Uptake of Individual Isomers of 2,6-Diaminopimelate and their Incorporation into Walls during Growth of *Bacillus megaterium*

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(Received 22 June 1978; revised 22 August 1978)

Bacillus megaterium NCIB 7581, when growing exponentially in a simple chemically defined medium (without 2,6-diaminopimelic acid), contained only 4 mM-diaminopimelate in the free amino acid pool; this diaminopimelate was 84% (w/w) *meso*-isomer and 16% *LL*-isomer. Growing organisms could take up any of the three isomers of diaminopimelate from the medium, though the *DD*-isomer was taken up at only half the rate of the other two isomers (*meso*- and *LL*-). When lysine was also present in the medium, most (70%, w/w) of the diaminopimelate that was taken up entered peptidoglycan; only about 5% of the total uptake was into the free amino acid pool. When *DD*-diamino[^{14}C]pimelate was supplied in the medium, 86% of the diamino[^{14}C]pimelate incorporated into peptidoglycan was present as the *meso*-isomer and 14% was the *DD*-isomer.

INTRODUCTION

The wall of *Bacillus megaterium* NCIB 7581 contains all three isomers of 2,6-diaminopimelic acid (A_2pm) in the following proportions (% w/w): *meso*-, 75; *LL*-, 18; *DD*-, 7 (Day & White, 1977). If free *DD*- A_2pm were to be found in the amino acid pool, this would support the view that *DD*- A_2pm was really present in the wall and had not arisen solely by isomerization during acid hydrolysis of the peptidoglycan. The concentrations of the individual isomers of A_2pm in the amino acid pool of this organism have therefore been studied. The uptake of each separate isomer of A_2pm and its subsequent metabolism have also been examined with growing cultures, and the conversion of *DD*- A_2pm into *meso*- A_2pm has been recognized for the first time.

METHODS

Organism. *Bacillus megaterium* NCIB 7581 was used throughout and was maintained as described by Day & White (1977).

Medium. Medium A2 (White, 1972) containing biotin ($20\text{ }\mu\text{g l}^{-1}$) was used.

Conditions of growth. Organisms were grown at 37°C in 500 ml medium in shaken flasks (2 l) and growth was assessed turbidimetrically as described previously (White, 1972).

Preparation of enzymes. Diaminopimelate decarboxylase (EC 4.1.1.20; *meso*-2,6-diaminopimelate carboxy-lyase) and diaminopimelate epimerase (EC 5.1.1.7; 2,6-*LL*-diaminopimelate 2-epimerase) were partially purified (for use in assays for isomers of A_2pm) as described by Day & White (1977).

Assays for isomers of A_2pm . Colorimetric and manometric methods (Day & White, 1977) were used. In addition, isomers of [^{14}C] A_2pm and [^{14}C]lysine were separated by paper chromatography (see below) and then the radioactivity in each was measured.

Preparation of isomers of A_2pm . The unlabelled isomers were isolated as described by Day & White (1977).

Isolation of isomers of [^{14}C] A_2pm . (i) *meso*-[^{14}C] A_2pm . [$1, (7)\text{-}^{14}\text{C}$] A_2pm ($5\text{ }\mu\text{Ci}$; about $100\text{ }\mu\text{g}$) was dis-

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solved in water (2 ml) containing *meso*-A₂pm (5 mg) and ethanol was added until a faint turbidity persisted. The solution was left overnight at room temperature and the precipitate was then collected by centrifuging. Ethanol was again added to the supernatant liquid until it was just turbid and next day the precipitate was collected by centrifuging. Each precipitate was washed in 1 ml water at 2 °C and the solid (*meso*-[¹⁴C]A₂pm) was collected by filtration and dried over P₂O₅. The filtrate was added to the supernatant liquids from the two earlier centrifugations and the solution was dried by evaporation at 35 °C to give crude LL-[¹⁴C]A₂pm plus DD-[¹⁴C]A₂pm.

(ii) LL-[¹⁴C]A₂pm and DD-[¹⁴C]A₂pm. The crude preparation (see above) was redissolved in 0.1 M-phosphate buffer, pH 6.8 (1.75 ml), and pyridoxal phosphate (10 µg), 2,3-dimercaptopropan-1-ol (to give 1 mM) and diaminopimelate decarboxylase (0.14 unit) were added in a final volume of 2.5 ml. A similar flask, containing *meso*-A₂pm (1 mg) in place of the radioactive material, was set up as a control for the completeness of the enzymic conversion of the *meso*-isomer to lysine. The flasks were shaken at 37 °C for 1.5 h, by which time no A₂pm remained in the control flask.

Ethanol (7.5 ml) was added to deproteinize the radioactive solution. After 1 h the precipitate was removed by centrifuging and unlabelled DD- and LL-A₂pm (each 100 µg) were added as carriers of the radioactive material. The mixture was then desalted on a column (6 × 0.5 cm) of Dowex 50 (H⁺-form, 8 % cross-linking). The A₂pm was eluted with 1 M-NH₄OH; the eluate was dried, taken up in water (0.2 ml) and applied as a streak to the origin of a paper chromatogram which was developed with solvent 2 (see below) overnight and then dried at room temperature in a stream of air for 24 h. A guide strip with markers of LL- and DD-A₂pm was cut off and treated with ninhydrin. Areas on the chromatogram that corresponded to the positions of the markers were cut out and eluted with water by descending chromatography. The separate eluates of LL-[¹⁴C]A₂pm and DD-[¹⁴C]A₂pm were dried.

Each radioactive isomer was dissolved in water, assayed colorimetrically for A₂pm and counted. A sample of each solution was incubated with diaminopimelate decarboxylase and then chromatographed (solvent 1). Proportions of LL- and DD-isomers were determined from the radioactivity in the appropriate areas of the chromatogram; *meso*-A₂pm was assessed from the radioactivity in the lysine area, which was doubled to correct for loss of ¹⁴CO₂ from the *meso*-[¹⁴C]A₂pm. The yield of *meso*-[¹⁴C]A₂pm was 71 % (of the *meso*-[¹⁴C]A₂pm present in the starting material); its specific activity was 0.087 µCi µmol⁻¹ and 88 % of this radioactivity was present as *meso*-A₂pm, 6 % was LL-A₂pm and 6 % was DD-A₂pm, although the contamination expressed as a percentage by weight would be negligible. The yield of LL-[¹⁴C]A₂pm was 68 %; its specific activity was 3.2 µCi µmol⁻¹ and 96 % of this radioactivity was present as LL-A₂pm and 4 % was DD-A₂pm. The yield of DD-[¹⁴C]A₂pm was 66 %; its specific activity was 3.4 µCi µmol⁻¹ and 95 % of this radioactivity was present as DD-A₂pm and 5 % was LL-A₂pm. No radioactive *meso*-A₂pm was detected in the labelled LL- or DD-isomers, probably because of the use of diaminopimelate decarboxylase in their isolation.

Paper chromatography and electrophoresis. Descending chromatograms on Whatman no. 1 paper were developed with solvent 1 (Rhuland *et al.*, 1955): methanol/water/11.6 M-HCl/pyridine (32:7:1:4, by vol.), or solvent 2 (Perkins, 1965): methanol/water/98 % (w/v) formic acid/pyridine (80:19:1:10, by vol.). Amino acids were detected by reaction with ninhydrin. In solvent 1, LL-A₂pm had a higher mobility than either the *meso*- or DD-isomers, which ran almost together, and lysine ran well in front of LL-A₂pm. Solvent 2 was used when A₂pm was subsequently to be eluted (for purposes other than measuring radioactivity), so as to avoid eluting pyridine hydrochloride, which interfered in further manipulations.

Electrophoresis was carried out on Whatman no. 1 or 3 MM paper at 45 V cm⁻¹ and about 50 mA for 1 h in a Savant FP22A apparatus with a Savant HV5000A power supply (Savant Instruments, 221 Park Avenue, Hicksville, N.Y., U.S.A.). The buffer (pH 6.5) was pyridine/acetic acid/water (100:4:896, by vol.).

Extraction of the free amino acid pool. The procedure of Tempest *et al.* (1970) was followed. The dry weight of organisms in a culture was determined turbidimetrically; then the organisms were collected by centrifuging at 2 °C and resuspended in ice-cold 0.25 M-HClO₄ (about 1 ml per mg dry wt bacteria). After 10 min at 2 °C the suspension was centrifuged and the supernatant liquid was collected. The organisms were again treated with 0.25 M-HClO₄ (one-quarter of the volume used for the first extraction) and the two extracts were combined and neutralized with 5 M-KOH. After 1 h at 0 °C the precipitated KClO₄ was removed and the supernatant liquid ('pool extract') was stored at -15 °C until assayed for amino acids in an analyser. The volume of the pool (in µl) was assumed to be numerically equal to four times the dry weight (in mg) of the bacteria extracted (Tempest *et al.*, 1970).

Radiochemical techniques. Labelled compounds were located by placing developed chromatograms in contact with Kodirex X-ray film for periods of up to 4 weeks. Radioactive areas detected in this way were counted with an end-window Geiger-Müller tube coupled to a scaler.

Radioactive samples were counted in a scintillation fluid (containing 7 g 2,5-diphenyloxazole, 0.3 g 1,4-di-2-(5-phenyloxazolyl)benzene, 100 g naphthalene and dioxan to 1 l) in a Nuclear Chicago apparatus (model 6801). Quench corrections were made by the channels ratio method.

Uptake of A₂pm by growing cultures. Organisms in the exponential phase of growth in medium A2 were harvested, washed and inoculated into fresh medium containing A₂pm (see Results). This suspension was shaken at 37 °C and samples were taken at intervals for measurement of turbidity and of A₂pm remaining in the medium (by colorimetric assay).

When [¹⁴C]A₂pm was used, a sample of medium (0.5 ml) was removed immediately before inoculation for measurements of the radioactivity added and of the concentration of A₂pm (by colorimetric assay). Further samples (2 ml) were removed at intervals; the turbidity of each was measured and then the organisms were collected on a Millipore membrane (0.45 µm pore size). The membrane was washed with 0.1 M-phosphate buffer, pH 6.8 (10 ml) containing unlabelled A₂pm (50 µg ml⁻¹; the same isomer as was added to the growth medium) to remove surplus radioactive medium. Radioactivity in the membrane plus organisms was then counted in scintillation fluid (10 ml, in which the membrane dissolved). The uptake of A₂pm was calculated from this radioactivity and the measured specific activity of the A₂pm. At the end of the incubation, the remaining culture was divided into two equal parts, and peptidoglycan was isolated from the organisms by the method of Park & Hancock (1960). The peptidoglycan was suspended in 6 M-HCl and heated at 105 °C for 18 h to liberate A₂pm. The A₂pm in the hydrolysate was eluted from Dowex 50 as described by Day & White (1977) before individual isomers were assayed.

In one experiment, organisms that had been grown with unresolved synthetic [¹⁴C]A₂pm were centrifuged, then washed with 0.1 M-phosphate buffer, pH 6.8 (10 ml) containing unlabelled synthetic A₂pm (50 µg ml⁻¹). The washed organisms were hydrolysed with 6 M-HCl as described above. Peptidoglycan was also isolated from these organisms and similarly hydrolysed.

Entry of [¹⁴C]A₂pm into the amino acid pool. Organisms were grown in medium A2 (20 ml) containing L-lysine (50 µg ml⁻¹) and either LL- or DD-A₂pm (50 µg ml⁻¹); early in the exponential phase of growth LL-[¹⁴C]A₂pm (0.34 µCi) or DD-[¹⁴C]A₂pm (0.33 µCi) was added. At intervals, the turbidities of the cultures were measured and samples (0.5 ml) were transferred to 0.1 M-phosphate buffer, pH 6.8 (5 ml) or to 5 % (w/v) trichloroacetic acid (5 ml) at 2 °C; after 10 min these suspensions were filtered through membrane discs, which were washed with cold buffer (15 ml) or 5 % trichloroacetic acid (15 ml) and then counted for radioactivity. The radioactivity present in the organisms after washing with buffer was taken to represent A₂pm in the free amino acid pool plus covalently linked A₂pm, while the radioactivity after extraction with trichloroacetic acid represented only covalently linked A₂pm in the organisms.

Chemicals. Diamino[1,(7)-¹⁴C]pimelic acid (10 mCi mmol⁻¹) was purchased from Calatomic (54984 Terminal Annex, Los Angeles, Calif., U.S.A.). This material contained approximately 50 % of its radioactivity as *meso*-A₂pm, 25 % as LL-A₂pm and 25 % as DD-A₂pm. Synthetic A₂pm (unlabelled) was made as described by Saleh & White (1976).

RESULTS

The free amino acid pool of B. megaterium 7581

Exponential phase organisms (grown in medium A2) contained only a few detectable free amino acids in the pool (mM): glutamate, 140; alanine, 12; glycine, 8; serine, 5; lysine, 5; ornithine, 4; A₂pm, 4. The A₂pm was made up of 84 % (w/w) *meso*- and 16 % LL-isomers; no DD-isomer was detected by chromatography or colorimetric assay after treatment of the pool A₂pm with diaminopimelate epimerase and diaminopimelate decarboxylase. The absence of DD-A₂pm does not prove that this isomer has no metabolic role; several amino acids that must be important intermediary metabolites (e.g. aromatic amino acids, aspartate, histidine) were also not detected in the amino acid pool of *B. megaterium* 7581.

Uptake of isomers of A₂pm during growth of B. megaterium

The rate of exponential growth of *B. megaterium* 7581 was unaltered by the presence of A₂pm. The rate of uptake of *meso*-A₂pm reached a maximum when 50 µg ml⁻¹ was used. About 30 µg *meso*-A₂pm was taken up during growth of 1 mg dry wt bacteria; this represents about half the total A₂pm needed by the organisms for synthesis of wall and protein. (About 500 µg protein, containing, say, 25 µg lysine, and about 250 µg wall, containing approximately 125 µg peptidoglycan, equivalent to 25 µg A₂pm, are present per mg bacteria.) Synthetic A₂pm (50 µg ml⁻¹) was taken up slightly more slowly. It was possible, therefore, that the organisms were not equally permeable to all three isomers of A₂pm, and

Fig. 1. Comparison of the uptakes of the three isomers of A₂pm (each initially 50 μg ml⁻¹) by growing cultures of *B. megaterium* 7581: (a) medium A₂ plus A₂pm; (b) medium A₂ plus A₂pm and L-lysine (50 μg ml⁻¹). The A₂pm that remained in the medium was measured at intervals and the amount that had disappeared (since zero time) was assumed to represent the uptake of the amino acid: ○, turbidity of the culture (colorimeter reading); Δ, *meso*-A₂pm; □, LL-A₂pm; ●, DD-A₂pm.

so the uptakes of the isomers were compared (Fig. 1*a*). The rates of uptake of *meso*- and LL-isomers were similar, but DD-A₂pm was taken up more slowly. When the organisms reached the stationary phase (about 1.5 mg dry wt ml⁻¹) 85% of the *meso*- or LL-A₂pm had been taken up, while only 30% of the DD-isomer had disappeared from the medium. Later experiments (see below) showed that the rate of uptake of radioactivity supplied as A₂pm in medium containing lysine closely matched the rate of disappearance of A₂pm, and that most of the radioactivity taken up remained in the form of A₂pm within the organisms.

Incorporation of A₂pm into peptidoglycan

The A₂pm that is taken up by growing organisms may enter the free amino acid pool, some may be incorporated into peptidoglycan and some may be decarboxylated to lysine and then incorporated into protein. In experiments with [¹⁴C]A₂pm, cultures were always grown with lysine added to the medium to repress or inhibit diaminopimelate decarboxylase (White *et al.*, 1964; Rosner, 1975). With *B. megaterium* 7581, the activities of this enzyme (assayed manometrically in the absence of lysine; White, 1971) were [nmol min⁻¹ (mg protein)⁻¹]: 6.3 in organisms from minimal medium A₂, and 3.6 in organisms from medium plus lysine and A₂pm (both 50 μg ml⁻¹). When lysine was present in the medium, the amount of *meso*-A₂pm taken up was decreased to less than half (Fig. 1*b*), presumably because A₂pm was no longer used as a precursor of lysine, though lysine might perhaps also compete with A₂pm for entry into the bacteria. The uptakes of the LL- and DD-isomers were also decreased in the presence of lysine.

When organisms were grown with synthetic [¹⁴C]A₂pm (50 μg ml⁻¹; 0.01 μCi ml⁻¹) and L-lysine (50 μg ml⁻¹) in 20 ml medium A₂, radioautograms (solvent 1) of the hydrolysates of the whole organisms (see Methods) showed some labelling of lysine (14% of the total radioactivity present, which implies that about 25% of the [¹⁴C]A₂pm taken up was decarboxylated) but most of the radioactivity was present as A₂pm. The hydrolysates of

Table 1. *Proportions of [¹⁴C]A₂pm isomers in the peptidoglycan of B. megaterium 7581 after growth with individual isomers of [¹⁴C]A₂pm and unlabelled lysine*

Organisms were grown in medium A2 (as described in Methods) with L-lysine (50 µg ml⁻¹) and individual isomers (each 50 µg ml⁻¹) of [¹⁴C]A₂pm. Peptidoglycan was isolated when growth had reached about 1 mg ml⁻¹ (late-exponential phase); the peptidoglycan was hydrolysed with acid and the proportions of the isomers of [¹⁴C]A₂pm were determined in the hydrolysate.

Isomers of [¹⁴ C]A ₂ pm supplied in medium (µCi ml ⁻¹)	Total uptake of [¹⁴ C]A ₂ pm (nCi ml ⁻¹)	Isomers of [¹⁴ C]A ₂ pm in peptidoglycan (% of total [¹⁴ C]A ₂ pm present in hydrolysate)		
		<i>meso</i> -	LL-	DD-
<i>meso</i> - (0.013)	5.9	89	7	4
LL- (0.011)	4.1	82	18	Trace
DD- (0.010)	1.6	86	0	14
Unresolved (0.010)	3.5	85	9	6

In every case, at least 70 % of the total [¹⁴C]A₂pm taken up was present in the isolated peptidoglycan, as [¹⁴C]A₂pm.

peptidoglycan (see Methods) contained at least 95 % of the radioactivity as A₂pm, the remainder being present as lysine, which might indicate some contamination of the peptidoglycan by protein.

When organisms were grown in medium A2 (20 ml) plus L-lysine (50 µg ml⁻¹) and individual isomers of [¹⁴C]A₂pm (each at 50 µg ml⁻¹; 0.01 Ci ml⁻¹), the observed uptakes of the [¹⁴C]A₂pm isomers, measured by scintillation counting, were similar to those of the unlabelled isomers in the presence of lysine (Fig. 1*b*). The peptidoglycan samples were hydrolysed with acid and assayed (see Methods) for the proportions of *meso*-, LL- and DD-[¹⁴C]A₂pm that they contained (Table 1).

When radioactivity was supplied in the medium as DD-A₂pm, most (86 %) of the labelling in the isolated peptidoglycan was in the form of *meso*-[¹⁴C]A₂pm, suggesting that DD-A₂pm may be enzymically converted to *meso*-A₂pm. The small amount of LL-[¹⁴C]A₂pm present in the DD-[¹⁴C]A₂pm could, of course, also give rise to *meso*-[¹⁴C]A₂pm by the action of diaminopimelate epimerase. However, the amount of radioactivity incorporated into peptidoglycan as *meso*-A₂pm after growth with DD-[¹⁴C]A₂pm was twice the total amount of radioactivity present initially in the medium as contaminating LL-isomer, and no labelled LL-A₂pm was found in the peptidoglycan, suggesting that little LL-[¹⁴C]A₂pm was actually taken up. Supplying DD-[¹⁴C]A₂pm in the medium also led to an increased proportion of the radioactivity in the peptidoglycan being in the form of DD-[¹⁴C]A₂pm. This result is consistent only with the DD-isomer being incorporated as such. If the DD-A₂pm in the hydrolysed peptidoglycan had arisen solely by acid-induced isomerization of *meso*- or LL-A₂pm residues, then DD-[¹⁴C]A₂pm would have been found in high proportion when *meso*-[¹⁴C]A₂pm or LL-[¹⁴C]A₂pm were supplied in the medium; DD-[¹⁴C]A₂pm would have only labelled the peptidoglycan after isomerization to *meso*- or LL-A₂pm.

The pattern of labelled isomers in the peptidoglycan after growth with radioactive LL- or *meso*-A₂pm is consistent with the known interconversion of these two isomers (White *et al.*, 1969). Some DD-[¹⁴C]A₂pm was found in the peptidoglycan after growth with *meso*-[¹⁴C]A₂pm, but the radioactivity in the DD-isomer was only one-third of that added to the medium as DD-[¹⁴C]A₂pm present as an impurity in *meso*-[¹⁴C]A₂pm. On the other hand, the LL-[¹⁴C]A₂pm contained nearly as much contaminating DD-[¹⁴C]A₂pm but gave very little DD-[¹⁴C]A₂pm in the peptidoglycan.

Entry of A₂pm into the free amino pool acid

The rate of uptake of DD-A₂pm by growing cultures was less than the rates of uptakes of the *meso*- or LL-isomers (see above), and this difference could have been due to a lower

rate of entry of the DD-isomer or to a lower rate of its incorporation, or to both. The uptakes of DD- and LL- A_2pm into pool and into covalently bound material were compared during exponential growth in medium A2 containing lysine (see Methods). With each isomer, less than 10% of the A_2pm taken up by the organisms was extractable by trichloroacetic acid at any time.

DISCUSSION

Separation of LL- and DD- $[^{14}C]A_2pm$ with relatively high specific activity and free of *meso*-isomer is fairly easy. It is more difficult to obtain pure *meso*- $[^{14}C]A_2pm$ of high specific activity and purity; the method described in this paper is not very satisfactory.

Unlike *E. coli* (Leive & Davis, 1965), *B. megaterium* can readily take up *meso*- or LL- A_2pm during growth (cf. Pitel & Gilvarg, 1970; Saleh & White, 1976). There is stereochemical specificity of uptake, the DD-isomer being taken up at less than half the rate of the LL-isomer. Little $[^{14}C]A_2pm$ is accumulated in a free amino pool, which could mean that the speed of entry rather than of utilization limits the rate of uptake.

The proportions of the different radioactive isomers found in the peptidoglycan in these experiments do not necessarily reflect closely the proportions of total A_2pm (^{14}C - and ^{12}C -) actually present in the wall, because the extents to which synthesis of the different isomers of A_2pm from glucose and their incorporation continue, even when A_2pm and lysine are supplied to the medium, are not known. However, with this reservation, the results suggest that *meso*- and DD- A_2pm may be directly interconvertible, while LL- and DD- A_2pm may not be, thus: Glucose $\rightarrow$ LL- $A_2pm \rightleftharpoons$ *meso*- $A_2pm \rightleftharpoons$ DD- A_2pm .

Metabolism of DD- A_2pm by growing organisms has not previously been reported. Mutants of *E. coli* that required A_2pm (plus lysine) for growth (Meadow *et al.*, 1957) did not respond to the DD-isomer, nor did a diaminopimelate-requiring mutant of *B. megaterium* (Pitel & Gilvarg, 1970). Saleh & White (1976) used the inability of a diaminopimelate-requiring mutant of *B. megaterium* 7581 to remove DD- A_2pm rapidly from the medium during its growth as a means of resolving DD- A_2pm from a racemic mixture with the LL-isomer. The present study shows that DD- A_2pm can be taken up by *B. megaterium* 7581 and incorporated as such into peptidoglycan. More interestingly, the DD- A_2pm was partly converted to *meso*- A_2pm , presumably by epimerization at C-2 of the DD-isomer. If, like LL-diaminopimelate 2-epimerase, this suggested enzyme-catalysed reaction were reversible, then it would provide a route for synthesis of the DD- A_2pm found in the walls of *B. megaterium*. Preliminary attempts (unpublished) to recognize such an enzyme in extracts of *B. megaterium* have been unsuccessful. Other enzymes that can use DD- A_2pm as a substrate have been described: Meadow & Work (1958) found that transamination between DD- A_2pm and 2-keto acids was catalysed by extracts of several bacteria; and Wickus & Strominger (1972) showed that an enzyme system from *B. megaterium* QMB1551 could add DD- A_2pm to a peptidoglycan precursor.

A.D. was supported by a postgraduate studentship from the Science Research Council. We thank Dr W. Ferdinand (Department of Biochemistry, University of Sheffield) for amino acid analysis, and Chas. Pfizer and Co., Groton, Conn., U.S.A. for a gift of diaminopimelic acid.

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