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

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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-diaminol[14C]pimelate was supplied in the medium, 86% of the diaminol[14C]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 (A2pm) in the following proportions (% w/w): meso-, 75; LL-, 18; DD-, 7 (Day & White, 1977). If free DD-A2pm were to be found in the amino acid pool, this would support the view that DD-A2pm 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 A2pm in the amino acid pool of this organism have therefore been studied. The uptake of each separate isomer of A2pm and its subsequent metabolism have also been examined with growing cultures, and the conversion of DD-A2pm into meso-A2pm 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 μg 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 A2pm) as described by Day & White (1977).

Assays for isomers of A2pm. Colorimetric and manometric methods (Day & White, 1977) were used. In addition, isomers of [14C]A2pm and [14C]lysine were separated by paper chromatography (see below) and then the radioactivity in each was measured.

Preparation of isomers of A2pm. The unlabelled isomers were isolated as described by Day & White (1977).

Isolation of isomers of [14C]A2pm. (i) meso-[14C]A2pm. [1,(7)-14C]A2pm (5 μCi; about 100 μg) was dis-
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 ml) and dianinopimelate 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 dianinopimelate 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 dianinopimelate 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 was more migratory than meso-A2pm (from 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 dianinopimelate decarboxylase in their isolation.

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

When [14C]A,pm 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 A,pm (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 A,pm (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 A,pm was calculated from this radioactivity and the measured specific activity of the A,pm. 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 A,pm. The A,pm 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]A,pm were centrifuged, then washed with 0.1 M-phosphate buffer, pH 6.8 (10 ml) containing unlabelled synthetic A,pm (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]A,pm into the amino acid pool. Organisms were grown in medium A2 (20 ml) containing L-lysine (50 μg ml⁻¹) and either L- or D,D-A,pm (50 μg ml⁻¹); early in the exponential phase of growth L-[14C]A,pm (0.34 μCi) or D,D-[14C]A,pm (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 A,pm in the free amino acid pool plus covalently linked A,pm, while the radioactivity after extraction with trichloroacetic acid represented only covalently linked A,pm in the organisms.

Chemicals. Diaminoproline acid (10 mCi mmol⁻¹) was purchased from Calatonic (54984 Terminal Annex, Los Angeles, Calif., U.S.A.). This material contained approximately 50 % of its radioactivity as meso-A,pm, 25 % as LL-A,pm and 25 % as DD-A,pm. Synthetic A,pm (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 (μM): glutamate, 140; alanine, 12; glycine, 8; serine, 5; lysine, 5; ornithine, 4; A,pm, 4. The A,pm 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 A,pm with diaminopimelate epimerase and diaminopimelate decarboxylase. The absence of DD-A,pm does not prove that this isomer has no metabolic role; several amino acids that must be intermediate 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 A,pm during growth of B. megaterium

The rate of exponential growth of B. megaterium 7581 was unaltered by the presence of A,pm. The rate of uptake of meso-A,pm reached a maximum when 50 μg ml⁻¹ was used. About 30 μg meso-A,pm was taken up during growth of 1 mg dry wt bacteria; this represents about half the total A,pm 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 A,pm, are present per mg bacteria.) Synthetic A,pm (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 A,pm, and...
Fig. 1. Comparison of the uptakes of the three isomers of A\textsubscript{2}pm (each initially 50 \(\mu\)g ml\(^{-1}\)) by growing cultures of \textit{B. megaterium} 7581: (a) medium A2 plus A\textsubscript{2}pm; (b) medium A2 plus A\textsubscript{3}pm and L-lysine (50 \(\mu\)g ml\(^{-1}\)). The A\textsubscript{3}pm 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: \(\circ\), turbidity of the culture (colorimeter reading); \(\triangle\), meso-A\textsubscript{3}pm; \(\Box\), LL-A\textsubscript{3}pm; \(\bullet\), DD-A\textsubscript{3}pm.

so the uptakes of the isomers were compared (Fig. 1a). The rates of uptake of meso- and LL-isomers were similar, but DD-A\textsubscript{2}pm 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-A\textsubscript{2}pm 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 A\textsubscript{2}pm in medium containing lysine closely matched the rate of disappearance of A\textsubscript{2}pm, and that most of the radioactivity taken up remained in the form of A\textsubscript{2}pm within the organisms.

\textit{Incorporation of A\textsubscript{2}pm into peptidoglycan}

The A\textsubscript{2}pm 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 \(^{14}\text{C}\)A\textsubscript{2}pm, cultures were always grown with lysine added to the medium to repress or inhibit diaminopimelate decarboxylase (White \textit{et al.}, 1964; Rosner, 1975). With \textit{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 A\textsubscript{2}pm (both 50 \(\mu\)g ml\(^{-1}\)). When lysine was present in the medium, the amount of meso-A\textsubscript{2}pm taken up was decreased to less than half (Fig. 1b), presumably because A\textsubscript{2}pm was no longer used as a precursor of lysine, though lysine might perhaps also compete with A\textsubscript{2}pm 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 \(^{14}\text{C}\)A\textsubscript{2}pm (50 \(\mu\)g ml\(^{-1}\); 0-01 \(\mu\)Ci ml\(^{-1}\)) and L-lysine (50 \(\mu\)g 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 \(^{14}\text{C}\)A\textsubscript{2}pm taken up was decarboxylated) but most of the radioactivity was present as A\textsubscript{2}pm. The hydrolysates of
Incorporation of diaminopimelate isomers

Table 1. Proportions of $[^{14}C]A_{2}pm$ isomers in the peptidoglycan of B. megaterium 7581 after growth with individual isomers of $[^{14}C]A_{2}pm$ and unlabelled lysine

Organisms were grown in medium A2 (as described in Methods) with L-lysine (50 $\mu$g ml$^{-1}$) and individual isomers (each 50 $\mu$g ml$^{-1}$) of $[^{14}C]A_{2}pm$. 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 $[^{14}C]A_{2}pm$ were determined in the hydrolysate.

<table>
<thead>
<tr>
<th>Isomers of $[^{14}C]A_{2}pm$ supplied in medium ($\mu$Ci ml$^{-1}$)</th>
<th>Total uptake of $[^{14}C]A_{2}pm$ (nCi ml$^{-1}$)</th>
<th>Isomers of $[^{14}C]A_{2}pm$ in peptidoglycan (% of total $[^{14}C]A_{2}pm$ present in hydrolysate)</th>
</tr>
</thead>
<tbody>
<tr>
<td>meso- (0-013)</td>
<td>5.9</td>
<td>meso-</td>
</tr>
<tr>
<td>LL- (0-011)</td>
<td>4.1</td>
<td>LL-</td>
</tr>
<tr>
<td>DD- (0-010)</td>
<td>1.6</td>
<td>DD-</td>
</tr>
<tr>
<td>Unresolved (0-010)</td>
<td>3.5</td>
<td></td>
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</table>

In every case, at least 70% of the total $[^{14}C]A_{2}pm$ taken up was present in the isolated peptidoglycan, as $[^{14}C]A_{2}pm$.

peptidoglycan (see Methods) contained at least 95% of the radioactivity as $A_{2}pm$, 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 $\mu$g ml$^{-1}$) and individual isomers of $[^{14}C]A_{2}pm$ (each at 50 $\mu$g ml$^{-1}$; 0-01 Ci ml$^{-1}$), the observed uptakes of the $[^{14}C]A_{2}pm$ isomers, measured by scintillation counting, were similar to those of the unlabelled isomers in the presence of lysine (Fig. 1b). The peptidoglycan samples were hydrolysed with acid and assayed (see Methods) for the proportions of meso-, LL- and DD-$[^{14}C]A_{2}pm$ that they contained (Table 1).

When radioactivity was supplied in the medium as DD-$A_{2}pm$, most (86%) of the labelling in the isolated peptidoglycan was in the form of meso-$[^{14}C]A_{2}pm$, suggesting that DD-$A_{2}pm$ may be enzymically converted to meso-$A_{2}pm$. The small amount of LL-$[^{14}C]A_{2}pm$ present in the DD-$[^{14}C]A_{2}pm$ could, of course, also give rise to meso-$[^{14}C]A_{2}pm$ by the action of dianminopimelate epimerase. However, the amount of radioactivity incorporated into peptidoglycan as meso-$A_{2}pm$ after growth with DD-$[^{14}C]A_{2}pm$ was twice the total amount of radioactivity present initially in the medium as contaminating LL-isomer, and no labelled LL-$A_{2}pm$ was found in the peptidoglycan, suggesting that little LL-$[^{14}C]A_{2}pm$ was actually taken up. Supplying DD-$[^{14}C]A_{2}pm$ in the medium also led to an increased proportion of the radioactivity in the peptidoglycan being in the form of DD-$[^{14}C]A_{2}pm$. This result is consistent only with the DD-isomer being incorporated as such. If the DD-$A_{2}pm$ in the hydrolysed peptidoglycan had arisen solely by acid-induced isomerization of meso- or LL-$A_{2}pm$ residues, then DD-$[^{14}C]A_{2}pm$ would have been found in high proportion when meso-$[^{14}C]A_{2}pm$ or LL-$[^{14}C]A_{2}pm$ were supplied in the medium; DD-$[^{14}C]A_{2}pm$ would have only labelled the peptidoglycan after isomerization to meso- or LL-$A_{2}pm$.

The pattern of labelled isomers in the peptidoglycan after growth with radioactive LL- or meso-$A_{2}pm$ is consistent with the known interconversion of these two isomers (White et al., 1969). Some DD-$[^{14}C]A_{2}pm$ was found in the peptidoglycan after growth with meso-$[^{14}C]A_{2}pm$, but the radioactivity in the DD-isomer was only one-third of that added to the medium as DD-$[^{14}C]A_{2}pm$ present as an impurity in meso-$[^{14}C]A_{2}pm$. On the other hand, the LL-$[^{14}C]A_{2}pm$ contained nearly as much contaminating DD-$[^{14}C]A_{2}pm$ but gave very little DD-$[^{14}C]A_{2}pm$ in the peptidoglycan.

Entry of $A_{2}pm$ into the free amino pool acid

The rate of uptake of DD-$A_{2}pm$ 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,pm 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,pm taken up by the organisms was extractable by trichloroacetic acid at any time.

DISCUSSION

Separation of LL- and DD-[14C]A,pm with relatively high specific activity and free of meso-isomer is fairly easy. It is more difficult to obtain pure meso-[14C]A,pm 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,pm 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 [14C]A,pm 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,pm (14C- and 15C-) actually present in the wall, because the extents to which synthesis of the different isomers of A,pm from glucose and their incorporation continue, even when A,pm and lysine are supplied to the medium, are not known. However, with this reservation, the results suggest that meso- and DD-A,pm may be directly interconvertible, while LL- and DD-A,pm may not be, thus: Glucose \rightarrow LL-A,pm \rightleftharpoons meso-A,pm \rightleftharpoons DD-A,pm.

Metabolism of DD-A,pm by growing organisms has not previously been reported. Mutants of E. coli that required A,pm (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,pm rapidly from the medium during its growth as a means of resolving DD-A,pm from a racemic mixture with the LL-isomer. The present study shows that DD-A,pm can be taken up by B. megaterium 7581 and incorporated as such into peptidoglycan. More interestingly, the DD-A,pm was partly converted to meso-A,pm, 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,pm 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,pm as a substrate have been described: Meadow & Work (1958) found that transamination between DD-A,pm 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,pm to a peptidoglycan precursor.

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Incorporation of diaminopimelate isomers


