HYDROXYLYSINE METABOLISM IN *STREPTOCOCCUS FAECALIS*

By

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

CHAPTER I

INTRODUCTION

The amino acid, 5-hydroxylysine, first isolated from gelatin in 1921 by Van Slyke, et al. (1) has been found by others in hydrolysates of animal collagen. Extensive studies on the source and state of hydroxylysine have shown that dietary lysine is the chief source of hydroxylysine in collagen (2), and that the carbon from hydroxylysine is not incorporated into collagen, as either hydroxylysine or lysine (3).

Hydroxylysine is not an "essential amino acid" for the growth of rats (4). However, it was found that while hydroxylysine was unable to replace lysine for the growth of Leuconostoc mesenteroides and Streptococcus faecalis, small amounts of hydroxylysine allow maximum growth of these organisms at reduced levels of lysine (5).

This study was initiated in an effort to find out how hydroxy-L-lysine is metabolized in the cells of S. faecalis. A modified procedure (6) for the isolation of hydroxylysine from gelatin hydrolysate and for separation of the naturally occurring isomer is described. Growth studies have confirmed and extended the observations regarding the sparing effect on the lysine requirement of S. faecalis.

Tritiated hydroxy-L-lysine was prepared by the Wilzbach procedure (+), and the incorporation of lysine-C^{14} and hydroxylysine-H^3 into the

cells of S. faecalis was investigated. Isotope analyses have shown that free hydroxylysine is not dehydroxylated to lysine, and lysine in the growth medium does not give rise to labeled hydroxylysine in the cells.

The distribution of labeled hydroxylysine in the cells was studied by means of fractionation procedures and it was found to be incorporated into the cells in combined form as protein and cell wall mucopeptide.

A series of uptake studies indicated a difference in the mode of uptake and utilization of lysine and hydroxylysine.

Lysine was taken up faster than hydroxylysine and the presence of one of these amino acids in the medium did not significantly affect the utilization of the other except that hydroxylysine seemed to stimulate lysine uptake in short term studies.

CHAPTER II

HISTORICAL

A. Occurrence

Hydroxylysine was first recognized among the acid hydrolysis products of gelatin by Van Slyke, et al. (1) and later isolated and identified by the same group (8, 9). Many other investigators have shown that hydroxylysine occurs in the collagen of terrestrial animals and the gelatin derived therefrom. Hamilton and Anderson (10) examined a number of protein hydrolysates and found that hydroxylysine was absent from all of the proteins examined other than collagen. Astrup (11) in confirmation of Gordon (12, 13) found the phosphate ester of hydroxylysine in a number of the tissues of adult and embryonic ox. Barbier and Lederer (14) reported a hydroxylysine phosphatide in Mycobacterium phlei. Very recently Viswantha and Irreverre (15) found hydroxylysine in trypsin.

B. Isolation of Hydroxylysine

Hydroxylysine was first isolated from gelatin by Van Slyke, Hiller, Dillon and MacFadyen (8). After removal of arginine and histidine from the gelatin hydrolysate, a "lysine fraction" was obtained by precipitation with phosphotungstic acid. The hydroxylysine formed a monopicrate (m.p. 225°) which was separated from lysine picrate by

fractional crystallization. A mono-hydrochloride was prepared from the pure picrate. Heathcote (16) separated a "lysine fraction" from a protein hydrolysate by electrodialysis after arginine and histidine had been removed with flavianic acid; pure hydroxylysine monopicrate was obtained by Van Slyke's (8) procedure.

More satisfactory isolation procedures eventually were based on the ion exchange techniques developed so extensively by Moore and Stein (17). In 1950 Weisiger employed Dowex-50 in the ammonium phase. Bergström and Lindstedt (18) used a series of Amberlite columns to separate basic amino acids from less basic amino acids, and then separated hydroxylysine from the other basic amino acids in low yield, but pure, on a column of "Hyflo Super Cel." Sheehan and Bolhofer (19) used chromatography on alumnia to separate hydroxylysine from lysine. Inskip (20) used Amberlite IR-C-50. The best yields have been obtained by the procedure of Hamilton and Anderson (6). Monoamino acids were removed by means of Amberlite IR-120, and the hydroxylysine was separated from the other basic amino acids on a column of Dowex-50 in the sodium phase prepared according to Moore and Stein (17). The yields were 80 to 90 per cent of the hydroxylyxine present in the protein.

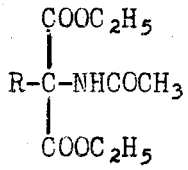
C. Quantitative Determination of Hydroxylysine in Proteins

The first analytical procedure employed was that of Van Slyke, Hiller and MacFadyen (9) who applied the Criegee (21) periodate procedure then determined the ammonia split from the epsilon amino group of recrystallized hydroxylysine phosphotungstates obtained from hexone bases of protein hydrolysates.

Rees (22) determined colorimetrically the formaldehyde evolved from hydroxylysine with chromotropic acid and Chinard (23) has described conditions for the determination of hydroxylyxine by the color formed with ninhydrin at pH 1 after separation from other amino acids.

D. Synthesis of Hydroxylysine

Several syntheses of hydroxylysine have been published, all by the malonic ester route. In each, a compound of the type,



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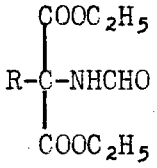

is prepared, where the R is $NH_2CH_2CH(OH)CH_2CH_2$. Hydrolysis with HCl is used to remove either the acetyl or formyl group, the C_2H_5 and one of the malonic carboxyl groups yielding hydroxylyxine. It will be noted that these syntheses would be expected to give equal proportions of the two racemates.

E. Structure and Isomerism of Hydroxylysine

Analyses by Van Slyke, et al. (24, 25) indicated that the compound contained one more oxygen atom than lysine and a positive ninhydrin reaction showed it to be an α-amino acid. Periodate liberated one mole of ammonia and one mole of formaldehyde, indicating that the hydroxyl and one of the amino groups were on adjacent carbon atoms at one end of the chain. The above evidence taken with the effect of the hydroxyl group on the pK of the adjacent NH_2 group (26) and the inability to form a lacetone indicated it was a straight chain diamino acid. Van Slyke's group proposed as probable structures for hydroxylysine either α-ε-diamino-δ-hydroxycaproic acid or α,δ-diamino-ε-hydroxycaproic acid.

Several syntheses of hydroxylysine by Weisiger and others established the structure of hydroxylysine as the α-ε-diamino-δ-hydroxycaproic acid. The first optical data on isolated hydroxylysine was reported by Sheehan and Bolhofer (27). The variation they observed was attributed to partial inversion during the isolation procedure. Fones (28) has prepared the optical antipodes of both hydroxylysine and allohydroxylysine by applying the asymetric enzymatic hydrolysis procedure of Greenstein, et al. (29, 30).

The hydroxylysine from an acid hydrolysate of gelatin was resolved into hydroxy-L-lysine and allohydroxy-D-lysine by Hamilton and Anderson (6) by ion exchange chromatography. These results agreed with those of Piez (31), who separated a synthetic mixture of hydroxy-D-lysine from allohydroxy-D-lysine similarly. Hamilton and Anderson (6) suggested that the variation in the rotation of the isolated hydroxylysine was due to the inversion around the α-carbon atom which occurred during prolonged refluxing with 6 N hydrochloric acid.

F. Biological Synthesis of Hydroxylysine

Hydroxylysine is not an "essential" amino acid for the growth of rats. This fact has been demonstrated by the numerous experiments of Rose and others in which rats were maintained on mixture of amino acids containing lysine, but lacking hydroxylysine. Since the collagen in these rats presumably contained hydroxylysine it was assumed these animals can synthesize hydroxylysine, either from lysine or from other substances present in the diet. Sinex and Van Slyke (2) showed that hydroxylysine and lysine in skin collagen of the rat had the same specific activity after lysine-C^{14} had been fed, indicating

that dietary lysine serves as chief, and probably only significant source of hydroxylysine in rat skin collagen. The results were further confirmed by Piez and Likins (32) with regard to the collagen of rat skin, bone and dentine and by Kao and Boucek (33) with regard to collagen formed in implanted polyvinyl sponges.

Sinex, Van Slyke and Christman (3) established the failure of free hydroxylysine to serve as a source of the hydroxylysine or lysine of collagen with the isotope studies with H^3-labeled hydroxylysine administered either orally or by injection to growing rats. These results were consistent with the deduction of Piez and Likins (32), who observed that the C^{14} content of the hydroxylysine isolated from collagen after injection of C^{14}-labeled lysine into rats was not altered by accompanying injections of unlabeled hydroxylysine.

That the reverse reaction, formation of lysine from hydroxylysine in the animal body, does not occur to a significant extent was demonstrated by Lindstedt (34). He showed that dietary supplements of synthetic hydroxylysine were ineffective in promoting growth of lysine-deficient rats. Bergström and Lindstedt (35) found that hydroxylysine was unable to replace lysine for the growth of Leuconostoc mesenteroides P-60. Similarly, Mitchell and Houlahan (36) found that, in a Neurospora mutant which requires lysine for growth, the lysine could not be replaced by hydroxylysine.

Peterson and Carroll (5) studied the ability of hydroxylysine to support growth of the lysine-requiring bacteria, Streptococcus faecalis and Leuconostoc mesenteroides. They found that the addition of hydroxylysine to a lysine-free medium failed to allow growth of either of these

organisms. However, hydroxylysine had considerable ability to lower the lysine requirements of these bacteria; thus small amounts of hydroxylysine allowed maximum growth of the organism with reduced levels of lysine. Higher levels of the hydroxy amino acid showed a marked growth-depressing effect. This growth depression was reversed by larger amounts of L-lysine.

Since Leuconostoc mesenteroides P-60 and Streptococcus faecalis are commonly used for microbiological assay of lysine in hydrolysates of foods and tissues, Peterson and Carroll (5) and Sauberlich (37) suggested that the presence of hydroxylysine in sample hydrolysates could interfere with the quantitative microbiological determination of lysine if the basal media contain no hydroxylysine.

Studies on the metabolic degradation of hydroxylysine in mammals was reported recently by Lindstedt and Lindstedt (38). Using the "metabolic overloading" technique, they found that 5-hydroxypipecolic acid was formed from 5-hydroxy-DL-lysine in rat kidney and liver preparations.

CHAPTER III

MATERIALS AND METHODS

A. Materials

A synthetic preparation of hydroxy-DL-lysine and allohydroxy--DL-lysine hydrochloride in unknown proportions was obtained from the California Foundation for Biochemical Research.

Hydroxy-L-Lysine was isolated from gelatin hydrolysates as described below.

Tritium labeled hydroxylysine was prepared by the Wilzbach procedure (7). Hydroxy-L-lysine isolated from gelatin was used. Paper chromatography of the tritium labeled hydroxy-L-lysine using the phenol:water:NH_4OH (80:20:0.3) system showed a single ninhydrin positive spot at R_f 0.62. By assaying the developed chromatograms with the automatic windowless chromatogram scanner it was noted that there were four radioactive areas, (1) a spot at the origin, (2) a spot at R_f 0.75, (3) the hydroxylysine (R_f = .62) and (4) a spot at the solvent front. Tritiated hydroxylysine was then separated on a 1.0 x 40 cm. column of Dowex-50 in the sodium phase as described in Section III C 2 below. Examination of the tritiated hydroxylysine either with the chromatographic scanner or by radioautography showed only one radioactive spot at R_f .62. The tritiated hydroxylysine was crystallized as the dihydrochloride and was used without further purification

The specific activity of the hydroxy-L-lysine used was 3.54 mc. per millimole.

L-lysine-C^{14} uniformly lableled was obtained from Volk Radio-Chemical Company. The specific activity was 88.44 mc. per millimole and 0.05 mc. per ampoule.

5-Hydroxypipecolic acid was kindly provided by Dr. B. Witkop from the National Institutes of Health.

B. Methods

1. Isolation of Hydroxy-L-Lysine

a. Preparation of the acid hydrolysate of gelatin

One hundred g. of gelatin (J. T. Baker Company) was hydrolyzed in the autoclave at 15 lbs. per square inch with 1500 ml. of 3 N hydrochloric acid for 5 hours.

b. Isolation of hydroxylysine

In general, the methods used were a modification of those devised by Hamilton and Anderson (6). The acidic and neutral amino acids were largely separated from the basic amino acids by ion-exchange chromatography on a 3.4 x 115 cm. column of Amberlite IR-125 (in hydrogen form) at room temperature. The developing solvent of 1.0-2.5 N hydrochloric acid was passed through the column at a rate of 0.05 ml. per ml. resin per minute. The later fractions, which contained chiefly lysine and hydroxylysine, were then passed through a 7.4 x 30 cm. column of sodium Dowex-50 (8 per cent cross-linked 200 to 400 mesh) at room temperature. The developing solvent was 0.3 M, pH 5.0 sodium acetate buffer and was used with a flow rate of 0.004 ml. per ml. of resin per minute. This column completely separated lysine and hydroxylysine.

Hydroxylysine monohydrochloride was crystallized as described by Hamilton and Anderson. (6)

c. Chromatographic resolution of hydroxylysine

The diastereoisomers hydroxylysine were chromatographed on a 3.4 x 115 cm. column of sodium Dowex-50, buffered at pH 3.4, and the column was developed at room temperature with 0.1 M sodium phosphate buffer, pH 7.5.

d. Quantitative and qualitative determination of hydroxylysine

After each 100 ml. of effluent had passed through the column, 0.5 ml. was collected, neutralized with NaOH, and treated with periodate at pH 7.5 according to the method of Nicolet and Shinn (39) for the determination of α-amino-β-hydroxy acids. The formaldehyde evolved from serine or hydroxylysine was determined colorimetrically with chromotropic acid by the procedure of Rees (22). The position of the emerging hydroxylysine was determined by analysis of 0.1 ml. of each fraction by the colorimetric ninhydrin method (40). Paper chromatograms were prepared as needed in all column operations to determine the presence of lysine and other contaminants.

2. Growth Experiments

The basal medium used in all determinations of the sparing effect of hydroxylysine, unless specifically stated otherwise, was medium III, Table I. A dry mixture of amino acids was ground in a mortar stored dry and dissolved when needed. The vitamin solution was prepared every two weeks and stored under refrigeration.

TABLE I

COMPOSITION OF THE BASAL MEDIA USED IN GROWTH STUDIES*

Compound	Medium I[1]	Medium II[2]	Medium III[3]
	mg.	mg.	mg.
DL-α-Alanine	40	200	40
L-Arginine·HCl	48.4	40	48.5
L-Asparagine	80		80
L-Aspartic Acid	20	100	20
L-Cysteine	10	(cystine) 20	10
L-Glytamic Acid	60	200	60
Glycine	20	20	10
L-Histidine·HCl	14	20	12.4
DL-Isoleucine	50	40	50
DL-Leucine	50	40	50
DL-Methionine	20	40	20
DL-Phenylalanine	20	40	20
L-Proline	20	20	20
DL-Serine	10	40	90
DL-Threonine	40	40	40
DL-Tryptophan	8	40	8
L-Tyrosine	20	20	20
DL-Valine	50	40	50
	gm.	gm.	gm.
Glucose	5.0	4.0	1.0
Sodium Acetate	4.0	0.2	0.60
Sodium Citrate	---	4.0	4.0
Ammonium Chloride	0.6	0.6	---
Ascorbic Acid	---	---	0.05
	mg.	mg.	mg.
KH_2PO_4	120	1000	50
K_2HPO_4	120	160	50
$MgSO_4 \cdot 7H_2O$	40	8	20
$FeSO_4 \cdot 7H_2O$	2	32	1
$MnSO_4 \cdot 4H_2O$	4	8	1
NaCl	2	2	1
Adenine Sulfate·H_2O	2	2	0.5
Guanine·HCl·$2H_2O$	2	2	0.5
Uracil	2	2	2.5
Xanthine	2	2	0.5
Guanylic Acid	---	---	10.0
Thiamine·HCl	0.1	0.2	0.04
Pyridoxine·HCl	0.2	---	---
Pyridoxamine·HCl	0.06	---	---

TABLE I (Continued)

Pyridoxal·HCl	0.06	0.04	0.08
Calcium-dl-Pantothenate	0.1	0.2	0.08
Riboflavin	0.1	0.2	0.08
Nicotinic Acid	0.2	0.2	0.08
p-Aminobenzoic Acid	0.02	0.04	0.04
Biotin	0.0002	0.002	0.0004
Folic Acid	0.002	0.002	0.002
Distilled H_2O to	100 ml.	100 ml.	100 ml.

* One ml. of the basal medium was diluted with 1 ml. of water or of sample

[1] Steele, Sauberlich, Reynolds and Baumann (41)

[2] Henderson and Snell (42)

[3] Leach and Snell (43) (modified by addition of Na citrate)

Streptococcus faecalis ATCC 8043 was grown as a stab culture on a medium composed of 1.5 per cent agar, 1 per cent glucose, and 1 per cent Difco Bacto-yeast extract. Transfers to fresh medium were made every two weeks and just prior to use in each growth study.

The organism was transferred to broth containing 1 per cent each of glucose, yeast extract and tryptone and grown for 20 hours to obtain an inoculum. In all studies, the final volume used was 2 ml. The tubes were inoculated with a drop of cell suspension made by suspending cells after one washing in a volume of sterile water equal to the volume of the broth in which they were grown. Incubation was at 37° C for 20 hours. Cell growth was determined photometrically with a Coleman Jr. spectrophotometer at 660 mμ. The cell yield was estimated from a calibration curve relating the weight of the dry cells to the optical density observed.

3. Incorporation of Hydroxylysine and Lysine

The microorganism was grown on 100 ml. of medium I diluted with 100 ml. of water containing 5 mg. of L-lysine-C^{14} or on the same amount of medium III plus 2 mg. of unlabeled L-lysine and 2 mg. of hydroxy-L-lysine-H^3 for 20 hours. About 50 mg. dry weight of washed cells thus grown were hydrolyzed with 4 ml. of 3.0 N HCl in an autoclave at 15 pounds pressure for 5 hours.

For the experiment with labeled lysine 50 mg. of carrier hydroxylysine was added and for the labeled hydroxylysine experiment 50 mg. of carrier lysine was added.

In general the methods of isolation were the same as those described in III B 1 above.

The hydrolysate from 40-50 mg. of cells plus the carrier was placed on a 1.0 x 30 cm. column of Amberlite IR-120 to separate most of the monoamino acids. The solvent was 1-2.5 N HCl, developed at a rate of 0.009 ml. per ml. resin per minute, hydroxylysine was then separated from the other basic amino acids on a 1.0 x 40 cm. column of Dowex-50 buffered at pH 3.4, the column was then developed with 0.1 M sodium acetate buffer, pH 5.0. Two ml. fractions were collected unless otherwise stated. One-tenth ml. aliquots were removed from each fraction for analysis with ninhydrin. Another 0.1 ml. was taken for either H^3 or C^{14} analysis.

4. Cell Fractionation and Cell Wall Preparations

a. Cell fractionation

Streptococcus faecalis was grown on 50 ml. of the basal medium III (TableI) diluted with 50 ml. of water containing 1 mg. of hydroxy-L-lysine-H^3 and 1 mg. of unlabeled lysine. Cells were harvested and washed with saline. The localization of the radioactivity in the cells from tritium labeled hydroxylysine has been determined by the classical cell fractionation procedure of Roberts, et al. (44). The method as outlined in Table II yields six fractions which have been characterized as shown.

The method devised by Park and Hancock (45) was also used. It is similar to the procedure of Roberts, et al. (44) with the important addition that protein and cell wall-mucopeptides in the residual fraction are separated. The results obtained with the latter method were too preliminary to be reported here. Each fraction obtained from cells grown on labeled hydroxylysine was analyzed for tritium.

TABLE II

SCHEME FOR FRACTIONATION OF STREPTOCOCCUS FAECALIS

Fraction		Treatment*	Contents of Fraction
1.	Cold TCA	Suspend 30 mg. wet weight of washed cells in 4 ml. of 5% TCA. After 30 minutes at 5°, centrifuged at 4,000 x g. for 5 minutes and decant extract.	All low M.W. compounds soluble in 5% (W/V) TCA
2.	Alcohol-Soluble	Suspend residue in 4 ml. of 75% ethanol; after 30 minutes at 40-50°, centrifuge (4,000 x g. 10 minutes) and decant extract.	Ethanol soluble "protein" and lipid
3.	Alcohol-Ether Soluble	Suspend residue in 4 ml. of a solution containing 2 ml. of ether and 2 ml. of 75% ethanol; after 15 minutes at 40-50°, centrifuge (4,000 x g. 10 minutes) decant extract.	Apparently the same as alcohol soluble
4.	Hot TCA-Soluble	Suspend residue in 4 ml. of 5% TCA and kept in a bath of boiling water for 20 minutes and centrifuged.	Breakdown products of nucleic acid and teichoic acid
5.	Residual	Washed twice to free of residual TCA by suspending in 4 ml. of acidified alcohol (pH = 5), decant extract. The precipitate is the principal protein fraction.	Protein

* The wall of the centrifuge tube is wiped dry after each decantation so as to decrease contamination of the subsequent extract to negligible amounts.

b. Cell wall preparation

The cells were prepared by growing S. faecalis on 1 liter of medium III diluted with 1 liter of water containing 20 mg. each of unlabeled L-lysine and hydroxylysine-H^3 for 20 hours at 37°.

Mechanical rupture of the bacterial cells was attempted with a sonic oscillator, the use of the French press, and by freezing and thawing in succession. None of these methods ruptured more than a small percentage of the cells so these cells were subjected to grinding with alumia. An excellant cell wall preparation was obtained which was then treated with crystalline trypsin and ribonuclease at pH 7.6 in phosphate buffer at 37° for 2 hours. This was followed by digestion with pepsin in 0.02 N HCl solution at 37° for 12 hours. Centrifugation of this preparation at 9,000 x g for 20 minutes gave a clear supernatant solution which was discarded. The residue was resuspended in saline and centrifuged at 1,500 x g for 10 minutes to remove heavier particles. The cell wall suspension after alumina grinding was examined under a phase microscope and found to be essentially free of whole cells.[4]

5. Uptake Studies

a. Cultures of Streptococcus faecalis

Streptococcus faecalis was grown on medium I (Table I). After 15 to 17 hours of growth at 37° the cells were harvested by centrifugation and were resuspended in a salt solution containing the salts of medium I (single strength) adjusted to pH 7.0.

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[4] The author is indebted to Dr. F. R. Leach and Dr. E. Grula for assistance in preparation of the cell walls.

6. Measurement of Uptake of L-lysine-C^{14} and Hydroxy-L-Lysine-H^{3}

The procedure similar to that of Leach and Snell (46) given below was followed in all experiments except where otherwise indicated. The suspension of cells in salt solution (usually 900 to 1,000 µg. dry weight of cells per ml.) was incubated at 37° for 15 minutes. Glucose (0.1%) was then added and after incubation for an additional 15 minutes the radioactive substrate was added. The accumulation of radioactive substrate was determined by the membrane filter technique of Britten, et al. (47) using Millipore filters (Millipore Filter Corporation, pore size 0.45 µ). At desired times, 0.5 ml. samples of the cell suspension were removed with a syringe fitted with an automatic stop, expelled onto the Millipore filter, and the cells were washed with 0.5 ml. of water from the syringe. Determination of the radioactivity of these cells gave the total uptake. A duplicate 0.5 ml. sample and the syringe rinse were added to 1.0 ml. of a 12% solution of trichloroacetic acid. After 15 minutes at 37° C., the insoluble material was collected on a Millipore filter. Determination of the radioactivity of this sample gave the incorporation into protein, cell wall material, and other fractions insoluble in cold trichloroacetic acid. Incorporation into insoluble material in these resting cells was low enough to make the differences between the uptake and incorporation meaningful in the case of lysine. This difference is designated as accumulation or "pool" content.

Since only 450 to 500 µg. of cells were present in the samples, no correction for self-absorption was necessary for C^{14} counting. However, the liquid scintillation procedure for tritium counting was very inefficient for whole cells. Certain samples of tritium-labeled whole

cells were counted by the zinc fusion method of Wilzbach (48) as a gas on the vibrating reed electrometer and by the liquid scintillation procedure routinely used to obtain accurate counting efficiencies for this type of material.

7. Paper Chromatography and Radioautography

a. Paper chromatography

The technique of paper chromatography is well adapted to these studies. Whatman No. 1 filter paper was used for both one and two dimensional ascending chromatography. Chromatograms were developed in a wooden cabinet. After the solvent had ascended to the top of the sheet, a process requiring 3 to 24 hours depending on the solvent employed, the chromatogram was dried in an air stream in a hood.

The amino acids which separated were then located by spraying the papergrams with 0.2% solution of ninhydrin in 95% ethanol or by means of radioautography when members of a mixture contained radioactive tracers.

A list of the solvent most frequently used in our studies is given in Table III.

b. Radioautography

The routine preparation of radioautograms of radioactive chromatograms is an extremely useful method for locating radioactive substances. Eastman "no-screen" x-ray film, 10 by 12 inches, with emulsion on both sides, is placed on top of the

TABLE III

COMPOSITION AND PRINCIPAL APPLICATION OF

SOLVENTS USED IN PAPER CHROMATOGRAPHY

	Solvent		Principal Application	Comment
1.	phenol	80 gm.	amino acids, peptides	routinely used as a qualitative method for separation of amino acids
	conc. NH_4OH	0.3 ml.		
	water	20 ml.		
2.	phenol	100 gm.	protein hydrolysates nucleic acid hydrolysates	routinely used in 2nd dimension
	buffered H_2O (6.3% Na citrate) 3.7% KH_2PO_4 0.5% ascorbic acid	20 ml.		
3.	n-butanol	40 ml.	protein hydrolysates	used in first dimension
	acetic acid (glacial)	10 ml.	amino acids and peptides	
	water	10 ml.		
4.	tert-butanol	70 ml.	nucleic acid hydrolysates	used in first dimension
	12 N HCl	6.7 ml.		
	water	23.3 ml.		
5.	sec-butanol	70 ml.	break down products of nucleic acid	routinely used in one dimensional
	water	20 ml.		
	formic	10 ml.		
6.	ethanol	75 ml.	amino acids peptides	routinely used for rapid identification of amino acids by one dimentional chromatography
	water	25 ml.		

chromatogram and held securely by weights in complete darkness for a period of 2-4 weeks. After being thus exposed the film was developed in Kodak D-19 developer for 5 minutes at 20° C. and treated in acid fixer for 8 minutes. The developed film was then washed in running water and dried.

8. Counting Technique

a. Tritium counting

(1) Liquid scintillation counting

The Packard Tri-Carb Liquid Scintillation Spectrometer was used for H^3 assays. Each counting bottle contained 10 ml. of a solution containing 100 mg. of 2,5-diphenyloxazole, 5 mg. of 1,4-di[2-(5-phenyloxazoyl)] benzene in 10 ml. ethanol and 15 ml. toluene. The solvents used for counting were of reagent grade. The samples were dissolved or suspended in the solvent and counted at -5° for 10 minutes or to 10,000 counts.

(2) Gas counting

Analysis of the tritium labeled compounds by the above method was calibrated against the Wilzbach zinc-fusion method (48). The gases were collected in a 250 cc. ionization chamber and counted with the vibrating reed electrometer.

b. C^{14} counting (solid counting of C^{14})

C^{14} labeled compounds were pipetted onto planchets and counted with a Nuclear-Chicago D-47 counter fitted with micromil window.

CHAPTER IV

RESULTS AND DISCUSSION

A. Isolation of Hydroxylysine

Isolation of hydroxylysine from gelatin hydrolysate was partially effected on a column of Amberlite IR-120 and was completed on a column of Dowex-50, as shown in Fig. 1. The separation of hydroxylysine was good and, when the flow rate was not excessive or the column was not overloaded, overlapping of lysine and hydroxylysine did not occur. With some columns, tailing of the hydroxylysine occurred with some mixing with lysine but, as these later fractions contained only a few mg. of this amino acid, they were discarded. The hydroxylysine-containing fractions were combined, and hydroxylysine monohydrochloride were crystallized by the procedure of Hamilton and Anderson (6).

In typical experiments, the yield of hydroxylysine monohydrochloride ranged from 35 to 45 per cent based on the highest value reported in the literature (22) of 1.2 gm. of free base per 100 gm. of protein (Table IV). The hydroxylysine monohydrochloride isolated was subjected to chromatographic resolution procedure described in III B 1 c above. In contrast to the reports of Hamilton and Anderson (6), who separated hydroxy-L-lysine from allohydroxy-D-lysine, only one peak was obtained (Fig. 2). Thus, no inversion of hydroxylysine appears to have occurred during

Figure 1.

Separation of hydroxylysine on a column of Dowex-50, 7.4 x 30 cm., operated in the sodium form at room temperature, with 0.3 M sodium acetate buffer, pH 5.0 as developing solvent. The amino acid mixture placed on the column is the hydroxylysine-containing effluent fraction from a column of Amberlite IR-120 employed to give a preliminary partial separation of hydroxylysine from other amino acids in gelatin hydrolysate.

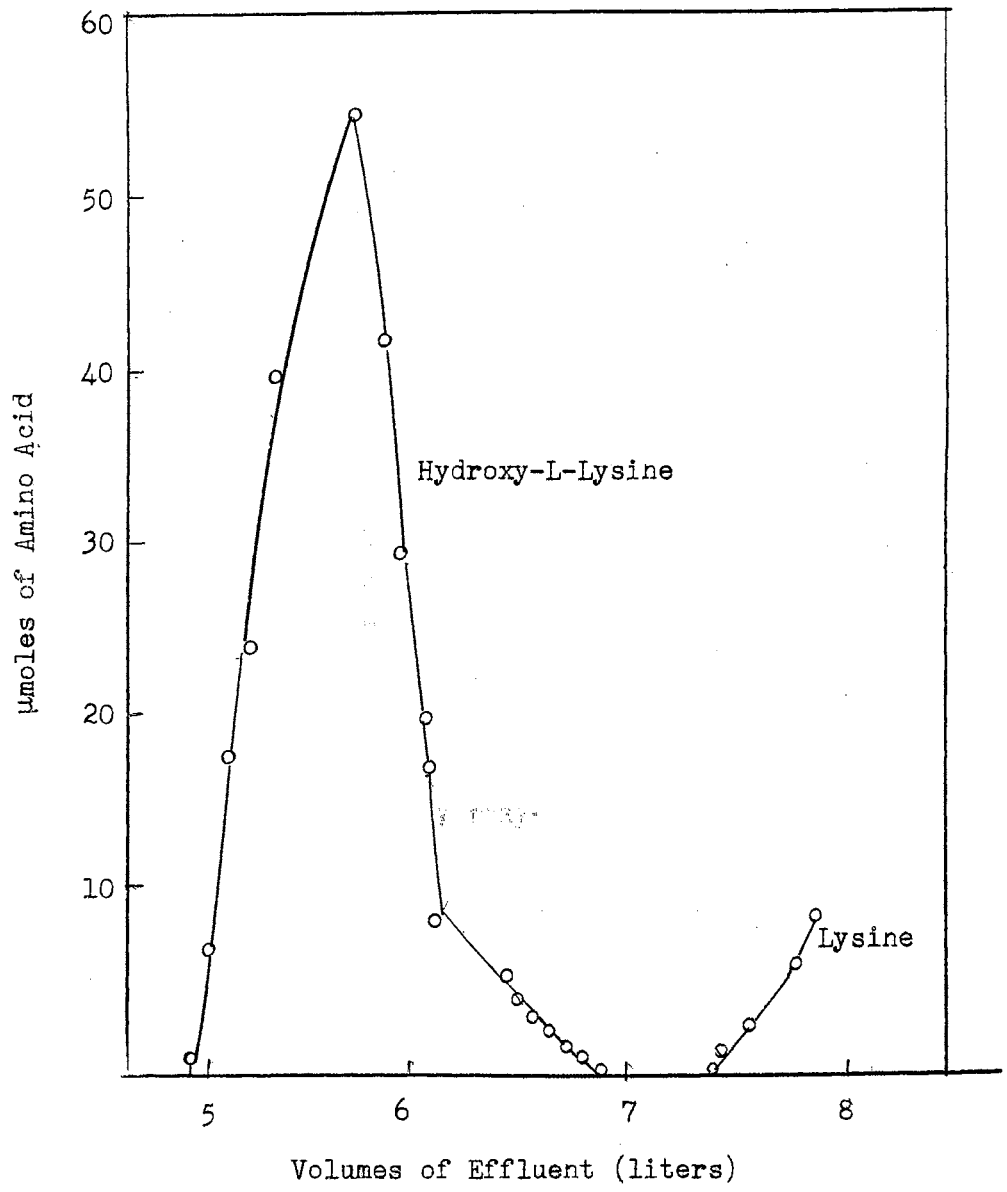

60
50
40
30
20
10
µmoles of Amino Acid
Hydroxy-L-Lysine
Lysine
5
6
7
8
Volumes of Effluent (liters)

TABLE IV

CHROMATOGRAPHIC ISOLATION OF HYDROXY-L-LYSINE

FROM HYDROLYZED GELATIN

For each experiment 100 grams of gelatin was used

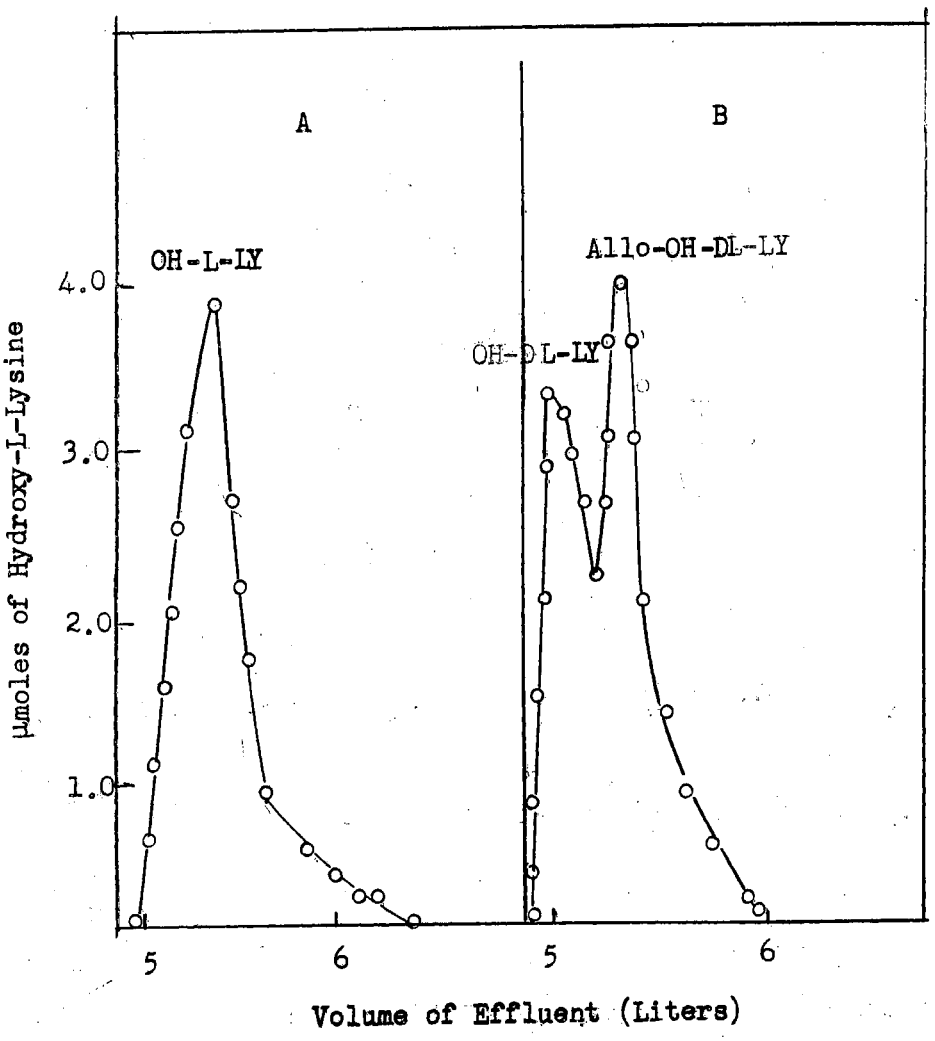
Comment	Experiment		
	1	2	3
Amberlite IR-120-- Separation of diamino acids			
Beginning peak volume (liters)	8.5	8.5	8.5
Peak volume (liters)	9.25	9.1	9.3
Sodium Dowex-50-- Separation of hydroxylysine			
Beginning peak volume (liters)	4.5	4.7	4.5
Peak volume (liters)	5.1	5.24	5.2
Sodium Dowex-50-- Resolution of hydroxylysine			
Beginning peak volume (liters)	5.0	5.1	---
Peak volume (liters)	5.4	5.4	---
Total yield of hydroxylysine·HCl (mg.)	448	550	389
% yield	37	45	33

Figure 2.

Results of chromatograms showing no epimerization of hydroxylysine, 200 mg. of hydroxylysine monohydrochloride from the second Dowex-50 column was placed on a 3.4 x 115 cm. column of sodium Dowex-50. Column was developed at room temperature with 0.1 M sodium phosphate buffer, pH 7.5

A. Hydroxylysine monohydrochloride separated from Dowex-50 was used.

B. Racemic hydroxylysine was used as a control.


A
B
OH-L-LY
Allo-OH-DL-LY
OH-DL-LY
4.0
3.0
2.0
1.0
μmoles of Hydroxy-L-Lysine
5
6
5
6
Volume of Effluent (Liters)

hydrolysis. Hydroxylysine monohydrochloride was rechromatographed through a 7.5 x 30 cm. column of sodium Dowex-50 and crystallized as described above.

The specific rotation of the hydroxylysine isolated from gelatin was $[\alpha]_D^{25} = 15.02°$[5]. Fones (28) reported $[\alpha]_D^{25} = + 17.8°$ for the hydroxyl-L-lysine, free base, and $[\alpha]_D^{25} = - 32.1°$ for the allohydroxy--D-lysine.

The major impurities in this hydroxylysine are probably inorganic salts. Quantitative chromotropic acid and ninhydrin procedures using the mixed isomer sample as a standard indicated that the isolated sample was 95% pure. If it is assumed that the optical impurity is allohydroxy-D-lysine the specific rotation data would suggest that the compound used was approximately 90% pure hydroxy-L-lysine. The rotation and colorimetric analysis data are subject to some variation, and the compound may be somewhat more or less pure than this. The purification of the labeled compound following exposure to tritium might lead to greater purity in this sample.

The present study indicated that little or no inversion of hydroxylysine occurred during the course of isolation and thus only a single L-isomer was detected. It appears that the epimerization around C-2 during prolonged boiling with 6 N hydrochloric acid, as observed by Hamilton and Anderson (6), did not occur under the conditions of hydrolysis employed in this study.

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[5]The author is indebted to Dr. M. Ikawa for optical rotation measurements.

B. Growth Studies

The results of growth studies (Fig. 3, 4, 5) duplicate the experimental findings in the literature on the sparing effect of hydroxy-L-lysine on the lysine requirement of S. faecalis. They are in general agreement with those of Bergstrom and Lindstedt (18) and Peterson and Carroll (5), in that the addition of hydroxylysine to a lysine-free medium failed to allow growth. However, hydroxylysine appears to lower markedly the lysine requirement of this organism.

The growth curves obtained with the three media used were so different (Fig. 3, 4, 5) that the further investigation of the cause of these differences was attempted. Using medium III (Fig. 3), which originally gave poor growth under conditions of optimum lysine supply, hydroxylysine stimulated very markedly. With medium I and medium II, which gave better growth of the microorganism under conditions of optimum lysine supply, gave similar growth curves and both permitted considerable sparing of lysine by hydroxylysine.

Each major component in medium III was replaced by the corresponding component in medium I or II. The relative proportions of certain amino acids vary greatly among the three media. Medium I and medium III are essentially the same in amino acid composition. The most conspicuous differences are the large amount of alanine and aspartic acid in medium II. Supplementation of medium III with alanine or aspartic acid to the same level as present in medium II did not improve the growth, whereas the increase of glucose and vitamins to the levels in medium II improved the bacterial growth (Fig. 6).

Figure 3.

Effect of addition of hydroxy-L-lysine to the basal medium III on response of Streptococcus faecalis to increments of L-lysine.

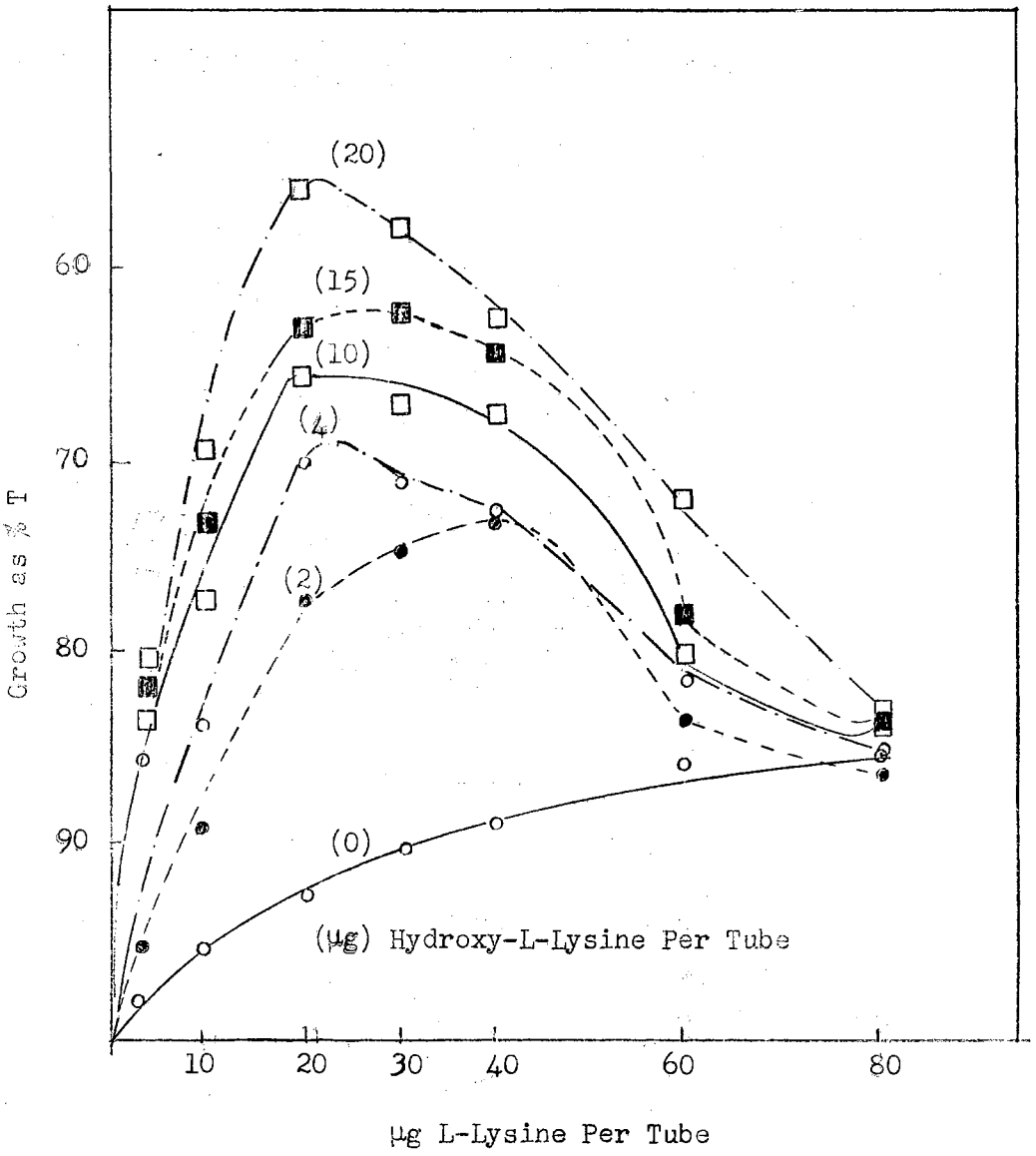

(20)
(15)
(10)
(4)
(2)
(0)
60
70
80
90
Growth as % T
(µg) Hydroxy-L-Lysine Per Tube
10
20
30
40
60
80
µg L-Lysine Per Tube

Figure 4.

Effect of addition of hydroxy-L-lysine to the basal medium I on the response of Streptococcus faecalis to increments of L-lysine.

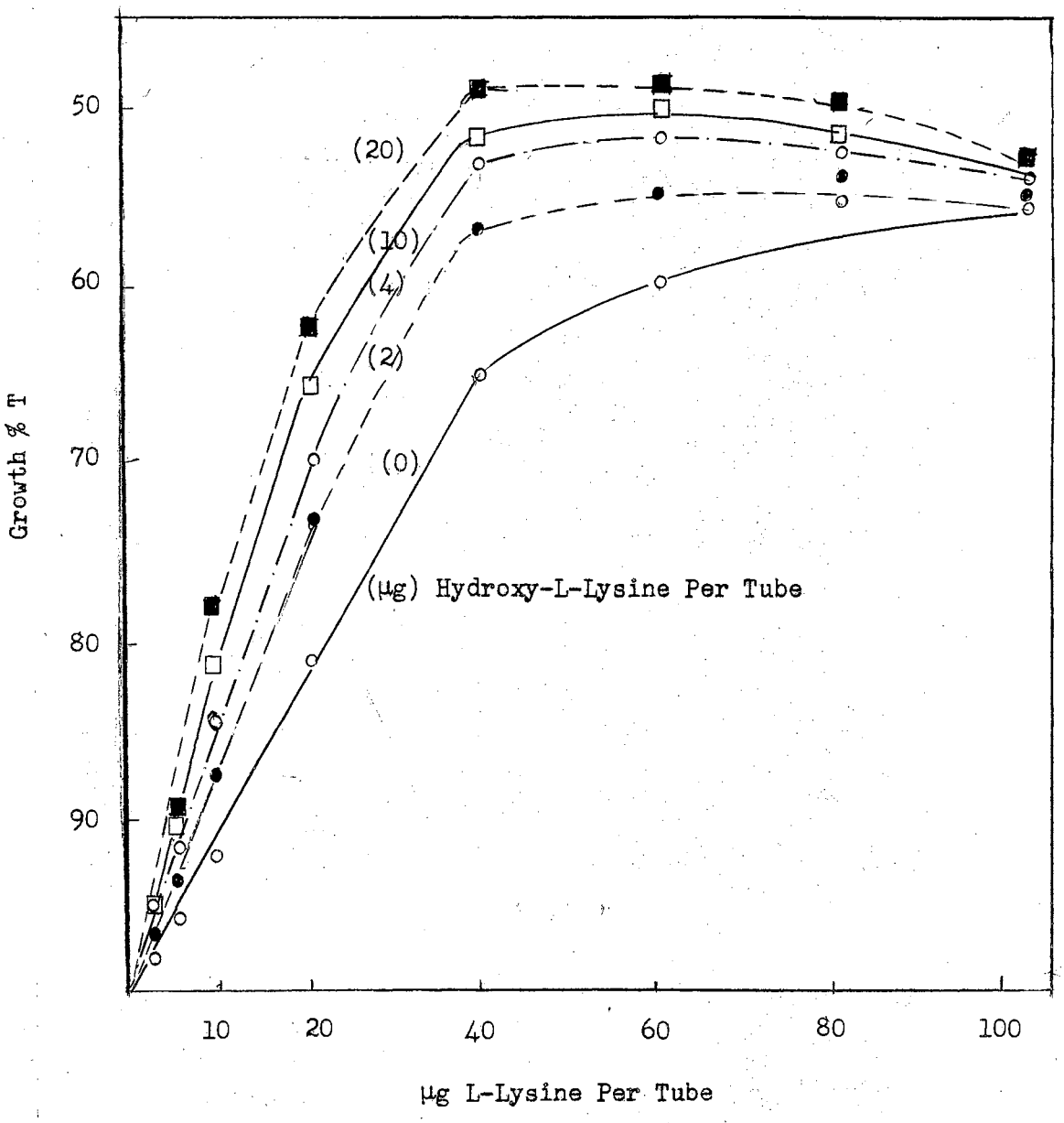

50
60
70
80
90
Growth % T
(20)
(10)
(4)
(2)
(0)
(µg) Hydroxy-L-Lysine Per Tube
10
20
40
60
80
100
µg L-Lysine Per Tube

Figure 5.

Effect of addition of hydroxy-L-lysine to the basal medium II on the response of Streptococcus faecalis to increments of L-lysine.

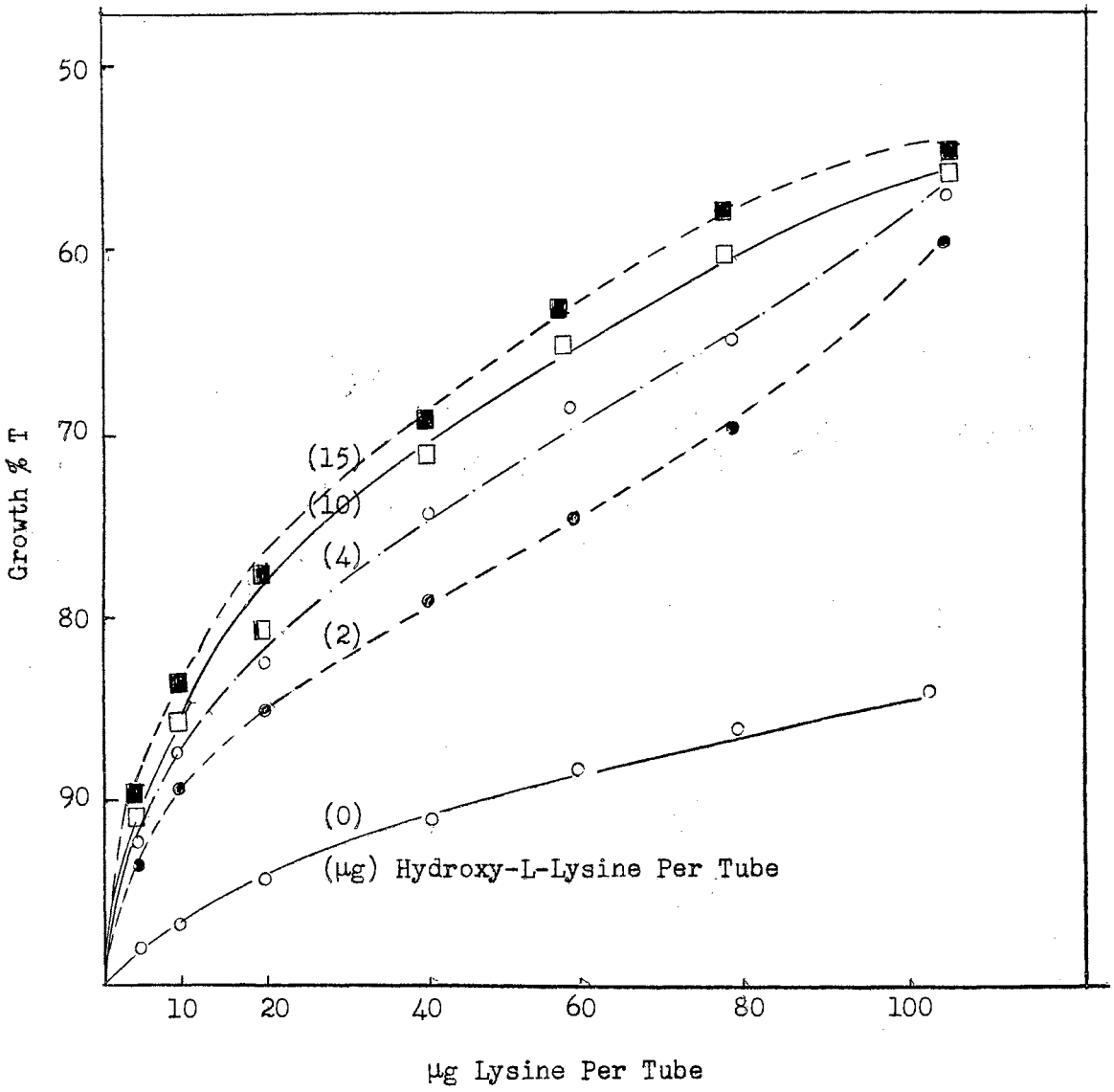
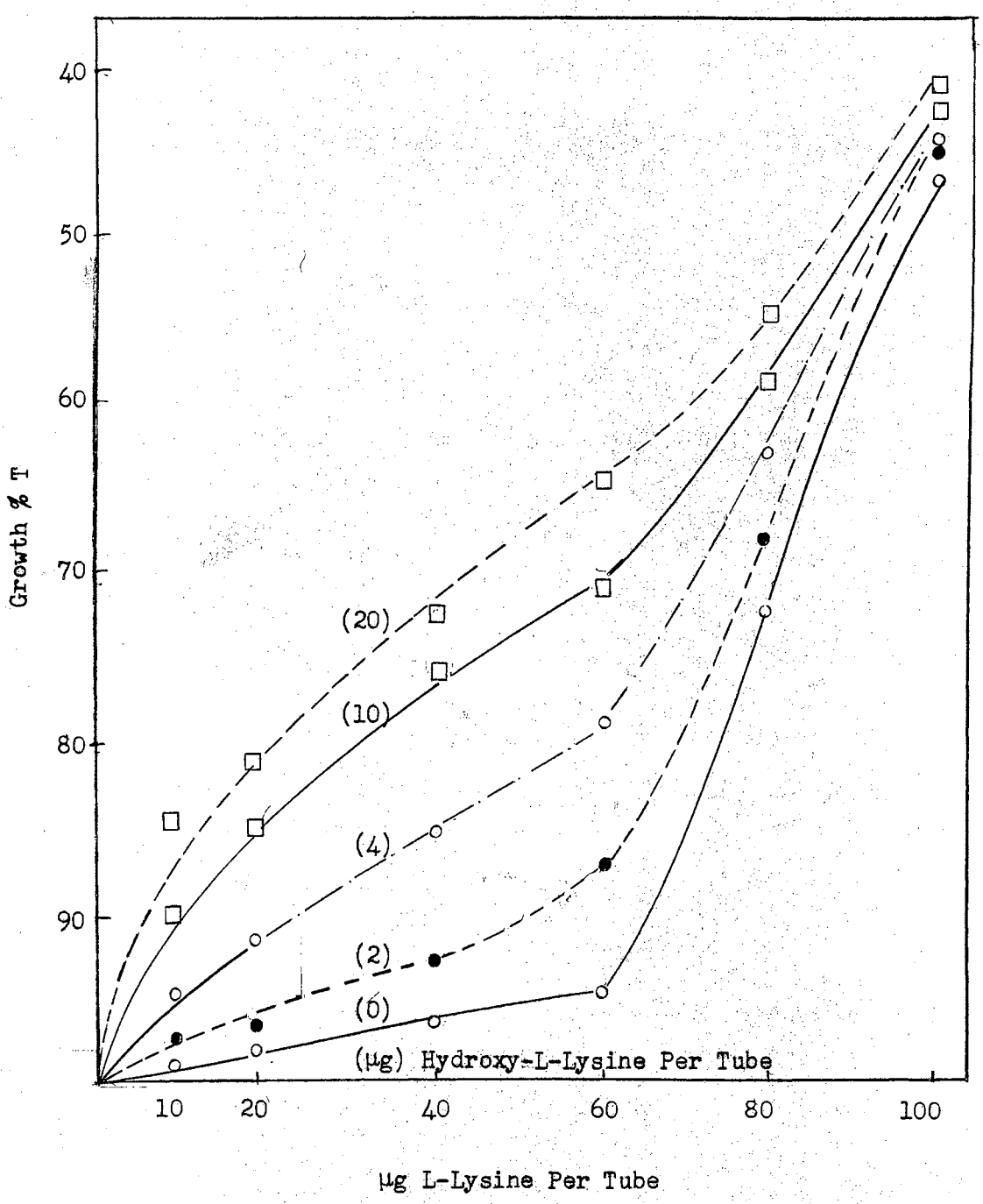

50
60
70
80
90
Growth % T
(15)
(10)
(4)
(2)
(0)
(µg) Hydroxy-L-Lysine Per Tube
10
20
40
60
80
100
µg Lysine Per Tube

Figure 6.

Effect of addition of hydroxy-L-lysine to the basal medium III in which vitamin solution and glucose were replaced by that of medium II.


40
50
60
70
80
90
Growth % T
(20)
(10)
(4)
(2)
(0)
(µg) Hydroxy-L-Lysine Per Tube
10
20
40
60
80
100
µg L-Lysine Per Tube

A rather complete investigation was made of the reason for the difference in growth with medium I and medium III. Preliminary results have shown that none of the components added in solution were responsible for the difference. Increasing the amount of sodium acetate and ammonium chloride with elimination of sodium citrate from medium III gave a growth curve similar to that obtained with medium I (Fig. 7). A complete examination of this matter was not completed and the exact cause of the difference in the growth observed remains unknown.

Figs. 8 and 9 compare the response to hydroxy-L-lysine and racemic hydroxylysine. The sparing effect of 4 μg. of racemic hydroxylysine corresponded to that of 1 μg. hydroxy-L-lysine; 10μg. racemic hydroxylysine per tube stimulated the bacterial growth to the same extent as 4 μg. hydroxy-L-lysine. This indicated that the racemic hydroxylysine contained approximately 25 per cent hydroxy-L-lysine and 75 per cent of the remaining three diasteroisomers. It further suggests that only the natural isomer is active in stimulating growth on low lysine media. This, in turn, served to indicate that the sample hydroxylysine isolated was a single, naturally occurring isomer.

Preliminary results showed that hydroxypipecolic acid produced a lysine sparing effect similar to that observed with hydroxylysine.

Lindstedt and Lindstedt (38) have studied the degradation of DL-lysine-6-C^{14} and hydroxy-DL-lysine-6-C^{14} by <u>in vitro</u> systems and obtained conversion of the two amino acids to pipecolic acid and 5-hydroxypipecolic acid, respectively. These results thus demonstrate a close parallelism in the catabolism of the two amino acids as well

Figure 7.

Effect of addition of hydroxy-L-lysine to the basal medium III in which the amount of dry components added was adjusted to the same level as that in medium I.

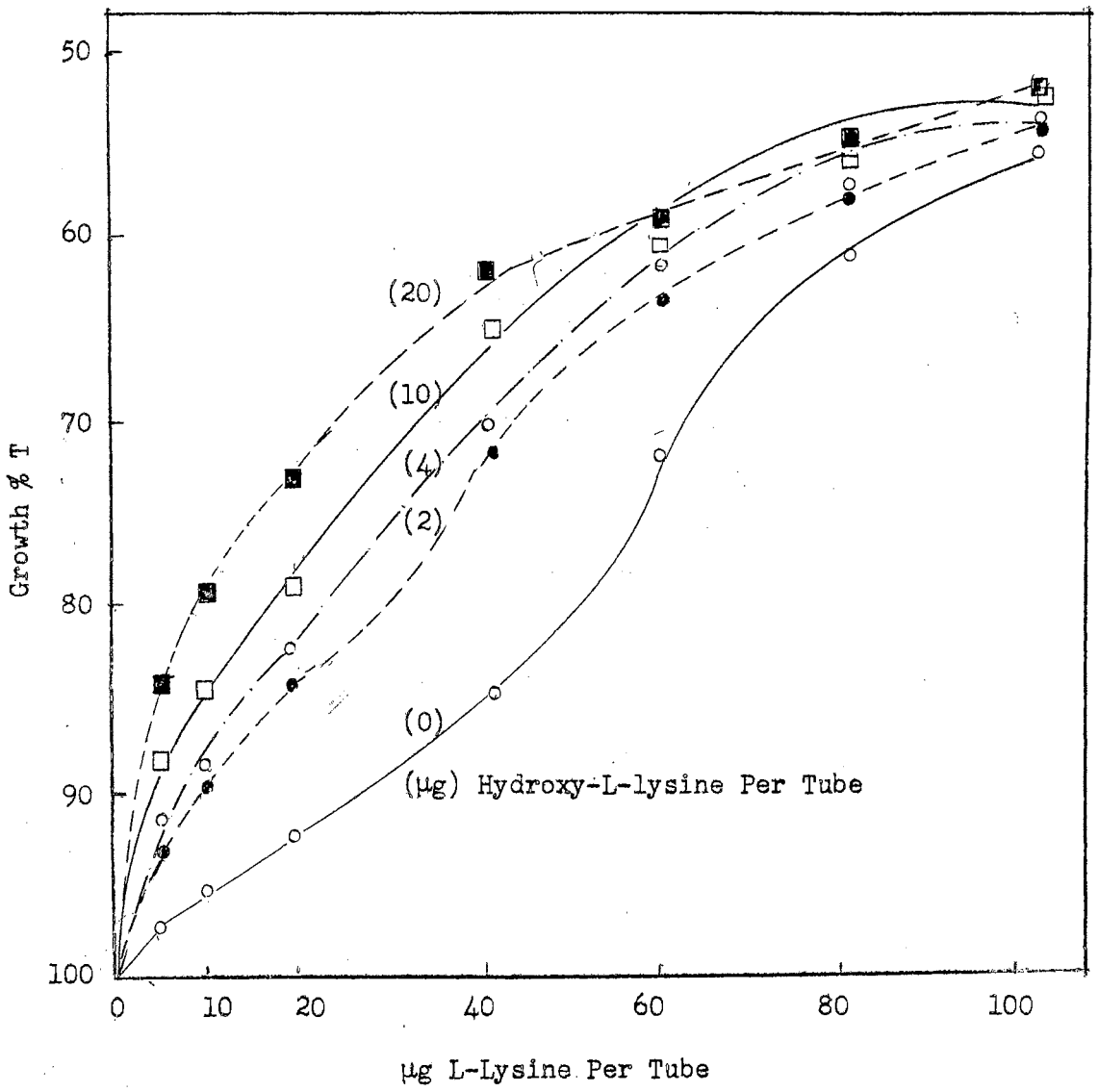

50
60
70
80
90
100
Growth % T
(20)
(10)
(4)
(2)
(0)
(µg) Hydroxy-L-lysine Per Tube
0
10
20
40
60
80
100
µg L-Lysine Per Tube

Figure 8.

Effect of addition of hydroxy-L-lysine or racemic (DL and allo) hydroxylysine.

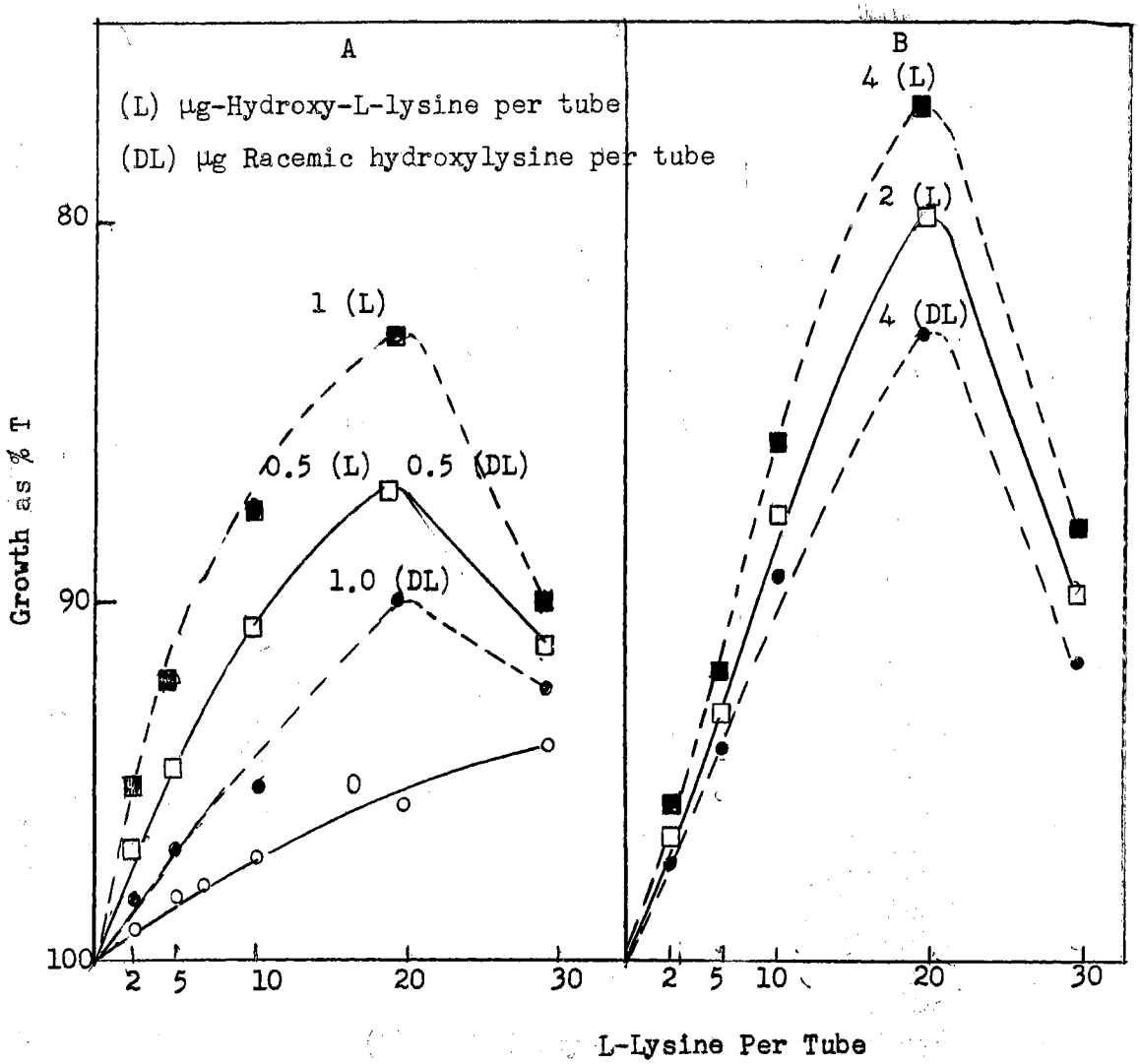
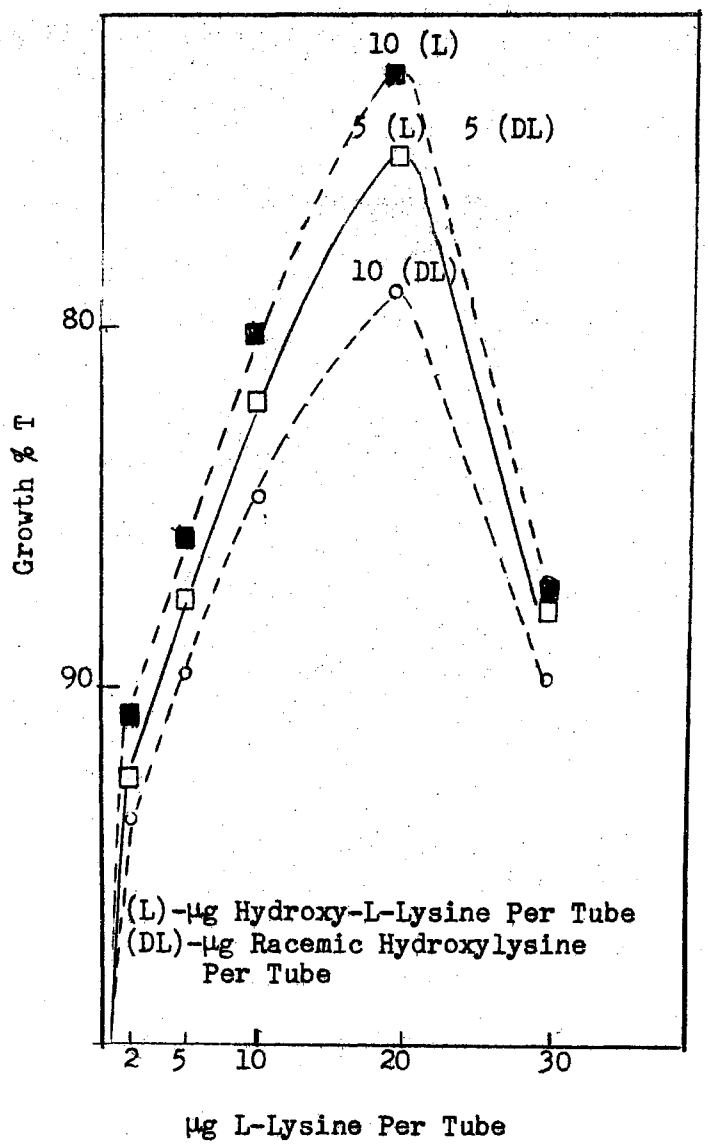

A
B
(L) µg-Hydroxy-L-lysine per tube
(DL) µg Racemic hydroxylysine per tube
80
90
100
Growth as % T
1 (L)
0.5 (L)
0.5 (DL)
1.0 (DL)
0
4 (L)
2 (L)
4 (DL)
2
5
10
20
30
L-Lysine Per Tube

Figure 9.

Effect of addition of hydroxy-L-lysine or racemic (DL and allo) hydroxylysine.


10 (L)
5 (L)
5 (DL)
10 (DL)
80
90
Growth % T
(L)-µg Hydroxy-L-Lysine Per Tube
(DL)-µg Racemic Hydroxylysine Per Tube
2
5
10
20
30
µg L-Lysine Per Tube

as the formation of hydroxypipecolic acid by mammalian tissue. The biological activity of hydroxypipecolic acid suggested that this compound might also be a degradation product in the lactic acid bacteria. The nature of the degradative process and the further investigation of both hydroxyamino and imino acid was not attempted.

C. Incorporation Studies

The results of a study of the incorporation L-lysine-C^{14} are shown in the first row of figures in Table V, and in a diagram, Fig. 10, showing the results of an ion exchange chromatographic separation of lysine and hydroxylysine from a hydrolysate of the S. faecalis cells grown on labeled lysine. The ordinates were drawn to make the resulting peaks given either by ninhydrin analyses or by radioactive analyses the same height. The C^{14} of lysine incorporated into the cells was found largely as lysine and there was essentially no radioactivity found in the carrier hydroxylysine separated.

The separation of lysine and hydroxylysine from the cell hydrolysates on a column of sodium Dowex-50 was essentially complete though a slight overlapping occurred. The tubes collected between 130 and 142 ml. contained the major part of the hydroxylysine, but these fractions contained no detectable C^{14}. Since no carrier lysine was added the ninhydrin color for the lysine-containing fractions was weak, but there was considerable C^{14} present. The radioautogram supported this result in that there was no radioactive spot found at the R_f corresponding to hydroxylysine. Consequently these results indicate that lysine is not converted to hydroxylysine or if it is

TABLE V

RADIOACTIVITY RECOVERED IN CELLS AND IN LYSINE AND HYDROXYLYSINE AFTER GROWING S. FAECALIS IN THE PRESENCE OF THESE LABELED AMINO ACIDS*

Added to the Medium		Found in Cells	Present in Separated Amino Acids
	CPM	CPM	CPM
L-Lysine-C^{14}	1.1×10^{7}	2.13×10^{6}	1.84×10^{6}
Hydroxylysine			6.0×10^{3}
Hydroxy-L-Lysine-H^{3}	2.09×10^{11}	6.15×10^{9}	3.98×10^{9}
L-Lysine			2.84×10^{3}

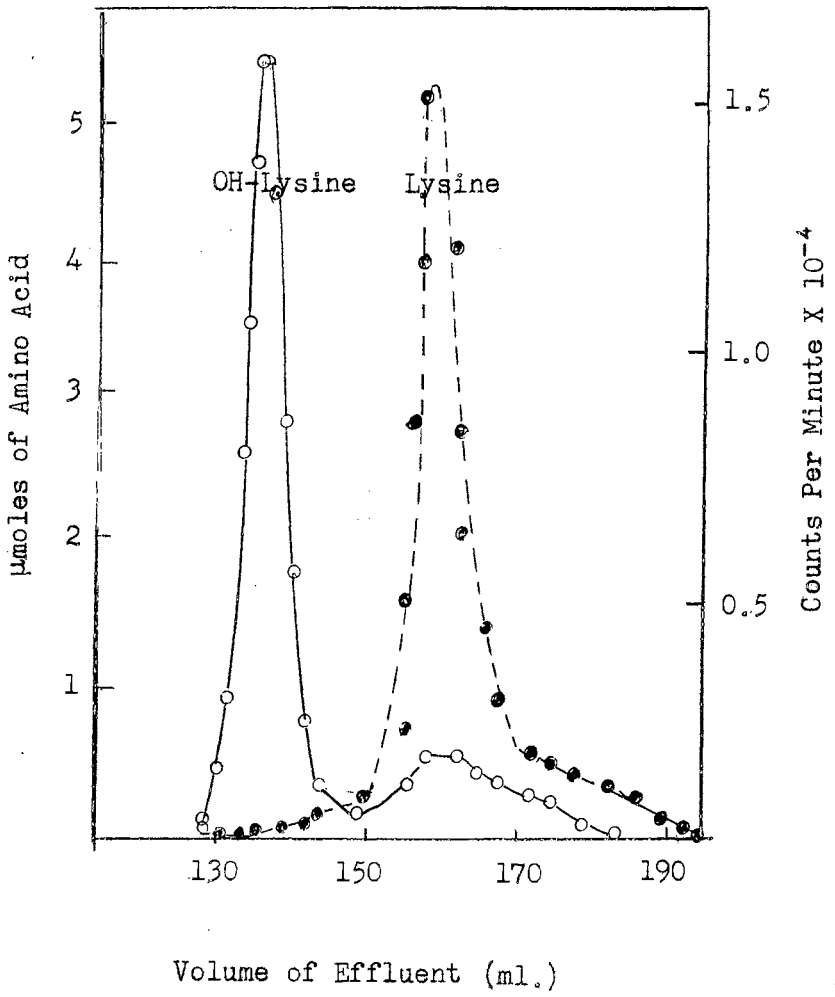
* C^{14} by thin window planchet counting, efficiency about 20%; H^{3} by liquid scintillation counting, efficiency about 5.5%.

Figure 10.

Separation of hydroxylysine and lysine on a column of Dowex-50, 1.0 x 40 cm., operated in the sodium form at room temperature, with 0.3 M acetate buffer, pH 5.0 as eluent. The amino acid mixture placed on the column is the hydroxylysine-containing effluent fraction from a column of Amberlite IR-120 employed to remove most of the monoamino acid of the acid hydrolysate of S. faecalis grown on medium I with L-lysine-$-C^{14}$. 50 mg. of racemic hydroxylysine was added as carrier.

● C^{14} analyses

o ninhydrin analyses


OH-Lysine
Lysine
μmoles of Amino Acid
Counts Per Minute X 10^{-4}
1
2
3
4
5
0.5
1.0
1.5
130
150
170
190
Volume of Effluent (ml.)

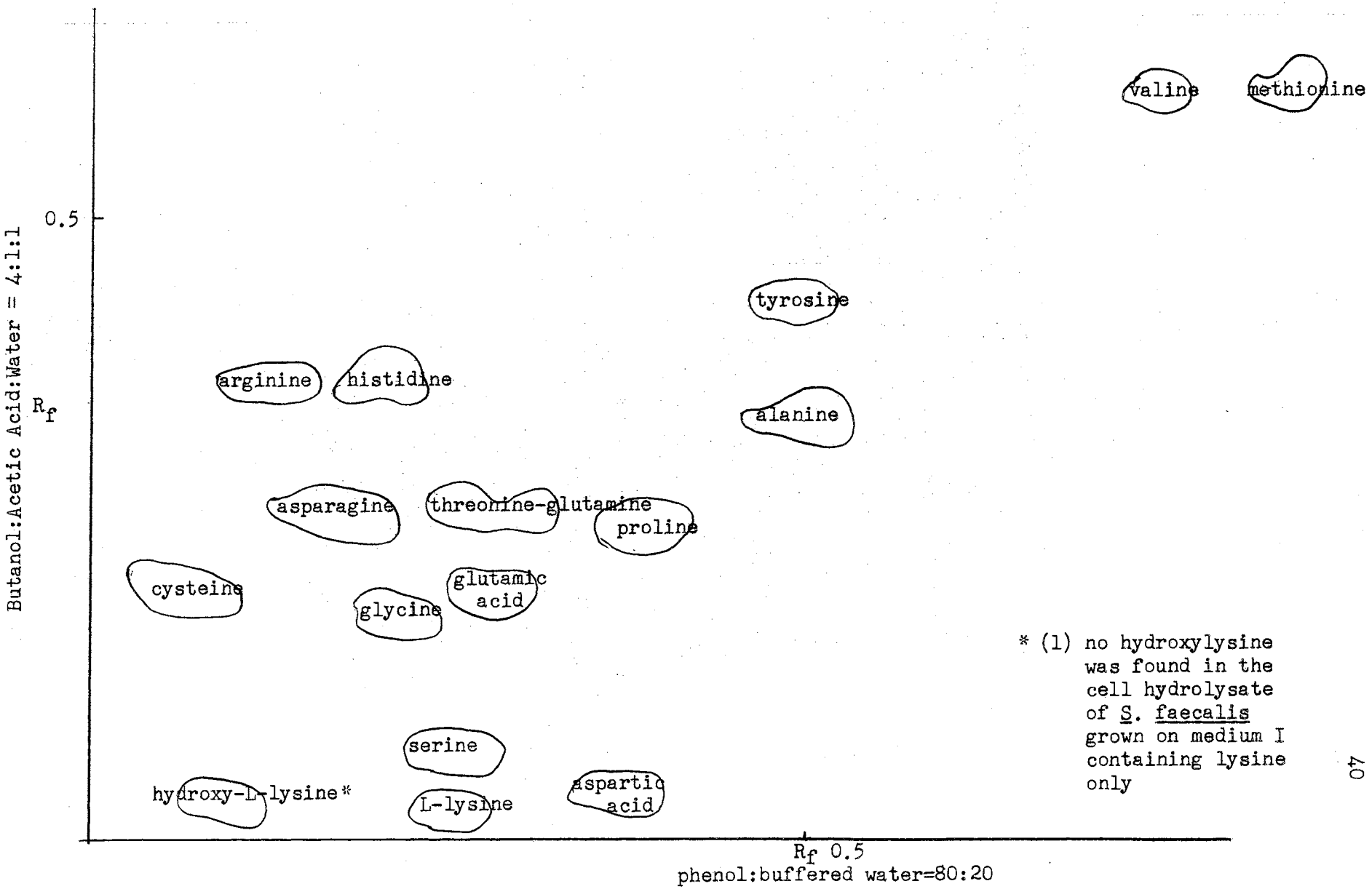
the hydroxylysine is rapidly metabolized to other compounds. This finding contrasts with the results reported by Sinex and Van Slyke (2) but in that case a substantial amount of hydroxylysine is stored in collagen. The results reported here are in keeping with the observations made in these studies and by Sinex (personal communication to L. M. Henderson) that lactic acid bacteria cells do not contain hydroxylysine.

As discussed in the growth studies above hydroxylysine markedly stimulated the growth of S. faecalis under conditions of limiting amounts of L-lysine in the basal medium. Hydroxylysine is probably metabolically related to lysine in this microorganism, but lysine may not be converted to hydroxylysine. Results from a two dimensional chromatogram (Fig. 11) indicate that hydroxylysine might not be a naturally occurring amino acid in S. faecalis since nothing was found at the R_f corresponding to hydroxylysine.

The isotope analyses of cell hydrolysates of S. faecalis grown on the medium III containing hydroxylysine-H^3 are shown in the second row of figures in Table V. A typical ion exchange chromatogram pattern, shown in Fig. 12, indicates a complete separation of hydroxylysine from lysine of the cell hydrolysate. Tritium analyses have shown that H^3 of hydroxylysine was incorporated into the cells chiefly as hydroxylysine. Lysine was found to contain no tritium and the radioautogram of a sample taken from the lysine peak showed no radioactive spots. This result agrees with the results in rats (3), where lysine was not formed from hydroxylysine. It differs from the results in rats in that S. faecalis did incorporate hydroxylysine directly into its cells.

Figure 11.

Two dimensional chromatogram of the acid hydrolysate of Streptococcus faecalis grown on medium I containing L-lysine only.



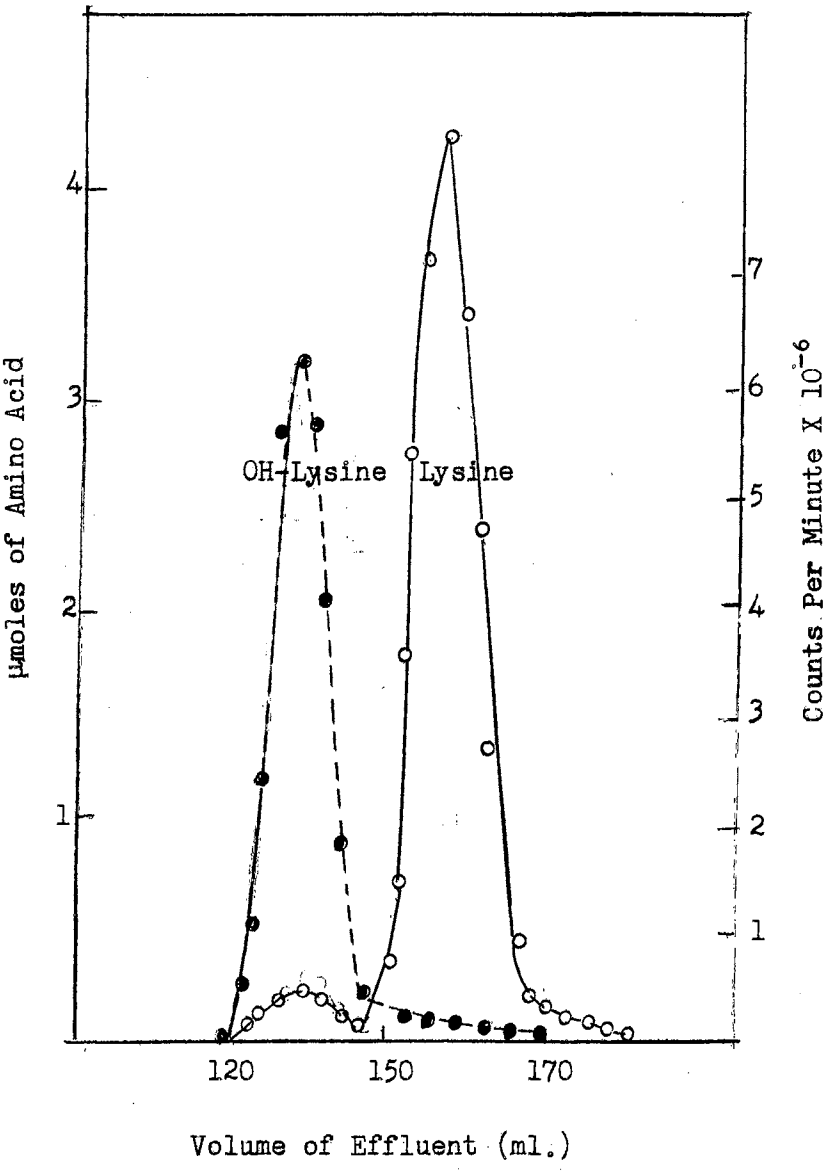
* (1) no hydroxylysine was found in the cell hydrolysate of S. faecalis grown on medium I containing lysine only

Figure 12.

Separation of hydroxylysine and lysine on a column of Dowex-50, 1.0 x 40 cm., operated in the sodium form at room temperature, with 0.3 M acetate buffer, pH 5.0 as eluent. The amino acid mixture placed on the column is the hydroxylysine-containing effluent fraction from a column of Amberlite IR-120 employed to remove most of the monoamino acid of the acid hydrolysate of S. faecalis grown on medium III with hydroxy-L-lysine-H^3 and unlabeled L-lysine. 50 mg. of L-lysine was added as carrier.

● H^3 activity

○ ninhydrin color


μmoles of Amino Acid
Counts Per Minute X 10^{-6}
OH-Lysine
Lysine
1
2
3
4
1
2
3
4
5
6
7
120
150
170
Volume of Effluent (ml.)

D. Cell Fractionation

Cells grown on medium III in the presence of labeled hydroxylyxine were harvested and fractionated by the method of Roberts, et al. (44) described above. Each fraction was counted by the liquid scintillation procedure (Table VI).

Nearly 85% of the tritium was found in the hot trichloroacetic acid soluble and residual protein fractions. An effort was made to determine the nature of the labeled compounds in the hot TCA soluble fraction. After removal of the TCA and hydrolysis in 0.1 N HCl for 1 hour at 90 to 100° C., an ascending paper chromatographic separation of the hydrolysis products was made in one dimension with 70 per cent t-butyl alcohol in 0.8 N. HCl (51). Also, two dimensional separations are made using sec-butanol/water/formic acid (70:20:10) as the second solvent. After 18 hours of development in the one direction and thorough air drying, the one dimensional papergrams were examined under an ultraviolet lamp and the ultraviolent-absorbing regions marked. Four spots corresponding to adenine, guanine, cytidylic and uridylic acids were found, the spots were cut out of the paper strip, extracted with liquid scintillation counting solvent-X-10 for 6 hours then counted. No radioactivity was found in either the purines or the pyrimidine nucleotides. A duplicate sheet of paper chromatogram was sprayed with ninhydrin and no ninhydrin positive compound was detected. Further work would be required to characterize the labeled compounds present in this fraction.

TABLE VI

DISTRIBUTION OF RADIOACTIVITY FROM HYDROXY-L-LYSINE-H^3

IN VARIOUS CELL FRACTIONS OF S. FAECALIS

Fraction	CPM*	Percentage of Radioactivity
original washed cells	313,000	100
cold-TCA-soluble	11,200	3.58
alcohol-soluble	1,200	0.384
alcohol-ether soluble	800	0.256
hot-TCA-soluble	102,000	32.6
acidified alcohol wash	12,400	3.96
ether wash	0	0.00
residual protein	162,660	52.1
total recovered	290,260	92.6

* Corrected for background but not corrected for efficiency which was about 5.5%.

The residual protein fraction was not subjected to a further analysis for isotopes, since this type of experiment had already been done.

Fractionation of the cells of S. faecalis indicated that hydroxy--L-lysine-H^3 were incorporated into the protein, presumably bound by peptide linkage. The residual protein was further fractionated to the solubilized protein and cell wall mucopeptide by the procedure of Park and Hancock (45). Preliminary data showed that the radioactivity of the hydroxylysine-H^3 was evenly distributed between soluble protein and cell wall mucopeptide. This observation is rather interesting for lysine is a known constituent of these mucopeptides.

The cell wall preparation made as described under "Methods" contained approximately 5% of the tritium in the harvested cells. This figure is minimal since many steps were used to get a pure cell wall preparation.

E. Uptake of Labeled Amino Acids

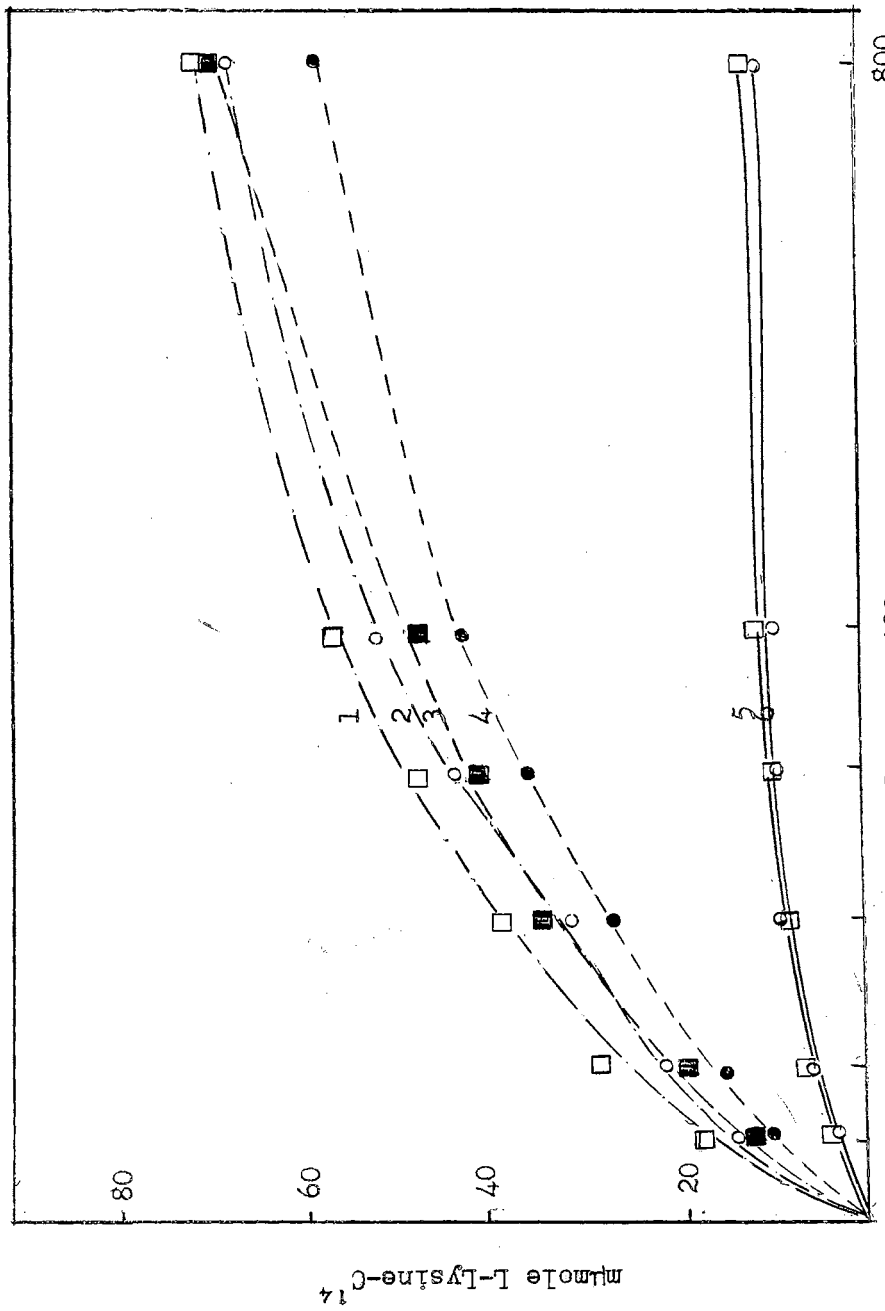
A sensitive approach to the relationships between various accumulating systems is to determine the effect of unlabeled compounds on the accumulation of another labeled compound. A preliminary study of lysine-C^{14} uptake by S. faecalis cells indicated that in a 30-minute test 300 mμmoles per ml. of lysine saturated the uptake system. In a second study of this kind the system did not appear to be saturated even with 800 mμmoles per ml. (Fig. 13). This figure shows data illustrating the effect of the concentration of lysine-C^{14} on its

Figure 13.

Effect of substrate concentration on the uptake, accumulation and incorporation of L-lysine-C^{14} in the presence or absence of unlabeled hydroxy-L-lysine (300 mμmoles per ml.).

Curve:

1. uptake without hydroxy-L-lysine
2. uptake with hydroxy-L-lysine
3. accumulation without hydroxy-L-lysine
4. accumulation with hydroxy-L-lysine
5. incorporation with hydroxy-L-lysine
6. incorporation without hydroxy-L-lysine


mμmole L-Lysine-C14
20
40
60
80
50
100
200
300
400
800
mμmole L-Lysine-C14 Per ml.
(Substrate)
1
2
3
4
5
6
c

uptake by resting cells in the presence and absence of unlabeled hydroxy-L-lysine (300 mμmole per ml.). The hydroxy-L-lysine reduced the uptake and accumulation of lysine-C^{14} approximately 15%.

A comparison of the rates of uptake, accumulation, and incorporation of radioactivity from a concentration of 300 mμmoles per ml. of L-lysine-C^{14} with and without unlabeled hydroxylysine is shown in Fig. 14. Accumulation from the samples with hydroxylysine reached a maximum in approximately 5 minutes, then slowly decreased whereas, samples without hydroxylysine reached a maximum in approximately 30 minutes, then more slowly decreased. This interesting stimulation of lysine uptake by hydroxylysine was observed only in the early minutes of an uptake experiment. Its significance in relation to the stimulatory effect of hydroxylysine on growth is not known.

Fig. 15 shows the effect of the concentration of hydroxy-L-lysine-H^3 on its uptake with and without unlabeled lysine. From these results it is difficult to evaluate the importance of the slight reduction in hydroxylysine uptake caused by lysine particularly in view of the slow uptake rate under both circumstances. However, it is striking that there are essentially no differences between the rates of uptake and incorporation indicating the absence of hydroxylysine accumulated as "pool" content. This suggested that hydroxylysine, though taken up very poorly by S. faecalis, was incorporated into protein as soon as it entered the cell.

The time course shown in Fig. 16 indicated that the system for the uptake hydroxylysine by S. faecalis was not affected by the presence

Figure 14.

The time course of uptake, accumulation and incorporation of L-lysine-C^{14} in the absence or presence of unlabeled hydroxy-L-lysine (300 mμmoles per ml.).

Curve:

1. uptake with hydroxy-L-lysine
2. uptake without hydroxy-L-lysine
3. accumulation with hydroxy-L-lysine
4. accumulation without hydroxy-L-lysine
5. incorporation without hydroxy-L-lysine
6. incorporation with hydroxy-L-lysine

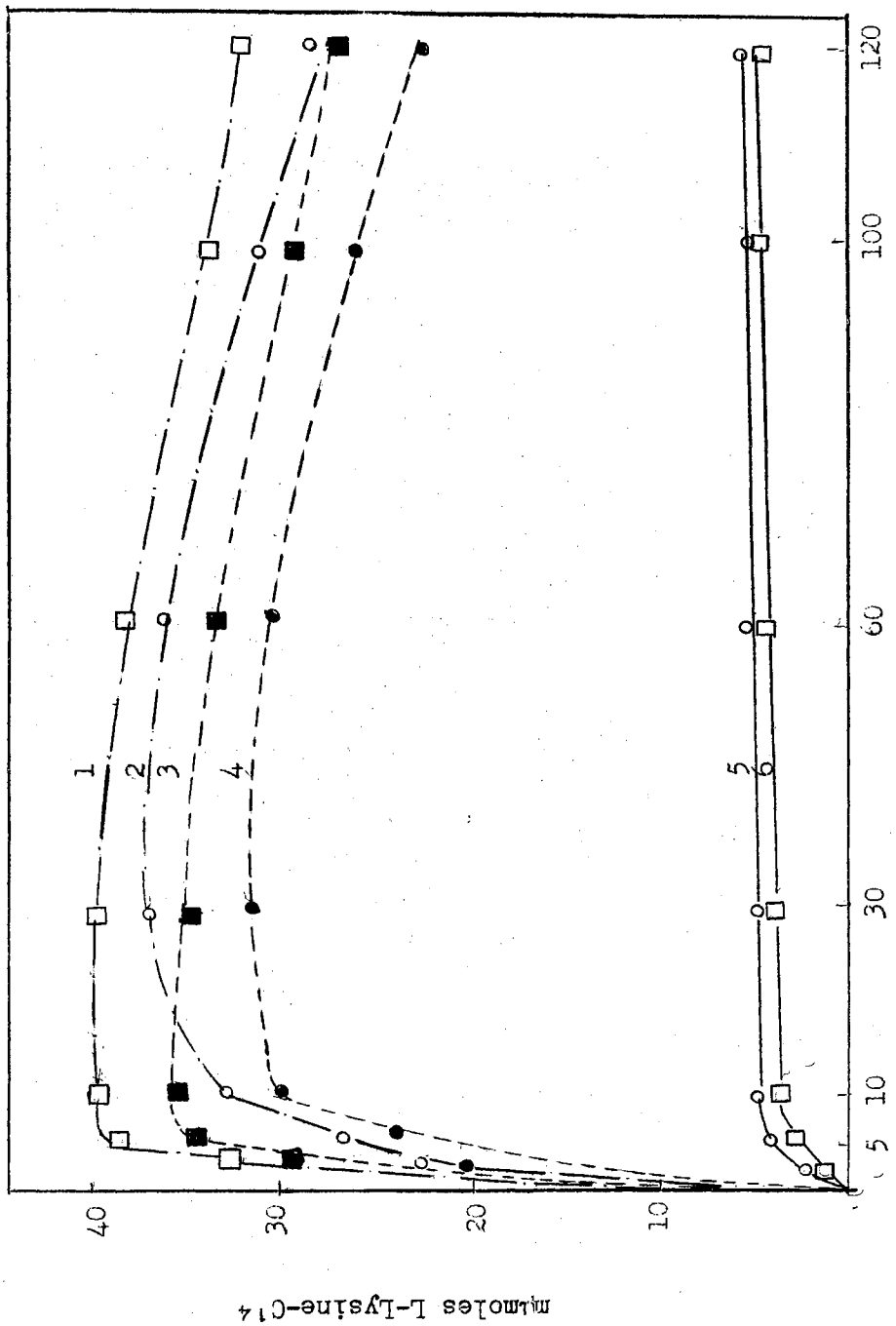

μmoles L-Lysine-C14
40
30
20
10
5
10
30
60
100
120
Time in Minutes
1
2
3
4
5
6

Figure 15.

The effect of substrate concentration on the uptake and incorporation of hydroxy-L-lysine-H^3 in the absence or presence of unlabeled L-lysine.

Curve:

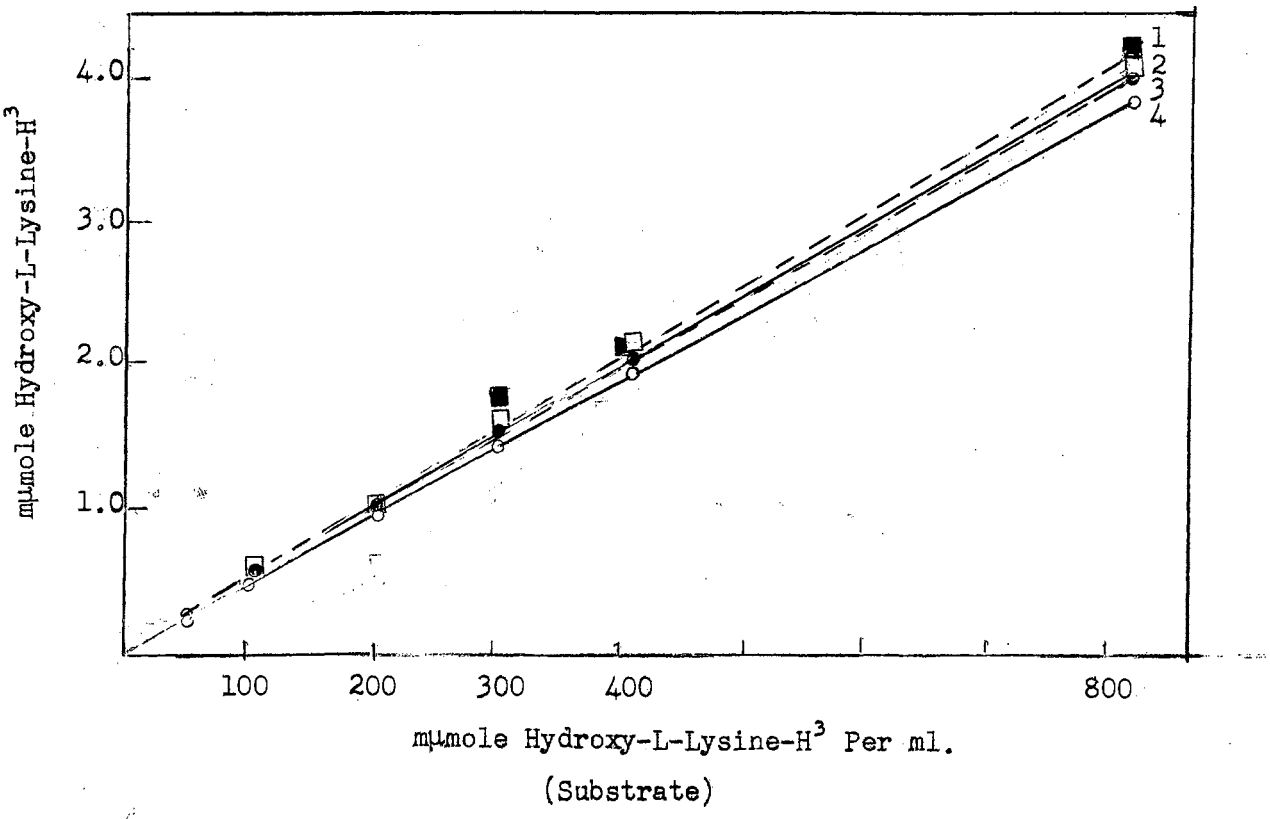
1. uptake without L-lysine
2. incorporation without L-lysine
3. uptake with lysine
4. incorporation with L-lysine

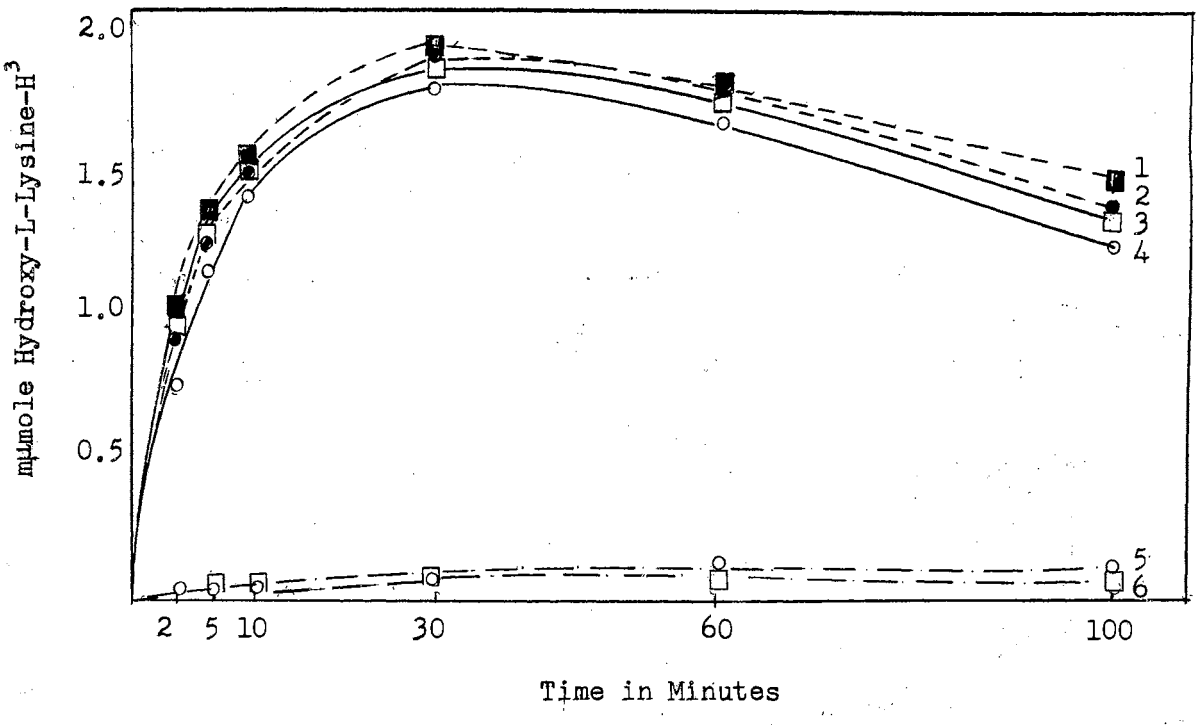
Figure 16.

The time course of uptake, accumulation and incorporation of hydroxy--L-lysine-H^3 in the absence or presence of unlabeled L-lysine (300 mμmoles per ml.).

Curve:

1. uptake without L-lysine
2. uptake with L-lysine
3. incorporation without L-lysine
4. incorporation with L-lysine
5. accumulation without L-lysine
6. accumulation with L-lysine


mμmole Hydroxy-L-Lysine-H3
4.0
3.0
2.0
1.0
100
200
300
400
800
1
2
3
4
mμmole Hydroxy-L-Lysine-H3 Per ml.
(Substrate)


mμmole Hydroxy-L-Lysine-H3
2.0
1.5
1.0
0.5
2
5
10
30
60
100
1
2
3
4
5
6
Time in Minutes

of unlabeled lysine. The rates of uptake and of incorporation were so similar that the curves nearly coincided. Thus, hydroxylysine-H^3 did not appear to accumulate as "pool" in this organism. The amount of hydroxylysine within the cells reached a maximum in 30 minutes and then some decrease in isotope within the cell occurred. This finding indicated that hydroxylysine-H^3 was very slowly taken up by S. faecalis, but as soon as it was taken up it was incorporated into protein leaving never more than traces in the pool. The decrease in radioactivity of the cells which were held for a longer period in the presence of excess labeled hydroxylysine is difficult to explain.

These experiments showed a difference in the mode of uptake or utilization of lysine and hydroxylysine. The slow uptake of hydroxylysine-H^3 was once thought to be due to the low efficiency of counting of tritium in bacterial cells. The hyamin technique was used, but the results were erratic probably due to variation in the intensity of the color produced. Wilzbach's (48) zinc fusion technique gave a better information from which to calibrate the counting efficiency of whole cells and trichloroacetic acid precipitates. The counting efficiency of the Liquid Scintillation spectrometer was found to be 5-6%.

The stimulatory effect of hydroxylysine on the uptake of lysine suggests that the observed sparing effect of hydroxylysine on the lysine requirement may be the result of its effect on lysine utilization. Other explanations seem more tenable in view of the magnitude of the lysine effect on hydroxylysine uptake.

CHAPTER V

SUMMARY

1. The mechanism by which hydroxylysine stimulates the growth of Streptococcus faecalis on a medium containing amounts of lysine normally limiting for growth has been investigated.

2. Natural hydroxy-L-lysine was isolated from gelatin hydrolysate, by ion exchange chromatography, and labeled with tritium by the Wilzbach procedure. Under the conditions of hydrolysis little or no epimerization around the α-carbon atom occurred.

3. Hydroxy-L-lysine was approximately four times as effective in sparing lysine as a synthetic mixture of the diasteroisomers commercially available indicating that only one of the stereoisomers is utilized.

4. L-Lysine-C^{14} did not lead to labeled hydroxylysine in cells grown in the absence of hydroxylysine. Likewise tritium labeled hydroxylysine did not label the lysine in the protein of S. faecalis grown on limiting amounts of lysine and a stimulatory level of the labeled hydroxylysine. These cells did contain tritium in the protein and largely as hydroxylysine.

5. Fractionation of the cells led to suggestive evidence that the wall of cells grown in the presence of labeled hydroxylysine contained this amino acid presumably as mucopeptides.

6. Measurement of the total, bound and pool levels of lysine-C^{14} taken up from the medium indicated that this amino acid is actively absorbed and that during rapid uptake a major part of the lysine is in the cell pool. Hydroxylysine increased the rate of uptake of lysine in the first few minutes but after 30 minutes the lysine uptake was 15% less when hydroxylysine was present.

7. Hydroxylysine-H^3 uptake was much slower and independent of the presence of lysine in the medium. In contrast to lysine, hydroxylysine was present largely in a trichloroacetic acid-insoluble form immediately after entering the cell.

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