*GLYCOPEPTIDE TRANSPEPTIDASE AND D-ALANINE CARBOXYPEPTIDASE: PENICILLIN-SENSITIVE ENZYMATIC REACTIONS**

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In 1929, Fleming discovered penicillins and observed that this group of substances kills gram-positive bacteria more effectively than gram-negative bacteria (thus implying some important physiological difference between the two groups of organisms).[1] Subsequent knowledge of the bacterial cell wall made it possible to establish both on physiological and chemical grounds that penicillins are specific and highly selective inhibitors of the biosynthesis of cell walls, both in gram-positive and in gram-negative bacteria.[2–6] The reason for the relative insensitivity of gram-negative bacteria to most penicillins has remained obscure.

More recent knowledge of the structure and biosynthesis of bacterial cell walls has suggested that, in a terminal reaction in cell wall synthesis, linear glycopeptide strands are cross-linked in a transpeptidation, accompanied by release of the terminal D-alanine of the pentapeptide precursor, with formation of a two- or three-dimensional network. Direct chemical analyses of cell walls prepared from cells treated with penicillin[7, 8] and isotopic studies of wall biosynthesis[9, 10] suggested that penicillin was interfering with this hypothetical glycopeptide cross-linking reaction. In the presence of penicillin, nascent glycopeptide, an uncross-linked monomeric unit of the wall, accumulated.[8] Pulse-labeling experiments have established that this nascent unit is an immediate precursor of the final cross-linked glycopeptide; penicillin completely inhibited its integration into the network.[10] Moreover, molecular models of penicillin resembled the acyl-D-alanyl-D-alanine in the linear glycopeptide and it was possible to suggest a molecular mechanism for the transpeptidation and its inhibition by penicillin.[8]

However, the actual reaction or reactions inhibited had not been demonstrated. In this paper we wish to report that a cell-free enzymatic system has been obtained from cells of *Escherichia coli* which catalyzes this cross-linking reaction. We call the enzyme which catalyzes this step glycopeptide transpeptidase, since it catalyzes a reaction in which the peptide chains of two linear glycopeptide strands are cross-linked by a transpeptidation, in which the terminal D-alanine residue of one of the strands is eliminated; this peptide bond synthesis proceeds without any other source of energy and is reversible. The terminal D-alanine residue of the other strand is also removed by hydrolysis catalyzed by a D-alanine carboxypeptidase in the preparation (Fig. 1). Both of these reactions are inhibited by low levels of penicillin G, other penicillins, and a cephalosporin.

Materials and Methods.—Preparation of substrates: UDP-MurNAc·L-ala·D-glu·H^3-meso-DAP·D-ala·D-ala and UDP-MurNAc·L-ala·D-glu·meso-DAP·C^{14}-D-ala·C^{14}-D-ala were prepared enzymatically by sequential addition of amino acids to the appropriate uridine nucleotides.[10a] UDP-GlcNAc-C^{14} is the same preparation used previously.

Preparation of enzyme: Two particulate enzymes, obtained from cells of *E. coli* strain Y-10 or *E. coli* strain B, were employed. The cells were ground with alumina. The fraction of the disintegrated bacteria sedimenting between 10,000 and 100,000 *g* was washed twice with 0.05 *M*

Tris-HCl buffer, pH 7.5, containing 10^{-4} *M* $MgCl_2$ and 10^{-3} *M* mercaptoethanol, resuspended in a small volume of the same buffer, and used as enzyme. The protein content was about 15 mg/ml.

Incubation mixture and assay: A typical incubation mixture contained, in a total volume of 25 μl, 5 μmoles of Tris-HCl buffer, pH 7.5, 1 μmole of $MgCl_2$, 10 mμmoles of UDP-GlcNAc, 0.5 mμmoles of UDP-MurNAc·L-ala·D-glu·meso-DAP·D-ala·D-ala (containing 20,000 cpm either of H^3-meso-DAP or of C^{14}-D-ala·C^{14}-D-ala), and 5 μl of the particulate enzyme, and was incubated at 37° for 1–3 hr. After incubation, the reaction mixtures were boiled for 1 min and then spotted as a 1-cm band on Whatman no. 1 filter paper. After descending paper chromatography in isobutyric acid:1 *N* NH_4OH (5:3) for 24 hr, radioautograms were prepared. Areas of the paper corresponding to the glycopeptide product (R_F = 0), alanine (R_F = 0.5), lipid intermediates (R_F = 0.9), or to other radioactive compounds were cut out and counted in a Packard Tri-carb liquid scintillation spectrometer.

Results.—Formation of glycopeptide product and liberation of free D-alanine with E. coli particles: When UDP-MurNAc·L-ala·D-glu·meso-DAP·D-ala·D-ala (labeled with C^{14}-D-ala·C^{14}-D-ala) and UDP-GlcNAc were incubated with *E. coli* particles, a glycopeptide product was formed[11] as in the case of similar particles prepared from *S. aureus* or from *M. lysodeikticus*.[12] However, the reaction with *E. coli* particles differed in two important respects, viz., free C^{14}-D-alanine was liberated simultaneously, and the glycopeptide product remaining at the origin of the paper chromatogram had a condensed appearance (Fig. 2). With particles from *E. coli* strain Y-10, half of the C^{14}-D-alanine in the original substrate remained in the product and half was liberated as free D-alanine (Table 1).

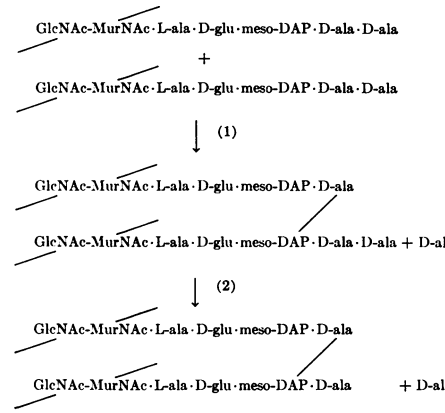

FIG. 1.—Formation of cross-linked dimer in *E. coli* from linear glycopeptide strands, catalyzed by (*1*) glycopeptide transpeptidase, and (*2*) D-alanine carboxypeptidase, the penicillin-sensitive enzymes. Reaction *2* might equally well precede reaction *1* in the sequence.

Similarly, when UDP-GlcNAc-C^{14} was employed as substrate, glycopeptide formation occurred on addition of unlabeled UDP-MurNAc·L-ala·D-glu·meso-DAP·D-ala·D-ala.[13] UDP-MurNAc·L-ala·D-glu·meso-DAP was virtually inactive but UDP-MurNAc·L-ala·D-glu·L-lys·D-ala·D-ala isolated from cells of *S. aureus* was almost as active as the nucleotide containing meso-DAP.

Effects of penicillin G, other penicillins, and a cephalosporin on the reaction: Four penicillins have been examined so far—penicillin G [6-(phenylacetamido)penicillanic acid], ampicillin [6-(D-α-aminophenylacetamido)penicillanic acid], methicillin [6-(2′,6′-dimethoxybenzamido)penicillanic acid], and cephalothin [7-(2-thienylacetamido)cephalosporanic acid]. All four of these substances irreversibly inhibited cross-linking. The irreversible nature of the inhibition was demonstrated by the fact that activity could not be recovered by centrifuging and washing penicillin-treated particles or by adding penicillinase to treated particles. The concentrations required to inhibit the liberation of D-alanine by 50 per cent were: penicillin G, 3 μg/ml; cephalothin, 50 μg/ml; ampicillin, 3 μg/ml; and methicillin, 1000 μg/ml (Table 1). It should be noted that the concentration of penicillin G is one tenth of the concentration required to inhibit growth of the *E. coli* cells. With the

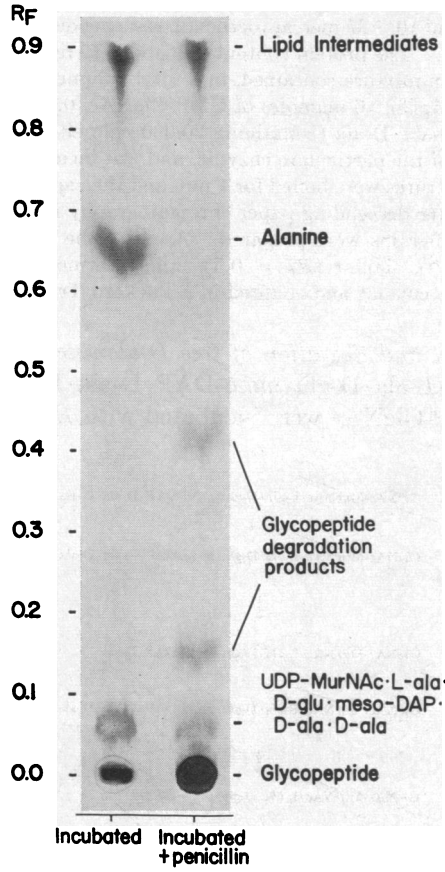

FIG. 2.—Separation of the products of glycopeptide synthesis catalyzed by enzyme from *E. coli* strain Y-10 in the absence and presence of penicillin. A radioautogram of the chromatogram is shown. UDP-MurNAc·L-ala·D-glu·meso-DAP·C^{14}-D-ala·C^{14}-D-ala was the labeled substrate. In the zero time control, only this labeled substrate could be seen. Incubation was for 1 hr.

other substances the growth inhibitory concentrations were similar to those required to inhibit the enzyme system.

In addition to this effect on D-alanine release, the nature of the glycopeptide product was changed on addition of penicillin G or cephalothin. In the first place, it contained twice as much radioactivity as that formed in the absence of these substances when the substrate contained C^{14}-D-ala·C^{14}-D-ala. Second, no increase in the radioactivity of the product was observed when the substrate contained H^3-meso-DAP. Moreover, instead of appearing on the paper chromatogram as a condensed band at the origin, the product appeared to spread from the point of application to the filter paper (Fig. 2) and is referred to as "spreading product."

Methicillin and ampicillin appeared to have additional effects on glycopeptide synthesis. Although these substances inhibited D-alanine release, this inhibition was not accompanied by any enhancement of total D-alanine incorporated into product (Table 1) and, in the case of methicillin, occurred only at relatively high concentrations. These two substances appeared to inhibit total glycopeptide synthesis in addition to inhibiting cross-linking. This phenomenon is being further investigated and extended to the study of other penicillins.

Analysis of products synthesiezd enzymatically in the absence or presence of penicillins: The condensed product formed in the absence of penicillins is a water-insoluble polymer. If the incubation mixture was heated and then centrifuged at 1000 × *g* for 10 min, 55–75 per cent of the product was centrifuged down with the heat-denatured proteins. By contrast, the spreading product formed in the presence of penicillins is a water-soluble polymer; 80–90 per cent of this material remained in the supernatant of heat-treated incubation mixtures (see Table 4). This striking difference in solubility is responsible for the appearance of these materials on the paper chromatograms. The condensed product remains exactly at its point of application to the filter paper, while the spreading product is washed into a larger circular area by succeeding applications of material and by the water used to rinse the incubation tubes.

In addition to the products which remain at the origin of the chromatograms,

TABLE 1

EFFECTS OF PENICILLINS ON GLYCOPEPTIDE SYNTHESIS IN *Escherichia coli* STRAIN Y-10

Amount added (μg/ml)	Penicillin G		Cephalothin		Ampicillin		Methicillin	
	GP	Ala	GP	Ala	GP	Ala	GP	Ala
0	4100	3730	—	—	—	—	—	—
1	4900	2740	4270	3500	4250	2610	4100	3420
10	7000	890	6200	2400	4220	1650	4300	2590
100	8600	235	7700	675	4780	680	3700	2380
1000	7300	150	7920	225	5710	255	4000	1400

Incubations were carried out with UDP-MurNAc-L-ala·D-glu.meso-DAP·C^{14}-D-ala·C^{14}-D-ala as substrate for 1 hr with varying amounts of penicillins. Data are expressed as cpm incorporated into glycopeptide (GP) or free D-alanine (Ala). The concentrations of the antibiotics required to inhibit growth by 50% were: penicillin G, 30 μg/ml; cephalothin, 50 μg/ml; ampicillin, 3 μg/ml; and methicillin, 1000 μg/ml.

small amounts of two products with relatively large mobilities were formed during incubation either in the absence or in the presence of penicillin (Fig. 3, C1 and C2 or P1 and P2; see also Fig. 2). These materials increased on prolonged incubation. They are products of hydrolysis of the radioactive glycopeptides by endogenous autolytic enzymes of *E. coli*,[14] and are probably formed by the acetylmuramidase (C1 and P1) and acetylmuramyl-L-alanine amidase (C2 and P2). These materials are formed more rapidly from the spreading product, presumably because the solubility of this polymer makes it a better substrate for the autolytic enzymes.

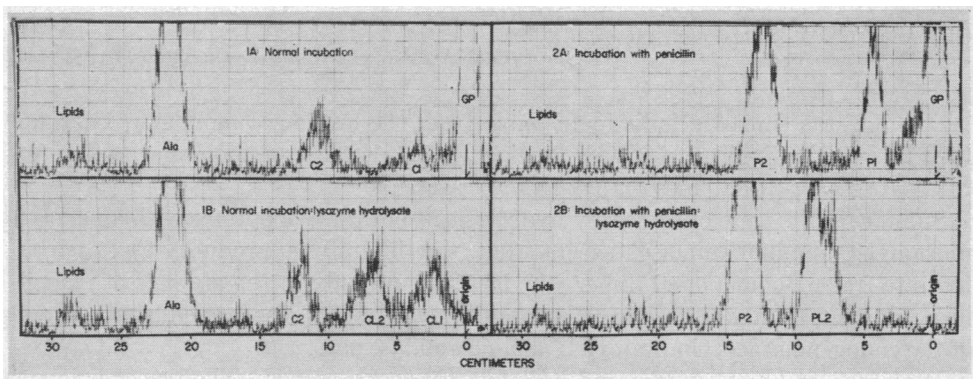

FIG. 3.—Separation of the products of glycopeptide synthesis with enzyme from *E. coli* strain Y-10 in the absence and presence of penicillin. Duplicate incubation mixtures were chromatographed directly or after incubation with 250 μg/ml of egg white lysozyme for 20 hr. A scan of the strips in the Vanguard paper strip scanner is shown. The original incubation was for 3 hr. Under these conditions the labeled substrate, UDP-MurNAc·L-ala·D-glu·H^3-meso-DAP·C^{14}-D-ala·C^{14}-D-ala, was exhausted and hence, did not interfere with detection of the products of hydrolysis by lysozyme.

Both glycopeptides, as well as C1 and P1, were degraded by treatment with acetylmuramidase from egg white (lysozyme). Two products were formed from the glycopeptide and C1 in the control incubation mixture, termed CL1 and CL2. However, only a single product, PL2, was formed in the lysozyme hydrolysate of the penicillin-treated incubation mixture. The lysozyme hydrolysates of these incubation mixtures also contained C2 and P2 which were unaffected by the treatment. It may be noted that P2 has a slightly greater chromatographic mobility than C2 and similarly, the lysozyme product PL2 is slightly faster than CL2.

These compounds were prepared in incubation mixtures containing a mixture of UDP-MurNAc·L-ala·D-glu·meso-DAP·D-ala·D-ala, labeled both with H^3-meso-

TABLE 2

ANALYSES OF PRODUCTS OF HYDROLYSIS BY LYSOZYME OF GLYCOPEPTIDES SYNTHESIZED IN THE PRESENCE OR ABSENCE OF PENICILLIN

Compound*	C^{14}-D-ala/H^3-meso-DAP	Internal DAP†/total DAP
CL1	1.02	0.40
CL2	1.29	0.07
C2	0.93	0.03
PL2	2.36	0.03
P2	2.02	0.02

* See text for description of compounds.
† The compounds were dinitrophenylated and then acid-hydrolyzed. H^3-mono-dinitrophenyl-DAP and H^3-DAP were then separated by thin-layer chromatography. The latter is "internal DAP," i.e., DAP not reactive with 2,4-dinitrofluorobenzene.

TABLE 3

INHIBITION BY PENICILLINS OF D-ALANINE CARBOXYPEPTIDASE IN *E. coli* STRAIN B

Amount added (μg/ml)	Cpm of D-ala Released in Presence of— Penicillin G	Cephalothin	Ampicillin	Methicillin
0	2230	—	—	—
0.04	527	2293	338	2166
0.4	319	1689	242	1427
4.0	87	322	164	503
40.0	37	87	58	225

Incubations were carried out as described in the text with enzyme from *E. coli* strain B, except that UDP-GlcNAc was omitted.

DAP and with C^{14}-D-ala·C^{14}-D-ala. Measurement of the isotope ratios established that CL1, CL2, and C2 contained one D-ala residue per meso-DAP residue, while PL2 and P2 contained two D-ala residues per meso-DAP residue (Table 2). All of the DAP had a free amino group in CL2, C2, PL2, and P2. However, in CL1 only about half of the DAP had a free amino group. Moreover, CL1 was chromatographically and electrophoretically indistinguishable from the authentic dimer,

GlcNAc-MurNAc·L-ala·D-glu·meso-DAP
·D-ala GlcNAc-MurNAc·L-ala·D-glu·meso-DAP·D-ala,

while CL2 was similarly indistinguishable from the authentic monomer, GlcNAc-MurNAc·L-ala·D-glu·meso-DAP·D-ala.[15] PL2 is also a monomer, GlcNAc-MurNAc·L-ala·D-glu·meso-DAP·D-ala·D-ala.

Similar results were obtained by hydrolysis of both glycopeptides with an acetylmuramyl-L-alanine amidase, except that the hydrolysis products were carbohydrate-free monomers and dimer (the latter found only in the absence of penicillin). In fact, C2 and P2 are small amounts of such monomers formed by the action of the endogenous acetylmuramyl-L-alanine amidase of *E. coli*.

D-alanine carboxypeptidase and its inhibition by penicillin: The presence in the glycopeptide formed in the absence of penicillin of a monomer containing one rather than two D-ala residues and of a similar dimer, composed of two tetrapeptides, only one of whose terminal D-ala residues was involved in a cross-link, indicated that some D-ala residues had been removed from the pentapeptide precursor by a mechanism not involving cross-link formation, presumably a D-alanine carboxypeptidase. It has not been possible to distinguish the two activities with particulate enzyme from *E. coli* strain Y-10. However, the particulate enzyme prepared from *E. coli* strain B contains an extremely active D-alanine carboxypeptidase (which, in fact, interferes with any attempts to establish equivalence between D-ala released and D-ala incorporated into glycopeptide, as in Table 1). This enzyme in *E. coli* strain B also utilizes as a substrate the uridine nucleotide, UDP-MurNAc·L-ala·D-glu·meso-DAP·D-ala·D-ala. Thus, it can be assayed by incubation of the particulate enzyme in the absence of UDP-GlcNAc (under which condition no glycopeptide synthesis and hence, no transpeptidation occurs) (Table 3). This carboxypeptidase is much more sensitive to penicillins than is the transpeptidase. The

TABLE 4

EFFECTS OF PENICILLIN AND D-AMINO ACIDS ON FORMATION OF SOLUBLE AND INSOLUBLE GLYCOPEPTIDE PRODUCTS

Addition	Glycopeptide Formed			Ala released
	Insoluble	Soluble	Total	
None	2700	2230	4930	3790
Penicillin G (800 μg/ml)	980	7460	8440	150
D-ala (40 μM/ml)	680	3710	4390	4000
D-met (40 μM/ml)	670	3290	3960	3830
L-ala (40 μM/ml)	2010	2890	4900	3930
L-met (40 μM/ml)	2140	2300	4440	3950

After 1 hr incubation, samples were heat-inactivated. Soluble and insoluble products were separated by centrifugation, chromatographed separately, and then counted. The soluble fraction includes both the material which remained at the origin and the products of autolysis (see Figs. 2 and 3).

concentrations required for 50 per cent inhibition were: penicillin G, 0.02 μg/ml; ampicillin, 0.004 μg/ml; methicillin, 1 μg/ml; and cephalothin, 1 μg/ml.

Effects of other antibiotics: Glycopeptide synthesis with *E. coli* particles was also inhibited by ristocetin (50% inhibition at 3 μg/ml), vancomycin (10 μg/ml), and bacitracin (40 μg/ml). By contrast, growth of the cells was inhibited only by 1000 μg/ml of ristocetin or bacitracin, or 100 μg/ml of vancomycin. As in the case of the *S. aureus* or *M. lysodeikticus* enzymes,[12] both ristocetin and vancomycin interfered with the utilization of lipid-phosphodisaccharide-pentapeptide for glycopeptide synthesis. Two- to fourfold accumulation of the lipid intermediates (which can be seen in Fig. 1) was obtained in the presence of an appropriate concentration of either of these substances. Chloramphenicol (100 μg/ml), novobiocin (40 μg/ml), D-cycloserine (100 μg/ml), streptomycin (100 μg/ml), and puromycin (100 μg/ml) had no effect on the reaction.

Effect of D-alanine and other D-amino acids on glycopeptide synthesis: Since the free energy change in a transpeptidation would be small, the effect of D-alanine and other D-amino acids on the reaction was investigated. Like penicillin, D-alanine, D-serine, and D-methionine, all resulted in the conversion of a water-insoluble to a water-soluble product (Table 4). The D-amino acids did not inhibit alanine release. L-amino acids had little or no effect on the reaction.

An incubation was carried out under these conditions in the presence of C^{14}-D-alanine with UDP-MurNAc·L-ala·D-glu·H^3-meso-DAP·D-ala·D-ala as substrate. A soluble polymer was formed which contained 0.2 C^{14}-D-ala residue per H^3-meso-DAP residue. On hydrolysis with lysozyme, the monomer GlcNAc-MurNAc·L-ala·D-glu·H^3-meso-DAP·D-ala·C^{14}-D-ala was formed from this soluble polymer and, as in the case of polymer formed in the presence of penicillin, no dimer was present. These experiments demonstrate the reversibility of the terminal transpeptidation step in cell wall synthesis and indicate that it can be reversed by D-amino acids other than D-alanine. These substances had no effect on C^{14}-D-alanine released from the substrate because formation of the water-soluble product is due to reversal of the transpeptidation, in contrast to penicillins which inhibit the forward reaction.

Discussion.—These experiments therefore establish that linear glycopeptide strands are cross-linked in a transpeptidation in which D-alanine is liberated. In the enzymatic system from *E. coli* studied, dimers of two disaccharide-tetrapeptide units linked to each other are formed. In addition, some disaccharide-tetrapeptide monomers are incorporated into the product. The nature of these enzymatically

synthesized polymers corresponds exactly to the compounds found after extensive analysis of the cell wall glycopeptide of *E. coli*.[15] The formation of these products from its precursors, UDP-GlcNAc and UDP-MurNAc-pentapeptide, requires, in addition to the transpeptidation, removal of some terminal D-alanine residues from the pentapeptide precursor by a D-alanine carboxypeptidase. This enzyme is especially active in the particulate preparation from *E. coli* strain B.

Both the glycopeptide transpeptidase and the D-alanine carboxypeptidase are inhibited by several penicillins. In the case of the transpeptidase, the sensitivity to ampicillin, cephalothin, and methicillin is virtually identical to the concentrations required to inhibit growth of the cells. However, the particulate enzyme system is ten times more sensitive to penicillin G than are the intact cells. This enzyme system is also inhibited at an earlier step by ristocetin (3 μg/ml), vancomycin (10 μg/ml), and bacitracin (40 μg/ml), although the intact cells are inhibited only by 10–300 times higher concentrations of these substances. The insensitivity of *E. coli* (and by inference other gram-negative bacteria) to these substances and to penicillin G is probably due to their failure to penetrate to the sensitive site in the microbial cell.

The D-alanine carboxypeptidase is an order of magnitude more sensitive to the penicillins than is the glycopeptide transpeptidase. The large difference in sensitivity suggests that it may be possible to find penicillin concentrations at which a tetrapeptide is cross-linked to a pentapeptide, or even in which extensive polymerization has occurred. Conceivably, the carboxypeptidase functions to regulate the extent of cross-linking, since removal of a terminal D-ala residue prevents cross-linking of that peptide unit. However, it may be pointed out that the cross-linking reaction and the carboxypeptidase could be different manifestations of the activity of one enzyme. It has been postulated that an acetylmuramyl-tetrapeptidyl-enzyme intermediate is formed in the transpeptidation with liberation of D-alanine.[8] The reaction of this intermediate with another peptide unit would be a transpeptidation, while its reaction with water would result in hydrolysis of the terminal D-alanine residue. However, the difference in the sensitivity of the two reactions to penicillins may suggest that they are catalyzed by different proteins.

The reversal of the reaction with D-alanine indicates that the free energy change in the transpeptidation is relatively small. Presumably, the reaction is driven by the fact that the glycopeptide product is relatively insoluble. These effects of D-amino acids probably provide the explanation for the formation of spheroplasts induced by D-amino acids.[16, 17] It has been demonstrated in one case that D-methionine, one of the inducing agents, was incorporated into a lysozyme-sensitive glycopeptide.[18] Under these conditions electron microscopy revealed the formation of disorganized fibrous materials in the glycopeptide. Presumably, these are the uncross-linked glycopeptide strands. A similar material has also been seen in cells treated with penicillin.[19]

Bacterial cell walls, including the glycopeptide and antigens, are synthesized in a complex reaction mechanism requiring a minimum of 30 enzymes, and perhaps more than 50 in some organisms. A major impetus to the elucidation of this reaction sequence has been the fact that somewhere in it lay the penicillin-sensitive reactions. It has turned out that the last steps in this sequence, catalyzed by a glycopeptide transpeptidase and a D-alanine carboxypeptidase, are the targets of

penicillin action. Much further work remains to be done to obtain these proteins in a relatively homogeneous form and to elucidate the precise mechanism of the inhibition, i.e., to identify the sites with which penicillins react, presumably through acylation by the β-lactam structure.

Summary.—A particulate enzyme preparation from *E. coli* strain Y-10 catalyzes the synthesis of *cross-linked* glycopeptide strands from UDP-GlcNAc and UDP-MurNAc·L-ala·D-glu·meso-DAP·D-ala·D-ala. The terminal reaction in the sequence is a transpeptidation by means of which two peptide units are cross-linked with elimination of D-alanine from the peptide. This transpeptidation can be reversed by addition of D-alanine or several other D-amino acids. A D-alanine carboxypeptidase present in the particulate preparation is also required for the terminal steps. Both of these enzymes are specifically inhibited by penicillin G, ampicillin, cephalothin, and methicillin. A comparison of the sensitivity of the enzymes and of the cells to these penicillins and to several other antibiotics suggests that the relative insensitivity of *E. coli* to some antibiotics is due to their failure to penetrate to the sensitive site.

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