AMINE METABOLISM IN MYCOBACTERIA ISOLATED FROM POIKILOTHERMIC ANIMALS

by

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AMINE METABOLISM IN MYCOBACTERIA ISOLATED FROM POIKILOTHERMIC ANIMALS

ABSTRACT

Three mycobacterial strains isolated from fish were studied to determine possible roles that amines might play in the physiology of these microorganisms.

An examination of cells of one mycobacterial strain which possessed some ability to metabolize putrescine when not previously exposed to this compound showed that no di- or polyamines were present. Amines, therefore, may not be involved in the metabolic processes occurring in such cells.

A number of amines were tested as substrates for mycobacteria. Only putrescine was metabolized by the strains tested. The ability to utilize putrescine was enhanced by growing the cells in the presence of the compound.

Studies with intact cells showed that putrescine was approximately 45% oxidized. During the reaction, the only products excreted were ammonia and carbon dioxide. The former accounted for 69-76% of the amino-nitrogen of putrescine and the latter for at least 37.5% of the putrescine-carbon under CO_2- free conditions.

Radioactive studies with 1,4-C^{14} putrescine indicated that approximately 25% of the C^{14} was assimilated at a point corresponding to 43-44% of the maximum level of oxidation. During the reaction, nucleic acid, lipids, and protein were found to be labelled.

An examination of cell-free extracts obtained from putrescine-adapted cells revealed the presence of four enzymes involved in the utilization of putrescine.

GRADUATE STUDIES

Field of Study: Bacteriology

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Other Studies:

Biology of Fishes	C.C. Lindsey

ABSTRACT

Three mycobacterial strains isolated from fish were tested for their ability to metabolize various amines, supplied individually as sources of carbon. A series of six aliphatic and two cyclic amines was used. Growth occurred only in putrescine.

The ability to oxidize putrescine was enhanced by growing the cells in the presence of this compound.

One mycobacterial strain appeared to possess some ability to oxidize putrescine without pre-exposure to the compound. However, when cells of this strain were examined for the presence of putrescine and related polyamines, no such compounds were detected.

In the mycobacterial strains tested, putrescine did not appear to affect the rate of endogenous respiration. Therefore, in reporting oxygen consumption due to putrescine, the endogenous oxygen consumption has been subtracted from the total oxygen uptake.

During the oxidation of putrescine, the oxygen uptake curves "broke" at a point corresponding to approximately 43% of the maximum level of oxidation. Thereafter, very small increases in the level of oxidation occurred, so that by the end of the experiment, over 50% of the molecule appeared to be assimilated. Up to 69-76% of the amino-nitrogen of the putrescine molecule was released as ammonia during the reaction, the rest of the nitrogen being assimilated. Measurement of carbon dioxide production

using 1,4-C^{14} putrescine revealed that under CO_2-free conditions, at least 1.5 μM of carbon dioxide were released per μM of putrescine.

When cells were incubated with 1,4-C^{14} putrescine, 25% of the radioactivity was assimilated. The remaining radioactivity was liberated as $C^{14}O_2$. Chemical fractionation of the cells indicated that fractions containing lipid, nucleic acid and protein were labelled.

Cell-free extracts obtained from putrescine-adapted cells contained four enzymes responsible for the conversion of putrescine to succinic acid. The enzymes occurred in the 10,000 X G. supernatants of the cell-free extracts and were all active at pH 9.0. Putrescine was oxidatively deaminated to yield Δ'-pyrroline. The latter compound was then oxidized to γ-aminobutyric acid by a dehydrogenase enzyme requiring NAD or NADP. Gamma-aminobutyric acid underwent a transamination with α-ketoglutaric acid to yield succinic semialdehyde and glutamic acid. Succinic semialdehyde was oxidized to succinic acid by an NAD- or NADP- requiring dehydrogenase.

The specificity for putrescine, shown by the tested mycobacterial strains, is discussed.

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INTRODUCTION

Amines appear to be natural constituents of biological materials. They are widely distributed in nature either in free or conjugated forms. This fact, together with an increasing awareness as to the multiplicity of effects that amines have on biological systems, has recently stimulated a substantial amount of research on the possible roles that amines may play in biology. A rapidly growing literature indicates that amines are required for, or stimulatory for, the growth of some microorganisms; that amines may in some manner be involved with nucleic acids and that they may play a part in maintaining the structural integrity of fragile biological units. The compounds are also known to have a number of other physiological and pharmacological effects but if a specific physiological role exists for these substances, it still remains to be discovered.

This study was therefore undertaken in an attempt to discover the possible roles that amines may play in the physiology of mycobacteria isolated from poikilothermic animals. Cells were tested initially for their ability to metabolize putrescine and certain other naturally-occurring amines. In addition, cells were examined for the presence of amines.

REVIEW OF LITERATURE

Distribution of amines

Normal animal tissues contain significant concentrations of the polyamines spermine and spermidine (1,23,24,35,42,82, 116) in addition to smaller amounts of the aliphatic diamines 1,3-diaminopropane, 1,4-diaminobutane (putrescine) and 1,5-diaminopentane (cadaverine) (1,116). In these tissues the amines appear to be free although Kosaki et al (53) have reported the presence of a possible spermine conjugate isolated from malignant tissues. Furthermore, the enzyme, transglutaminase, exists in many animal tissues and is capable of catalyzing the exchange of the amide group of some proteins with a number of amines. It is therefore possible that protein-amine conjugates are more widespread in animal tissues than is now realized (14,15,66,67,69,70,86).

In microorganisms, substantial amounts of spermine, spermidine, 1,3-diaminopropane and putrescine have been reported in the free form (8,17,31,32,37,38,107,116). Gram-negative bacteria contain, in general, much higher concentrations of amines than Gram-positive bacteria and yeasts (37,38,107). Conjugated amines are more frequently encountered in microorganisms than in animal tissues. Acetylated derivatives of putrescine, spermidine and spermine have been reported for Escherichia coli and Staphylococcus aureus (22,79,80). The former organism is also reported to produce

a peptide containing spermidine (21).

Among the viruses, significant amounts of putrescine and spermidine have been reported for certain phages of E. coli (2,3,49) while the apparent lack of amines in other bacteriophages and plant viruses (2) may have been due to the release of the amines to the suspending medium.

Plant materials also contain amines. Herbst and Snell (36) have reported the presence of putrescine in orange juice.

Amines as growth factors and stabilizers

Putrescine serves as a growth factor for a mutant of Aspergillus nidulans (95) and for the bacterium Haemophilus parainfluenzae (36). Amine requirements have also been reported for Neisseria perflava (64), Pasteurella tularensis (112) and Achromobacter fischeri (22). Spermine, spermidine and putrescine stimulate the growth of Lactobacillus casei (51). With P. tularensis, N. perflava and A. fischeri the growth-furthering effect of the added polyamine appears not to be due to specific growth factor activity, but rather to a decrease in the sensitivity of the microorganism to osmotic lysis conferred by the polyamine (55,56,57,59). The stabilizing effect of amines has been demonstrated for protoplasts of E. coli, Micrococcus lysodeikticus and Pseudomonas aeruginosa (33,56,99,100,101). Mitochondrial swelling induced by hypotonic media and various chemicals has in many cases been inhibited by various poly- and diamines

(39,100,101). The dissociation of ribosomes into their various components has been inhibited by spermidine (17). Stabilization of phage preparations against shock has also been accomplished with 1,5-diaminopentane (27) and spermine and spermidine (105). A similar resistance to shock is conferred on the transforming DNA preparation of Bacillus subtilis by spermine, spermidine and putrescine (104).

Although the exact mechanism of the stabilization effect is not known, it is probable that the high concentrations of phospholipids in bacterial protoplasts and mitochondria allow the formation of stable phospholipid-amine complexes. Similar complexing of the nucleic acids of phages, transforming principles and ribosomal ribonucleic acid with amines could also conceivably produce more stabilized structures. Such complexes are not difficult to conceive of since the cationic amines should be attracted to anionic materials like phospholipids and nucleic acids. Indeed, such complexes have readily been demonstrated (2,25,27,50,60,61,77).

Other effects of amines

Apart from their growth-promoting properties, amines have been shown to protect against the adverse effects of drugs and chemicals in bacteria, yeast cells and phage (13, 49,65,92,96). On the other hand, amines or their degradation products exert toxic effects on a number of biological systems. In bacteria, yeasts and phage tested, spermine

was found to be more toxic than spermidine, while putrescine exhibited little or no toxicity (49,75,77,85,102). In certain bacteriophages a decrease is noted in the burst size when the systems are exposed to spermine and spermidine (49).

Among the mycobacteria, the oxidation product(s) of spermine appear(s) to exert a greater toxicity than the parent polyamine (40,41,42). Similar observations have been made with E. coli and S. aureus (102) and with malignant mammalian cells (1).

A precise mechanism which explains the toxicity of polyamines is not immediately available, but there is growing support for the idea that amines may be involved in the cation balance in certain organisms (22,77). The displacement of non-toxic metal cations by polyamines may partially explain toxicity (34,58). In E. coli, S. aureus and yeasts (22,75,85) amines exert a more toxic effect under alkaline conditions. Under these conditions, acetylation of the di- and polyamines is accelerated in E. coli (22). Acetylation could serve to reduce a rise in cation concentration. In addition, acetylated spermine is reported to be less toxic than free spermine and a significant reduction in the synthesis of putrescine and spermidine in E. coli occurs when the organism is exposed to exogenous spermidine (22). The plant studies of Smith and Richards (94) also lend support to the cation balance theory. These workers discovered that under conditions of potassium

deficiency, barley and red clover plants contain greater concentrations of putrescine and agmatine. These data would suggest that the lack of an inorganic cation led the plants to synthesize a substitute organic cation.

In animals, amines, particularly spermine, are reported to cause necrosis of the renal tubules (81,102) while in plants, abnormal chromosomal behaviour was observed when the tissues were exposed to spermine, cadaverine and putrescine (19,63).

For a more detailed discussion of the biological significance of amines, the reader is referred to the excellent review by Tabor et al (110).

Amines: their synthesis and degradation

Apart from the general phenomena elicited by biological materials when exposed to various amines, the synthesis and degradation of this group of compounds is also of interest.

Synthesis of amines

The relatively simpler amines usually arise from the corresponding amino acids by decarboxylation. Gale (30a, 30b,30c,30d) showed that the naturally occurring amines histamine, tyramine, putrescine, cadaverine and agmatine were produced from the analogous amino acids by a number of bacteria. Information bearing on this topic has been adequately reviewed by Gale (28).

The more complex polyamines, spermidine and spermine are probably derived from simpler compounds. Most studies involving polyamine synthesis have employed microorganisms, although minced rat prostate showed some ability to synthesize spermidine (109). Studies with growing E. coli and Aspergillus nidulans (109) and with Neurospora crassa (31, 32) employing labelled substrates, indicated C^{14}-N^{15} putrescine and 2-C^{14} methionine to be precursors of spermidine and spermine. When other microorganisms and their extracts were studied using 2-C^{14} methionine, labelled spermidine was also isolated (8). From such studies it was concluded that putrescine was incorporated into spermidine as an intact unit, while on the other hand, methionine served by donating the three-carbon moiety of the spermine carbon chain. Enzymes isolated from E. coli (98,108,110) were capable of catalyzing the following reactions:

(1) ATP + L-methionine $\xrightarrow{Mg^{++}}$ S-adenosylmethionine + H_3PO_4

(2) S-adenosylmethionine $\xrightarrow{Mg^{++}}$ CO_2 + "decarboxylated adenosylmethionine"*

(3) "Decarboxylated methionine" + putrescine $\longrightarrow$ methylthioadenosine + spermidine

The synthesis of spermine has not yet been elaborated, but it is reasonable to assume that spermidine may accept a propylamine group from another molecule of "decarboxylated

* 5' deoxyadenosyl-(5'), 3-aminopropyl-(1) methyl sulfonium salt.

adenosylmethionine" to form spermine.

The degradation of amines

The initial step in the degradation of monoamines has been the subject of a review by Zeller (117). The degradation of other amines, chiefly diamines, has been reviewed by Zeller (117) and Tabor (106).

It is generally agreed that mono- and diamines are first oxidatively deaminated to yield ammonia and hydrogen peroxide:

(1) $RCH_2NH_2 + O_2 + H_2O \longrightarrow RCHO + NH_3 + H_2O$

In the presence of catalase, the net reaction becomes:

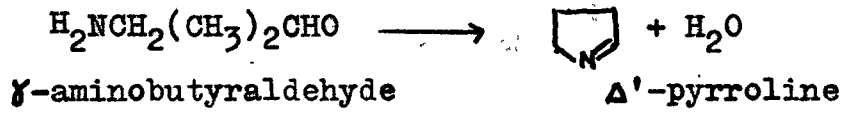
(2) $RCH_2NH_2 + 0.5\ O_2 \longrightarrow RCHO + NH_3$

The production of H_2O_2 during the reaction has been demonstrated by coupled oxidation using substrates not attacked by the enzyme but which are oxidized by the H_2O_2 (48,97) or by destruction of the H_2O_2 formed, with catalase (106).

Unlike diamine oxidases, monoamine oxidases are not sensitive to carbonyl reagents and therefore it has been possible to demonstrate that aldehydes are products of the reaction by trapping the latter with carbonyl reagents and identifying the derivatives so formed (117). In the case of diamine oxidases, this technique is not generally applicable

as these enzymes are usually inhibited by carbonyl reagents. The same difficulty exists for the enzymes which oxidize the polyamines, spermine and spermidine (76,115). Since the polyamines are structurally more complex than the diamines, products derived from the former have only recently been partially characterized.

In the case of the diamines putrescine and possibly cadaverine, the aminoaldehyde formed by the action of diamine oxidase undergoes a spontaneous cyclization with the elimination of water to form Δ'-pyrroline and Δ'-piperideine (46,62,106):

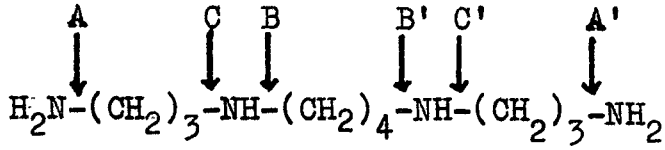


γ-aminobutyraldehyde Δ'-pyrroline

Such cyclic compounds are unstable under the best of conditions and techniques for their preparation, assay and characterization have recently been described (5,43,46,72).

With the polyamines, the points at which an initial enzymatic attack are likely, are between the carbon-nitrogen bonds. Since there are four of these for spermidine and six for spermine, it can readily be visualized that the products that might be expected, are several. Tabor et al (103) found that during the oxidation of spermine by beef plasma 2.0 μM of NH_3 and 2.0 μM of H_2O_2 were produced for every 2.0 μM of O_2 consumed when catalase was absent from the reaction mixture. These workers therefore concluded

that spermine was deaminated at both ends of the molecule (positions A and A'):

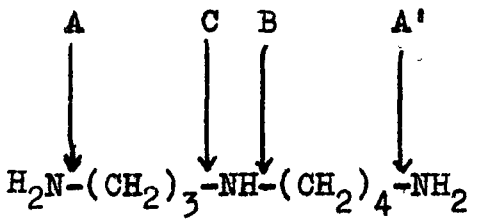


spermine

However, the appearance in their reaction mixtures of putrescine and possibly spermidine and an aminoaldehyde (102,103) indicated that the initial conclusions were doubtful since the latter products required that spermine be split at C and C'. It is very likely that the beef plasma preparation contained several enzymes for spermine degradation.

More recently, Bachrach and Bar-Or (7) demonstrated that sheep serum contained an enzyme which cleaved spermine at C or C' to yield spermidine and an unidentified aminoaldehyde. The spermidine was then further degraded.

Weaver and Herbst (115) have reported that N. perflava extracts degrade spermine and spermidine with the consumption of 0.5 μM O_2 and the production of 1.0 μM of an aminoaldehyde and 1.0 μM of 1,3-diaminopropane. This would require that spermine or spermidine be split at B or B'. In the case of spermidine such a split would yield γ-aminobutyraldehyde which they assumed to exist as such or to be converted spontaneously to Δ'-pyrroline.



More recently Bachrach (5) reported findings similar to Weaver and Herbst. Using a partially purified cell-free extract from Serratia marcescens, it was found that spermidine was degraded to equimolar amounts of 1,3-diaminopropane and Δ'-pyrroline with the consumption of 0.5 equivalents of O_2 when catalase was present. No ammonia was produced.

If the report of putrescine (102) as an intermediate in spermine breakdown is valid, there may be two general types of degradative enzymes for polyamines— one type yielding 3-carbon aminoaldehydes and 4-carbon diamines and the other producing 4-carbon aminoaldehydes and 3-carbon diamines.

Amines as substrates for bacteria

Gale (29) reported on the ability of washed cell preparations to oxidize a variety of amines including putrescine, cadaverine, agmatine, histamine and tyramine. Among the organisms tested by this worker, Ps. pyocyanea was found to be the most active while other organisms tested, including E. coli, attacked fewer amines. In the case of the former bacterium, putrescine, cadaverine and agmatine were completely oxidized with none of the substrate being assimilated.

Satake et al (87) tested the ability of a number of microorganisms to oxidize primary amines. All but one of the organisms tested were found to be capable of oxidizing putrescine after adaptive lag periods. Members of the following genera were tested: Serratia, Proteus, Escherichia, Flavobacter, Pseudomonas, Bacillus, Achromobacter and Micrococcus. In addition, Achromobacter liquidum, when adapted to putrescine appeared to be simultaneously adapted to γ-aminobutyric acid.

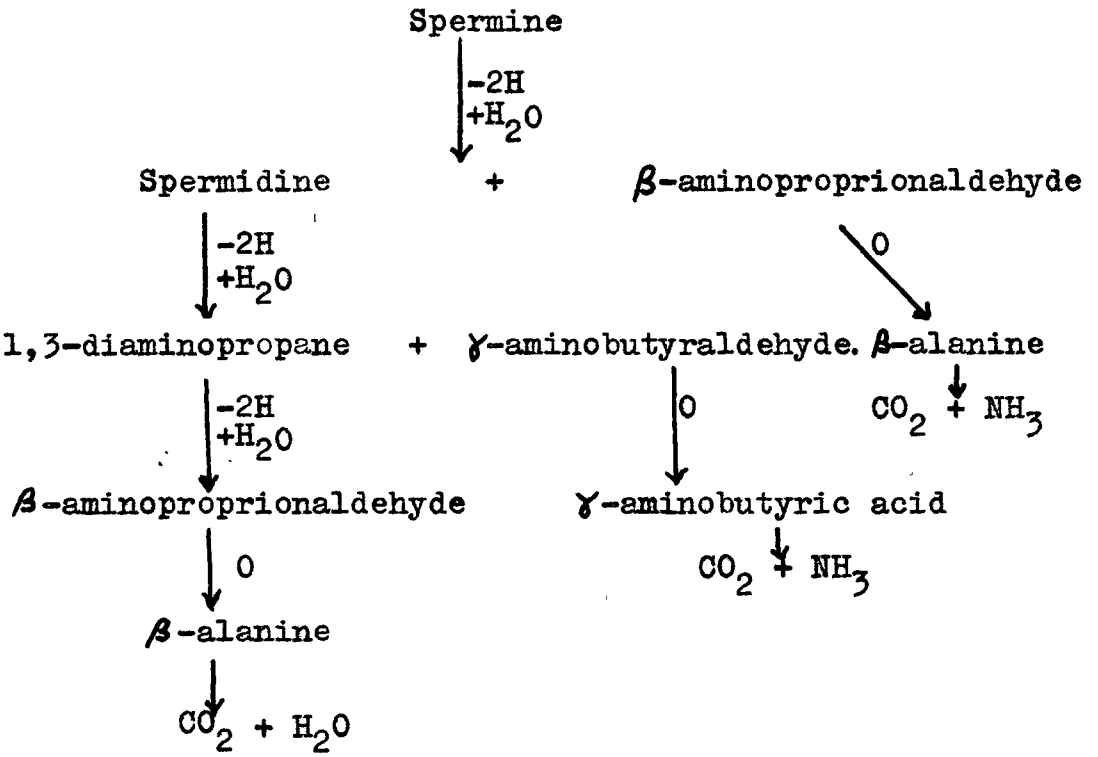
Razin et al (76) surveyed some twenty strains of microorganisms to test their ability to utilize di- and polyamines. Of the strains tested, putrescine and spermine were not metabolized by E. coli (2 strains), Klebsiella (1 strain), Proteus vulgaris (1 strain), Shigella (4 species), Salmonella (4 species), Staphylococcus (1 species), Micrococcus (1 species), Streptococcus (2 species) and Mycobacterium phlei. Ps. aeruginosa was found to oxidize spermine, spermidine, putrescine, agmatine and cadaverine. S. marcescens oxidized spermidine, putrescine and agmatine while Corynebacterium pseudodiphtherium oxidized only putrescine. Jakoby and co-workers (46,47,89,90) reported that Ps. fluorescens oxidized putrescine and studied the degradation of this compound in detail. Bachrach et al (9) reported that Myc. smegmatis oxidized spermine, spermidine and putrescine. This confirmed the observations of Roulet and Zeller (84) that this microorganism attacked spermine and

putrescine. Other microorganisms reported as oxidizing spermine and/or spermidine are: Ps. pyocyanea (92) H. parainfluenzae, N. perflava and P. tularensis (115,116).

Studies with washed cell preparations have usually employed conventional manometric techniques in CO_2-free gas atmospheres. Such studies have indicated that, with certain exceptions (29), only portions of the amines are oxidized to CO_2, H_2O and NH_3, the remainder of the molecules presumably being assimilated. Thus Roulet and Zeller (84) reported that putrescine was only partially oxidized. Similar results are reported by Razin et al (76) and Bachrach et al (9) for the oxidation of various di- and polyamines by a number of bacteria. It would therefore appear that amines may serve not only as sources of energy for many bacteria, but also as structural building blocks. This fact has been proven conclusively for Ps. fluorescens which can grow on putrescine as a sole carbon, nitrogen and energy source (46).

Detailed elucidation of the pathways for the utilization of di- and polyamines in bacteria are largely due to the work of Razin et al (74,76), Bachrach et al (9), Jakoby and colleagues (46,47,89,90) and Weaver and Herbst (115). The work of the last authors has already been mentioned in connection with polyamine metabolism. Studies with Ps. aeruginosa, Myc. smegmatis and S. marcescens indicated that along with O_2 consumption and the production of NH_3 and CO_2,

certain intermediates were produced which were either later utilized, or accumulated in the reaction mixture. Thus Ps. aeruginosa degraded spermine by a pathway with spermidine, 1,3-diaminopropane, β-alanine and γ-aminobutyric acid as intermediates while S. marcescens produced 1,3-diaminopropane as an intermediate which accumulated during the oxidation of spermidine. Freeze-dried cells of Myc. smegmatis metabolized spermine by a similar pathway to Ps. aeruginosa. The net result of this research led to the following degradative pathway formulated by Razin et al (76):



The details for the mechanism of the oxidation of γ-aminobutyric acid and β-alanine were studied by Bachrach

et al (9), Bachrach (6) and Jakoby and colleagues (46,89, 90) using freeze-dried cells and cell-free extracts.

In Ps. aeruginosa and Myc. smegmatis, γ-aminobutyric acid and β-alanine undergo transamination with keto acids to form succinic and malonic semialdehydes. The semialdehydes are then oxidized to succinic and malonic acids. A similar process for the utilization of putrescine exists in Ps. fluorescens (46). The diamine is first deaminated oxidatively to yield γ-aminobutyraldehyde. The latter which is thought to exist in equibrium with its spontaneous cyclization product, Δ'-pyrroline, is dehydrogenatively oxidized to γ-aminobutyric acid which in turn undergoes a transamination with α-ketoglutaric acid to form glutamic acid and succinic semialdehyde. The latter compound is then oxidized via an NAD-or NADP-requiring enzyme to succinic acid whence it can enter the tricarboxylic acid cycle and be further degraded and/or assimilated. This series of reactions has been studied in some detail and because of the remarkable specificity of the enzymes involved in Ps. fluorescens, the enzymes are suitable for assay methods (44).

A rather novel mechanism for the metabolism of putrescine in an E. coli mutant has recently been reported (52). In this system putrescine is not oxidatively deaminated as is usual during the initial degradative steps. Instead, putrescine undergoes a transamination with α-ketoglutaric acid to yield glutamic acid and γ-aminobutyraldehyde.

From this stage on, the remaining reactions are identical with those of the other systems discussed. In this system the transaminase appeared to be constitutive while in most of the other systems examined where putrescine and other amines were oxidized, the systems usually appeared to be induced (9,46,76,87,115).

MATERIALS AND METHODS

I Organisms

Strains of mycobacteria obtained for this study were kindly supplied by Dr. H. Vogel, Department of Health, City of New York, N.Y., and A.J. Ross, Western Fish Disease Laboratory, Seattle, Washington. Table 1 lists the cultures obtained which proved suitable for the immediate purposes of this project because of their relatively rapid rate of growth.

Table 1

List of cultures obtained for study.

Mycobacterium sp.	Host	Supplier	Designation
N.C.T.C. strain #2291	Halibut	H. Vogel	H.A.L.
Neon tetra, strain N	Neon tetra*	H. Vogel	N.T.N.
Chambers Creek strain	Steelhead trout	A.J. Ross	C.C.
Myc. salmoniphilum	Chinook salmon	A.J. Ross	S.C.

* Species of tropical fish.

Some of the characteristics of the microorganisms listed in Table 1 are described in papers by Vogel (114) and Ross (83).

All mycobacterial strains were initially assayed for their ability to metabolize putrescine (1,4-diaminobutane), since according to Zeller et al (118) this compound is one of the better substrates for demonstrating diamine oxidase

activity in bacteria. However, the studies that followed this general survey were concentrated on strains N.T.N., C.C. and S.C.

II Growth conditions

Preliminary work showed that growth in static broth media was granular and in pellicular form. The addition of surface active agents to such static cultures did little towards producing uniform suspensions of cells. Uniform suspensions of mycobacterial cells suitable for quantitative studies were found to be most readily prepared by growing the cells on a wrist-action shaker in fluid media containing Tergitol NPX at a final concentration of 0.01% (v/v). Although growth was equally good at 30°C, incubation was routinely conducted at room temperature in cotton-stoppered erlenmeyer flasks.

A 5% inoculum (v/v) of an actively growing culture usually produced a satisfactory yield of log-phase cells in three to four days under the conditions already described.

III Media

In studies designed to demonstrate the ability of the microorganisms to grow on amines as the sole carbon source, a minimal salts medium was used (see Appendix, Table 1). When it was necessary to show whether the system responsible for the metabolism of putrescine was constitutive or induced,

larger volumes of cells were required since such studies involved observations on both intact cells and their cell-free extracts. In such cases, a richer synthetic medium was employed which could be fortified with putrescine (see Appendix, Table 2).

Where large batches of cells were required for preparing cell-free extracts containing putrescine-metabolizing enzymes, tryptic soy broth (Difco), supplemented with putrescine at a final concentration of 5.0 μM/ml. of medium, was employed. This medium resulted in faster growth, and better yields of cells, than either of the previously listed media.

IV Preparation of washed cell suspensions

Since the mycobacterial cells used in these experiments were hydrophobic, all manipulations involving intact cells were carried out in suspending media containing 0.01% (v/v) Tergitol NPX.

Cells were harvested by centrifugation. The packed cells were washed twice in large volumes of 0.85% (w/v) saline and resuspended in buffer at pH 7.2. Intact cells were studied manometrically at pH 7.2 in sodium-potassium phosphate or tris-(hydroxymethyl)aminomethane (tris) buffer. Oxygen uptake studies on intact cells acting on putrescine in either buffer gave identical results. Varying the concentration of the suspending buffer from 0.1 M to 0.03 M also failed to have an effect on putrescine oxidation. Cells

were therefore routinely suspended in 0.1 M tris buffer, pH 7.2.

V Standardization of washed cell suspensions

It was empirically determined that a cell suspension in 0.1 M tris buffer adjusted on a Klett-Summerson photoelectric colorimeter to 250 Klett units (filter #42) contained the equivalent of 0.8-1.0 mg. of dried cells/ml. The volume of buffer required to adjust 1.0 ml. of a heavy cell suspension to 250 Klett units was then noted and from this value it was possible to calculate an appropriate dilution so that 1.0 ml. of diluted cell suspension added to a Warburg respirometer vessel would contain the desired number of mg. (dry weight) of cells.

Cells were dried to constant weight at 100°C using 5.0 ml. samples. Corrections were made for the weight of dried buffer salts.

VI Preparation of cell-free extracts

Cell-free extracts were obtained by grinding frozen cell-glass bead mixtures in a mortar and pestle. Wet, packed cells and glass beads (1.0 g. to 8.0 g.) were thoroughly mixed and frozen at -20°C. The mixture was then ground at 4°C for 5-6 minutes in 10 g. amounts. Ground preparations were pooled and extracted with 0.05 M phosphate buffer, pH 7.2, containing 0.005 M mercaptoethanol

such that 200 mg. of wet cells were extracted with 1.0 ml. of buffer. The supernatant fluid was collected and the glass bead - cell mixture was extracted once more with an equal volume of buffer. Both extracts were then combined and centrifuged at 0°C for 30 minutes at 10,000 X G. The supernatant fluid was then decanted and dialyzed for 36 hours at 0-4°C in three changes of distilled water totalling 15-20 litres. The dialyzed extracts were then centrifuged as before, and the supernatants divided into 10 ml. quantities for storage at -20°C. Such dialyzed extracts typically contained 5.0-6.0 mg./ml. of protein when the trichloroacetic acid (TCA) precipitable material was converted to nitrogen and the latter converted to protein values by using a factor of 6.25. No TCA soluble nitrogen was present. When protein was determined by the turbidimetric method (91), using bovine albumen (Armour) as the standard, the extracts were found to contain 6.0-7.0 mg. protein/ml.

Glass beads used were Superbrite, type 110, (regular). The beads were covered with 12 N HCl and allowed to sit overnight at room temperature. They were then washed free of acid with distilled water, rinsed with 0.1 M phosphate buffer, pH 7.2, and finally rinsed with distilled water.

VII Manometric measurements

Gas exchange was measured at 30°C.

1 Intact cells.

Unless otherwise specified, each Warburg vessel received 3.0-5.0 mg. of cells (dry weight) in 1.0 ml. of 0.1 M tris buffer. Substrate additions were usually in 0.5 ml. amounts. The total volume of fluid in each vessel was always 3.2 ml. In O_2 uptake studies, 0.2 ml. of 20% (w/v) KOH was placed in the centre well. Where CO_2 output was to be determined, 0.2 ml. of 6 N H_2SO_4 replaced the alkali and was put in the second sidearm of a double sidearm vessel. CO_2 production was determined by the direct "two cup" method followed by an "acid tip" (113).

In experiments where inhibitors were used, the cells were preincubated with inhibitor for 30 minutes prior to the addition of substrate. Under these conditions, substrate was added in 0.1 ml. amounts to avoid any drastic dilution of inhibitor.

Following prolonged manometric experiments, smears of the reaction mixtures were stained by the Ziehl-Neelson acid-fast method to ensure that the results were not influenced by the appearance of contaminants during the experiment.

2 Cell-free extracts.

Warburg vessels usually contained 1.0 ml. of extract and 1.0 ml. of 0.1 M buffer at the desired pH. The remaining volume was brought up to 3.0 ml. with water and/or aqueous substrate and/or aqueous inhibitor. The centre well contained 20% KOH (w/v).

VIII Studies with intact cells

1 Ability of washed cells to oxidize putrescine.

Cells for this study were grown in tryptic soy broth. Washed, standardized cells were assayed in Warburg respirometer vessels in order that oxygen consumption could be followed. Four mycobacterial strains were assayed on 0.001 M and 0.01 M putrescine dihydrochloride (Nutritional Biochemical Corp.). Since putrescine at each concentration resulted in identical rates of oxygen uptake initially, it was concluded that the compound did not affect the cells adversely. The oxygen uptake values in Table 2 represent oxygen consumption in the presence of 0.01 M putrescine at 4.5 hours. The corresponding endogenous oxygen uptake values are also listed. The latter values represent oxygen consumption with water substituted for putrescine.

2 Influence of pre-exposure to putrescine on the oxidation of putrescine.

The object of this experiment was to determine whether the initial rate of putrescine oxidation could be enhanced by growing cells in the presence of the compound.

Cells were grown in the synthetic medium described in Table 2 of the Appendix. During the final 16 hours of growth, half of each culture was supplemented with a sterile solution of putrescine (5.0 µM putrescine/ml. medium). The remaining half of each culture received no additions. The cells were then harvested, washed, standardized, and assayed on 0.01 M putrescine in Warburg respirometer vessels. Results for the three mycobacterial strains tested are presented in Figs. 1-3. The curves represent oxygen uptake due to added putrescine, endogenous oxygen uptake values having been subtracted from those in the presence of putrescine.

3 Ability of washed cells to oxidize aliphatic amines.

Previous results had indicated that washed cells of various mycobacterial strains could oxidize putrescine and that pre-exposure to the compound enhanced the initial rate of putrescine oxidation. This experiment was therefore designed to determine whether similar results could be obtained for the related diamines 1,3-diaminopropane and 1,5-diaminopentane.

Strain C.C. cells were grown in the synthetic medium described in Table 2 of the Appendix. The medium was supplemented with 5.0 µM/ml. of either 1,3-diaminopropane or

1,5-diaminopentane. Log-phase cells were harvested, washed, standardized and assayed manometrically on 15 μM of each of the following amines: 1,3-diaminopropane, putrescine and 1,5-diaminopentane. The results recorded in Table 3 represent the oxygen uptake at six hours.

In a related experiment, strain C.C. cells were grown in the same medium supplemented with spermidine (5.0 μM/ml. medium). It was expected that if the cells could be adapted to spermidine they might simultaneously be adapted to putrescine and/or 1,3-diaminopropane. The cells were therefore assayed manometrically with spermine, putrescine and 1,3-diaminopropane as substrates. The results are graphically depicted in Fig. 5.

The reaction mixture supernatants were examined by paper chromatography at the conclusion of the experiments to determine whether the substrates had disappeared or had been modified in any manner.

4 Amines as sole carbon sources.

This experiment was designed to test the ability of mycobacterial strains to utilize a number of amines as sole carbon sources for growth.

The basal medium used is described in Table 1 of the Appendix. The following amines were tested as their hydrochloride salts at 0.4% (w/v): 1,3-diaminopropane, putrescine, cadaverine, pyrrolidine, piperidine and

1-aminobutane. The phosphate salts of the polyamines, spermidine and spermine, were also tested on a smaller scale in test tubes at a concentration of 0.1% (w/v). A dilute suspension of washed cells (0.2 ml.) in 0.067 M phosphate buffer, pH 7.2, was used as inoculum and the cultures were incubated for 17 days. Controls, with 0.4% glucose (w/v) as the sole source of carbon were also included. The results for the three mycobacterial strains tested, are presented in Table 4.

5 Occurrence of amines in mycobacteria.

This experiment was conducted to determine whether mycobacterial cells contain amines in detectable quantities. Because of the lengthy procedure involved, only one mycobacterial strain was examined.

Strain C.C. cells, grown in tryptic soy broth, were harvested and washed. The packed cells were combined with glass beads and the mixture thoroughly ground. The mixture was then extracted at room temperature with several volumes of 5% TCA containing HCl at a final concentration of 0.1 N. A protein-free extract, equivalent to 2.0 g. of wet, packed cells was then combined with 10 g. of NaOH pellets and the mixture steam-distilled for 2.5 hours. At this time, no further detectable base appeared to be coming over in the distillate. The distillate (approximately 1 litre) was acidified with dilute HCl and the volume reduced by boiling.

Final drying of the distillate to free it of excess acid, was accomplished overnight by means of a warm blast of air from a fan. The acid-free residue was dissolved in a small volume of water and applied, with washings, to a column of Dowex 50 H^+ resin. The column (110 X 12 mm.) had a hold-up capacity of approximately 12 ml. for 2 N NaOH. The sample was eluted with a linear concentration gradient of H^+ ions supplied by 550 ml. of water and 550 ml. of 3 N HCl. Fractions were collected in 5.0 ml. amounts on a fraction collector. These were fan-dried overnight in a wind tunnel and redissolved in 1.0 ml. of water. Aliquots (0.1 ml.) were then assayed for the presence of amines with the 2,4-dinitroflourobenzene technique of Dubin (20). Fractions which were positive for amines were subjected to paper chromatography in order to identify the compounds.

Identical analyses were performed on a 5.0 g. sample of tryptic soy broth powder and on a synthetic mixture of amines containing 1.5 μM of spermine and 1.5 μM of putrescine.

The results are discussed under the appropriate sections of "Experimental Results and Discussion". Fig. 6 shows the elution patterns obtained with the strain C.C. extract, tryptic soy broth and the synthetic amine mixture respectively.

6 Influence of putrescine on endogenous respiration.

Indirect techniques were used to determine whether

putrescine influenced the rate of endogenous respiration.

Oxygen consumption by strain S.C. was measured in an experiment where a constant quantity of cells (approximately 2.0 mg.) was allowed to oxidize 1.5, 3.0 and 6.0 µM of putrescine respectively. Strain N.T.N. was also tested by this technique with 1.5 and 3.0 µM of putrescine. In another experiment, three different quantities of strain S.C. cells (approximately 1.0 mg., 2.0 mg. and 3.0 mg.) were each allowed to oxidize 3.0 µM of putrescine. Oxygen consumption was followed on the Warburg apparatus until the curves levelled off at 4.5 hours. Cells of both strains had previously been grown in the presence of putrescine to ensure a satisfactory rate of oxidation of the compound. The results of these experiments are presented in Tables 5 and 6.

7 Oxygen, ammonia and carbon dioxide relationships during putrescine oxidation.

Cells used in these experiments were adapted to putrescine. The studies were designed to determine, quantitatively, the degree of assimilation occurring during the oxidation of putrescine by washed cells. Strains N.T.N., C.C. and S.C. were tested.

A. Oxygen consumption.

Washed cells were allowed to oxidize a given amount of

putrescine and the oxygen uptake was followed manometrically. Putrescine oxidation, in these studies, occurred in a CO_2-free atmosphere.

B. Ammonia production.

Ammonia produced during the oxidation of putrescine was quantitatively determined on reaction mixtures obtained from Warburg respirometer vessels after the oxygen uptake curves had levelled off. In addition, larger scale reaction mixtures of similar proportions to those used in the Warburg respirometer vessels, were run in parallel on the Warburg respirometer with shaking. The latter reaction mixtures were run in cotton-stoppered erlenmeyer flasks which facilitated sampling at desired intervals, but it should be noted that in these flasks reactions occurred under atmospheric conditions. Ammonia determinations were also conducted on reaction mixtures withdrawn from Warburg reaction vessels which contained no alkali and which had been used for CO_2 determinations. Such determinations were made after the oxygen uptake curves had levelled off.

C. Carbon dioxide.

Carbon dioxide was determined by the direct "two-cup" method followed by an "acid tip". This technique does not take into account the influence that the presence, or absence, of CO_2 may have on the course of the reaction.

Oxygen consumption curves were used to determine when to stop the reaction. Acid was tipped into the reaction mixture after the oxygen uptake curve had levelled off, or at the region of the "break" of the oxygen uptake curve.

D. Paper chromatography of reaction mixture supernatants.

In order to determine whether products accumulated in the reaction mixtures during the oxidation of putrescine, the reaction mixture supernatants were subjected to paper chromatography. The reaction mixtures were chromatographed directly, or when necessary, after the removal of buffer salts by ion exchange.

In order to detect nitrogenous compounds, chromatograms were sprayed with ninhydrin or 2,4-dinitroflourobenzene. Organic acids were sought by spraying chromatograms with an indicator dye.

Reaction mixture supernatants from Warburg respirometer vessels were examined chromatographically at the end of the reaction period, while those from erlenmeyer flasks were examined at various intervals during the course of putrescine oxidation.

The results of these studies are discussed under "Experimental Results and Discussion". Figs. 7 and 8 present the oxygen uptake and ammonia curves obtained for two strains while Fig. 9 shows the course of oxygen uptake for a third strain. The curves have been corrected for

endogenous oxygen and ammonia values since earlier studies indicated endogenous respiration to be independent of putrescine oxidation.

8 Inhibitor studies with washed cells.

Cells used for the following experiments were adapted to putrescine. The effects of a number of inhibitors on two mycobacterial strains were tested.

Strain C.C. cells (approximately 5.0 mg. dry weight/ reaction vessel) were allowed to oxidize putrescine (3.0 µM) in the presence of sodium arsenite (0.003 M), sodium azide (0.003 M), p-phenylenediamine (0.0045 M) and malonic acid (0.005 M), respectively. The effects of these inhibitors on the oxidation of putrescine and on the endogenous respiration were measured manometrically. The oxygen consumption values for the various treatments are presented in Table 7. The values represent oxygen consumption after the curves had levelled off.

Table 8 records the results of similar studies with strain S.C. cells (approximately 3.0 mg./reaction vessel). In addition to arsenite, the effects of dihydrostreptomycin (DHS) (0.001 M and 0.006 M) and of isonicotinic acid hydrazide (INH) (0.001 M and 0.006 M) were measured.

Valuable information might have been obtained from a paper chromatographic analysis of the various reaction mixtures obtained with the inhibitors tested. However, the time required to prepare clean samples suitable for

paper chromatography precluded such studies. Instead, tests with all other inhibitors were abandoned in favour of a more detailed examination of the affects of arsenite. This compound was the only inhibitor which caused hydrazone-forming substances to accumulate in the reaction mixtures. In addition, arsenite had the most pronounced affects on corrected oxygen uptake values.

Fig. 10 represents the effect of 0.003 M arsenite on the oxygen uptake of strain S.C. cells acting on 3.0 µM of putrescine. Parallel quantitative determinations on the concentration of carbonyl compounds in the reaction mixture are presented in terms of Klett units.

Fig. 11 records the effect of 0.009 M arsenite on the oxygen uptake and ammonia production by strain S.C. cells (3.0 mg. dry weight/Warburg vessel) acting on 3.0 µM of putrescine. The results of similar studies with strain C.C. (2.0 mg./Warburg vessel) are presented in Fig. 12.

In these experiments, samples for analysis were taken from cotton-stoppered erlenmeyer flasks which were run in parallel with the Warburg respirometer vessels as previously described.

Samples of the reaction mixtures were used for paper chromatography after the removal of sodium arsenite by ion exchange. Paper chromatography was also employed to identify the hydrazones formed when the carbonyl compounds in the reaction mixtures reacted with 2,4-dinitrophenylhydrazine

(DPNH).

At various times during the course of the reaction, cells obtained from the various reaction mixtures were washed once with cold water and the sedimented cells extracted with cold 0.1 N HCl for 30 minutes. Chromatography of such extracts was conducted to obtain some idea as to the nature of the amino acid pool within the cells and to determine whether certain amino acids might be derived from putrescine.

IX. Studies with cell-free extracts

Crude, dialyzed, cell-free extracts were examined for the presence of enzymes responsible for the dismutation of putrescine. No attempts were made to purify, or to characterize in detail, the enzymes encountered. Cell-free extracts were obtained from strain C.C. cells adapted to putrescine during growth in tryptic soy broth.

1 Diamine oxidase (D.O.).

The presence of D.O. in cell-free extracts was established by following the oxygen consumption resulting from its action on known amounts of putrescine. During the reaction, ammonia and Δ^1-pyrroline were produced. The latter compound was demonstrated qualitatively by paper chromatography. Techniques for the quantitative assay of both compounds are dealt with under Analytical Methods.

The sensitivity of D.O. to heat and several inhibitors

was briefly investigated. The specificity of the enzyme was determined for a series of aliphatic mono-, di- and polyamines. The effect of pH on the rate of the reaction was also investigated.

In heat stability experiments, the cell-free extract (2.0-3.0 ml.), was placed in tubes containing an equal volume of 0.1 M phosphate buffer, pH 7.2, at 55°C. After incubation for the desired period, the buffered extract was chilled immediately and assayed.

During inhibitor experiments, enzyme was incubated with inhibitor at 30°C for 20 minutes before the addition of substrate.

A typical reaction mixture contained, in a total of 3.0 ml.: Cell-free extract, 1.0 ml.; substrate, 6.0-30 μM; buffer, 100 μM at the desired pH; water or aqueous inhibitor.

2 Δ'-pyrroline dehydrogenase (P.D.).

The occurrence of the P.D. enzyme in crude, dialyzed, cell-free extracts could only be demonstrated if reducing materials were present. This requirement was satisfied by adding mercaptoethanol to the reaction mixture.

Two techniques were used to follow P.D. activity. The first method was based on the increase in optical density at 340 mμ due to the reduction of added nicotinamide adenine dinucleotide (NAD). Appropriate controls were employed to cancel out the effect on optical density of

added substrate or cofactor. Such studies were conducted on a Beckman model DU spectrophotometer. A typical reaction mixture contained, in a total of 3.0 ml.: Cell-free extract, 1.0 ml.; buffer, pH 9.0, 100 μM; Δ'-pyrroline, 3.0 μM; cofactor, 0.3 μM; mercaptoethanol, 15 μM.

The second method used to follow P.D. activity, involved coupling dehydrogenase activity with atmospheric oxygen by means of methylene blue (final concentration, 4.45×10^{-5} M). Under such conditions, the course of the reaction could be followed by measuring oxygen consumption manometrically. The ability of the enzyme to catalyze the reaction was tested at pH 7.2 and 9.0 with Δ'-pyrroline and Δ'-piperideine as substrates.

The manometric method required that two reaction vessels be used, since the oxidation of mercaptoethanol by methylene blue resulted in a considerable endogenous oxygen uptake. For this reason, it was also necessary to add higher concentrations of mercaptoethanol since this compound was progressively oxidized as the reaction proceeded. Theoretical oxygen consumption values were obtained routinely by correcting for endogenous oxygen consumption when the following 3.0 ml. reaction mixtures were employed: Cell-free extract, 1.0 ml.; buffer, 100 μM at the desired pH; mercaptoethanol, 50 μM; methylene blue, 1.34×10^{-1} μM; cofactor, 0.3 μM; substrate, 6.0 μM, or water.

In manometric experiments, the reaction was initiated

by adding putrescine to the complete reaction mixture, in which case, the oxygen uptake represented the oxygen consumption due to both D.O. and P.D. activity. Alternatively, methylene blue was added to the reaction mixture after the oxygen consumption due to added putrescine was completed. Under the latter conditions, the oxygen consumption due to the dehydrogenation of enzymatically synthesized Δ^1-pyrroline could be measured independently.

The transformation of Δ^1-pyrroline to γ-aminobutyric acid during the P.D. catalyzed reaction, was demonstrated by paper chromatography.

3 Gamma-aminobutyric acid - α-ketoglutaric acid transaminase.

Cell-free extracts did not appear to contain oxidative or dehydrogenative enzymes capable of catalyzing the deamination of γ-aminobutyric acid to yield succinic semialdehyde and ammonia.

Gamma-aminobutyric acid was found to undergo a transamination with α-ketoglutaric acid to yield succinic semialdehyde and glutamic acid. Succinic semialdehyde produced by the reaction was demonstrated by paper chromatography as its 2,4-dinitrophenylhydrazone derivative. Glutamic acid was shown to be the other product, by paper chromatography of the deproteinated, deionized reaction mixture.

The reaction was followed routinely by assaying aliquots

of the reaction mixture for glutamic acid. For this purpose, a specific glutamic decarboxylase preparation was used (see Analytical Methods).

The optimum pH was determined by conducting the reaction at various pH values between 7.0 and 9.0 in phosphate and tris buffers. Thirty-minute samples of each reaction mixture were analysed for the amount of glutamic acid produced. The effect of pH on the amount of glutamic acid produced in these systems, was also studied after more extended reaction periods.

The specificity of the transaminase enzyme was also investigated. Pyruvic and oxalacetic acids were tested with γ-aminobutyric acid to determine whether they could serve as alternative amino-group acceptors. The following amino acids were tested as alternative amino-group donors with α-ketoglutaric acid: glycine, β-alanine, δ-aminovaleric acid and ε-aminocaproic acid. The specificity studies were conducted at pH 9.0 in tris buffer for 60 minutes at 30°C.

A typical reaction mixture for the optimum pH experiments contained in a total of 3.0 ml.: Cell-free extract, 1.0 ml.; phosphate or tris buffer at the desired pH, 200 μM; γ-aminobutyric acid, 30 μM; α-ketoglutaric acid, 15 μM.

A typical reaction mixture for the specificity experiment, contained in a total of 3.0 ml.: Cell-free

extract, 1.0 ml.; tris buffer, pH 9.0, 100 μM; amino acid, 15 μM; keto acid, 15 μM.

Reaction mixtures from the specificity studies were examined qualitatively by paper chromatography. In the case of the latter experiment where α-ketoglutaric acid served as the amino-group acceptor, the amount of glutamic acid formed was determined quantitatively.

4. Succinic semialdehyde dehydrogenase (S.S.D.).

Succinic semialdehyde dehydrogenase activity in dialyzed, cell-free extracts, was demonstrated by following the increase in optical density at 340 mμ due to the reduction of NAD or NADP (nicotinamide adenine dinucleotide phosphate) in the presence of succinic semialdehyde.

Succinic semialdehyde was prepared chemically (see Analytical Methods) or enzymatically by the transaminase reaction earlier described.

The dehydrogenative oxidation of succinic semialdehyde yielded succinic acid. The product of the reaction was shown to be succinic acid by paper chromatography of the ether extract obtained from the acidified, protein-free, reaction mixture, and by the use of a succin-oxidase enzyme, the preparation of which is described under Analytical Methods.

In an initial experiment, paper chromatography indicated that during the transamination reaction between α-ketoglutaric acid and γ-aminobutyric acid, a new hydrazone-

forming compound accumulated. The addition of substrate amounts of NAD prevented the accumulation of this compound and allowed the transamination reaction to go to completion (as determined by the amount of glutamic acid produced).

A second experiment was therefore devised in which oxalacetic acid was included in the reaction mixtures to inhibit any breakdown of the succinic acid formed. Reaction mixtures were made to contain, in a total of 6.0 ml.: Cell-free extract, 2.0 ml.; tris buffer, pH 8.5, 200 µM; γ-aminobutyric acid, 6.0 µM; α-ketoglutaric acid, 12 µM; NAD, 6.0 µM. An identical reaction mixture, with water substituted for cofactor, was included as a control. The reaction was allowed to proceed at 30°C for 4 hours under an atmosphere of air at which time a 2.0 ml. sample was taken, adjusted to pH 4.8, and analysed quantitatively for glutamic acid. The reaction in the remaining 4.0 ml. of each reaction mixture was stopped by adding 0.1 ml. of 6 N HCl. Each reaction mixture was then heated in a boiling water bath for 15 minutes in order to precipitate the protein and to destroy both the reduced cofactor and oxalacetic acid. The mixtures were then centrifuged, the supernatants collected and adjusted to pH 7.4, and representative aliquots assayed for their succinic acid content with the succin-oxidase preparation.

X Analytical methods

1 Ammonia.

Ammonia was determined after micro-diffusion, by Nesslerization or by titration (18). Nesslerization was employed mainly in studies with cell-free extracts where under certain circumstances, the presence of volatile amines might have led to erroneus results if measured by the titration method.

2 Δ'-pyrroline.

A standard curve for the quantitative determination of Δ'-pyrroline was prepared. The assay was based on the yellow colour produced when Δ'-pyrroline and o-aminobenzaldehyde were allowed to react (43,46).

Known quantities of Δ'-pyrroline (0.1 to 0.5 μM), prepared from putrescine by D.O. action at pH 7.2, were reacted with a constant amount of o-aminobenzaldehyde at pH 6.5 in a total volume of 5.0 ml. After incubation at room temperature for 90 minutes, the absorbance of the solutions was read on a Klett-Summerson photoelectric colorimeter (Filter #42). Under the conditions of the assay, the colour developed bore a linear relationship to the concentration of Δ'-pyrroline. An aliquot of the Δ'-pyrroline solution used to prepare the standard curve was checked for

its Δ'-pyrroline content by the manometric method outlined earlier under the Δ'-pyrroline dehydrogenase section and the concentration of the compound was found to be within 3% of the theoretical value.

The reagent, o-aminobenzaldehyde, used for the colorimetric assay of Δ'-pyrroline, was prepared by the chemical reduction of commercially available o-nitrobenzaldehyde (46).

Chemically synthesized Δ'-pyrroline and Δ'-piperideine were used in the manometric assay method for Δ'-pyrroline dehydrogenase. These compounds were prepared by reacting ornithine and lysine, respectively, with N-bromosuccinimide (44,46). Chemically synthesized Δ'-pyrroline was also used to characterize the product of putrescine degradation due to D.O. activity.

3 Glutamic acid.

Quantitative determinations for L-glutamic acid were conducted in KOH-free Warburg reaction vessels. The assay was based on the stoichiometric production of 1.0 μM of CO_2 for each μM of glutamic acid present. Carbon dioxide production was determined manometrically at pH 4.8 in 0.1 M sodium-acetate buffer. A specific L-glutamic decarboxylase was employed to catalyze the reaction. L-glutamic decarboxylase was prepared as an acetone-dried powder from a prototrophic E. coli K 12 strain, grown according to the directions of

Cohen (16). A number of amino acids tested, including aspartic acid, lysine, histidine, arginine and D-glutamic acid, were not attacked under the conditions of the assay. Under the same conditions, there was no oxygen uptake in the presence of glutamic and γ-aminobutyric acids. Organic acids (succinic and α-ketoglutaric acids) and other amino acids, singly or in mixtures, served to decrease the rate of the reaction, but at completion, theoretical carbon dioxide values were obtained.

For assay, aliquots of reaction mixtures were adjusted to pH 4.8 with sodium-acetate buffer to stop the reaction. After temperature equilibration at 30°C, the enzyme (approximately 2.0 mg./reaction vessel) was added from the reaction vessel sidearm. Endogenous controls were always included, although corrections for endogenous carbon dioxide production were usually unnecessary.

A single preparation of the acetone-dried cells, stored in a tightly stoppered vial at -20°C, retained high decarboxylase activity for more than a year.

4 Succinic acid.

Succinic acid was determined quantitatively by measuring the oxygen consumption resulting from the enzymatic oxidation of succinic acid to fumaric acid. During the reaction, 0.5 μM O_2 were consumed for each μM of succinic acid oxidized. The reaction was catalyzed by a succin-oxidase enzyme which

was prepared from pig heart muscle by the method outlined by Cohen (16). A number of organic acids were tested as substrates: malonic, succinic, glutaric, α-ketoglutaric, oxalacetic and pyruvic acids. Succinic acid was the only compound attacked. The oxidation of succinic acid was completely inhibited by equimolar amounts of oxalacetic acid, while pyruvic acid was inactive in this respect. When, therefore, reaction mixtures containing oxalacetic acid were to be assayed for succinic acid, the former was first converted to pyruvate by heating in the presence of HCl. The same technique was used to destroy reduced cofactors (NADH or NADPH) in such reaction mixtures, since the succin-oxidase preparation contained a system for reoxidizing these compounds which would otherwise have interfered with succinic acid determinations. The latter fact was readily established when it was found that the succin-oxidase enzyme preparation could replace methylene blue in the manometric method for the assay of Δ'-pyrroline dehydrogenase activity.

For assay, a representative aliquot of the protein-free reaction mixture was assayed at pH 7.4 in 0.1 M phosphate buffer in Warburg reaction vessel containing 0.2 ml. of 20% (w/v) KOH in the centre well. Approximately 4.0 mg. (dry weight) of the preparation were used/assay. Corrections for endogenous oxygen consumption were usually unnecessary, even after extended runs.

A batch of the pig heart preparation, stored at - 20°C in sealed polyethylene bags, retained high activity with low blank oxygen uptake values for over a year.

5 2,4-dinitrophenyl hydrazones.

Pyruvate, α-ketoglutarate and succinic semialdehyde were determined qualitatively by paper chromatography as their 2,4-dinitrophenyl hydrazine (DPNH) derivatives.

Pyruvic and α-ketoglutaric acids were determined quantitatively as their DPNH derivatives. Standard curves were prepared for this purpose by reacting 0 to 1.0 μM of either acid with excess DPNH (54). The hydrazone(s) formed was (were) extracted quantitatively into ethyl acetate (3 X 2.0 ml. aliquots). The pooled ethyl acetate extracts were then quantitatively extracted with 10% (w/v) Na_2CO_3 (3 X 2.0 ml. aliquots). The pooled Na_2CO_3 extracts were then washed free of traces of unreacted DPNH with 1.0 ml. of ethyl acetate and the latter was discarded. The volume of the Na_2CO_3 solution was then brought up to 10 ml. with 4.0 ml. of 2 N NaOH, and the colour formed was read within 10 minutes on a Klett-Summerson photoelectric colorimeter (Filter #54). Under the conditions of the assay, there was a linear relationship between the colour developed and the keto acid concentration.

A reaction mixture to be examined for the presence of hydrazone-forming compounds was first freed of cells or

protein. The reaction mixture was treated with excess DPNH in 2 N. HCl for 1.5 hours at room temperature. The hydrazones were then trapped in 10% Na_2CO_3 by the procedure already described. A representative portion of the Na_2CO_3 solution was then assayed for absorbance as earlier described and the remaining solution was acidified at $0^{\circ}C$ with concentrated HCl. The hydrazones were then extracted into peroxide-free ethyl ether for spotting on paper chromatograms.

Accurate quantitative determinations of pyruvate or α-ketoglutarate using the Klett-Summerson photoelectric colorimeter, were only possible when the keto acid occurred alone, or where interference due to colour produced by other hydrazone derivatives, was negligible. When paper chromatography indicated the presence of more than one hydrazone derivative, the results were expressed in Klett units, and the relative concentration of each derivative could only be judged grossly by the density of the spots obtained by paper chromatography.

Hydrazone derivatives for use as standards in paper chromatography were always freshly prepared. Standards used were: α-ketoglutarate, oxalacetate, pyruvate and succinic semialdehyde. Since the latter compound was not available commercially, it was synthesized chemically by the crossed Claisen condensation technique described by Jakoby (44).

6 Paper chromatography.

A. Solvents.

Routine paper chromatography of amines and amino acids was conducted in n-butanol:acetic acid:water (2:1:1 v/v) (10). A second solvent employed to characterize amines was n-propanol:concentrated HCl:water (3:1:1 v/v) (22). Amino acids were also separated in water-saturated phenol prepared by dissolving 500 g. of phenol crystals in 225 ml. of water, allowing the layers to separate and using the lower layer to irrigate the paper. Amines and amino acids were run as their hydrochloride salts.

Organic acids were chromatographed as their ammonium salts in ethanol:NH_4OH:water (16:1:3 v/v) (93).

2,4-dinitrophenylhydrazones were separated in n-butanol saturated with 5 N NH_4OH. The lower phase was used to saturate the chromatographic chamber. As opposed to other chromatograms, hydrazones were developed by the ascending technique.

Whatman #4 paper was used for all paper chromatography.

B. Detection reagents.

Nitrogenous compounds were detected by spraying with 0.5% (w/v) ninhydrin in n-butanol, or with 2,4-dinitroflourobenzene (DNFB). The latter reagent was prepared by dissolving 0.65 ml. of DNFB in 50 ml. of acetone and using 10 ml. of this solution dissolved in 90 ml. of 2.5% (w/v) sodium

tetraborate (20). The DNFB reagent gave yellow spots with aliphatic mono- and diamines and brown spots with the polyamines spermidine and spermine. It suffered the disadvantages of not being as sensitive as ninhydrin and in not being capable of detecting volatile amines.

Organic acids were detected with an indicator dye solution: Brom cresol purple 0.04% (w/v) in a 1:5 (v/v) mixture of commercial formalin and ethanol. The pH was adjusted to 5.0 with 0.1 N NaOH (91).

C. Preparation of samples for paper chromatography.

Hydrochloric acid extracts of intact cells were dried to remove excess HCl. The dried salts were redissolved in water and the aqueous solutions were used for spotting chromatograms.

While it was possible to chromatograph small samples of cell-free reaction mixtures directly, with larger samples the buffer resulted in poor separations. Sodium arsenite used to poison intact cells, and protein precipitants like zinc sulphate and TCA, also resulted in poor separations. In order to obtain reproducible chromatograms it was therefore necessary to free samples of such interfering substances.

Δ^1-pyrroline was freed of zinc sulphate by ethyl ether extraction from alkaline solution. Succinic acid was obtained free from zinc sulphate by extraction into ethyl ether from acid solution. Δ^1-pyrroline was then converted

to its hydrochloride salt and succinic acid to its ammonium salt, for paper chromatography.

Cell-free reaction mixtures were examined for organic acids after the removal of tris buffer. For this purpose, the sample was applied to a Dowex 50 H^+ column which retained the tris buffer while the organic acids appeared in the water wash. The latter was concentrated to a suitable volume for paper chromatography after neutralization with dilute NH_4OH.

Cell-free reaction mixtures were examined for the presence of amino acids after the elimination of tris buffer by ion exchange on Dowex 1 OH^- columns. In this technique, the column was washed free of tris buffer with water. The amino acids which remained on the column were then eluted with HCl. The acid eluate was then dried and the salts were redissolved in water for spotting on the chromatograms.

Amino acids were freed of sodium arsenite and tris buffer by ion exchange on both Dowex resins. The sample was first applied to a Dowex 1 OH^- column. Washing with water removed sodium ion and tris buffer. The amino acids were then eluted with HCl and the eluate was dried. The redissolved salts were then applied to a Dowex 50 H^+ column where the arsenite ion was removed by the water wash leaving the amino acids behind on the column. These were then eluted with HCl. The acid eluate was then dried and the salts were dissolved in water for chromatography. A similar technique

was used to free amino acid-containing samples from TCA and tris buffer, except that the bulk of the TCA was usually first removed by one or two extractions with ethyl ether.

Dowex 50 H^+ columns employed for deionizing purposes, had a hold-up capacity of 1.5-2.0 ml. for 2 N NaOH. Dowex 1 OH^- columns had a similar hold-up capacity for 2 N HCl. Ions not exchanged by these columns, were usually completely washed out of the columns by 3-4 column volumes of water. A 10 ml. aliquot of 2 N HCl was sufficient to elute all the amino acids encountered from either type of column. Further washing with HCl failed to elute any nitrogenous compounds when the fractions were analyzed for their presence by the DNFB technique of Dubin (20).

XI Isotopic studies with intact cells

Assimilation studies with uniformly labelled C^{14} putrescine were not conducted since the compound was not readily available commercially. Instead, assimilation was studied using 1,4-C^{14}. Labelled putrescine was supplied by the New England Nuclear Corporation.

1 Incubation with substrate.

Strain N.T.N. cells, adapted to putrescine, were used for these studies.

Washed N.T.N. cells (approximately 5.0 mg./Warburg reaction vessel) were allowed to oxidize 3.0 μM of putrescine (1.75 μc). The oxygen uptake was followed manometrically and at various times, samples were taken for analysis. At each sampling, counts of the radioactivity were made on the following samples: (1) KOH-trapped $C^{14}O_2$ (2) cells and supernatant and (3) supernatant. Cells obtained from the samples were also fractionated immediately by a modification of the procedure outlined by Roberts et al (78). The cell fractions were analysed to determine the distribution of the radioactivity within the cells.

Aliquots of the various samples were plated in duplicate on stainless steel planchets at infinite thinness. Samples were dried under an infra red lamp. Counts were recorded with a Nuclear-Chicago scaler, Model 181A, equipped with a gas-flow counter having a thin-end-window Geiger tube. Corrections were made for background and a minimum of 2000 counts were recorded to reduce the reliable error to less than 5%.

Cell-fractions were chromatographed and the radioactive areas on the chromatograms were located by running chromatogram strips through a Nuclear-Chicago Model C 100 B Actigraph II equipped with a gas-flow counter and a Model 1620 B analytical Count Rate Meter and Chart Recorder.

2 Sampling and cell fractionation procedure.

At given times, the reaction was "stopped" by pipetting the reaction mixture (3.0 ml.) into ice-cold centrifuge tubes. As a precaution against loss by breakage, 1.0 ml. of each reaction mixture was withdrawn and frozen immediately at -20°C. Of the remaining 2.0 ml. of the reaction mixture, 0.2 ml. were taken for plating on planchets (0.02 ml./planchet). Counts recorded for such samples represented cells and supernatant. The remaining 1.8 ml. of suspension was centrifuged immediately in the cold, and the supernatant was collected and counted (0.02 ml./planchet). Traces of supernatant remaining on the walls of the centrifuge tube were removed with a paper swab and the cell pellet was resuspended in 1.8 ml. of ice-cold 0.1 N HCl. The cells were extracted with HCl for one hour at 0°C after which the sample was centrifuged in the cold. The supernatant (cold HCl-soluble fraction) was collected and counted (0.02 ml./planchet). The walls of the centrifuge tube were again dried with a paper swab and the extracted pellet was resuspended in 1.8 ml. of 75% ethanol containing HCl at a final concentration of 0.01 N. The sample was incubated for 30 minutes at 45°C and then centrifuged. The supernatant (acid alcohol-soluble fraction) was collected and counted (0.02 ml./planchet). Residual acid ethanol was removed from the centrifuge tube and the pellet was resuspended in 1.8 ml. of 5% (w/v) TCA.

The suspension was extracted for 30 minutes at 90°C and then centrifuged. The supernatant (hot TCA-soluble fraction) was counted (0.02 ml./planchet). The centrifuge tube was again wiped dry and the cell residue was "dissolved" in 1.8 ml. of 0.5 N NaOH for counting (0.02 ml./planchet).

$C^{14}O_2$ determinations were made on the KOH from the centre well of each reaction vessel. Filter paper and KOH were transfered to a 5.0 ml. volumetric flask and the volume was brought up to 5.0 ml. with water rinses from the centre well. The contents of each flask were then thoroughly mixed and transfered to large test tubes. The stoppered tubes were held at -20°C until 0.05 ml. aliquots were counted. Counts on KOH samples were recorded as soon after plating as possible to avoid any CO_2 exchange with atmospheric CO_2. The possibility of such an exchange was further minimized by keeping the plated samples dry.

EXPERIMENTAL RESULTS AND DISCUSSION

I Studies with intact cells

(1) Ability of washed cells to oxidize putrescine.

The results summarized in Table 2 indicate that all four strains of mycobacteria tested were capable of oxidizing putrescine. Washed cells of strain C.C. appeared to be most active in this respect.

Table 2

Oxygen uptake by washed cells acting on 0.01 M putrescine at pH 7.2 at 4.5 hours.

Mycobacterial strain	O_2 uptake (µl) with putrescine	O_2 uptake (µl) with water	O_2 uptake (µl) corrected
H.A.L.	305	190	115
N.T.N.	325	200	125
C.C.	810	220	590
S.C.	330	210	120

(2) Influence of pre-exposure to putrescine on the oxidation of putrescine.

The results of three adaptation studies shown in Figs. 1-3, indicate that each of the three mycobacterial strains tested, oxidize putrescine more rapidly after prior exposure to the compound.

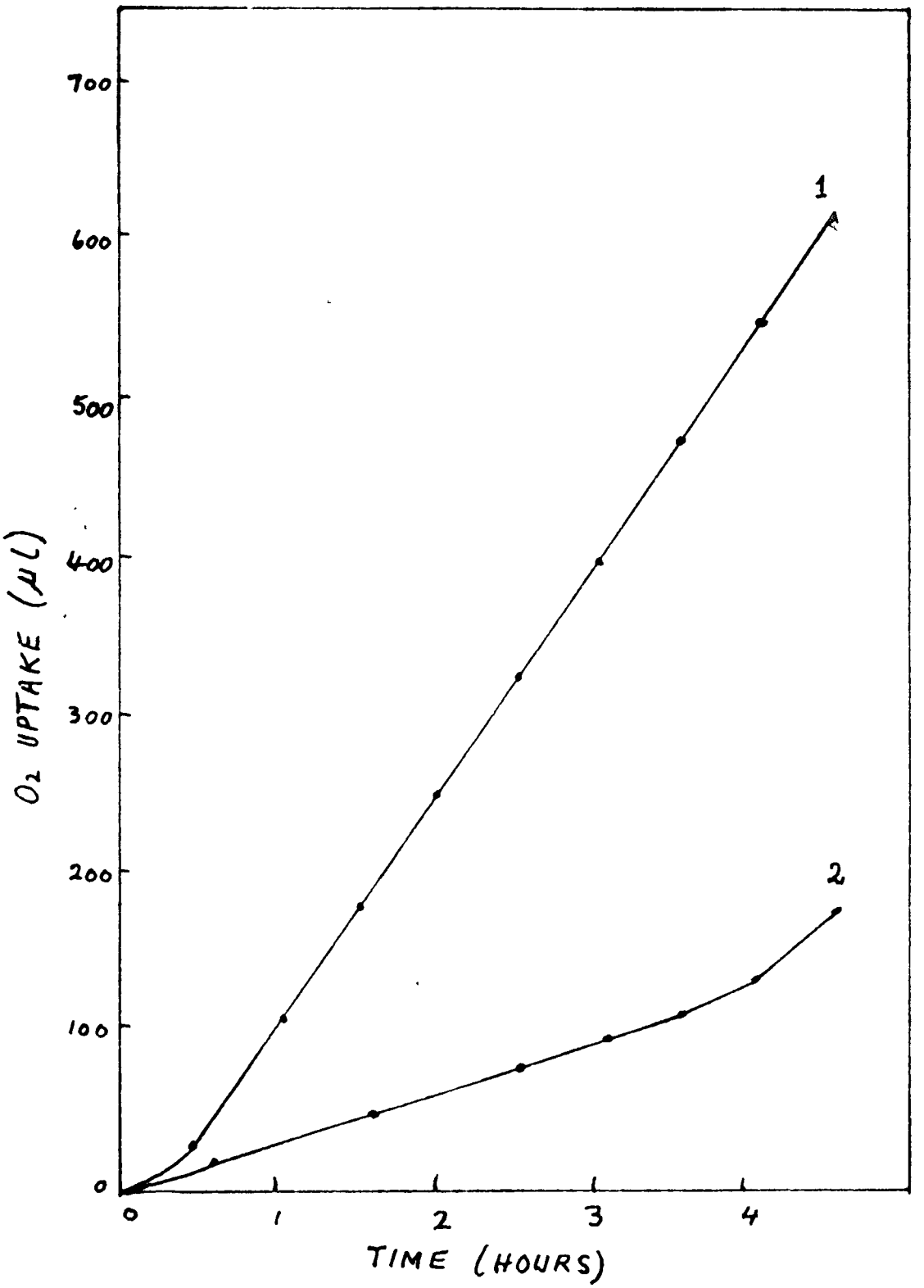


FIG. 1. Oxidation of putrescine by putrescine-adapted (curve 1) and unadapted (curve 2) strain N.T.N. cells.

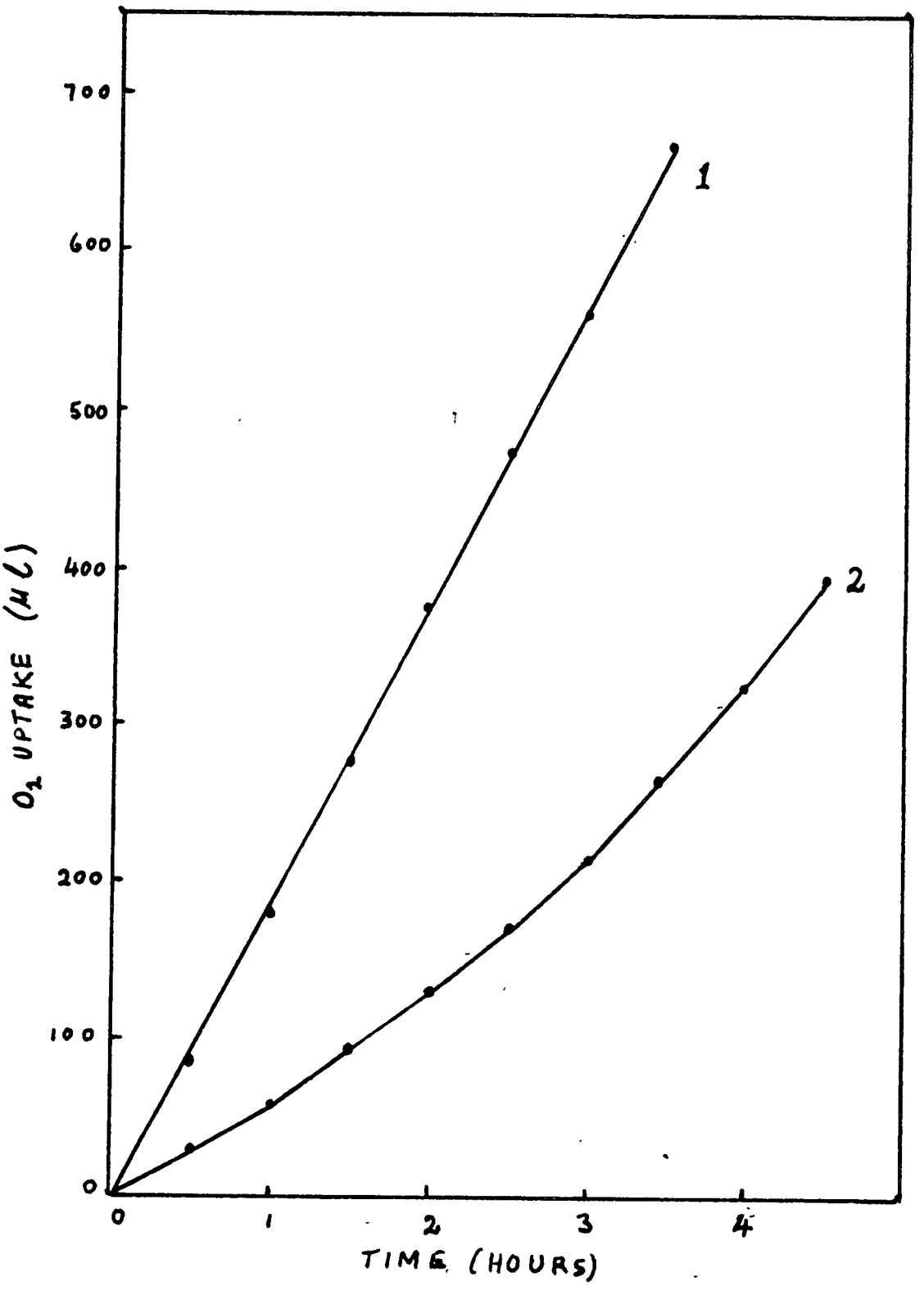


FIG. 2. Oxidation of putrescine by putrescine-adapted (curve 1) and unadapted (curve 2) strain C.C. cells.

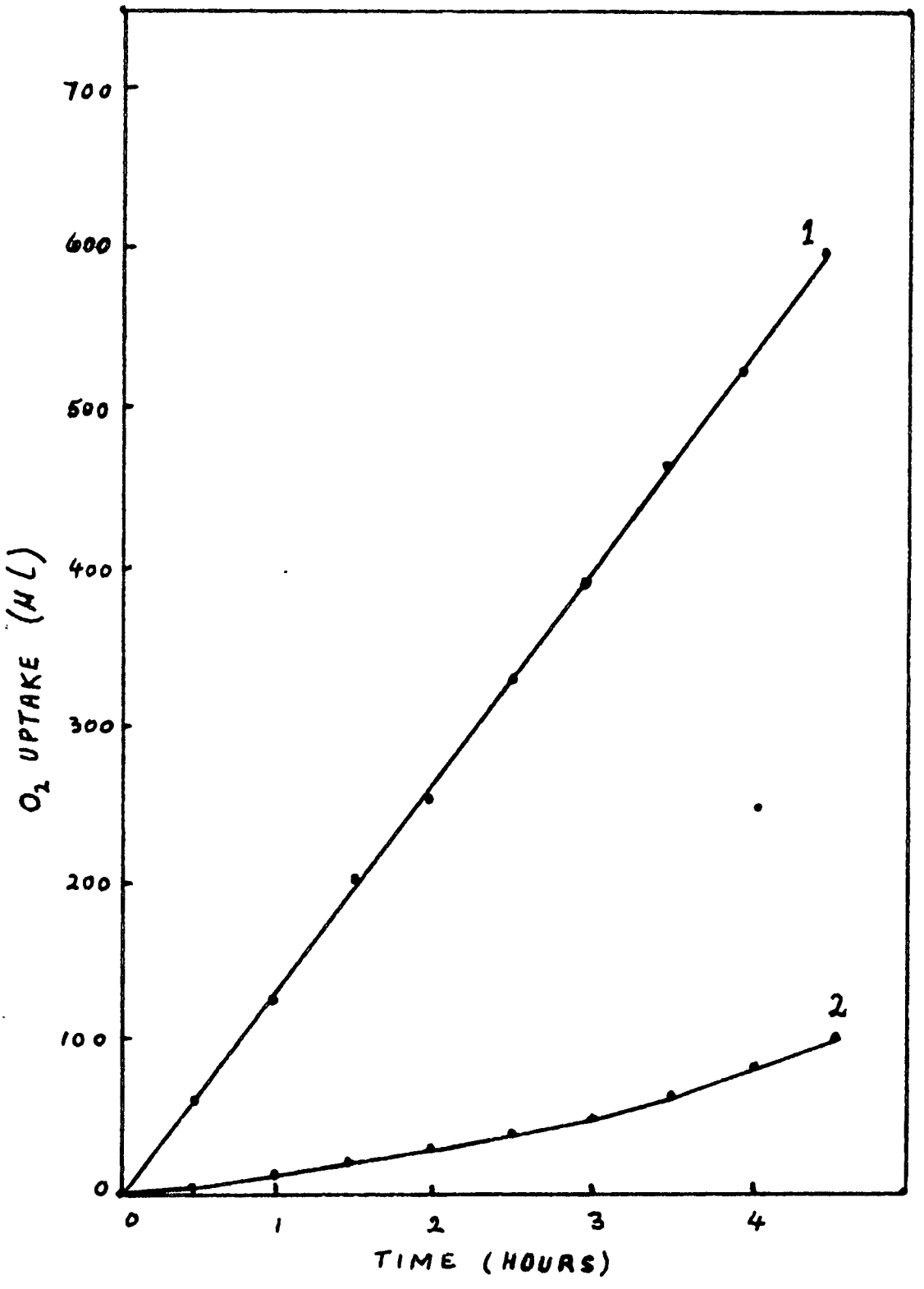


FIG. 3. Oxidation of putrescine by putrescine-adapted (curve 1) and unadapted (curve 2) strain S.C. cells.

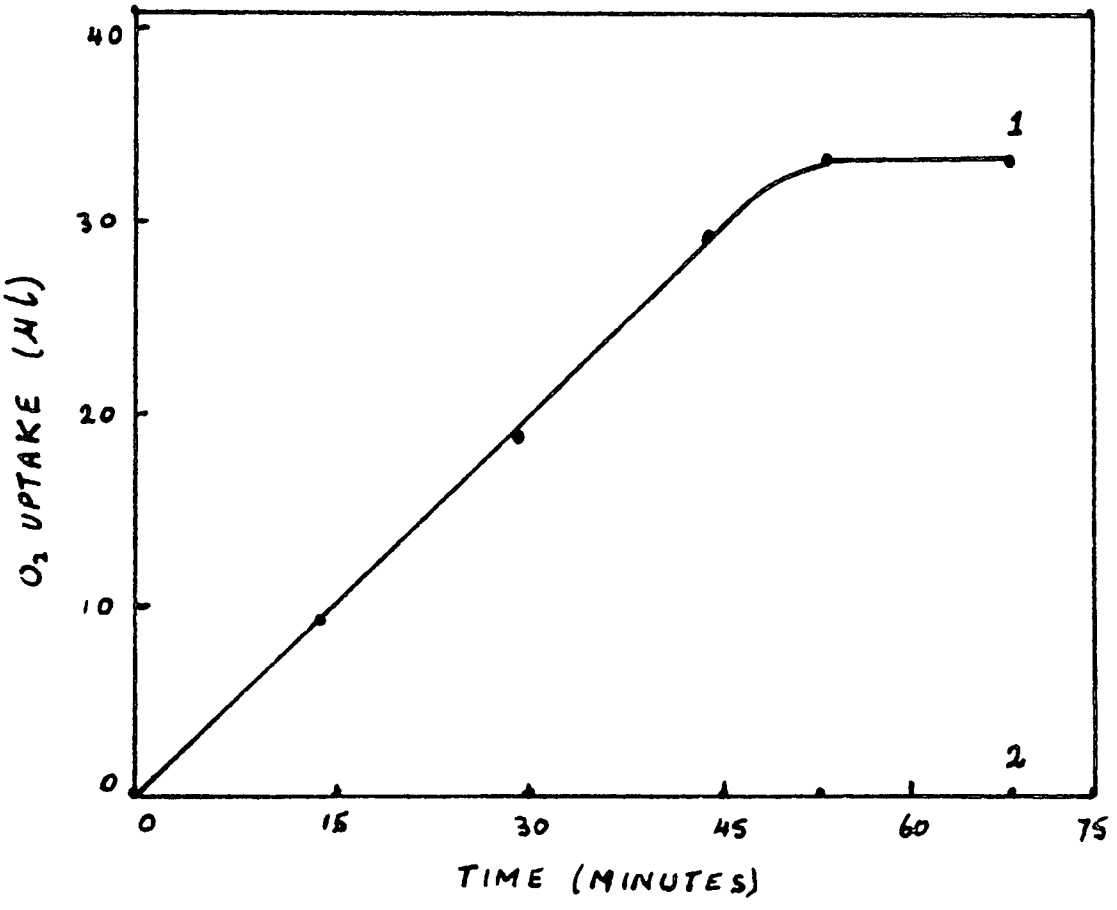


FIG. 4. Diamine oxidase activity in cell-free extracts obtained from putrescine-adapted (curve 1) and unadapted (curve 2) strain S.C. cells. Reaction mixtures contained in a volume of 3.0 ml.: Cell-free extract, 1.0 ml., equivalent to 3.0 mg. protein; putrescine, 3.0 μM; tris buffer, pH 9.0, 100 μM; H_2O.

Strain C.C. cells appear to contain a significant amount of activity for putrescine even without pre-exposure to putrescine. However, adaptation to putrescine caused a three-fold increase in the Q_{O_2} value (µl O_2 consumed/mg. dry weight of cells/hour). The adaptive nature of the putrescine-metabolizing system is most pronounced with strain S.C. cells (Fig. 3). In a similar, but independent experiment with the latter strain, an increase in the Q_{O_2} value of over eleven-fold was obtained after preincubating with putrescine. Because of the remarkable increase in activity obtained after adaptation with this strain, cell-free extracts derived from putrescine-adapted and unadapted cells were assayed for D.O. activity with putrescine as substrate. The curves in Fig. 4 indicate that D.O. activity only occurred in cell-free extracts obtained from putrescine-adapted cells.

If one defines an induced system as one in which the level of activity may be raised by prior exposure to inducing substrate, then it would appear that the putrescine-metabolizing enzymes constitute an induced system in the mycobacterial strains tested. These findings agree with those of other workers for amine-metabolizing systems in a variety of bacteria (9,46,76,87,115).

(3) Ability of washed cells to oxidize aliphatic amines.

In order to determine whether mycobacterial strains possessed the ability to utilize various amines, diamines

closely related to putrescine were tested. Cells of strain C.C. were grown in a basal medium (see Appendix, Table 2) supplemented with either 1,3-diaminopropane or 1,5-diaminopentane and assayed subsequently to determine whether these compounds were oxidized. The results of these experiments are recorded in Table 3.

Table 3

Effect of adaptation to amines on their subsequent oxidation by strain C.C. cells.

Cells grown in basal medium plus:	Oxygen uptake (µl) after 6 hours with 0.005 M			
	1,3-diamino-propane	1,4-diamino-butane	1,5-diamino-pentane	water
No amines	175	667	210	244
1,3-diaminopropane	204	569	182	218
1,5-diaminopentane	161	552	171	206

It was apparent that cells grown in the presence of 1,3-diaminopropane or 1,5-diaminopentane, were not adapted to either of these compounds even though such cells were capable of oxidizing putrescine. Oxidation of putrescine by such cells was not as rapid as that obtained with cells grown in the basal medium alone. In addition, exposure of cells during growth to the 3- and 5-carbon diamines resulted in a slight decrease in the amount of oxygen consumed when

compared with that for endogenously respiring cells. Any adverse effects that these diamines may have caused with growing cells was not evident as the yields of cells from the diamine-containing media were comparable to those obtained from the basal medium. Tests on the cell-free culture media with the DNFB technique (20) indicated that substantial amounts of the added diamines were still present. It would therefore appear that 1,3-diaminopropane and 1,5-diaminopentane were present in the media as relatively inert ingredients. Under similar conditions with putrescine as inducing substrate, the putrescine disappeared completely.

Fig. 5 shows the result of adaptation during growth to spermidine. It was thought that if cells could be adapted to spermidine, a simultaneous adaptation to 1,3-diaminopropane and/or putrescine might be accomplished. The curves in Fig. 5 indicate, however, that such cells fail to oxidize both spermidine and 1,3-diaminopropane. Spermidine in the growth medium did not appear to inhibit growth. An examination of the growth medium after the cells were harvested for study, showed the presence of much residual spermidine. Spermidine "adapted" cells were capable of oxidizing putrescine after a lag period. Equimolar amounts of 1,3-diaminopropane did not affect the rate of putrescine oxidation. In the presence of 7.5 μM of putrescine, the oxygen consumption at the end of the experiment was 425 μl (equivalent to 46% of the maximum theoretical oxygen

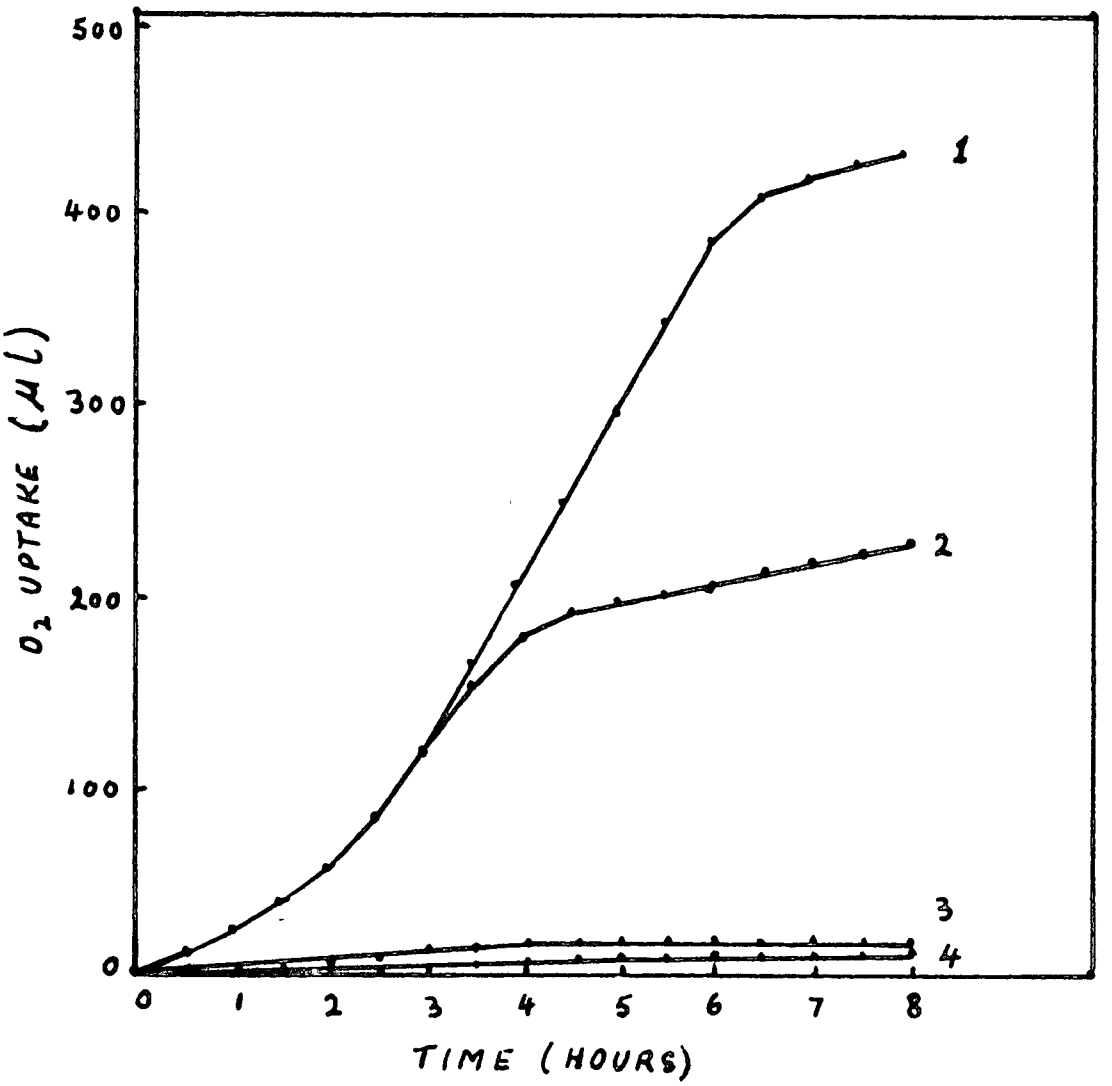


FIG. 5. Effect of pre-exposure to spermidine on the subsequent oxidation by strain C.C. cells of 7.5 μM of putrescine (curve 1), 3.25 μM of each of putrescine and 1,3-diaminopropane (curve 2), 7.5 μM of 1,3-diaminopropane (curve 3), and 7.5 μM of spermidine (curve 4).

consumption*). With 3.25 μM of putrescine in the presence of an equal amount of 1,3-diaminopropane, the oxygen consumption at the end of the experiment amounted to 225 μl (or 48.7% of the maximum theoretical value). This slight increase in the percentage of the maximum possible oxygen uptake was not necessarily entirely due to 1,3-diaminopropane oxidation, but probably also reflected the fact that with twice as much putrescine to be oxidized, the time required to reach a similar level of oxidation would be longer.

Paper chromatography of the reaction mixture supernatants obtained from the Warburg respirometer vessels at the conclusion of the experiment showed that while putrescine had disappeared, 1,3-diaminopropane and spermidine were still present.

These experiments indicate that strain C.C. cells are relatively limited in the range of amines that can be utilized as substrates. Whether inability of such cells to adapt to 1,3-diaminopropane, 1,5-diaminopentane and spermidine reflects a complete lack of the genetic capability to synthesize the system(s) which metabolize the compounds, or whether the cells only lack the ability to synthesize a permease, is not known. As will be shown later, cell-free extracts obtained from putrescine-adapted cells, oxidize 1,5-diaminopentane at a significant rate. Since unadapted

* Based on a value of 5.5 μM O_2/μM putrescine.

strain C.C. cells possess some ability to oxidize putrescine, a certain amount of activity could have been expected when such cells were incubated with 1,5-diaminopentane. The failure to demonstrate such activity with the latter compound could indicate that the cells are not permeable to 1,5-diaminopentane, or that intermediates derived from it are strongly inhibitory to oxidative processes occurring within the cells.

(4) Amines as sole carbon sources.

Various amines were tested individually for their ability to support the growth of three mycobacterial strains when present as the sole carbon source. The results of this study are recorded in Table 4.

Table 4

Amines as sole carbon sources for growth*.

	Mycobacterial strain		
Carbon source	N.T.N.	C.C.	S.C.
1,3-diaminopropane	-	-	-
1,4-diaminobutane (putrescine)	+	+	+
1,5-diaminopentane (cadaverine)	-	-	-
1-aminobutane	-	-	-
Pyrrolidine	-	-	-
Piperidine	-	-	-
Spermidine	-	-	-
Spermine	-	-	-
Glucose	+	+	+

* + = Growth within 17 days; - = No growth within 17 days.

Good growth was obtained for each strain tested with glucose and with putrescine within 5-8 days. The experiment was concluded after 17 days, at which time none of the other amines had produced any detectable growth.

Among the mycobacterial strains tested, the ability to utilize amines appeared to be restricted to putrescine. The fact that putrescine was the only amine attacked supports the findings of Zeller et al (118) who reported this compound to be one of the better bacterial diamine substrates. Razin et al (76) also found that putrescine was the most frequently utilized amine among the naturally occurring amines tested with various bacteria.

Among the mycobacteria, the ability to oxidize amines varies considerably. Putrescine and histamine were reported not to be attacked by Myc. tuberculosis, M. rubrum, M. smegmatis and M. phlei (26). The latter organism was also found not to oxidize spermidine (76). On the other hand, strains of M. smegmatis studied by Roulet and Zeller (60) and Bachrach et al (9) were found to oxidize histamine, putrescine, cadaverine, agmatine, spermidine and spermine. Avian tubercle bacilli (4,73) and M. tuberculosis var. hominis (73) oxidized putrescine while M. lacticola attacked both putrescine and histamine (26).

(5) Occurrence of amines in mycobacteria.

Fig. 6 shows the elution patterns obtained for

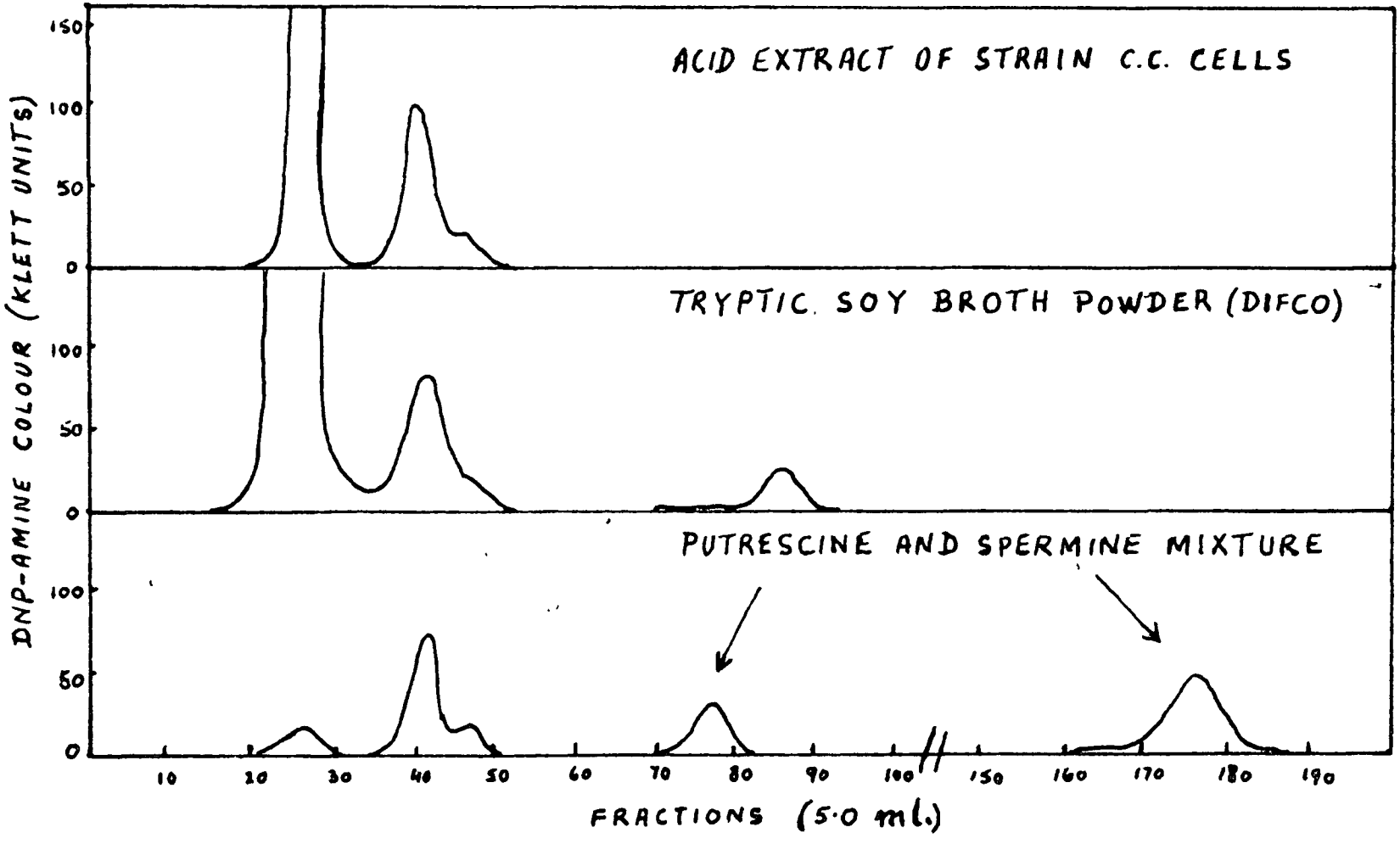


FIG. 6. SEPARATION OF AMINES BY ION EXCHANGE. SAMPLES WERE ELUTED FROM A COLUMN OF DOWEX 50 H^+ RESIN WITH A LINEARLY INCREASING CONCENTRATION OF HCl (0 – 1·5 N).

nitrogenous compounds contained in steam-distillates obtained from strain C.C. cell extracts, tryptic soy broth powder and a synthetic mixture of putrescine and spermine.

The first peaks (fractions 20 to 30) represent ammonia. Both nesslerization and the DNFB technique gave corresponding peaks. In the case of tryptic soy broth, ammonia (as the hydrochloride salt) accounted for over 99.9% of the weight of the dried salts obtained from the steam-distillate.

Fractions 35-50 contained relatively large amounts of pyrrolidine and trace quantities of piperidine and 1-aminobutane. Pyrrolidine gave a yellow colour initially with ninhydrin on paper chromatograms. Later the colour turned brown. The Rf value in the solvent n-butanol:acetic acid: water (2:1:1 v/v), was 0.69. These properties were shared by commercially obtained pyrrolidine which was used as a standard. Piperidine and 1-aminobutane had Rf values of 0.74 and 0.72 respectively, in the same solvent. The commercially available compounds migrated with identical Rf values. The colour reactions with ninhydrin were also characteristic. Piperidine gave lilac-purple spots while 1-aminobutane yielded mauve-red spots. The coloured spots due to these compounds tended to fade gradually while colour with pyrrolidine was more permanent. Pyrrolidine, piperidine and 1-aminobutane were not readily detectable on paper chromatograms when sprayed with alkaline-DNFB solution, indicating that these compounds were relatively

volatile under alkaline conditions. These compounds also formed derivatives with DNFB that were soluble in aqueous acid-dioxane.

While it was reasonable to expect that pyrrolidine and 1-aminobutane might occur in cell-extracts and tryptic soy broth powder, possibly as decomposition products of compounds containing related structures, it was difficult to conceive of a mechanism by which a 5-carbon structure like piperidine could be derived from compounds like putrescine and spermine which constituted the synthetic amine mixture. It was therefore concluded that the presence of such compounds was due to contamination (possibly from the washed corks used in the steam distillation apparatus).

Tryptic soy broth powder contained an amine, the DNP-derivative of which was only very slightly soluble in aqueous acid-dioxane. Such derivatives are typical of those of di- and polyamines. The amine was collected in fractions 81-89. Commercial putrescine was eluted in fractions 72-82 but slight differences in the concentration gradient of the eluting fluid may have accounted for this. The amine had an Rf of 0.23 in common with putrescine in the n-butanol: acetic acid:water solvent. In the solvent n-propanol: 12 N HCl:water (3:1:1 v/v), the amine migrated like putrescine (Rf 0.34). Unfortunately, not enough sample was obtained for enzymatic assay with D.O. prepared from strain C.C. putrescine-adapted cells.

Acid extracts of strain C.C. cells did not contain any di- or polyamines. Herbst et al (37) reported similar findings with Myc. phlei and Myc. smegmatis. If, therefore, di- and polyamines occur in mycobacterial cells, they must be present in extremely small quantities.

(6) Influence of putrescine on endogenous respiration.

In order to determine whether putrescine affected the rate of endogenous respiration in mycobacterial cells, two variations of the indirect manometric technique were employed.

In the first method, a single concentration of strain S.C. or N.T.N. cells was allowed to oxidize various concentrations of putrescine. If putrescine had a significant effect on the rate of endogenous respiration, then the effect should be reflected in the degree of substrate oxidation when the latter value was based on the oxygen uptake corrected for endogenous oxygen consumption. The results of such studies are recorded in Table 5.

The data indicate that if endogenous oxygen uptake is neglected, with strain S.C. cells, there is a 33.5% variation in the degree of putrescine oxidation depending on the concentration of the substrate. With N.T.N. cells, a variation of 37% was obtained. The latter strain also consumed more oxygen than was theoretically possible with 1.5 µM of putrescine. When, however, the degree of substrate oxidation was based on the oxygen uptake corrected

Table 5

Effect of putrescine on endogenous respiration of strain S.C. and N.T.N. cells: one concentration of cells acting on various concentrations of putrescine.

Mycobacterial strain	Putrescine (μM/cup)	Total uptake (μl O_2)	Maximum theoretical uptake* (μl O_2)	% theoretical uptake	corrected uptake (μl O_2)	% theoretical corrected uptake
S.C.	1.5	172	184.8	93.5	87	47.1
	3.0	262	369.6	71.0	177	47.9
	6.0	443	739.2	60.0	358	48.5
N.T.N.	1.5	219	184.8	118.7	88	47.6
	3.0	302	369.6	81.7	171	46.4

* Based on 5.5 μM O_2/μM putrescine.

for endogenous oxygen consumption, fairly constant values for the degree of substrate oxidatiom were obtained. The data strongly suggest that putrescine, at the concentrations employed, had little or no effect on the rate of endogenous respiration.

In the second method, strain S.C. cells were used at three concentrations with substrate (putrescine) at a single concentration. In this technique, the level of endogenous respiration should vary with the cell concentration. If the endogenous respiration was not affected by putrescine, the total oxygen consumed with a constant amount of substrate

should be the same when the latter values were corrected for their respective endogenous oxygen uptake values. The results are summarized in Table 6.

Table 6

Effect of putrescine on endogenous respiration of strain S.C. cells: three concentrations of cells acting on one concentration of putrescine.

Cell concentration	Endogenous O_2 uptake (µl)	Putrescine 3 µM O_2 uptake (µl)	% maximum* theoretical uptake	corrected uptake (µl)	% maximum theoretical uptake
1 X	70	231	62.5	161	43.6
2 X	127	290	78.5	163	44.2
3 X	186	353	95.5	167	45.3

* Based on 5.5 µM O_2/µM putrescine or 396.6 µl O_2/3.0 µM putrescine.

The results in Table 6 indicate that a variation of 33% in the degree of substrate oxidation occurs if the endogenous respiration is not taken into account. If, however, the endogenous respiration is assumed to continue unabated in the presence of putrescine, and the oxygen uptake due to putrescine oxidation is calculated by subtracting the endogenous oxygen consumption from the total oxygen uptake, the degree of substrate oxidation appears to be relatively constant for all cell concentrations. Stated

in another way, the total oxygen uptake in the presence of a single concentration of substrate, increases proportionately with an increase in endogenous respiration. The data in Tables 5 and 6 are consistent with the fact that endogenous respiration continues normally in the presence of putrescine. In reporting oxygen uptake, it therefore appears justifiable to report oxygen uptake values corrected for endogenous respiration. Similar techniques have been used to determine the status of endogenous respiration in the presence of substrate (71) although Blumenthal (12) has pointed out that the premise on which the manometric techniques are based, requires further testing.

(7) Oxygen, ammonia and carbon dioxide relationships during putrescine oxidation.

Data showing in parallel the consumption of oxygen and the production of ammonia by N.T.N. cells during the oxidation of 3.0 μM putrescine are illustrated in Fig. 7. Oxygen consumption was rapid initially. The break in the oxygen uptake curve occurred at 160 μl O_2 (equivalent to 2.38 μM O_2/μM putrescine or to 43.3% of the maximum theoretical level of oxidation). At the end of the experiment, the oxygen consumption had proceeded to 44.2% of the maximum degree of oxidation. At this point, the curve for ammonia which had already levelled off, showed the reaction mixture supernatant to contain 1.52 μM NH_3/ml. Since the

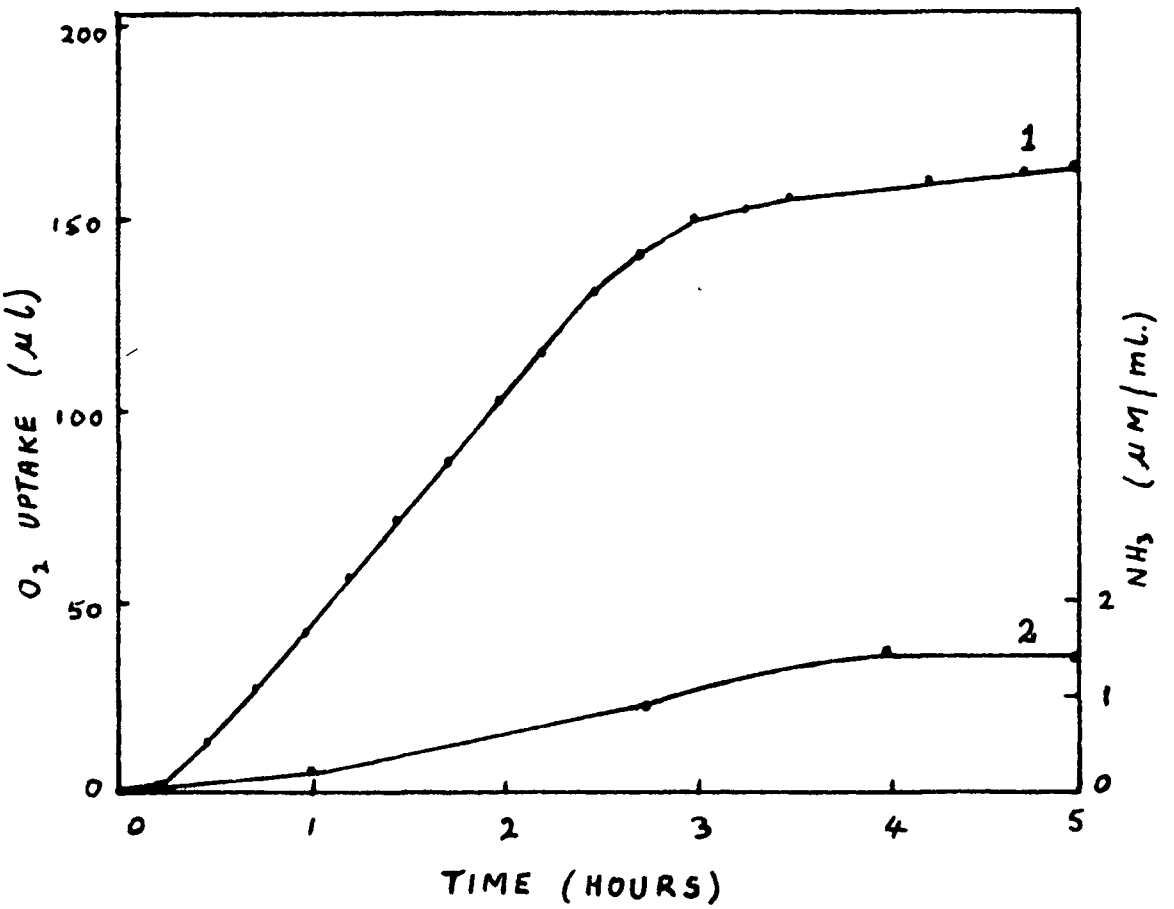


FIG. 7. Oxygen consumption and ammonia production during putrescine oxidation by strain N.T.N. cells. Curve 1: O_2 uptake with 3.0 µM putrescine (1.0 µM/ml.). Curve 2: NH_3 production with putrescine (1.0 µM/ml.).

maximum possible concentration of ammonia was 2.0 μM NH_3/ml., than at this stage of the reaction, 76% of the ammonia had been released from the putrescine molecule.

Ammonia production was not influenced by the presence or absence of carbon dioxide in the reaction mixtures. During the oxidation of putrescine by N.T.N. cells in the presence of atmospheric carbon dioxide, 1.52 μM NH_3/ml. of reaction mixture were produced. The corresponding value for ammonia production under CO_2-free conditions was 1.50 μM NH_3/ml. At the end of the 5-hour experiment, ammonia in the endogenous flasks amounted to 0.30 μM NH_3/ml. of reaction mixture. However, since zero time ammonia determinations were not made, endogenous ammonia production was probably somewhat less than indicated above.

In an independent study to determine carbon dioxide production by N.T.N. cells, the cells were allowed to oxidize 3.0 μM of putrescine. At the break in the oxygen uptake curve (160 μl O_2 or 43.3% of the maximum level of oxidation), the carbon dioxide production amounted to 1.06 μM CO_2/μM putrescine. At a point further along the oxygen uptake curve corresponding to 46.2% oxidation, the CO_2 output was 1.14 μM CO_2/μM putrescine. Thus, as expected, there is a sharp decrease in the rate of carbon dioxide production after the break in the oxygen uptake curve. During the rapid oxygen uptake portion of the curve, the respiratory quotient (R.Q.) was 0.445. However, in reality the R.Q. may be somewhat

higher (at least 0.60) since radioactive studies conducted subsequently with 1,4-C^{14} putrescine showed that under CO_2-free conditions, approximately 72% of the terminal carbons of putrescine were evolved as carbon dioxide at the break region of the oxygen uptake curve. During studies on the oxidation of putrescine by Myc. smegmatis, Roulet and Zeller (60) determined carbon dioxide by the indirect manometric method. Since these workers did not know to what extent the presence and absence of carbon dioxide in their reaction systems might have influenced the course of the oxidation, these workers were concerned about the validity of the carbon dioxide values they obtained. They observed, however, that values for ammonia obtained from the reaction mixtures, with and without carbon dioxide, were identical and therefore inferred that differences in the carbon dioxide pressure in their reaction systems had not influenced carbon dioxide production. In the light of the present results, however, such an assumption may have been invalid. Thus under CO_2-free conditions, more carbon dioxide is actually evolved than is indicated by the indirect manometric method, even though ammonia production in the reaction flasks, with and without carbon dioxide, may be identical.

Fig. 8 shows the oxygen uptake curve obtained with strain S.C. cells acting on 6.0 µM of putrescine together with ammonia production determined in parallel. The oxygen uptake curve broke at 305 µl O_2 (2.27 µM O_2/µM putrescine

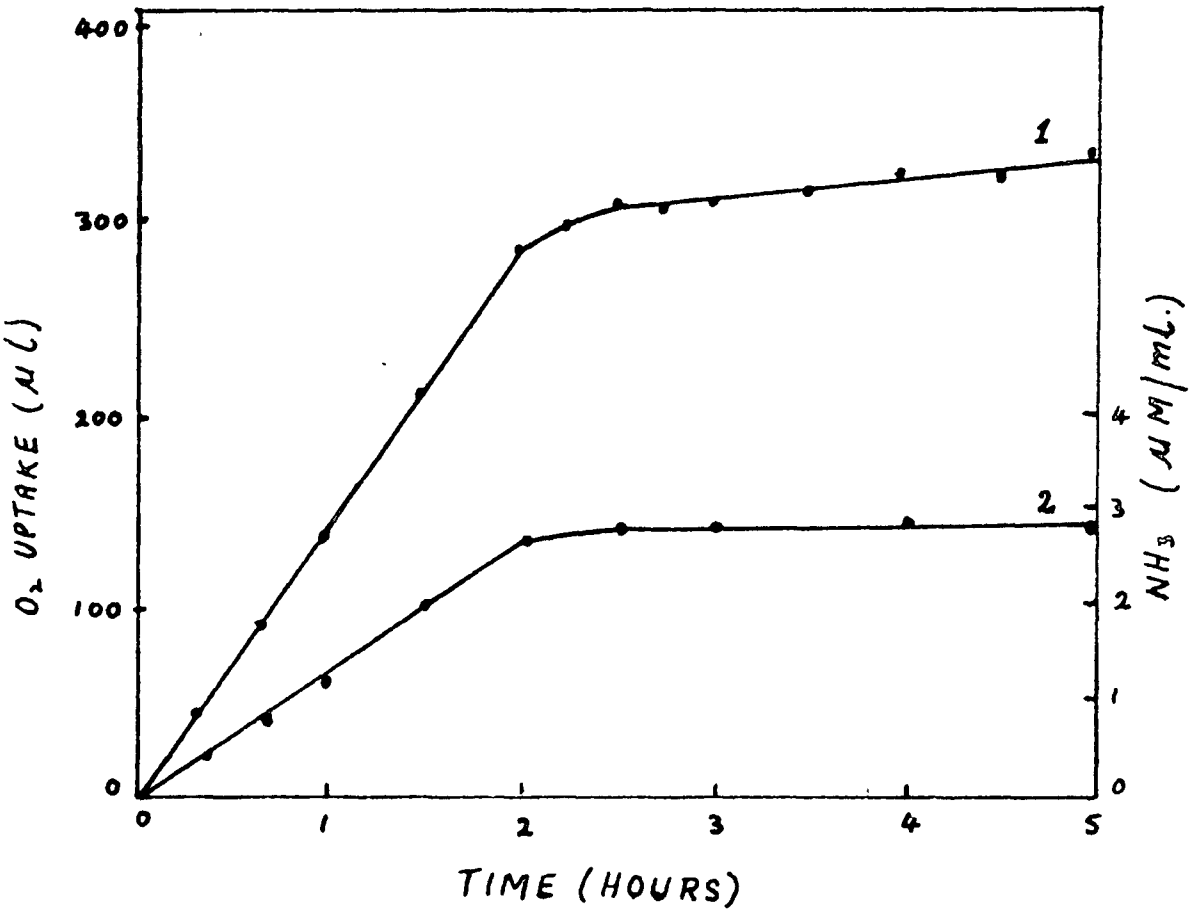


FIG. 8. Oxygen consumption and ammonia production during putrescine oxidation by strain S.C. cells.
Curve 1: O_2 uptake with 6.0 µM putrescine (2.0 µM/ml.).
Curve 2: NH_3 production with putrescine (2.0 µM/ml.).

or at 41.3% of the maximum level of oxidation). At the conclusion of the experiment, the net oxygen consumption amounted to 2.48 μM O_2/μM putrescine (or 45.1% of the maximum degree of oxidation). The break in the ammonia output curve coincided with that of the oxygen uptake curve and remained essentially at the same level thereafter. At the end of the experiment, a total of 2.84 μM NH_3/ml. were produced out of a possible 4.0 μM NH_3/ml. of reaction mixture. This meant that 1.42 μM NH_3/μM putrescine (or 71% of the ammonia) had been released from the putrescine molecule. At the end of the 5-hour experiment, ammonia in the control flasks amounted to 0.37 μM/ml. of reaction mixture.

Carbon dioxide production with strain S.C. cells was determined in an independent experiment. At a point after the break in the oxygen uptake curve corresponding to 47.2% of the maximum level of oxidation, carbon dioxide production amounted to 1.14 μM CO_2/μM putrescine.

With this organism, the presence or absence of carbon dioxide had no effect on the amount of ammonia produced since 69% of the theoretical maximum amount of ammonia was produced in each of the Warburg reaction vessels used to determine carbon dioxide production by the indirect manometric method.

In other independent studies with this mycobacterial strain, the break in the oxygen consumption curve occurred

at 2.4 μM O_2/μM putrescine, and the percentage of the maximum ammonia production ranged from 69 to 76%.

Fig. 9 shows the oxygen uptake curve obtained for strain C.C. cells acting on 6.0 μM of putrescine. By extrapolation, the curve appeared to break at 305 μl O_2 (equivalent to 2.27 μM O_2/μM putrescine or to 41.3% of the theoretical maximum oxidation level). This strain seldom gave sharp breaks in the oxygen uptake curves and consequently determinations of the break-points were more difficult. Oxygen consumption continued after the break until at the end of the experiment 342 μl O_2 were consumed. This corresponded to 2.54 μM O_2/μM putrescine or to 46.3% of the maximum level of oxidation. At this point, a total of 1.26 μM of CO_2 were produced per μM of putrescine.

Parallel ammonia determinations were not conducted during this experiment but at the end of the reaction, of a possible 4.0 μM NH_3/ml. of reaction mixture, 2.75 μM NH_3/ml., or 69% of the maximum possible ammonia were actually liberated. With this strain, the ammonia curve broke in the region of the oxygen uptake curve break, after which the level of ammonia remained relatively constant. An example of an ammonia curve for strain C.C. is shown in the next section (Fig. 12) where the effect of arsenite on ammonia production was studied. At the end of the experiment, ammonia in the endogenous flasks amounted to 0.30 μM/ml. of reaction mixture.

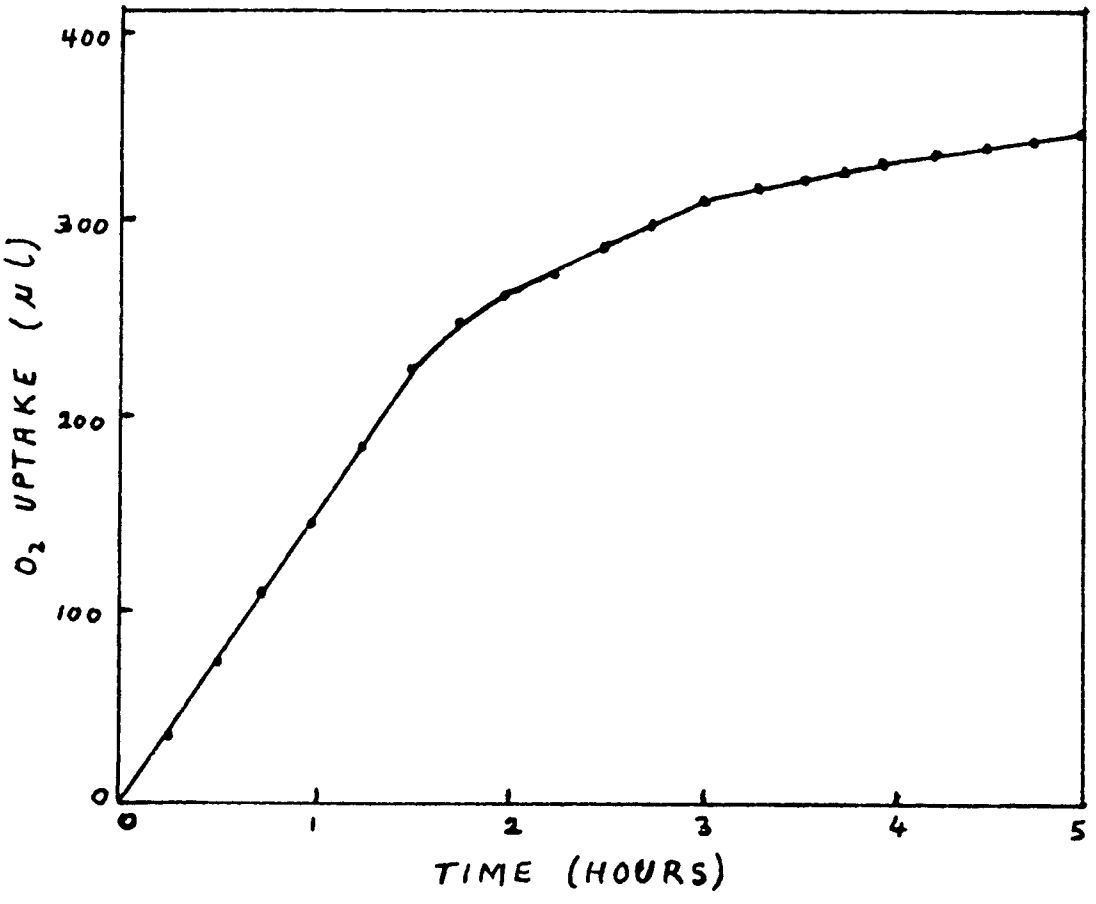


FIG. 9. Oxygen consumption during the oxidation of 6.0 μM of putrescine by strain C.C. cells.

An examination of the reaction mixture supernatants by paper chromatography failed to reveal the presence of any organic acids or nitrogenous compounds. Such examinations were made at various times during the course of the reaction with each strain.

To summarize, all three mycobacterial strains oxidized putrescine after adaptation to the compound. The break in the oxygen uptake curves occurred at points ranging from 2.27 to 2.40 μM O_2/μM putrescine. Thereafter oxygen consumption proceeded more slowly until at the conclusion of the experiments the level of the maximum theoretical oxidation had reached 43.3 to 47.2%. During the experiments, only two products appeared in the supernatants: NH_3 and CO_2. Ammonia production reached a maximum level and remained at this level corresponding to 69-76% of the theoretical maximum for ammonia output. Presumably, the rest of the ammonia was assimilated by the cells since no other nitrogenous compounds were detectable in the supernatants. All putrescine-carbon not assimilated by the cells appeared to be accounted for as carbon dioxide. Cells at the conclusion of the experiments appeared to have assimilated somewhat over 2.0 μM of carbon out of a possible 4.0 μM of carbon.

The finding that part of the putrescine molecule is assimilated, is in agreement with growth studies with putrescine as a sole source of carbon. Since the cells also

assimilated 24%, or more, of the nitrogen, it is possible that putrescine could serve as both a carbon and nitrogen source. This is reported to be the case with a strain of Ps. fluorescens (46). The incomplete oxidation of putrescine by Myc. smegmatis (60) is in accord with the results obtained in this study. During the oxidation of putrescine by Myc. smegmatis, the compound was 65% oxidized. Carbon dioxide and ammonia production accounted for 39% and 72%, respectively, of the putrescine molecule (60). Putrescine and related polyamines were also found to be incompletely oxidized by other bacteria tested (76). However, while the incomplete oxidation of amines may be the general rule, Ps. pyocyanea was shown to oxidize putrescine to completion (29).

(8) Inhibitor studies with intact cells.

Table 7 summarizes the results obtained from studies with strain C.C. cells acting on putrescine in the presence of various inhibitors. The data represent oxygen consumption after the oxygen uptake curves had levelled off.

Malonic acid and p-phenylenediamine did not exert any significant effects on putrescine oxidation or on endogenous respiration. This was possibly due to the fact that the cells were not readily permeable to the compounds. These agents have been reported to inhibit succinic dehydrogenase activity (88) and were used in an attempt to demonstrate that succinic acid was produced as an intermediate during putrescine degradation.

Table 7

The effect of various inhibitors on the oxygen consumption by strain S.C. cells acting on 3.0 μM of putrescine at 4 hours.

Treatment* Cells plus:	O_2 uptake (μl)	Net O_2 uptake (μl)
PUT	490	188
H_2O	302	-
PUT + MAL (0.005 M)	501	185
H_2O + MAL (0.005 M)	316	-
PUT + PPD (0.0045 M)	493	193
H_2O + PPD (0.0045 M)	300	-
PUT + Azide (0.003 M)	560	160
H_2O + Azide (0.003 M)	400	-
PUT + Arsenite (0.003 M)	315	123
H_2O + Arsenite (0.003 M)	192	-

* PUT = putrescine; MAL = malonic acid; PPD = p-phenylenediamine.

Sodium azide stimulated endogenous oxygen consumption. A similar effect on the oxygen uptake in the presence of putrescine was also observed. The net effect of azide, was to prevent the slow increase in oxygen consumption that normally occurred after the break in the oxygen uptake curve. Thus,

after this point, the oxygen uptake remained at a level corresponding to 160 µl O_2.

Of the inhibitors tested, sodium arsenite exerted the most pronounced effects on the net oxygen consumption. Both endogenous respiration, and oxygen consumption due to putrescine, were inhibited.

Reaction mixture supernatants from each of the inhibitor studies with the C.C. strain were examined for the presence of carbonyl compounds. It was found that substances reacting with 2,4-dinitrophenylhydrazine (DPNH) only accumulated when arsenite was present. Endogenously respiring cells poisoned with arsenite, excreted α-ketoglutaric acid (0.13 µM/ml. of reaction mixture) in addition to traces of pyruvate. Under similar conditions, but with putrescine as substrate, only traces of α-ketoglutarate accumulated. Under the latter conditions, pyruvic acid was the dominant keto acid released by the cells (0.18 µM/ml.). Such results suggested that strain C.C. cells contained a relatively arsenite-insensitive system which utilized α-ketoglutaric acid when putrescine, or an intermediate of putrescine degradation, was present. Cell-free extracts contained a transaminase enzyme for which α-ketoglutaric acid and γ-aminobutyric acid were substrates. Since the latter compound was also shown to be an intermediate in the dissimilation of putrescine, it is probable that the conversion of α-ketoglutaric acid to glutamic acid by this system explained the observed facts.

The increase in the concentration of excreted pyruvate observed when putrescine was present as substrate, indicated that at least a portion of the putrescine molecule was metabolized via pyruvic acid.

Table 8 records the results of inhibitor studies with strain S.C. cells acting on putrescine. The data represent the oxygen uptake after 4 hours at which time the break in the oxygen uptake curves had already occurred.

Dihydrostreptomycin and isonicotinic acid hydrazide exerted no significant effects on the net oxygen consumption due to putrescine oxidation. The former may have inhibited the net oxygen uptake slightly while the latter appeared to produce a small increase in the net oxygen consumption. Neither of the reaction mixtures treated with these anti-mycobacterial agents contained any compounds reacting with DPNH.

The effect of arsenite at 0.003 M on the net oxygen uptake by S.C. cells respiring endogenously, and in the presence of putrescine, was not very marked. However, an examination of the reaction mixtures obtained at the conclusion of the experiment indicated that endogenously respiring cells, poisoned with 0.003 M arsenite, had excreted 0.16 μM of pyruvate/ml. The corresponding value for cells with putrescine as substrate, was 0.33 μM of pyruvate/ml. At this concentration of inhibitor, no leakage of α-ketoglutarate occurred. Arsenite at 0.009 M exerted a

Table 8

The effect of various inhibitors on the oxygen consumption by strain S.C. cells acting on 3.0 µM of putrescine.

Treatment* Cells plus:	O_2 uptake (µl)	Net O_2 uptake (µl)
PUT	337	171
H_2O	166	-
PUT + DHS (0.001 M)	324	157
H_2O + DHS (0.001 M)	167	-
PUT + DHS (0.006 M)	322	164
H_2O + DHS (0.006 M)	158	-
PUT + INH (0.001 M)	355	184
H_2O + INH (0.001 M)	171	-
PUT + INH (0.006 M)	354	193
H_2O + INH (0.006 M)	161	-
PUT + Arsenite (0.003 M)	264	160
H_2O + Arsenite (0.003 M)	104	-
PUT + Arsenite (0.009 M)	228	143
H_2O + Arsenite (0.009 M)	85	-

* DHS = dihydrostreptomycin;
INH = isonicotinic acid hydrazide.

more pronounced effect on the net amount of the oxygen consumed but there was a concurrent reduction in the quantity of DPNH-reacting materials excreted into the reaction mixtures. Table 9 expresses the concentrations of the coloured DPNH-reacting substances found in the reaction mixture supernatants in terms of Klett units (Filter #54).

Table 9

The effect of arsenite on strain S.C. cells: accumulation of DPNH-reacting materials.

Treatment Cells plus:	Colour due to DPNH derivatives in 1.0 ml. of reaction mixture at 4 hours (Klett units)
PUT*	14
H_2O	13
PUT + Arsenite (0.003 M)	195
H_2O + Arsenite (0.003 M)	85
PUT + Arsenite (0.009 M)	68
H_2O + Arsenite (0.009 M)	56
Blank	15

* PUT - putrescine (0.001 M).

Endogenously respiring cells treated with arsenite at 0.009 M produced both pyruvate and α-ketoglutarate in approximately equal amounts as judged by the density of the

spots obtained on paper chromatograms. The same was true for cells incubated with putrescine and arsenite at 0.009 M. It therefore appeared that by increasing the concentration of inhibitor from 0.003 M to 0.009 M, not only was there a decrease in the amount of the excreted keto acids, but also there must have been an accumulation of some other oxidizable material, the loss of which from the cell might account for the reduction in the net oxygen uptake. The fact that putrescine did not prevent the accumulation of α-ketoglutarate, suggested that under the conditions of higher levels of arsenite, the system that utilized α-ketoglutarate was now being inhibited. Concurrent studies demonstrated that arsenite decreased ammonia output (Figs. 11 and 12). It therefore appeared likely that the suspected leakage material might be nitrogenous. As will be shown later, several amino acids were found to be excreted by cells poisoned with arsenite.

Fig. 10 shows the oxygen consumption by arsenite-poisoned strain S.C. cells acting on putrescine in comparison with that for unpoisoned cells. The coincident accumulation of carbonyl compounds in the reaction mixture supernatant is also illustrated. Endogenously respiring cells (1.0 mg. dry weight/Warburg vessel), poisoned with 0.003 M arsenite, excreted traces of α-ketoglutaric acid along with measurable amounts of pyruvic acid (0.08 μM/ml. of reaction mixture). When putrescine was present as substrate, similarly treated cells excreted only pyruvic acid (0.33 μM/ml. of reaction

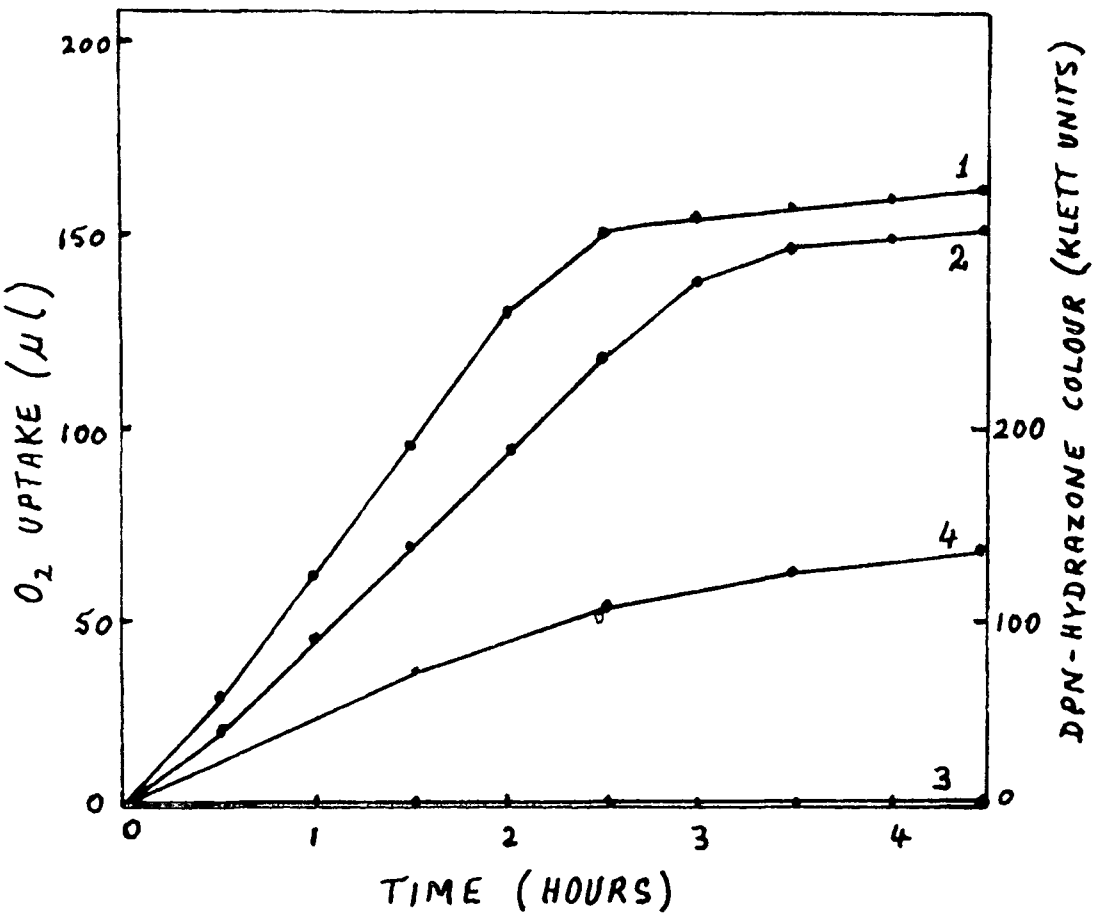


FIG. 10. Effect of arsenite on the metabolism of putrescine by strain S.C. cells.
Curve 1: O_2 uptake with 3.0 μM putrescine (1.0 μM/ml.).
Curve 2: Same as curve 1, but arsenite (3.0 μM/ml.) present.
Curve 3: Leakage of pyruvate with putrescine (1.0 μM/ml.).
Curve 4: Same as curve 3, but arsenite (3.0 μM/ml.) present.

mixture). Curves 3 and 4 in Fig. 10 represent hydrazone colour with added substrate measured in Klett units (Filter #54) corrected for hydrazone colour due to endogenously produced pyruvate. At the end of the experiment, the net amount of pyruvate excreted amounted to 0.25 μM/ml. of reaction mixture. Unpoisoned cells did not excrete carbonyl compounds.

Fig. 11 shows the effect of 0.009 M arsenite on the oxygen uptake and ammonia production by strain S.C. cells acting on 3.0 μM of putrescine. Both oxygen consumption and ammonia output were decreased. Similar effects on the oxygen consumption and ammonia production are recorded in Fig. 12 for the oxidation of putrescine by strain C.C. cells in the presence of arsenite. With strain S.C. cells, arsenite reduced the ammonia output from 71.1% of a possible maximum for unpoisoned cells, to 53.1% with poisoned cells. The corresponding values for poisoned and unpoisoned strain C.C. cells were 68.7% and 50.3% respectively. Oxygen uptake and ammonia output were plotted as the total oxygen uptake or ammonia output observed with putrescine and arsenite, corrected for the corresponding endogenous values obtained for cells incubated with arsenite.

The data recorded in Figs. 11 and 12 were obtained by using high enough levels of arsenite to cause the accumulation of only α-ketoglutarate in reaction mixtures containing endogenously respiring cells. With putrescine as substrate

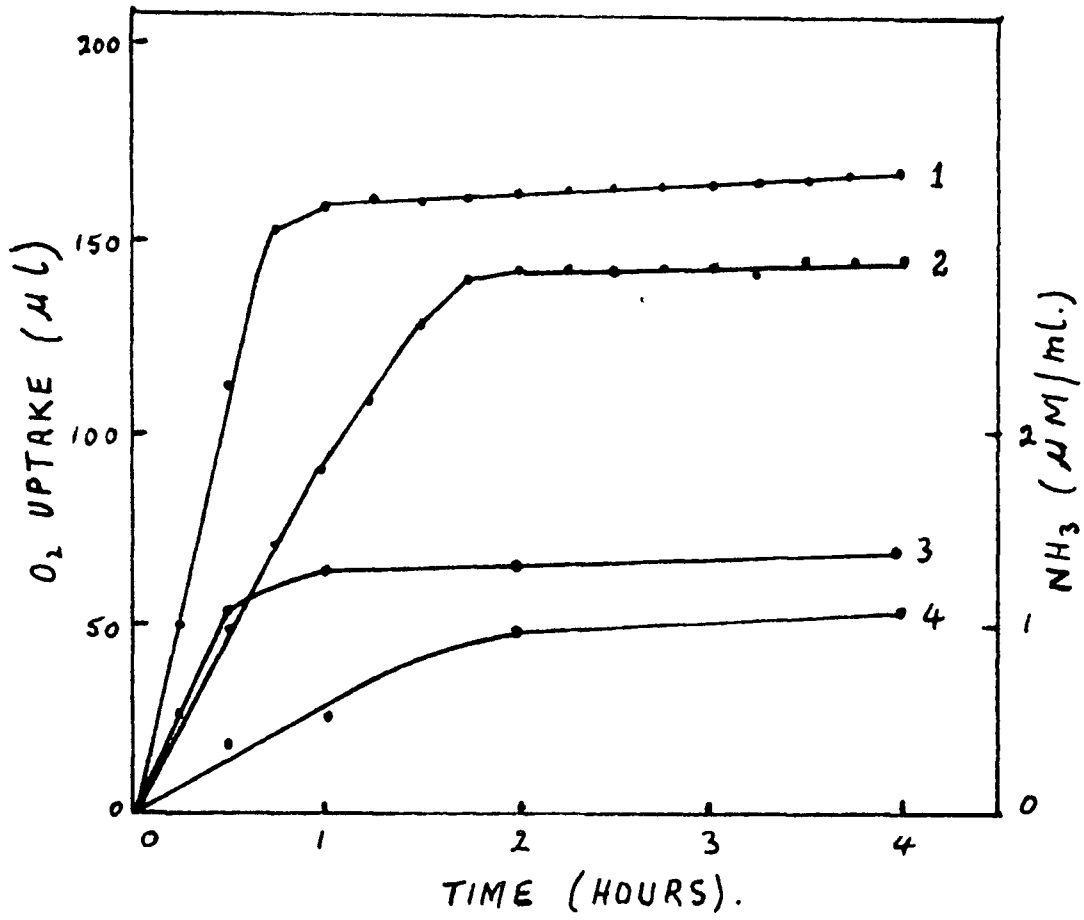


FIG. 11. Effect of arsenite on oxygen consumption and ammonia output by strain S.C. cells acting on putrescine.
Curve 1: O_2 uptake with 3.0 μM putrescine (1.0 μM/ml.).
Curve 2: Same as curve 1, but arsenite (9.0 μM/ml.) present.
Curve 3: NH_3 output with putrescine (1.0 μM/ml.).
Curve 4: Same as curve 3, but arsenite (9.0 μM/ml.)present.

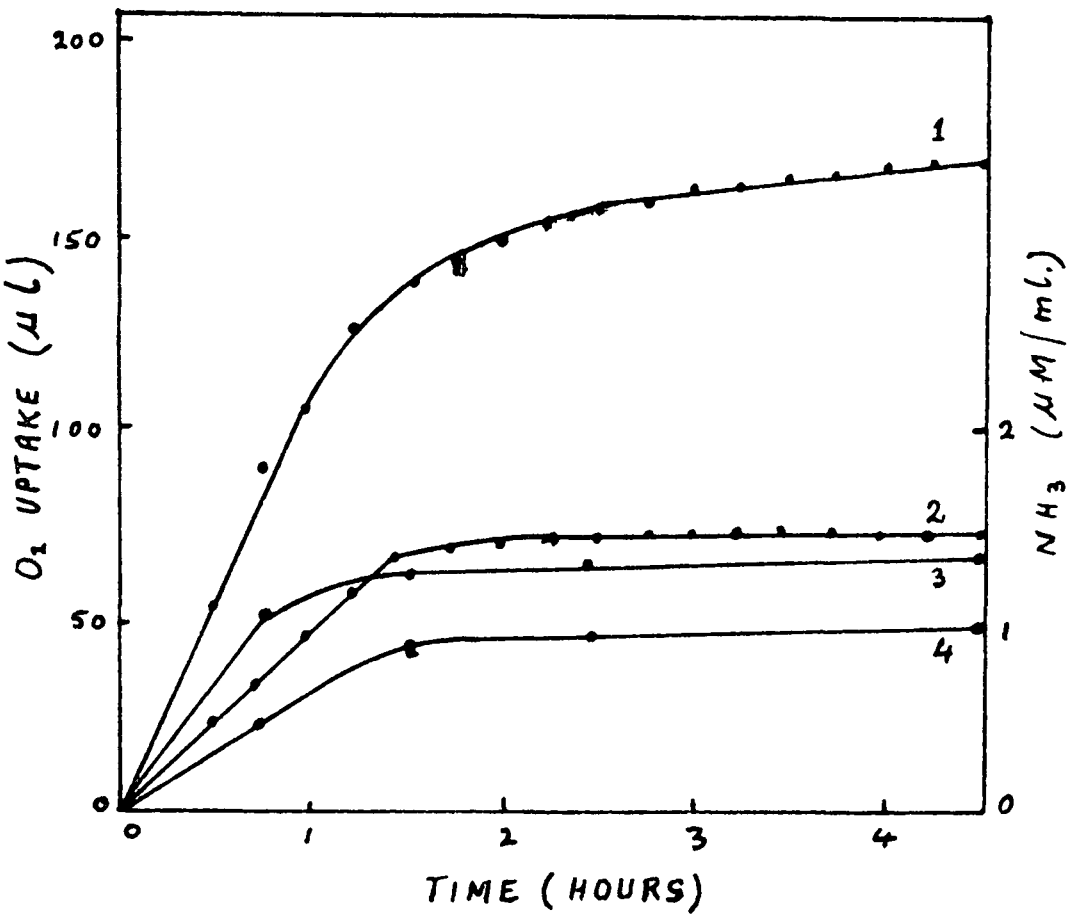


FIG. 12. Effect of arsenite on oxygen consumption and ammonia output by strain C.C. cells acting on putrescine.
Curve 1: O_2 uptake with 3.0 μM putrescine (1.0 μM/ml.).
Curve 2: Same as curve 1, but arsenite (3.0 μM/ml.) present.
Curve 3: NH_3 production with putrescine (1.0 μM/ml.) present.
Curve 4: Same as curve 3, but arsenite (3.0 μM/ml.) present.

similarly treated cells excreted pyruvate in addition to α-ketoglutarate. Since the percentage of the ammonia released had been reduced to 50-53% of the possible maximum, the data supported the view that only one out of a possible two micromoles of ammonia had been released from the putrescine molecule. Thus, if one postulated that γ-aminobutyric acid was the intermediate retaining the residual nitrogen, this compound, or amino-compounds derived from it, should be present in the reaction mixture as the excreted product(s). A paper chromatographic examination of the reaction mixtures obtained at the conclusion of the experiments with strain C.C. and S.C. cells showed that in the presence of putrescine and arsenite, the cells had excreted trace amounts of aspartic acid, glutamic acid and valine, together with larger amounts of alanine and γ-aminobutyric acid. In the case of strain C.C. cells, γ-aminobutyric acid was the amino acid leaked in highest concentration. With strain S.C. cells, the concentration of alanine approached that of γ-aminobutyric acid as judged by the density of the ninhydrin-positive areas on paper chromatograms. Supernatants from endogenous reaction mixtures containing arsenite contained no γ-aminobutyric acid, but traces of aspartic acid, glutamic acid, alanine and valine were detected. Cells not incubated with arsenite did not excrete any amino acids into the reaction mixture supernatants.

In order to determine the nature of the amino acid pool

within the cells, and to determine whether certain amino acids were derived from putrescine, washed samples of cells, obtained at various periods during the course of the reaction, were extracted with HCl and the acid extracts were chromatographed. The separated compounds were developed with ninhydrin. Cells examined in this manner included endogenously respiring cells, with and without arsenite, and cells oxidizing putrescine, with and without arsenite. Acid extracts corresponding to 2.0 mg. dry weight of strain C.C. cells were examined by paper chromatography and the amino compounds observed are listed in Table 10.

Table 10

Ninhydrin-positive areas on chromatograms* obtained from HCl extracts of variously treated strain C.C. cells.

Ninhydrin-positive spot		Treatment: cells plus:			
Rf	Probable identity	H_2O	PUT	H_2O+Arsenite	PUT+Arsenite
0.416	Glutamic acid	+	+	+	+
0.582	γ-aminobutyric acid	-	+	-	+
0.692	Valine	+	+	+	+

* Solvent: n-butanol:acetic acid:water (2:1:1 v/v).

The data in Table 10 indicated that γ-aminobutyric acid was an intermediate in putrescine degradation since the former

compound only occurred when putrescine was present as substrate. Unfortunately, too little material was spotted on chromatograms with the result that only those amino compounds present in relatively large quantities were detected. However, the chromatograms demonstrated that while the concentrations of glutamic acid and valine remained essentially constant, γ-aminobutyric acid disappeared completely in unpoisoned cells. The disappearance coincided with the region of the break of the oxygen uptake curve. In cells poisoned with arsenite, traces of γ-aminobutyric acid were still evident at the conclusion of the experiment. The absence of alanine on these chromatograms was somewhat surprising in view of the fact that relatively large amounts of the amino acid were excreted into the external medium by poisoned cells metabolizing putrescine. It is possible, however, that inhibited cells excreted most, or all of the alanine formed during putrescine dissimilation, and that in uninhibited cells, alanine was utilized as rapidly as it was synthesized.

Table 11 records the results of paper chromatographic studies on acid extracts obtained from various treatments of strain S°C. cells. In this experiment, an amount of acid extract corresponding to about 6.0 mg. dry weight of cells was applied to each spot on the chromatogram so that a more complete picture of the acid-soluble pool might be obtained.

The data in Table 11 again suggested that γ-aminobutyric acid was an intermediate of putrescine dissimilation since

Table 11

Ninhydrin-positive areas on chromatograms* obtained from HCl extracts of variously treated strain S.C. cells.

Ninhydrin-positive spot		Treatment: cells plus			
Rf	Probable identity	H_2O	PUT	H_2O+Arsenite	PUT+Arsenite
0.178	Unknown	+-	+-	+-	+
0.252	Putrescine	-	+	-	+
0.318	Aspartic acid	+	+	+	+
0.414	Glutamic acid	+	+	+	+
0.515	Alanine	+	+	+	+
0.582	γ-aminobutyric acid	-	+	-	+
0.680	Valine	+	+	+	+
0.771	Leucine	+-	+-	+-	+-

* Solvent: n-butanol:acetic acid:water (2:1:1 v/v).

the former only occurred when putrescine was present. Gamma-aminobutyric acid disappeared more slowly in acid extracts obtained from arsenite-poisoned cells. The latter observation was equally true for the disappearance of putrescine. Glutamic acid occurred in high concentrations throughout the experiment. Aspartic acid, valine and leucine were present in lower, but constant amounts. The most notable effect observed with arsenite was a large accumulation of alanine in acid-extracts from poisoned cells. This build-up of alanine was particularly marked in the case of poisoned cells where putrescine was present as substrate. A similar

accumulation of an unidentified ninhydrin-positive compound (Rf 0.178) occurred with poisoned cells which was more noticeable when putrescine was present. Unfortunately insufficient sample was available to allow any further characterization of the compound and the experiment was not repeated. However, the unknown was not detected as an excreted product - perhaps because the product was not excreted. Or, since reaction mixtures were examined by paper chromatography after anion and cation exchange, it was possibly lost with the water wash of the anion exchange column - in which case the compound might have been an amine.

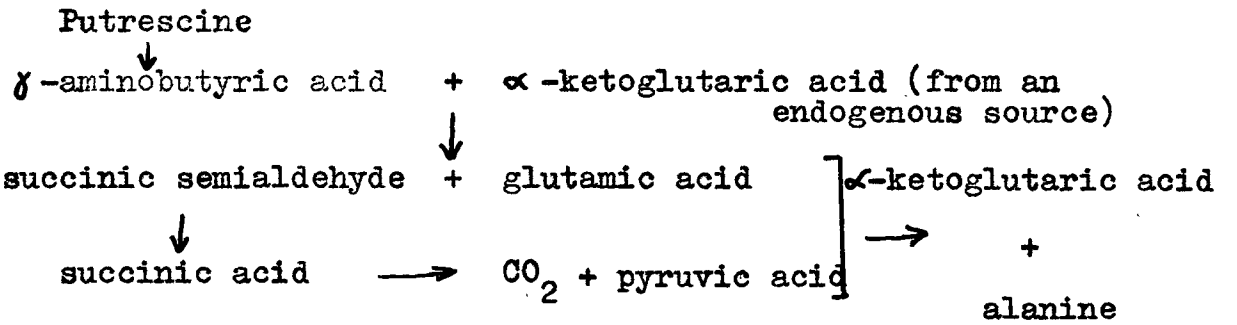
Arsenite inhibition of putrescine oxidation by N.T.N. cells, produced results that differed from those obtained with cells of strains S.C. and C.C. With N.T.N. cells, arsenite at 0.001 M and 0.003 M caused the oxygen uptake to proceed very slowly so that by the end of a 4-hour experiment, the oxygen uptake had not yet approached the break-point in the oxygen uptake curve. No carbonyl compounds or amino acids were detected in the reaction mixture supernatants. This was probably because the system most sensitive to arsenite in these cells did not involve the utilization of these compounds. It therefore seemed likely that the arsenite-sensitive system occurred early in the reaction sequence, possibly at the Δ^1-pyrroline dehydrogenase level. A block at this point would not be unexpected since arsenite has

been reported to inhibit aldehyde dehydrogenases (45). Furthermore, if Δ'-pyrroline had accumulated in arsenite containing reaction mixtures, it would have been lost during the anion and cation exchange procedure employed to eliminate Na^+ ions and AsO_2^- ions from the reaction mixtures and therefore would not have been detected on paper chromatograms.

Hydrochloric acid extracts of non-poisoned N.T.N. cells metabolizing putrescine contained six ninhydrin-positive areas corresponding to: putrescine (which disappeared eventually), aspartic acid, glutamic acid, alanine, γ-aminobutyric acid (which later vanished) and valine. Similar extracts from endogenously respiring cells were qualitatively identical except that putrescine and γ-aminobutyric acid were absent. The oxidation of putrescine by N.T.N. cells which had been frozen and thawed once, proceeded normally when judged by oxygen consumption. However, reaction mixtures were found to contain excreted alanine. Freezing and thawing therefore appeared to impair to some extent the ability of the cells to metabolize alanine. The fact that the excretion of alanine by such cells did not affect the oxygen consumption indicated that either alanine was not metabolized oxidatively or that the amount of the compound excreted was too small to be reflected in the oxygen uptake. An examination of the reaction mixtures obtained from similar cells respiring endogenously, failed to reveal the presence of any amino acids. During the oxidation of putrescine by freshly prepared cells, no excretion

products other than ammonia and carbon dioxide were detected. N.T.N. cells therefore appear to metabolize putrescine in a similar manner to that of the other two mycobacterial strains. During the degradation of putrescine, γ-aminobutyric acid was a common intermediate produced by each strain. Another amino acid which appeared to be derived from putrescine was alanine. The build-up of an unidentified ninhydrin-positive compound (Rf 0.178) on inhibition with arsenite was only observed with the S.C. strain.

Alanine formation probably occurred as follows:



Cell-free extracts obtained from strain C·C. cells adapted to putrescine, were capable of catalyzing the transamination of oxalacetic and pyruvic acids with glutamic acid to yield aspartic and alanine respectively. Under the conditions of the assay (pH 9.0) the reactions were not as rapid as the transamination between α-ketoglutaric acid and γ-aminobutyric acid. It is therefore not difficult to visualize that putrescine may become equilibrated rapidly with a number of amino acids.

II Studies with cell-free extracts

(1) Diamine oxidase (D.O.).

When crude, dialyzed, cell-free extracts were incubated with putrescine, oxygen was consumed and ammonia was produced. The stoichiometry of the reaction was determined by allowing the extract to oxidize 15 µM of putrescine. At completion of the reaction, 167.2 µl O_2 were consumed. The expected O_2 uptake (assuming catalase to be present in the extract) was 168.0 µl. Measurements for ammonia indicated that 14.94 µM were produced instead of a theoretical 15.0 µM. The other product of the reaction was shown by paper chromatography to be Δ'-pyrroline. In the solvent, n-butanol:acetic acid:water, chemically synthesized Δ'-pyrroline had an Rf value of 0.30. The compound could be eluted from the chromatogram and reacted with o-aminobenzaldehyde to produce the characteristic yellow complex. On chromatograms, the colour produced with ninhydrin was initially yellow. Later, the colour turned tan-brown. These properties were also shared by the compound produced from putrescine by D.O. action. Extraction of Δ'-pyrroline into ethyl ether from alkaline solution appeared to cause some destruction of the compound. Two additional ninhydrin-positive areas occurred on paper chromatograms: a tan-brown spot, Rf 0.15, and a grey-brown spot, Rf 0.60. The degradation of Δ'-pyrroline was accompanied

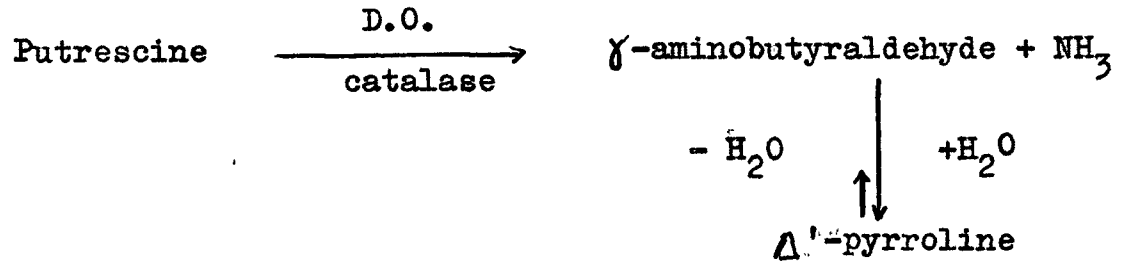
by a certain amount of streaking on chromatograms and the decomposition could not be prevented by conducting the extraction at 0°C.

The instability of Δ'-pyrroline has been well documented (44,72). When, therefore the stoichiometric production of Δ'-pyrroline from putrescine was studied, the reaction was conducted at pH 7.2 to avoid the decomposition of the former compound. The oxygen uptake due to D.O. action on 6.0 μM of putrescine was followed in order to determine when the reaction had proceeded to completion. At this time, the reaction was stopped with zinc sulphate, the precipitated protein removed by centrifugation, and a representative aliquot of the supernatant assayed for Δ'-pyrroline with o-aminobenzaldehyde. Instead of a theoretical 6.0 μM of Δ'-pyrroline, 5.7 μM of Δ'-pyrroline were actually found to be present.

The possibility that Δ'-pyrroline existed in equilibrium with γ-aminobutyraldehyde (44), with the former compound as the quantitatively dominant species, was briefly investigated with inconclusive results. Protein-free reaction mixtures containing Δ'-pyrroline, were allowed to react with DPNH at room temperature for up to 6 hours in order to trap γ-aminobutyraldehyde as its hydrazone derivative. All colour was extractable into ethyl acetate from acid solution. Since the hydrazone should have possessed a positively charged amino group under acid conditions, it should have had a tendency

to remain in the aqueous phase unless the large hydrophobic portion of the molecule was able to overcome the ionic effect. That some hydrazone formation may have occurred, was suggested by the fact that one or two extra extractions with ethyl acetate were required to remove all colour from the aqueous phase. In the absence of Δ'-pyrroline, similarly treated controls required fewer extractions with ethyl acetate for removal of DPNH.

The results support the conclusion that during the oxidation of 1.0 μM of putrescine by the D.O. of crude, cell-free extracts, 0.5 μM O_2 are consumed and 1.0 μM of NH_3 and 1.0 μM of Δ'-pyrroline are produced:



Gamma-aminobutyraldehyde has been included in the reaction illustrated even though its existence was not conclusively demonstrated. By analogy with related reactions it is assumed to be the immediate product of the oxidative deamination (43,44,46,115).

That catalase was present in crude cell-free extracts was indicated in an earlier experiment which was initially intended to investigate the heat stability of the D.O. enzyme. The results in Table 12 not only demonstrate that the enzyme

is relatively heat-stable, but also that heat treatment for 15 minutes at 55°C caused a doubling in the oxygen consumption without affecting the expected ammonia production. The latter observation strongly suggested that while the heat treatment did not affect the D.O. enzyme, catalase activity in the cell-free extract was completely destroyed.

Table 12

The effect of heat on D.O. activity* in cell-free extracts.

Treatment of cell-free extract	O_2 uptake (μl) at 30 min.	Expected O_2 uptake with catalase: present	absent	NH_3 (μM) at 30 min.	Expected NH_3 (μM)
Unheated	33	33	66	2.92	2.95
Heated 15 mins., 55°C	64	33	66	2.90	2.95
Heated 30 mins., 55°C	61	33	66	3.01	2.95

* Activity measured at pH 7.2 in phosphate buffer with putrescine (0.01 M) as substrate.

To further characterize the putrescine-metabolizing enzyme as typical of a D.O. enzyme, the effects of several inhibitors were briefly studied. The results are recorded in Table 13.

The data in Table 13 indicate that the oxidation of putrescine was inhibited by carbonyl reagents like

Table 13

The effects of various inhibitors on D.O. activity* in cell-free extracts.

Inhibitor	Inhibitor Concentration	O_2 uptake (µl) at 15 minutes
Hydroxylamine	0.01 M	0
	0.001 M	0
Semicarbazide	0.01 M	0
	0.001 M	9
Ephedrine	0.01 M	20
	0.001 M	21
Water	-	20

* Activity measured at pH 7.2 in phosphate buffer with putrescine (0.01 M) as substrate.

hydroxylamine and semicarbazide. Such results are in accord with the reports of D.O. susceptibility to carbonyl reagents (117). The fact that ephedrine sulphate failed to inhibit D.O. activity was not surprising since this compound is reported to be a monoamine oxidase inhibitor (117).

The curve relating D.O. activity to pH is shown in Fig. 13. D.O. activity was studied over the pH range 4.5-9.0. Citrate buffer was employed from pH 4.5 to pH 6.0. Phosphate and tris buffers were used for the pH ranges 6.0 to 8.0 and 7.5 to 9.0 respectively.

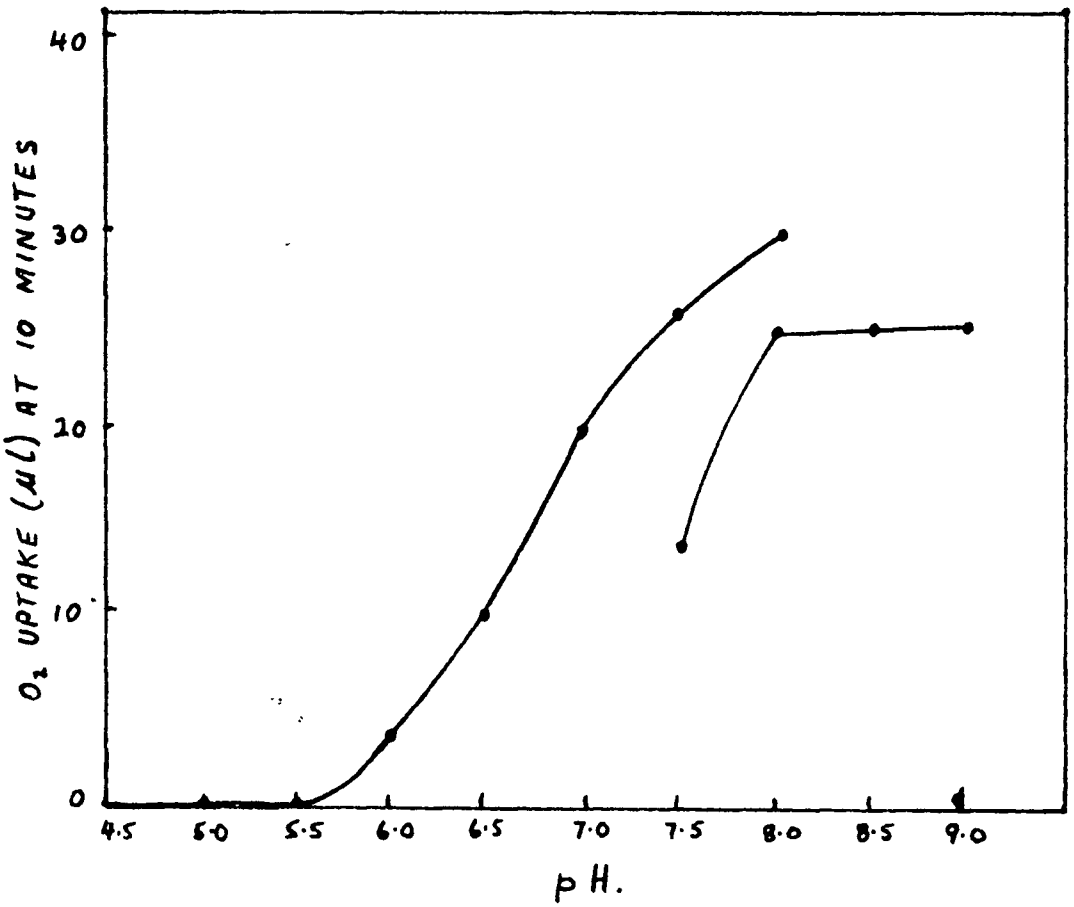


FIG. 13. The optimum pH for diamine oxidase activity. Reactions conducted in citrate (pH 4.5 - 6.0) and phosphate (pH 6.0 - 8.0) buffers (curve 1) and in tris buffer (pH 7.5 - 9.0) (curve 2).

The pH optimum for D.O. activity was found to lie in the pH region 8.0 to 9.0. Tris buffer exerted a marked inhibitory effect at pH 7.5 which was correlated with a significant amount of precipitation of cell-free extract material. This phenomenon occurred consistently in different batches of tris buffer at pH 7.5 but was not observed with phosphate buffer at the same pH. At pH 8.0, D.O. activity was greater in phosphate buffer than in tris buffer but at this pH no protein precipitation was evident with the latter buffer. It may be that some enzyme denaturation occurred with tris buffer at pH 8.0 which went undetected, or it is possible that phosphate buffer stimulated D.O. activity. Stimulation of D.O. activity by phosphate ions has been reported by other workers (11). At pH 8.5-9.0 in tris buffer, the oxygen uptake curves proceeded upwards in a linear fashion and levelled off abruptly when substrate was exhausted. At pH 8.0 in phosphate buffer, the levelling off of the oxygen uptake curve due to depletion of substrate, occurred more gradually. These observations were made during short and extended experiments although the effects were not as marked during the shorter reaction periods. It was therefore felt that enzyme inactivation could not entirely explain this phenomenon. It appeared likely that at higher pH values, some of the product (Δ'-pyrroline) was removed from the reaction by spontaneous decomposition. In the presence of oxygen, and under alkaline

conditions, Δ^1-pyrroline is reported to be very unstable (44,72). During assays for D.O. activity, the net result of Δ^1-pyrroline decomposition would be to cause the reaction to proceed to completion more rapidly. An alternative explanation could be that the equilibrium constant of the reaction favoured completion of the reaction at higher pH values.

Table 14 records the results of a study of the substrate specificity of the D.O. enzyme contained in cell-free extracts obtained from putrescine-adapted strain C.C. cells. The reaction was conducted at pH 8.5 with each substrate at 0.005 M (except spermine 0.001 M).

Table 14

Aliphatic amines as substrates for D.O. in cell-free extracts obtained from putrescine-adapted cells.

Substrate*	O_2 uptake (µl) at 15 minutes	O_2 uptake (µl) at 120 minutes
1-aminoethane	2	4
1-aminopropane	1	2
1-aminobutane	2	4
1-aminopentane	3	5
1-aminohexane	1	3
1,2-diaminoethane	1	5
1,3-diaminopropane	2	5
1,4-diaminobutane (putrescine)	20	134
1,5-diaminopentane (cadaverine)	7	52
1,6-diaminohexane	1	1
Spermidine	1	0
Spermine	1	0
Water	0	3

* Mono- and diamines as their hydrochloride salts; spermidine and spermine as their phosphate salts.

The data in Table 14 indicate that the D.O. enzyme is fairly specific. No mono- or polyamines were attacked. Of the diamines, only putrescine and cadaverine were oxidized with the latter compound being attacked at an approximately one-third of the rate for that of putrescine.

D.O. activity was not appreciably reduced by dialysis. Prolonged dialysis (48 hours at 4°C) versus 100 volumes of distilled water resulted in a decrease of only 5-8% of the original activity. Such losses in activity were more likely attributable to the precipitation of some active material rather than to a loss of cofactor. It was concluded that, as is typical of other D.O. preparations (117), the prosthetic group of the D.O. enzyme is tightly bound to the enzyme.

D.O. activity was not sedimented by centrifugation for 1 hour at 10,000 X G. When the unwashed pellet (debris) was resuspended in the original volume and an aliquot assayed for D.O. activity with putrescine, it was found to be one-nineteenth as active as a comparable portion of the supernatant fraction. Much of the activity in the sedimented debris may have been due to unbroken cells since smears of this material showed numerous intact acid-fast bacilli to be present. Cell-free extracts did not contain NADH oxidizing ability which indicated that intact mitochondria were eliminated by centrifugation. It is possible, however, that the D.O. enzyme was attached to particulate material since the gravitational forces used would not have sedimented

ribosomes and small mitochondrial fragments.

(2) Δ'-pyrroline dehydrogenase (P.D.)

Fig. 14 shows the P.D. catalyzed reduction of NAD and NADP (curves 1 and 2, respectively) with chemically synthesized Δ'-pyrroline as substrate. The reactions were conducted at pH 9.0 in the presence of 0.005 M mercaptoethanol and assayed spectrophotometrically at 340 mμ. The enzyme employed for the assay was obtained from putrescine-adapted strain C.C. cells. When the initial reaction rates were compared, the enzyme was found to be about six times more active with NAD than with NADP. The effect on the reaction rate of omitting mercaptoethanol from the system is shown in curves 3 and 4 for NAD and NADP, respectively. The marked decreases in P.D. activity observed in the absence of mercaptoethanol, have been reported for the same enzyme obtained from Ps. fluorescens (44).

The results of identical studies with cell-free extracts obtained from putrescine-adapted strain S.C. cells, are presented in Fig. 15. In the presence of 0.005 M mercaptoethanol, the P.D. enzyme was approximately 3.3 times as active with NAD as with NADP. Δ'-pyrroline employed as substrate for this assay, was synthesized from putrescine with the D.O. enzyme contained in strain S.C. cell-free extracts.

Δ'-pyrroline dehydrogenase activity was also measured

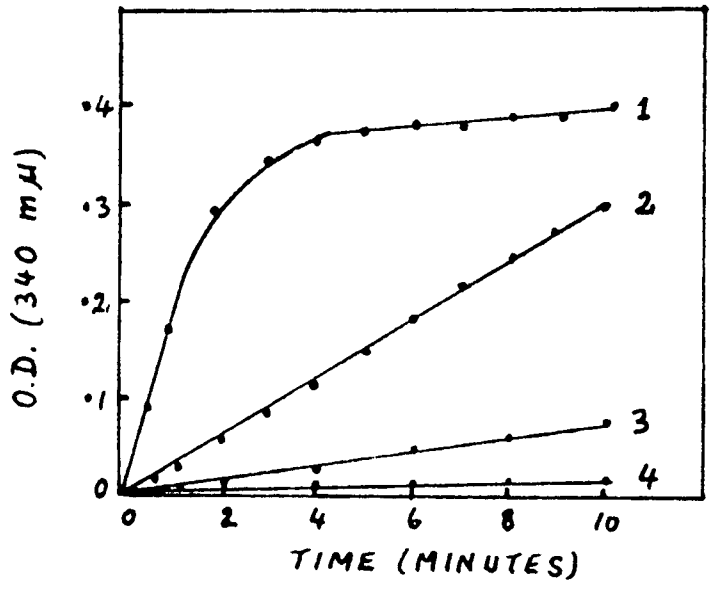


FIG. 14. Strain C.C. Δ'-pyrroline dehydrogenase activity (pH 9.0).
Curve 1: Mercaptoethanol + NAD.
Curve 2: Mercaptoethanol + NADP.
Curve 3: NAD.
Curve 4: NADP.

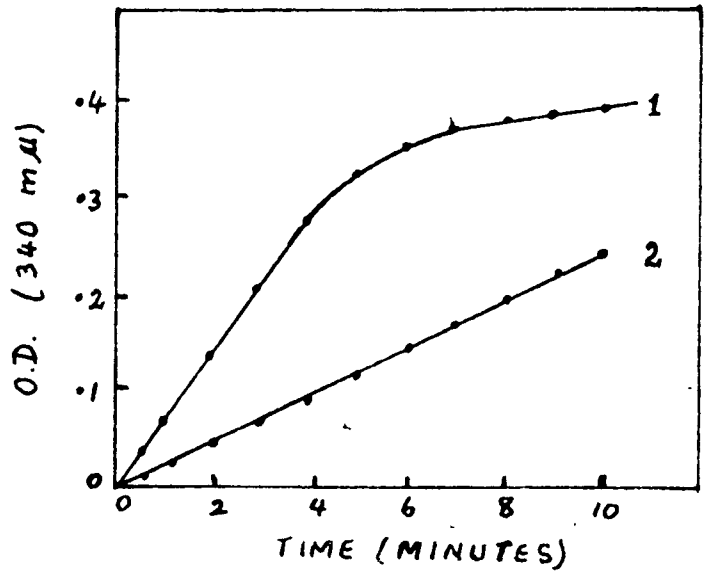


FIG. 15. Strain S.C. Δ'-pyrroline dehydrogenase activity (pH 9.0).
Curve 1: Mercaptoethanol + NAD.
Curve 2: Mercaptoethanol + NADP.

manometrically using methylene blue (MB) to couple the reaction to molecular oxygen. The substrate, Δ'-pyrroline, was either synthesized chemically, or it was prepared enzymatically from putrescine using crude, dialyzed cell-free extracts obtained from putrescine-adapted strain C.C. cells:

$$\text{1 Putrescine} \xrightarrow[\text{D.O.}\ \text{catalase}]{\text{A}} \text{1 Δ'-pyrroline} \xrightarrow[\text{P.D.}\ \text{NAD}\ \text{catalase}\ \text{M.B.}\ \text{R-SH.}]{\text{B}} \text{1 γ-aminobutyric acid}$$

In the reactions shown, the oxidation of 1.0 μM of putrescine to 1.0 μM of γ-aminobutyric acid would require the consumption of 22.4 μl O_2 (1.0 μM O_2) since each step (A,B) consumes 11.2 μl O_2. Alternatively, the reactions A and B could be separated so that the oxygen uptake due to each step could be observed individually.

Fig. 16 illustrates the results obtained at pH 7.2 from manometric studies on P.D. activity with putrescine (6.0 μM) as the precursor of Δ'-pyrroline. The cell-free extract used, was obtained from strain C.C. cells. Curve 1 shows the oxygen uptake with the "complete system": Cell-free extract, mercaptoethanol, NAD, MB, and putrescine. The oxygen consumption levelled off at 136 μl O_2 (theory: 134.4 μl O_2). Curve 2 illustrates the oxygen uptake obtained with an identical reaction mixture except that NAD was omitted. It

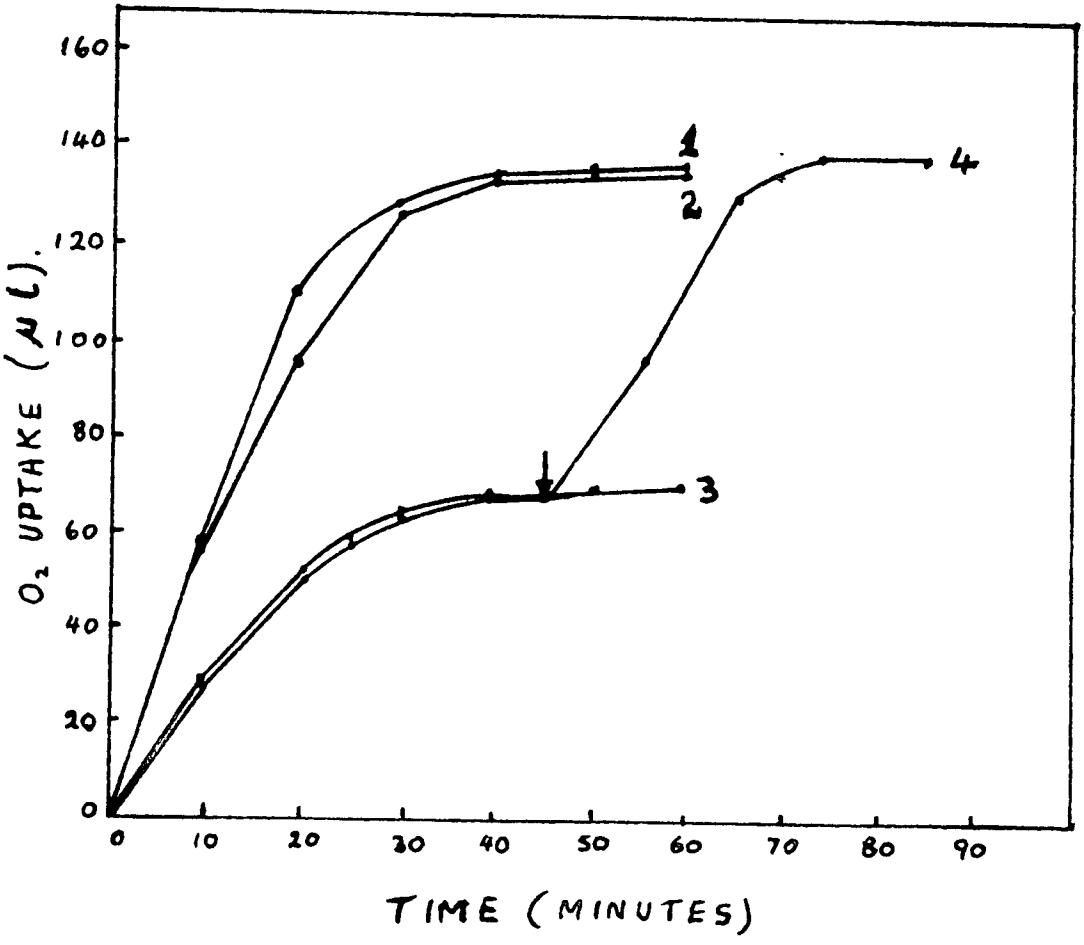


FIG. 16. Manometric assay of strain C.C. Δ^1-pyrroline dehydrogenase activity (pH 7.2). See text for details.

can be seen that the oxygen consumption proceeded to completion (133 µl O_2) although in the upper portion of the curve, it did so more slowly. This finding was surprising since the ommision of NAD should have reduced the final oxygen uptake by 50% (67.2 µl O_2). Furthermore, previous spectrophotometric studies with the dialyzed enzyme preparation had failed to reveal an increase in absorbance at 340 mµ when Δ'-pyrroline was added as substrate. This was taken to indicate that the extract was free of NAD or NADP. In the light of the manometric results, however, it appeared that amounts of cofactor small enough to go undetected by spectrophotometry remained in the cell-free preparation. Under conditions where the cofactor was continually reoxidized by MB, its presence was demonstrated.

Curve 3, Fig. 16 demonstrates the almost absolute requirement of the P.D. enzyme for reducing substances. The reaction system contained: Cell-free extract, NAD, MB and substrate (putrescine). Mercaptoethanol was not present. The oxygen uptake curve proceeded to 69 µl O_2 instead of 134.4 µl O_2 indicating that Δ'-pyrroline was not attacked. Curve 4 shows the result of allowing the oxidation of putrescine to go to completion in the presence of mercaptoethanol and NAD (68 µl O_2 instead of 67.2 µl O_2). At this stage (indicated by the arrow), the addition of MB from the second sidearm of a double sidearm reaction vessel, allowed the accumulated Δ'-pyrroline to be oxidized quantitatively.

Fig. 17 records the results of similar manometric studies on the P.D. enzyme at pH 9.0. Curve 1 represents the oxygen uptake due to 6.0 μM of putrescine in the presence of the "complete system". The oxygen consumption levelled off at 141 μl O_2 (4.9% higher than the expected value). Curve 2 demonstrates the effect of omitting mercaptoethanol from an otherwise "complete system". Once again, the oxygen uptake at completion, was somewhat higher than expected although the requirement of the P.D. enzyme for mercaptoethanol was clearly demonstrated. Although no attempt was made in this experiment to separate D.O. activity from that of the P.D. enzyme, an idea of the reaction rate due to the latter enzyme is obtained from curve 3. Curve 3 was calculated by subtracting the oxygen uptake values for curve 2 from those of curve 1. The calculated reaction rate for the P.D. enzyme is valid since the rate of oxygen uptake in the "complete system" was double that for the mercaptoethanol-deficient system.

No activity was demonstrated when chemically synthesized Δ'-piperideine was tested as substrate at pH 9.0. However, it is not known whether this was due to substrate specificity on the part of the P.D. enzyme. The possibility exists that traces of bromine, formed during the chemical synthesis of Δ'-piperideine, may have remained in the substrate solution. If such was the case, the P.D. enzyme may have been inactivated by bromine.

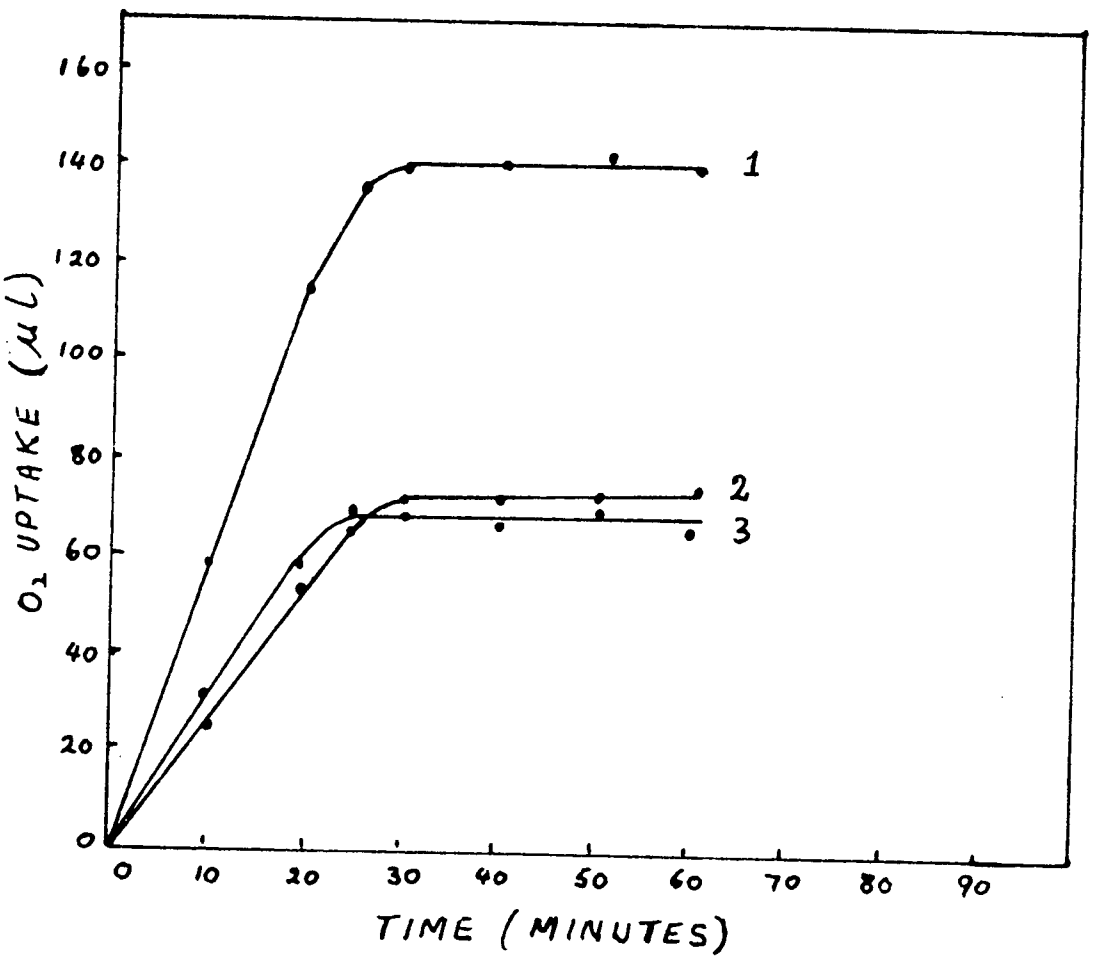


FIG. 17. Manometric assay of strain C.C. Δ'-pyrroline dehydrogenase activity (pH 9.0). See text for details.

The results indicated that the P.D. enzyme was active at pH 7.2 and 9.0. NAD was the preferred cofactor. The enzyme exhibited an almost absolute requirement for free thiol groups.

Paper chromatography of "complete reaction mixtures" obtained at the end of the experiments, showed the presence of a single ninhydrin-positive area which corresponded to γ-aminobutyric acid (Rf 0.582 in butanol:acetic acid: water, 2:1:1 v/v; Rf 0.750 in water-saturated phenol). This compound contained acidic and basic functional groups since it was retained by anion and cation exchange resins. Cell-free extracts therefore contain two enzymes responsible for the synthesis of γ-aminobutyric acid from putrescine.

(3) Gamma-aminobutyric acid - α-ketoglutaric acid transaminase.

Intact cells were shown to release up to 1.5 μM NH_3/μM putrescine. Since the D.O. enzyme could only account for the production of 1.0 μM NH_3/μM putrescine, it appeared that the remaining 0.5 μM NH_3 might possibly have been derived from γ-aminobutyric acid. Consequently, cell-free extracts derived from putrescine-adapted strain C.C. cells were examined for their ability to metabolize this compound.

Cell-free extracts did not consume oxygen at pH 7.0, 8.0 or 9.0 with γ-aminobutyric acid as substrate. Under similar conditions in the presence of NAD or NADP, there was

no increase in absorbance at 340 mμ. An examination of the reaction mixtures obtained from such experiments, indicated that ammonia was not produced. It was therefore concluded that intact cells produced ammonia from γ-aminobutyric acid indirectly, since cell-free extracts were not capable of catalyzing the oxidative, or dehydrogenative deamination of the compound. Similar results have been reported by Bachrach (6) for cell-free preparations obtained from Ps. aeruginosa.

Gamma-aminobutyric acid was metabolized by cell-free extracts when α-ketoglutarate was present. An initial experiment in which 3.0 μM of γ-aminobutyric acid and 6.0 μM of α-ketoglutaric acid were incubated at pH 8.5 for 4 hours at 30°C showed that transamination had occurred. Paper chromatography of the deproteinated, deionized reaction mixture demonstrated glutamic acid to be a product (Rf 0.414 in the n-butanol:acetic acid:water, 2:1:1 v/v solvent; Rf 0.231 in water-saturated phenol solvent). A measurement of the glutamic acid produced during the reaction showed that the reaction had proceeded 76.3% to completion. Paper chromatography of the hydrazones formed with DPNH indicated the appearance of a second hydrazone (Rf 0.439 in n-butanol saturated with 5 N NH_4OH). This compound migrated like the hydrazone prepared from chemically synthesized succinic semialdehyde.

The optimum pH for transaminase activity was determined for the pH range 7.0-9.0 employing phosphate buffer (pH 7.0)

and tris buffer (pH 8.0-9.0). Transaminase activity was determined by measuring quantitatively, the amount of glutamic acid produced. The results illustrated in Fig. 18 indicate that the optimum pH lies in the pH range 8.5-9.0. However, on prolonging the reaction time, the reaction proceeded further to completion at pH 9.0 than at lower pH values. This fact is shown in Figs. 18 and 19. This effect was not further investigated but it may have been due to enzyme inactivation at lower pH values, or to destruction of one of the reaction products at higher pH (succinic semialdehyde?). Alternatively, the equilibrium point may have favoured completion of the reaction at alkaline pH.

The results of substrate specificity studies with the transaminase enzyme are summarized in Table 15.

Table 15

The ability of strain C.C. cell-free extracts to catalyze the transamination between various ω-amino acids and α-ketoglutaric acid.

Amino acid	Glutamic acid (μM) synthesized at 1 hour
Glycine	0
β-alanine	0
γ-aminobutyric acid	4.95
δ-aminovaleric acid	2.80
ε-aminocaproic acid	0.27

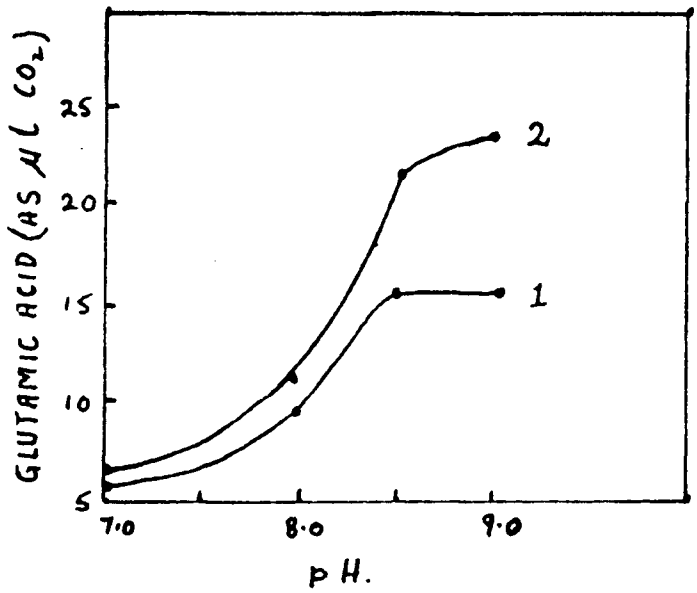


FIG. 18. γ-aminobutyric acid - α-ketoglutaric acid optimum pH curve. Glutamic acid synthesized at 30 mins. (curve 1) and at 60 mins. (curve 2).

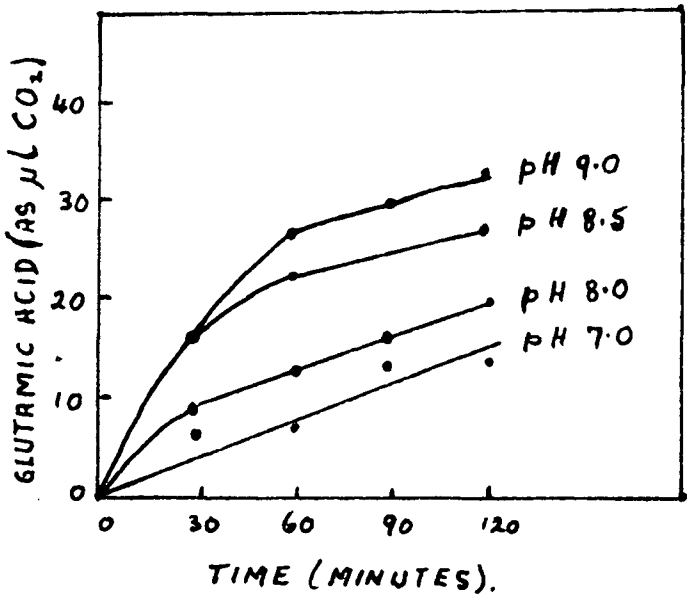


FIG. 19. Effect of pH and reaction time on γ-aminobutyric acid - α-ketoglutaric acid transaminase activity.

The results in Table 15 were further confirmed qualitatively by paper chromatography. Although γ-aminobutyric acid appeared to be the most efficient amino group donor, other ω-amino acids were capable of undergoing transamination in this system. The results disagree with those for a similar transaminase enzyme obtained from Ps. fluorescens in which the enzyme was specific for γ-aminobutyric acid (44).

When γ-aminobutyric acid was incubated with oxalacetate or pyruvate, no aspartate or alanine was detected by chromatography. The enzyme therefore appeared to be specific in the requirement for α-ketoglutarate. Similar results were obtained for the same transaminase in Ps. fluorescens (18) but cell-free preparations from Ps. aeruginosa were capable of transaminations between γ-aminobutyric acid and pyruvate, oxalacetate and α-ketovalerate in addition to α-ketoglutarate (6).

(4) Succinic semialdehyde dehydrogenase (S.S.D.).

Fig. 20 shows the S.S.D.-catalyzed reduction of NAD and NADP (curves 1 and 2, respectively) by a cell-free preparation obtained from putrescine-adapted strain C.C. cells. Succinic semialdehyde used in this assay, was prepared by incubating equimolar amounts of γ-aminobutyric acid and α-ketoglutaric acid with the cell-free extract for 30 minutes prior to the addition of cofactor. The reaction was conducted at pH 9.0. The curves demonstrate that NAD is the more

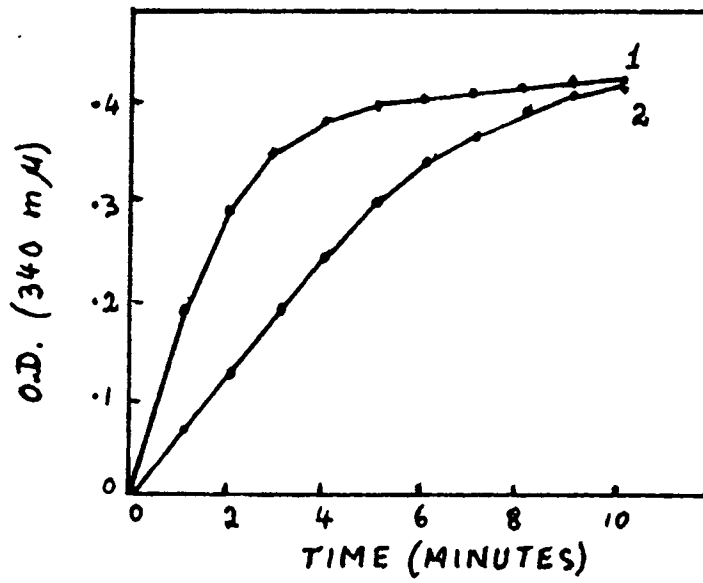


FIG. 20. Strain C.C. succinic semialdehyde dehydrogenase activity (pH 9.0).
Curve 1: NAD
Curve 2: NADP.

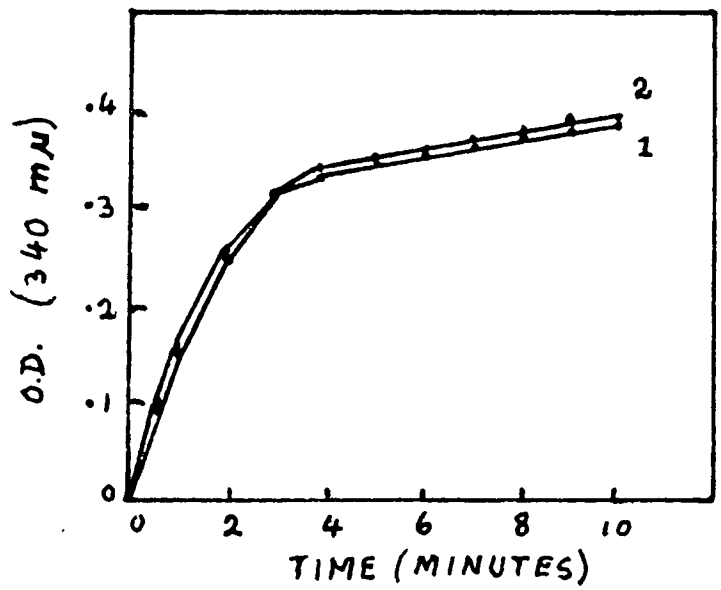


FIG. 21. Strain S.C. succinic semialdehyde dehydrogenase activity (pH 9.0).
Curve 1: NAD.
Curve 2: NADP.

efficient cofactor in the S.S.D. system. The results of similar experiments with the S.S.D. enzyme obtained from putrescine-adapted strain S.C. cells are illustrated in Fig. 21. With this strain, NAD and NADP appeared to be equally as efficient as cofactors in the dehydrogenation. Chemically synthesized succinic semialdehyde was employed as substrate for the reaction shown in Fig. 21. Although Jakoby (18) reported that S.S.D. from Ps. fluorescens required mercaptoethanol for optimal activity, the effect of this compound on the activity of mycobacterial S.S.D. was not tested since good activity was obtained in its absence, even after prolonged storage of the enzyme at -20°C. In Ps. fluorescens (147) and Ps. aeruginosa (106), NADP appeared to be the more efficient cofactor for the S.S.D. enzyme. In the latter organism, there appeared to be two S.S.D. enzymes, one requiring NAD, and the other requiring NADP (68).

In an initial experiment, the product of the S.S.D.-catalyzed reaction was shown to be succinic acid. For this experiment, the substrate, succinic semialdehyde, was prepared enzymatidally using cell-free extract obtained from putrescine-adapted strain C.C. cells. The reaction mixture contained: γ-aminobutyric acid (12 μM), α-ketoglutaric acid (6.0 μM), NAD (6.0 μM) and malonic acid (12 μM). Malonic acid was employed to inhibit the further possible breakdown of any succinic acid formed. The reaction was conducted at

pH 8.5 for 2.5 hours at 30°C, at which time a representative aliquot was examined for its succinic semialdehyde content. Succinic semialdehyde was determined by measuring the amount of glutamic acid present since these compounds would have been produced in equimolar amounts during the transamination. The remaining reaction mixture was freed of protein with zinc sulphate (95) and a portion was examined for DPNH-reacting materials. The rest of the reaction mixture was acidified, extracted with ethyl ether, and the ether extract was chromatographed to demonstrate the presence of succinic acid. Similar techniques were employed to examine a control reaction mixture in which water was substituted for NAD.

The results of the glutamic acid assay indicated that transamination had proceeded to completion in the NAD-containing system. The corresponding value for the control reaction mixture was 66.5%. Paper chromatography of the hydrazones formed with DPNH showed that the NAD-containing system was devoid of both succinic semialdehyde and α-ketoglutarate while both of these compounds remained in the cofactor-deficient system. These results were consistent with the fact that succinic semialdehyde was quantitatively removed from the reaction system when substrate amounts of cofactor were present, and coincident with the dismutation of succinic semialdehyde, the transaminase reaction was induced to proceed to completion. Paper chromatography of the ether extracts demonstrated that succinic acid was the product of the

dehydrogenative oxidation (Rf 0.254 in ethanol:NH_4OH:H_2O, 16:1:3 v/v).

In the preceding experiment, no quantitative estimation was made on the succinic acid produced because of the tedious procedure required to separate malonate from succinate before the assay of the latter could be accomplished by enzymatic means. In a subsequent experiment, however, it was shown that during the S.S.D.-catalyzed reaction, 1.0 μM of succinate was produced from 1.0 μM of succinic semialdehyde. In this experiment oxalacetate was used in place of malonate, since the former was readily destroyed and since the products of its destruction were shown not to interfere with the enzymatic assay of succinate. The actual result obtained showed that in the presence of 1.04 μM of succinic semialdehyde (measured as glutamic acid), 0.93 μM of succinic acid were produced during the S.S.D. reaction.

III Isotopic studies with intact cells

Table 16 records the distribution of radioactivity that occurred in the reaction system when putrescine-adapted strain N.T.N. cells were allowed to oxidize 1,4-C^{14} putrescine under CO_2-free conditions.

Table 16

Distribution of C^{14} occurring during the oxidation of 1,4-C^{14} putrescine (3.0 µM) by putrescine-adapted strain N.T.N. cells.

Time (minutes)	% of total counts added to the reaction system in:				
	Cells + Supernatant	Supernatant	Cells (difference)	CO_2	Cells + Supernatant + CO_2
25	72.0	31.0	41.0	19.5	91.5
50	41.8	1.3	40.5	62.1	103.9
65	33.0	1.2	31.8	72.2	105.2
120	29.5	1.2	28.3	78.6	108.2

Table 17 shows the incorporation of the radioactivity that occurred in the various cell fractions during the course of the reaction. By summation of the counts contained in the various cell fractions that comprised the intact cell, a second estimate of the cell radioactivity was made possible. When these counts for cells were added to the counts contained in supernatant and carbon dioxide samples, the percentage of the counts obtained approximated more closely the counts actually added to the reaction system than did the corresponding

values shown in Table 16.

Table 17

Incorporation of C^{14} into various N.T.N. cell fractions during the oxidation of 1,4-C^{14} putrescine (3.0 μM).

% of total counts in cell fraction:	Time (minutes)			
	25	50	65	120
Cold HCl	24.5	8.1	5.3	2.9
Acid alcohol	14.7	10.6	7.9	6.5
Hot TCA	4.9	6.5	6.8	5.4
Residue	4.8	11.2	11.8	10.0
% of total counts in:				
Combined cell fractions	48.9	36.4	31.8	24.8
Supernatants	31.0	1.3	1.2	1.2
CO_2	19.5	62.1	72.2	78.6
% of total counts obtained	99.4	99.8	105.2	104.6

The data presented in Table 16 indicated that by 50 minutes, no substrate remained in the reaction mixture supernatant and that no subsequent release of non-volatile C^{14}-material occurred. This finding confirmed the results obtained from earlier studies with non-radioactive putrescine.

During the period of active oxygen consumption, the cells contained up to 41-48.9% (Tables 16 and 17) of the radioactivity supplied. As will be shown later (Table 18), as much as 50% of this radioactivity occurred in the cold HCl-soluble fraction as undegraded putrescine and as amino acids. As the reaction proceeded, the level of the counts in the cells decreased until at the end of two hours, the cells contained between 21.4-28.3% of the radioactivity supplied. The average of these values (24.8%), is the value obtained by adding the counts contained in the various cell fractions for this sampling time, (Table 16). All C^{14} lost by the cells appeared in the alkali in the centre well as carbon dioxide. Fig. 22 illustrates the distribution of C^{14} that occurred during the reaction among cell, supernatant and carbon dioxide samples. In addition, oxygen consumption that occurred is shown in parallel.

The amount of terminal C^{14} assimilated by the cells agreed very well for the amount of putrescine-nitrogen incorporated by cells of this strain. As indicated by previous studies 24-25% of the amino-nitrogen of putrescine was assimilated at this stage of the reaction. Such results suggested that this portion of the putrescine molecule might have been assimilated into nitrogenous materials. Unfortunately, the fate of carbons 2 and 3 of the putrescine molecule is uncertain since they were unlabelled and since the indirect technique for carbon dioxide production gave lower values

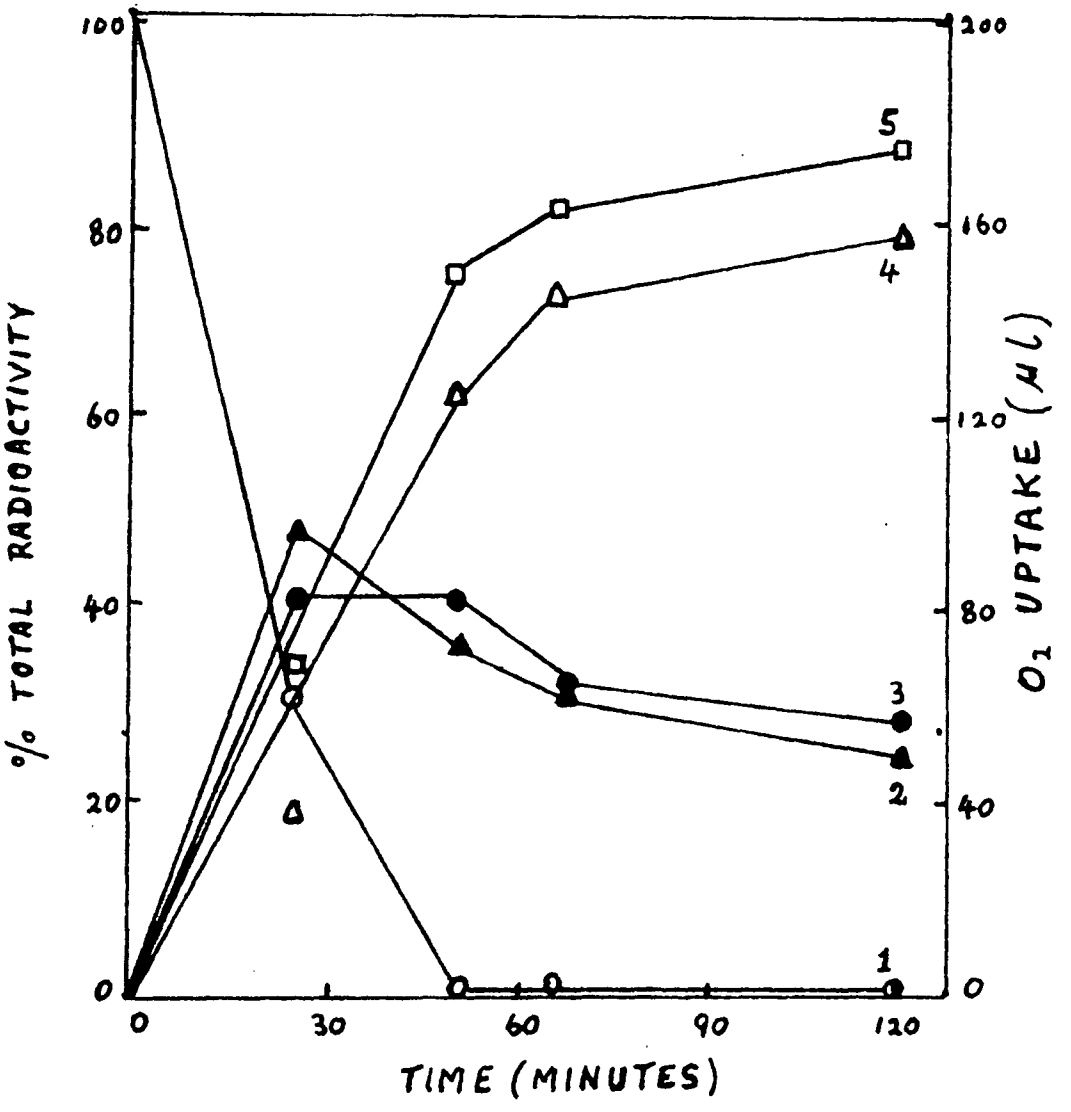


FIG. 22. Distribution of radioactivity during the oxidation of 1,4-C^{14} putrescine by putrescine-adapted strain N.T.N. cells. Reaction mixture supernatant (curve 1); combined cell fractions (curve 2); cells, calculated as the difference between cells + supernatant and supernatant (curve 3); $C^{14}O_2$ (curve 4); O_2 uptake occurring during the reaction (curve 5).

than actually were true for carbon dioxide production under CO_2-free conditions. However, only a small portion of these carbons may have been released as carbon dioxide since the oxygen uptake at the conclusion of the experiment corresponded to 44.4% of the level for maximum oxidation.

Table 18 expresses the radioactivity of the cell fractions as a percentage of the counts occurring within the intact cells.

Table 18

Distribution of incorporated C^{14} in various cell fractions obtained from strain N.T.N. cells incubated with 1,4-C^{14} putrescine (3.0 µM).

Cell fraction:	Counts per fraction as a % of counts in whole cells at time (minutes):			
	25	50	65	120
Cold HCl	50.1	22.2	16.7	11.7
Acid alcohol	30.1	29.2	24.8	26.2
Hot TCA	10.0	17.8	21.4	21.8
Residue	9.8	30.8	37.1	40.3
% Total counts in cells	100.0	100.0	100.0	100.0

It can be seen that initially, 50.1% of the counts contained in the cells occurred in the cold HCl-soluble fraction. At the end of two hours, the level of C^{14} in this

fraction was reduced to 11.7%. Coincident with the loss in radioactivity in the cold HCl-soluble fraction, there was a gain in that of the hot TCA-soluble and residue ffactions. In E. coli, the former fraction was found to consist largely of nucleic acid, while the latter contained mainly protein (78). Presumably the loss in radioactivity in the cold HCl-soluble fraction was partly due to the formation of macromolecules (nucleic acid and protein). The acid alcohol-soluble fraction retained a fairly constant proportion of the cell radioactivity throughout the reaction. According to Roberts et al (78), the latter fraction, in E. coli consisted largely of protein and lipid materials.

Efforts were made to determine the composition of the fractions obtained from N.T.N. cells.

Paper chromatography of the cold HCl-soluble fraction (in n-butanol:acetic acid:water 2:1:1 v/v), demonstrated the presence initially of six ninhydrin-positive compounds which migrated like putrescine, aspartic acid, glutamic acid, alanine, γ-aminobutyric acid and valine. At 25 minutes, five peaks of radioactivity occurred on the chromatograms. These peaks corresponded to the first five compounds listed. Radioactivity in the sample obtained at 120 minutes revealed that all the counts of the fraction were present in glutamic acid. No materials absorbing in the ultra-violet range were detected.

Paper chromatography of the unhydrolysed, two-hour,

acid-ethanol soluble fraction, was conducted in sec-butanol: formic acid:water (7:1:2 v/v) (78). Most of the radioactivity in this fraction remained at the origin of the chromatogram while a lesser amount of C^{14} material migrated with the solvent front. The activity occurring at the origin probably represented protein since the material was feebly ninhydrin-positive. The material migrating with the solvent front did not react with ninhydrin. According to Roberts et al (78), the materials at the origin and at the solvent front represent protein and lipid, respectively. Three additional ninhydrin-positive areas occurred on the chromatogram at Rf values corresponding to: 0.155, 0.380, and 0.580. Low levels of radioactivity were associated with the compound at Rf 0.380. If these compounds represented amino acids that were not completely removed by the first fractionation step, then the compound at Rf 0.380 was probably glutamic acid. No U.V.-absorbing materials were evident on the chromatogram.

The hot TCA-soluble fraction obtained at two hours, was freed of the bulk of the TCA by extracting with ethyl ether. The sample was chromatographed after partial hydrolysis in 1.0 N HCl at 100°C for 1 hour. Chromatography was conducted in sec-butanol:formic acid:water (7:1:2 v/v). When the dried chromatogram was viewed under ultra-violet light, three absorbing areas were detected (Rf: 0.188, 0.267 and 0.420). Radioactivity was associated with the absorbing areas. In addition, C^{14} occurred at the origin and at

ninhydrin-positive areas with Rf values of 0.151 and 0.455. The fraction therefore appeared to contain protein in addition to nucleic acid materials.

Satisfactory fractionation of the residue was not achieved. The residue could not be completely dissolved in warm 0.5 N NaOH. Extraction of the residue from basic or acid solution with ethyl ether, resulted in the formation of a solid layer of material at the interface of the liquids. Although a small percentage (3-5%) of the counts in the fraction was extractable into the organic phase, the bulk of the counts was associated with the non-ether-soluble residue. When the acid-aqueous and organic phases were centrifuged in the cold, the liquids separated cleanly with a layer of compacted residue remaining at the interface. Hydrolysis of this material was conducted overnight at 100°C in 5 N HCl. Paper chromatography of the hydrolysate (in n-butanol:acetic acid:water 2:1:1 v/v) showed the presence of ninhydrin-positive areas from Rf 0.20 to 0.80. This fraction therefore contained large amounts of protein but since the material appeared to possess definite lipophilic characteristics, the protein was probably associated with hydrophobic materials-presumably the waxy substances responsible for the hydrophobic nature of intact mycobacterial cells.

When putrescine-adapted cells were allowed to oxidize 1,4-C^{14} putrescine, the radioactivity disappeared from the medium and appeared in the cells and in carbon dioxide. An

analysis of various cell fractions showed that all fractions were rapidly labelled. Initially, the bulk of the radioactivity appeared to be associated with simple nitrogenous compounds (amino acids), but as the reaction was allowed to progress, larger proportions of the C^{14} appeared in fractions containing nucleic acid, lipid and protein. These results were consistent with those of earlier studies which showed that putrescine could support growth when present as the sole source of carbon. The liberation of $C^{14}O_2$ and the labelling of glutamic acid indicated that at least a portion of the putrescine molecule was oxidized via the tricarboxylic acid cycle. Judging from oxygen uptake occurring during the reaction, over 50% of the added putrescine was assimilated. However, since the substrate was not uniformly labelled, the fate of carbons 2 and 3 remains in some doubt.

GENERAL CONCLUSIONS AND DISCUSSION

Mycobacteria isolated from poikilothermic animals appear to be remarkably restricted in their ability to utilize amines as carbon sources for growth. Of a series of aliphatic and cyclic amines tested, only putrescine was found to support growth.

In mycobacterial cells, the system responsible for the oxidation of putrescine, was shown to be adaptive. This fact, together with the finding that such cells do not appear to contain any di- and polyamines, probably indicates that such compounds are not normally present in mycobacterial cells as metabolic intermediates. The failure to demonstrate the presence of amines in mycobacterial cells in this and other studies (37) may mean that mycobacteria do not store amines. If this is so, the role (or roles) that these compounds play in other microorganisms are not required in mycobacteria. Alternatively, in mycobacteria, some other substance (or substances) may function in place of the amines.

During the oxidation of putrescine, the endogenous respiration appeared to function normally. Calculations for the degree of putrescine - oxidation were therefore made possible. When putrescine was oxidized, oxygen consumption usually proceeded to approximately 45% of the maximum level of oxidation. During this process, 69-76% of the amino-nitrogen of the putrescine molecule was released as ammonia, the remaining 24-31% being assimilated. Carbon dioxide production, estimated by the "direct method", resulted in carbon dioxide values which were unreliable since in the

absence of carbon dioxide there appeared to be increased carbon dioxide production. When 1,4-C^{14} putrescine was incubated with cells under CO_2-free conditions, approximately 75% (or 1.5 μM CO_2/μM putrescine) of the terminal carbons were evolved as carbon dioxide. The possibility exists that the release of carbon dioxide may have even been higher since the fate of the two remaining unlabelled carbons of the putrescine molecule is uncertain.

Studies with cell-free extracts obtained from putrescine-adapted cells indicated the presence of four enzymes which were involved in converting putrescine into succinic acid. Enzymes performing a similar series of reactions in Ps. fluorescens have recently been described in detail by Jakoby (44).

Putrescine was first oxidatively deaminated to yield γ-aminobutyraldehyde. This compound was detected in its Δ'-pyrroline form. The latter compound was then oxidized to γ-aminobutyric acid by a Δ'-pyrroline dehydrogenase enzyme which required NAD or NADP. Gamma-aminobutyric acid was found to undergo a transamination with α-ketoglutaric acid to yield succinic semialdehyde and glutamic acid. It is assumed that intact cells supply the α-ketoglutarate from endogenous sources since cell-free extracts appeared to contain no alternative system for the metabolism of γ-aminobutyric acid. In addition, inhibitor studies with arsenite showed that endogenously respiring cells produced α-ketoglutarate.

Succinic semialdehyde produced as a result of the transamination, was oxidized to succinic acid by a

dehydrogenase enzyme requiring NAD or NADP.

Up to this stage, no reactions responsible for the production of carbon dioxide had occurred. Decarboxylation reactions that occurred later, resulted from the further utilization of products derived from putrescine.

Amino-nitrogen derived from putrescine was assimilated indirectly by way of glutamic acid which yielded other amino acids (aspartic acid and alanine) by other transaminase reactions. Transaminase enzymes were demonstrated in the cell-free extracts which could accomplish the transaminations required to produce these amino acids.

That γ-aminobutyric acid was produced by intact cells during the degradation of putrescine was demonstrated by paper chromatography of HCl extracts obtained from cells metabolizing putrescine. When 1,4-C^{14} putrescine was employed as substrate, the γ-aminobutyric acid produced was radioactive.

Cells inhibited with arsenite were shown to produce pyruvic acid. When putrescine was included in such systems, the amount of pyruvic acid formed was increased. This finding suggested that part of the putrescine molecule was metabolized by way of pyruvate. Cells incubated with putrescine showed the presence of aspartic acid, glutamic acid and alanine in HCl extracts. When 1,4-C^{14} putrescine was employed as substrate these amino acids were found to be radioactive. Since aspartic and glutamic acids are derived from oxalacetate and α-ketoglutarate respectively, and since

the latter compounds are intermediates in the tricarboxylic acid cycle, the finding that the listed amino acids were radioactive, indicated that some of the putrescine carbon-skeleton was metabolized via the tricarboxylic acid cycle. Carbon dioxide production was to be expected when intermediates of putrescine degradation were metabolized via this cycle.

When mycobacterial cells were allowed to oxidize 1,4-C^{14} putrescine under CO_2-free conditions the cells assimilated approximately 25% of the radioactivity. All C^{14} not assimilated by the cells appeared as $C^{14}O_2$. The 25% of assimilated radioactivity was distributed among fractions containing lipid, nucleic acid and protein. These findings are consistent with the ability of mycobacterial cells to utilize putrescine as the sole source of carbon for growth.

Since amines are reported to occur in nature usually in relatively low concentrations, it might be of advantage for bacteria to utilize only those amines which (within their genetic capacity) require the minimum expenditure of stored energy and reserve materials. Putrescine meets these requirements, since the synthesis of only four enzymes is required to produce succinic acid - an intermediate metabolite which the cell machinery can normally handle. On the other hand, if diamines like 1,3-diaminopropane and cadaverine were metabolized by an analogous series of reactions,

they would yield malonic and glutaric acids, respectively. But since these intermediate products do not normally occur within cells, their effective utilization would require additional enzyme synthesis. Otherwise, a situation would arise in which an initial waste of energy led to the accumulation of substances, some of which might be detrimental to the cell. Malonic acid, for instance, is known to impair the efficient operation of the tricarboxylic acid cycle.

The inability of the tested mycobacterial strains to utilize certain amines appears at first sight to be a handicap to survival. But actually when the cell environment includes certain amines, in addition to acceptable substrates,, a complete inability (rather than partial ability) to utilize the former may be more advantageous to the cell.

APPENDIX

Table 1

Composition of the medium employed to test the ability of mycobacterial cells to utilize amines as sole sources of carbon.

Amine (as hydrochloride salt or phosphate salt)	1.0 g. or 4.0 g.
Ferric ammonium citrate	50.0 mg.
Magnesium sulphate	10.0 mg.
Calcium chloride	0.5 mg.
Zinc sulphate	0.1 mg.
Copper sulphate	0.1 mg.
Disodium phosphate	2.5 g.
Monopotassium phosphate	1.0 g.
Ammonium nitrate	1.0 g.
Aqueous Tergitol (1% v/v)	10.0 ml.
Water	1000.0 ml.

The medium was sterilized at 121°C for 15 minutes.

Table 2

Composition of the medium used for growing mycobacterial cells in order to test for the inductive nature of the D.O. enzyme.

Asparagine (Difco)	5.0 g.
Glucose*	2.0 g.
Ferric ammonium citrate	50.0 mg.
Magnesium sulphate	10.0 mg.
Calcium chloride	0.5 mg.
Zinc sulphate	0.1 mg.
Copper sulphate	0.1 mg.
Disodium phosphate	2.5 g.
Monopotassium phosphate	1.0 g.
Aqueous Tergitol (1% v/v)	10.0 ml.
Water	1000.0 ml.

* Glucose was prepared as a 50% w/v solution and sterilized separately at 121°C for 15 minutes.

The medium was autoclaved at 121°C for 20 minutes in 100 ml. amounts. To each 100 ml. of medium was added 0.4 ml. of sterile glucose solution.

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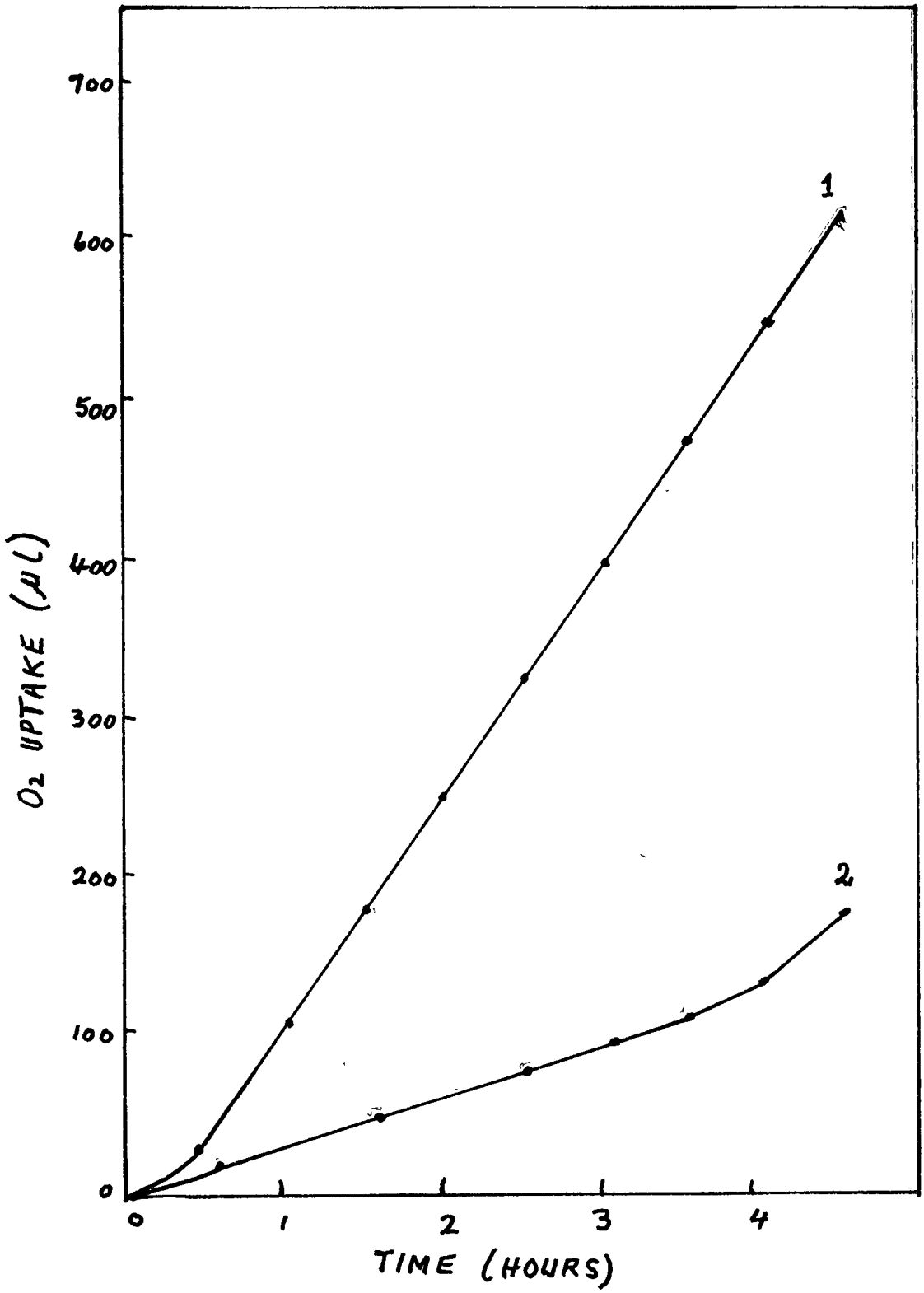

FIG. 1. Oxidation of putrescine by putrescine-adapted (curve 1) and unadapted (curve 2) strain N.T.N. cells.

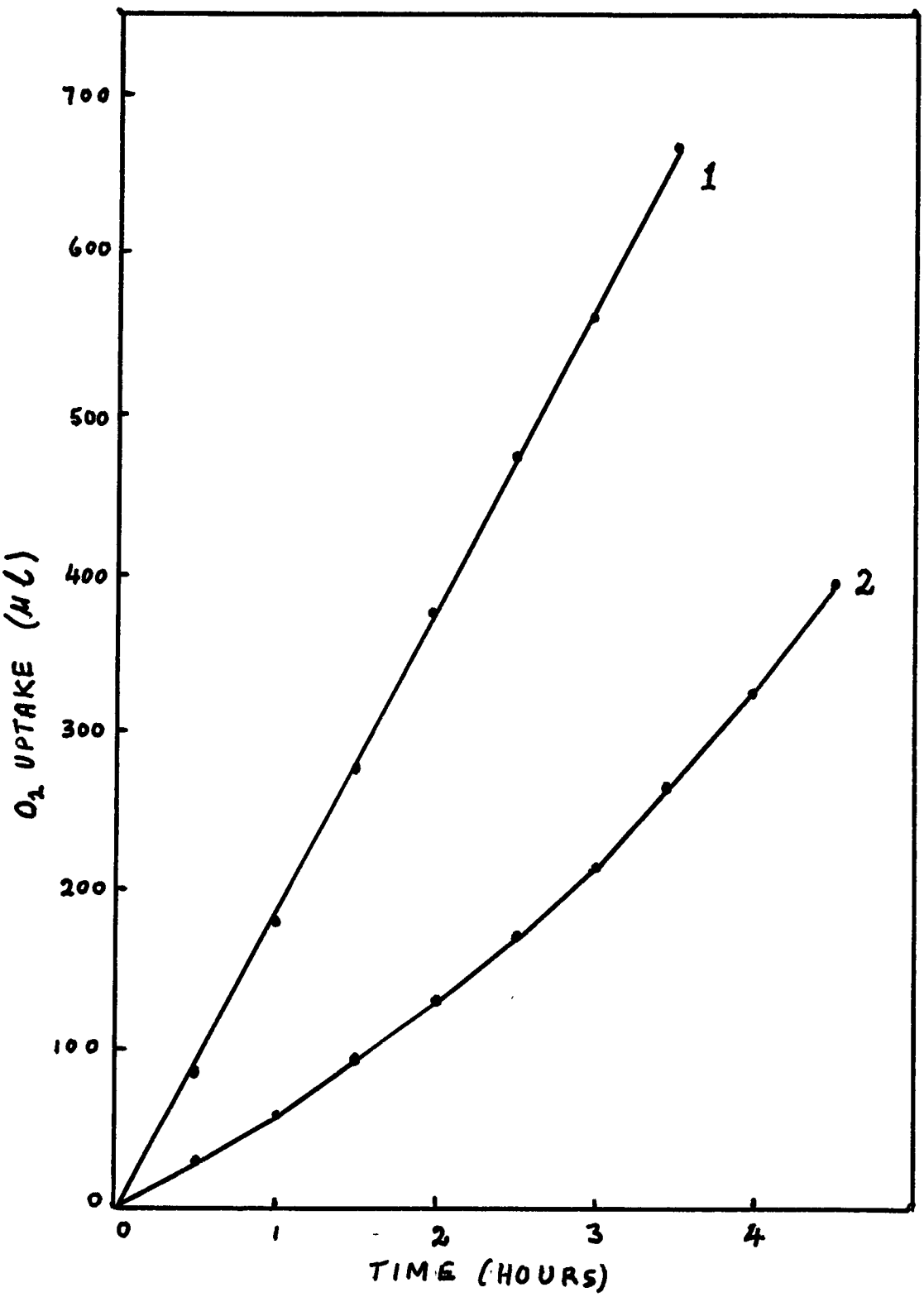

FIG. 2. Oxidation of putrescine by putrescine-adapted (curve 1) and unadapted (curve 2) strain C.C. cells.

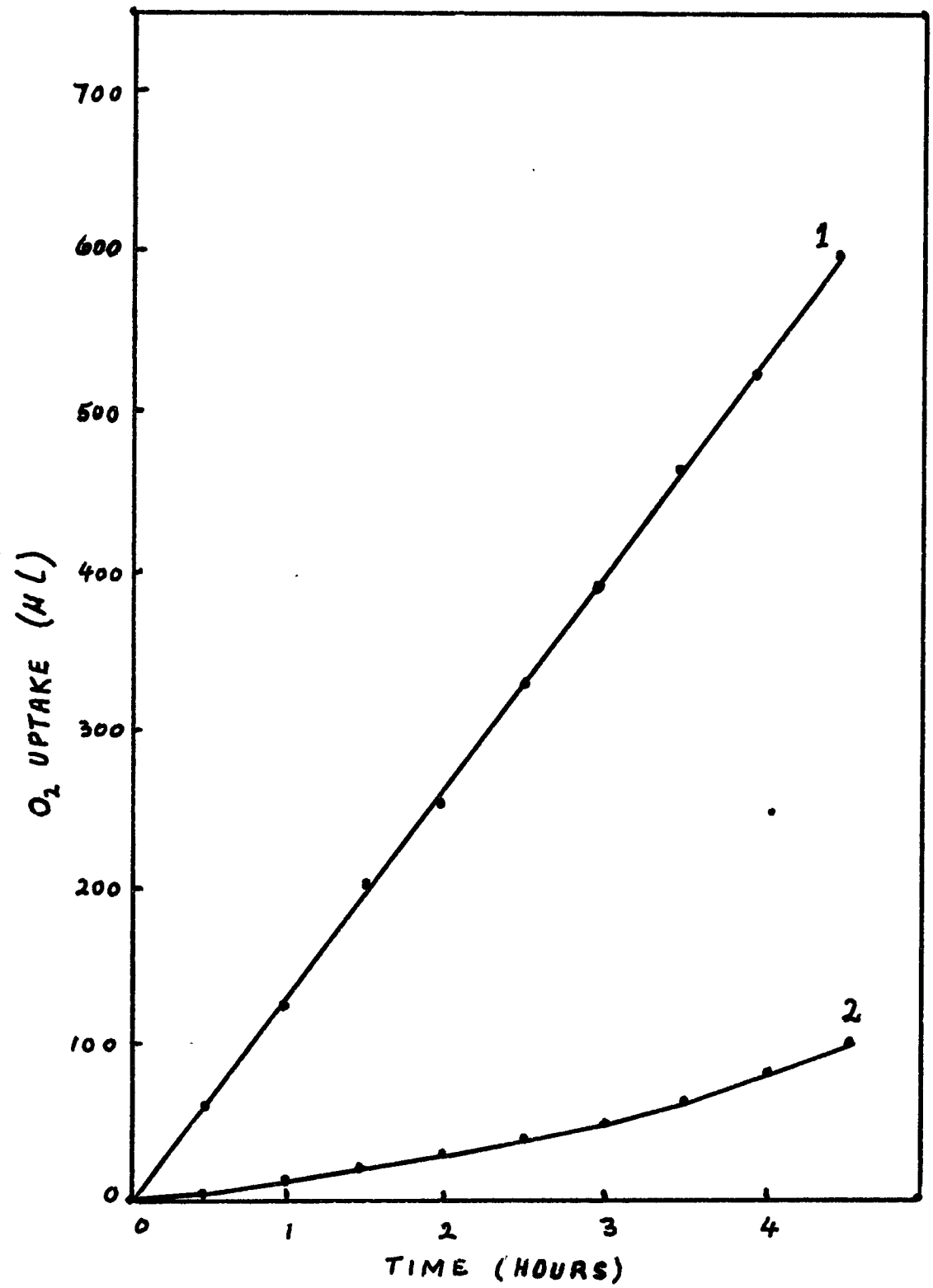

FIG. 3. Oxidation of putrescine by putrescine-adapted (curve 1) and unadapted (curve 2) strain S.C. cells.

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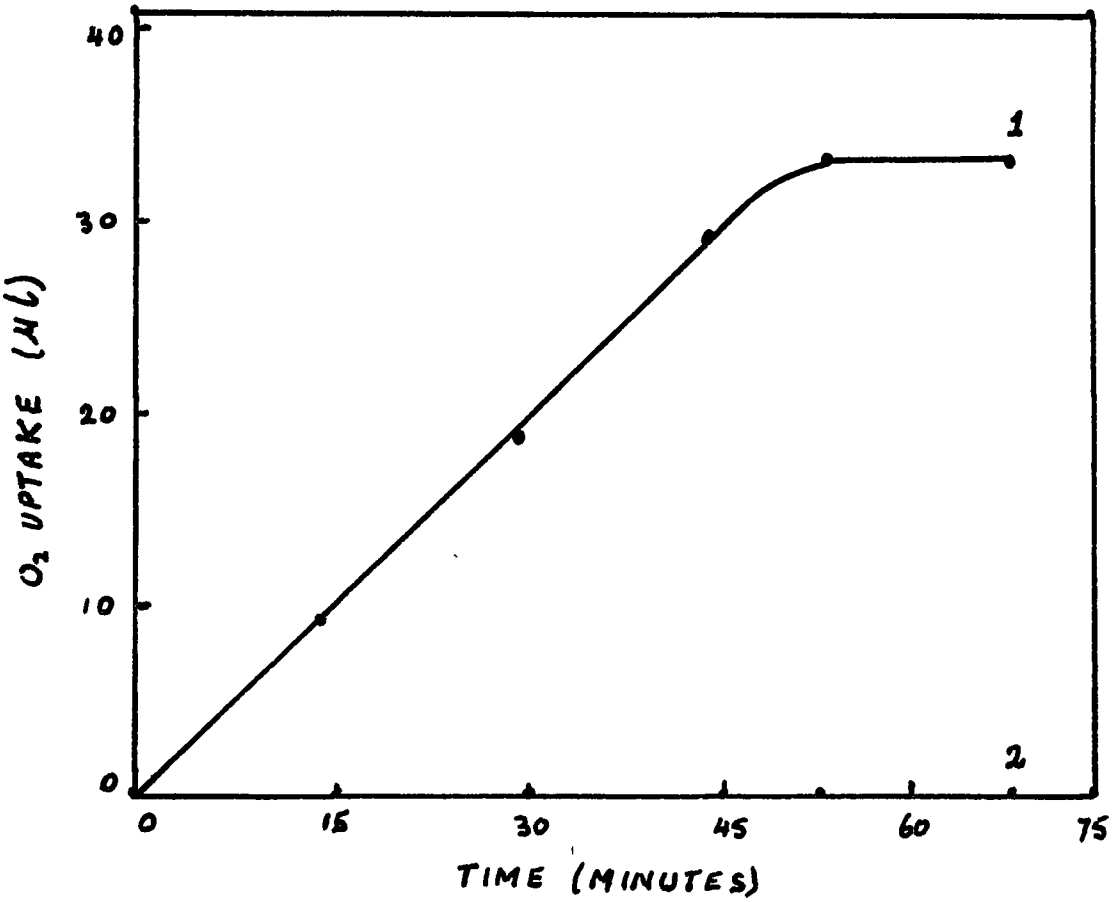

FIG. 4. Diamine oxidase activity in cell-free extracts obtained from putrescine-adapted (curve 1) and unadapted (curve 2) strain S.C. cells. Reaction mixtures contained in a volume of 3.0 ml.: Cell-free extract, 1.0 ml., equivalent to 3.0 mg. protein; putrescine, 3.0 μM; tris buffer, pH 9.0, 100 μM; H_2O.

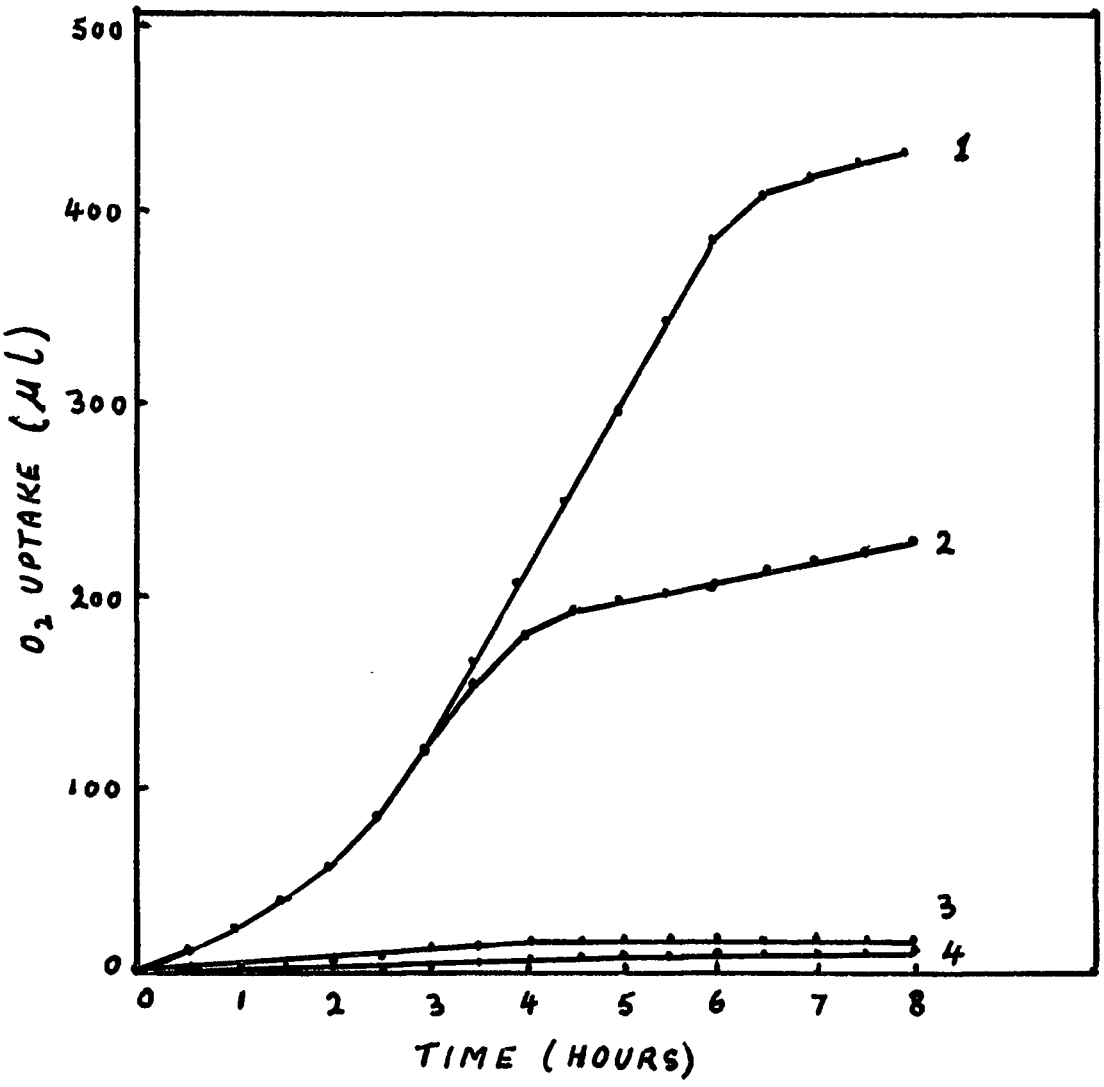

FIG. 5. Effect of pre-exposure to spermidine on the subsequent oxidation by strain C.C. cells of 7.5 μM of putrescine (curve 1), 3.25 μM of each of putrescine and 1,3-diaminopropane (curve 2), 7.5 μM of 1,3-diaminopropane (curve 3), and 7.5 μM of spermidine (curve 4).

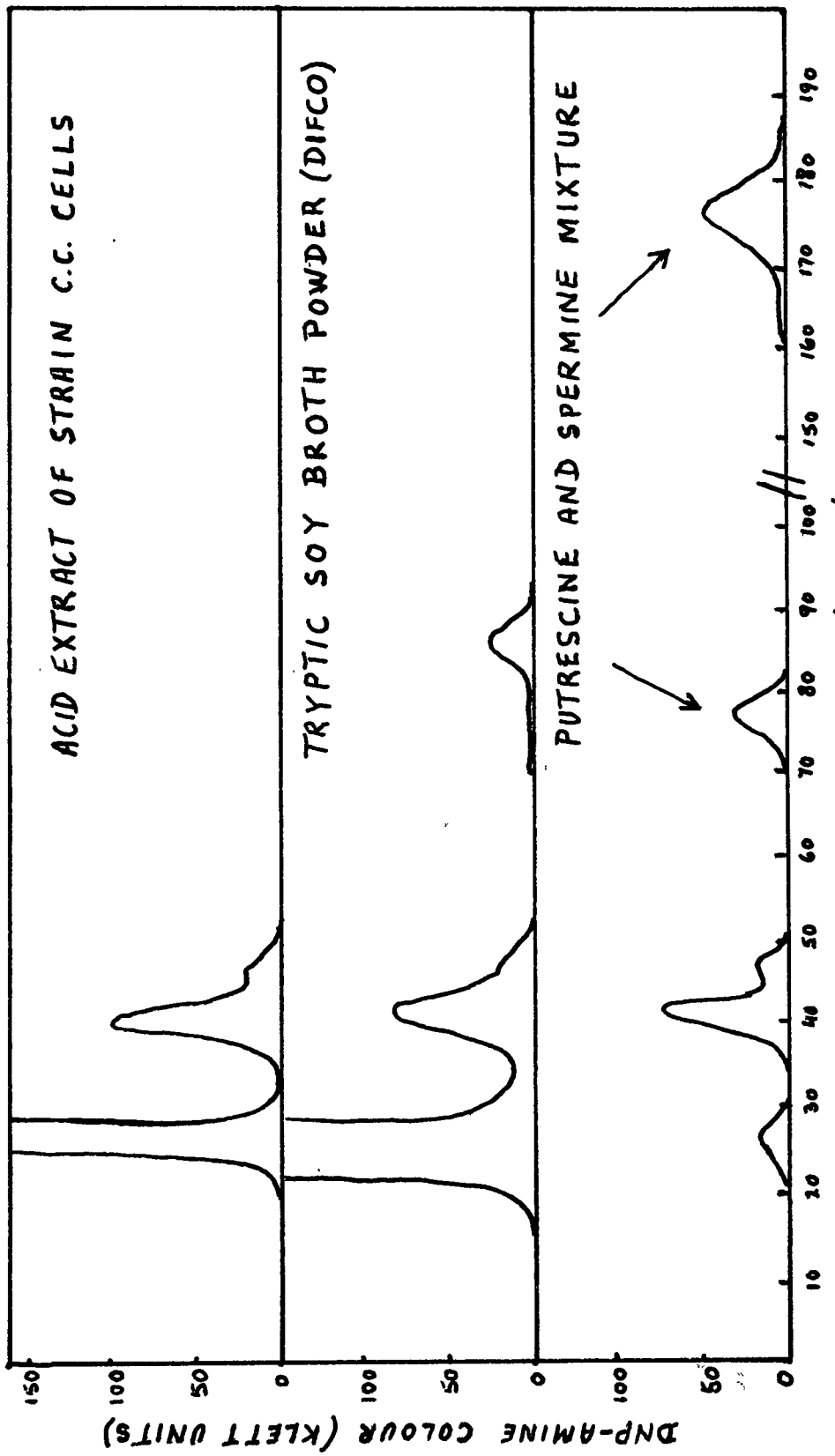

FIG. 6. SEPARATION OF AMINES BY ION EXCHANGE. SAMPLES WERE ELUTED FROM A COLUMN OF DOWEX 50 H^+ RESIN WITH A LINEARLY INCREASING CONCENTRATION OF HCL (0 – 1·5 N).

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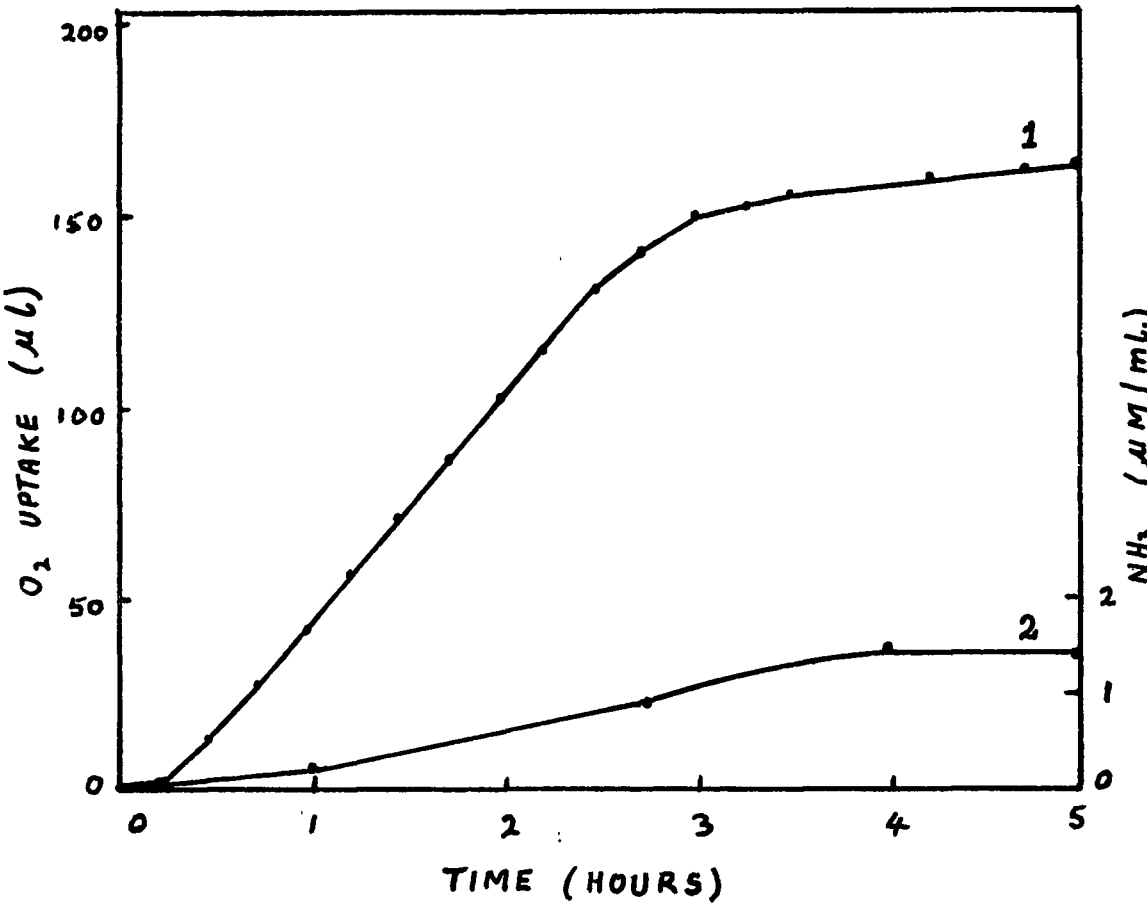

FIG. 7. Oxygen consumption and ammonia production during putrescine oxidation by strain N.T.N. cells. Curve 1: O_2 uptake with 3.0 µM putrescine (1.0 µM/ml.). Curve 2: NH_3 production with putrescine (1.0 µM/ml.).

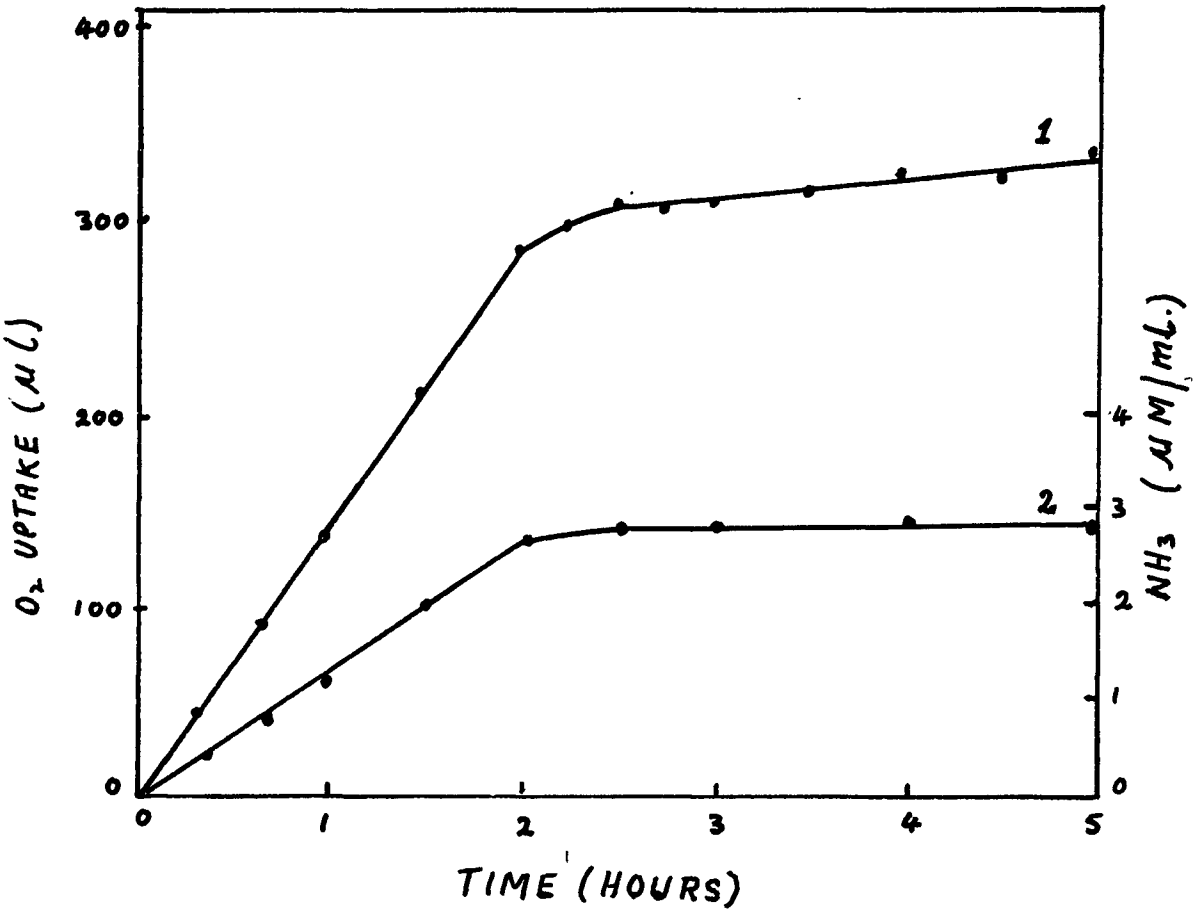

FIG. 8. Oxygen consumption and ammonia production during putrescine oxidation by strain S.C. cells. Curve 1: O_2 uptake with 6.0 μM putrescine (2.0 μM/ml.). Curve 2: NH_3 production with putrescine (2.0 μM/ml.).

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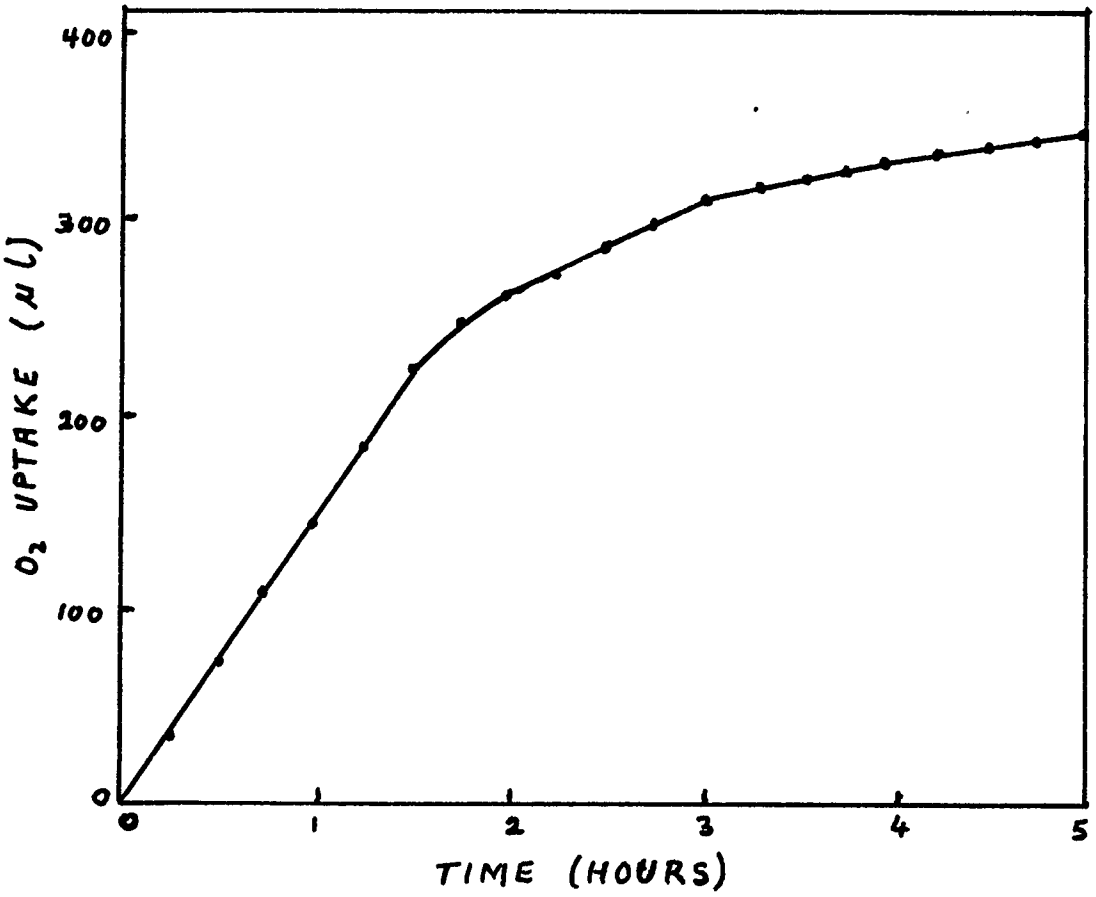

FIG. 9. Oxygen consumption during the oxidation of 6.0 μM of putrescine by strain C.C. cells.

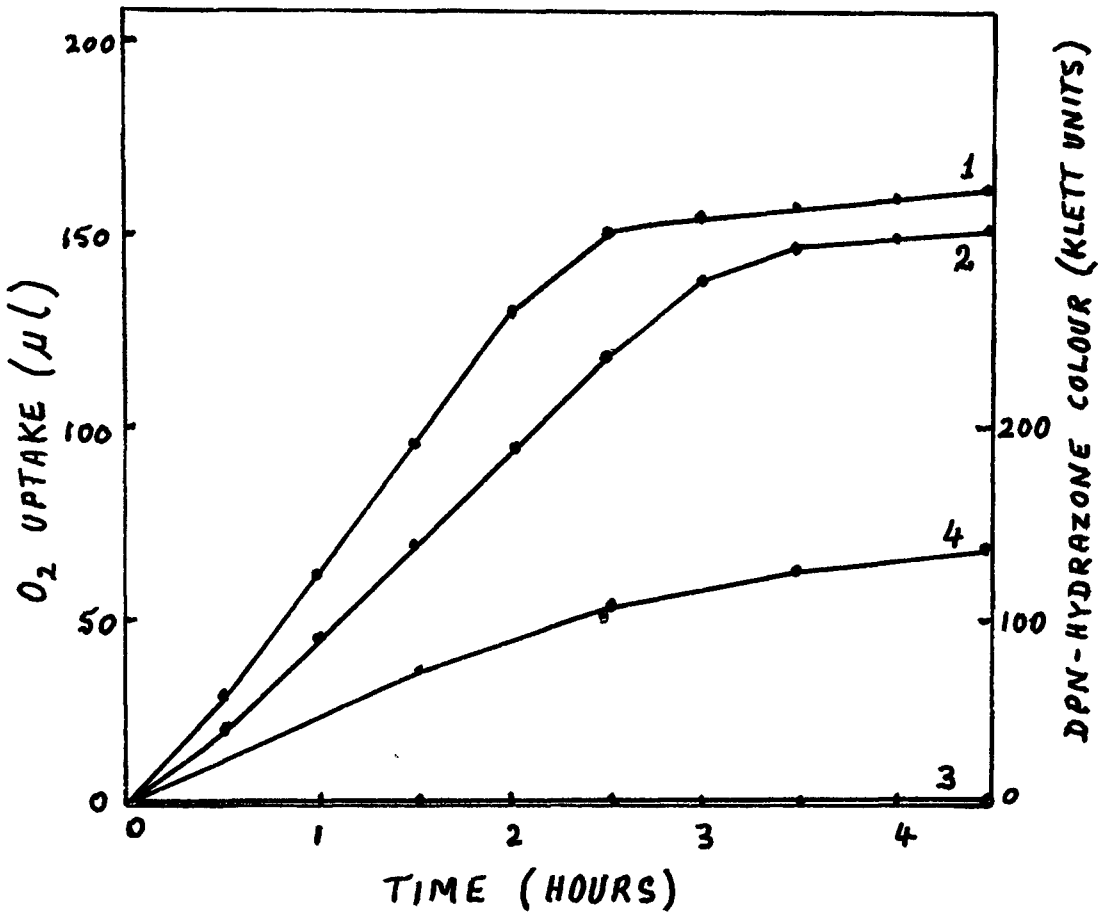

FIG. 10. Effect of arsenite on the metabolism of putrescine by strain S.C. cells.
Curve 1: O_2 uptake with 3.0 µM putrescine (1.0 µM/ml.).
Curve 2: Same as curve 1, but arsenite (3.0 µM/ml.) present.
Curve 3: Leakage of pyruvate with putrescine (1.0 µM/ml.).
Curve 4: Same as curve 3, but arsenite (3.0 µM/ml.) present.

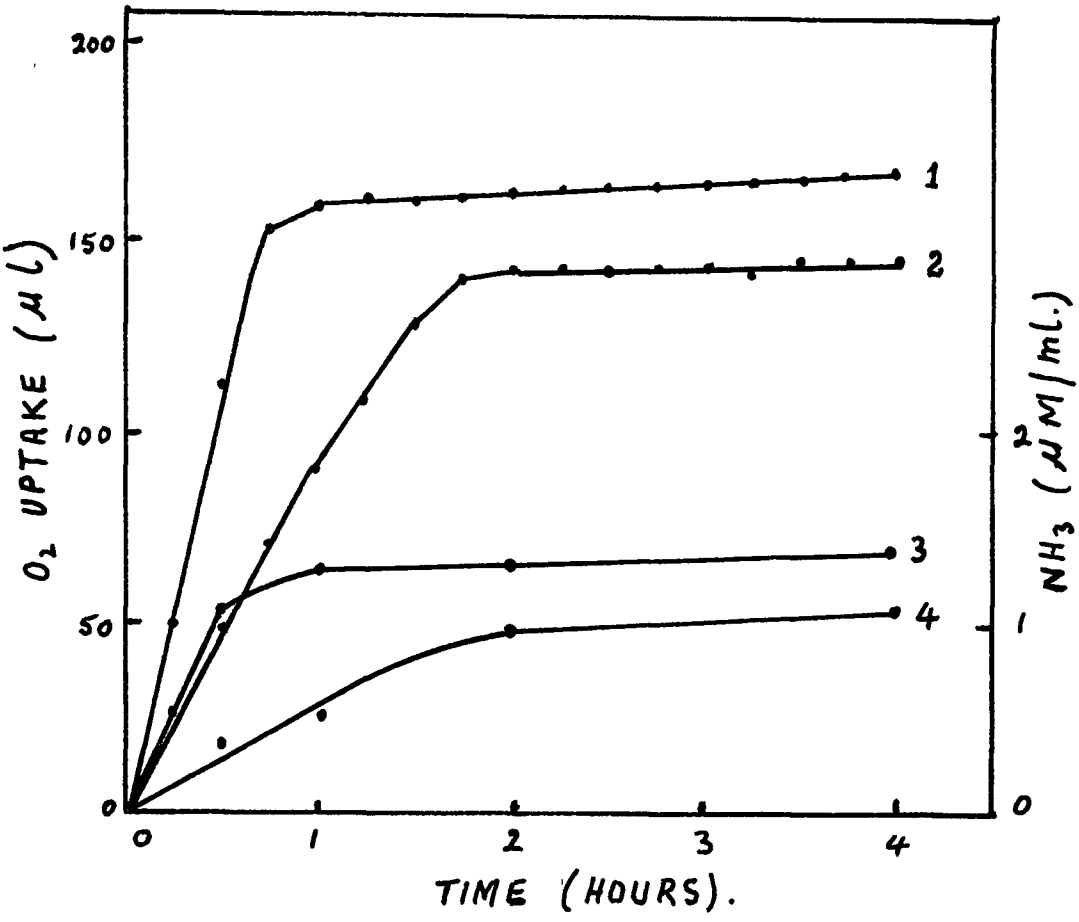

FIG. 11. Effect of arsenite on oxygen consumption and ammonia output by strain S.C. cells acting on putrescine.
Curve 1: O_2 uptake with 3.0 μM putrescine (1.0 μM/ml.).
Curve 2: Same as curve 1, but arsenite (9.0 μM/ml.) present.
Curve 3: NH_3 output with putrescine (1.0 μM/ml.).
Curve 4: Same as curve 3, but arsenite (9.0 μM/ml.)present.

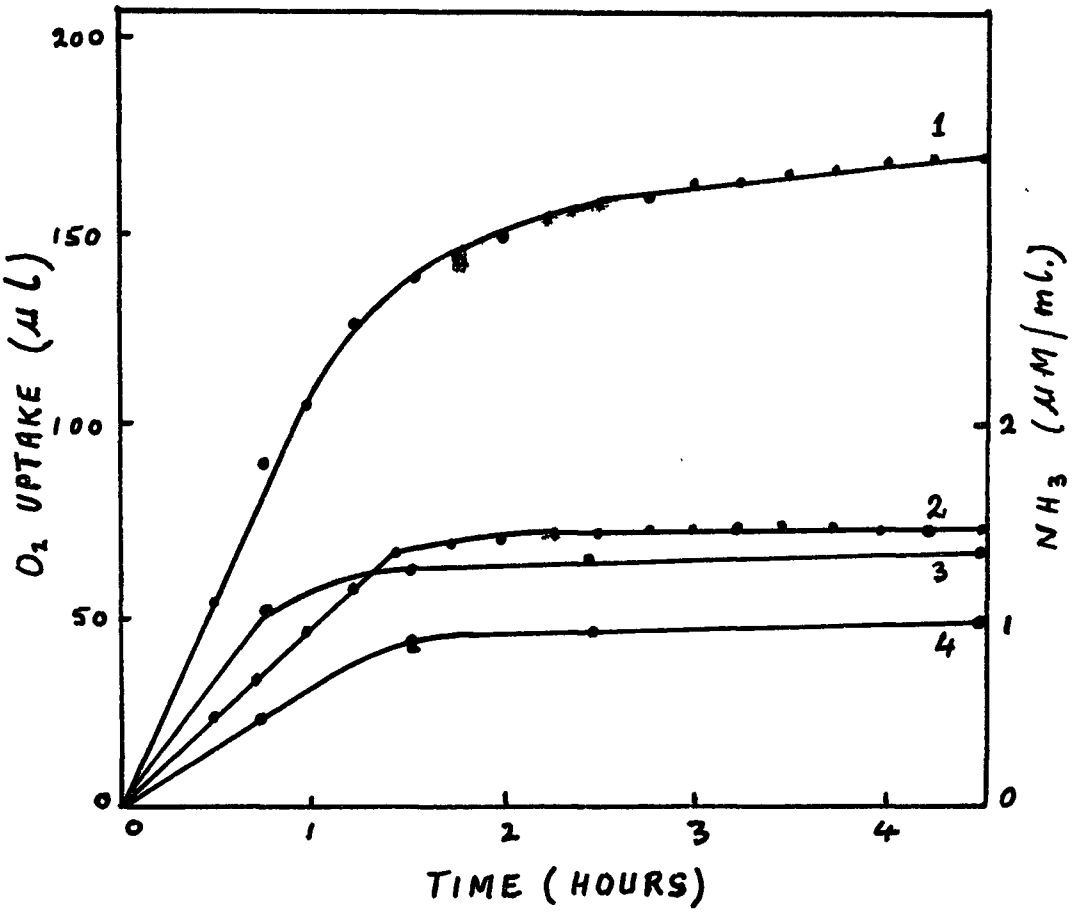

FIG. 12. Effect of arsenite on oxygen consumption and ammonia output by strain C.C. cells acting on putrescine. Curve 1: O_2 uptake with 3.0 μM putrescine (1.0 μM/ml.). Curve 2: Same as curve 1, but arsenite (3.0 μM/ml.) present. Curve 3: NH_3 production with putrescine (1.0 μM/ml.) present. Curve 4: Same as curve 3, but arsenite (3.0 μM/ml.) present.

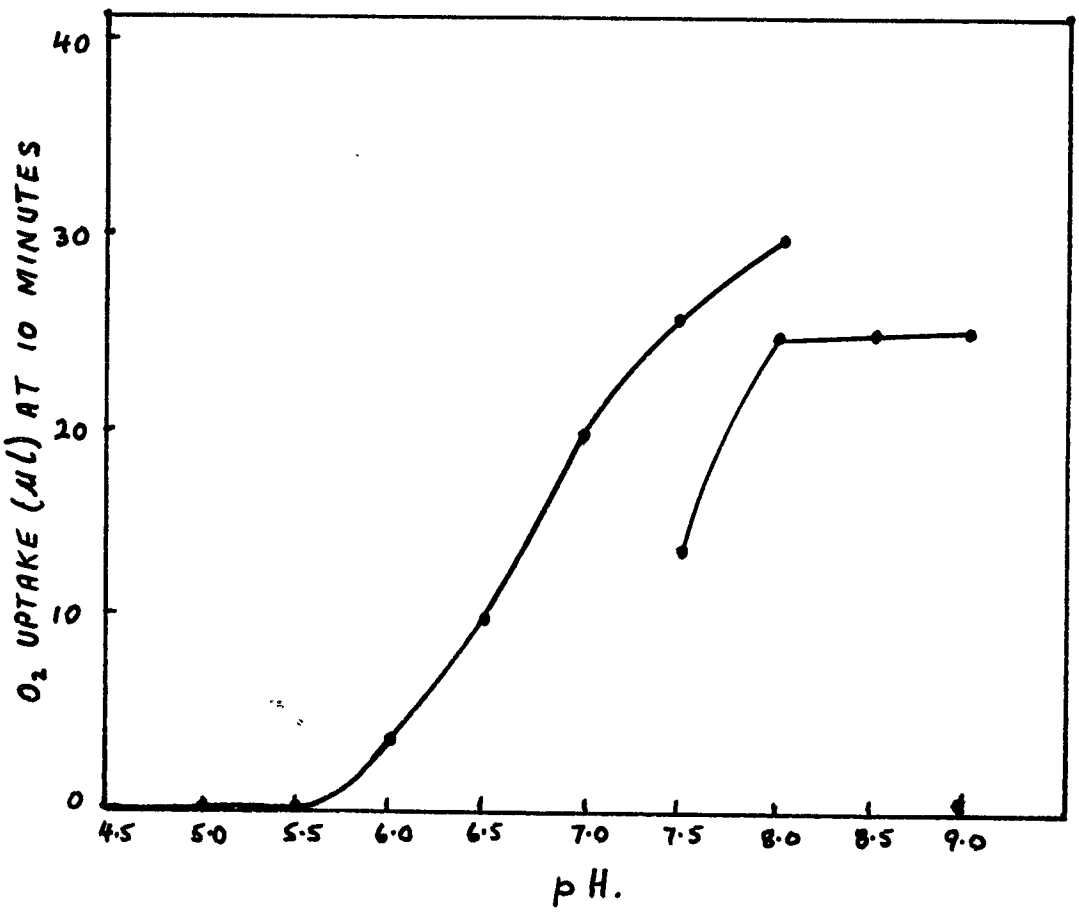

FIG. 13. The optimum pH for diamine oxidase activity. Reactions conducted in citrate (pH 4.5 - 6.0) and phosphate (pH 6.0 - 8.0) buffers (curve 1) and in tris buffer (pH 7.5 - 9.0) (curve 2).

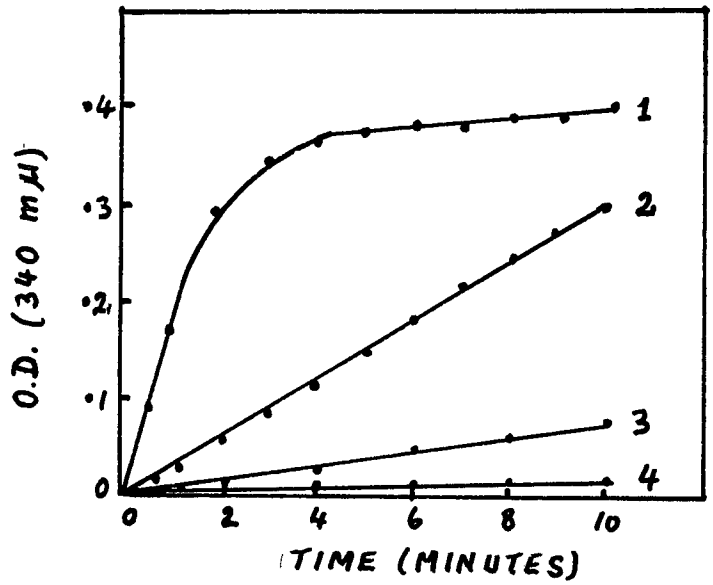

FIG. 14. Strain C.C. Δ'-pyrroline dehydrogenase activity (pH 9.0).
Curve 1: Mercaptoethanol + NAD.
Curve 2: Mercaptoethanol + NADP.
Curve 3: NAD.
Curve 4: NADP.

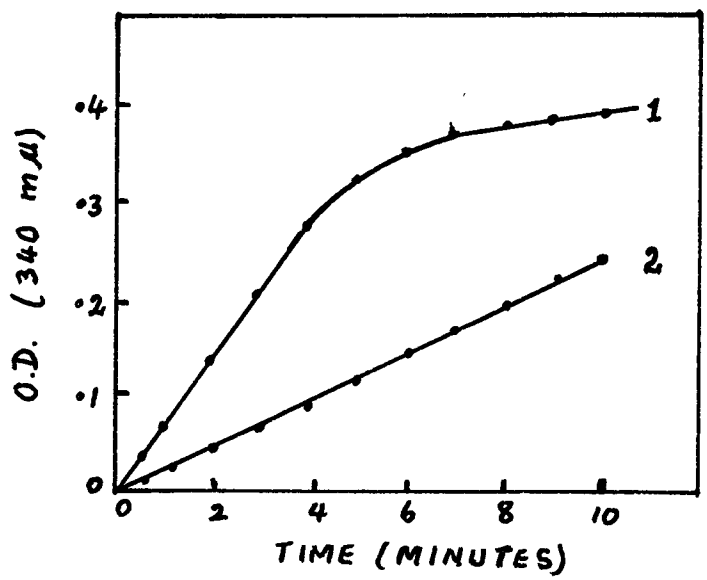

FIG. 15. Strain S.C. Δ'-pyrroline dehydrogenase activity (pH 9.0).
Curve 1: Mercaptoethanol + NAD.
Curve 2: Mercaptoethanol + NADP.

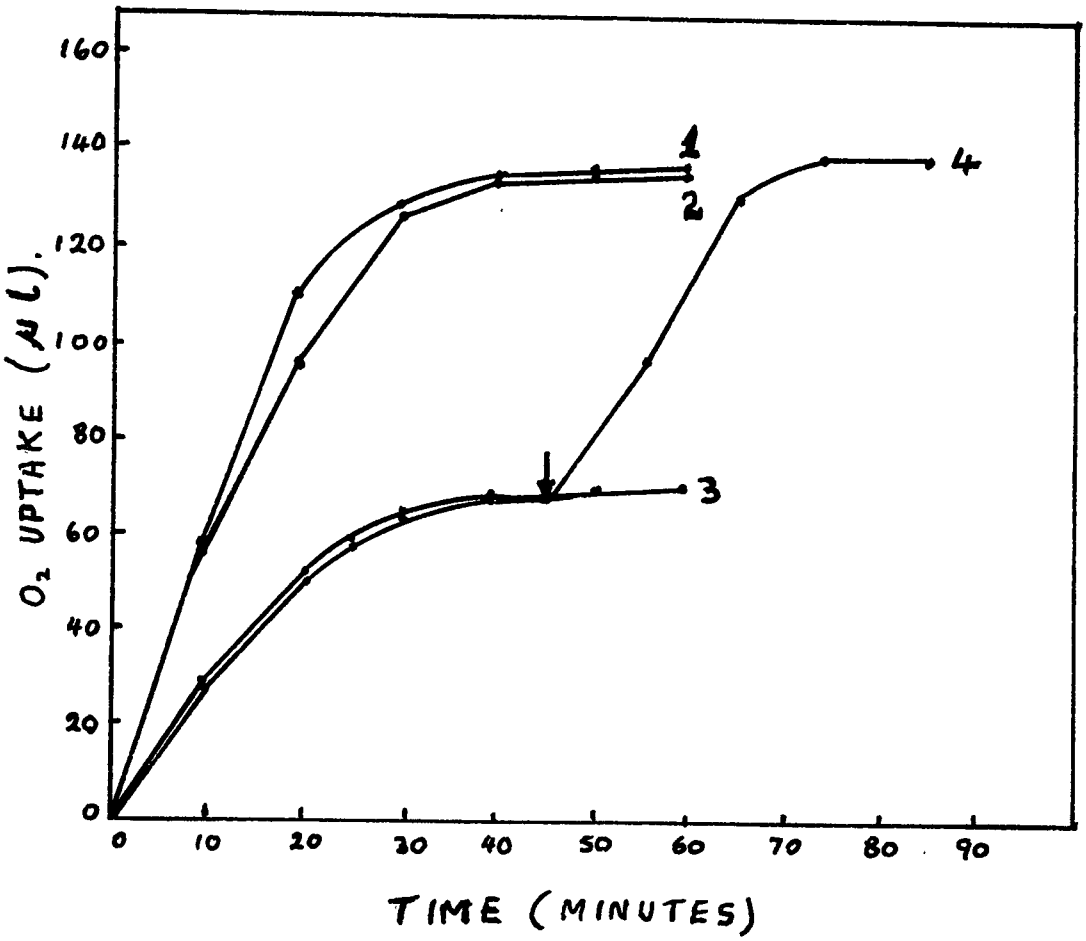

FIG. 16. Manometric assay of strain C.C. Δ'-pyrroline dehydrogenase activity (pH 7.2). See text for details.

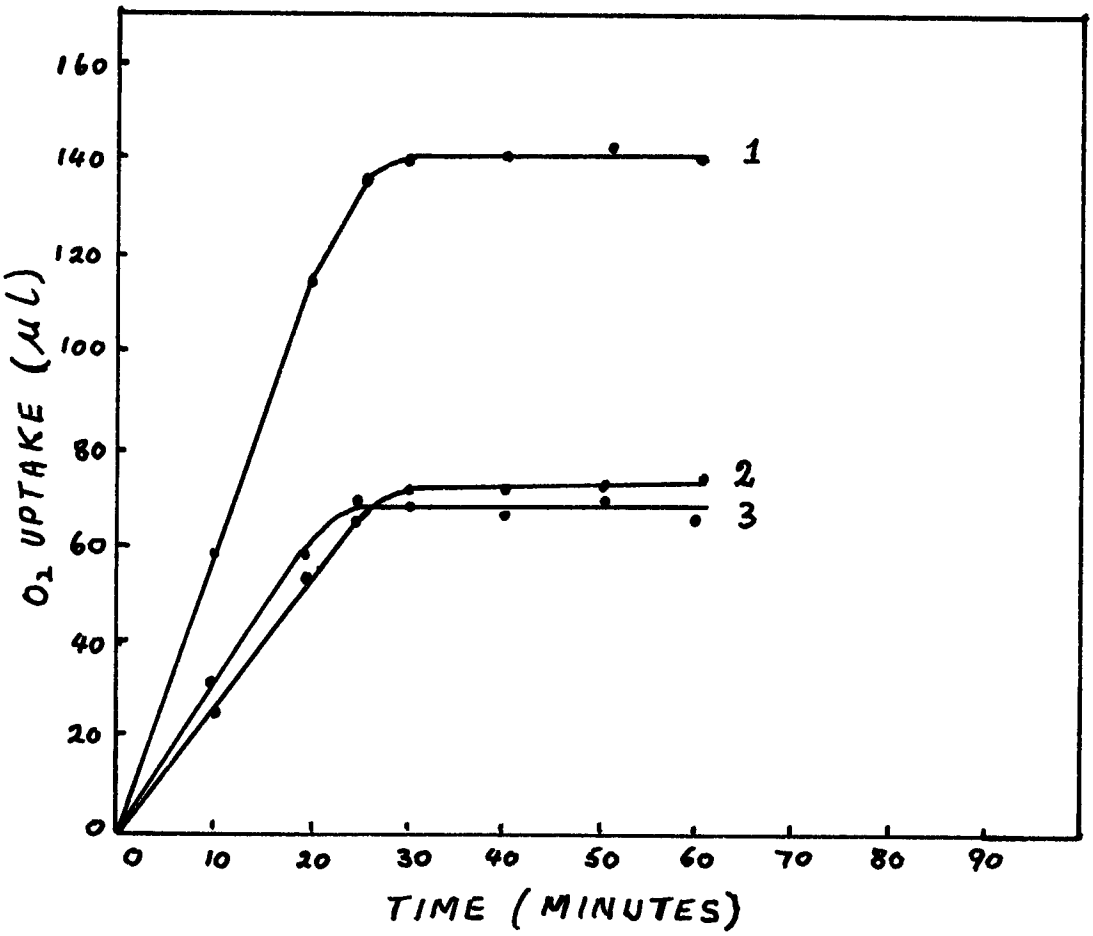

FIG. 17. Manometric assay of strain C.C. Δ'-pyrroline dehydrogenase activity (pH 9.0). See text for details.

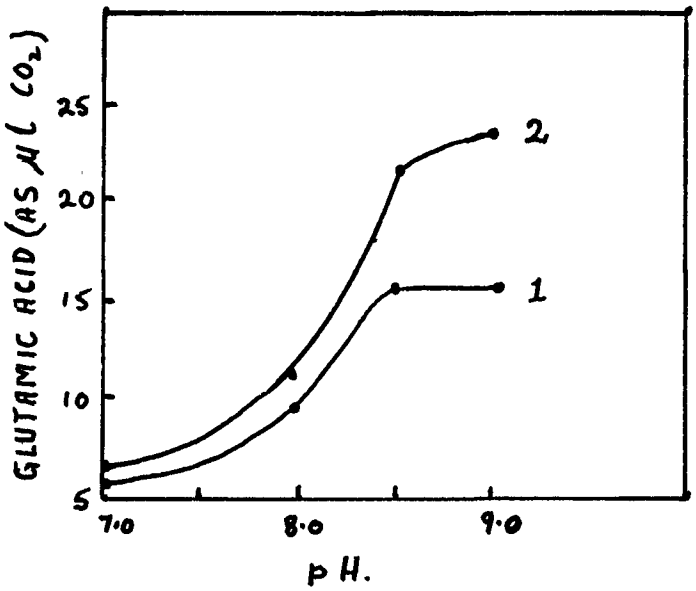

FIG. 18. γ-aminobutyric acid - α-ketoglutaric acid optimum pH curve. Glutamic acid synthesized at 30 mins. (curve 1) and at 60 mins. (curve 2).

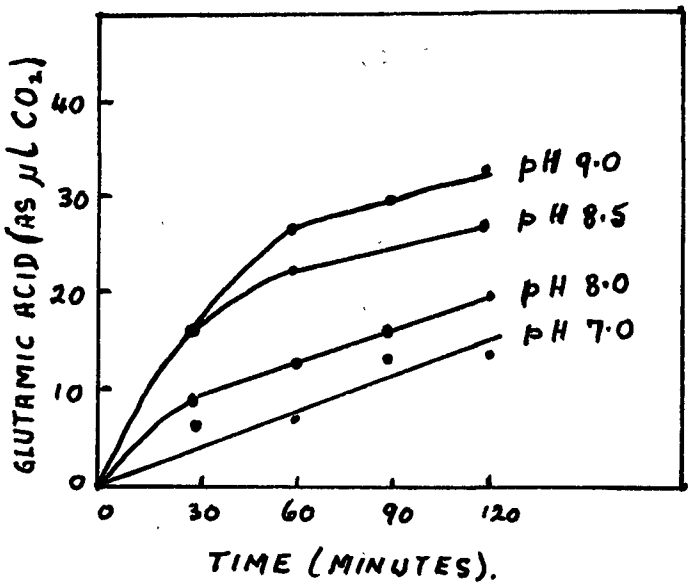

FIG. 19. Effect of pH and reaction time on γ-aminobutyric acid - α-ketoglutaric acid transaminase activity.

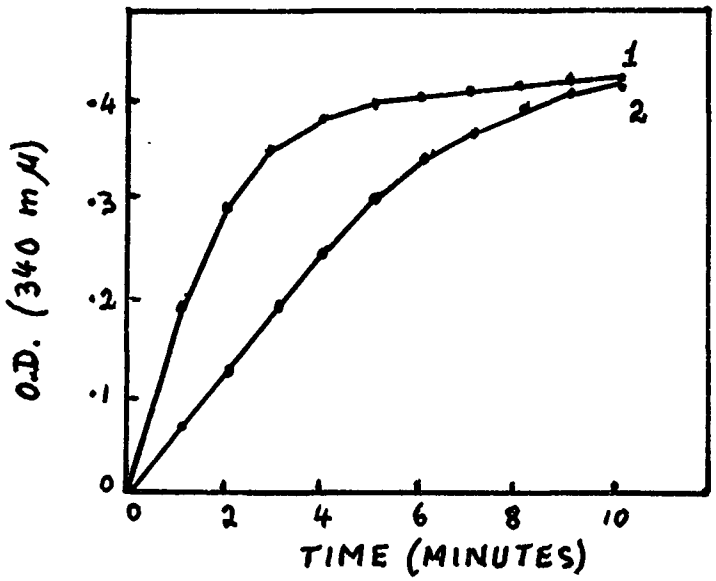

FIG. 20. Strain C.C. succinic semialdehyde dehydrogenase activity (pH 9.0).
Curve 1: NAD
Curve 2: NADP.

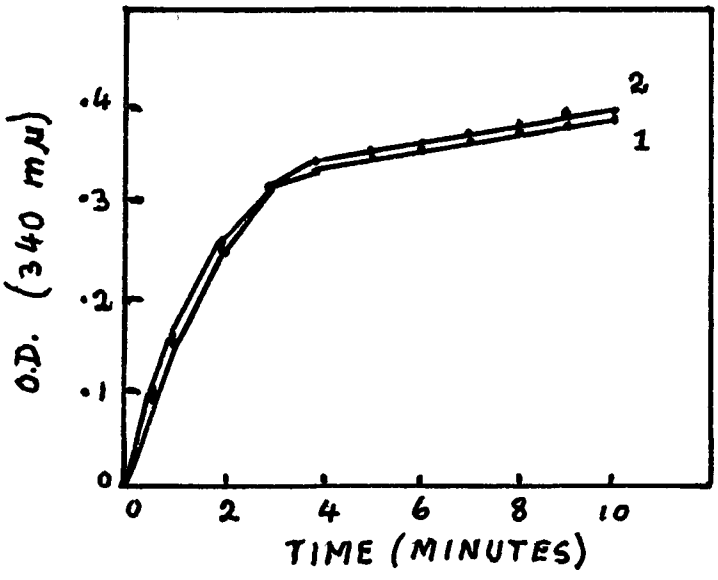

FIG. 21. Strain S.C. succinic semialdehyde dehydrogenase activity (pH 9.0).
Curve 1: NAD.
Curve 2: NADP.

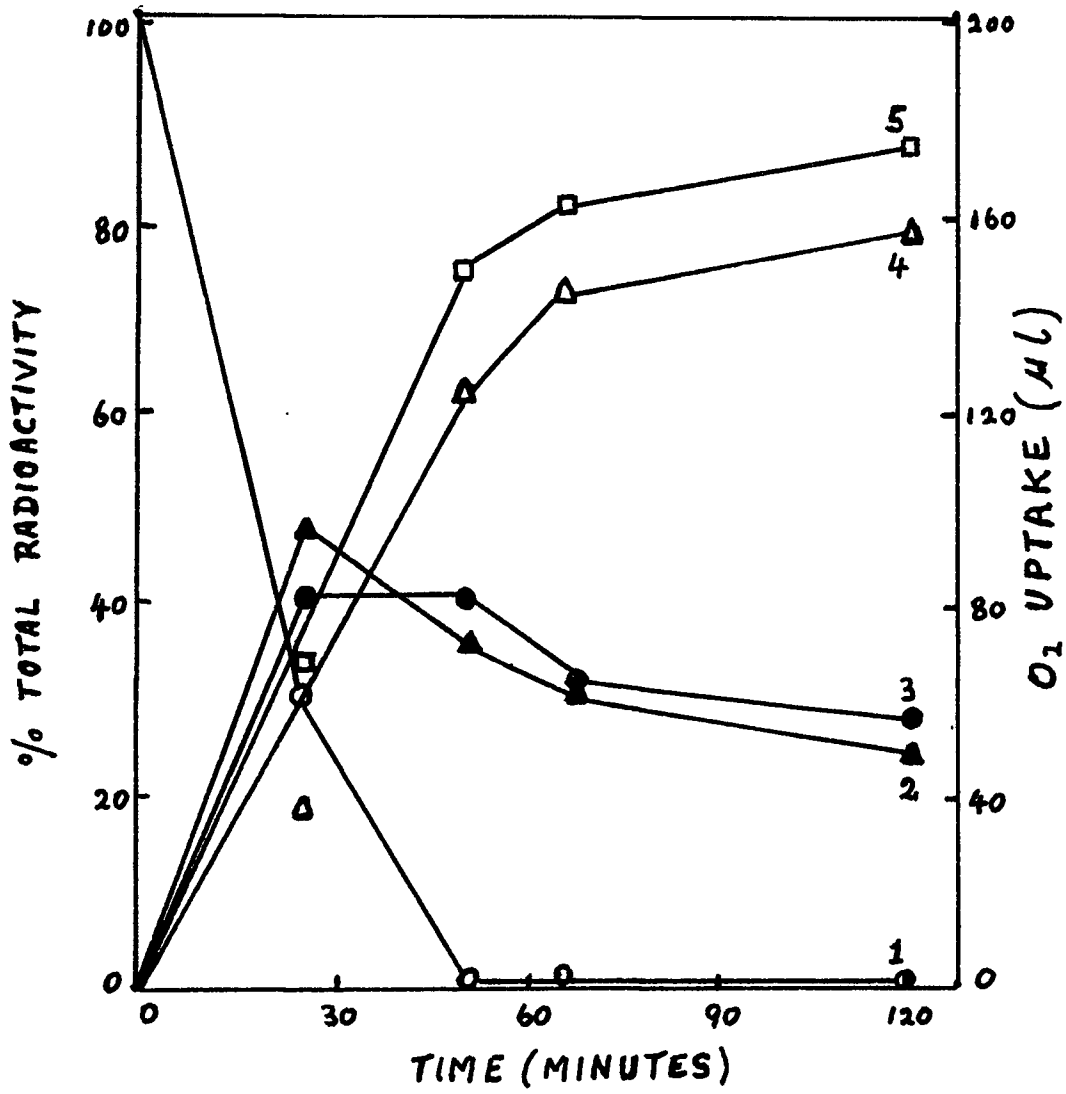

FIG. 22. Distribution of radioactivity during the oxidation of 1,4-C^{14} putrescine by putrescine-adapted strain N.T.N. cells. Reaction mixture supernatant (curve 1); combined cell fractions (curve 2); cells, calculated as the difference between cells + supernatant and supernatant (curve 3); $C^{14}O_2$ (curve 4); O_2 uptake occurring during the reaction (curve 5).