STUDIES ON THE BACTERIAL UTILIZATION OF AMINES

by

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I

INTRODUCTION

A number of enzymes catalyzing reactions which lead to *in vitro* destruction of amines have been shown to be present in animal and plant tissues, but comparatively little is known about the distribution and properties of such enzymes in bacteria. In addition to contributing to our knowledge of nitrogen metabolism of microorganisms,studies of the bacterial enzymatic processes which bring about the utilization of amines may help to interpret other phenomena. For example, many antibiotic agents are monoamines or polyamines, hence the effect of these agents on bacteria may be explained by their ability to interfere with amine metabolism. Recently, a diamine has been added to the list of required growth substances for some bacteria, however the reason for the need of this accessory growth factor is not known. While the importance of the removal of amines formed in the large intestine is realized, the role that the bacteria play in their destruction is not fully known.

Not only are the enzymes involved in the utilization of amines of importance but also the bacteria themselves are of interest. Presently, the genus *Protaminobacter* are classified on the basis of the degradation of alkyl amines. Other genera have been shown also to possess this biochemical function.

It is the purpose of this thesis to investigate the extent of the bacterial utilization of amines and establish the role of these enzymes in bacterial metabolism.

II

HISTORICAL

Any attempt to assess significance of the utilization of amines by bacteria must first consider the formation of these compounds. With this in mind, a brief survey of published work on the bacterial production of amines will precede the literature review on utilization of these compounds.

The presence of amines due to action of bacterial decarboxylases on amino acids has been reported at different times, first by mixed putrefactive bacteria and later by pure cultures. Müller in 1857 obtained isoamylamine from putrefied yeast. Probably this amine was formed from the decarboxylation of leucine (Schales, 1951).

Putrescine and cadaverine were isolated by Ellinger (1900), after ornithine and lysine, respectively, had undergone degradation. Work showing the formation of amines in mixed cultures has been cited by others (Schales, 1951; Porter, 1946).

Various workers using pure cultures studied the biological production of amines. This ability has been shown to be characteristic of action on a variety of amino acids by bacteria belonging to widely separated genera (Gale, 1946; Porter, 1946; Schales, 1951).

Gale (1946) reported that the production of amines by bacteria is due to specific amino acid decarboxylases formed

within bacterial cells in response to well defined conditions of growth. The enzymes have been obtained in a cell-free state and their properties have been investigated in detail. The amino acid decarboxylases are formed and are active only when the organisms are grown at low pH values.

Although the formation of amines by bacteria had been known for some years, published reports on the further degradation of the amines were not available until 1926. At this time the classical studies of den Dooren de Jong (1926, 1927) showed that a large number of compounds can serve as the sole source of carbon in a synthetic medium for a group of common organisms which can utilize ammonia as the sole source of nitrogen. Of the 200 compounds tested, 41 were amines. The group tried were not able in general to utilize the amines as sources of carbon and nitrogen under the conditions used. These organisms were: _Bacillus subtilis_; _Bacillus mycoides_; _Bacillus polymyxa_; _Aerobacter aerogenes_; _Escherichia coli_; _Serratia marcesens_; _Bacillus herbicola_; _Proteus vulgaris_; _Mycobacterium phlei_; _Micrococcus pyogenes_ var. _albus_; _Pseudomonas fluorescens_; _Spirillum tenue_ and _Pseudomonas aminovorans_ α. When glucose was added and the amine was the source of nitrogen, many of the organisms were able to grow. _Pseudomonas aminovorans_ α was most active in decomposing amines. Den Dooren de Jong did not study the dissimilation products of the various amines, but merely their ability to support visible growth.

Den Dooren de Jong (1926) proposed a separate genus of bacteria, _Protaminobacter_, differentiated from other bacteria on the basis of their ability to dissimilate alkyl amines.

In the present edition of _Bergey's Manual_ (Breed _et al._, 1948) the _Protaminobacter_ comprise a genus of the Family _Pseudomonadaceae_; there are two species: _Protaminobacter alboflavum_ and _Protaminobacter rubrum_.

Weaver _et al._, (1938) isolated a culture from a sewage disposal plant having characteristics of _Protaminobacter rubrum_ den Dooren de Jong, except that the organisms were actively motile by means of single polar flagella. Although the original species had been reported as non-motile, _Bergey's Manual_ changed the description upon the recommendation of these investigators.

Gale (1942) studied the oxidation by washed cells of _Pseudomonas aeruginosa_ of several amines that are formed upon the decarboxylation of some amino acids. This species carries out the oxidation of putrescine, cadaverine and agmatine to completion, i.e., to carbon dioxide, water, and ammonia. The oxidation of tyramine and histamine was found to be incomplete. The oxidations took place more rapidly at pH values between 7.5 and 9.5 and all oxidations but the oxidation of tyramine were found to be inhibited by M/100 semicarbazide or M/10000 cyanide. In the sense of Karström (1930), the enzymes appear to be constitutive while the primary stages of histamine and tyramine oxidation are adaptive. Some strains of _E. coli_ could oxidize tyramine, putrescine, and cadaverine, but in

no case was the activity marked. The organisms used were not able to utilize primary amines such as methyl amine or ethyl amine.

Zeller _et al._, (1951) found that ethylene diamine, 1,3-trimethylene diamine, 1,3-diaminopropanol, 1,3-diaminobutane, putrescine, cadaverine, and hexamethylene diamine undergo oxidative deamination by _Mycobacterium smegmatis_ due to the presence of diamine oxidase. This diamine oxidase is inhibited by direct interaction by 1,4-diguanidinobutane (arcaine), streptomycin, and dihydrostreptomycin.

This inhibition of diamine oxidase by these same compounds was also found to occur in _Mycobacterium avium_, _Mycobacterium tuberculosis_ var. _hominis_, _Pseudomonas aeruginosa_ and _Micrococcus pyogenes_ var. _aureus_.

Other antibiotic agents such as pyocyanin, streptothricin, chloramphenicol, and a wide variety of synthetic amines, also gave this inhibition (Owen _et al._, 1951). Interest is shown in the inhibition of diamine oxidase by dihydrostreptomycin because of the possible explanation of the therapeutic action of this drug.

An unidentified factor necessary for the growth of _Hemophilus parainfluenzae_ was found later to be putrescine, a diamine. Other compounds containing the 1,4-diaminobutane residue, such as spermidine and spermine were effective. The requirement is not satisfied by ornithine, hence it is assumed that the enzyme which catalyzes the reaction, ornithine to putrescine, ornithine decarboxylase, is absent (Herbst and Snell, 1949a, 1949b).

Other workers have found putrescine to be a growth requirement of Neisseria perflava; spermidine, agmatine, and cadaverine also satisfy this need (Martin et al., 1951).

Animal and plant tissues contain two enzymes which oxidize amines: monoamine oxidase which is specific for amines containing one NH_2 group; and diamine oxidase which is specific for amines containing two NH_2 groups. These enzymes split off NH_3 from the amine with the utilization of 1 atom of oxygen per molecule to form the corresponding aldehyde and hydrogen peroxide in the following manner:

$$RCH_2NH_2 + O_2 \longrightarrow RCH = NH + H_2O_2$$

Monoamine
or
Diamine

$$RCH = NH + H_2O \longrightarrow RCHO + NH_3$$

The two enzymes are not only differentiated by their specificity but also by the following: the monoamine oxidase is the less soluble and is active in the presence of semicarbazide while the diamine oxidase is relatively more soluble and is inhibited by semicarbazide and other carbonyl fixatives (Sumner and Somers, 1947; Lardy et al., 1949; Zeller, 1951).

Kenten and Mann (1951), in an investigation of enzymes catalyzing reactions which lead to the formation of hydrogen peroxide in extracts of pea seedlings, found that not

only diamines but also certain monoamines were oxidized by these extracts with formation of hydrogen peroxide. They believe that the pea-seedling enzyme which attacks mono-amines differs from the previously described plant and animal monoamine oxidases.

Amines have also been studied in regards to their ability to prevent growth. Tilley and Schaffer (1928) re-ported on the relation between the chemical constitution and the bactericidal activity of some alkyl and aryl amines and found that low molecular weight amines had poor germici-dal qualities but that the disinfectant powers increased as the homologous series increased. Since then, long-chain monoamines and diamines have been found to be strongly bactericidal (Fuller, 1942).

III

MATERIALS AND METHODS

Media. The basal medium used in preliminary isolation work of organisms was that of den Dooren de Jong (1926). This consisted of 0.1 per cent KCl; 1.0 per cent $MgHPO_4.7H_2O$; tap water. This medium was considered unsatisfactory due to the use of tap water and because of a precipitate formed by the $MgHPO_4$.

A basal medium of Gray and Thornton (1928) modified by the omission of a nitrogen source, composed of K_2HPO_4 0.10 per cent; $FeCl_3$ trace; distilled water; was next used. Each ingredient was made up and sterilized separately but upon mixing of the ingredients there was a slight turbidity and a flocculent precipitate formed.

Before turbidimetric measurements could be made, a clear medium was required. Such a medium was the medium of Bushnell and Haas (1941) modified by the omission of a nitrogen source and reduction in the concentration of $CaCl_2$. This medium was composed of $MgSO_4$ 0.02 per cent; $CaCl_2$ trace; KH_2PO_4 0.10 per cent; K_2HPO_4 0.10 per cent; $FeCl_3$ trace; distilled water.

Cultures. Two known cultures, Protaminobacter rubrum ATCC 8457 and Protaminobacter alboflavum ATCC 8458 were acquired from the American Type Culture Collection, Washington, D.C. Enrichment culture techniques were used to isolate other cultures. The method consisted of adding 0.5 g of

soil or chick feces to 25 ml autoclaved basal medium plus amine concentrations of 0.01 M and 0.001 M of the HCl or HBr salt of various amines previously sterilized by Seitz filtration. Adjustment to pH 7.0 was made by the addition of either NaOH or HCl. These flasks were incubated at 28 C for 5 days. After being subcultured three times by transferring 0.1 ml of each flask to new medium, the cultures were placed on solid medium consisting of basal salts, the appropriate concentration of the amine and 2.0 per cent agar. Isolated colonies were transferred to a liquid medium containing the amine used originally for isolation and were subsequently transferred to amine agar and nutrient agar slants.

Morphology and physiology of cultures. Morphological studies were performed using a 48 hour culture grown in the basal medium plus 0.01 M ethyl amine. The hanging drop technique was used to determine motility of cultures and flagella were demonstrated using the method of Bailey (1929). The gram stain technique and biochemical tests employed were those suggested in the Manual of Methods for Pure Culture Study (1948).

Fermentation of carbohydrates were tested by the addition of the previously filtered carbohydrate to sterile nutrient broth containing phenol red indicator and Durham tubes. These determinations were made after 14 days at 30 C.

Optimum temperature requirements of organismswere determined by inoculating one loopful of bacteria from tubes

containing basal and ethyl amine into tubes of the same medium. These were incubated at 6 days at 4 C, 20 C, 30 C, 37 C and 55 C, and examined for growth as indicated by turbidimetric measurements using the Klett-Summerson photoelectric colorimeter using a blue filter (400-465 mμ).

Optimum pH requirements for organisms were determined by inoculating one loopful of bacteria into tubes of various pH units containing basal and ethyl amine. These tubes were adjusted to the proper pH by the addition of NaOH or HCl and the pH was determined by the use of a Beckman glass electrode pH meter. These tubes were incubated at 6 days at 28 C and examined for growth turbidimetrically as given previously.

Growth curve experiments were made by pipetting 0.1 ml of 4 day old ethyl amine-glucose cultures into 5.0 ml of the same medium in matched Klett-Summerson tubes. Growth was determined at various intervals by turbidity readings on the Klett-Summerson photoelectric colorimeter using the blue filter (400-465 mμ).

Estimation of Growth on Test Compounds. Washed cells from an ethyl amine agar slant were transferred to liquid synthetic media prepared by the addition of salts and the specific carbon compound or compounds. These tubes were incubated at 30 C for 6 or 13 days depending on the organism used and the growth was estimated turbidimetrically with the aid of a Klett-Summerson photoelectric colorimeter using a blue filter (400-465 mμ). Approximately 0.05 ml from tubes showing growth was placed into new media. Growth in at

least three serial transfers was considered the criterion for utilization of the carbon compounds added.

Cell Crop for Respiration Studies. For respiration studies organisms were grown at 30 C for 48 hours in a modified Bushnell and Haas liquid medium containing 0.01 M ethyl amine and 0.10 per cent glucose in addition to the basal salts. 0.25 ml of this culture was spread on plates of an agar medium of the same composition by means of a sterile bent glass rod. After incubation at 30 C for 48 hours the cells were harvested by the addition of water followed by a dislodging of the growth with a bent glass rod. After filtering through two layers of cheese cloth to remove particles of agar medium, the cells were centrifuged at 4250 g for 10 min with a Sorvall type SS1 centrifuge. The supernatant was discarded and the cells were resuspended in water and washing was repeated twice to remove as much oxidizable material as possible. The washed cells were resuspended in M/10 phosphate buffer pH 6.75 to obtain a suspension of such turbidity that 0.1 ml added to 9.9 ml distilled water gave a reading on the Klett-Summerson photoelectric colorimeter using the blue filter (400-465 mμ) corresponding to the desired concentration in terms of bacterial nitrogen per ml of suspension as determined by the micro-kjeldahl method (figure 1).

Micro-kjeldahl Determination of Bacterial Nitrogen. Cell nitrogen was determined by the micro-kjeldahl method wherein the cell protein is digested to ammonium sulphate with

sulfuric acid and sulfates in the presence of selenium catalyst. In a micro-distillation apparatus the ammonia evolved by treating with NaOH is absorbed in boric acid which is then titrated with sulfuric acid. A minor modification of the method of Kabat and Mayer (1948) was followed.

Manometric Measurements. Oxygen utilization was measured by means of Warburg constant volume respirometers of both the single and double arm type according to standard techniques (Umbreit *et al.*, 1949). The fluid volume of 3.1 ml consisted of 1.0 ml M/10 potassium phosphate buffer pH 6.75, 1.0 ml cell suspension, 0.5 ml substrate and distilled water to give a total volume of 3.0 ml in the main well and side arm; while the center well contained a strip of filter paper folded in accordion fashion moistened with 0.1 ml 25 per cent KOH to absorb the CO_2 evolved during the oxidation. The flasks were shaken in an atmosphere of air in a constant temperature bath at 30 C.

An endogenous flask consisting of no substrate was included to check respiration of cells alone and to permit corrections for various chemical determinations. Gas exchanges and analyses were corrected for endogenous activity except where indicated in the results.

Substrates were made up as 0.01 M solutions in distilled water and neutralized to pH 6.8 with NaOH or HCl.

Inhibitors, where used, were added from a second side arm at the same time as the substrate.

The indirect method of Warburg was used for the determination of CO_2. At the end of an experiment involving CO_2 determination, 0.2 ml of 10 N H_2SO_4 was added to liberate dissolved CO_2.

Storage of Cells. In the early studies fresh cells were prepared for each separate manometric determination. Experiments were carried out to determine the effect of storage of cells at a low temperature. The cells were prepared as given previously, stored in M/10 phosphate buffer pH 6.75 at 5 C, and tested for their ability to oxidize ethyl amine.

Lyophilization of Cells. Lyophilized cells were prepared by quick freezing and vacuum drying according to the standard method used for the preservation of cultures (Flosdorf and Mudd, 1935).

Vacuum Dried Cells. A heavy suspension of cells was placed in a flat dish over concentrated H_2SO_4 for 12 hours in a dessicator which was evacuated for 4 hours using a pump.

Acetone Preparations of Cells. A bacterial suspension consisting of a thick cream was added dropwise to 20 ml ice cold anhydrous acetone. This mixture was stirred vigorously and allowed to settle after flocking of the cells had occurred. The supernatant was discarded and the remainder was filtered through a Buchner funnel by suction. The residue was treated with a small portion of anhydrous cold acetone and then was dried under a vacuum (Umbreit et al., 1949).

Alumina Preparation of Cells. A cell suspension obtained as described above was recentrifuged in a chilled cup 10 min at 4600 g. The recovered paste of wet bacterial cells of a total weight of 1.5 g was transferred to a chilled mortar with the aid of 2 ml chilled buffer. After the addition of 4.5 g alumina the mixture was ground for 4 min, mixed with 6 ml more of cold buffer and recentrifuged for 10 min at 4600 g. The resulting supernatant was retained for further study (Stanier, 1951).

Ammonia Determination. Ammonia was determined by a modification of the Conway diffusion method (Tonhazy, 1952). To the Warburg flask 0.3 ml 100 per cent trichloroacetic acid was added to precipitate the cells. After mixing, the suspension was centrifuged for 10 min at 1457 g in a Clay-Adams safety-head centrifuge. One ml of the supernatant was placed in the outer chamber of a Conway vessel. In the inner chamber one ml of 0.1 N H_2SO_4 was distributed. To the side opposite that of the sample in the outer chamber was placed 1 ml 50 per cent KOH. The ground glass cover, smeared with petroleum jelly to make an adequate air-tight seal, was fixed over the vessel. The KOH and the sample were mixed and the vessel was incubated at room temperature for two hours. At the end of this time 0.6 ml was withdrawn from the center chamber and was pipetted into a Klett-Summerson colorimeter tube. The volume was made up to 8 ml with distilled water followed by the addition of 1 ml gum arabic and 1 ml Nessler's reagent. The ingredients were

mixed by inverting the tubes and after 5 min colorimetric measurements were made using a Klett-Summerson photoelectric colorimeter using the blue filter (400-465 mμ). The samples were compared with ammonia standards treated in the same manner.

Other Chemical Tests. Attempts to bind aldehyde possibly formed during the oxidation of amines were made by the addition of various concentrations of aqueous solutions of 2,4-dinitrophenylhydrazine or sodium bisulfite directly to the Warburg flasks added in the same manner as the inhibitors as given previously.

Possible hydrogen peroxide formation was studied by the addition of other oxidizable substances assuming that hydrogen peroxide formed causes a secondary oxidation through the action of another enzyme such as peroxidase thereby giving increased oxygen consumption (Stevenson, 1943). Oxidizable substances which were used included ethyl alcohol, hemoglobin, and indigodisulfonate.

During and at the completion (30 and 150 min) of the oxidation of ethyl amine and putrescine, several additional chemical analyses were made. Most of these tests were in the nature of qualitative spot tests done according to methods of Feigl (1946).

The reaction mixtures in the Warburg flasks were treated to remove the cells as given previously for ammonia determination except when the malachite green test was performed. The trichloracetic acid interfered with this test, therefore the

reaction mixtures were removed from the flask and immediately centrifuged in a Sorvall type SS 1 centrifuge at 4250 g for 10 min. The test was then run on the supernatant.

Attempts to show aldehydic or ketonic groups were made by the use of a malachite green test and a bisulfite test. The former is carried out by placing a drop of neutralized (pH 7.0) test solution on a dry filter paper impregnated with an alkali sulfite decolorized solution of malachite green. This paper is prepared by suspending 0.8 g malachite green in 5 ml water and slowly adding 5.0 g sodium sulfite until dissolved. After filtering, this solution is used to bathe thin filter paper which is used as the reagent paper. If the test solution contains aldehyde, a green spot develops on the paper. The color reaction will reveal 20 to 300 μg aldehyde. In the bisulfite test, one drop of the test solution is mixed on a spot plate with 1 drop 0.001 N sodium bisulfite. After 5 min one drop of 0.001 N iodine and a drop of 1.0 per cent starch solution are added resulting in a blue color. If the blue color remains, the test is positive indicating the presence of aldehyde or ketone.

In an attempt to show acetate formation the lanthanum nitrate test was selected. The presence of acetic acid is indicated by the formation of a blue precipitate when lanthanum salts are mixed with iodine and ammonia. A drop of the test solution was mixed on a spot plate with a drop of 5 per cent aqueous lanthanum nitrate and a drop of 0.01 N iodine solution followed by the addition of a drop of 1 N ammonia.

A spot test for hydrogen peroxide in which the hydrogen peroxide reduces ferricyanide to form Prussian blue was performed. One drop of the reagent solution consisting of a mixture of equal parts of 0.4 per cent ferric chloride and 0.8 per cent potassium ferricyanide and a drop of water are placed on a spot plate. In an adjacent depression a drop of reagent solution and a drop of the test solution are used for comparison. An intense blue color or precipitate indicates hydrogen peroxide.

In addition to these spot tests, the 2,4-dinitrophenylhydrazine test, in which aldehyde presence is shown by a rose color obtained upon the addition of 3 ml 2,4-dinitrophenylhydrazine reagent to one drop of test solution, was attempted (Shriner and Fuson, 1948). The reagent is prepared by dissolving 2 g of 2,4-dinitrophenylhydrazine in 15 ml concentrated sulfuric acid. This solution is then added, with stirring, to 150 ml of 95 per cent ethanol, and the solution is diluted to 500 ml with distilled water. The solution is mixed thoroughly and filtered.

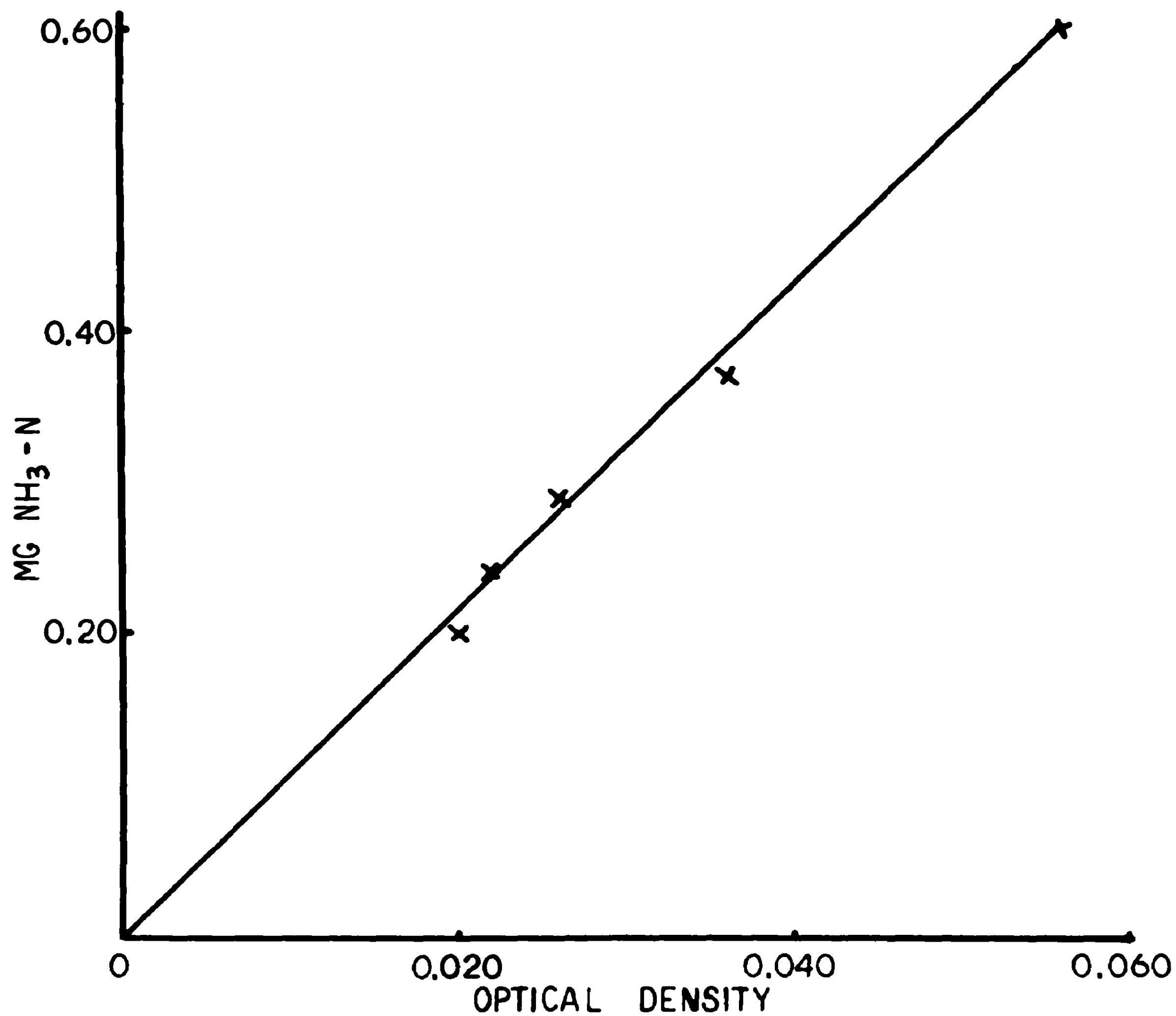

Figure 1. Relationship between NH_3-N content and optical density of organism 34A cells diluted 0.1 ml to 10 ml with distilled water.

IV

RESULTS

Results of Isolation Procedures. Thirteen different amines which were used in the original isolation work are listed in table 1. It can be seen that eight amines supported growth of mixed cultures in the lowest concentration used (0.001 M) while four were able to do so at a high concentration (0.01 M).

From flasks exhibiting visible growth a total of 23 organisms were isolated. A summary of the reactions used to differentiate these organisms and the two known *Protaminobacter* species is given in table 2. Only one of the isolates resembled a *Protaminobacter* species. Two of the strains differed from the majority in that they were gram positive, while the remainder were small gram negative rods. All of the strains gave a slight acid reaction in glucose medium.

Since the aim of the isolation procedure was to obtain satisfactory growing cultures having the ability to utilize amines, only the three organisms giving the greatest turbidity in ethyl amine broth and the *Protaminobacter* species were retained for further study.

Comparison of Growth Between Organism 34A and *Protaminobacter alboflavum* and *Protaminobacter rubrum*. Isolate 34A, an organism giving the best growth in the amine it was initially isolated from, namely, ethyl amine, was compared with *Protaminobacter rubrum* 8457 and *Protaminobacter albo-*

TABLE 1

Growth of enrichment cultures in amines used in original isolation procedure

Amine (HCl Salt)	Final Concentration of Amine in Medium	
	0.001 M	0.01 M
Methyl amine	+	+
Dimethyl amine	+	-
Ethyl amine	+	+
Diethyl amine	+	-
Triethyl amine	+	+
Di-n-propyl amine·HBr	-	-
Di-n-butyl amine·HBr	+	+
Di-n-amyl amine	+	-
Ethylene diamine·HBr	+	-
p-aminodimethylaniline	-	-
2,5-diaminotoluene	-	-
Tetramethyl-p-phenylene diamine	-	-
α-napthyl amine	-	-

\+ Growth
\- No growth

TABLE 2

Morphological and physiological characteristics of enrichment culture isolates, *Protaminobacter alboflavum* and *Protaminobacter rubrum*

Number of Strains Isolated and Known Cultures	Description of Colonies on Nutrient Agar	Action on:				Gram Reaction	Morphology
		Dextrose	Lactose	Sucrose	Litmus Milk		
2	bright orange mucoid	+	alk	+	P	-	long & short rods
2	gray granular	+	alk	alk	-	-	long & short rods
2	white mucoid	+	alk	alk	-	-	long & short rods
5	white mucoid	+	alk	+	-	-	short rods
3	white mucoid	+	alk	alk	-	+	short rods
1, *P. alboflavum*	white mucoid	+	-	-	-	-	short rods
2, includes *P. rubrum*	red mucoid	+	+	+	-	-	short rods
1	red mucoid	+	+	+	P	-	short & long rods
2	yellow mucoid	+	-	+	-	+	short rods
1	yellow mucoid	+	-	-	P	-	short rods
2, includes organism 34A	yellow mucoid	+	+	+	P	-	short rods
1	yellow mucoid	+	alk	+	-	-	short rods
1	yellow mucoid	+	alk	alk	-	-	short rods

+ acid on carbohydrates
- no reaction
alk alkaline reaction

flavum 8458 for ability to grow on various amines. Amines selected were those capable of supporting growth of mixed cultures. Di-n-propyl amine and other amines made available since the isolation experiments began were also examined. These results, presented in table 3, show that 34A can grow on methyl, dimethyl, ethyl, diethyl, di-n-amyl and di-n-butyl amine; while 8457 grows on only methyl and ethyl amine and 8458 only on ethyl amine. Slower growing organisms 8457 and 8458 were incubated for 13 days rather than 6 days as 34 A.

Table 3 gives the results obtained when the amine was the sole source of carbon and nitrogen. To check the possibility that the amine may serve as a N source or that the amine may be the C source if N is supplied, a growth experiment was undertaken using glucose and ammonium sulfate in various combinations with the amine. The data for this experiment are presented in table 4. The data has been corrected for controls. For example, optical density on glucose-ammonium sulfate has been subtracted from the optical density on glucose-ammonium sulfate-amine medium. For organism 34A, methyl, dimethyl, ethyl and di-n-butyl amine can serve as sole source of C and N; methyl, dimethyl, ethyl, diethyl, di-n-butyl, ethylene diamine and trimethyl amine are a source of N if glucose is supplied as the C source; and methyl, dimethyl, ethyl, di-n-butyl amine can serve as a source of C if ammonium sulfate is given as the N source. All amines tested except dimethyl amine, triethyl amine and

TABLE 3

Comparison of growth on various amines of organism 34A with *Protaminobacter alboflavum* 8457 and *Protaminobacter rubrum* 8458

HCl Salt of Amine Incorporated in Growth Medium	Organism and Concentration of Amine					
	34A*		8457#		8458#	
	0.001 M	0.01 M	0.001 M	0.01 M	0.001 M	0.01 M
	Optical Density					
Methyl amine	0.041	0.137	0.032	0.149	0.000	0.000
Dimethyl amine	0.027	0.215	0.000	0.000	0.000	0.000
Trimethyl amine	0.000	0.000	0.000	0.000	0.000	0.000
Ethyl amine	0.022	0.252	0.071	0.229	0.041	0.268
Diethyl amine	0.000	0.149	0.000	0.000	0.000	0.000
Triethyl amine	0.000	0.000	0.000	0.000	0.000	0.000
Triethanol amine	0.000	0.000	0.000	0.000	0.000	0.000
Di-n-propyl amine•HBr	0.000	0.000	0.000	0.000	0.000	0.000
Di-n-butyl amine•HBr	0.102	0.538	0.000	0.000	0.000	0.000
Di-n-amyl amine	0.066	0.000	0.000	0.000	0.000	0.000
Ethylene diamine	0.000	0.000	0.000	0.000	0.000	0.000
Ethylene diamine•HBr	0.000	0.000	0.000	0.000	0.000	0.000
0.1% Glucose - 0.1% $(NH_4)_2SO_4$	0.328*		0.432*		0.409*	

* 6 days incubation at 30 C
# 13 days incubation at 30 C

TABLE 4

Growth of organism 34A on various combinations of amine, glucose and ammonium sulfate

Compound	Amine	Amine - Glucose	Amine - $(NH_4)_2SO_4$	Amine - Glucose - $(NH_4)_2SO_4$
	Optical Density			
Methyl amine	0.092	0.276	0.086	0.032
Dimethyl amine	0.108	0.131	0.076	0.000
Trimethyl amine	0.000	0.056	0.000	0.058
Ethyl amine	0.268	0.469	0.222	0.146
Diethyl amine	0.013	0.215	0.000	0.076
Triethyl amine	0.004	0.000	0.000	0.000
Triethanol amine	0.000	0.004	0.000	0.024
Di-n-propyl amine*	0.000	0.000	0.000	0.032
Di-n-butyl amine*	0.328	0.284	0.347	0.247
Di-n-amyl amine	0.000	0.000	0.041	0.000
Ethylene diamine*	0.000	0.000	0.000	0.040
Ethylene diamine	0.000	0.131	0.000	0.076

*HBr salt; others, HCl salt
Concentrations: amine, 0.01 M; Glucose, 0.10 per cent; $(NH_4)_2SO_4$, 0.10 per cent

di-n-amyl amine gave increased growth over the glucose-ammonium sulfate controls. At a concentration of 0.01 *M*, triethyl amine and di-n-amyl amine were toxic to the organism as evidenced by the fact that the growth obtained in their presence when glucose and ammonium sulfate were supplied was lower than the controls of glucose-ammonium sulfate alone.

Comparison of Growth of Organism 34A with Organisms 26, 30A, 8457 and 8458. Incomplete multiplication curves of five organisms grown on glucose ethyl amine liquid medium are given in figure 2. Isolate 34A is the most rapidly growing organism reaching its maximum growth in liquid medium in 6 days; while 8457, 8458 and 26 approach equal turbidity in 14 to 17 days. Organism 30A is the slowest growing organism showing the least amount of growth at 17 days.

The organisms grow much faster on solid media. On glucose-ethyl amine agar, 48 hours incubation gives a sufficient cell suspension of organism 34A, while longer time is required for the other four organisms. When grown on nutrient agar or in nutrient broth dense growth is obtained by all organisms in 24 hours.

On the basis of the results of these studies, organism 34A was the organism of choice for the respiration experiments conducted during the investigation. The morphological, cultural and physiological characteristics of this organism are as follows: gram negative motile rod possessing one polar flagellum; small yellow mucoid colonies on nutrient

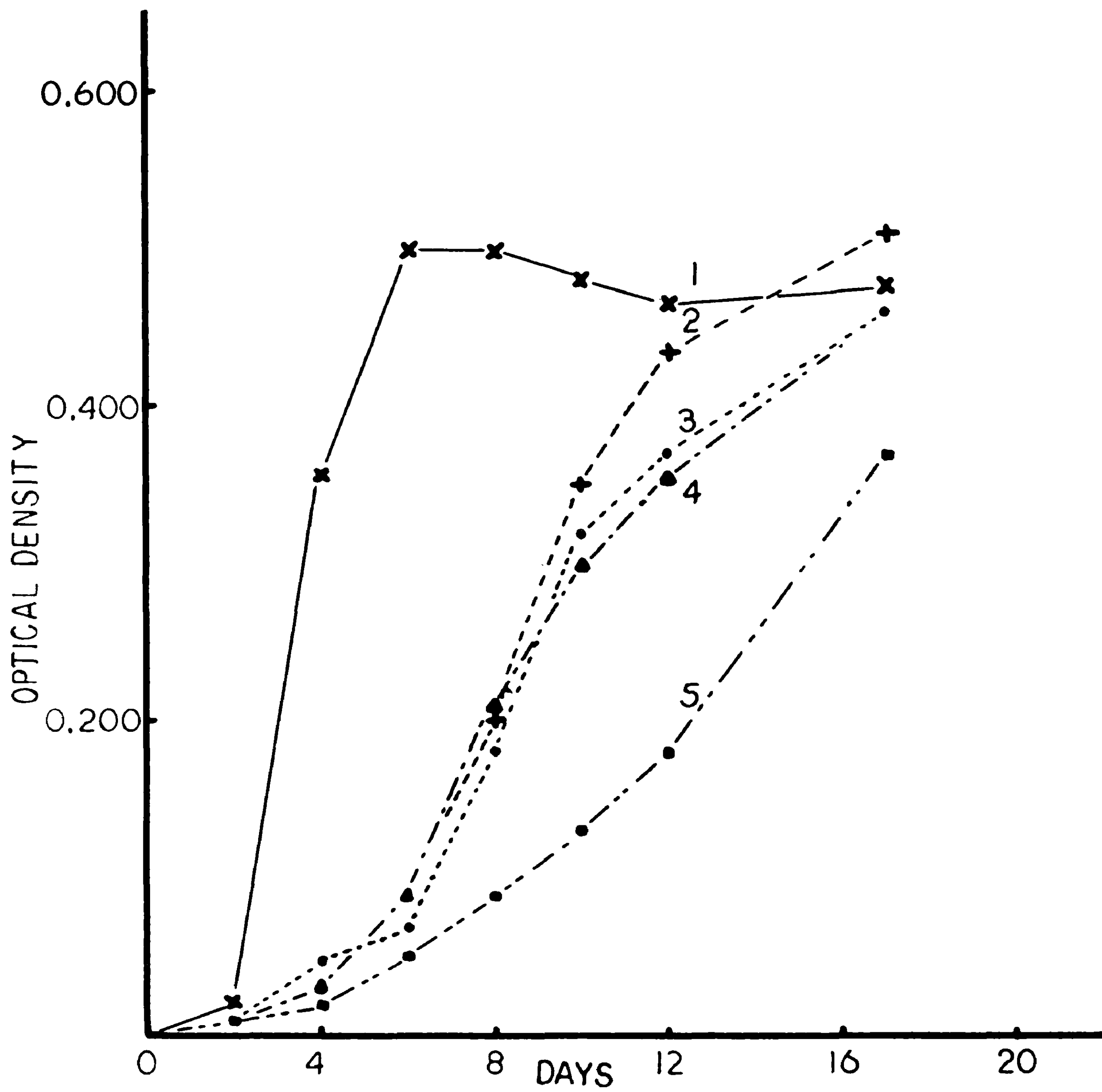

Figure 2. Multiplication curves of various strains on glucose ethyl amine medium: 1, 34A; 2, 8458; 3, 26; 4, 8457; 5, 30A.

agar; optimum temperature 28 C; optimum pH 6.8; citrate, positive; gelatin, positive; litmus milk, peptonization; MRVP, negative; H_2S, negative; slight acid but no gas on dextrose, lactose, maltose, sucrose and mannite. This organism most closely resembles a species of the genus *Pseudomonas*.

Effect of Storage of Cells of Organism 34A at 5 C. Table 5 shows the effect of storage of cells at 5 C in M/10 phosphate buffer pH 6.75 on the oxidation of ethyl amine by glucose-ethyl amine grown cells of organism 34A. There was no appreciable difference in the activity of cells stored by this method up to 28 days, therefore cells were stored at 5 C and used within this time.

The Adaptive Pattern in the Oxidation of Monoamines by Organism 34A. When cells grown on a specific compound show an immediate oxygen uptake when tested manometrically against this compound, it is considered that the compound is oxidized by adaptive enzymes. During preliminary studies, it was noticed that the oxidation of the amine upon which the organism was grown was immediate while oxidation of other amines was preceded by a long lag period. This suggested that adaptive enzymes played a part in the oxidation and experiments were undertaken to study this possibility. Cells were grown on glucose ammonium sulfate agar, nutrient agar, glucose ethyl amine agar, glucose dimethyl amine agar, and glucose di-n-butyl amine agar. Cells were separately tested for oxidation of methyl, dimethyl, ethyl and di-n-butyl

TABLE 5

Effect of storage at 5 C in M/10 phosphate buffer pH 6.75 on the oxidation of 5 μM ethyl amine by 34A cells grown on glucose-ethyl amine agar

Days Stored	μLO_2 Uptake at 45 Min
3	175
7	170
20	187
28	168
44	105
58	63

amines. These results are given in figure 3. Cells grown on ethyl amine could immediately oxidize ethyl amine although long lag periods were found in the oxidation of methyl, dimethyl and di-n-butyl amine. Cells harvested from methyl amine agar oxidized methyl and ethyl amine without delay but did not oxidize dimethyl and di-n-butyl amine in 120 min. Dimethyl amine grown cells promptly oxidized methyl, dimethyl, and ethyl amines but oxidized di-n-butyl amine very slowly. Cells having di-n-butyl amine as growth substrate oxidized di-n-butyl amine immediately, ethyl and methyl amine slowly but not dimethyl amine. Cells grown on glucose ammonium sulfate agar oxidized the amines tested to a very slight degree and only after an extended lag period.

Tables 6 and 7 also summarize these experiments. Table 6 shows oxygen uptake when the experimental time is extended to 5 hours. In addition, data are presented for cells grown on nutrient agar and nutrient agar plus ethyl amine. With nutrient agar grown cells methyl amine and ethyl amine are only slightly oxidized while dimethyl amine and di-n-butyl amine are not oxidized after 5 hours. With nutrient agar plus ethyl amine cells, the substrates tested are not oxidized after three hours.

Table 7 gives the rate of oxidation, indicated by $Q_{O_2}^{(N)}$; i.e.; microliters oxygen per hour per mg of bacterial nitrogen computed using the maximum slope of the oxidation curve. The rates are given for the oxidations of the substrates by organism 34A cells grown on different media.

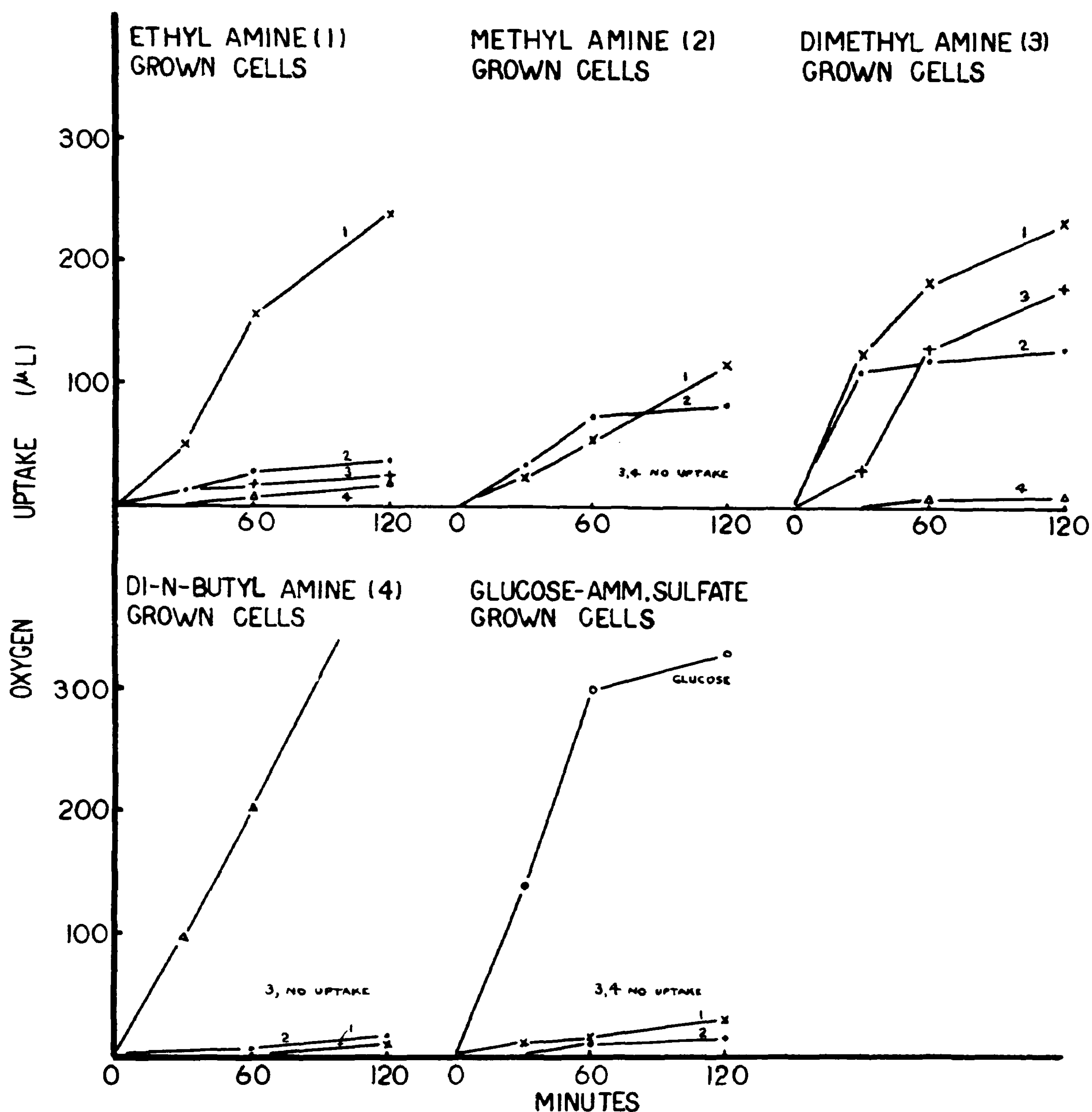

Figure 3. The effect of conditions of growth on adaptive patterns with respect to 5 μM of the following compounds: 1, ethyl amine; 2, methyl amine; 3, dimethyl amine; 4, di-n-butyl amine; by organism 34A.

TABLE 6

Effect of growth substrate on oxidation of various amines by organism 34A

Amine Incorporated in Growth Medium	$\mu L O_2$ Uptake at 5 Hours			
	5 μM Substrate			
	Methyl Amine	Ethyl Amine	Dimethyl Amine	Di-n-butyl Amine
Methyl amine	100	196	57	6
Ethyl amine	90	239	40	233
Dimethyl amine	147	248	203	39
Di-n-butyl amine	44	16	0	805
Glucose - ammonium sulfate	64	102	18	0
Nutrient agar	17	27	0	0
Nutrient agar - ethyl amine*	0	0	0	0

*3 hours

TABLE 7

Effect of growth medium on rate of oxidation of various amines by organism 34A

Amine Incorporated in Growth Medium	$Q_{O_2}^{(N)}$				
	5 μM Substrate				
	Methyl Amine	Ethyl Amine	Dimethyl Amine	Di-n-butyl Amine	Glucose
Methyl amine	95	74	10	0	not run
Ethyl amine	54	165	36	48	152
Dimethyl amine	545	443	333	18	not run
Di-n-butyl amine	45	14	0	890	not run
Nutrient agar	5	25	0	0	not run
Glucose - $(NH_4)_2SO_4$	80	96	68	0	1200
Nutrient agar - ethyl amine	0	0	0	0	not run

Oxidation of Other Amines by Organism 34A. The data for oxidations of various amines by organism 34A as measured by Warburg technique are presented in figures 4 and 5. A wide variety of amines are oxidized: monoamines, ethyl amine, trimethyl amine, triethyl amine, triethanol amine, tyramine, histamine, di-n-amyl amine, benzyl amine; diamines, ethylene diamine, putrescine, cadaverine; and the polyamine, spermine. In most cases, the longer the carbon chain of the amine, the greater the oxidation of the amine. Exceptions are ethyl amine, which was the amine upon which the cells were grown and benzyl amine. In the latter case, the benzene ring may be oxidized to give the increased oxygen uptake. In the sense of Karström (1930), only the oxidations of ethyl amine and putrescine are by constitutive enzymes while the remainder are oxidized by adaptive enzymes.

Oxidation of Amino Acids by Organism 34A. The data for oxidations of various amino acids by organism 34A as measured manometrically are presented in figures 6 and 7. Amino acids chosen were among those known to be decarboxylated to amines by bacteria. The order of increasing oxygen uptake at 180 min. was as follows: L-tryptophane; L-histidine; L-tyrosine; glycine; L-glutamic acid; L-leucine: DL-valine; DL-ornithine; DL-alanine: L-arginine; and L-lysine. Qualitative determinations at the completion of the experiment indicated ammonia formation during the oxidation of all amine acids.

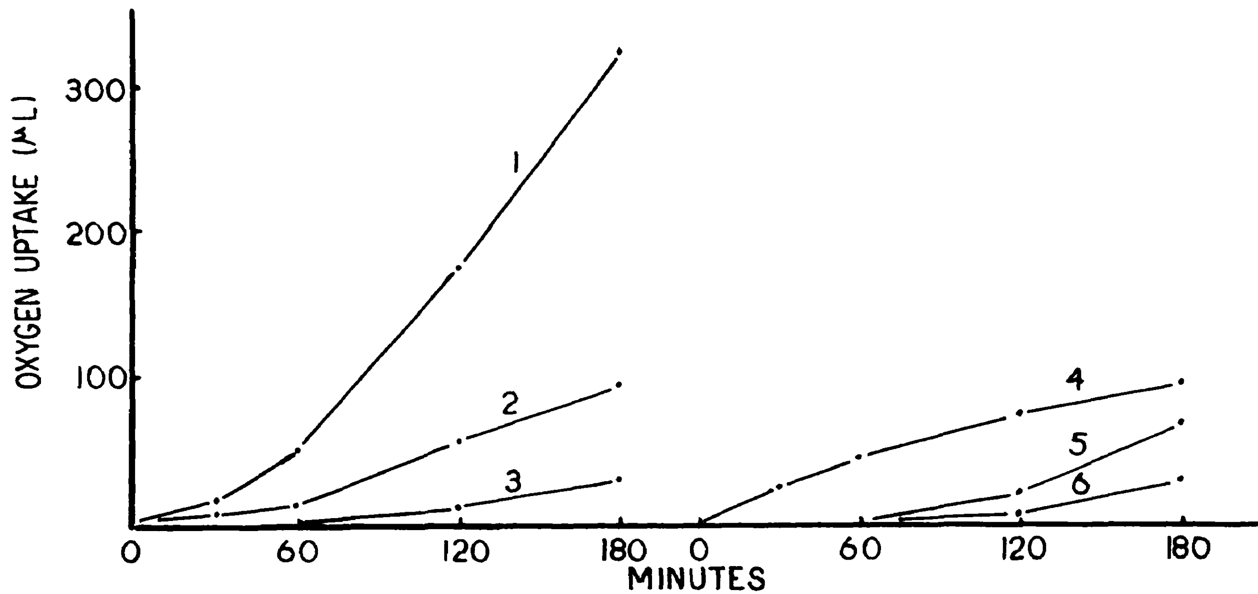

Figure 4. Oxidation by 34A cells grown on glucose ethyl amine agar on 5 μM of the following amines: 1, di-n-amyl amine; 2, tyramine; 3, ethylene diamine; 4, triethanol amine; 5, triethyl amine; 6, trimethyl amine.

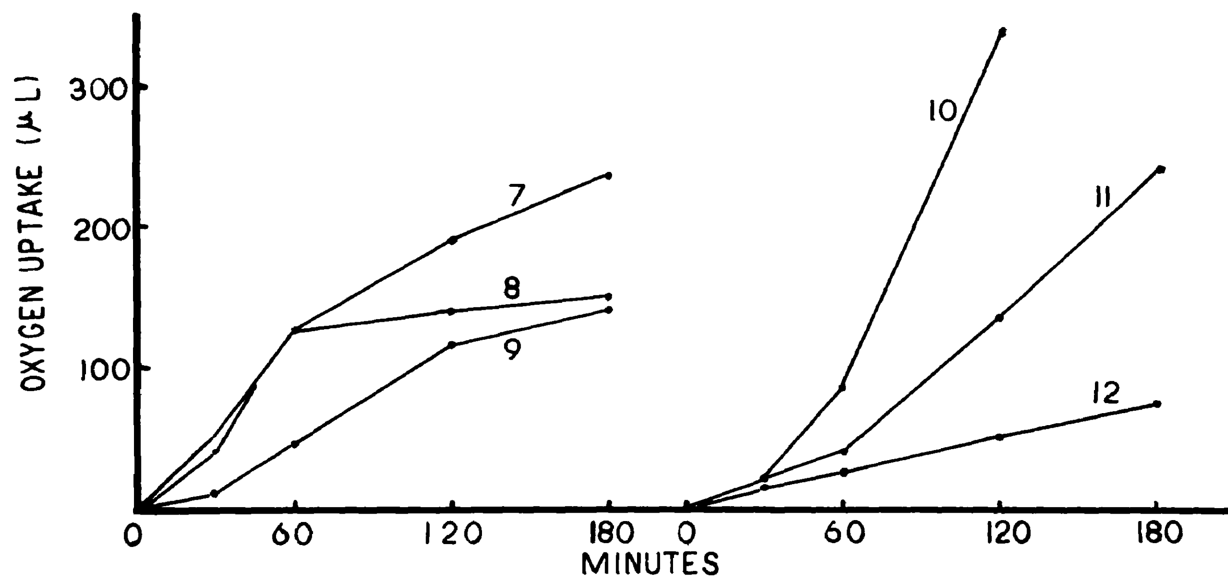

Figure 5. Oxidation by 34A cells grown on glucose ethyl amine agar on the following amines: 7, 5.0 μM ethyl amine; 8, 2.5 μM putrescine; 9, 2.5 μM cadaverine; 10, 5.0 μM benzylamine; 11, 5.0 μM histamine.

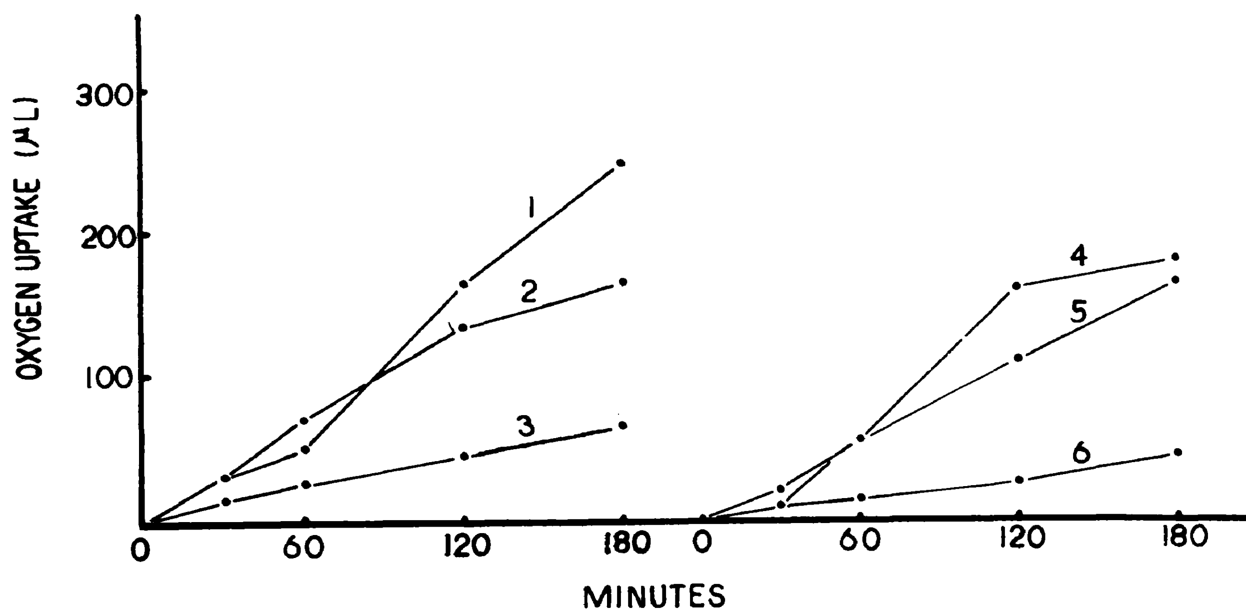

Figure 6. Oxidation by 34A cells grown on glucose ethyl amine agar on 2.5 μM of the following amino acids: 1, L-lysine; 2, DL-alanine; 3, glycine; 4, L-arginine; 5, DL-ornithine; 6, L-histidine.

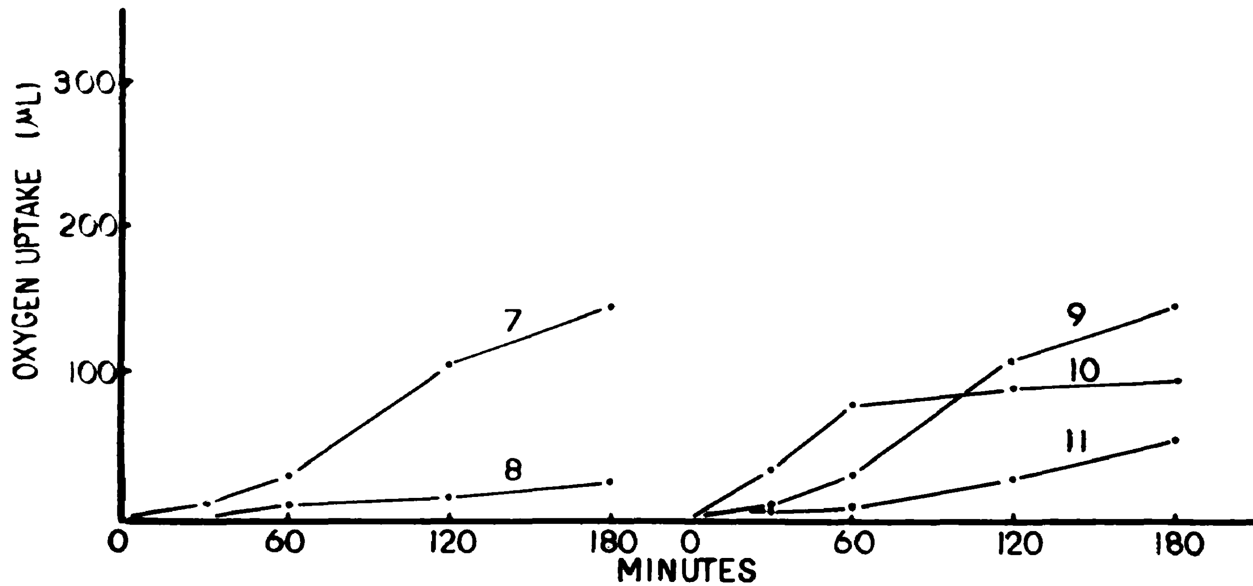

Figure 7. Oxidation by 34A cells grown on glucose ethyl amine agar on 2.5 μM of the following amino acids; 7, L-leucine; 8, L-tryptophane; 9, DL-valine; 10, L-glutamic acid; 11, L-tyrosine.

Optimum pH for Oxidation of Ethyl Amine by Organism 34A. Using M/10 phosphate buffer with a pH range of 5.3 to 9.0, oxidation of 5 µM ethyl amine by organism 34A cells grown on glucose-ethyl amine medium was found to occur over this range of pH with an optimum pH at 7.5.

The Effect of Added Glucose and Ammonium Sulphate to Growth Medium on the Oxidation of Ethyl Amine. In the adaptive enzyme studies, glucose was added to the growth substrate since this led to an increased cell crop. If glucose was omitted, more medium was needed to obtain an equivalent amount of cells. Data are presented in table 8 showing the effect of added glucose and ammonium sulfate to the growth medium on the oxidation of ethyl amine. Cells grown on ethyl amine alone oxidize ethyl amine to maximum at a high rate. When glucose is added to the growth medium, the oxidation is carried to maximum at a decreased rate. Upon the addition of ammonium sulfate to the growth medium the rate and the total oxygen uptake are lessened. The elimination of ethyl amine from the growth medium results in practically no oxidation of ethyl amine. These data indicate further the adaptive nature of the enzymes and suggest that as the need for ethyl amine as an energy source by organism 34A is eliminated its oxidative activity on ethyl amine is reduced.

In the study of enzyme formation it is possible that the suspected adaptive enzyme formation is due to a permeability effect and that one is measuring the time for the substrate to enter the cell. One way to overcome such an effect

TABLE 8

Effect of medium composition on oxidation of ethyl amine by organism 34A

Additions to Medium	$Q^{(N)}_{O_2}$	$\mu L O_2$ Uptake at 150 Min
Ethyl amine	442	225
Glucose-ethyl amine	196	222
Glucose-ammonium sulphate-ethyl amine	164	191
Glucose-ammonium sulphate	18	20

is by the use of dried cell preparations or cell extracts. Lyophilized, acetone dried, vacuum dried and alumina ground cells from both glucose-ethyl amine and nutrient agar grown cells were investigated. Only with lyophilized glucose-ethyl amine grown cells was activity obtained; after 7.5 hours 112 microliters of oxygen were taken up.

The Effect of Inhibitors on the Oxidations of Ethyl Amine and Putrescine by Organism 34A. Since it had been shown that organism 34A could oxidize both monoamines and diamines, questions arose as to the specificity of the enzyme system or systems involved. Figures 8, 9, and 10 give results showing the effect of several inhibitors on the oxidation of ethyl amine and putrescine.

Arcaine (1,4-diguanidinobutane), a strong inhibitor of mammalian diamine oxidase and diamine oxidase of *Mycobacterium smegmatis* (Zeller, 1951), was found to inhibit oxidation of the monoamine, ethyl amine, more than that of the diamine, putrescine (figure 8). It is also noted that the endogenous respiration is greatly enhanced suggesting that the cells can oxidize the inhibitor, arcaine.

Sodium cyanide, an inhibitor of cytochrome enzyme systems, inhibited both ethyl amine and putrescine only in the highest concentration used, M/500. At a concentration of M/5000, no inhibition of the amine oxidations occured; however an increased oxygen uptake was noted, possibly due to the inhibition of catalase preventing further action on hydrogen peroxide which may be formed in the reaction (figure 9).

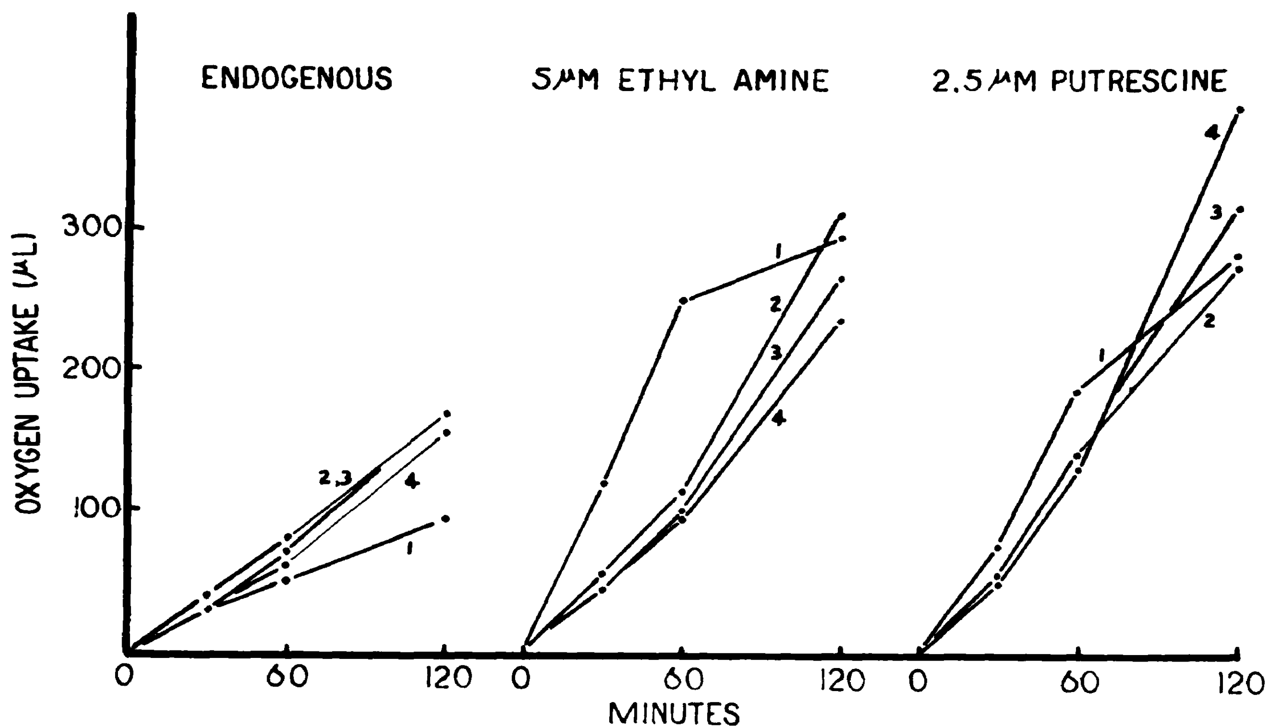

Figure 8. The effect of various concentrations of arcaine on endogenous respiration and on oxidation of ethyl amine and putrescine by glucose ethyl amine grown cells of organism 34A: 1, no inhibitor; 2, 15 µM; 3, 25 µM; 4, 50 µM. (not corrected for endogenous)

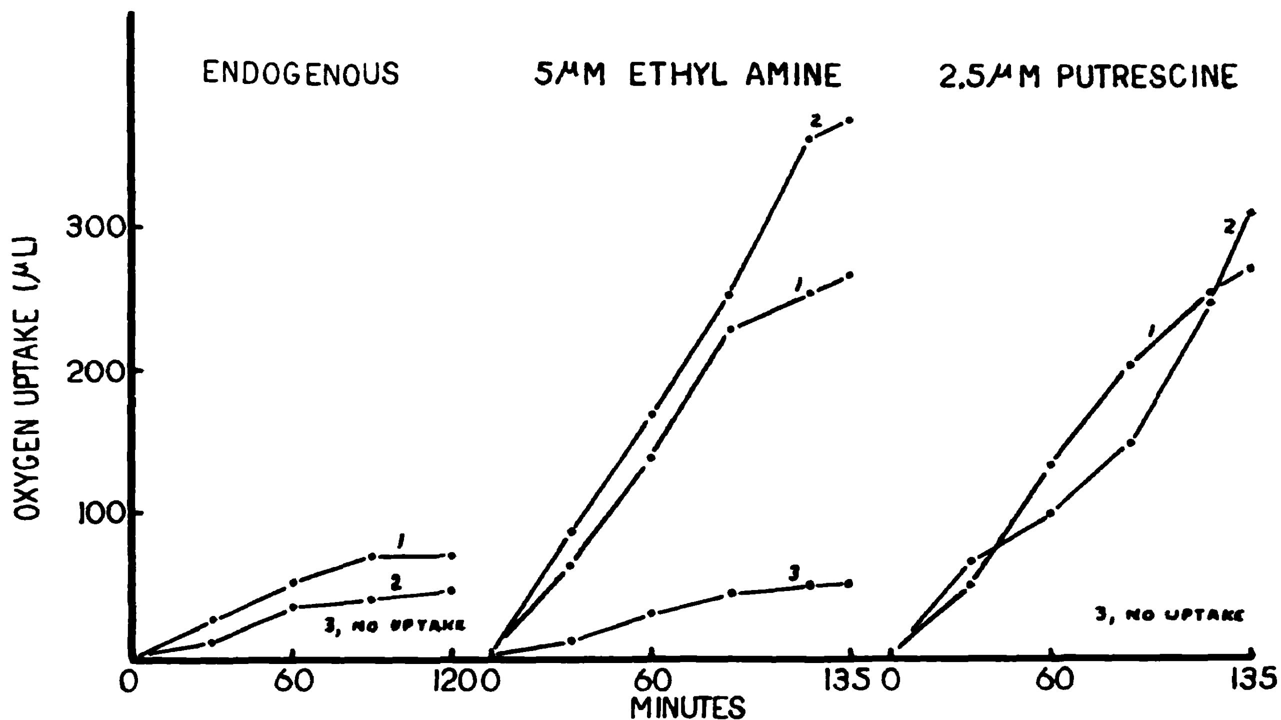

Figure 9. The effect of sodium cyanide on endogenous respiration and on oxidation of ethyl amine and putrescine by glucose ethyl amine grown cells of organism 34A: 1, no inhibitor; 2, M/5000; 3, M/500. (not corrected for endogenous)

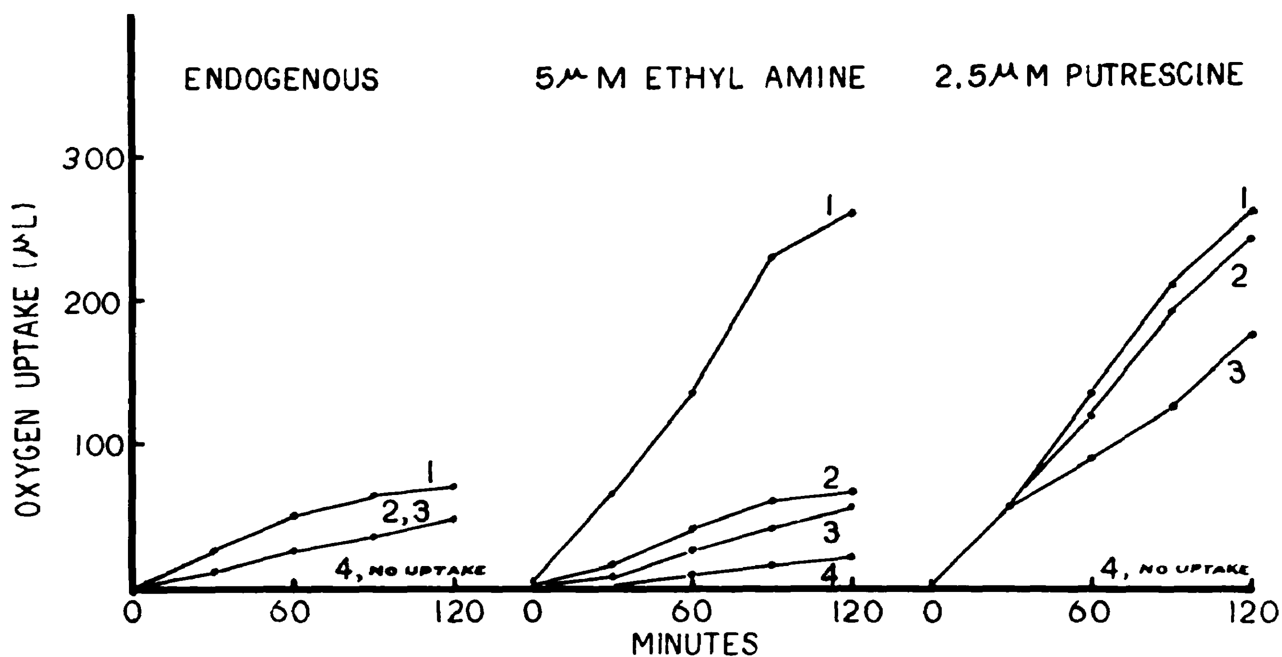

Figure 10. The effect of semicarbazide on endogenous respiration and on oxidation of ethyl amine and putrescine by glucose ethyl amine grown cells of organism 34A: 1, no inhibitor; 2, M/2000; 3, M/200; 4, M/20. (not corrected for endogenous)

Semicarbazide, a carbonyl fixative and inhibitor effective with mammalian and bacterial diamine oxidases (Gale, 1941; Zeller, 1951), was found to inhibit ethyl amine oxidation at a concentration of M/2000 and the oxidation of putrescine at a concentration of M/200 (figure 10).

Mode of Action of Oxidation of Amines by Organism 34A. The first attempts to determine the metabolic pathway in the oxidation of amines were made by measuring carbon dioxide evolution and ammonia production during the oxidation of the monoamine, ethyl amine and the diamines, putrescine and cadaverine. These data are presented in table 9.

With an increase in concentration of the amine there is a concomitant increase in the rate of oxidation of all amines. The rate is greatest on the amine upon which the cells were grown, ethyl amine; while the oxidation rate on putrescine where no lag period is found is greater than the oxidation rate on cadaverine which shows a short lag period.

As the carbon chain of the amine is lengthened there is found an increase in the amount of oxygen taken up and, consequently, an increase in the μM O_2 per μM substrate.

Ammonia determinations indicate that with the monoamine, ethyl amine, less than one mole of ammonia is produced from one mole of substrate while more than one mole of ammonia is produced from one mole of the diamines, putrescine and cadaverine.

The amount of carbon dioxide obtained suggests that the substrates are not being oxidized to completion but that other end products are formed.

TABLE 9

Comparative oxidation rates, carbon dioxide and ammonia production during oxidation of ethyl amine, putrescine, and cadaverine by glucose-ethyl amine grown cells of organism 34A

Substrate and Concentration	$Q_{O_2}^{(N)}$ at Maximum Rate	Maximum μLO_2 Uptake	μM O_2/μM Substrate	μM CO_2/μM Substrate	μM NH_3/μM Substrate
Ethyl amine					
2.50 μM	110	104	1.86	0.88	0.44
5.00 μM	194	184	1.64	0.91	0.75
Putrescine					
1.25 μM	56	60	2.14	0.50	1.31
2.50 μM	155	161	2.88	1.91	1.38
Cadaverine					
1.25 μM	42	96	3.43	2.92	1.53
5.00 μM	157	358	3.20	2.45	1.65

These data indicate that the amines are being oxidatively deaminated with the formation of ammonia, carbon dioxide, and other unidentified end products.

Investigation as to Formation of Other End Products in the Oxidations. One of the suspected intermediates of the oxidation of amines is the corresponding aldehyde (Lardy, et al., 1949; Zeller, 1951). Sodium bisulphite and 2,4-dinitrophenylhydrazine, compounds known to have aldehyde binding properties, were added directly to the respiration flask in an attempt to prevent further oxidation of the postulated aldehyde. No decrease in the oxidation of ethyl amine or putrescine by glucose-ethyl amine grown cells was found by the concentrations of 2,4-dinitrophenylhydrazine employed (figure 11).

With increasing concentrations of sodium bisulphite a decrease in the amount of oxygen consumed by suspensions of glucose ethyl amine cells on putrescine was detected (figure 12). This may be due to either binding of formed aldehyde or to a binding of aldehyde groups on the enzyme itself. Also noted was the enhancement of the endogenous respiration and oxidation of ethyl amine.

Another suggested intermediate in amine oxidation reactions is hydrogen peroxide (Lardy et al., 1949; Zeller, 1951). A method of detecting this compound is by coupled oxidation wherein extra oxygen consumption in manometric experiments, observed when other oxidizable substances are added to the substrate, is explained by assuming that hydrogen peroxide

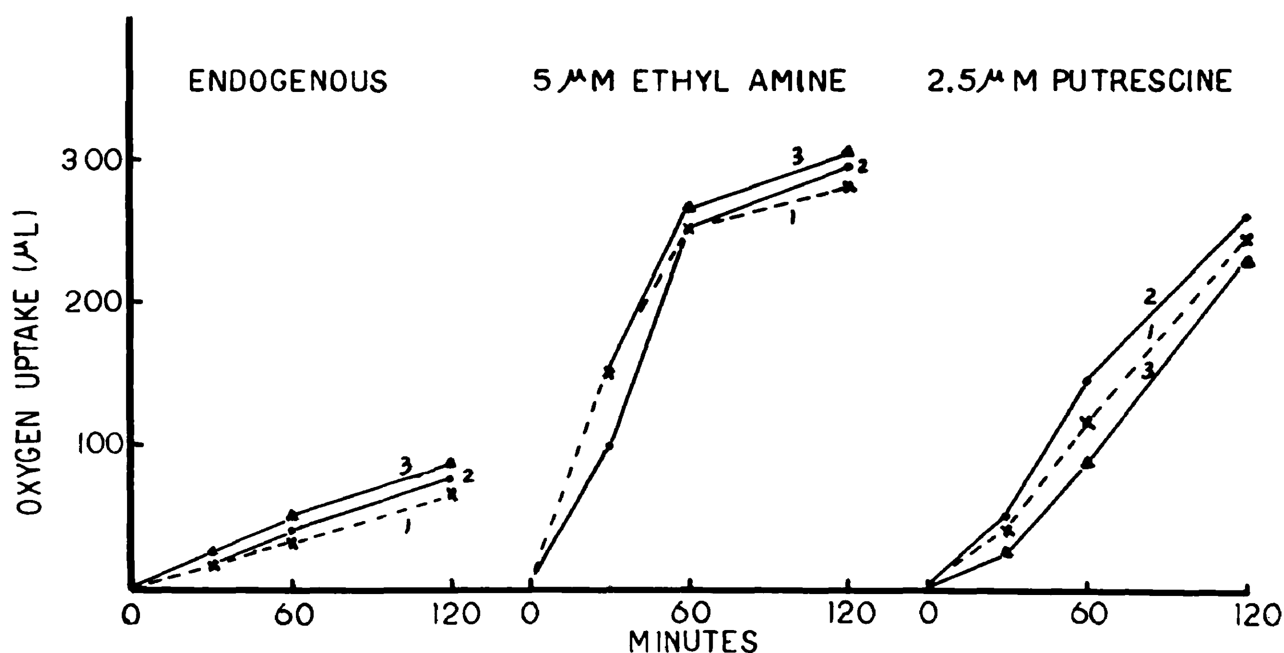

Figure 11. The effect of 2,4-dinitrophenylhydrazine on endogenous respiration and on oxidation of ethyl amine and putrescine by glucose ethyl amine grown cells of organism 34A: 1, 0; 2, 0.002 mg; 3, 0.125 mg. (not corrected for endogenous)

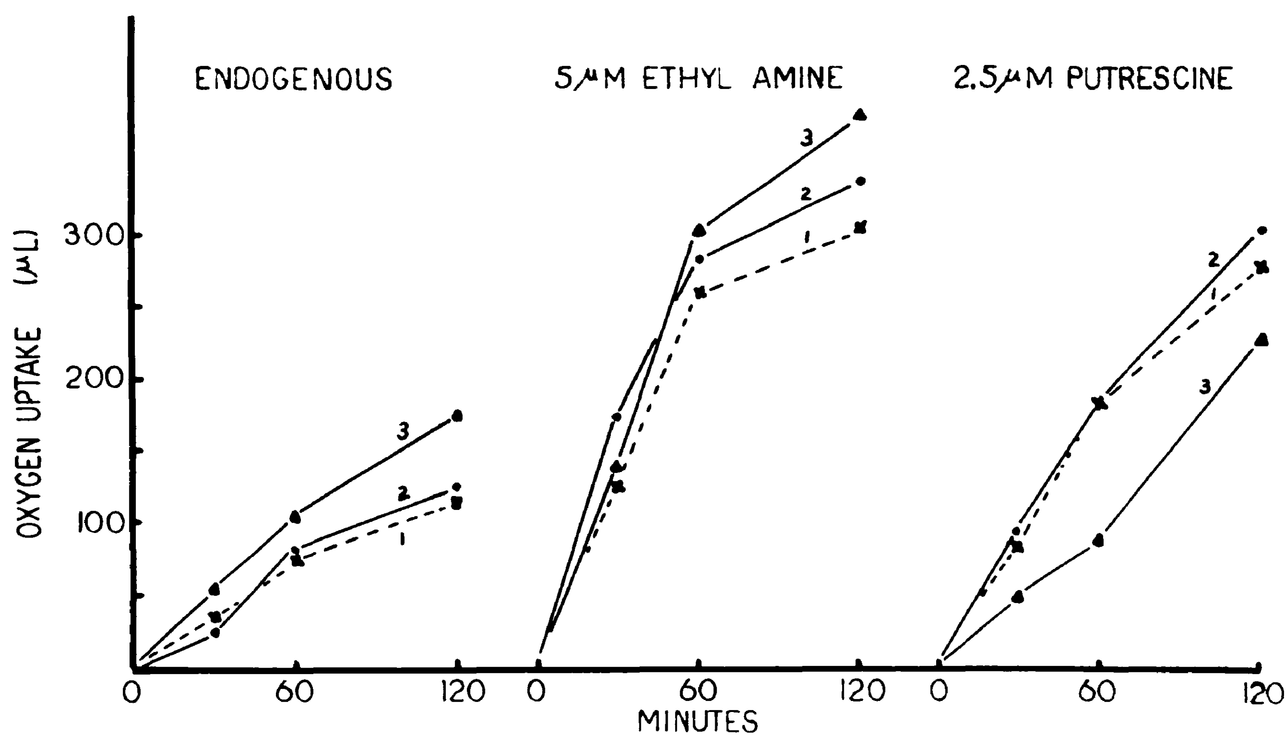

Figure 12. The effect of the addition of sodium bisulfite on endogenous respiration and on oxidation of ethyl amine and putrescine by glucose ethyl amine grown cells of organism 34A: 1, 0; 2, M/100; 3, M/20. (not corrected for endogenous)

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formed causes a secondary oxidation through the action of another enzyme such as peroxidase (Stevenson, 1943). Oxidizable substances which were tried are listed in table 10.

Ethyl alcohol was found to be unsatisfactory since this organism can oxidize ethyl alcohol, therefore it is not known whether the increased oxidation is a coupled oxidation or additive oxidation due to the oxidation of both the substrate and the oxidizable compound. In view of the results obtained with ethyl alcohol, hemoglobin and indicarmine were next used.

With hemoglobin at a concentration of 0.2 mg there is an equivalent increase in the oxygen taken up by the cells alone and the substrates employed, ethyl amine and putrescine.

Concentrations of 0.002 and 0.200 mg of indicarmine (indigodisulfonate) gave no appreciable increase in the oxidation of ethyl amine or putrescine when added with the substrate.

In addition to introducing compounds directly to the respiration flask during the oxidation of ethyl amine and putrescine, chemical determinations were made on the reaction mixtures at 30 and 150 min in an attempt to explain the reactions. The results are as follows: malachite green spot test for aldehydes, negative; bisulfite spot test for aldehydes, negative; 2,4-dinitrophenylhydrazine test for aldehydes, negative; lanthanum nitrate spot test for acetate, negative; and the ferric-ferricyanide spot test for hydrogen peroxide, negative.

TABLE 10

Coupled oxidation of ethyl amine and putrescine with added compounds by glucose-ethyl amine cells of organism 34A

Oxidizable Material Added	Concentration	Substrate	μLO_2 Uptake at 120 min	
			Without Test Material	With Test Material
Ethyl alcohol	0.036 M	No substrate	59	295
"	"	Ethyl amine	270	460
Hemoglobin	0.200 mg	No substrate	85	165
"	"	Ethyl amine	295	365
"	"	Putrescine	255	340
Indicarmine	0.002 mg	No substrate	55	67
"	"	Ethyl amine	285	290
"	"	Putrescine	245	280
"	0.200 mg	No substrate	55	80
"	"	Ethyl amine	285	290
"	"	Putrescine	245	275

(Not corrected for endogenous)

The other end products formed in the reaction remain unidentified. It may be that the compounds suspected are formed in trace amounts preventing their chemical determination. Larger amounts of substrate and cells may be required to give end products in quantities which would permit easier chemical analysis. Also, the possibility remains that the end products may be other than those tested for.

V

DISCUSSION

It is significant in this investigation that the *Protaminobacter* species examined were unable to grow to any extent on alkyl amines. This is in contrast with the report of the original investigator who found that alkyl amine utilization by these organisms was sufficient to warrant them status of generic rank (den Dooren de Jong, 1926). It may be that these organisms once possessed this ability but that continued cultivation in the absence of amines has given rise to the present inactive strains. Similar observations on the loss of a biochemical characteristic have been reported in studies on the utilization of hydrocarbons by bacteria (Stone, *et al.*, 1942) and in the bacterial degradation of phenolic compounds (Kramer, 1950).

Gale (1942); Zeller, *et al.* (1951) and Owen, *et al.* (1951) reported that other bacterial genera either can grow *in vitro* on amines or oxidize these compounds; these genera included *Pseudomonas*, *Mycobacterium*, *Micrococcus* and *Escherichia*.

Furthermore, organisms growing on amines are isolated with ease using enrichment techniques. Of 23 cultures isolated, only 1 culture resembled *Protaminobacter* biochemically and morphologically; while the majority of the remainder closely resembled *Pseudomonas* with 1 culture definitely identified as such.

It is obvious that amine decomposition is not a special function of organisms in the genus Protaminobacter but that this characteristic is found in many common soil forms which possess the ability to oxidize a wide variety of organic compounds. These organisms preferentially will utilize certain compounds which are usually available in their environment, but upon depletion or omission of these compounds, they must utilize the organic compounds present if they are to survive.

Since amine utilization is not a stable characteristic or limited to the organisms included in the present genus Protaminobacter, it is felt that the taxonomic validity of this genus may be questioned.

It was pointed out by Karström (1930, 1937) that bacterial enzymes could be considered constitutive and adaptive. The application of this concept has been made to bacterial activities on various substrates. The substrates most commonly found in the environment are broken down by enzymes found in the cell at all times, hence are called constitutive enzymes. Those enzymes which are produced as a specific response to the presence of an homologous substrate were referred to by Karström as adaptive. More recently, these enzymes have been termed inductive enzymes (Cohn and Monad, 1953; Stanier, 1953). Elaboration of inductive enzymes is especially likely when organisms are placed in an environment where growth depends on the development of an enzyme able to initiate the attack on a particular compound.

Some enzymes elaborated by the organisms studied in this investigation were found to be under adaptive or induced control. This was suggested in growth studies where a slower rate of growth was noted on a synthetic medium containing the amine as compared with a more complete medium such as nutrient agar. Also, adaptive enzyme formation was apparent from manometric experiments. In most cases oxygen uptake on a substrate by cells grown on a medium containing the homologous amine was immediate, while long lag periods were found with other amines. Exceptions were as follows: methyl amine grown cells were adapted to methyl and ethyl amines; dimethyl amine grown cells were adapted to methyl, dimethyl and ethyl amine; and ethyl amine grown cells were adapted to ethyl amine and putrescine. The question immediately arises as to why ethyl amine grown cells were not adapted to methyl and dimethyl amine. No logical explanation can be given.

It is noted that the addition of glucose and ammonium sulfate in various combinations to ethyl amine in the growth medium results in a change in the rate of the oxidation of ethyl amine. When the organism is completely dependent on ethyl amine for carbon and nitrogen, the oxidation rate is greatest. When the amine is the sole nitrogen source the oxidation rate on ethyl amine is lessened. Upon the addition of other carbon and nitrogen sources, the rate of oxidation is reduced further. However, only slight activity is noted upon the elimination of the amine from the growth medium. This not only indicates adaptive nature of the enzymes

involved, but also indicates that the amount of enzyme produced or the activity of the enzyme may be affected by the growth medium.

Other cases of adaptive control in the oxidation of various organic compounds are cited in recent literature. For example, Levine and Krampitz (1952) found that a soil diptheroid had the ability to oxidize acetone only when the organism was grown in its presence. Walker and Wiltshire (1953) studied the breakdown of napthalene using a soil bacterium adapted to this compound.

The premise that the long lag period obtained on substrates other than the growth substrate indicates adaptive or induced enzyme formation rests on the assumption that the cell is equally permeable to all substrates (Stanier, 1950). Attempts made in this investigation to eliminate the possibility of permeability as a cause of lag by the use of dried cell preparations or cell extracts were unsuccessful. The oxidations catalyzed by dried cells resulted in no uptake or in a much smaller oxygen uptake per mole of substrate than the equivalent oxidations by living cells. This is comparable to other work with microbial enzymes. Lower activity on cells treated by freezing, lyophilization and acetone treatment were found in studies with _Ashbya gossypi_ (Mickelson, 1950).

Sleeper _et al._, (1950) found that not all enzyme systems were extracted from _Pseudomonas fluorescens_ and perhaps amine oxidases are of a similar nature being labile to drying or extraction by the methods employed. It is interest-

ing to note, however that the enzymes are stable after storage at 5 C for 28 days.

Previous reports (Gale, 1942; Zeller, 1951) established that a number of bacteria are able to oxidize diamine compounds and that these organisms contain diamine oxidase. The oxidation of tyramine and benzylamine (Gale, 1942) is the only known report concerning the oxidation of monoamines by bacteria. Gale states that it may be the lack of some active group other than the $-NH_2$, such as the -OH in tyramine or the _para_-H in benzylamine, that prevents the attack of most monoamines. Organism 34A studied here attacks a wide variety of amines, both diamines and monoamines. The latter with and without active polar groups are actively oxidized.

This is similar to the situation described with decarboxylating enzymes. According to Gale (1952) only those amino acids having at least one chemically active (polar) group in the molecule other than the terminal -COOH and the α NH_2 groups are decarboxylated to the corresponding amine. Hence, Gale claims that amino acid decarboxylases exist only for arginine, lysine, ornithine, histidine, tyrosine, glutamic acid and aspartic acid giving agmatine, cadaverine, putrescine, histamine, tyramine, γ-aminobutyric acid and β-alanine, respectively. However, as pointed out in the historical section (Porter, 1946) other workers have reported the decarboxylation of monoaminomonocarboxylic acids. For example, glycine can be decarboxylated to methyl amine and alanine can give rise to ethyl amine as a result of

bacterial decarboxylation. Both of these amines were found to be readily oxidized in this investigation. Monoamines may be widespread in nature and therefore, the utilization of monoamines may not be solely a laboratory observation but may be of importance in nature.

Amino acids are readily oxidized, however those with strong polar groups such as histidine, tyrosine, and tryptophane are oxidized to a lesser degree than those not possessing such polar groups. Apparently the amino acids tested are oxidatively deaminated by organism 34A. The enzyme may be amino acid oxidase which is similar to amine oxidase.

The results obtained with inhibitors indicate that separate enzymes are active in the oxidation of mono- and diamines. This is in agreement with studies of animal monoamine and diamine oxidases (Zeller, 1951); however the inhibition by specific inhibitors is of a different pattern when compared with previous work.

Arcaine (1,4-diguanidinobutane) inhibited the monoamine oxidase but not the diamine oxidase of organism 34A. It was also noted that organism 34A can degrade arcaine. This is contrary to Zeller et al., (1951) who found arcaine to be a strong inhibitor of diamine oxidase of Mycobacterium smegmatis. It may be that in the oxidation of ethyl amine in the presence of arcaine, the inhibitor is the preferred substrate, therefore only the oxidation of the inhibitor was noted. In the oxidation of putrescine in the presence of

arcaine, the inhibitor may not be the preferred substrate. The oxygen uptake was similar to that of the control flasks with putrescine alone, however when the putrescine was oxidized to maximum, there was an increase in the oxygen uptake in the flasks containing inhibitor. This suggests oxidation of the inhibitor after putrescine was oxidized.

Gale (1942) found the oxidation of histamine, putrescine, cadaverine and agmatine to be inhibited by M/100 semicarbazide or M/10000 cyanide. In this investigation oxidation of ethyl amine was inhibited by M/2000 semicarbazide while the oxidation of putrescine was inhibited by M/200 semicarbazide. Animal diamine oxidase is completely inhibited by semicarbazide so that it is suggested (Zeller, 1951) that the active group of the enzyme is of a ketonic nature. Animal diamine oxidase is also inhibited by cyanide. On the other hand, animal monoamine oxidase is not inhibited by carbonyl fixatives or cyanide (Zeller, 1951). No explanation can be given for this inhibition by semicarbazide of the monoamine oxidation by the organism studied here.

The oxidation of ethyl amine and putrescine was found to be inhibited by M/500 cyanide, while a concentration of M/5000 cyanide resulted in an increased oxygen uptake. One explanation for this increased oxygen uptake may be in the stimulation effect found by low concentrations of some inhibitors (Pratt and Dufrenroy, 1949), although the concentration used here is higher than that found to be inhibitory by Gale (1942). Another explanation for this increased

activity is that it may be due to the inhibition of catalase. Cyanide is known to inhibit iron enzyme systems such as catalase (Gale, 1952). If hydrogen peroxide is formed from amine breakdown, catalase would convert this compound to water and oxygen which may in turn be used in the oxidation of the amine. Upon inhibition of catalase, oxygen for the amine oxidation would have to come from the flask, resulting in higher recorded oxygen uptake.

An optimum pH of 7.5 was found for the oxidation of ethyl amine. This is similar to findings of Gale (1942) who reported the oxidation of putrescine, cadaverine, agmatine, histamine and tyramine to occur optimally between pH 7.5 and 9.5.

Although possibly five different amine oxidizing enzymes have been described in the literature (Gale, 1942; Lardy _et al_., 1949; Zeller, 1951; Kenten and Mann, 1951), the resulting reactions involving the breakdown of the amine is limited to two. Either the amine, mono- or di-, is broken down to the corresponding aldehyde, ammonia and hydrogen peroxide (Zeller, 1951) or the amine is completely oxidized to ammonia, carbon dioxide and water (Gale, 1942). In the former case, there may be accompanying reactions of the end products of the reaction. For example, the aldehyde may be further oxidized to the corresponding acid or the hydrogen peroxide will be decomposed to water. These secondary reactions will involve other enzyme systems such as aldehyde oxidase and catalase or peroxidase. The reactions observed

in this investigation do not seem to fit in either pattern. In the first case, no aldehyde or hydrogen peroxide formation could be demonstrated. It must be recognized that, whereas a positive finding is significant, a negative finding does not necessarily exclude the possibility of the formation of these compounds. In the second case, the carbon dioxide formation does not indicate a complete oxidation of the substrates. If 1 mole of either ethyl amine or putrescine were oxidized to completion approximately 2 or 4 moles of carbon dioxide, respectively, would be formed. The possibility should not be overlooked that oxidative assimilation (Clifton, 1937) may occur. This is probable in the case of the oxidation of ethyl amine, where fewer carbon atoms are unaccounted for; however, with the diamines, putrescine and cadaverine, the number of unaccounted carbon atoms is too large to account for by oxidative assimilation.

It appears that neither pathway is followed here, however it is unwise to assume that a new mode of breakdown occurs. The basic similarity of metabolic patterns in all living organisms as has been emphasized in comparative physiology should not be cast aside. Perhaps in this study the use of the whole cell rather than cell extracts has masked the mode of action of amine oxidizing organisms. In this type of investigation, conclusions concerning a given enzyme must be drawn cautiously since the whole bacterium has been used instead of isolated enzymes. Experiments with mammalian and plant amine oxidases are carried out with extracted enzymes while the bacterial enzymes are investigated _in situ_.

Other factors, such as permeability, rather than the enzyme reactions under investigation, may be limiting. Other concomitant reactions, given previously, may prevent accurate study of the main reaction. The use of inhibitors and fixatives to eliminate these reactions has not been successful.

Amines and amine decomposition may have biological significance for man and other animals. The presence of active monoamine and diamine oxidase in intestinal mucosa suggests that the enzymes carry out a protective function in preventing amines formed in the intestine by bacterial decarboxylases from entering the general circulation (Zeller, 1951). Of the amines formed by decarboxylation of amino acids, methyl amine, ethyl amine, putrescine and cadaverine are believed to be physiologically inert, however histamine, tryptamine and tyramine are known to be of importance physiologically. Tyramine is a pressor substance, i.e., it raises the blood pressure while histamine reduces arterial pressure. Chick feces served as a source of organisms able to degrade amines. It is probable that these organisms aid in the detoxification of amines in the intestine.

Amines may find their way to the soil by excretion as such; by subsequent degradation of excreted nitrogenous compounds; or by dissimilation of animal and plant protein. The further dissimilation of amines in the soil by bacterial species either individually or synergistically may play an important part in contributing to the cycle of elements in nature.

VI

SUMMARY

Cultures of organisms capable of using amines as the sole source of carbon and nitrogen were isolated by enrichment techniques. One organism, a pseudomonad, was found to utilize methyl, dimethyl, ethyl, diethyl, di-n-amyl and di-n-butyl amines in a synthetic medium. Methyl, dimethyl, ethyl, diethyl, di-n-butyl and trimethyl amines serve as a nitrogen source for this organism if glucose is supplied in the medium. If ammonium sulfate is included, the organism can use methyl, dimethyl, ethyl, di-n-amyl, and di-n-butyl amines as the carbon source.

Protaminobacter _rubrum_ ATCC 8257 and _Protaminobacter alboflavum_ ATCC 8258, organisms differentiated from others on the basis of their ability to degrade amines, were shown to have little activity as compared with freshly isolated organisms. Methyl amine and ethyl amine were the only amines utilized by 8257 while 8258 utilized only ethyl amine. For this and various other reasons characterization of _Protaminobacter_ on a biochemical basis was questioned.

Respiration studies using organism 34A cells, the most active against amines, grown on glucose ammonium sulfate agar, nutrient agar, glucose methyl amine agar, glucose ethyl amine agar, glucose dimethyl amine agar, and glucose di-n-butyl agar revealed that the enzymes are "adaptive" in nature. Methyl amine grown cells were adapted to methyl

and ethyl amine; dimethyl amine grown cells were adapted to methyl, dimethyl and ethyl amines; ethyl amine grown cells were adapted to ethyl amine; and di-n-butyl amine grown cells were adapted to di-n-butyl amine. Cells grown on glucose ammonium sulfate agar oxidized the amines tested only slightly and after an extended lag period. No enzymatic activity against amines was noted with cells grown on nutrient agar or nutrient agar plus amine.

Evidence was obtained indicating that the addition of other utilizable carbon and nitrogen sources with ethyl amine to the growth medium affected the rate of oxidation of ethyl amine.

In addition to the oxidation of amines studied above, a wide variety of other amines also are oxidized by organism 34A: monoamines, ethyl amine, trimethyl amine, triethyl amine, triethanol amine, tyramine, histamine, di-n-amyl amine, benzylamine; diamines, ethylene diamine, putrescine, cadaverine; and the polyamine, spermine. Only the oxidations of ethyl amine and putrescine are by constitutive enzymes while the remainder are oxidized by adaptive enzymes.

The following amino acids were oxidized by organism 34A: L-tryptophane; L-histidine; L-tyrosine; glycine; L-glutamic acid; L-leucine; DL-valine; DL-ornithine; DL-alanine; L-arginine; and L-lysine. Ammonia formation during the oxidation of amino acids suggests that these compounds were oxidatively deaminated possibly by amino acid oxidases.

Optimum pH for the oxidation of ethyl amine by organism 34A was found to be 7.5.

The use of inhibitors showed that the monoamine, ethyl amine and the diamine, putrescine, were oxidized by separate enzymes. The oxidation of ethyl amine was inhibited by 15 μM arcaine; M/500 cyanide and M/2000 semicarbazide while the oxidation of putrescine, although not inhibited by arcaine, was inhibited by M/500 cyanide and M/200 semicarbazide.

Acetone dried, vacuum dried or alumina ground cells of organism 34A were inactive against ethyl amine while decreased activity was found with lyophilized preparations of this organism. Whole cells stored in M/10 phosphate buffer pH 6.75 at 5 C maintained activity for 20 to 25 days.

Studies on the mode of oxidation of ethyl amine, putrescine and cadaverine indicate that these amines were oxidized to ammonia, carbon dioxide and other unidentified end products. Attempts made to identify these end products were unsuccessful.

As a result of this investigation it has been established that the bacterial utilization of amines is a characteristic of organisms easily isolated from soil and that in nature this process probably occurs to a greater extent than is generally recognized.

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