Biosynthesis of Peptidoglycan in the One Million Molecular Weight Range by Membrane Preparations from Bacillus megaterium*

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SUMMARY

Peptidoglycan made in vitro by membrane preparations of Bacillus megaterium had a buoyant density in CsCl different from that of native cell wall. This newly made material was not covalently attached to substantial amounts of previously formed cell material such as wall. From sedimentation velocity centrifugation, the molecular weight of much of the in vitro product was found to be greater than $6 \times 10^6$. Peptidoglycan made in the presence of penicillin had smaller molecular weights suggesting that transpeptidation was in part responsible for the large size of the product. With reaction conditions in which transpeptidation was presumed to have occurred, release of alanine was observed. This release was inhibited by penicillin.

It has been reported that Bacillus megaterium membrane preparations can synthesize peptidoglycan cross-linked in two different patterns (1). One of these patterns involves the removal of the terminal alanine from a peptide side chain and the attachment of the rest of the peptide to a diaminopimelic acid residue of another peptide. Since this type of transpeptidation is also seen in many other organisms, experiments were undertaken to study the physical properties of the peptidoglycan made in vitro under conditions where only such cross-linking could occur. It was found that B. megaterium membrane preparations could make a substantial amount of cross-linked peptidoglycan of molecular weight greater than approximately $6 \times 10^6$. This material was not linked to previously existing cell wall.

One method used to study peptidoglycan made in vitro is electron microscopy. However, for this technique to be useful, it is advantageous for the product to be as large as possible. In order to make a large product resembling closely that made in vivo, the isolated membranes should obviously be damaged as little as possible. Therefore, a new method of cell breakage was employed for preparing membranes. In this method bacteria were cut cleanly in two in the frozen state. This procedure was presumed to be more gentle than techniques employed previously.

EXPERIMENTAL PROCEDURE

Bacterial Strain and Culture Conditions—Bacillus megaterium 899 was grown to early stationary phase (turbidity at 540 nm of 1.8 to 2.2) in either H2O or D2O in antibiotic medium No. 3 (Difco) as described in the accompanying paper (2). Unless otherwise stated, all cultures were grown in H2O medium.

Cell Breakage—The bacteria were suspended in Buffer A (0.1 M Tris-HCl, pH 7.4) for all manipulations following the harvesting of the cells until the beginning of the incubations for peptidoglycan synthesis. The cells were broken by sonic oscillation at 20,000 cps for 1 min, grinding with alumina, or freeze-fracturing. For this last procedure, the bacteria were first treated with toluene as described elsewhere (2). The cells were then washed with Buffer A with toluene added to 1%. The bacteria were centrifuged, and the pellet was transferred with a spatula to a mortar filled with liquid nitrogen. The mortar was kept cold in a bath of liquid nitrogen. The bacterial pellet froze rapidly and was ground to a fine powder using a pestle. The frozen powder was transferred with a spatula to a beaker at room temperature with a volume of Buffer A equivalent to 10 to 20 times the volume of the original pellet. When the powder had thawed, a few micrograms of deoxyribonuclease were added to increase the viscosity.

Membrane Preparation from Broken Cells—Cells disrupted by one of the three methods described above were suspended in Buffer A. The suspension was then centrifuged at 1,000 × g for 5 min to remove the unbroken cells. The supernatant was centrifuged at 4,000 × g for 5 min to remove the cell wall material. The remaining supernatant was centrifuged at 48,000 × g for 5 min to collect the large membrane fragments. For the in vitro synthesis experiments, the membrane pellets were suspended in 2 volumes of Buffer A for each volume of the pellet. By visual observation, unbroken cells comprised less than 10% of the pellet volume. There were usually 10 to 15 mg of protein per ml of membrane suspension as measured by the Lowry technique (3).

In vitro Peptidoglycan Synthesis—Typically, 10 µl of a membrane preparation in Buffer A were mixed with 10 µl of 0.5 µl Tris buffer, pH 8.5, containing 0.08 M MgCl2, and, when desired, 1 mg per ml of benzylpenicillin. After 5 min preincubation at 30°C, the UDP-
GlcNAc and UDP-MurNAc-Ala-Dap-Ala-Ala were each added to a final concentration of 0.4 mM. The final reaction mix-
ture contained approximately a 30 μl volume. The incubation
was continued for another 60 min at 30°C unless otherwise stated.
In order that the extent of peptidoglycan synthesis might be
determined, one of the substrates was radioactively labeled in each
experiment.

Chemicals—[9C]Glucose with specific activity of 268 mCi per
mmole and UDP-[9C]GlcNAc with specific activity of 276 mCi per
mmole were purchased from Amersham-Searle Corp. UDP-
[9H]GlcNAc with specific activity of 8 Ci per mmole was purchased
from New England Nuclear. UDP-MurNAc-Ala-Glu-Dap-
Ala-[9C]-Ala-[9C] Ala with specific activity of 186 mCi per mmole was prepared
from Escherichia coli B. This enzyme preparation was
made by proteolytic subtilisin precipitation and subsequent elution
with phosphate buffer essentially according to the method of
Mizuno and Ito (4). The preparation of the substrates UDP-
MurNAc-d-Ala-d-Glu and UDP-MurNAc-L-Ala-L-Glu from the few cells that were broken. Since
preparation described above was added to 4.8 ml of CsCl in Buffer
B (0.1 M KCl-0.1 M Tris, pH 8.6) with a refractive index for the
density gradient of 1.553 g as measured in an Abbé refrac-
tometer. CsCl solutions with this refractive index have a density
of 1.522 g per cm (8). When desired, B. megaterium walls prepared
as described above were added to the CsCl solution to provide a
density marker. The usual method of addition was to sediment
the walls by centrifugation and resuspend the pellet in the CsCl
solution. All samples of peptidoglycan made in vitro together
with density marker walls were thoroughly mixed with the entire
volume of CsCl solution required to form the density gradients
prior to centrifugation. All gradients were centrifuged at 180,000
× g (SW 50.1 rotor at 39,000 rpm in a Spinco ultracentrifuge) for
16 to 18 hours at 20°C. Fractions were collected through the hypo-
dermic syringe needle used to puncture the bottoms of the tubes.

Sometimes the refractive index was measured for the samples, and
the corresponding densities were obtained from a table of den-
tities (8). The position of the marker walls was obtained by dilut-
ing the fractions with water and reading the turbidity at 540 nm
in a spectrophotometer. For measurements of radioactivity in
peptidoglycan, the gradient fractions were collected directly
onto Whatman No. 3MM filter paper and chromatographed. In an alternate
procedure, an aliquot from the reaction was spotted on the chro-
matograms and then radioactivity was added in the same manner.

In order that the extent of peptidoglycan synthesis might be
determined, one of the substrates was radioactively labeled in each
experiment.

Chromatographic Analysis—Aliquots from the synthetic reac-
tions were added to an equal volume of isobutyric acid to stop
incorporation. The inactivated reactions were spotted on What-
man No. 3MM filter paper and chromatographed. In an alternate
procedure, an aliquot from the reaction was spotted on the chro-
matograms and then radioactivity was added in the same manner.

To differentiate the various products of the reactions, the chro-
matograms were developed by descending chromatography for 12
hours in Solvent A (isobutyric acid-1 N HCl-0.1 M KCl-0.1 M
MgCl (5.3, v/v)). In this solvent system the substrates are widely separated from im-
mobile product and from alanine. To measure only the produc-
tion of nonmigrating peptidoglycan, the chromatograms were
developed by descending chromatography for 12 hours in Solvent B
(isobutyric acid-1 M NH4OH-H2O (2:2:1, v/v)), which could not
resolve the migrating small molecules from each other. The drier
chromatograms were examined in a radiochromatogram scanner to
determine the positions of various radioactively bound compo-
unds. Quantitative measurements were made by cutting appropri-
ate segments from the chromatograms and counting these segments in a liquid
scintillation counter.

Bacillus megaterium Walls—Cells broken by sonication were
centrifuged at 1,000 × g for 5 min to remove remaining whole
peptidoglycan was determined in a liouid scintillation counter. The final super-
natant having a volume of approximately 200 μl was used for the approximat-
ely 10% of the bacteria were broken. The frozen
trifugation steps were pooled, and the amount of radioactivity
was determined in a liquid scintillation counter. All the after the centrifugation steps had been col-
b Spectrum Analysis—Fifteen microliters of concentrated
were added to the synthetic reactions prepared for centrifu-
gation analysis. The fractions were collected, and the radioactivity
was determined as described for the CsCl gradients. In addition, the band
of the gradient fractions on E. coli C (obtained from A. Burton, St. Olaf College) according to published procedures (9).

RESULTS

Biological Studies—For experiments in peptidoglycan syn-
these, B. megaterium cells were broken by any of three tech-
niques, sonoceussion, grinding with alumina, or freeze-fract-
turing. This last technique has not been generally used and
will be discussed in some detail. Electron micrographs of cells
preparations, the membranes from broken cells could be damaged mechanically only at the position of cell fracture. The only other possible source of mem-
brane damage was the process of freezing and thawing.

Often, the freeze-fractured bacteria were also exposed to tol-
ulene before the freezing step. Prior treatment with toluene
was performed to ensure that membrane fragments from the

1 The abbreviations used are: UDP-GlcNAc, UDP-N-acetyl-
N-glucosamine; UDP-MurNAc-Ala-Glu-Dap-Ala-Ala, UDP-N-acet-
yl-DL-alanine-DL-glutamyl-DL-meso-diaminopimelyl-D-alanine-DL-
alanyl-DL-alanine; D2O walls, walls made from cells grown in D2O
medium; H2O walls, walls made from cells grown in H2O medium;
D2O membranes, membranes prepared from cells grown in D2O
medium; H2O membranes, membranes prepared from cells grown in H2O
medium.
broken cells would be able to utilize exogenously added peptido-

glycan substrates. It was expected that these fragments would

have formed closed vesicles (11). If these vesicles behaved like

whole cell membranes then in the absence of toluene exposure

they also would have been impermeable to peptidoglycan pre-

ursors (2).

In initial experiments, membranes from freeze-fractured B. me-

gaterium cells treated with toluene were used to synthesize

peptidoglycan from the substrates UDP-[14C]GlcNAc and UDP-

MurNAc-Ala-Glu-Dap-Ala-Ala. The product was measured as

ch chromatographically immobile material. The time course of

the reaction was followed at various temperatures (Fig. 1). All

subsequent reactions were performed at 30° where the rate and

extent of incorporation were greatest. As expected, the omission

of UDP-MurNAc-Ala-Glu-Dap-Ala-Ala completely inhibited

the synthesis (Fig. 1). Chromatographic analyses of the reaction

mixtures in Solvent A showed that the substrate was either

unchanged or was transformed to nonmigrating peptidoglycan.

The product was also completely digestible by 0.25 mg per ml of

lysozyme treatment for 15 min at 30° and pH 8.0. The depend-

ence of synthesis on pH (Fig. 2) and magnesium ion concentra-

tion (Fig. 3) was also determined. The effect of the toluene

treatment before the freeze-fracture step was examined as shown

in Fig. 4. This treatment was seen to be slightly stimulatory.

To check the possibility that the peptidoglycan made \textit{in vitro}

was linked by peptide cross-bridges, UDP-MurNAc-Ala-Glu-

Dap-[14C]Ala-[14C]Ala was used instead of UDP-[14C]GlcNAc as

the radioactive precursor in the incubation mixtures (Fig. 5).

In the absence of UDP-GlcNAc, there was no synthesis of pep-
tidoglycan but carboxypeptidase(s) did cleave the terminal 1 or

2 alanine residues from the unused substrate (Fig. 5B). When

both precursors were present (Fig. 5A), immobile product was

formed. Furthermore, more alanine was released than when

UDP-GlcNAc was absent. This increase in free alanine in the

presence of both substrates was consistent with the interpreta-
tion that in addition to carboxypeptidase activity there was also

transpeptidase activity which could liberate alanine while

making peptide cross-links. The experiment of Fig. 5C shows

that all alanine release was blocked by penicillin as had been

found previously (1). Therefore, the peptidoglycan made in

the presence of penicillin was uncross-linked and retained both

radioactive alanine residues at the carboxyl end of each peptide

side chain. The specific radioactivity per disaccharide subunit

was adjusted to give the indicated final pH. The radioactive

substrate was UDP-[14C]GlcNAc. Following a 30-min incorpora-

tion the radioactive precursor in the incubation mixtures (Fig. 5).

Time courses of synthesis in the standard reaction were followed by

incorporation of radioactivity from UDP-[14C]GlcNAc into non-
migrating product. Solvent B was used for the chromatographic

analysis.

\begin{figure}[h!]
\centering
\includegraphics[width=\textwidth]{fig1.png}
\caption{(left). Temperature dependence of peptidoglycan syn-

thesis by \textit{Bacillus megaterium} membranes. Membranes used in

the reactions were prepared from tolune-treated cells by the

freeze-fracture technique. The reactions were performed under

standard conditions at various temperatures. The labeled sub-

strate was UDP-[14C]GlcNAc. At the times indicated, samples

were withdrawn and chromatographed in Solvent B. The non-
migrating peptidoglycan was plotted: 20° (△—△), 30°

(●—●), 37° (▲—▲), 30° (○—○) with UDP-MurNAc-

Ala-Glu-Dap-Ala-Ala omitted from the reaction.

\caption{(right center). Dependence of peptidoglycan synthesis by

\textit{Bacillus megaterium} membranes on magnesium ion concentra-

tion. Membranes used in the reactions were prepared from tolune

treated cells by the freeze-fracture technique. The reactions

were performed under the standard conditions with penicillin

present except that the MgCl₂ used in the preincubation was

adjusted to give the indicated final magnesium ion concentra-

tion. The radioactive substrate was UDP-MurNAc-Ala-Glu-Dap-[14C]-

Ala-[14C]Ala. After 30-min incubation the reactions were

analyzed chromatographically in Solvent B.

\caption{(right). Effect of tolune treatment of peptidoglycan syn-

thesis by \textit{Bacillus megaterium} membranes. A \textit{B. megaterium}

culture was divided in two portions. One portion was treated with

tolune (●—●) and the other (○—○) was not. Membranes

were made from both portions by freeze-fracturing, and the time

courses of synthesis in the standard reaction were followed by

incorporation of radioactivity from UDP-[14C]GlcNAc into

nonmigrating product. Solvent B was used for the chromatographic

analysis.}
Fig. 5. Time course of peptidoglycan synthesis and alanine release by *Bacillus megaterium* membranes. Membranes used in the reactions were prepared from toluene-treated cells by the freeze-fracture technique. A, standard reaction conditions; B, standard reactions with UDP-GlcNAc omitted; C, standard reaction with penicillin added. The labeled substrate was UDP-MurNAc-Ala-Glu-Dap-[\(^{14}\)C]Ala-[\(^{14}\)C]Ala. At the times indicated, samples were withdrawn and chromatographed in Solvent A. O--O, free radioactive alanine released from peptidoglycan and residual substrate. ⚫⚫, radioactive alanine in non-migrating peptidoglycan.

Fig. 6. Buoyant density in CsCl of walls made from *Bacillus megaterium* cells grown in H\(_2\)O or D\(_2\)O media. Walls were prepared from *B. megaterium* cells grown in H\(_2\)O or D\(_2\)O media and then centrifuged in CsCl gradients as described under "Experimental Procedures." O--O, walls from cells grown in H\(_2\)O; ⚫⚫, walls from cells grown in D\(_2\)O; ▲▲, mixture of the walls from cells grown in H\(_2\)O and walls from cells grown in D\(_2\)O. The fractions from the bottom of the centrifuge tube are at the left. Only that portion of the gradient containing significant turbidity was analyzed.

from cells grown in H\(_2\)O had a buoyant density of 1.510 g per cc, and the density of the walls from cells grown in D\(_2\)O was 1.519 g per cc. The densities of H\(_2\)O walls were sometimes as great as 1.530 g per cc, and the densities of D\(_2\)O walls were observed to reach 1.540 g per cc. Most other cell constituents, if they banded in the CsCl gradient, would have been separated from either D\(_2\)O or H\(_2\)O walls. Proteins in general have a density of 1.3 g per cc, whereas nucleic acids have a typical density of 1.7 g per cc or greater.

Peptidoglycan synthesized by membranes prepared by freeze-fracturing from cells grown in H\(_2\)O was freed from membranes by treatment with Sarkosyl followed by treatment with NaOH. The treated radioactive peptidoglycan was mixed with carrier walls isolated from growing bacteria and centrifuged in a CsCl gradient (Fig. 7A). The radioactivity banded at a density lighter than that of walls made *in vivo*. This result suggested that the newly made peptidoglycan was not attached to any
large pieces of previously existing wall. Very little previously formed wall should have been present in the preparation of membranes used in the incubation. However, the radioactive peptidoglycan formed in vitro may have been attached to some cell component present in the membrane preparation other than wall. Therefore CsCl gradient analysis was performed on product from a synthetic reaction in which membranes from cells grown in H2O were replaced by those from cells grown in D2O (Fig. 7B). Since the synthetic reaction contained non-deuterated substrates in an H2O solution, the radioactive peptidoglycan should have had the same density in the experiments of both Fig. 7A and B. However, if peptidoglycan synthesized in vitro were attached to some cell component formed before cell breakage, then the material made from D2O membranes would have had an apparent density greater than that made from H2O membranes. The observed position of the radioactivity relative to the carrier walls was the same in Fig. 7, A and B. In both cases the peak positions were separated by 30% of the gradient length. To verify that the carrier walls did not affect the sedimentation, peptidoglycan made by both kinds of membranes were also centrifuged together with carrier D2O walls (Fig. 7, C and D). In these cases the separations of the peak positions were 55% of the gradient length. The most sensitive check of the co-sedimentation of peptidoglycan synthesized by D2O and H2O membranes is presented in Fig. 7E. Here radioactive material from both incubations were mixed in equal amounts. If these substances had different densities, the width of the radioactive peak would have been broader than that of any peak where peptidoglycan from only one incubation was centrifuged (Fig. 7, A to D).

A computation can be made as to how much broader the peaks should have been. The gradients in Fig. 6 show that the displacement between the peaks of the D2O and H2O walls was about the same as the peak width of the D2O or H2O walls centrifuged separately. As a result, the peak width of the mixture of the two types of walls was twice as great as that for either type alone. Since the gradients of Figs. 6 and 7 were centrifuged under identical conditions using the same material, the density separation between the H2O and D2O walls in Fig. 7 would also be equivalent to the width of either the H2O or D2O wall peak, a difference of approximately 10 fractions. If the newly synthesized peptidoglycan were attached to old cell material that was affected by the medium change in the same way as cell wall, the peaks of radioactive material made by D2O membranes should be about 10 fractions denser than the same material made by H2O membranes. This computation is based on the fact (Fig. 9) that the density gradient is approximately linear in the region between walls and radioactive peptidoglycan. Therefore, in the gradient where the two types of radioactive peptidoglycan were mixed, the peak should have been 10 fractions or 50% broader than the profile of either type of peptidoglycan centrifuged alone. In fact, the peak width measured at the inflection point in Fig. 7E was slightly narrower than that in Fig. 7A, indicating that the peptidoglycan made by B. megaterium membranes prepared by freeze-fracturing was not covalently attached to a significant amount of previously existing cell material.

The experiments described in Figs. 8 and 9 provide additional evidence for this conclusion. In these experiments, B. megaterium cells were grown in the presence of [14C]glucose to radioactivity labeled cell walls and other molecules. In order to ensure that transient intermediates as well as stable compounds were labeled, it was essential that growth be exponential and that the radioactivity not be exhausted from the medium. The increase in turbidity in Fig. 8B showed that the culture was indeed in the exponential growth phase at the time of harvest. The fact that the increase in acid-precipitable radioactivity was also linear (Fig. 8A) also showed that the cells were collected while ample [14C]glucose was still present in the medium. Consistent with this conclusion was the finding that only 15% of the total radioactivity in the culture was acid-precipitable at the time of harvest. The radioactive cells were freeze-fractured without prior toluene treatment. Then the membrane vesicles were harvested for synthesis of peptidoglycan using UDP-[3H]GlcNAc as the radioactive substrate. The incorporation mixtures were then extracted with Sarkosyl and NaOH and centrifuged in a CsCl gradient (Fig. 9B). In this experiment the CsCl solution was adjusted to 1.380 instead of 1.383 before centrifugation in order to shift the bands toward the center of the tube. The gradient tube also contained walls isolated from the cells which were broken by the freeze-fracture procedure. Therefore, the [14C]labeled material in the gradient was cell walls and membrane fragments from cells broken by the freeze-fracture technique. The amount of [14C]labeled walls in the gradient represented the same proportion of broken cells as was used to obtain membranes for the incubation. The UDP-[3H]GlcNAc-labeled material resulted from peptidoglycan synthesis in vitro. The profiles in Fig. 9B showed that there was no detectable [14C] radioactivity in the region of the peak of the [3H]-labeled peptidoglycan confirming the conclusion that the bulk of the newly made material was not linked in appreciable amounts to either old cell wall or old membrane components. The small amount of [14C] radioactivity at the top of the gradient was probably due to membrane components such as proteins solubilized by the Sarkosyl and NaOH extraction steps. This was the region where Sarkosyl and any associated lipophilic material banded in the gradient. Also, free proteins would have been found in this density region. There also was a small amount of [3H]-labeled...
material at this light density which could easily have been newly synthesized peptidoglycan which was not fully removed from membrane material. A control experiment (Fig. 9A) showed that, in the absence of peptidoglycan synthesis, the profile of $^{14}$C radioactivity was the same.

Two separate sets of experiments, (Figs. 7 and 9) then, have both demonstrated that the newly synthesized peptidoglycan was not linked to any substantial amounts of old wall. In the experiment of Fig. 9B, only membrane fragments would have accounted for the synthesis because any contaminating whole cells would not have been able to incorporate the externally added precursors (2). The same result was found in this experiment as ones in which the freeze-fractured cells were first incubated without prior toluene treatment. The unbroken cells were re-

FIG. 9. Buoyant density of peptidoglycan made by membranes of B. megaterium grown in [Wlglucose-containing medium. Cells harvested from the experiment of Fig. 8 were freeze-fractured without prior toluene treatment. The unbroken cells were re-

mixture was separated by centrifugation at 4000 $\times$ g for 5 min. The $^{14}$C glucose-labeled walls were collected by centrifugation at 4000 $\times$ g for 5 min, mixed with carrier nonradioactive walls, sonicated to insure complete cell breakage and then extracted with sarkosyl and NaOH as described under "Experimental Procedures." After the 4000 $\times$ g centrifugation to harvest cell walls, the supernatant solution from the freeze-fractured cells was processed to obtain membranes. One-half of the membrane preparation was incubated with UDP-MurNAc-Ala-Glu-Dap-Ala-Ala but no UDP-GlcNAc (A). The other half of the membrane preparation was identically incubated except that UDP-[H]GlcNAc was also added (B). After 60 min at 30° the two incubation mixtures were extracted separately with Sarkosyl and NaOH and analyzed by CsCl density gradient centrifugation. Unlike other experiments of this kind, the refractive index of the CsCl solution used in the gradient was adjusted to 1.380. Also included in each gradient was one-half of the sonicated and extracted $^{14}$C-glucose-labeled walls. $\bigcirc$,$\bigcirc$, $^1$H-radioactivity in newly synthesized peptidoglycan; $\bullet$,$\bullet$, $^1$C-radioactivity in previously formed walls and membranes; $\Delta$$\Delta$, density as deduced from refractive index measurements.

Since it has already been shown that B. megaterium membranes can make cross-linked peptidoglycan (1), it seemed likely that the formation of peptide cross-bridges might have been at least partly responsible for the density characteristics of the peptido-

glycan synthesized in vitro. Therefore, if penicillin were added to block cross-linking, the density profile of the synthetic product might have been changed. The results of the experiment of Fig. 10A showed that peptidoglycan made by membranes from cells broken by grinding with alumina banded similarly to pepti-
doglycan made from membranes prepared by freeze-fracturing (Figs. 7 and 9). When penicillin was added to the reaction, the amount of product synthesized was diminished only by approxi-

mately 30%, but this material did not form a discrete band when centrifuged in a CsCl density gradient (Fig. 10B). The failure to band could have resulted from the peptidoglycan being heterogeneous in density and hence banding throughout the gradient. Another explanation of the disperse profile is that the material had a small size which would have increased both the band width and time required to reach equilibrium. In any case, the experiment does suggest that cross-linking affects the physical characteristics of the in vitro product. As predicted, in the absence of the second substrate no acid-precipitable radio-

activity was found in the gradient (Fig. 10). The results in Fig. 10 were also found for incubations using membranes from cells broken by sonic oscillation and freeze-fracturing.

Before addition of the CsCl for the gradients, all samples were centrifuged to remove membranes denatured by the Sarkosyl and NaOH treatments. This centrifugation was necessary because substrate was trapped by the denatured membranes. If the material seen as nonmigrating product after chromatography
phage was added to the samples to provide a sedimentation
Bacillus megaterium.

The radioactive peptidoglycan in the incubations
used for the gradients of Fig. 10, A and B were also centrifuged
through 10 to 50% sucrose gradients. Escherichia coli bacterio-
phage φX174 was added to the samples to provide a sedimentation
marker. The gradients of A and B had the same material as was
in the gradients in Fig. 10, A and B, respectively. The fractions
from the bottom of the centrifuge tube are on the left. All radio-
active material to the left of the vertical line was considered larger
than φX174. For both frames: O—O, radioactivity in peptido-
glycan; •—•, φX174 plaque-forming units.

was defined as 100% of the peptidoglycan made in those reaction
mixtures where both substrates were present, then the radio-
activity recovered at the various steps involved in the CsCl
gradient analysis were divided typically as follows: 50% was re-
covered from the gradient fractions and 15% was recovered in the
pellets from the preliminary centrifugations after the Sarkosyl and NaOH treatments. All almost of the radioactive ma-
terial was of homogeneous density in the gradients of reaction
mixtures to which no penicillin was added. These recovery
figures were essentially identical regardless of whether penicillin
was present in the synthetic reactions. The remaining 35% of
the chromatographically immobile radioactivity was unac-
counted for and may have been due to the liberation of very
short peptidoglycan chains from the membrane-bound enzyme
by Sarkosyl and NaOH. This small material might have been
chromatographically immobile but not precipitable by trichloro-
acetic acid.

It is possible to estimate the size of the peptidoglycan made
in vitro by sedimentation velocity centrifugation in a sucrose
gradient. The radioactive product made by membranes was fre-
ed from the synthetic enzymes by Sarkosyl and NaOH

treatment. The remaining material, which by CsCl gradient
analysis (Fig. 10) was shown to be peptidoglycan of a unique
density, was centrifuged through a 10 to 35% sucrose gradient.
The E. coli bacteriophage φX174 was layered with the peptido-
glycan sample to provide an internal marker for molecular
weight determination. As expected, the peptidoglycan was
heterogeneous in size but approximately 35% of all the peptido-
glycan sedimented more rapidly than φX174 (Fig. 11). If these
two compounds had the same sedimentation characteristics,
then the peptidoglycan in the heavier fraction would have had
molecular weights greater than the $6 \times 10^4$ typical of φX174
(11). By comparison, only 13% of the material made in the
presence of penicillin sedimented faster than φX174 (Fig. 11B).

This decrease in the amount of rapidly sedimenting radioactivity
suggested that the large size of the peptidoglycan made in the
absence of penicillin was due in part to cross-linking.

DISCUSSION

In vitro synthesis of peptidoglycan from the substrates UDP-
MurNAc-Ala-Glu-Dap-Ala-Ala and UDP-GlcNAc catalysed by
membrane preparations obtained from B. megaterium was dem-
onstrated by the incorporation of radioactivity from either sub-
strate into chromatographically immobile product. Both sub-
strates had to be present for peptidoglycan synthesis to occur.
As expected, the reaction product formed in vitro was degraded
by lysozyme.

In experiments conducted with UDP-MurNAc-Ala-Glu-Dap-
Ala-Ala labeled in the 2 terminal alanine residues, the pattern
of release of alanine was consistent with previous reports (1)
that B. megaterium could make cross-linked peptidoglycan.

Sedimentation velocity experiments have established that a
large portion of the peptidoglycan made in vitro by B. mega-
terium membranes had molecular weights greater than $6 \times 10^4$.
These same experiments comparing peptidoglycan made in the
presence and absence of penicillin suggested that cross-linking
was at least partly responsible for the large size of the material
made in the absence of the antibiotic. The CsCl gradient pro-
files of the same material also indicated that transpeptidation
could affect the physical properties of the biosynthetic product.
Two types of density gradient experiments have demonstrated
that the peptidoglycan made in vitro was not attached to sig-
nificant amounts of previously formed wall or membrane ma-
terial.

The difference between the density of the peptidoglycan made
in vitro and that of walls isolated from growing cells could be due
to the fact that free diaminopimelic acid was not added to the in
vitro reaction mixtures. Therefore, the transpeptidation depend-
ent on free diaminopimelic acid (1) was not permitted in the in
vitro product. The resulting peptidoglycan could have had a
cross-linking pattern different from that of the walls made in
vitro. Also, the chemical composition would certainly have been
different. In addition, any other wall polymers made by B.
megaterium such as a teichuronic acid would only have been
present in the walls made from growing cells because any neces-
sary substrates were not present in the in vitro incubations.
Experiments are now in progress to study the physical properties
of the peptidoglycan made in vitro in which both B. megaterium
transpeptidation reactions are permitted.

Substances with molecular weights in the range of $10^4$ to $10^7$
can be observed with the electron microscope. Therefore, it
should be possible to examine the differences between walls
made in vitro and the peptidoglycan made in vitro. Since newly
made material can be radioactively labeled with tritium, we have
begun autoradiographic experiments to locate and positively
identify peptidoglycan made in vitro.

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