Oxidation of *meso*- α,ϵ -Diaminopimelic Acid by Certain Sporulating Species of Bacteria

BY MANI ANTIA* AND ELIZABETH WORK†

*Department of Chemical Pathology, University College Hospital
Medical School, London, W.C.1*

(Received 2 February 1961)

SUMMARY

Investigations were made of the transformations undergone by the stereoisomers of α,ϵ -diaminopimelic acid in suspensions of acetone-dried organisms of two species of sporulating bacteria, *Sporosarcina ureae* and *Bacillus sphaericus*, both of which contain diaminopimelic acid in their spores but not in their vegetative cells. *Meso*-diaminopimelic acid was rapidly decarboxylated by vegetative organisms of both species; it was also utilized by some other unidentified anaerobic reaction. The vegetative organisms also oxidized *meso*-diaminopimelic acid with release of ammonia. L-Lysine was oxidized by *S. ureae*, but not by *B. sphaericus*. Neither LL- nor DD-diaminopimelic acid was attacked by either organism.

Disintegrated spores of *Bacillus sphaericus* did not oxidize *meso*-diaminopimelic acid, but decarboxylated it and also utilized it by the unidentified anaerobic reaction. The decarboxylation, but not the oxidation, of diaminopimelic acid by *Sporosarcina ureae* was greatly stimulated by pyridoxal phosphate; both reactions were inhibited by the same compounds. Study of the oxidation was complicated by the side reactions which occurred with *S. ureae*, but a simpler system was provided by an asporogenous variant of *B. sphaericus* which did not decarboxylate diaminopimelic acid without added pyridoxal phosphate. Only one equivalent of ammonia was produced, a small amount of CO₂ was evolved and two equivalents of oxygen were utilized; no oxidation product was identified. The methods of attacking diaminopimelic acid by these two atypical species are compared and discussed in relation to other species in their respective families.

INTRODUCTION

α,ϵ -Diaminopimelic acid differs from the majority of the common natural amino acids in being confined almost exclusively to bacteria where it occurs mainly in the cell walls (Work & Dewey, 1953; Work, 1957*a*, 1961). However, certain of its stereoisomers undergo enzymic reactions resembling those of other amino acids: thus, in many types of bacteria the *meso*-isomer is decarboxylated to L-lysine (Dewey, Hoare & Work, 1954) and is also racemized to the LL-isomer (Antia, Hoare & Work, 1957); all three isomers can transaminate (Meadow & Work, 1958*a*) while L-amino acid oxidases of *Neurospora* and snake venoms oxidize *meso*- and LL-diaminopimelic acid (Work, 1955). Studies on the decarboxylation of *meso*-diamino-

* Present address: Glaxo Laboratories (Ceylon), Ltd., Ratmalana, Ceylon.

† Present address: Twyford Laboratories, Twyford Abbey Road, London, N.W.10.

pimelic acid by acetone-dried bacteria (Dewey *et al.* 1954) have not so far suggested that many of these preparations can carry out other types of reaction with the amino acid. Thus, the rate of decarboxylation was not usually affected by the presence of oxygen, and the volume of gas evolved, both aerobically and anaerobically, corresponded to that expected from the release of one mole CO_2 /mole *meso*-diaminopimelic acid added. In the two species now used, *Sporosarcina ureae* and *Bacillus sphaericus*, neither of these conditions was found; the rate of CO_2 evolution from *meso*-diaminopimelic acid by acetone-dried organisms was apparently lowered by the presence of air, and the amount of CO_2 produced was less than stoichiometric even under anaerobic conditions.

Certain relationships between bacterial classification and the distribution of diaminopimelic acid and of its decarboxylase and racemase have been established in a few families (Work & Dewey, 1953; Antia *et al.* 1957; Dewey, 1954; Hoare & Work, 1957; Cummins & Harris, 1956*a, b*; Cummins, 1956). As a consequence, the species studied in this communication can be differentiated from other members of their families by their content of diaminopimelic acid and of enzymes attacking it (Table 2). *Sporosarcina ureae* is a spore-forming member of the family Micrococcaceae. Typical organisms in this family show diaminopimelic acid decarboxylase and racemase activities, but do not contain the amino acid itself (Antia *et al.* 1957; Cummins & Harris, 1956*b*); *S. ureae* has an active decarboxylase but no racemase. As described in this paper it has diaminopimelic acid only in its spores and not in its vegetative cells. *Bacillus sphaericus* is classified with the other aerobic spore-forming bacilli, most members of which contain diaminopimelic acid and its racemase but no decarboxylase. No diaminopimelic acid racemase was found by Powell & Strange (1957) in *B. sphaericus*, but there was a very active decarboxylase; diaminopimelic acid itself was not present in vegetative cells, but was present in the spores.

This paper describes the reactions undergone by diaminopimelic acid in suspensions of acetone-dried vegetative cells of these two species of bacteria which are atypical with respect to diaminopimelic acid content and metabolism. The anomalies of aerobic decarboxylation were due to oxidation, which has not been hitherto observed in bacteria.

METHODS

Organisms. The strain of *Sporosarcina ureae* studied originated from the Microbiological Laboratory, Technical High School, Delft. Another strain from the Edinburgh and East of Scotland College of Agriculture was examined briefly. The organism was subcultured weekly in a medium (called hereafter urea medium) containing urea 0.5% (w/v), Lab-Lemco 1% (w/v), peptone (Oxoid) 1% (w/v), adjusted to pH 7.0 with NaOH and filtered. The organism was grown at room temperature (18°) and cultures were stored at 0°. Other media used for this organism were nutrient broth fortified with tryptic digest of casein (0.5 g. nitrogen/100 ml.) called TMB broth, the CCY medium of Gladstone & Fildes (1940), and the meat extract-peptone of Tarr (1933). Preparations of the vegetative form were grown with shaking at 18°; for each 100 ml. of medium, 1 ml. of a 48 hr. culture in the urea medium was used as inoculum after adjusting to a standard optical density. After 24 or 48 hr. growth, the organisms were harvested by centrifugation,

washed twice with 0.9% (w/v) NaCl solution and acetone-dried in the cold. They were stored at -15° .

Bacillus sphaericus was var. *fusiformis* NCTC 7582, and its asporogenous variant; these strains were studied by the late Mrs J. F. Powell and her colleagues (Powell & Strange, 1957; Powell 1958; Powell & Hunter, 1955). The organisms were grown at 37° by Mrs Powell in potato-extract medium enriched with CCY (Meadow & Work, 1958*b*); for vegetative preparations the growth period was 10 hr.; for spore preparations it was 48 hr. The harvested organisms were washed three times with water, and either acetone-dried or freeze-dried and stored at -15° . The spores were disintegrated mechanically in a Mickle disintegrator for 40 min. at 0° (20 mg. dry wt./ml.) in the presence of thiolacetic acid (mM) with octyl alcohol as antifoaming agent.

Other organisms. The strains and growth conditions were described by Antia *et al.* (1957).

Enzymic reactions. Decarboxylation and oxidation were measured in Warburg manometers at 37° . In both cases 0.1 M-phosphate buffer (pH 6.8) was used with 20–40 mg. dried organism suspended in a total volume of 2.5 ml. Unless otherwise stated, the substrates (tipped from side arm) were either *meso*-diaminopimelic acid (2 mg.; final concentrations of 4.2 mM) or L-lysine (15 mM); pyridoxal phosphate, when present, was $10\mu\text{M}$. Decarboxylation was carried out in an atmosphere of N_2 ; when gas evolution had ceased, m-citric acid (0.1 ml.) was tipped in from a second side arm to release CO_2 from solution (Hoare & Work, 1955). Oxidation was carried out in air with 20% KOH (0.2 ml.) and filter paper in the centre well. Control flasks without substrate were included, and were corrected for when calculating the final reaction rates. Carbon dioxide output (Q_{CO_2}) was expressed as $\mu\text{l. CO}_2$ produced/mg./hr. (not corrected for gas retention), and oxygen uptake (Q_{O_2}) as $\mu\text{l. O}_2$ taken up/mg./hr.

The manometric balance experiments were carried out essentially according to the method described under 'direct method for estimation of CO_2 ' (Umbreit, Burris & Stauffer, 1957). Pairs of flasks were set up, aerobically and anaerobically, containing either KOH or water in the centre well; this was done in the presence and in absence of synthetic diaminopimelic acid (8 mg./2.8 ml.). By this means the amounts of CO_2 evolved by oxidation and of O_2 absorbed were calculated. At the same time, the suspensions were incubated in open flasks with identical proportions of diaminopimelic acid, and samples were withdrawn at intervals for estimation of ammonia and diaminopimelic acid.

Examination of reaction mixtures. When not investigated immediately, the final reaction mixtures from the Warburg flasks were frozen rapidly and stored at -15° . Measured amounts of the thawed mixtures were examined as required.

Paper chromatography. The reaction mixtures were usually deproteinized and freed from organisms by treatment with 2 vol. ethanol and centrifugation before paper chromatography without preliminary hydrolysis; under certain circumstances the mixture was used after removal of the organisms by centrifugation only. The equivalent of 0.1 ml. of reaction mixture was examined by two-dimensional chromatography on Whatman no. 4 paper using as solvents aqueous phenol (NH_3 atmosphere) and *n*-butanol (4) + acetic acid (1) + water (5). Alternatively, $33\mu\text{l.}$ was examined for diaminopimelic acid on one-dimensional chromatograms on

no. 1 paper with the solvent methanol (80) + pyridine (10) + 10 N-HCl (2.5) + water (17.5) (Hoare & Work, 1955). Amino acids were revealed by dipping the chromatograms in ninhydrin in acetone (0.1%, w/v) and heating at 102°. When required, hydrolysis of reaction mixtures or of bacteria was carried out with 6 N-HCl for 24 hr.

Estimation of diaminopimelic acid. Ninhydrin in strong acetic acid was used to estimate diaminopimelic acid colorimetrically (Work, 1957*b*). For the balance experiments, synthetic diaminopimelic acid (mixture of *meso*, LL- and DD-isomers) was used, in preference to the *meso* isomer (the only form decarboxylated), in order to decrease the amount of reaction mixture necessary to give a measurable colour, and so to avoid high blank values due to the intracellular amino acids. Two flasks were used, one contained diaminopimelic acid, the other was a control. The mixtures were shaken at 37°, and at intervals samples were deproteinized with an equal volume of acetic acid and centrifuged after coagulation was complete; 0.1 ml. of the supernatant solution was mixed with water (0.4 ml.), acetic acid (0.5 ml.) and ninhydrin reagent (0.5 ml.). Ninhydrin reagent b was used (Work, 1957*b*); it consisted of ninhydrin (A.R. Grade) 250 mg., acetic acid 6 ml., 0.6 M-phosphoric acid 4 ml. The solutions were heated at 100° for 2 min., cooled and diluted with acetic acid (3.5 ml.); the optical density at 440 m μ was read against the mixture from the control flask. A standard curve was constructed by adding known amounts of synthetic diaminopimelic acid to the deproteinized contents of the control flask. The short heating time of 2 min. was used to minimize colour formation from intracellular amino acids and also from lysine formed by decarboxylation of *meso*-diaminopimelic acid. Estimations on other reaction mixtures were carried out by essentially the same method, with slight differences introduced according to conditions.

Estimation of ammonia. The reaction mixture (0.5 ml.) was aerated for 1 hr. at room temperature in the presence of 4 ml. water + 1 ml. saturated K₂CO₃ + 1 drop triamylcitrate. The ammonia carried over was trapped in 15 ml. of 0.04 N-H₂SO₄ and measured colorimetrically after treating 5 ml. of the distillate (diluted with an equal volume of water) with Nessler's reagent (0.5 ml.). The optical density at 450 m μ , read within 2 min. against that of a reagent blank, was compared against that of a standard curve previously constructed with solutions from known amounts of ammonium sulphate treated identically. The ammonia contents of the control suspensions incubated without added substrate were subtracted from those of the test solutions.

Qualitative examination for keto-acids. Keto-acids were examined in the reaction mixtures by a modification of the method of el Hawary & Thompson (1953). Some of the yellow dinitrophenylhydrazones of keto-acids were apparently unstable in the K₂CO₃ solution used to extract them from ethyl acetate, therefore the alkaline extractions were carried out at 0° and the extracts neutralized immediately with cold 3 N-HCl. After re-extraction into ethyl acetate and drying over anhydrous K₂CO₃, the extracts were examined by ascending paper chromatography in *n*-butanol (70) + ethanol (10) + 0.5 N-ammonia (20).

Examination for dipicolinic acid. This was carried out as suggested by Powell & Strange (1957); after deproteinization of the reaction mixtures with perchloric acid and dilution with 50 volumes of buffer (pH 7.3) the light absorption between 240 and 280 m μ was measured in the Beckmann spectrophotometer.

RESULTS

Sporosarcina ureae

Diaminopimelic acid in *Sporosarcina ureae*. Both strains of *S. ureae*, examined shortly after their arrival in the laboratory, yielded organisms which contained small amounts of diaminopimelic acid: subsequently, no diaminopimelic acid was found in whole or fractionated organisms grown in liquid culture. One culture, grown on solid medium, contained a trace of diaminopimelic acid and was found to consist of a mixture of vegetative organisms and spores. All further attempts in our laboratory to produce sporulation of either strain failed, but later Powell & Hunter (private communication) succeeded once in obtaining spores from the Edinburgh strain. These spores contained *meso*-diaminopimelic acid, the vegetative organisms had none.

Table 1. *Degradation of meso-diaminopimelic acid and L-lysine by acetone-dried Sporosarcina ureae*

Experiments were carried out in Warburg manometers with 40 mg. dried organism in total volume of 2.5 ml. 0.1 M-phosphate buffer (pH 6.8). Anaerobic experiments in nitrogen, aerobic in air with KOH in centre cup. Pyridoxal phosphate when present was 10 μ M. Other conditions as in Methods.

Manometric experiment												
Culture				<i>meso</i> -Diaminopimelic acid								
Expt. no.	Growth conditions		Yield (g. dry wt./l.)	Pyridoxal phosphate	Anaerobic		Aerobic			L- Lysine Aerobic		
	Medium	Time (hr.)			Q_{CO_2}	$V^*_{CO_2}$	Lag (min.)	Q_{O_2}	$V^*_{O_2}$	$V^*_{H_2}$	Q_{O_2}	$V^*_{O_2}$
1	Urea	24	1.46	+	9.3	92	.	.	.	.	3.0	87
				0	3.4	.	.	.	.	.	3.0	87
1	Urea	48	1.45	+	8.7	89	15	3.8	.	.	5.5	87
				0	4.5	.	.	.	.	.	5.5	94
2	Urea	48	1.10	+	6.9	77	16	2.3	106	.	.	.
				0	5.4	56	16	2.0	117	38	.	.
3	CCY	24	1.01	+	8.8	87	30	1.3	> 142	.	0.6	.
3	CCY	48	1.60	+	8.6	94	19	1.6	> 165	36	0.6	.
4	CCY	48	1.92	+	9.3	78	18	2.6	154	50	3.7	.
				0	7.4	63	20	2.5	157	50	.	.
5	TMB broth	48	1.48	+	7.7	85	37	0.8	32	21	.	.
				0	3.1	40	15	0.3	.	.	.	.
6	Tarr spore broth	48	0.73	+	7.0	86	27	0.7	.	.	.	.
				0	4.3	54	24	0.7	.	.	.	.

* Volume of reactants used or produced expressed as % of theoretical, based on 1 equivalent/mole amino acid present.

Q_{CO_2} = μ l. CO_2 evolved/hr./mg. dry wt. Q_{CO_2} = μ l. oxygen absorbed/hr./mg. dry wt.

Anaerobic transformations of diaminopimelic acid. Acetone-dried organisms from both strains of *Sporosarcina ureae* grown on urea medium decarboxylated *meso*-diaminopimelic acid; the rate with the Delft strain was about double that with the Edinburgh strain. Thereafter all experiments were carried out with the Delft strain. The rate of decarboxylation did not vary greatly with change in growth conditions

(Table 1); the highest activities ($Q_{\text{CO}_2} = 9.3$) were observed with organisms grown either on urea medium or on CCY medium. Omission of pyridoxal phosphate from the Warburg flasks lowered the decarboxylation rate. There was no decarboxylation reaction with LL-diaminopimelic acid, indicating the absence of diaminopimelic racemase (Antia *et al.* 1957).

The identifiable products of reaction were CO_2 and lysine. The organisms contained no lysine decarboxylase; an equimolar volume of CO_2 should therefore have been liberated from *meso*-diaminopimelic acid, but this was seldom the case (Table 1). Gas evolution stopped at 80–90 % of the theoretical value when pyridoxal phosphate was added, and at 40–80 % of theoretical without added pyridoxal phosphate. In spite of this, only 1–5 % of the original *meso*-diaminopimelic acid remained in the final reaction mixtures. Additional *meso*-diaminopimelic acid caused resumption of gas evolution, even in the absence of added pyridoxal phosphate. These results suggested that, in addition to decarboxylation, *meso*-diaminopimelic acid was undergoing some anaerobic reaction which was not stimulated by pyridoxal phosphate. Paper chromatography showed that the diaminopimelic acid unaccounted for had not been incorporated as a peptide or other form liberated by hydrolysis, either in the soluble or the insoluble portions of the reaction mixtures.

Aerobic breakdown of diaminopimelic acid. When the decarboxylation of *meso*-diaminopimelic acid by acetone-dried *Sporosarcina ureae* was carried out in air, the gas output was slower and lower than under anaerobic conditions (Fig. 1, curves A and B). In spite of this diminished CO_2 production, both diaminopimelic acid and lysine disappeared completely from the aerobic reaction mixture, while no other amino acids were produced. When the CO_2 evolved was absorbed by KOH, a steady uptake of gas occurred after a short lag entailing a slight gas output (curve D). A gas uptake also occurred with L-lysine (curve E and Table 1), but there was no lag. No gas uptake occurred with LL-diaminopimelic acid (curve C), DD-diaminopimelic acid, D-lysine, L-alanine, L-glutamic acid or glucose. It was evident that oxidative reactions had occurred with both *meso*-diaminopimelic acid and L-lysine. In the case of diaminopimelic acid, simultaneous oxidation and decarboxylation could result in curves such as B and D; in curve B where no KOH was present, oxygen uptake would produce a decrease in overall gas evolution. The preliminary lag in curve D can be attributed to the inability of KOH to absorb all the CO_2 released by decarboxylation in the first few minutes; when KOH was present during anaerobic decarboxylation of *meso*-diaminopimelic acid, there was also a considerable delay in the absorption of CO_2 , but finally there was no over-all change in volume. There is, however, a possibility that only lysine was oxidized by the preparation, and that the lag in oxygen uptake with diaminopimelic acid was due to preliminary decarboxylation to lysine. The reactions were therefore studied further. Fresh suspensions or broken-cell suspensions could not be used because of their high endogenous respiration rate; in acetone-dried organisms this was not unduly high ($Q_{\text{O}_2} = 1.1$ – 1.8 for organisms grown on CCY medium, 0.5 – 0.8 for other organisms), and was allowed for in calculating oxidation rates of the various substrates.

The rate of oxidation of *meso*-diaminopimelic acid was raised only slightly by the addition of pyridoxal phosphate (Table 1), that of lysine was unaffected. The rates

and extents of oxygen consumption varied with the conditions of growth, particularly in the case of L-lysine, but they also varied from one preparation to another of organisms grown under the same conditions. The total oxygen consumed was usually between 80 and 100 % of the theoretical value calculated for one atom of oxygen taken up by one molecule of either amino acid; but in some cases, particularly when the organisms were grown on CCY medium, the oxygen consumed

Fig. 1. Behaviour of diaminopimelic acid and lysine in the Warburg apparatus in presence of acetone-dried *Sporosarcina ureae*. Each flask contained 40 mg. dried organism (grown 48 hr. in urea medium) in 0.1 M-phosphate buffer (pH 6.8); total volume 2.5 ml.; present $10\mu\text{M}$ pyridoxal phosphate. Substrates tipped from side arms were as follows: curves A, B and D, *meso*-diaminopimelic acid (4.2 mM); curve C, L-diaminopimelic acid; curve E, L-lysine (5.5 mM). Curve A, atmosphere N_2 ; curve B, atmosphere air, no KOH present; curves C, D, E, atmosphere air, KOH present. Changes in volume were corrected for those in control flasks without substrate.

by *meso*-diaminopimelic acid exceeded this value. There was no constant relation between the rates of oxidation of L-lysine and the decarboxylation of *meso*-diaminopimelic acid by different batches of organisms. The minimum concentrations of *meso*-diaminopimelic acid and L-lysine required to produce maximum oxidation rates were 2 and 15 mM, respectively.

The effects of inhibitors on the decarboxylation and oxidation of *meso*-diaminopimelic acid were investigated in the hope that decarboxylation might be inhibited specifically. Compounds which bind thiol or carbonyl groups inhibit diaminopimelic acid decarboxylase from *Aerobacter aerogenes* (Dewey *et al.* 1954; Hoare, 1956). Both oxidation and decarboxylation by *Sporosarcina ureae* were inhibited to the same extent by any one of these inhibitors.

Ammonia was always produced by these aerobic reactions, whereas none was produced anaerobically. Ammonia could only be estimated reliably in the products from *meso*-diaminopimelic acid oxidation; in the case of L-lysine, duplicate estimations did not agree, suggesting that a reaction product was unstable under the

Fig. 2. Balance experiment showing aerobic decomposition of *meso*-diaminopimelic acid by acetone-dried *Sporosarcina ureae*. Final reaction mixture contained dried organism (grown CCY medium for 48 hr.) 1.6 mg./ml. in 0.1 M-phosphate buffer (pH 6.8) containing 10 μ M pyridoxal phosphate. Pairs of Warburg flasks were used containing total volumes of 2.8 ml.: in one flask of each pair there was 8 mg. (42 μ mole) of synthetic diaminopimelic acid, the other contained no substrate. In two pairs the reaction was carried out aerobically; KOH (0.2 ml. of 20 %) was in the centre well of only one pair. In a third pair of flasks KOH was present but the atmosphere was N₂. The same suspension was also incubated in two open flasks, with and without diaminopimelic acid, at the same concentration as in the Warburg flasks; samples were withdrawn at intervals for estimation of ammonia and diaminopimelic acid. Changes in gas volumes were calculated as in Umbreit *et al.* (1957). Results are expressed as change in amounts of reactants in a Warburg flask. x—x = oxygen taken up; O—O = CO₂ evolved (not corrected for retention in solution); ●—● = diaminopimelic acid disappearing; Δ—Δ = ammonia formed.

alkaline conditions of the ammonia distillation. The amount of ammonia produced during oxidation of diaminopimelic acid bore no constant relation to the oxygen uptake or to the amount of diaminopimelic acid utilized, but the molar ratio of

oxygen or diaminopimelic acid consumed to ammonia produced was always greater than unity (Table 1).

When the reaction products from diaminopimelic acid oxidation were examined by chromatography in methanol + water + pyridine + HCl and revealed with ninhydrin, a yellow spot having a pink fluorescence in ultraviolet radiation was often observed just behind the solvent front. The substance giving this spot has not been identified; it was not dipicolinic acid or piperidine-2:6-dicarboxylic acid, but it might have been a keto-acid, as suggested by the colour and fluorescence of its ninhydrin reaction product on paper (Rabson & Tolbert, 1958). Examination of the keto-acid dinitrophenyl hydrazones in the reaction mixtures showed very small amounts of oxoglutaric and pyruvic acids and also of an unidentified keto-acid with R (pyruvate) = 0.7; these were insufficient in amount to account for the diaminopimelic acid used.

Balance experiments were carried out, as described in methods, with synthetic diaminopimelic acid. One such experiment is illustrated in Fig. 2. During the first 40 min., in which rapid decarboxylation took place, diaminopimelic acid disappeared rapidly; after this it was utilized more slowly, finally reaching a constant value representing 50 % of the original amount present (this is the probable proportion of LL- and DD-isomers in a synthetic mixture). A steady uptake of oxygen continued even after utilization of diaminopimelic acid had stopped; it had not appreciably slowed by the end of the experiment (5 hr.), when 1.25 atom of oxygen had been used per mole of diaminopimelic acid consumed. This high oxygen consumption, typical of organisms grown on CCY medium, showed that in these cells, at any rate, the oxygen uptake was due in part to a secondary reaction. Ammonia production had only reached $2.6 \mu\text{mole}$ at 3 hr. when $20 \mu\text{mole}$ diaminopimelic acid had disappeared.

Degradation of diaminopimelic acid by dried cells of other species of bacteria

In the family Micrococcaceae, the marked stimulation by pyridoxal phosphate of anaerobic decarboxylation of *meso*-diaminopimelic acid was not peculiar to *Sporosarcina ureae*, although the low output of CO_2 was more specific. For example, with *Sarcina lutea*, although the decarboxylation rate was doubled by added pyridoxal phosphate, the CO_2 output was 97 % of theoretical even in the absence of added pyridoxal phosphate. Aerobic experiments were difficult to carry out on most Micrococcaceae, because of their high endogenous respiration, even after acetone-drying. With acetone-dried *Sarcina lutea*, this rate was not altered by synthetic diaminopimelic acid or L-lysine; *Staphylococcus citreus* had $Q_{\text{O}_2} = 8.0$ with no added substrate or with L-lysine, and in the presence of *meso*-diaminopimelic acid $Q_{\text{O}_2} = 8.3$.

The majority of Bacillaceae do not decarboxylate *meso*-diaminopimelic acid (Antia *et al.* 1957); the known exceptions being *Clostridium tetani* and *Bacillus sphaericus*. *C. tetani* decarboxylated *meso*-diaminopimelic acid faster under anaerobic conditions ($Q_{\text{CO}_2}^{\text{N}_2} = 3.0$) than in presence of air ($Q_{\text{CO}_2}^{\text{air}} = 1.4$). Even anaerobically, the gas output stopped after only 50 % of the theoretical amount of CO_2 had been evolved. This suggests that *C. tetani* utilized diaminopimelic acid by the unidentified anaerobic reaction and by oxidation, but no further work was done with this organism.

Bacillus sphaericus. The metabolism of diaminopimelic acid by *B. sphaericus* has been studied in detail, and is described here and elsewhere (Powell & Strange, 1957; Meadow & Work, 1958*b*). Diaminopimelic acid decarboxylase activity was very high in freeze-dried and acetone-dried vegetative *B. sphaericus*, and was greatly stimulated by pyridoxal phosphate, rates of the order of $Q_{CO_2}^{N_2} = 30$ being found under optimal conditions. Anaerobic decarboxylation of *meso*-diaminopimelic acid evolved suboptimal amounts of CO_2 , especially in the absence of pyridoxal phosphate when only about 60 % of the theoretical volume was produced (Fig. 3A, curve 1).

Fig. 3. Behaviour of *meso*-diaminopimelic acid in Warburg apparatus in presence of acetone-dried *Bacillus sphaericus*. Each flask contained a suspension of dried vegetative organisms (20 mg.) in 0.1 M-phosphate buffer (pH 6.8); final volume 2.5 ml. A; *B. sphaericus*, var. *fusiformis* NCTC 7582, normal strain. B; Asporogenous variant of this strain. All solid curves represent substrate *meso*-diaminopimelic acid (2 mg.), × --- × = substrate L-lysine (6 mg.). Curve 1, decarboxylation, atmosphere N_2 , no KOH; curve 2, atmosphere air, no KOH; curves 3 and 5, oxidation, atmosphere air, KOH present; curves 1, 2, 3 and 5, no pyridoxal phosphate added. Curve 4, oxidation, atmosphere air, KOH present, pyridoxal phosphate (10 μM) added.

The rate of gas evolution was almost unaffected by the presence of air, but in this case the volume of gas evolved was lower (Fig. 3A, curve 2). In all experiments, 95–100 % of the added diaminopimelic acid disappeared; with the exception of lysine no other amino acids were formed. No evidence for binding of diaminopimelic acid was obtained. Aerobic experiments in the presence of KOH resulted in slow gas uptakes with *meso*-diaminopimelic acid (Fig. 3A, curves 4 and 5). These

results resembled those found in acetone-dried *Sporosarcina ureae*, and suggested that in *B. sphaericus* meso-diaminopimelic acid was subjected to both the unidentified anaerobic reaction and oxidation. However, owing to very rapid decarboxylation, even in the absence of pyridoxal phosphate, the direct observation of oxidation of diaminopimelic acid was even more difficult than in the case of *S. ureae*. On the other hand, *B. sphaericus* did not oxidize L-lysine (Fig. 3A, curve 3), so that any observed oxygen uptake could be attributed directly to diaminopimelic acid even if decarboxylation were occurring at the same time. The oxygen uptakes in the absence of substrate were negligible with acetone-dried organisms. Disintegrated suspensions of fresh spores of *B. sphaericus* showed lower values for diaminopimelic acid decarboxylase ($Q_{\text{CO}_2} = 4.1$) than did the vegetative organisms. Neither the rate nor the extent of decarboxylation by spores was affected by the presence of oxygen or of pyridoxal phosphate; the volume of CO_2 evolved was always about 60% if theoretical. Diaminopimelic acid was evidently not oxidized by spores, but the decarboxylase was active and fully saturated with pyridoxal phosphate; the unidentified anaerobic reaction also occurred.

The asporogenous variant of *Bacillus sphaericus* is known to have no diaminopimelic acid decarboxylase activity (even when acetone-dried) unless pyridoxal phosphate is added (Meadow & Work, 1958*b*). In the absence of this coenzyme, the unknown anaerobic reaction evidently also does not occur, since meso-diaminopimelic acid was unchanged in concentration after anaerobic incubation for 5 hr. with acetone-dried cell suspensions (Work, 1957*b*). However, meso-diaminopimelic acid was oxidized (Q_{O_2} between 2.0 and 3.0), whereas L-lysine and the other isomers of diaminopimelic acid were not attacked (Fig. 3B). The gas uptake with meso-diaminopimelic acid started immediately, without a preliminary lag (Fig. 3B, curve 5). Since pyridoxal phosphate had already been found to have little effect on the oxidation of diaminopimelic acid by *Sporosarcina ureae*, it was decided that the asporogenous variant of *B. sphaericus* would be a good material for a study of the reaction.

The pH optimum for the oxidation of meso-diaminopimelic acid by a suspension of acetone-dried *Bacillus sphaericus* (asporogenous) lay between 6.8 and 7.4, outside this range a rapid fall in reaction rate was noted (at pH 6.0 and 7.8 the rates were only 14% of the maximum).

Balance experiments showed that the reaction with asporogenous *Bacillus sphaericus* was more straightforward than in the case of *Sporosarcina ureae*, as the amounts of ammonia produced and oxygen taken up were proportional to the diaminopimelic acid utilized. For example, in an oxidation, not carried to completion, of 20.6 μmole synthetic diaminopimelic acid by 30 mg. acetone powder, in 165 min. 5.5 μmole ammonia were produced, 5.8 μmole of diaminopimelic acid disappeared and 13 $\mu\text{equivalents}$ of oxygen were taken up. In another experiment, in 45 min., 2 $\mu\text{equivalents}$ of oxygen were used by 20 mg. of acetone powder and 0.6 μmole CO_2 produced; during the same period, only 0.2 μmole CO_2 was evolved anaerobically. No dipicolinic acid was formed during the aerobic reaction. There were trace amounts of the keto acid with a dinitrophenyl hydrazone having a mobility of R (pyruvate) = 0.7 on paper chromatograms.

These experiments indicate that the reaction involved in meso-diaminopimelic acid oxidation by the asporogenous strain of *Bacillus sphaericus* is probably oxidative removal of one amino group, followed perhaps by oxidative splitting of the

carbon chain or by decarboxylation. Another batch of asporogenous cells was grown, but the preparation showed very weak oxidase activity, although it still retained an active apodecarboxylase. Since this organism has been found to change very much in successive subculture (Meadow & Work, 1958*b*), it was not considered to be suitable for further study of diaminopimelic acid oxidation. The work has been temporarily abandoned.

DISCUSSION

The experiments described showed that *meso*-diaminopimelic acid, but not the LL- or DD-isomers, is oxidized by acetone-dried vegetative organisms of two species of sporulating bacteria which are atypical in their contents of diaminopimelic acid and in their methods of utilizing this substance (summarized in Table 2). This stereochemical specificity of the oxidation, easily demonstrable owing to the unusual absence of diaminopimelic acid racemase from the organisms in question, distinguishes the reaction from the less stereospecific oxidations which both the LL- and *meso*-isomers undergo with L-amino acid oxidases from *Neurospora* or snake venoms (Work, 1955). The reaction probably involves a deamination, but whether this is the primary reaction is not yet known. For example, a transamination, catalysed by small amounts of keto acids found in the organisms, could remove one amino group, and the resulting α -keto- ϵ -aminopimelic acid might then be oxidized with or without loss of its amino group. The stereochemical specificity of the oxidation does not support this theory, since both LL- and *meso*-diaminopimelic acid are transaminated by the organisms in question (see Meadow & Work, 1958*a*, for transamination by *Bacillus sphaericus*). The virtual independence of added pyridoxal phosphate of the oxidative reaction by *Sporosarcina ureae* also suggests that it was not connected with transamination, which requires high concentrations ($40\mu\text{M}$) of this coenzyme in acetone-dried organisms. The neutral pH value at which the oxidation occurs also does not favour transamination.

The failure to find significant amounts of keto acids in the reaction mixtures is not contra-indicative of an oxidative deamination, since α -keto- ϵ -aminopimelic acid would be expected to cyclize spontaneously by condensation of the α -keto and ϵ -amino groups, as in the case of α -keto acid resulting from oxidation of lysine (Meister, 1954). α -Keto- ϵ -aminopimelic acid is known to be an intermediate in the biosynthesis of LL-diaminopimelic acid in *Escherichia coli*, but in this case it is present as the *N*-succinyl derivative and is thereby protected against cyclization (Gilvarg, 1960).

Neither piperidine-2:6-dicarboxylic acid, a reduced product of ring closure, nor dipicolinic acid, the fully unsaturated derivative which occurs in bacterial spores (Powell, 1953), was identified among the reaction products.

Although *Sporosarcina ureae* and *Bacillus sphaericus* are organisms belonging to different families, their methods of utilizing diaminopimelic acid (Table 2) are sufficiently similar to enable them to be compared and to be differentiated from other organisms in their respective families. *Clostridium tetani* may be a similar exception: it is the only other member of the Bacillaceae known to decarboxylate diaminopimelic acid and sometimes to lack it in its vegetative cells.

Table 2. *Comparison of certain characteristics in typical and atypical members of families micrococcaceae and bacillaceae*

Characteristic	Micrococcaceae		Bacillaceae		
	Typical species	<i>Sporosarcina ureae</i>	Typical species	<i>Bacillus sphaericus</i>	<i>Clostridium tetani</i>
DAP* in vegetative cells	—	—	+	—	Not invariably
DAP in spores	No spores	+	+	+	?
DAP decarboxylase†	+	+	—	+	+
Alternative anaerobic reaction†	—	+	?	+	+
DAP racemase†	+	—	+	—	Slight
DAP oxidation†	—	+	—	+	Possible

* Diaminopimelic acid.

† Reactions investigated in acetone-dried vegetative organisms.

Diaminopimelic acid is an important constituent of the mucopeptide of the cell walls of many Gram-positive bacteria such as Bacillaceae; whenever it is absent, as in the Micrococcaceae, it is replaced in the wall by lysine (Cummins & Harris, 1956*b*). In the case of *Bacillus sphaericus*, Powell & Strange (1957) found that the soluble mucopeptides obtained by enzymic degradation of walls of vegetative organisms and spores had similar compositions except for the presence of lysine in the former and diaminopimelic acid in the latter. They also showed variations in the cellular activities of diaminopimelic acid decarboxylase throughout the growth and sporulation cycle, finding a marked decrease in activity in ageing cultures coincident with the appearance of spores and soluble mucopeptides containing diaminopimelic acid. We found that, in contrast to the vegetative cells, spores of this organism did not oxidize diaminopimelic acid.

It is possible that diaminopimelic acid is synthesized in vegetative cells of both *Bacillus sphaericus* and *Sporosarcina ureae*, but that before it can be inserted into the walls it is degraded by the three types of enzymes present in these cells. It is notable that the activities of decarboxylase found in normal dried vegetative cells of *B. sphaericus* and of *S. ureae*, are respectively, 10 and 8 times higher than those found in most other bacteria (Antia *et al.* 1957); however, considerably lower quantities were present in the asporogenous variant of *B. sphaericus*, which also has lysine in its cell wall. Nothing is known of the metabolic determinant which causes the change from a vegetative cell containing lysine in its wall mucopeptides to a spore containing diaminopimelic acid.

REFERENCES

- ANTIA, M., HOARE, D. S. & WORK, E. (1957). The stereoisomers of $\alpha\epsilon$ -diaminopimelic acid: III. Properties and distribution of diaminopimelic acid racemase, an enzyme causing interconversion of the LL and *meso* isomers. *Biochem. J.* **65**, 448.

- CUMMINS, C. S. (1956). Chemical composition of the bacterial cell wall. *Int. Rev. Cytol.* **5**, 25.
- CUMMINS, C. S. & HARRIS, H. (1956*a*). The chemical composition of the cell wall in some gram-positive bacteria and its possible value as a taxonomic character. *J. gen. Microbiol.* **14**, 583.
- CUMMINS, C. S. & HARRIS, H. (1956*b*). The relationships between certain members of the Staphylococcus-Micrococcus group as shown by their cell wall composition. *Int. Bull. bact. Nomen. & Taxon.* **6**, 111.
- DEWEY, D. L. (1954). The distribution of diaminopimelic acid decarboxylase among some organisms of the coli-aerogenes group and certain other bacteria. *J. gen. Microbiol.* **11**, 307.
- DEWEY, D. L., HOARE, D. S. & WORK, E. (1954). Diaminopimelic acid decarboxylase in cells and extracts of *Escherichia coli* and *Aerobacter aerogenes*. *Biochem. J.* **58**, 523.
- GILVARG, C. (1960). Biochemistry of diaminopimelic acid. *Fed. Proc.* **19**, 948.
- GLADSTONE, G. P. & FILDES, P. (1940). A simple culture medium for general use without meat extract or peptone. *J. exp. Path.* **21**, 161.
- EL HAWARY, M. F. S. & THOMPSON, R. H. S. (1953). Separation and estimation of blood keto acids by paper chromatography. *Biochem. J.* **53**, 340.
- HOARE, D. S. (1956). The progressive reaction of isonicotinyl hydrazide with two bacterial amino acid decarboxylases. *Biochim. biophys. Acta*, **19**, 141.
- HOARE, D. S. & WORK, E. (1955). The stereoisomers of $\alpha\epsilon$ -diaminopimelic acid: their distribution in nature and behaviour towards certain enzyme preparations. *Biochem. J.* **61**, 562.
- HOARE, D. S. & WORK, E. (1957). The stereoisomers of $\alpha\epsilon$ -diaminopimelic acid: II. Their distribution in the bacterial order Actinomycetales and in certain Eubacteriales. *Biochem. J.* **65**, 441.
- MEADOW, P. & WORK, E. (1958*a*). Bacterial transamination of the stereoisomers of diaminopimelic acid and lysine. *Biochim. biophys. Acta*, **28**, 596.
- MEADOW, P. & WORK, E. (1958*b*). The effects of vitamin B₆ and its derivatives on diaminopimelic acid decarboxylase in *Bacillus sphaericus* asporogenous. *Biochim. biophys. Acta*, **29**, 180.
- MEISTER, A. (1954). The α -keto analogues of arginine, ornithine, and lysine. *J. biol. Chem.* **206**, 577.
- POWELL, J. F. (1953). Isolation of dipicolinic acid (pyridine-2:6-dicarboxylic acid) from spores of *Bacillus megatherium*. *Biochem. J.* **54**, 210.
- POWELL, J. F. (1958). The changes in the total vitamin B₆ and the pyridoxal phosphate content of cells of *Bacillus sphaericus* during growth and sporulation: their possible relationships with $\alpha\epsilon$ -diaminopimelic acid metabolism. *Biochem. J.* **70**, 91.
- POWELL, J. F. & HUNTER, J. R. (1955). The sporulation of *Bacillus sphaericus* stimulated by association with other bacteria: an effect of carbon dioxide. *J. gen. Microbiol.* **13**, 54.
- POWELL, J. F. & STRANGE, R. E. (1957). $\alpha\epsilon$ -Diaminopimelic acid metabolism and sporulation in *Bacillus sphaericus*. *Biochem. J.* **65**, 700.
- RABSON, R. & TOLBERT, N. E. (1958). Detection of α -ketoglutaric acid and other keto-acids on paper chromatograms with ninhydrin. *Nature, Lond.* **181**, 50.
- TARR, H. L. A. (1933). Some observations on the respiratory catalysts present in the spores and vegetative cells of certain aerobic bacilli. *Biochem. J.* **27**, 186.
- UMBREIT, W. W., BURRIS, R. H. & STAUFFER, J. F. (1957). *Manometric Techniques*, 3rd ed. p. 28. Minneapolis, U.S.A.: Burgess Publ. Co.
- WORK, E. (1955). The action of L-amino acid oxidases on the optical isomers of α,ϵ -diaminopimelic acid. *Biochim. biophys. Acta*, **17**, 410.
- WORK, E. (1957*a*). Biochemistry of the bacterial cell wall. *Nature, Lond.* **179**, 841.
- WORK, E. (1957*b*). Reaction of ninhydrin in acid solution with straight-chain amino acids containing two amino groups and its application to the estimation of $\alpha\epsilon$ -diaminopimelic acid. *Biochem. J.* **67**, 416.
- WORK, E. (1961). Mucopeptides of bacterial cell walls. *J. gen. Microbiol.* **25**, 166.
- WORK, E. & DEWEY, D. L. (1953). The distribution of $\alpha\epsilon$ -diaminopimelic acid among various micro-organisms. *J. gen. Microbiol.* **9**, 394.