INDUCTION OF AN ENZYME FOR INCORPORATION OF D-SERINE INTO THE CELL WALL MUCOPEPTIDE OF MICROCOCCUS LYSODEIKTICUS.[a]

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Recently incorporation of D-serine into the growing cell wall mucopeptide of Micrococcus lysodeikticus was reported (Whitney and Grula, 1964). When serine addition occurred, amounts of glycine in the mucopeptide decreased. Several amino acids (D- or L-alanine, L-serine, or glycine) brought about varying reversal of the growth inhibition and cell wall modification caused by D-serine. Incorporation of D-serine into the cell wall could be mediated by the "glycine-adding" enzyme or by an enzyme specific for D-serine which could be either constitutive or induced. Induction of an enzyme for incorporation of an amino acid into the cell wall of bacteria has never been reported.

One approach for obtaining data relative to the inducible nature of the serine-adding enzyme would involve incorporation of serine into the cell wall in the absence of protein synthesis. Incorporation could be accepted as evidence for a constitutive enzyme (glycine- or serine-adding), whereas lack of incorporation could indicate the need for synthesis of an induced enzyme.

Three known inhibitors of protein synthesis, actinomycin-D, puromycin, and D-chloramphenicol (CAP) were selected. The latter, CAP, has been reported by Hancock and Park (1958) to allow mucopeptide synthesis while inhibiting synthesis of protein. Data will be presented showing that actinomycin-D and puromycin also allow continued mucopeptide synthesis while inhibiting protein synthesis.

Results

To determine if the three antibiotics inhibited protein synthesis while allowing synthesis of mucopeptide in M. lysodeikticus, incorporation of glycine-2-C^{14} into protein and mucopeptide was studied in the presence and absence of the antibiotics. This amino acid was chosen because it is a normally occurring component of the cell wall and protein of this organism.

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Representative data are shown in Table I. All antibiotics drastically inhibit protein synthesis. However, incorporation of glycine into mucopeptide is not inhibited; instead a stimulation in the presence of all antibiotics occurs. A possible explanation for the stimulation could be a sparing effect due to inhibition in protein synthesis.

The effect of the antibiotics on the incorporation of D-serine-3-C^{14} into mucopeptide and protein was next studied. Data in Table I show that incorporation of D-serine into both protein and mucopeptide fractions is greatly inhibited, particularly in the presence of puromycin and CAP.

It was possible that lack of D-serine incorporation into cell wall in the presence of these antibiotics could have been due to inhibition in synthesis of an enzyme necessary for entry of D-serine into the cell. Therefore, the C^{14}-content of intracellular pools was determined after growth in the presence of D-serine-3-C^{14} and the antibiotics. Cell pools contain appreciable amounts of label from D-serine-3-C^{14} (Table II); therefore, lack of incorporation into mucopeptide is not

TABLE I

Effect of Chloramphenicol, Actinomycin-D, and Puromycin on Synthesis of Cell Wall Mucopeptide and Protein

	Incorporation of C^{14} (cpm/mg Dry Wt. Cells Fractionated)							
	Glycine-2-C^{14}				D-Serine-3-C^{14}			
Additions	Cell Wall Muco-	% Change	Protein	% Change	Cell Wall Muco-peptide	% Change	Protein	% Change
None	10996	---	35484	---	970	---	1330	---
Chloramphe-nicol	14247	+29	2195	-93	126	-87	168	-87
Actinomy-cin-D	16158	+46	1452	-95	563	-41	168	-87
Puromycin	15412	+40	4690	-87	222	-77	260	-80

Test system consisted of cells grown under defined conditions (Grula, 1962) at 30°C in a Dubnoff shaker. At OD of 0.5 (540 mμ, Spectronic 20), D-glucose was added to a final concentration of 0.2%. After 30 min either actinomycin D (10 μg/ml medium), puromycin (45 μg/ml), or D-chloramphenicol (100 μg/ml) was added. D-Serine-3-C^{14} (0.1 μC/ml) or glycine-2-C^{14} (0.04 μC/ml medium) purchased from Calbiochem 3625 Medford St., Los Angeles, was added after an additional 30 min incubation. Unlabeled D-serine or glycine was also added such that the final concentration was 5×10^{-4} M. After 1 hr, OD readings were made, the samples immediately immersed in an ice bath, washed in cold water, and cells fractionated by the method of Park and Hancock (1960) for cell wall mucopeptide. All centrifugations were done in an RC-2 at 27,000 x G for 15 min.

due to inhibition in synthesis of a permease necessary for entry of the amino acid into the cells.

TABLE II

Effect of Actinomycin-D, Puromycin, and D-Chloramphenicol on the Intracellular Pool Content of Label from D-Serine-3-C^{14} and Glycine-2-C^{14}

Medium Addition	Control	CAP	Actinomycin-D	Puromycin
D-Serine-3-C^{14}	5700	4111	5033	7075
Glycine-2-C^{14}	1658	3838	1203	1772

Technique for growth as given under Table I. Cell pools were obtained by extraction with cold 5% TCA for 10 min. Counts were made using a Picker gas-flow automatic planchet counter.

TABLE IV

Effect of Various Amino Acids Upon the Uptake and Incorporation of Label from D-Serine-3-C^{14}

Fraction	D-Ser-3-C^{14}	D-Ser-3-C^{14} + D-Alanine	% Inhibition	D-Ser-3-C^{14} + L-Alanine	% Inhibition	D-Ser-3-C^{14} + L-Serine	% Inhibition	D-Ser-3-C^{14} + Glycine	% Inhibition
Cell Pools	3917	576	85	1441	63	1567	60	1919	51
Protein	625	206	67	262	58	81	94	136	78
Cell Wall	363	108	70	120	66	158	56	177	51

Cells were grown to an OD of 0.7 as given under Table I, and 19.5 ml of cell suspension were then added to each of five flasks containing the amino acid combinations shown. Amino acid additions were made in a volume of 0.5 ml such that final concentration of each amino acid was 5 X 10^{-4} M. D-Serine-3-C^{14} was added to a concentration of 0.05 μC/ml medium and incubation continued for 30 additional min in a Dubnoff water bath shaker. Isolation of cell pools, protein, and cell walls was accomplished by the method of Park and Hancock (1960). Radioactivity is expressed as cpm/mg dry wt of cells fractionated.

Because all data are based on the counting of radioactivity in pure cell walls, it was possible that glycine and particularly D-serine were being metabolized and their carbon entering the cell wall as other compounds. Data in Table III reveal that the cell wall preparations were pure, in that no contaminating amino acids were present and also that glycine and D-serine were incorporated essentailly unchanged.

Previously, we reported that four amino acids partially annulled growth inhibition due to D-serine and the incorporation of D-serine into the cell wall mucopeptide (Whitney and Grula, 1964). Data in Table IV reveal that all four amino acids, D- and L-alanine, L-serine, and glycine inhibit the uptake of label from D-serine into intracellular pools. Therefore at least part of the reversal of

TABLE III

Qualitative Amino Acid Analysis of Cell Wall Mucopeptide

Medium Additions	Cell Wall Amino Acids detected with Ninhydrin in 60 min Samples					Cell Wall Amino Acids Labeled in 60 min Samples				
	Ala	Glut	Gly	Lys	Ser	Ala	Glut	Gly	Lys	Ser
None	+	+	+	+	-	-	-	-	-	-
Glycine-2-C^{14}	+	+	+	+	-	V.S.*	-	+	-	-
Glycine-2-C^{14} +CAP	+	+	+	+	-	-	-	+	-	-
Glycine-2-C^{14} + Puro.	+	+	+	+	-	V.S.	-	+	-	-
Glycine-2-C^{14} + Actino.D	+	+	+	+	-	V.S.	-	+	-	-
D-Ser-3-C^{14}	+	+	+	+	+	-	-	-	-	+
D-Ser-3-C^{14} +CAP	+	+	+	+	-	-	-	-	-	V.S.
D-Ser-3-C^{14} + Puro.	+	+	+	+	-	-	-	-	-	V.S.
D-Ser-3-C^{14} + Actino.D	+	+	+	+	-	-	-	-	-	V.S.

Technique for growth and fractionation as given under Table I. The two-dimensional chromatographic system was that of Redfield (1953). Amino acids were detected using ninhydrin spraying (0.5% in acetone) and heating at 105°C for 5 min. Presence or absence of radioactivity in the amino acids was determined by exposing chromatograms (before ninhydrin treatment) to Blue Brand X-ray film for 21 days and developing in Diafine. This procedure permits detection of less than 100 cpm.

*Very slight labeling observed.

the effects of D-serine by these amino acids appears to be due to their competition with D-serine at the cell entry level. In no instance is incorporation or entry of D-serine completely annulled as might be expected from our previous report.

As a check on the reproducibility of the specific activity figures obtained from our chemically isolated cell wall preparations, five cell wall preparations were made from the same flask culture. The greatest deviation from an average specific activity of 10,996 was ± 600 cpm.

Discussion

Addition of CAP, actinomycin-D, or puromycin to growing cells of M. lysodeikticus causes an inhibition in protein synthesis while mucopeptide synthesis continues. This is further supported by the fact that during exposure to the antibiotics the optical density of the cell suspensions increases slightly. This would be expected since growth or increase in cell mass is not completely stopped due to continued cell wall synthesis. Incorporation of D-serine into the cell wall mucopeptide in the presence of these antibiotics is greatly reduced during a 1 hr incubation indicating a need for protein synthesis in order for D-serine addition to occur. In the absence of these antibiotics low levels of D-serine can be detected, both isotopically and chemically, in the mucopeptide after 15 minutes of exposure. Therefore, we interpret our results to mean that the major incorporation of D-serine into the mucopeptide of this organism occurs because of enzyme induction by D-serine.

Actinomycin-D, which gave the least inhibition in D-serine incorporation into the cell wall (41%) was also tested at increased concentrations. When the antibiotic was present at 40 μg/ml of medium, inhibition in protein synthesis was increased to 98% and inhibition of D-serine incorporation into mucopeptide was increased to 59%. These data may indicate a concentration effect by this antibiotic on synthesis of RNA involved in protein fabrication.

Pollock (1963) reported that actinomycin-D manifests a difference in affinity for genetic sites depending on the incidence of guanine residues. It is possible that the genetic site for synthesis of the D-serine-adding enzyme is low in guanine and is therefore less susceptible to actinomycin-D.

The amino acids D- and L-alanine, L-serine, and glycine reverse to varying degrees growth inhibition and cell wall modification caused by D-serine (Whitney and Grula, 1964). At least a portion of this reversal is due to decreased uptake of D-serine in the presence of these amino acids. A good correlation also exists between the reversal of growth inhibition and the inhibition in incorporation of label from D-serine into the protein fraction. L-Serine, which is most effective in overcoming growth inhibition caused by D-serine, is also the most effective inhibitor of incorporation of label from D-serine into protein. This relationship also holds true for the other three amino acids. These data indicate that synthesis of "fraudulent" proteins may occur in the presence of D-serine since growth inhibition cannot be directly correlated with incorporation of D-serine into the cell wall of this organism (Whitney and Grula, 1964). Further information to support this view comes from data presented in Table I where it can be seen that the three antibiotics inhibiting protein synthesis significantly decrease incorporation of label from D-serine into protein. Also, preliminary experiments (unpublished) reveal that: (1) Labeled serine is present in cell protein fractions from cells incubated with

D-serine-3-C^{14}. (2) Labeling patterns of L- and D-serine appear to be different for this organism.

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