Biochemistry

Volume 12, Number 7 March 27, 1973

Structure of the Wall Peptidoglycan of *Streptomyces* R39 and the Specificity Profile of Its Exocellular DD-Carboxypeptidase–Transpeptidase for Peptide Acceptors†

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ABSTRACT: The peptide units in the wall peptidoglycan of *Streptomyces* R39 had the sequence L-alanyl-D-isoglutaminyl-(L)-*meso*-diaminopimelyl-(L)-D-alanine and were cross-linked through D-alanyl-(D)-*meso*-diaminopimelic acid linkages. The carboxyl group located on the D carbon of *meso*-diaminopimelic acid was free so that the interpeptide bonds were at a C-terminal position. The substrate requirements of the exocellular DD-carboxypeptidase–transpeptidase of *Streptomyces* R39 for peptide acceptors which were either similar to or identical with the natural peptide were studied by using the synthetic tripeptide N^{α},N^{ϵ}-diacetyl-L-lysyl-D-alanyl-D-alanine as a donor in transpeptidation reactions. The nonamidated disaccharide tetrapeptide β-1,4-*N*-acetylglucosaminyl-*N*-acetylmuramyl-L-alanyl-γ-D-glutamyl-(L)-*meso*-diaminopimelyl-(L)-D-alanine, the corresponding disaccharide-free tetrapeptide, and the tripeptide L-alanyl-γ-D-glutamyl-(L)-*meso*-diaminopimelic acid had very similar properties as acceptors for transpeptidation. Amidation of the (D)-carboxyl group of *meso*-diaminopimelic acid prevented the peptide from being recognized by the enzyme. Amidation of the α-carboxyl group of D-glutamic acid exerted strong influences on both hydrolysis and transpeptidation activities. Control mechanisms included inhibition by excess acceptor and by transpeptidated product, and sensitivity of the transpeptidated product to hydrolysis through the carboxypeptidase activity of the enzyme.

There are two enzymic activities involved in the last stages of the bacterial wall peptidoglycan biosynthesis. One of them is a transpeptidase activity which catalyzes cross-linking between the peptide units. During this reaction, the carboxyl group of the penultimate D-alanine residue of a peptide donor ending in a C-terminal L-R_3-D-alanyl-D-alanine sequence is transferred to the ω-amino group of the L-R_3 diamino acid residue of a peptide acceptor of the same composition. Interpeptide linkages are formed and equivalent amounts of D-alanine residues are liberated from the donor peptides (Wise and Park, 1965; Tipper and Strominger, 1965). The second activity is a carboxypeptidase activity which catalyzes the hydrolysis of the C-terminal D-alanyl-D-alanine peptide bond without concomitant transpeptidation, hence preventing further propagation of the cross-linking system (Strominger, 1970). Although the possibility that the carboxypeptidases might be uncoupled transpeptidases was recognized (Izaki *et al.*, 1966), data obtained from more recent experiments carried out with unpurified particulate preparations from several bacterial species were interpreted as suggesting the existence of two distinct enzymes (Suginaka *et al.*, 1972). This question, however, can probably only be resolved by solubilization and purification of these activities and, to date, no bacterial membrane-bound transpeptidase has been isolated to protein homogeneity and fully characterized.

Among the enzymes liberated into the culture medium during growth of *Streptomyces* strains R61, K11, and R39 there is one which acts on peptides ending in a C-terminal D-alanyl-D-alanine sequence and functions either as carboxypeptidase or transpeptidase depending upon the availability of nucleophiles (H_2O or NH_2–R). These exocellular enzymes were purified. Their specificity profile for peptide donors is such that they exhibit high carboxypeptidase activity (*i.e.*,

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TABLE I: Peptide Acceptors and Transpeptidated Products.[a]

No.	Acceptors	Transpeptidated Products	Electrophoretic Mobility of Transpeptidated Products[b] (cm)
1	L-Ala-D-Glu-(OH) L -D-Ala-(OH) DAP -(OH) D	L-Ala-D-Glu-(OH) L -D-Ala-(OH) DAP Ac_2-L-lys-D-Ala- -(OH) D	85
2	L-Ala-D-Glu-(OH) L -(OH) DAP -(OH) D	L-Ala-D-Glu-(OH) L -(OH) DAP Ac_2-L-lys-D-Ala- -(OH) D	87
3	disaccharide[d] L-Ala-D-Glu-(OH) L -(D-Ala)-(OH) DAP -(OH) D	disaccharide L-Ala-D-Glu-(OH) L -(D-Ala)-(OH) DAP Ac_2-L-lys-D-Ala- -(OH) D	58
4	L-Ala-D-Glu-(OH) L -(OH) DAP -(amide) D	L-Ala-D-Glu-(OH) L -(OH) DAP Ac_2-L-lys-D-Ala- -(amide) D	35[c]
5	L-Ala-D-Glu-(amide) L -D-Ala-(OH) DAP -(OH) D	L-Ala-D-Glu-(amide) L -D-Ala-(OH) DAP Ac_2-L-lys-D-Ala- -(OH) D	35
6	L-Ala-D-Glu-(OH) L -(OH) L-Ala-D-Glu-(OH) DAP L -D-Ala- D -(amide) DAP -(OH) D		

[a] Peptides no. 1, 2, and 3 (in this latter case, a mixture of disaccharide tetrapeptide and disaccharide tripeptide in equimolar amounts) were prepared from walls of *Bacillus megaterium* (Van Heijenoort *et al.*, 1969). Peptides no. 4 and 6 were prepared from walls of *Bacillus subtilis* and were the gift of Dr. Alan Warth (Warth and Strominger, 1971). Peptide no. 5 was prepared from walls of *Streptomyces* R39 (this paper). [b] The reaction products were spotted at 30 cm from the cathode. The electrophoreses were carried out for 4 hr at 60 V/cm and at pH 6.5 (see text). All products migrated toward the anode. The mobility of N^{α},N^{ϵ}-diacetyl-L-lysyl-D-alanyl-D-alanine (peptide donor) was 65 cm and the mobility of N^{α},N^{ϵ}-diacetyl-L-lysyl-D-alanyl (product of hydrolysis) was 75 cm. When the incubations were carried out in 0.5 M K_2HPO_4, the samples (15 μl) were diluted with water (40 μl) and then spotted as bands, 4-cm width, on Whatman 3MM paper strips, before electrophoresis. [c] As determined after transpeptidation with the DD-carboxypeptidase–transpeptidase from *Streptomyces* R61. This peptide is not a substrate for the R39 enzyme. [d] Disaccharide, β-1,4-*N*-acetylglucosaminyl-*N*-acetylmuramic acid.

release of the C-terminal residue) providing the peptides have a C-terminal D-alanyl-D sequence preceded by an L-R_3 group with a long side chain (Leyh-Bouille *et al.*, 1971, 1972). The specificity profile for amino acid and peptide acceptors in transpeptidation reactions has also been studied (Pollock *et al.*, 1972; Perkins *et al.*, 1973). N^{α},N^{ϵ}-Diacetyl-L-lysyl-D-alanyl can be transferred from N^{α},N^{ϵ}-diacetyl-L-lysyl-D-alanyl-D-alanine (the carboxyl donor) to either [^{14}C]glycine, D-[^{14}C]alanine or *meso*-[^{3}H]diaminopimelic acid (the acceptor). The terminal D-alanine of the peptide donor is released and, concomitantly, N^{α},N^{ϵ}-diacetyl-L-lysyl-D-alanyl-D-[^{14}C]alanine, N^{α},N^{ϵ}-diacetyl-L-lysyl-D-alanyl[^{14}C]glycine, or N^{α},N^{ϵ}-diacetyl-L-lysyl-D-alanyl-(D)-*meso*-[^{3}H]diaminopimelic acid are formed (Pollock *et al.*, 1972). L-Alanine was

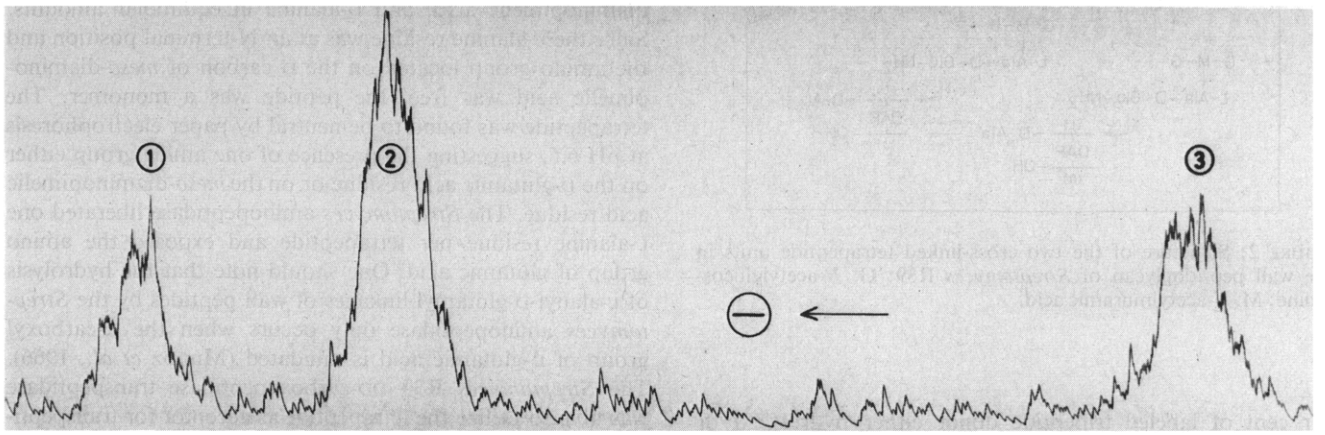


FIGURE 1: Separation by electrophoresis at pH 6.5 (4 hr, 60 V/cm) of a mixture of, from left to right, ① N^{α},N^{ϵ}-diacetyl-L-lysyl-D-alanine (product of hydrolysis; migration, 75 cm); ② N^{α},N^{ϵ}-diacetyl-L-lysyl-D-alanyl-D-alanine (tripeptide donor; migration, 65 cm); ③ product of transpeptidation obtained with peptide acceptor no. 5 (see Table I) (migration, 35 cm). For details, see the text and legend of Table I.

not an acceptor in transpeptidation reactions. Penicillins and cephalosporins inhibit transpeptidase activity at those concentrations which inhibit carboxypeptidase activity, and, with strain R61, the lethal action of these antibiotics was shown to occur at those concentrations which inhibit the activity of the membrane-bound enzyme (Dusart *et al.*, 1973).

In marked contrast to the exocellular DD-carboxypeptidases–transpeptidases from strains R61 and K11 which utilized a series of peptides as acceptors for transpeptidation, the exocellular enzyme from strain R39 was unable to catalyze transpeptidation reactions when peptides, instead of amino acids, were tested as possible acceptors (Perkins *et al.*, 1973). If this enzyme were a solubilized form of the membrane-bound transpeptidase, *i.e.*, were the one that, when integrated in the membrane, catalyzes and controls the extent of peptide cross-linking in the biosynthesis of the wall peptidoglycan, then it should be able to catalyze transpeptidation reactions with acceptors other than simple amino acids. Therefore, experiments were carried out in order to establish the structure of the wall peptidoglycan in *Streptomyces* R39 and to study the specificity profile of the exocellular R39 enzyme for acceptors by using peptides which were either similar to or identical with the natural peptide acceptors. In these experiments N^{α},N^{ϵ}-diacetyl-L-lysyl-D-alanyl-D-alanine was always used as a peptide donor.

Materials and Methods

Structure of the Wall Pepidoglycan of Streptomyces R39. Walls were prepared from 72-hr cultures of *Streptomyces* grown under the conditions given by Leyh-Bouille *et al.* (1972). The structure of the wall peptidoglycan was established by using those techniques and methods that had been used before for the establishment of many other bacterial peptidoglycans (Ghuysen, 1968). Reducing groups, acetamido sugars, amino acids, D-alanine, and N-terminal groups were measured as described in Ghuysen *et al.* (1966, 1968). Identification of diaminopimelic acid isomers was carried out as described in Bricas *et al.* (1967). Aminopeptidase from *Streptomyces albus* G, DD-carboxypeptidase from *S. albus* G (*i.e.*, KM endopeptidase), *Myxobacter* AL1 enzyme, *Chalaropsis endo-N*-acetylmuramidase (a gift from Dr. N. A. Hash), and egg-white lysozyme (Armour) were used. The *endo-N*-acetylmuramidases (*Chalaropsis* and lysozyme enzymes) hydrolyze the glycan strands into β-1,4-*N*-acetylglucosaminyl-*N*-acetylmuramic acid disaccharides. *Myxobacter* AL1 enzyme hydrolyzes *N*-acetylmuramyl-L-alanine linkages between the glycan and peptide moieties. DD-Carboxypeptidase from *S. albus* G hydrolyzes C-terminal D-alanyl-D linkages. *Streptomyces* aminopeptidase hydrolyzes N-terminal L-alanyl-D-isoglutaminyl linkages. For more details, see Ghuysen (1968).

Exocellular DD-Carboxypeptidase–Transpeptidase from Streptomyces R39. The isolation of this enzyme and some of its properties have been described (Leyh-Bouille *et al.*, 1972; Pollock *et al.*, 1972; Dusart *et al.*, 1973; Perkins *et al.*, 1973). One unit of enzyme catalyzes the hydrolysis of 1 nmol of N^{α},N^{ϵ}-diacetyl-L-lysyl-D-alanyl-D-alanine (release of the C-terminal D-alanine) per hour at 37° when 8 mM peptide is incubated with the enzyme in the absence of amino group acceptor, in 0.03 M Tris-HCl buffer, pH 7.5, plus 3×10^{-3} M $MgCl_2$. The K_m value of enzyme R39 for the tripeptide in such a carboxypeptidase assay is 0.8 mM.

Transpeptidation Assays. Radioactive N^{α},N^{ϵ}-diacetyl-L-lysyl-D-alanyl-D-alanine (with both acetyl residues labeled with ^{14}C; specific activity, 10,000 dpm/nmol) was always used as acyl donor. The donor (4 nmol of radioactive tripeptide supplemented as required with nonradioactive tripeptide) was incubated with the R39 enzyme under various conditions, in the presence and absence of peptide acceptor. Donor and acceptor were first dissolved in the buffer solution and the enzyme was then added. The donor tripeptide left unused at the end of the incubation, the product of hydrolysis (diacetyl-L-lysyl-D-alanine), and the product of transpeptidation (diacetyl-L-lysyl-D-alanyl acceptor) were separated from each other by electrophoresis on Whatman No. 3MM paper, at pH 6.5 (collidine–acetic acid–water, 9.1:2.65:1000), for 4 hr at 60 V/cm, using a Gilson High Voltage (10,000 V) Electrophorator Model DW. The radioactive compounds were located on the strips by using a Packard Radiochromatogram Scanner. The radioactivity was determined by cutting strips into sections of 10 mm which were counted as described by Pollock *et al.* (1972) in a Packard Tri-Carb liquid scintillation spectrometer. All the peptides used as possible acceptors were obtained by enzymatic degradation of bacterial cell walls. They are listed in Table I. A typical separation by paper electrophoresis of tripeptide donor, the product of hydrolysis, and the product of transpeptidation is shown in Figure 1. The results were expressed as the mole

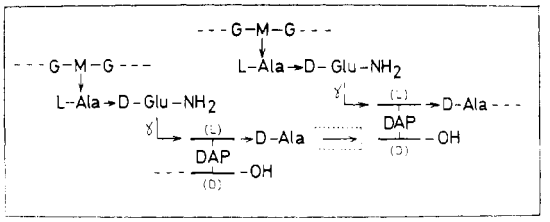


FIGURE 2: Structure of the two cross-linked tetrapeptide units in the wall peptidoglycan of *Streptomyces* R39: G, *N*-acetylglucosamine; M, *N*-acetylmuramic acid.

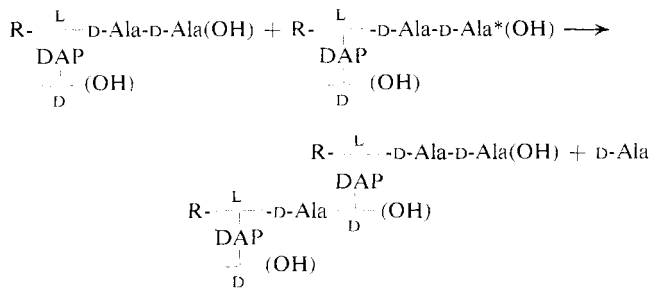
per cent of labeled tripeptide donor either hydrolyzed or converted into the transpeptidation product.

Experimental Section

Composition of the Wall Peptidoglycan of Streptomyces R39 and Its Sensitivity to the Lytic Enzymes. The walls contained muramic acid, glucosamine, L-alanine, *meso*-diaminopimelic acid, D-glutamic acid, and D-alanine in equimolar amounts. There were approximately 600 nequiv of disaccharide tetrapeptide units per milligram of walls. Approximately 10% of the *meso*-diaminopimelic acid residues had one free amino group. At pH 9 (0.02 M Veronal buffer), the walls autolyzed through the action of an endogenous *N*-acetylmuramyl-L-alanine amidase resulting in the exposure of N-terminal L-alanine residues. Similarly, and under the same conditions, *Myxobacter* AL1 enzyme solubilized the heat-inactivated walls mainly through the hydrolysis of the *N*-acetylmuramyl-L-alanine amide bonds. At completion of the reaction, 83% of the L-alanine residues were in a N-terminal position. Hen's egg-white lysozyme (in 0.01 M phosphate buffer, pH 6.1), at a ratio of enzyme to wall of 1:40, solubilized 44% of the heat-inactivated walls. *Chalaropsis endo-N*-acetylmuramidase (in 0.01 M acetate buffer, pH 4.5), at a ratio of enzyme to wall of 1:100, solubilized 80% of the heat-inactivated walls and liberated, per milligram of wall, 470 nmol of disaccharide peptide units.

The lytic D-alanyl-D-carboxypeptidase from *S. albus* G (in 0.02 M Tris-HCl buffer, pH 7.5, plus 0.002 M $MgCl_2$), at a ratio of enzyme to wall of 1:2500, solubilized the walls. At the end of the treatment (48 hr at 37°), 77% of the *meso*-diaminopimelic acid residues had one free amino group. Since this *Streptomyces* enzyme is specific for C-terminal D-alanyl-D linkages (Leyh-Bouille *et al.*, 1970b), the interpeptide bonds in the R39 wall peptidoglycan are mediated through D-alanyl-(D)-*meso*-diaminopimelic acid linkages.

Isolation and Characterization of the Tetrapeptide Monomer from the Wall Peptidoglycan of Streptomyces R39. Fresh walls (25 mg) were incubated for 48 hr at 37°, in 0.02 M Tris-HCl buffer plus 0.002 M $MgCl_2$, with 10 μg of DD-carboxypeptidase from *S. albus* G. Complete solubilization occurred through the hydrolysis of D-alanyl-(D)-*meso*-diaminopimelic acid linkages as well as *N*-acetylmuramyl-L-alanine linkages (site of action of the endogenous amidase). Filtration of the degraded products on a 120-ml column of Sephadex G-25 in 0.1 M LiCl yielded a peptide monomer fraction (K_D = 0.6) well separated from the nondegraded glycan moiety of the peptidoglycan and the nonpeptidoglycan polymers of the walls (K_D = 0). After desalting by filtration on Sephadex G-25 in water, the peptide monomer (yield, 300 nequiv/mg of walls) was shown to contain L-alanine, D-glutamic acid, *meso*-diaminopimelic acid, and D-alanine in equimolar amounts. Since the L-alanine residue was at an N-terminal position and the amino group located on the D carbon of *meso*-diaminopimelic acid was free, the peptide was a monomer. The tetrapeptide was found to be neutral by paper electrophoresis at pH 6.5, suggesting the presence of one amide group either on the D-glutamic acid residue or on the *meso*-diaminopimelic acid residue. The *Streptomyces* aminopeptidase liberated one L-alanine residue per tetrapeptide and exposed the amino group of glutamic acid. One should note that the hydrolysis of L-alanyl-D-glutamyl linkages of wall peptides by the *Streptomyces* aminopeptidase only occurs when the α-carboxyl group of D-glutamic acid is amidated (Munoz *et al.*, 1966). The *Streptomyces* R39 DD-carboxypeptidase–transpeptidase was able to utilize the tetrapeptide as acceptor for transpeptidation (*vide infra*). Since this enzyme requires its peptide acceptors to have a free α-carboxyl group (Perkins *et al.*, 1973), this result demonstrated that the amino group located on the D carbon of *meso*-diaminopimelic acid in the tetrapeptide was in the α position relative to a free carboxyl group (*vide infra*) and, therefore, the C-terminal D-alanine residue was located on the L carbon of *meso*-diaminopimelic acid. Hence, the tetrapeptide monomer had the sequence L-alanyl-γ-D-isoglutaminyl-(L)-*meso*-diaminopimelyl-(L)-D-alanine and the structure of the wall peptidoglycan of *Streptomyces* R39 was that shown in Figure 2. This structure is very different from that found in the wall peptidoglycans of *S. albus* G, R61, and K11 where LL- rather than *meso*-diaminopimelic acid occurs and one glycine residue forms the link between the D-alanine of one tetrapeptide and the LL-diaminopimelic acid of another tetrapeptide (Leyh-Bouille *et al.*, 1971). Owing to the structure of the wall peptidoglycan, the *in vivo* peptide cross-linking in *Streptomyces* R39 must occur through the following transpeptidation reaction



The asterisked D-alanine residue in the peptide acceptor is not essential to the reaction. The acceptor amino group involved in the reaction is located on the D carbon of *meso*-diaminopimelic acid so that the interpeptide bond which is synthesized is a D-alanyl-(D)-*meso*-diaminopimelic acid linkage in α position to a free carboxyl group.

Transpeptidation with the Nonamidated Tetrapeptide (No. 1, Table I) as Acceptor. Both hydrolysis of N^{α},N^{ϵ}-diacetyl-L-lysyl-D-alanyl-D-alanine to N^{α},N^{ϵ}-diacetyl-L-lysyl-D-alanine and transpeptidation of N^{α},N^{ϵ}-diacetyl-L-lysyl-D-alanyl from the above tripeptide donor to the nonamidated tetrapeptide acceptor were found to be very much influenced by the environmental conditions, *i.e.*, pH, ionic strength, and the amount of water present in the reaction mixture.

EFFECTS OF pH AND IONIC STRENGTH. The effects of pH at low ionic strength (μ 0.015) in cacodylate, phosphate, and Tris-HCl buffers and at high ionic strength (μ, 1.5) in phosphate buffers are shown in Figure 3. The optimum pH for hydrolysis (*i.e.*, the conversion of tripeptide donor into di-

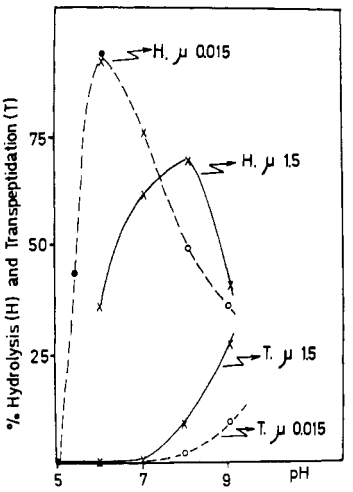


FIGURE 3: Effects of pH and ionic strength on hydrolysis and transpeptidation by the DD-carboxypeptidase-transpeptidase from *Streptomyces* R39 (acceptor, peptide no. 1, Table I). The following buffers were used: cacodylate (●); phosphate (×); Tris-HCl (○). Conditions of incubation at low ionic strength: ^{14}C-Ac$_2$-L-Lys-D-Ala-D-Ala (6 nmol) and acceptor (18 nmol) were incubated for 30 min at 37° in a final volume of 15 μl in the presence of 80 units of R39 enzyme. Conditions of incubation at high ionic strength: ^{14}C-Ac$_2$-L-Lys-D-Ala-D-Ala (4 nmol) and acceptor (18 nmol) were incubated for 45 min at 37° in a final volume of 15 μl in the presence of 40 units of R39 enzyme. For the separation and estimation of the product of hydrolysis (^{14}C-Ac$_2$-L-Lys-D-Ala) and the product of transpeptidation (Table I, peptide no. 1) see Materials and Methods.

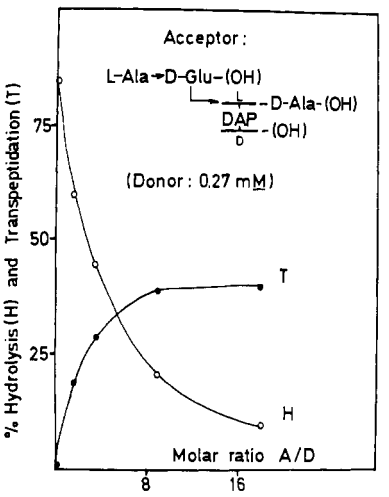


FIGURE 4: Effect of variations in acceptor concentrations, in the presence of a fixed amount of donor (0.27 mM), on hydrolysis and transpeptidation by the DD-carboxypeptidase-transpeptidase from *Streptomyces* R39 (acceptor, nonamidated tetrapeptide no. 1, Table I). ^{14}C-Ac$_2$-L-Lys-D-Ala-D-Ala (4 nmol) and acceptor (from 7.3 to 73 nmol) were incubated in 0.5 M K_2HPO_4 for 30 min at 37° in a final volume of 15 μl in the presence of 40 units of R39 enzyme. For the separation and estimation of the product of hydrolysis and the product of transpeptidation, see Materials and Methods.

peptide) is 6 at low ionic strength and 8 at high ionic strength. The optimum pH for transpeptidation (*i.e.*, the conversion of tripeptide donor into N^{α},N^{ϵ}-diacetyl-L-lysyl-D-alanyl acceptor) is 9 or above 9 (Figure 3). The effects of K_2HPO_4 concentration (from 0.05 to 0.5 *M*) at pH 9 were also studied. The ionic strength of K_2HPO_4 had little or no influence on the yield of hydrolysis but greatly influenced the yield of transpeptidation and its optimum value is 1.5 (0.5 M K_2HPO_4) or above. The enhancement of the yield of transpeptidation product which was observed when the phosphate concentration of the reaction mixture was increased could not be attributed to the effect of the high phosphate concentration on divalent cations since transpeptidase activity in 0.05 M Tris-HCl buffer, pH 8.5, was not affected by the addition of 10^{-3} M sodium ethylenediaminetetraacetate. Because of this apparently specific effect of high ionic strength in phosphate, all the ensuing experiments were carried out in 0.5 M K_2HPO_4.

EFFECTS OF INCREASING CONCENTRATIONS OF ACCEPTOR. A fixed amount of tripeptide donor (0.27 mM) was incubated in 0.5 M K_2HPO_4 for 30 min at 37° with 40 enzyme units (final volume, 15 μl) in the presence of various amounts of tetrapeptide acceptor (Figure 4). Under these conditions, the yield of transpeptidation product was maximal at a 2.4 mM concentration in acceptor which corresponded to a ratio of acceptor to donor of 9:1. At this concentration of acceptor, there was a decrease in hydrolysis greater than could be accounted for by the yield of transpeptidation. A further increase in acceptor concentration did not affect the transpeptidation reaction but caused further inhibition of the hydrolysis.

EFFECTS OF ETHYLENE GLYCOL–GLYCEROL AND OF TRITON X-100. Tripeptide donor (0.27 mM) and tetrapeptide acceptor (1.5 mM) were incubated in 0.5 M K_2HPO_4 with 40 units of enzyme (final volume, 15 μl), for increasing times at 37°. In a parallel experiment, half of the water in the reaction mixtures was replaced by an equal volume of a solution of ethylene glycol and glycerol (65:35, v/v). In a third experiment, the reaction mixtures contained 0.5% Triton X-100 (final concentration) in addition to the other reagents. In aqueous media (0.5 M K_2HPO_4), the rate of hydrolysis was higher than that of transpeptidation. Expressed as per cent of tripeptide donor, hydrolysis was 60% and transpeptidation was 29% after 60 min of incubation. Replacement of half of the water by the ethylene glycol–glycerol mixture inhibited the hydrolysis more than the transpeptidation so that, under these conditions, both reactions proceeded at the same rate (25% after 60 min of incubation). The results in the presence of ethylene glycol and glycerol and added Triton X-100 were identical with those obtained in the absence of Triton X-100.

Transpeptidation with the Nonamidated Tripeptide (No. 2, Table I) and the Nonamidated Disaccharide Peptide (No. 3, Table I) as Acceptors. The effect of increasing the concentrations of these acceptors in the presence of a fixed amount of donor (0.27 mM) was tested under the same conditions as those used with the nonamidated tetrapeptide acceptor. The results were similar to those previously observed with the tetrapeptide. However, the yields of transpeptidation products were maximal at a 4.36 mM concentration in tripeptide and at a 2.4 mM concentration in disaccharide peptide which corresponded to molar ratios of acceptor to donor of 16:1 with the tripeptide and of 9:1 with the disaccharide peptide. Since the disaccharide peptide was an acceptor for the R39 enzyme, it follows that the N-terminal L-alanine residue of the peptides is not the amino group involved in transpeptidation but that this reaction occurs on the amino group located on the D carbon of *meso*-diaminopimelic acid. Other studies (Pollock *et al.*, 1972; Perkins *et al.*, 1973) have also shown that an asymmetric carbon of the L configuration can never be an acceptor for the enzyme.

Effect of Amidation of the Carboxyl Group Located on the D Carbon of meso-Diaminopimelic Acid of the Peptide Acceptor

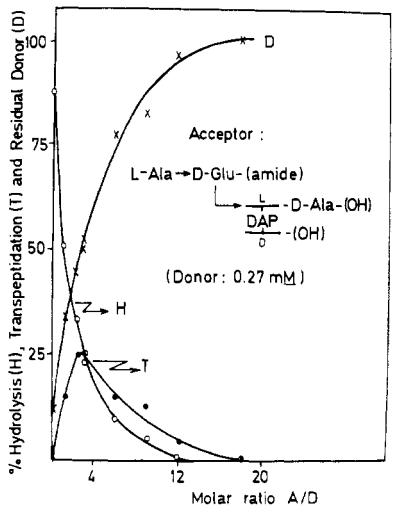


FIGURE 5: Effect of variations of acceptor concentration, in the presence of a fixed amount of donor (0.27 mM), on hydrolysis and transpeptidation by the DD-carboxypeptidase–transpeptidase from *Streptomyces* R39 (acceptor, Glu-amidated tetrapeptide, no. 5, Table I). ^{14}C-Ac$_2$-L-Lys-D-Ala-D-Ala (4 nmol) and Glu-amidated tetrapeptide (4–72 nmol) were incubated in 0.5 M K_2HPO_4 for 30 min at 37° in a final volume of 15 μl in the presence of 34 units of R39 enzyme. For the separation and estimation of the product of hydrolysis and the product of transpeptidation, see Materials and Methods.

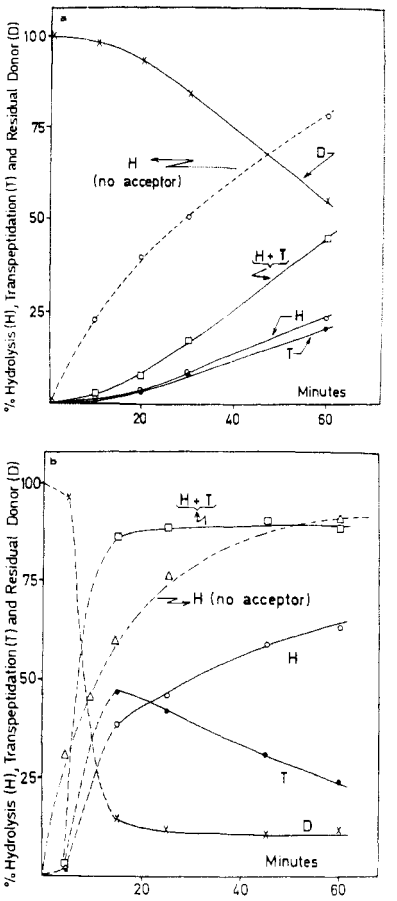


FIGURE 6: (a) Kinetics of hydrolysis and transpeptidation by the DD-carboxypeptidase–transpeptidase from *Streptomyces* R39 in 0.5 M K_2HPO_4 (acceptor, Glu-amidated tetrapeptide no. 5, Table I). For conditions, see text; 15 units of enzyme were used; H (no acceptor), hydrolysis of the tripeptide donor in the absence of Glu-amidated tetrapeptide acceptor. For the separation and estimation of the product of hydrolysis and the product of transpeptidation, see Materials and Methods. (b) Kinetics of hydrolysis and transpeptidation by the DD-carboxypeptidase–transpeptidase from *Streptomyces* R39 in 0.5 M K_2HPO_4 (acceptor, Glu-amidated tetrapeptide no. 5, Table I). For conditions, see text; 40 units of enzyme were used; H (no acceptor), see legend of Figure 6a.

on Transpeptidation. The amidation of the carboxyl group on the D carbon of *meso*-diaminopimelic acid completely prevented the peptide (No. 4, Table I) from being recognized by the R39 enzyme so that the L-alanyl-γ-D-glutamyl-(L)-*meso*-diaminopimelic acid-(D) amide tripeptide was not a substrate for transpeptidation and, moreover, did not inhibit the hydrolysis of the N^{α},N^{ϵ}-diacetyl-L-lysyl-D-alanyl-D-alanine tripeptide donor. In these assays [^{14}C]diacetyl-L-lysyl-D-alanyl-D-alanine (4 nmol) and *meso*-diaminopimelic acid amidated tripeptide (5.76–64 nmol) were incubated in 0.5 M K_2HPO_4 for 30 min at 37° in a final volume of 15 μl in the presence of 34 units of R39 enzyme. In all cases, the yield of hydrolysis, expressed as conversion of the donor tripeptide into [^{14}C]-diacetyl-L-lysyl-D-alanine and free D-alanine, was 90–95%. The demonstration that no transpeptidation product was formed by the action of the R39 enzyme contrasted with the action of the exocellular DD-carboxypeptidase–transpeptidase from *Streptomyces* R61, for which the above-mentioned amidated tripeptide (as well as the nonamidated peptides and disaccharide peptide) is a substrate for transpeptidation reactions (unpublished results).

Transpeptidation with the Glu-Amidated Tetrapeptide (No. 5, Table I) as Acceptor. EFFECTS OF INCREASING CONCENTRATIONS OF ACCEPTORS. The natural Glu-amidated tetrapeptide, *i.e.*, the peptide isolated from *Streptomyces* R39 (peptide no. 5, Table I), was first tested as a possible acceptor in transpeptidation reactions, under conditions identical with those described above for the nonamidated peptides and the diaminopimelic acid amidated tripeptide. The effects of various concentrations of Glu-amidated tetrapeptide are shown in Figure 5. In marked contrast with the results obtained with the nonamidated acceptors, the yield of transpeptidation was maximal within a narrow range of concentrations of Glu-amidated tetrapeptide (about 0.81 mM) corresponding to a ratio of acceptor to donor of 3:1. At higher concentrations of acceptor, both transpeptidation and hydrolysis reactions were progressively inhibited until eventually they were completely abolished. Under these latter conditions, the tripeptide donor present in the reaction mixtures remained unused.

The same experiment was repeated in the presence of a fivefold increase in the concentration of the tripeptide donor (1.35 mM instead of 0.27 mM) and the same amount of enzyme (34 units for 15 μl, final volume). Maximal yield of transpeptidation (23%) occurred at 1.35–2.70 mM concentrations in Glu-amidated tetrapeptide or at ratios of acceptor to donor of 1–2 to 1. Hence, the concentration in acceptor that yields maximal transpeptidation depends on the concentration of donor used.

TIME COURSE EXPERIMENTS. Tripeptide donor (0.27 mM) and Glu-amidated tetrapeptide acceptor (0.81 mM) were incubated in 0.5 M K_2HPO_4 for increasing times at 37° in the presence of 40, 15, and 7.5 enzyme units (final volumes, 15 μl). The following observations were made (Figure 6). (1) At the beginning of the incubations, there was a lag phase during which the tripeptide donor was used at a very low rate or remained intact. These lag phases were about 5 min with 40 units of enzyme, 10–15 min with 15 units of enzyme, and more than

60 min with 7.5 units of enzyme (not shown). (2) At the two lower concentrations, the sum of the products produced by hydrolysis and by transpeptidation was lower than the amount of product produced by hydrolysis of the peptide donor in the absence of peptide acceptor (Figure 6a), but at the highest enzyme concentration after the lag period, overall breakdown of the donor was greater in the presence of acceptor (Figure 6b). (3) At high enzyme concentrations, the transpeptidated product was hydrolyzed into the dipeptide N^{α},N^{ϵ}-diacetyl-L-lysyl-D-alanine and the Glu-amidated tetrapeptide through the hydrolysis of the C-terminal D-alanyl-D-*meso*-diaminopimelic acid linkage formed by transpeptidation. Hence, the dipeptide N^{α},N^{ϵ}-diacetyl-L-lysyl-D-alanine can be produced either directly by hydrolysis of the tripeptide donor or indirectly by hydrolysis of the transpeptidated product. Figure 6b shows that the dipeptide N^{α},N^{ϵ}-diacetyl-L-lysyl-D-alanine which appeared in the reaction mixtures between 15 and 60 min of incubation was produced exclusively by the hydrolysis of the transpeptidated product. It should be noted that in the experiment presented in Figure 6b the hydrolysis of the residual donor is inhibited (10% is left all the time). This inhibition may be caused either by the transpeptidation product or by the amount of free D-alanine present in the reaction mixtures which is probably enough to keep the residual donor in a "transpeptidation equilibrium" (according to the equation: Ac_2-L-Lys-D-Ala-D-Ala + D-*Ala → Ac_2-L-Lys-D-Ala-D-*Ala + D-Ala).

Amidated Peptide Dimer (No. 6, Table I) as Possible Acceptor in Transpeptidation and Its Effect on Transpeptidation with the Glu-Amidated Tetrapeptide (No. 5, Table I). The amidated peptide dimer (in which the D-alanyl-(D)-*meso*-diaminopimelic acid interpeptide linkage was protected against hydrolysis by amide substitution of the α-carboxyl group) was not a substrate of the R39 enzyme for transpeptidation in spite of having a free amino group at a D center of *meso*-diaminopimelic acid that would apparently be suitable for transpeptidation, but it inhibited the hydrolysis of the tripeptide donor. In these experiments, amidated peptide dimer (4–100 nmol) and N^{α},N^{ϵ}-diacetyl-L-lysyl-D-alanyl-D-alanine (4 nmol) were incubated in 0.5 M K_2HPO_4 for 30 min at 37° in a final volume of 15 μl in the presence of 34 units of R39 enzyme. Under these conditions, the extent of hydrolysis of the tripeptide donor which was 90–95% in the absence of amidated peptide dimer was only 5% when 64 nmol of amidated peptide dimer was added to the reaction mixture. Moreover, when added to the donor–acceptor system N^{α},N^{ϵ}-diacetyl-L-lysyl-D-alanyl-D-alanine (0.27 mM)–Glu-amidated tetrapeptide (0.81 mM) the amidated peptide dimer inhibited both the hydrolysis of the tripeptide donor and the transpeptidation of the Glu-amidated tetrapeptide by the R39 enzyme (34 units for 15 μl, final volume) (Figure 7). The concentrations of tripeptide donor, Glu-amidated tetrapeptide and enzyme used in this latter experiment were those which gave a maximal yield of transpeptidation (see Figure 5). Under these conditions, there was a parallel inhibition of hydrolysis and transpeptidation and inhibition of both activities began at a concentration of the peptide dimer (referred to as I, for inhibitor) which was about half that of the Glu-amidated tetrapeptide (A for acceptor).

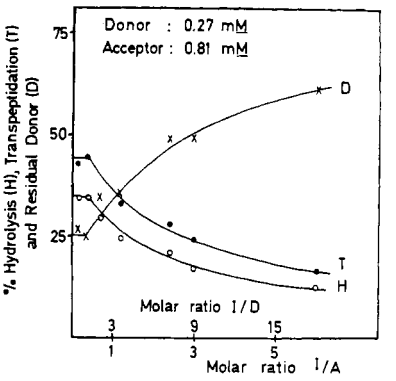


FIGURE 7: Effect of the amidated peptide dimer (no. 6, Table I) on transpeptidation with the Glu-amidated tetrapeptide (no. 5, Table I) by the DD-carboxypeptidase–transpeptidase from *Streptomyces* R39. ^{14}C-Ac_2-L-Lys-D-Ala-D-Ala (4 nmol), Glu-amidated tetrapeptide (12 nmol), and peptide dimer (2.4–72 nmol) were incubated in 0.5 M K_2HPO_4 for 30 min at 37° in a final volume of 15 μl in the presence of 34 units of R39 enzyme. For separation and estimation of product of hydrolysis and product of transpeptidation, see Materials and Methods: I, inhibitor, *i.e.*, peptide dimer; A, acceptor, *i.e.*, Glu-amidated tetrapeptide; D, donor, *i.e.*, Ac_2-L-Lys-D-Ala-D-Ala.

Discussion

As shown by previous studies on the wall composition of various actinomycetes (Yamaguchi, 1965), the wall peptidoglycans of *Streptomyces* sp contain LL-diaminopimelic acid and glycine whereas those of other actinomycetes, such as *Streptosporangium* sp, contain *meso*- and/or DD-diaminopimelic acid and only traces of glycine. In agreement with these results, the wall peptidoglycans of *Streptomyces albus* G, R61, and K11 (Leyh-Bouille *et al.*, 1970a) were found to be formed of L-alanyl-D-isoglutaminyl-[L_1]-LL-diaminopimelyl-(L_1)-D-alanine units that were cross-linked through single glycine residues (peptidoglycans of chemotype II; Ghuysen, 1968). *Streptomyces* strain R39, however, is quite different in that its wall peptidoglycan is composed of cross-linked L-alanyl-γ-D-isoglutaminyl-(L)-*meso*-diaminopimelyl-(L)-D-alanine tetrapeptide units (Figure 2) (peptidoglycan of chemotype I; Ghuysen, 1968). It is possible that the actinomycete strain R39 was erroneously characterized as a *Streptomyces* sp.

The exocellular DD-carboxypeptidase–transpeptidase from actinomycete strain R39 has been extensively studied with respect to its requirements for peptide donor in carboxypeptidase assays (Leyh-Bouille *et al.*, 1972), its requirements for amino acid acceptor in transpeptidase assays (Pollock *et al.*, 1972; Perkins *et al.*, 1973), and the inhibition of its carboxypeptidase and transpeptidase activities by β-lactam antibiotics (Leyh-Bouille *et al.*, 1972; Dusart *et al.*, 1973). The inability of the R39 enzyme to utilize a series of peptides as acceptors for transpeptidation—*i.e.*, the inability of the enzyme to catalyze the synthesis of a peptide bond in an endo position (Perkins *et al.*, 1973)—prompted us to study the structure of the wall peptidoglycan of the corresponding strain in order to determine the nature of the interpeptide bond which must be synthesized *in vivo* by transpeptidation. It has now been established that this interpeptide bond is a D-alanyl-(D)-*meso*-diaminopimelic acid linkage in α position to a free carboxyl group.

When D-[^{14}C]alanine was used as acceptor at saturating concentrations (molar ratio of acceptor to donor of 100:1; Pollock *et al.*, 1972), the time course of transpeptidation by the R39 enzyme paralleled the time course of hydrolysis to the donor peptide when no acceptor other than water was present in the reaction mixture. The use of peptides which were either similar or identical with the natural wall peptide has revealed that the requirements of the R39 enzyme for

peptide acceptors actually reflected specific structural features of the wall peptidoglycan of strain R39. These studies have also revealed the existence of control mechanisms in the transpeptidase activity of the enzyme. These demonstrations rest upon a number of observations. When the nonamidated tetrapeptide L-alanyl-γ-D-glutamyl-(L)-*meso*-diaminopimelyl-(L)-D-alanine, the nonamidated tripeptide L-alanyl-γ-D-glutamyl-(L)-*meso*-diaminopimelic acid, and the nonamidated disaccharide peptide were compared as acceptors in transpeptidation reactions, it appeared that the disaccharide moiety and the C-terminal D-alanine residue were not important features of the acceptor molecule. However, in contrast to what had been observed with D-alanine as acceptor (Pollock *et al.*, 1972), when increasing amounts of the above-mentioned peptides were used, the yield of transpeptidation increased until it reached a maximal value and then remained constant. Under these conditions of saturation in acceptor concentration, only part of the donor peptide was utilized for transpeptidation. Moreover, the decrease in hydrolysis of the tripeptide donor was always greater than could be accounted for by the yield of transpeptidation.

Amidation of the peptide acceptor either on the D carbon of *meso*-diaminopimelic acid or on the α-carboxyl group of D-glutamic acid exerted drastic and specific influences on the transpeptidase activity of the R39 enzyme. Amide substitution of the (D)-COOH of *meso*-diaminopimelic acid completely prevented the diaminopimelic acid amidated tetrapeptide from being recognized by the enzyme, thus demonstrating the requirement of the enzyme for the presence of a free carboxyl group in α position to the amino group acceptor (and therefore providing an explanation of the inability of this enzyme to catalyze the synthesis of peptide bonds in an endo position).

Amide substitution of the α-carboxyl group of D-glutamic acid of the tetrapeptide acceptor considerably limited the conditions of concentrations in donor, acceptor, and enzyme under which hydrolysis and transpeptidation occurred. (i) The yield of transpeptidation was maximal only within a narrow range of concentrations in acceptor, which itself depended upon the concentration of the donor (Figure 5). Large excess in acceptor completely inhibited both hydrolysis and transpeptidation. (ii) The interpeptide bond D-alanyl-(D)-*meso*-diaminopimelic acid which is synthesized through the transpeptidase activity of the enzyme is at a C-terminal position and hence can be hydrolyzed through the carboxypeptidase activity of the same enzyme. These antagonistic activities of the R39 enzyme readily explain the fact that under conditions of prolonged incubation in the presence of a large amount of enzyme, the product initially formed by transpeptidation was reconverted into peptide monomers (Figure 6b). The original peptide donor, however, is liberated as N^{α},N^{ϵ}-diacetyl-L-lysyl-D-alanine dipeptide and, hence, cannot undergo further transpeptidation. (iii) The peptide dimer formed of one L-alanyl-γ-D-glutamyl-(L)-*meso*-diaminopimelyl-(L)-D-alanine tetrapeptide and one L-alanyl-γ-D-glutamyl-(L)-*meso*-diaminopimelic acid tripeptide (and in which the D-alanyl-(D)-*meso*-diaminopimelic acid interpeptide bond was protected against hydrolysis by amide substitution) was not a substrate for transpeptidation reaction. However, it inhibited the hydrolysis of the tripeptide donor and when added to the system tripeptide donor–Glu-amidated tetrapeptide acceptor, it also inhibited transpeptidation (Figure 7). (iv) The properties of the R39 enzyme—*i.e.*, its dependence for activity upon a strict relative concentration in acceptor and donor, its inhibition by excess of acceptor and by product of transpeptidation, and its capability of hydrolyzing the transpeptidated product—make it clear that kinetics of hydrolysis and transpeptidation are complex phenomena (Figure 6).

All the above observations point to the existence of multiple substrate sites on the exocellular R39 enzyme and of mechanisms that control the hydrolyzing and synthesizing activities of the enzyme. In this respect, the amide substituent on the α-carboxyl group of D-glutamic acid of the peptide acceptor plays a role of prime importance. The enhancement of the transpeptidase activity of the R39 enzyme *vs.* its carboxypeptidase activity by high concentrations in phosphate (Figure 3) and by increasing the hydrophobic character of the reaction mixture (by addition of ethylene glycol and glycerol) also points to the enormous influence which is exerted on the functioning of the enzyme by the environmental conditions. It is also worth noting that some of the "control mechanisms" suggested above for the R39 enzyme and the influence exerted by the hydrophobic character of the reaction mixture match the results obtained with the exocellular DD-carboxypeptidase–transpeptidase from *Streptomyces* R61 (Perkins *et al.*, 1973; Frère *et al.*, manuscript in preparation). Finally, it should be emphasized that all the experiments described in the present paper were carried out with an artificial synthetic peptide donor N^{α},N^{ϵ}-diacetyl-L-lysyl-D-alanyl-D-alanine. The question as to whether or not a natural peptide donor would exert more specific influences on the functioning of the enzyme is currently under study.

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Characterization of a Phosphorylated Pentasaccharide Isolated from *Hansenula holstii* NRRL Y-2448 Phosphomannan†

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ABSTRACT: The phosphomannan secreted by the yeast *Hansenula holstii* NRRL Y-2448 has been degraded by mild acid hydrolysis of the hemiacetal phosphodiester linkages. This procedure yields: (1) a phosphorylated pentasaccharide which accounts for 65% of the carbohydrate in the intact polymer, (2) a high molecular weight phosphorylated core fragment which is resistant to further mild acid hydrolysis and which accounts for 9% of the carbohydrate in the intact polymer, and (3) other small fragments (tetrasaccharide and smaller) which have not been characterized. The structure of the phosphorylated pentasaccharide, determined by chemical, physical, and enzymatic methods, was concluded to be P-6-Man*p*-α-(1→3)-Man*p*-α-(1→3)-Man*p*-α-(1→3)-Man*p*-α-(1→2)-Man. No evidence for structural heterogeneity of this compound was apparent.

The phosphomannan produced exocellularly by the yeast *Hansenula holstii* NRRL Y-2448 has the constituents D-mannose and phosphorus in a molar ratio of 5:1 (Jeanes *et al.*, 1961). The phosphate is present in diester linkage between carbon-6 hydroxyl of one mannose unit and carbon-1 hemiacetal hydroxyl of another mannose unit (Slodki, 1962).

Jeanes and Watson (1962) proposed from periodate oxidation studies that the structure of this polymer may consist of a repeating unit of ten mannose units distributed on an average of five between a phosphodiester group linked between carbon-6 of one mannose unit and carbon-1 of another which is mannosidically linked at carbon-2. The remaining three mannosidic linkages in each pentamer unit were suggested to be 1→3′ in one of the mannopentaose units and two 1→3′ and one 1→2′ linkages in the other mannopentaose unit. That sequences of at least three 1→3′ linkages occur was demonstrated by isolation of the 1→3′-linked trisaccharide from periodate-oxidized phosphomannan Y-2448 (Jeanes *et al.*, 1962).

The questions as to whether this phosphomannan is comprised solely of such repeating monophosphomannopentaose units and whether these units are structurally homogeneous are of interest from the viewpoint of biosynthesis. Slodki (1962) suggested that these phosphomannans could be synthesized by appropriate transfers of mannosyl and mannose 1-phosphoryl units from GDP-mannose to the growing polysaccharide. We have since demonstrated both such transfer reactions from GDP-mannose with a particulate enzyme fraction from *H. holstii* (Bretthauer *et al.*, 1969; Kozak and Bretthauer, 1970), although it has not been established that the mannose 1-phosphoryl transfer was to exocellular phosphomannan. If a repeating unit structure exists in the phosphomannan, one may further suggest that this repeating unit is preassembled during biosynthesis on an intermediate acceptor molecule with subsequent polymerization of the repeating unit, analogous to the biosynthesis of bacterial peptidoglycan, the antigenic side chain of lipopolysaccharide, and capsular polysaccharide (for review, see Rothfield and Romeo, 1971).

This report presents evidence for the existence in phosphomannan Y-2448 of an apparently homogeneous monophosphomannopentaose unit which accounts for at least 65% of the mannose and phosphate in the native polymer. A core polysaccharide which lacks phosphodiester linkages accounts for about 10% of the remaining polymer.

Materials and Methods

Production of Phosphomannan Y-2448. H. holstii Y-2448 was grown at 25° in a medium containing 1 g of corn steep liquor, 1 g of tryptone, 5 g of KH_2PO_4, 5 ml of salts solution, and 40 g of glucose per l. (Anderson *et al.*, 1960). The shake flasks were inoculated with a 5% volume of a culture grown for 24 hr. After growth for 72–96 hr, the potassium salt of phosphomannan was isolated and purified as described by Jeanes *et al.* (1961). To obtain uniformly labeled [^{14}C]phosphomannan, 500 μCi of uniformly labeled [^{14}C]glucose was added to 2.5 ml of the above growth medium. This yielded, after 72-hr growth, 50 mg of phosphomannan having a specific radioactivity of 7×10^6 cpm/mg.

Hydrolysis of Phosphomannan. The solution contained

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