	480 sec.	840 sec.	840 sec.

TABLE V

Growth Curves Obtained Using Cells Grown On Benzylamine

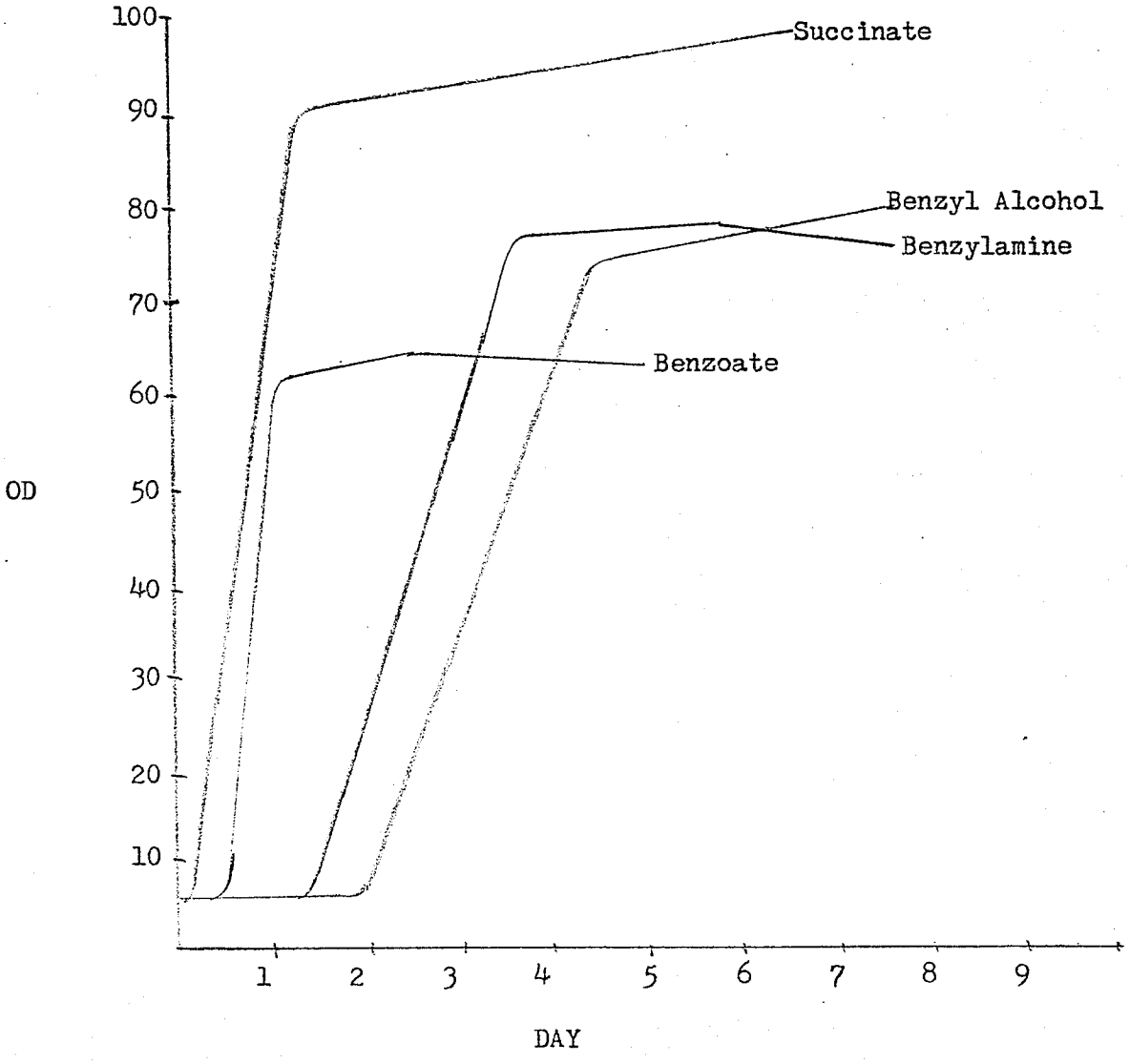


Fig. 5

Growth Curves Obtained Using Cells Grown On
Nutrient Broth

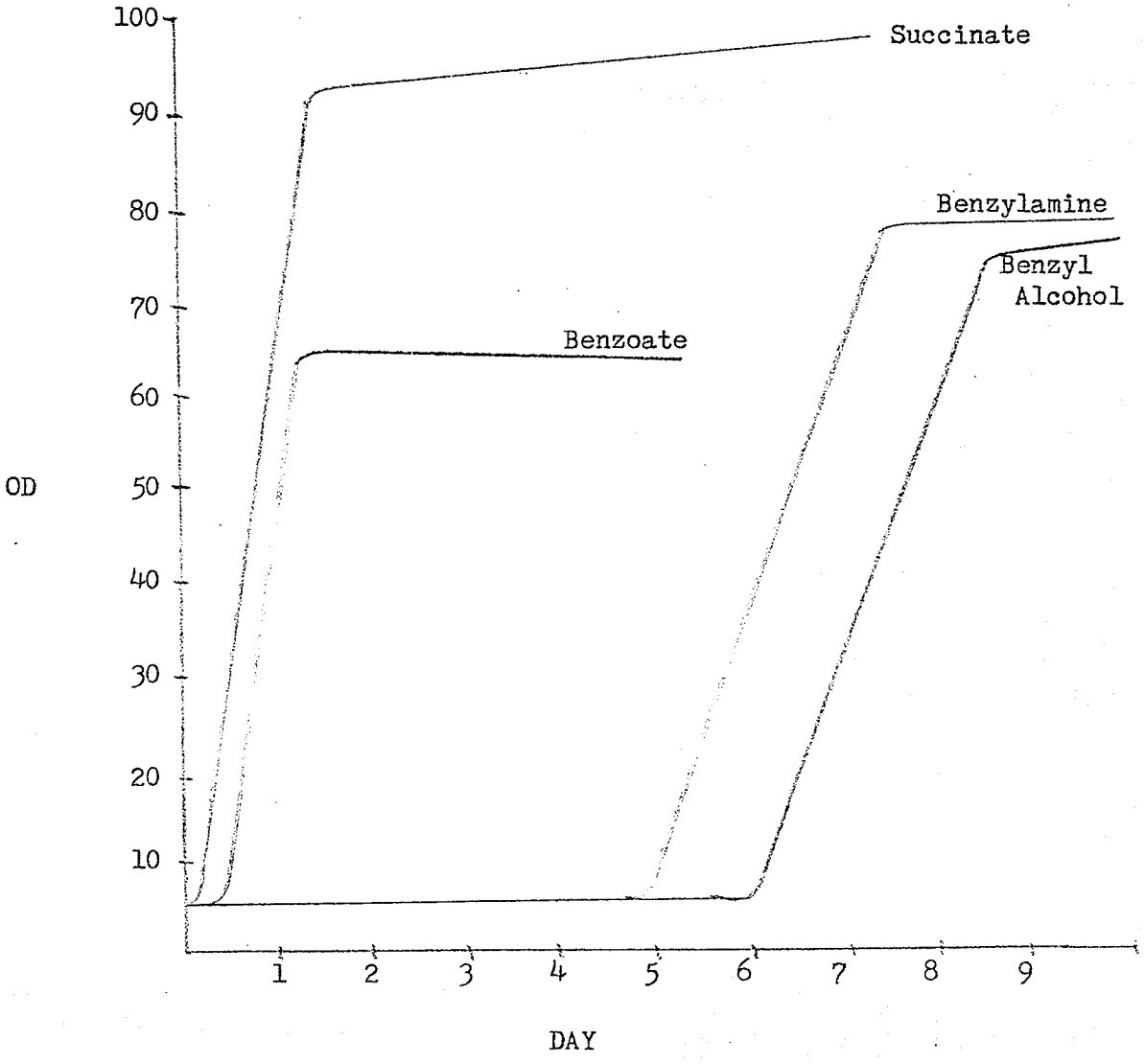


Fig. 5

(e) Comparative growth curves

B = benzylamine subculture
N = nutrient broth subculture

	Benzylamine		Benzyl alcohol		Benzoic acid		Succinate	
Day	B.	N	B.	N	B.	N	B.	N
0	5.0	5.0	5.0	5.0	5.0	5.0	5.0	5.0
0.5	5.0	5.0	5.0	5.0	9.0	9.0	32.0	32.0
1.0	5.0	5.0	5.0	5.0	60.0	60.0	68.0	68.0
1.5	9.0	5.0	5.0	5.0	63.0	62.0	90.0	90.0
2.0	25.0	5.0	6.0	5.0	64.4	65.0	92.0	92.0
2.5	39.0	5.0	19.0	5.0	65.0	65.0	92.8	92.6
3.0	54.0	5.0	33.0	5.0	65.0	65.0	93.0	93.0
3.5	69.0	5.0	46.0	5.0	65.0	64.0	94.0	94.0
4.0	77.5	5.0	60.0	5.0	64.5	64.0	95.0	95.0
4.5	77.8	5.0	73.0	5.0	64.0	64.0	96.0	96.0
5.0	77.9	8.5	75.0	5.0	63.0	63.0	97.0	97.0
5.5	78.0	21.0	77.0	5.0	61.0	60.0	97.1	97.0
6.0	78.0	37.0	77.0	6.5			97.3	97.2
6.5	77.0	53.0	77.0	18.0			97.1	97.0
7.0	76.0	68.0	76.5	31.0			97.0	97.0
7.5	75.0	77.0	76.0	45.0				
8.0		77.6		59.0				
8.5		77.8		73.0				
9.0		78.0		76.0				
9.5		78.0		77.0				
10.0		76.0		77.0				

TABLE VI

4.0 DISCUSSION

The simple salts medium chosen for this series of experiments proved to be quite satisfactory although in some respects this was unexpected. The medium contained no added iron and it has been shown that Pseudomonas aeruginosa has a definite requirement for iron (55). Studies with Enterobacter aerogenes, Escherichia coli, and Klebsiella pneumonia showed a requirement for about 0.03 ppm of iron for optimal growth while Pseudomonas aeruginosa required to the order of 0.12 ppm of iron (55). This is a reasonable finding considering the very high levels of iron-containing cytochromes and enzymes in the cells (29, 30). In our experiments, however, the addition of even very small amounts of iron (ferric ions) led to the formation of a precipitate which would have interfered with our optical density readings for growth evaluation. Since the cells grew well using this medium, it is most probable that the water used, although distilled, contained trace amounts of iron sufficient for the needs of the cells. This iron may have been leeched from the pipes or fixtures carrying the water or it is also possible that trace amounts of iron may have been present in one or more of the other components in the medium.

The choice of growth parameters was done quite arbitrarily. A pH of 7.0 was chosen even though growth occurred to the same extent at pH 6.5 and 7.5. It was thought that at pH 7.0 the

suspected metabolites would be most stable and since both acidic and basic components would be present, at neutrality they would all be more likely to remain in solution. Since growth occurred at 30, 35 and 40° C., 35° C. was chosen as it was median. The choice of substrate concentration was somewhat more rational since one of the objectives of this study was to detect the metabolic intermediates. The concentrations of the intermediates would be quite low in any case, but the higher the initial substrate concentration, the higher the level of intermediates and thus the better the chance of detecting them. For this reason a substrate concentration of 0.5% was chosen even though a heavier yield of cells were obtained at concentrations of 0.4% and 0.3%.

These tests were by no means intended to be definitive but rather to act as very rough guides as to what set of conditions would be most advantageous to our purposes. Other conditions were also determined in this rather empirical fashion--for example, it was found that aeration seemed to speed the growth rate which was in agreement with Calam (7) and the flow rates used for the air were arbitrary.

The microorganism was identified as probably being Pseudomonas aeruginosa. This identification was made on the basis of cell and colony morphology, growth at 42° C., oxidative metabolism, of glucose, oxidase and catalase production, pigment production and citrate utilization.

Although more than 140 species of the genus Pseudomonas

are listed in Bergey's Manual (5) the characters examined for this microorganism seem to be distinctive for Pseudomonas aeruginosa as described by several authors (5, 27, 28, 35). The genus is very wide spread with at least three-quarters of the members being found in soil and/or water. The group as a whole is also known for their metabolic versatility (35), with many able to grow on mineral salts medium with an inorganic nitrogen source and a simple carbon source. This versatility probably contributes to some extent to the terrible confusion existing at present regarding their taxonomy.

During the course of the experimental work, an attempt was made to grow the organism in medium using benzylamine as sole source of carbon, energy and nitrogen. Since very slight growth was noted, it was assumed that an additional source of nitrogen was necessary as the amount of nitrogen liberated from the benzylamine was so low as to become a growth-limiting factor.

The oxygen uptake studies showed that benzylamine, benzyl alcohol, benzoic acid and catechol were oxidized with no lag indicating that the enzymes catalyzing their oxidation were present in the cells. Phenol and aniline showed no immediate oxygen uptake indicating that the enzymes which would oxidize these materials were not present in the cells. This information indicated that neither phenol nor aniline were intermediates in the pathway by which benzylamine was utilized by these cells (48, 51).

Quantitative measurements of the metabolite concentrations were done using a Beckman GC-5 and these values were plotted against time (Figure 3). Since the concentrations were calculated by using a triangulation procedure, considerable error was introduced as can be seen in Table IV. Because of the error factors inherent in the procedures used, the results of these experiments are termed semi-quantitative and not meant to be taken as absolute values but rather as indications of the relative changes occurring in the medium with time. The efficiency of the complete assay procedure was found to be to the order of 92%. To arrive at this figure, solutions of known metabolite concentrations were extracted and assayed using the same procedures as was used for the cultures. An average of 92% of the original amounts were detected. Although the value is for a very artificial set of conditions, it did show to some extent how efficient our system was.

The data obtained showed the time relative to growth of the cells that the maximum levels of the metabolic intermediates were reached and also gives the order in which they appeared in the medium and thus the order in which they are probably formed.

The qualitative results using gas-liquid chromatography were of course much more satisfactory than the quantitative values. On both systems used, the metabolites formed single peaks with added standards of the corresponding authentic materials and the retention times for each set of peaks were quite

different on the different columns. A more satisfactory procedure would have been that of peak-shift where derivatives of the metabolites are prepared and these co-chromatographed with corresponding standards. The formation of tri-sil (19) derivatives was attempted but running conditions for their satisfactory peak resolution could not be established. The retention times were extremely short and all the peaks extremely close together on Degs 1%, OU-17, and Amine-220.

Qualitative results were not felt to be essential in this study as the levels reached, and rates of production, accumulation and breakdown are functions of the cells used and of the conditions under which they are grown. The important aspect was that the intermediates be detected.

The first intermediate detected was benzyl alcohol which was quite interesting. Benzyl alcohol is considered to have an antiseptic effect (38), and has been incorporated as a preservative in ampuls of injectible drugs (36) usually to the order of 1%. It is interesting to note that benzyl alcohol is no longer commonly used as a preservative.

The maximum level of benzyl alcohol was detected on day three of growth, corresponding roughly to about the mid-point of the log phase of cell growth, and the maximum level was to the order of 0.3% w/v under the conditions used. The conversion of benzylamine to benzyl alcohol is probably mediated by an oxidase enzyme of some type, with the release of an ammonium ion. The

degradation of benzylamine by mammalian microsomes follows a different pathway, going first to benzylimine and then to benzaldehyde with no formation of benzyl alcohol (56).

The second metabolite to be detected was benzaldehyde. This material was detectible only for a short period of time on day 3.5 and although the characteristic almond-like odor of benzaldehyde was very noticeable in the vicinity of the culture, only a small amount was detected on analysis--to the order of 0.01%. Because the material is somewhat volatile (38) and air was being passed through the culture, some of the material may have been lost. A control trial was set up in which air was passed through a 0.1% solution of benzaldehyde in simple salt medium at 35° C. with an air flow rate of 50 ml/min. After 48 hours, about 30% of the benzaldehyde was still present. This rate of disappearance was much slower than that observed in the growing culture and it was concluded that an enzymatic process was involved. It has been shown that brewer's yeast is able to form benzaldehyde from benzyl alcohol by means of a pyridine nucleotide-linked dehydrogenase (47) and it has also been shown that benzaldehyde is an intermediate in the conversion of mandelic acid to benzoic acid (22) by Pseudomonas fluorescens.

The next metabolite to be detected was benzoic acid. This material reached a maximum level on day five and in the system used reached a level to the order of 0.25%. The conversion of benzaldehyde to benzoic acid is quite easy and can occur

by atmospheric oxidation. A blank trial showed, however, that atmospheric oxidation alone could not account for the rapid conversion to benzoic acid. An enzyme system such as benzaldehyde: NADP oxidoreductese--E.C.1.2.1.7 (15) would account for the rapid conversion of benzaldehyde to benzoic acid. The metabolism of benzoic acid has been well studied and the pathway established (18, 40, 41) as conversion to catechol followed by ortho cleavage.

The last metabolite detected was catechol. The maximum level was detected on day 6.5 and it was to the order of 0.09%. The degradation of catechol and its formation from benzoic acid has been well established (10, 11, 18, 24, 40, 41) and was discussed earlier.

Growth assessment was based on:

(i) optical density measurements

(ii) dry weight of the cells

(iii) viable count

For the optical density readings, blanks consisting of membrane-filtered cell suspensions were used against each sample examined. This was essential as pigments were produced which would have lead to a very large error (39).

In the dry weight determinations, a membrane filter technique was used (45). Since it was noted that benzyl alcohol could dissolve cellulose acetate (38) and thus possibly dissolve a portion of the membrane filter, a blank was done using a 1% solution of benzyl alcohol in salt medium. A filter was washed,

dried to constant weight and then used to filter the solution. The filter was then again dried to constant weight. No significant change in the weighings was noted indicating that the metabolite had no significant effect on the dry weight values obtained.

To check the validity of the optical density procedure with the dry weight procedure, standards were prepared from dilutions of a heavy cell suspension. First the optical density was recorded and then the dry weight determined. Both methods showed very good co-relations and it was found that one optical density unit was equivalent to about 0.009 mgm dry weight of cells. Some distortions were expected at the end limits (3).

An attempt was made to co-relate optical density to the viable count but it was found that the viable count values showed a low level of reproducibility. In the procedure used, dilutions of the cells were prepared and then a measured volume spread over the surface of a plate and incubated for 48 hours then counted. Since it is known that high phosphate levels favor the production of slime by Pseudomonas aeruginosa (31), a medium with much lower phosphate levels was tried but there was still a low level of reproducibility. No satisfactory explanation for this could be found.

The growth curves on benzylamine, benzyl alcohol, benzoic acid and succinate, using cells maintained in nutrient broth and cells maintained in benzylamine were compared on the basis of

their growth lag phase. The cells maintained on benzylamine showed lag times of the following order:

benzylamine	1.3 days
benzyl alcohol	1.9 days
benzoic acid	0.4 days
succinate	0.1 days

The cells subcultured into nutrient broth prior to transfer to benzylamine medium showed lag times of the following order:

benzylamine	5.0 days
benzyl alcohol	6.0 days
benzoic acid	0.4 days
succinate	0.1 days

As can be seen there was no measurable difference in the lag times for succinate and benzoate indicating that these enzymes are probably constitutive. There was a dramatic increase in the lag times for benzylamine and benzyl alcohol, however, indicating that the enzymes involved in their metabolism are probably inducible (44). It has been shown that several pseudomonads have constitutive benzoate oxidase (35), for example Pseudomonas aeruginosa, Pseudomonas chlororaphis, Pseudomonas pseudomallei, Pseudomonas fragi, Pseudomonos statzeri, Pseudomonas denitrificans, Pseudomonas iodinum and Pseudomonas taetrolens.

From the data obtained the following pathway may be

proposed; benzylamine to benzyl alcohol, to benzaldehyde, to benzoic acid, to catechol. (Figure 6)

A great deal of work would have to be done to confirm this pathway, as in this study only enough information was presented to indicate that this may be the catabolic pathway used by Pseudomonas aeruginosa to utilize benzylamine.

That the enzymes involved in this pathway are inducible, cannot be concluded from the data presented here. Increased lag times alone do not provide any clear cut indication of inducibility. Further work using resting cells and measurement of oxygen uptake would have to be done and increased lag in oxygen uptake would be considered as indication that the enzyme systems are inducible.

PROPOSED PATHWAY

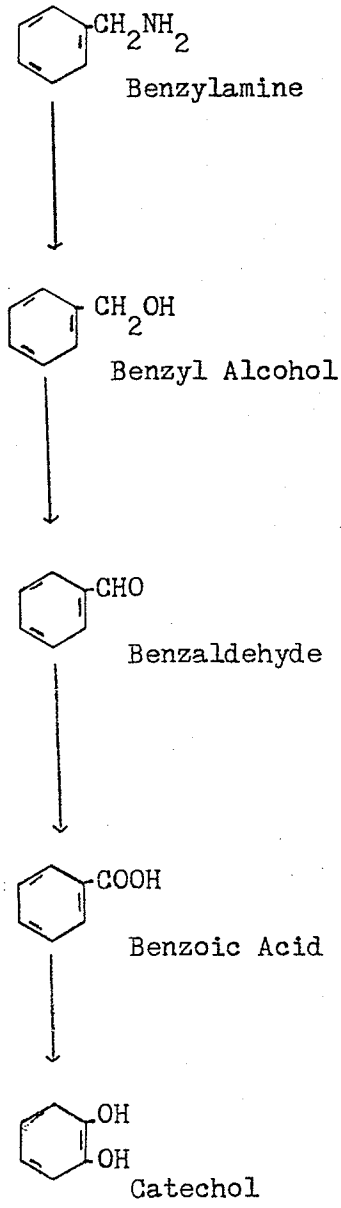


Fig. 6

5.0 SUMMARY

A strain of Pseudomonas aeruginosa isolated from soil was examined for its ability to utilize benzylamine as sole source of carbon and energy. Growth curves showed an inducible enzyme system was probably involved. By the use of gas-liquid chromatography a series of metabolites were identified and their probable sequence of production determined. It was found that this pathway was possibly benzylamine to benzyl alcohol, to benzaldehyde to benzoic acid to catechol.

APPENDIX

CHEMICAL MATERIALS

All materials used were of analytical grade as supplied by the manufacturer. The catechol used was purified using a sublimation technique and the redistilled benzaldehyde was kindly supplied by Dr. J. Templeton.

Agar	Difco Co.
Amine-220	Applied Science Co.
Ammonium chloride	BDH
Ammonium dihydrogen orthophosphate	BDH
Benzaldehyde	
Benzoic acid	BDH
Benzyl alcohol	BDH
Benzylamine	Baka
Calcium chloride	BDH
Catechol	Matheson, Coleman and Bell
Degs	Applied Science Co.
Diethyl ether (anhydrous)	Fisher
Dipotassium hydrogen orthophosphate	BDH
Magnesium sulphide	BDH
Nutrient broth	Oxoid
Succinic acid	BDH

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