EFFECT OF HYDROXYLYSINE ON BACTERIA

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CHAPTER I

INTRODUCTION

Since the discovery of hydroxylysine in 1921 by Van Slyke and co-workers, it has become apparent that this amino acid has a relatively restricted range of occurrence. Collagen and the gelatin derived from it are the principal sources of hydroxylysine in nature, although its occurrence has been reported in the proteins, trypsin and chymotrypsin, and among the free amino acids of several other sources. The formation of hydroxylysine in mammals is analagous to the formation of hydroxyproline. These two hydroxyamino acids are formed from the dietary lysine and proline. The hydroxyamino acids themselves are not essential in the diet nor are they utilized in collagen biosynthesis. Hydroxylysine will not replace lysine in mammalian or bacterial nutrition. Hydroxylysine will, however, produce a growth response in certain lactic acid bacteria growing in a medium limited in, but not devoid of, lysine. Hydroxylysine appeared to be much more effective in producing growth under these conditions than did an equimolar amount of lysine. The mechanism of this growth-promoting effect of hydroxylysine in Streptococcus faecalis and Leuconostoc mesenteroides is the subject of this investigation.

The effect of hydroxylysine on growth, cell stability, and cell wall synthesis was studied in comparison with lysine. The incorporation of hydroxylysine-^{3}H and lysine-^{14}C into various cellular components

was investigated. The mutual competition of lysine and hydroxylysine for a place in cellular macromolecules and the effects of chloramphenicol and penicillin on this process were studied.

CHAPTER II

LITERATURE REVIEW

Part A. Hydroxylysine

Occurrence of hydroxylysine in nature

Hydroxylysine (α,ϵ-diamino-δ-hydroxy caproic acid) was first discovered by Van Slyke and Hiller (1) among the acid hydrolysis products of gelatin. Subsequent work established that collagen was the only hydroxylysine containing protein (2-9). Recently Visawanatha and Irreverre (10) reported the occurrence of hydroxylysine in trypsin and chymotrypsin.

Hydroxylysine has been reported to occur as a free phosphate ester in several tissues of the ox (11-13); as a phosphatide in Mycobacterium phlei (14). Wirtschafter (15) reported the occurrence of hydroxylysine in amniotic fluid. Other reports place hydroxylysine in the purple pigment of sea anemone Adamsia rondeletti (16), associated with a protein-carbohydrate complex isolated from compact bone (17), in ethanol extracts of Corynebacterium diptheriae (18), in muscle extracts of the snake Agkistrodon blomhoffi (19) and possibly in date pericarp (20-21). Other doubtful reports also exist (22-24).

Isolation of hydroxylysine

Hydroxylysine was first isolated from gelatin by Van Slyke et al. (25) as the picrate which was separated from lysine picrate by fractional

crystallization. The two picrates were obtained from the "lysine fraction" of gelatin which was isolated by precipitation with phosphotungstic acid after removal of histidine and arginine.

Heathcoate (26) obtained pure hydroxylysine monopicrate by Van Slyke's procedure (25) from a lysine fraction separated by electrodialysis after histidine and arginine had been removed from the mixture with flavianic acid.

Martin and Synge (27) obtained a hydroxylysine monopicrate from hydroxylysine purified by the acetylation-benzoylation procedure of Synge. Sheehan and Bolhofer (28) used chromatography on alumina to separate lysine and hydroxylysine. Bergstrom and Lindstedt (29) employed partition chromatography on Hy-flo super-cel.

Ramachandran (30) isolated hydroxylysine as the oxazoline derivative and Robson and Selim (31) employed a carbon column to separate the picrates of lysine and hydroxylysine. Sorm and Mikes (32) used a combination of paper chromatography and cation exchange procedures to separate hydroxylysine.

The ion exchange technique of Moore and Stein (33, 34) has been applied by Hamilton and Anderson (4, 35, 36) to separate hydroxylysine from other basic amino acids.

Determination of hydroxylysine

Van Slyke *et al.* (2) determined hydroxylysine by measuring the ammonia evolved from the phosphotungstate precipitate when heated with periodate. Rees (37) and Ramchandran (30) also applied periodate oxidation to the determination of hydroxylysine. Chinard (38) described a ninhydrin procedure at pH 1.0 for hydroxylysine estimation.

Structure and synthesis of hydroxylysine

From periodate oxidation, ninhydrin reaction, and electrometric titration data, Van Slyke (25, 39, 40) proposed either α,ϵ-diamino-δ-hydroxy caproic acid or α,δ-diamino-ϵ-hydroxy caproic acid as the probable structure for hydroxylysine.

Sheehan and Bolhofer (41) showed the structure to be α,ϵ-diamino-δ-hydroxy caproic acid by conversion to methyl-α,ϵ-diphthalimido-δ-keto-DL-caproic acid, which was prepared from glutamic acid by an unambiguous route.

Several syntheses for hydroxylysine have been published (42-45), most via the malonic ester route and all leading to a mixture of four isomers. Fones (46) prepared the optical antipodes of hydroxylysine and allohydroxylysine and reported the optical rotation for both the hydrochloride salts and the free bases.

Witkop (47) applied Hudson's rule and proposed that the configuration of natural hydroxylysine was related to L-glyceraldehyde at C-2 and L-serine at C-6.

Hamilton and Anderson (35) have shown that racemization of hydroxylysine occurs predominately at the α carbon with prolonged refluxing in HCl.

Metabolism of hydroxylysine

Despite its occurrence in collagen, hydroxylysine is not an essential amino acid for animal nutrition (48, 49). Sinex and Van Slyke (50, 51) have shown that lysine is the source of collagen hydroxylysine. The relative rate of labeling of the two amino acids indicated that lysine was hydroxylated nearly simultaneously with incorporation. Both feeding and injecting of lysine-^{14}C led to lysine and hydroxylysine

of the same specific activity in rat skin collagen, indicating that lysine is the chief source of hydroxylysine. A similar conclusion was reached by Piez and Likens (52). Feeding of hydroxylysine did not alter the extent of *in vivo* incorporation of isotope from lysine-^{14}C into hydroxylysine of rat collagen of skin, bone, tail, and dentin. Kao and Boucek (53) arrived at the same conclusion with regard to collagen formed in implanted sponges. Lindstedt (54) showed that lysine was not formed from hydroxylysine in the animal body and that hydroxylysine would not replace lysine in maintaining growth of rats. Sinex *et al.* (55) found that radioactivity from 6-^{14}C-hydroxylysine was not incorporated into lysine or hydroxylysine of collagen to a significant extent.

Thus the formation of collagen hydroxylysine is presumably analagous to the formation of collagen hydroxyproline. Stetten and Schoenheimer (56, 57) have shown that proline is the precursor of collagen hydroxyproline and that hydroxylation apparently occurs after proline is incorporated into some bound form.

Lindstedt and Lindstedt (58) using the metabolite overloading technique have shown that hydroxylysine is converted to 5-hydroxypipecolic acid by rat liver or kidney. Turkey liver L-amino acid dehydrogenase has been reported to deaminate hydroxylysine to a product that can be reduced catalytically to 5-hydroxypipecolic acid (59). Cohen *et al.* (60) have reported this conversion to occur chemically.

Rabinovitz and Tune (61) have reported that hydroxylysine inhibits *in vitro* incorporation of lysine-^{14}C into rat bone marrow protein non-competitively. Rabinovitz *et al.* (62) have also shown that hydroxylysine

inhibits leucine, lysine, phenylalanine, and methionine incorporation into Ehrlich ascites tumor protein as an indirect result of its inhibition of glutamine synthesis. Inhibition seemed to be due to the L-allo isomer.

Bergstrom and Lindstedt (29) reported that hydroxylysine will not replace lysine in Leuconostoc mesenteroides growth as measured by lactic acid titration. Peterson and Carrol (63) found that small amounts of hydroxylysine greatly stimulated growth of L. mesenteroides P-60 and Streptococcus faecalis with sub-optimal levels of lysine. High levels of hydroxylysine inhibited L. mesenteroides but the inhibition could be reversed by additional lysine.

Mitchell and Houlahan (64) reported that hydroxylysine would not replace lysine in a lysine-requiring Neurospora mutant. Hydroxylysine (65) has also been shown to inhibit utilization of lysine in carrot tissue cultures. β-Alanyl-5-hydroxylysine has been reported to be synthesized by young chick pectoral muscle enzymes (66).

Leach (67) reported that hydroxylysine could neither spare nor replace lysine in the growth of ascites H cells in suspension. Radioactivity from labeled hydroxylysine was found in the cell pool indicating that hydroxylysine is taken up by these cells. Hydroxylysine did, however, stabilize cells following inoculation into fresh medium.

Tsung (68) has shown that there is no interconversion of lysine and hydroxylysine in S. faecalis since radioactivity from labeled lysine is not incorporated into hydroxylysine and vice-versa.

Part B. Biochemistry of Streptococcus faecalis

Cell wall composition of S. faecalis

In recent years it has become increasingly evident that a mucopeptide containing N-acetylglucosamine and N-acetylmuramic acid in its backbone structure forms the rigid layer of the bacterial cell wall (69-71). Major species' differences occur in the peptide linked via a peptide bond to the lactyl carboxyl group of muramic acid. This mucopeptide is the principal part of the cell wall of gram-positive organisms. Gram-negative bacteria have, in addition to mucopeptide, protein and lipid in their wall. Several gram-positive bacteria also possess various types of teichoic acid in their wall studies. The wall of S. faecalis also contains antigenic protein and carbohydrate groups (specific sugars, e.g. rhamnose).

Salton (72) reported paper chromatographic evidence indicating that glutamic acid, lysine, and alanine were the principal amino acids in the cell wall of S. faecalis NCTC 6782. Ninhydrin positive spots corresponding to serine, glycine, threonine, aspartic acid, valine and methionine, leucine and isoleucine were also detected. D-galactose, D-glucose, L-rhamnose, and a hexosamine were also found. Some other ninhydrin positive substances were detected, one of which, in the light of current knowledge, undoubtedly corresponds to muramic acid.

Investigations by Cummins and Harris (73, 74) showed a similar composition for the streptococci of Lancefield's groups A-G. They suggest that cell wall composition may be useful as a taxanomic aid.

Snell et al. (75) showed that the occurrence of D-alanine in S. faecalis ATCC 8043 and subsequently reported (76) that the D-alanine in S. faecalis existed in two forms. Forty per cent of the D-alanine was extractable by hot trichloracetic acid; the remainder was firmly bound in the insoluble residue from which it could be freed by vigorous

acid hydrolysis. These two forms undoubtedly correspond to the D-alanine present in teichoic acid and in mucopeptide. Snell et al. (76) also reported the replacement of D-alanine by D-α-aminobutyric acid in a growing D-alanine-requiring strain of S. faecalis. However, this replacement was not mole for mole and growth was decreased. Some L-α-aminobutyric acid was also taken up.

Ikawa and Snell (77) reported the occurrence of D-glutamic acid in the cell wall of S. faecalis Dunn. Aspartic acid, lysine, and alanine were also major components.

Ikawa and Snell (78) reported an extensive investigation of lactic acid bacterial cell walls, including those of S. faecalis and L. mesenteroides. Qualitatively, the cell walls of these two species are very similar; both contain glucose, rhamnose, glycerol, ribitol, glucosamine, and muramic acid. S. faecalis contains galactosamine but L. mesenteroides does not. The walls of both possess the same principal amino acids, i.e. glutamic acid, alanine, aspartic acid, and lysine. Serine, valine, and leucine were detected as minor amino acids in S. faecalis and glycine, serine, valine, threonine, histidine, and leucine in L. mesenteroides. The D-forms of both glutamic and aspartic acids predominate. Lysine is present exclusively in the L-form. Alanine is one-half in the D-form and one-half in the L-form.

The significance of the trace amino acids in walls of gram-positive bacteria is not known. They may be contaminants from adhering residues of the cell membrane or some other cellular structure or may represent an integral component of the wall.

Salton and Pavlik (79) reported the lysine:glutamic:alanine ratio in S. faecalis NCTC 6782 to be 1.0:0.9:4.0.

Shockman et al. (80) reported the ratio of D-glutamic:D-aspartic: L-lysine:L-alanine:D-alanine to be 1.0:0.9:1.1:1.1:1.0.

Ikawa (81) has separated purified cell walls of S. faecalis into a mucopeptide fraction, a glucose-aminosugar-rhamnose-ribitol phosphate fraction, a glucose-rhamnose-glycerol phosphate fraction and D-alanine by different chemical agents.

S. faecalis contains both ribitol phosphate and glycerol phosphate type teichoic acid (82). L. mesenteroides appears to contain a ribitol type teichoic acid and possibly a glycerol type as well (78).

Abrams (83) reported that S. faecalis ATCC 9790 cell walls contain approximately one per cent O-acetyl ester groups. Purified soluble macromolecular polysaccharide-amino acid preparations derived from cell walls migrated in paper electrophoresis experiments as a single band which contained O-acetyl groups. Abrams' organism was susceptible to N-acetylmuramide glycanohydrase (lysozyme, muramidase E.C. 2.3.1.17) and did not contain galactose; whereas, another strain investigated by Salton (84) contained galactose and was resistant to muramidase.

Effect of amino acid depletion

Extensive investigations by Toennies and Shockman (85) have indicated that incubation of S. faecalis beyond the depletion point of a given amino acid leads to marked changes in the cell. These changes are dependent on the amino acid depleted. Three distinct types of depletion were recognized. Depletion of lysine is followed by termination of the exponential phase and the prompt onset of rapid lysis which runs to completion. When valine is the limiting amino acid, its depletion is also followed by termination of exponential growth followed by a slow increase in dry weight of the culture which reaches

50% of the original weight after about twenty hours of incubation. This increase is due to formation of additional cell wall material. If threonine is limiting, its depletion is followed by cessation of exponential growth followed in 40 hr by a dry weight gain of 100%. Depletion of other amino acids leads to behavior intermediate in character between the above mentioned. In post exponential growth following either threonine or valine depletion, about seventy per cent of the total weight gain can be accounted for by new cell wall substance (80, 86). After threonine depletion, there is considerable nucleic acid formation but not after valine depletion (80). A role for the membrane in post exponential growth has been suggested. Indications were obtained (80) that evolution of exponential cells into post exponential cells is accompanied by a significant increase in cell membrane. Toennies et al. (87) have examined cytoplasm-membrane-wall ratios in S. faecalis. Threonine depletion post exponential growth involves nucleic acid synthesis, more wall synthesis, and less membrane synthesis; whereas, valine depletion post exponential growth does not involve nucleic acid synthesis and has less wall and more membrane synthesis compared to threonine depletion. Their data suggest that diverse shifts in cell morphology can occur as a result of withdrawal of single nutrients.

Lysis of S. faecalis

MacLeod (88) used biological purification to obtain Mn^{++} and Mg^{++} free media and demonstrated a Mn^{++} and Mg^{++} requirement for S. faecalis ATCC 8043. In his experiments, S. faecalis cells lysed at a greatly increased rate between the sixteenth and twenty-fourth hour when grown on sub-optimal levels of Mn^{++} and Mg^{++}. A small amount of Mn^{++} or a

large amount of Mg^{++} prevented lysis. Citrate interfered with the response to Mn^{++} at low Mg^{++} concentrations.

Van Eys and Pearson (89) studied early autolysis of S. faecalis (onset after about eighteen hours of growth) and found that Mn^{++} and citrate were involved. Autolysis was induced by large quantities of citrate due to its chelation of Mn^{++}. Autolysis was reversed by Mn^{++}, but high concentrations inhibited growth. Growth was inhibited by high concentrations of Mn^{++} even in filter sterilized medium and could be counteracted with the reducing agent, ascorbic acid. "Browning" was proportional to Mn^{++} concentration which in turn was proportional to growth depression; however, browning did not appear to be the cause of growth depression.

The work of Toennies and Shockman has amply demonstrated that S. faecalis undergoes lysis as a result of lysine depletion. Shockman et al. (90) have studied the factors which lead to lysis prone cells and factors which allow expression of the lytic phenomena. Lysis prone cells may be either exponential phase cells or cells depleted of a cell wall nutrient while in the exponential phase. Exponential cells become resistant to lysis after post exponential wall synthesis has occurred. The pH of the medium is important in the expression of lysis; lysis is more rapid at pH 6.5 than at pH 5.9. Ionic strength is also a factor; lysis is more rapid in phosphate buffer or sodium chloride solutions than in water.

Depletion of cell wall amino acids other than lysine results in lysis only under special conditions. For lysis to occur as a result of glutamic acid depletion, aspartic acid must also be absent (91) or the organism must be vitamin B_6-depleted in order for lysis to occur

as a result of alanine depletion.

S. faecalis cells also lyse as a result of glucose depletion (85). Glucose is the presumed precursor of cell wall polysaccharides as well as an energy source.

Shockman et al. (90) feel that lysis is due to a lytic enzyme system attacking the cell wall. He proposes that normal growth includes a balance between synthesizing systems and lytic systems. The lytic systems are believed to be necessary for growth, i.e. they function during cell division or enlargement of the wall. Depletion of a cell wall nutrient during exponential growth would then stop the synthesizing processes and allow the lytic process to predominate. In this regard, Shockman et al. (90) showed that cell walls from exponentially growing S. faecalis cells lysed when suspended in water or phosphate buffer; whereas, walls from threonine depleted organisms (post exponential wall synthesis had occurred) were stable under these conditions.

Montague (92) has found that S. faecalis walls contain a wall lysing system which releases all typical constituents of the wall into the soluble fraction. This system could not be removed from the walls by washing with water, salt solutions, or EDTA; it was little affected by Fe^{++}, Mg^{++}, or Mn^{++}. Heating at 100° for 10 min destroyed the autolytic activity.

Cell wall synthesis in S. faecalis

Shockman et al. (91) have studied the nutritional requirements for cell wall synthesis in S. faecalis. Cells of S. faecalis harvested in the exponential phase (log cells) and suspended in a simple medium consisting of buffer, glucose, salts, acetate, and five amino acids will engage in cell wall synthesis resulting in a turbidity increase

of about forty per cent. Ammonium sulfate and cysteine were necessary for the maximum turbidity increase. Deletion of acetate from the medium results in slow lysis. Deletion of glutamic acid, aspartic acid, lysine, and alanine leads to rapid lysis. Deletion of DL-alanine does not lead to lysis unless the cells have been depleted of vitamin B_6. The absence of L-glutamic acid results in lysis only if aspartic acid is also deleted. In vitamin B_6-depleted organisms, exogenous aspartic acid is necessary for wall synthesis, and the absence of either D- or L-alanine leads to lysis (93). Despite the fact that D-glutamic and D-aspartic acids occur in the cell wall of S. faecalis, the D-forms will not replace the L-forms nutritionally. D-alanine has, however, been shown to be required by S. faecalis in the absence of vitamin B_6 (79, 94). Toennies and Shockman (85) state, however, that the D-forms are not utilized efficiently for cell wall synthesis. Phenomena of this type might be explained by assuming that there are stereospecific permeases (95).

α-Ketoglutarate will replace glutamic acid for wall synthesis in the presence of aspartate but not in its absence nor in the presence of oxaloacetic acid (91). This indicates the involvement of a conventional transaminase.

Chloramphenicol, an inhibitor of protein synthesis, does not affect the turbidity increase which occurs when log cells are suspended in the simplified wall medium. Penicillin, an inhibitor of cell wall synthesis, leads to rapid lysis of the cells (93).

Mandelstam et al. (96) have recently reported that S. faecalis contains all the five enzymes necessary for synthesis of the nucleotide-peptide which accumulates in Staphylococcus aureus Copenhagen as a

result of penicillin inhibition. They also report the isolation of this nucleotide-peptide (UDP--N-acetylmuramyl--L-ala--D-glu--L-lys--D-ala--D-ala) from a strain of S. faecalis as a result of penicillin inhibition. In addition to the usual compound, a novel nucleotide-peptide was also isolated. It appears to have the structure uridine-diphospho-N-acetylmuramyl--L-ala--D-glu--L-lys(ϵ-L-ala)--D-ala--D-ala. This result provides an explanation for the 1:1 D-alanine:L-alanine ratios reported in S. faecalis (78, 80).

CHAPTER III

EXPERIMENTAL PROCEDURE

Growth studies

Media: The media of Henderson and Snell (97) or Leach and Snell (98) were used with the appropriate level of L-lysine or hydroxylysine (either L or the mixed racemate containing all four isomers). In some experiments hydroxylysine was added aseptically after growth had progressed for a period of time. Media were routinely incubated a minimum of 24 hr after sterilization as a check for possible contamination.

Organisms: The organisms used in this study were as follows: Streptococcus faecalis ATCC 8043, Leuconostoc mesenteroides P60 ATCC 8042, and a wild type of Staphylococcus aureus obtained from the Microbiology Department of Oklahoma State University.

Sterilization: Media were sterilized by autoclaving for 5 to 10 min (for 2 to 10 ml volumes) at 121° and 15 psi. Glucose was usually sterilized separately and combined with the sterile media aseptically; although with short sterilization times, browning, due to the presence of glucose, was not sufficient to interfere with the experiments. In the experiments involving attempts to demonstrate nucleotide-muramyl-peptide accumulation, in media containing 0.5 M sucrose, the media were filter sterilized to prevent browning and hydrolysis of sucrose. Chloramphenicol and penicillin solutions were also filter sterilized and combined aseptically with the appropriate sterile media.

TABLE I

COMPOSITION OF MEDIA USED IN GROWTH EXPERIMENTS

Compound	Henderson and Snell medium	Leach and Snell medium
	mg	mg
DL-alanine	200	40
L-arginine·HCl	40	48.5
L-asparagine	--	80
L-aspartic acid	100	20
L-cysteine	--	10
L-cystine	20	--
L-glutamic acid	200	60
Glycine	20	10
L-histidine·HCl	20	12.4
DL-isoleucine	40	50
DL-leucine	40	50
DL-methionine	40	20
DL-phenylalanine	40	20
L-proline	20	20
DL-serine	40	90
DL-threonine	40	40
DL-tryptophan	40	8
L-tyrosine	20	20
DL-valine	40	50
	g	g
Glucose	4.0	1.0
Sodium acetate	0.2	0.6
Sodium citrate	4.0	4.0
Ammonium chloride	0.6	--
Ascorbic acid	--	0.05
	mg	mg
KH_2PO_4	1000	50
K_2HPO_4	160	50
$MgSO_4 \cdot 7H_2O$	8	20
$FeSO_4 \cdot 7H_2O$	32	1
$MnSO_4 \cdot 4H_2O$	8	1
NaCl	2	1
Adenine sulfate·H_2O	2	0.5
Guanine·HCl·$2H_2O$	2	0.5
Xanthine	2	0.5
Uracil	2	2.5
Guanylic acid	--	10.0
Thiamine·HCl	0.2	0.04
Pyridoxal·HCl	0.04	0.08

TABLE I (CONTINUED)

Compound	Henderson and Snell medium	Leach and Snell medium
	mg	mg
Calcium pantothenate	0.2	0.08
Riboflavin	0.2	0.08
Nicotinic acid	0.2	0.08
p-Aminobenzoic acid	0.04	0.04
Biotin	0.002	0.0004
Folic acid	0.002	0.002
Distilled water	100 ml	100 ml

One ml of the basal medium was diluted with 1 ml of water

Inoculation and maintenance of stock cultures: Stock cultures of S. faecalis and L. mesenteroides were maintained in yeast extract-glucose-agar stabs. The lysine-independent S. faecalis culture was maintained in 2% agar stabs made from lysine-free Henderson and Snell medium. The hydroxylysine-tolerant S. faecalis culture was maintained as above except that 200 μg/ml of racemic hydroxylysine was added to the lysine-free medium.

Inocula were grown in 5 ml cultures of either 1% glucose, 1% peptone, and 1% yeast extract for S. faecalis and L. mesenteroides or the appropriate medium for the lysine-independent and hydroxylysine-tolerant organisms. Inocula were grown 18-24 hr, harvested by centrifugation and washed with and suspended in either 5 ml of cold, sterile water or 5 ml of cold, sterile physiological saline solution. One drop of this suspension was used to inoculate 2 to 10 ml cultures or 2.5 ml for large batches of media.

Measurement of growth: Turbidity of cultures in optically matched tubes was measured at 660 mμ on a Coleman Jr. Model 6 spectrophotometer. Appropriate uninoculated blanks were used in all cases. In some experiments growth was reported as mg dry-weight of cells. This was determined by measuring the absorbancy of a diluted aliquot of the cell suspension at 660 mμ and determining dry weight from an absorbance versus dry weight curve.

Cell stability studies

Susceptibility to autolysis and muramidase: Cells were grown in the medium of Henderson and Snell. One culture was grown in the presence of 100 μg of L-lysine per ml (grown on lysine) and another in the presence of 20 μg of L-lysine and 20 μg of hydroxy-L-lysine per ml

(grown on hydroxylysine). Cells were harvested by centrifugation during the exponential phase of growth and washed twice with cold 0.9% NaCl solution. At this point, each culture was divided in half. One-half was used to study the effect of muramidase and the other to study autolysis. Cells equivalent to 5 mg dry weight were suspended in 10 ml of the following solutions: 0.9% NaCl, 0.85% KCl, 0.3 M sodium phosphate buffer, pH 6.5 and water. The resulting suspensions were incubated at 37° and the absorbancy determined at intervals with a Coleman model 6 spectrophotometer. The experiments involving muramidase were conducted as mentioned except that the solutions contained 5 μg per ml of muramidase and 133 μg per ml of ethylenediaminetetracetic acid tetrasodium salt (EDTA). Aseptic technique was used throughout, with the exception that muramidase was made up in sterile water and used immediately.

Susceptibility to disruption by sonic oscillation: Two 5 liter cultures of S. faecalis were grown on the medium of Leach and Snell (98), one containing 100 μg per ml of L-lysine and the other containing 20 μg per ml of L-lysine plus 20 μg per ml of hydroxy-L-lysine. After 12 hr of growth at 37°, the cells were harvested by centrifugation and washed with 0.9% NaCl solution. They were then suspended in sufficient 0.9% NaCl solution to give a standard absorbancy. A 50 ml sample of each batch of cells was subjected to sonic oscillation in a Raytheon 10 kc sonic oscillator set at 1.2 ampere output current. Samples were removed at various times and the absorbancy of an aliquot was determined at 660 mμ. The remaining sample was centrifuged for 5 min to remove whole cells and debris, and the absorbancies at 280 and 260 mμ were then determined on a diluted aliquot of the supernatant solution to

measure the release of soluble protein and nucleic acids.

Cell wall synthesis

Cultures of S. faecalis were grown as previously described. Cells were harvested while in the exponential phase of growth, washed twice in cold 0.9% NaCl solution, and suspended in a medium containing only the compounds found essential for cell wall synthesis in this organism (91). The composition of the medium used is shown in Table II. These are the same concentrations as are used in the Henderson and Snell medium (97). During the experiments, one or more of the amino acids was omitted and replaced by hydroxy-L-lysine at a level of 20 μg per ml. These experiments were conducted in the presence or absence of chloramphenicol (100 μg per ml) or penicillin G (33 μg per ml, 55 units).

Tritium and carbon-14 experiments

Counting procedure: Radioactivity was determined by counting in a Packard Tri-Carb model 314 A liquid scintillation spectrometer. One-tenth ml of a solution of the material to be counted was added to either 0.1 ml of 2 N NaOH or 0.1 ml of hyamine (1 M in methanol) solution in a counting vial. To this was added 10 ml of solvent consisting of 60% toluene and 40% absolute ethanol. The phosphor was 0.5% 2,5-diphenyl-oxazole and 0.2% 1,4-bis-2'(5'-phenyloxazolyl-)benzene. This system has an efficiency of approximately six per cent for tritium and forty per cent for ^{14}C. Radioactivity on paper chromatograms was detected by scanning with a Radiological Service Co. chromatogram scanner.

Labeled compounds: Uniformly labeled L-lysine was obtained from Volk Radiochemical Corporation; the specific activities were 1.17 mc/mmole and 4.64 mc/mmole. Hydroxy-L-lysine dihydrochloride was isolated from gelatin by C. M. Tsung (68) and labeled with tritium by the

TABLE II

COMPOSITION OF MEDIUM USED IN CELL WALL SYNTHESIS EXPERIMENTS

	Concentration
	mg/ml
L-lysine	0.2
DL-alanine	1
L-glutamic acid	1
L-cystine	0.1
L-aspartic acid	0.5
Sodium citrate	20
Glucose	20
Sodium acetate	1
K_2HPO_4	5
NH_4Cl	3
$MgSO_4 \cdot 7H_2O$	0.8
$FeSO_4 \cdot 7H_2O$	0.04
NaCl	0.04
$MnSO_4 \cdot 4H_2O$	0.16
	Adjusted to pH 6.8

Wilzbach procedure. It had a specific activity of 3.54 mc/mmole.

Distribution of radioactivity under growing conditions: Cells were grown either in the medium of Henderson and Snell or Leach and Snell containing the appropriate levels of labeled compound (L-lysine-^{14}C or hydroxy-L-lysine-^{3}H) and unlabeled compound (hydroxy-L-lysine, the mixed racemate of hydroxylysine or L-lysine) for the desired time. See the "Results" section for details concerning individual experiments. Cells were harvested by centrifugation, washed twice with cold water or cold 0.9% NaCl, and an aliquot fractionated by the method of Park and Hancock (99). Aliquots of the extracts were counted as previously described. In some experiments only the cold trichloracetic acid precipitable material was counted to determine total incorporation. This material was solubilized with trypsin and muramidase prior to counting.

Distribution of radioactivity under resting conditions: Cells were grown at 37° in flasks fitted with a side arm which could be inserted in a Klett colorimeter. Cells were harvested during the log phase at about half maximal growth as determined from a growth curve. Harvesting and washing were carried out at 0° as rapidly as possible, never exceeding 2 hr, to prevent damage to the cells. Washing was done with water. These precautions were observed whenever log cells were harvested. Shockman et al. (90) have shown that log cells are not damaged under these conditions. The concentration of the cell suspension was determined from an absorbance versus dry weight curve so that a suitable aliquot could be suspended in the experimental medium. Log cells so harvested were suspended in the complete or wall medium containing labeled compounds and/or inhibitors and incubated for a

suitable length of time. Harvesting and fractionation were then conducted as previously described.

Interconversion of lysine and hydroxylysine: Cells were grown in the presence of the appropriate labeled compound, harvested and washed as previously described. Aliquots of the cells were hydrolyzed in 3 N HCl in an autoclave at 121° and 15 psi. HCl was removed in a rotary evaporator under vacuum. The hydrolysate was decolorized with Norite A and filtered. The compound to which conversion was expected was added as carrier and lysine and hydroxylysine separated as described by Hamilton and Anderson (35). Aliquots of fractions were counted in the scintillation spectrometer and quantitative ninhydrin determinations made to locate compounds emerging from the column.

Chemical and preparative procedures

Cell fractionations: The fractionation scheme employed was that of Park and Hancock (99). Two and one-half to 5 mg of cells were fractionated by successive extractions with 2.5 to 5 ml of the following: cold 5% trichloracetic acid at 0° for 10 min, 75% aqueous ethanol at room temperature for 10 min, and 5% trichloracetic acid at 90° for 6 min. Cells were centrifuged and washed with the extracting media or the tube walls were wiped dry between extractions. The material remaining after the hot trichloracetic acid step was suspended in ammonium bicarbonate buffer (0.05 M NH_4HCO_3, 0.005 M NH_4OH) and trypsin (E.C. 3.4.4.4) added at a substrate to enzyme ratio of 50:1 by weight. The resulting suspensions were incubated at 37° and the turbidity measured at 700 mμ at intervals. Digestion was considered complete when additional trypsin failed to bring any further turbidity decrease. The mixture was then centrifuged and washed. The supernatant solution is the

trypsin-soluble protein and the residue is cell wall mucopeptide. Cold trichloracetic acid extracts the cell pool and some teichoic acid, ethanol extracts lipid and some protein-like material, and hot trichloracetic acid extracts the teichoic and nucleic acids.

Preparation of mucopeptide: Large amounts of mucopeptide were prepared by a scaled up Park and Hancock procedure. Extractions of cells from a one liter culture were done in 100 ml volumes with constant stirring. Extraction times were 30 min, except for hot trichloracetic acid which was 15 min. Trypsin digestion occurred overnight in 0.05 M ammonium bicarbonate--0.005 N ammonium hydroxide buffer, which also contained 100 μg/ml of chloramphenicol and penicillin to prevent contaminating growth. The residue was washed six times by centrifugation in NH_4HCO_3 buffer. The entire sequence of digestion and washing was repeated once and mucopeptide material was centrifuged slowly to remove debris. The mucopeptide was then sedimented by centrifugation (10,000 x g 10 min) and the pellet dried by washing with acetone.

Column chromatography techinques: Cell wall hydrolysates were added to a 1 x 10 cm column of Dowex-1-acetate and the neutral and basic amino acids were washed through with water. The glutamic and aspartic acids were eluted from the Dowex-1-acetate with 0.5 M acetic acid. The solution of neutral and basic amino acids was concentrated in vacuo, dissolved in pH 3.4, 0.1 M citrate buffer, and put onto a Dowex-50x8-Na^+, 200-400 mesh, 1 x 13 cm column previously equilibrated with 0.1 M, pH 3.4 citrate. Elution was accomplished with 0.1 M, pH 5 citrate buffer. Quantitative ninhydrin and radioactivity determinations were made on appropriate aliquots. Deionized water was used throughout.

Paper chromatography techniques: Material to be chromatographed was spotted on Whatman No. 1 paper and chromatographed in the appropriate solvent. Butanol:acetic acid:water 4:1:1 was used in the first dimension and phenol saturated with an aqueous solution of 6.3% Na citrate-3.7% K_2HPO_4 for the second dimension. Amino acids were located by spraying with an ethanol solution of ninhydrin or a mixture consisting of 0.1 g ninhydrin, 100 ml ethanol, 30 ml acetic acid, and 4 ml γ-collidine. Other solvent systems used were: phenol:water:ammonia 80:20:0.3, butanol:acetic acid:water 3:1:1, pyridine:water 3:1, and ethanol:water 3:1.

Quantitative ninhydrin method: This procedure is essentially that of Rosen (100) except for some slight modifications (101). To a sample volume of 1.5 ml add 0.25 ml of cyanide-acetate solution (0.2 ml of 0.01 M KCN diluted to 10 ml with a solution of 360 g of sodium acetate$\cdot 3H_2O$ and 67 ml glacial acetic acid in 1 liter). Add 0.25 ml of 0.3% ninhydrin in methylcellosolve and heat in a boiling water bath for 15 min. Then 1 ml of isopropanol:water 1:1 was immediately added and mixed. After cooling the absorbancy was read at 570 mμ in the spectrophotometer. Deionized water was used throughout.

Muramidase digestion of mucopeptide: Mucopeptide was suspended in 0.1 M ammonium acetate solution and muramidase added. Digestion was followed by measuring turbidity at 700 mμ. When digestion was complete (no further turbidity decrease with additional muramidase), the solution was concentrated to a viscous syrup at 50° in vacuo. This material was diluted to 20 ml with water and passed through a column of Sephadex G-25 and eluted with water.

Uridine diphosphate-N-acetylmuramyl-peptide accumulation: Cells

were harvested in the log phase with previously mentioned precautions and suspended in various lysine-free media which contained 0.5 M sucrose in some cases. Incubation was carried out for 90 min, and cells were harvested and washed with cold water or with 0.5 M sucrose if it was present in the medium. The cells were then extracted with 10% trichloracetic acid for 1 hr at 0°. Trichloracetic acid was removed from the extract by extraction with water-saturated ether. Cells were also exposed to penicillin in the log phase for 90 min and treated as above. N-acetylamino sugar determinations were carried out on the extract by the method of Strominger (102).

N-acetylamino sugar determination: The following steps were used: 1) to 0.1 ml extract add 0.1 ml of 0.25 N HCl; 2) heat in boiling water bath for 5 min; 3) add 0.2 ml of 0.125 N NaOH in 2% sodium tetraborate; 4) heat in boiling water bath for 7 min; 5) add 1.6 ml glacial acetic acid, 0.2 ml 16% p-dimethylaminobenzaldehyde in 95% acetic acid-5% 11.6 N HCl; 6) incubate at 37° for 20-25 min and read at 550 mμ immediately. Omission of the acid heat step gives the blank value for each determination.

Immunological techniques

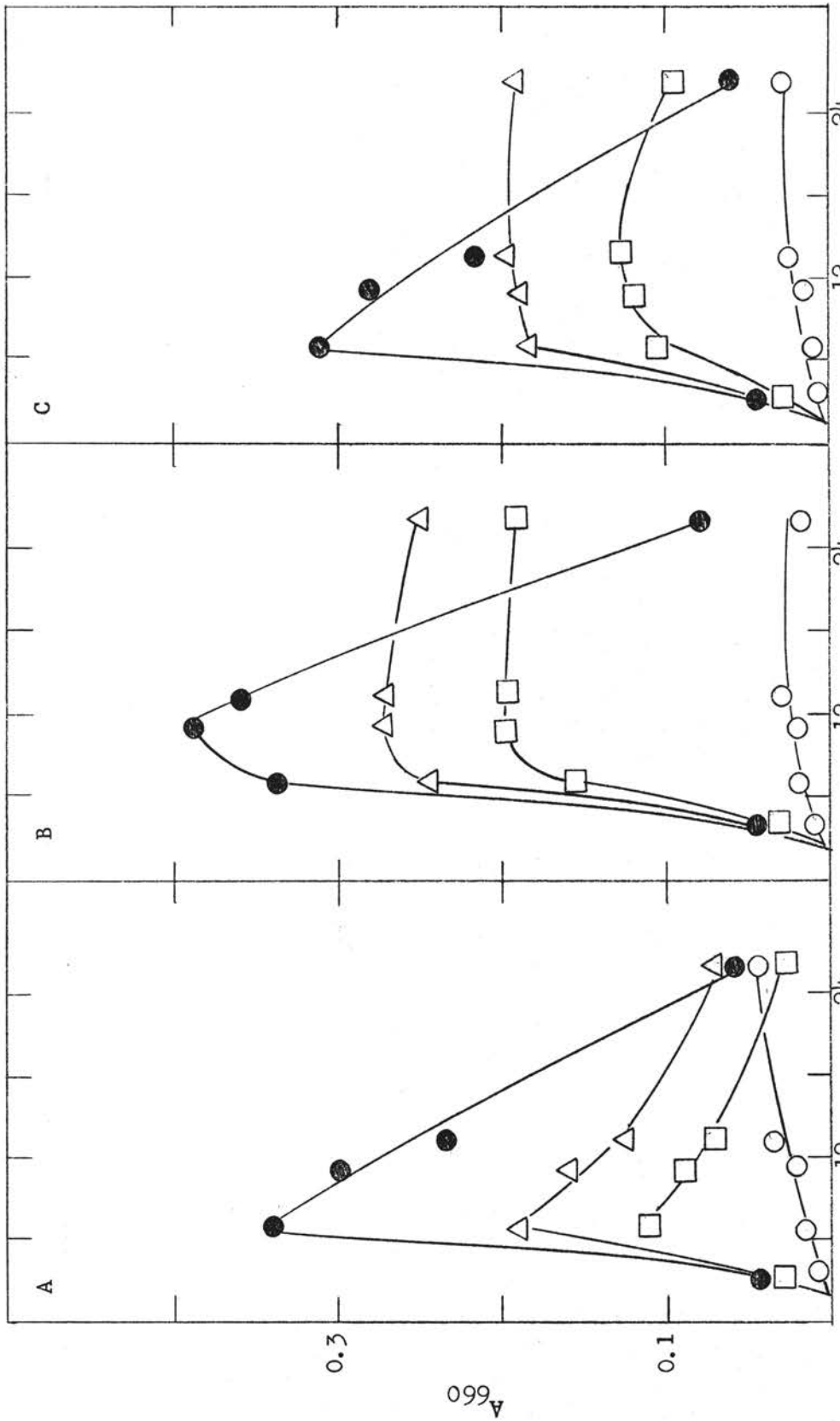
Rabbits were injected 2-3 times weekly over a period of 2 months with a physiological saline suspension of either lysine mucopeptide or hydroxylysine mucopeptide. At the end of this time they were bled by heart puncture, the blood allowed to clot, and the serum obtained by centrifugation. Antibody was titered by adding antigen to serial dilutions of sera, heating for 1 hr at 56°, then incubating overnight at 37°. Hydroxylysine, lysine, and glucose were checked for inhibition of agglutination.

CHAPTER IV

RESULTS AND DISCUSSION

Growth studies

In the results of other investigations (63, 68) concerning the growth-promoting effect of hydroxylysine on lactic acid bacteria in the presence of sub-optimal lysine levels, growth was measured after a fixed time. In view of the well-documented (90) lytic behavior of S. faecalis, it seemed desirable to investigate the effect of hydroxylysine during the entire growth cycle. Figures 1A and 1B show the results of such an experiment using the medium of Leach and Snell (98) with varying levels of lysine. After about eight hours of growth, the onset of rapid lysis was observed (Figure 1A), presumably due to depletion of lysine from the medium. In Figure 1B it can be seen that 10 μg/ml of hydroxy-L-lysine prevented lysis at the lower levels of lysine but had no effect at the high (40 μg) level. From Figure 1C it will be noted that similar results were obtained if the addition of hydroxylysine were delayed until growth had progressed for 7 hr. In other experiments in which up to 200 μg/ml of lysine was present (Figure 2), lysis still occurred after about twelve hours of growth. This is well above the optimum levels of lysine for S. faecalis. The complete medium of Toennies and Shockman (85) contains only 110 μg/ml. In the experiments reported here hydroxylysine was effective in preventing lysis only at the lower levels of lysine (10-20 μg/ml). Some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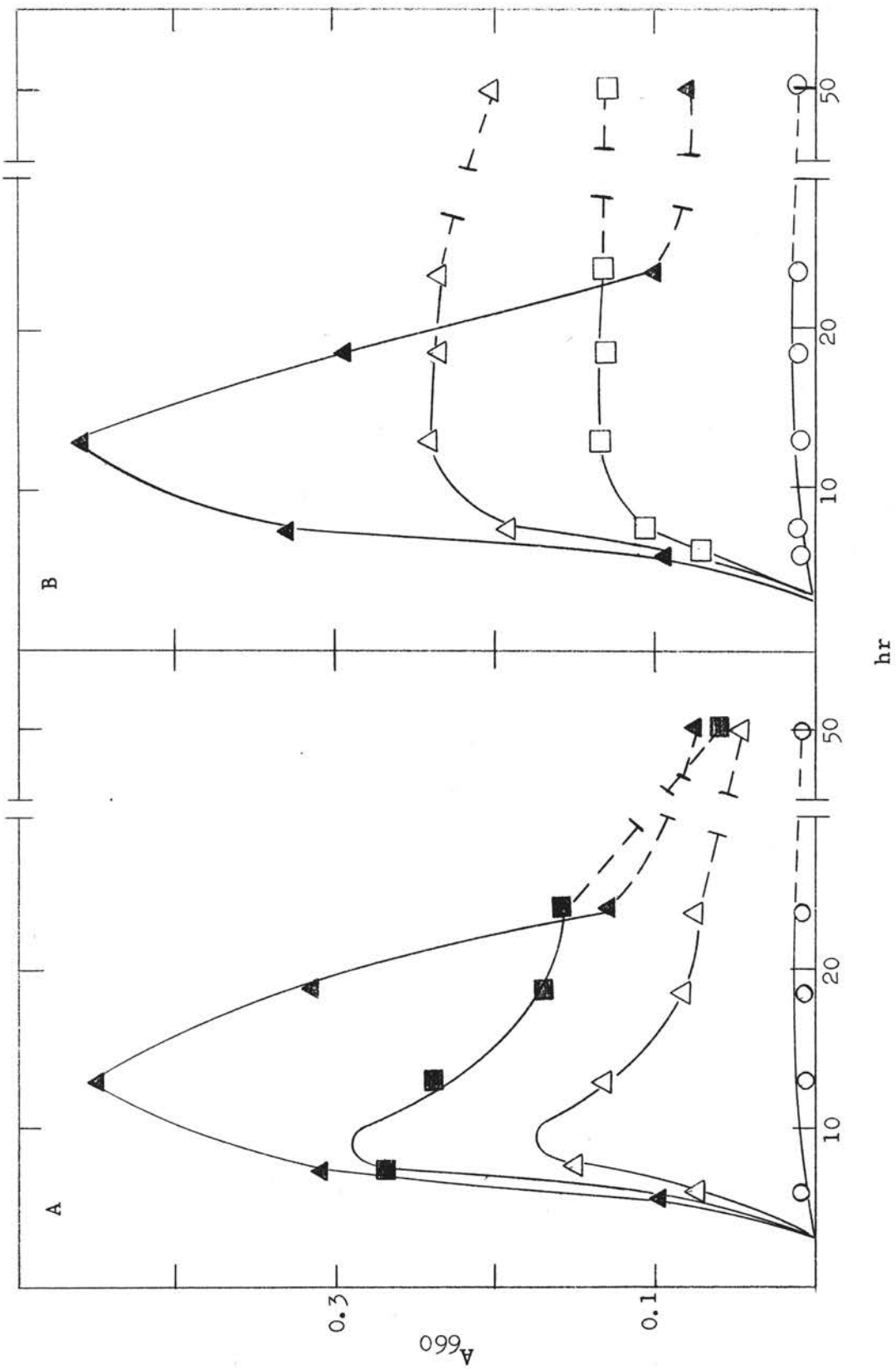
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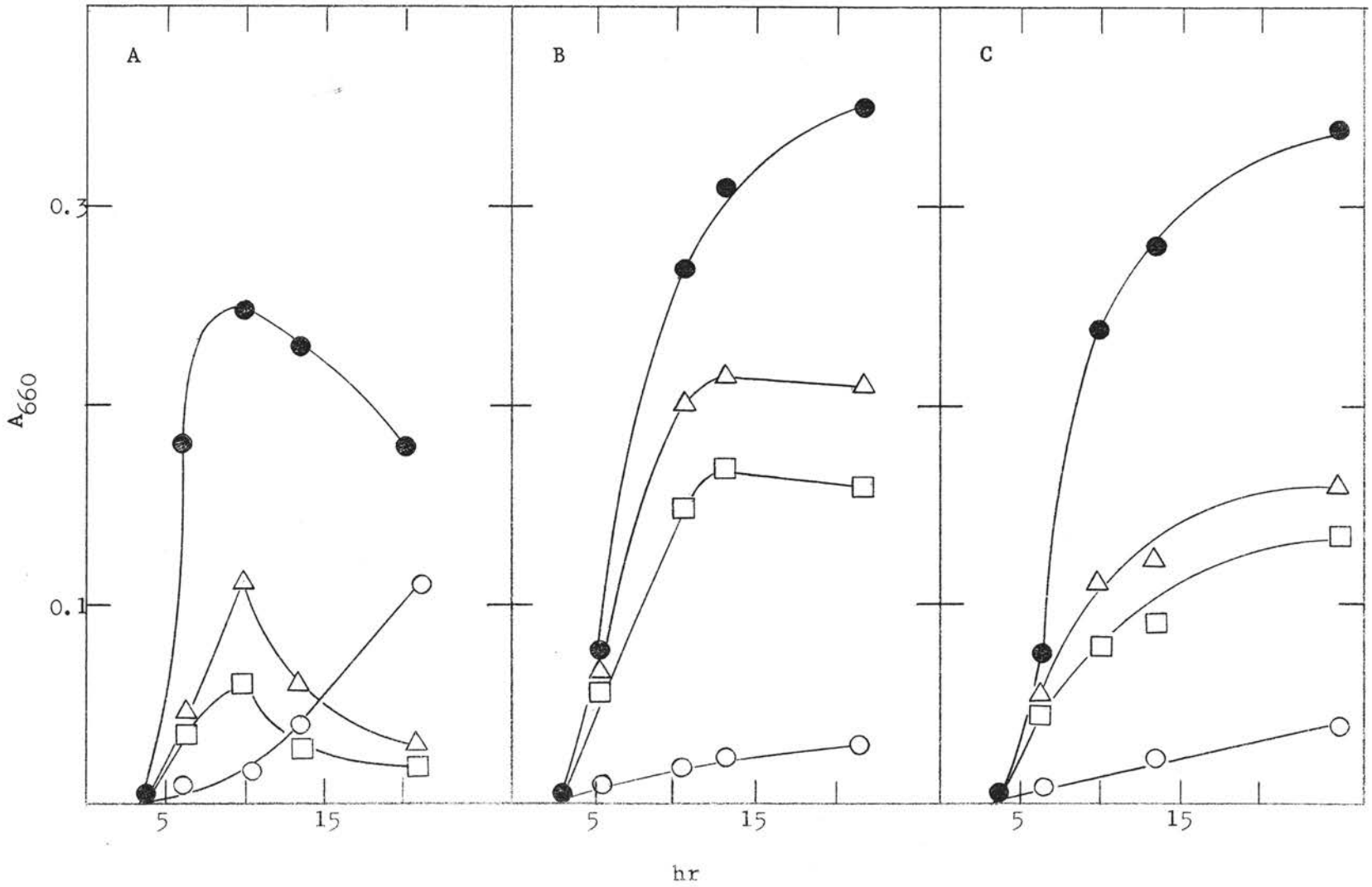
protection was afforded at 30 μg/ml of lysine, but the cells lysed between the twenty-fourth and fiftieth hours. However, at 10-20 μg/ml the cells were stable for at least 50 hr when hydroxylysine was present.

These results suggest that hydroxylysine can protect S. faecalis from lysis if the lysine concentration of the Leach and Snell medium does not exceed 20 μg/ml. It should be pointed out, however, that the Leach and Snell medium may be deficient in some wall precursor other than lysine, probably glucose. This medium contains only 5 mg/ml of glucose; whereas, the Henderson and Snell (97) medium and Toennies and Shockman (85) medium contain 20 mg/ml. Curves similar to those shown in Figure 2 can be obtained on the Henderson and Snell media if the glucose concentration is decreased to 5 mg/ml.

The results depicted in Figure 3 are from an experiment identical to that which gave the results shown in Figure 1 except that the Henderson and Snell medium was used. Ten μg/ml of hydroxylysine protected (Figure 3B) the cells from lysis even at the higher (40 μg/ml) level of lysine. Similar results were obtained if the hydroxylysine were added after 7 hr of growth (Figure 3C). Contrary to the findings with the Leach and Snell medium, an optimum level of lysine could be reached in the Henderson and Snell medium at which no lysis occurred (Figure 4A). As shown in Figure 4B hydroxylysine prevented lysis at the 40 μg/ml lysine level; whereas, it did not with the other medium (Figure 1B). The results shown in Figure 4A indicate that 60 μg/ml of lysine was sufficient to allow S. faecalis to grow out of the log phase and support post-exponential cell wall synthesis, in which case the organism lost its susceptibility to autolysis (90).

In the experiments reported in Figure 5A, 20 μg/ml of hydroxylysine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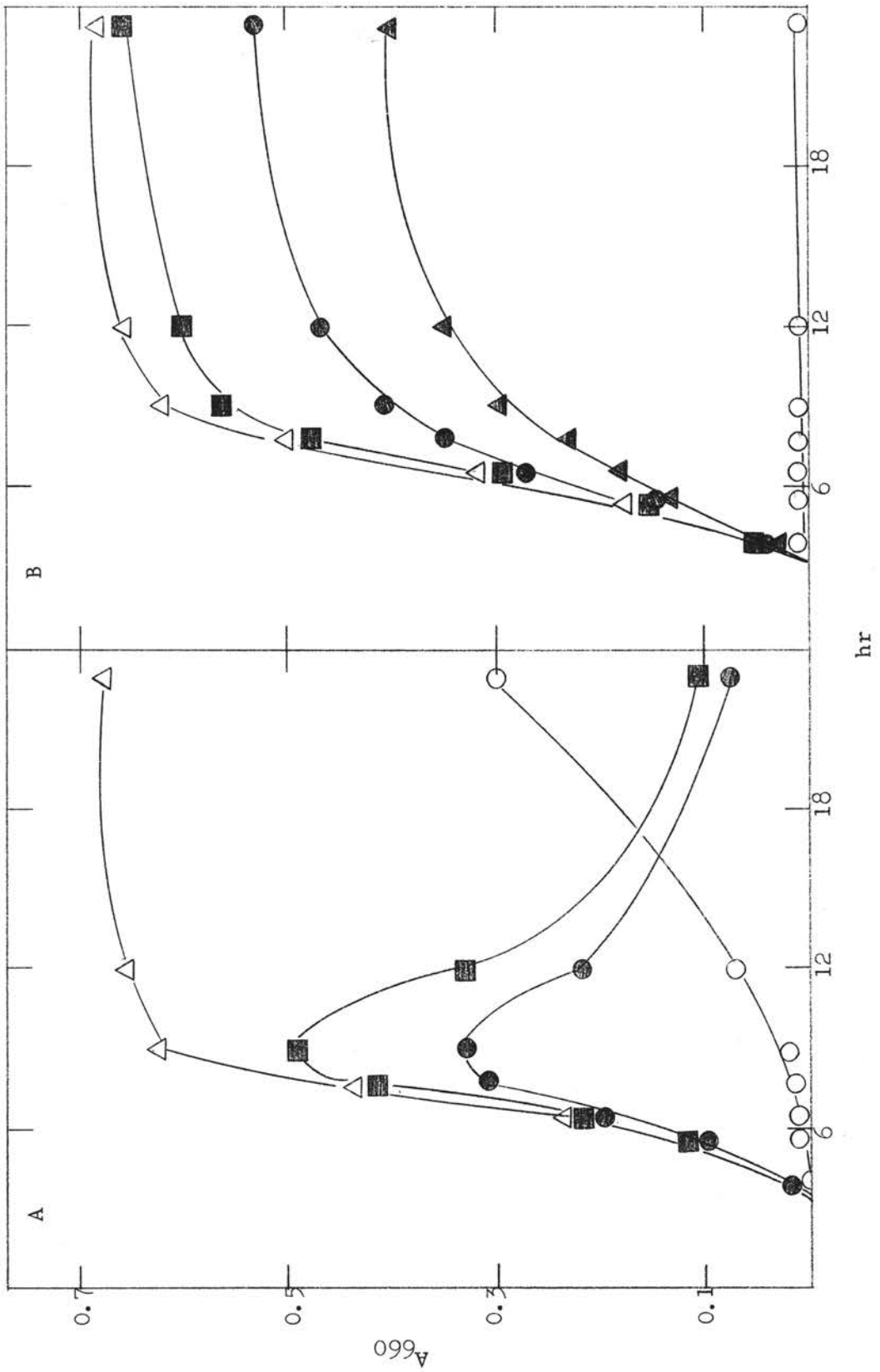
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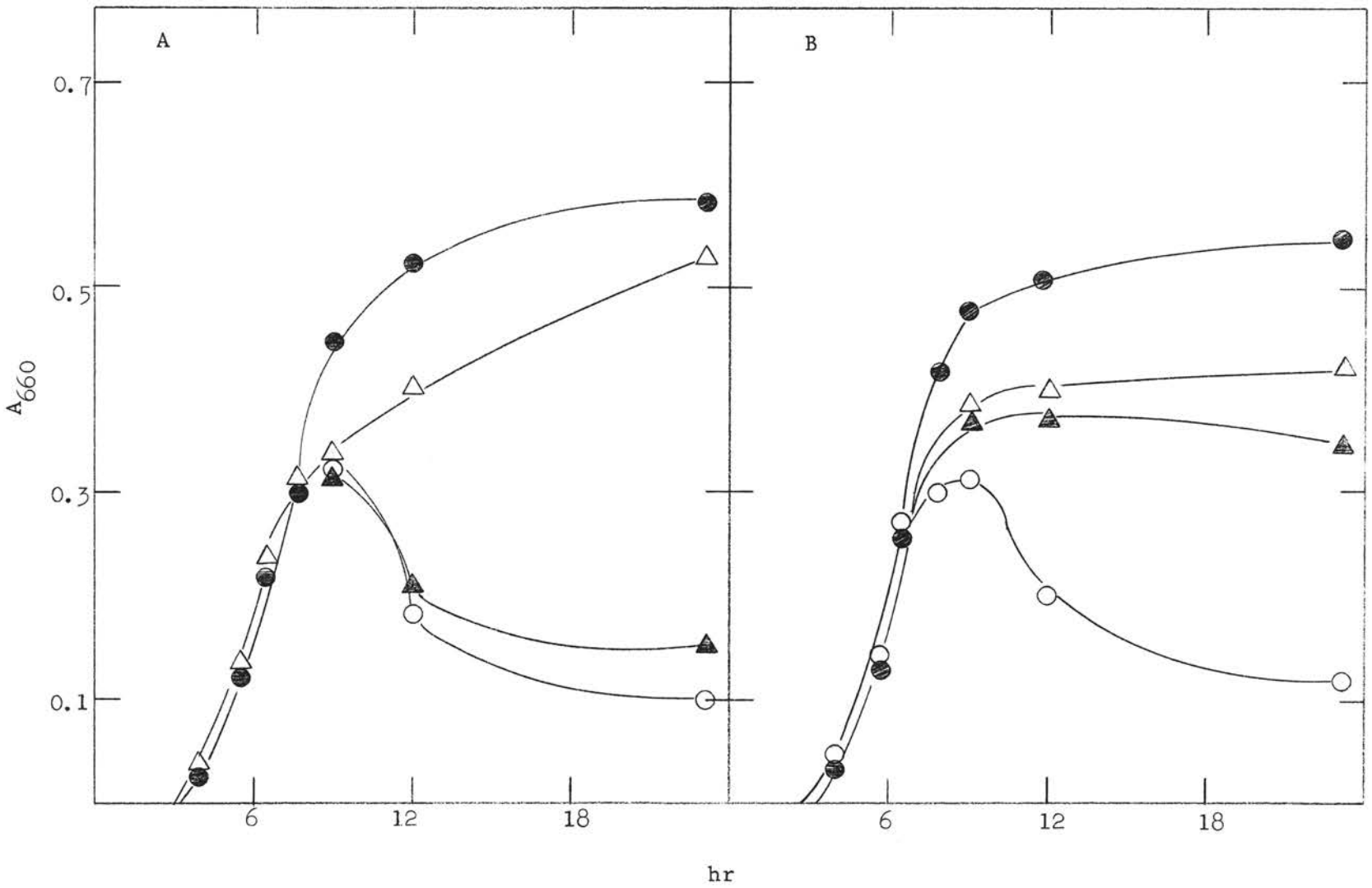
was added to the cultures aseptically at the indicated times. Hydroxylysine added during the log phase was almost as effective as if it were present initially (compare Figures 5A and 4B). Figure 5B illustrates the growth response of S. faecalis to increments of hydroxylysine. It is evident that the time at which absorbancy was observed had a marked effect on the amount of growth stimulation attributed to hydroxylysine. The response appeared much greater when absorbancy was determined after 20 hr of growth than when absorbancy was determined after only 12 hr of growth.

The above experiments were all conducted with hydroxy-L-lysine, but the mixed racemate gave similar results. About forty μg/ml of mixed racemate was required to afford maximum protection from lysis in the presence of 20 μg/ml of lysine; whereas, only 5 to 10 μg/ml of hydroxy-L-lysine gave adequate protection. When the two compounds were compared with regard to total growth response, the L isomer was about two and one-half times as effective as the mixture of isomers. This relative activity of the L isomer was effective over the range of 15 μg to 50 μg of lysine per ml and indicated that the naturally occurring isomer was utilized more efficiently by this organism.

Hydroxylysine is also known to elicit a similar growth response in L. mesenteroides. The time course of this response was investigated and the results presented in Figure 6. As demonstrated in Figure 6A there was no lysine-depletion lysis with L. mesenteroides; however, a comparison with Figure 6B shows that hydroxylysine produced a growth response with the lower levels of lysine, although possibly not so dramatic as that obtained with S. faecalis. These results indicated that while protection from lysine-depletion lysis accounts for much of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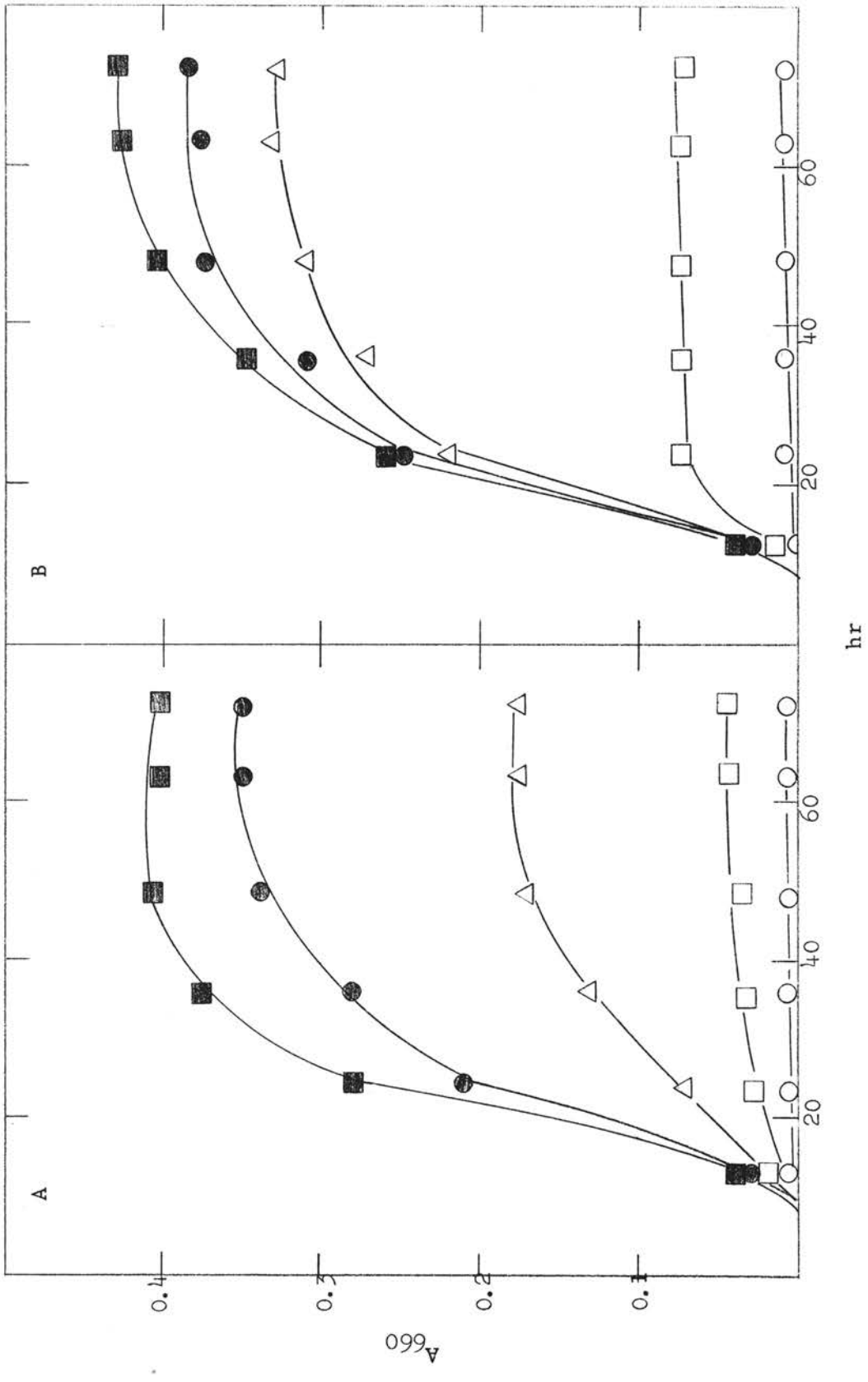
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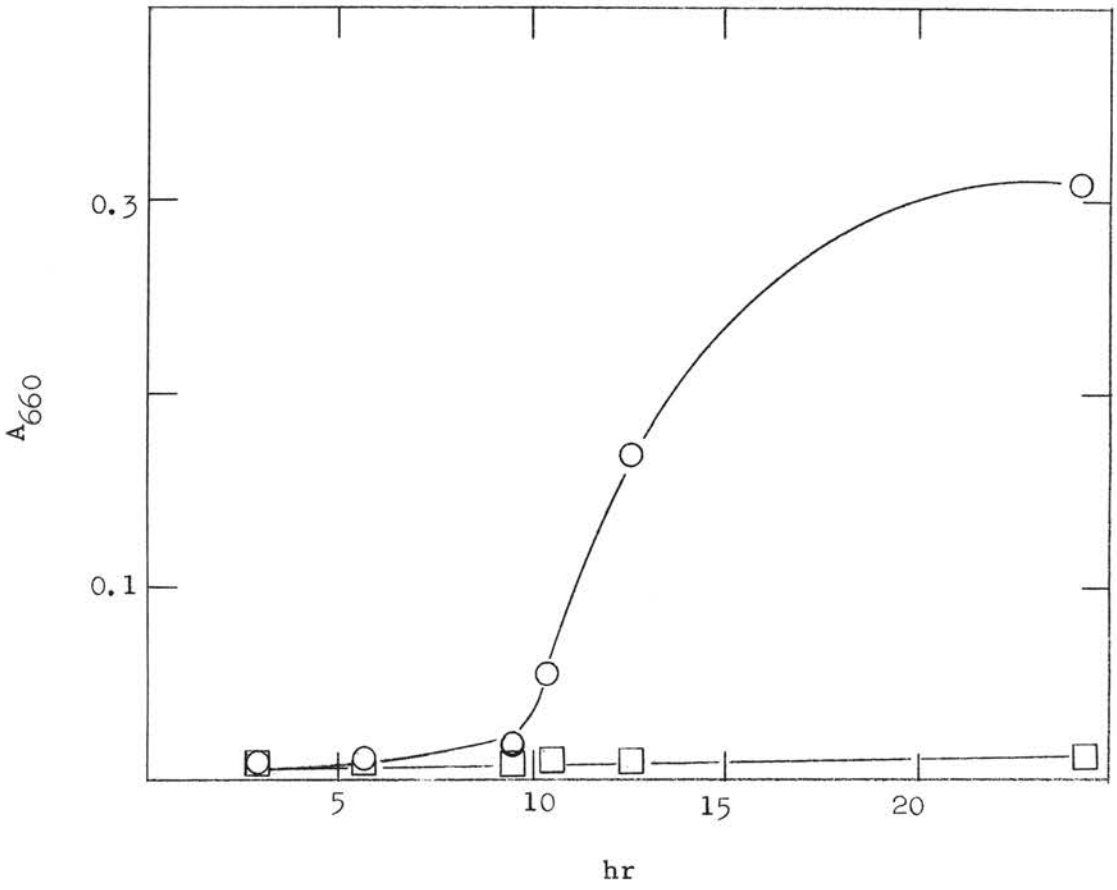
60
hr

the observed growth response to hydroxylysine in S. faecalis (Figure 5B), it apparently cannot explain any of the response in L. mesenteroides unless the lysis is not observable by the method used.

During the course of the previous experiments it was noted that S. faecalis would begin to grow in the absence of lysine after 12 to 24 or more hours and that hydroxylysine would prevent this growth. Figure 7 shows the results of an experiment designed to test this observation. The organism which grows under these conditions appears identical with S. faecalis when examined microscopically. Repeated streaking of the stock culture and isolation of individual colonies and their subsequent use as inocula did not alter the results. The media were incubated at room temperature for up to three weeks prior to inoculation to check for possible contamination. Both the mixed racemate and the L isomer were effective growth inhibitors in the absence of lysine. The data presented in Figure 8 indicate that there is some relationship between the concentration of hydroxylysine and the time required for maximum growth to occur in the absence of lysine. This growth has a sudden onset and proceeds rapidly to its maximum extent in 8 to 10 hr. There seems to be an almost linear relationship between time required to attain maximum growth and hydroxylysine concentration within the range of 0 to 5 μg/ml. Above 5 μg/ml the results were somewhat erratic. No growth occurred at hydroxylysine concentrations above 10 μg/ml during the course of the experiments. A probable explanation for the growth inhibitory effect of hydroxylysine in the absence of lysine is that hydroxylysine represses formation of enzymes involved in or otherwise interferes with the synthesis of lysine.

During the course of these studies, the S. faecalis which grew on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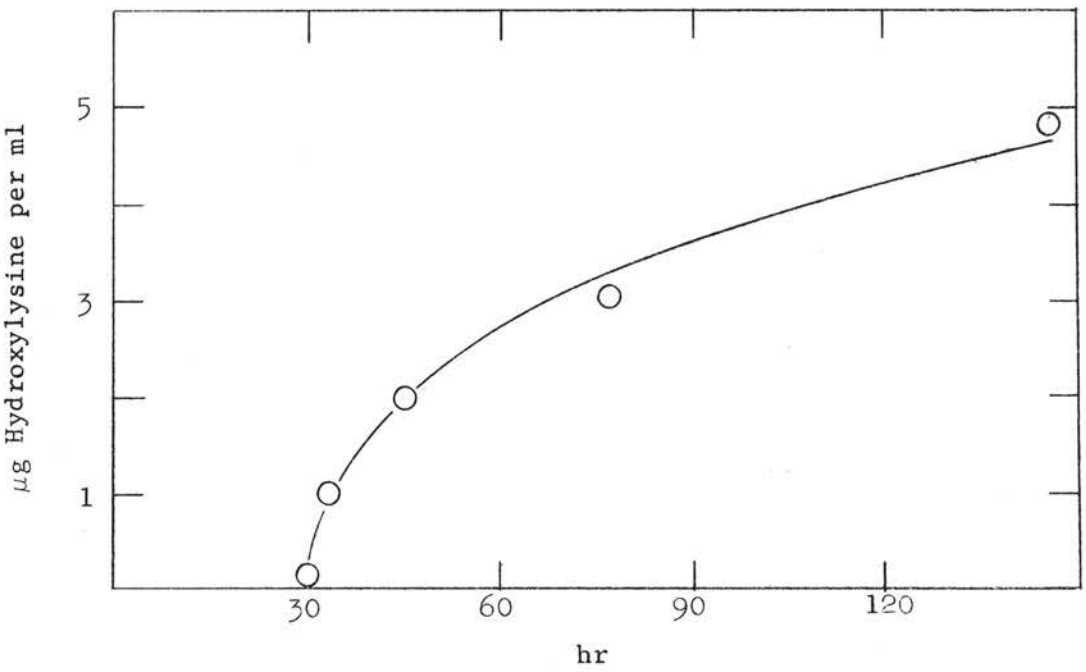

μg Hydroxylysine per ml
5
3
1
30
60
90
120
hr

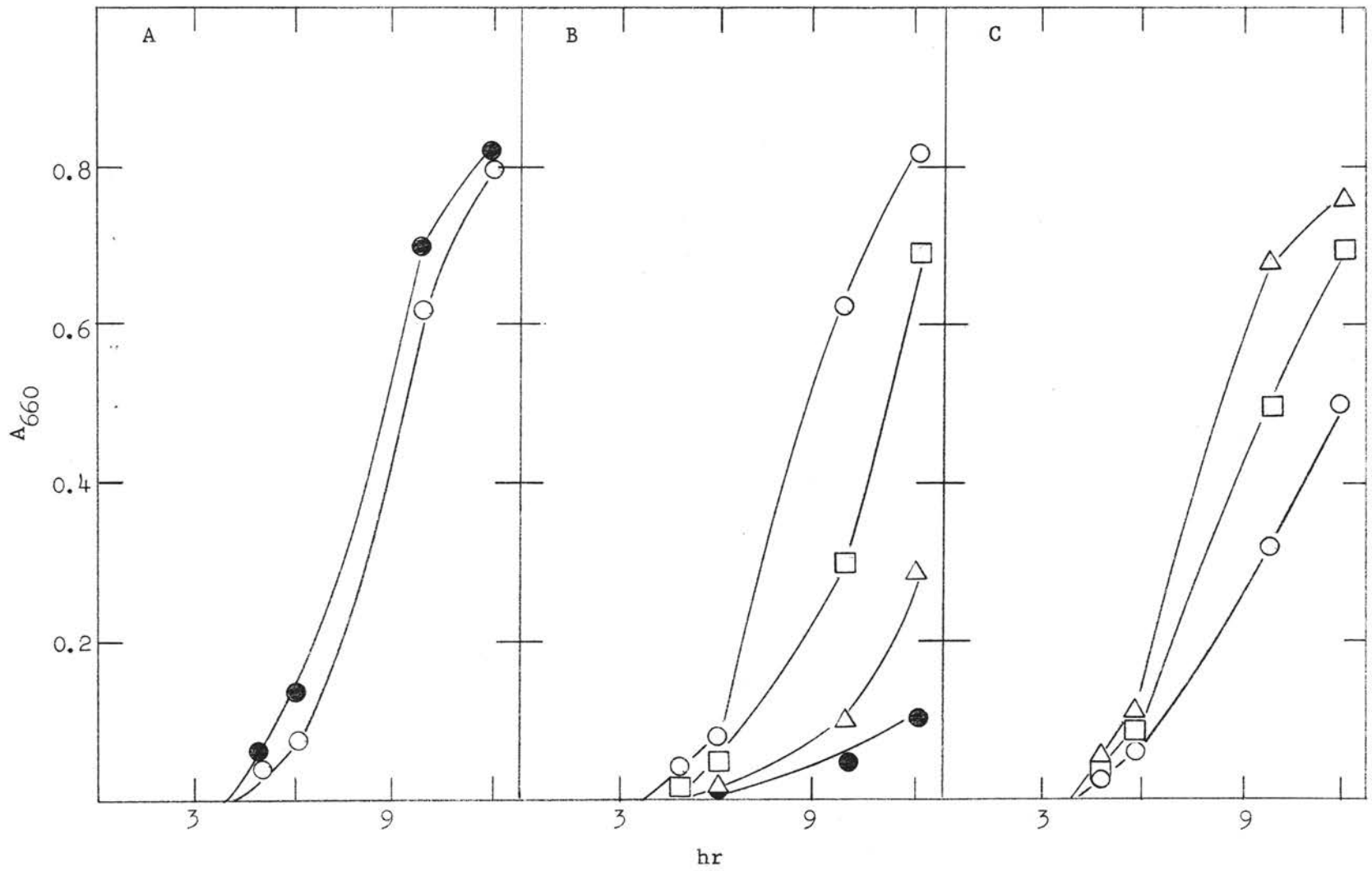
low levels of hydroxylysine was transferred to media containing higher levels of hydroxylysine. After a few transfers with streaking on hydroxylysine-containing agar and single colony isolations between transfers, the organism gave maximum growth within 24 hr in the presence of 200 μg/ml of the mixed racemate. This organism was designated "hydroxylysine-tolerant" (hylys-tol).

The S. faecalis which grows in absence of lysine will also attain maximum growth in 24 hr after being treated as above except that lysine-free agar and media were used for growth and colony isolation. This organism was designated "lysine-independent" (lys-i).

The hydroxylysine-tolerant organism will grow equally well in the presence or absence of hydroxylysine, but it responds to additional lysine. Growth of the lysine-independent organism is inhibited by hydroxylysine, and this inhibition can be overcome by additional lysine. These data are summarized in Figures 9 and 10. Neither of these organisms display any lytic phenomena under the conditions employed, presumably because they do not depend on an exogenous supply of lysine.

Cell stability

Susceptibility to autolysis and muramidase: The data presented in Figures 11-14 show that exponential cells grown in the presence of hydroxylysine are lysed at a slower rate than cells grown on lysine. The phosphate buffer suspension of hydroxylysine-grown cells after 1 3/4 hr was 70% as turbid as it was initially; whereas, the suspension of cells grown on lysine was only 38% as turbid. Similar results were obtained for the NaCl, KCl and water suspensions; however, the suspending medium influenced the rate of lysis. Hydroxylysine-grown cells were lysed slower in all cases. Lysis was most rapid in phosphate buffer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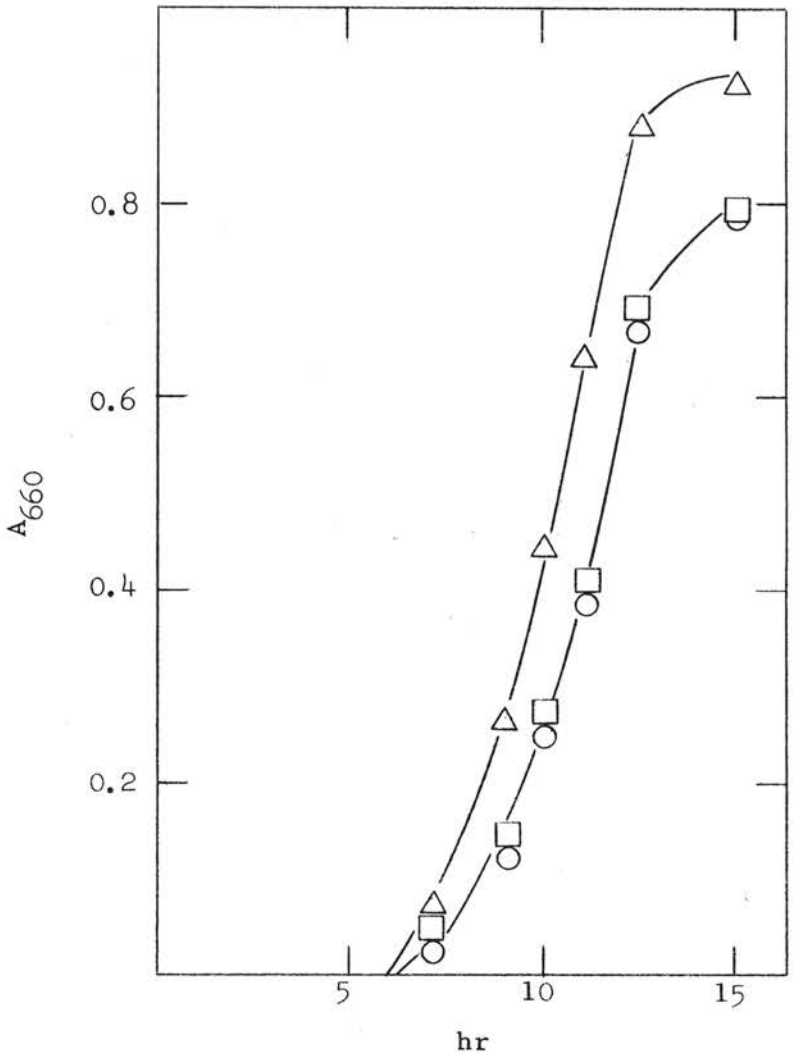
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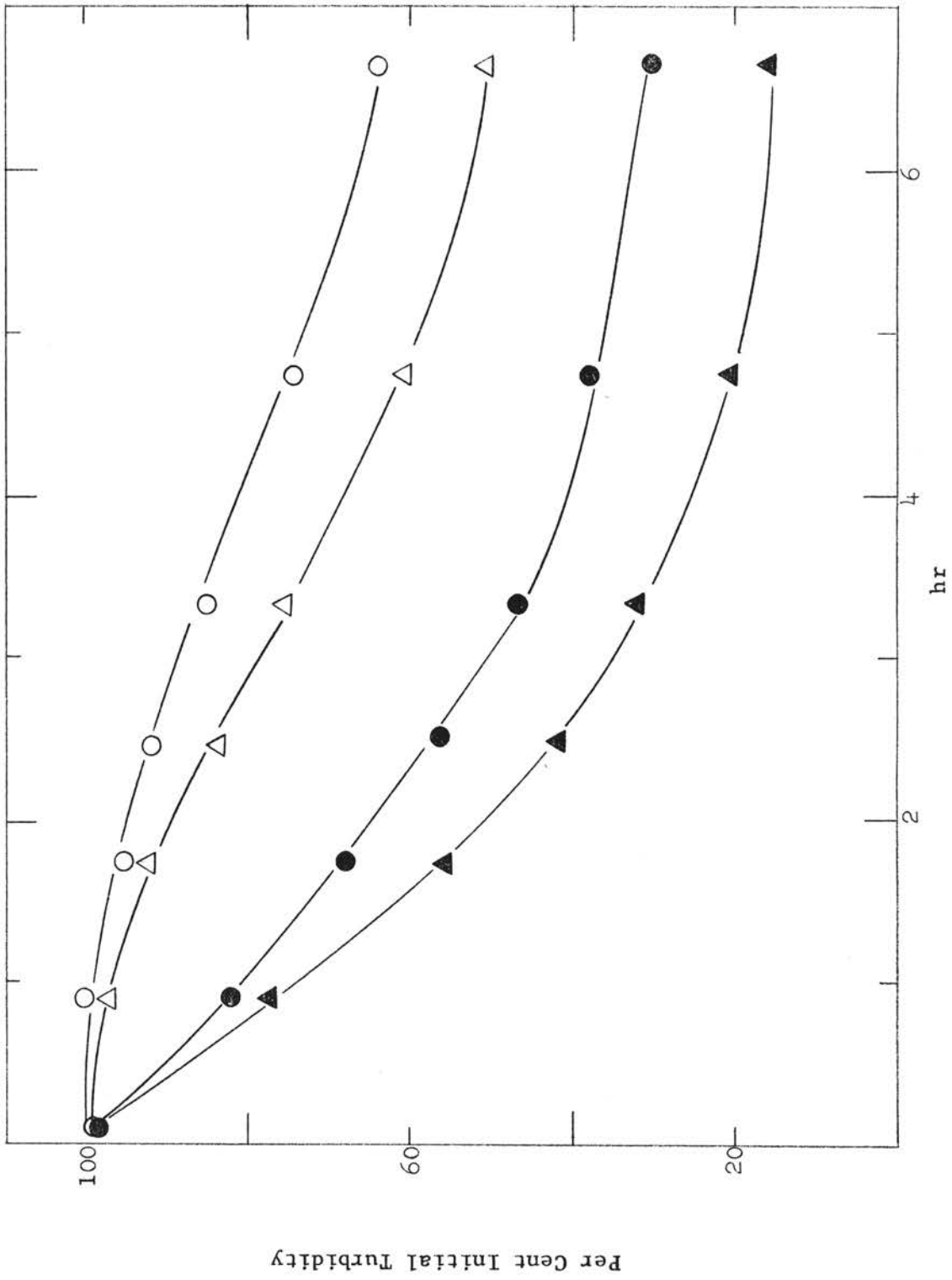
Per Cent Initial Turbidity
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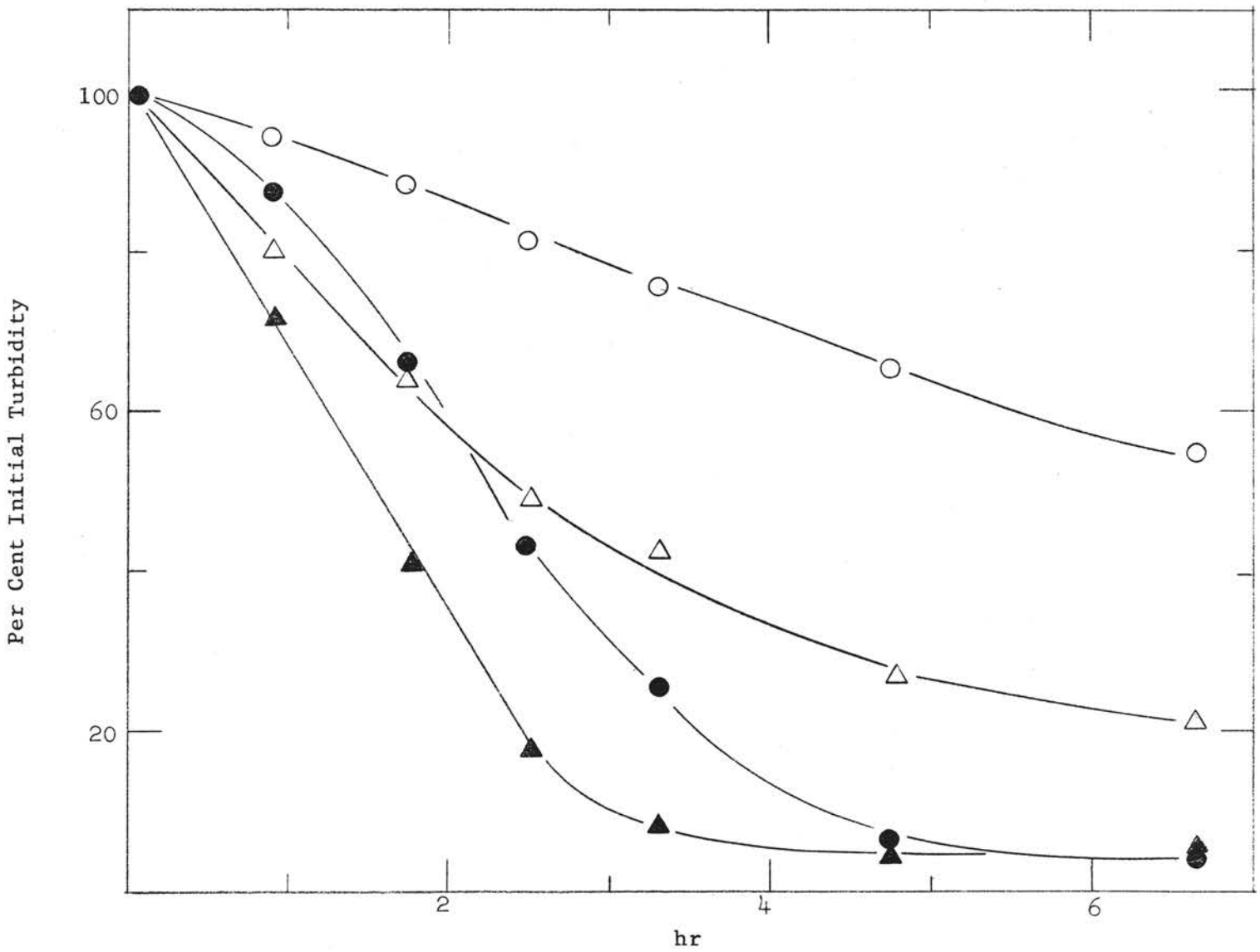
60
20
2
4
6
hr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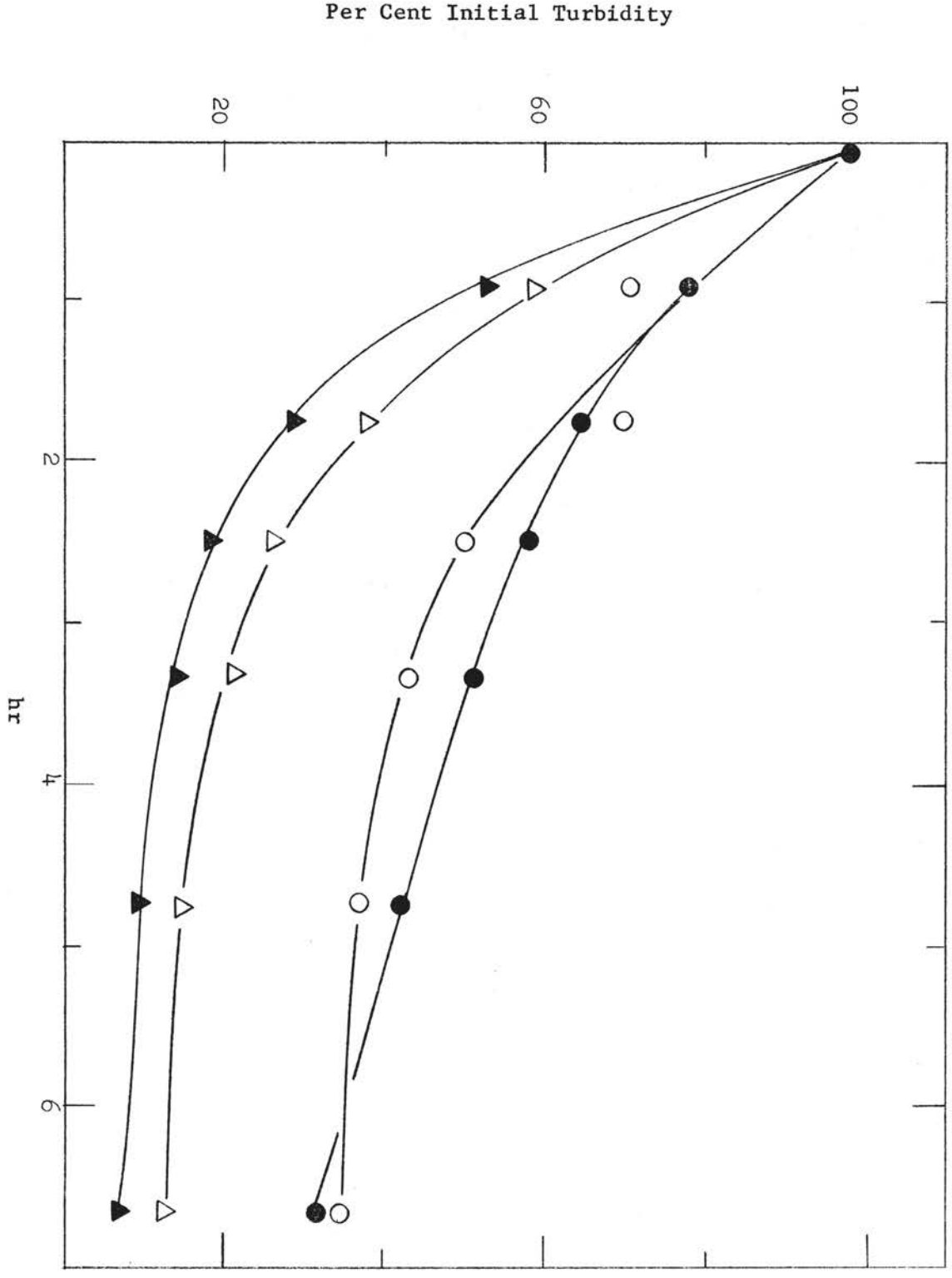
Per Cent Initial Turbidity
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and slowest in water; whereas, in NaCl and KCl suspensions the stability of the cells lay between that observed in water and buffer. Muramidase and EDTA increased the rate of lysis of both types of cells in all media with the possible exception of the phosphate buffer suspension. Hydroxylysine cells lysed slower in the presence of EDTA and muramidase than did lysine cells. This should not be construed to mean that hydroxylysine cells are resistant to muramidase, since log phase cells were used and the total lysis measured would be the sum of autolysis and that due to muramidase action.

These results suggest that incorporation of hydroxylysine into the cell leads to a stronger cell wall. It may be that hydroxylysine in the cell wall makes it a less suitable substrate for the lytic enzymes of the cell or that the presence of extra hydroxyl groups strengthens the cell wall through hydrogen bonding.

Susceptibility to disruption by sonic oscillation: Two cultures of cells, one grown on lysine and the other on lysine plus hydroxylysine, were harvested and subjected to sonic oscillation. Disruption was determined by the decrease in turbidity of the culture at 660 mμ and by the increase in release of material which absorbs at 280 and 260 mμ into the supernatant solution. The slope of the linear curve obtained from plotting absorbancy versus time was determined and the ratio reported in Table III. Lysine cells were disrupted at a rate about 1.7 times as fast as were hydroxylysine cells.

Cell wall synthesis studies

Since lysis is related to the stability of the cell wall and since isotope tracer studies have indicated that hydroxylysine is incorporated into the cell wall of S. faecalis, experiments were designed to determine

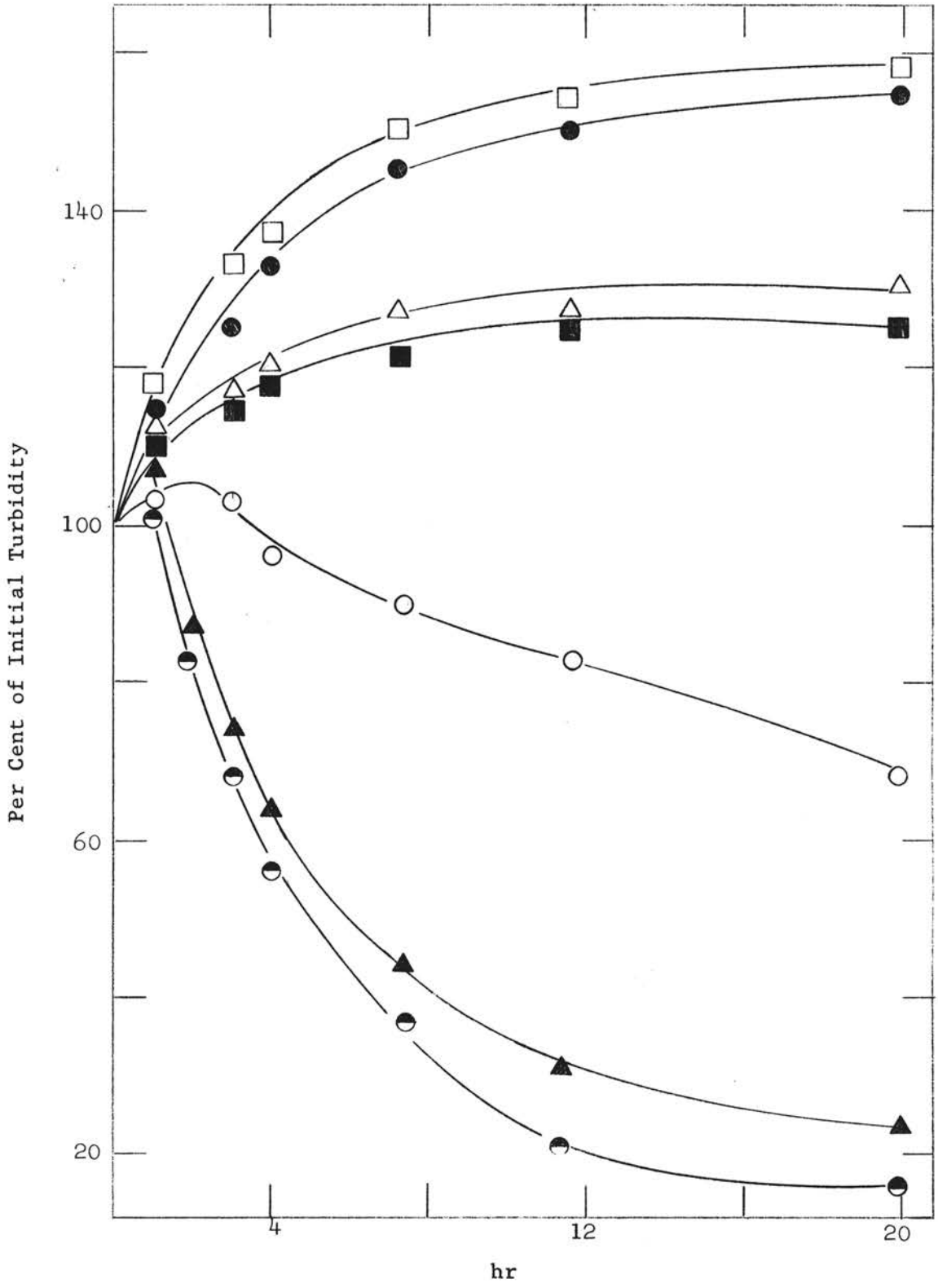
TABLE III

COMPARISON OF CELL STABILITY

Cells Grown On	Relative rate change in absorbancy/min		
	660 mμ	280 mμ	260 mμ
Lys	0.012	0.062	0.120
Lys + (OH)Lys	0.0067	0.035	0.069
Ratio $\frac{\text{Lys}}{\text{Lys + (OH)Lys}}$	1.79	1.77	1.74

S. faecalis cells grown with or without added hydroxylysine were harvested by centrifugation after 11 hr of growth. They were washed with 0.9% NaCl and then suspended in 0.9% NaCl at a standardized density. A 50-ml sample of each batch of cells was subjected to sonic oscillation in a Raytheon 10kc Sonic Oscillator set at 1.2 A output current (at the start with each culture). Samples were removed at the various times and the absorbancy of an aliquot was determined at 660 mμ. The remaining sample was centrifuged for 5 min to remove whole cells and debris then the absorbancies at 280 and 260 mμ were determined on a diluted aliquot of the supernatant solution. The relative rate is expressed at the change in absorbancy/min as determined from the linear portion of the absorbancy versus time curves.

if hydroxylysine could replace lysine in cell wall synthesis. Shockman et al. (91) have shown that the increase in turbidity of cultures of log phase cells suspended in a simplified medium is due to synthesis of new cell wall material. This turbidity increase is resistant to chloramphenicol and is sensitive to penicillin. To minimize effects of changes in the medium, the same levels of cell wall precursors and energy sources as were present in the growth medium were used. Cells grown in the presence and absence of hydroxylysine were harvested in the exponential phase and suspended in this wall synthesis medium at a standardized concentration, and the absorbancy was determined at intervals. Figures 15 and 16 show the results of these experiments. When lysine was not present in the wall medium, the cells lysed. The addition of lysine or hydroxylysine (20 μg/ml) resulted in an increase in turbidity, indicating that hydroxylysine can replace lysine for the synthesis of cell walls. Chloramphenicol, an inhibitor of protein synthesis but not of cell wall formation (103, 104), did not decrease the turbidity increment in either case. In fact, chloramphenicol enhanced the turbidity increment in both cases. Penicillin led to rapid lysis of cells grown under both conditions, but a comparison of the figures will show that cells grown in the presence of hydroxylysine were more resistant to lysis resulting from penicillin action. In other experiments, hydroxylysine would not replace any of the other essential amino acids for wall synthesis. In accord with the results of Shockman, the deletion of alanine from the wall medium did not lead to lysis, presumably because the cells had an adequate internal supply of vitamin B_6. It was necessary to delete aspartic acid also, in order for lysis to occur as a result of glutamic acid deletion. These results show


Per Cent of Initial Turbidity
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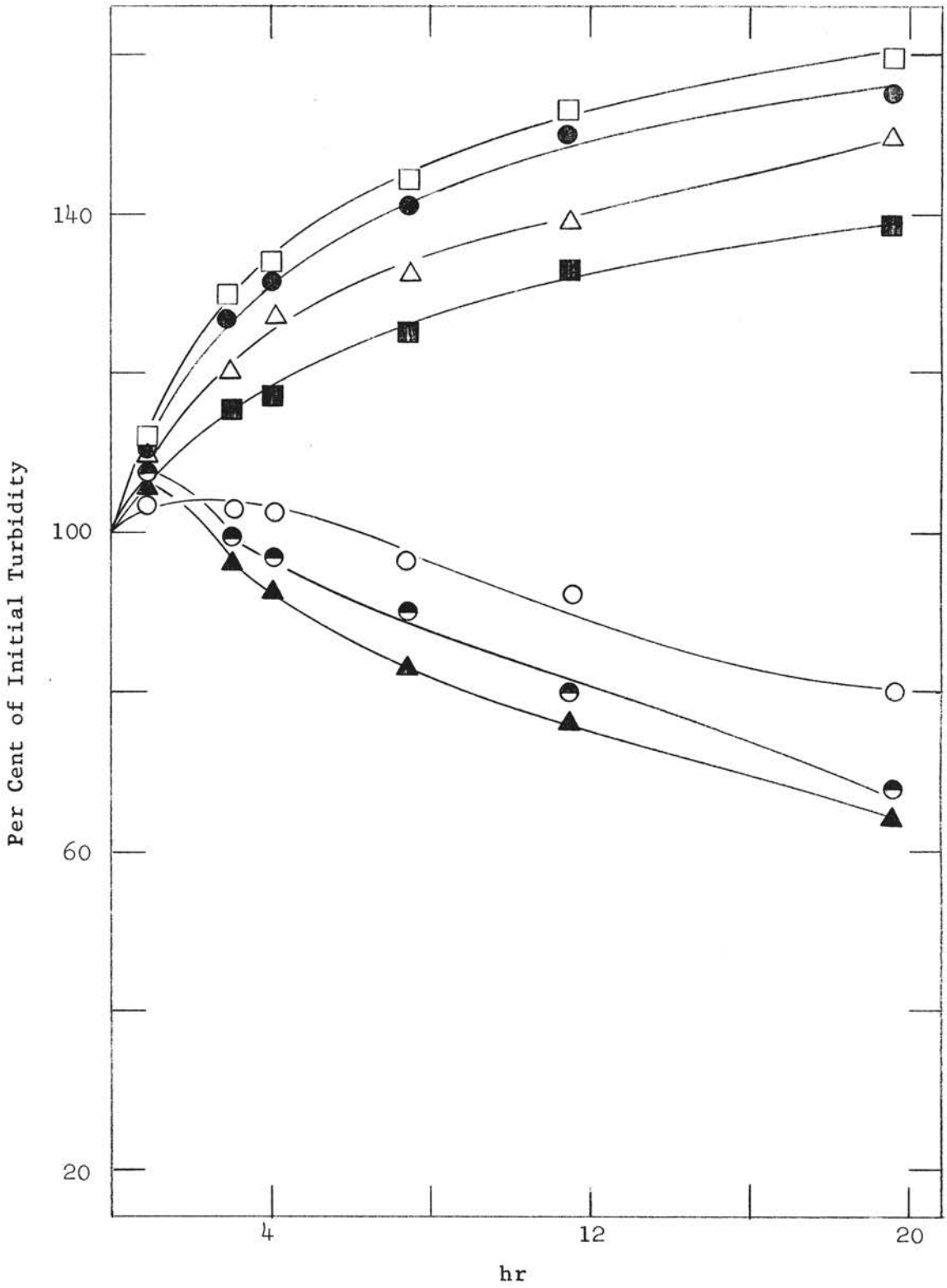
Per Cent of Initial Turbidity
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that hydroxylysine can replace lysine in cell wall synthesis. The fact that the turbidity increase was resistant to chloramphenicol and sensitive to penicillin confirms the supposition that the turbidity change reflects cell wall synthesis. The presence of chloramphenicol consistently resulted in an increase in turbidity. This may be due to the blockage of the incorporation of amino acids into protein via the amino acid-protein exchange mechanism that would require the transfer of the amino acid from soluble-RNA to the ribosome. This area involving the latter stages of protein synthesis is the apparent site of chloremphenicol inhibition, and it is possible that chloramphenicol inhibits messenger RNA (105-108). The breakdown of protein might still occur and thereby result in higher pool levels of the amino acids. Under these conditions, more of the cell wall amino acids would be available for wall synthesis.

Accumulation of cell wall precursors

Strominger has amply demonstrated that penicillin or lysine deprivation results in accumulation of UDP-muramyl-peptide cell wall precursors in Staphylococcus aureus. These compounds can be detected by reaction with p-dimethylaminobenzaldehyde according to the method of Strominger (102). Studies were initiated to determine if hydroxylysine could be found in any of these wall precursors. However, as can be seen in Table IV no such compounds accumulated in S. faecalis as a result of penicillin action or lysine deprivation under a variety of conditions. Some accumulation occurred in a wild type S. aureus treated with penicillin but only about one-fourth as much as Strominger obtains with strain Copenhagen. In spite of the use of high concentrations of sucrose to protect the osmotically sensitive S. faecalis, no detectable levels of amino sugar esters could be found. Mandelstam et al. (96)

TABLE IV

ACCUMULATION OF CELL WALL PRECURSORS IN STREPTOCOCCUS FAECALIS AND STAPHYLOCOCCUS AUREUS

	μmoles N-acetyl amino sugar per liter culture of A = 1.0						
	Organism						
	S. faecalis			S. aureus			
Experiment Number	1*	2*	3**	1*	2**	3***	4***
Log Cells	--	--	--	--	3.55	5.67	8.2
Penicillin Treated		--	--	--	8.75	10.4	11.15
Addition to suspending medium							
lysine			--	--	7.77	5.04	9.12
hydroxylysine			--	--	10.0	6.87	9.53
lysine + chloramphenicol			--			4.16	10.05
hydroxylysine + chloramphenicol			--			3.92	9.4
broth						1.25	
broth + chloramphenicol						5.4	
lysine free wall media			--				9.03
lysine free wall media + chloramphenicol			--				8.95
Lysine Free Cells (log cells suspended in lysine free media)	--		--	--			6.45
Addition to suspending medium							
lysine	--	--	--				5.14
hydroxylysine	--	--	--				6.85
lysine + chloramphenicol	--	--	--				5.33
hydroxylysine + chloramphenicol		--					5.80
lysine free wall		--					6.15
lysine free wall + chloramphenicol		--					5.87

TABLE IV (CONTINUED)

*Suspending media have 0.5 molar sucrose, lysine free media is Henderson Snell media without lysine.
**Suspending media have no sucruse, wall medium is patterned after Henderson Snell
***Suspending media have no sucrose, wall medium is that of Mandelstam and Rogers (109).

Note:

1. S. aureus will not grow well in Henderson Snell medium.
2. Broth is 1% glucose, 1% pentone, 1% yeast extract.
3. Material given following "suspended in" denotes compound(s) substitutes for lysine in cell wall synthesizing medium except in case of "broth."
4. -- denotes that concentration of material was too low to detect with accuracy.
5. All cells grown in still cultures and suspended in still cultures except Staphylococcus Experiment No. 3 which was suspended in shake culture.
6. Penicillin treatment and suspension all for 1 1/2 hr.
7. Penicillin concentration 30 μg/ml.
   Chloramphenicol concentration 95 μg/ml.

have recently reported the accumulation of such compounds in a strain of S. faecalis and high concentrations of sucrose were necessary for maximum accumulation. The failure of lysine deprivation to cause accumulation of UDP-muramyl-peptides in S. aureus may indicate that it can synthesize lysine. When penicillin-treated S. aureus were suspended in a wall medium containing either lysine or hydroxylysine, the concentration of amino sugar esters returned to that of the normal exponential cells. This cannot be taken as evidence that hydroxylysine substitutes for lysine because a similar reduction was noted when the suspending medium contained neither amino acid. The failure of amino-sugar esters to accumulate does not indicate that the organism does not utilize them in wall synthesis. Indeed, their accumulation may be the reflection of the failure of some control mechanism, the lack of control not being obvious until their incorporation into wall material is blocked by penicillin or some other factor.

Incorporation and distribution studies using lysine-^{14}C and hydroxy-lysine-^{3}H

Tables V and VI show the distribution of radioactivity in L. mesenteroides and S. faecalis when grown in the presence of lysine-^{14}C or hydroxylysine-^{3}H. The fractionation procedure used was that of Park and Hancock (98). Relatively, radioactivity from hydroxylysine becomes incorporated into cell wall mucopeptide and the cell pool of S. faecalis to a greater extent than does carbon-14 from lysine. When the Henderson and Snell medium was used, the high level of tritium from hydroxylysine did not appear in the pool of S. faecalis. Compared to S. faecalis, L. mesenteroides incorporates tritium from hydroxylysine into its cell wall to a much greater extent. Values are expressed as

TABLE V

DISTRIBUTION OF RADIOACTIVITY FROM LYSINE-^{14}C AND HYDROXYLYSINE-^{3}H IN S. FAECALIS

	Percentage of Total Counts in Cells							
Experiment No.	1		2		3		4	
Fraction	^{14}C	^{3}H	^{14}C	^{3}H	^{14}C	^{3}H	^{14}C	^{3}H
Cold trichloracetic acid	2.3	21.0	5.5	27.0	4.3	16.7	2.0	3.5
Ethanol	3.5	2.8	3.1	1.7	3.0	0.8	2.4	0.4
Hot trichloracetic acid	3.2	4.2	7.5	2.4	2.0	3.1	0.7	3.2
Trypsin	58.0	52.0	73.0	46.0	82.7	57.3	76.5	60.5
Residue	21.0	10.5	17.5	25.5	7.5	24.1	20.0	32.6
Recovery	88.0	90.5	108.6	102.6	99.5	102.0	101.6	100.2

1. Leach and Snell medium, lysine-^{14}C 50 μg/ml, hydroxylysine-^{3}H 20 μg/ml with 10 μg/ml lysine, 24 hr culture

2. As above, 20 hr culture

3. Leach and Snell medium, lysine-^{14}C 56 μg/ml, hydroxylysine-^{3}H 27.8 μg/ml with 10.0 μg/ml lysine, 12 hr culture

4. Henderson and Snell medium, lysine-^{14}C 56 μg/ml, hydroxylysine-^{3}H 20 μg/ml with 10 μg/ml lysine, 12 hr culture

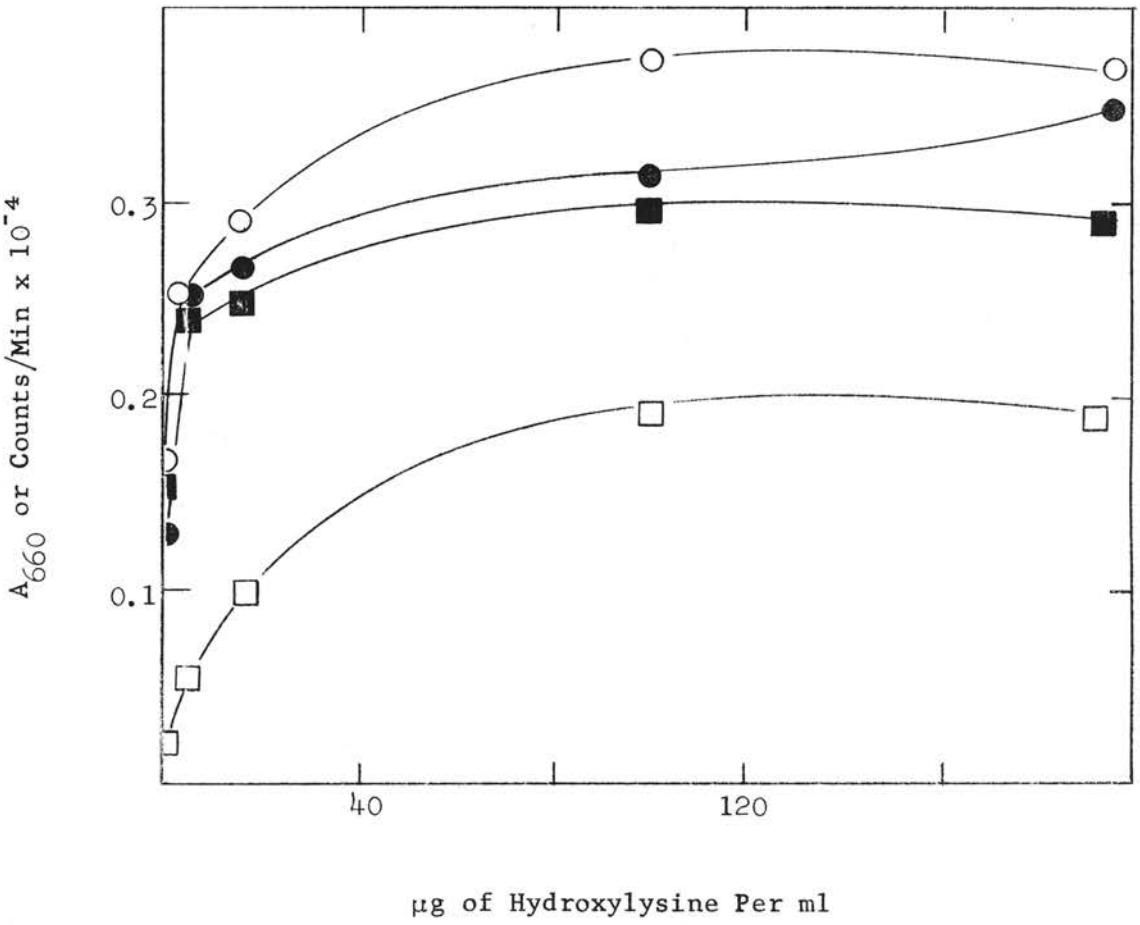
TABLE VI

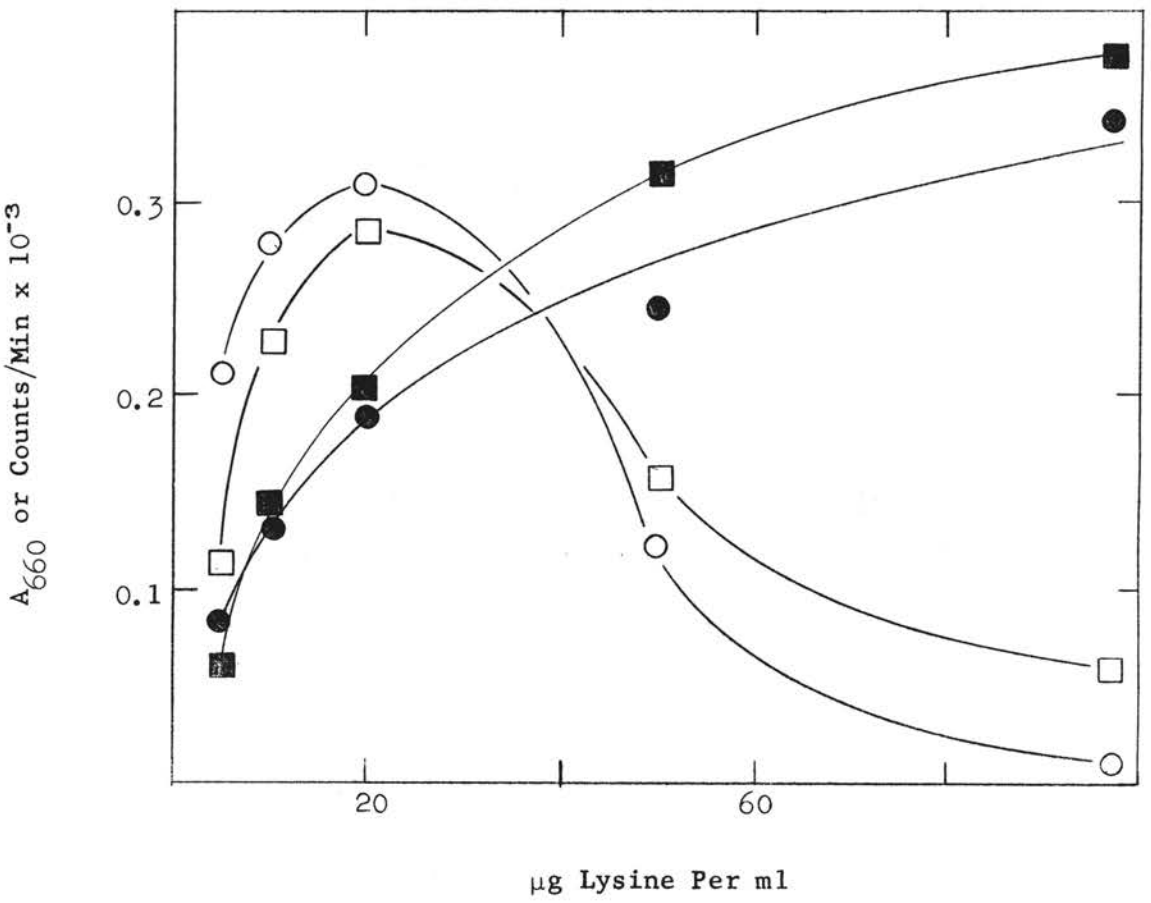
DISTRIBUTION OF RADIOACTIVITY FROM LYSINE-^{14}C AND HYDROXYLYSINE-^{3}H IN LEUCONOSTOC MESENTEROIDES

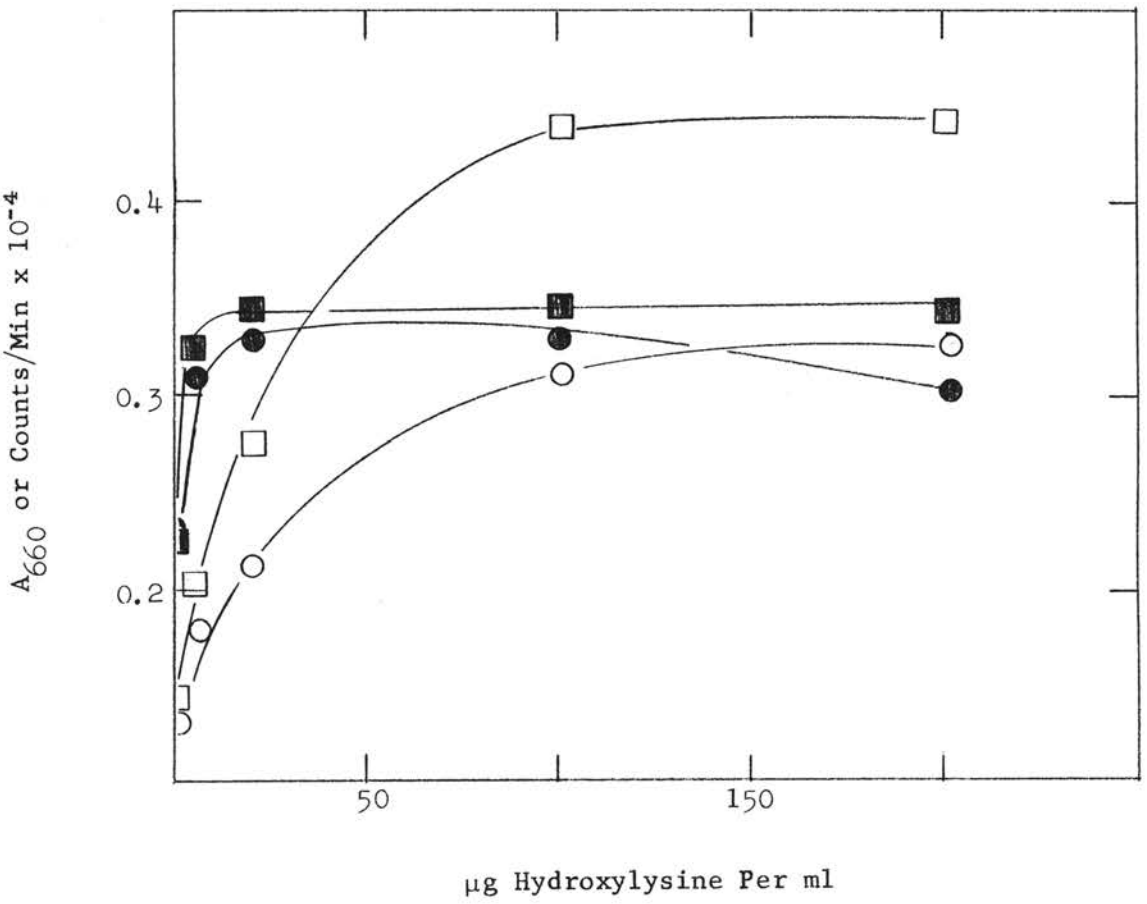
Fraction	Cellular Constituents	% of Total ^{14}C	% of Total ^{3}H
Cold trichloracetic acid (5%)	Cell pool	0.8	0.2
Ethanol (75%)	Lipid and protein	0.1	0.7
Hot trichloracetic acid (5%)	Nucleic and teichoic acids	1.2	3.3
Trypsin	Protein	60.6	18.3
Residue	Mucopeptide of cell wall	32.1	72.4

percentages of total radioactivity present in the cell. When the residue and trypsin soluble fractions from S. faecalis grown on hydroxylysine were hydrolyzed and chromatographed two dimensionally on paper and sprayed with ninhydrin, hydroxylysine was detected. When these chromatograms were assayed for tritium with a chromatogram scanner, the only radioactive area detected corresponded to the R_f value of hydroxylysine. This is in agreement with Tsung (68), who found no interconversion of lysine and hydroxylysine in S. faecalis. Thus, it appears that hydroxylysine is incorporated as such into protein and mucopeptide without being metabolized. This is supported by the failure to find any radioactive peaks other than hydroxylysine-^{3}H when hydrolysates of whole cells were chromatographed by ion exchange procedures.

To investigate the effect of lysine on incorporation of hydroxylysine-^{3}H and of hydroxylysine on incorporation of lysine-^{14}C, cells were grown for 24 hr (S. faecalis) or 48 hr (L. mesenteroides) on a constant level of the labeled compound and varying levels of the unlabeled compound in the Henderson and Snell medium. The cells were harvested, washed, and extracted with cold 5% trichloracetic acid for 10 min. The amount of tritium or carbon-14 incorporated into the trichloracetic acid-precipitable material was then determined by counting on the liquid scintillation counter. The results of these experiments are shown in Figures 17, 18, and 19. From the data in Figure 17, it will be noted that high concentrations of hydroxylysine did not inhibit incorporation of lysine-^{14}C into either S. faecalis or L. mesenteroides. The results presented in Figure 18 show that high concentrations of lysine markedly inhibited incorporation of hydroxylysine-^{3}H into both organisms. At the lower levels of lysine, incorporation of hydroxylysine


A_{660} or Counts/Min x 10^{-4}
0.3
0.2
0.1
40
120
µg of Hydroxylysine Per ml


A_{660} or Counts/Min x 10^{-3}
0.3
0.2
0.1
20
60
μg Lysine Per ml


A_{660} or Counts/Min x 10^{-4}
0.4
0.3
0.2
50
150
µg Hydroxylysine Per ml

increased as did growth as the lysine concentration was increased. At a lysine:hydroxylysine ratio above one, incorporation of hydroxylysine decreased rapidly with increasing lysine concentrations. In contrast, lysine incorporation was not inhibited at a hydroxylysine:lysine ratio of approximately thirteen in L. mesenteroides or at a ratio of four in S. faecalis (Figure 17).

In Figure 19 hydroxy-L-lysine and the mixed racemate were compared with regard to their ability to inhibit incorporation of lysine-^{14}C. Practically identical results were obtained with hydroxy-L-lysine or an equal amount of the mixed racemate with regard to inhibition of lysine-^{14}C incorporation. Growth stimulation, however, was greater when the L-isomer alone was present, indicating that the L-isomer is used more efficiently.

These experiments show that hydroxylysine is not utilized by either S. faecalis or L. mesenteroides when the lysine supply is adequate and thus explain why hydroxylysine's growth-stimulating effect is manifest only at low lysine concentrations. Experiments of the type just discussed give no indication of the internal distribution of radioactivity. The results of the experiment in Figure 20, in which the complete Park and Hancock fractionation scheme was performed on S. faecalis cells grown on hydroxylysine-^{3}H and various levels of lysine, indicate that incorporation of hydroxylysine into protein is inhibited first. Incorporation into the cell pool remained proportional to growth up to a lysine:hydroxylysine-^{3}H ratio of four before any inhibition was noted. Incorporation into the mucopeptide was inhibited but there was some incorporation even up to a ratio of 16; whereas, incorporation into protein was completely inhibited at a ratio of four. From the results

Counts/Min x 10^{-4} or mg Dry Weight of Cells x 0.23
12
9
6
3
2
6
10
14
Lysine:Hydroxylysine-^{3}H Weight Ratio

of this experiment, the uptake process does not appear to be the primary site of inhibition of hydroxylysine incorporation by lysine. This observation was tested under conditions wherein log cells of S. faecalis and L. mesenteroides were suspended in a medium containing hydroxylysine-^{3}H with or without lysine. The results of this experiment appear in Table VII and confirm the findings of the previous experiment. However, hydroxylysine-^{3}H was not incorporated into protein of L. mesenteroides under these conditions nearly to the extent that it was in S. faecalis. Hydroxylysine did not affect the internal distribution of lysine-^{14}C in experiments of the type presented in Figure 20.

Table VIII shows the results of experiments designed to investigate the effect of chloramphenicol and penicillin on the incorporation of lysine-^{14}C and hydroxylysine-^{3}H into mucopeptide and protein of S. faecalis and L. mesenteroides. The results are as would be expected from a consideration of the properties of these two antibiotics. Incorporation of both amino acids into the mucopeptide of both organisms was inhibited by penicillin but unaffected by chloramphenicol; whereas, incorporation into protein of both organisms was inhibited by chloramphenicol but unaffected by penicillin. These results show that hydroxylysine is utilized in a manner similar to other cell wall amino acids.

Table IX shows that distribution of radioactivity from hydroxylysine-^{3}H in S. faecalis which grew in the absence of lysine and in the presence of hydroxylysine. The medium was inoculated with the original S. faecalis. Growth occurred between the tenth and eleventh days after inoculation. These results indicate that S. faecalis utilizes hydroxylysine under these conditions. When this mucopeptide was examined for

TABLE VII

EFFECT OF LYSINE ON THE DISTRIBUTION OF HYDROXYLYSINE-^{3}H IN S. FAECALIS AND L. MESENTEROIDES

Fraction	Lysine Absent		Lysine Present (12 μg/ml)			
	S. faecalis	L. mesenteroides	S. faecalis		L. mesenteroides	
	Counts/Min	Counts/Min	Counts/Min	%I*	Counts/Min	%I
Cold trichloracetic acid	5,260	15,640	2,420	54.0	6,240	60.0
Ethanol	740	360	160	78.4	120	66.7
Hot trichloracetic acid	640	180	300	56.7	80	50.6
Trypsin	6,810	130	110	98.4	80	38.4
Residue	7,180	5,550	1,880	74.0	1,170	79.0

$$*\%I = \frac{(\text{counts/min - lysine}) - (\text{counts/min + lysine})}{(\text{counts/min - lysine})} \times 100$$

TABLE VIII

EFFECT OF CHLORAMPHENICOL AND PENICILLIN ON INCORPORATION OF LYSINE-^{14}C AND HYDROXYLYSINE-^{3}H INTO PROTEIN AND MUCOPEPTIDE OF *LEUCONOSTOC MESENTEROIDES* AND *STREPTOCOCCUS FAECALIS*

Conditions			Total Counts Per Minute In			
Medium Used	Labeled Compound Present	Inhibitor Used	*L. mesenteroides*		*S. faecalis*	
			Protein	Mucopeptide	Protein	Mucopeptide
Wall	HYLYS-^{3}H	None	410	6,870	370	6,900
Wall	HYLYS-^{3}H	Chloramphenicol	270	6,160	120	5,820
Wall	LYS-^{14}C	None	14,410	148,560	1,305	3,613
Wall	LYS-^{14}C	Chloramphenicol	4,800	112,100	1,025	4,559
Wall	LYS-^{14}C	Penicillin	10,170	10,120	1,048	582
Complete	HYLYS-^{3}H	Chloramphenicol	180	5,070	140	3,850
Complete	HYLYS-^{3}H	Penicillin	110	440	2,850	1,100
Complete	LYS-^{14}C	Chloramphenicol	5,120	125,650	769	2,700
Complete	LYS-^{14}C	Penicillin	42,100	11,100	3,170	186

TABLE IX

DISTRIBUTION OF RADIOACTIVITY FROM HYDROXYLYSINE-^{3}H IN *S. FAECALIS* GROWN IN THE ABSENCE OF LYSINE

Fraction	Constituents	Counts/Min/ Culture	Per Cent
Cold trichloracetic acid (5%)	Cell pool	1,880	2.4
Ethanol (70%)	Lipid and alcohol soluble protein	500	0.6
Hot trichloracetic acid (5%)	Nucleic aid teichoic acids	1,580	2.0
Trypsin solubilized	Protein	31,320	40.1
Residue	Mucopeptide of cell wall	42,000	54.3

its amino acid content by ion exchange chromatography, the only radioactivity found in the effluent was in the area occupied by hydroxylysine.

Muramidase digestion of S. faecalis mucopeptide

Another approach taken toward finding how hydroxylysine occurs in the cell wall was to digest the mucopeptide with muramidase and attempt to isolate a glucosaminyl-muramyl-peptide as has been done from muramidase digests of Micrococcus lysodeikticus (110). Treatment of S. faecalis mucopeptide from cells grown on lysine-^{14}C in 0.1 M ammonium acetate with muramidase for 24 hr resulted in a turbidity decrease of 75%. This digestion mixture was chromatographed on Sephadex G-25. The radioactivity was washed from the column within the first few fractions, indicating that the carbon-14 remained associated with a fairly high molecular weight material. The fractions containing radioactivity were also turbid. These results indicate that no small molecular weight compounds containing lysine are released by muramidase. The products of muramidase action on S. faecalis mucopeptide, therefore, appear not to include disaccharide-peptides such as those from M. lysodiekticus. Since all previous evidence indicates that hydroxylysine is incorporated into the mucopeptide in lieu of lysine, no experiments were done with the mucopeptide from cells grown on hydroxylysine-^{3}H.

Amino acid analysis of mucopeptides

Since no positive results could be obtained regarding incorporation of hydroxylysine into a UDP-muramyl-peptide or into a glucosaminyl-muramyl-peptide isolatable from muramidase digests of the wall, direct analysis of the amino acid content of the mucopeptide was made. The mucopeptide was hydrolyzed and the amino acids separated by ion exchange chromatography. The amount of each amino acid was determined

quantitatively by the ninhydrin procedure. The results are expressed in Table X as molar ratios. The glutamic acid to aspartic acid ratio is in good agreement with the literature values.

In the method employed, all neutral amino acids are obtained from the ion exchange column in one peak. This peak contained predominantly alanine as would be expected but this fraction also contained traces of two or three other amino acids. The alanine:glutamic acid ratio was about two without any correction for contamination. This would be in fair agreement with the reports of alanine:glutamic acid ratios of four that were based on analysis of walls which contained teichoic acids; whereas, the hot trichloracetic acid extraction used herein removes the teichoic acids which contain about forty per cent of the alanine of the cell wall. The lysine:glutamic acid ratio in mucopeptide which contains no hydroxylysine is in agreement with the theoretical value of 1.0. In mucopeptide containing hydroxylysine, the ratio of lysine + hydroxylysine:glutamic acid is also approximately 1.0. This observation agrees with the hypothesis that hydroxylysine replaces lysine in the cell wall mucopeptide and suggests that care should be exercised in using cell wall composition as a taxanomic key.

Mucopeptide containing hydroxylysine-^{3}H was treated with either basic or acidic periodate for 24 hr in an attempt to cleave the C_5-C_6 bond of hydroxylysine. This would be expected to occur if hydroxylysine were bound via a peptide bond with the remainder of the molecule free. However, when the periodate treated mucopeptide was removed by centrifugation, washed well, and hydrolyzed, hydroxylysine was the only radioactive compound detected in the eluate from an ion exchange column. About fifty per cent of the mucopeptide was solubilized by this treatment.

TABLE X

MOLAR RATIOS OF AMINO ACIDS IN CELL WALL MUCOPEPTIDE OF *S. FAECALIS* GROWN IN THE PRESENCE AND ABSENCE OF HYDROXYLYSINE

	Hydroxylysine Present	Hydroxylysine Absent
Glutamic acid	1.00	1.00
Aspartic acid	0.76	0.77
Lysine	0.27	1.14
Hydroxylysine	0.66	0.00
Hydroxylysine + lysine	0.93	1.14

Thus, it appears that the ϵ-amino and/or δ-hydroxyl group of hydroxylysine may be involved in some linkage. However, the hydroxylysine may not be exposed in the cell wall structure and thereby may be inaccessable to attack by periodate. This result agrees with the failure of others (84) to get the ϵ-amino group of lysine to react with dinitrofluorobenzene.

Immunological character of the mucopeptide

Injection intravenously or subcutaneously of lysine or hydroxylysine mucopeptide into rabbits over a period of several weeks gave rise to antibodies in the blood serum which could be detected by their agglutinating activity toward the original antigen. The systems, however, were completely cross reactive, that is, antisera prepared against either lysine or hydroxylysine mucopeptide reacted equally well with both types of mucopeptide. Some hapten inhibition experiments were carried out in an attempt to identify the immunologically determinate group present in the mucopeptide. However, lysine, hydroxylysine, and glucose were all ineffective as inhibitors of agglutination. Thus some group in the mucopeptide other than lysine, hydroxylysine or the glucose portion of glucosamine would have to be the immunologically determinant group. This group must also occur in both types of mucopeptide.

CHAPTER V

SUMMARY AND CONCLUSIONS

The results of the growth studies indicate that much of the growth response of S. faecalis attributed to hydroxylysine can be explained by its protective action against lysine depletion lysis. There is apparently an additional growth response over and above that due to lytic protection, since increments of hydroxylysine above that required for protection from lysis give an additional growth response.

The experiments with L. mesenteroides show that it does not undergo lysis consequent to lysine depletion, unless of course the lysis is not made manifest by a turbidity decrease. At present then the growth response in L. mesenteroides can be explained only by hydroxylysine performing some of the functions of lysine.

Hydroxylysine had a growth inhibitory action in a lysine-independent strain of S. faecalis, probably as a result of its repressing one or more enzymes involved in lysine synthesis and this can be overcome by lysine.

Another strain of S. faecalis was also isolated which grew well in the presence of hydroxylysine and apparently utilized hydroxylysine.

Cell wall synthesis experiments with S. faecalis indicated that hydroxylysine can substitute for lysine as a cell wall amino acid. This is in agreement with tracer studies indicating that hydroxylysine becomes incorporated into the cell wall mucopeptide.

As evidenced by the cell stability experiments, the presence of hydroxylysine in the cell wall of S. faecalis results in log cells which are more resistant to autolysis and to lysis by muramidase. Hydroxylysine-grown S. faecalis cells were also more resistant to disruption by sonic oscillation.

The results of isotope experiments indicated that hydroxylysine becomes incorporated into the cell only when lysine is limiting. Hydroxylysine is incorporated mainly into the protein and mucopeptide fractions of both S. faecalis and L. mesenteroides. Hydroxylysine has a greater tendency to become incorporated into protein than into cell wall in the former organism and the reverse situation in the latter. Hydroxylysine behaves as a typical cell wall amino acid in both organisms. It is incorporated mainly into mucopeptide when only the wall amino acids are present, and its incorporation into protein is inhibited by chloramphenicol but not by penicillin. However, its incorporation into mucopeptide is inhibited by penicillin but not by chloramphenicol.

Scanning of chromatograms of hydrolysates of protein and mucopeptide fractions of S. faecalis cells grown on hydroxylysine-^{3}H revealed only one radioactive area which corresponded in R_f value to hydroxylysine. The only radioactive fractions from ion exchange chromatography of mucopeptide hydrolysates from L. mesenteroides and S. faecalis cells grown on hydroxylysine-^{3}H appeared to be hydroxylysine. Treatment of the mucopeptide from hydroxylysine-^{3}H grown S. faecalis with periodate did not give rise to any other labeled compound.

Mucopeptide from either lysine-grown or hydroxylysine-grown S. faecalis cells were antigenic in rabbits, but neither lysine nor

hydroxylysine appeared to be involved in the immunologically determinant group.

A SELECTED BIBLIOGRAPHY

1. Van Slyke, D. D., and Hiller, A., Proc. Natl. Acad. Sci., 7, 185 (1921).

2. Van Slyke, D. D., Hiller, A., and MacFayden, D. A., J. Biol. Chem., 141, 681 (1941).

3. Macpherson, H. T., Biochem. J., 40, 470 (1946).

4. Hamilton, P. B., and Anderson, R. A., J. Am. Chem. Soc., 77, 2892 (1955).

5. Ackerman, D., Hoppe-Seylers Z. Physiol. Chem., 287, 46 (1951).

6. Solmones, C. C., and Irving, J. J., S. African J. Med. Sci., 20, 143 (1955).

7. Kulonen, E., Ann. Med. Exptl. Biol. Fenniae (Helsinki), 33, No. 3 (1955).

8. Bose, S. M., and Das, B. M., J. Am. Leather Chemists' Assoc., 51, 647 (1956).

9. Piez, D. A., and Gross, J., J. Biol. Chem., 235, 995 (1960).

10. Viswanatha, T., and Irreverre, F., Biochim. Biophys. Acta, 40, 564 (1960).

11. Astrup, T., Carlstrom, G., and Stage, A., Acta Physiol. Scand., 24, 202 (1951).

12. Gordon, A. H., Nature, 162, 778 (1948).

13. Gordon, A. H., Biochem. J., 45, 99 (1949).

14. Barbier, M., and Lederer, E., Biochim. Biophys. Acta, 8, 590 (1952).

15. Wirtschafter, Z. T., Am. J. Obstet. Gynecol., 75, 718 (1958).

16. Christomanus, A., Nature, 171, 886 (1953).

17. Eastoe, J. E., Nature, 173, 540 (1954).

18. Work, E., Biochim. Biophys. Acta, 3, 400 (1949).

19. Tomita, T., Hoppe-Seylers Z. Physiol. Chem., 304, 72 (1956).

20. Virtanen, A. I., and Kari, S., Acta Chem. Scand., 8, 1290 (1954).

21. Grobbelaar, N., Pollard, J. K., and Steward, F. C., Nature, 175, 703 (1955).

22. Holman, R. T., Penzer, F., and Schweigart, B. S., Arch. Biochem., 26, 99 (1950).

23. Hulme, A. C., J. Sci. Food Agri., 2, 160 (1951).

24. Roberts, E. A., and Wood, D. J., Current Sci. (India), 20, 151 (1951).

25. Van Slyke, D. D., Hiller, A., Dillon, R. T., and MacFadyn, D. A., Proc. Soc. Exptl. Biol. Med., 38, 548 (1938).

26. Heathcote, J. G., Biochem. J., 42, 305 (1948).

27. Martin, J. P., and Synge, R. L. M., Biochem. J., 35, 294 (1941).

28. Sheehan, J. C., and Bolhofer, W. A., J. Am. Chem. Soc., 72, 2466 (1950).

29. Bergstrom, S., and Lindstedt, S., Acta Chem. Scand., 5, 157 (1951).

30. Ramchandran, L. K., J. Sci. Ind. Research (India), 12B, 9 (1953).

31. Robson, W., and Selim, A. S., Biochem. J., 53, 431 (1953).

32. Sorm, F., and Mikes, O., Collection Czechoslov. Chem. Commun., 15, 288 (1950).

33. Moore, S., and Stein, W. H., J. Biol. Chem., 178, 53 (1949).

34. Moore, S., and Stein, W. H., J. Biol. Chem., 192, 663 (1951).

35. Hamilton, P. B., and Anderson, R. A., J. Biol. Chem., 213, 249 (1955).

36. Hamilton, P. B., and Anderson, R. A., J. Biol. Chem., 211, 95 (1954).

37. Rees, M. W., Biochem. J., 40, 632 (1946).

38. Chinard, F. B., J. Biol. Chem., 199, 91 (1952).

39. Van Slyke, D. D., MacFadyn, D. A., Hiller, A., Hastings, A. B., and Klemperer, F. W., J. Biol. Chem., 133, 287 (1940).

40. Klemperer, F. W., Hastings, A. B., and Van Slyke, D. D., J. Biol. Chem., 143, 433 (1942).

41. Sheehan, J. C., and Bolhofer, W. A., J. Am. Chem. Soc., 72, 2469 (1950).

42. Sheehan, J. C., and Bolhofer, W. A., J. Am. Chem. Soc., 72, 2472 (1950).

43. Weisiger, J. R., J. Biol. Chem., 186, 591 (1950).

44. Van Zyl, G., van Tamelen, E., and Zuidema, G. D., J. Am. Chem. Soc., 73, 1765 (1951).

45. Lindstedt, S., Acta Chem. Scand., 7, 340 (1953).

46. Fones, W. S., J. Am. Chem. Soc., 75, 4865 (1953).

47. Witkop, B., Experentia, 12, 372 (1956).

48. Rose, W. C., Science, 86, 2231 (1957).

49. Rose, W. C., Nutr. Rev., 8, 118 (1950).

50. Sinex, F. M., and Van Slyke, D. D., J. Biol. Chem., 216, 245 (1955).

51. Van Slyke, D. D., and Sinex, F. M., J. Biol. Chem., 232, 797 (1958).

52. Piez, K. A., and Likens, R. C., J. Biol. Chem., 229, 101 (1957).

53. Kao, K. Y. T., and Boucek, R. J., Proc. Soc. Exptl. Biol. Med., 98, 526 (1958).

54. Lindstedt, S., Acta Physiol. Scand., 27, 377 (1952).

55. Sinex, F. M., Van Slyke, D. D., and Christman, D. R., J. Biol. Chem., 234, 918 (1959).

56. Stetten, M. R., and Schoenheimer, R., J. Biol. Chem., 153, 113 (1944).

57. Stetten, M. R., J. Biol. Chem., 181, 31 (1949).

58. Lindstedt, S., and Lindstedt, G., Arch. Biochem. Biophys., 85, 565 (1959).

59. Boulanger, P., Osteux, R., and Bertrand, J., Biochim. Biophys. Acta, 29, 534 (1958).

60. Cohen, L. A., Irreverre, F., Piez, K. A., Witkop, B., and Wolff, H. C., Science, 123, 842 (1956).

61. Rabinovitz, M., and Tuve, R. K., Proc. Soc. Exptl. Biol. Med., 100, 222 (1959).

62. Rabinovitz, M., Olson, M. E., and Greenberg, D. M., Cancer Res., 17, 885 (1957).

63. Peterson, C. S., and Carrol, R. W., Science, 123, 546 (1956).

64. Mitchell, H. K., and Houlahan, M. B., J. Biol. Chem., 174, 883 (1948).

65. Steward, F. C., Pollard, J. K., Patchett, A. A., and Witkop, B., Biochim. Biophys. Acta, 28, 308 (1958).

66. Kalyankar, G. D., and Meister, A., J. Biol. Chem., 234, 3210 (1959).

67. Leach, F. R., Exptl. Cell Res., 29, 162 (1963).

68. Tsung, C. M., Masters Thesis, Oklahoma State University, 1960.

69. Salton, M. R. J., Microbial Cell Walls, John Wiley and Sons, New York, 1960.

70. Work, E., J. Gen. Microbiol., 25, 167 (1961).

71. Zilliken, F., Federation Proc., 18, 966 (1959).

72. Salton, M. R. J., Biochim. Biophys. Acta, 8, 510 (1952).

73. Cummins, C. S., and Harris, H., J. Gen. Microbiol., 13, piii (1955).

74. Cummins, C. S., and Harris, H., J. Gen. Microbiol., 14, 583 (1956).

75. Holden, J. T., and Snell, E. E., J. Biol. Chem., 178, 799 (1949).

76. Snell, E. E., Radin, N. S., and Ikawa, M., J. Biol. Chem., 217, 803 (1955).

77. Ikawa, M., and Snell, E. E., Biochim. Biophys. Acta, 19, 576 (1956).

78. Ikawa, M., and Snell, E. E., J. Biol. Chem., 235, 1376 (1960).

79. Salton, M. R. J., and Pavlik, J. G., Biochim. Biophys. Acta, 39, 398 (1960).

80. Toennies, G., Bakay, B., and Shockman, G. D., J. Biol. Chem., 234, 3269 (1959).

81. Ikawa, M., J. Biol. Chem., 236, 1087 (1961).

82. Armstrong, J. J., Baddiley, J., Buchanan, J. G., Davison, A. L., Kelemen, M. V., and Neuhaus, F. C., Nature, 184, 247 (1959).

83. Abrams, A., J. Biol. Chem., 230, 949 (1958).

84. Mandelstam, M. H., and Strominger, J. L., Biochem. Biophys. Res. Commun., 5, 466 (1961).

85. Toennies, G., and Shockman, G. D., in Fourth Int. Cong. Biochem., Vol. XIII, p. 365, Pergamon Press, London, 1958.

86. Shockman, G. D., Kolb, J. J., and Toennies, G., J. Biol. Chem., 230, 961 (1958).

87. Toennies, G., Shockman, G. D., and Kolb, J. J., Biochemistry, 2, 294 (1963).

88. MacLeod, R. A., J. Bacteriol., 62, 337 (1951).

89. Van Eys, J., and Pearson, W. N., J. Bacteriol., 68, 362 (1954).

90. Shockman, G. D., Conover, M. J., Kolb, J. J., Phillips, P. M., Riley, L. S., and Toennies, G., J. Bacteriol., 81, 36 (1961).

91. Shockman, G. D., Conover, M. J., Kolb, J. J., Riley, L. S., and Toennies, G., J. Bacteriol., 81, 44 (1961).

92. Montague, M. D., Biochem. J., 87, 29P (1963).

93. Shockman, G. D., Proc. Soc. Exptl. Biol. Med., 101, 693 (1959).

94. Snell, E. E., J. Biol. Chem., 158, 497 (1945).

95. Cohen, G. N., and Monod, J., Bacteriol. Rev., 21, 169 (1957).

96. Mandelstam, P., Loercher, R., and Strominger, J. L., J. Biol. Chem., 237, 2683 (1962).

97. Henderson, L. M., and Snell, E. E., J. Biol. Chem., 172, 15 (1948).

98. Leach, F. R., and Snell, E. E., J. Biol. Chem., 235, 2523 (1960).

99. Park, J. T., and Hancock, R., J. Gen. Microbiol., 22, 249 (1960).

100. Rosen, H., Arch. Biochem. Biophys., 67, 10 (1957).

101. O'Neal, R. M., Ph.D. Dissertation, Oklahoma State University, 1963.

102. Strominger, J. L., J. Biol. Chem., 224, 509 (1957).

103. Mandelstam, J., and Rogers, H. J., Nature, 181, 956 (1956).

104. Hancock, R., and Park, J. T., Nature, 181, 1050 (1956).

105. Brock, T. D., Bacteriol. Rev., 25, 32 (1961).

106. Mandelstam, J., Bacteriol. Rev., 24, 289 (1960).

107. Brenner, S., Jacob, F., and Meselson, M., Nature, 190, 576 (1961).

108. Gros, F., Hiatt, H., Gilbert, W., Kurland, C. G., Risebrough, R. W., and Watson, J. D., Nature, 190, 581 (1961).

109. Mandelstam, J., and Rogers, H. J., Biochem. J., 72, 655 (1959).

110. Ghuyson, J. M., Biochim. Biophys. Acta, 40, 473 (1960).

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