2. The yellow pigment has been identified as riboflavin which occurred at an average concentration of 19 $\mu\text{g./ml.}$ in pigmented samples compared with 0.5 $\mu\text{g./ml.}$ in colourless samples. Similar amounts of chloroform-soluble fluorescent material were also present.

3. The niacin, thiamine and fructose levels of the yellow samples were not significantly elevated.

4. Exposure to light caused the yellow semen to fade and this was accompanied by the breakdown of riboflavin to lumichrome. Ribitol, which might be expected to form in the process, is not oxidized by bull spermatozoa.

5. When riboflavin was added to normal white ejaculates at a concentration of 20 $\mu\text{g./ml.}$ it decreased the oxygen uptake and decreased the motility of the spermatozoa.

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Metabolism of Amines

2. THE OXIDATION OF NATURAL POLYAMINES BY *MYCOBACTERIUM SMEGMATIS**

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The degradation of natural polyamines by various bacteria has been described by Razin, Gery & Bachrach (1959). *Pseudomonas aeruginosa* was found to oxidize spermine and spermidine, γ -aminobutyric acid and β -alanine being intermediates in the reaction. Roulet & Zeller (1945) had already reported that *Mycobacterium smegmatis* was capable of oxidizing spermine. It was therefore decided to study polyamine oxidation by this

organism, and to compare the oxidation pathway with that of *P. aeruginosa* (Razin *et al.* 1959).

The degradations of γ -aminobutyric acid and β -alanine, which are intermediates in the oxidation of the polyamines, are also described in this paper.

MATERIALS AND METHODS

Organisms. *Mycobacterium smegmatis* strain 10143 was obtained from the American Type Culture Collection. *Pseudomonas aeruginosa* was the strain used previously (Razin *et al.* 1959).

* Part 1: Razin, Gery & Bachrach (1959).

Media. *M. smegmatis* was grown on the medium of Youmans & Karlson (1947), distributed in quantities of 100 ml. in Roux bottles. For adaptation, spermine was added to the medium to give a final concentration of 0.1%. *P. aeruginosa* was grown on the adaptation medium, as described by Razin *et al.* (1959).

Cell suspensions. *M. smegmatis* was grown at 37° for 3–4 days. During this incubation period a pellicle was formed on the surface of the medium. This pellicle was dispersed by shaking with glass beads and the resulting cells were washed three times with 0.85% NaCl solution and suspended in 0.067 M-phosphate buffer, pH 7.2; this suspension contained an equivalent of 10 mg. dry wt. of cells/ml. Cell suspensions of *P. aeruginosa* were prepared as described by Razin *et al.* (1959). Dried cell preparations were obtained by drying the cells over P₂O₅ at a pressure of 0.3 mm. Hg in a centrifugal freeze-drier (W. Edwards and Co. Ltd., Crawley, Sussex, England).

Chemicals. Succinic semialdehyde was prepared according to Prescott & Waelsch (1946). Malonic semialdehyde was prepared by the same method, aspartic acid being used instead of glutamic acid. All other chemicals were commercial products.

Manometric methods. Uptake of O₂ was measured by the conventional Warburg manometric technique, with air as the gas phase and a bath temperature of 30° (Umbreit, Burris & Stauffer, 1957). In anaerobic experiments H₂ served as the gas phase.

Identification of amines. Paper chromatography was used for qualitative and quantitative determination (Giri, Radhakrishnan & Vaidyanathan, 1952) of the amines and their degradation products. The cells of the tested reaction mixture were removed by centrifuging and 60 mm.³ of the supernatant was applied to each spot on Whatman no. 1 filter paper. Butanol-acetic acid-water (50:25:25, by vol.; Baker, Harborne & Ollis, 1952) was the standard solvent used. Other solvents included: 70% (v/v) ethanol (Miller & Rockland, 1952); pyridine-acetic acid-water (50:35:15, by vol.; Decker & Riffart, 1950); 95% ethanol-amylic alcohol-water (70:50:30, by vol.). The papers were sprayed with 0.2% (w/v) ninhydrin in butanol. β -Alanine was also identified by the bioassay method of Billen & Lichstein (1949) with *Saccharomyces fragilis* as test organism. Propane-1:3-diamine was separated by passage through an Amberlite IRC 50 column (Bergström & Hansson, 1951).

Identification of succinic and malonic semialdehydes. 2:4-Dinitrophenylhydrazones derivatives of the semialdehydes were prepared and analysed by paper chromatography according to Block, Durrum & Zweig (1958). The solvents used were: butanol-ethanol-0.5 N-ammonia solution (70:10:20, by vol.; El Hawary & Thompson, 1953); butanol-ethanol (80:20, v/v; Cavallini, Frontali & Toschi, 1949); butanol-N-NaHCO₃ (66:33, v/v; Seligson & Shapiro, 1952). The papers were sprayed with 2% (w/v) KOH in ethanol.

The semialdehydes were oxidized to the corresponding acids by alkaline H₂O₂ according to Shriner & Fuson (1948).

Identification of succinic acid and malonic acid. The acids were separated and identified by paper chromatography with ethanol-water-ammonia (80:10:10, by vol.) as solvent. The papers were sprayed with 0.04% (w/v) of bromocresol green in ethanol. Hydroxamate derivatives of the organic acids were prepared according to Feigl (1954) and separated by paper chromatography with water

saturated with phenol as solvent (Fink & Fink, 1949). The papers were sprayed with 1% (w/v) FeCl₃ solution. The acids were also separated and identified by ion-exchange chromatography by the method of Bush, Hulbert & Potter (1952), with Amberlite IRA 400 instead of Dowex 1.

RESULTS

Oxidation of polyamines by washed cells

Cells grown on Youmans-Karlson medium oxidized spermine, spermidine and putrescine after a lag period. The addition of spermine to this medium enhanced the initial rate of oxidation (Fig. 1). Cells grown on spermine were simultaneously adapted to spermidine and putrescine. During the oxidation of spermine 6 μ moles of oxygen were taken up and 2 μ moles of ammonia were set free for each μ mole of substrate oxidized. When spermidine served as substrate 8 μ moles of oxygen were taken up and 2.5 μ moles of ammonia released for each μ mole of substrate oxidized.

Oxidation of polyamines by freeze-dried cells

No ninhydrin-positive intermediates could be detected by paper chromatography during the oxidation of the polyamines by washed cells. When freeze-dried cells were employed, several ninhydrin-positive compounds accumulated during spermine oxidation. Fig. 2 shows that after 90 min. of incubation all spermine was oxidized, and that compounds corresponding to spots with R_f values 0.13, 0.24, 0.50 and 0.58 were formed, by using the standard butanol-acetic acid-water solvent system.

The compound with R_f 0.13 was identified as spermidine, by using all the solvents employed for the identification of the amines. The compound giving a spot with R_f 0.24 was isolated from the reaction mixture by Amberlite IRC 50 and identified as propane-1:3-diamine by paper chromatography with the standard solvent. The chloroplatinate derivative prepared from this compound (Wrede, 1926) blackened at 230° and sintered at 240° in the same way as the chloroplatinate of propane-1:3-diamine.

The compound having R_f 0.50 was identified as β -alanine by paper chromatography and by bioassay, and that giving the spot with R_f 0.58 was identified as γ -aminobutyric acid by paper chromatography, by using all the solvents employed for the identification of the amines.

Degradation of γ -aminobutyric acid and β -alanine

The fate of γ -aminobutyric acid and β -alanine, which were found to be intermediates in the oxidation of the polyamines by both *P. aeruginosa* (Razin *et al.* 1959) and *M. smegmatis*, was further investi-

gated. Transamination was found to be the first step in their degradation. A suspension of *P. aeruginosa* cells, containing the equivalent of approx. 5 mg. dry wt. of cells, was incubated with 10 μ moles of γ -aminobutyric acid and 10 μ moles of α -oxoglutaric acid in phosphate buffer, pH 7.2, under anaerobic conditions. After 90 min. of incubation, 3.4 μ moles of glutamate were formed, as determined by quantitative paper chromatography. During the transamination reaction succinic semialdehyde was produced. β -Alanine was converted into malonic semialdehyde in the presence of α -oxoglutarate or pyruvate under anaerobic conditions, by washed or freeze-dried cells of *P. aeruginosa*. Under aerobic conditions these semialdehydes were further metabolized. The semialdehydes were identified by paper chromatography of the 2:4-dinitrophenylhydrazone derivatives.

Succinate and malonate were obtained from the corresponding semialdehydes after oxidation with alkaline hydrogen peroxide. The acids formed were separated on an Amberlite IRA 400 column and finally identified by paper chromatography.

Transamination was also shown to be the first step in the degradation of γ -aminobutyric acid and

β -alanine by freeze-dried cells of *M. smegmatis*. Freeze-dried cells (50 mg.) were incubated with 20 μ moles of γ -aminobutyric acid and 20 μ moles of α -oxoglutarate in phosphate buffer, pH 7.2, under anaerobic conditions. After 90 min. of incubation, 2.2 μ moles of glutamate were produced as determined by quantitative paper chromatography. During the transamination reaction succinic semialdehyde was produced. When β -alanine was used instead of γ -aminobutyric acid, malonic semialdehyde was formed. Under aerobic conditions succinate and malonate were formed from γ -aminobutyric acid and β -alanine respectively. The acids formed were separated from the reaction mixture by Amberlite IRA 400, and finally identified as their hydroxamate derivatives.

Effect of inhibitors on polyamine oxidation

The effect of various inhibitors on the oxidation of spermine by *M. smegmatis* is shown in Table 1. Semicarbazide and isonicotinic acid hydrazide inhibited the oxidation of spermine. Dihydrostreptomycin (0.04 mM) did not inhibit the oxidation of putrescine. A partial inhibition was obtained with 2.3 mM-dihydrostreptomycin. The growth of the

Fig. 1

Fig. 1. Oxidation of polyamines by washed cells of *Mycobacterium smegmatis*. Each Warburg vessel contained 2.5 μ -moles of spermine (○) or spermidine (●), 1 ml. of cell suspension (approx. 10 mg. dry wt.) and 1 ml. of 0.067M-phosphate buffer, pH 7.2. In the centre well there was 0.2 ml. of 15% (w/v) KOH; the total volume was 3.2 ml. Incubation was carried out in air at 30°. —, Cells grown on Youmans-Karlson medium; - - -, cells grown on Youmans-Karlson medium containing 0.1% (w/v) of spermine.

Fig. 2

Fig. 2. Oxidation of spermine by freeze-dried cells of *Mycobacterium smegmatis*. Cells were grown on Youmans-Karlson medium containing 0.1% (w/v) of spermine. Experimental conditions were as given in Fig. 1, but freeze-dried cells (approx. 20 mg. dry wt.) and 2.5 μ moles of spermine were used. The figure represents a photograph of a chromatogram obtained with the butanol-acetic acid-water solvent.

Table 1. *Effect of inhibitors on the oxidation of amines by Mycobacterium smegmatis*

Experimental conditions were as given in Fig. 1, but the cells were grown on Youmans-Karlson medium containing 0.1% (w/v) of spermine and pre-incubated with the inhibitor for 30 min. Experiments were carried out for 3 hr.

Substrate (μ moles)	Inhibitor	Concn. (mm)	Inhibition of O ₂ uptake (%)
Spermine 2.5	Semicarbazide	15	86
		7.5	77
	<i>iso</i> Nicotinic acid hydrazide	7.5	64
		3.75	8.5
	Dihydrostreptomycin	2.3	60
		0.26	13
0.13		0	
Putrescine 10	Dihydrostreptomycin	2.3	16
		0.04	0

strain used was found to be inhibited by 0.3 μ g. of dihydrostreptomycin/ml. in Nutrient Broth (Difco).

DISCUSSION

The degradation of polyamines in *M. smegmatis* is similar to that found in *P. aeruginosa* (Razin *et al.* 1959). γ -Aminobutyric acid, β -alanine and propane-1:3-diamine appear to be intermediates in the reaction. These findings support the view that the pathway described for the bacterial degradation of the polyamines is a general one. Formation of propane-1:3-diamine from spermine by *Haemophilus parainfluenzae* and *Neisseria perflava* has been reported by Weaver & Herbst (1958*a, b*).

Transamination was found to be the first step in the breakdown of γ -aminobutyric acid and β -alanine by both *M. smegmatis* and *P. aeruginosa*. The transamination of γ -aminobutyric acid by *Mycobacterium avium* has already been described (Shoji, Mori & Ito, 1958). Succinic and malonic semialdehydes obtained as transamination products were further oxidized by freeze-dried cells of *M. smegmatis* to succinate and malonate respectively. These acids were not detected when freeze-dried cells of *P. aeruginosa* were used (U. Bachrach, unpublished work). Oxygen uptake during the oxidation of the polyamines by *M. smegmatis* was lower than by *P. aeruginosa*, indicating a difference in the final steps of the degradation of spermine and spermidine by these organisms.

The higher oxygen uptake during the oxidation of spermidine as compared with spermine, already described for *P. aeruginosa* (Razin *et al.* 1959), was found to occur also with *M. smegmatis*. No explanation can yet be given for this observation; similar unexplained high oxygen uptakes during the oxidation of several amines have been reported (Gale, 1942).

The oxidation of polyamines by *M. smegmatis* was found to be inhibited by compounds also

inhibiting polyamine oxidation by *P. aeruginosa* (Razin *et al.* 1959). Zeller, Owen & Karlson (1951) found that dihydrostreptomycin inhibited the oxidation of putrescine and other amines by *M. smegmatis*. Their results could not be confirmed by us; even at high concentrations of dihydrostreptomycin the oxidation of putrescine by the same organism was hardly affected. However, dihydrostreptomycin inhibited the oxidation of putrescine by cells of *M. smegmatis* unadapted to this amine (Persky, 1959). These findings suggest that the inhibitory effect of dihydrostreptomycin described by Zeller *et al.* (1951) might be due to inhibition of adaptive amine-oxidase formation, and not a direct effect on oxidation (*cf.* Geronimus, 1951). The inhibition of adaptive-enzyme formation by streptomycin is well known (Eagle & Saz, 1955).

SUMMARY

1. Spermine and spermidine were oxidized by cells of *Mycobacterium smegmatis*. The oxidation was enhanced by the incorporation of spermine into the culture medium.

2. Spermidine, γ -aminobutyric acid, propane-1:3-diamine and β -alanine were found to be intermediates in the oxidation of spermine by freeze-dried cells.

3. Cells of *M. smegmatis* and *Pseudomonas aeruginosa* degraded γ -aminobutyric acid and β -alanine by transamination with the formation of succinic and malonic semialdehydes. The semialdehydes were further oxidized to succinate and malonate by freeze-dried cells of *M. smegmatis*.

4. Semicarbazide and isonicotinic acid hydrazide inhibited the oxidation of spermine by *M. smegmatis*. Dihydrostreptomycin did not affect the oxidation of putrescine.

5. The possibility of a general pathway for the bacterial degradation of spermine and spermidine is discussed.

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Oxidative Metabolism of Phthalic Acid by Soil Pseudomonads

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Phthalic acid is removed from chemical waste effluents by biological purification through filter beds. This has led to an investigation of the oxidative metabolism of this aromatic compound by the micro-organisms responsible for this action industrially, and from the soil.

In mammals, e.g. dog and man, phthalic acid is largely excreted unchanged, and does not appear to form glycine conjugates (Shemyakin & Schukina, 1944). Jacobs (1931), on very slender evidence, suggested that phthalic acid was an intermediate in the dissimilation of naphthalene by soil micro-organisms; Fernley & Evans (1958) have shown this view to be incorrect.

From young liquid cultures of a soil pseudomonad growing on phthalate, Evans (1955) identified, by chromatography, the presence of 4:5-dihydroxyphthalate. Application of the technique of sequential induction revealed that this acid and protocatechuic acid were immediately metabolized

by phthalate-grown cells. The adaptation to protocatechuic acid was not conclusive evidence of its being an intermediate, since it might have arisen from *p*-hydroxybenzoic acid, a known anabolic product of most bacteria. Further, a hitherto undescribed enzyme reaction (a non-oxidative aromatic decarboxylation) would have been implied. We have extended this study, a preliminary report of which has been presented (Ribbons & Evans, 1959a, b).

EXPERIMENTAL

Isolation of organism. Microbes isolated from filter beds actively detoxicating phthalic acid at Monsanto Chemicals, Ruabon, were kindly made available through the courtesy of Dr G. Wilson and Miss M. Harrison. Our own isolates were obtained by the elective-culture method from garden soil, manure and coniferous litter. The enrichment medium, adjusted to pH 7 with NaOH, contained the following (g./l.): phthalic acid, 1; $(\text{NH}_4)_2\text{SO}_4$, 1; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.5;