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THE METABOLIC EFFECTS AND ILLICIT TRANSPORT OF 3,4-DIHYDROXYBUTYL-1-PHOSPHONATE IN ESCHERICHIA COLI

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THE METABOLIC EFFECTS AND ILLICIT TRANSPORT OF 3,4-DIHYDROXYBUTYL-1-PHOSPHONATE IN *ESCHERICHIA COLI*

by

MARY MARGARET CONNOLLY

A dissertation submitted to the Graduate Faculty in Biochemistry in partial fulfillment for the degree of Doctor of Philosophy, the City University of New York.

1983

This manuscript has been read and accepted for the Graduate Faculty in Biochemistry in satisfaction of the dissertation requirement for the degree of Doctor of Philosophy.

August 11, 1983
date

Burton E. Tropp
Chairman of Examining Committee

August 11, 1983
date

Aaron Lukton
Executive Officer

Jules Golubow

Thomas C. Strekas

Corinne Michels

Robert Engel

Supervisory Committee

The City University of New York

ABSTRACT

THE METABOLIC EFFECTS AND ILLICIT TRANSPORT OF
3,4-DIHYDROXYBUTYL-1-PHOSPHONATE
IN *ESCHERICHIA COLI*

by

Mary Margaret Connolly

Advisor: Professor Burton E. Tropp

3,4-Dihydroxybutyl-1-phosphonate (DHBP), an inhibitor of phosphoglyceride synthesis, exerts a bacteriostatic effect on the growth of *Escherichia coli*. Inhibition of growth by the analog is lessened or offset in strains having a large glycerol-3-phosphate pool. The effect of 3,4-dihydroxybutyl-1-phosphonate on growth was also examined in conjunction with deoxycholate, a bile salt present in the intestine, the natural habitat of the organism. The DHBP and deoxycholate together, exert a bactericidal effect on growth as determined by viability studies. This synergistic effect of the analog and deoxycholate also appears to depend on the intracellular glycerol-3-phosphate pool.

DHBP was also examined for its *in vivo* effect on the fatty acyl CoA: *sn*-glycerol-3-phosphate acyltransferase. When assayed for acyltransferase activity, membrane preparations isolated from DHBP treated cells have approximately the same K_m value (8.4×10^{-5} M) as those isolated from untreated cells (9.5×10^{-5} M).

The metabolism of DHBP labeled with tritium in the 1,2 position was studied. Chromatographic analysis of either the formic acid soluble or water soluble cell fractions reveals the presence of a metabolite of DHBP, as yet unidentified.

The oligopeptide transport system in *E. coli* was studied for possible exploitation as a means of phosphonic acid analog uptake. DHBP as well as the phosphonic acid analogs of dihydroxyacetone phosphate and phosphomevalonic acid were coupled to several different tripeptides and their effect on the growth of *E. coli* investigated. The analogs of dihydroxyacetone phosphate and phosphomevalonate do not appear to be transported in the strains tested unless coupled to peptides in which case they inhibit growth. These results indicate possible transport of the phosphonate coupled peptides via the oligopeptide transport system.

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My parents and family, last in this list, but my first and best teachers. The wealth of knowledge that I have gained from them could be learned from no books and no university. I owe them all that I am or will be.

DEDICATION

In memory of my Mother

MARY NOLAN CONNOLLY

Who taught me of love, laughter and life

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ABBREVIATIONS

Abbreviation	Compound
1. G3P	*sn*-Glycerol-3-phosphate
2. DHBP	3,4-Dihydroxybutyl-1-phosphate
3. DHAP	Dihydroxyacetone phosphate
4. DHAPa	4-Hydroxy-3-oxobutyl-1-phosphonate
5. PE	Phosphatidylethanolamine
6. PG	Phosphatidylglycerol
7. PGP	Phosphatidylglycerol phosphate
8. CL	Cardiolipin
9. [3-^{3}H]-DHBP	3,4-Dihydroxy[3-^{3}H]butyl-1-phosphonate
10. [1,2-^{3}H]-DHBP	3,4-Dihydroxy[1,2-^{3}H]butyl-1-phosphonate
11. F1Pa	Fructose-1-phosphate analog
12. MevPa	5-Phosphomevalonate analog
13. MevP	5-Phosphomevalonic acid

CHAPTER 1

The Metabolic Effects of 3,4-Dihydroxybutyl-1-Phosphonate

Introduction

L-Glycerol-3-phosphate (G3P), an important metabolic intermediate in *Escherichia coli* (1-7), is used for both lipid synthesis and as a precursor of glycolysis (Figure 1, adapted from reference 6). The genes for the proteins involved in the transport and metabolism of G3P and glycerol are under the control of a single repressor which can exert negative control over what has been termed the glp regulon (7).

Glycerol-3-phosphate, unlike many other phosphate esters in nature, is transported into cells of *E. coli* without hydrolysis of the phosphate ester (2,5). In an alkaline phosphatase negative strain of *E. coli*, Lin *et al.* (5) observed growth on glycerol, DL-G3P or L-G3P as carbon sources but not on glycerol-2-phosphate, which did support the growth of a strain with an active alkaline phosphatase. Hayashi *et al.* (2) showed that in strain 9 (negative for alkaline phosphatase, glycerol kinase and the L-G3P dehydrogenase) there is no exchange of either the glycerol or the phosphate moieties of L-G3P during transport.

FIGURE 1

Metabolic Pathways for Glycerol-3-Phosphate Metabolism in *E. coli*.

Adapted from reference 6. The following abbreviations are used for the intermediates involved in the metabolism of glycerol-3-phosphate: L-G3P, glycerol-3-phosphate; DHAP, dihydroxyacetone phosphate; GAP, L-glyceraldehyde 3-phosphate; F-1,6-diP, fructose 1, 6-diphosphate. The enzymes involved in the metabolism of G3P are as follows: 1) L-glycerol-3-phosphate transport system; 2) fatty acylCoA: *sn*-glycerol-3-phosphate acyltransferase; 3) membrane bound catabolic L-glycerol-3-phosphate dehydrogenase; 4) anaerobic L-glycerol-3-phosphate dehydrogenase; 5) anabolic L-glycerol-3-phosphate dehydrogenase (G3P synthase); 6) triose phosphate isomerase; 7) aldolase; 8) glycerol transport (facilitator); 9) glycerol kinase.

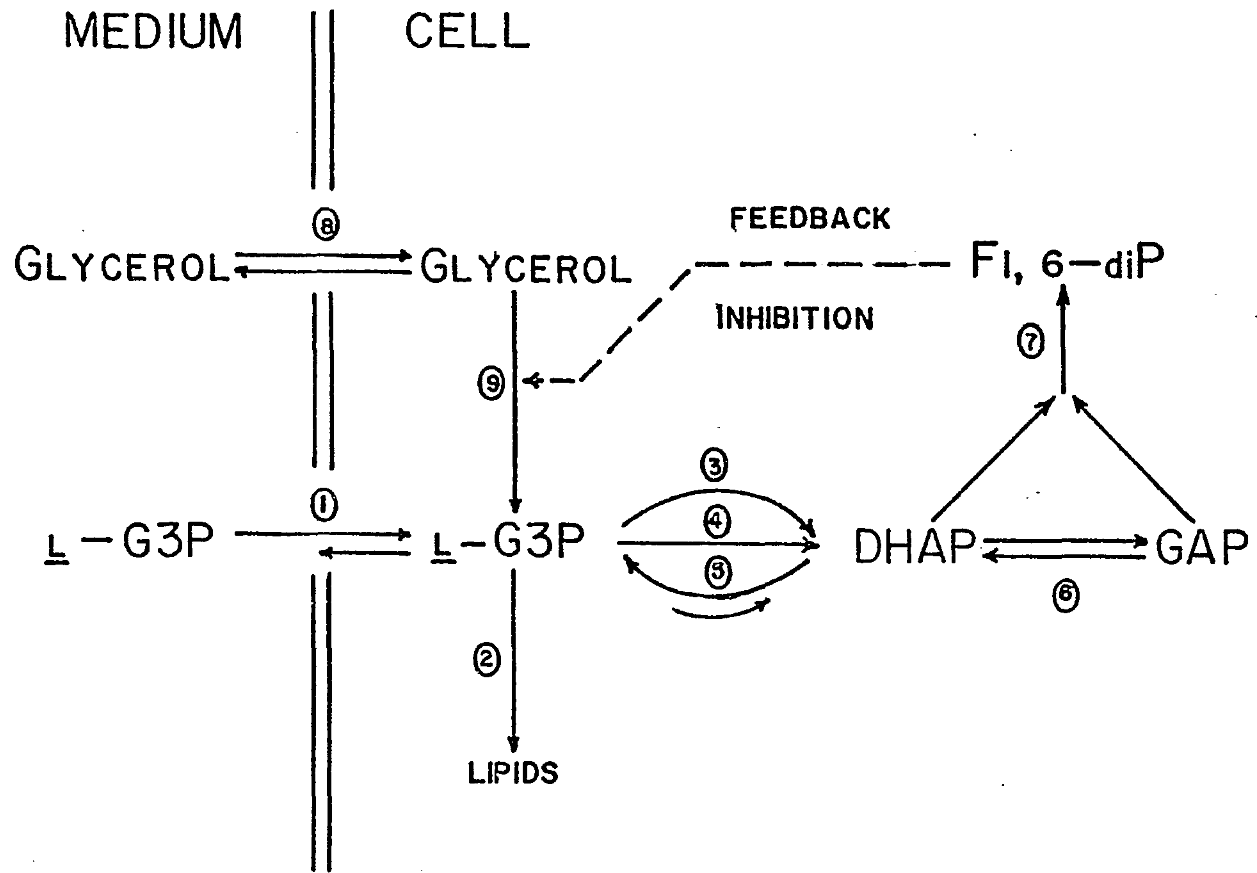


FIGURE 1

The uptake of L-G3P is via active transport which is inhibited by azide or dinitrophenol (2). The transport system also acts as an exit port for G3P since cells pre-loaded with radioactive G3P rapidly lose the label when cold G3P is added to the medium. The active transport of G3P is also competitively inhibited by glyceraldehyde-3-phosphate and inorganic phosphate but not by dihydroxyacetone phosphate (2).

Glycerol and G3P can induce at least three of the genes of the glp regulon, namely glpK (glycerol kinase, number 9 in Figure 1), glpD (L-G3P dehydrogenase, number 3 in Figure 1) and glpT (the L-G3P transport system, number 1 in Figure 1) in wild type strain 1 of *E. coli* (4). Glycerol induces the enzymes only in a strain possessing an active glycerol kinase and it was therefore assumed that the true inducer for the system is G3P. These three genes (glpR, glpD, glpK) are also subject to catabolite repression in the wild type strain. When glucose and G3P are added to strain 1 ($glpR^+$) cultured in casein hydrolysate medium, there is little or no detectable activity for the three enzymes. In a constitutive strain (strain 7, $glpR^c$), the activity of all three enzymes is reduced by about 50% when glycerol was added, but glucose depresses the activity of the dehydrogenase 2-fold, the kinase 20-fold and the transport system 8-fold. Koch *et al.* (4) concluded from these results that the three genes belong to different operons but share a

common inducer-repressor system.

Wild type strains of E. coli can utilize either glycerol or G3P as the sole carbon source. Hayashi et al. (2) studied the growth of several strains constitutive for the glp regulon. Strain 7 (glpR^c) is able to utilize either glycerol or G3P as a carbon source. The growth of strain 7 on other carbon sources is not inhibited by the addition of either glycerol or G3P. Strain 8, on the other hand, cannot utilize either G3P or glycerol as carbon source and its growth is inhibited when either of these metabolites is added to the medium. Strain 9 was able to grow in the presence of glycerol in the medium but not G3P. The genotypes and phenotypes of the strains mentioned are listed below.

Strain	Genotype	Phenotype
7	glpR^c	constitutive transports G3P utilizes G3P, glycerol
8	glpR^c glpD	lacks the G3P-dehydrogenase inhibited by glycerol inhibited by G3P
9	glpR^c glpD glpK	lacks G3P dehydrogenase and glycerol kinase not inhibited by glycerol inhibited by G3P

Phosphonic acid analogs of phosphates have been found

to occur in nature. Phosphonomycin, (-)(1R,2S)-1,2-epoxypropylphosphonic acid, has been isolated from *Streptomyces* (8,9) and has been shown to be bactericidal to both gram-negative and gram-positive bacteria (8). Kittredge and Roberts have reviewed the literature on other phosphonates found in nature (10).

3,4-Dihydroxybutyl-1-phosphonic acid (11,12), the phosphonic acid isostere (an analog of nearly the same size and shape as the natural substrate) of G3P, has been used in this laboratory to examine the pathways involved in the metabolism of G3P and its incorporation into lipids. The phosphonate is sterically similar to the natural phosphate but the substitution of the $-CH_2PO_3H_2$ for the $-OPO_3H_2$ inteferes with some aspects of the metabolism of the phosphonate as compared to tha of the natural phosphate.

3,4-Dihydroxybutyl-1-phosphonate (DHBP) inhibits the growth of strains of *E. coli* which have an active *L*-G3P transport system (glpT) (13), a hexose-phosphate transport system (*uhp*) (14) or a second transport system for G3P (*ugp*) (15). Leifer *et al*. (16) showed that mutants of *E. coli* strain 8 which are defective for G3P transport (number 1, Figure 1) do not transport DHBP. In strains inhibited by *L*-G3P ($glpR^c$, glpD, number 3, Figure 1), DHBP inhibition cannot be offset by glucose in the medium or by high concentrations of inorganic phosphate which does offset G3P

inhibition. DHBP also inhibits strains with an active catabolic dehydrogenase which are not inhibited by G3P (13).

Initial studies by Shopsis _et al_. (13,17,18) showed that DHBP inhibits the synthesis of lipids more than the synthesis of either RNA or protein. [^{14}C]Acetate incorporation into phospholipid is markedly decreased in the presence of concentrations of DHBP which have only a slight effect on growth. The amount of label incorporated into phosphatidylglycerol (PG) is inhibited by almost 50%, that into cardiolipin (CL) increases by about 2-fold, and the amount of label incorporated into phosphatidylethanolamine (PE) is only slightly inhibited (13).

The rate of total phospholipid synthesis decreases when strain 8 is pulsed with [^{32}P]-phosphate after addition of 0.03 mM DHBP (17). Phosphatidylglycerol (PG) synthesis is the most severely affected even at the earliest time points studied. There is little effect on cardiolipin (CL) synthesis and PE is inhibited at later time points. Accumulation of phospholipids in the presence of DHBP was also studied by pre-labeling cells with [^{33}P]-phosphate. Again, both total phospholipids and PG specifically are the most inhibited. Turnover of PG is unaffected by DHBP. The drop in the rate of PG synthesis combined with its continued turnover in the presence of DHBP leads to a drop in total PG content.

The synthesis of 3,4-dihydroxy[3-^{3}H]butyl-1-phosphonate (12) provided a valuable tool for demonstrating the *in vivo* synthesis of a novel and highly polar lipid. Shopsis *et al*. (17) showed that DHBP is incorporated into the polar lipid. Upon alkaline hydrolysis of the labeled lipid which is formed, the same chromatographic behavior as native DHBP is obtained.

Since DHBP is an analog of G3P, it was necessary to do a study of the enzymes involving G3P metabolism as well as those involving the incorporation of G3P into lipids in order to determine at what point(s) DHBP exerts its inhibitory action. DHBP inhibits both strains 7 and 8 which differ only in that strain 8 lacks the membrane-bound catabolic *L*-G3P dehydrogenase (2). Strain 8, although sensitive to DHBP, is more resistant to inhibition than is strain 7 (unpublished results). The differences in the growth response of strains 7 (glpR^{c}) and 8 (glpR^{c}, glpD) to DHBP must be attributed to the presence of the catabolic dehydrogenase in strain 7 even though crude extracts of strain 7 fail to oxidize DHBP. The phosphonate is not oxidized even when the purified enzyme is used. The anabolic G3P dehydrogenase (G3P synthase) was also assayed for its activity with dihydroxyacetone phosphate and its phosphonic acid isostere, 4-hydroxy-3-oxobutyl-1-phosphonate. The K_m for the phosphonate is 1.82×10^{-1} mM which is comparable to the

reported value for the natural phosphate (20). DHBP appears to act as a feedback inhibitor of the G3P synthase although G3P is slightly more effective (18).

Since DHBP inhibits total phospholipid synthesis, the effect of DHBP on the fatty acyl CoA: *sn*-glycerol-3-phosphate acyltransferase was also investigated and it was determined that DHBP is neither substrate nor inhibitor of this reaction *in vitro* (18). DHBP, on the other hand, does act as a substrate for the CDP-diglyceride: *sn*-glycerol-3-phosphate phosphatidyltransferase (K_m DHBP 4.5×10^{-4} M, K_m G3P 1.4×10^{-4} M) as well as an inhibitor (K_i DHBP 7.4×10^{-4} M) (18). DHBP has no effect on the CDP-diglyceride: serine phosphatidyl-transferaseferase.

Chromatographic analysis of the chloroform-soluble material (labeled with [3-^{3}H]-DHBP) formed *in vitro* revealed one material having an R_f of 0.52 on Sil-N HR thin-layer plates (18). Hydrolysis of the labeled material by phospholipase C followed by treatment of the water-soluble spot with alkaline phosphatase gives one radioactive material which migrates with an R_f identical to that of native DHBP. Cheng *et al*. (18) therefore concluded that DHBP is incorporated into the phosphonic acid analog of phosphatidylglycerol phosphate (PGPa) *in vitro*.

Tyhach *et al*. (21) further studied the incorporation of the radioactive DHBP and determined that DHBP is degraded *in*

vivo. Assay of the phosphatidyltransferase with the [3-^{3}H]-DHBP results in one labeled material, but fractionation on a DEAE column of the lipids formed *in vivo*, when strain 8 was incubated with the radioactive DHBP, results in the formation of four labeled materials. The major peak formed *in vivo* elutes with that formed *in vitro*. The three other peaks formed *in vivo* were identified as native PE, CL, and PG. There is no detectable precursor product relationship between the polar analog formed (PGPa) and these three lipids. It was concluded that the D-DHBP (the isostere which corresponds to the L-G3P) is incorporated, intact, into the analog of phosphatidylglycerol.

Degradation of the other three labeled lipids formed *in vivo* results in the formation of glycerol and not DHBP indicating that DHBP is not incorporated into the backbone of the lipids. It also seems possible that the labeling pattern observed might result from the metabolism of DHBP *in vivo*. Using rabbit muscle L-G3P dehydrogenase (L-G3P: NAD oxidoreductase, E.C.1.1.1.8) on the glycerol-3-phosphate obtained from the fraction migrating as PE indicated that the tritium is present on carbon 2. Since strain 8 lacks the membrane-bound catabolic dehydrogenase, this enzyme was ruled out as a possible participant in the metabolism of DHBP. Further studies using mutants suggest that neither

the anaerobic catabolic dehydrogenase nor the anabolic dehydrogenase contribute to the labeling pattern observed.

Tyhach *et al.* (21) also studied the incorporation of the label from DHBP into chloroform-soluble, water-soluble and residue remaining after extraction, as well as distillable material. Little label is found in the insoluble fraction, a large quantity of label remains unchanged in the intracellular pool and a significant amount of radioactive material can be distilled from the cultures, presumably as tritiated water. It was uncertain whether growth inhibition is caused by aberrations in phospholipid metabolism or by the intracellular buildup of DHBP or its metabolites.

Leifer *et al.* (16) had previously shown that G3P and DHBP share the same intracellular pool. It therefore seemed necessary to investigate the effect of the size of the G3P pool on DHBP inhibition. Strain 244 ($glpK^{fr}$, number 9, Figure 1), which has a glycerol kinase insensitive to feedback inhibition by fructose-1,6-diphosphate, should have a larger intracellular G3P pool than strains 7 or 8. Therefore strain 244, cultured in the presence of glycerol, should be more resistant to DHBP inhibition. When cultured in medium containing glycerol as the sole carbon source, strain 244 is almost insensitive to 2.5 mM DHBP. Strain 7 is completely inhibited under these conditions. In order to determine if glycerol is responsible for the resistance of

strain 244 to DHBP, other carbon sources were investigated. When the two strains were cultured in the presence of glucose, both strain 7 and strain 244 are completely inhibited indicating that the glycerol was responsible for offsetting DHBP inhibition (21).

The studies discussed above raised many questions. The effect of DHBP on the synthesis of phosphatidylglycerol in *E. coli* is explained by the competitive inhibition between DHBP and G3P for the CDP-diglyceride: *sn*-glycerol-3-phosphate phosphatidyltransferase. The delayed effect on PE synthesis has, however, not been explained. Although the activity of the acyltransferase is unaffected by the presence of DHBP *in vitro*, the membrane bound enzyme might be affected by 1) the presence of the highly polar analog of phosphatidylglycerol phosphate in the membrane, 2) the decreased content of PG in the membrane or a combination of both factors. In order to determine if DHBP has any effect on the activity of the acyltransferase *in vivo*, the enzyme was isolated from DHBP-treated and untreated cultures of strain 8 and assayed for activity with the natural substrate.

Low concentrations of DHBP have a pronounced effect on phosphatidylglycerol synthesis but only a slight effect on growth. This observation seemed puzzling. Why should cells synthesize more phosphatidylglycerol than is necessary? *E. coli* evolved to live in the human gut, not in the

laboratory. It was therefore decided to examine the effect of deoxycholate, a detergent present in the intestine, on the growth of *E. coli*.

The findings of Tyhach *et al*. (21) that the tritium of the labeled DHBP appears at carbon-2 of the glycerol phosphate backbone of phospholipids as well as in water suggests that DHBP is being metabolized *in vivo*. The presence of a phosphonatase (which would convert DHBP to glycerol) has not been demonstrated despite attempts at growing the cells on DHBP as carbon or phosphorus source (C. Shopsis, this laboratory, unpublished results). Since it has been reported (22) that *E. coli* can utilize phosphonic acids as a source of phosphorus, the existence of such an enzyme has not been ruled out. The second possiblity for the distribution of the tritium is that DHBP is oxidized *in vivo*. If DHBP is oxidized by either the aerobic or anaerobic catabolic dehydrogenases, then the tritium of the labeled DHBP would be transferred to either NAD or FAD and hence to other cell intermediates. DHBP is not a substrate for the purified membrane-bound catabolic *L*-G3P dehydrogenase found in *E. coli* (18). While it is unlikely that the anaerobic dehydrogenase plays a role in DHBP metabolism when the cells are grown aerobically, the high intracellular concentration of DHBP (16) may cause this or other enzymes to act in a nonphysiological manner.

Further study of the oxidation of DHBP *in vivo* presented a problem. Oxidation of the radioactive DHBP (labeled in the C-3 position) would result in the loss of the tritium. We also wished to isolate and identify any intracellular metabolites of DHBP. In order to accomplish this, the [1,2-^{3}H]-DHBP was synthesized and its metabolism examined in strain 8. In this labeled compound, oxidation occuring at the C-3 position, would not result in the loss of the ^{3}H label.

The formic acid and water soluble pool of cells treated with [1,2-^{3}H]- and [3-^{3}H]-DHBP were isolated and chromatographed to examine the soluble labeled material. The [1,2-^{3}H]-DHBP gives rise to two materials while only one is observed when cells are labeled with the [3-^{3}H]-DHBP.

MATERIALS AND METHODS

CHEMICALS: Each of the phosphonic acid analogs used was a generous gift from the laboratory of Dr. Robert Engel. *rac*-3,4-Dihydroxybutyl-1-phosphonate was synthesized as described previously (11) and unless specified, the racemic form was used. *rac*-3,4-Dihydroxy[3-^{3}H]butyl-1-phosphonate was prepared by the method of Goldstein *et al.* (12). *S*-3,4-dihydroxybutyl-1-phosphonate was prepared by T. Latham as described by Tang *et al.* (23) and the 3,4-O-isopropylidinebut-1-enyl-1-phosphonate diisopropyl ester was prepared by N. Lalinde from 3,4:5,6-O-diisopropylidine-*D*-mannitol as the starting material (24). The 3,4-O-isopropylidinebut-1-enyl-1-phosphonate diisopropyl ester (100 mg, 0.373 mmoles) was reduced with 5 Curies of $^3H_2/PtO_2$ by Amersham, Inc. The total amount of radioactivity recovered (44 mCi) was returned in four vials each containing 6 mL of labeled material in ethanol. The isopropyl esters and the isopropylidine group were removed as described.

Carrier-free [^{32}P]-phosphate was purchased from ICN. [^{14}C]-*DL*-G3P was obtained from New England Nuclear. The following were obtained from Sigma, St. Louis, Mo.: tris(hydroxymethyl)aminomethane (TRIS), N,N'-dithiobis-(2-nitrobenzoic acid) (DTNB),

sn-glycerol-3-phosphate, casein hydrolysate, rabbit muscle *sn*-glycerol-3-phosphate dehydrogenase, Dowex 50 H^+ and Triton X-100. Whatman 3 MM chromatography paper and Difco bactoagar were purchased from Scientific Products. Millipore filters were obtained from the Millipore Corp. and diethylaminoethyl cellulose (DE 52) from Whatman, Inc. All other chemicals were of reagent grade or better.

BACTERIA AND GROWTH CONDITIONS: All bacteria used for these studies are derivatives of *E. coli* K 12. The relevant genotype, source and reference are given in Table 1. For growth studies, the strains were incubated overnight in either the minimal medium of Garen and Levinthal (G and L) (25) supplemented with 0.5% potassium succinate and 0.6 mM phosphate, pH 7.5, or in casein hydrolysate medium (CH) consisting of 1.0% casein hydrolysate and 0.5% sodium chloride, pH 7.4. Overnight cultures incubated at 37 ^oC and 200 rpm in a New Brunswick Scientific Metabolyte Water Bath Shaker Model G77 were diluted into fresh medium and allowed to grow until the desired cell density was reached. Growth was followed turbidimetrically using a Klett-Summerson colorimeter fitted with a 660 nm filter. Unless otherwise indicated, additions of drugs were made when the cell cultures reached 15-20 Klett Units (K.U.) and all data is as time after addition (0 time is the time of drug addition).

TABLE I. BACTERIAL STRAINS

BACTERIA	GENOTYPE	SOURCE AND REFERENCE
7	glpR^{c}2 phoA8 tonA22 T2^{R} rel-1 (λ)	E.C.C Lin (2)
8	same as 7,glpD3	J.E. Cronan, Jr. (2)
T 7	same as 7, uhpR^{c}	Y.-W. Hwang (26)
BB 6	same as 8, gpsA^{fr}	R. Bell (45)

Pre-conditioned G and L medium was prepared by filtration of a culture (either untreated or DHBP-treated) at 20-40 K.U. The filtrate was maintained at 37 °C.

Viability Studies: For viability studies, cultures were diluted in G and L medium lacking a carbon source. Dilutions were carried out at room temperature unless otherwise indicated. The final dilutions (0.1 mL) were then plated on CH plates containing casein hydrolysate medium and 1.5% non-nutritive agar, incubated at 37 °C for 18-20 hours and scored for viability.

Preparation of 3,4-Dihydroxy[1,2-^{3}H]butyl-1- phosphonate. Approximately 6 mL of an ethanol solution containing 11 mCi of 3,4-O-isopropylidine-[1,2-^{3}H]-butyl-1-phosphonic acid diisopropyl ester (0.086 mmoles) was dried under a stream of N_2. The residue was dissolved in 1.0 mL of 2N HCl, kept in a boiling H_2O bath with constant stirring for 4 hours and then lypholyzed. Saturated LiOH (1.0 mL) was then added and the solution autoclaved for a total of 18 hours (20 psi, 131 °C). The milky precipitate was dissolved in water and passed through a Dowex 50 x 4 (50-100 mesh) column (approximately 30 meq) in the H^+ form. The resin had been washed previously with 1N NaOH, distilled water, 1N HCl and then water to equilibrate the resin. Approximately 40 mL of column eluate was collected and lypholyzed. The

lypholyzed material was dissolved in a small amount of water and applied to 1 x 10 cm DE 52 column in the NH_4^+ form. Prior to packing the column, fines had been removed by decantation. The cellulose was washed in 0.2 M NH_4HCO_3 buffer (pH 8.5) until the filtrate had the the same pH and conductivity as the buffer. The cellulose was then washed with water until the pH and conductivity were that of the water. A slurry of the cellulose in water was then made and a column packed by gravity. After applying the sample, a linear gradient of NH_4HCO_3 (0.00-0.2 M) was then applied to the column. DHBP elutes at approximately 40 mM (R. Tyhach, unpublished results). Fractions (2.0 mL) were collected and those containing radioactivity were chromatographed on Whatman 3 MM paper. The descending solvent was n-butanol/acetic acid/H_2O (6/3/1) (27). Those fractions which co-chromatographed with DHBP (R_f 0.47) were combined, lypholyzed, and resuspended in water. The concentration of the [1,2-^{3}H]-DHBP was determined enzymatically using rabbit muscle L-glycerol-3-phosphate dehydrogenase. The concentration of the S-[1,2-^{3}H]-DHBP was 1.375 x 10^{-2} mmoles/mL. Radioactivity was determined to be 1.22 mCi/mL, for a final specific activity of 88.7 mCi/mmole.

ENZYME ASSAYS:

sn-*Glycerol*-*3*-*phosphate Dehydrogenase*. *sn*-Glycerol-3-phosphate and DHBP concentration were assayed using the spectrophotometric G3P dehydrogenase assay as described by Hohorst (28). The assay mixture contained the following in a final volume of 1.0 mL: 0.2 mmoles hydrazine sulfate, 0.5 mmoles glycine, 3×10^{-3} mmoles EDTA, pH 9.5, 2.5 mmoles NAD, 2 units of enzyme, and various concentrations of substrate. When the *L*-G3P was assayed for substrate activity, it was present at a concentration of 5.0×10^{-2} mmoles. The spectrophotometric assay was initiated by the addition of enzyme and the absorbance change at 340 nm followed. By using either *L*- or *DL*-G3P as a standard for the assay, it was determined that better than 98% of the *L* isomer reacted under these conditions. Only the *S* isomer of DHBP acts as a substrate for this enzyme.

Acyltransferase. After isolating the membrane fraction of cells treated with DHBP by the procedure of Cheng *et al* (19), the activity of the acyltransferase was assayed by following the incorporation of radioactive *sn*-G3P into trichloroacetic insoluble material. The assay mixture contained the following in a final volume of 0.125 mL: 1.25×10^{-2} mmoles Bicine, pH 8.5, 1.0×10^{-3} mmoles $MgCl_2$, 7.5 nmoles palmitoyl CoA, approximately 4.0 x

10^{-2} mg enzyme, and 1.0-5.0 x 10^{-3} mCi of *sn*-[^{14}C]-glycerol-3-phosphate (specific activity 20 mCi/mmole) at the indicated concentrations (29). The incorporation was linear for at least 15 minutes at 25-30 ^{o}C. Incorporation was monitored by placing 0.05 mL of the assay mixture on Whatman 3 MM filter disks and drying for about 10 minutes under a heat lamp. After the last sample was collected, the drying was continued for another 10 minutes (total drying time was about 20 minutes). The filters were washed in ice-cold trichloroacetic acid (TCA) and then acetone according to the method of Goldfine (30). After the original samples were dried under the heat lamp, the filters were placed in an ice-cold 10% TCA bath for 30 minutes. The filters were then transferred to a cold 5% TCA bath for 30 minutes. After the second TCA bath, the filters were washed once with ice-cold distilled water (30 minutes). The filters were washed for 30 minutes in acetone and the acetone wash repeated. All washes contained at least 5-10 mL of TCA solution (water or acetone)/filter. After the second acetone wash, the filters were dried under a heat lamp and counted in toluene-based scintillation fluid in a Nuclear Chicago Isocap 300 liquid scintillation counter.

Scintillation Fluid. Two scintillation fluids were used throughout these studies. Toluene based scintillation fluid was used for non-aqueous samples and consists of 17 gms of

2,5-diphenyloxazole (PPO) and 0.85 gms of (1,4-bis [2-(5-phenyloxazolyl)] benzene;2,2-p-phenylene-bis[5-phenyloxazole]) (POPOP) per 4 L of toluene. After addition of the scintillation fluors to a fresh bottle of toluene, the solution was stirred for several hours to dissolve the fluors and stored in the dark before use. Patterson-Green (31) scintillation fluid is used for aqueous samples and consists of toluene-based scintillation fluid/Triton X-100 (2/1). Triton X-100 was added to the toluene scintillation fluid and mixed. For aqueous samples, water or solution (buffer, formic acid, *etc*.) is present in the samples to be counted to a final concentration of approximately 10%.

Lipid Extraction. Lipids were extracted from cultures by the procedure of Bligh and Dyer (32) as modified by Ames (33). Chloroform/methanol (1/2) (7.5 mL) was added to a 2.0 mL sample of cells. After vortexing the mixture, 2.5 mL of chloroform and 2.5 mL of water was added with mixing after each addition. Phases were separated by centrifugation at room temperature, and the upper layer removed by aspiration. The chloroform layer was washed once with 2.0 mL of 2M KCl and twice with 2.0 mL of water. After each wash step, the phases were separated by centrifugation and the upper phase removed by aspiration. Care was taken to remove as little of the chloroform layer and the interface as possible.

Methanol (2.0 mL) then added to the final chloroform layer to make one phase and after the volumes of the individual samples were measured, a portion was dried and counted in toluene based scintillation fluid as described (13) in a Beckman LS 200 liquid scintillation counter. Note: while the addition of methanol is valid under these conditions, it would be advisable not to add methanol to the chloroform layer since some chloroform may be lost during extraction and washing by aspiration. In this case, the same volume of methanol was added to all the samples and the final volume of the samples taken into consideration.

Where indicated, a portion of the final chloroform-methanol layer was dried under a stream of N_2, taken up in a small volume of chloroform and chromatographed on silica gel TLC plates in $CHCl_3/CH_3OH$/acetic acid (65/25/8) as described previously (18).

Cell Fractionation. Cells were separated into soluble and insoluble materials as follows:

(1) Formic Acid Extraction. After treating cells with DHBP as indicated, cultures were filtered through 0.45 micron Millipore filters, washed with warm (37 ^{o}C) pre-conditioned G and L medium and allowed to air dry for approximately one minute. The cells were then extracted

with ice-cold 2N formic acid by the modified procedure of Tang _et al_ (34). One mL of ice-cold formic acid was placed on the filters (with 1.0 mL of cells) without suction for 2 minutes. Suction was then applied and the filters washed twice more with formic acid. A portion of the combined formic acid filtrates was counted in Patterson-Green scintillation fluid (31) in a Nuclear Chicago Isocap 300 liquid scintillation counter. The filters were also counted after drying and the remainder of the formic acid extract lypholyzed, resuspended in a small volume of water and chromatographed as described.

(2) Osmotic Shock. After incubation with DHBP and washing with pre-conditioned G and L medium as above, cells were subjected to cold osmotic shock. Ice-cold distilled water was placed on the filters without suction for approximately 2 minutes. After filtering, the filters were washed twice more with cold distilled water and the filtrates combined. A portion of the water-soluble extract was counted as described above and the remainder lypholyzed, resuspended in a small volume of water and chromatographed. The filters were also counted after drying.

Chromatographic Analysis. Portions of either the formic acid soluble or water soluble cell fractions as well as unlabeled standards were subjected to descending chromatography at room temperature in either

n-propanol/conc. ammonia/water (6/3/1) (26, 35) or ethyl acetate/formic acid (88%)/water (7/2/1) (27) on Whatman 3 MM paper. After applying the samples, the paper was equilibrated with solvent for at least 8 hours before freshly prepared solvent was added to the troughs. After drying, the chromatographs were then sprayed with a mixture of $FeCl_3 \cdot 6H_2O$ and 7% sulfosalicyclic acid in 80% aqueous ethanol (36) to visualize the unlabeled phosphorus containing compounds. Radioactive material was detected by scanning the chromatograms on a Packard Radiochromatogram Scanner Model 7220/7221. When the n-propanol system was used for development, solvent was rountinely run off the edge of the paper. In this system, mobility of the standards relative to the mobility of G3P (R_{G3P}) is recorded rather than the actual R_f. In neither system did any compound migrate more than half-way down the paper. The R_f and R_{G3P} of the standards is recorded in Table 2.

TABLE 2

Chromatographic Behavior of Phosphonic Acid Analogs

	Solvent System [a,b]	
	Ethyl Acetate	*n*-Propanol
Compound [c]	R_f	R_{G3P}
G3P	.297	1.00
DHBP	.338	1.11
PGAa	.335	.730
GAPa	.127	.285
DHAPa	.376	.240
F1Pa	.138	.810

[a] Ethyl Acetate: ethyl acetate/formic acid (88%)/water (7/2/1) (27). *n*-Propanol: *n*-propanol/ammonia (conc)/water (6/3/1) (27,35).

[b] Descending at room temperature on Whatman 3 MM paper.

[c] Abbreviations: G3P (*L*-glycerol-3-phosphate); DHBP (3,4-dihydroxybutyl-1-phosphonate); PGAa (3-phosphoglyceric acid analog, 3-carboxy-3-hydroxy-propyl-1-phosphonate); GAPa (glyceraldehyde-3-phosphate analog, 4-oxo-3-hydroxybutyl-1-phosphonate); DHAPa (dihydroxyacetone phosphate analog, 4-hydroxy-3-oxobutyl-1-phosphonate); F1Pa (fructose-1-phosphate analog, 1-deoxy-1-dihydroxyphosphinylmethylfructose).

[d] R_{G3P} is the average distance from the origin of the standard/the average distance from the origin of the G3P.

RESULTS AND DISCUSSION

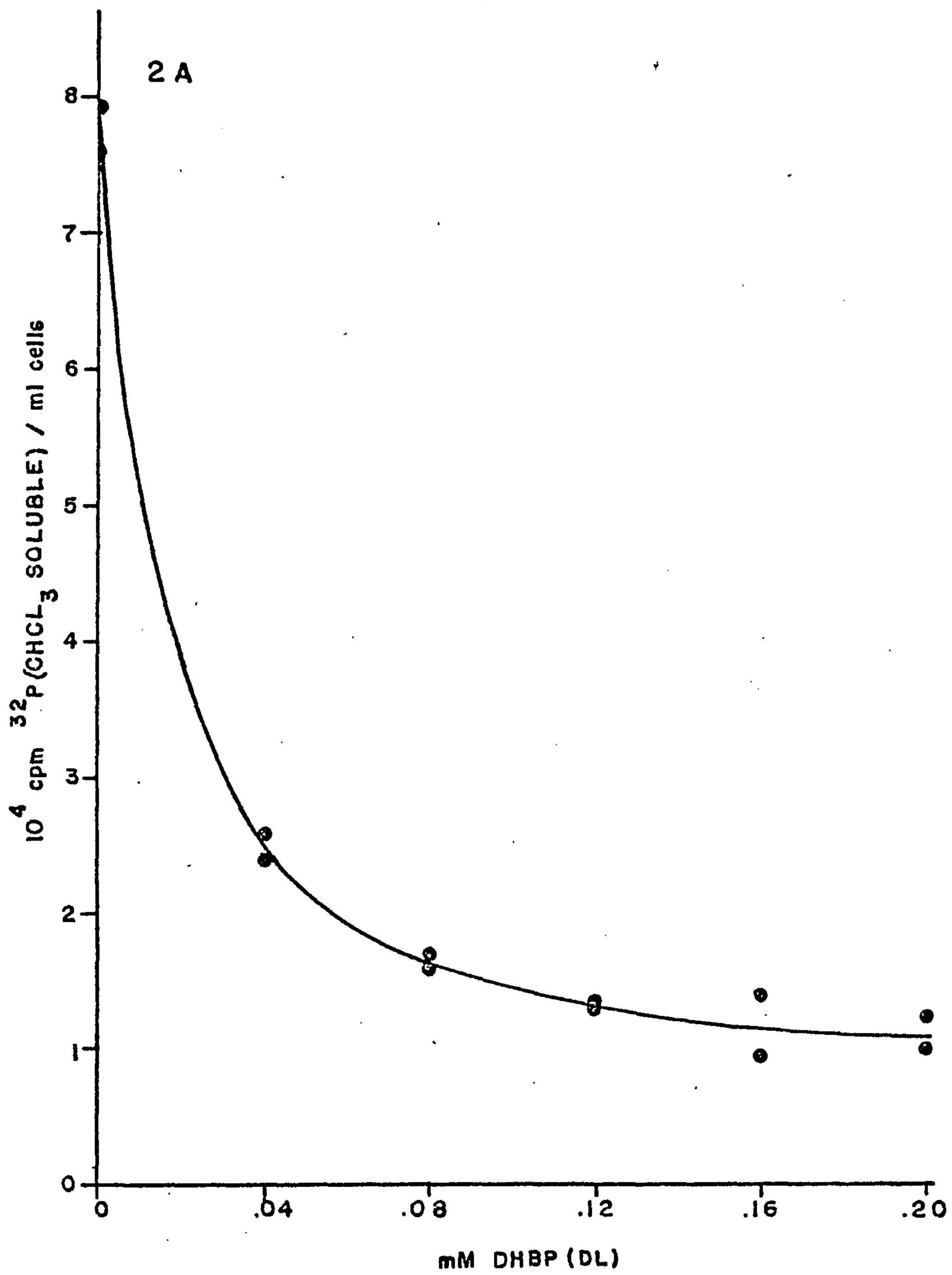
Effect of DHBP Concentration on the Incorporation of [^{32}P]-Phosphate into Lipids. Strain 8 cultured in G and L medium with succinate was treated with the indicated concentrations of DHBP for 30 minutes. Carrier-free [^{32}P]-phosphate was then added to the cultures to a final concentration of 1.2×10^{-2} mCi/mL (final specific activity in G and L, 20.1 mCi/mmole). The cultures were then incubated for an additional 30 minutes. At this time, 2.2 mL of culture was pipeted into test tubes, centrifuged, washed with 2.0 mL of potassium phosphate buffer, (pH 7.0) containing cold carrier cells and then resuspended in the original volume with G and L medium. Lipids were then extracted by the procedure of Bligh and Dyer (32) as modified by Ames (33) as described.

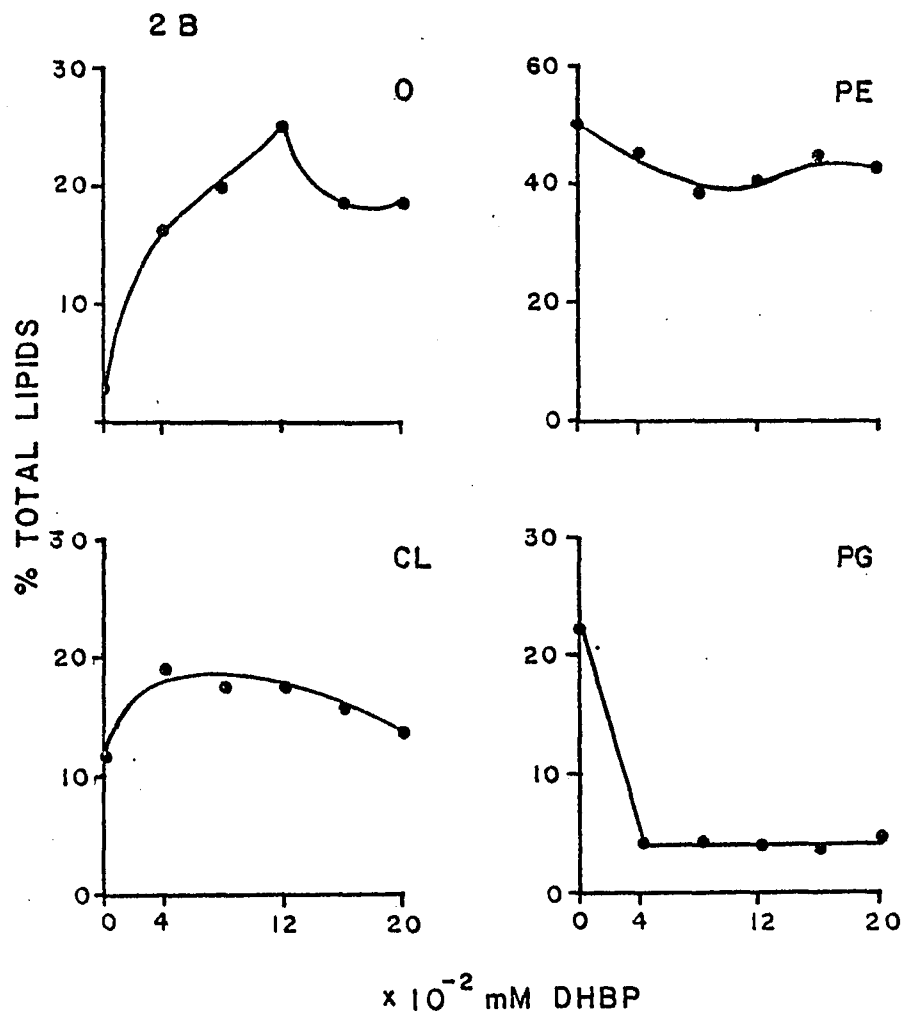
Further studies have been undertaken in order to determine the mechanism(s) of inhibition by DHBP. In a variation of the procedure of Shopsis *et al.* (17), *E. coli* strain 8 was treated with the indicated concentrations of DHBP and [^{32}P]phosphate and the lipid fraction extracted as described. Figure 2 A shows that the rate of synthesis of total phospholipids is markedly inhibited even at 0.04 mM DHBP, the lowest concentration tested. At this concentration, DHBP has little effect on growth (18). In

FIGURE 2

Effect of DHBP Concentration on the Incorporation of $[^{32}P]$-Phosphate into Chloroform-soluble Material.

Strain 8 cultured in G and L medium with succinate as the carbon source was treated with the indicated concentrations of DHBP for 30 minutes. $[^{32}P]$-Phosphate was then added and, after an additional 30 minutes of incubation, the amount of label incorporated into the chloroform-soluble material was determined as described in the text (A). (B), A portion of the final chloroform-methanol extract was dried under a stream of N_2, taken up in a small volume of chloroform and chromaographed on silica gel plates in $CHCl_3/CH_3OH$/acetic acid (65/25/8) as described previously (18). Abbreviations: O, origin; PE, phosphatidylethanol amine; CL, cardiolipin; PG, phosphatidylglycerol.


2 A
10^4 cpm ^{32}P ($CHCL_3$ SOLUBLE) / ml cells
0
1
2
3
4
5
6
7
8
0
.04
.08
.12
.16
.20
mM DHBP (DL)


2 B
O
PE
CL
PG
% TOTAL LIPIDS
30
20
10
60
40
20
0
0
4
12
20
x 10^-2 mM DHBP

this experiment, 0.04 mM DHBP caused a 70% inhibition in the amount of ^{32}P incorporated into lipids. At 0.2 mM DHBP, the inhibition was increased to about 90%. Chromatographic analysis of the membrane fraction resulted in a distribution of lipids similar to that obtained previously (18, Figure 2 B).

In vivo Effect of DHBP on the Fatty Acyl CoA: *sn*-Glycerol-3-Phosphate Acyltransferase. Strain 8 was cultured in G and L medium supplemented with succinate and, where indicated, treated with 0.03 mM DHBP for 90 minutes. The procedure of Cheng *et al* (19) was used to isolate the membrane-bound acyltransferase. Protein was determined by the method of Lowry *et al*. (37). The specific activity of the DHBP-treated or the untreated enzyme was determined by the spectrophotometric assay of Cronan (38). The V_{max} and K_m values for the enzyme preparations were determined by measuring the incorporation of *sn*-[^{14}C]-glycerol-3-phosphate into trichloroacetic acid insoluble material as described.

DHBP has been shown to act as a competitive inhibitor of the CDP diglyceride: *sn*-glycerol-3-phosphate phosphatidyltransferase (19). It is incorporated into the phosphatidylglycerol phosphate analog (21). These two findings explain the inhibition of PG synthesis, but the delayed effect on PE synthesis has not been explained,

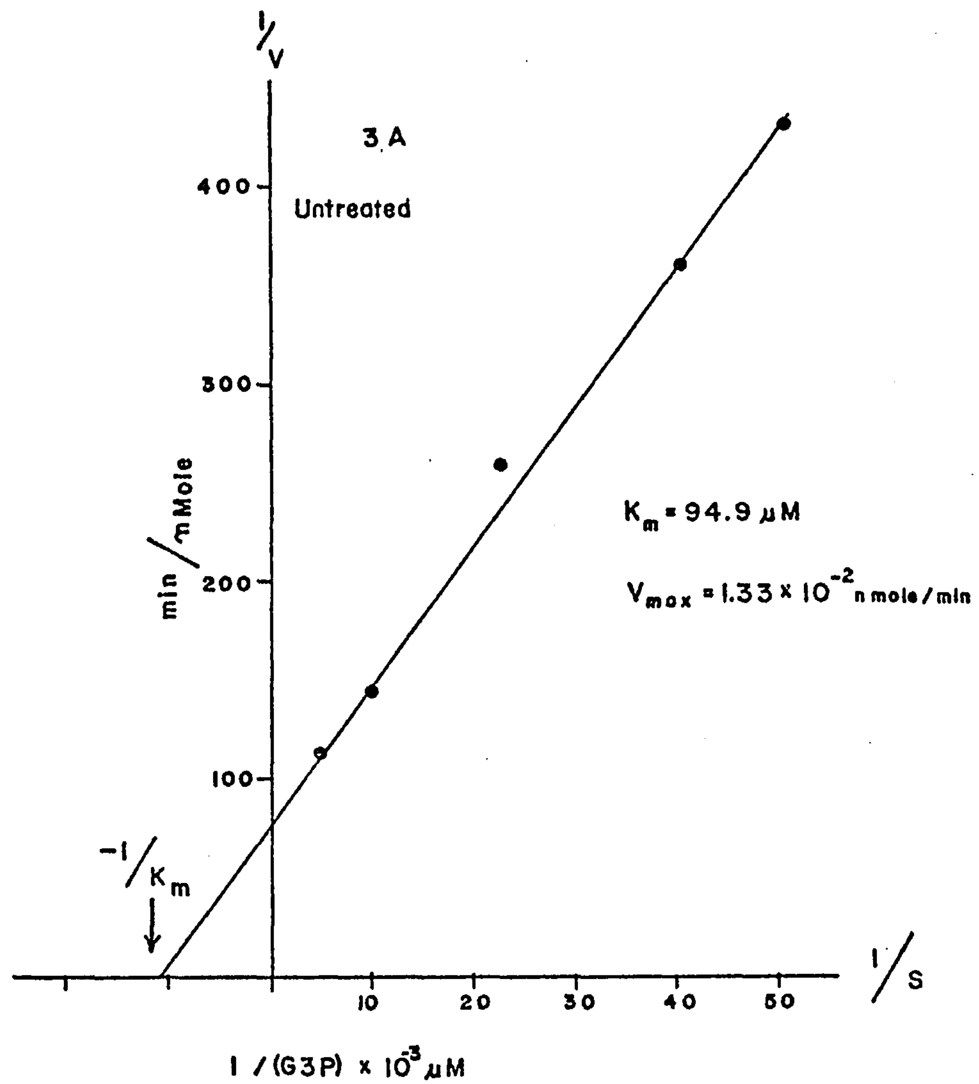
especially in light of the work of Cheng _et al_ (19) who determined that DHBP is not recognized by the fatty acyl CoA: _sn_-glycerol-3-phosphate acyltransferase.

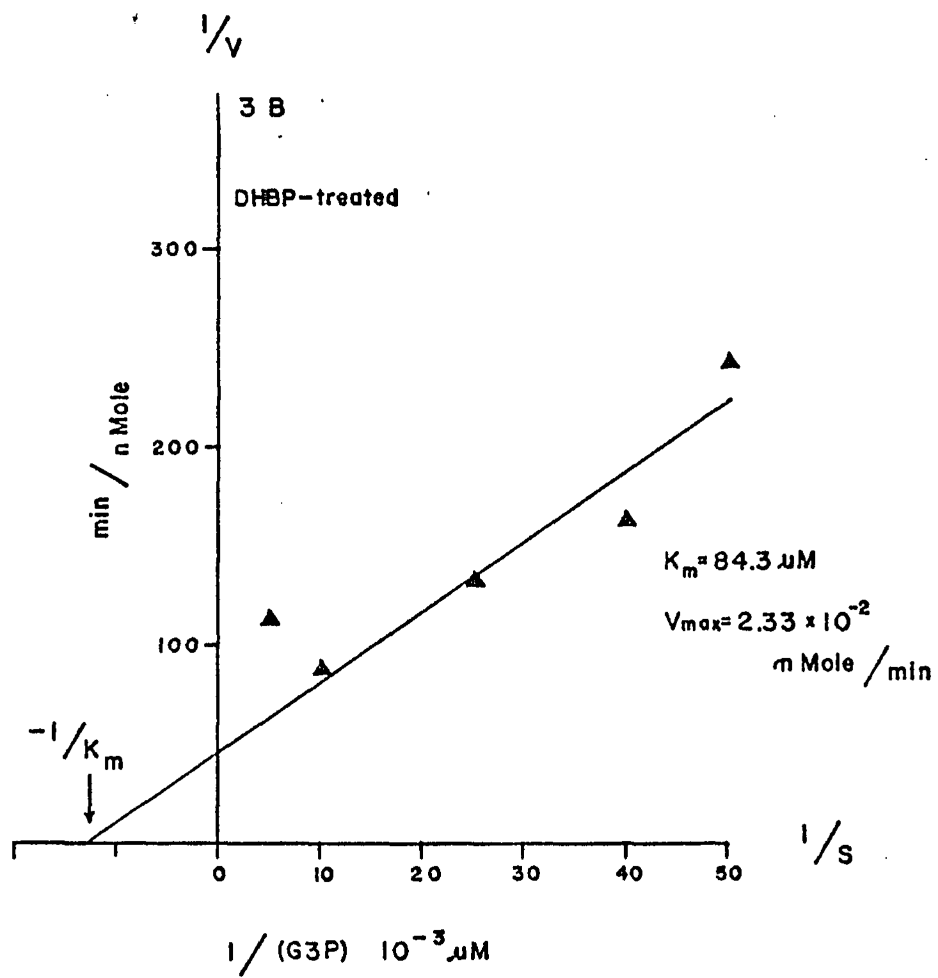
Since Raetz _et al_ (39-41) had shown that an alteration of the polar head groups in the membrane could alter the properties of the membrane, an attempt was made to determine if the presence of the polar phosphonate analog of phosphatidylglycerol phosphate in the membrane and the decreased PG content of the membrane would affect the activity of the membrane bound acyltransferase. The enzyme was isolated from DHBP-treated cultures of strain 8. Lineweaver-Burk plots (42) for K_m and V_{max} (Figure 3 A,B) show that the values obtained from treated cells are comparable to those previously reported (43,44). The K_m values obtained are as follows: from the DHBP treated cells, 8.4×10^{-5} M and from untreated cells, 9.5×10^{-5} M. Bell and Cronan previously reported a value of 8.8×10^{-5} M (43,44). The V_{max} values for the enzyme isolated from DHBP-treated cells (2.33×10^{-2} nmoles/min) and untreated (1.33×10^{-2} nmoles/min) are comparable (for assay of either the DHBP-treated or the untreated membrane preparations, approximately 4.0×10^{-2} mg of protein was used). The specific activity of the enzyme previously reported (3.5 nmoles/min/mg) is somewhat higher than that reported here. This difference might be attributable to the diffence in cell disruption (French Pressure Cell _vs_. sonication).

FIGURE 3

Lineweaver-Burk Plots (44) for the Fatty Acyl CoA: *sn*-Glycerol-3-Phosphate Acyltransferase.

Strain 8 cultured in G and L medium with succinate as the carbon source was incubated with 0.03 mM DHBP for 90 minutes. The acyltransferase was isolated from untreated and DHBP-treated cultures by the procedure of Cheng *et al.* (19). Enzyme activity was determined by measuring the incorporation of the indicated concentrations of *sn*-[^{14}C]-G3P (20 mCi/mmole) into trichloroacetic acid (TCA) insoluble material (33). The assay mixture was incubated (with shaking) for 10 minutes at 25 ^{o}C, at which time, 0.05 mL of the assay mixture was transferred to Whatman 3 MM filter disks (approximately 1 sq. in.). After drying under a heat lamp for 10 minutes, the filters were washed in TCA according to the procedure of Goldfine (34) as described in the text. After drying, the filter disks were transferred to toluene-based scintillation fluid and the amount of [^{14}C]-G3P determined as described. A, untreated enzyme preparation; B, DHBP-treated enzyme preparation.


1/V
3.A
Untreated
400
300
200
100
min / nMole
K_m = 94.9 μM
V_max = 1.33 × 10^-2 n mole/min
-1/K_m
1/S
10
20
30
40
50
1 / (G3P) × 10^-3 μM


1/V
3 B
DHBP-treated
300
200
100
min / n Mole
K_m = 84.3 uM
Vmax = 2.33 x 10^-2
n Mole / min
-1/K_m
0
10
20
30
40
50
1/S
1/ (G3P) 10^-3 uM

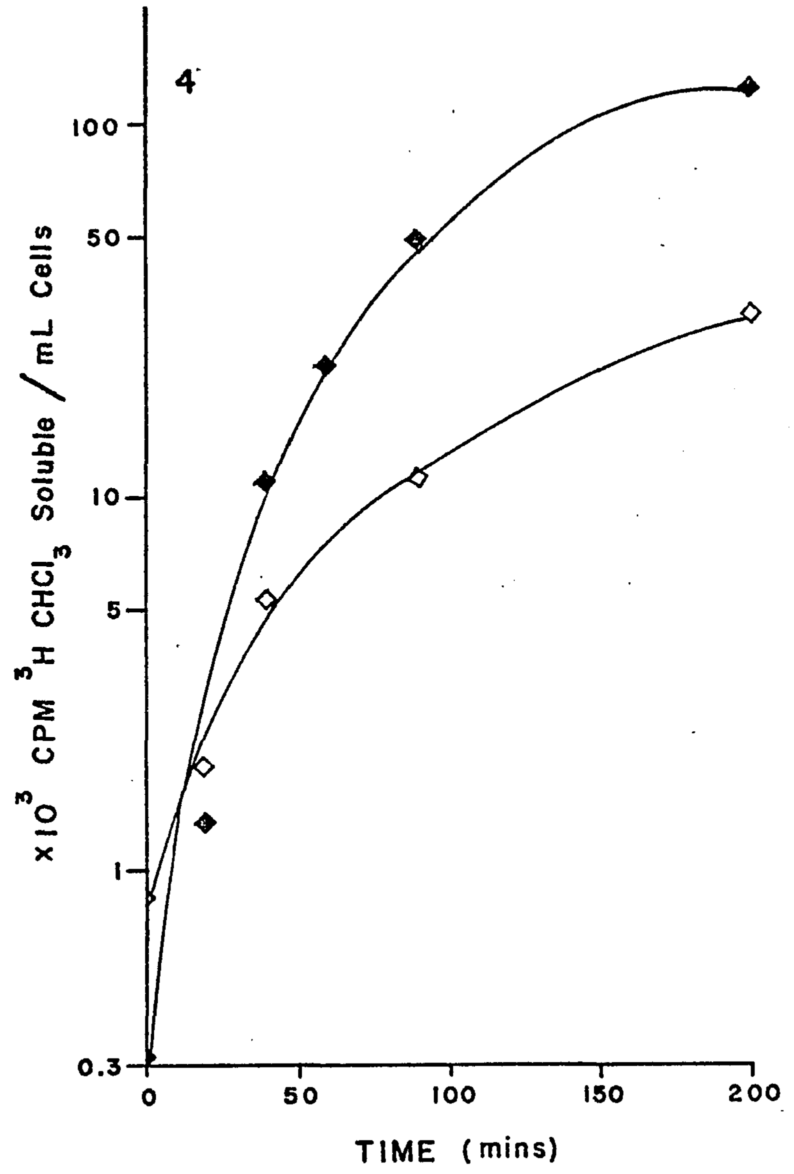
Incorporation of DL-[3-^{3}H]DHBP into Lipids. Strains 7 and 8 were cultured in CH medium as described. [3-^{3}H]-DHBP was added to a final concentration of 1.0 x 10^{-3} mCi/mL and 0.03 mM (specific activity, 31 mCi/mmole). At various times after addition, 1.0 mL of cells were mixed with 0.1 mL of cold carrier cells and the lipids extracted as described above up to the wash step. The chloroform layer was washed once with 1.0 mL of 2M KCl, twice with 1.0 mL of 1 mM DHBP, and twice with 1.0 mL of water. The chloroform layer was then treated as described and a portion of the final chloroform-methanol mixture was dried under a stream of warm air and counted as described previously (21). Cold carrier cells were prepared by treating 400 mL of culture (at 15-20 K.U.) with 0.03 mM DHBP for 90 minutes. After incubation, cultures were centrifuged, washed and resuspended in a volume of 10 mL for 40-fold concentration of cold carrier cells.

Other studies have been undertaken to examine the effects of DHBP on lipid synthesis in *E. coli*. [3-^{3}H]-DHBP incorporation into lipids in strains 7 and 8 cultured in CH medium indicates that, while the two strains transport G3P (and presumably DHBP) at the same rate under these conditions (2), there is a great difference in the incorporation of the DHBP into lipids (Figure 4). Under these growth conditions, strain 7 and 8 differ only in that strain 7 has an active catabolic G3P dehydrogenase. Since

FIGURE 4

[3-^{3}H]-DHBP Incorporation into Chloroform-soluble Material.

Strains were incubated in CH medium and treated with [3-^{3}H]-DHBP as described. At the indicated times after addition, 2.0 mL of culture was extracted by the procedure of Bligh and Dyer (28) as modified by Ames (29) and the amount of label in the chloroform-soluble material determined as described in the text. Strain 7, (◆-◆-◆); strain 8, (◇-◇-◇).


4
x10³ CPM ³H CHCl₃ Soluble / mL Cells
100
50
10
5
1
0.3
0
50
100
150
200
TIME (mins)

the intracellular G3P pool in strain 7 should be much smaller than that in strain 8, the tritiated DHBP would have to compete with a larger intracellular concentration of glycerol-3-phosphate in strain 8. Consistent with this prediction is the observation that strain 7 incorporates DHBP at a higher rate than strain 8 (Figure 4).

Effect of DHBP, Deoxycholic Acid (DOC) or Both on Growth and Viability. Strains, as indicated, were cultured in either G and L medium supplemented with succinate or in CH medium as described. When the desired cell density was reached, DHBP, DOC, or both, were added to the cultures to a final concentration of 0.03 mM for DHBP and 0.25% for DOC and the growth followed. Where indicated, viability was determined as described.

DHBP has been shown to have different inhibitory properties, depending upon the strain studied and the growth medium employed. The relation between the G3P pool size and DHBP inhibition was investigated further. Strain BB 6 has a non-feedback sensitive anabolic G3P dehydrogenase (gpsA) in a strain 8 background (R. Bell, personal communication) and has a larger G3P pool than strain 8. The G3P pool in strain BB 26-36 R2 (the gpsA donor for the BB 6) is approximately 12 times larger than that in strain 8 (43,44). Strain 7 differs from strain 8 in the presence of the catabolic G3P dehydrogenase and should have a smaller G3P pool than strain

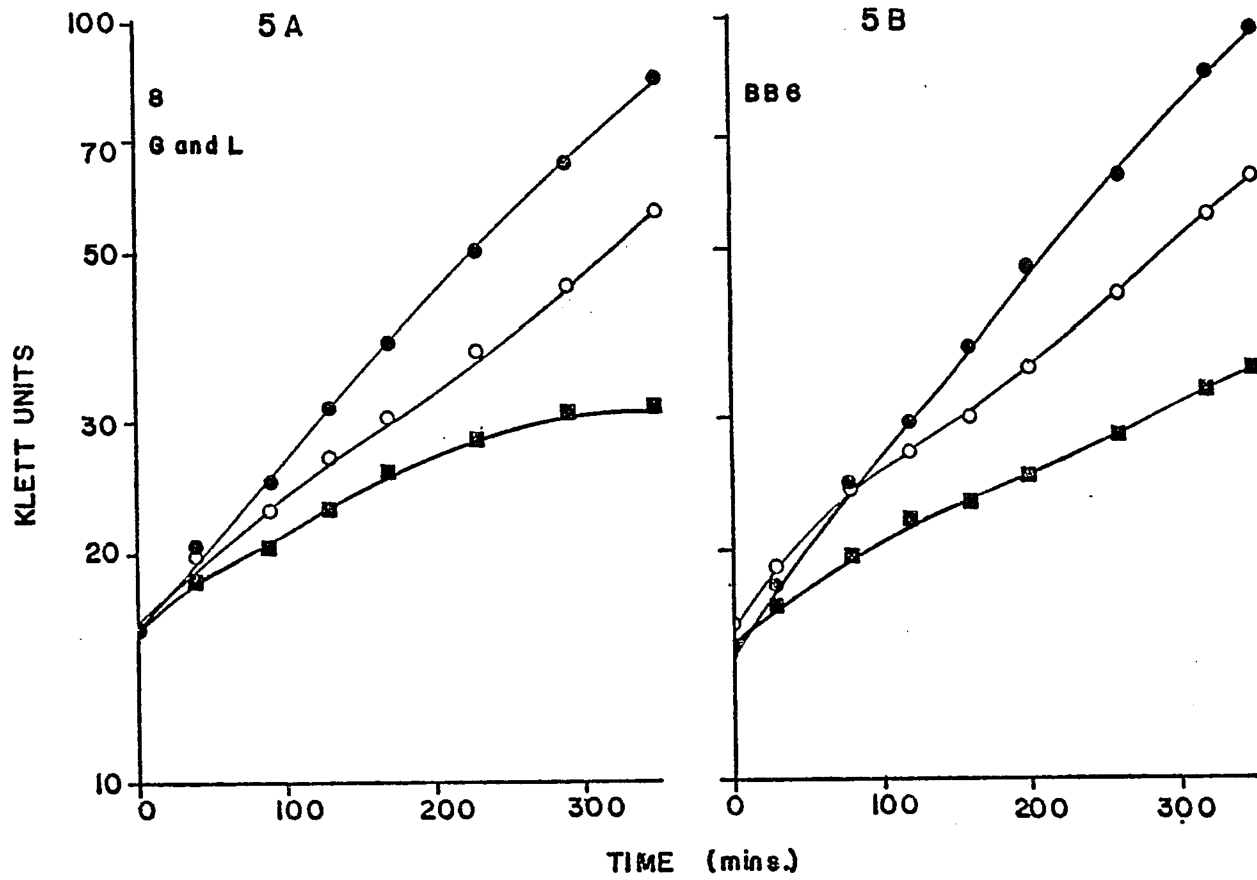
8. The order of the G3P pool size is BB 6, strain 8, strain 7 (largest to smallest). If this hypothesis, that the G3P pool size affects DHBP inhibition, is correct, then BB 6 should be more resistant to DHBP than strain 8 which should, in turn, be more resistant than strain 7. In either strain 8 or strain BB 6, cultured in G and L medium, DHBP slightly inhibits growth (Figure 5 A, B). At a concentration of 0.12 mM, DHBP totally inhibits the growth of strain 8 while BB 6 continues to grow. When incubated in CH medium, strain 8 is completely resistant to the lower concentration of DHBP while strain 7 is completely inhibited (Figure 5 C, D). Strain 7, with the active catabolic dehydrogenase, has a smaller intracellular G3P pool than strain 8 and this may account for the greater sensitivity of strain 7 to the DHBP. The anabolic dehydrogenase (in BB 6) is not sensitive to feedback inhibition by G3P (45) and the reduction of dihydroxyacetone phosphate is not as readily inhibited by DHBP in strain BB 6 as it is in strain 8 (19). This difference in the gpsA gene accounts for the difference in sensitivity of strain 8 and BB 6.

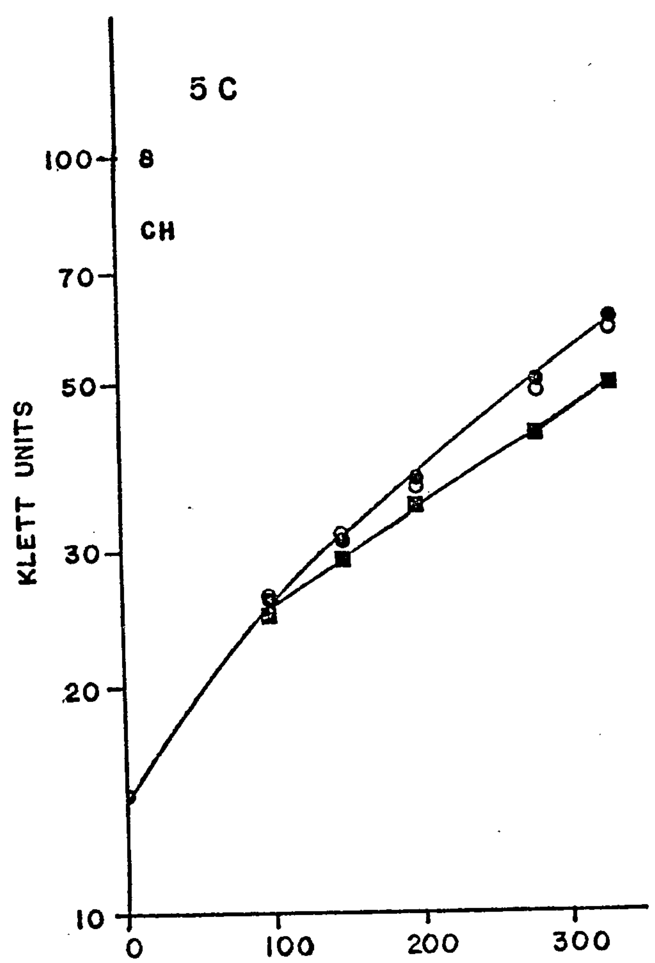
At low concentrations, DHBP has a pronounced effect on the synthesis of phosphatidylglycerol. At these same concentrations, there is little inhibition of growth. This observation raised many questions, since such a major perturbation in membrane composition or in phospholipid metabolism should have a much greater effect on cellular

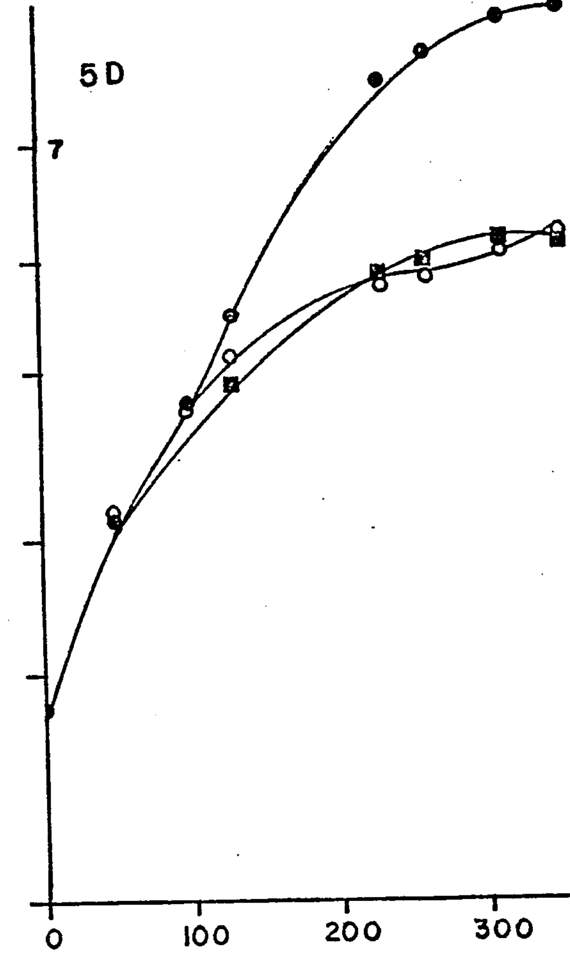
FIGURE 5

Effect of DHBP Concentration on Growth.

Strains were incubated in either G and L medium with succinate as the carbon source (A, B) or in CH medium (C, D) as described. DHBP was added to the indicated concentrations when the cultures reached 15-20 K.U. and the growth followed. Strain 8 (A, C); strain BB 6, (B); strain 7 (D). Concentrations of DHBP, (●-●-●), 0.0 mM; (○-○-○), 0.03 mM; (■-■-■), 0.12 mM.


5 A
5 B
8
G and L
BB6
KLETT UNITS
100
70
50
30
20
10
0
100
200
300
TIME (mins.)





TIME (mins)

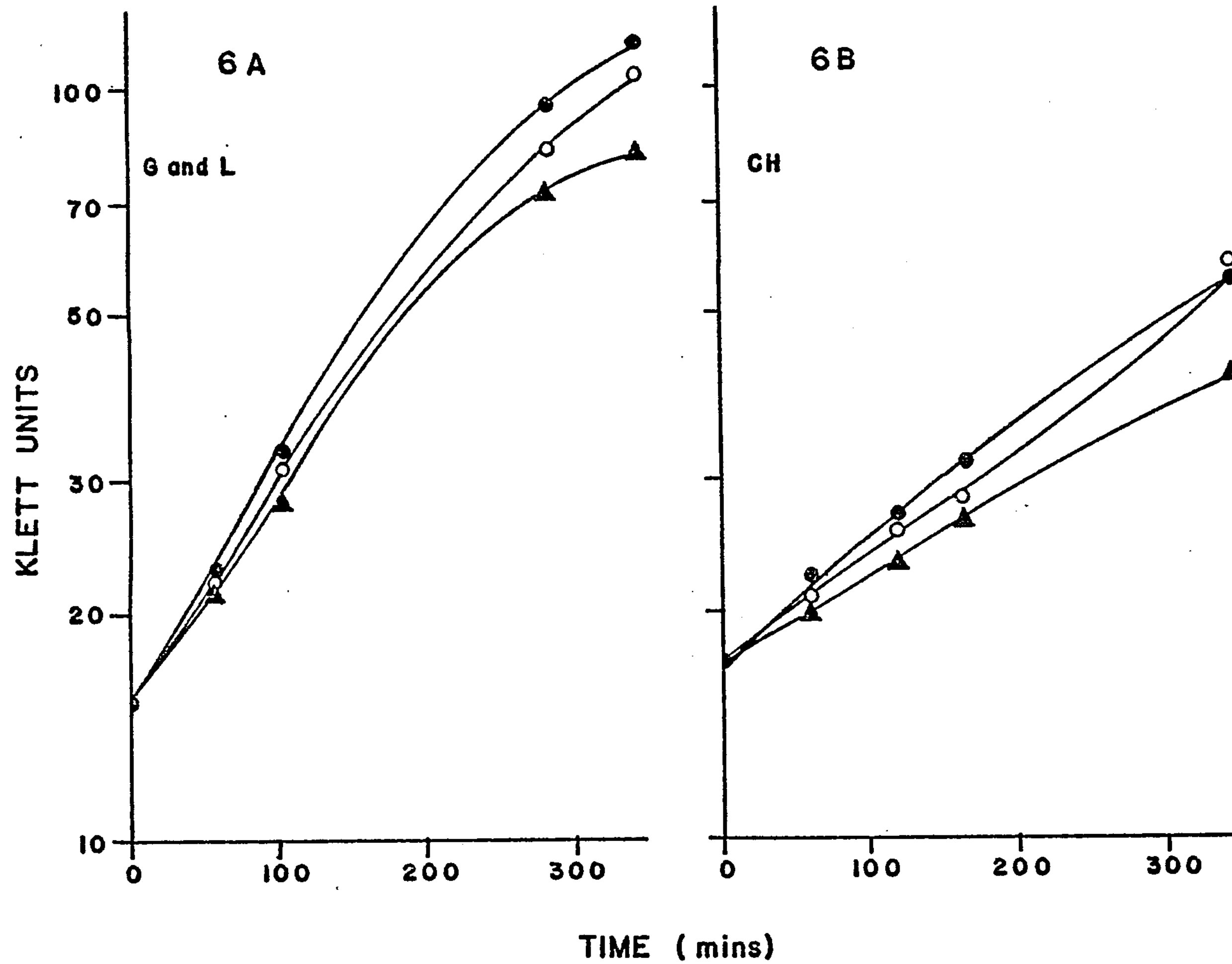
growth. It was therefore decided to examine the effects of DHBP in an environment more similar to its natural habitat. As an enteric bacteria, E. coli is exposed to cholic acid. It was decided to examine the effects of adding deoxycholate to cells incubated in the presence of DHBP. Deoxycholic acid has been used to study membrane structure in Salmonella typhimurium (46,47). Both Roantree et al (46) and Wilkinson et al (47) had demonstrated that there is an increased sensitivity to deoxycholate (DOC) in mutants of S. typhimurium with an altered lipopolysaccharide. Coleman and Leive (48) found a similar sensitivity in lipopolysaccharide mutants of E. coli. Sodium taurodeoxycholate (a conjugated bile salt) has been reported to increase the amount of lipids, protein and phosphorus released from everted rat intestines (49). Lipopolysaccharide is found in the outer membrane of gram-negative organisms and it is in this outer membrane that the barrier function of the cell resides (50). There has been no experimental work done on the effect of DHBP on lipopolysaccharide metabolism.

DOC, itself, has little effect on the growth of strain 8 in either G and L medium (Figure 6 A) or in CH medium (Figure 6 B). This lack of inhibition by DOC was not surprising since as a bacterium found in the intestinal flora, E. coli is constantly exposed to cholic acid (51). The combination of DHBP and DOC was tested on strain 8 in G and L medium supplemented with succinate (Figure 7A). Both

FIGURE 6

The Effect of DOC on the Growth of Strain 8.

Strain 8 was incubated in either G and L medium supplemented with succinate or in CH medium as described. DOC was added at 0 time. A, G and L medium; B, CH medium. DOC concentrations: (○-○-○), 0.10%; (▲-▲-▲), 0.25%; (●-●-●), no addition.


6 A
G and L
6 B
CH
KLETT UNITS
100
70
50
30
20
10
0
100
200
300
0
100
200
300
TIME (mins)

strain 8 and strain 7 were also incubated in CH medium (Figure 8 A, B). Strain BB 6 was also cultured in both media and tested for the synergistic effect (Figure 7 B, 8 C). In G and L medium with succinate, strain 8 is quite sensitive to both drugs together (Figure 7 A). In CH medium, there is little synergistic effect observed with strain 8 (Figure 8 A) while strain 7 is very sensitive (Figure 8 B). While 0.25% DOC has no effect on the strain 7, the 0.03 mM DHBP by itself inhibits. In strain BB 6, cultured in G and L medium with succinate, there is marked (but not total) inhibition of growth with the addition of both drugs (Figure 7 B) and when cultured in CH medium, there is no effect by either DHBP or DOC, alone or in combination (Figure 8 C).

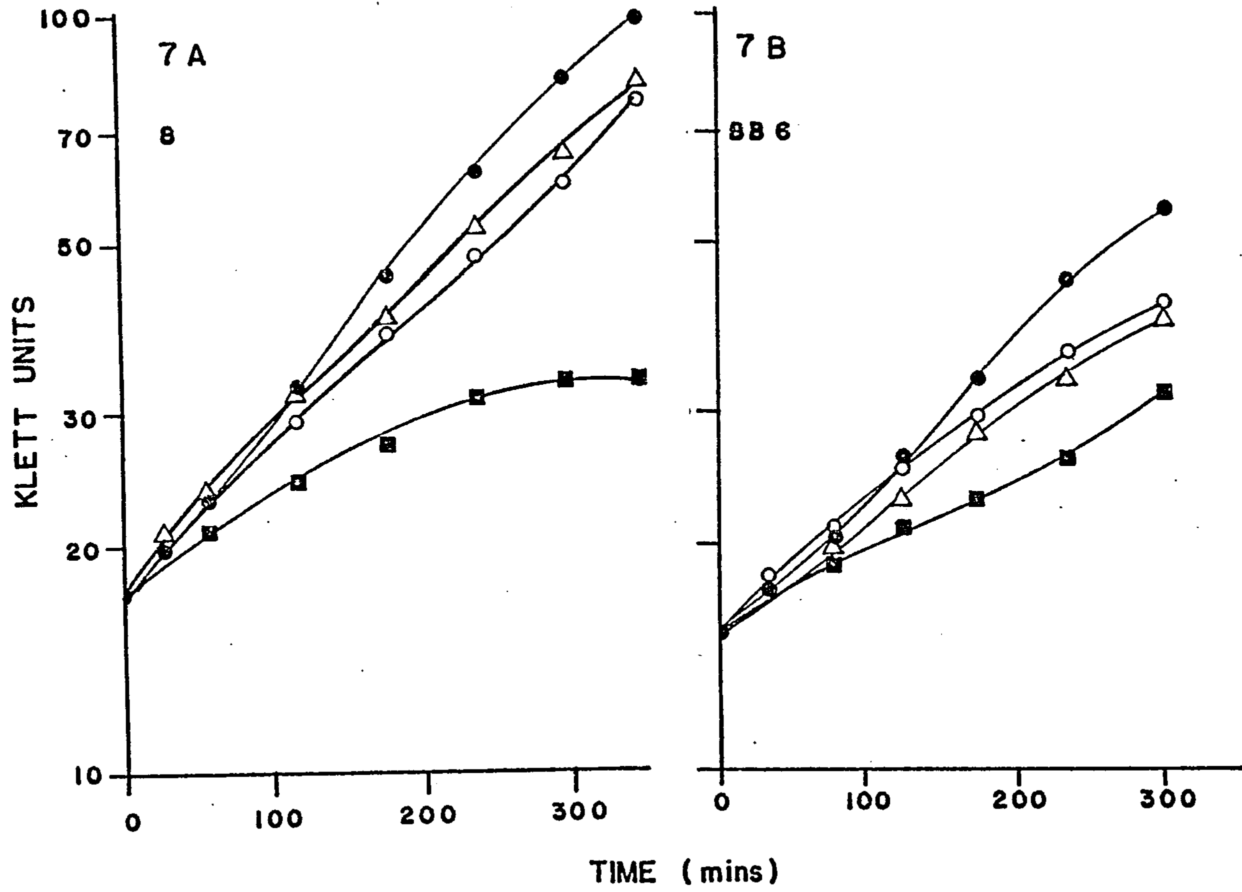
In order to determine if the synergistic effect of DHBP and DOC was bacteriostatic or bactericidal, cells from liquid cultures were diluted and the viability determined. The synergistic effect appears to stop growth immediately, as determined by measurement of turbidity (Figure 9 A). The viability, however, decreases with DHBP and DOC in combination (Figure 9 B) and is thus indicative of a somewhat bactericidal effect.

Effect of the DHBP Concentration on the Synergistic Effect of DHBP was DOC. Strain 8 cultured in G and L medium with succinate as the carbon source and treated with DHBP, DOC,

FIGURE 7

Synergistic Effect of DHBP and DOC.

Strains, as indicated, were cultured in G and L medium with succinate as the carbon source as described. DHBP, DOC, or both drugs were added to the final concentrations indicated and the turbidity followed. A, strain 8; B, strain BB 6. No addition, (●-●-●); 0.25% DOC, (△-△-△); 0.03 mM DHBP, (○-○-○); 0.03 mM DHBP plus 0.25% DOC, (■-■-■).

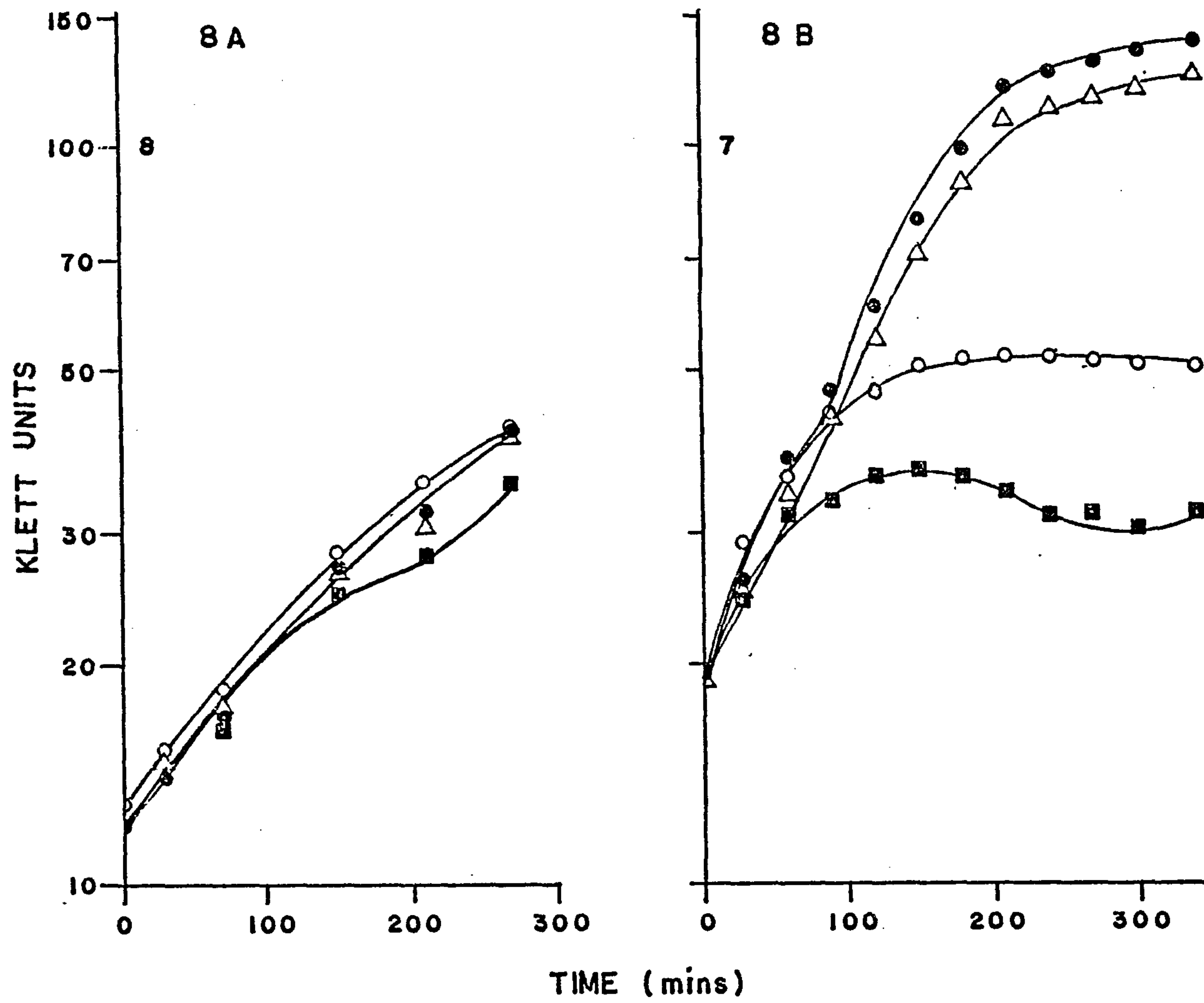


7 A
8
7 B
8B 6
KLETT UNITS
100
70
50
30
20
10
0
100
200
300
TIME (mins)

FIGURE 8

Synergistic Effect of DHBP and DOC in CH Medium.

Strains, as indicated, were cultured in CH medium as described. When the cultures reached 15-20 K.U., either DHBP, DOC, or both drugs were added and the turbidity followed. A, strain 8; B, strain 7; C, BB 6. No addition, (●-●-●); 0.25% DOC, (△-△-△); 0.03 mM DHBP, (○-○-○); 0.25% DOC plus 0.03 mM DHBP, (■-■-■).


8 A
8 B
150
100
70
50
30
20
10
8
7
KLETT UNITS
0
100
200
300
TIME (mins)

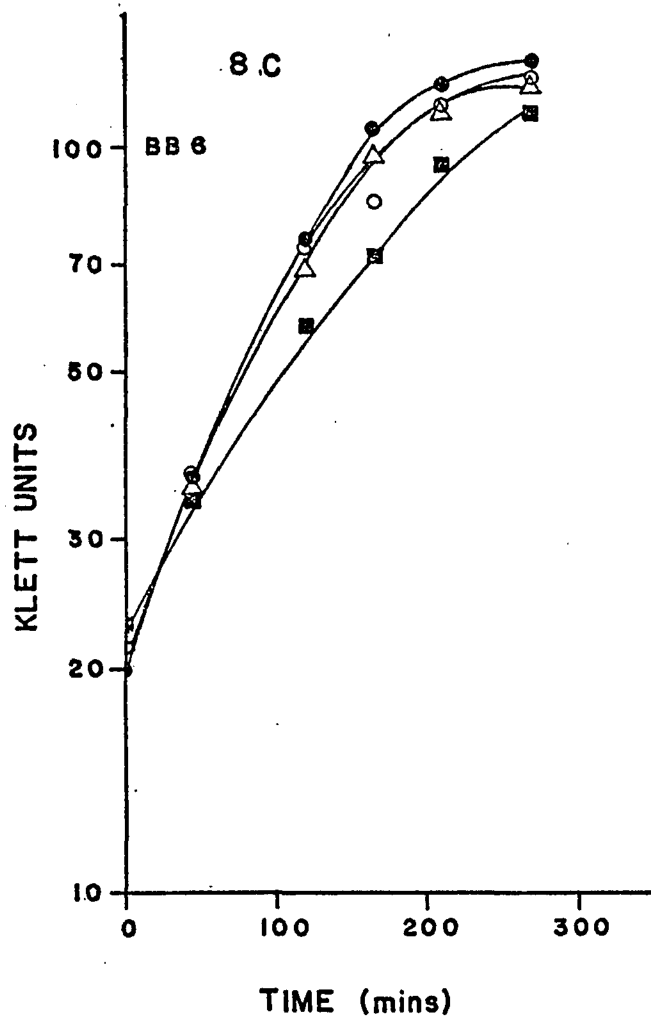
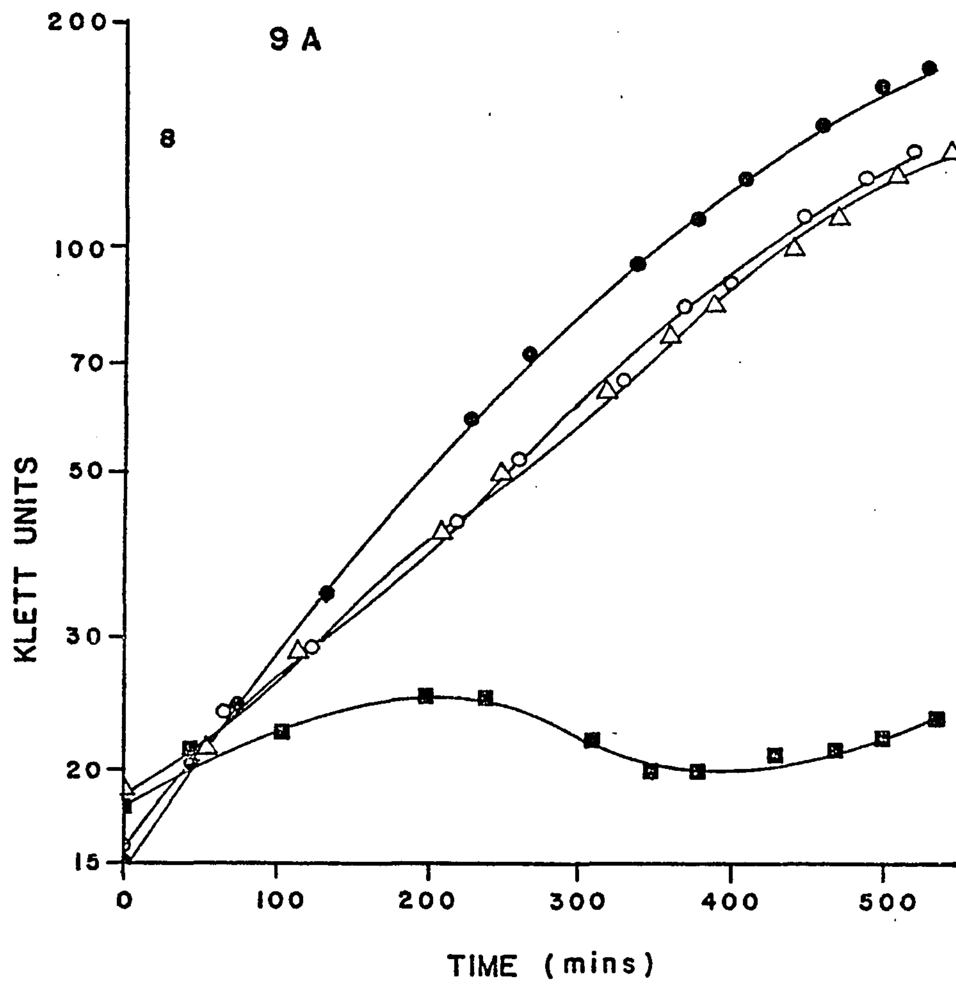

8.C
BB 6
KLETT UNITS
100
70
50
30
20
10
0
100
200
300
TIME (mins)

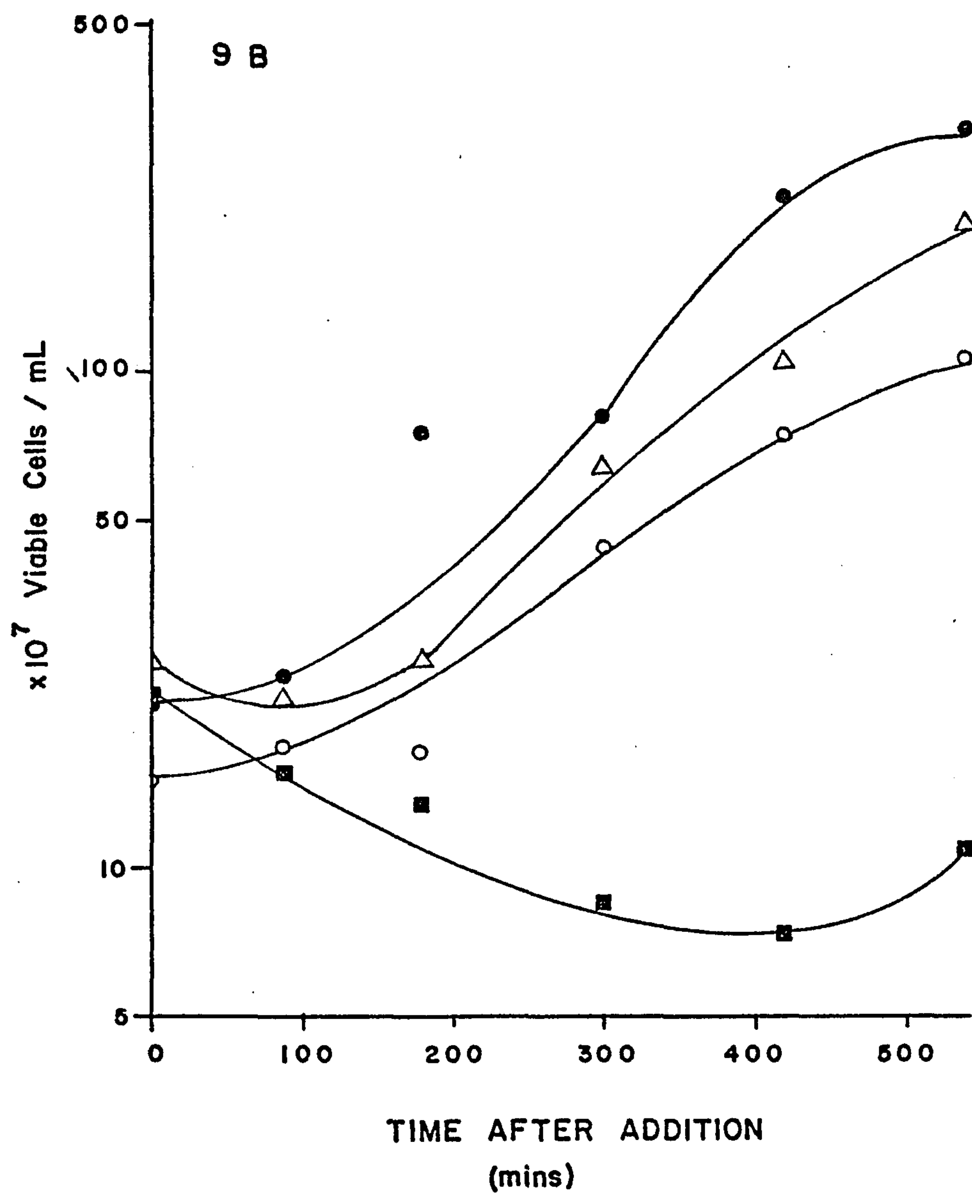
FIGURE 9

Effect of DHBP and DOC on the Viability of Strain 8.

(A), Strain 8 cultured in G and L medium with succinate as the carbon source was treated with either DHBP, DOC, or both drugs when the cultures reached 15-20 K.U. and the turbidity followed after addition of drugs. At the indicated times after addition, 0.1 mL of cells was serially diluted in G and L medium minus succinate and plated on CH medium. After an 18-20 hour incubation, the number of viable cells/mL was determined (B). No addition, (●-●-●); 0.25% DOC, (△-△-△); 0.03 mM DHBP, (○-○-○); 0.25% DOC plus 0.03 mM DHBP, (■-■-■).



9 A
8
KLETT UNITS
200
100
70
50
30
20
15
0
100
200
300
400
500
TIME (mins)


9 B
500
100
50
10
5
x10^7 Viable Cells / mL
0
100
200
300
400
500
TIME AFTER ADDITION
(mins)

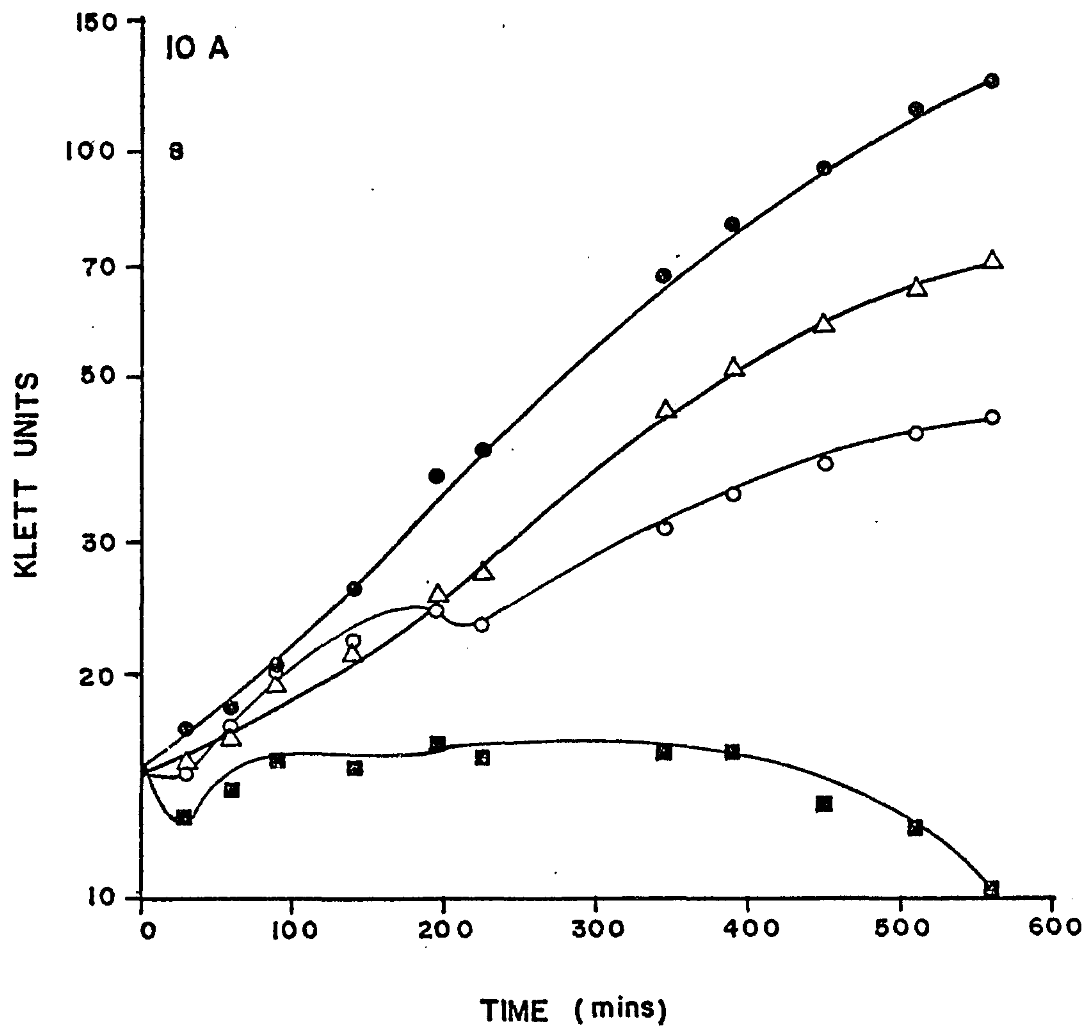
or both, in two ways. In the first experiment, the DHBP concentration was raised to 0.06 mM. DHBP, DOC, or both, were added to cultures at 15-20 K.U. In the second experiment, the DHBP concentration was 0.03 mM and was added to cultures at 5-7 K.U. In both experiments, the DOC concentration was kept at 0.25%. Growth was followed after addition. The cell viability was determined in the second experiment.

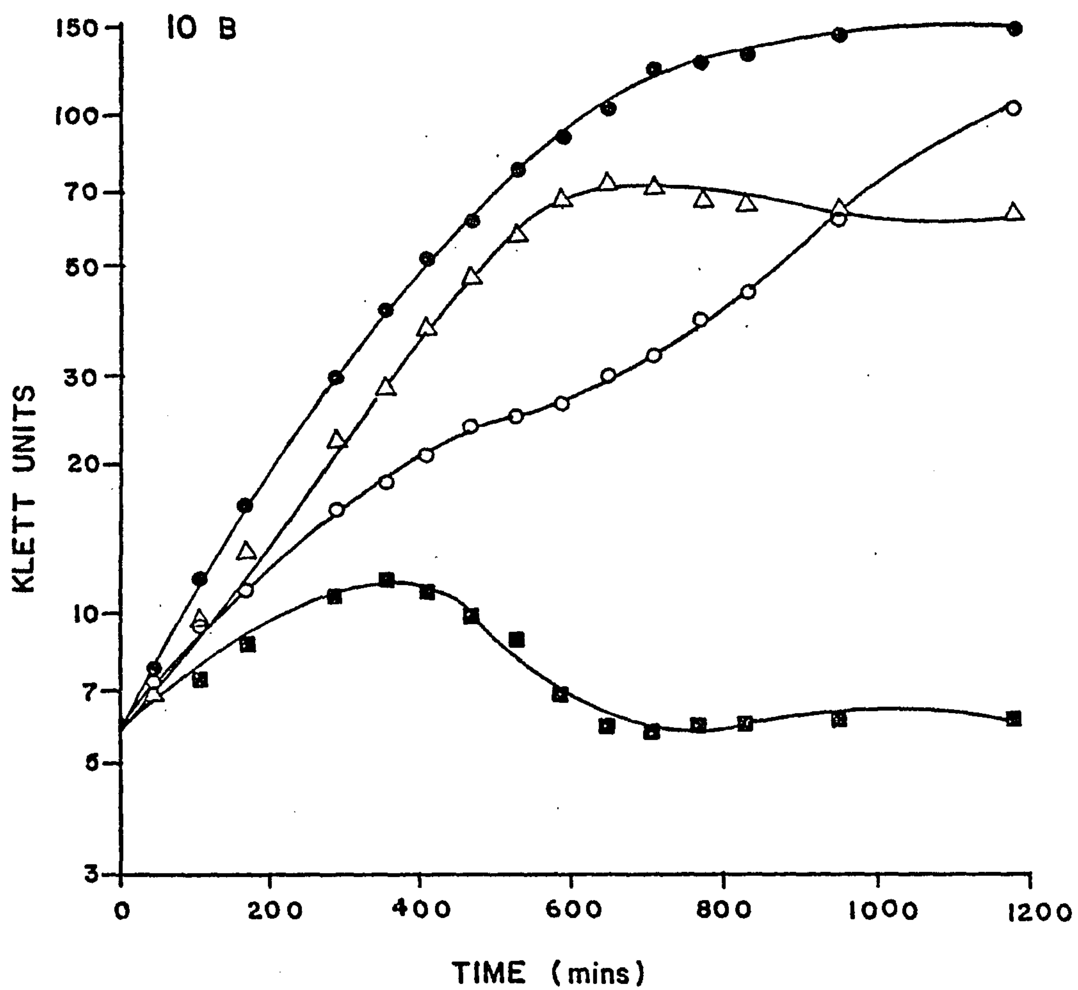
Further studies of the synergistic effect of DHBP and DOC on strain 8 were performed to determine if the concentration of the DHBP had any effect on the bactericidal action of the two drugs together. The concentration of the DOC remained at 0.25%, while the DHBP was raised to 0.06 mM (Figure 10 A). A second culture of strain 8 in G and L medium with succinate was treated with 0.03 mM DHBP, but drugs were added to the cultures at a lower concentration of cells (5-10 K.U.) (Figure 10 B). In both cases, when both DHBP and DOC are added to cultures, the rate of growth of strain 8 in G and L medium with succinate is more inhibited (than either drug alone) and remains inhibited for a longer period of time. DHBP or DOC added to the cells at the lower turbidity resulted in more inhibition of growth. The viability, however, is demonstrably affected when both DHBP and DOC were added to cells at the lower turbidity (Figure 10 C).

FIGURE 10

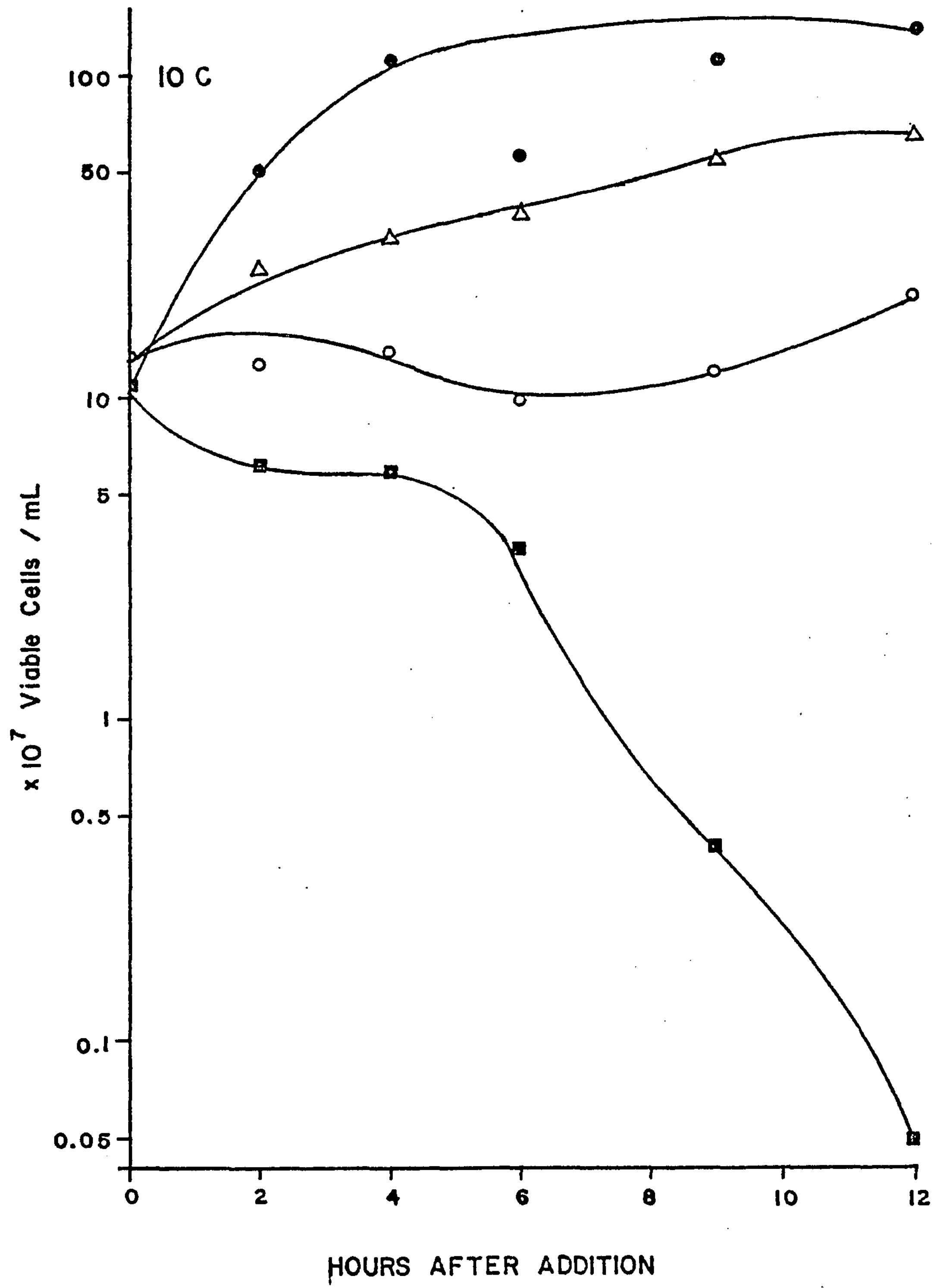
Effect of DHBP Concentrations on the Synergistic Effect of DHBP and DOC.

Strain 8 was incubated in G and L medium supplemented with succinate as described and treated with either DHBP, DOC, or both drugs. The DOC concentration was maintained at 0.25%. DHBP (0.06 mM) was added to cultures at 15-20 K.U. (A) or 0.03 mM DHBP was added to cultures at 5-7 K.U. (B). Turbidity was followed after addition. (C), At the indicated times after addition, 0.1 mL of cells from cultures at the lower turbidity (B) were serially diluted in G and L minimal medium minus succinate (at room temperature) and the viability determined. A, Untreated, (●-●-●); 0.06 mM DHBP, (○-○-○); 0.25% DOC, (△-△-△); 0.06 mM DHBP plus 0.25% DOC, (■-■-■). B, C, No addition, (●-●-●); 0.03 mM DHBP, (○-○-○); 0.25% DOC, (△-△-△); 0.03 mM DHBP plus 0.25% DOC, (■-■-■).


150
100
70
50
30
20
10
10 A
8
KLETT UNITS
0
100
200
300
400
500
600
TIME (mins)



10 B
150
100
70
50
30
20
10
7
5
3
KLETT UNITS
0
200
400
600
800
1000
1200
TIME (mins)


10 C
x 10^7 Viable Cells / mL
100
50
10
5
1
0.5
0.1
0.05
0
2
4
6
8
10
12
HOURS AFTER ADDITION

Incorporation of S-[1,2-^{3}H]-DHBP and rac-[3-^{3}H]-DHBP into Formic Acid Soluble and Insoluble Cell Fractions. Strain 8 cultured in G and L medium supplemented with succinate was treated with 5.0 x 10^{-4} mCi/mL of the *S*-[1,2-^{3}H]-DHBP or 1.8 x 10^{-3} mCi/mL of the *rac*-[3-^{3}H]-DHBP. The concentrations of the two tracers were 0.015 and 0.030 mM respectively (specific activities of 88 and 55 mCi/mmole). The [1,2-^{3}H]-DHBP was present as the *S* isomer while the [3-^{3}H]-DHBP was used as the racemic form. At various times after addition, 1.0 mL of cells were filtered, washed, and the formic acid soluble and insoluble fractions isolated as described. Where indicated, the formic acid soluble fraction was chromatographed as described.

Tyhach *et al* (21) had demonstrated that label from [3-^{3}H]-DHBP appears in a distillable fraction, presumably as tritiated water. DHBP is not a substrate for the G3P dehydrogenase from *E. coli* (19). If there is some other intracellular enzyme that can oxidize DHBP, then the tritium on the third carbon of the DHBP would be lost. For this reason, the 1,2 tritiated DHBP was synthesized (24) since oxidation of this molecule would not result in the loss of the tritium label. The [1,2-^{3}H]-DHBP was tested along with the [3-^{3}H]-DHBP according to the procedure of Tyhach *et al* (21) and the cells fractionated into chloroform-soluble, water soluble and chloroform-water

insoluble materials. Results similar to those with the [3-^{3}H]-DHBP of Tyhach were obtained with the [1,2-^{3}H]-DHBP (data not shown).

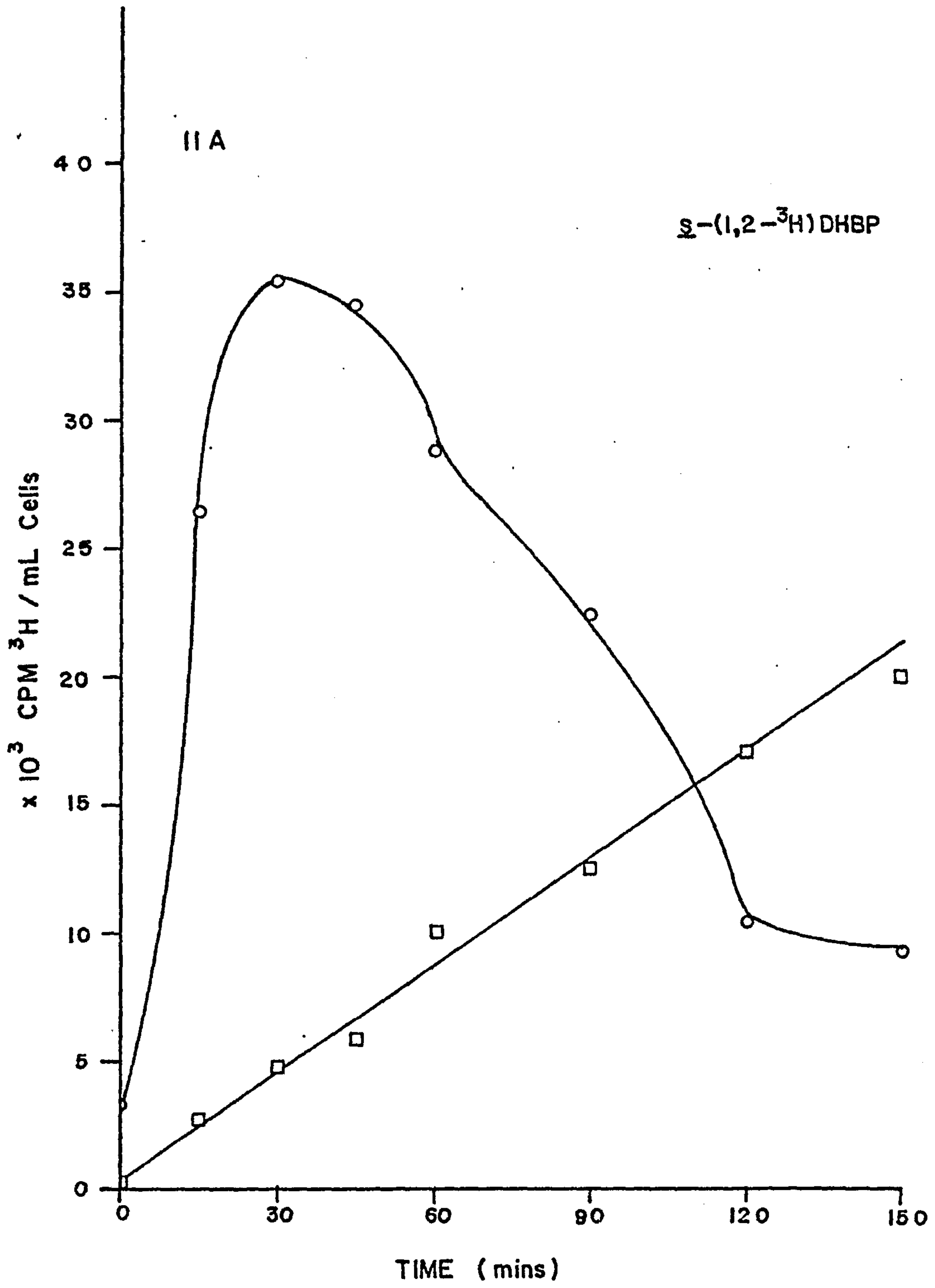
In an attempt to identify the water soluble material, strain 8 was incubated with 0.015 mM S-[1,2-^{3}H]- or 0.030 RS-[3-^{3}H]-DHBP (specific activities of 88 and 55 mCi/mmole, respectively). At various times after addition, the cells were treated with formic acid as described. The amount of ^{3}H incorporated into the formic acid soluble and insoluble pools was then determined. The amount of ^{3}H incorporated into the soluble pool was rapid and reached a maximum at approximately 30 minutes after addition. The total soluble pool then decreased steadily to reach a steady-state after about two hours (Figure 11 A, B). The uptake into the insoluble material was linear over the entire assay period.

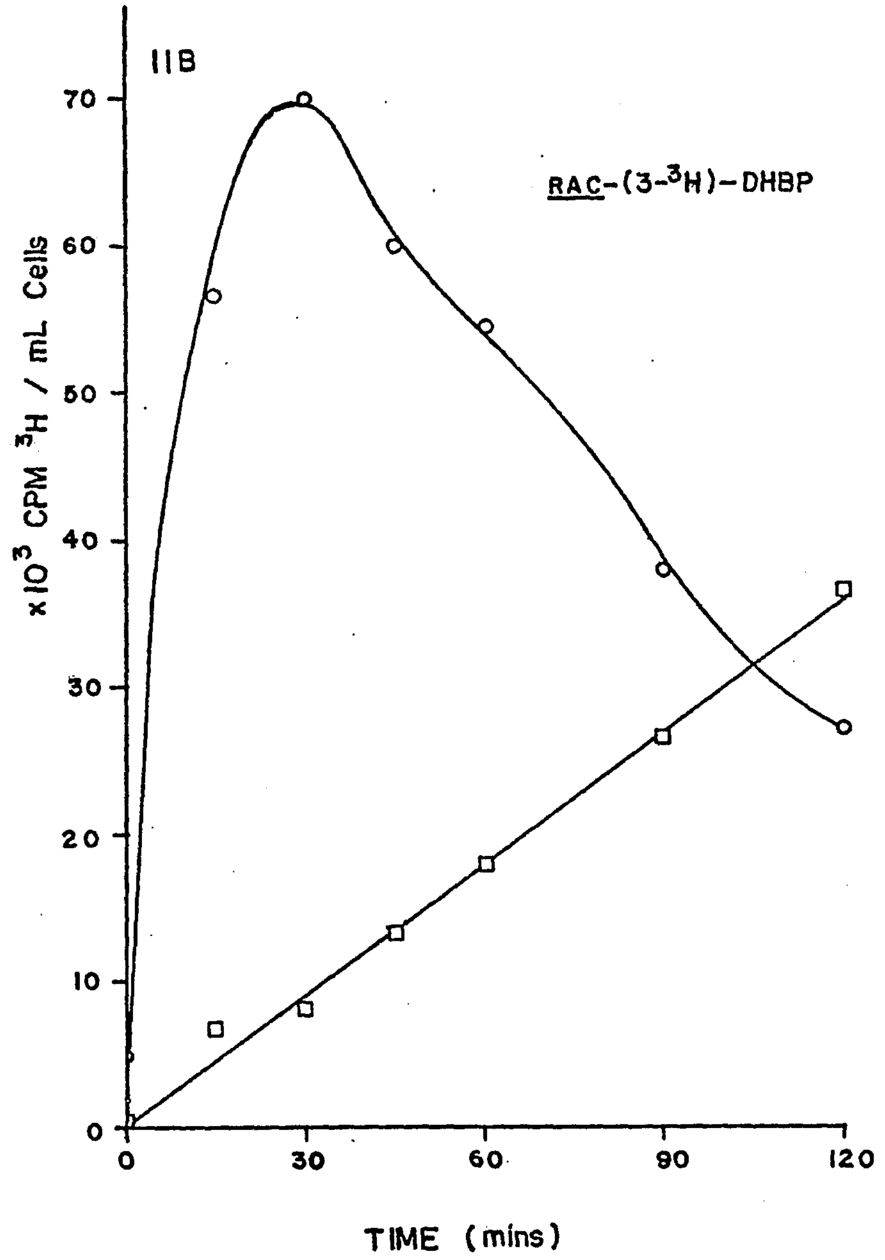
To identify any labeled metabolites of DHBP, the formic acid soluble fraction from cells treated for 30 minutes with DHBP labeled in the 1,2 position was lypholyzed and resuspended in a small volume of water and chromatographed in n-propanol/NH_3 (conc)/H_2O (6/3/1). As expected, the formic acid extract from cells labeled for 30 minutes with the [3-^{3}H]-DHBP showed only one material which migrated with either the cold or radioactive DHBP standard. The formic acid extract from cells labeled with the [1,2-^{3}H]-DHBP for 30 minutes also showed only one

FIGURE 11

Incorporation of [^{3}H]-DHBP into Formic Acid Soluble and Insoluble Material.

Strain 8 incubated in G and L medium with succinate as the carbon source was treated with either 1.0×10^{-3} mCi/mL of S-[1,2-^{3}H]-DHBP or 1.8×10^{-3} mCi/mL of rac-[3-^{3}H]-DHBP (0.015 and 0.030 mM, specific activity of 88 and 55 mCi/mmole, respectively). At the indicated times after addition, 1.0 mL of cells were filtered, washed and treated with ice-cold 2N formic acid as described in the text. A portion of the total formic acid soluble fraction as well as the filters (insoluble) were counted in Patterson- Green scintillation fluid as described. A, [1,2-^{3}H]-DHBP; B, [3-^{3}H]-DHBP. Formic acid soluble fraction (○-○-○); insoluble fraction (□-□-□).


11 A
S-(1,2-3H)DHBP
x 10
3
CPM 3H / mL Cells
40
35
30
25
20
15
10
5
0
0
30
60
90
120
150
TIME (mins)


11B
RAC-(3-³H)-DHBP
x10³ CPM ³H / mL Cells
0
10
20
30
40
50
60
70
0
30
60
90
120
TIME (mins)

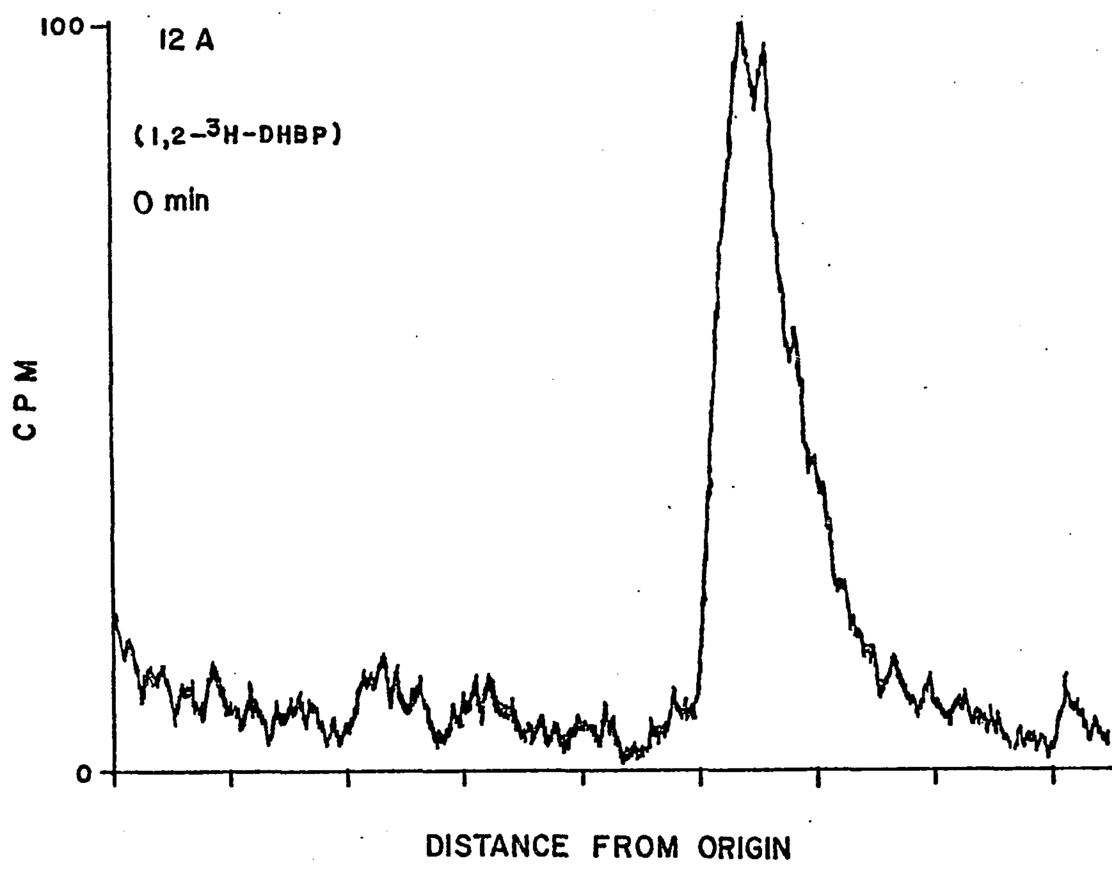
compound (data not shown). The 90 minutes extracts (Figure 12) have a second labeled material (peak II) migrating slower than DHBP (peak I) from the [1,2-^{3}H]-DHBP treated cells. There is, as expected, no second material in the extract from the [3-^{3}H]-DHBP treated cells. The nature of the material remaining at the origin is unknown.

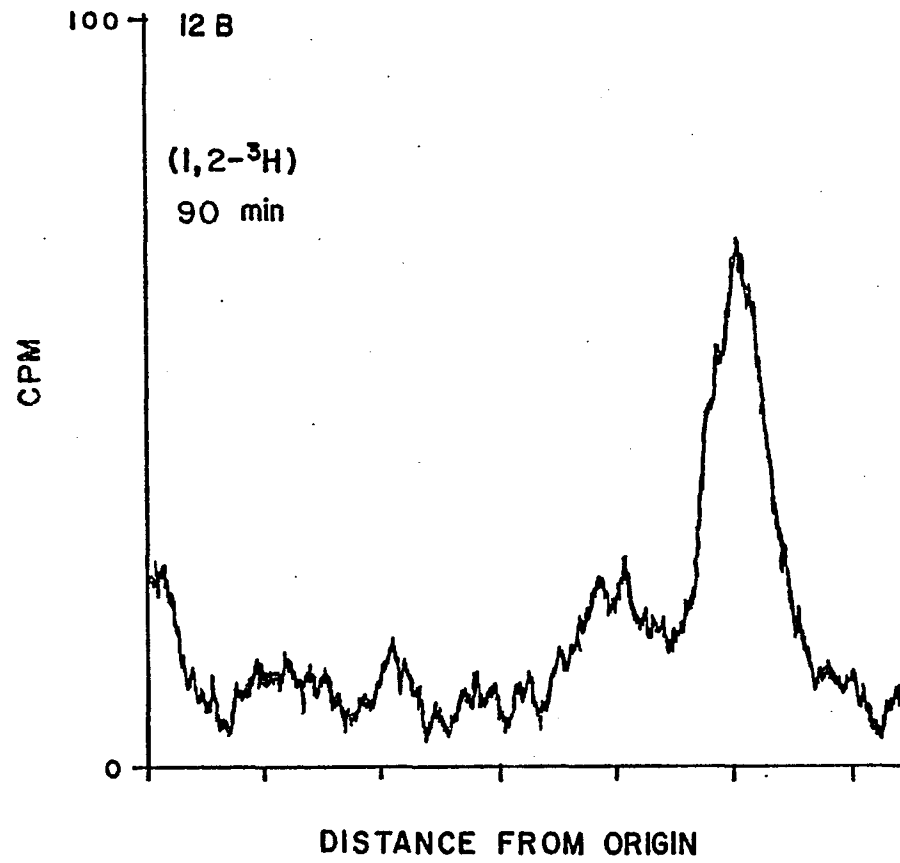
Descending chromatography in ethyl acetate/formic acid (88%)/H_2O (7/2/1) separated the two peaks from cells treated with the [1,2-^{3}H]-DHBP for 90 minutes (Figure 13). In order to determine the identity of the unknown material (peak II), the phosphonic acid analogs of G3P metabolites were chromatographed in the two solvent systems to determine which, if any, migrated with or close to the unknown material. The analog of fructose-1-phosphate was also chromatographed. Table 2 indicates the R_f and R_{G3P} values of several authentic phosphonic acid analogs in both the n-propanol and ethyl acetate solvent systems. Only the fructose-1-phosphate analog (52) has the same R_f or R_{G3P} value as the unknown in both solvent systems. Further efforts to demonstrate that the unidentified material is, in fact, the fructose-1-phosphate analog were inconclusive. Attempts at reduction of the material in peak II with $NaBH_4$ or oxidation with periodate in order to change its chromatographic behavior have been unsuccessful.

FIGURE 12

Chromatographic Analysis of the Formic Acid Soluble Fraction.

Strain 8 cultured in G and L medium supplemented with succinate was treated with either the [1,2-^{3}H]-DHBP or [3-^{3}H]-DHBP as described. The formic acid soluble fractions were extracted and chromatographed descending in *n*-propanol/NH_3 (conc)/H_3O (6/3/1). After drying, the chromatograms were scanned to locate radioactivity. Unlabeled DHBP was visualized as described. A, 0 minute incubation with the [1,2-^{3}H]-DHBP; B, 90 minute incubation with [1,2-^{3}H]-DHBP; C, 90 minute incubation with [3-^{3}H]-DHBP.


100
12 A
(1,2-3H-DHBP)
0 min
CPM
0
DISTANCE FROM ORIGIN


100
12 B
(1,2-³H)
90 min
CPM
0
DISTANCE FROM ORIGIN

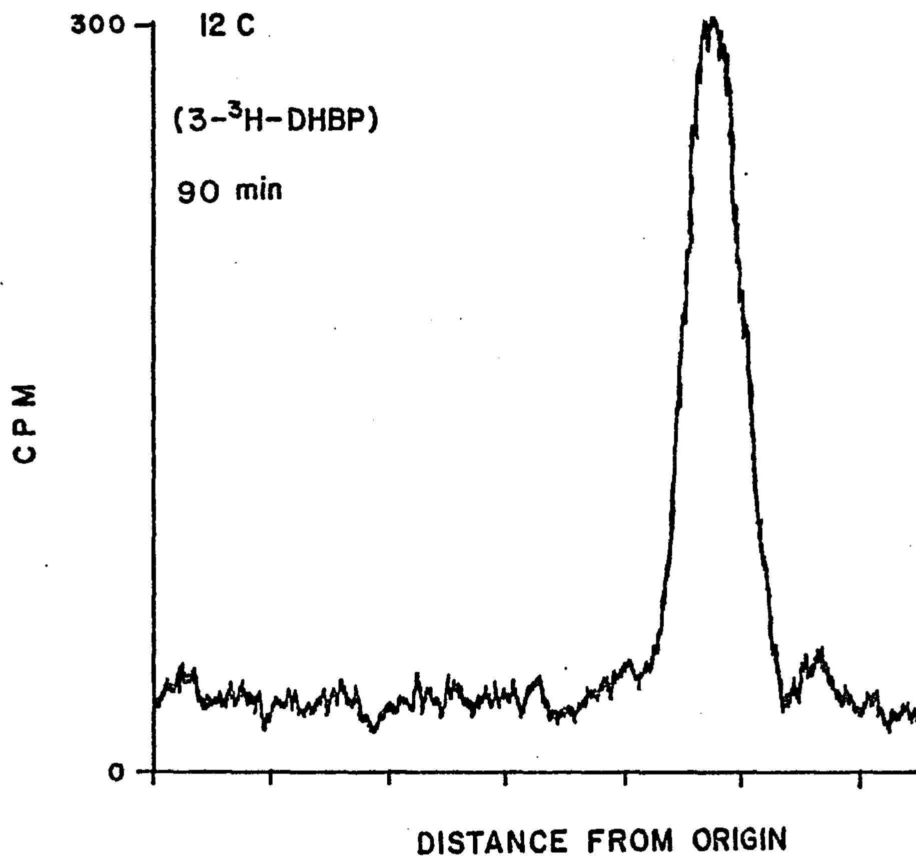
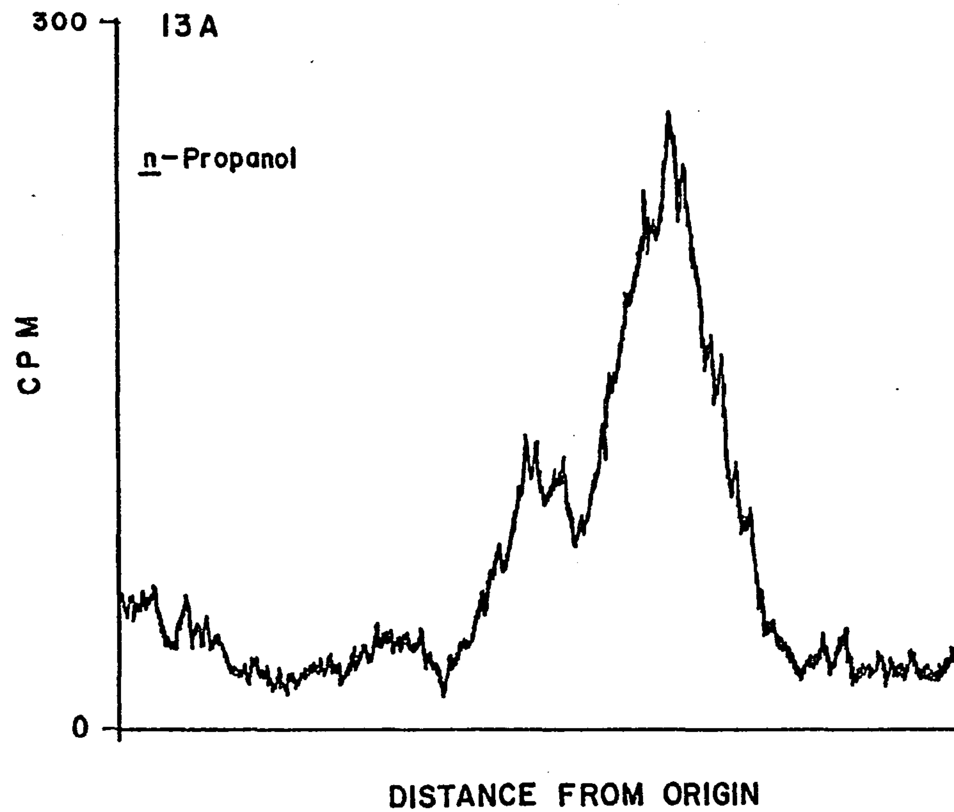
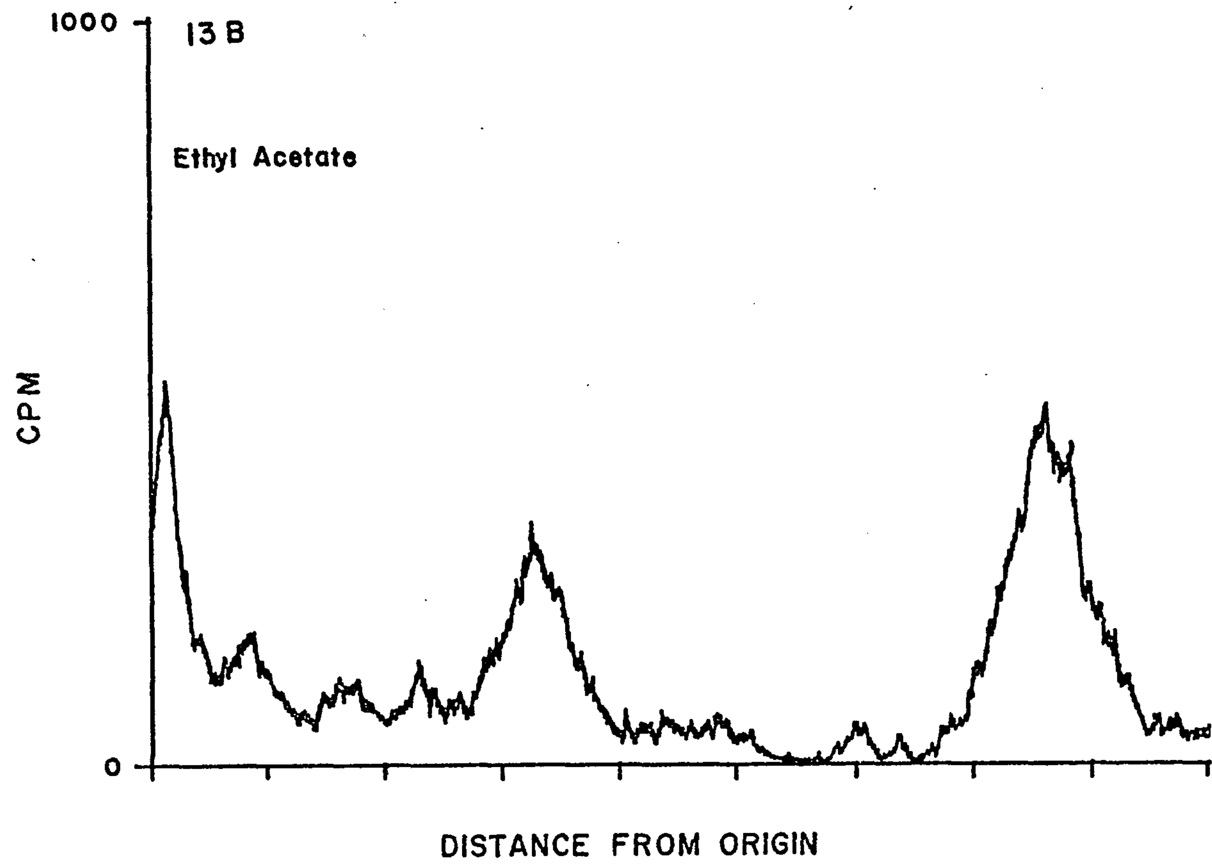

300
12 C
(3-³H-DHBP)
90 min
CPM
0
DISTANCE FROM ORIGIN

FIGURE 13

Chromatographic Separation of the Formic Acid Soluble Fraction of Strain 8.

Strain 8 cultured in G and L medium supplemented with succinate was treated with the [1,2-^{3}H]-DHBP as described and the formic acid soluble (intracellular pool) extracted. After lypholyzing the soluble fraction, the residue was resuspended in a small volume of water and chromatographed descending in either n-propanol/NH_3 (conc)/H_2O (6/3/1) (A) or in ethyl acetate/formic acid (88%)/H_2O (7/2/1) (B). After drying the chromatograms, unlabeled compounds were visualized as described and the radioactive material located by scanning.


300
13 A
n-Propanol
CPM
0
DISTANCE FROM ORIGIN


1000
13 B
Ethyl Acetate
CPM
0
DISTANCE FROM ORIGIN

Incorporation of S-[1,2-^{3}H]-DHBP into the Water Soluble Cell Fraction. Strain 8 cultured in G and L medium supplemented with succinate was incubated with 1.1 x 10^{-3} mCi/mL of *S*-[1,2-^{3}H]-DHBP (0.015 mM, 88.7 mCi/mmole) for 90 minutes. Cells (50 mL) were filtered and washed as described with 3 x 10 mL of warm pre-conditioned G and L medium (37 ^{o}C) and the filters air dried for a few minutes. The cells on the filter were then subjected to cold osmotic shock as described. After resuspension, the water soluble cell fraction was chromatographed.

Strain T 7 (strain 7, *uhp*R^c) (26) and strain 8 were also cultured in CH medium and treated as above and the water soluble pools chromatographed.

Treatment of the cells with ice-cold water releases more than 95% of the soluble pools and is comparable to formic acid treatment.

Since it is possible that the formic acid used for extraction affects the intracellular pool, cells were also extracted by cold osmotic shock with ice-cold distilled water. Chromatography of labeled DHBP as well as the water soluble extracts in either system gives the same pattern as that obtained when the cells are extracted with formic acid. The amount of material remaining at the origin is reduced (data not shown).

It has been demonstrated that strain 8 is more resistant to DHBP when cultured in CH medium than when

cultured in G and L medium supplemented with succinate. The differences observed in the growth patterns for strain 8 in G and L medium with succinate as compared to CH medium, might be attributed to the G3P pools. If the unidentified material (peak II) is the true growth inhibiting agent, then it is possible that there would be more of this compound present when strain 8 is cultured in G and L medium with succinate than when cultured in CH medium. A comparison of strain 8 cultured in G and L medium with succinate and CH medium was made according to the procedure outlined. The extract from cells cultured in G and L medium shows the same chromatographic behavior as obtained previously (Figure 13). However, there is no second material (peak II) in extracts from cells cultured in CH medium (data not shown). A comparison of strains 8 and 7 cultured in CH medium was also made in order to determine if the unknown material (peak II) was present in strain 7. It was thought that, since strain 7 is more sensitive to DHBP than strain 8 when cultured in CH medium (Figure 5 C, D), there should be more of this unknown in the strain 7. However, when the water-soluble extracts were chromatographed, in either system, there was little or no peak II present. As yet, we have no explanation for this difference.

What has been generally observed, is that approximately 10% of the <u>total</u> amount of radioactivity added to the cultures could be extracted from the intracellular pool,

whether the cells were extracted with formic acid or with cold water. There is no apparent difference in the amount of labeled material released from the cells by either cold formic acid or osmotic shock (43).

The following conclusions can be drawn from the results obtained in these studies on the effect of DHBP in *E. coli*. While DHBP markedly alters the phosphoglyceride composition of the membrane, it does not appear to have an *in vivo* effect on the activity of the fatty acyl CoA: *sn*-glycerol-3- phosphate acyltransferase. The K_m of the enzyme for G3P is approximately the same whether it is isolated from cells exposed to DHBP or from untreated cells and both are comparable to the value previously reported.

The effect of DHBP on the inhibition of growth in *E. coli* appears to be mediated, in part, by the size of the intracellular G3P pool for the following reasons. Previous investigation had shown that DHBP and G3P share the same pool. The rate of incorporation of DHBP into lipids is higher in a strain with a smaller intracellular G3P pool. DHBP inhibition is also partially offset in strains which have a larger G3P pool. The bactericidal effect of DHBP and DOC on cells is also dependent on the size of the G3P pool, since strains with a larger intracellular concentration of G3P are less sensitive to the synergistic effect. The investigation of the metabolism of DHBP has led to the conclusion that DHBP is not only incorporated into a

phosphatidylglycerol phosphate analog, but is also converted into an as yet unidentified water soluble intermediate. Of all the phosphonic acid analogs tested, only the fructose-1-phosphate analog migrates with the same R_f or R_{G3P} as the unknown material. Attempts at proving its identity chemically have been unsuccessful to date.

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CHAPTER 2

The Illicit Transport of Phosphonic Acid Analogues via the Oligopepide Permease System.

INTRODUCTION

Two books (1, 2) and several reviews (3-10) have appeared concerning peptide transport and metabolism in bacteria. Since it would be almost impossible to review all of the facets of peptide transport, only those subjects concerning peptide transport which are relevant to the work described here will be considered in detail. For information on topics not discussed in this review, the reader is referred to the above reviews.

A. Peptides in Microbial Nutrition

The nutritional requirement of peptides by bacteria was first demonstrated, if somewhat unknowingly, by Koch in his studies with the anthrax bacillus. The addition of peptones

(an enzyme digest of meat) as well as meat extract to nutrient broth served as a rich medium for the culture of many, but not all microorganisms. It was later determined that some bacteria are unable to utilize the peptones (3). It was not until about 1940, when Woolley (11) first reported the isolation of the bacterial growth factor streptogenin, that an investigation of the role of peptides in microbial nutrition was undertaken. Streptogenin is required by several different organisms for rapid growth and it could be isolated from several different sources: tomato juice for *Lactobacillus arabinosus* (12), yeast or peptone for *Streptococcus lactis* (13), or peptone for *L. casei* (14). Sprince and Woolley (15) concluded that the same substance(s) is required by the three organisms. This is the substance(s) that was named "streptogenin". Because of its properties, it was proposed (16) that streptogenin is a peptide.

There have been many reviews written on the subject of peptides, their role in nutrition and their transport (1-10). Most, if not all of these mention or discuss streptogenin. Woolley (11) first reported the existence of streptogenin in an aqueous extract of liver that was used for culturing a strain of hemolytic streptococcus. There was no growth in the basal medium employed, even when acid hydrolyzed casein was present at a concentration of 1%. This indicated that the requirement is not an amino acid.

The addition of the liver extract to the basal medium, however, promoted growth. The active fraction of the liver was insoluble in alcohol and acetone, was not precipitated by lead and was adsorbed by $BaSO_4$ but not by charcoal. The properties of streptogenin and some of the other growth factors were further studied and it was shown that they are the same or closely related substances. Since the factor(s) were soluble in acidic or alkaline alcohol, it was concluded that the substance(s) are amphoteric and the solubility is due to the formation of soluble acidic or basic salts. While the extraction of streptogenin from partially hydrolyzed casein originally puzzled Sprince and Woolley (it was not found in unhydrolyzed or completely hydrolyzed casein), this fact is no longer surprising since casein is a protein and partial hydrolysis results in the formation of peptides. The streptogenin "molecule" is considered to be a peptide because of 1) its chemical properties, 2) most sources of streptogenin were proteins and 3) it could be released from proteins by enzyme digestion.

Woolley and Merrifield (10) summarized the work done on streptogenin. The conclusions were the following: 1) it is not the sequence or amino acid content that gives a peptide streptogenin (growth promoting) activity and 2) the size of the peptide (i.e. the number of amino acid residues) is important for streptogenin activity. In general, peptides are viewed as a means of getting amino acids into cells.

B. Amino Acid vs. Peptide Transport

For the sake of simplicity, the standard three letter abbreviations for both free and peptide bound amino acids will be used (17). Unless otherwise specified, amino acids are of the L configuration.

That amino acid transport and peptide transport are separate and distinct cellular functions has been well documented. Most of the evidence for this distinction has arisen from the use of amino acid transport mutants. The ability of di- or oligopeptides containing the required amino acid(s) can be used as a criterion for demonstrating that the peptides are transported into the cells.

Growth supplementation of amino acid auxotrophs by peptides or inhibition of growth by toxic peptides are also used as criteria for transport in bacteria. A mutant of *Escherichia coli* deficient in the transport of [^{14}C]valine was isolated and found to be sensitive to dipeptides containing val (18). Other amino acid auxotrophs were also studied. Kessel and Lubin (19, 20) studied a strain of *E. coli* deficient in pro transport (20). A pro auxotroph as well as a mutant of this strain deficient in pro transport which required high exogenous concentrations of pro for growth were tested for growth on gly-pro and pro-gly. Both strains grew equally well on the peptides.

The use of radioactive amino acids and peptides has been a valuable tool with which to study transport and incorporation. Much work has been done using these labeled probes to distinguish between the transport of amino acids and peptides, and to study the incorporation of amino acids from peptides. [^{14}C]Gly and [^{14}C]gly-gly uptake in E. coli was studied by Levine and Simmonds (21). A mutant deficient in gly uptake was shown to incorporate the labeled gly-gly and to use it as a source of gly for growth.

Studies with L. casei (22-26) have also demonstrated the independence of amino acid and peptide transport. Snell et al (22-26) have investigated the uptake of ala, gly and alanyl and glycyl dipeptides and evidence has been presented to substiantiate the idea that transport of ala is by a different mechanism than is transport of alanyl peptides (24). The reason for this hypothesis is that D-ala antagonizes L-ala uptake but not the uptake of ala peptides when L. casei is grown in vitamin B_6 deficient medium. Leach and Snell (22) showed that the rate of uptake of L-ala-[^{14}C]gly is about 10 times that of [^{14}C]gly. Substantial loss of labeled intracellular [^{14}C]gly pool occurs when either unlabeled ala-gly or gly is added to the cultures. This decrease in pool size is faster with the peptide than with the amino acid. It was also demonstrated that unlabeled glycine does not lower the rate of transport of labeled peptide, but it does decrease the amount of

[^{14}C]gly incorporated from the labeled peptide. This indicates that the peptide must be hydrolyzed before amino acids can be incorporated into protein.

Three separate transport systems exist in L. casei (23), for gly, D- or L-ala, and gly-ala. An energy dependent system which accumulates label faster from peptides than from amino acids was demonstrated. According to Leach and Snell (23), if one or more systems exists, one for gly and one for peptide, then the amount of label accumulated when both labels are present should equal the sum of each label. If only one transport system exists then an amount of label intermediate between that found with each label alone should be obtained. When L. casei is incubated with both [^{14}C]gly and [^{14}C]gly-ala, the amount of radioactivity found is equivalent to the sum of each alone (control cultures were incubated with either amino acid or peptide). This result indicates the existence of at least two systems, one for the amino acid and one for the peptide. By competition studies, it was determined that L-ala, D-ala and gly-D-ala has no effect on the incorporation of [^{14}C]gly-L-ala. None of the 17 other amino acids tested compete with the peptide. Pro-phe and his-his also fail to lower the incorporation of label indicating the possibility of two or more dipeptide transport systems. L-Ala-gly effectively competes with the transport of gly-ala as measured by incorporation of either [^{14}C]gly-ala or

gly-[^{14}C]ala (23). In certain experiments (25,26), it was shown that there was a different growth response to different peptides containing ala or gly. Hydrolysis of these peptides was investigated and it was determined that different dipeptides were hydrolyzed at different rates.

In some cases, it has been demonstrated that peptides are better at fulfilling an amino acid requirement than are the free amino acids themselves. Peters *et al*. (27) studied peptide transport in *L. delbrueckii* and found that histidinyl peptides containing an unnatural amino acid (*D*-ala, beta-ala, or alpha amino-N-butyric acid) are far better growth promoters than is free histidine. The presence of the unnatural amino acid eliminates the possibility of direct incorporation of the dipeptide into protein. Since the strain fails to concentrate histidine when present at low concentrations in the medium, a mutant which accumulated higher amounts of his than the parent was isolated and both strains tested for growth on carnosine (his-beta-ala). Both the parent and mutant grow equally well on the carnosine as a source of histidine.

In other experiments, the ability of a peptide or a free amino acid to offset growth inhibition by some amino acid was also found to be better when a peptide was used. The addition of *DL*-ala to *L. delbrueckii* causes growth cessation (28). *L*-Ser offsets this inhibition but dipeptides containing serine are 15-90 fold better at

reversing the inhibition than is the free serine. The peptides tested are all hydrolyzed intracellularly.

The ability of peptides to offset amino acid inhibition, or to surpass free amino acids in supplementation tests is not limited to the peptides mentioned. Other peptides have also been studied. The growth promoting activity of tyrosyl peptides was studied in S. faecalis (29). Both gly-tyr and leu-tyr far surpassed tyr in the presence of B_6 in the assay. Both of the tyrosine containing peptides which were tested are hydrolyzed intracellularly. In Leuconostoc mesenteroides, beta-2-thienylalanine inhibits utilization of phe and L-ala inhibits gly utilization. With both L-ala and L-thienylalanine present, growth inhibition can be offset by 1) large amounts of phe and gly in the medium, 2) small amounts of peptides containing either phe or gly, or 3) a small amount of a single peptide containing both gly and phe (30). In other studies with L. casei (31) a serine requirement is fulfilled by dipeptides containing serine, cysteine is furnished by peptides of cys and the requirement for both cys and gln by either a tripeptide of gln-aspn-cys or a hexapeptide gln-aspn-cys-pro-leu-gly-amide.

C. The Role of Peptidases in Peptide Transport and Metabolism

Assuming that one of the roles of peptides (either di- or oligopeptides) is to furnish amino acids to cells, then the cells must have a means of hydrolyzing the peptides to their component amino acids before, during or after transport across the cell membrane. Since extracellular peptidases have not been found in bacteria (if they did exist, there would be little use for peptide transport systems) hydrolysis before transport can be ruled out. The isolation of a gly-gly dipeptidase mutant (32) would tend to eliminate the possibility of hydrolysis during transport and support the idea of intracellular hydrolysis. Oligopeptidase mutants have also been isolated (6, 33). These mutants transport but do not hydrolyze tri- or higher order peptides.

The transport and hydrolysis of peptides (either dipeptides or oligopeptides) are separate and distinct functions. This has been demonstrated by use of either transport negative mutants or peptidase mutants. Mutants which have lost the ability to transport oligopeptides but retain the capacity to hydrolyze them (transport mutants) (34) have been isolated. Reports have also shown that some oligopeptides as well as dipeptides can be toxic if absorbed

by the cells but not hydrolyzed to their amino acids (peptidase mutants) (5-9, 33-36).

Dipeptide transport and hydrolysis has been studied in strains of _E. coli_. In a strain requiring glycine, growth can be supplemented by gly-gly (32). Two types of mutants of this strain were isolated, one which fails to hydrolyze the dipeptide and another which lacks the ability to transport [^{14}C]gly-gly. Kessel and Lubin (32) therefore concluded that the transport and hydrolysis of the dipeptide are separate functions. It is interesting to note here (and it will be mentioned later on in relation to the transport of di- and oligopeptides) that other dipeptides containing gly inhibit accumulation (transport) of the radioactive gly-gly (some to the extent of 80-85%) but gly-gly-gly does not markedly inhibit uptake (15%). The hydrolysis of peptides is not limited to glycyl peptides. The lack of a trilysine specific peptidase in a lysine auxotroph does not allow trilysine to be utilized by a mutant (33).

Most of the peptidase activity is located in the soluble fraction of the cell within the plasma membrane. Some evidence has been presented which indicates that some activity is bound to or associated with the ribosomal fraction (37, 38). Simmonds and Toye (39, 40) also studied peptidase activity and found that there are three dipeptidases which hydrolyze peptides containing either leucine or glycine. One enzyme hydrolyzes gly-leu and

gly-phe and is resistant to EDTA. Two other EDTA sensitive systems were found, one which acts on phe-gly and is inhibited by Mn^{+2} and another hydrolyzes leu-gly and is insensitive to Mn^{+2}. The leu-gly activity is found in the soluble as well as the ribosomal fraction. Both fractions catalyze the hydrolysis of tripeptides as well as dipeptides (38, 41).

For cells to have an individual dipeptidase for each combination of amino acids would require the production of over 400 enzymes and one for each tripeptide would require 8000. It is conceivable that cells have one or more enzymes with varying specificities capable of hydrolyzing either or both dipeptides and oligopeptides. Simmonds (36, 41) has demonstrated the existence of several dipeptidases as well as several aminopeptidases (oligopeptidases).

The characteristics of the intracellular peptidases have been studied (42-44). In a glycine auxotroph of _E. coli_, Payne tested the effectiveness of gly-sarcosine (gly-_n_-Methyl-gly) and gly-gly-sarc as a source of glycine for growth. While both the di- and tripeptide are transported, only the gly-gly-sarc serves as a source of glycine (42). The growth yield on the gly-gly-sarc is compatible with the utilization of only one glycine residue. The gly-sarc moiety from either the gly-sarc or gly-gly-sarc is not hydrolyzed into glycine and sarcosine. Apparently, this peptidase requires an unsubstituted peptide

linkage in order to hydrolyze the peptide.

The nutritional state of *E. coli* has been shown to affect the activity of the intracellular peptidases (39). Both di- and tripeptidase activity of extracts of chemostat grown cultures were studied by Payne (44). For the dipeptidase activity with gly-gly, pro-gly or val-val, there are different effects on the enzymes if Co^{+2}, Mn^{+2}, Zn^{+2}, EDTA, tris or PO_4^{-3} are added to the assay mixture. With the tripeptidase and gly-gly-gly as substrate and Co^{+2} there is no stimulation in contrast to that found in the dipeptidase. Zn^{+2} inhibits both the tripeptidase and dipeptidase activity. The conclusions drawn from these results are that there were both di- and oligopeptidase (both amino- and carboxypeptidases) and that there are, perhaps, several of each.

Prior to Simmonds (39) work with glycine containing peptides, leucyl peptides had been studied in relation to their inhibitory effect on *E. coli* (45, 46). It had been shown that these leucyl peptides inhibit the initiation of growth (45). Meisler and Simmonds (46) studied uptake and hydrolysis of gly-leu in both "young" cultures (30 hour incubation) and "old" cultures (150 hour incubation). It was determined that the old cultures lack the high peptidase activity found in the younger cultures and are more sensitive to the bacteriostatic effect of gly-leu. An aged, static culture (unshaken), has less peptidase activity than

does the same culture if shaken. Organisms incubated on the surface of a complete agar slant also show a lack of peptidase activity. It was believed that, during the aging process, a cell component is produced which inhibits the peptidase. Extracts of whole cells were heated and then added to assay mixtures containing either whole young cells or cell-free extracts from aged cultures. Both assays result in a decrease in the hydrolysis of gly-leu (46) indicating that a heat stable component of the cell extract can inhibit peptidase activity. The identity of the compound(s) was not reported (46).

An aminopeptidase from _E. coli_ B has been isolated and purified (47). This enzyme, designated aminopeptidase P, will release the N-terminal amino acid residue if adjacent to proline. The enzyme digests poly-_L_-pro, but not poly-hydroxypro. The small peptides hydrolyzed are pro-pro-pro, pro-pro-ala, pro-pro-ala-OMe, gly-pro-gly, and pro-pro. Val-pro, ala-pro and gly-pro are hydrolyzed, but at a much slower rate.

A dipeptidocarboxypeptidase was isolated from _E. coli_ B and purified by Yaron (48). The enzyme is capable of releasing a dipeptide from the COOH-terminus of an N-blocked tripeptide or from tetra or higher order peptides. Unprotected ala-ala-ala is not hydrolyzed, but ala-ala is released from Z-N-ala-ala-ala. The enzyme requires a free COOH group and has strict stereospecificity for the _L_ forms.

The enzyme does not cleave between glycine residues and will not hydrolyze a peptide if the N-terminus of the resulting dipeptide is proline (48).

A different aminopeptidase was isolated from a K 12 strain of E. coli by Vogt (49) and purified. This enzyme appeared to be specific for N-terminal methionyl residues. While it was originally thought that this enzyme was responsible for cleaving the N-met residue from nascent polypeptide chains, the enzyme, however, shows no absolute specificity for N-met. Tripeptides containing N-terminal leucyl residues are also hydrolyzed, usually at rates faster than the corresponding peptides containing met. There is no endopeptidase activity present in this enzyme (49).

Oligo- and dipeptide transport and hydrolysis are not limited to E. coli but have been found in many other bacteria as well as in yeast. In a leucine auxotroph of Salmonella typhimurium, four peptidase activities have been distinguished based on substrate specificity: pepA, pepN, pepB, pepD (50). Two other peptidases have been isolated from proline auxotrophs: pepP and pepQ (51). These mutants fail to grow on leu-pro or gly-pro-ala as a source of proline. Jackson et al (52) further studied growth supplementation by prolyl peptides in the pepQ and pepP mutants (51). Strain TN 87 ($pepQ^-$, $pepP^-$) is capable of growing on pro-leu, pro-ala, pro-gly-gly and pro-val-gly indicating that it has functional transport

systems and that that the lack of growth on leu-pro or gly-pro-ala is due to failure to hydrolyze the peptides. It was demonstrated that ala-pro-[^{14}C]gly is transported but not hydrolyzed. Several other peptides which do not support growth in the absence of proline were shown to inhibit growth in the presence of proline.

Peptidases (both di- and tripeptidases) have been isolated from S. pneumoniae (53), from Saccharomyces cerevisiae (54, 55), Lactobacilli (10, 14, 17-19, 22-28), S. faecalis (29), Neurospora (56) as well as from mammalian cells (57).

The role of peptidases in yeast has been studied by Becker et al (54). The growth of a methionine auxotroph of S. cerevisiae is supported by met-met, met-met-met and met-gly-met-met. Gly-met-gly is inactive as a met source. A lysine auxotroph was isolated which is unable to use lysyl peptides for its lysine requirement. In both the lys and met auxotrophs, peptidases were found which can hydrolyze the peptides (54). A number of met or gly di- or tripeptides with the amino terminus either acetylated or protected by a t-butoxycarbonyl group were found to support growth (55). This is in contrast to acetylated peptides in E. coli which neither support nor inhibit growth (58).

The role of peptidases in peptide transport and metabolism may be summarized as follows. The transport of a peptide is not dependent on its intracellular hydrolysis.

The hydrolysis of the peptides is not dependent on transport. This is supported by the isolation of mutants which can transport peptides (as demonstrated by growth supplementation or by incorporation of labeled peptides) but not hydrolyze them, as well as mutants (or cell-free extracts) which hydrolyze peptides but do not transport them. Care must be taken when testing for transport via growth supplementation, however, since lack of growth may be due to lack of peptidase activity. It has also been demonstated that some peptides (either di- or tripeptides may be toxic to cells if not hydrolyzed intracellularly to the amino acids (36,45,46,52).

D. Oligopeptide *vs* Dipeptide Transport Systems Multiplicity of Oligopeptide Transport Systems

Oligopeptide (opp) and dipeptide transport systems can be distinguished by 1) competition studies between di- and tripeptides, 2) the isolation of mutants, especially those that are resistant to triornithine (35) and 3) the structural requirements for the two systems. The first two points will be taken up here. The third (structural requirements) will be considered in the next section.

The competition between di- and tripeptides for the oligopeptide transport systems and the competition between tri- and dipeptides for the dipeptide transport systems has been the subject of numerous investigations. The best characterized systems are those in *E. coli* and, for the most part, only *E. coli* will be considered here.

Growth supplementation has been the most extensively used method for detecting compeptition between di- and tripeptides. If two peptides share the same transport system (either di- or oligopeptide) then supplementation of the auxotrophs by one peptide will be reduced or eliminated by the presence of the second peptide. The following evidence with auxotrophs of gly, lys, leu and thr can, in part, demonstrate this reasoning. Other evidence that some di- or tripeptides can reverse inhibition by toxic peptides (if transported by the same system) is also presented. In glycine or lysine auxotrophs of *E. coli* (to follow growth supplementation by the peptides) it was determined that sensitivity to triornithine can be reversed by gly-gly-gly but not by gly-gly (in the glycine auxotroph (58-60). Gly-gly-norleu inhibition could be overcome with gly-gly-gly but not gly-gly. In the lysine auxotroph, gly-gly-gly but not gly-gly competes with lys-lys-lys (58). The parent strain can use either pro-phe-gly or pro-gly-gly for its glycine requirement, but a triornithine mutant is unable to utilize these two peptides. Both the peptides pro-phe-gly

and pro-gly-gly are able to overcome triornithine sensitivity indicating that triornithine, pro-phe-gly and pro-gly-gly are transported by the same system. Growth on pro-gly or pro-gly-gly can be inhibited by lys-lys and lys-lys-lys respectively, but lys-lys has no effect on growth with pro-gly-gly and lys-lys-lys does not interfere with growth on pro-gly. These results indicate that, for the most part, dipeptides are transported by one system and tripeptides are transported by a second system (58-60).

One other interesting observation was made by Payne and Gilvarg (60). In a gly auxotroph of strain M 123, lys-lys severely inhibits the utilization of gly-gly-gly. Lys-lys also partially offsets triornithine inhibition of this strain. This appears to suggest that dipeptides may also be transported via the oligopeptide system.

The above mentioned TOR mutants of *E. coli* (34, 58-60) as well as the oligopeptide permease mutants of *S. typhimurium* isolated by Ames *et al* (61) are either resistant to growth inhibiting tripeptides or are unable to use some tripeptides as amino acid sources. These mutants of *E. coli* or *S. typhimurium* are, however, able to use dipeptides or are still sensitive to growth inhibiting dipeptides (or dipeptides containing a toxic amino acid). One such example is evident from work by Barak and Gilvarg (62). In a proline auxotroph that was resistant to triornithine pro-phe but not pro-phe-lys could be utilized as a pro souce. The

parent strain (sensitive to triornithine) was inhibited by either lys-p-F-phe or lys-lys-p-F-phe. The sensitivity to lys-p-F-phe is retained in the mutant (triornithine resistant) while lys-lys-p-F-phe does not inhibit growth. The mutant no longer transports the tripeptide, indicating that the lys-lys-p-F-phe is transported via the same system as triornithine. The parent and mutant strains, however, still have the capacity to transport the dipeptide.(62).

Evidence has been presented for a dipeptide transport system which is specific for dipeptides and an oligopeptide system which may transport dipeptides as well as tripeptides or higher order peptides. These results are indicative of two separate and distinct transport systems. It is also evident that dipeptides may compete with tripeptides for entry into cells via the oligopeptide transport system, but that tripeptides do not compete with dipeptides for entry via a dipeptide system.

The multiplicity of dipeptide systems has already been mentioned (23). Naider and Becker (63) studied two different sets of amino acid auxotrophs of _E. coli_ (opp^+) and their TOR mutants (opp^-) and the results demonstrate the existence of multiple oligopeptide permeases. In strain 4212 TOR BN1 (a lys, met, leu auxotroph) lys-lys-lys does not supply the requirement for lysine and the strain is insensitive to val-val-val. Apparently, these two peptides and triornithine are

transported by the same system in this strain. Met-met-met will, however, support growth in the absence of methionine (in the TOR mutant), but unlike the parent strain 4212 BN1, gly-met-gly is a poor source of the amino acid. The TOR mutant also fails to utilize met-met-gly-met as a source of methionine. In the absence of leucine, both 4212 BN1 and the TOR mutant can be supplemented with leu-leu-leu. The peptides, triornithine, val-val-val and gly-met-gly appear to be transported by one system, while met-met-met and leu-leu-leu are transported by a different system in this strain.

The multiplicity of the opp system has been demonstrated in other strains of E. coli with different amino acid requirements. In the leucine, threonine requiring strain used by Barak and Gilvarg (64) leu-leu-leu supports the growth of both TD-V and TD-V-TOR (TD-V is a derivative of E. coli K 12). With leu-leu-leu in the presence of met-met-met, both the parent and the TOR mutant show a lag in growth response thus indicating competition between leu-leu-leu and trimethione for an oligopeptide system (which differs from the one which transports triornithine). In the presence of lys-lys-lys, there is no lag when cells were incubated with leu-leu-leu. This lack of competition between leu-leu-leu and lys-lys-lys indicates the presence of two oligopeptide transport systems. Leu-leu-leu and met-met-met are transported by one system

and triornithine and lys-lys-lys are transported by a different system in this strain (64). Although strain TD-V is a threonine auxotroph, trithreonine suppresses growth in the parent and increasing concentrations of trithreonine caused an increase in the lag phase in mutant. A second strain, TL 3 (a threonine, lysine requiring derivative of *E. coli* W) was also studied and found to be resistant to trithreonine. Strain TL 3 is able to utilize trithreonine and lys-lys-lys for the required amino acids. The TOR mutant of TL 3 however, is unable to utilize lys-lys-lys while it can still be supplemented wih trithreonine. Leu-leu-leu and val-val-val do not compete with trithreonine while met-met-met does. A trithreonine resistant mutant of TD-V (TD-V-TTR) and its TOR mutant were isolated. Both strains exhibit normal growth when pro-phe is used to supply the proline requirement and both are inhibited by val-val (64). The parent TD-V-TTR is able to utilize pro-phe-lys and is sensitive to val-val-val and lys-lys-p-F-phe indicating a normal opp^+ phenotype. The parent strain showed a normal response to trithreonine. Lys-lys-lys is not utilized by TD-V-TOR-TTR (64).

In conclusion, since there are tripeptides which will not effectively compete with other tripeptides for entry into cells, it is quite logical to infer the existence of more than one oligopeptide transport system. These different systems may have different side-chain specificities.

E. Characteristics of the Dipeptide and Oligopeptide Transport Systems.

The requirement for a free N-terminal alpha group or a C-terminal carboxyl group, the stereospecificity and the side chain specificity of the oligopeptide and dipeptide transport systems have been studied in many organisms. The initial studies with E. coli have been summarized (9). In general, the requirements of the di- and tripeptide transport systems are the same; the only difference between the two systems is the requirement of the free COOH terminus by the dipeptide system. This difference is the distinguishing factor between the two systems (5, 8, 9, 65).

1. N-terminal Alpha Amino Group.

Payne (58) had shown that the presence of an unsubstituted alpha amino group is important for transport via both the oligo- and dipeptide transport systems in E. coli. Acetylation of the N-terminus of either lysine or arginine peptides destroys the growth promoting ability of the peptides. N-Acyl-glycine neither inhibits nor supports growth of a glycine auxotroph which can be supported by

sarc-ser. Inhibition of growth by triornithine, lys-lys-lys or gly-gly-norleu can be overcome with N-methyl-gly-gly-gly. However, N-acetyl-, N-succinyl- or N-glutamyl-gly-gly or gly-gly-gly do not reverse the inhibition by either triornithine, lys-lys-lys or gly-gly-norleu. This indicates that the acetylated di- and tripeptides are not transported via the opp system. Naider and Becker (63) showed that the alpha-N-acetylated peptides of met-met-met and met-gly-met do not support the growth of an opp^+ and opp^- strain even though the free peptides could be used.

The work of Gilvarg and Katchalski (66) with alpha amino N-acetylated lysyl and arginyl peptides in E. coli and Payne's work with either N-methylated or N-acetylated di- or tripeptides (58) has demonstrated the requirement for the positively charged alpha amino group. Losick and Gilvarg (67) showed that M 26-26 (a lysine auxotroph) could be supplemented with N-acetyl-lys-lys-lys but the growth is linear rather than logarithmic. Growth is also concentration dependent. Several systematic studies of the requirement for the N-terminus have been carried out in E. coli and reviewed (8, 9).

Payne's work (68) with a homologous series of N-alkylated-gly-gly and -gly-gly-gly showed that M 123 (a glycine auxotroph) could be supplemented with ethyl-, propyl-, and to a lesser extent, with butyl-gly-gly-gly. These peptides are also able to reverse triornithine

inhibition. Ethyl- and propyl-gly-gly can partially inhibit the utilization of gly-gly. The conclusion drawn from this work is that the presence of an acylated N-terminus on either di- or tripeptides renders the peptides inactive due to the loss of the positive charge on the N-terminal group rather than to a steric hindrance. Differences in growth rates observed for different alkyl groups probably reflects differences in rates of uptake and hydrolysis of the peptides which may be influenced by the size of the alkyl group as well as the pK_a of the amino terminus.

The requirement for the positively charged N-terminus has also been found with _S. typhimurium_ (50, 52) and _Lactobacillus_ (10).

Studies with the yeast _S. cerevisiae_, by Becker _et al._ (54) have shown that the transport system for oligopeptides in yeast appears to be different from that in bacteria. Boc-met-met-met, Boc-met-met, Boc-gly-met as well as Boc-gly-met-met and Boc-gly-gly-met support the growth of a met auxotroph. The acetylated dipeptides met-met and gly-met as well as the acetylated tripeptides met-met-met, and met-gly-met are also utilized by the yeast. These results clearly show that the bacterial and yeast systems differ in the requirement for a free amino group.

2. C-Terminal COOH Group.

The most striking difference between the di- and oligopeptide transport systems is the requirement for a free carboxyl group by the dipeptide transport system. Di- and tripeptides with either esterified C-terminal carboxyl groups or C-terminal amide groups have been used to demonstrate this. Amino acid homologues (homoserine for serine, etc.) or substituted amino acids have also been used (61, 69). Transport of these peptides is assumed if the peptides can be used for amino acid requirements or if the C-terminal substituted peptide (homologue, substitued amino acid, amide, ester etc.) competes with a growth supplementing pepide.

Kessel and Lubin (32) demonstrated that gly-gly-OMe is a poor competitive inhibitor of gly-gly transport in *E. coli*. Payne and Gilvarg (5) used leu-gly-amide and gly-leu-amide on a glycine auxotroph and found the amides to be poorer at growth supplementation than the free dipeptides. Lysylcadavarine (lys-lys lacking the COOH terminus) is utilized by a lysine auxotroph (58) but, by using oligopeptide transport mutants, the authors showed that the lysylcadavarine is transported via the oligopeptide system and not the dipeptide system.

Fickel and Gilvarg (69) used lys-lys-homoserine phosphate to fulfill the threonine requirement of *E. coli*

strain M 145 in the absence of alkaline phosphatase. Homoserine phosphate itself does not support growth. Lys-lys-homoserine phosphate was also tested in another threonine requiring strain of *E. coli* (TL3) and its TOR mutant. The peptide supports growth in the parent but not the mutant strain indicating transport via the oligopeptide transport system.

Hirshfield and Price also studied dipeptide and dipeptide amides as supplements (70). It was found that peptides with the structure leu-X-amide (X is any amino acid) are better leucine sources than are peptides with the strucure X-leu-amide (X is not leucine) presumably because leucine is not quickly liberated from the amide bond. This is also the case for X-ileu-amide and X-phe-amide if ileu and phe auxotrophs are used. The transport of the dipeptides leu-leu-amide, leu-ala-amide as well as leu-leu-leu and leu-gly-gly is inhibited by val-gly-gly. These results are indicative of a common transport system for these five peptides. The transport system involved, must be the oligopeptide system rather than the dipeptide since val-gly-gly inhibits uptake of the amides and leu-leu-leu.

In *Salmonella*, few systematic studies have been carried out. One of the few reports of dipeptide transport in *S. typhimurium* by Ames *et al*. (61) makes use of histidine requiring strains and the peptide gly-gly-histidinol

phosphate (gly-gly-HOLP) to supplement growth. Mutants of these strains were tested for growth on gly-gly-HOLP. Since the parent strain can use both gly-his-gly and gly-gly-HOLP as sources of histidine and the mutants could not, the gly-gly-HOLP must be transported via the opp system.

The oligopeptide transport system, unlike the dipeptide transport system, has no specific requirement for the free COOH. Dipeptide amides (70) or substituted amino acids (61, 69) at the C terminus are transported via the opp system in either _E. coli_ or _S. typhimurium_. There is also no absolute requirement for a particular number of amino acids. In a methionine requiring strain of _E. coli_, Becker and Naider (71) found growth on met-met-met-OMe, and tetra peptides with methionine or their methyl esters. The experiments with the lys-lys-cadavarine (60) as well as the inhibition of cells by triornithine-amide support the absence of a need for a free COOH. Payne's work (72) with gly-gly-beta-ala (which can be considered to be gly-gly-asp devoid of the C -terminal COOH) has also demonstrated that the COOH is not required for transport via the opp system. It is because of this nonessential nature of the COOH that has allowed the opp system to be used for the illicit transport of otherwise impermeant substances (61,69).

The lack of specificity for the COOH group has also been demonstrated in _Lactobacilli_ by growth supplementation and competition (73, 74).

In yeast, the support of growth by the methyl esters of tri-, tetra- and penta-methionine (55) is not as good as that by the free peptides. After long lag phases, growth rates are similar for free and esterified peptides. However, no competition studies were done, nor was the possibility of extracellular hydrolysis of the esters ruled out (55).

3. Stereospecificity.

The question of the stereospecificty of the peptide transport systems has been investigated using either radioactive peptides or competition studies. Since most microorganisms are unable to convert D to L amino acids, growth tests have been of limited value.

In the gly-gly peptidase mutant of *E. coli* isolated by Kessel and Lubin (32) [^{14}C]gly-gly transport is not inhibited by dipeptides containing D residues. Levine and Simmonds (75) showed that leu-gly transport is unaffected by D-leu-gly. This lack of competition between the dipeptides indicates that these dipeptides containing D-amino acids do not appear to be recognized by the transport system.

The inability of DDD-ala-ala-ala to overcome inhibition by triornithine or val-val-val in *E. coli* was shown by Payne (65). The D isomer of ala-ala-ala also fails to inhibit gly-gly-gly uptake in a glycine auxotroph. It had been

postulated earlier (5) that the presence of the D isomer at the COOH terminus should not prohibit transport and some evidence has been presented to support this idea (71). LLL and LLD met-met-met peptides support growth of a methionine auxotroph. Most of the other tripeptides (LDL, DLL, DDD, DDL, DLD and LDD) are not hydrolyzed, and no conclusions were drawn as to transport. A mutant capable of growth on DLL met-met-met was isolated and it is determined that this tripeptide was transported via the opp system (71).

In *Lactobacilli*, there appears a specificity similar to *E. coli*. LLD val-val-val inhibits *Pediococcus cerevisiae* and the LLL isomer of ala-ala-ala will reverse inhibition by the LLD val-val-val. The tripeptide is actively transported (as measured by radioactive tripeptide) but not readily hydrolyzed (73, 74).

While it appears that the oligopeptide transport system is specific for the L configuration, substitution of a D isomer in some (but not all) positions on the peptide may allow transport.

4. Side Chain Specificity

In general, no evidence has been presented which would demonstrate that the oligopeptide transport system has any specificity with regard to amino acid side chains. Results with competition studies or with mutants have shown that,

for the most part, there appears to be a wide diversity of peptides which can be transported via the dipeptide or oligopeptide systems. Failure, in some instances, to show competition may, in part, be due to differing affinities of the transport systems. The use of opp mutants may be more clear cut. The simultaneous loss of ability to transport two or more peptides has been offered as evidence that there is a common transport system.

Levine and Simmonds (75) demonstrated the common transport of leu-gly and gly-leu. Inhibition of radioactive gly-gly transport by dissimilar peptides was studied (32). Payne's work (34, 42, 59) with lys-lys, gly-val, and gly-sarc shows competition with the peptides pro-gly, gly-gly and gly-sarc and gly-pro. These competition studies have helped to substantiate the lack of specificity for the dipeptide system in E. coli.

The oligopeptide transport system(s) have been shown to accept such nonprotein amino acids as ornithine (34, 35, 62), norleucine and norvaline (58) as well as acetylated lysine (65, 66). Mutants resistant to triornithine (opp^-) also show a resistance to val-val-val and dileucyl-p-F-phe (62). An opp^- mutant of S. typhimurium (61) was isolated from a histidine auxotroph and was found resistant to lys-lys-lys, norleu-gly-gly as well as triornithine. The TOR mutant also failed to grow on gly-his-gly which the parent could utilize as a source of

his. The common transport of these diverse peptides in the parent and the loss of transport in the TOR mutant indicates that the opp system does not have (or has very little) ability to distinguish between tripeptides (61).

Since there is no apparent specificity for side chains, it has been possible to use the oligopeptide transport system to transport nonprotein amino acids. Those compounds not normally tranported might gain access to the cell if attached to an oligopeptide. The proposed name for this type of illicit transport is "smugglin" (7).

Homoserine phosphate is not transported by E. coli but lys-lys-homoserine phosphate is transported via the opp system (69). Histidinol phosphate is not transported by S. typhimurium but gly-gly-histidinol phosphate can be used for growth by a histidine mutant (61).

In E. coli, N-(phosphonoacetyl)-L-ornithine (PALO) will inhibit growth in liquid medium or in disk sensitivity tests on plates but only if transported. By coupling the PALO to gly-gly, Penninckx and Gigot (76) were able to demonstrate inhibition. TOR mutants were insensitive to gly-gly-PALO, thus indicative of transport via the opp system.

Two peptide antibiotics, L-phosphonothricyl-alanyl-alanine and L-(N^5-phosphono)methionyl-S-sulfoximinyl-alanyl-alanine were tested by Diddens et al. (77) and found to be transported into E. coli. Both peptide antibiotics have been shown to be more inhibitory than the corresponding

amino acid (either phosphonothricin or the sulfoximinine) (77). Since competitive inhibition is found between either of the above tripeptide antibiotics and tri- alanine, serine, glycine and lysine, these peptides are also transported via an oligopeptide system.

The naturally occuring phosphonic acid, aminoethyl phosphonic acid (78), can also be transported into cells (79), presumably via the oligopeptide system when coupled to amino acids. The L-alanyl-L-1-amino-ethylphosphonic acid was shown to be a potent inhibitor of several gram-negative or gram-positive organisms (79).

In our laboratory, we have used phosphonic acid analogs of naturally occuring phosphates to study intermediary metabolism in E. coli. In these studies, we have taken advantage of the oligopeptide transport system and used this system to transport three phosphonic acid analogs, two of which do not appear to be transported (or transported very poorly). The phosphonate analogs of glycerol-3-phosphate, dihydroxyacetone phosphate and phosphomevalonate were coupled to the tripeptides indicated, added to cultures of E. coli as described and growth inhibition was monitored. The metabolic effects of 3,4-dihydroxybutyl-1-phosphonate (G3P analog) have been reviewed in Chapter 1. No prior work on the metabolic effects of the 4-hydroxy-3-oxobutyl-1-phosphonate had been done although it had been reported that the analog does not appear to be transported (80). The

phosphomevalonate analog, 5-carboxy-4-hydroxy-4-methylpentyl-1-phosphonate has been shown to be a potent inhibitor of the 5-phosphomevalonate kinase from rat liver (81). The results of the growth studies with the three analogs (free or peptide coupled) are reported here.

MATERIALS AND METHODS

CHEMICALS: *rac* 3,4-Dihydroxybutyl-1-phosphonate (DHBP) was synthesized as described (82) and unless specified, the racemate was used. 4-Hydroxy-3-oxobutyl-1-phosphonate (DHAPa) was prepared by D. Braksmayer by the method of Goldstein *et al.* (83). The *S*-3,4-dihydroxybutyl-1-phosphonate was synthesized by T. Latham by method of Tang *et al.* (84). The 5-phosphomevalonic acid analog, 5-carboxy-4-hydroxy-4-methylpentyl-1-phosphonate (MevPa) was prepared by M. Sheik by the method of Sarin *et al.* (85).

The tripeptides alanylaspartylalanine (ala-asp-ala), aspartylalanylalanine (asp-ala-ala), aspartylleucylalanine (asp-leu-ala), and alanylserylalanine (ala-ser-ala) as well as the phosphonate-coupled tripeptides (β-O-[*S*]-2-hydroxy-4-dihydroxyphosphinyl-1-butyl ester)-aspartylalanylalanine ((DHBP)asp-ala-ala), alanyl-aspartyl-(β-O-[*S*]-2-hydroxy-4-dihydroxyphosphinyl-1-butyl ester)-alanine (ala-(DHBP)asp-ala), aspartyl(β-O-2-oxo-4-dihydroxy-phosphinyl-1-butyl ester)alanylalanine ((DHAPa)asp-ala-ala), alanylaspartyl(β-O-2-oxo-4-dihydroxyphosphinyl-1-butyl ester)alanine (ala-(DHAPa)asp-ala), aspartyl(β-O-2-hydroxy-4-dihydroxy-1-butyl ester)leucylalanine ((DHBP)asp-leu-ala), as well as alanylseryl (O-[*RS*]-3-hydroxy-3-methy-6-dihydroxyphosphinyl

hexanoate)alanine (ala-(MevPa)ser-ala), were a gift of Dr. R. Engel's laboratory and were synthesized by M. Sheik (86). The synthesis is briefly described as follows. Either the $-NH_2$ or the -COOH terminus was protected by t-BOC (tert butoxy carbonyl) or t-Butyl ester. Protected amino acids were usually coupled using dicyclohexylcarbodiimide in CH_2Cl_2. The third amino acid was then added to either the $-NH_2$ or the COOH terminus after removal of the respective protecting group. For studies involving free peptide (uncoupled to a phosphonate) t-BOC and t-butyl groups were removed by treatment with trifluoroacetic acid (TFA). The butyl ester on the beta -COOH of the aspartate removed by H_2/Pd-C. For coupling of the phosphonates, the alkyl esters of the phosphonic acids were used. The alkyl esters were removed by treatment of the coupled tripeptide with $(CH_3)_3SiCl$ and the $-NH_2$ and -COOH protecting groups removed with TFA.

Whatman SG 81 and 3 MM chromatography papers were obtained from Scientific Products. Casein hydrolysate and tris(hydroxymethyl) amino methane (TRIS base), thiamine, adenine and amino acids were purchased from Sigma, St. Louis, Mo. Triornithine was a generous gift of Dr. J. Becker, University of Tennessee. All other chemicals used were of reagent grade or better.

BACTERIA AND GROWTH CONDITIONS: *E. coli* strain 8, originally isolated by Hayashi *et al*. (87) was a gift of Dr. J. E. Cronan, Jr., University of Illinois, Urbana. The genotype of this strain is HfrH glpR^{c} phoA8 ton22 T2^{r} rel-1-(λ) glpD3. Strains 3-3, 4-9, and 5-6 were derived from strain 8 by nitrosoguanidine treatment (88). These strains are phenotypically negative for G3P and DHBP transport but have not been characterized genetically. Strain 4212 and its triornithine resistant mutant 4212 TorA were a gift of Dr. J. Becker, University of Tennessee. The genotype of the parental 4212 is as follows: thi-1 metE70 lysA22 trypE42 proC32 leu-6 (71). 4212 TorA carries the additional lesion opp^{-}. Strain MV12 and its triornithine resistant mutant MV12 Tor were a gift of Dr. C. Gilvarg, Princeton University. These strains are derived from strain C600 and require leucine, tryptophan, thiamine and threonine for growth.

Strain 8 and its derivatives were usually cultured in the medium of Garen and Levinthal (89) supplemented with 0.6 mM phosphate and 0.5% potassium succinate, pH 7.5 (G and L medium), or in casein hydrolysate medium (CH) which consists of 1.0% casein hydrolysate and 0.5% sodium chloride, pH 7.4.

Strains 4212 and MV12 were occasionally cultured in CH medium supplemented with 1.0 x 10^{-3} mg/mL thiamine for both sets of strains and 1.0 x 10 $^{-2}$ mg/mL adenine for 4212. These two sets of isogenic strains were usually

incubated in Vogel and Bonner minimal medium Medium E (90) with the following supplements added: for 4212: leucine, adenine, proline and tryptophan (0.02 mg/mL), lysine, methionine (0.03 mg/mL) and thiamine (1.0 x 10^{-3} mg/mL); and for MV 12: leucine, tryptophan, threonine (0.05 mg/mL), and thiamine (1.0 x 10^{-3} mg/mL). The amino acids were sterilized by filtration through 0.45 micron Millipore filters and added aseptically to the medium to give the concentrations indicated. Carbon sources, as indicated, are present at a final concentration of 0.5%.

Strains were cultured overnight in minimal medium and diluted into fresh medium in the morning and allowed to grow until the desired cell density was reached. Growth was followed turbidimetrically using a Klett-Summerson colorimeter fitted with a 660 nm filter. All incubations were carried out at 37 °C and 200 rpm.

Phosphonate-coupled or free tripeptides were routinely lypholyzed before weighing, suspended in sterile water and added aseptically to the cultures at 15-20 Klett units (K.U.) unless specified. Growth was followed after addition.

To determine if the asp-leu-ala would serve as a source of leucine for the auxotrophic strains, the cultures were centrifuged, washed once with 37 °C medium lacking leucine and resuspended in the original volume of 37 °C medium minus leucine. Asp-leu-ala or leucine was then added

and the growth followed.

<u>Hydrolysis of the Phosphonate-coupled Tripeptides</u>. To 0.02 mL of a 30 mM (DHBP)asp-ala-ala (0.0006 mmoles) in the bottom of a melting point capillary tube was added 0.02 mL of 2N NaOH. After sealing the capillary tube in a flame, the capillary was placed in a screw-capped test tube containing approximately 1.0 mL of boiling hot water. The test tube was then immersed in a boiling water bath and heated for 30 minutes. S-DHBP (0.0006 mmoles) was similarly treated with NaOH. After heating, the capillary tubes were scored and unsealed. The contents of the tubes (0.04 mL) was then diluted to a volume of 1.0 mL to give a final concenration of 0.6 mM S-DHBP or 0.6 mM (DHBP)asp-ala-ala. Unhydrolyzed (DHBP)asp-ala-ala was also diluted to this final concentration. The solutions were then assayed for DHBP concentration using the rabbit muscle G3P dehydrogenase according to the procedure of Hohorst (91).

The assay mixture contained the following in a final volume of 1.0 mL: hydrazine sulfate, 0.2 moles; glycine, 0.5 moles; EDTA, 3×10^{-3} mmoles; (pH 9.5); NAD, 2.5 mmoles; 2 units of enzyme and 30 nmoles of the unhydrolyzed or hydrolyzed (DHBP)asp-ala-ala or 30 nmoles of S-DHBP. The assay mixure was pre-incubated at 25-30 ^{o}C. The reaction was initiated by the addition of enzyme and the reduction of NAD followed at 340 nm. The reaction was

allowed to proceed to completion (a constant absorbance). The overall change in absorbance was then calculated and the amount of DHBP determined using an extinction coefficient of 6.22×10^{-3} mL/nmole. In order to determine if an inhibitor from the unknown DHBP-tripeptide solutions was present in the reaction mixture, a known amount of L-G3P was added to the reaction and the G3P dependent change in absorbance determined.

RESULTS AND DISCUSSION

The metabolism of glycerol-3-phosphate (G3P) and its incorporation into phospholipids has been the subject of investigation in this laboratory for several years. We have made use of phosphonic acid analogs of G3P and dihydroxyacetone phosphate (DHAP) to study those pathways in *E. coli* which involve G3P. The results of the studies with 3,4-dihydroxybutyl-1-phosphonate (DHBP), the analog of G3P, are summarized in Chapter 1.

One problem which has arisen from our work with the phosphonates is that of transport. It has been demonstrated that DHBP inhibits *E. coli* but only if transported into the cells. DHBP is usually transported via three systems which actively transport G3P (88, 92, 93). The DHAP analog, 4-hydroxy-3-oxobutyl-1-phosphonate, acts as a substrate for the anabolic G3P dehydrogenase and is converted to DHBP *in vitro*, but does not inhibit intact cells, presumably because it is not transported across the membrane (81). Evidence has been presented to support the transport of otherwise impermeant substances via the oligopeptide permease system. Homoserine phosphate (69), histidinol phosphate (61) and N-(phosphonoacetyl)-*L*-ornithine (76) as well as phosphonothricin and *L*-(N^5-phosphono)methionine-S-sulfoximine (77) are substances which are not usually

transported in either *E. coli* or *S. typhimurium*. However, these otherwise impermeant substances can be transported via the oligopeptide system when coupled to a tripeptide since the the oligopeptide transport system does not have an apparent specificity for either the amino acid side chains or for the terminal COOH group (1, 3, 5, 7-9). For this reason, tripeptides containing the phosphonates that we have studied previously were synthesized and their effect on cell growth investigated. Other peptides containing aminoethylphosphonic acid have been studied (79) and it appears that these are also transported via the opp system. It was hoped that, by use of the tripeptide vector, otherwise impermeant phosphonates might gain access to the cells and exert an inhibitory effect. Since other peptides containing phosphonic acids had been studied (79), we felt that the presence of a phosphonic acid on an amino acid side chain should not preclude transport of the tripeptide.

An earlier attempt was made to determine whether the DHAP analog linked to triglycine had biological activity (94). Triglycine was chosen since its synthesis presented the fewest complications with regard to side chain reactions and stereospecificity. The DHAP analog was chosen since it was known to be a substrate for the anabolic G3P dehydrogenase (81). If the DHAP analog was transported via the oligopeptide transport system, it would be bacteriostatic to cells upon conversion to DHBP *in vivo*.

The DHAP analog was esterified to the terminal carboxyl group of gly-gly-gly, and the phosphonate-coupled tripeptide tested for its effect on bacterial growth. Unfortunately, the initial studies with this system proved unsatisfactory (94) and a second peptide was synthesized using aspartic acid and alanine. DHBP was coupled to the tripeptide through the beta-COOH of the aspaartic acid residue (86).

The phosphonic acid analog of phosphomevalonate has also been synthesized (85). The phosphomevalonate is a cholesterol precursor in animal systems (95). The analog was to be used as a possible cholesterol inhibitor and it has been demonstrated that the analog inhibits the 5-phosphomevalonate kinase from rat liver (81). The analog, however, is not transported into intact liver. The tripeptide, ala-ser-ala, was synthesized as a possible vector to be used for transport of the phosphomevalonate analog (MevPa). The phosphomevalonate analog is not transported by E. coli or transported very poorly.

The abbreviations used and the structures for the phosphonates, tripeptides and phosphonate-coupled tripeptides are given in Table 1.

Prior investigations with DHBP have shown that it exerts a bacteriostatic effect in E. coli strain 8. Our initial studies with DHBP linked to asp-ala-ala ((DHBP)asp-ala-ala) were done with strain 8 in the hope that the (DHBP)asp-ala-ala might prove inhibitory towards this

TABLE 1

Abbreviations and Structures of Phosphonic Acid Analogs, Tripeptides and Phosphonate-linked Tripeptides.

The following abbreviations are used in the table and throughout the text: L-G3P (sn-glycerol-3-phosphate), DHBP (3,4-dihydroxy-butyl-1-phosphonate, in the S configuration when coupled to tripeptides); DHAP (dihydroxyacetone phosphate); DHAPa (4-hydroxy-3-oxobutyl-1-phosphonate, DHAP analog); MevP (phosphomevalonic acid); MevPa (5-carboxy-4-hydroxy-4-methylpentyl-1-phosphonate, the phosphomevalonic acid analog); asp-ala-ala (aspartylalanylalanine); ala-asp-ala (alanylaspartylalanine); asp-leu-ala (aspartylleucylalanine); ala-ser-ala (alanylserylalanine). The peptides are of the L configuration. The DHBP and MevPa, when used in the uncoupled form, are as the racemic mixtures, unless specified.

TABLE I

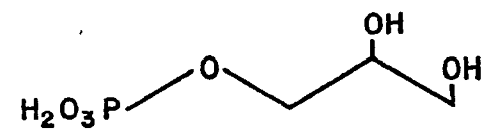
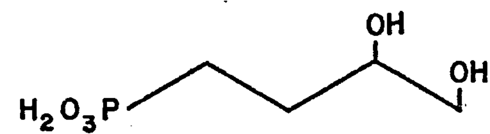
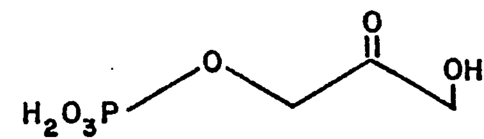
ABBREVIATIONS	STRUCTURES
<u>L</u>-G3P	
<u>DL</u>-DHBP	
DHAP	

TABLE I (cont)

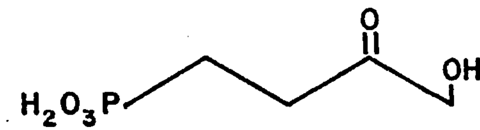
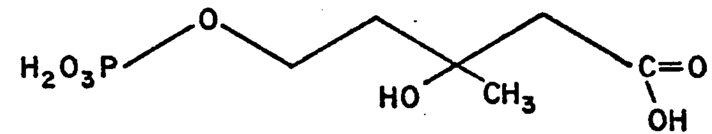
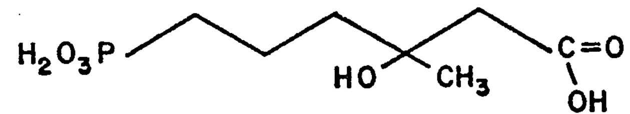
ABBREVIATIONS	STRUCTURES
DHAPa	
MevP	
MevPa	

TABLE I (cont)

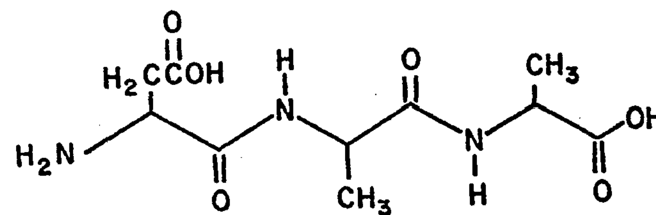
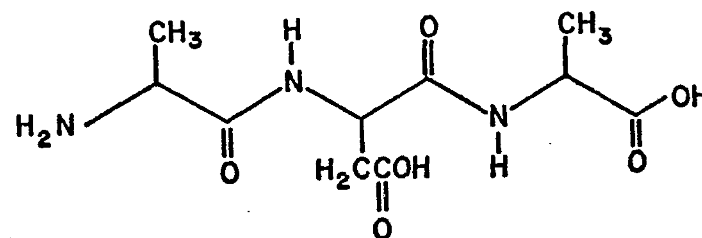
ABBREVIATIONS	STRUCTURES
asp-ala-ala	
ala-asp-ala	

TABLE I (cont)

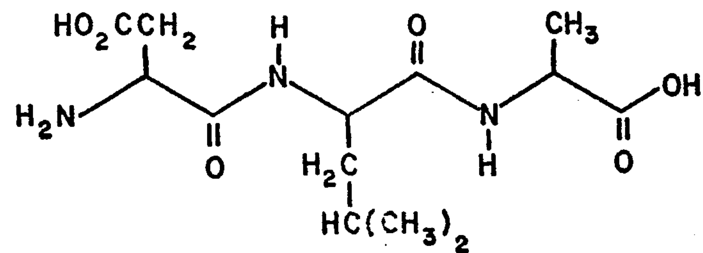
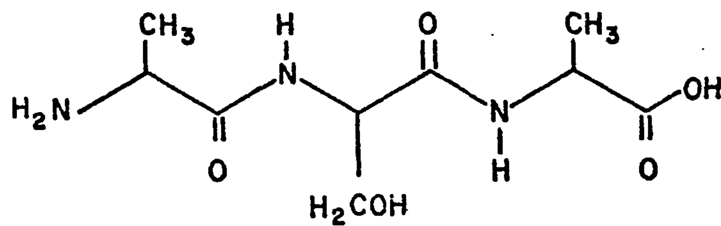
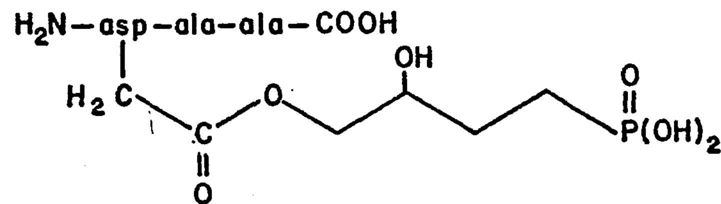
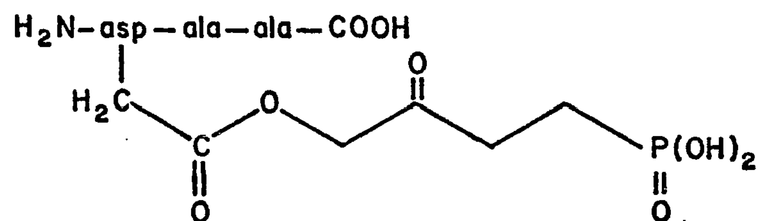
ABBREVIATIONS	STRUCTURES
asp–leu–ala	
ala–ser–ala	
(DHBP)asp–ala–ala	

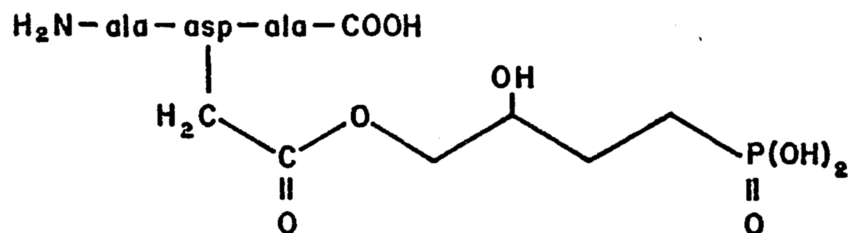
TABLE I (cont)

ABBREVIATIONS | **STRUCTURES**

(DHAPa)asp–ala–ala



ala–(DHBP)asp–ala



ala–(DHAPa)asp–ala

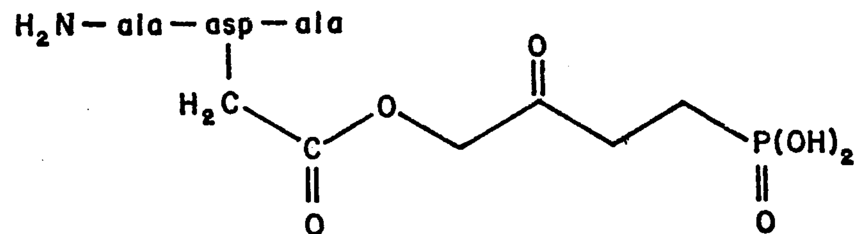
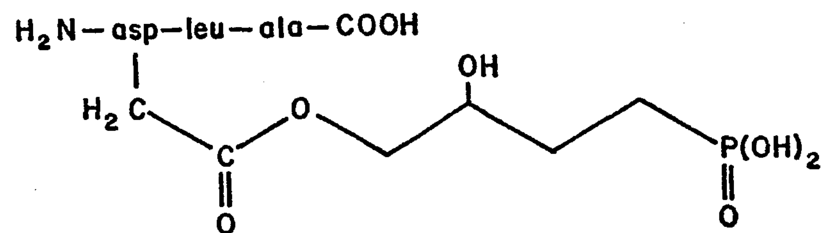
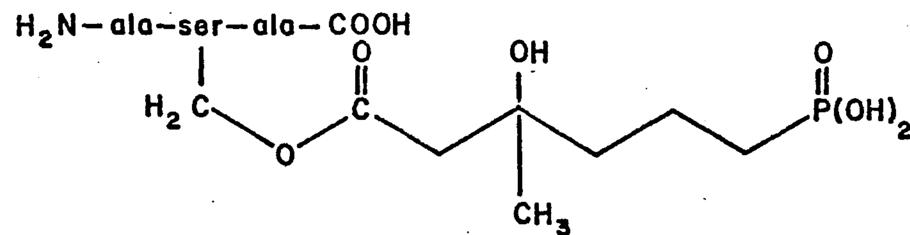


TABLE I (cont)

ABBREVIATIONS	STRUCTURES
(DHBP) asp — leu — ala	
ala — (Mev Pa)ser — ala	

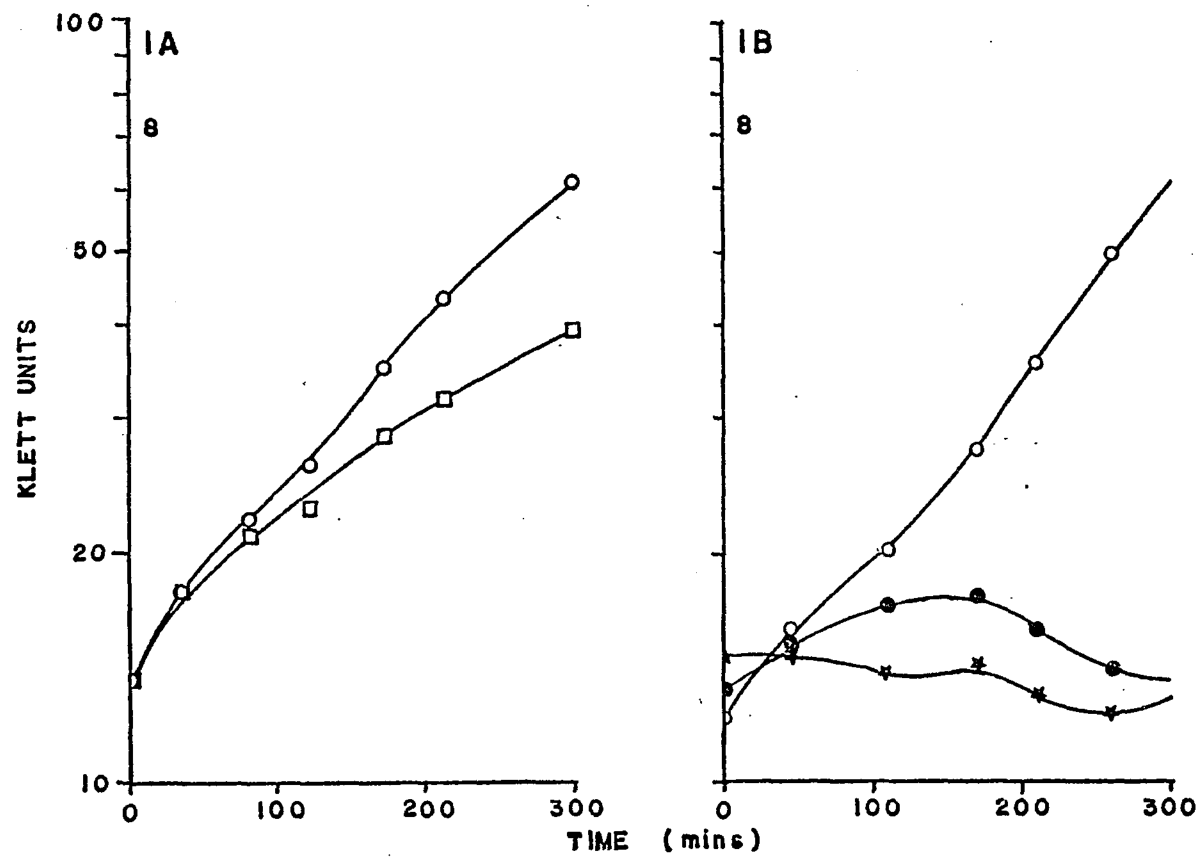
strain. The results of these studies are shown in Figure 1. At a concentration of 0.3 mM, the (DHBP)asp-ala-ala begins to inhibit the growth of strain 8 approximately 80 minutes after addition. The rate of growth is also slowed. At a concentration of 2.5 mM (DHBP)asp-ala-ala, total inhibition of growth occurs upon addition. The free tripeptide, asp-ala-ala, itself does not inhibit strain 8, even at a concentration of 2.5 mM.

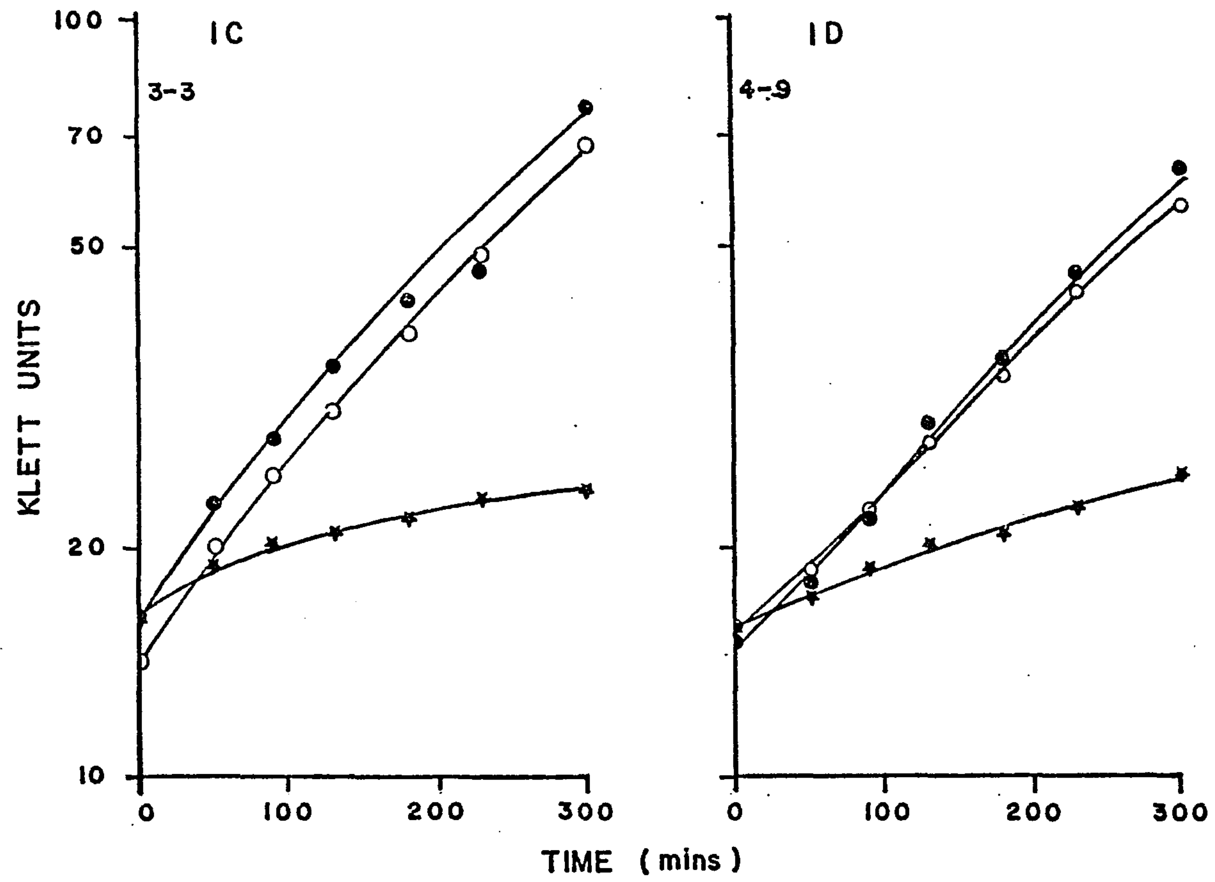
Strain 8 is inhibited by DHBP itself and it was therefore necessary to check the ability of the tripeptide to act as a carrier system for the DHBP. Since DHBP can be transported into strain 8 via the glpT system, the inhibition of strain 8 by (DHBP)asp-ala-ala might result if the phosphonate-tripeptide were hydrolyzed extracellularly and the DHBP transported via the glpT system. In order to eliminate this possiblilty, three independently isolated mutants of strain 8 (phenotypically glpT$^-$) were tested for growth on (DHBP)asp-ala-ala. Strains 3-3, 4-9 and 5-6, cultured in G and L medium with succinate are not inhibited by DHBP alone, even at a concentration of 2.5 mM (Figure 1 C-E). Under these conditions, strain 8 is completely inhibited. It is evident from Figure 1 (C-E) that that 2.5 mM (DHBP)asp-ala-ala inhibits the growth of the three mutant strains. Under these conditions, strain 5-6 appears to be less sensitive to the DHBP-tripeptide than is either strain 3-3 or strain 4-9. Inhibition of these strains by

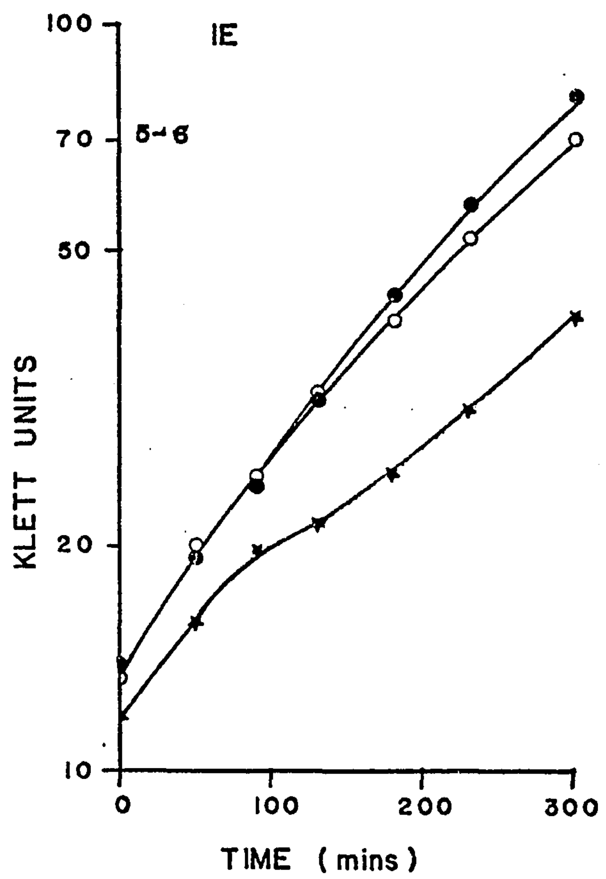
FIGURE 1

The Effect of (DHBP)asp-ala-ala on Growth.

Strains were cultured in G and L medium supplemented with succinate as described in the text. When the cultures reached 15-20 K.U., either 2.5 mM DHBP, 0.3 mM (DHBP)asp-ala-ala or 2.5 mM (DHBP)asp-ala-ala were added and the growth followed. A, B, strain 8; C, 3-3; D, 4-9; E, 5-6. Untreated cultures, (○-○-○); 0.3 mM (DHBP)asp-ala-ala, (□-□-□); 2.5 mM (DHBP)asp-ala-ala, (★-★-★); 2.5 mM DHBP, (●-●-●).


1A
1B
8
8
100
50
20
10
KLETT UNITS
0
100
200
300
0
100
200
300
TIME (mins)


1C
3-3
1D
4-9
100
70
50
20
10
KLETT UNITS
0
100
200
300
TIME (mins)


IE
5-6
100
70
50
20
10
KLETT UNITS
0
100
200
300
TIME (mins)

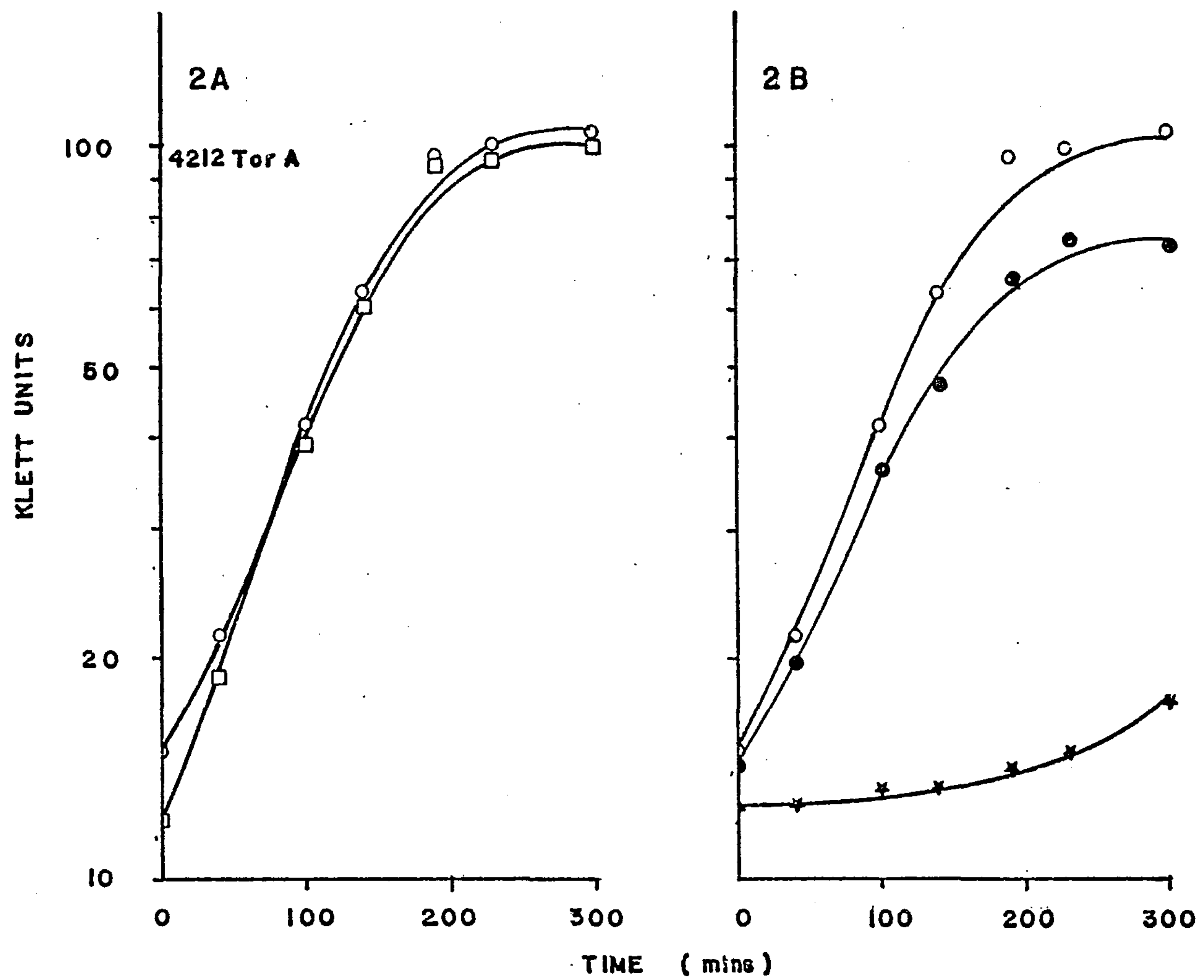
(DHBP)asp-ala-ala does not appear to be by a cooperative effort between DHBP and free tripeptide since DHBP plus asp-ala-ala (2.5 mM each) does not inhibit the growth of the glpT$^-$ strains and in fact, appears to stimulate growth. This stimulation (albeit slight) may be due to the gluconeogenic character of both aspartic acid and alanine or to the increase of intracellular nitrogen which is furnished by the peptide.

The results of the studies with strain 8 and its glpT$^-$ mutants demonstrate that the (DHBP)asp-ala-ala must be transported intact into the cells. However, the inhibition of these strains by the (DHBP)asp-ala-ala, does not prove that the coupled tripeptide is transported by the oligopeptide transport system. In order to show transport of the (DHBP)asp-ala-ala via the opp system, a triornithine resistant mutant (opp$^-$), strain 4212 TorA, was tested for its sensitivity to the (DHBP)asp-ala-ala. It was thought that, since this strain lacked the ability to transport some peptides used to satisfy the amino acid requirement of the parent it might be less sensitive to the DHBP tripeptide. In this strain cultured in Medium E supplemented with glucose, 0.3 mM (DHBP)asp-ala-ala does not inhibit growth (Figure 2 A). At this concentration, the growth rate of strain 8 is slowed, but not totally inhibited (Figure 1 A). Unlike strain 8 which is immediately inhibited by 2.5 mM DHBP (Figure 1 B), strain 4212 TorA

FIGURE 2

The Effect of (DHBP)asp-ala-ala on The Growth of Strain 4212 TorA.

Strain 4212 TorA was cultured in Medium E supplemented with glucose and the required amino acids as described in the text. When the cultures reached 15-20 K.U., DHBP or (DHBP)asp-ala-ala was then added and the growth followed. A, Untreated, (O-O-O); 0.3 mM (DHBP)asp-ala-ala. B, Untreated, (O-O-O); 2.5 mM DHBP, (●-●-●); 2.5 mM (DHBP)asp-ala-ala, (★-★-★).



2A
2B
4212 Tor A
KLETT UNITS
100
50
20
10
0
100
200
300
TIME (mins)

continues to divide for several hours after the addition of 2.5 mM DHBP (Figure 2 B). However when 2.5 mM (DHBP)asp-ala-ala is added to strain 4212 TorA, growth stasis is immediate.

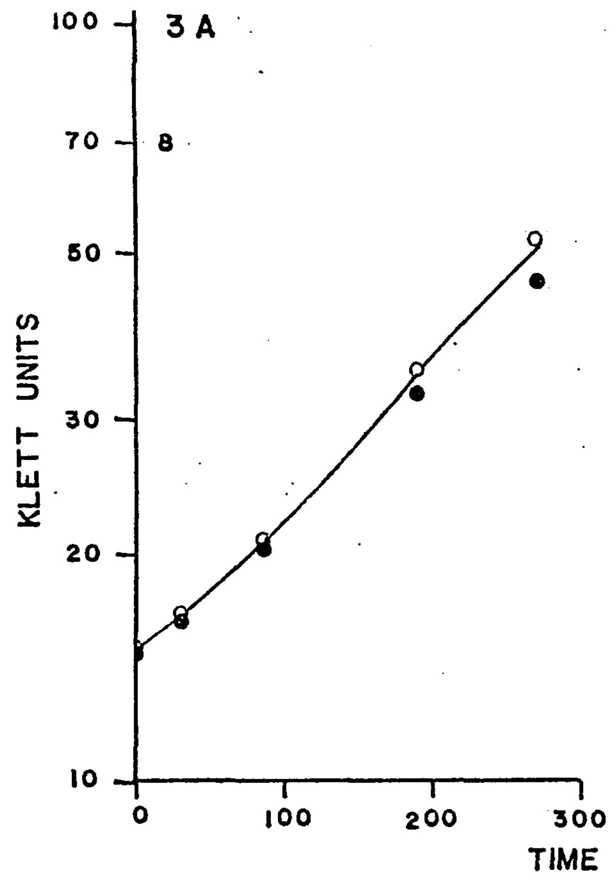
The second tripeptide studied, ala-asp-ala, was synthesized and coupled to DHBP and its effect on growth investigated. The free tripeptide ala-asp-ala does not appear to affect the growth of strains 8 and 5-6 (Figure 3 A, B). At a concentration of 0.3 mM, DHBP itself inhibits the growth of strain 8 but not strain 5-6 when cultured in G and L medium with succinate (Figure 4 A, B). This is not surprising since strain 5-6 lacks the glpT system and cannot transport DHBP. The tripeptide ala-(DHBP)asp-ala, at a concentration of 0.3 mM, however, inhibits neither strain 8 nor strain 5-6. Higher concentrations of the ala-(DHBP)asp-ala were then tested in these strains (Figure 5 A-D). At 1.5 or 1.8 mM, ala-(DHBP)asp-ala inhibits both strain 8 and 5-6 when cultured in G and L medium with succinate. Strain 8 is inhibited by either 1.5 or 1.8 mM DHBP (Figure 5 A, C) while DHBP alone has no effect on 5-6 at these concentrations (Figure 5 B, D). Under these conditions, strain 8 appears to be more sensitive to the 1.5 mM ala-(DHBP)asp-ala than strain 5-6 and both strains are completely inhibited by 1.8 mM ala-(DHBP)asp-ala.

Previous work by Cheng _et al._ (78) had shown that the phosphonic acid analog of DHAP acts as a substrate for the

FIGURE 3

Effect of Ala-asp-ala on Growth.

Strain 8 (A) and strain 5-6 (B) were incubated in G and L minimal medium supplemented with succinate as the carbon source. When the cultures reached 15-20 K.U., 2.5 mM ala-asp-ala (●-●-●) was added and the growth followed. No addition (○-○-○).


3 A
8
KLETT UNITS
100
70
50
30
20
10
0
100
200
300

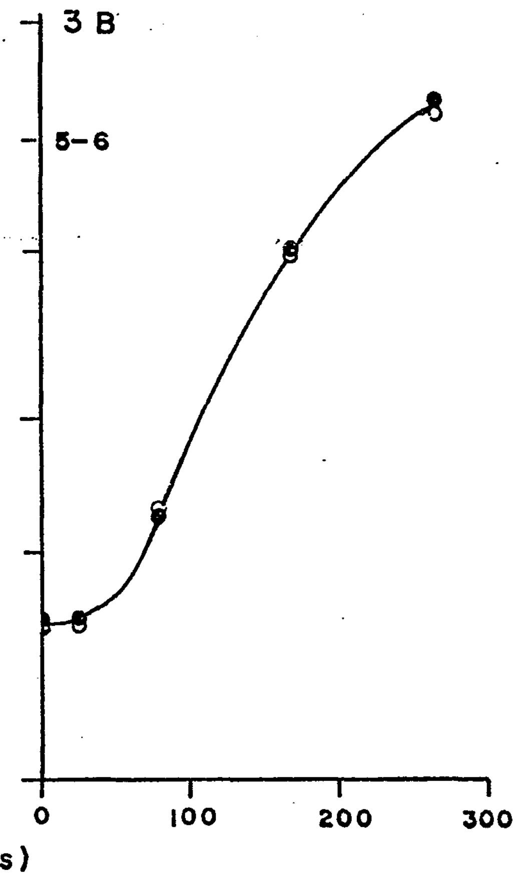
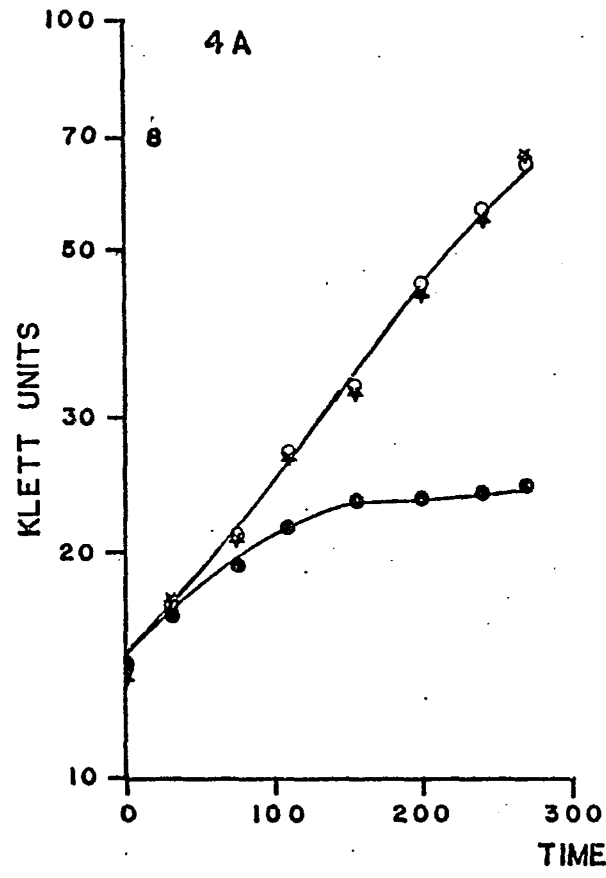

3 B
5-6
0
100
200
300
TIME (mins)

FIGURE 4

Effect of Ala-(DHBP)asp-ala on the Growth of
Strains 8 and 5-6.

Strain 8 (A) and strain 5-6 (B) were incubated in G and L medium supplemented with succinate as described in the text. Cultures were treated with either 0.3 mM ala-(DHBP)asp-ala (✱-✱-✱) or 0.3 mM DHBP (●-●-●) as described and the growth followed. No addition, (○-○-○).


4A
8
KLETT UNITS
100
70
50
30
20
10
0
100
200
300

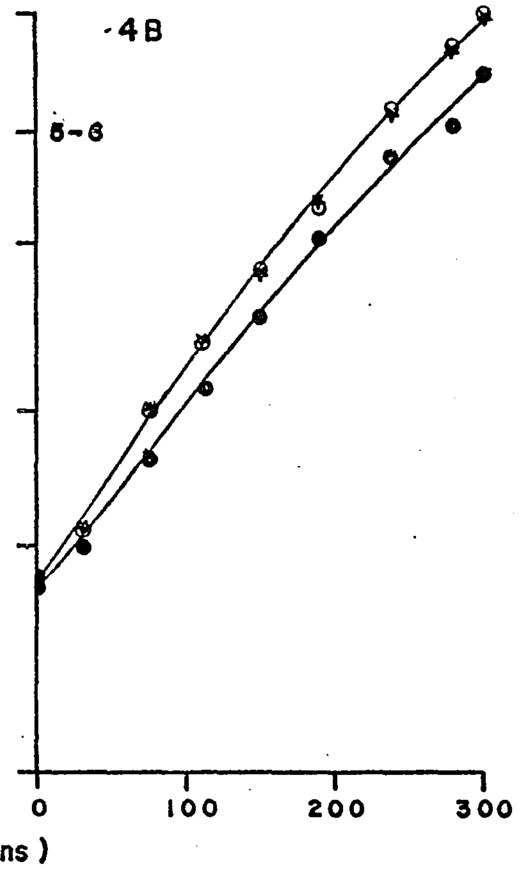
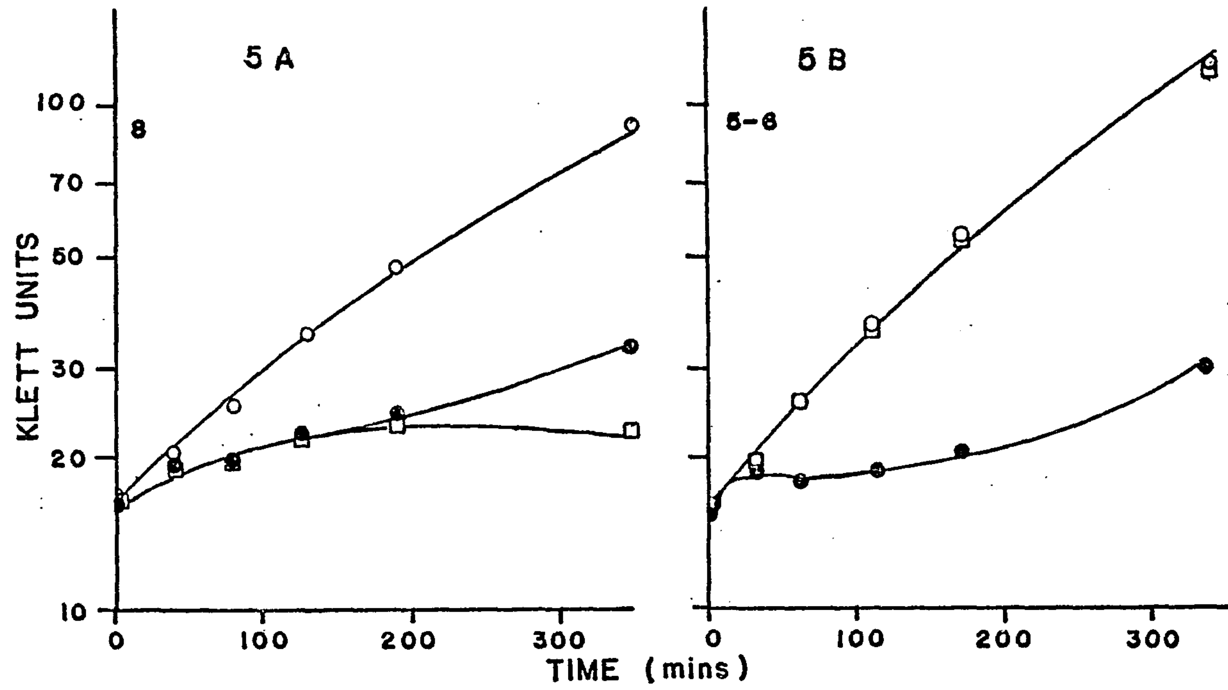

4B
5-6
0
100
200
300
TIME (mins)

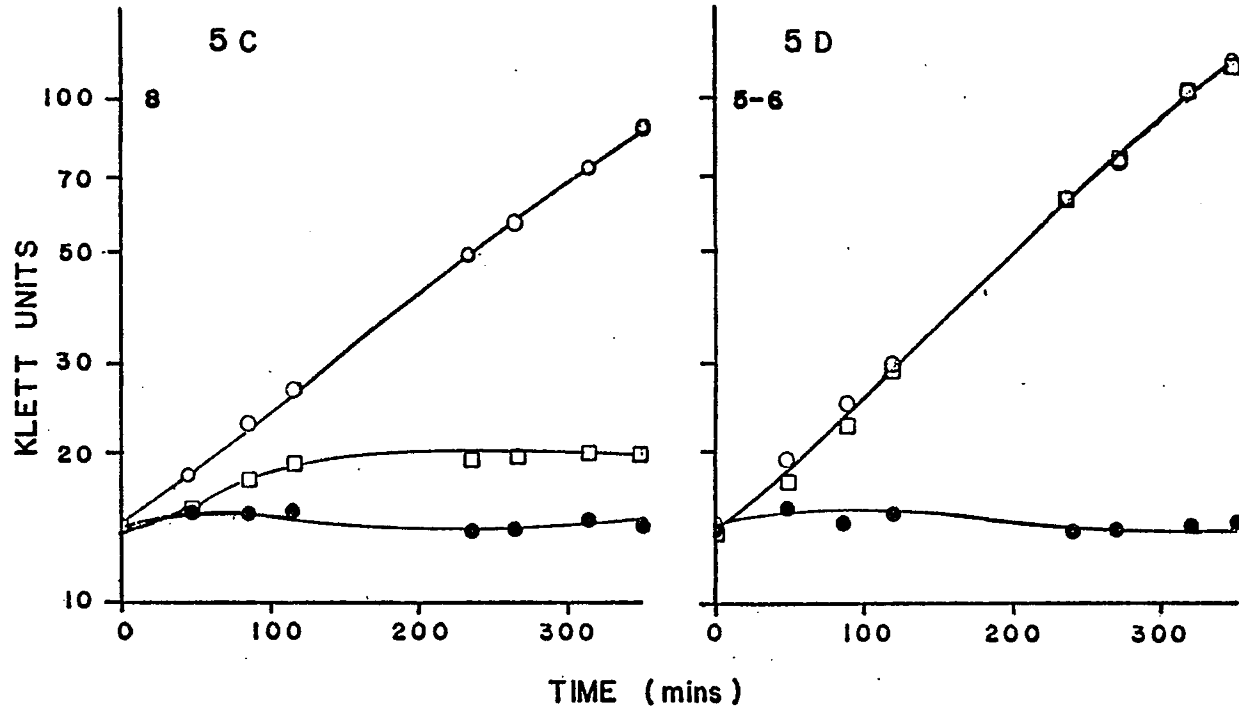
FIGURE 5

Effect of Higher Concentrations of Ala-(DHBP)asp-ala on the Growth of Strains 8 and 5-6.

Strains 8 (A, C) and 5-6 (B, D) were cultured in G and L minimal medium supplemented with succinate as described. Cultures were treated with either DHBP or ala-(DHBP)asp-ala at a concentration of 1.5 mM (A, B) or 1.8 mM (C, D). Growth was followed after addition. Figures A, B, untreated , (○-○-○); 1.5 mM DHBP, (□-□-□); 1.5 mM ala-(DHBP)asp-ala, (●-●-●). Figures C, D; No addition, (○-○-○); 1.8 mM DHBP, (□-□-□); 1.8 mM ala-(DHBP)asp-ala, (●-●-●).



5 A
5 B
8
5-6
KLETT UNITS
100
70
50
30
20
10
0
100
200
300
TIME (mins)


5 C
8
5 D
5-6
KLETT UNITS
100
70
50
30
20
10
0
100
200
300
TIME (mins)

anabolic G3P dehydrogenase *in vitro*. However, the analog does not appear to be transported into *E. coli* (78). We therefore, decided to determine if the analog, coupled to a tripeptide would exert a bacteriostatic effect. The analog (DHAPa) was coupled to the two tripeptides previously mentioned and its effects studied in strain 8 and the glpT$^-$ mutants. At a concentration of 2.5 mM, (DHAPa)asp-ala-ala exerts a very slight inhibition in strain 8 when cultured in CH medium (Figure 6 A). At 2.5 mM DHBP there is a marked inhibition of strain 8 but not to the extent of that seen in G and L medium supplemented with succinate. Strain 5-6 cultured in CH medium, however, is completely resistant to both 2.5 mM DHBP (as expected) and 2.5 mM (DHAPa)asp-ala-ala (Figure 6 B).

When incubated in G and L medium supplemented with succinate, strain 8 is quite sensitive to 2.5 mM (DHAPa)asp-ala-ala (Figure 7 A). At this same concentration, DHAP analog alone causes no inhibition. However, strains 3-3, 4-9 and 5-6, under the same growth conditions as strain 8, are completely insensitive to the (DHAPa)asp-ala-ala (Figure 7 B-D). Strain 8 is not as sensitive to the DHAP analog linked to asp-ala-ala when cultured in CH medium as it is in G and L medium with succinate. Strain 8 is more sensitive to DHBP itself when cultured in G and L medium with succinate and since the DHAP analog would be converted to DHBP this result with the

FIGURE 6

Effect of (DHAPa)asp-ala-ala on the Growth of Strains 8 and 5-6.

Strain 8 (A) or 5-6 (B) were cultured in CH medium as described. When the cultures reached 15-20 K.U., either 2.5 mM DHBP (□-□-□) or 2.5 mM (DHAPa)asp-ala-ala (△-△-△) was then added and the growth followed. Untreated, (○-○-○).

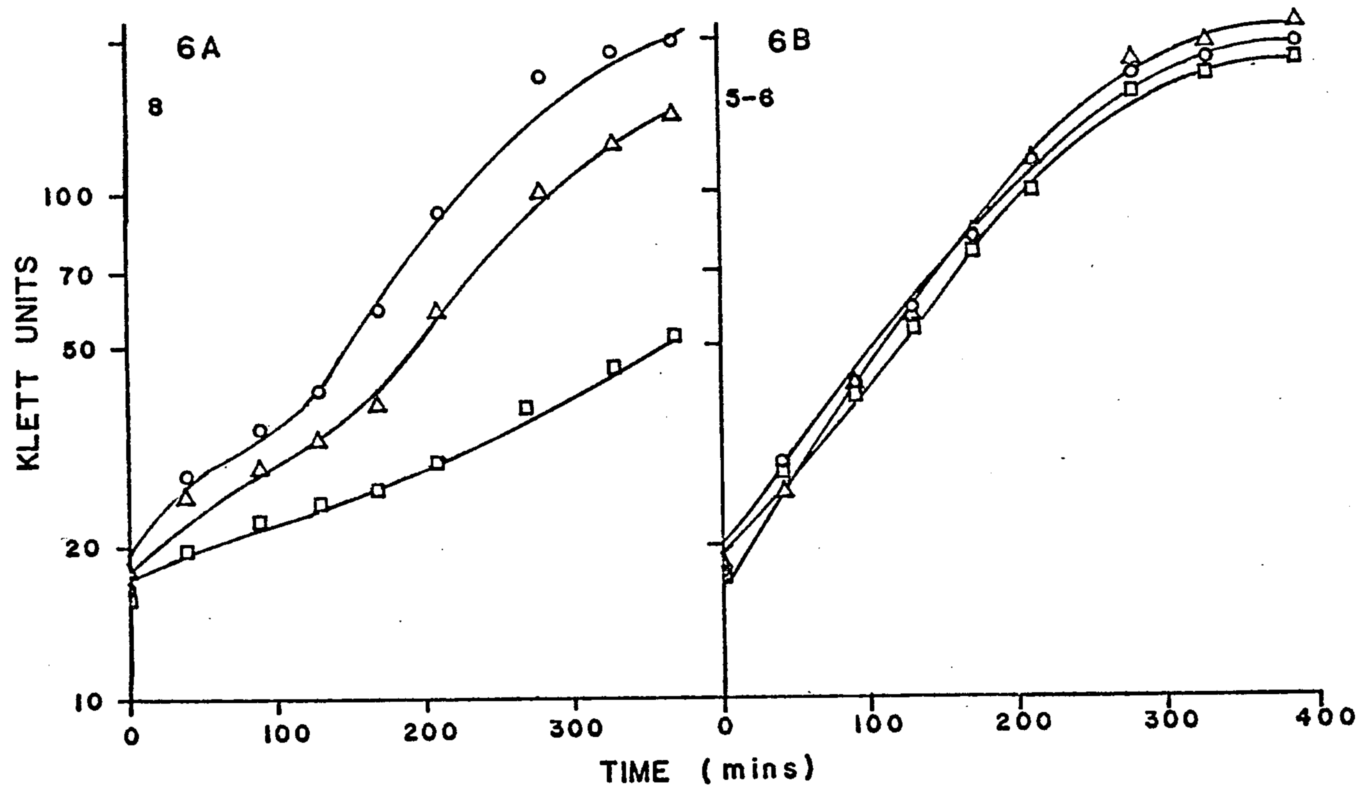
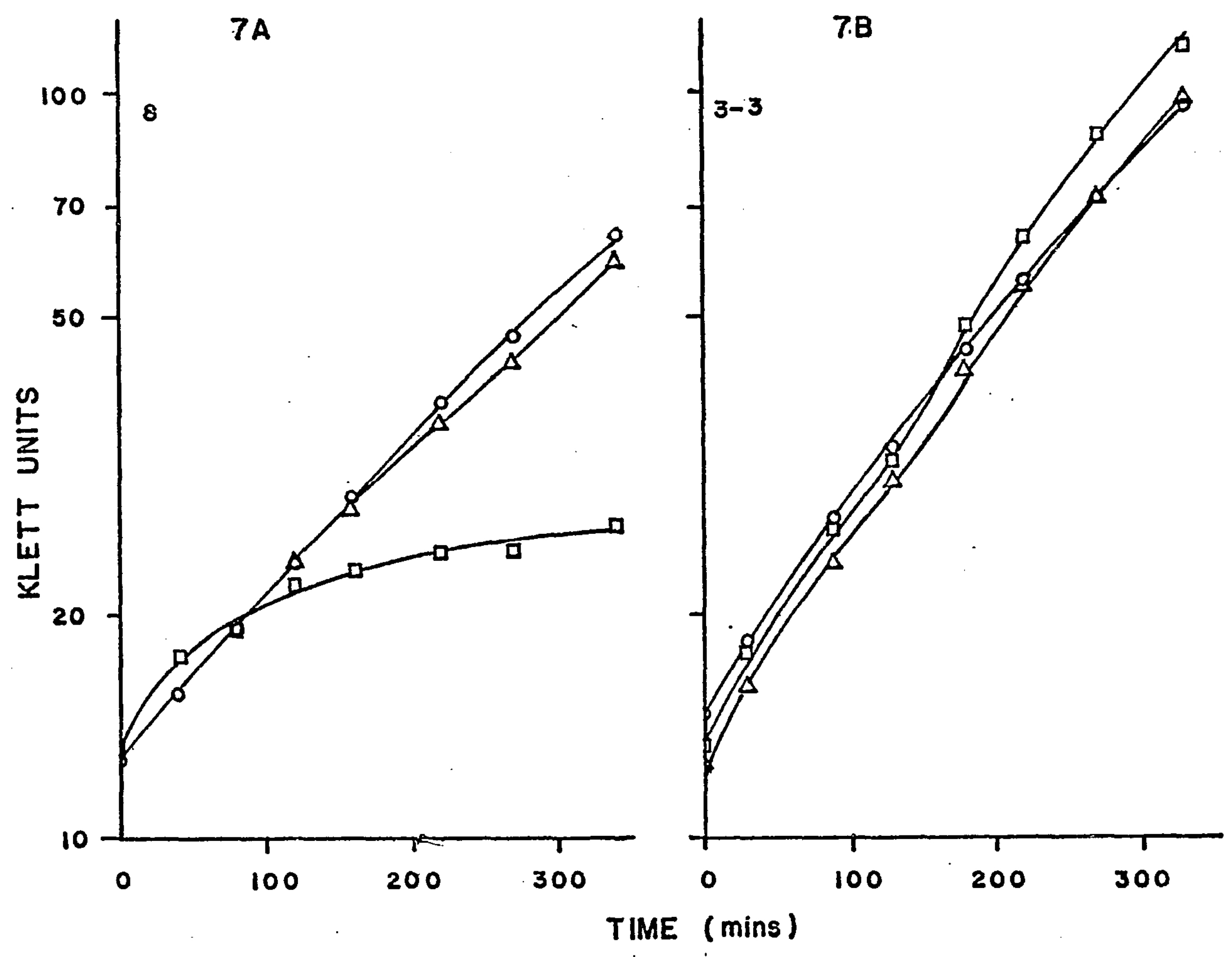
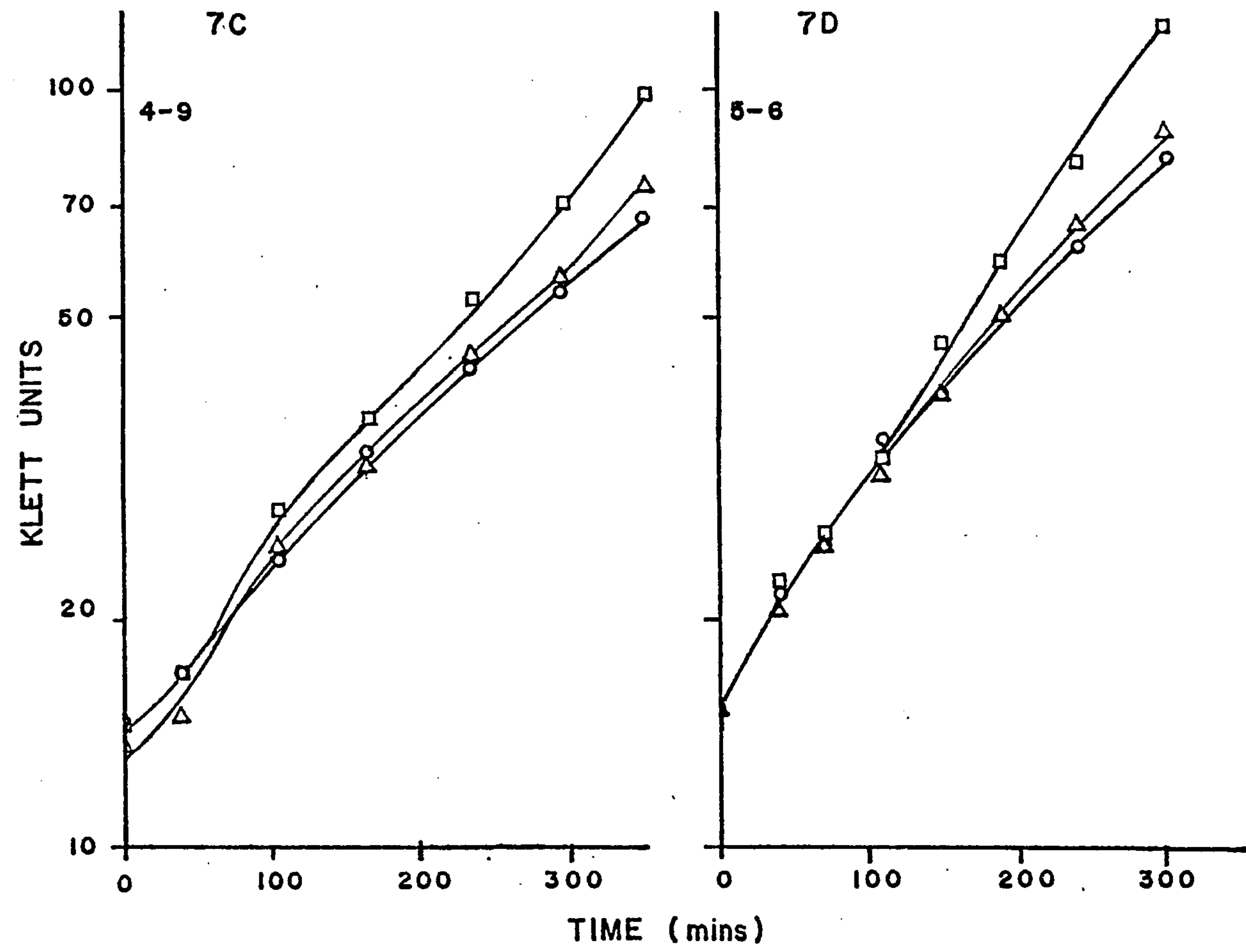

6A
8
6B
5-6
KLETT UNITS
100
70
50
20
10
0
100
200
300
400
TIME (mins)

FIGURE 7

Effect of DHAPa and (DHAPa)asp-ala-ala on the Growth of Strain 8 and its $GlpT^-$ Mutants.

Strains 8 (A), 3-3 (B), 4-9 (C) and 5-6 (D) were cultured in G and L minimal medium with succinate as the carbon source. When the cultures reached 15-20 K.U., either 2.5 mM DHAPa or 2.5 mM (DHAPa)asp-ala-ala was added and the growth followed. Untreated, (○-○-○); 2.5 mM DHAPa, (△-△-△); 2.5 mM (DHAPa)asp-ala-ala, (□-□-□).


7A
7B
8
3-3
100
70
50
20
10
KLETT UNITS
0
100
200
300
TIME (mins)


7C
4-9
7D
5-6
KLETT UNITS
100
70
50
20
10
0
100
200
300
TIME (mins)

tripeptide is not unreasonable. However, we have no explanation for the difference in response to the DHAPa-tripeptide between strain 8 and the glpT mutants.

The DHAP analog linked to the second tripeptide, ala-(DHAPa)asp-ala, has no effect on either strain 8 or strain 5-6 when cultured in G and L medium supplemented with succinate, even at a concentration of 2.5 mM (Figure 8).

In order to determine if the tripeptides themselves would be transported, the opp^+ and opp^- strains (leucine auxotrophs) tested for their ability to grow on asp-leu-ala as a source of leucine. In the absence of an exogenous supply of leucine, we hoped to prove transport of the asp-leu-ala by the ability of the strains to use the tripeptide for growth. Overnight cultures of 4212 and 4212 TorA cultured in Medium E with glucose as carbon source were washed and resuspended in medium lacking leucine. Either leucine or asp-leu-ala was added to the cultures to final concentrations of 0.02 mg/mL leucine or leucine equivalent. Since the parent strain has the oligopeptide transport system, it should be capable of transporting the asp-leu-ala. The Tor mutant should not transport the tripeptide and hence no growth should occur in the absence of exogenous leucine. In Medium E supplemented with glucose (Figure 9 A, B) asp-leu-ala is not able to fulfill the leucine requirement for the parent strain and served as a poor source in the mutant. However, the results obtained

FIGURE 8

The Effect of Ala-(DHAPa)asp-ala on the Growth of Strains 8 and 5-6.

Strains 8 (A) and 5-6 (B) were incubated in G and L minimal medium supplemented with succinate as described and treated with either 2.5 mM DHBP or 2.5 mM ala-(DHAPa)asp-ala and the growth followed. Untreated, (○-○-○); 2.5 mM ala-(DHAPa)asp-ala, (●-●-●); 2.5 mM DHBP, (△-△-△).

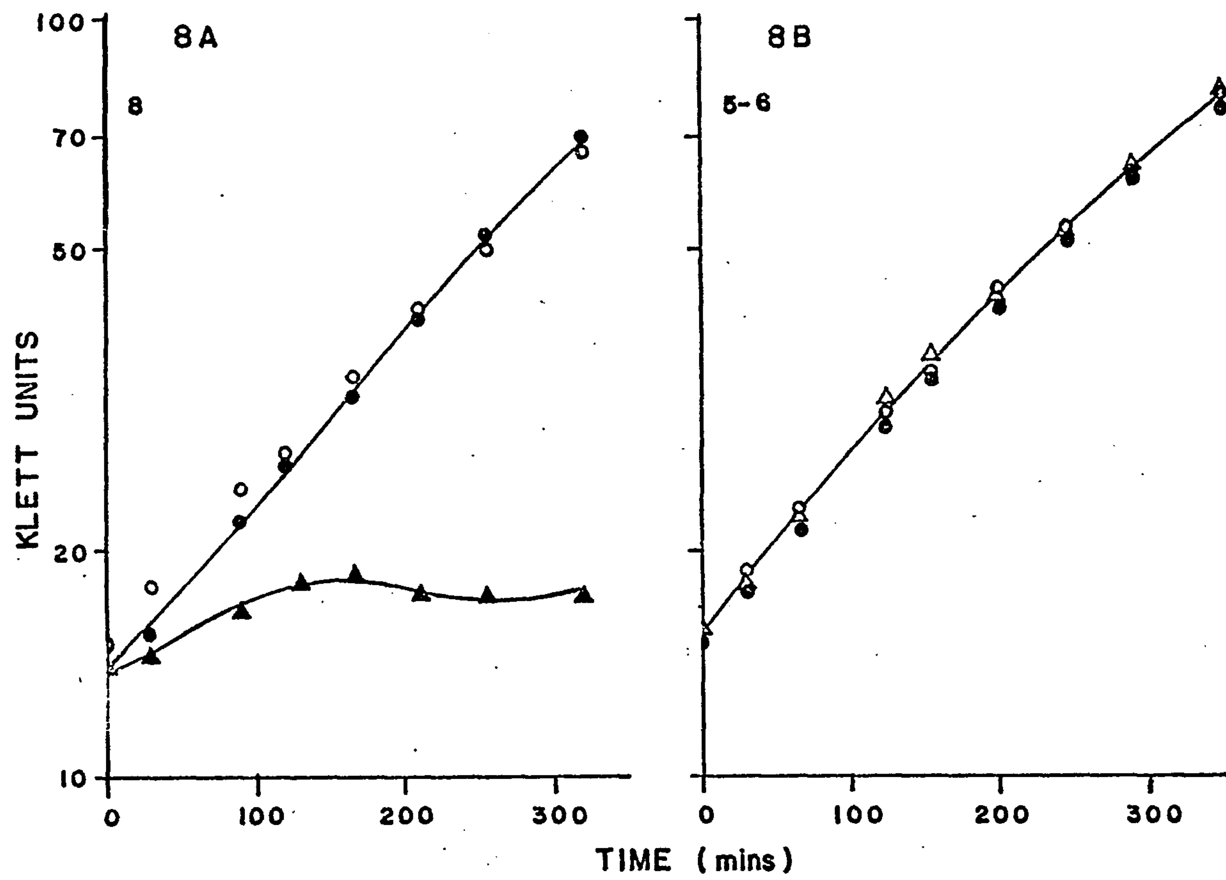
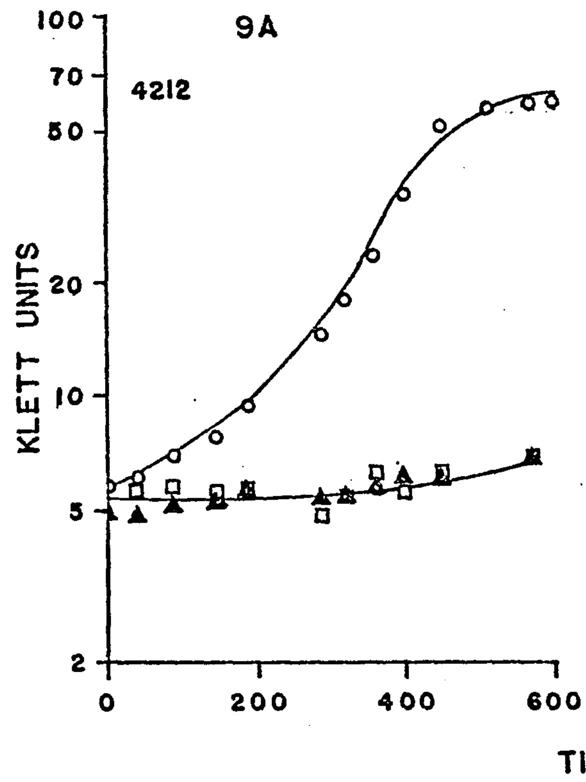
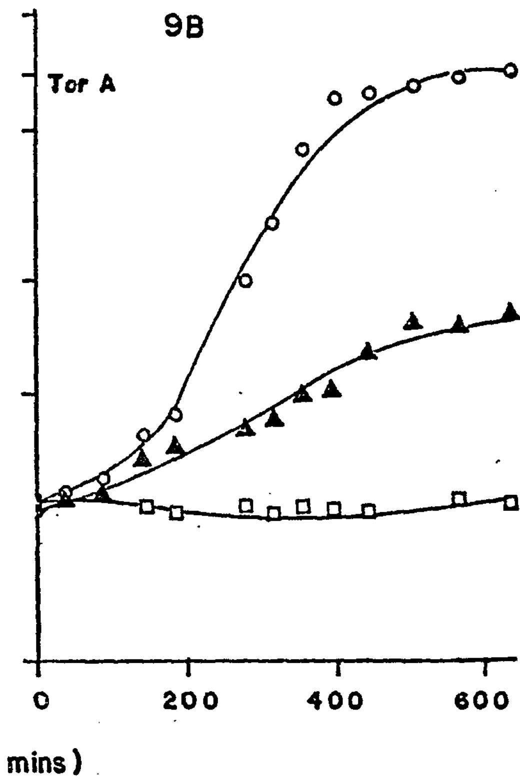

8A
8
8B
5-6
KLETT UNITS
100
70
50
20
10
0
100
200
300
0
100
200
300
TIME (mins)

FIGURE 9

Utilization of Asp-leu-ala as a Source of Leucine by Strains 4212 and 4212 TorA.

Strains 4212 (A) and 4212 TorA (B) were cultured overnight in Medium E supplemented with glucose and the required supplemens as described. Cultures were filtered, washed with sterile minimal medium minus leucine and resuspended in the original volume of warm (37 ^{o}C) medium without leucine. Either leucine (○-○-○) or asp-leu-ala (▲-▲-▲) was then added to a final concentration of 0.02 mg/mL leucine equivalent. Growth was followed after addition. No addition (□-□-□).


9A
4212
KLETT UNITS
100
70
50
20
10
5
2
0
200
400
600


9B
Tor A
0
200
400
600
TIME (mins)

indicate that the opp$^-$ strain appears to transport the asp-leu-ala slightly better than the parent. When supplemented with a concentration of 0.38 mM asp-leu-ala (the equivalent of 0.05 mg/mL leucine) the growth of the opp$^-$ mutant is equivalent to wild-type culture supplemented with 0.05 mg/mL leucine (data not shown). The parent strain, on the other hand, grows very poorly even when given the higher concentration of the tripeptide (data not shown). In order to determine if the presence of glucose in the medium causes this apparent anomaly in growth response, several carbon sources were investigated. Galactose was chosen since the growth rates of strain 4212 and the Tor mutant were the same as that in glucose. The strains were treated in the same manner as for glucose. The results of the supplementation of 4212 and its Tor mutant by asp-leu-ala in Medium E with galactose is shown in Figure 10. The same anomalous growth response towards asp-leu-ala was obtained whether glucose or galactose was employed as a carbon source. We have no explanation for why asp-leu-ala appears to be a better leucine source for the opp$^-$ mutant than it is for the wild type parent.

In contrast to the results obtained with strains 4212 and 4212 TorA, the other set of isogenic strains MV 12 and MV 12 Tor are both unable to grow on asp-leu-ala as a source of leucine (Figure 11 A, B). The reason for this is unclear but may be due to genetic differences between 4212 and MV

FIGURE 10

Effect of Carbon Source on the Utilization of Asp-leu-ala by Strain 4212 and 4212 TorA.

Strain 4212 (A) and 4212 TorA (B) were cultured in Medium E with galactose and the required supplements as described in Figure 9. After resuspension of the cultures, either leucine (○-○-○) or asp-leu-ala (▲-▲-▲) was then added to a final concentration of 0.02 mg/mL leucine equivalent and the growth followed. No addition, (□-□-□).

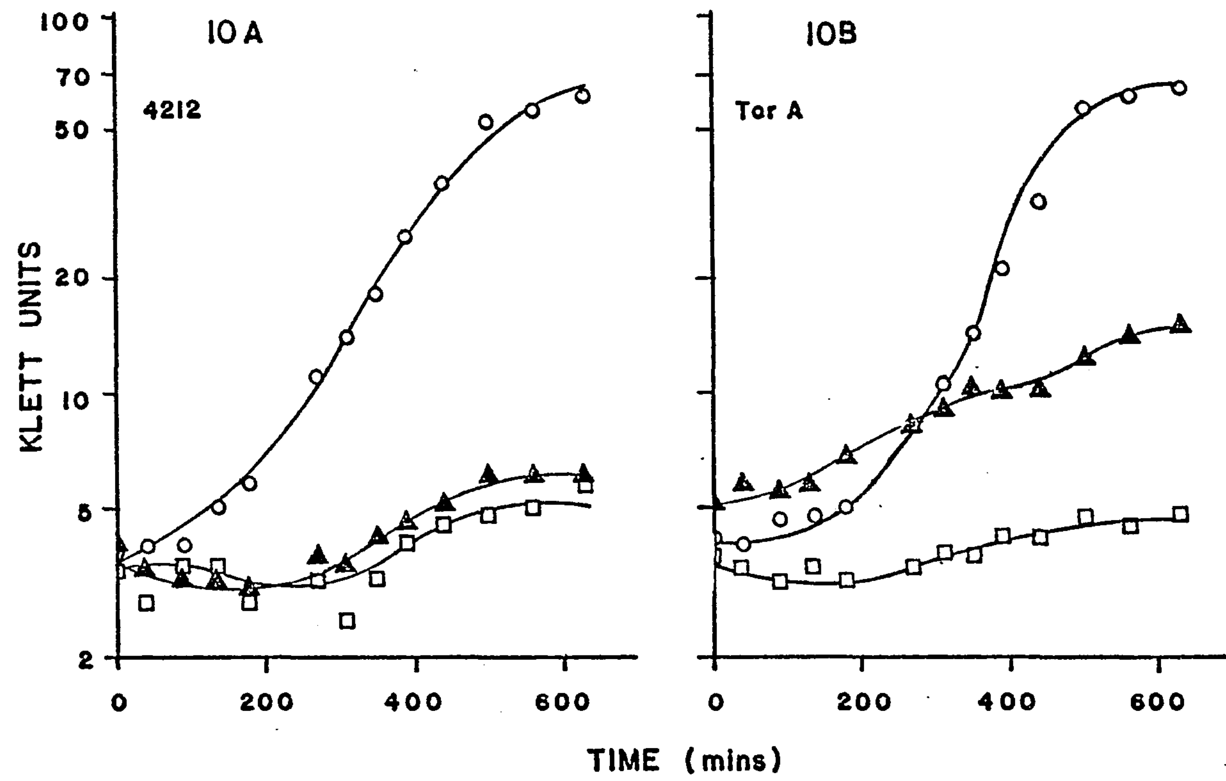
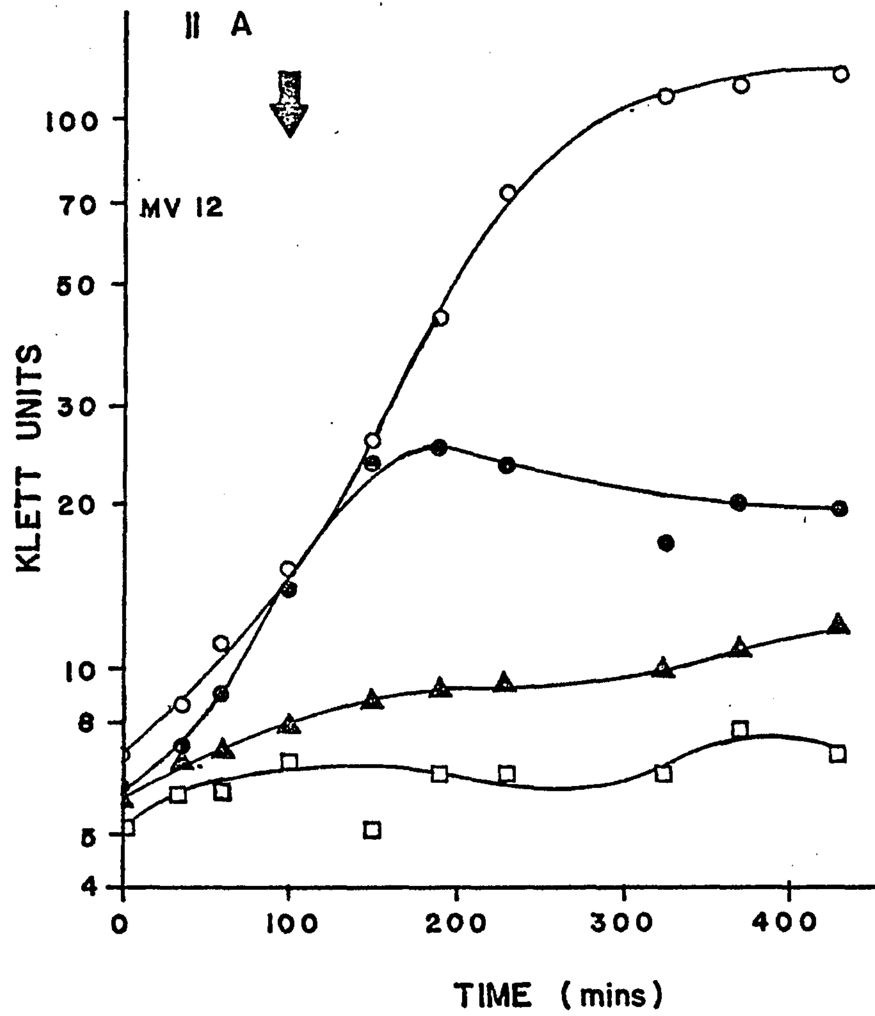
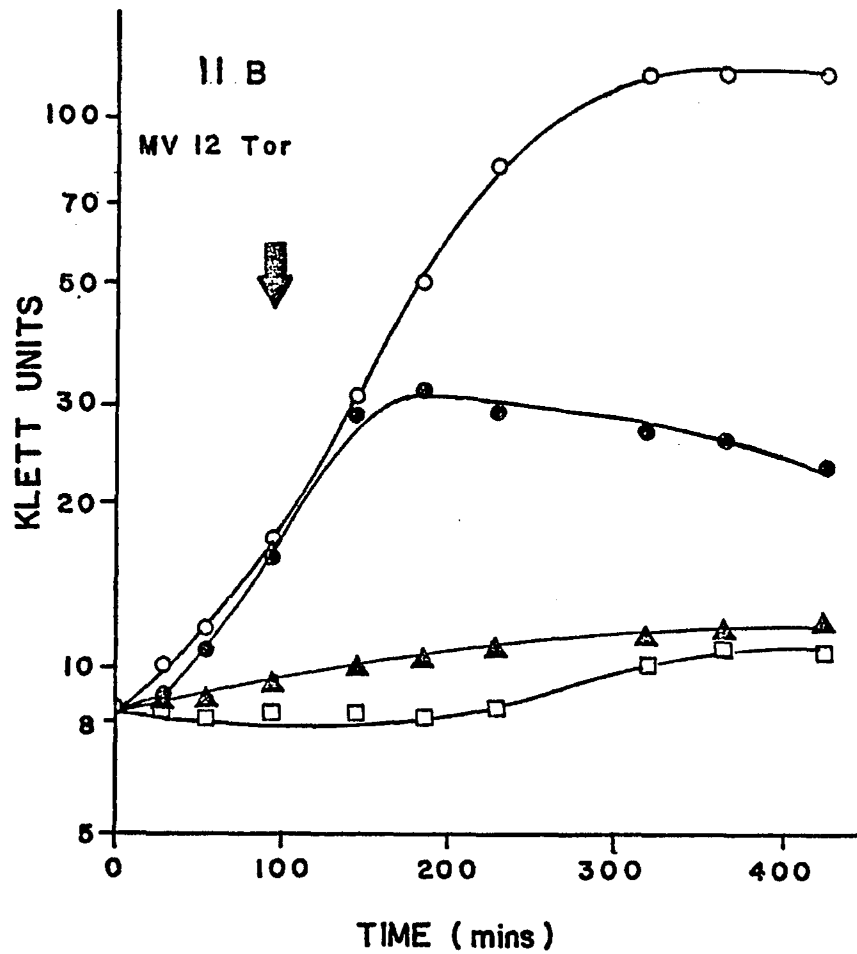

10 A
4212
10B
Tor A
KLETT UNITS
100
70
50
20
10
5
2
0
200
400
600
TIME (mins)

FIGURE 11

Utilization of Asp-leu-ala by Strains
MV 12 and MV 12 Tor.

Strains MV 12 (A) and MV 12 Tor (B) were incubated as described in Medium E with glucose and the required supplements. Overnight cultures were treated with either 2.5 mM DHBP at the time indicated by the arrow, or were filtered, washed and resuspended in minimal medium minus leucine (at 37 ^{o}C). Leucine or asp-leu-ala was then added to a final concentration of 0.02 mg/mL leucine equivalent and the growth followed. Plus leucine, (○-○-○); plus 2.5 mM DHBP, (●-●-●); plus asp-leu-ala, (▲-▲-▲); minus leucine, (□-□-□).


II A
MV 12
KLETT UNITS
100
70
50
30
20
10
8
5
4
0
100
200
300
400
TIME (mins)


11 B
MV 12 Tor
KLETT UNITS
100
70
50
30
20
10
8
5
0
100
200
300
400
TIME (mins)

12. The strains were also checked for their sensitivity to DHBP and found to be completely inhibited by 2.5 mM DHBP.

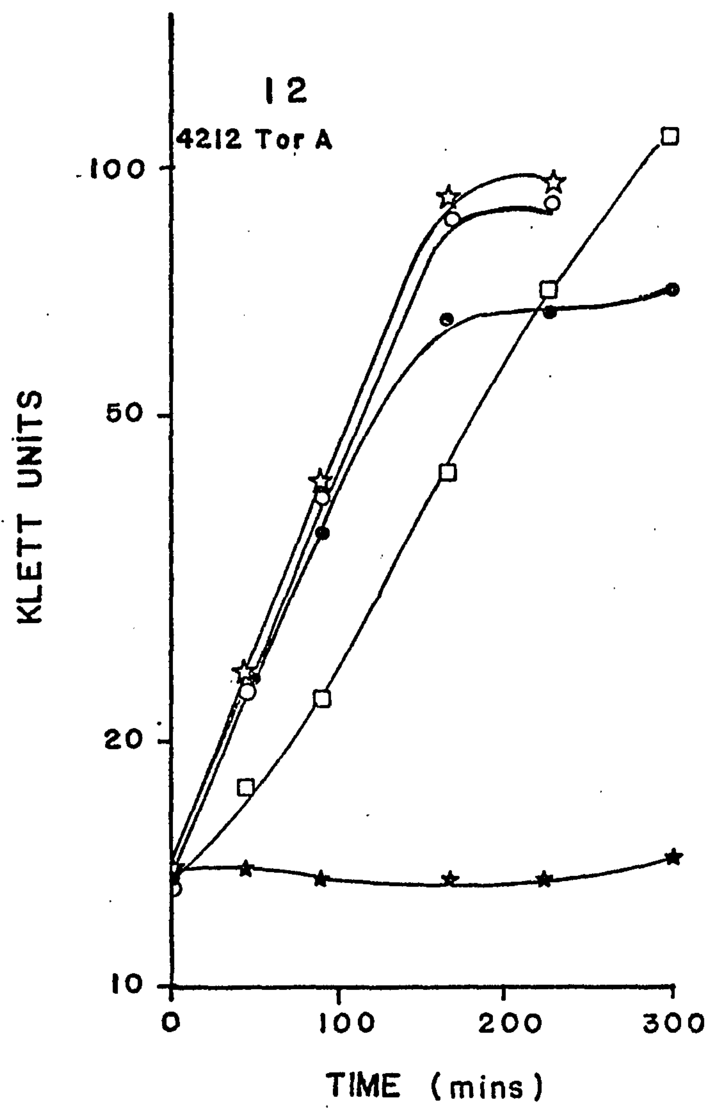
Although the asp-leu-ala (at low concentrations) is a poorer leucine source in 4212 than the Tor mutant, it was decided to investigate the efect of (DHBP)asp-leu-ala on the growth of 4212 TorA. At a concentration of 2.5 mM, (DHBP)asp-ala-ala totally inhibits the growth of 4212 TorA when cultured in Medium E supplemented with glucose (Figure 12). Under these same conditions, however, 2.5 mM (DHBP)asp-leu-ala has only a slight effect on growth. We felt it was necessary to determine if the opp^- genotype had been lost. In order to confirm the presence of the opp^- mutation, 4212 TorA was tested for its response to triornithine (orn_3) and found to be resistant (Figure 12) while the parent was totally inhibited (data not shown).

The tripeptides and phosphonate-linked tripeptides given to us were reported to be chromatographically pure. Late in these investigations, we had reason to chromatograph the peptides and discovered evidence for degradation. Apparently, some of the trifluoroacetic acid used to cleave the protecting groups was not totally removed and catalyzed degredation of the peptides or phosphonate-linked peptides. It is also possible that the acidity of the phosphonic acids themselves may catalyze degradation (R. Engel, personal communication). Therefore, it is likely that the undegraded material will prove to be more potent than the values

FIGURE 12

The Effect of (DHBP)asp-leu-ala *vs.* (DHBP)asp-ala-ala on the Growth of Strain 4212 TorA.

Strain 4212 TorA was cultured in Medium E with glucose as the carbon source and the required supplements as described in the text. When the cultures reached the desired cell density (15-20 K.U.) either (DHBP)asp-leu-ala or (DHBP)asp-ala-ala was then added and the growth followed. Untreated, (○-○-○); 2.5 mM DHBP, (●-●-●); 2.5 mM (DHBP)asp-ala-ala, (✱-✱-✱); 2.5 mM (DHBP)asp-leu-ala, (□-□-□); 0.075 mg/mL triornithine, ✡-✡-✡.


12
4212 Tor A
KLETT UNITS
100
50
20
10
0
100
200
300
TIME (mins)

reported here. However, two problems do arise. It is conceivable that some of the phosphonate analog may be carried into the cells by dipeptides. The other problem is exactly how much phosphonate remains linked to the tripeptide.

We have learned, subsequent to these studies, that the benzyl group used to protect the beta-COOH of the aspartyl residue is quite labile to either acid or base catalyzed hydrolysis (96). Protecting groups on the phosphonate-coupled tripeptides used for this investigation were routinely removed using trifluoroacetic acid. According to Bodansky et al. (96), the use of acid or base to remove these groups can result in the formation of aminosuccinyl peptides and the loss of the protecting group on the beta-COOH of the aspartyl residue. The DHBP and DHAPa on our tripeptides can, in a sense, be compared with the benzyl group on the aspartate and the possible loss of the phosphonate was considered.

Since we discovered that it was possible that the DHBP was removed from the peptides during deprotection, the amount of DHBP (if any) remaining on the peptide had to be determined. The concentration of DHBP after base hydrolysis of the (DHBP)asp-ala-ala was assayed using the rabbit muscle G3P dehydrogenase. Since the S-DHBP was linked to the tripeptide it was necessary to subject free S-DHBP to base hydrolysis in order to determine if racemization of DHBP

occurs under these conditions. The R-DHBP, corresponding to D-G3P, is not oxidized by the enzyme. Analysis of the coupled tripeptide resulted in a concentration of DHBP that was approximately 15% of what was expected if calculated based on the amount of material weighed after lypholyzation. There was little DHBP activity in unhydrolyzed (DHBP)asp-ala-ala (less than 2%). The decreased amount of activity was not due to the presence of an enzyme inhibitor in the unknown solutions since the addition of a known amount of L-G3P to the reaction mixture (subsequent to the reduction of DHBP) resulted in the expected yield of NADH.

This finding that the DHBP concentration in the tripeptides is lower than expected, may account for some of results that were obtained. At a concentration of 0.3 mM, (DHBP)asp-ala-ala does not totally inhibit the growth of strain 8 (Figure 1 A). The same concentration of DHBP, however, is bacteriostatic (97) (Figure 4 A). If there is only about 15% DHBP activity with the 0.3 mM DHBP-tripeptide, then the actual concentration of DHBP would be about 0.05 mM. This concentration of DHBP exerts a slight inhibitory effect on strain 8 cultured in G and L with succinate (97).

As can be seen from Figure 4 A, there is no inhibition of strain 8 by 0.3 mM ala-(DHBP)asp-ala. At a concentration of 1.5 mM, there is a pronounced decrease in growth (Figure 5 A), but complete growth stasis does not occur until the

concentration of the peptide is raised to 1.8 mM (Figure 5C). At these same concentrations, DHBP is immediately bacteriostatic. Although the actual content of DHBP in the ala-(DHBP)asp-ala was not determined, these observations (as well as those from the DHAPa-tripeptides and the DHBP-asp-leu-ala) may arise, in part from the decreased amount of phosphonate in the coupled tripeptides.

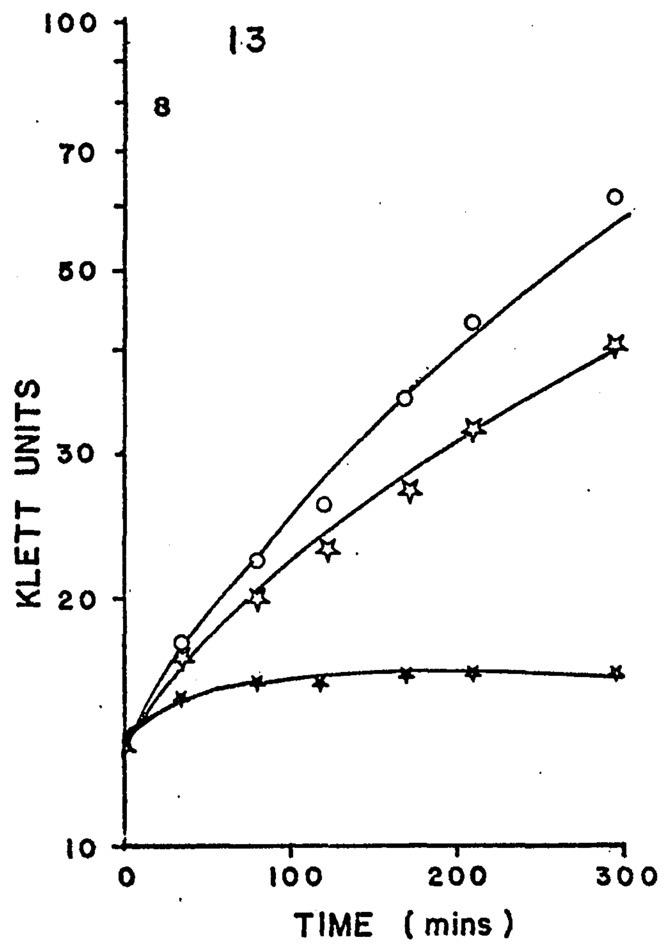
Subsequent studies will be done with peptides containing glutamate (which does not undergo the cyclization, (96)), with peptides deprotected by other routes or with phosphonate-linked peptides in the form of salts.

Mevalonic acid is a cholesterol precursor in animals (95) and a precursor for the polyisoprenes found in bacteria (95, 98, 99). Strain 8 does not transport (or transports very poorly) the phosphonic acid analog of 5-phosphomevalonic acid (5-carboxy-4-hydroxy-4-methylpentyl-1-phosphonate) (Figure 13). In the hope of facilitating its transport, the phosphonic acid analog of phosphomevalonate (MevPa) was coupled to the tripeptide ala-ser-ala through the -COOH of the analog and the -OH of the serine residue. The effect of the analog on the growth of E. coli was then tested. At 2.5 mM, ala-(MevPa)ser-ala completely inhibits the growth of strain 8 (Figure 13) and strain 3-3 (Figure 14) when cultured in G and L medium supplemented with succinate. Strain 4212 TorA (Figure 15) when cultured in

FIGURE 13

Effect of MevPa and Ala-(MevPa)ser-ala on the Growth of Strain 8.

Strain 8 cultured in G and L minimal medium supplemented with succinate was treated wih either 2.5 mM MevPa (☆-☆-☆) or 2.5 mM ala-(MevPa)ser-ala (✱-✱-✱) as described in the text and the growth followed. No addition, (○-○-○).



100
13
8
70
50
30
20
10
KLETT UNITS
0
100
200
300
TIME (mins)

FIGURE 14

Effect of Ala-(MevPa)ser-ala on the Growth of Strain 3-3.

Strain 3-3 was cultured in G and L minimal medium with succinate as described in the text. When the cultures reached the desired cell density (15-20 K.U.), 2.5 mM ala-(MevPa)ser-ala (●-●-●) was added and the growth followed. No addition, (○-○-○).

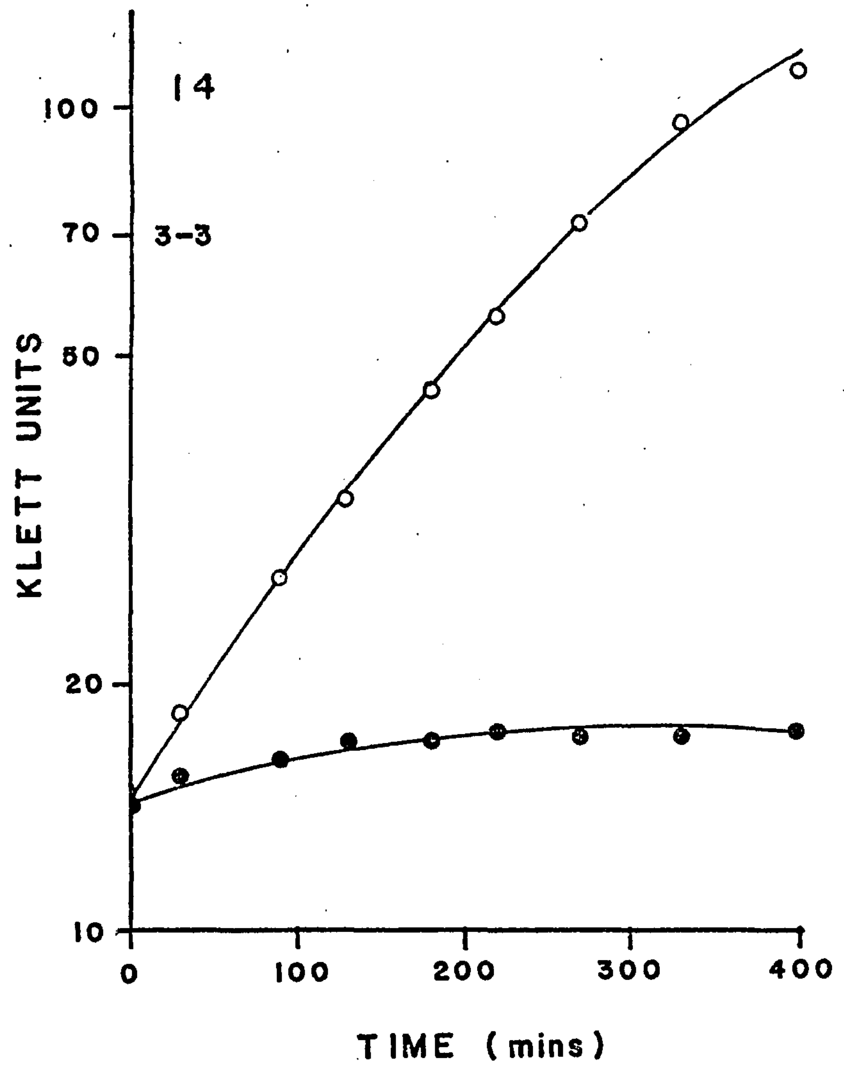
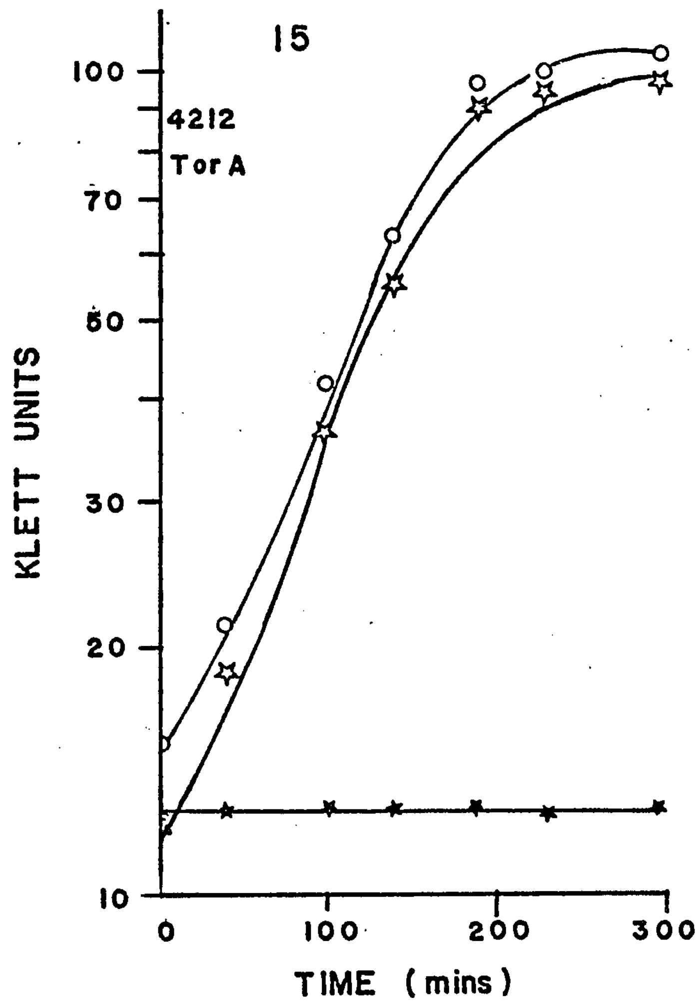

14
3-3
KLETT UNITS
100
70
50
20
10
0
100
200
300
400
TIME (mins)

FIGURE 15

Effect of MevPa and Ala-(MevPa)ser-ala on the Growth of Strain 4212 TorA.

Strain 4212 TorA was cultured in minimal Medium E with glucose and the required supplements. When the cultures reached the desired cell density (15-20 K.U.) either 2.5 mM MevPa (☆-☆-☆) or 2.5 mM ala-(MevPa)ser-ala (✱-✱-✱) was added and the growth followed. Untreated, (O-O-O).


15
100
4212
Tor A
70
50
30
20
10
KLETT UNITS
0
100
200
300
TIME (mins)

Medium E with glucose is also inhibited. At a concentation of 2.5 mM, MevPa alone slightly inhibits the growth of strain 8 but has no effect on 4212 TorA. Ala-ser-ala also has no effect on any of the strains tested. The effect of the concentration of the ala-(MevPa)ser-ala on the growth of strain 8 is shown in Figure 16. At a low concentration (0.1 mM), ala-(MevPa)ser-ala is less inhibitory towards strain 8 cultured in G and L medium supplemented with succinate than is 2.5 mM MevPa alone. At a concentration of 0.3 mM MevPa-tripeptide, the growth of strain 8 is markedly inhibited, while a concentration of 2.5 mM ala-(MevPa)ser-ala results in complete growth stasis (Figure 16).

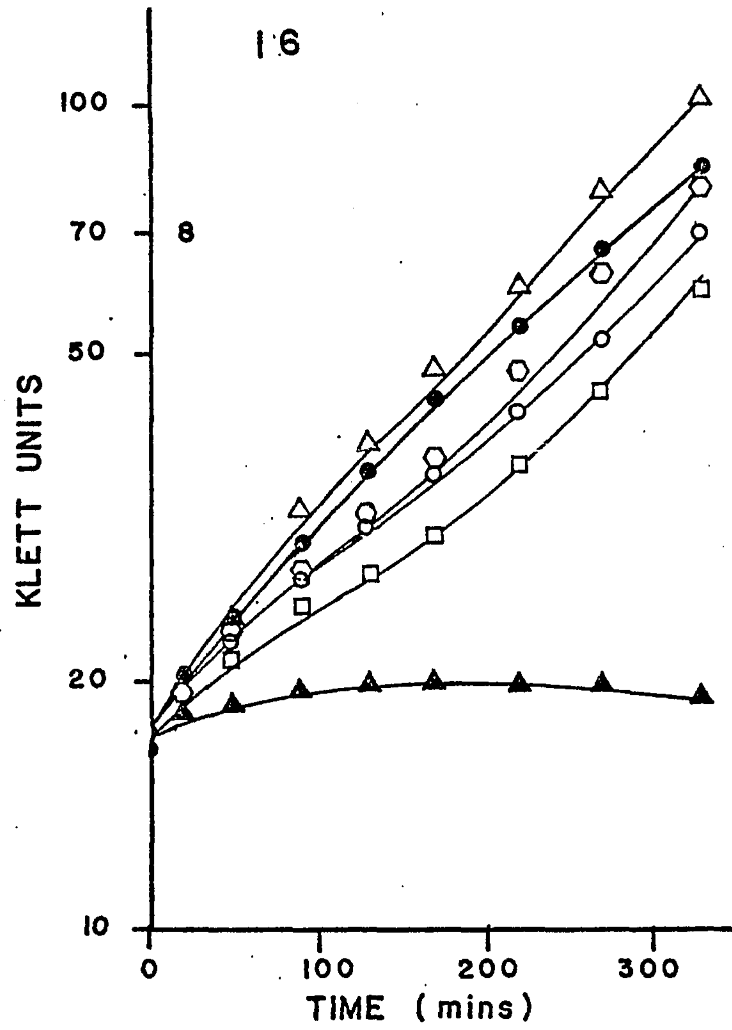
The results obtained using the phosphomevalonate analog raised many questions. While the lack of inhibition by free MevPa was not surprising, the immediate bacteriostatic effect of 2.5 mM ala-(MevPa)ser-ala in strains 8, 3-3 and the 4212 TorA was quite unexpected. The analog is an inhibitor of phosphomevalonate kinase in rat liver (81) and as such can inhibit the synthesis of cholesterol (81). What then is the mechanism of inhibition by the ala-(MevPa)ser-ala?

Mevalonate is a precursor for the isoprenoids found in bacteria (98, 99). The C_{55} isoprenoid, bactoprenol, acts as a carrier in the synthesis of peptidylglycan, the O-antigen in gram-negative bacteria, the capsular

FIGURE 16

Effect of Concentration of Ala-(MevPa)ser-ala on the Growth of Strain 8.

Strain 8 was cultured in G and L minimal medium with succinate as the carbon source. When the cultures reached 15-20 K.U., cultures were treated with 0.1 mM (⬡-⬡-⬡), 0.3 mM (□-□-□) 2.5 mM (▲-▲-▲) ala-(MevPa)ser-ala, 2.5 mM MevPa alone (○-○-○), or with 2.5 mM ala-ser-ala (△-△-△) alone and the growth followed. Untreated, (●-●-●).



1·6
8
KLETT UNITS
100
70
50
20
10
0
100
200
300
TIME (mins)

polysaccharide in gram-positive organisms and a mannan of *Micrococcus lysodiekticus* (100, 101). This isoprenoid (bactoprenol) is similar to the dolichols found in mammalian cells (102). Mevalonate is also incorporated into dolichol and ubiquinones (103) and isopentenyl-tRNA in mammalian cells (103, 104). Bacteria also contain vitamin K (105) and ubiquinones, especially coenzyme Q (CoQ) (106). The inhibition of the synthesis of any of these compounds would not be expected to result in the immediate inhibition of growth. The mechanism of inhibition of the ala-(MevPa)ser-ala cannot be attributed to the ala-ser-ala, but the reason for the inhibition by the coupled tripeptide is unknown. We are not even sure, at this point, that the phosphomevalonate analog is inhibiting isoprene or polyisoprene synthesis. Further investigation must be done in order to determine which apparently vital cellular process(es) is involved.

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