INFLUENCE OF DIVISION-INHIBITING AGENTS ON PROPERTIES AND CHEMICAL COMPOSITION OF AN *ERWINIA* SPECIES

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CHAPTER I

INTRODUCTION

Coleman (29) was first to report that D-amino acids inhibited cell division. Since Coleman's report, many types of compounds and treatments have been shown to inhibit bacterial cell division. These include p-fluorophenylalanine (189), magnesium deprivation (258, 259), aminopterin (88), S-(1,2-dichlorovinyl)-L-cysteine (45), penicillin (5, 69, 214, 243), vancomycin (116), cycloserine (91), mitomycin c (93), ultraviolet light (16, 50, 236, 237), x-irradiation (2, 3), and increased hydrostatic pressure (277).

Although division inhibition is frequently reported, very little work has been done on the mechanism of this inhibition. Inhibition of deoxyribonucleic acid synthesis was reported to be responsible for division inhibition in Pseudomonas aeruginosa (78) and Escherichia coli (278). However, this has been questioned by other workers (16, 50, 89, 236, 237). Previc and Binkley (189) reported that division inhibition by p-fluorophenylalanine was due to interference with tyrosine metabolism, principally by repression of enzymes early in tyrosine biosynthesis.

It seems unlikely that all inhibitory agents are working by a similar mechanism; however, results of Grula and Grula suggest that this is the case. These workers found that division inhibition due to D-amino acids, penicillin, aminopterin, S-(1,2-dichlorovinyl)-L-cysteine, ultraviolet light (88), mitomycin c (93), cycloserine and

vancomycin (91) is prevented by addition of pantoyl lactone to the growth medium. Also, division inhibition is overcome by addition of osmotic agents (91). Tomasz and Borek (247) also reported that the action of 5-fluorouracil was prevented by osmotic agents. The observation that division inhibition brought about by many different types of inhibitors is overcome by a single compound, pantoyl lactone, or by addition of inert osmotic agents, strongly suggests that a common mechanism or that a common site in the cell is involved. The action of osmotic agents to prevent division inhibition suggests an osmotic instability in the cells which would, of course, involve the bacterial cell wall and cell membrane.

The purpose of this study was to survey the biochemical properties of division-inhibited cells, to locate the site of action of several inhibitors, and to define more clearly the mechanism of action of pantoyl lactone.

CHAPTER II

LITERATURE REVIEW

Part A. Possible mechanisms of action of several inhibitors

Penicillin.

Penicillin may cause many morphological aberrations including swelling and bulb formation (187). A common aberration is inhibition of cell division. This was noticed early in E. coli both in vivo (5) and in vitro (214, 243) and in Staphylococcus, Streptococcus, and Pneumococcus species (69). Lately, inhibition of cell division has been reported in Erwinia sp and a method found to prevent and reverse penicillin-induced division inhibition (88).

Work on the proposed mechanisms of action of penicillin prior to 1948 has been adequately reviewed by Pratt and Dufrenoy (187). Early mechanisms included a change in the distribution of electrostatic charges at the cell surface (57), interference with the assimilation of an essential growth factor(s) (75, 77), and stimulation of oxidation of sulfhydryl compounds eventually depleting the cell of available reduced sulfhydryl materials (59, 60).

In 1949, Park and Johnson (184) reported that penicillin induced the accumulation of labile phosphate in Staphylococcus aureus. Park (181-183), Park and Strominger (185), and Strominger (227, 228) reported the pile-up of uridine diphospho amino sugar compounds some of

which contained peptides composed of one to three amino acids. Strominger (228) concluded that these uridine compounds were intermediates in the biosynthesis of nucleic acids and some other structure which he called "X". Penicillin induced the accumulation of the uridine nucleotides by interfering with the conversion of nucleotides to nucleic acids and "X".

Although not working with penicillin, Strange and Powell (225) found a peptide released from germinating spores which contained diaminopimelic acid (DAP), glutamic acid, alanine, acetylglucosamine, and an unidentified sugar amine. Strange and Dark (224) crystallized this unidentified sugar amine, characterized it by chemical reactions, and called it muramic acid. Kent (126), however, was the first to prove the structure of muramic acid. This work has been reviewed by Strange (223).

Salton (204-208), Cummins and Harris (41-43), and Weidel (270) in their studies on the structure of the bacterial cell wall demonstrated the presence of a mucopeptide very similar to that reported by Strange and Powell (225). The mucopeptide contained glucosamine, muramic acid, and a restricted number of amino acids (lysine or DAP, alanine, glutamic acid, and glycine).

Lederberg (143) was able to correlate these observations and in a short communication was the first to report that cell wall synthesis was the major site of action of penicillin and to identify "X" as the bacterial cell wall.

Strominger and co-workers (107-110, 159, 173, 229-234) and others (8, 106, 223) have demonstrated that a uridine nucleotide is actually

involved in cell wall synthesis. Recent reviews on the structure and biosynthesis of the bacterial cell wall have accepted this viewpoint (186, 209).

Park and Strominger (230) developed the theory that the uridine diphosphate compound carried the mucopeptide precursor (N-acetyl-muramyl-alanyl-glutamyl-lysyl-alanyl-alanine) to the wall where it was inserted. This was supported by the data of Brooks, Crathorn, and Hunter (21) who suggested that the peptide component of the wall was probably synthesized in the cell membrane. Penicillin could then prevent the passage of the uridine-mucopeptide precursor through the membrane or attach to the wall and prevent the insertion of the new mucopeptide fragment into the wall (230). The lysis induced by penicillin (18, 188) would then be due to increased pressure on the cell membrane caused by continued cytoplasmic growth. Addition of osmotic agents at the time of exposure to penicillin prevents cellular lysis with the formation of protoplasts or spheroplasts (142).

Other workers have also reported that penicillin inhibits mucopeptide synthesis (91, 170, 198, 274). Collins and Richmond (30) proposed that the structural similarity between penicillin and N-acetyl muramic acid might account for the biological activity of penicillin.

First reports indicated that binding of penicillin in the cell was not required for antibiotic activity (99). However, work in the laboratories of Johnson (44, 151, 210) and Cooper (34, 35, 38, 68, 204) have shown beyond any doubt that penicillin is irreversibly bound to some lipo-protein which probably resides in the cell membrane. These articles have been reviewed by Cooper (37).

Lately, Duerksen (58) reported that 95% of the radioactivity in cells exposed to penicillin-S^{35} was released by treatment with lysozyme. The remaining 5% was bound to lipo-protein and released only by the combined action of lipase and trypsin. Duerksen speculated that the penicillin receptor lipo-protein was located between the membrane and the wall and was lost during protoplast formation since protoplasts did not bind penicillin as did whole cells.

Similar results were reported by Hancock and Fitz-James (94). They demonstrated that penicillin did not inhibit the growth of protoplasts of Bacillus megaterium. It was further concluded that the only significant process affected by penicillin was cell wall synthesis.

Edwards and Panos (64) observed that penicillin could induce the formation of stable L-forms of Streptococci. After cultivation in the absence of exogenous penicillin, organisms remained as L-forms and continued to accumulate both nucleic acids and cell wall precursors. Since growth should have eventually diluted the bound penicillin to a non-toxic level, it appears that penicillin induced some lesion which could not be repaired by the cell. This could possibly involve the loss of a primer molecule required for wall synthesis as suggested by Landman and Halle (133).

Grula and Grula (91) reported that penicillin also inhibited mucopeptide synthesis in Erwinia sp . However, neither pantoyl lactone (88) nor osmotic agents (91), which completely reverse division inhibition due to penicillin, prevented the inhibition of mucopeptide synthesis. This suggests that the direct cause of division inhibition by penicillin is not inhibition of cell wall synthesis. The fact that various osmotic

agents prevent division inhibition due to penicillin suggests that a penicillin-induced modification of the cell membrane might be responsible for division inhibition.

Prestidge and Pardee (188) reported that penicillin induced leakage of large molecules from the cell. Extracts of penicillin-treated E. coli caused lysis when added to suspensions of protoplasts of B. megaterium. It was also observed that compounds which inhibit protein synthesis prevented penicillin-induced leakage. These data were interpreted as demonstrating the induction of an enzyme by penicillin which attacked the cell membrane.

Trucco and Pardee (248) reported that penicillin did not specifically inhibit the incorporation of C^{14}-amino acids into the cell wall. No differences were observed in the electron microscope between normal and penicillin-treated walls. Therefore, Trucco and Pardee concluded that penicillin did not specifically inhibit wall synthesis.

Other reports have attempted to show that penicillin inhibits RNA, DNA, and protein synthesis (138, 188) and an unidentified intracellular enzyme (141).

Vancomycin.

In 1959, Jordan and Inniss (116) reported that vancomycin inhibited cell division while permitting protoplasmic growth to continue. It was demonstrated that vancomycin specifically inhibited RNA synthesis.

In 1961, Jordan proposed that the primary site of action of vancomycin was inhibition of mucopeptide synthesis and that the previously noted inhibition of RNA synthesis was probably due to secondary damage.

Other workers have also shown that vancomycin inhibits mucopeptide synthesis in S. aureus (197) and Bacillus subtilis (14).

Grula and Grula (91) reported that vancomycin inhibited mucopeptide synthesis in Erwinia sp ; however, as with penicillin, neither pantoyl lactone nor osmotic agents prevented this biochemical lesion although they did nullify division inhibition caused by vancomycin.

It has been suggested that vancomycin affects the cell membrane (14). Shockman and Lampen (218) concluded that vancomycin affects the membrane since it inhibited growth of protoplasts.

Hancock and Fitz-James (94) also reported that the primary site of action of vancomycin was at the cell membrane and that inhibition of cell wall synthesis was a secondary effect. They further demonstrated that neither bacitracin nor vancomycin competed with penicillin for cellular binding sites. Thus, these antibiotics appear to have different primary sites of action.

Yudkin (276) was not able to demonstrate a specific effect of vancomycin on cell membrane synthesis. He concluded that inhibition of wall synthesis was not due to prior membrane damage.

Cycloserine.

Ciak and Hahn (27) reported that cycloserine caused the formation of protoplasts (spheroplasts) from E. coli when cells were exposed to the antibiotic in a hypertonic medium. This indicated that, similar to penicillin, cycloserine inhibited mucopeptide synthesis.

Strominger, Threnn, and Scott (235) concluded that cycloserine competed with D-alanine for incorporation into a uridine nucleotide in S. aureus. The activity of both alanine racemase and D-alanyl-D-alanine

synthetase was inhibited. The inhibitory effects of cycloserine are competitively reversed by addition of D-alanine to the suspending medium (217, 279).

Thus, it appears that cycloserine inhibits mucopeptide synthesis by preventing the synthesis of D-alanyl-D-alanine. That this is not the primary mechanism of division inhibition by cycloserine has been shown by Grula and Grula (91). Division inhibition but not inhibition of mucopeptide synthesis was prevented by pantoyl lactone and osmotic agents.

D-amino acids.

Either isomer of several amino acids may inhibit growth (201) or cause morphological aberrations (55). However, the D-isomers appear generally to have more toxic effects.

Fox, Fling, and Norris (72), Fling and Fox (70), Kobayashi, Fling, and Fox (130), and Yaw and Kakavas (275) first noted that D-amino acids inhibited growth. Coleman (29) was the first to report that several D-amino acids inhibited both growth and cell division in Rhodospirillum rubrum. Tuttle and Gest (249) reported morphological aberrations due to D-amino acids also using R. rubrum.

In 1960, Grula (86) reported that the D-isomers of serine, methionine, phenylalanine, threonine, tryptophan, and histidine inhibited cell division in Erwinia sp and suggested that these interfered with cell wall synthesis. Division inhibition due to D-serine could be prevented by the addition of ammonium ion, D- or L-alanine, or para- aminobenzoic acid. Tanaka (241) and Morrison (167) also reported that D-alanine reversed growth inhibition due to either carbamyl-D-serine or

D-serine.

Maas and Davis (150) concluded that D-serine inhibited pantothenic acid biosynthesis by interfering with the condensation of beta-alanine and pantoic acid.

L-serine stimulates the production of pantothenic acid from L-valine and beta-alanine. D-serine antagonizes this stimulation according to the data of Altenbern and Ginoza (4).

Grula and Grula (90) demonstrated that D-serine inhibited the conversion of aspartic acid to beta-alanine by inhibiting the alpha-decarboxylase. This inhibition was partially responsible for lowering the cellular content of pantothenic acid and coenzyme A (CoA) in Erwinia sp. These same workers also demonstrated that D-serine inhibited the conversion of alpha-ketoisovaleric acid to ketopantoic acid and the conversion of ketopantoic acid to pantoic acid (92). However, they were not able to confirm the findings of Maas and Davis (150) relating to inhibition of the condensing enzyme for pantoic acid and beta-alanine by D-serine. Inhibition of beta-alanine synthesis by D-serine has been suggested for a species of Flavobacterium (62, 63).

The findings of Grula and Grula were significant since either pantoyl lactone or pantoic acid completely prevent division inhibition due to D-serine (88). Addition of pantoyl lactone plus beta-alanine completely restored normal pantothenic acid and CoA levels in the cell while pantoyl lactone alone allowed a partial restoration (92). However, Grula and Grula could not obtain a direct correlation between the ability of a compound to restore pantothenic acid levels and to prevent division inhibition. Thus, inhibition in pantothenic acid biosynthesis by D-serine does not appear to be the cause of division inhibition.

Kavanagh, Tunin, and Wild (122) reported that D-methionine increased the synthesis of cephalosporin N in Cephalosporium sp. D-methionine, however, had no stimulatory effect on the biosynthesis of penicillin.

Murachi and Tashiro (169) reported that D-lysine inhibited the activity of D-amino acid oxidase by competing with the substrate for the apo-oxidase protein.

Eisenstadt, Grossman, and Klein (65) reported that D-aspartic acid inhibited protein synthesis. The conversion of inosine monophosphate (IMP) to adenosine monophosphate (AMP) via transamination with L-aspartic acid was inhibited. This resulted in a decrease in adenosine triphosphate (ATP) biosynthesis. The energy supply of the cell was thereby lowered. The authors claim that protein synthesis was inhibited, therefore, because of a decreased ATP level.

In 1959, Lark and Lark (139) reported that D-methionine induced spheroplast formation in Alcaligenes fecalis. D-methionine acted synergistically with penicillin in decreasing the mucopeptide content of the cell. Lark and Lark (136) also reported that D-methionine inhibited the incorporation of L-methionine into the cell wall but not into protein. They postulate two intracellular amino acid pools. The expandable pool is maintained by exogenous amino acids while the internal pool is not affected by exogenous amino acids. D-methionine displaced L-methionine from the expandable pool but not from the internal pool. Therefore, if cell wall synthesis depends on the expandable pool for a source of amino acids and protein synthesis depends on the internal pool, addition of D-methionine would inhibit cell wall synthesis but not protein synthesis.

In 1961, Lark and Lark (140) reported that a chloramphenicol-resistant incorporation of D-methionine into the mucopeptide portion of the cell wall was responsible for spheroplast formation.

In 1963, Lark, Bradley, and Lark (137) reported that the D-methionine incorporated into the mucopeptide could be released by lysozyme treatment. Peptides released by lysozyme from cells grown in the presence of D-methionine migrated differently in paper chromatographic systems than those from control cells. Data suggested a mechanism other than incorporation of D-methionine was responsible for inhibition of mucopeptide synthesis. D-methionine was incorporated into the mucopeptide only when added to the growth medium in low concentrations and not when added in high levels.

Neuhaus (174) found that D-serine could be used as a substrate by D-alanyl-D-alanine synthetase resulting in the synthesis of D-alanyl-D-serine. In addition, the dipeptide, D-alanyl-D-serine, inhibited the functioning of this enzyme.

Whitney and Grula (271) reported that D-serine was incorporated into the mucopeptide of Micrococcus lysodeikticus. There appeared to be a partial replacement of glycine in the mucopeptide; however, no decrease in alanine incorporation was detected.

Other workers have reported that D-serine inhibits salt uptake (67, 239). Thus, it appears that D-serine has an additional effect on the cell membrane.

Mitomycin c.

Shiba, Terawaki, Taguchi, and Kawamata (216) and Sekiguchi and Takagi (212) first reported that mitomycin c inhibited deoxyribonucleic

acid (DNA) synthesis in E. coli. Sekiguchi and Takagi (213) and Reich, Shatkin, and Tatum (195, 196) later found that mitomycin c caused the breakdown of DNA and release of deoxyribosides into the culture medium. Nakata, Nakata, and Sakamoto (171) attributed this breakdown to an increase in deoxyribonuclease (DNase) production. They found that the specific activity of the DNase extracted from the cell was increased 180%. Similarly, Kersten (128) found that mitomycin c caused the release of DNase from ribosomes. This seemed to explain the mechanism of action of mitomycin c , however, workers could not explain why mitomycin c neither caused the breakdown of DNA nor activated DNase in vitro (168).

Iyer and Szybalski (112) found by measuring the physical constants of DNA obtained from cells grown both in the presence and absence of mitomycin c that this antibiotic prevented the uncoupling of complementary strands necessary for DNA synthesis.

Iyer and Szybalski (113) later reported that mitomycin c could intercalate between complementary strands, binding them together, and preventing their separation. Intercalation, however, had to be preceded by activation of the mitomycin c molecule. This involved either an enzymic or chemical reduction which rendered the molecule active as a bifunctional alkylating agent. Thus activated, the molecule was active both in vitro and in vivo. Intercalation was favored by a high content of guanine and cytosine residues. It appears, therefore, that mitomycin c binds complementary strands of DNA by combining with guanine of one strand and cytosine of the other strand.

Grula and Grula (93) reported that mitomycin c-induced growth inhibition could be overcome by addition of several cations ($Ca^{++} = Mn^{++} > Mg^{++} > Fe^{+++}$) or by increasing the pH of the growth medium prior to

inoculation. Calcium and mangenese partially prevented division inhibition; however, pantoyl lactone was more effective in overcoming division inhibition by mitomycin c . Based on observed changes in permeability and cellular lysis induced by mitomycin c , these workers suggested that the membrane and/or the cell wall were damaged.

Later, Grula and Grula (91) reported the inhibition of mucopeptide synthesis by mitomycin c . They were unable, however, to prevent this damage by addition of either pantoyl lactone or osmotic agents to the growth medium although these agents are able to prevent division inhibition by mitomycin c.

Ultraviolet light.

In addition to being bactericidal, ultraviolet light (UV) is mutagenic (114). It is generally conceded that the biological lesions induced by UV irradiation are due to a direct effect on DNA and/or DNA synthesis (15, 56, 178, 191, 220, 238).

Smith (220) found two types of DNA based on UV sensitivity. The greater the exposure to UV the less DNA which could be extracted from the cell. This was interpreted as showing complex formation between DNA and protein induced by UV irradiation. DNA was precipitated as a protein conjugate from UV-treated cells during extraction.

It has been reported that "thymineless death" and UV killing involve the same site in the cell (78). This is supported by Rasmussen and Painter (191) who found that addition of thymidine protected cells after UV irradiation.

Beukers and Berends (15) reported that UV irradiation caused the formation of thymine dimers and interchain crosslinking. Opara-Kubinska,

Kurylo-Borowska, and Szybalski (178) also found cross linking between complementary strands of DNA induced by UV irradiation.

Sussenbach and Berends (238) reported that irradiation damaged DNA by causing the release of C-8 of guanine as carbon dioxide. Kubinski (132), however, reported that UV caused the breakdown of highly polymerized RNA. He found an increased retention of irradiated RNA on methylated albumin columns and postulated that irradiation either caused the exposure or formation of new binding sites in the RNA molecule.

Hewitt and Billen (98) observed a change in the sequence of DNA biosynthesis following UV irradiation.

Gale, Kendall, McLain, and DuBois (78) reported that inhibition of DNA synthesis was responsible for cell division inhibition in P. aeruginosa. Similar conclusions were reached by Zobell and Cobet (278) using E. coli.

The inhibition of DNA synthesis does not appear to be the primary cause of inhibition of mitosis in fibroblasts (96). In fact, the division mechanism is more sensitive to UV irradiation than is DNA synthesis (16). Several workers have reported that inhibition of cell division was not accompanied by inhibition of DNA synthesis (50, 236, 237).

Grula and Grula (89) have questioned the need for either intact DNA or DNA synthesis for cell division to occur in Erwinia sp. Division inhibition in Erwinia sp due to UV irradiation was prevented by pantoyl lactone (88). Pantoyl lactone also prevented division inhibition due to UV irradiation (190) or x-irradiation (2) in E. coli.

Ultraviolet irradiation inhibited mucopeptide synthesis in Erwinia sp (91). This particular inhibition was not prevented by addition of pantoyl lactone. Also, osmotic agents prevented division

inhibition caused by UV irradiation but did not repair inhibition of mucopeptide synthesis.

Summary.

At present, it is undisputed that penicillin binds to a lipoprotein component which is probably located in the cell membrane. It is assumed by some workers that such a binding could result in inhibition of mucopeptide synthesis. However, it has never been established that binding of penicillin to the cell membrane is the cause of division inhibition.

Vancomycin was first thought to inhibit RNA synthesis selectively, however, evidence soon pointed to the cell wall as the target organelle. More conclusive evidence is required before the primary site of action of vancomycin is known.

Cycloserine inhibits mucopeptide synthesis by inhibiting the functioning of D-alanyl-D-alanine synthetase and D-alanine racemase.

D-amino acids may act in several ways. The best documented mechanisms, however, include the inhibition of pantothenic acid synthesis by D-serine and inhibition of mucopeptide synthesis. Also, incorporation of the D-isomer of either serine or methionine into the cell wall mucopeptide in place of either L-methionine or glycine has been well documented.

Mitomycin c has been reported to inhibit DNA synthesis, to cause its breakdown by activating DNase, and,more recently, to cause coupling of complementary DNA strands by intercalation between the guanine and cytosine residues. Evidence has also been presented, however, showing that mitomycin c inhibits mucopeptide synthesis. Inhibition of mucopeptide

synthesis does not appear to be the primary cause of division inhibition (91) while data to be presented in this thesis will demonstrate that mitomycin c inhibits division at concentration levels which have no effect on DNA synthesis.

Ultraviolet irradiation damage may express itself as division and/or growth inhibition, or as mutation. Data tend to indicate that the expression of damage depends on the degree of irradiation. DNA appears to be affected both in its method of biosynthesis and in its structure. However, substantial evidence is presented to demonstrate that damage more subtle than inhibition of DNA synthesis is responsible for division inhibition.

Although it has been shown that all agents discussed affect mucopeptide synthesis to some degree, only penicillin, vancomycin, cycloserine, and D-amino acids are generally considered to have their major site of action on mucopeptide synthesis. Mitomycin c and UV irradiation are thought to be effective mainly on DNA biosynthesis and structure; however, Grula and Grula have reported that these agents also inhibit mucopeptide synthesis.

In addition to inhibiting mucopeptide synthesis, all agents discussed inhibit cell division. In every case division inhibition is overcome by addition of pantoyl lactone; however, in no instance does pantoyl lactone restore normal mucopeptide synthesis. Normal division can occur while mucopeptide synthesis is impaired. Thus, division inhibition is not a direct result of the ability of these agents to inhibit mucopeptide synthesis. The cause of division inhibition must necessarily be located in some other reaction or structure. There is some evidence that vancomycin attacks the cell membrane and it seems logical

that attention should next be focused on the membrane as the target organelle of these various division inhibitors.

Part B. Composition of the bacterial cell membrane

Most work on the chemical composition of bacterial membranes has been done using Gram-positive organisms due to ease of isolation and purification of their membranes. In most cases rather pure membrane preparations may be obtained by osmotic lysis of bacterial suspensions treated with lysozyme (82, 221, 222), ethylene diaminetetraacetic acid and DNase (252), or from autolysates (169). The great difficulty in working with Gram-negative organisms has led several workers to suggest that these organisms do not have separate cell membranes and walls (155, 156).

Several reviews have been published which discuss the nature of the bacterial cell membrane (103, 158, 209, 267). The membrane composes 15 to 20% of the dry weight of Bacillus sp [266, cited in O'Leary (177), p. 436] and 35% of Mycoplasma cells (193). It appears to consist of two parallel outer protein layers surrounding a lipid layer (103). Several recent studies of the membrane using the electron microscope have been published (32, 124, 176).

The bacterial membrane is generally considered to be mainly lipoprotein (158, 177). In fact, most of the cell's lipids are found in the membrane [266, cited in O'Leary (177), p. 436] and consist mainly of phospholipids (103). Due to the abundance of lipoidal material, a study of the cell membrane should focus initially, at least, on the phospholipids since technics for assay of these compounds, although tedious and sophisticated, are not impossible.

In addition to containing lipo-protein, the membrane may contain polysaccharide, various enzymes, and cytochromes (209). Also, ribosomes are associated with the cell membrane (76).

Bacterial lipids differ markedly from lipids of higher organisms in that they contain many free and unusual fatty acids, are generally low in or devoid of sterols, lecithins, and cephalins, and possess phospholipids low in nitrogen and high in carbohydrate (177). Carbohydrate, inositol, and glycerol containing lipids are common while ethanolamine and choline containing phospholipids are the most common of those that contain nitrogen. Lovern [147, cited in O'Leary (177), p. 435] has described bacterial phospholipids as being mainly "fatty acid esters of phosphorylated carbohydrates." Asselineau [9-11, cited in O'Leary (177), p. 428] has published several reviews on the chemical nature of bacterial lipids.

In 1951, Mitchell and Moyle (163) reported that the "small particle" fraction from S. aureus was 41% protein and 22.5% lipid. The protein contained an abundance of glycine, alanine, and glutamic acid probably due to the presence of cell wall mucopeptide. The lipid was found to contain 1.3% nitrogen (N) and 1.85% phosphorus (P).

Weibull [266, cited in O'Leary (177), p. 436] reported that 55-75% of the lipids in Bacillus sp were located in the cell membrane. The major lipid constituents were phosphatidic acid and neutral fat and accounted for 13-21% of the membrane by weight. The membrane contained 10.3-10.9% N, 15.9-20.9% lipid, 1.8-9.8% hexose, less than 0.7% amino sugar, and less than 0.1% DAP (269). Traces of nucleic acids were found in the membrane. Vennes and Gerhardt (252) also detected nucleic acids in membranes of B. megaterium.

Gilby, Few, and McQuillen (82) found that membranes of M. lysodeikticus contained 8.4% N, 1.16% P, 28.0% lipid, 18.9% mannose, 2.7% amino sugar, and no glucose. These same workers also demonstrated the presence of carotenoids which imparted a characteristic yellow color to purified membrane pellets (81, 82).

Razin, Argaman, and Avigan (193) found that membranes of Mycoplasma contained 47-60% protein, 35-37% lipid, and 4-7% carbohydrate.

Ikawa (105) found in lactic bacteria that the total lipid was 2-8% and total phospholipid 0.3-2.5% of the dry weight of the cell. Lactobacillus casei and Streptococcus fecalis lacked neutral glycerides while Lactobacillus plantarum, Leuconostoc mesenteroides, and Pediococcus cerevisiae contained glycolipids, glucose, and galactose. All lacked serine, ethanolamine, and choline containing phosphatides. The major ninhydrin positive material in L. mesenteroides was D-alanine while the major reacting material in the other organisms was L-lysine.

In addition to its structural lipo-protein composition, the cell membrane contains several supplemental components. The cytochrome system has been shown to be located in the membrane (164, 261, 262) as well as other electron transport enzymes (39, 221).

Enzymes found in the cell membrane include diphosphopyridine nucleotide oxidase (164, 242, 268), acid phosphatase (164), succinic acid dehydrogenase (104, 164, 242, 268), nicotinic acid hydroxylase (104), malic dehydrogenase (164, 242, 268), and lactic, alpha-ketoglutaric acid, and dihydroorotic dehydrogenases (242).

Part C. Physiological role of the cell membrane

Weibull (263) was the first to establish that a structure other than the wall was the primary osmotic barrier of the cell. He demonstrated

that the wall was permeable to small molecular weight compounds, however, an osmotic barrier did exist at the surface of the protoplasm. It has since been well documented through work dealing with the permeability characteristics of protoplasts that the cell membrane is actually the major osmotic barrier of the cell (165, 166, 264, 265).

In addition to passive entry of solutes into the cell, the functioning of active transport mechanisms involving permeases which operate at the cell membrane has been well documented and reviewed by Cohen and Monod (28) and by Kepes and Cohen (127). More recent reports include a study of the beta-galactosidase system of E. coli (131) and amino acid transport in Salmonella typhimurium (6).

The membrane is important in energy metabolism in that it contains the cytochrome system (164, 261, 262), electron transport enzymes (39, 221), and many dehydrogenases (104, 164, 242, 268).

It has also been reported that the mucopeptide is synthesized in the cell membrane (21). This was supported by Edwards and Panos (64) who reported that stable Streptococcal L-forms continued to synthesize uridine-muramyl peptide.

McNamara and Abrams (157) reported that S. fecalis cell membranes are capable of synthesizing polyribonucleotides. Thus, it appears that RNA synthesizing enzymes are also located in the membrane.

Grula and Grula (91) recently focused attention on the cell membrane as the possible site of initiation of the division process.

Part D. Biosynthesis of phospholipids

In 1956, Kennedy and Weiss (125) made a major breakthrough in studies on phospholipid biosynthesis. They found that cytidine diphos-

phocholine was an essential cofactor for lecithin biosynthesis. Later, Schneider and Behki (211) reported an increase in deoxycytidine diphosphate choline in extracts of Novikoff hepatoma which had an impaired lecithin synthesis. These data suggested that a cytidine diphosphate (CDP)-containing compound was involved in lecithin biosynthesis in a manner analogous to the carrier UDP compounds necessary for mucopeptide synthesis.

In 1963, Kiyasu, Pieringer, Paulus, and Kennedy (129) reported the biosynthesis of phosphatidylglycerol by enzymes from chicken liver mitochondria. The reaction sequence was as follows:

CDP-diglyceride + L-alpha-glycerophosphate ⟶ cytosine monophosphate (CMP) + phosphatidylglycerophosphate

phosphatidylglycerophosphate ⟶ phosphatidylglycerol + inorganic phosphate.

Free glycerol would not react with the CDP-diglyceride. The second reaction was inhibited by sulfhydryl poisons and resulted in the accumulation of phosphatidylglycerophosphate.

Wilgram and Kennedy (272) demonstrated the intracellular localization of the enzymes in rat liver that catalyze the biosynthesis of lipids. Phosphorylcholine-glyceride transferase and diglyceride acyl transferase were located entirely in the microsomal fraction while phosphorylcholine-cytidyl transferase was found in both the microsomal and soluble fractions. These data indicate that phosphatidylcholine is synthesized mainly in the membrane. It is possible that the enzyme activity found in the soluble fraction was liberated from the microsomes during preparation.

Using extracts of E. coli , Kanfer and Kennedy (120) demonstrated the cell-free synthesis of phosphatidylserine and phosphatidylethanolamine. D-serine would not serve as a substrate for the synthesis of phosphatidylserine. The reaction sequence was as follows:

CDP-diglyceride + L-serine ⟶ phosphatidylserine + CMP

phosphatidylserine ⟶ phosphatidylethanolamine + CO_2.

The biosynthesis of phosphatidylglycerol reported previously (129) was confirmed using cell-free preparations. It was noted that L-serine-CMP-phosphatidyl transferase required both octanol and sodium sulfate for activity. Ammonium sulfate or sodium chloride could substitute for sodium sulfate while diisobutyl ketone could replace octanol. These compounds may be required for solubilization of either the substrates or the end products in order to allow the enzyme to function. Enzyme activity was not affected by potassium fluoride, iodoacetate, N-methyl maleimide, hydrazine, or hydroxylamine.

Miras, Mantzos, and Levis (162) reported that L-serine-3-C^{14} was incorporated into phosphatidylserine only in the presence of ATP, CoA, and magnesium ion.

Hokin and Hokin (101) reported the incorporation of radioactive phosphorus into phosphatidic acid from P^{32}-labeled ATP. Thompson and DeLuca (244) reported that vitamin D stimulated the incorporation of radioactive phosphorus into phospholipids of the intestinal mucosa.

Phosphatidylcholine may be synthesized by two different methods (40). The first involves the reaction of CDP-choline with a diglyceride (125). Second, phosphatidylcholine may be synthesized by methylation of phosphatidylethanolamine. The enzyme catalyzing the latter

reaction has been purified from Agrobacterium tumefaciens and characterized by Kaneshiro and Law (118).

The biosynthesis of phosphatidylinositol has not been well described; however, Keenan and Hokin (123) reported the reaction of fatty acid thioesters of CoA with lysophosphatidylinositol to yield phosphatidylinositol. This reaction was catalyzed by an enzyme isolated from pigeon pancreas ribosomal and mitochondrial fractions.

Part E. Biological reactions and characteristics of phospholipids

Phospholipids aggregate in vitro to form globular micelles and bimolecular leaflets (12, 148). Lucy and Glauert (148) reported that the lipid core of the micelle has a diameter of approximately 40 to 45 angstroms. The hydrophilic portions of the lecithin molecules extend outward on all sides. Cholesterol molecules are oriented with hydroxyl groups at the outer boundry of the lipid core. Adjacent micelles may be held together by hydrogen bonds between hydroxyl groups of the cholesterol molecules. Also, there may be electrostatic interactions between lecithin molecules in adjacent micelles. Small globular micelles and bimolecular leaflets may function as building blocks in the formation of lipid-containing structures; however, it has not been shown that these characteristics are actually retained in vivo.

It has been reported that phospholipids are involved in transport of ions into the cell (100). The inositol phosphatides have been implicated in transport mechanisms (48, 66, 80, 255). A role has also been reported in the transport of monosaccharides (144). Sugars dissolved in non-polar solvents only when phospholipids were present. Complex

formation between the sugars and phospholipids was suggested.

Abramson, Katzman, and Gregor (1) implicated phosphatidylserine in transport mechanisms. They prepared aqueous dispersions of phosphatidylserine and noted formation of micelles. These micelles had an average molecular weight of 4×10^6 and were strongly acidic with an isoelectric point at pH 1.2 . Also, the micelles possessed ion exchange properties. The cation exchange nature of phosphatidylserine has also been studied by Nash and Tobias (172).

Das and Crane (46) reported complex formation between cytochrome c and phospholipid. The size of the complex depended on the ratio of the reactants. Monovalent cations inhibited complex formation proportional to ionic strength while divalent cations inhibited completely. Binding appeared to involve attractive forces between the negatively charged lipid and the positively charged protein molecules. Also, DeKoning (52) reported that peptides may be bound to phospholipid by salt linkages.

Phospholipids have also been associated with activity of several enzymes. Tobari (246) reported the requirement of phospholipid for activity of malate dehydrogenase isolated from Mycobacterium avium while others have shown involvement of phospholipids in the reaction catalyzed by beta-hydroxybutyrate dehydrogenase (117).

Vignais, Vignais, and Lehninger (253) reported that a protein-bound lipid was required for mitochondrial contraction. This lipid was identified as phosphatidylinositol (254). The authors offered the following mechanisms as explanation of this requirement.

Phosphatidylinositol may serve as a specific fatty acid acyl donor or may serve to remove some compound that inhibits contraction. Lysophosphatides accumulate upon aging in mitochondria and inhibit contraction.

Phosphatidylinositol could detoxify these lysophosphatides by donating an acyl group. It may be possible that palmitoyl Co A and palmitoyl carnitine, which also support contraction, are capable of acylating lysophosphatides (134, 135).

Phosphatidylinositol could, however, associate in some way with the lipids of the mitochondrial membrane to bring about contraction. It might serve to stabilize the tertiary or quaternary conformation of the membrane proteins or act as an allosteric effector for some enzyme.

Bruce, Giles, and Jain (22) reported that phosphatidylinositol could complex with protein through hydrogen bonds. Similarly, Webb (260) demonstrated that phosphatidylinositol protected bacteria from dessication and death.

It has also been well documented that lipids are functional in antibody formation. This aspect has been recently reviewed by Brady and Tramms (20).

The physiological role of phospholipids appears to be extremely complex. Apparently they are involved in transport mechanisms, enzyme activities, and react readily with a variety of compounds to form complexes.

CHAPTER III

MATERIALS AND METHODS

Test organism.

The organism used throughout this study was a stock culture of a soft rot-producing Erwinia. The biochemical and morphological characteristics most nearly resemble those described for Erwinia carotovora in the 7th edition of Bergey's Manual of Determinative Bacteriology (85). The organism is Gram-negative, motile, and rod-shaped. Optimum temperature for growth is approximately 25 C; however, growth is readily obtained on nutrient agar slants at 30 C . Acid is produced from glucose, lactose, and maltose.

Stock cultures were maintained on nutrient agar containing 0.5% sodium chloride with and without 1.0% glucose. Transfers were made daily alternating media with glucose and media without glucose. To insure purity the culture was periodically streaked on nutrient agar plates.

Media.

The basal medium used in this study contained the following per 100 ml : DL-aspartic acid (450 mg), D-glucose or D-mannose (300 mg), KH_2PO_4 (136 mg), K_2HPO_4 (174 mg), and $MgSO_4$ (3.0 mg). The following were added as trace mineral salts : H_3BO_3 (0.5 µg), $CaCO_3$ (10.0 µg), $CuSO_4 \cdot 5\ H_2O$ (1.0 µg), $FeSO_4(NH_4)SO_4 \cdot 6\ H_2O$ (50 µg), KI (1.0 µg),

$MnSO_4 \cdot H_2O$ (2.0 µg), MoO_3 (1.0 µg), and $ZnSO_4 \cdot 7\ H_2O$ (5.0 µg). When desired D-serine (0.031 M) or DL-serine (0.062 M) and pantoyl lactone (0.056 M) were added. All solutions were adjusted to pH 6.8 to 7.0. Glucose, mannose, D-serine, and pantoyl lactone were sterilized by filtration. Phosphates, aspartic acid, magnesium sulfate, and mineral salts were sterilized by autoclaving for 12 minutes at 110 C .

Growth of cells.

Nutrient agar slants containing 0.5% sodium chloride were inoculated from a stock culture and incubated 24 hours at 25 C. The cells were suspended in sterile distilled water and washed two times with distilled water by centrifugation. Cells from one nutrient agar slant were then resuspended in 5 ml distilled water and one drop used to inoculate 5.0 ml of basal medium. Volumes of medium less than 25 ml were incubated on a Dubnoff Metabolic Shaking Incubator at 25 C in 50 ml Erlenmeyer flasks. Volumes greater than 25 ml were incubated at 25 C in 250 ml Erlenmeyer flasks on a rotary shaker. In general, cells were harvested by centrifugation after 12 hours incubation.

Cell fractionation procedure.

After growth in basal medium, cells were harvested and washed two times with a mineral salts solution at the concentration used in the basal medium. Cellular pools and other soluble materials were extracted with 5% trichloroacetic acid at 25 C for 15 minutes. After centrifugation, the pellet was extracted with chloroform:methanol (2:1) at 25 C for 30 minutes. Nucleic acids were then removed from the residue with 5% trichloroacetic acid at 100 C for 30 minutes. The insoluble protein-mucopeptide fraction was resuspended in 0.1 N sodium hydroxide.

Ultraviolet light irradiation procedure.

Cells which had grown for 20 hours on five nutrient agar slants at 25 C were washed two times with mineral solution. Cells were resuspended in 6 ml saline and poured into the bottom of a petri dish. The suspension was irradiated for 40 seconds at a distance of 25.5 cm from a 15-W Sylvania ultraviolet light-emitting germicidal lamp in the dark with shaking. Irradiated cells were inoculated into desired media and incubation continued at 25 C in the dark.

DNA determination.

DNA was determined as deoxyribose by the method of Burton (23). An aliquot of the hot trichloroacetic acid soluble material was diluted with an equal volume of N perchloric acid and made to 2.0 ml volume with distilled water. Four ml of diphenylamine reagent was then added. The diphenylamine reagent consisted of 1.5 g diphenylamine dissolved in 100 ml glacial acetic acid containing 2.0 ml concentrated sulfuric acid and 0.5 ml of a solution containing 16 mg acetaldehyde per ml. The mixture was incubated for 20 hours at 30 C. Absorbancy was then measured at 600 mμ using a Coleman Junior Spectrophotometer. The DNA standard was prepared by dissolving 0.4 mg of commercially prepared DNA in 1.0 ml of 0.005 M sodium hydroxide. Before use this solution was diluted with an equal volume of N perchloric acid and heated at 70 C for 15 minutes. The standard curve was determined using a concentration of 3.3 to 33.0 μg DNA per ml.

RNA determination.

RNA was determined as ribose by the orcinol method described by Morse and Carter (168). An aliquot of the hot trichloroacetic acid

soluble material was made to 2.0 ml with distilled water. Five ml of a solution containing 0.1 g of anhydrous ferric chloride in 500 ml of concentrated hydrochloric acid were added. This was followed by 0.3 ml of a solution containing 2.0 g orcinol in 20 ml of 95% ethanol. After mixing, the tubes were heated at 100 C for 20 minutes. Volumes were adjusted to 7.3 ml with distilled water. Absorbancy was measured at 660 mμ using a Coleman Junior Spectrophotometer. The standard solution consisted of 400 μg RNA dissolved in 1.0 ml of 5% trichloroacetic acid. The standard curve was determined using a concentration of 5.5 to 55.0 μg RNA per ml.

Protein determination.

Protein was determined by the method of Sutherland, Cori, Hayes, and Olsen (240). An aliquot of the protein-mucopeptide fraction was made to 0.5 ml with distilled water. Five ml of a solution consisting of 1.0 ml of 2% copper sulfate, 1.0 ml of 4% sodium tartrate, and 100 ml of 4% sodium carbonate were added and mixed. Commercial Folin-Ciocalteau reagent was diluted with two volumes of distilled water and 0.5 ml added to each tube. After standing at 25 C for 15 minutes, absorbancy was measured at 660 mμ using a Coleman Junior Spectrophotometer. The standard consisted of 500 μg/ml crystalline bovine serum albumin dissolved in 0.1 N sodium hydroxide. The standard curve was determined using a concentration of 8.0 to 33.0 μg protein per ml.

Dry weight determinations.

The relation between dry weight and absorbancy at 540 mμ on a Coleman Junior Spectrophotometer was determined for cells grown in basal medium with and without D-serine. Cells which had grown for 12 hours

were washed three times with a mineral solution and once with distilled water. Serial dilutions of the cell suspension were made and absorbancy of each sample was measured. Aliquots were pipetted into preweighed aluminum dishes and dried to a constant weight at 100 C in a dry air oven. Results were plotted as mg dry weight of cells versus absorbancy at 540 mμ.

Isotopic uptake experiments.

Cells were grown in 6.0 ml of basal medium with and without additions for 11 hours. At that time 0.1 μc of the desired labeled compound was added. Incubation was continued for various time intervals from 10 minutes to 2 hours. The radioactive compound was not diluted with non-labeled carrier. Five ml aliquots were taken. After measuring absorbancy at 540 mμ on a Coleman Junior Spectrophotometer, the cells were washed two times with mineral salts solution, resuspended in distilled water, and radioactivity determined. Results are expressed as count per minute per mg dry weight of cells.

Manometric experiments.

Manometric experiments were performed using Warburg techniques according to the procedure of Umbreit et al. (251) at 25 C with air as the gas phase. Cells were grown for 11 hours in basal medium at 25 C. After washing, cells were either placed in a starvation medium containing only minerals and magnesium sulfate or in fresh basal medium containing added inhibitors. Incubation was then carried out for 2 hours at 25 C prior to manometric experiments. The cell suspension was placed in the main chamber of the Warburg flask, the substrates and inhibitors in the side arms. Phosphate buffer (0.01 M, pH 7.0) was added to give

a final volume of 2.4 ml. Endogenous respiration was determined in each experiment by measuring oxygen uptake in the absence of substrate.

Hexose determination.

Hexose was determined by the anthrone procedure. The sample was made to 2 ml volume with distilled water and cooled in an ice bath. Four ml of a 0.2% solution of anthrone in 95% sulfuric acid were added and the mixture heated for 10 minutes at 100 C. After cooling to room temperature, absorbancy was measured at 630 mμ on a Coleman Junior Spectrophotometer. The standard consisted of 90 μg glucose per ml. The standard curve was determined using a concentration of 1.5 to 30 μg glucose per ml.

Lipid extraction procedure for column chromatography.

Cells were grown in basal medium containing 0.16 μc of the desired radioactive compound per ml for 12 hours. After washing three times with 5% trichloroacetic acid at 25 C, the residue was resuspended in 20 ml methanol to dissociate lipo-protein complexes, flushed with either methane or nitrogen to prevent oxidation, capped, and incubated at 55 C for 30 minutes. Forty ml of chloroform were added and after flushing with gas and mixing, the suspension was allowed to stand at 25 C for 12 hours. The insoluble residue was removed by filtration using a Millipore filter apparatus. The chloroform:methanol soluble fraction was then washed according to the procedure of Folch, Lees, and Sloane-Stanley (71). The solution was equilibrated two times against equal volumes of 2 M potassium chloride and once against distilled water. In all cases, the top aqueous layer was removed by aspiration. After concentration to approximately 4 ml in a rotary evaporator at 45 C under nitrogen, the

sample was ready to be applied to the silicic acid column.

Silicic acid column chromatography.

A column was prepared by pouring a slurry containing 10 g of silicic acid (Mallinckrodt analytical reagent, 100 mesh) into a glass tube 12 mm in diameter and fitted with a teflon stop-cock (153). The column was washed with 20 ml of 1:1 chloroform:methanol and then with 25 ml chloroform. The sample was applied to the column and elution carried out with 50 ml chloroform followed by 250 ml of chloroform: methanol (4:1). A positive pressure head was maintained on the column. The pressure was controlled to give a constant flow rate through the column of about 0.75 ml per minute. Samples of 240 drops (about 4 ml) were collected using a Gilson automatic fraction collector equipped with a drop counter.

Organic phosphorus determination.

Organic phosphorus was determined according to the procedure of Chen, Toribara, and Warner (26). The sample in chloroform:methanol was taken to dryness in a boiling water bath. Four drops of concentrated sulfuric acid were added and the sample heated in a sand bath which had been pre-heated for three hours over an open flame. This was continued until the white fumes of sulfur trioxide appeared at the mouth of the tube at which time the tube was cooled to 25 C and two drops of 72% perchloric acid added. The tube was then heated over an open flame until the solution became clear. Distilled water was carefully added to 25 ml, an aliquot transferred to a graduated conical centrifuge tube, and the volume adjusted to 4.0 ml. Four ml of a solution containing one volume 6 N sulfuric acid, two volumes distilled water, one volume 2.5%

ammonium molybdate, and one volume 10% ascorbic acid were then added. After mixing, the tubes were incubated at 37 C for 1.5 hours at which time the absorbancy was measured at 820 mμ on a Beckman DU spectrophotometer. The standard consisted of 136 μg K_2HPO_4 per ml. The standard curve was determined using a concentration of 1.7 to 5.0 μg K_2HPO_4 per ml.

Determination of ninhydrin-positive material in solution.

The lipid sample in chloroform was diluted with an equal volume of methanol and made to 5.0 ml with chloroform:methanol (1:1). The ninhydrin reagent (1.5 ml) described below was added, mixed, and the tube heated in a boiling water bath for 20 minutes. After cooling to room temperature, 8.0 ml of aqueous 50% n-propanol were added with vigorous mixing. After sitting at 25 C for 10 minutes, the absorbancy at 570 mμ was determined using a Coleman Junior Spectrophotometer. The standard consisted of 0.5 mg phosphatidylethanolamine per ml in chloroform: methanol (1:1). The standard curve was determined using a concentration of 25 to 250 μg phosphatidylethanolamine per ml. The ninhydrin reagent was made by dissolving 400 mg stannous chloride in pH 5 citrate buffer, 0.2 M (made by dissolving 4.3 g citric acid and 8.7 g sodium citrate in 250 ml distilled water and adjusting the pH to 5.0 using potassium hydroxide and hydrochloric acid). This was then added to 250 ml of methyl cellosolve containing 10 g ninhydrin.

Amino acid hydrolysis procedure.

Amino acid hydrolysis was performed by placing 0.2 to 2.0 ml of sample in an 8 x 100 mm test tube. An equal volume of 12 N hydrochloric acid was added and the tube sealed in vacuo. Hydrolysis was allowed to

proceed for 18 hours at 100 C.

Alkaline hydrolysis of lipids.

Lipids were hydrolyzed according to the procedure of Dawson (49). The lipid sample was placed in a round bottom flask and dried in vacuo at 45 C. After drying, the residue was dissolved in 0.8 ml carbon tetrachloride and treated with 7.5 ml ethanol, 0.65 ml water, and 0.25 ml aqueous N sodium hydroxide at 37 C for 20 minutes. The sample was then placed on an Amberlite-IRC 50 column (0.8 x 12.0 cm) which had been washed with 50 ml of 80% ethanol at 4 C. The hydrolysis products were washed through the column with 25 ml of 80% ethanol. The eluate was taken to dryness in vacuo at 45 C. One volume of distilled water and two volumes of isobutanol:chloroform (1:2) were added. After shaking at 50 C for 5 minutes, the mixture was allowed to settle and then centrifuged. The upper aqueous phase was saved for analysis by paper chromatography.

Acid hydrolysis of nucleic acids.

Nucleic acids and compounds suspected of containing purines and/or pyrimidines were hydrolyzed in 6 N hydrochloric acid at 100 C for 4 hours in a sealed and evacuated tube. After hydrolysis, the contents were taken to dryness under a stream of warm air and dissolved in distilled water prior to paper chromatography.

Alkaline hydrolysis of nucleic acids.

Samples were hydrolyzed in 0.5 N potassium hydroxide at 25 C for 16 hours. After hydrolysis, the potassium ion was removed by precipitation with 0.1 N perchloric acid. The precipitate was removed by centri-

fugation and the sample applied to paper for chromatography.

Paper chromatography.

Chromatography and detection of amino acids.

Paper chromatograms for amino acid analysis were developed in the two-dimensional system of Redfield (194). The first solvent consisted of methanol:water:pyridine (80:20:4) and the second of tertiary butanol:methyl ethyl ketone:water:diethylamine (40:40:20:4). Diethylamine was redistilled before use. Samples of 10 to 200 µl were spotted on an 8 x 8 inch sheet of Whatman #1 filter paper and dried under a stream of warm air. Development in the first solvent required approximately 1.5 hours while approximately 4 hours were required for the second solvent. After development, chromatograms were autoclaved at 121 C for 30 minutes to remove residual diethylamine. Amino acids were detected by spraying with a solution of 0.5% ninhydrin in 95% acetone containing 5% distilled water (V/V). After spraying, the chromatograms were heated at 100 C for approximately 3 minutes. Amino acids appeared as blue, yellow, or reddish-brown spots on a white background.

Chromatography and detection of nucleic acids.

Nucleic acids and hydrolysis products were spotted on 8 x 8 inch sheets of Whatman #1 filter paper and developed ascendingly in the two-dimensional system of Strominger (257). The first solvent consisted of isobutyric acid:0.5 N ammonium hydroxide (5:3) and the second of ethanol: 1 M ammonium acetate, pH 7.0 (7.5:3). Development of the first solvent required approximately 4 hours while the second required 5 to 6 hours. Compounds containing purines and pyrimidines were detected by their

ability to quench ultraviolet light.

A differential procedure for detection of purines, pyrimidine, and imidazoles was used on occasion (7). The chromatogram was sprayed with a solution containing 0.25% mercuric acetate in 95% ethanol containing 0.6% acetic acid. After 30 seconds, the chromatogram was sprayed with 0.05% diphenylcarbazone in 95% ethanol. The chromatogram was then heated over a hot plate at 90 C. Specific compounds react with the reagents to form a blue complex which is detectable after heating.

Chromatography and detection of lipid hydrolysis products.

The water soluble hydrolysis products of cellular phospholipids were spotted on $1\frac{1}{2}$ x 20 inch strips of Whatman #1 filter paper and the chromatogram developed in a descending fashion in either aqueous phenol:acetic acid:ethanol (100:10:12) or in aqueous phenol containing 0.1% ammonium hydroxide. Ninhydrin was used to detect the amino compounds as described previously. Rhodamine 6 G was used as a general indicator for lipoidal compounds (149). The stock solution of rhodamine was made by dissolving 240 mg in 1 liter of distilled water and permitting to stand for 8 hours at 25 C. The working solution was prepared by diluting 50 ml of stock solution with 950 ml distilled water. After the chromatogram had thoroughly dried, it was immersed in 200 ml of working solution for 3 minutes at 25 C. The strip was then rinsed in distilled water for 1 to 2 minutes and viewed under ultraviolet light while wet. Anionic phosphatides such as phosphatidylinositol, phosphatidylserine, and phosphatidic acid appear blue while others such as phosphatidylethanolamine, lecithin, and sphingomyelin fluoresce yellow (153).

Amino acid quantitation.

Amino acids were quantitated according to the procedure of Giri, Radhakrishnan, and Vaidyanathan (83). Protein samples were hydrolyzed and chromatographed as described previously. The chromatograms were sprayed with 0.5% ninhydrin in 95% acetone and heated at 65 C for 30 minutes. The ninhydrin-positive spots were cut out and shredded into a test tube. Four ml of 75% ethanol containing 0.05 mg of copper sulfate per ml were added. The tubes were allowed to stand approximately 5 minutes with frequent shaking. The supernatant fluid was decanted and absorbancy measured at 540 mμ. Standard curves were prepared for each individual amino acid after chromatography in each of the solvent systems. The standard curve was determined using a concentration of 10 to 200 mμmoles of amino acid per spot.

Radioautography.

Radioautography was performed by placing paper chromatograms next to Blue Brand medical x-ray film and allowing to stand 1 to 4 weeks at 25 C. Samples containing various known amounts of radioactivity were spotted, chromatographed, and prepared for radioautography. A C^{14}-labeled compound emitting 100 counts per minute as detected on a Picker gas-flow automatic planchet counter operated windowless (40% of theoretical) could be resolved within 16 days.

Conversion of pantoyl lactone to pantoic acid.

Since pantoyl lactone but not pantoic acid is available commercially, it was necessary to hydrolyze pantoyl lactone in order to obtain pantoic acid. This hydrolysis was brought about by heating 2.0 g pantoyl lactone in 20 ml distilled water containing 0.56 g sodium hydroxide at 100 C

for 10 minutes. After cooling to 25 C, the pH of this solution was 6.8 to 7.2 with no adjusting. In solution this reaction is reversible so in order to minimize interconversion of the lactone and the acid, the stock solution of pantoyl lactone was maintained at pH 6.8 while the stock solution of pantoic acid was maintained at pH 7.2.

Tritiation of pantoic acid.

Pantoic acid was tritiated by Dr. Robert Suhadolnik, previously of the Department of Biochemistry, Oklahoma State University, using the Wilzbach procedure (273).

Purification of tritiated pantoyl lactone.

The tritiated pantoic acid obtained from Dr. Suhadolnik was chromatographed on $1\frac{1}{2}$ x 20 inch strips of Whatman #1 filter paper. The chromatogram was developed in a descending fashion in methanol:water: pyridine (80:20:4). Two areas of radioactivity were detected using a chromatogram scanner. One zone which contained about 90% of the total radioactivity had an Rf of approximately 0.48 while the other had an Rf of about 0.89. The slower zone gave a yellow color when sprayed with a pH indicator containing brom-cresol purple indicating an acidic nature (19). Only the more rapidly moving zone reacted with hydroxylamine to form a hydroxamate indicating an ester linkage (24). Similar Rf values and chemical reactions were found using known pantoic acid and pantoyl lactone. It was concluded that the radioactive spot with an Rf of 0.48 was pantoic acid and that the spot with an Rf of 0.89 was pantoyl lactone. The tritiated sample was chromatographed as described and the two radioactive zones cut out and eluted with distilled water prior to use.

Tritiation of sodium penicillin G.

Sodium penicillin G (Calbiochem) was tritiated by Volk Radiochemical Company, Skokie, Illinois, using the Wilzbach procedure at 4 C (273).

Purification of tritiated sodium penicillin G.

An aliquot of the tritiated penicillin was spotted on a $1\frac{1}{2}$ x 20 inch strip of Whatman #1 filter paper and developed descendingly in methanol:water:pyridine (80:20:4). Two radioactive zones were located on a chromatogram scanner. One remained at the origin and contained no more than 5% of the total radioactivity. The major radioactive zone had an Rf of 0.8 to 0.9. When placed on the surface of an autobiographic plate containing nutrient agar seeded with a penicillin sensitive strain of E. coli B , only one zone of antibiotic activity could be detected. This zone had an Rf similar to the rapidly moving radioactive area. A corresponding zone of antibiotic activity was found when non-labeled penicillin was chromatographed and placed on autobiographic plates. The rapidly moving area was cut out, eluted with distilled water, and was used as the stock solution of tritiated penicillin.

Isolation of penicillin and vancomycin resistant mutant.

Dr. Mary Grula isolated a strain of Erwinia resistant to 20 µg vancomycin per ml. Resistance was obtained by continued cultivation in the presence of the antibiotic. Upon testing, this strain was found to be resistant also to 50 units of penicillin per ml. Daily transfers for 14 days on nutrient agar slants in the absence of any antibiotic did not result in loss of resistance. Addition of vancomycin or penicillin at the above concentrations to wild type organisms results in a decreased

growth rate as well as inhibition of division. Vancomycin and penicilin do not inhibit growth or division of the mutant organism.

Leakage experiments.

Cells were prelabeled by growth in 50 ml of basal medium containing 0.2 μc of the desired isotopic compound per ml. At 11 hours, the cells were washed three times with a mineral solution and resuspended in fresh non-radioactive basal medium with and without D-serine and/or pantoyl lactone. Incubation was continued at 25 C on a Dubnoff Metabolic Shaking Incubator. At timed intervals, 3 ml aliquots were taken, optical density measured at 540 mμ on a Coleman Junior Spectrophotometer, and the cells removed by centrifugation at full speed for 5 minutes in an International Clinical Centrifuge. The supermatant fluids were frozen awaiting future radioactivity determinations.

Charcoal procedure for nucleotide isolation.

Cells were grown in basal medium containing D-serine. At 12 hours, the cells were removed by centrifugation and the spent medium retained. Charcoal-adsorbing materials were isolated by a modification of the procedure described by Ito and Strominger (109). Twelve ml of 50% trichloroacetic acid were added to 120 ml of spent medium followed by 24 ml of a 5% suspension of charcoal (Norite A). This was placed on a magnetic stirring apparatus for 3 hours at 25 C. Charcoal was removed by centrifugation and washed four times with 0.1 M ammonium acetate. Elution of nucleotides from the charcoal was carried out by stirring in 0.05 M ammonium hydroxide in 50% ethanol for 4 hours at 4 C. Elution was repeated and the eluates combined. The sample was then placed in a rotary evaporator and concentrated at 45 C to about 1 ml.

Counting procedure.

Samples of spent growth medium were counted in a Packard "Tri-Carb" model 314-A liquid scintillation spectrometer using 10 ml per vial of the following phosphor solution: diphenyloxazole (4.0 g), 1,4-bis-2(5-phenyloxoyl)benzene (0.2 g), absolute ethanol (400 ml), sulfur-free toluene (600 ml). Scintillation vials were acid cleaned and used a maximum of three times. All caps were discarded after one use.

Other samples were plated at infinite thinness and counted using a Picker automatic gas-flow planchet counter operated windowless. A counting efficiency of approximately 40% was obtained with this unit. Samples dissolved in organic solvents were plated on ringed aluminum planchets while others were plated on plain stainless steel planchets. All planchets were discarded after one use.

CHAPTER IV

RESULTS AND DISCUSSION

Inhibition of isotopic uptake by D-serine.

Since the first step in the metabolism of a compound is entry into the cell, the effect of D-serine on uptake and incorporation of several C^{14}-amino acids was studied. Dickie and Schultze (53) reported that S-(1,2-dichlorovinyl)-L-cysteine, which inhibits cell division in *E. coli*, also inhibits the uptake of phenylalanine-1-C^{14} and glycine-2-C^{14}. Roodyn and Mandel (200) reported that 8-azaguanine stimulated uptake and incorporation of alanine, glycine, serine, and diaminopimelic acid in *B. cereus*.

When tested as described in Materials and Methods , cells inhibited from dividing by D-serine took up only 4% as much DL-alanine-2-C^{14} during the first 20 minutes of exposure as control cells. A similar inhibition was noticed using glycine-1-C^{14} and to a lesser extent with DL-glutamic-2-C^{14}. These data are shown in Table I. D-serine did not affect the uptake of DL-leucine-2-C^{14} or DL-lysine-2-C^{14}.

These inhibitions can be separated from the cause of division inhibition based on the following observations. Addition of D-serine to log phase control cells caused observable division inhibition only after 2 to 3 hours incubation. However, inhibition of uptake of DL-alanine and glycine occurred within 20 minutes after addition of D-serine. Additional preincubation with D-serine was not required to demonstrate uptake inhibition.

TABLE I

INHIBITION OF AMINO ACID UPTAKE AND INCORPORATION

Inhibitor compound*	Labeled compound**	Specific activity[a]		Percent inhibition
		Control system	Inhibited system	
D-serine	DL-alanine-2-C^{14}	66,727	2,677	96.0
	glycine-1-C^{14}	60,218	3,130	94.8
	L-serine-U-C^{14}	32,700	15,800	51.7
	D-serine-3-C^{14}	1,925	268	86.1
	DL-glutamic acid-2-C^{14}	27,210	6,333	76.7
	DL-leucine-2-C^{14}	2,556	2,817	0.0
	DL-lysine-2-C^{14}	2,536	2,495	1.7
L-serine	L-serine-U-C^{14}	32,700	4,400	86.5
	D-serine-3-C^{14}	1,925	806	58.1
	DL-alanine-2-C^{14}	4,350	1,540	62.3
	glycine-1-C^{14}	14,760	5,800	60.7
glycine	DL-serine-3-C^{14}	8,333	5,066	39.2
	DL-alanine-2-C^{14}	61,600	12,440	79.8
DL-alanine	glycine-2-C^{14}	19,885	3,456	82.1
L-alanine	D-serine-3-C^{14}	4,260	1,590	62.7
DL-aspartic acid	DL-glutamic acid-2-C^{14}	29,150	360	98.8

* Added inhibitor to 3.3 mg per ml.

** Added 0.2 μc per ml; 2.6 to 3.0 μg per ml; incubation continued for 20 minutes.

[a] Counts per minute per mg dry weight.

Pantoyl lactone or DL-alanine, which prevent division inhibition due to D-serine, did not prevent uptake inhibition.

After cells have grown in the presence of D-serine for 20 hours, cell division occurs and the cells return to a normal size. When either C^{14}-glycine or C^{14}-alanine was added at this stage of growth, the same degree of uptake inhibition was observed.

Cells inhibited from dividing by mitomycin c (0.2 µg/ml) or vancomycin (10 µg/ml) showed an increased uptake of glycine and not an inhibition as would be expected if uptake inhibition were related to cell division inhibition (Table II). This stimulation may be due to damage to the permeability barriers of the cell. If so, mitomycin c damaged these barriers within 15 minutes after addition whereas vancomycin did not. The effect of these antibiotics on the cell membrane will be discussed later.

It appears, therefore, that inhibition of amino acid uptake is neither the cause nor an expression of the cause of division inhibition by D-serine.

Other workers have reported that the D-isomers of valine, phenylalanine, proline, and methionine did not inhibit the uptake of the L-isomers (127). Boezi and DeMoss (17) , however, reported inhibition of L-tryptophan uptake by D-tryptophan.

Oxender and Christensen (180) using Ehrlich cells reported that amino acids may be classified into groups based on their ability to serve as substrates for a given permease. Alanine, glycine, serine, threonine, proline, asparagine, and glutamine were transported into the cell by the same enzyme while leucine, isoleucine, valine, and phenylalanine were carried into the cell by a separate enzyme. Methionine

TABLE II

EFFECT OF MITOMYCIN C AND VANCOMYCIN ON THE UPTAKE AND INCORPORATION OF GLYCINE-2-C^{14}

Antibiotic*	Time of addition**	Specific activity[a]
none	-	17,650
mitomycin c	inoculation	33,400
mitomycin c	log[b]	32,200
vancomycin	inoculation	33,650
vancomycin	log[b]	20,100

* Mitomycin c was added to 0.2 µg/ml and vancomycin to 10 µg/ml.

** Antibiotics were added either at time of inoculation or in early log phase.

[a] Counts per minute per mg dry weight; glycine-2-C^{14} (0.2 µc/ml) was added and incubation continued for 20 minutes.

[b] Labeled compound was added 15 minutes after addition of the antibiotic.

could serve as a substrate for either permease.

Since inhibition of uptake of alanine and glycine by D-serine could merely be substrate competition for a specific permease, various combinations of a non-radioactive inhibitor and a radioactive substrate amino acid were tested (Table I). Although addition of non-labeled D-serine to isotopic D-serine and non-labeled L-serine to isotopic L-serine effected an 86% inhibition of uptake, the L-isomer inhibited the D-isomer only 58.1% and the D-isomer inhibited uptake of the L-isomer 51.7%. These data could be interpreted as demonstrating the presence of three separate enzymes for the transport of D- and L-serine. An enzyme specific for each of the isomers could be present along with one capable of transporting either isomer. Addition of D-serine would inhibit the uptake of D-serine-3-C^{14} by the D-serine specific permease and by the non-specific permease, however, only the uptake of L-serine by the non-specific permease would be inhibited by D-serine. However, it is more likely that there exists only two enzymes for the transport of D- and L-serine. One would have a greater affinity (K_m) for one isomer and the other would have a greater affinity for the other isomer.

It was also noted that L-serine inhibited uptake of glycine and alanine although not as efficiently as did D-serine. L-alanine inhibited uptake of D-serine to the same extent that L-serine inhibited the uptake of alanine. Glycine almost completely inhibited the uptake of DL-serine while glycine and alanine inhibited uptake of each other approximately 80%. Also, aspartic acid effectively inhibited the uptake of glutamic acid.

Qualitatively, these data were expected from the patterns established by Oxender and Christensen (180). However, because of the large ratio of

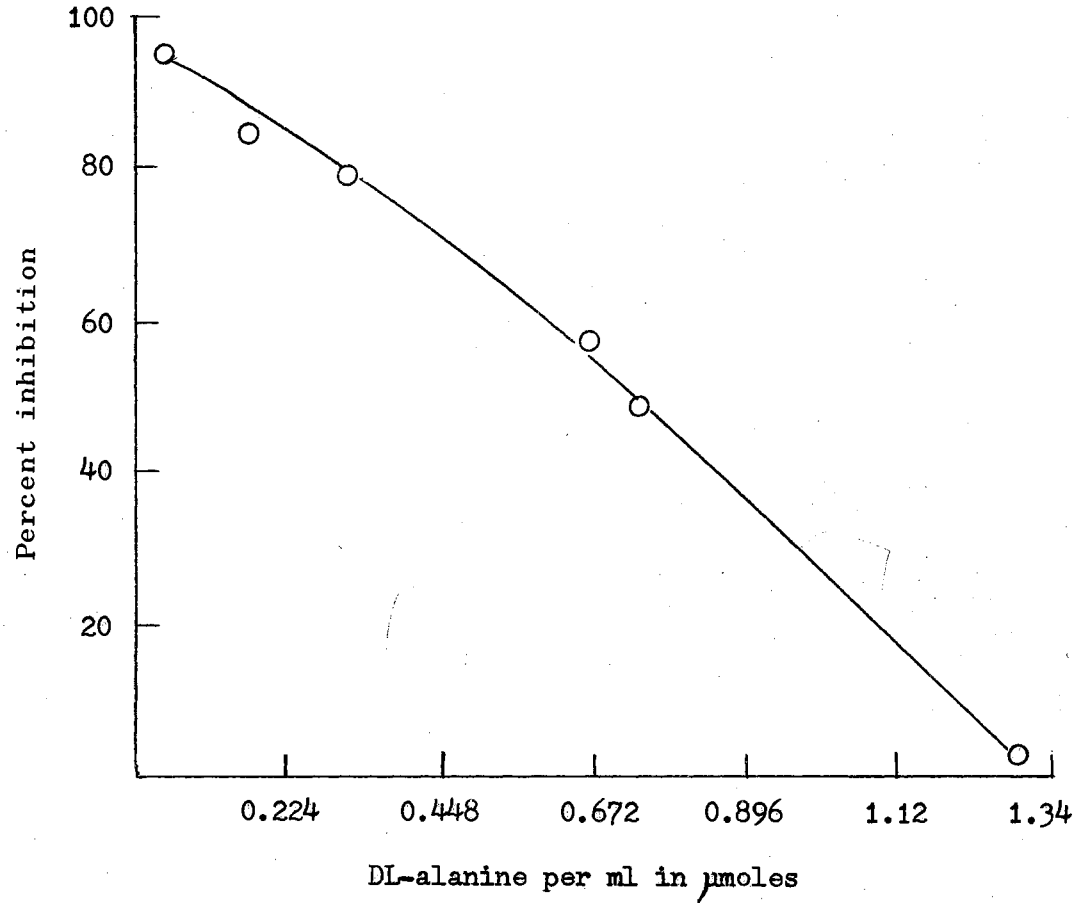
inhibitor to test compound required to demonstrate uptake inhibition, these data cannot be interpreted as showing substantial uptake of more than one amino acid by a given permease. In fact, uptake inhibition could not be detected if the concentration ratio of inhibitor amino acid to test amino acid was less than 24:1 . This is illustrated in Figure 1. The uptake of DL-alanine-2-C^{14} at varying specific activities was tested in basal medium containing D-serine (31.4 µmoles per ml). A ratio of about 43:1 allowed approximately 50% inhibition of uptake while a ratio of 892:1 was required to demonstrate 95% inhibition. Similar observations were made using other combinations of inhibitor and test amino acids. It appears, therefore, that each amino acid is transported mainly by an individual permease. To a small degree, these enzymes are capable of transporting other amino acids within a group into the cell. However, the affinity of the enzyme was approximately 43 times greater for one specific amino acid than for the others.

These inhibitions may also be due in part to inhibition of mucopeptide synthesis by D-serine. Grula and Grula (91) have reported that D-serine inhibits the incorporation of glucosamine and muramic acid into the cell wall mucopeptide. The mucopeptide also contains aspartic acid, glutamic acid, alanine, glycine, and diaminopimelic acid but not lysine or leucine (Grula and Grula, unpublished data).

Also,these inhibitions could be due to isotopic dilution by the carbons of D-serine. As will be shown later, D-serine is converted to glutamic acid, glycine, alanine, isoleucine, and valine. Inhibition of glutamic acid, alanine, and glycine incorporation could be due to dilution of the radioactive carbon by non-labeled D-serine. However, it would be expected that time would be required for these mechanisms to be operating

Figure 1

Inhibition of uptake of DL-alanine-2-C^{14} by D-serine at varying concentrations of alanine. Growth medium contained 0.2 μc DL-alanine-2-C^{14} per ml, 31.4 μmole D-serine per ml and varying amounts of non-labeled DL-alanine.


100
80
60
40
20
Percent inhibition
0.224
0.448
0.672
0.896
1.12
1.34
DL-alanine per ml in μmoles

since D-serine was metabolized much slower than glycine, alanine, or glutamic acid. As mentioned previously, inhibition of incorporation of these materials by D-serine was noticed within 20 minutes. Therefore, it is concluded that the major mechanism by which D-serine is inhibiting the uptake and incorporation of alanine, glycine, and glutamic acid is competition for entry. These data do not necessarily support the theory that D-serine disturbs the permeability barriers of the cell. However, this does not appear to be the case with mitomycin c and vancomycin.

Synthesis of protein and nucleic acid in division-inhibited cells

It has been reported that inhibition of DNA synthesis is responsible for or associated with inhibition of cell division (61, 78, 111, 278). It was necessary, therefore, to determine the effects of several division-inhibiting agents on the synthesis and cellular content of nucleic acids and protein.

A typical growth curve for Erwinia sp in basal medium is presented in Figure 2. Figure 3 shows the synthesis of protein in control cells growing in log phase. Similar results were obtained when DNA or RNA were followed. During this phase of growth, the increase in DNA, RNA, and protein closely followed the increase in cell mass.

Figure 4 shows the change in percent dry weight of the cells as RNA, DNA, and protein in log phase control cells. Protein remained constant while the percent dry weight as RNA and DNA decreased slightly.

Data obtained from cells inhibited from dividing by UV irradiation, D-serine (0.031 M), or mitomycin c (0.2 μg/ml) did not deviate from those found in rapidly dividing cells (Figure 5). Addition of D-serine

Figure 2

Growth of Erwinia sp in basal medium. Absorbancy was measured at 540 mμ using a Coleman Junior Spectrophotometer.

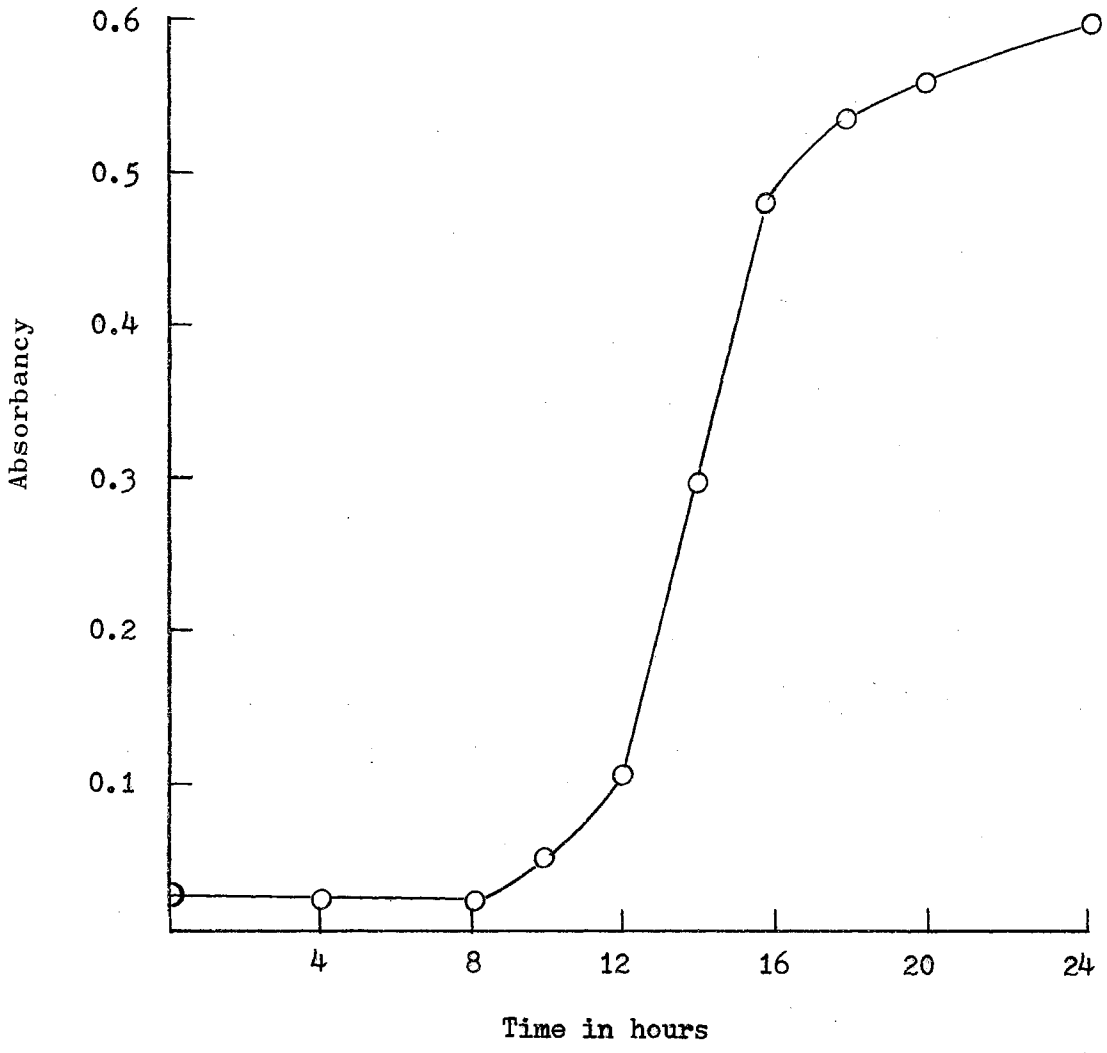

0.6
0.5
0.4
0.3
0.2
0.1
Absorbancy
4
8
12
16
20
24
Time in hours

Figure 3

Protein synthesis in Erwinia sp.

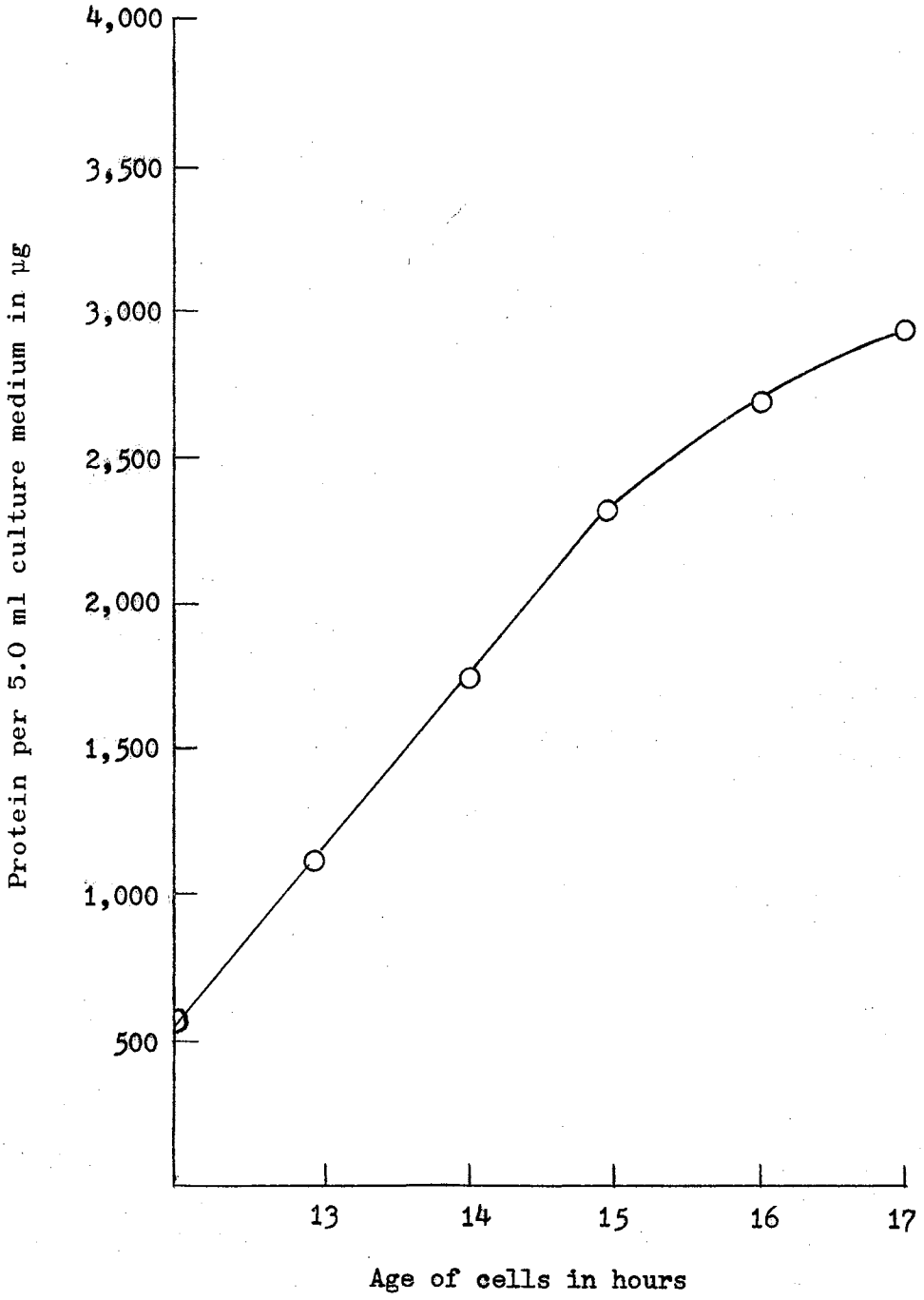

4,000
3,500
3,000
2,500
2,000
1,500
1,000
500
Protein per 5.0 ml culture medium in µg
13
14
15
16
17
Age of cells in hours

Figure 4

Cellular content of RNA, DNA, and protein in rapidly dividing Erwinia sp . ○ RNA; △ DNA; □ protein.

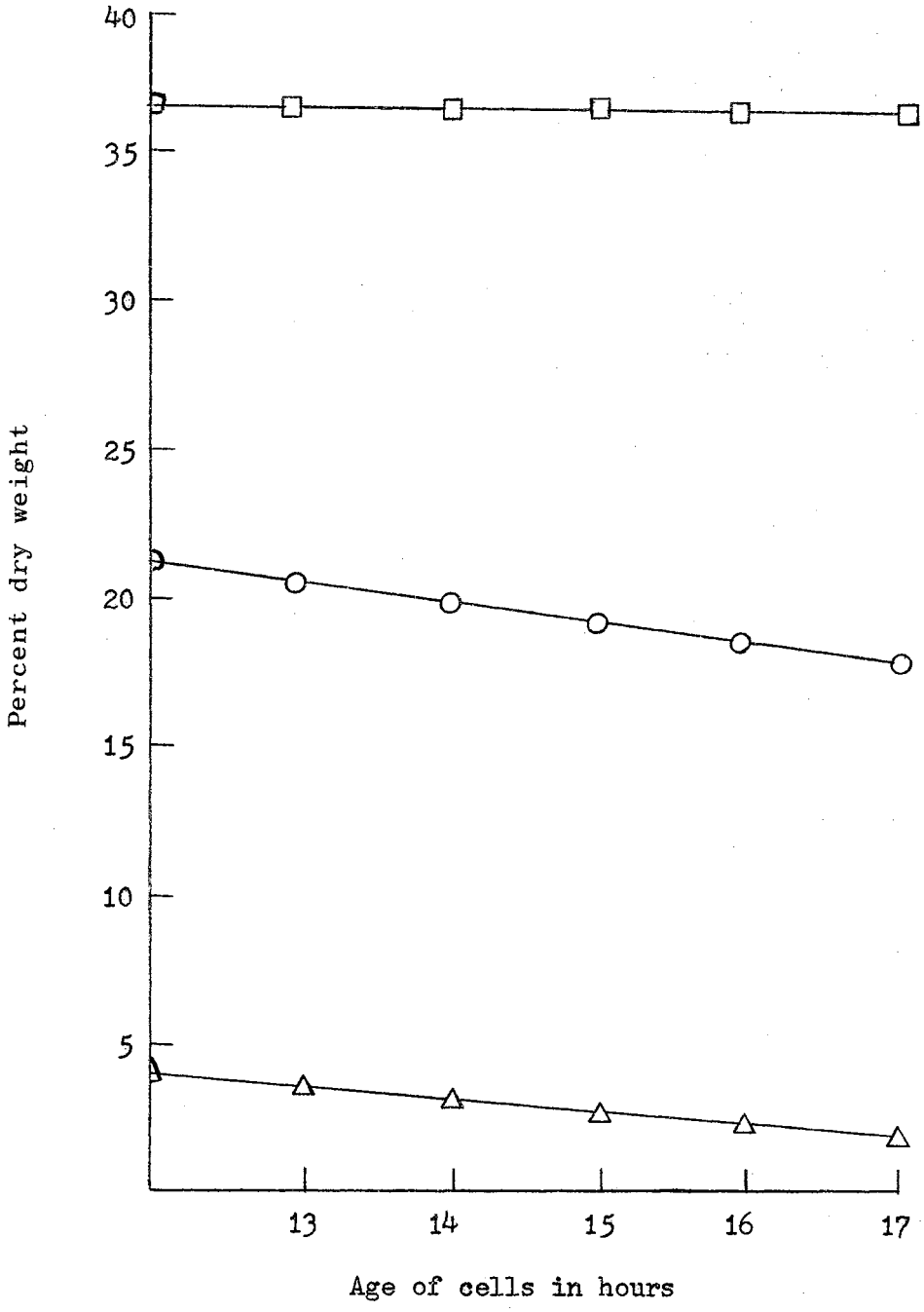
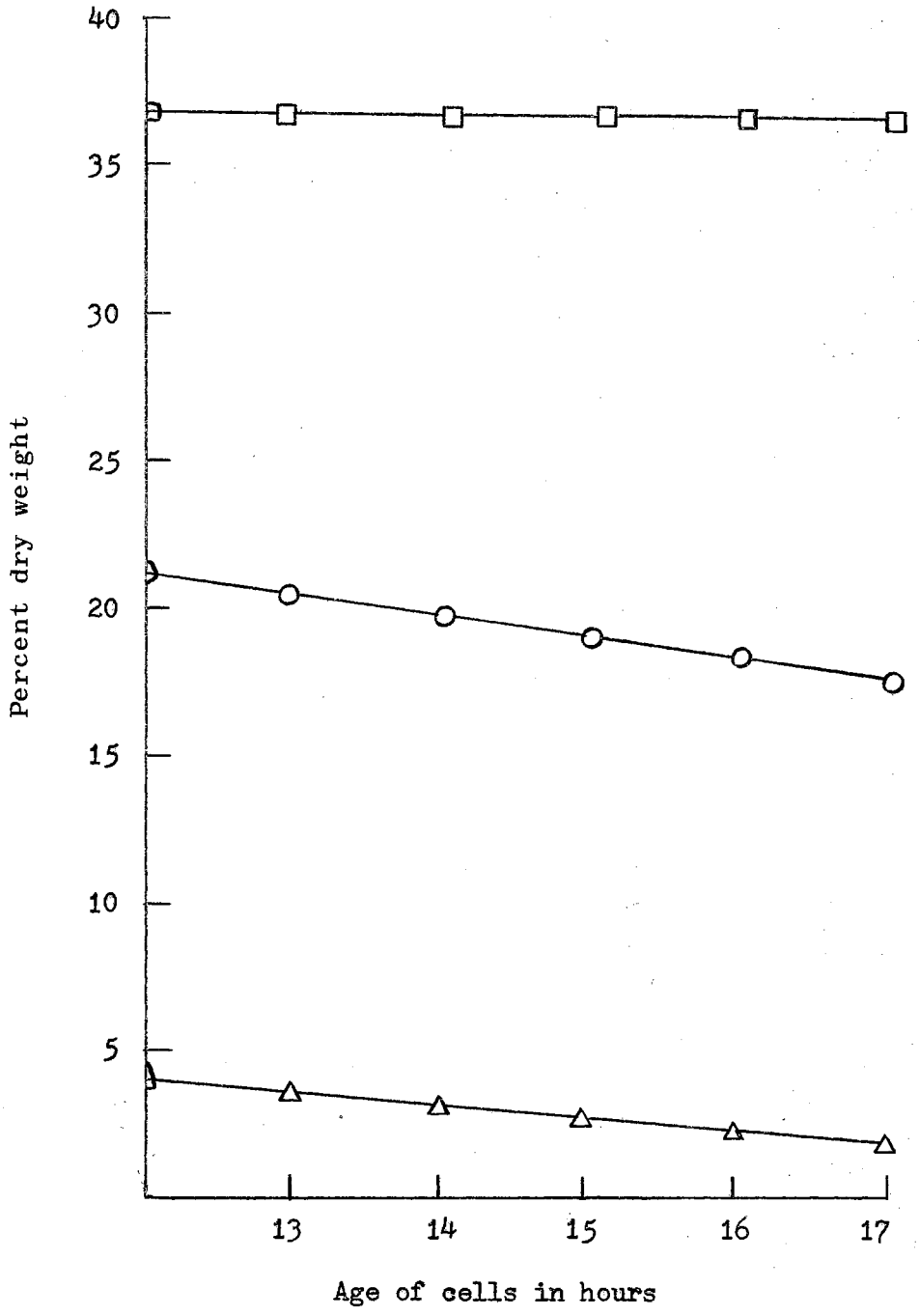

40
35
30
25
20
15
10
5
Percent dry weight
13
14
15
16
17
Age of cells in hours

Figure 5

Cellular content of RNA, DNA, and protein in division inhibited Erwinia sp. ○ RNA; △ DNA; □ protein.


40
35
30
25
20
15
10
5
Percent dry weight
13
14
15
16
17
Age of cells in hours

to rapidly growing cells did not affect the synthesis of RNA, DNA, or protein within five hours. No change was observed when mitomycin c was added to log phase cells; however, general lysis occurred after 2.5 hours at which time the cells had increased their normal length approximately five-fold.

These data discount neither the possibility of the synthesis of defective RNA, DNA, or protein nor damage occurring to them after a normal synthesis. Mennigmann and Szybalski (160) reported that damage to the molecular structure of DNA preceded thymineless death induced by 5-fluorodeoxyuridine. Beukers and Berends (15) reported that UV irradiation caused the formation of thymine dimers and interchain cross-linking. Iyer and Szybalski (112, 113) reported that mitomycin c intercalates between complementary strands of DNA. Alterations such as these would not have been detected using our test procedures.

Reich et al. (195, 196) and Nakata et al. (171) reported that mitomycin c caused the breakdown of DNA and inhibited its synthesis. Data presented in this thesis do not necessarily conflict with these findings. In general, previous reports have described the effects of 1.0 to 20.0 µg of mitomycin c per ml. The low levels (0.2 µg/ml) used in the present study give support to the theory that mitomycin c does not inhibit cell division by inhibiting DNA synthesis.

Although UV irradiation is generally assumed to affect DNA synthesis (56, 191, 220), no deviation in the synthesis or cellular content could be found in cells inhibited from dividing by exposure to UV irradiation. These data confirm the apparently overlooked (or disregarded) findings of Deering (50) and Stuy (236, 237).

Deering reported no change in RNA or DNA per unit length in UV-induced filaments of E. coli. Stuy (236) reported that UV irradiation of B. cereus in dosages sufficient to kill 99.7% of the organisms had no post-irradiation effects on RNA, DNA, or protein synthesis. Also, there was no change in the base ratios. Using Haemophilus influenzae, Stuy (237) reported an inhibition of DNA synthesis and in transforming activity immediately after irradiation. However, these damages were repaired after 40 minutes with a resumption of DNA synthesis and in transforming activity.

Several workers have attempted to show a direct correlation between DNA synthesis and cell division (51, 61, 78, 95, 111, 278). An obligatory coupling between DNA synthesis and cell division is generally assumed to occur because many morphological observations on mammalian cells have shown that mitosis and consequently a doubling of DNA occurs immediately prior to cell division. No one has proven, by chemical procedures, that cell division is automatically triggered following DNA synthesis. In fact, amoeba that have been enucleated still possess the capacity for cell division (146). The cytoplasm, at least in part, appears to have some control over cell division. Ord and Danielli (179) reported that if the nucleus were removed, the cytoplasm treated with some mutagenic agent, and the nucleus replaced, cell division would be inhibited.

Grula (87) reported that non-dividing cells possess many well-spaced nuclei throughout their length. Grula and Grula (89) questioned the need of intact DNA or DNA synthesis for cell division in Erwinia sp. Daniel and Schultze (45) reported that S-(1,2-dichlorovinyl)-L-cysteine, which inhibits cell division in E. coli , did not greatly affect the

synthesis of RNA, DNA, or protein.

These observations clearly show that cell division and nuclear division (DNA synthesis) can be separated into two distinct cellular events. DNA synthesis is necessary to insure genetic continuity within the new daughter cell but is not a sufficient condition to insure that cell division will follow. That is, synthesis of RNA, DNA, and protein can occur in what appears to be a normal manner in these cells, yet they do not divide.

Amino acid content of division-inhibited cells.

Although no quantitative differences in the protein content of division-inhibited cells could be detected, differences could exist in amino acid composition. The pronounced effect of D-serine on the uptake of glycine, alanine, and glutamic acid might be reflected in the molar ratios of amino acids found in protein hydrolyzates. Dickie and Schultze (54) reported that S-(1,2-dichlorovinyl)-L-cysteine changed the amino acid content of protein in E. coli and also inhibited the uptake of phenylalanine and glycine (53). Protein of inhibited cells contained less phenylalanine and lysine and more proline, serine, and threonine.

The Redfield system (194) for paper chromatography of amino acids did not provide adequate separation of valine-methionine and leucine-isoleucine to allow for quantitation of the individual amino acids. Therefore, the figures reported in Table III represent the sum of valine plus methionine and leucine plus isoleucine. Also, since amino acids were quantitated after hydrolysis of cellular protein in 6 N hydrochloric acid at 100 C for 18 hours, the figures represent only the amino acids not destroyed by such hydrolytic procedures. Tryptophan and

cysteine are completely destroyed by acid hydrolysis whereas serine and threonine are only partially destroyed (74).

No significant differences in the molar ratios of protein amino acids could be detected in D-serine-inhibited and non-inhibited cells (Table III). Thus, division-inhibited cells synthesize protein similar both qualitatively and quantitatively to non-inhibited cells. However, properties of these proteins such as conformation and function remain to be studied.

Leakage experiments.

Grula and Grula (92) reported that extensive cellular leakage accompanied cell division inhibition. In particular, D-serine induced the accumulation of keto acids, principally pyruvic acid, while penicillin also caused the leakage of pantothenic acid.

Leakage of cellular materials induced by D-serine is illustrated in Figure 6. Cells were grown in basal medium with and without D-serine. Cells were removed by centrifugation and the absorbancy of the medium at 260 and 280 mμ measured using a Beckman model DU spectrophotometer. It is recognized that centrifugation probably did not remove all cells from the medium. However, since large differences were noted and since filamentous cells appeared to sediment faster than normal cells although they did not pack as tightly as normal cells, it is thought that experimental error due to incomplete removal of the cells is negligible. Milligrams of nucleic acid per ml were calculated using a nomogram designed by E. Adams and distributed by California Corporation for Biochemical Research, Los Angeles. Leakage was detectable soon after the cells entered the exponential growth phase. These data are significant only in that

TABLE III

AMINO ACID CONTENT OF DIVISION-INHIBITED CELLS

Amino acid*	Molar ratio based on glycine** Control cells	Inhibited cells[a]	Average deviation
alanine	1.16	1.18	0.11
arginine	0.63	0.71	0.08
aspartic acid	1.18	1.27	0.09
glutamic acid	0.94	0.94	0.07
glycine	1.00	1.00	0.0
leucine-isoleucine	1.12	1.06	0.07
lysine	0.33	0.38	0.04
phenylalanine	0.26	0.30	0.04
serine	0.40	0.44	0.05
threonine	0.25	0.25	0.03
tyrosine	0.24	0.25	0.05
valine-methionine	0.75	0.75	0.04

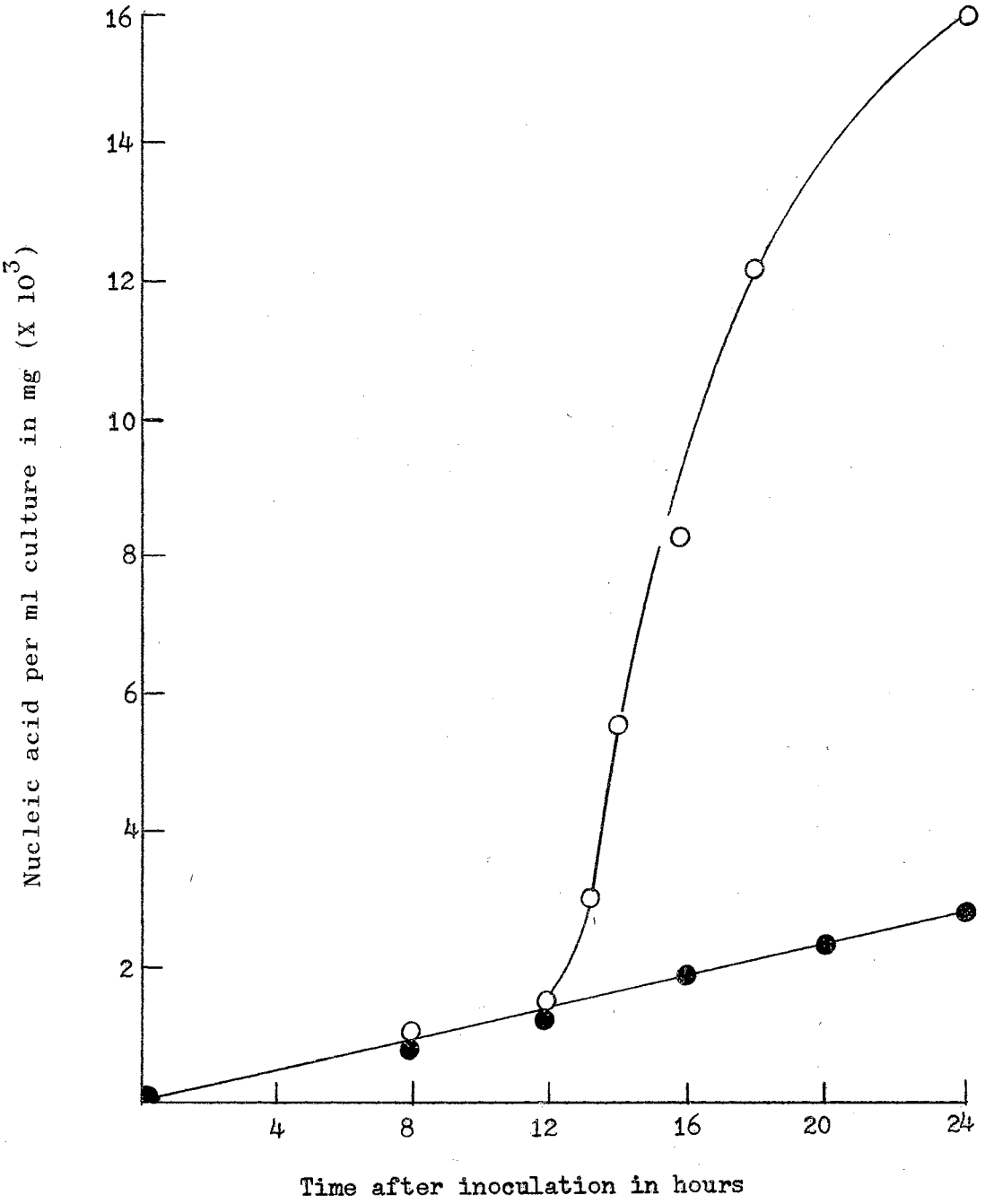
* Histidine, proline, tryptophan, and cysteine were not detectable.

** Figures represent the average of at least 12 experiments.

[a] Cells were grown in basal medium containing 0.031 M D-serine.

Figure 6

Leakage of nucleic acids from cells inhibited from dividing by D-serine. ● control; ○ D-serine-inhibited.


16
14
12
10
8
6
4
2
Nucleic acid per ml culture in mg (X 10^3)
4
8
12
16
20
24
Time after inoculation in hours

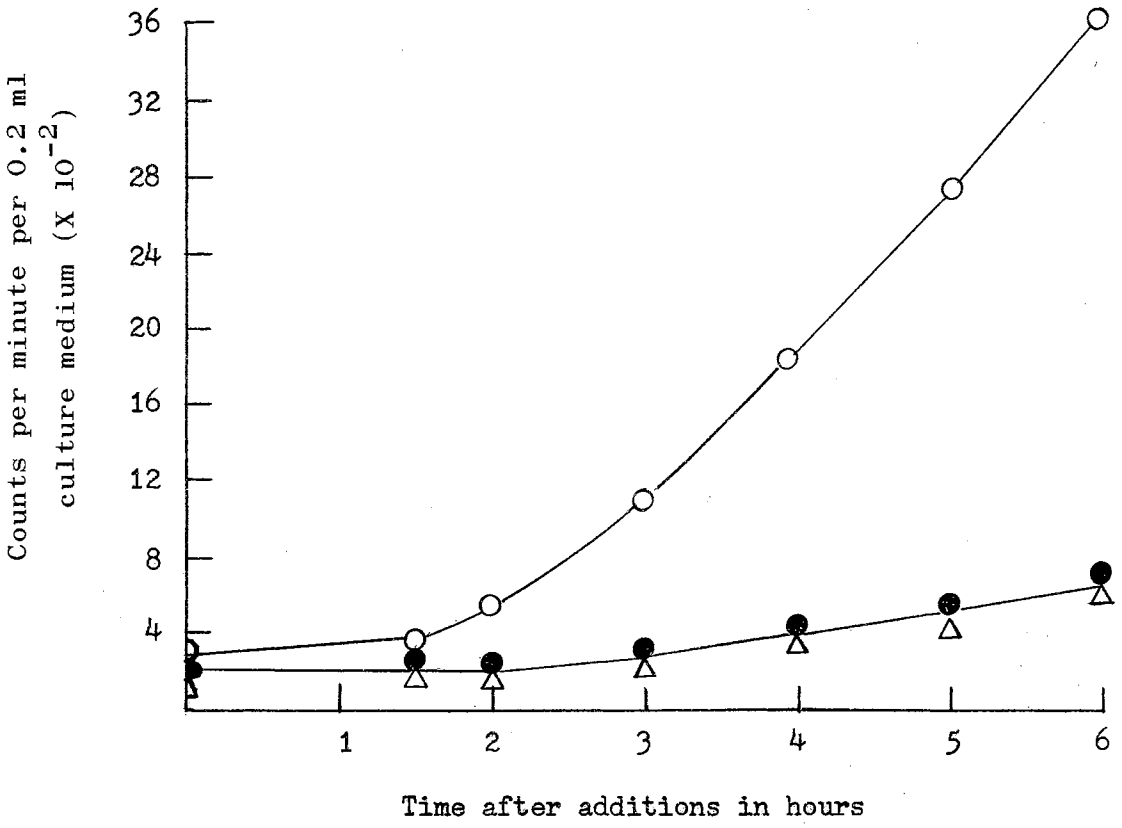
they show leakage of material which absorbs predominantly at 260 mμ. Although data are calculated as mg nucleic acid, they should not be considered as being a quantitative measure of RNA and/or DNA.

Release of cellular materials was also demonstrated using cells prelabeled by growth in the presence of D-glucose-U-C^{14} (Figure 7). D-serine induced leakage 1.5 to 3 hours after its addition. Also, division inhibition became evident based on morphological observations at about the same time. Addition of pantoyl lactone at the time of addition of D-serine completely prevented D-serine-induced leakage. Similar results were obtained if cells were prelabeled by either DL-aspartic acid-4-C^{14} or uracil-2-C^{14}. However, leakage could not be demonstrated from cells prelabeled using thymidine-2-C^{14}. A specific activity of approximately 50,000 counts per minute per mg dry weight of cells was obtained using uracil-2-C^{14} while 3,000 was the maximum obtainable using thymidine-2-C^{14}. A specific activity of about 300 was the highest obtained after 11 hours growth in the presence of thymine-2-C^{14}. Therefore, thymidine was taken into the cell much more readily than was thymine although not as efficiently as was uracil. The amount of thymidine-2-C^{14} taken into the cell, however, should have been sufficient to demonstrate leakage. Fractionation of cells revealed that approximately 95% of the radioactivity from uracil-2-C^{14} and 84% from thymidine-2-C^{14} was extracted by 5% trichloroacetic acid at 100 C for 30 minutes.

These data show that D-serine induces the leakage of uracil-containing compounds but not thymine-containing compounds. This may be due, in part, to either a stimulation of synthesis of uracil-containing compounds or their breakdown coupled with damage to the permeability barrier (cell membrane). Damage to the cell membrane is

Figure 7

Leakage of cellular material from cells prelabeled by growth in the presence of D-glucose-U-C^{14}. ● control; ○ D-serine added; △ D-serine plus pantoyl lactone added.


Counts per minute per 0.2 ml culture medium (X 10^{-2})
36
32
28
24
20
16
12
8
4
1
2
3
4
5
6
Time after additions in hours

occurring but is subtle in that not all compounds are released.

D-serine induced the leakage of uracil-containing compounds into the medium yet did not alter the normal cellular content of RNA. There could, therefore, be a stimulated synthesis of uracil compounds induced by D-serine. However, these data could also be due to loss of uracil-containing compounds from cellular pools.

These data are not the result of cell lysis induced by D-serine. Cellular lysis could not be detected by following the absorbancy at 540 mμ or by observations using a phase contrast microscope. Also, the lack of release of thymidine into the medium demonstrates that a generalized cellular lysis did not occur.

Accumulation of charcoal-adsorbable material

Accumulation of radioactivity from cells prelabeled with uracil-2-C^{14} may be due to release of the uridine nucleotide precursors of mucopeptide synthesis. To test this possibility, these materials were isolated by adsorption onto charcoal. Five UV-quenching compounds were separated using the solvent system of Strominger (257). These compounds were not found in media from control cells and were effectively diminished by addition of pantoyl lactone. The Rf values of these compounds are presented in Table IV. In general, these values are intermediate between those of free bases and the various nucleotides. Only compound B migrated similarly to any of the known mucopeptide precursors. Compound B had an Rf value similar to those of UDP-muramyl-alanyl-glutamyl-lysine and UDP-muramyl-alanyl-glutamate. Ninhydrin-positive material, however, could not be detected either before or after acid hydrolysis in 6 N hydrochloric acid at 100 C for 18 hours. Apparently B is not

TABLE IV

Rf VALUES OF VARIOUS COMPOUNDS IN THE SOLVENT SYSTEM OF STROMINGER (257)

Compound	Solvent 1*	Solvent 2**
A	0.40	0.34
B	0.36	0.50
C	0.50	0.27
D	0.53	0.42
E	0.57	0.66
xanthosine	0.42	0.35
uridine	0.52	0.65
uracil	0.62	0.65
UDP	0.20	0.05
UMP	0.31	0.12
cytosine	0.82	0.58
cytidine	0.65	0.55
CMP	0.49	0.09
CDP	0.31	0.04
dCMP	0.52	0.12
adenine	0.87	0.61
AMP	0.50	0.08
ADP	0.40	0.02
ATP	0.30	0.04
d-adenosine	0.84	0.59
d-AMP	0.60	0.12
thymine	0.72	0.68

TABLE IV (Continued)

Compound	Solvent 1*	Solvent 2**
guanosine	0.55	0.51
orotic acid	0.33	0.40
UDP-N-acetyl-glucosamine[a]	0.35	0.68
UDP-N-acetyl-glucosamine-pyruvate[a]	0.32	0.66
UDP-muramyl-alanine[a]	0.37	0.75
UDP-muramyl-alanyl-glutamate[a]	0.33	0.58
UDP-muramyl-alanyl-glutamyl-lysine[a]	0.32	0.53
UDP-muramyl-alanyl-glutamyl-lysyl-alanyl-alanine[a]	0.42	0.63

* Isobutyric acid:0.5 N ammonium hydroxide (5:3).

** Ethanol:1 M ammonium acetate, pH 7.0 (7.5:3).

[a] Rf values calculated from data of Ito and Strominger (109).

identical to these mucopeptide precursors.

Compound A migrated similarly to xanthosine while D corresponded most nearly to guanosine and E to uridine or uracil. None of these compounds reacted with ninhydrin. Identification of A as xanthosine and E as uridine was supported by cochromatography with known compounds.

Paper chromatograms were dipped in a solution containing 2,4-dinitrophenylhydrazine (1 mg/ml) in 3 N hydrochloric acid. After hanging for 3 minutes, the chromatograms were washed thoroughly with distilled water and examined under UV light. Only one zone which was yellow in visible light and quenched UV light was detectable. Compounds which contain a functional carbonyl group react with hydrazines to form hydrazones. Uracil, 2,6-diketopyrimidine, therefore, should form a hydrazone derivative. The reactive zone corresponded to compound E (uridine).

After either acid or alkaline hydrolysis, compounds C, D, and E were lost. Compounds A and B remained unchanged.

A solution containing the five unknown compounds was chromatographed on a column of Dowex 50 according to the procedure described by Katz and Comb (121). Dowex 50 X2 (100-200 mesh) was washed with 3 N hydrochloric acid and then with distilled water until neutral. A column 1.2 x 8.0 cm was packed in water and washed with 20 ml of 0.05 N hydrochloric acid. The sample was made to 0.05 N with hydrochloric acid and applied to the column. Elution was carried out with 6.0 ml of 0.05 N hydrochloric acid followed by 25 ml distilled water. Sixty-drop fractions were collected using a Gilson automatic fraction collector equipped with a drop counter. Each fraction was adjusted to 0.05 N with hydrochloric acid and the absorbancy at 250, 260, and 280 mμ measured using a Beckman model DU spectrophotometer.

Two major and two minor peaks were observed (Figure 8). The 250/260 and 280/260 ratios of the material in the peak tube of each of these areas are presented in Table V. These values do not correspond to any previously published probably due to the fact that these fractions still contain several compounds and are not pure solutions.

Chromatography of each fraction revealed that compound E eluted from the column in tubes 3 to 5 while compounds A and B eluted in tubes 7 to 9. Compounds C and D could not be recovered. A new compound was found in tubes 3 to 5 which had an Rf identical to UDP. Katz and Comb (121) reported that uracil-containing compounds eluted from the column as one peak which corresponds to that found in tubes 3 to 5.

The contents of tubes 3 to 5 were pooled and hydrazone derivatives prepared (226). Two water soluble compounds were separated after paper chromatography in n-butanol:0.5 N ammonium hydroxide:ethanol (70:20:10) with Rf values of 0.43 and 0.91. Previously reported Rf values for known compounds in this solvent are presented in Table VI. The compound with Rf of 0.43 corresponds closely with that of 0.46 reported for the hydrazone derivative of pyruvic acid. The hydrazone derivative of known pyruvic acid was prepared. This compound had the same Rf and cochromatographed with the derivative of the unknown compound. However, the pyruvate hydrazone characteristically forms a dark brown color when sprayed with a 10% solution of sodium hydroxide. The unknown derivative did not turn brown indicating that although it migrates similar to the pyruvate hydrazone, they are not the same compound.

The unknown hydrazone with an Rf of 0.91 was present in such a small quantity that further identification was not possible.

Thus, D-serine induced the accumulation of at least five UV-quenching

Figure 8

Elution pattern of ultraviolet absorbing material from Dowex 50 columns. ● absorbancy at 250 mμ; ○ absorbancy at 260 mμ; △ absorbancy at 280 mμ.

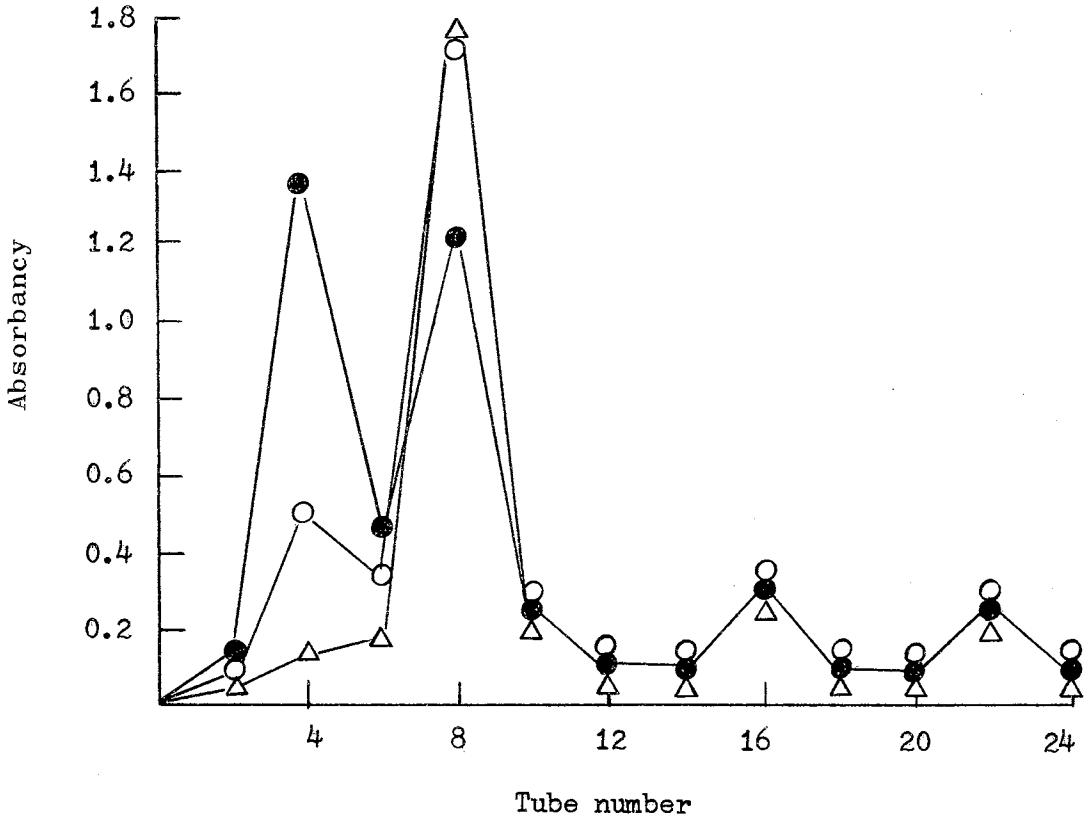

1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
Absorbancy
4
8
12
16
20
24
Tube number

TABLE V

250/260 AND 280/260 RATIOS OF FRACTIONS
ELUTED FROM DOWEX 50 COLUMNS

Fraction eluted in tubes	250/260*	280/260*
3-5	2.9	0.23
7-9	0.695	1.03
16	1.0	0.825
21	1.0	1.42

* Solvent was 0.05 N hydrochloric acid.

TABLE VI

Rf VALUES OF HYDRAZONE DERIVATIVES

Parent compound	Rf of hydrazone*
oxalsuccinic acid	0.09
alpha-ketoglutaric acid	0.19
oxalacetic acid	0.24
mesoxylic acid	0.34
glyoxylic acid	0.30 (0.51)**
pyruvic acid	0.46 (0.60)**
alpha-acetolactic acid	0.64
beta-mercaptopyruvic acid	0.40
alpha-ketobutyric acid	0.66
alpha-ketoisovaleric acid	0.73
alpha-ketovaleric acid	0.74
hydroxypyruvic acid	0.40
acetoacetic acid	0.54
beta-ketovaleric acid	0.58
beta-ketocaproic acid	0.61
beta-ketocaprylic acid	0.70
p-hydroxyphenylpyruvic acid	0.70
beta-ketocapric acid	0.77
beta-hydroxyphenylpyruvic acid	0.76

TABLE VI (Continued)

Parent compound	Rf of hydrazone*
phenylpyruvic acid	0.74 (0.80)**
acetone	0.95

* Data of Bassett and Harper (13); solvent was n-butanol:0.5 ammonium hydroxide:ethanol (70:20:10).

** Second figure represents Rf of second spot often found with these compounds.

materials which adsorb onto charcoal. Two of these have tentatively been identified as xanthosine and uridine; however, the identity of the others remains unknown. None appeared to be identical to the uridine nucleotides described by Ito and Strominger (109). Grula and Grula (91) have shown that D-serine inhibits mucopeptide synthesis in Erwinia sp. This being true, the precursors of mucopeptide synthesis should have been found in the growth medium. Also, the occurrence of uracil compounds was not unexpected since leakage of uracil-C^{14} has been demonstrated.

These compounds are associated with the division inhibition process since they were not found in media from normally dividing cells. Also, their accumulation was effectively prevented by addition of pantoyl lactone.

Leakage of nucleic acids and/or bases from inhibited cells may indicate that the free nucleotide levels in the cell are altered. This could be important in view of the theories proposed by Commoner (31) that the relative concentration of free nucleotides in the cell may serve as regulatory components in determining metabolic pathways functional in the cell.

Accumulation of radioactive sulfur compounds.

Cook and Hess (33) reported the presence of sulfur-containing nucleotides which were associated with cell division in synchronized cultures of Euglena gracilis. Since several of the unknown compounds which accumulate in the growth medium of D-serine-inhibited Erwinia did not appear identical to any of the common purine- and pyrimidine-containing compounds, they could be similar to the sulfur compounds

discussed by Cook and Hess.

To determine if such a similarity existed, the medium was examined for charcoal adsorbing compounds which contained sulfur. Cells were grown in 100 ml basal medium containing 50 μc of S^{35}-sodium sulfate with and without D-serine. The magnesium sulfate levels in the basal medium were lowered to 18 μg/ml (60% of normal) to decrease dilution of the isotope by non-labeled sulfate. After growth for 12 hours, cells were removed by centrifugation and charcoal adsorbable material isolated as described in Materials and Methods. The material was chromatographed ascending in one direction using both solvents of Strominger (257). Chromatograms were examined under UV light and by using a chromatogram scanner to detect radioactive zones. Two radioactive areas were found in both solvents. In solvent one these had Rf values of 0.32 and 0.61; in solvent two the Rf values were 0.19 and 0.54. Ultraviolet-quenching material was detected with an Rf of 0.32 in solvent one and 0.59 in solvent two. Thus, there appeared to be at least one compound with an Rf of approximately 0.32 in solvent one and 0.59 in solvent two which contained sulfur and quenched UV light. However, it must be remembered that detection of radioactivity is more sensitive than detection by UV quenching. The Rf values of this one compound most nearly matched those of compound B (0.36 in solvent one and 0.50 in solvent two). However, the radioactive compound was not identical to compound B based on the following observations. Accumulation of the S^{35}-containing material was not induced by D-serine whereas compound B accumulated in the growth medium only in the presence of D-serine. Similar radioactive material was found in media from control cells and from cells grown with D-serine and pantoyl lactone. The majority of the radioactive material

eluted from a Dowex 50 column in fraction 3 to 5 while compound B eluted in fraction 8 (Figure 9). Thus, the radioactive compound behaved like uracil-containing compounds when chromatographed on Dowex 50 columns. Also, examination of radioactive material by radioautography after two-dimensional chromatography both in the systems of Strominger (257) and Redfield (194) failed to demonstrate a similarity between the UV-quenching compounds and the sulfur materials. In fact, chromatography in the Redfield system separated the sulfur compounds into at least five spots (Table VII). Thus, instead of aiding in the identification of the previous compounds, five more compounds, all of which contain sulfur and do not react with ninhydrin, are added to the list of materials found in the spent growth medium. However, in contrast to the previous compounds, accumulation of sulfur materials in the growth medium did not depend on the presence of D-serine and was not a result of division inhibition. Therefore, the sulfur-containing nucleotides of Cook and Hess (33) do not appear to be important in the Erwinia system.

Intracellular sulfur-containing compounds.

Cells which had been grown in S^{35}-sodium sulfate-containing medium to study the accumulation of sulfur compounds were examined for intracellular differences. Grula and Grula (92) have previously reported that D-serine lowers the intracellular concentration of Co A.

After extraction with boiling water for 20 minutes, the cells were hydrolyzed in 6 N hydrochloric acid at 100 C for 18 hours in sealed and evacuated tubes. The hydrolyzates were chromatographed in the system of Redfield (194) and examined by radioautography. Seven distinct radioactive areas (compounds 6 to 12) were found (Table VIII). None quenched

Figure 9

Elution of S^{35}-containing materials from Dowex 50 columns.

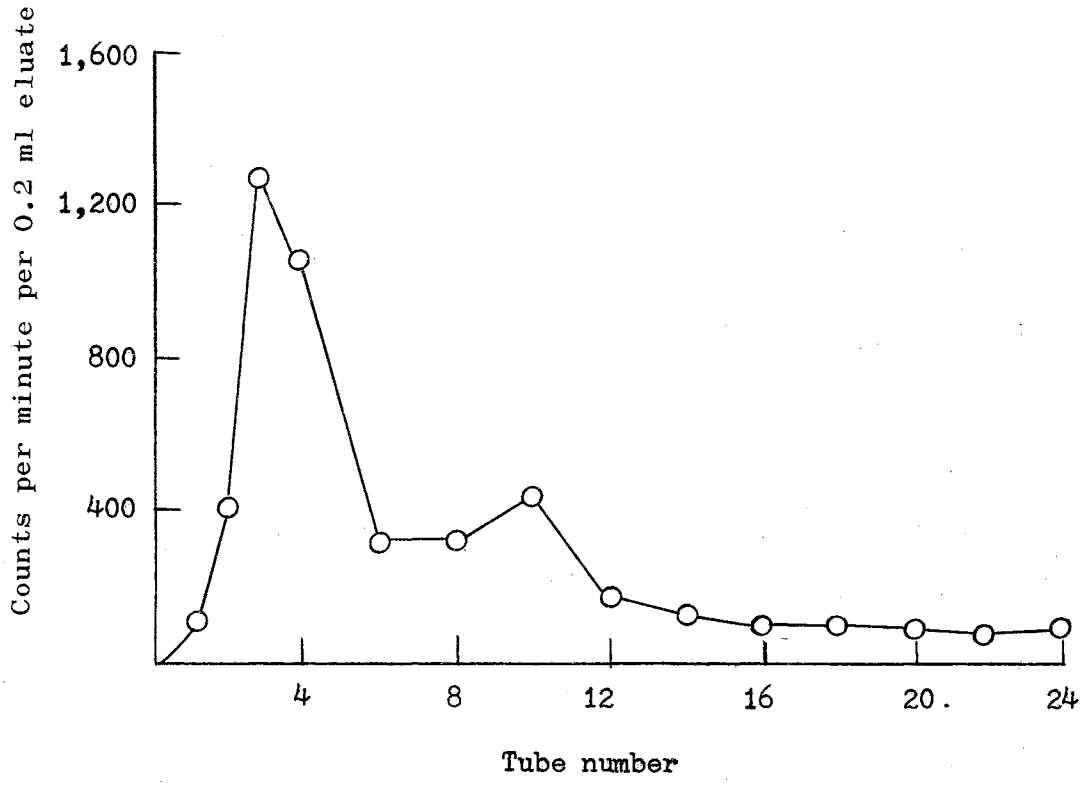

Counts per minute per 0.2 ml eluate
1,600
1,200
800
400
4
8
12
16
20
24
Tube number

TABLE VII

Rf VALUES OF S^{35}-LABELED MATERIALS
IN REDFIELD SOLVENTS (194)

Compound	Solvent 1*	Solvent 2**
1	0.18	0.20
2	0.27	0.11
3	0.39	0.28
4	0.73	0.37
5	0.63	0.44

* Methanol:water:pyridine (80:20:4).

** t-butanol:methyl ethyl ketone:water:diethylamine (40:40:20:4).

TABLE VIII

Rf VALUES OF INTRACELLULAR S^{35}-LABELED COMPOUNDS*

Compound	Solvent 1[a]	Solvent 2[b]
6	0.17	0.04
7	0.17	0.08
8	0.47	0.13
9	0.41	0.16
10	0.48	0.23
11	0.79	0.15
12	0.78	0.58

* Material was hydrolyzed in 6 N hydrochloric acid at 100 C for 18 hours.

[a] Methanol:water:pyridine (80:20:4).

[b] t-butanol:methyl ethyl ketone:water:diethylamine (40:40:20:4).

UV light or reacted with ninhydrin.

Of the seven, spots 6, 7, and 11 were found in the same proportion in media from either control cells, D-serine-inhibited cells, or cells inhibited by D-serine but reversed by pantoyl lactone. Material in spots 9 and 12 was increased by D-serine while that in spot 10 was decreased. These materials were returned to normal levels by addition of pantoyl lactone. More significant, however, is the observation that material in spot 8 was present only in inhibited cells. It was found neither in control cells nor in cells sparked to divide by pantoyl lactone.

It was possible that these seven compounds represented breakdown products of acid hydrolysis and did not exist in the forms studied here in vivo. Cells were hydrolyzed in 2 N hydrochloric acid for 2 hours at 100 C and examined by radioautography. Eight radioactive spots were found, seven of which differed from any previously found (Table IX). Only spot 11 was found to be unaltered by the harsher hydrolysis procedure. Spots 13, 14, 16, 17, and 18 did not move in solvent two. Accurate Rf values for these areas could not be obtained due to extensive trailing and overlapping. This also made it difficult to discern differences in the relative amounts of these compounds. However, spots 13, 14, 15, and 17 did appear to be increased by D-serine. These were returned to a normal level by pantoyl lactone.

Identification of these materials was attempted using the chromatographic system of Roberts, Cowie, Abelson, Bolton, and Britten (199). Rf values of known compounds are presented in Table X. Values obtained by radioautography of the S^{35}-containing materials are presented in Tables XI and XII. Although several compounds appear similar to cystine, lan-

TABLE IX

Rf VALUES OF INTRACELLULAR S^{35}-LABELED COMPOUNDS*

Compound	Solvent 1[a]	Solvent 2[b]
11	0.74	0.15
13	0.44	0.0
14	0.57	0.0
15	0.50	0.16
16	0.69	0.0
17	0.80	0.0
18	0.85	0.0
19	0.68	0.41

* Material was hydrolyzed in 2 N hydrochloric acid at 100 C for 2 hours.

[a] Methanol:water:pyridine (80:20:4).

[b] t-butanol:methyl ethyl ketone:water:diethylamine (40:40:20:4).

TABLE X

Rf VALUES OF KNOWN SULFUR-CONTAINING COMPOUNDS*

Compound	Solvent 1[a]	Solvent 2[b]
glutathione (-SO_3H)	0.09	0.26
cystine	0.12	0.21
lanthionine	0.12	0.21
S-methyl cysteine	0.14	0.28
cystathionine	0.14	0.28
glutathione (-S-S-)	0.23	0.41
glutathione (-SH)	0.60	0.79
homocysteine	0.28	0.50
cysteic acid	0.40	0.53
taurine	0.45	0.46
S-acetyl glutathione	0.70	0.87
methionine	0.84	0.88

* Rf values calculated from data of Roberts et al. (199).

[a] t-butanol:hydrochloric acid:water (70:6.7:23.3).

[b] Isopropanol:water:formic acid (70:20:10).

TABLE XI

Rf VALUES OF INTRACELLULAR S^{35}-LABELED COMPOUNDS*

Compound	Solvent 1[a]	Solvent 2[b]
1a	0.14	0.11
2a	0.22	0.19
3a	0.28	0.31
4a**	0.38	0.18
5a	0.41	0.37
6a	0.47	0.27
7a	0.51	0.49
8a	0.67	0.59
9a**	0.89	0.70
10a**	0.97	0.65

* Material was hydrolyzed in 6 N hydrochloric acid at 100 C for 18 hours.

[a] t-butanol:hydrochloric acid:water (70:6.7:23.3).

[b] Isopropanol:water:formic acid (70:20:10).

** Material in spots 4a and 10a was increased 2 to 3 fold by D-serine while material in spot 9a was found only in cells inhibited by D-serine. All alterations were reversed by addition of pantoyl lactone.

TABLE XII

Rf VALUES OF INTRACELLULAR S^{35}-LABELED COMPOUNDS*

Compound	Solvent 1[a]	Solvent 2[b]
1b	0.24	0.27
2b	0.47	0.27
3b	0.47	0.42
4b	0.69	0.30
5b	0.76	0.42
6b	0.78	0.60
7b	0.83	0.49

* Material was hydrolyzed in 2 N hydrochloric acid at 100 C for 2 hours.

[a] t-butanol:hydrochloric acid:water (70:6.7:23.3).

[b] Isopropanol:water:formic acid (70:20:10).

thionine, and taurine, none of the materials whose relative concentrations were affected by D-serine could be identified.

Thus, D-serine induced the synthesis of a new intracellular sulfur-containing compound and changed the concentration ratios of at least two others. These apparently existed in the cell as more complex molecules and were broken down by acid hydrolysis. These alterations appear to be associated with cell division since all were prevented by pantoyl lactone. D-serine may, in some way, cause the synthesis of these abnormal sulfur-containing compounds, which in turn function to inhibit cell division. This could be due to changes in the oxidation-reduction potential of the cell. Studies are underway at present to identify these compounds and to determine their significance in the division process.

Incorporation of C^{14}-diaminopimelic acid in division-inhibited cells.

Grula and Grula (89) have suggested that mucopeptide synthesis may be important in cell division. In fact, cell division appears to require at least some mucopeptide synthesis. An attempt, therefore, was made to determine if division inhibitors also inhibited mucopeptide synthesis in Erwinia sp. Since this organism contains DAP instead of lysine in its mucopeptide (Grula and Grula, unpublished data), the incorporation of DAP-2-C^{14} into the hot trichloroacetic acid insoluble fraction was used to study mucopeptide synthesis.

Cells were grown in basal medium containing the inhibitor plus DL-lysine (1.0 mg/ml). Lysine was added to inhibit the incorporation of lysine formed from C^{14}-DAP into protein. After 11 hours, cells were harvested, washed three times in mineral solution, and resuspended in fresh medium identical to that in which the cells had been grown except that

DAP-2-C^{14} (0.01 μc/ml) was added. Incubation was continued for 1 hour at 25 C on a Dubnoff shaking water bath at which time incorporation was stopped by addition of trichloroacetic acid to 5%. The cells were extracted with 5% trichloroacetic acid at 100 C for 30 minutes and the insoluble residue resuspended in 0.1 N sodium hydroxide. Aliquots were plated and counted.

As shown in Table XIII, addition of DL-lysine reduced to a great extent the incorporation of radioactivity from DAP-2-C^{14}. In the absence of lysine, no difference was detected in the amount of radioactivity incorporated into the cells inhibited by D-serine and non-inhibited cells. However, addition of lysine revealed that D-serine inhibited the incorporation of radioactivity approximately 37%. Pantoyl lactone completely prevented division inhibition and also prevented the inhibition of DAP incorporation by D-serine.

Thus, first observations suggest that D-serine inhibited mucopeptide synthesis and that this inhibition was related to division inhibition. However, further studies using mitomycin c, vancomycin, and penicillin to inhibit division revealed that incorporation of radioactivity from DAP-C^{14} was actually stimulated and not inhibited as with D-serine. This stimulation was probably due to damage to the cellular permeability barriers allowing more DAP to enter, thus, making more available for metabolism.

In order to determine if this stimulation represented an increase in DAP incorporation or a stimulation in DAP metabolism followed by incorporation of metabolic products, samples were hydrolyzed for amino acids and examined by radioautography. Two radioactive zones of approximately equal size were found in all cases. One corresponded to DAP,

TABLE XIII

INCORPORATION OF DIAMINOPIMELIC ACID-2-C^{14} IN ERWINIA SP

Additions*	Specific activity**	Percent change
none (no lysine)	8,440	-
D-serine (0.031 M; no lysine)	8,690	+ 2.8
none (control)	586	-
D-serine (0.031 M)	371	-37
D-serine + pantoyl lactone (0.056 M)	580	- 1
vancomycin (20 µg/ml)	962	+ 64
penicillin (50 units/ml)	693	+ 18
mitomycin c (0.2 µg/ml)	729	+ 24

* All situations contained DL-lysine (1.0 mg/ml) unless otherwise stated; additions were made at time of inoculation.

** Counts per minute per mg dry weight; DAP-2-C^{14} (0.01 µc/ml) was added to log phase cells and incubation continued for 1 hour; figures represent radioactivity incorporated into the protein-mucopeptide fraction.

the other to lysine.

Although the exogenous lysine greatly lowered the amount of DAP taken into the cell, it neither prevented the conversion of DAP to lysine nor effectively diluted the lysine after it had been formed. These data suggest that DAP is converted to lysine in a bound form and that this lysine is incorporated into protein in such a manner as to be protected from dilution by non-labeled exogenous lysine.

Since there appeared to be an equal amount of radioactive DAP and lysine in all cases, it is concluded that mitomycin c, vancomycin, and penicillin stimulated the uptake of DAP-C^{14}. It has been reported that 8-azaguanine has the same effect in B. cereus. Data presented previously have shown that vancomycin and mitomycin c stimulate the uptake of glycine-C^{14}. Therefore, these antibiotics appear to disturb the cellular permeability characteristics which would strongly indicate an effect on the cell membrane.

Several other workers have used the incorporation of DAP-C^{14} as a measure of mucopeptide synthesis (94, 190). In view of these data, however, it is concluded that incorporation of DAP-C^{14} is not a valid measure of mucopeptide synthesis in the absence of proper controls showing that the carbon of DAP is not further metabolized.

Although these data cannot be interpreted as showing inhibition of mucopeptide synthesis by D-serine, it is concluded that D-serine does inhibit mucopeptide synthesis in Erwinia sp based on data of Grula and Grula (91). Inhibition of mucopeptide synthesis has been separated from the cause of division inhibition since division inhibition but not inhibition of mucopeptide synthesis is prevented by pantoyl lactone or osmotic agents.

Fractionation experiments.

The effect of D-serine on the distribution of radioactivity from labeled carbon compounds into the various cellular fractions and the distribution of radioactivity from D-serine-C^{14} might indicate the site in the cell where D-serine is exerting its effects. To obtain data relevant to this point, cells were grown in the presence of a radioactive carbon source and fractionated as described in Materials and Methods. The percent of the total radioactivity taken into the cell which was incorporated into each of the major cellular fractions is shown in Table XIV.

Radioactivity from D-glucose-U-C^{14} and L-serine-3-C^{14} was distributed evenly among the cellular fractions. This was expected since L-serine is probably metabolized by conversion to glycolytic intermediates (73, 161, 250). The equal distribution of glucose carbon into the fractions does not indicate that the fractions were present in the cell in equal quantities. Some radioactivity was spared from protein synthesis by the presence of aspartic acid in the growth medium.

Addition of D-serine did not affect the distribution of radioactivity from L-serine-3-C^{14}. However, D-serine did induce a change in the distribution of glucose carbon. There was a significant drop in the percent incorporated into the protein-mucopeptide fraction with an increase in the lipid and nucleic acid fractions.

Using L-aspartic acid-U-C^{14}, D-serine also increased the percent incorporated into the lipid fraction while decreasing that recovered in the protein-mucopeptide fraction. There was, however, no significant change in the amount taken into the nucleic acid fraction.

Using sodium acetate-2-C^{14}, a decrease in both the nucleic acid and

TABLE XIV

DISTRIBUTION OF RADIOACTIVITY INTO CELLULAR FRACTIONS

Radioactive compound*	Other additions**	Percent of radioactivity in lipid	nucleic acid	protein
D-glucose-U-C^{14}	none	36	34	30
	D-serine	39	42	19
	D-serine + pantoyl lactone	38	38	24
L-aspartic acid-U-C^{14}	none	36	17	47
	D-serine	43	16	40
	D-serine + pantoyl lactone	40	17	43
sodium acetate-2-C^{14}	none	53	16	31
	D-serine	78	9	13
	D-serine + pantoyl lactone	65	15	20
D-serine-3-C^{14}	none	45	17	39
	D-serine	76	5	20
	D-serine + pantoyl lactone	60	11	29
	L-serine	47	16	37
L-serine-3-C^{14}	none	32	34	34
	L-serine	34	35	31
	D-serine	30	33	37

* Radioactive compound added to 0.01 μc/ml at time of inoculation.

** D- and L-serine (0.031 M) and pantoyl lactone (0.056 M) added at time of inoculation.

the protein-mucopeptide fractions were observed. There also was a great increase in the percent incorporated into the lipid fraction.

These data indicate that D-serine has altered carbon metabolism. In general, this alteration involves an increased incorporation into lipoidal material and a decrease into the protein-mucopeptide fraction. Pantoyl lactone allowed for a partial restoration to normal patterns in every case.

Radioactivity from non-inhibitory levels of D-serine-3-C^{14} was recovered mainly in the lipid and protein-mucopeptide fractions. These data support previous findings in the rat that D-serine is metabolized differently than L-serine (73, 161). Most significant, however, is the observation that addition of carrier D-serine to inhibitory concentrations changed the distribution of D-serine-3-C^{14}. The majority of radioactivity was shifted into the lipid fraction. This response was specific for D-serine since D-serine did not alter the distribution of L-serine-3-C^{14} nor did non-labeled L-serine alter the distribution of D-serine-3-C^{14}. Once again, pantoyl lactone partially prevented this shift into the lipid fraction. It was further noticed that pantoyl lactone stimulated the metabolism of D-serine (Table XV). Addition of pantoyl lactone increased the specific activity of whole washed cells approximately four-fold. Pantoyl lactone may cause D-serine to be metabolized at a rate fast enough to prevent D-serine from exerting its toxic effects. Pantoyl lactone could be stimulating or inducing the activity of serine racemase and allowing the conversion of D-serine to the L-isomer. If this were happening, D-serine would be oxidized similar to L-serine in the presence of pantoyl lactone in manometric experiments. Cells were grown in basal medium, washed, and tested for the ability to oxidize

TABLE XV

INCORPORATION OF D-SERINE-3-C^{14}

Additions*	Specific activity**
none	15,760
D-serine (0.031 M)	5,718
D-serine (0.031 M) + pantoyl lactone (0.056 M)	19,235

* Additions were made to basal medium containing 0.05 μc D-serine-3-C^{14} per ml at non-inhibitory concentrations at time of inoculation. Incubation was continued for 12 hours at 25 C on a Dubnoff Metabolic Shaking Incubator.

** Counts per minute per mg dry weight.

D- and L-serine. As shown in Figure 10, L-serine was rapidly oxidized by whole cells; however, D-serine was oxidized at only about 9% the rate of L-serine. Oxidation of D-serine was not stimulated by addition of pantoyl lactone. It can be calculated from data in Table I that D-serine-C^{14} was metabolized at approximately 17% the rate of L-serine-C^{14}. This demonstrates that the low rate of oxidation of D-serine may not be due to a slow rate of uptake into the cell. Pantoyl lactone, therefore, does not stimulate the metabolism of D-serine by stimulating the activity of serine racemase or by causing the conversion of D-serine to L-serine.

Data in Table XV further show that addition of carrier D-serine, which decreased the isotopic specific activity of the growth medium by a factor of 1,000 , only decreased the specific activity of whole cells by a factor of 3. This may be due to damage to the cell membrane induced by the higher levels of D-serine allowing more D-serine to be taken into the cell and metabolized.

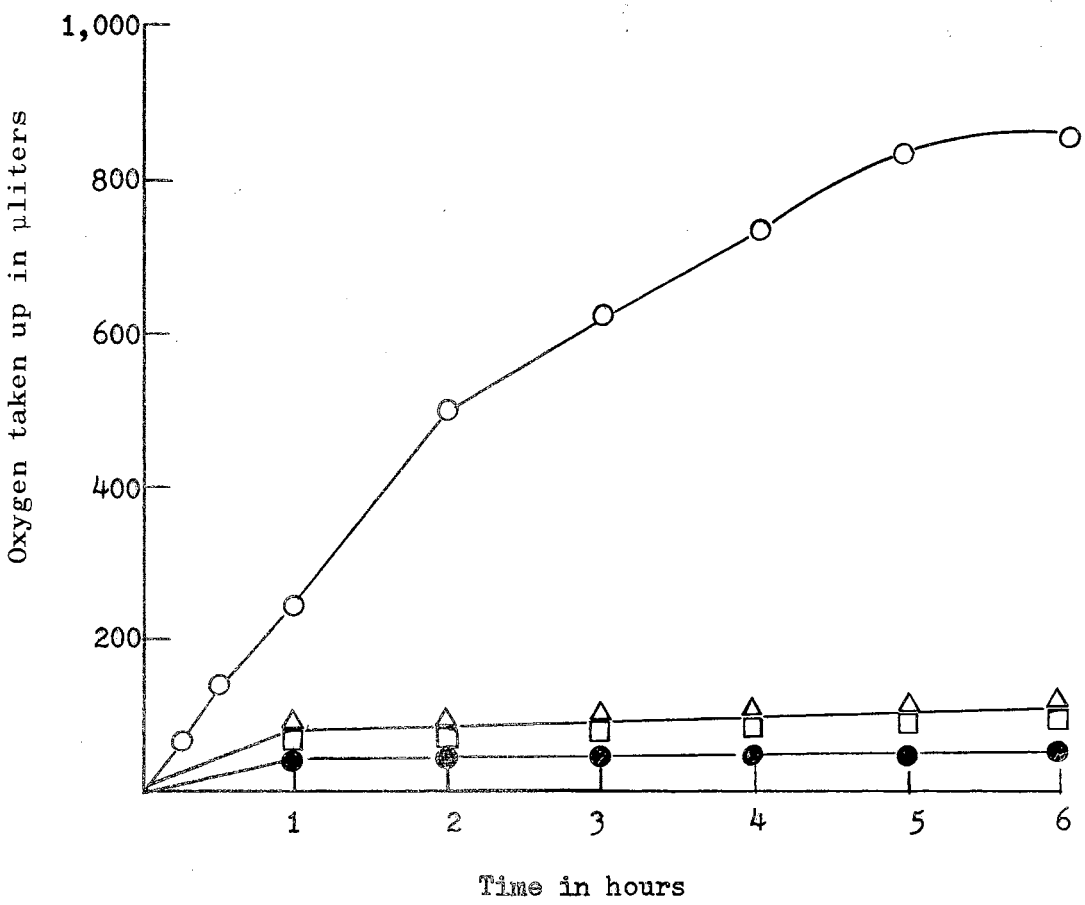
Incorporation of D-serine-3-C^{14} into lipids.

The increased incorporation of D-serine-3-C^{14} into lipids induced by inhibitory concentrations of D-serine could be due to stimulation of incorporation of D-serine or some product of D-serine metabolism. Also, there could be a shift in biosynthetic properties of inhibited cells so that more lipid is being made in proportion to other cellular constituents or an inhibition of incorporation of radioactivity from D-serine-3-C^{14} into protein.

To test the hypothesis that high levels of D-serine induce the incorporation of D-serine per se into lipids, lipids extracted from inhi-

Figure 10

Oxidation of D- and L-serine. ○ L-serine (9.4 μmoles); △ D-serine (9.4 μmoles); □ D-serine (9.4 μmoles) plus pantoyl lactone (9.4 μmoles); ● endogenous. Cells grown in basal medium. Concentrations given as μmoles per flask. Volume in Warburg flask was 2.4 ml.


1,000
800
600
400
200
Oxygen taken up in μliters
1
2
3
4
5
6
Time in hours

bited cells were examined for the presence of D-serine. Cells were grown in basal medium containing 0.05 μc D-serine-3-C^{14} per ml with and without inhibitory levels of carrier D-serine. Lipids were extracted and hydrolyzed for amino acids. After chromatography in the two-dimensional system of Redfield (194), the hydrolyzates were examined by radioautography. Only one major radioactive spot was found with an Rf of 0.69 in solvent one and 0.56 in solvent two. In this system, serine has Rf values of 0.37 in solvent one and 0.28 in solvent two. Thus, radioactivity from D-serine-3-C^{14} was not incorporated into lipids as serine and must have been incorporated as some metabolic product.

Since phosphatidylserine is the direct precursor of phosphatidylethanolamine, the radioactive spot could be ethanolamine. Commercial samples of phosphatidylethanolamine were hydrolyzed and chromatographed. Ethanolamine was found to have Rf values of 0.67 in solvent one and 0.34 in solvent two. Thus, the radioactive material was not ethanolamine and it can be concluded that D-serine does not serve as a substrate for phosphatidylethanolamine biosynthesis in Erwinia sp. Kanfer and Kennedy (120) found similar results in E. coli although they had not studied phosphatidylethanolamine biosynthesis at inhibitory levels of D-serine. The ethanolamine moiety of phosphatidylethanolamine must arise, therefore, from endogenously synthesized L-serine. These data possess further significance in that they indicate that D-serine does not dilute or interfere with incorporation of L-serine into phospholipid.

Since serine is metabolized through carbohydrate pathways, the radioactive material could be glycerol. Glycerol-2-C^{14} was found to have Rf values of 0.68 in solvent one and 0.51 in solvent two. Therefore, radioactivity from D-serine-3-C^{14} is incorporated into lipid as

glycerol-C^{14}. This was supported by cochromatography of the unknown radioactive compound and known glycerol-2-C^{14}. Apparently, inhibitory levels of D-serine stimulate the conversion of D-serine-C^{14} to glycerol. However, since D-serine also stimulates incorporation of radioactivity from glucose, aspartic acid, and acetate into lipids, the increased conversion of D-serine-C^{14} to glycerol-C^{14} may be coupled with an increased rate of lipid synthesis.

Lipid content of division-inhibited cells.

To determine if cells inhibited from dividing contained larger amounts of phospholipid, phosphorus assays were performed on lipid fractions extracted from inhibited and non-inhibited cells.

As shown in Table XVI, D-serine did not significantly alter the lipid phosphorus content of the cells. Therefore, D-serine did not increase the cellular content of phospholipids. The increased incorporation of carbon compounds into the lipid fraction must represent a stimulation in biosynthesis of non-phosphatides. Similar results were reported by Challindor, Power, and Tonge (25). Inositol-deficient yeast had an increased lipid content; however, lipid phosphorus was decreased. The increase in total lipid, therefore, was due to non-phosphorus-containing lipids.

Cells inhibited from dividing by penicillin or mitomycin c had an increased lipid phosphorus content (Table XVI). These cells appeared to possess a membrane unusually rich in phospholipids.

To determine if increases in lipid phosphorus were accompanied by a general increase in lipid carbon, cells were grown in basal medium containing inhibitor and 0.16 μc D-glucose-U-C^{14} per ml. Lipids were

TABLE XVI

LIPID PHOSPHORUS CONTENT OF DIVISION-INHIBITED CELLS

Additions*	Percent of dry weight as lipid phosphorus**
none	0.127
D-serine (0.031 M)	0.129
mitomycin c (0.2 ug/ml)	0.150
penicillin (50 units/ml)	0.187

* Additions were made at time of inoculation to basal medium.

** Lipids were extracted as for column chromatography as described in Materials and Methods.

extracted and radioactive content determined. As shown in Table XVII, all agents but D-serine significantly increased the incorporation of radioactivity into lipid material. These agents apparently increase the overall lipid composition of the cell. The D-serine result was not unexpected since D-serine is converted to glycerol and the incorporation of this non-labeled glycerol would dilute the glycerol-C^{14} from glucose-C^{14}. Therefore, these data do not indicate that D-serine-inhibited cells have a normal lipid content.

Since phosphatidylethanolamine was the only ninhydrin-positive material in lipid extracts, it was possible to quantitate this particular phospholipid by measuring the ninhydrin-reacting material in the chloroform:methanol extracts using the procedure described in Materials and Methods. As shown in Table XVIII, all division inhibitors tested increased the cellular content of phosphatidylethanolamine. Although a significant increase in lipid phosphorus or incorporation of C^{14}-glucose could not be demonstrated using D-serine as the inhibitor, it is noted that D-serine did increase cellular phosphatidylethanolamine. This agrees with the increased incorporation of C^{14}-acetate and aspartic acid into lipids induced by D-serine. The observation that total lipid phosphorus is not increased whereas phosphatidylethanolamine is increased probably indicates that other phosphorus-containing lipids are decreased.

Thus, division-inhibited cells appear to contain an increased content of lipid, both phosphorus- and non-phosphorus-containing, based on lipid phosphorus determinations, incorporation of radioactivity from C^{14}-carbon sources, and direct measurement of phosphatidylethanolamine. The significance of this finding in the division inhibition process will be discussed later.

TABLE XVII

INCORPORATION OF D-GLUCOSE-U-C^{14} INTO LIPIDS IN THE PRESENCE OF DIVISION-INHIBITING AGENTS

Inhibitor*	Specific activity**	Percent increase
none	2,240	-
mitomycin c (0.2 µg/ml)	3,760	68
penicillin (50 units/ml)	4,520	102
D-serine (0.031 M)	2,278	1.6
vancomycin (20 µg/ml)	3,078	37

* Inhibitor added at time of inoculation to basal medium containing 0.16 µc D-glucose-U-C^{14} per ml.

** Counts per minute per mg dry weight. Lipids were extracted after 11 hours growth at 25 C.

TABLE XVIII

PHOSPHATIDYLETHANOLAMINE CONTENT OF DIVISION-INHIBITED CELLS

Additions*	µg phosphatidyl-ethanolamine per mg dry weight[a]	Percent increase
none	22.2	-
mitomycin c (0.2 µg/ml)	40.4	82.0
penicillin (50 units/ml)	42.8	92.4
D-serine (0.031 M)	31.2	40.6
vancomycin (20 µg/ml)	30.2	36.1

* Additions made at time of inoculation to basal medium.

[a] Phosphatidylethanolamine determined as ninhydrin-reacting material in lipid extracts.

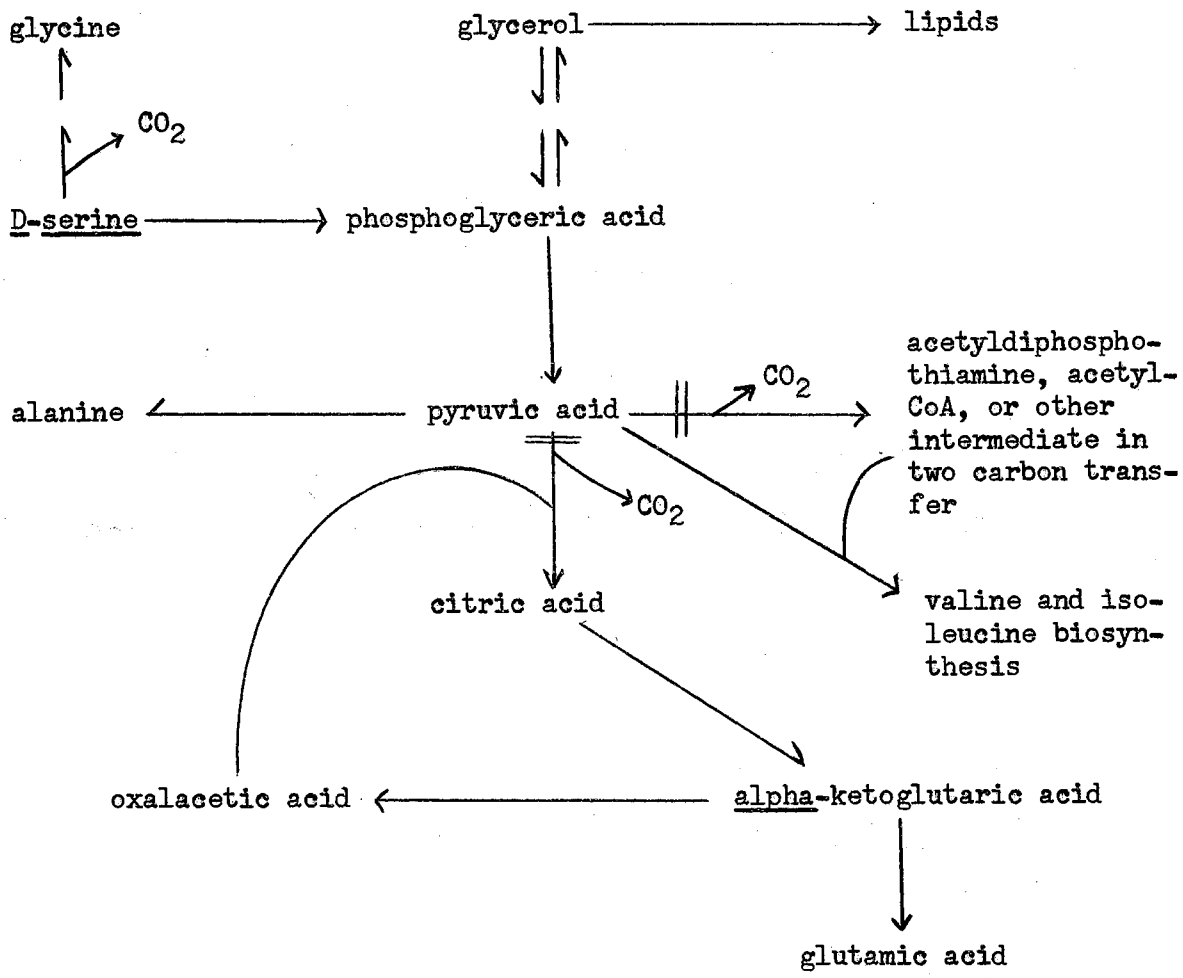
Conversion of D-serine to other amino acids

Since inhibitory levels of D-serine lowered the percent of radioactivity from D-serine-3-C^{14} incorporated into the protein-mucopeptide fraction, the effect of inhibitory levels of D-serine on the conversion of D-serine-3-C^{14} to other amino acids was studied. Radioautograms of protein hydrolyzates from cells grown with 0.05 μc undiluted D-serine-3-C^{14} per ml demonstrated that radioactivity could be recovered in approximately equal amounts in glutamic acid, glycine, alanine, valine, serine, and isoleucine. When sufficient non-labeled D-serine was added to bring the concentration to inhibitory levels, most of the radioactivity was found in serine, glycine, and alanine only. The conversion of D-serine to glutamic acid, valine, and isoleucine was greatly inhibited by the higher levels of D-serine. Thus, the observed increase in incorporation of D-serine-C^{14} into lipids could be associated with an inhibition of incorporation into protein glutamic acid, valine, and isoleucine.

If D-serine were being converted to alanine, glycine, valine, and isoleucine via primary conversion to 3-phosphoglyceric acid and if division-inhibitory levels of D-serine blocked this conversion at some point between phosphoglyceric acid and glutamic acid, valine, and isoleucine, most of the radioactivity from D-serine-3-C^{14} would be spared from amino acid synthesis and could be shunted into lipids as glycerol-C^{14} (Figure 11). Since high levels of D-serine inhibited the conversion of D-serine-C^{14} to glutamic acid, valine, and isoleucine, a block could be placed at the decarboxylation of pyruvic acid. The carbon of D-serine could reach alanine by amination or transamination of pyruvate, however, if the decarboxylation of pyruvic acid were inhibited, the flow into

Figure 11

Proposed mechanism for inhibition of the conversion of D-serine to glutamic acid, valine, and isoleucine by division-inhibitory levels of D-serine.


glycine
glycerol
lipids
CO_2
D-serine
phosphoglyceric acid
acetyldiphospho-thiamine, acetyl-CoA, or other intermediate in two carbon trans-fer
alanine
pyruvic acid
CO_2
CO_2
citric acid
valine and iso-leucine biosyn-thesis
oxalacetic acid
alpha-ketoglutaric acid
glutamic acid

isoleucine, valine, and glutamic acid would also be inhibited. This hypothesis is supported by the observation that D-serine induces the accumulation of pyruvic acid in the growth medium (92).

Lipid profiles on silicic acid columns.

Previous results have suggested that division-inhibiting compounds affect the lipid content of the cell and the cell membrane. Shatkin and Tatum (215) noted that mutant strains of Neurospora possessing an impaired phospholipid synthesizing mechanism with regard to inositol metabolism contained an abnormal amount of phospholipid and were subject to morphological aberrations. Tickner (245) reported that UV irradiation of mouse skin caused a decrease in ethanolamine-containing phospholipids. To study further the relation of phospholipid composition and cell division, the lipid content of inhibited and non-inhibited cells was examined by column chromatography using silicic acid.

Lipids from cells grown in basal medium containing 0.16 μc of D-glucose-U-C^{14} per ml eluted from the column as three radioactive peaks (Figure 12). Continued elution with chloroform:methanol (1:1) and methanol did not remove additional material from the column. Radioactivity was distributed among the three peaks in the ratio 18:2:80.

To determine if measurement of the total radioactivity in each peak was a measure of the amount of phospholipid in that peak, the organic phosphorus content of each was determined (Table XIX). Peak one contained very little phosphorus, however, the ratio of radioactivity in peak two to that in peak three was nearly identical to the ratio of phosphorus in the two peaks. Thus, measurement of radioactivity is a valid measure of phospholipid material in peaks two and three. Also, these

Figure 12

Elution of lipids from silicic acid columns. Lipids were extracted from cells grown in basal medium containing 0.16 μc D-glucose-U-C^{14} per ml. Elution was carried out with 50 ml chloroform followed by 250 ml chloroform:methanol (4:1). Arrow marks solvent change.

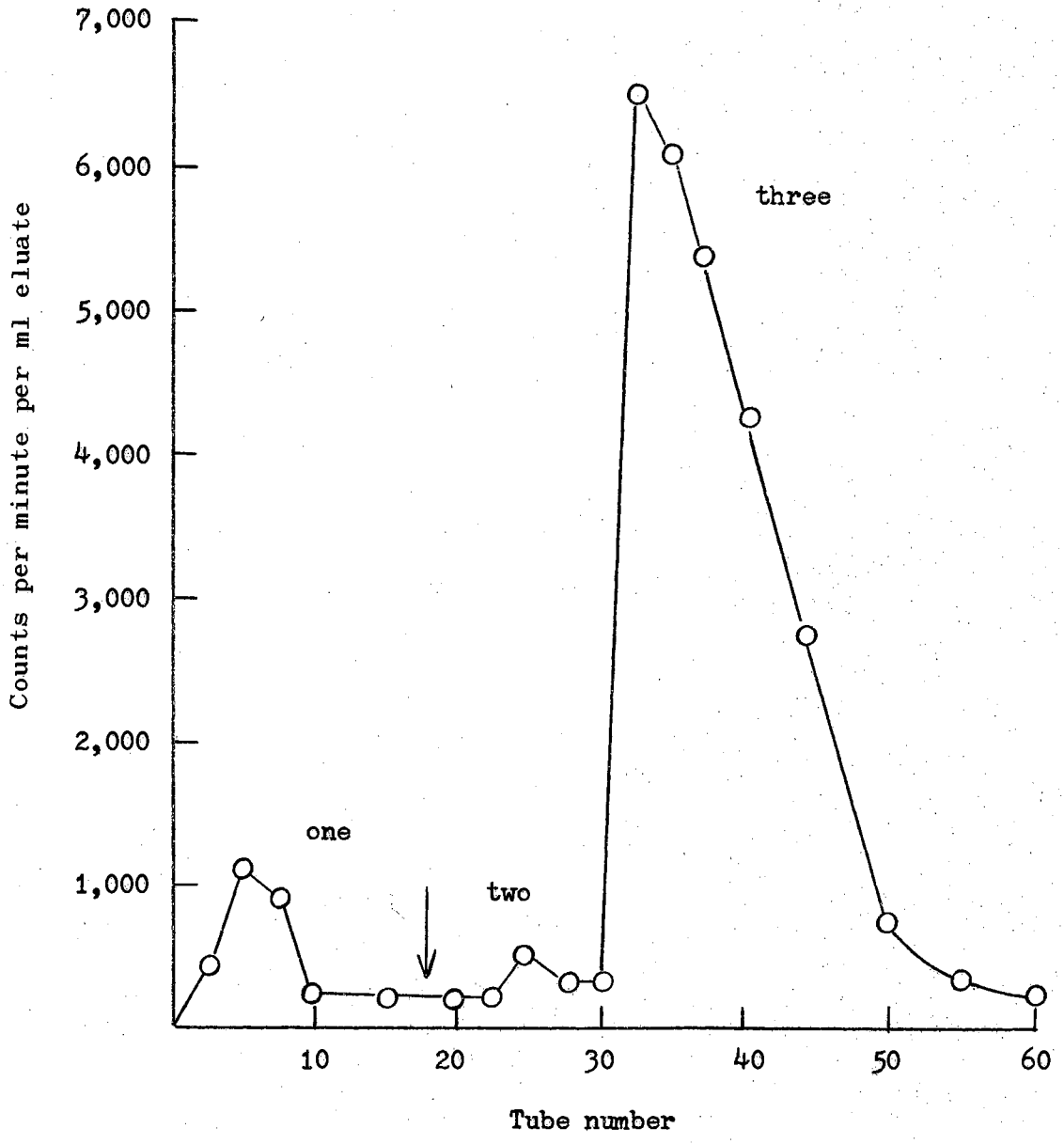

7,000
6,000
5,000
4,000
3,000
2,000
1,000
Counts per minute per ml eluate
one
two
three
10
20
30
40
50
60
Tube number

TABLE XIX

COMPARISON OF CARBON AND PHOSPHORUS CONTENT OF LIPID FRACTIONS FROM SILICIC ACID COLUMNS

Fraction	Total μg phosphorus*	Total counts per minute	μg phosphorus three/two	counts per minute three/two
one	2.01	3,958		
two	5.74	462		
three	239.20	18,818	41.6	40.7

* Phosphorus determined by the procedure of Chen, Toribara, and Warner (26).

data suggest that peak one contained mainly non-phosphatides while peaks two and three contained only phosphatides. This is based on the assumption that the phosphorus content of the individual phosphatides in the fractions did not change significantly. Similar elution data were reported by Marinetti, Erbland, and Kochen (153).

All division-inhibited cells possessed an altered lipid profile as shown in Table XX. In every case, the amount of material in peak two was increased at least two-fold. All inhibitors, except mitomycin c, decreased the size of peak one. Agents which commonly prevent division inhibition were not able to restore the normal profiles. In fact, most "reversers" actually exaggerated the changes in lipid patterns. Partial restoration was demonstrated only by L-alanine with D-serine-inhibited cells. Peak two was returned to normal; however, peak one was greatly decreased and peak three increased. To determine if this effect was associated with the process by which alanine overcomes division inhibition caused by D-serine, L-alanine was added at concentrations which do not prevent division inhibition (0.011 M). The same ratios were found. The L-alanine effect, therefore, cannot be correlated with its ability to prevent division inhibition due to D-serine. L-alanine probably served as a source of some component(s) in peaks one and two and diluted radioactivity from glucose-C^{14} going into these materials.

Distribution of D-serine-3-C^{14} among lipid fractions.

Data have indicated that D-serine-3-C^{14} is incorporated into lipids as glycerol-C^{14}. Also, it has been noted that inhibitory levels of D-serine stimulate the incorporation of radioactivity from D-serine-3-C^{14} into lipid materials. To study further the incorporation of D-serine

TABLE XX

LIPID PROFILES ON SILICIC ACID COLUMNS

Additions*	Percent of total radioactivity recovered in peak		
	one	two	three
none	18	2	80
D-serine (0.031 M)	14	5	81
+ pantoyl lactone (0.056 M)	15	7	78
+ NH_4Cl (0.074 M)	13	4	83
+ propylene glycol (0.66 M)	12	6	82
+ L-alanine (0.034 M)	6	2	92
+ L-alanine (0.011 M)	6	2	92
penicillin (50 units/ml)	10	4	86
+ pantoyl lactone (0.056 M)	6	7	87
+ NaCl (0.25 M)	8	5	87
+ $MgSO_4$ (4×10^{-4} M)	13	5	82
+ $MnCl_2$ (1.8×10^{-5} M)	9	11	80
vancomycin (20 ug/ml)	6	5	89
+ pantoyl lactone (0.056 M)	6	10	84
+ NaCl (0.25 M)	9	8	83
mitomycin c (0.2 ug/ml)	19	10	71
+ pantoyl lactone (0.056 M)	17	9	74
ultraviolet light-inhibited	8	5	87
cycloserine (1.7×10^{-5} M)[a]	14	5	81

TABLE XX (Continued)

* Additions made at time of inoculation into 120 ml basal medium containing 20 μc D-glucose-U-C^{14}.

[a] Actual concentration of cycloserine not known since the commercial sample (Calbiochem) contained an unknown amount of talc and lubricant.

into lipids, cells were grown in basal medium containing 0.16 μc D-serine-3-C^{14} per ml. Lipids were extracted and chromatographed using silicic acid. At non-inhibitory concentrations, radioactivity was distributed among the three peaks in the ratio 15:1.5:83.5. This is similar to the ratio of 18:2:80 found for cells grown with D-glucose-U-C^{14}. These data support previous observations that serine is metabolized via carbohydrate pathways. Also, these data would be expected if D-serine-C^{14} were incorporated into lipids as glycerol-C^{14}.

Addition of non-labeled D-serine to division-inhibitory levels (0.031 M) caused a change in the distribution of radioactivity from D-serine-3-C^{14} in the lipid fractions. A ratio of 25:5:75 was found. This represents a shift mainly from peak three into peaks one and two. Although high levels of D-serine caused an increased incorporation of glucose-C^{14} into peak two, it had little effect on incorporation into peaks one and three. These data suggest that inhibitory concentrations of D-serine change the form in which D-serine is incorporated into lipid and that this new form is incorporated preferentially into material which elutes as peak one. However, as stated previously, only glycerol-C^{14} could be detected in these fractions. As will be shown later, peak one contains a polyglycerol phospholipid in addition to non-phosphatides. Since high levels of D-serine apparently stimulate the conversion of D-serine-C^{14} to glycerol-C^{14}, it is not surprising that D-serine should increase the incorporation of radioactivity from D-serine-C^{14} into a lipid material rich in glycerol. Peak one probably contains a higher concentration of glycerol than the other peaks.

Identification of lipid components.

Thin-layer chromatography of lipids.

Each of the fractions separated on silicic acid columns was examined by thin-layer chromatography to obtain qualitative data relative to their composition. Silica gel G was spread 0.25 mm thick on a glass plate using the Desaga-Brinkmann apparatus obtained from Brinkmann Instrument Company, Westbury, New York. After drying at 25 C for 15 minutes, the plates were activated by heating for 1 hour at 100 C. Chromatograms were developed in chloroform:methanol:0.85% sodium chloride (80:25:0.5-3.0). The saline concentration was varied depending on the particular fraction under study. Data are summarized in Table XXI. Only phosphatidylethanolamine in fraction three could be positively identified. Similar identification was made by Marinetti et al. (153). Standard phosphatidylethanolamine (Mann Research Laboratories, New York) contained several components; however, the major portion of the material chromatographed as one ninhydrin-positive spot. This spot fluoresced yellow after treatment with rhodamine 6 G and had an Rf of 0.61 to 0.72. A choline-containing compound was detected using the Dragendorf reagent described by Skidmore and Entenman (219) and had an Rf of approximately 0.72. Material reacting with the Dragendorf reagent was also detected at the solvent front in all fractions. Commercial samples of standard phosphatides were badly contaminated and prevented identification of the other compounds. It was apparent, however, that each of the three fractions obtained from silicic acid columns contained several different compounds.

TABLE XXI

THIN-LAYER CHROMATOGRAPHY OF LIPIDS IN FRACTIONS SEPARATED ON SILICIC ACID COLUMNS

Fraction	Rf values*	Ninhydrin reaction	rhodamine reaction**	Dragendorf reaction[a]
one	0.77	-	b	-
	0.81	-	b	-
	0.94	-	b	-
	1.00	-	y	+
two	0.45	-	b	-
	1.00	-	y	+
three	0.48	-	b	-
	0.57	-	b	-
	0.61-0.72	+	y	-
	0.72	-	b	+
	0.89	-	b	-
	1.00	-	w	+

* Solvent was chloroform:methanol:0.85% sodium chloride (80:25:0.5-3.0). Three parts of saline were used for fraction three, 0.5 parts for fraction two, and 2 parts for fraction one.

** b - blue fluorescence under UV light; y - yellow fluorescence; w - white fluorescence.

[a] Dragendorf spray for choline-containing compounds prepared as described by Skidmore and Entenman (219).

Alkaline hydrolysis products.

Lipid samples were subjected to mild alkaline hydrolysis according to the procedure of Dawson (49). The water soluble products were chromatographed on paper. Results are presented in Table XXII. Values for known compounds are presented in Table XXIII. Tentative identifications are as follow. Peak one contained a polyglycerol phospholipid in addition to non-phosphatide components. Peak two was mainly phosphatidylglycerol. Peak three possibly contained phosphatidylcholine, phosphatidylethanolamine, and phosphatidylinositol. However, these identifications cannot be presented as definite. The other compounds were not identified due to lack of proper standards. These identifications have been supported by other workers (Kennedy, personal communication). Marinetti et al. (153), using a similar column, reported that phosphatidylinositol eluted as a separate peak immediately after phosphatidylethanolamine. However, due to the use of different collection procedures and different amounts of materials, the coalescence of these two peaks could easily be effected. In fact, a fourth peak appeared occasionally in about tube 43. At no time, however, could this peak be separated from peak three.

Very little work has been done on the phospholipid composition of bacteria. This is the first report of the phospholipid composition of Erwinia sp and may be compared with that reported for E. coli (119). The major phospholipids of E. coli are phosphatidylethanolamine and phosphatidylglycerol. Phosphatidylglycerol is approximately 21% of the total phospholipid content. Phosphatidylserine and phosphatidic acid were minor components. Erwinia and E. coli are similar in that the majority of the phospholipids were present as phosphatidylethanolamine,

TABLE XXII

CHROMATOGRAPHY OF LIPID HYDROLYSIS PRODUCTS

Fraction	Rf values in		Ninhydrin reaction	Rhodamine reaction[a]
	solvent 1*	solvent 2**		
one	0.0	-	-	b
	0.30	0.27	-	b
	0.56	-	-	b
two	0.38	0.39	-	b
	0.44	0.49	-	b
three	-	0.18	-	b
	0.60	0.63	+	y
	-	0.75	-	b

* 88% phenol containing 0.1% ammonium hydroxide.

** 88% phenol:acetic acid:water (100:10:12).

[a] b - blue fluorescence under UV light; y - yellow fluorescence.

TABLE XXIII

Rf VALUES OF LIPID HYDROLYSIS PRODUCTS

Parent compound	Solvent 1*	Solvent 2**
phosphatidylserine	0.17	0.30
phosphatidylethanolamine	0.62	0.63
phosphatidylcholine	0.88	0.77
phosphatidic acid	0.19	0.33
phosphatidylglycerol	0.37	-
monophosphoinositide	-	0.20
brain inositide	-	0.12
polyglycerol phospholipid	-	0.28

* Data of Dawson (47); solvent was 88% phenol containing 0.1% ammonium hydroxide.

** Data of Dawson (49); solvent was 88% phenol:acetic acid:water (100: 10:12).

however, E. coli contains approximately 10 times more phosphatidylglycerol. Erwinia may also contain phosphatidylinositol and a choline-containing phosphatide; these were not reported in E. coli.

Effect of D-serine on glucose oxidation.

Alteration of membrane structure due to changes in lipid composition might be expected to interfere with energy metabolism. These effects may be reflected in the glucose oxidation patterns. Cells grown in basal medium were treated as described in Materials and Methods and tested for their ability to oxidize glucose in manometric experiments. As shown in Table XXIV, D-serine stimulated the oxidation of both glucose and mannose. Residual hexose was determined at the termination of the experiment by the anthrone reaction. Data in Table XXV demonstrate that D-serine inhibited the utilization of glucose. These effects were partially prevented by addition of pantoyl lactone. Apparently, D-serine stimulated glucose and mannose oxidation whereas their assimilation into cellular materials was inhibited. D-serine may be functioning in a manner similar to uncouplers of oxidative phosphorylation,e.g. 2,4-dinitrophenol. These agents cause the complete oxidation of glucose, however, whereas only about 60% oxidation was obtained in the presence of D-serine. Dinitrophenol inhibits pantoyl lactone-sparked growth and cell division (89); however, it does not inhibit division when added to a culture at the time of inoculation. Therefore, the actions of D-serine and dinitrophenol are not completely analogous.

Commoner (31) postulated that changes in nucleotide levels in the cell might alter oxidative metabolism. As shown previously, D-serine induced leakage of purine and/or pyrimidine-containing compounds from

TABLE XXIV

EFFECT OF D-SERINE ON THE OXIDATION OF GLUCOSE AND MANNOSE

Treatment*	Substrate**	μliters O_2 taken up at inflection point	percent oxidized of theoretical
none	glucose	227	42.2
	mannose	236	43.9
D-serine (0.031 M)	glucose	316	58.7
	mannose	326	60.6
D-serine (0.031 M) + pantoyl lactone (0.056 M)	glucose	262	48.6
	mannose	269	50.0

* Cells were grown in basal medium with and without D-serine and/or pantoyl lactone and treated as described in Materials and Methods.

** Four μmoles of each substrate added to Warburg flask. Oxidation measured at 25 C. Total volume of each flask was 2.4 ml.

TABLE XXV

EFFECT OF D-SERINE ON UTILIZATION OF GLUCOSE AND MANNOSE

Treatment*	Substrate**	µg recovered per ml[a]	Percent recovered
none	glucose	26.0	7.95
	mannose	-	-
D-serine (0.031 M)	glucose	83.2	25.4
	mannose	100.0	30.6
D-serine (0.031 M) + pantoyl lactone (0.056 M)	glucose	37.4	11.4
	mannose	47.4	14.5

* Cells were treated as for manometric experiments described in Materials and Methods.

** Substrates added to 327 µg per ml.

[a] Residual hexose determined by the anthrone procedure.

inhibited cells. Therefore, according to the theory of Commoner, the D-serine-induced stimulation of glucose oxidation would be due to a decreased level of nucleotides inside the cell. This mechanism may or may not be operating in this system, however, D-serine did have a significant effect on glucose and mannose oxidation and assimilation. These data may be interpreted as supporting an hypothesis that D-serine interferes with energy metabolism. Apparently this interference is associated with the division inhibition process since both are effectively prevented by addition of pantoyl lactone.

Data of Grula and Grula (89) suggest that the division process is more sensitive to alterations in energy metabolism than other cellular functions. If so, a slight interference in energy production or utilization could prevent cell division without significantly affecting other cellular processes. It is possible that alteration in synthesis of the cell membrane could change carbon and energy metabolism which in turn could lead to division inhibition.

Penicillin binding.

Intracellular distribution.

It is well documented that penicillin binds to lipo-protein presumed to be in the cell membrane (34-38). However, the specific compound to which penicillin binds has not been identified. Also, it has never been shown that binding is the direct cause of division and/or mucopeptide synthesis inhibition. This section was designed to show binding of penicillin to lipid materials, to identify the lipid component, and to determine, if possible, the physiological importance of binding.

Cells were grown in basal medium containing H^3-penicillin (0.33 μc/ml; 13.7 units/ml). After washing, the cells were fractionated. Eighteen percent of the radioactivity taken into the cell was extractable into 5% trichloroacetic acid at 100 C for 30 minutes. Chloroform:methanol (2:1) at 55 C for 30 minutes removed 76%; the remaining 6% stayed in the protein-mucopeptide complex. Thus, at least 76% of the radioactivity was bound to lipoidal material. The radioactivity extracted with hot trichloroacetic acid may be due to partial hydrolysis of the penicillin-lipid complex. However, the complex was largely resistant to treatment with 5% trichloroacetic acid at 100 C for 30 minutes.

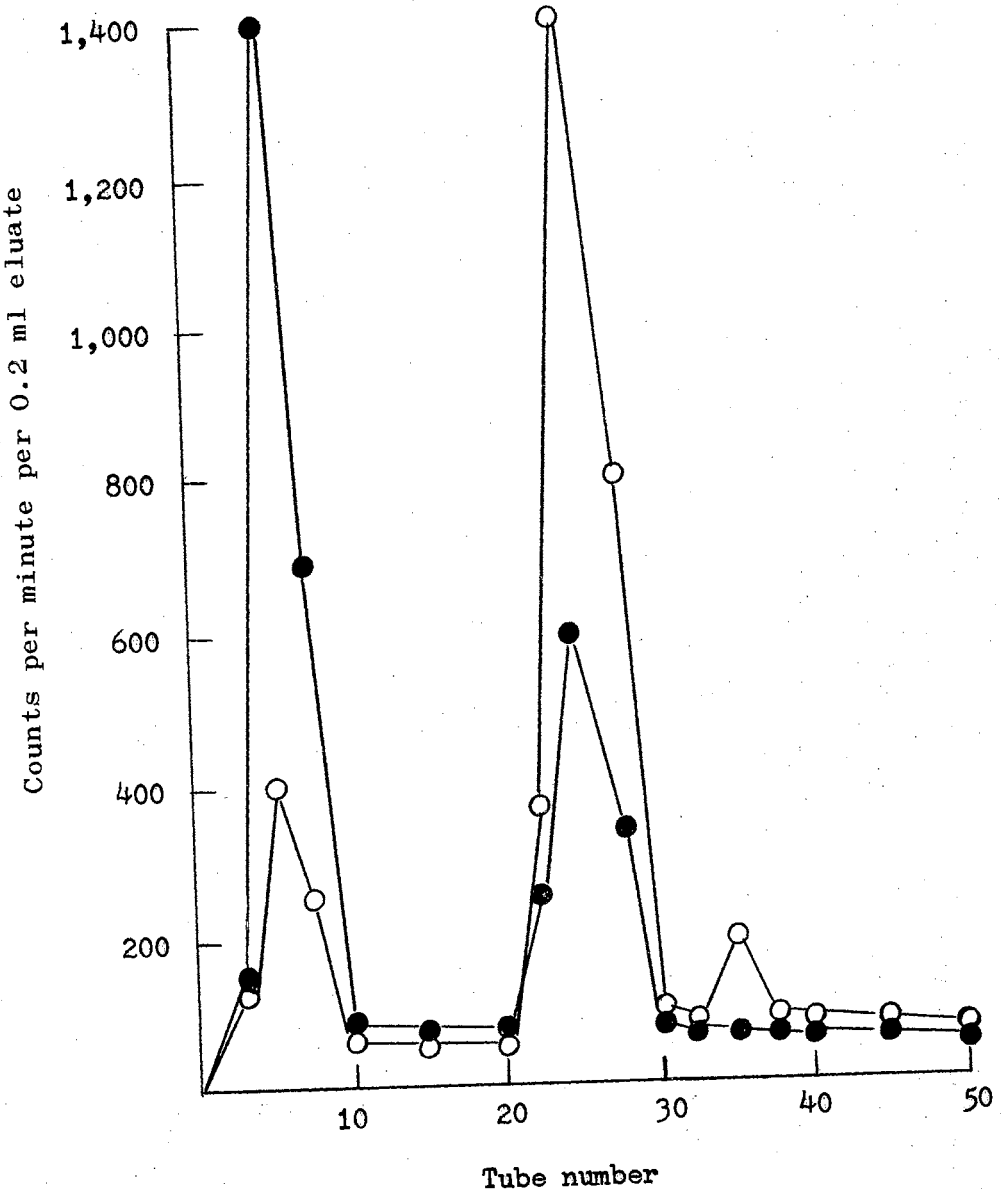
These data are in contrast with the findings of Duerksen (58) who reported that 95% of the penicillin taken up by B. cereus remained in the soluble state and could be released by treatment with lysozyme. The remaining 5% was bound to lipo-protein and could be removed by the combined action of lipase and trypsin. These differences could, however, be due to the large amount of lipid in the Gram-negative Erwinia cells compared with the Gram-positive Bacillus organism used by Duerksen.

Binding in vivo.

Chromatography of H^3-penicillin dissolved in chloroform:methanol (2:1) on silicic acid columns revealed that radioactivity eluted from the column in two peaks (Figure 13). One was identical to peak one and contained 70% of the radioactivity applied to the column. The other was similar to peak two and contained 30% of the radioactivity. Material in both peaks migrated with an Rf of approximately 0.82 in methanol: water:pyridine (80:20:4) and both demonstrated antibiotic activity on

Figure 13

Elution of H^3-penicillin from silicic acid columns. ● standard solution of H^3-penicillin in chloroform:methanol (2:1); ○ lipids extracted from cells grown with H^3-penicillin.


1,400
1,200
1,000
800
600
400
200
Counts per minute per 0.2 ml eluate
10
20
30
40
50
Tube number

autobiographic plates seeded with E. coli B. Therefore, both peaks appeared to contain biologically active penicillin. This effect may be similar to that reported by Rathbone (192) and Marinetti, Erbland, and Stotz (154). They found that different ionic forms of phosphatidylserine eluted from silicic acid columns at different time intervals. However, all forms appeared similar using paper chromatography.

Lipids extracted from cells grown with H^3-penicillin were also chromatographed on silicic acid columns. As shown in Figure 13, 70% of the radioactivity now eluted in peak two. Twenty percent eluted as peak one and 10% as peak three. These data demonstrate in vivo binding of H^3-penicillin to material in peak two (phosphatidylglycerol). Binding also occurred to material in peak three; however, since peak three contained approximately 40 times more phospholipid than peak two, these data show preferential binding to peak two.

Binding of penicillin to material in peak two is supported by the following observations. As shown previously, D-serine doubled the amount of material in peak two. Therefore, cells inhibited by D-serine should bind twice as much H^3-penicillin as non-inhibited cells. This was tested by growing cells in basal medium containing H^3-penicillin with and without D-serine. The control cells had a specific activity (counts per minute per mg dry weight) of approximately 735. D-serine-inhibited cells had a specific activity of about 1,600. Thus, cells inhibited by D-serine contained twice as much material in peak two and bound twice as much H^3-penicillin as non-inhibited cells.

Binding was also demonstrated by washing the chloroform:methanol solution of H^3-penicillin by the procedure of Folch, Lees, and Sloane-Stanley (71). The percent of radioactivity extracted into the aqueous

washes was considered to be representative of the amount of non-bound penicillin while that remaining in the organic phase was considered to be bound. When a solution of H^3-penicillin in chloroform:methanol (2:1) was washed, approximately 74% of the radioactivity was removed indicating 26% binding (Table XXVI). This represents background or false binding. Washing of lipids extracted from cells grown with H^3-penicillin revealed that 72% of the penicillin was bound. Correction for background binding shows that 46% of the radioactivity extracted from cells by chloroform:methanol (2:1) was actually bound to lipids. However this is a minimum figure since washing or the chloroform:methanol extraction procedure, which dissociates lipo-protein complexes, may have partially dissociated the penicillin-lipid complex. Also, it is assumed that the degree of background binding remains the same in the presence of phospholipid. There may be no need to subtract for background binding when testing lipids extracted from cells grown in the presence of H^3-penicillin.

Although others have shown penicillin binding to lipo-protein, these data demonstrate for the first time binding to the lipid component of lipo-protein complexes. This is concluded since all protein was removed from the chloroform solution during washing. Therefore, any penicillin left in the chloroform had to be bound to lipid.

Penicillin binding in vitro.

The non-enzymic nature of the binding of penicillin to lipids was demonstrated in vitro. Lipids extracted from cells grown in basal medium were treated in vitro with H^3-penicillin. After remaining at 25 C for 30 minutes, the solution was washed as described previously. Approximately

TABLE XXVI

BINDING OF H^3-PENICILLIN TO LIPID MATERIALS AS DEMONSTRATED BY WASHING TECHNIQUE

Sample	Total counts per minute before wash	Total counts per minute after wash*	Percent remaining in chloroform: methanol layer
H^3-penicillin-grown[a]	37,221	26,688	71.7
standard H^3-penicillin[b]	22,380	5,908	26.4
in vitro binding[c]	20,161	10,121	50.2

* Samples were washed according to the procedure of Folch, Lees, and Sloane-Stanley (71).

[a] Lipids were extracted from cells grown for 11 hours in the presence of H^3-penicillin (0.33 μc/ml ; 13.7 units/ml).

[b] Standard H^3-penicillin was dissolved in chloroform:methanol (2:1).

[c] Lipids extracted from control cells were treated *in vitro* with H^3-penicillin at 25 C for 30 minutes.

50% of the radioactivity was extracted into the aqueous phase as compared to 74% in the absence of lipid material (Table XXVI). About 50% of the penicillin (24% after correction for background binding) was bound to lipid material *in vitro*.

Descending chromatography of the above materials on 1½ x 20 inch strips of Whatman #1 filter paper (methanol:water:pyridine; 80:20:4) and examination using a chromatogram scanner revealed two radioactive zones. One was at the origin and contained approximately 25% of the total radioactivity. The remaining 75% was in one spot with an Rf of approximately 0.85. Chromatography of a standard solution of H^3-penicillin demonstrated two similar radioactive areas, however, material at the origin composed no more than 3 to 5% of the total radioactivity. It is proposed, therefore, that increased retention of radioactivity at the origin represented lipid-bound H^3-penicillin.

Lipids extracted from cells grown in basal medium and treated with H^3-penicillin *in vitro* were also chromatographed on silicic acid columns. Approximately 45% of the radioactivity eluted as peak one and 55% as peak two. As shown in Figure 13, non-bound penicillin eluted from the column also in two peaks; however, 70% was found in peak one and 30% in peak two. This shift of radioactivity into peak two is interpreted as a further demonstration of *in vitro* binding of penicillin to material in peak two.

Thus, penicillin was bound *in vivo* and *in vitro* to some component of peak two which is predominantly phosphatidylglycerol. This binding was non-enzymic and the bond formed was largely resistant to 5% trichloroacetic acid at 100 C for 30 minutes.

Effect of pantoyl lactone on binding.

Pantoyl lactone (0.056 M) completely prevents division inhibition due to penicillin in Erwinia sp (88). Based on data reported above, pantoyl lactone could be preventing either the binding of penicillin to the lipids in the membrane or causing its removal once it is bound. Cells were inoculated into basal medium containing pantoyl lactone and H^3-penicillin (0.33 μc/ml; 13.7 units/ml) to test if pantoyl lactone could prevent penicillin binding. The cells were permitted to grow for 12 hours at 25 C on a rotary shaker. Pantoyl lactone did not decrease the amount of penicillin taken into the cell which was extractable by chloroform:methanol (2:1).

To determine if pantoyl lactone caused the release of bound penicillin, cells were grown in basal medium containing H^3-penicillin for 11 hours, washed carefully two times with a mineral solution to remove non-bound penicillin, and resuspended in fresh basal medium containing pantoyl lactone. Non-labeled penicillin (50 units/ml) was added to the incubation medium to dilute any H^3-penicillin that was removed from the membrane and prevent re-adsorption. Aliquots were taken at 30 minute intervals for 5 hours and the supernatants examined for radioactivity. No radioactivity above background could be detected in the medium.

It is concluded, therefore, that pantoyl lactone neither prevents the binding of penicillin to lipids nor does it cause the release of penicillin from lipids once bound. These data are a further demonstration of the non-reversible nature of penicillin binding.

Binding of penicillin to the membrane does not necessarily result in division inhibition. Although it is tempting to speculate that binding of penicillin to phospholipids is intimately associated with

inhibition of cell division, speculation must be tempered since pantoyl lactone, a potent reverser of penicillin-induced division inhibition, neither prevented the binding nor caused the removal of bound penicillin. It is possible, however, to offer the following hypotheses to explain how the binding of penicillin could be physiologically important in the division inhibition process even though binding is not prevented by pantoyl lactone. First, a charge alteration on the membrane may result from penicillin binding. This has been suggested by Dorfman (57). Addition of pantoyl lactone might restore the initial charges sufficiently to permit the necessary reactions to occur which lead to cell division.

Second, pantoyl lactone may bind to the penicillin-lipid complex in such a manner as to "cover-up" the penicillin molecule and to prevent sterically its functioning which results in division inhibition.

Third, binding of penicillin could result in conformational changes in the cell membrane. Such changes could deter enzymic activities or simply weaken the membrane. Pantoyl lactone might in some way redirect these conformational changes to permit enzyme activities and/or structural functions to continue.

Fourth, the binding of penicillin to phospholipid may be gratuitous and not related to division inhibition. Data to be presented later will suggest that binding functions as a detoxifying mechanism.

Lipids of vancomycin and penicillin resistant mutant.

Wahlström and Wahlström (256) reported that protein and nucleic acid synthesis changed when organisms acquired resistance to penicillin. The resistant organisms had a decreased nitrogen and pyrimidine content.

Pyrimidine metabolism in susceptible organisms was inhibited by penicillin. Resistant strains, however, possessed mechanisms for pyrimidine metabolism which were resistant to penicillin. Also, strains possessing the resistant pathways for pyrimidine metabolism were able to maintain the osmotic barrier intact and did not demonstrate penicillin-induced leakage as did susceptible organisms.

Since penicillin binds to the cell membrane and since both penicillin and vancomycin alter the normal lipid profiles, resistance could also be effected by loss of the penicillin-binding material (peak two).

Examination of the lipid content of the mutant organism revealed that the mutant did, indeed, possess an altered lipid profile (Table XXVII). However, instead of lacking material in peak two, the size of this peak was increased more than five-fold. The amount of material in peak three was unchanged while that in peak one was decreased about 61%.

When the mutant organism was grown with either penicillin or vancomycin, the profiles were similar to those of wild-type cells which are inhibited from dividing by penicillin or vancomycin. It was also found that lipids extracted from the mutant organism were still capable of binding H^3-penicillin in vitro. Since cell division of the mutant was not inhibited by these antibiotics, data suggest that alteration of lipid profiles and penicillin binding are not directly responsible for division inhibition. That is, cells may have an altered lipid content, yet divide normally. This supports previous observations that agents that prevent division inhibition do not prevent alterations in the lipid profiles.

Since resistant organisms possessed a greatly increased peak two and since penicillin binds to material in peak two, it is interesting to

TABLE XXVII

LIPID PROFILES OF VANCOMYCIN AND PENICILLIN RESISTANT MUTANT

Organism	Growth medium*	Percent of radioactivity in peak**		
		one	two	three
wild-type	basal	18	2	80
	+ penicillin	10	4	86
	+ vancomycin	6	5	89
mutant	basal	7	11	82
	+ penicillin	10	6	84
	+ vancomycin	5	4	91

* Cells were grown for 12 hours in basal medium containing 0.16 μc D-glucose-U-C^{14} per ml. Penicillin (50 units per ml) or vancomycin (20 μg per ml) added at time of inoculation.

** Lipids were extracted and chromatographed on silicic acid columns as described in Materials and Methods.

speculate that binding represents a detoxifying mechanism. Perhaps, the mutants are more resistant to penicillin than wild-type organisms because they possess more phosphatidylglycerol in their membranes. To determine if penicillin lost its antibiotic activity when bound to lipid, H^3-penicillin was treated *in vitro* with lipids extracted from control cells. The complex formed was chromatographed in methanol:water:pyridine (80:20:4) and the chromatogram placed on the surface of an autobiographic plate seeded with *E. coli* B. No inhibitory zones could be detected. This appeared to demonstrate that penicillin lost its biological activity when bound to phospholipid. However, it was doubtful that sufficient penicillin was bound to demonstrate antibiotic activity even if activity were not destroyed. Various dilutions of H^3-penicillin were chromatographed and tested for detectable antibiotic activity. Sufficient H^3-penicillin to give 1,500 counts per minute on a Picker gas-flow chromatogram scanner was the minimum which could be detected by autobiographic assay. A maximum of 200 counts per minute could be bound to lipid *in vitro*. Therefore, these data cannot be interpreted as supporting a detoxification mechanism.

Pantoyl lactone binding.

Intracellular distribution.

A study of the intracellular fate of pantoyl lactone might provide evidence for its site and mechanism of action. In order to determine the amount of pantoyl lactone taken up, cells were grown in basal medium for 11 hours in the presence of a known amount of H^3-pantoyl lactone. A total of 13,080 detectable counts per minute out of 6,063,000 was taken into the cells and not removed by washing with mineral solution. Thus,

0.216% or 98 µg of the pantoyl lactone added was taken up and retained inside the cells. After 11 hours of growth, a total of 2.6 mg dry weight of cells was present. It is calculated, therefore, that one mg of cells took up approximately 37.6 µg of pantoyl lactone. Apparently very little pantoyl lactone was able to penetrate the permeability barriers of the cell. It has been difficult to explain a catalytic role for pantoyl lactone in the division process due to the high levels which must be added to the growth medium. These data demonstrate that although rather high concentrations (0.056 M) of pantoyl lactone must be added to the growth medium to effect reversal of division inhibition, the interior of the cell is not exposed to unphysiological amounts.

Upon fractionation, approximately 36% of the radioactivity from H^3-pantoyl lactone was removed by 5% trichloroacetic acid at 100 C for 30 minutes. Chloroform:methanol (2:1) removed 36%; the remaining 28% was found in the protein-mucopeptide complex. Thus, it was not possible to locate the major site of action of pantoyl lactone in the cell using this approach.

To determine if pantoyl lactone was actually bound into lipid and protein material, these fractions were chromatographed on 1½ x 20 inch strips of Whatman #1 filter paper in methanol:water:pyridine (80:20:4) before and after hydrolysis for amino acids. Strips were examined for radioactivity using a chromatogram scanner. Similar results were found in both the lipid and the protein-mucopeptide fractions. In non-hydrolyzed samples only one radioactive zone was found which remained at the origin. Failure to migrate in this chromatographic system indicated that pantoyl lactone was bound on or into a larger, more complex molecule. After hydrolysis, radioactivity migrated as one spot with an

Rf of 0.88 to 0.94 which is similar to that of standard pantoyl lactone. Pantoyl lactone, therefore, was bound into the lipid and protein-mucopeptide fractions and released as pantoyl lactone by acid hydrolysis. These data further show that the radioactivity from H^3-pantoyl lactone was not randomized throughout the cell and that measurement of radioactivity constituted measurement of pantoyl lactone.

Binding to lipids.

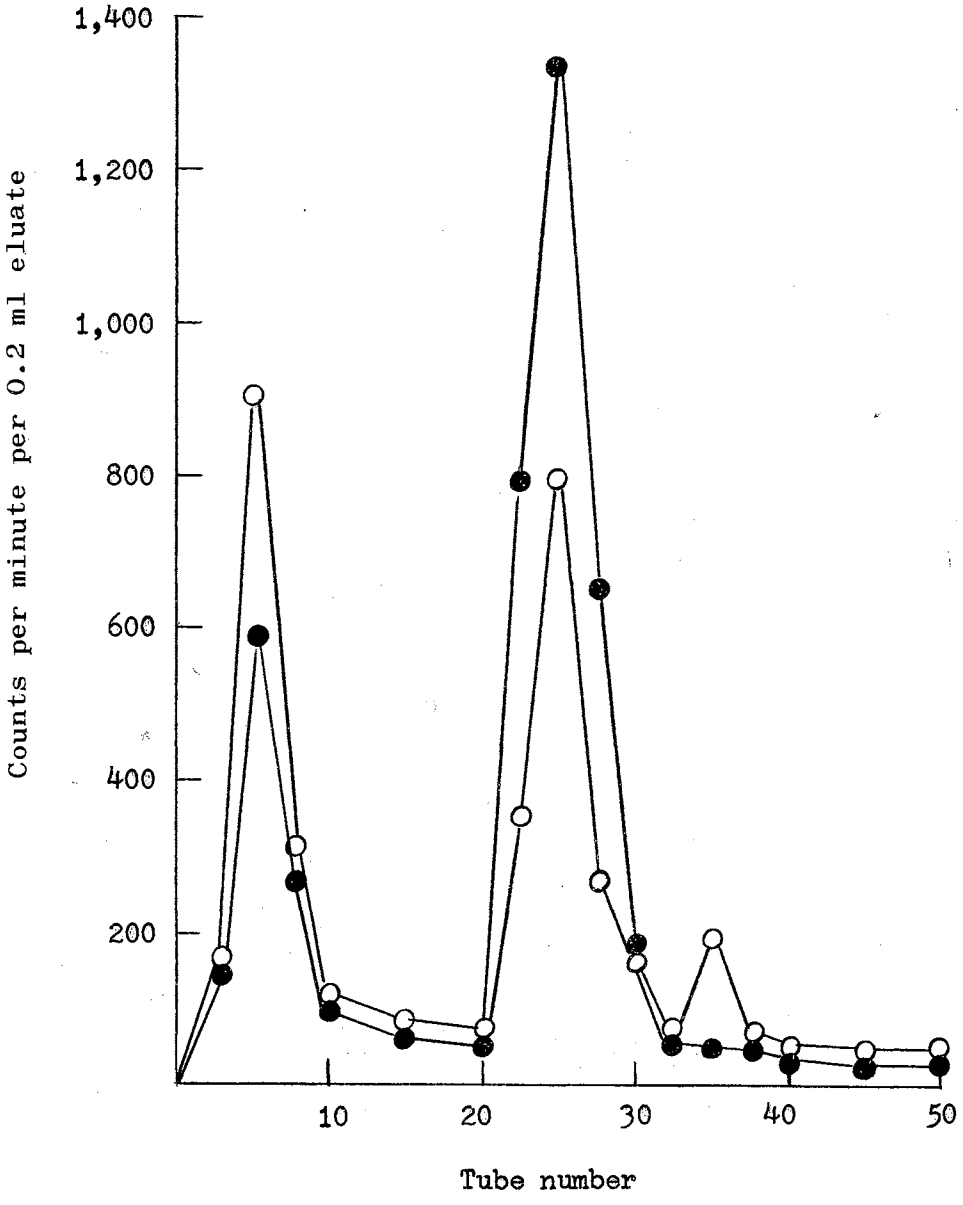
A chloroform:methanol (2:1) solution of H^3-pantoyl lactone was washed according to the procedure of Folch, Lees, and Sloane-Stanley (71); 95 to 98% of the radioactivity was removed from the organic layer indicating a background binding of 2 to 5%. If, however, lipids extracted from cells grown with H^3-pantoyl lactone were washed, only 77% was removed. This demonstrates that 23% of the pantoyl lactone was bound to lipid materials. The radioactivity extracted into the aqueous washes may have been due to partial dissolution of the pantoyl lactone-lipid complex either by the lipid extraction procedure or by the washing procedure. Also, it has been shown that pantoyl lactone was bound to protein. Considerable protein was solubilized during lipid extraction. This protein was completely removed by the washing procedure. Loss of radioactivity into the aqueous washes, therefore, may also be due to removal of protein-bound pantoyl lactone.

Chromatography of a chloroform:methanol (2:1) solution of H^3-pantoyl lactone on silicic acid yielded two radioactive peaks (Figure 14). Thirty percent of the radioactivity eluted as peak one while 70% eluted as peak two. Chromatography of lipids extracted from cells grown with H^3-pantoyl lactone demonstrated that approximately 45% of the

Figure 14

Elution of H^3-pantoyl lactone from silicic acid columns.

● standard solution of H^3-pantoyl lactone; O lipids extracted from cells grown with H^3-pantoyl lactone.


1,400
1,200
1,000
800
600
400
200
Counts per minute per 0.2 ml eluate
10
20
30
40
50
Tube number

radioactivity eluted as peak one, 42% as peak two, and 13% as peak three. Although there was significant binding to all three fractions, pantoyl lactone was bound preferentially to material in peak two. This is concluded since peak one contains approximately nine times and peak three forty times more material than does peak two; however, 42% of the pantoyl lactone was bound to material in peak two. Thus, both penicillin and pantoyl lactone bind to material in peak two which is mainly phosphatidylglycerol. Several attempts were made to demonstrate binding of pantoyl lactone to lipids in vitro. It appears, however, that pantoyl lactone, unlike penicillin, does not react non-enzymically with lipid material in vitro.

These data support the several hypotheses presented earlier concerning the mechanism by which pantoyl lactone overcomes the effects of penicillin. Binding of penicillin to phospholipid could still be important in both division inhibition and inhibition of mucopeptide synthesis. It appears, however, that binding to phospholipids per se is not responsible for division inhibition. Binding probably causes some alteration in the cell membrane. This alteration then could be responsible for division inhibition. Pantoyl lactone may prevent division inhibition by overcoming this alteration or by nullifying the effects of this alteration which lead to inhibition of cell division.

Proposed mechanisms for division inhibition.

Houtsmuller and Van Deenen (102) found in S. aureus that glucose caused the production of phosphatidylglycerolornithine. Lipid patterns were significantly changed. The authors suggest that balancing of charges at the membrane was involved in these alterations. Penicillin

has also been reported to alter the charge distribution on the cell membrane (57).

Data indicate that division-inhibiting agents have an effect which may be associated with charge alterations. Grula and Grula (93) have reported that the action of mitomycin c may be partially prevented by several divalent cations. Similar results have been found using D-serine and penicillin (91 ; Grula and Grula, unpublished data). It has also been observed that the action of D-serine, vancomycin, and mitomycin c may be overcome by slight alterations in the initial pH of the growth medium. Ions and pH changes would be expected to affect the action of inhibitors whose mechanisms of action involve charge alterations.

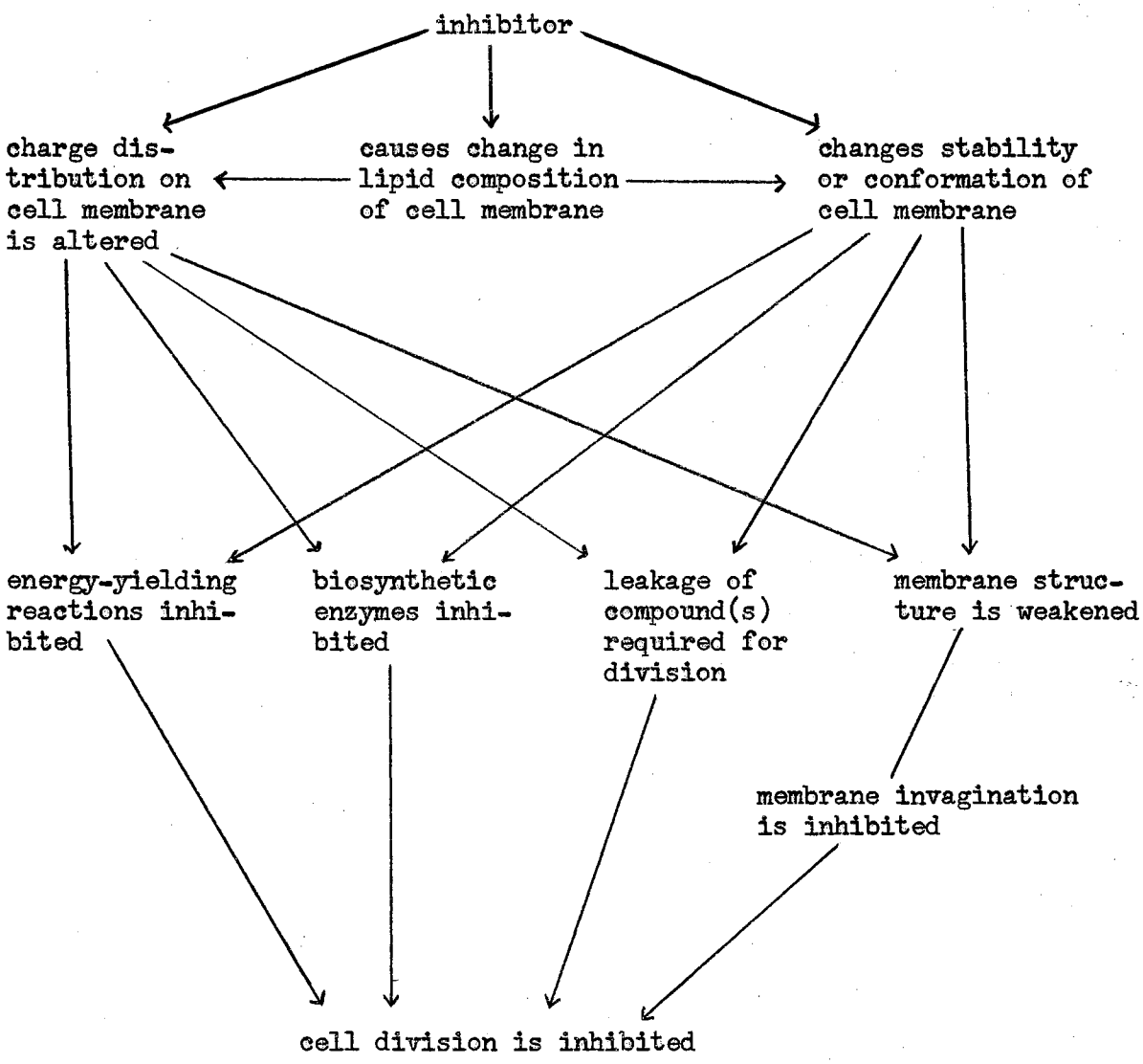
Phospholipids may exist in the membrane as bimolecular leaflets (12, 148). These leaflets are capable of reversible structural modifications (149). Furthermore, these leaflets become unstable upon incorporation of non-phospholipid molecules (97). This could lead to a loss of membrane stability and/or conformation.

A change in charge distribution and/or a change in membrane stability or conformation could impair activity of enzymes catalyzing energy-yielding and biosynthetic reactions, cause the leakage of some compound(s) required for cell division, and/or weaken the membrane so that invagination, hence, cell division, would be impaired (Figure 15). Any or all of these mechanisms could be responsible for division inhibition.

Energy is required for cell division (89) and energy-producing systems are located at the cell membrane (104, 164, 242, 261). Charge distribution alterations could alter the ionic nature either of the active site on the enzyme protein molecule or on the substrate to render

Figure 15

Proposed mechanisms for division inhibition.


inhibitor
charge distribution on cell membrane is altered
causes change in lipid composition of cell membrane
changes stability or conformation of cell membrane
energy-yielding reactions inhibited
biosynthetic enzymes inhibited
leakage of compound(s) required for division
membrane structure is weakened
membrane invagination is inhibited
cell division is inhibited

them inactive. Alternatively, the conformation of the membrane could be changed so that enzyme active sites and/or substrates are less accessible. This could result in decreased energy levels in the cell. Observations on the effect of D-serine on the oxidation and assimilation of glucose and mannose support this hypothesis. However, since inhibited cells continue to increase in mass, respire, and perform other functions necessary for maintenance of life, there cannot exist a general inhibition of energy production. It is necessary to postulate, therefore, that energy is channeled into certain areas and that division inhibitors interfere only with production of energy which is specifically channeled toward cell division. Supporting data include the observations of Grula and Grula (89) which suggest that the division mechanism is more susceptible to alterations in energy metabolism than other cellular functions. Slight damage to energy-producing mechanisms, therefore, could prevent cell division without significantly affecting other processes.

It is conceivable that ions and charged molecules, e.g. L-alanine, which prevent division inhibition could act in some way to restore normal charges sufficiently to permit these reactions to continue. However, it is difficult to imagine how osmotic agents could be functioning to prevent division inhibition if charge alterations are involved. Changes in membrane conformation and stability could, however, easily be corrected by osmotic agents.

It has also been reported that division inhibitors interfere with mucopeptide synthesis and that this damage is not repaired by pantoyl lactone or osmotic agents (91). The mucopeptide-synthesizing enzymes appear to be located at the cell membrane (21). Perhaps charge or conformational changes interfere with activity of the mucopeptide-

synthesizing enzymes. However, the mechanism of this particular inhibition should be separated from that of division inhibition since division inhibition, but not inhibition of mucopeptide synthesis, is overcome by pantoyl lactone and osmotic agents. Other biosynthetic enzymes are also located at the membrane, e.g. RNA synthesizing enzymes (157), and perhaps their functioning is affected. Previous data have shown that D-serine induces the accumulation of a uridine compound. This could have resulted from a block in pyrimidine and/or RNA metabolism.

Leakage of cellular materials from division-inhibited cells has been demonstrated (91). One possibility is that division-inhibiting agents destroy membrane stability and permit the leakage of a compound required for cell division. Pantoyl lactone could bind to the membrane in such a manner as to restore its "normal" permeability characteristics. Osmotic agents would also prevent leakage of this material. Leakage is prevented by pantoyl lactone. Grula and Grula (91) have shown that osmotic agents also prevent leakage.

Observations have indicated that filaments resulting from division inhibition are completely coenocytic. Membranes from inhibited cells could be weakened sufficiently to prevent septa formation. This, in turn, would result in division inhibition. Once again, pantoyl lactone and other reversing agents could either bind to the membrane and strengthen it or protect it osmotically.

Although it is not possible with a great deal of certainty to state which, if any, of these mechanisms is operating, any and all appear to be possible.

CHAPTER V

SUMMARY AND CONCLUSIONS

The effect of D-serine on the uptake and incorporation of several amino acids was studied. D-serine inhibited the incorporation of alanine, glycine, and glutamic acid but not lysine or leucine. Grula and Grula (91) have shown that D-serine inhibits mucopeptide synthesis 30 to 40% in Erwinia and this lesion may partially account for inhibition of incorporation of these compounds. The amount of radioactivity incorporated from C^{14}-alanine, glycine, and glutamic acid could have been diminished due to isotopic dilution with carbon from non-labeled D-serine although D-serine was metabolized at a rate much slower than these compounds. Inhibition of incorporation was observed within 20 minutes after addition of D-serine and did not appear to require as long a lag period as would be expected if inhibition of incorporation were due entirely to inhibition of mucopeptide synthesis and/or isotopic dilution. It is concluded, therefore, that inhibition of uptake and incorporation was mainly due to competition for entry into the cell.

Inhibition of incorporation of alanine, glycine, and glutamic acid has been separated from the cause of division inhibition by D-serine. Mitomycin c and vancomycin stimulated uptake and incorporation of glycine-C^{14}. This stimulation was probably due to damage to the cell membrane.

Neither D-serine, ultraviolet light, nor mitomycin c inhibited the synthesis of RNA, DNA, or protein at concentrations which inhibit cell

division. The relationship between nuclear division and cell division has been discussed. These have been separated into two distinct cellular events.

Protein of cells inhibited from dividing by D-serine has the same amino acid composition as non-inhibited cells. Apparently the protein of division-inhibited cells is similar qualitatively and quantitatively to that of non-inhibited cells although no information on conformation of protein structure or specific enzymes was obtained.

D-serine induced the leakage of cellular materials into the growth medium. After $1\frac{1}{2}$ to 3 hours, D-serine induced leakage of radioactivity from cells prelabeled with either D-glucose-U-C^{14}, DL-aspartic acid-4-C^{14}, or uracil-2-C^{14}. Leakage could not be detected from cells prelabeled with either thymine-2-C^{14} or thymidine-2-C^{14}. Leakage was prevented by addition of pantoyl lactone. Significant cellular lysis was not observed. This is probably due to damage to the cell membrane with the release of pool materials; however, it could also be due, in part, to a stimulated synthesis of uracil-containing compounds.

Examination of charcoal-adsorbable materials released into the growth medium from cells inhibited by D-serine revealed at least five UV-quenching compounds. None appeared to be identical to the uridine peptide precursors involved in mucopeptide synthesis. Two of these compounds were tentatively identified as xanthosine and uridine. These are associated with the division inhibition process since they were not released from control cells or cells sparked to divide by pantoyl lactone.

Five sulfur-containing compounds were found in spent growth media. However, their accumulation was not induced by D-serine. Similar compounds

were released from control cells. D-serine did, however, alter the intracellular content of sulfur-containing compounds. The relative concentration of several compounds was changed while an entirely new compound was found in division-inhibited cells. All but one of these sulfur compounds were broken into smaller fragments by acid hydrolysis. Their identity remains unknown. Perhaps this alteration in intracellular sulfur metabolism could alter the oxidation-reduction potential of the cell and lead to division inhibition by interfering with activity of an enzyme(s) required for cell division.

D-serine inhibited the incorporation of diaminopimelic acid-2-C^{14} into the protein-mucopeptide fraction approximately 37%. The obvious interpretation is that D-serine inhibits mucopeptide synthesis since Grula and Grula have shown that diaminopimelic acid and not lysine is present in the mucopeptide of this organism (unpublished data). However, vancomycin, penicillin, and mitomycin c, which have been reported to inhibit mucopeptide synthesis in this organism (91), stimulated the incorporation of radioactivity from diaminopimelic acid-2-C^{14}. Radioautography revealed that in all cases diaminopimelic acid-C^{14} was being rapidly converted to lysine-C^{14} which was incorporated into protein. Therefore, this is not a valid measure of mucopeptide synthesis. The stimulation of uptake by vancomycin, penicillin, and mitomycin c suggests damage to the cell membrane.

D-serine increased the amount of radioactivity from D-glucose-U-C^{14}, L-aspartic acid-U-C^{14}, and acetate-2-C^{14} incorporated into the lipid fraction of the cell. This was accompanied by a decrease in the amount incorporated into the protein-mucopeptide fraction. Partial restoration occurred in the presence of pantoyl lactone. Possible mechanisms for

this increase have been discussed. Radioactivity from non-inhibitory concentrations of D-serine-3-C^{14} was found mainly in the lipid and protein-mucopeptide fractions. Addition of non-labeled D-serine to division-inhibitory levels shifted the metabolism of D-serine-3-C^{14} resulting in a channeling of most of the radioactivity into the lipid fraction. However, only glycerol-C^{14} could be recovered from lipid hydrolyzates. Thus, D-serine was incorporated into lipids mainly as glycerol. Apparently D-serine increases the lipid content of the cell. Since most of the cellular lipids are located in the cell membrane, this implies that D-serine alters the lipid composition of the membrane. D-serine, however, did not alter the lipid phosphorus content of the cells. It is concluded, therefore, that D-serine increases the cellular content of non-phosphatides. Mitomycin c and penicillin did increase the lipid phosphorus composition of the cells. Penicillin, mitomycin c , and vancomycin stimulated the incorporation of C^{14}-glucose into lipids and increased cellular content of phosphatidylethanolamine. These antibiotics appear to increase the lipid content of the cell membrane.

Radioactivity from D-serine-C^{14} may be recovered in glutamic acid, alanine, glycine, serine, valine, and isoleucine. Addition of inhibitory concentrations of D-serine, however, interfered with the conversion of D-serine-C^{14} to glutamic acid, valine, and isoleucine. It is proposed that high concentrations of D-serine inhibit the decarboxylation of pyruvic acid. The carbon of D-serine then is shunted into lipids as glycerol. Conversion of D-serine to alanine and glycine would not be inhibited; however, conversion to glutamic acid, valine, and isoleucine would be inhibited.

All division inhibitors altered the cellular lipid profiles as

determined by chromatography on silicic acid columns. In general, this consisted of a decrease in the amount of material which eluted from the column as peak one (polyglycerol phospholipids and non-phosphatides) and an increase in that which eluted as the second peak (phosphatidylglycerol). Agents which reverse division inhibition did not cause the lipid profiles to return to normal.

The lipid composition of Erwinia sp has not been reported prior to this study. Approximately 80% of the extractable lipids are composed of phosphatidylethanolamine and two phosphatides thought to be phosphatidylcholine and phosphatidylinositol. Eighteen percent appear to be polyglycerol phospholipids and non-phosphatides while phosphatidylglycerol accounts for approximately 2%. Gas chromatography would be useful in identification of the non-phosphatides since they probably contain a large amount of free fatty acids.

Binding of penicillin to phospholipid has been demonstrated in vitro and in vivo. Binding occurred mainly to material in peak two (phosphatidylglycerol). Pantoyl lactone did not prevent binding of penicillin to phospholipid nor did it cause the removal of penicillin once bound. Several mechanisms are presented to explain how pantoyl lactone could reverse division inhibition by penicillin although it does not prevent binding of penicillin in the cell.

The lipid composition of a mutant strain of Erwinia sp which is resistant to vancomycin and penicillin differed from that of the wild-type organism. The mutant contained approximately five times more material in peak two than did the wild-type strain. Also, lipids from this mutant were still capable of binding penicillin. Although division of the mutant was not inhibited by penicillin or vancomycin, these anti-

biotics altered lipid profiles to the same extent as in the wild-type. It is speculated that binding of penicillin to material in peak two constitutes a detoxification mechanism; however, experimental results neither confirmed nor denied this hypothesis.

Cells of Erwinia sp were only slightly permeable to pantoyl lactone. Once taken up, however, it was incorporated into the lipid, nucleic acid, and protein fractions in approximately equal amounts. Pantoyl lactone may be released unchanged from lipid and protein by hydrolysis in 6 N hydrochloric acid at 100 C for 18 hours. Pantoyl lactone, like penicillin, was bound to material in peak two in vivo. However, this binding could not be demonstrated in vitro. It is postulated that the binding of penicillin to phospholipids causes some alteration in the cell membrane. This alteration then could be directly responsible for division inhibition. Pantoyl lactone could prevent division inhibition by overcoming or nullifying the effects of this alteration.

All agents tested which inhibit cell division had a profound effect on the cellular lipid content and membrane stability. However, all agents have also been shown to inhibit mucopeptide synthesis (91). This damage is not repaired by pantoyl lactone or osmotic agents. The fact that osmotic agents prevent division inhibition further implies a role for the cell membrane in the division inhibition process. Possibly the change in ratio of phospholipid to non-phospholipid coupled with a change in membrane conformation brings about a loss of stability and an inhibition of enzyme activities necessary for cell division. This could also involve leakage of a compound(s) which is (are) required for cell division.

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