VITAMIN AND AMINO ACID INTERRELATIONSHIPS IN THE METABOLISM OF A MUTANT STRAIN OF *ESCHERICHIA COLI*.

By

JOHN MURRAY McLAUGHLAN

A THESIS

Submitted to the Faculty of Graduate Studies and Research, McGill University, in partial fulfillment of the requirements for the degree of Master of Science.

Macdonald College

October, 1956.

TABLE OF CONTENTS

INTRODUCTION

Most strains of Escherichia coli have simple nutritional requirements. This fact indicates that these organisms are able to synthesize all their requirements for amino acids, purines, pyrimidines, vitamins and other substances necessary for growth in a mixture of a few inorganic salts and glucose. Davis and Mingioli (8), however, have isolated mutant strains of E. coli that require either methionine or vitamin B_{12} for growth.

Although the exact function of vitamin B_{12} in metabolism is not clearly understood many studies of animals and bacteria indicate that this vitamin is related either directly or indirectly with the biosynthesis of desoxyribosides and of methionine. Evidence for a function of vitamin B_{12} in the production of desoxyribosides is based mainly upon studies with lactic acid bacteria. On the other hand the results of studies concerning methionine synthesis indicate that vitamin B_{12} is required for this process in chicks, rats, and E. coli.

Numerous investigators have studied interrelationships of vitamin B_{12}, other vitamins and sulphur-containing amino acids, both with animals and bacteria. The work of McLaughlan et al (34) indicated that an interrelationship seemed to exist between vitamin B_{12} and thiamine in the metabolism of a mutant strain of E. coli developed by Davis and Mingioli. The present work is a continuation of that study and is concerned primarily with interrelationships of vitamin B_{12}, thiamine, pantothenic acid and sulphur-containing amino acids in the metabolism of this mutant strain of E. coli.

REVIEW OF LITERATURE

1. Biosynthesis of Methionine

Lampen et al (27) investigated the sulphur metabolism of several mutant strains of E. coli that were unable to utilize inorganic sulphate for growth. Some mutants grew when supplied with either inorganic sulphide or a sulphur-containing amino acid; a few grew only when the medium contained l-methionine. After determining the variety of different sulphur compounds utilized by each mutant, Lampen et al suggested that cysteine, homocysteine and cystathionine were intermediate compounds in methionine synthesis but the exact position of these compounds in the process was not determined. Fling and Horowitz (13) and Teas et al (49) also studied methionine synthesis but they used mutant strains of Neurospora. Teas et al postulated the following scheme for methionine synthesis:

L-homoserine + L-cysteine ⟶ L-cystathionine ⟶ 3 carbon fragment + L-homocysteine $\xrightarrow{+CH_3}$ L-methionine.

Several workers, including Simmonds (46), Gots and Koh (14), Davis and Mingioli (8) and Kalan and Ceithaml (22) found that the intermediate steps in the synthesis of l-methionine by E. coli appeared to be identical to those postulated by Teas et al for Neurospora.

Davis and Mingioli (8) discovered that l-methionine was replaceable by vitamin B_{12} in the nutrition of some methionine-requiring strains of E. coli. Mutants that were unable to synthesize cysteine, cystathionine or homocysteine did not grow when the medium contained vitamin B_{12} in place of methionine whilst those mutants that were

unable to synthesize methionine from homocysteine did respond to vitamin B_{12}. Davis and Mingioli concluded that vitamin B_{12} was required for the methylation of l-homocysteine to produce methionine. Helleiner and Woods (18) also found that vitamin B_{12} was required for the synthesis of methionine by cell-free extracts of a vitamin B_{12}-requiring strain of E. coli.

Kalan and Ceithaml (22) stated that "the function of vitamin B_{12} may be interpreted as being involved in methyl transfer or methyl group production which permits indirectly a more efficient utilization of the four-carbon precursor in the formation of methionine". On the other hand Dubnoff (9, 10) suggested a different function for vitamin B_{12} in the synthesis of methionine. Working with a mutant strain of E. coli he found that this organism grew anaerobically in the absence of vitamin B_{12} if homocysteine or certain reducing agents, such as glutathione or cysteine, that could reduce homocystine were present in the medium. He concluded that vitamin B_{12} was related either directly or indirectly with the maintenance of the reduced forms of certain sulphydryl compounds, such as homocysteine.

In 1955, Stekol (48) reviewed the literature concerning the biosynthesis of methionine. He stated that "vitamin B_{12} does not appear to be involved in any of the processes of methionine formation with the exception of the synthesis of the methyl group de novo from the α - carbon of glycine. The nature of the involvement of vitamin B_{12} appears to be indirect, since the deficiencies in either folic acid,

pyridoxine, or pantothenic acid in rats similarly affected the extent of synthesis of the methyl group of methionine from the α-carbon of glycine".

Although several workers have studied the function of vitamin B_{12} in methionine formation there is disagreement as to the function of vitamin B_{12} in this process. It appears, therefore, that the exact function of vitamin B_{12} in methionine synthesis is not yet understood.

2. The Use of E. coli for the assay of Vitamin B_{12}

Davis and Mingioli (8) found that certain of the mutant strains of E. coli that they isolated could be used for the assay of vitamin B_{12}. One of these mutant strains, designated as E. coli 113-3, has been widely used for the assay of vitamin B_{12}; reports of both plate assays (16, 51) and tube assays (8, 3, 5, 20) with this organism have appeared in the literature. Burkholder (3) described a tube assay with a more complex medium than that of Davis and Mingioli (8). Chiao and Peterson (5) compared the amount of growth of E. coli 113-3 in the two media and found that this organism grew equally well in either medium; therefore, they recommended the simpler medium of Davis and Mingioli. They also recommended that the cultures be shaken during incubation. Other workers (20, 34) also proposed minor modifications of Davis and Mingiolis' tube assay for vitamin B_{12}.

Several factors seem to affect the extent of the growth response of E. coli 113-3 to vitamin B_{12}. Chiao and Peterson (5) found that a ratio of methionine to vitamin B_{12} (on a weight basis) greater than 50,000 invalidated the assay for vitamin B_{12}, since methionine stimulated growth. They also studied the effect of other nutrients that might be present in vitamin B_{12}-containing materials. The growth-response was unaffected by the addition of 9 water-soluble vitamins, or of a combination of several purines and pyrimidines or of 15 amino acids, exclusive of methionine, added singly to the medium. Other workers (20, 34) reported, however, that certain vitamins such as ascorbic acid and thiamine inhibited the growth of E. coli 113-3.

3. Biochemical Reactions of Thiamine

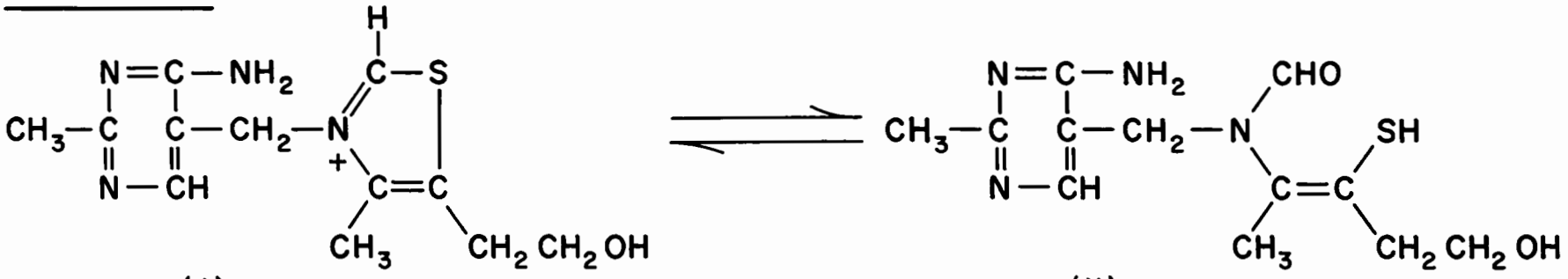
Thiamine has long been known to be concerned with the decarboxylation of pyruvic acid, but its exact function in metabolism is still unknown. Korkes et al (26) showed that E. coli required diphosphothiamine for the dismutation of pyruvate to acetyl phosphate, lactate, and carbon dioxide. Reed (38) reported that lipothiamide, which contains both lypoic acid and thiamine, is required by a mutant strain of E. coli as a bio-catalyst in the following reaction:-

$$CH_3 - \underset{\underset{O}{\|}}{C}O_2^- + CoA\text{-}SH + DPN \xrightarrow{+\ \text{lipothiamide}} CoA\ S\text{-}\underset{\underset{O}{\|}}{C}\text{-}CH_3 + CO_2 + DPNH$$

CoA-SH represents co-enzyme A and DPN represents diphosphopyridine nucleotide.

One suggested mechanism (40) for the biochemical action of thiamine is based upon the findings of Zima and Williams (55) who reported that thiamine is subject to reversible oxidation and reduction in physiological conditions. At a slightly alkaline reaction either hydrogen peroxide or oxygen, from the air oxidizes the thiol form of thiamine to a disulphide form of thiamine. Zima et al (56) found that the disulphide (B_1 - S-S-B_1) is reduced by hydrogen sulphide, glutathione or cysteine. Matsukawa and Yurugi (32) and Sahashi et al (42) reported that the thiol and disulphide forms of thiamine react with various sulphydryl compounds to produce mixed disulphides. Matsukawa and Yurugi postulated that the following reactions occur when thiamine and cystine are dissolved in a phosphate buffer at a slightly alkaline reaction.

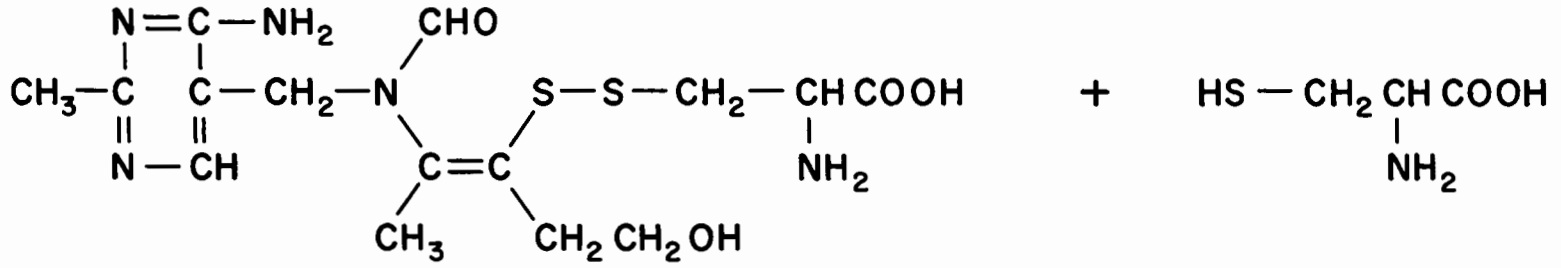
Reaction A.



(I) (II)

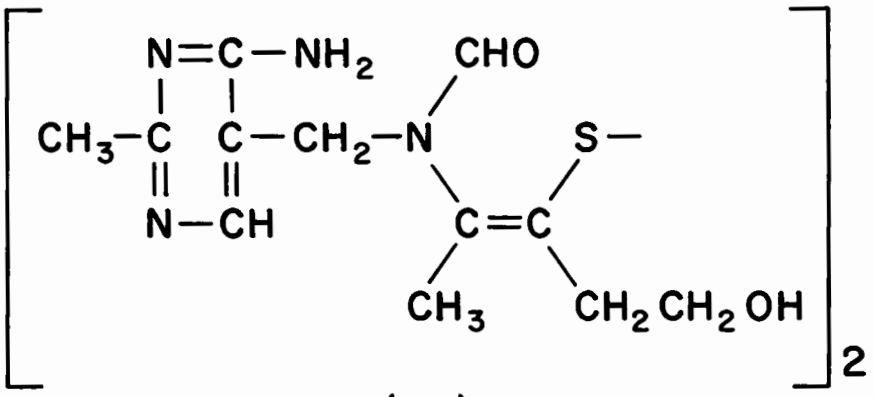
Reaction B.

(II) (III)



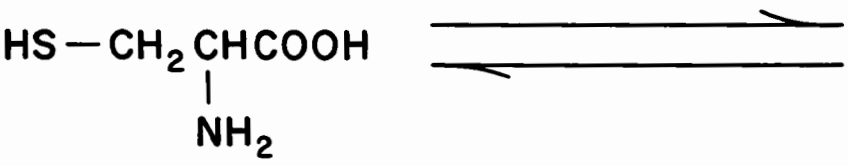
(IV) (V)

Reaction C.

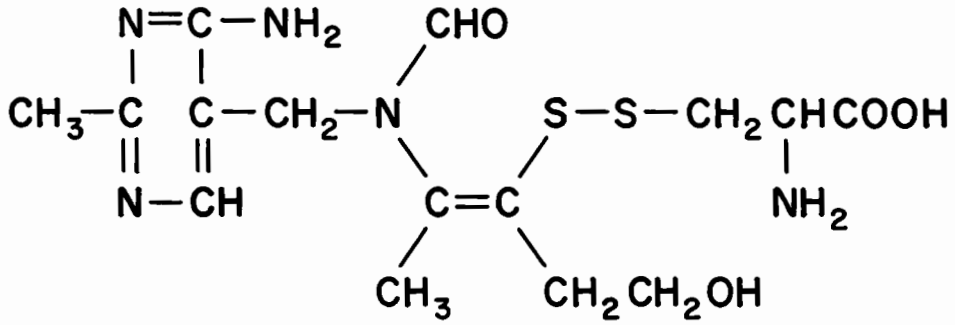


(VI)

+

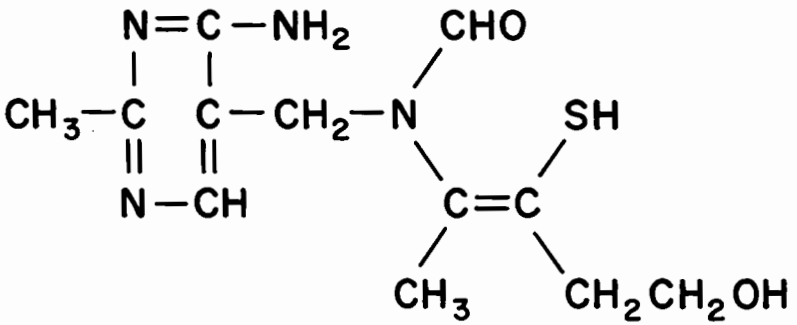


(V)



(IV)

+



(II)

In reaction A, thiamine in solution at pH 7.5, forms an equilibrium mixture of the thiazole (I) and thiol (II) forms of thiamine.

In reaction B, thiol thiamine (II) and cystine (III) react to produce the mixed disulphide thiamine cysteine (IV) and cysteine (V).

In reaction C, the disulphide form of thiamine (VI) reacts with cysteine (V) to produce thiaminecysteine (IV) and the thiol form of thiamine (II). Matsukawa and Yurugi (32) stated, however, that this reaction is unlikely to occur *in vivo* since the ratio of thiamine to cysteine is very small. Sahashi *et al* (42) reported an *in vitro* reaction similar to that of Matsukawa and Yurugi's reaction "C" occurring between thiamine disulphide and pantetheine and producing a mixed disulphide of thiamine and pantetheine.

Saxena *et al* (43) reported that the synthesis of thiamine by "wild" strains of *E. coli* was inhibited by the addition of vitamin B_{12} to the growth medium, but they did not offer any explanation for this effect.

Lang and Chow (28) found that thiamine destroyed the microbiological activity of vitamin B_{12} in solution particularly at a reaction close to neutrality. Although McLaughlan *et al* (34) found that the addition of thiamine to the medium depressed the growth-response curve with vitamin B_{12}, the effect did not appear to result from partial destruction of vitamin B_{12}. In view of the reported reactions of thiamine (55, 32, 42) and of Dubnoff's reports (9, 10) concerning the function of vitamin B_{12}, McLaughlan *et al* (34) suggested a mechanism that might explain the inhibitory action of thiamine on the growth of *E. coli* 113-3. In the highly aerobic conditions of the shaken

assay, thiamine added to the medium becomes oxidized to thiamine disulphide. The disulphide interacts with cysteine or homocysteine synthesized by the organism to produce a mixed disulphide and effectively diminishes the extent of growth in media containing suboptimal amounts of vitamin B_{12}.

4. Toxicity of Cystine for Bacteria

Several investigators have reported that cystine is toxic for various bacteria (particularly when autoclaved with media). Schuhardt et al (44) investigated the toxicity of cystine for Brucella abortus. They showed that cystine was not toxic when it was autoclaved at pH 1.5 or when it was sterilized by filtration and added to media aseptically, but that it was toxic when autoclaved in media at a neutral reaction. Schuhardt et al (44) isolated elemental sulphur from autoclaved cystine solutions, and they found that colloidal dispersions of this elemental sulphur at concentrations as low as 0.06 μg per ml. was toxic for certain strains of Brucella. They postulated the following scheme for the breakdown of cystine during autoclaving of media.

(1) A heat-induced rupture of the disulphide linkage with an associated dismutation of a portion of the cystine to cysteine and cysteine sulfinic acid; an intermediate unstable cysteine sulfenic acid is probable in this dismutation.

$$2\ R\text{-}S\text{-}S\text{-}R + 2H_2O + \text{Heat} \longrightarrow 2R\text{-}SH + 2R\text{-}SOH$$

$$2\ R\text{-}SOH + \text{Heat} \longrightarrow R\text{-}SO_2H + R\text{-}SH.$$

(2) The cysteine is decomposed to pyruvic acid, ammonia and hydrogen sulphide by way of an unstable imino acid, or by way of alpha-amino acrylic acid.

$$R\text{-}SH + \text{Heat} \longrightarrow H_2S + CH_2 = \underset{\displaystyle NH_2}{\overset{\displaystyle |}{C}} - COOH \text{ or } CH_3 - \underset{\displaystyle NH}{\overset{\displaystyle \|}{C}} - COOH$$

$$CH_3 - \underset{\displaystyle NH}{\overset{\displaystyle \|}{C}} - COOH + H_2O \longrightarrow CH_3 - \underset{\displaystyle O}{\overset{\displaystyle \|}{C}} - COOH + NH_3$$

(3) The hydrogen sulphide reacts with excess cystine to form sulphur and additional cysteine.

$$H_2S + R\text{-}S\text{-}S\text{-}R \longrightarrow 2R\text{-}SH + S^{o}$$

Schuhardt et al stated that "this last reaction is readily reversible, which fact might be expected to account for the ease of neutralization of sulfur toxicity by excess SH compounds".

Heathcote (17) suggested that cystine was somewhat toxic for Leuconostoc mesenteroides P 60, which is the test organism used for the microbiological assay of cystine. He did not offer an explanation for this effect. Rose et al (39) found that cystine autoclaved with the medium was toxic for Lactobacillus bifidus. The presence of a reducing sugar such as lactose, during autoclaving prevented the toxicity. The addition of cysteine, thioglycollic acid or thiomalic acids to the medium after autoclaving also reversed the inhibitory effect of autoclaved cystine. They tested colloidal sulphur but found that relatively large amounts were necessary to produce inhibition of growth and it appeared therefore that the cystine toxicity differed from that reported by Schuhardt et al (44).

Woiwod (52) reported that copper sulphides inhibited the growth of Staphylococcus aureus and several other Gram-positive organisms but that it had little or no effect on several Gram-negative organisms. He found that copper sulphide was formed during autoclaving of media containing cystine and copper. The inhibition did not occur, however, when the medium was autoclaved in screw-capped bottles or sealed ampoules. He suggested that possibly the decomposition of cystine on heating is a reversible reaction and that an equilibrium is attained when the

heating is done in a closed system. Under any given conditions of temperature and pressure a limited amount of hydrogen sulphide is produced; if this amount is insufficient to exceed the solubility product of copper sulphide the resulting solution would not be toxic for bacteria. He suggested that specific enzyme systems were being interfered with, possibly those concerned with the utilization of sulphydryl groups.

Konowalchuk et al (24, 25) reported that autoclaved media containing cysteine and ferric iron were toxic for Mycobacterium tuberculosis. They isolated the toxic substance and found that it appeared to be a colloidal complex of iron and sulphur. Prince and Cleverdon (36) reported that cysteine inhibited the growth of Flavobacterium species in simple chemically defined media. Histidine reversed the inhibition but several other amino acids were also partially effective in reversing the inhibition.

Rowley (41) collected 356 strains of E. coli that were capable of growing in the simple medium of Davis and Mingioli (8) and found that the growth of all strains was inhibited by certain amino acids. Cystine inhibited the growth of 40 strains of E. coli; methionine effectively reversed this inhibition with all strains tested. He stated "Since all the organisms examined grew readily on medium lacking amino-acids, they must all possess the enzymes necessary for the synthesis of these amino-acids from ammonium salts and glucose. Yet in the case of some strains the addition of a given amino-acid prevented or delayed growth. The presence of the amino-acid which abolished this

inhibition presumably supplies an end product of the particular inhibited pathway and the organism grows freely". Apparently Rowley did not autoclave the cystine solution.

Ravel and Shive (37) reported that cysteic acid, which is an oxidation product of cysteine, inhibited the growth of E. coli. They postulated that cysteic acid, which resembles aspartic acid, competes with aspartic acid in the formation of the "substrate-enzyme complex" that normally produces β- alanine. The resulting inhibition of growth may be reversed by increasing sufficiently the aspartic acid - cysteic acid ratio so that the competition for the specific enzyme greatly favors the formation of the aspartic acid enzyme complex i.e. reversal in a competitive manner. The inhibition may also be reversed by addition of β-alanine, the product of the aspartic acid enzyme reaction i.e. reversal in a non-competitive manner.

5. Inhibition of Pantothenic Acid Synthesis

The steps in the biosynthesis of Coenzyme A (CoA), which is the active form of pantothenic acid, are reasonably well established. The following scheme, compiled from reports in the literature outlines some of the probable steps in CoA synthesis.

1. Aspartic acid ⟶ β-alanine. (21)
2. Keto-valine ⟶ Ketopantoic acid ⟶ pantoic acid. (31)
3. β-alanine + pantoic acid ⟶ pantothenic acid. (30)
4. Pantothenic acid + cysteine ⟶ pantothenylcysteine. (2)
5. Pantothenylcysteine ⟶ pantetheine ⟶ CoA. (2)

Several compounds which inhibit growth of E. coli in simple media do so apparently because of interference with pantothenic acid synthesis. Wright and Skeggs (54) and King and Cheldelin (23) observed that propionic acid inhibited the growth of E. coli; the inhibition was reversed by either β-alanine or pantothenic acid. King and Cheldelin concluded that propionate inhibited growth by competing with β-alanine for a specific enzyme that united pantothenic acid moieties.

Ravel and Shive (37) and Shive and Macow (45) reported that both cysteic acid and hydroxyaspartic acid inhibited growth of E. coli; the inhibitions were reversed competitively by aspartic acid and non-competitively by either β-alanine or pantothenic acid. These workers postulated that because cysteic acid and hydroxyaspartic acid resembled aspartic acid structurally that they competitively inhibited the decarboxylation of aspartic acid to produce β-alanine which appears to be a precursor of pantothenic acid.

Maas and Davis (29) found that D-serine inhibited the growth of E. coli by interfering with the conversion of β-alanine to pantothenic acid, but they stated that the mechanism of the inhibition was obscure. Maas (30) reported that salicylates also interfere with pantothenic acid synthesis.

Jakobovits et al (19) studied the inhibitory effect of certain amino acids on the utilization of β-alanine by strains of Saccheromyces cerevisiae. Methionine, glutamic acid, asparagine and α-alanine particularly inhibited growth in media containing suboptimal amounts of β-alanine, but these amino acids were much less inhibitory in the presence of pantothenic acid. These workers suggested that α-alanine competed with β-alanine in pantothenic acid formation; they also suggested that glutamic acid and asparagine may inhibit growth indirectly through increased synthesis of α-alanine resulting from transamination reactions.

Several compounds seem to inhibit the biosynthesis of pantothenic acid. Hydroxyaspartic acid and cysteic acid may inhibit β-alanine formation, whilst propionic acid and α-alanine appear to prevent the utilization of β-alanine. Certain other compounds also inhibit pantothenic acid synthesis but the mechanisms are obscure. It is clear, however, that several compounds added singly to simple media may inhibit growth of microorganisms due to interference with pantothenic acid synthesis.

METHODS

1. Assay for Vitamin B_{12} Activity with E. coli 113-3.

In most tests the effect of various compounds on the growth of E. coli 113-3 was evaluated in a semi-quantitative manner by adding the compound or compounds to a standard solution of vitamin B_{12} and determining the apparent vitamin B_{12} activity of this "sample" by microbiological assay.

Reagents

(a) Standard cobalamin stock solution - Sufficient 25% alcohol was added to a suitable quantity of cyanocobalamin reference standard to make a solution containing 1 μg. of vitamin B_{12} per ml. This solution was stored in a refrigerator and was used no longer than 6 months.

(b) Standard cobalamin solution - Sufficient distilled water was added to 1 ml. of "standard cobalamin stock solution" so that the final solution contained 0.05 mμg. of vitamin B_{12} per ml. for a stationary assay or 0.2 mμg. of vitamin B_{12} per ml. for shaken assay.

(c) Basal medium stock solution - For the preparation of 500 ml. of double strength basal medium the amounts of ingredients listed were placed in a 600 ml. beaker.

Dextrose	2.0 gm.
$(NH_4)_2SO_4$	1.0 "
$MgSO_4 7H_2O$	0.1 "
Na-citrate. $3H_2O$	0.5 "
KH_2PO_4	3.0 "
K_2HPO_4	7.0 "
Distilled H_2O	500 ml.

This is Davis and Mingioli's medium (8).

(d) Inoculum broth - Two ml. of "vitamin-free" casein hydrolysate (10% solution) and 5 mμg. of vitamin B_{12} were added to 100 ml. of single strength basal medium stock solution. This inoculum broth was dispensed in 10 ml. amounts into 50 ml. Erlenmeyer flasks; the flasks were plugged with cotton and autoclaved for 10 minutes (121 - 123°C.)

(e) Agar slant culture medium - One and one half grams of agar were added to 100 ml. of inoculum broth and the mixture was heated on a steam bath with occasional stirring until the agar was in solution. Five ml. aliquots were dispensed into 16 mm. rimless pyrex test tubes; the tubes were plugged with cotton and autoclaved for 10 minutes (121 - 123°C.). The agar was cooled in a sloping position.

(f) Stock culture of E. coli 113-3 - The test organism E. coli 113-3 was subcultured twice weekly on the agar slant culture medium. Cultures were incubated for 8 hours at 31°C. and were then stored in a refrigerator.

(g) Inoculum .- A transfer of cells was made using a heavy inoculum from a fresh (24 - 72 hour) agar slant culture of E. coli 113-3 to a flask of inoculum broth. The culture was incubated for 8 hours at 31°C., and was then transferred aseptically to a suitable sterile centrifuge tube and centrifuged for 5 minutes. The supernatant medium was decanted and the cells were resuspended in 10 ml. of sterile single strength basal medium; this suspension was added dropwise to 10 ml. of medium until a final transmittancy of approximately 80% (Coleman Model 11) was obtained for the second suspension; this suspension was used for the inoculum.

Procedure

(a) Preparation of tubes - Hard glass test-tubes, 22 x 150 mm., and other necessary glassware were cleaned meticulously because of the sensitivity of the test organism to minute amounts of vitamin B_{12} activity.

Into clean test tubes 0.0, 1.0, 2.0, 3.0, 4.0 and 5.0 ml. respectively of standard cobalamin solution was added using 3 replicate tubes at each dosage level. Sufficient distilled water was added to make 5.0 ml.

Into clean test tubes 1.0, 2.0, 3.0, 4.0 and 5.0 ml. respectively of the sample was added using 3 replicate tubes at each dosage level. Sufficient distilled water was added to make 5.0 ml.

Two tubes were prepared, each containing 5.0 ml. of distilled water; these were used for diluting and suspending the inoculum.

All tubes were transferred to another rack and arranged in a random order. Five ml. of basal medium stock solution was added to each tube; tubes were covered with a cotton pad and heated in an autoclave for 3 minutes at 121 - 123°C. and they were cooled as rapidly as practicable.

One drop of inoculum was added aseptically to each tube. The cultures were incubated for 16 hours at a temperature of 31°C. Those used for shaken assays were incubated on a Yankee Kahn Test shaker with a speed of 275 oscillations per minute.

(b) Measurement of growth. - The tubes were chilled to arrest growth and the contents of each tube was transferred to optical colorimeter tubes. Using an uninoculated tube of culture medium and a filter

setting of 540 mμ the Coleman (Model 11) spectrophotometer was adjusted to read 100% transmission of light. The percent transmission values for all cultures was determined.

(c) Calculation of apparent vitamin B_{12} activity - A standard response curve was prepared by plotting the absorbance (2 - log G) readings for each dose of the standard vitamin B_{12} solution against mμg. of vitamin B_{12} contained in respective tubes. A ruler was used to draw the line that appeared to best fit the plotted points. The amount of vitamin B_{12} activity for each culture was determined by interpolation from this standard curve.

In microbiological assays since occasional inexplicable aberrant values are obtained in certain tubes, the series of values was inspected and any which varied markedly from most of the series were discarded. Usually the potency of the sample was expressed as a percentage of the standard response.

2. Assay for L-methionine with E. coli 113-3.

The assay for L-methionine was done in essentially the same way as the vitamin B_{12} assay (shaken) with E. coli 113-3. The standard solution of vitamin B_{12} was replaced by a standard solution of methionine containing 5 μg of DL-methionine per ml. The effect of various compounds on the growth of E. coli 113-3 with methionine was evaluated by adding the compound(s) either to an aliquot of the standard solution of methionine or to the medium before its addition to the tubes, and determining the apparent methionine content of this "sample" by microbiological assay.

3. "One dose" assay for vitamin B_{12} with E. coli 113-3.

Wood and Finney (53) described the one dose assay design; this design is only valid for assay purposes when the growth-response curves with the standard and test preparations are approximately linear and meet at the origin when they are plotted on an arithmetic scale. The growth-response curves for samples containing vitamin B_{12} and cystine in the shaken assay were not linear, however, and usually the highest dose of the sample gave the lowest apparent "potency".

The data for the one dose assay were plotted with an arithmetic scale; the average absorbance value for the one dose of the standard was plotted and a straight line was drawn from it to the origin. The apparent "potency" of the samples was determined by interpolation to the "standard curve" and was expressed as a percent of the standard response.

4. The pad-plate assay for vitamin B_{12} with E. coli 113-3.

The pad-plate assay was done as described by Williams et al (51). In this assay filter paper discs containing vitamin B_{12} are placed upon a flat surface of agar "seeded" with E. coli 113-3. Growth of the organism occurs around the discs and the size of the zones of growth is dependent upon the concentration of vitamin B_{12} in the paper disc.

5. Direct toxicity tests with E. coli 113-3.

The apparent toxicity of various compounds was evaluated in the following way. A series of tubes was prepared containing graded amounts of the compound being tested. In certain tests (e.g. cystine) the compound was dissolved in N/500 HCl. The volume of the liquid

in each tube was adjusted to 5.0 ml. and then 5.0 ml. of double strength medium were added to each tube; the medium contained sufficient vitamin B_{12} or methionine for optimal growth. A control series of tubes was also prepared containing similar graded amounts of a "blank" solution of either water or N/500 HCl; five tubes were used at each dosage level. In some tests the compound being tested (and the "blank solution") was sterilized by Seitz filtration and was added aseptically to the tubes of medium after these were autoclaved.

Tubes of media were autoclaved and inoculated as described in Method 1. After 16 hours the turbidities of cultures were determined and the average absorbance for the growth with each dosage level was plotted as the ordinate against the dose of the compound as the abscissa.

6. The *Lactobacillus leichmannii* assay for vitamin B_{12}.

The *L. leichmannii* turbidimetric assay was done as described by Campbell *et al* (4); this is a modification of the U.S.P. XIV (50) microbiological "tube" assay for vitamin B_{12}.

7. The thiochrome method for the assay of thiamine.

The thiochrome method for the assay of thiamine was done as described in "Methods of Vitamin Assay" (35). The thiochrome method depends upon the oxidation of the thiamine to thiochrome which fluoresces in ultra violet light. The fluoresence of the thiochrome solution is determined with a photofluorometer.

RESULTS

A. Studies Relating to the Thiamine Inhibition.

Experiment I. Test to detect destruction of vitamin B_{12} by thiamine.

This experiment was done to confirm the previous finding (34) that thiamine does not destroy vitamin B_{12} in the E. coli assay medium. Samples of vitamin B_{12} with and without thiamine were diluted with E. coli 113-3 basal medium to contain either 0.001 mg. of vitamin B_{12} per ml. (sample A) or 0.001 mg. of vitamin B_{12} and 0.5 mg. of thiamine per ml. (sample B). Duplicate 10.0 ml. aliquots or samples of each solution were pipetted into regular assay tubes, plugged with cotton and heated in the autoclave for 3 minutes at 15 lbs. pressure. The samples were shaken at 31° for 16 hours and then the potencies were determined with the L. leichmannii assay for vitamin B_{12}. Results of the assays are shown in Table I and are expressed as percentages of the standard vitamin B_{12} solution.

TABLE I

THE EFFECT OF THIAMINE ON THE STABILITY OF VITAMIN B_{12} IN CONDITIONS SIMILAR TO THOSE OF THE SHAKEN ASSAY.

Sample	Sample contained	Potency found (percent of the standard)
AI	Vitamin B_{12}	99
A2	Vitamin B_{12}	103
BI	Vitamin B_{12} + thiamine	104
B2	Vitamin B_{12} + thiamine	101

The data in Table I indicate that thiamine did not destroy vitamin B_{12} in conditions similar to those of the shaken assay for vitamin B_{12} with E. coli 113-3.

Experiment 2. The effect of shaking the culture medium before inoculation and incubation.

Another test was designed to confirm the finding that thiamine does not destroy vitamin B_{12} in the conditions of the shaken assay; this test was also designed to indicate the possible formation of toxic products. The effect of shaking the tubes of culture medium at 31° for 16 hours before inoculation and incubation was determined. In order to obtain precise assays a total of 120 cultures was used in this experiment. Results of this test are shown in Table II.

TABLE II

THE EFFECT OF SHAKING THE CULTURE MEDIUM FOR 16 HOURS BEFORE INOCULATION AND INCUBATION ON THE VITAMIN B_{12}-THIAMINE INTERRELATIONSHIP.

Treatment of medium and type of assay.	Relative response (Expressed as % of standard response)	
	Expt. I	Expt. 2
Culture medium shaken for 16 hours before inoculation - stationary assay.	99	97
Culture medium held without shaking for 16 hours before inoculation - stationary assay.	95	--
Culture medium held without shaking for 16 hours before inoculation - shaken assay.	--	73

It is evident from Table II that shaking the culture medium containing thiamine and vitamin B_{12} for 16 hours before inoculation and incubation of cultures had little or no effect on the growth response to vitamin B_{12}.

The inhibitory effect observed in experiment 2 is the usual effect obtained with samples containing both vitamin B_{12} and thiamine in the shaken assay. This test confirmed the previous finding that thiamine does not destroy vitamin B_{12} in the conditions of the shaken assay; from this experiment, it also appeared unlikely that a toxic degradation of thiamine is formed during the 16 hours incubation period of the shaken assay.

Experiment 3. Test for the possible conversion of vitamin B_{12} to vitamin B_{12b} by thiamine.

This test was made to determine if thiamine converts vitamin B_{12} to vitamin B_{12b} since various workers (5, 20, 34) reported that vitamin B_{12b} was only 75% as active as vitamin B_{12} for E. coli 113-3 in tube assays. McLaughlan et al (33) found that vitamin B_{12b} produced considerably larger zones of growth than vitamin B_{12} in the E. coli pad-plate assay. Conversion of vitamin B_{12} to vitamin B_{12b} by thiamine would be indicated by an apparent increase in potency of the sample containing thiamine.

In this test a sample containing 1.0 mg. of thiamine and 1.0 μg. of vitamin B_{12} was diluted with water and added to pads at the dosage levels of 0.01 and 0.02 μg. of vitamin B_{12} per pad. Twenty-four pads were used on both the standard and test preparations. Thiamine had no effect on the size of the zones of growth and therefore the potency obtained was nearly 100% of the standard indicating that thiamine did not convert vitamin B_{12} to vitamin B_{12b} in aerobic conditions. Although thiamine did not affect the size of the zones of growth a direct inhibitory effect of thiamine on the slight growth that occurs throughout the

whole plate was observed. Very large faint zones of inhibition surrounded each pad containing vitamin B_{12} and thiamine.

Experiment 4. The effect of aseptic addition of thiamine autoclaved separately in acid solution.

Since thiamine is partially destroyed by autoclaving in solutions at a neutral reaction (35) it appeared that degradation products of thiamine might be toxic for E. coli 113-3 in aerobic conditions. An experiment was done therefore in which thiamine was added aseptically to the autoclaved medium after the thiamine was autoclaved separately at an acid reaction.

A solution of thiamine was adjusted to pH 3.1 with hydrochloric acid and diluted to contain 20 μg. of thiamine per ml.; an appropriate acid solution containing no thiamine was also prepared. The thiamine solution and the "blank" acid solution were autoclaved and 0.1 ml. of each solution was added aseptically to each tube of the test and standard series respectively. A control test was also done in which thiamine was autoclaved with the medium.

The inhibitory effect of thiamine amounted to approximately 37% in the control test, i.e., thiamine autoclaved with the medium, and 35% in the test in which thiamine was autoclaved separately in an acid solution before addition to the medium. It would appear therefore that the inhibitory effect of thiamine in aerobic conditions is not due to the formation of toxic degradation products by autoclaving thiamine with the medium.

Experiment 5. Growth of variants of E. coli 113-3.

Dubnoff (10) reported that his culture of E. coli 113-3 contained variants that had vitamin B_{12} requirements differing from

the original culture. Therefore the culture used in this study was examined for the presence of similar variants. The stock culture was plated and several isolated cultures were tested in vitamin B_{12} assays. Only three of seven cultures tested grew with vitamin B_{12}. Since the stock culture of E. coli 113-3 was maintained on a medium containing both vitamin B_{12} and methionine presumably the other isolated cultures required methionine for growth. The three cultures that grew with vitamin B_{12} were identical to the original culture with respect to both vitamin B_{12} requirement and thiamine inhibition.

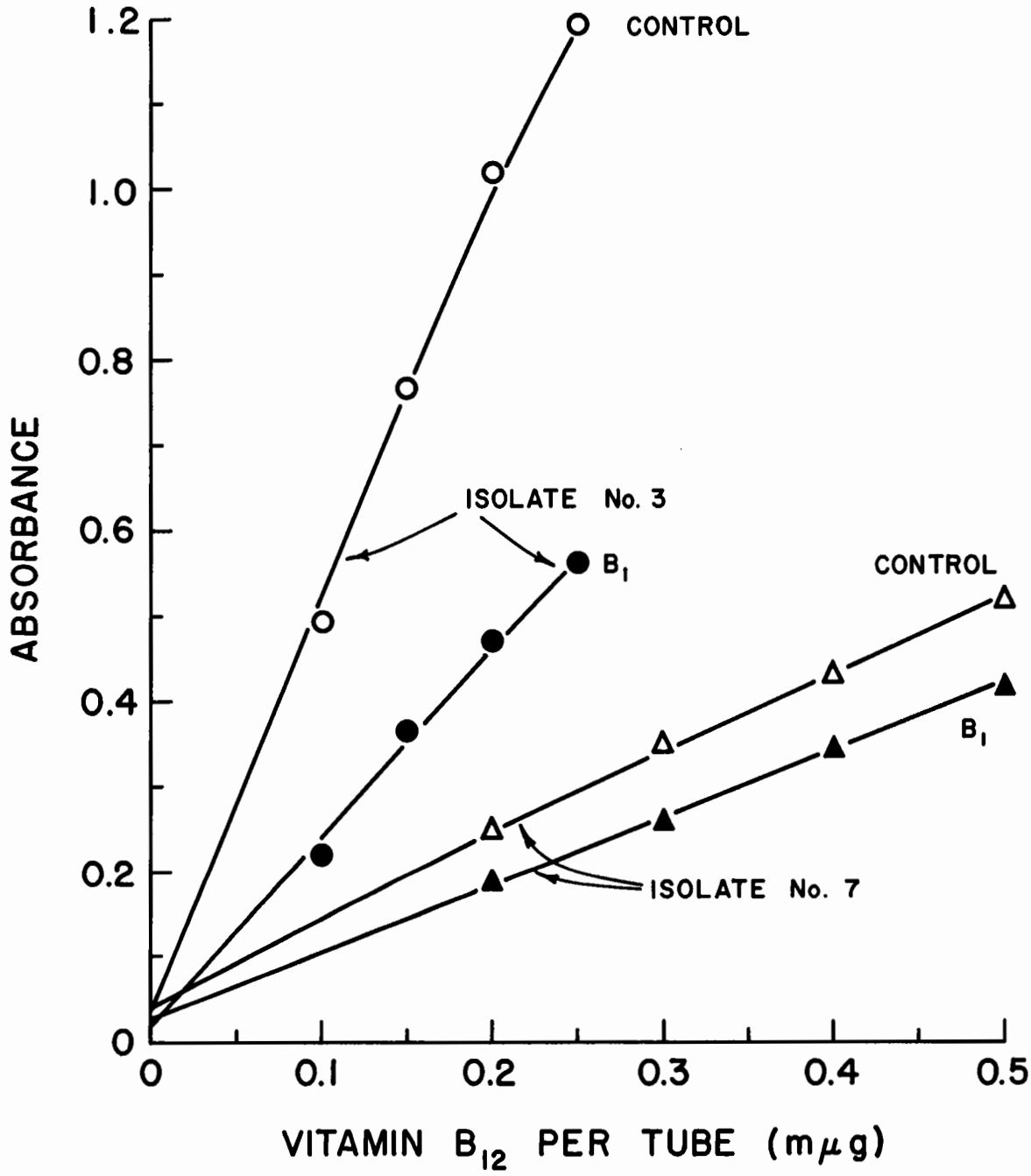
Several months later the culture was plated again and the isolates were tested in vitamin B_{12} assays. Two of the five cultures tested, required considerably less vitamin B_{12} for growth than the original strain of E. coli. The other three cultures appeared to be identical to the original culture. Growth of the typical and atypical cultures was examined in the presence and absence of thiamine; the growth-response curves with vitamin B_{12} are given in Fig. I. The atypical culture, designated as No. 3, had a relatively small requirement for vitamin B_{12}; thiamine depressed the growth-response curve of this variant by about 50%. Isolate No. 7 appeared to be identical to the original culture with respect to both vitamin B_{12} requirement and thiamine inhibition.

Experiment 6. The uptake of thiamine by E. coli 113-3.

Since relatively small amounts of thiamine were just as effective as much larger amounts in producing the inhibition, the effect appeared to be directly related to the metabolism of the organism. It was possible, however, that a physical factor such as an altered oxidation-reduction potential of the medium might be responsible for the effect

Fig. 1. Growth-response curves of "variants" of E. coli 113-3 to vitamin B_{12} with and without thiamine; the cultures were shaken during growth.

Fig. 1. Growth-response curves of "variants" of E. coli 113-3 to vitamin B_{12} with and without thiamine; the cultures were shaken during growth.


1.2
1.0
0.8
0.6
0.4
0.2
0
ABSORBANCE
CONTROL
ISOLATE No. 3
B1
CONTROL
B1
ISOLATE No. 7
0
0.1
0.2
0.3
0.4
0.5
VITAMIN B12 PER TUBE (mμg)

with added thiamine. A test was done therefore to determine whether or not E. coli 113-3 removed thiamine from the medium during growth.

Tubes of culture medium were prepared containing the basal medium and graded amounts of vitamin B_{12}. One µg. of thiamine was added to each tube. After 16 hours incubation the cultures were boiled for a few minutes to disrupt the cells; the cells were then centrifuged. The thiamine content of the cells was estimated by the thiochrome method following a takadiastase treatment (35). The results of this test are given in Table III.

TABLE III

UPTAKE OF THIAMINE FROM THE MEDIUM BY GROWING CELLS OF E. COLI 113-3.

Vitamins added (µg. per 10 ml.)		Thiamine content (µg.)		Total % recovery
B_{12}	Thiamine	Of supernatant	Of cells	
0	1.0	0.64	0*	64
0.2	1.0	0.41	0.27	68
0.4	1.0	0.27	0.39	66
0.6	1.0	0*	0.71	71
0.8	1.0	0*	0.61	61
1.0	1.0	0*	0.71	71
1.0	0	-	0.17	--

* Too low to estimate.

It is evident from Table III that E. coli 113-3 (original culture) removed thiamine from the medium during growth; with relatively small amounts of vitamin B_{12}, however, growth of the organism was limited and

thiamine was incompletely removed from the medium. The total recovery of thiamine was low but this was probably due to the instability of thiamine in solution at a neutral reaction.

Experiment 7. Relation between incubation time and the thiamine effect.

Although the turbidities of cultures in both the shaken and stationary assays are usually determined after 16 hours of incubation, a different phase of growth is reached in the two assays; growth is essentially complete only in the stationary assay. It appeared possible therefore that the difference between the thiamine inhibition in the shaken and stationary assays might be related to the length of the incubation period.

The thiamine inhibition was tested using a 12 hour incubation time for the stationary assay and a 24 hour incubation time for the shaken assay. The data for this experiment are given in Table IV.

TABLE IV

RELATION BETWEEN THE INCUBATION TIME AND THE THIAMINE EFFECT.

	Response in presence of thiamine (expressed as % of standard)		
Incubation time (hours)	12	16	24
Shaken assay	--	71	64
Stationary assay	97	95*	--

* Average value for several assays.

The data indicate that thiamine produced little or no detectable inhibition of growth in the stationary assay even with a short incubation time. The inhibitory effect of thiamine did not decrease with a longer

incubation time in the shaken assay. The data indicate therefore that the difference in the thiamine effect with shaken and stationary assays is not related to the different growth phases reached in the two assays when growth determinations are made.

Experiment 8. The effect of aeration on (a) the thiamine inhibition and (b) the growth response to vitamin B_{12}.

(a) Repeated tests (34) have indicated that the thiamine inhibition amounted to approximately 5% in the stationary assay and 25% in the shaken assay with 22 mm. culture tubes. The difference between the thiamine effect with the two assays appeared therefore to be related to the oxygen tension of the medium. The oxygen tension is increased by agitation of the medium and by increasing the ratio of the area of the liquid-air interface to the volume of the medium.

The results of an experiment to evaluate the magnitude of the thiamine inhibition when cultures were grown in various sized culture tubes and flasks are given in Table V. Each value in Table V represents an assay for vitamin B_{12} with thiamine; 133 cultures were used in this test).

TABLE V.

RELATIONSHIP BETWEEN THE THIAMINE INHIBITION AND OXYGEN TENSION.

Type of assay	Percent inhibition (expressed as % of standard response)				
	Diameter of tubes mm.				
	16	18	20	22	45*
Stationary	3	--	--	--	11
Shaken	21	26	25	30	--

* 50 ml. Erlenmeyer flasks.

The intermediate effect observed when cultures were grown with thiamine in 50 ml. Erlenmeyer flasks was confirmed in a separate test. The data indicate that the extent of the thiamine inhibition is directly related to the oxygen tension of the medium; within certain limits increasing the degree of aerobiosis produces an increase in the inhibitory effect of thiamine.

(b) With relatively small amounts of vitamin B_{12}, it was frequently observed that more growth occurred within 16 hours in the semi-anaerobic conditions of the stationary assay than in the aerobic conditions of the shaken assay. Although the incubation temperature was the same for the shaken and stationary assay, different incubators were used. A test was done therefore in more controlled conditions to learn whether or not semi-anaerobic conditions stimulated growth with relatively small amounts of vitamin B_{12}.

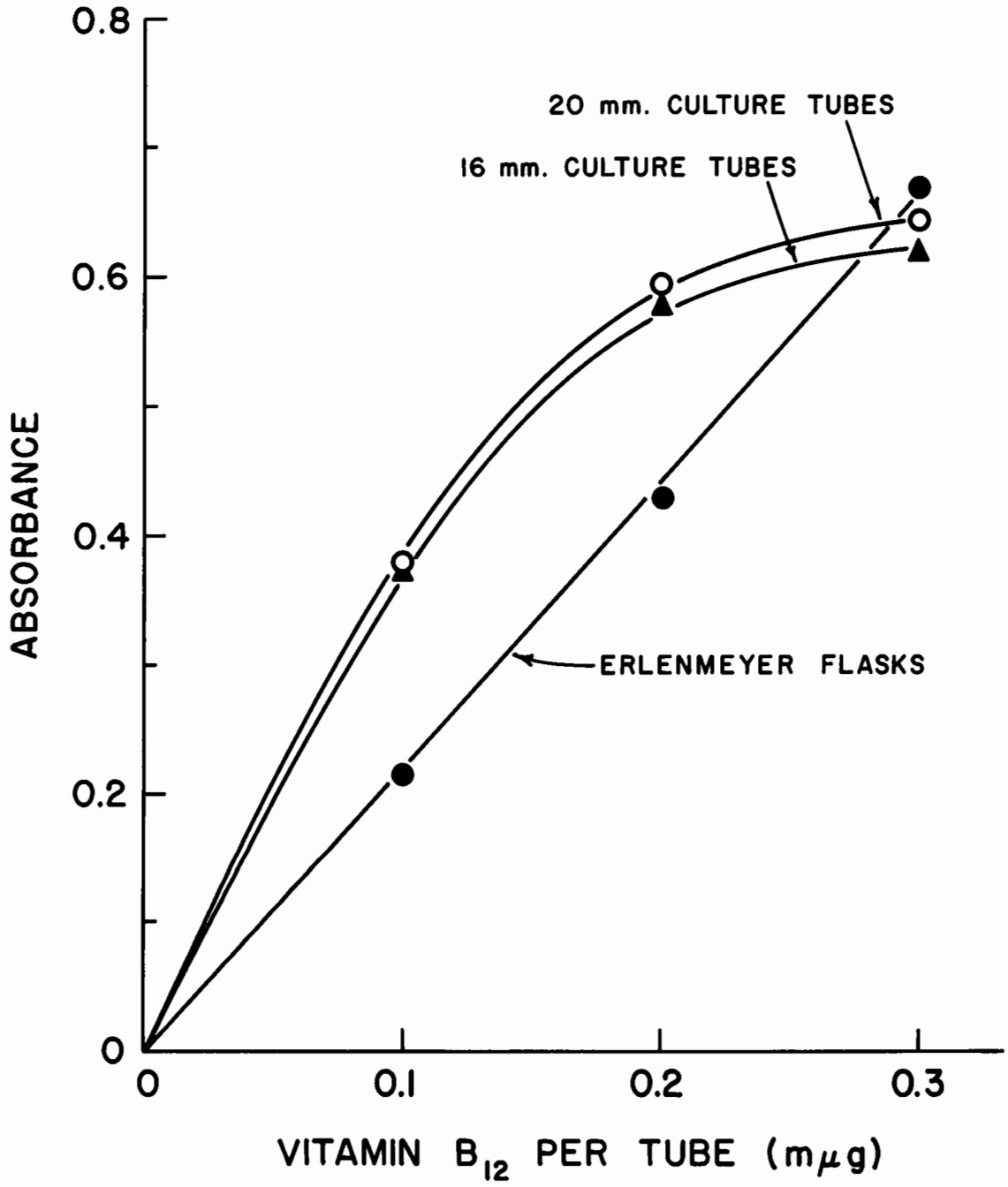
Cultures of E. coli 113-3 were grown with four concentrations of vitamin B_{12}, without shaking in 16 and 22 mm. tubes and in 50 ml. Erlenmeyer flasks; each vessel contained 10 ml. of medium. Results of this test are given in Fig. 2. It is evident that more growth was obtained with small amounts of vitamin B_{12} in semi-anaerobic than in aerobic conditions.

Experiment 9. An attempt to detect disulphide formation.

McLaughlan et al (34) have suggested that thiamine may react with essential sulphydryl compounds, producing mixed disulphides. Cysteine and homocysteine are sulphydryl compounds that appear to be intermediate compounds in the biosynthesis of methionine; therefore, reactions producing mixed disulphides may compete with reactions

Fig. 2. The effect of the degree of aerobiosis on the growth-response of *E. coli* 113-3 to relatively small amounts of vitamin B_{12}. The degree of aeration depended upon the diameter of the culture vessels; the cultures were not shaken during the incubation.

Fig. 2. The effect of the degree of aerobiosis on the growth-response of E. coli 113-3 to relatively small amounts of vitamin B_{12}. The degree of aeration depended upon the diameter of the culture vessels; the cultures were not shaken during the incubation.


0.8
0.6
0.4
0.2
0
ABSORBANCE
20 mm. CULTURE TUBES
16 mm. CULTURE TUBES
ERLENMEYER FLASKS
0
0.1
0.2
0.3
VITAMIN B_{12} PER TUBE (mμg)

producing methionine for these essential sulphydryl compounds. If cultures grown aerobically with thiamine contained a "pool" of inactive disulphides it seemed likely that the disulphides would be reduced when the cultures were placed in semi-anaerobic conditions. Cultures were grown aerobically with thiamine and sub-optimal amounts of vitamin B_{12} for 15 hours and then the cultures were placed in semi-anaerobic conditions and the growth of cultures with thiamine was compared with the growth of the control cultures.

Twenty cultures were incubated aerobically in media containing 0.4 mμg of vitamin B_{12} per tube (Evelyn colorimeter tubes); 10 cultures contained also 0.5 μg. of thiamine per tube. After 15 hours the turbidities of all cultures were read with an Evelyn colorimeter containing a 540 mμ filter. Five control cultures and 5 of the cultures with thiamine were then transferred to another incubator and grown semi-anaerobically for 10 hours; the other cultures were grown aerobically during the same period. The turbidities of all cultures were determined at intervals with an Evelyn Colorimeter.

The growth curves for this experiment are given in Fig. 3. It appears that cultures containing thiamine grew more than the control cultures during the period of semi-anaerobic growth, but there did not seem to be any increase in the rate of growth of cultures containing thiamine when they were placed in semi-anaerobic conditions. This experiment did not appear to indicate that a "pool" of disulphides containing essential sulphydryl compounds existed within organisms grown with thiamine.

Fig. 3. Growth curves of E. coli 113-3 with a growth-limiting amount of vitamin B_{12}, with and without thiamine (B_1). All cultures were shaken for the first 15 hours of the incubation period but half the cultures were incubated without shaking for the last 10 hours.

Fig. 3. Growth curves of E. coli 113-3 with a growth-limiting amount of vitamin B_{12}, with and without thiamine (B_1). All cultures were shaken for the first 15 hours of the incubation period but half the cultures were incubated without shaking for the last 10 hours.

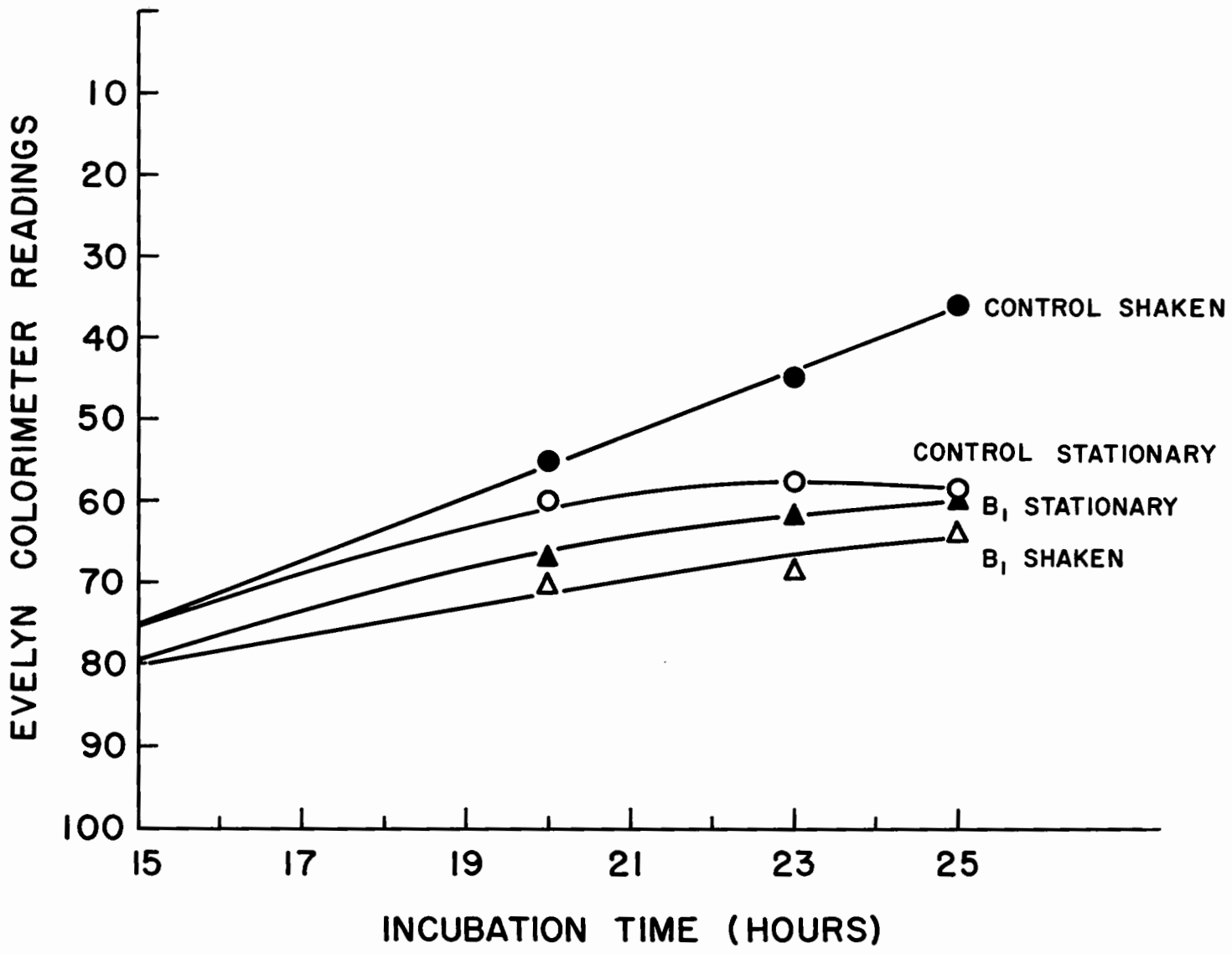
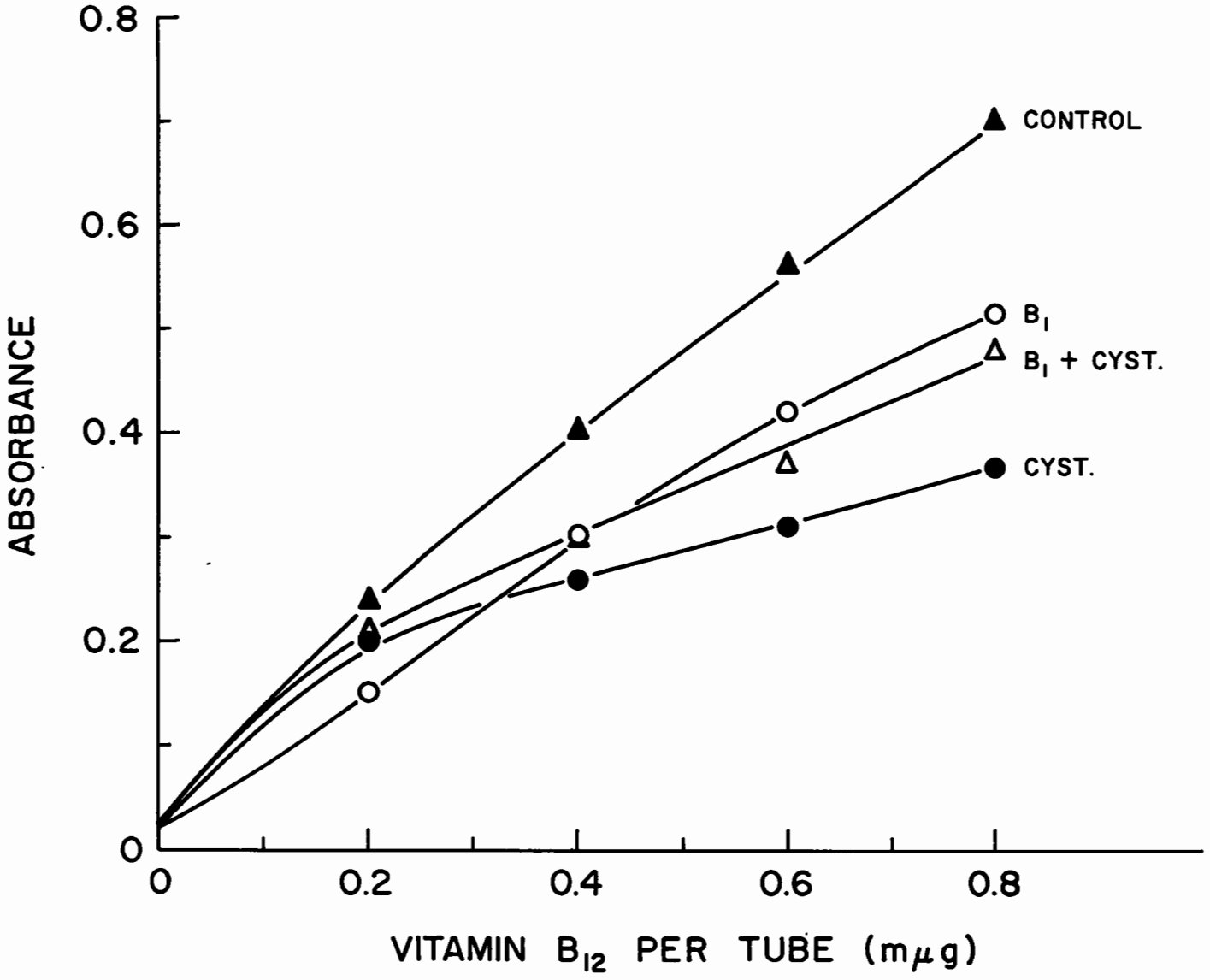

EVELYN COLORIMETER READINGS
10
20
30
40
50
60
70
80
90
100
15
17
19
21
23
25
INCUBATION TIME (HOURS)
CONTROL SHAKEN
CONTROL STATIONARY
B1 STATIONARY
B1 SHAKEN

Fig. 4. The effect of thiamine (B_1), of L-cystine (CYST), and of thiamine plus cystine, on the growth-response of *E. coli* 113-3 to vitamin B_{12} in the shaken assay.


0.8
0.6
0.4
0.2
0
ABSORBANCE
0
0.2
0.4
0.6
0.8
VITAMIN B_{12} PER TUBE (mμg)
CONTROL
B_1
B_1 + CYST.
CYST.

B. Studies Relating to the Cystine Inhibition.

Experiment 1. The thiamine-cystine interrelationship.

Matsukawa and Yurugi (32) found that cystine reacted with the thiol form of thiamine to produce a mixed disulphide. If cystine inhibited growth due to disulphide formation with thiamine synthesized by the organism, it appeared likely that thiamine would reverse the cystine inhibition. In preliminary tests added thiamine seemed to partially reverse the cystine inhibition but only a few cystine-thiamine ratios were tested; therefore the effect of a range of cystine-thiamine ratios on the growth of E. coli 113-3 was studied.

In the first two tests all tubes contained 0.4 mμg. of vitamin B_{12} per tube and assays were done as single dose assays with 4 replicate tubes for each assay. The data for the 1st test are given in Table VI.

TABLE VI

EFFECT OF THIAMINE AND CYSTINE ON THE RESPONSE OF E. COLI 113-3 TO VITAMIN B_{12}.
(RESPONSE EXPRESSED AS PER CENT OF STANDARD RESPONSE)

Thiamine added	Cystine added (μg/tube)			
(μg/tube)	0	0.2	1.0	10.0
0	100	103	100	90
0.25	78	78	76	78
0.5	77	76	74	74
2.0	78	76	74	74

From the data it appeared that cystine was not inhibitory at concentrations of 1 μg or less per tube. With the relatively low concentration of vitamin B_{12} the cystine inhibition with 10 μg per tube was less marked than the thiamine inhibition and there was no apparent interrelationship of thiamine and cystine. The data for the 2nd test are given in Table VII.

TABLE VII.

EFFECT OF THIAMINE AND CYSTINE ON THE RESPONSE OF *E. COLI* 113-3 TO VITAMIN B_{12}.
(Response expressed as percent of standard response).

Thiamine added	Cystine added μg/tube			
(μg/tube)	0	10	20	40
0	100	79	76	80
0.02	93	90	80	80
0.1	77	75	78	80
0.5	75	72	75	75

It seemed that thiamine partially reversed the cystine inhibition with cultures containing 0.02 and 10 μg of thiamine and cystine respectively.

In the 3rd test all cultures contained 0.6 mμg of vitamin B_{12} per tube and 5 replicate cultures were used for each of the 16 assays. The results of this test are given in Table VIII.

TABLE VIII.

EFFECT OF THIAMINE AND CYSTINE ON THE RESPONSE OF *E. COLI* 113-3 TO VITAMIN B_{12}.
(Response expressed as percent of standard response).

Thiamine added	Cystine added (μg/tube)			
(μg/tube)	0	25	50	100
0	100	40	40	36
0.1	107	55	50	69
0.5	103	52	48	64
1.0	94	51	46	66

In this test growth was more abundant than usual and the thiamine inhibition was not evident, whilst the cystine inhibition was quite marked. Thiamine appeared to reverse partially the cystine inhibition particularly with cultures containing 0.1 μg of thiamine per tube. From these tests it appeared that thiamine only slightly reversed the cystine inhibition.

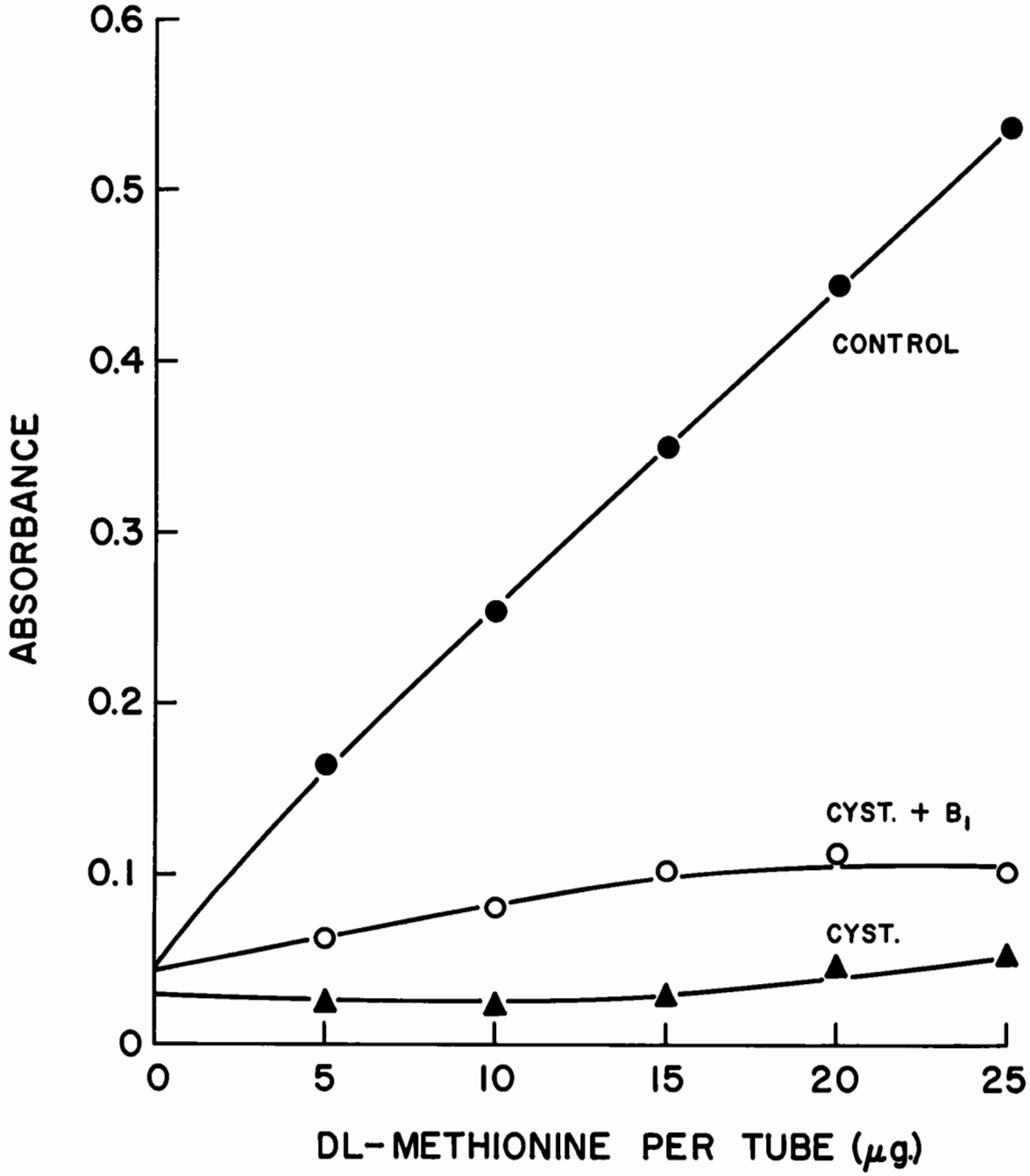
Experiment 2. The cystine inhibition with methionine.

Thiamine did not inhibit growth of *E. coli* 113-3 when the medium contained methionine; therefore the effect of cystine was determined with methionine replacing vitamin B_{12} in the medium. Cystine was tested at a concentration of 100 μg per tube and a series of cultures containing 0.5 μg of thiamine was also included to test for the possible reversal of the cystine inhibition.

The growth-response curves with methionine are given in Fig. 5. It seemed that cystine was toxic when the medium contained methionine and that thiamine slightly reversed the cystine inhibition.

Fig. 5. The effect of L-cystine (CYST) and of L-cystine plus thiamine (B_1) on the growth-response of E. coli 113-3 to DL-methionine in the shaken assay.

Fig. 5. The effect of L-cystine (CYST) and of L-cystine plus thiamine (B_1) on the growth-response of E. coli 113-3 to DL-methionine in the shaken assay.


0.6
0.5
0.4
0.3
0.2
0.1
0
ABSORBANCE
CONTROL
CYST. + B1
CYST.
0
5
10
15
20
25
DL-METHIONINE PER TUBE (μg.)

Experiment 3. The effect of several concentrations of cystine on growth with methionine.

The inhibitory effect of cystine appeared to be greater when the medium contained methionine in place of vitamin B_{12}. The effects of several concentrations of cystine on the growth-response curves with methionine were tested and the curves are given in Fig. 6. It appeared that the inhibitory effect of cystine increased with increasing amounts of cystine.

Experiment 4. Comparison of the cystine inhibition with vitamin B_{12} and with methionine.

There is always some variation from one assay to another in the growth-response with definite amounts of a growth-factor, and possibly this might account for the apparent difference of the inhibitory effect of cystine with vitamin B_{12} and with methionine. Therefore, the toxicity of cystine with vitamin B_{12} and with methionine was tested in as nearly identical conditions as possible. Direct toxicity tests with cystine were done; all tubes contained relatively large amounts of either vitamin B_{12} (0.05 μg) or DL-methionine (100 μg).

E. coli 113-3, isolate No. 3, was used in this experiment and the inhibition curves are given in Fig. 7. It appeared that cystine was more toxic when E. coli 113-3 was growing with methionine than when it was growing with vitamin B_{12}.

Experiment 5. The effect of cystine and homocystine in shaken and stationary assays.

Thiamine had only a slight inhibitory effect in stationary assays and therefore the effect of cystine was also studied in the stationary assay. Homocystine, the next higher homologue of cystine

Fig. 6. The effects of several concentrations of L-cystine on the growth-response of E. coli 113-3 to DL-methionine in the shaken assay.

Fig. 6. The effects of several concentrations of L-cystine on the growth-response of E. coli 113-3 to DL-methionine in the shaken assay.

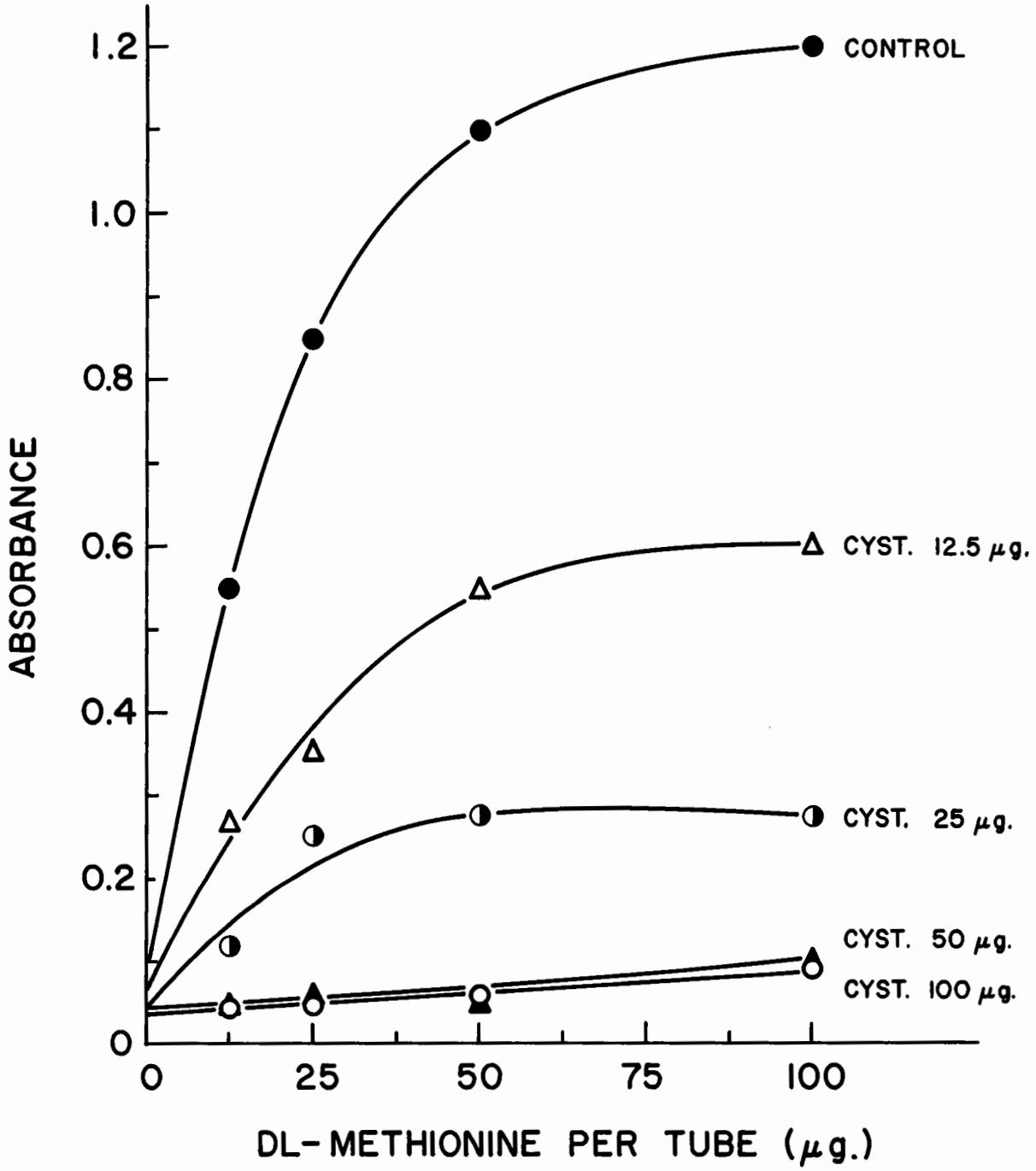
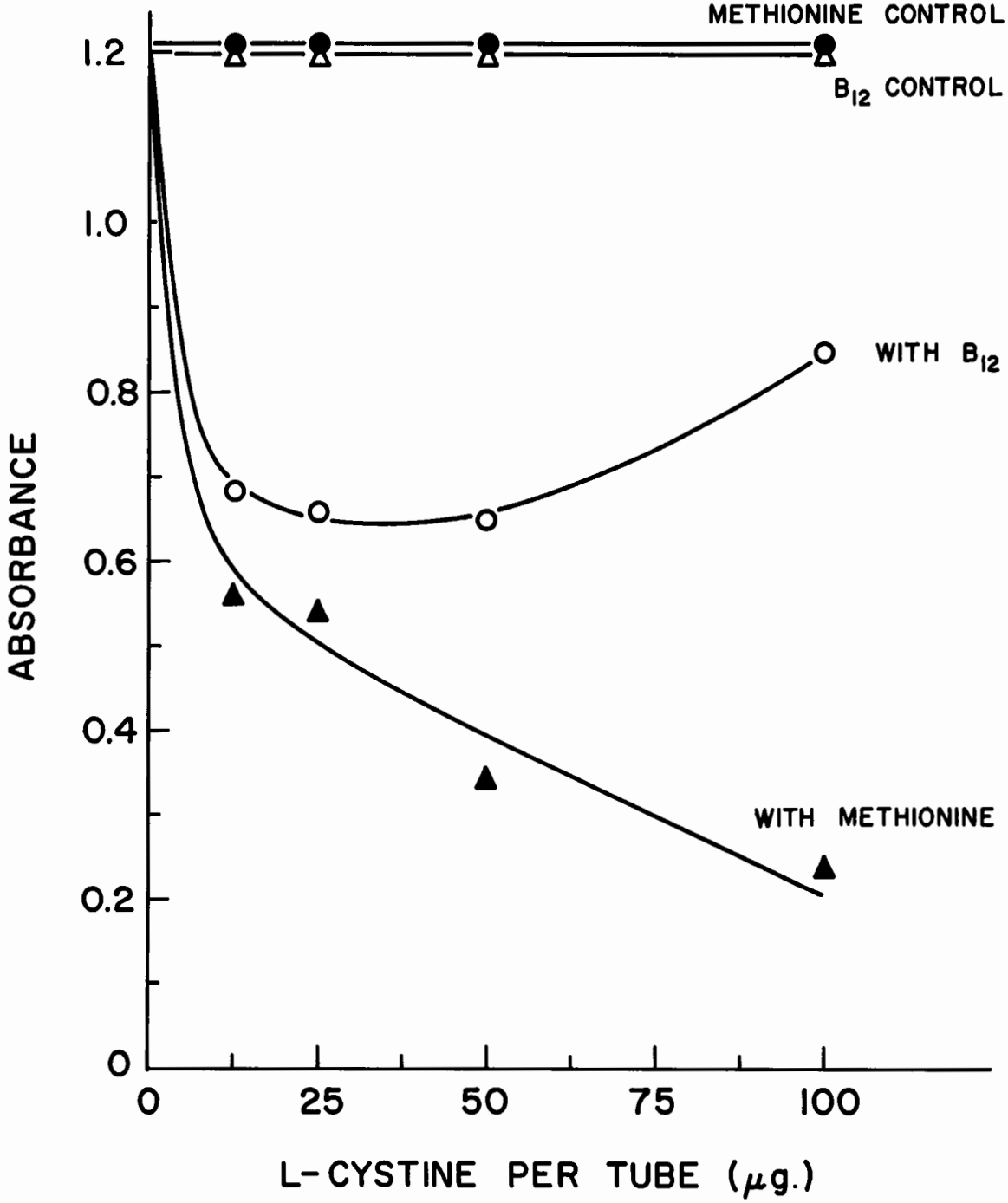

1.2
1.0
0.8
0.6
0.4
0.2
0
ABSORBANCE
0
25
50
75
100
DL-METHIONINE PER TUBE (μg.)
CONTROL
CYST. 12.5 μg.
CYST. 25 μg.
CYST. 50 μg.
CYST. 100 μg.

Fig. 7. The toxicity of autoclaved L-cystine for E. coli 113-3 (isolate No. 3) with optimal amounts of vitamin B_{12} or DL-methionine for growth. The cultures were shaken during the incubation.

Fig. 7. The toxicity of autoclaved L-cystine for E. coli 113-3 (isolate No. 3) with optimal amounts of vitamin B_{12} or DL-methionine for growth. The cultures were shaken during the incubation.


METHIONINE CONTROL
B12 CONTROL
WITH B12
WITH METHIONINE
ABSORBANCE
1.2
1.0
0.8
0.6
0.4
0.2
0
0
25
50
75
100
L-CYSTINE PER TUBE (μg.)

was also included in this experiment.

The growth-response curves to vitamin B_{12} with E. coli 113-3 (isolate 3) are given in Figs. 8 and 9 for the shaken and stationary assays respectively. It appeared that both cystine and homocystine inhibited growth in the shaken assay but it seemed that homocystine inhibited growth whilst cystine stimulated growth in the stationary assay.

Experiment 6. Toxicity of autoclaved cystine solutions.

There are several reports in the literature indicating that cystine is toxic for various bacteria, particularly when autoclaved with media. The effect of cystine was tested, therefore, after it was sterilized by Seitz filtration and added aseptically to the medium.

A solution of cystine (200 μg/ml.) in N/50 hydrochloric acid was sterilized by Seitz filtration; 0.5 ml. of the cystine solution was added aseptically to assay tubes containing only 9.5 ml. of media after these were autoclaved as usual. A N/50 hydrochloric acid solution was also sterilized by Seitz filtration and 0.5 ml. of the dilute acid was added to each tube in the standard (or control) series of tubes. For comparison a similar assay was done in which the cystine was autoclaved with the medium.

The growth curves for this experiment are given in Fig. 10. It appeared that cystine was inhibitory only when it was autoclaved with the medium; in fact cystine seemed to stimulate growth when it

Fig. 8. The effect of L-cystine and of L-homocystine on the growth-response of E. coli 117-3 (isolate No. 3) to vitamin B_{12} in the absence assay.

Fig. 8. The effect of L-cystine and of L-homocystine on the growth-response of *E. coli* 113-3 (isolate No. 3) to vitamin B_{12} in the shaken assay.

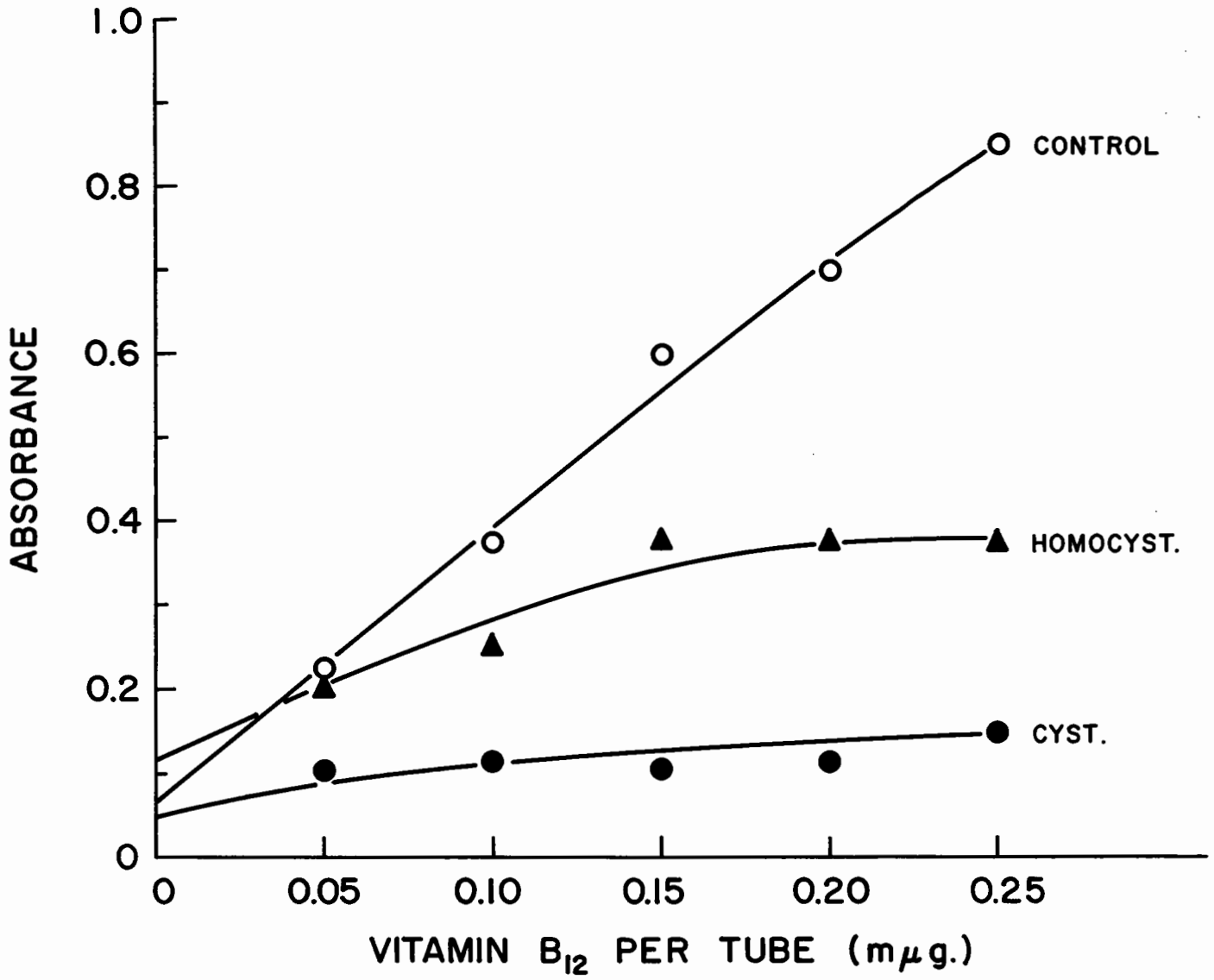

1.0
0.8
0.6
0.4
0.2
0
ABSORBANCE
CONTROL
HOMOCYST.
CYST.
0
0.05
0.10
0.15
0.20
0.25
VITAMIN B_{12} PER TUBE (mμg.)

Fig. 9. The effect of L-cystine and L-homocystine on the growth-response of *E. coli* 113-3 (isolate No. 3) to vitamin B_{12} in the stationary assay.

Fig. 9. The effect of L-cystine and L-homocystine on the growth-response of *E. coli* 113-3 (isolate No. 3) to vitamin B_{12} in the stationary assay.

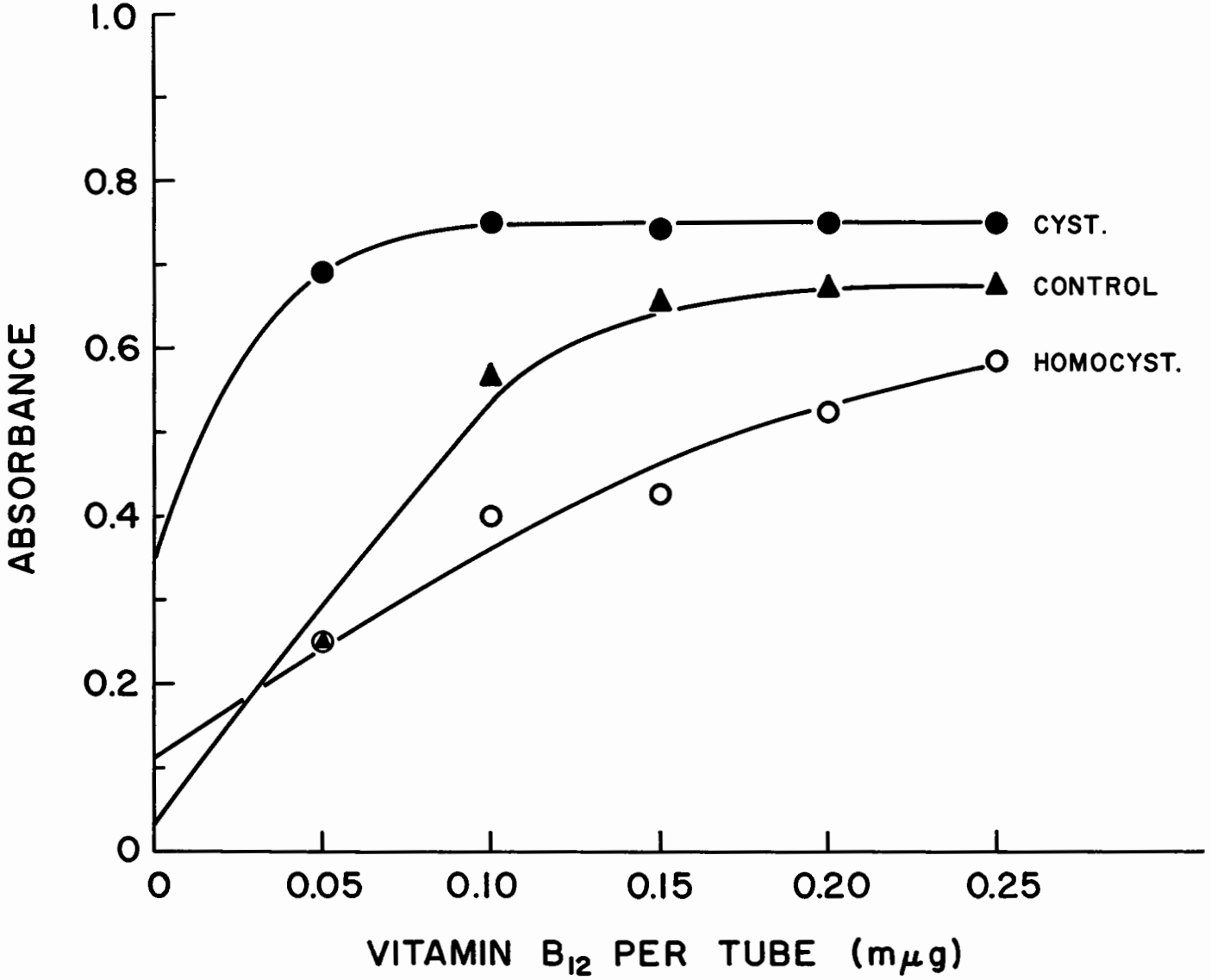
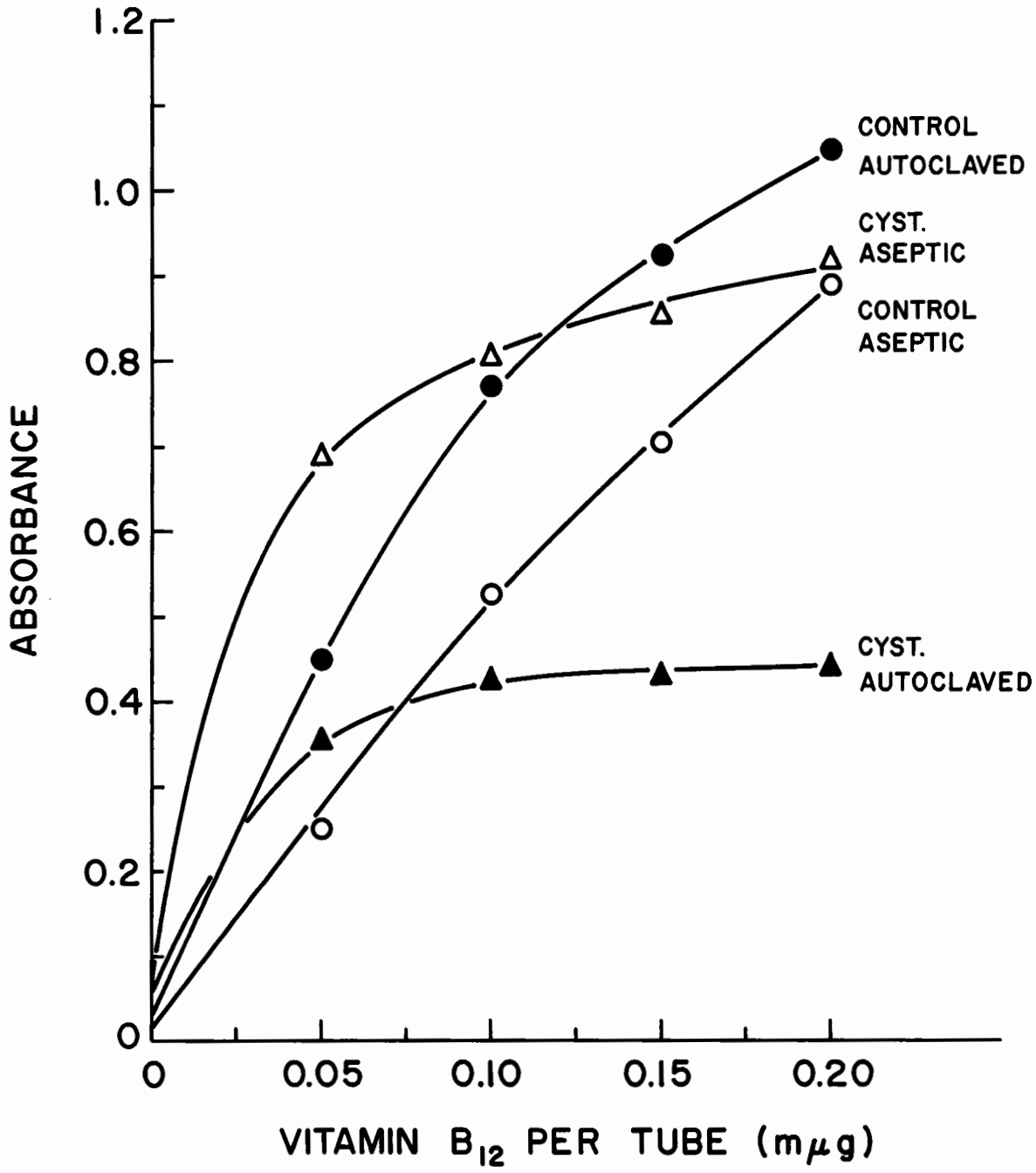

1.0
0.8
0.6
0.4
0.2
0
ABSORBANCE
0
0.05
0.10
0.15
0.20
0.25
VITAMIN B_{12} PER TUBE (mμg)
CYST.
CONTROL
HOMOCYST.

Fig. 10. The effect of L-cystine, on the growth-response of *E. coli* 113-3 (isolate No. 3) to vitamin B_{12} when added aseptically and when autoclaved with the medium.

Fig. 10. The effect of L-cystine, on the growth-response of *E. coli* 113-3 (isolate No. 3) to vitamin B_{12} when added aseptically and when autoclaved with the medium.


1.2
1.0
0.8
0.6
0.4
0.2
0
ABSORBANCE
0
0.05
0.10
0.15
0.20
VITAMIN B_{12} PER TUBE (mμg)
CONTROL AUTOCLAVED
CYST. ASEPTIC
CONTROL ASEPTIC
CYST. AUTOCLAVED

was added aseptically to the medium after Seitz filtration. Despite the apparent stimulatory effect of cystine the shape of the growth-response curve appeared to indicate that some factor other than vitamin B_{12} was limiting the amount of growth with the high dose of vitamin B_{12}.

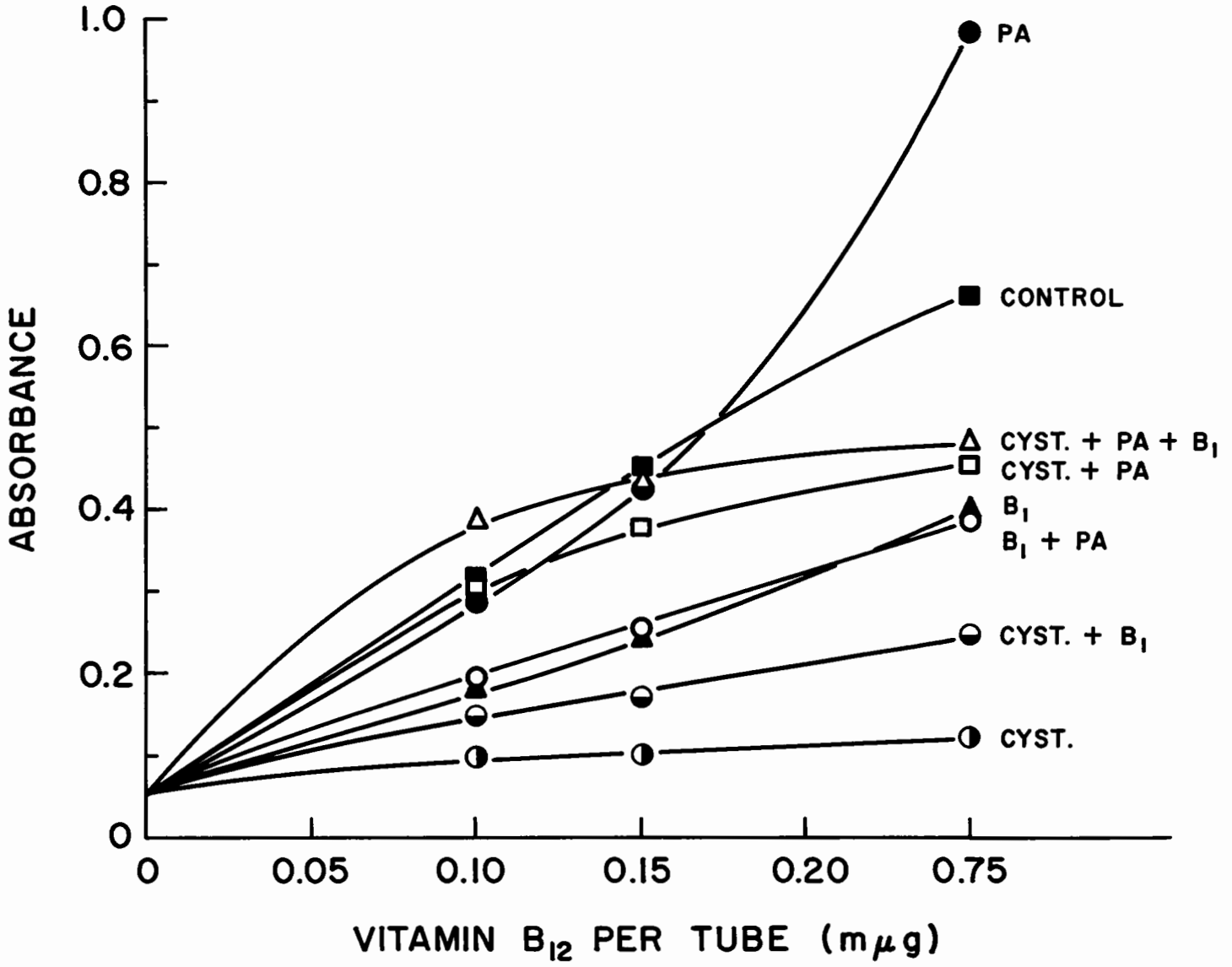
Experiment 7. Partial reversal of "autoclaved cystine" toxicity by pantothenic acid.

It appeared possible that some cysteic acid might be produced during autoclaving of cystine solutions since Ravel and Shive (37) found that thiamine partially reversed the cysteic acid inhibition with E. coli. They also found that pantothenic acid and certain compounds that appeared to be precursors of pantothenic acid completely reversed the cysteic acid inhibition; therefore the effect of pantothenic acid on the toxicity of autoclaved cystine was studied.

Pantothenic acid, with and without thiamine was tested for ability to reverse the cystine inhibition. The amounts of pantothenic acid and thiamine added per tube were 1.0 and 0.2 μg respectively. The growth-response curves for this experiment are given in Fig. 11; due to the relatively large number of assays involved only 3 replicate cultures were used for each dosage level of vitamin B_{12}. Pantothenic acid appeared to be more effective than thiamine in reversing the "cystine toxicity" but pantothenic acid and thiamine may have been more effective than pantothenic acid. Pantothenic acid also seemed to stimulate growth with the highest dose of vitamin B_{12}.

Fig. 11. The reversing effect of thiamine (B_1), of pantothenic acid (PA) and of a mixture of thiamine and pantothenic acid on the inhibitory effect of L-cystine (CYST) with E. coli 113-3 (isolate No. 3).

Fig. 11. The reversing effect of thiamine (B_1), of pantothenic acid (PA) and of a mixture of thiamine and pantothenic acid on the inhibitory effect of L-cystine (CYST) with E. coli 113-3 (isolate No. 3).


1.0
0.8
0.6
0.4
0.2
0
ABSORBANCE
0
0.05
0.10
0.15
0.20
0.75
VITAMIN B12 PER TUBE (mμg)
PA
CONTROL
CYST. + PA + B1
CYST. + PA
B1
B1 + PA
CYST. + B1
CYST.

Experiment 8. The effect of a mixture of glutamic acid, aspartic acid, pantothenic acid and thiamine on the cystine inhibition.

Ravel and Shive (37) found that aspartic acid and glutamic acid which appeared to be precursors of pantothenic acid reversed the cysteic acid inhibition with E. coli. An attempt was made, therefore, to completely reverse the cystine inhibition by adding to the medium several of the compounds that Ravel and Shive found effective in reversing the cysteic acid inhibition. The combination of compounds added and the amounts per tube were: aspartic acid, 10 μg; glutamic acid, 10 μg; pantothenic acid, 2 μg; and thiamine, 0.01 μg.

The growth response curves for this experiment are given in Fig. 12. In this experiment pantothenic acid alone did not appear to affect the cystine inhibition, nor did it stimulate growth with the high dose of vitamin B_{12}. The combination of compounds, however, appeared to reverse almost completely the cystine inhibition, but the shape of the growth-response curve seemed to indicate that some factor other than a deficiency of vitamin B_{12} was limiting the growth to some extent.

Experiment 9. The reversing effects of pantothenic acid and β-alanine on the cystine inhibition with methionine.

Since the cystine inhibition appeared to be more severe when the medium contained methionine in place of vitamin B_{12}, the reversing effects of pantothenic acid and of β-alanine were tested with the methionine assay. Relatively small amounts of DL-methionine were

Fig. 12. The reversing effect of pantothenic acid (PA) and of a mixture of pantothenic acid, pyruvic acid (PA), aspartic acid (AA) and thiamine (B_1) on the inhibitory effect of L-cystine (CYST) with E. coli 113-3 (isolate No. 3).

Fig. 12. The reversing effect of pantothenic acid (PA) and of a mixture of pantothenic acid, glutamic acid (GA), aspartic acid (AA) and thiamine (B_1) on the inhibitory effect of L-cystine (CYST) with E. coli 113-3 (isolate No. 3).

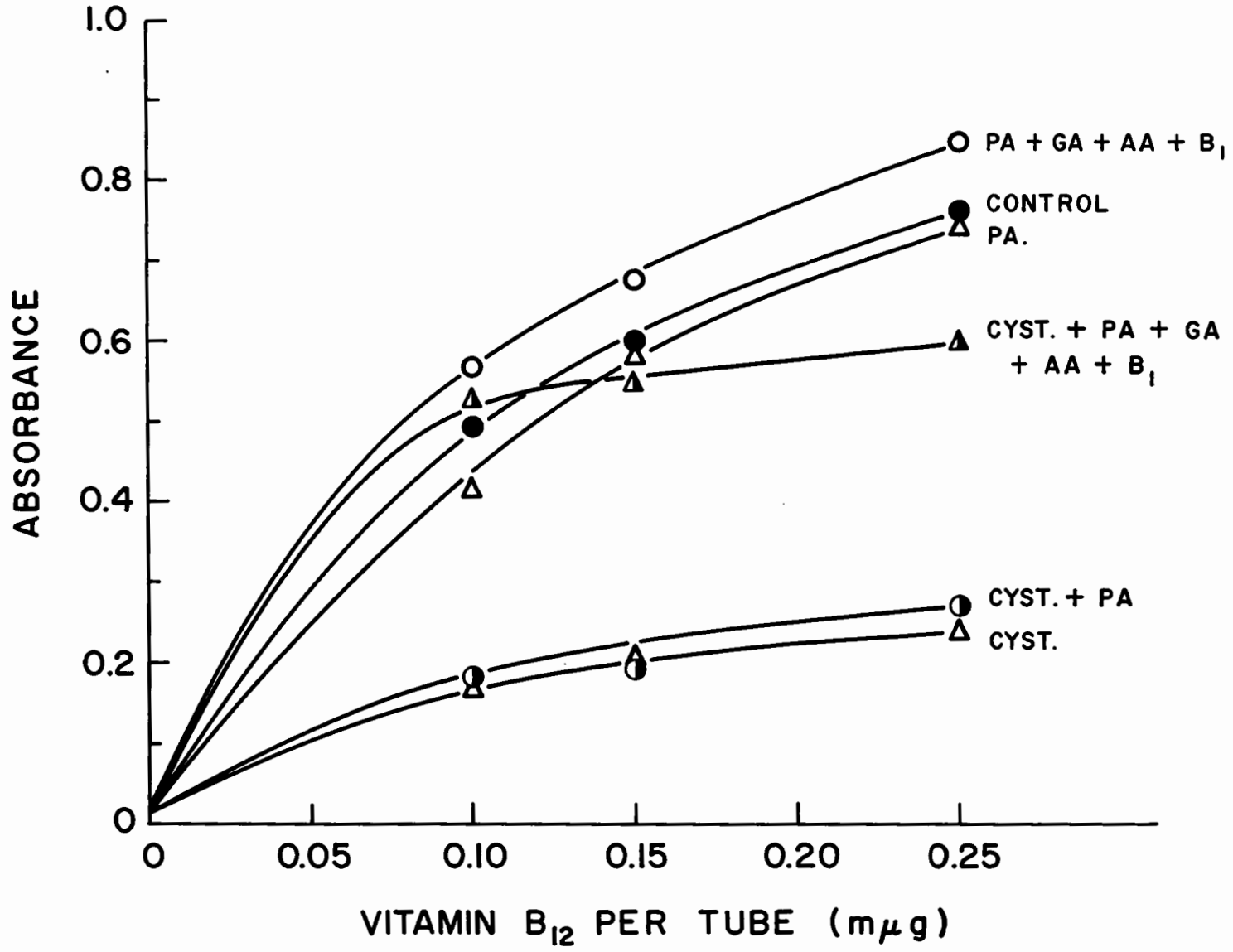

1.0
0.8
0.6
0.4
0.2
0
ABSORBANCE
0
0.05
0.10
0.15
0.20
0.25
VITAMIN B_{12} PER TUBE (mμg)
PA + GA + AA + B_1
CONTROL
PA.
CYST. + PA + GA + AA + B_1
CYST. + PA
CYST.

Fig. 13. The reversing effect of pantothenate acid (PA) and of β-alanine (β-ala) on the inhibitory effect of L-cycstine (CYST) with E. coli 113-3 (isolate No. 3). The medium contained relatively small amounts of DL-methionine.

Fig. 13. The reversing effect of pantothenic acid (PA) and of β-alanine (β-alan) on the inhibitory effect of L-cystine (CYST) with E. coli 113-3 (isolate No. 3). The medium contained relatively small amounts of DL-methionine.

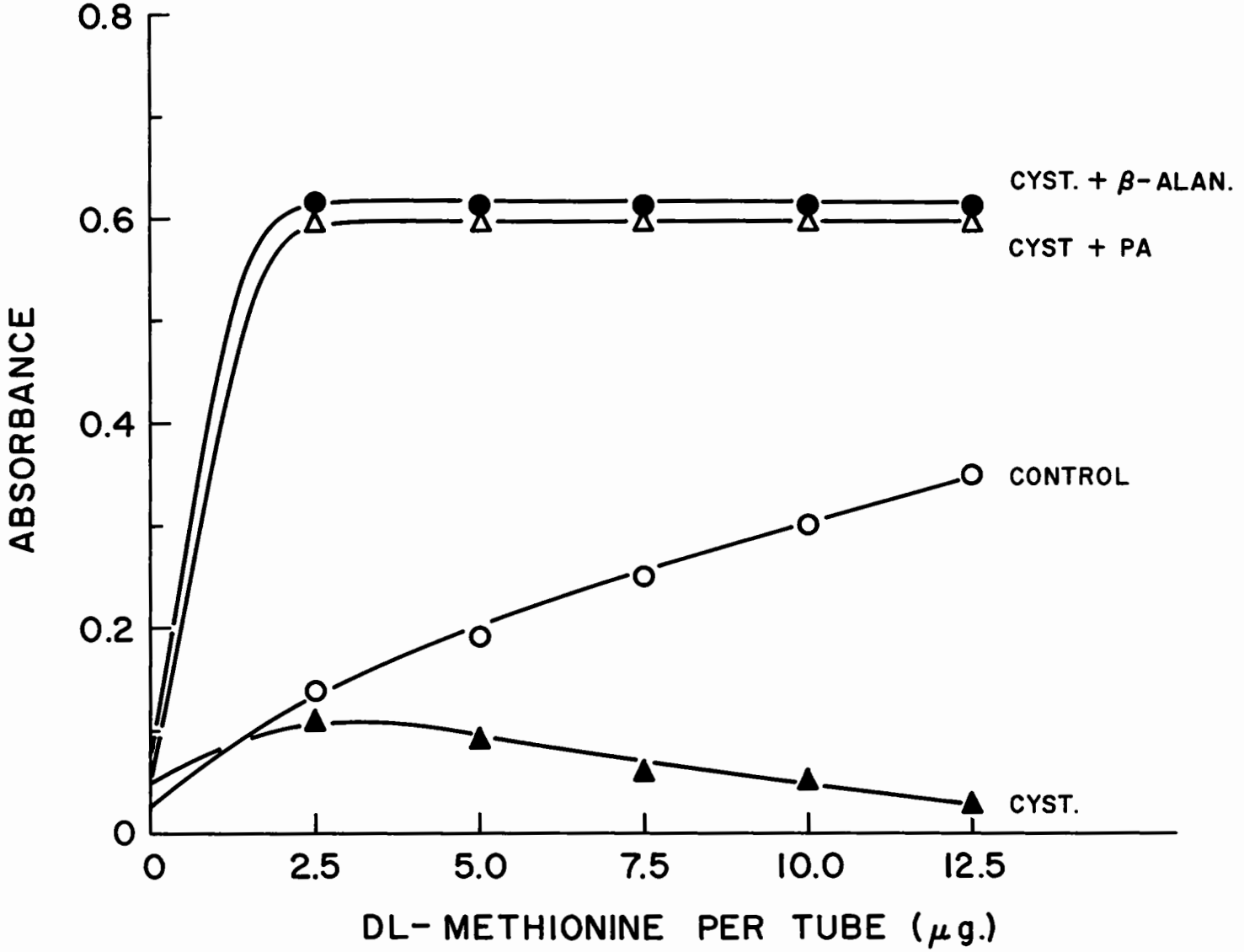
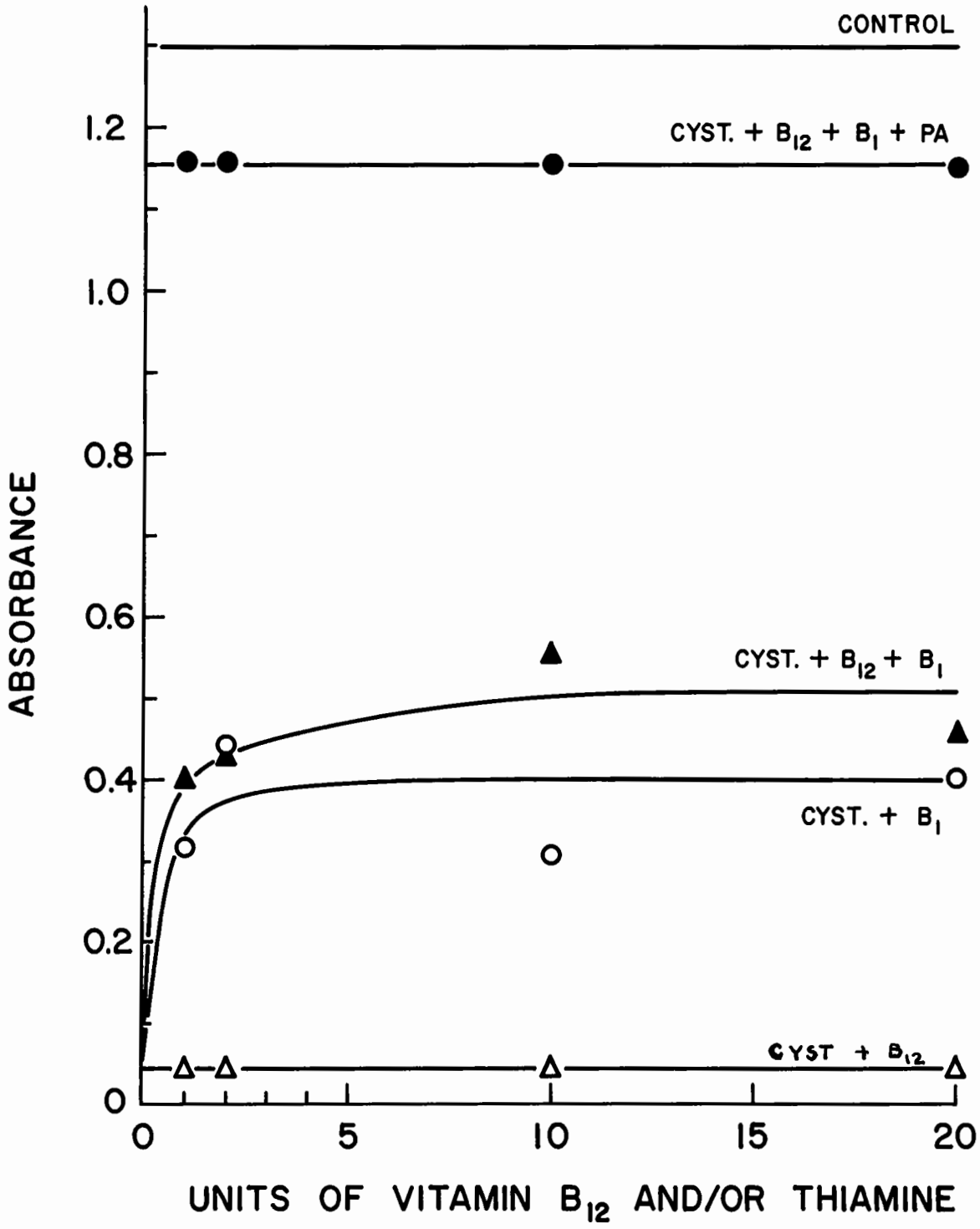

0.8
0.6
0.4
0.2
0
ABSORBANCE
CYST. + β-ALAN.
CYST + PA
CONTROL
CYST.
0
2.5
5.0
7.5
10.0
12.5
DL-METHIONINE PER TUBE (μg.)

Fig. 14. The reversing effect of thiamine (B_1) and of pantothenic acid (PA) on the L-cystine (CYST) inhibition with *E. coli* 113-3 (isolate No. 3). The medium contained an optimal amount of DL-methionine for growth and most tubes also contained vitamin B_{12}.

Fig. 14. The reversing effect of thiamine (B_1) and of pantothenic acid (PA) on the L-cystine (CYST) inhibition with E. coli 113-3 (isolate No. 3). The medium contained an optimal amount of DL-methionine for growth and most tubes also contained vitamin B_{12}.


CONTROL
CYST. + B12 + B1 + PA
CYST. + B12 + B1
CYST. + B1
CYST + B12
ABSORBANCE
0
0.2
0.4
0.6
0.8
1.0
1.2
0
5
10
15
20
UNITS OF VITAMIN B12 AND/OR THIAMINE

Experiment 11. The effect of cysteic acid on the growth of E. coli 113-3.

Since the toxicity of autoclaved cystine appeared to be similar to the toxicity for cysteic acid the inhibitory effects of cystine and cysteic acid were compared. Direct toxicity tests with the two compounds were done in as nearly identical conditions as possible; all tubes contained 20 μg of DL-methionine per tube. The inhibition curves for this experiment are given in Fig. 15.

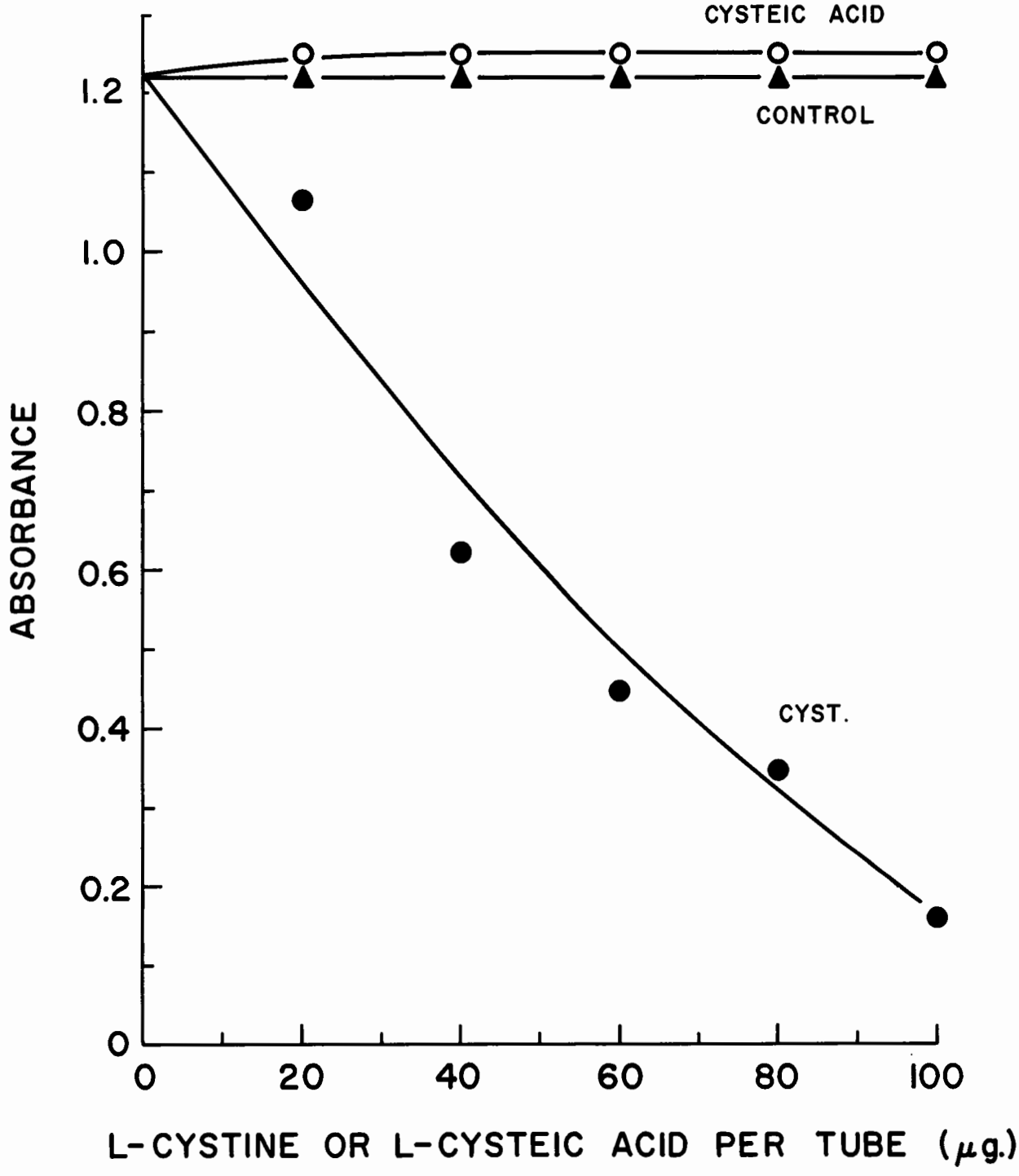
It appeared that cysteic acid was not toxic for E. coli 113-3 with concentrations of 100 μg or less per tube. Since cysteic acid might possibly be affected by autoclaving, the experiment was repeated except that cysteic acid was sterilized by Seitz filtration and added aseptically to the tubes. Cysteic acid had no apparent effect on growth. It appeared unlikely therefore that the toxicity of autoclaved cystine solutions was due to the formation of small amounts of cysteic acid during autoclaving.

Experiment 12. The effect of cysteinesulphinic acid (CSA) on the growth of E. coli 113-3.

A preliminary toxicity test was done to evaluate the effect of cysteinesulphinic acid (CSA) on the growth of E. coli 113-3 in a medium containing optimal amounts of methionine for growth. CSA appeared to inhibit growth of E. coli 113-3 quite markedly at concentrations as low as 5 μg. of CSA per tube and 10 μg. seemed completely to inhibit growth. A combination of pantothenic acid (1 μg) and aspartic acid (20 μg) seemed to reverse this inhibition. In two other tests CSA was added aseptically to the culture medium after Seitz filtration

Fig. 15. The toxicity of autoclaved L-cysteine and of L-cysteic acid for *E. coli* 113-3 (isolate No. 3) with an optimal amount of DL-methionine for growth.

Fig. 15. The toxicity of autoclaved L-cystine and of L-cysteic acid for E. coli 113-3 (isolate No. 3) with an optimal amount of DL-methionine for growth.


CYSTEIC ACID
CONTROL
CYST.
ABSORBANCE
1.2
1.0
0.8
0.6
0.4
0.2
0
0
20
40
60
80
100
L-CYSTINE OR L-CYSTEIC ACID PER TUBE (μg.)

and although growth was somewhat erratic the inhibitory effect of CSA seemed to be quite evident. There seems to be little doubt that relatively small amounts (5 - 20 μg) of CSA inhibit the growth of E. coli 113-3.

DISCUSSION

There are several possible explanations for the inhibitory effect of thiamine on the growth of E. coli 113-3, but the studies reported here appear to have eliminated some of the possible mechanisms for the action of thiamine. Although Lang and Chow (28) reported that thiamine destroyed the microbiological activity of vitamin B_{12}, the first 3 experiments described in this report indicated that the lowered growth-response in the presence of thiamine was not due to partial destruction of vitamin B_{12}. It is perhaps important to note that the criteria for judging destruction were different for each experiment. In the first test the potencies of the thiamine-vitamin B_{12} samples were determined with the L. leichmannii assay. In the second experiment the actual assay tubes were shaken for 16 hours before inoculation and incubation of the E. coli assay cultures. If thiamine destroyed vitamin B_{12}, particularly in aerobic conditions, the lowered growth-response should have been evident whether shaken or stationary assays were done, although the effect should have been particularly marked with the shaken assay. In the third experiment the size of the zones of growth in the pad-plate assay with E. coli 113-3 was used as the criterion for destruction of vitamin B_{12}. In other tests it was noted also that the thiamine effect differed considerably with different variants of E. coli 113-3. Considering the available evidence it seems very unlikely that the lowered growth-response produced by thiamine in the vitamin B_{12} assay results from partial destruction of vitamin B_{12}.

Thiamine is quite unstable in solution at pH 7.0, particularly during autoclaving of thiamine solutions (35). Experiments described in this report indicated, however, that toxic degradation products of thiamine were not formed either during autoclaving of the medium or during the period of the shaken assay. Although Berger and Lardy (1) found that purines and pyrimidines inhibited the growth of a "biotinless" mutant of E. coli, McLaughlan et al (34) reported that meta-bisulphite which splits thiamine into its component pyrimidine and thiazole moieties abolished the inhibitory effect of thiamine on the vitamin B_{12} assay with E. coli 113-3. From the available evidence, therefore, it does not seem that the inhibitory effect of thiamine results from formation of a toxic degradation product of thiamine.

Since E. coli 113-3 removed thiamine from the medium and as little as 0.1 μg of thiamine produced the maximum inhibitory effect, it appeared that added thiamine actually altered the metabolism of the organism. It seemed possible that thiamine absorbed by E. coli 113-3 might be phosphorylated to cocarboxylase thereby increasing the energy requirements of the organism, but tests indicated that both thiamine and cocarboxylase produced a similar effect on growth of E. coli 113-3. The function of thiamine in metabolism is known to be concerned with the reactions of Keto-acids and in particular with the decarboxylation of pyruvic acid. Pyruvic acid is an important basic chemical for many syntheses and it appeared possible that added thiamine might produce excessive decarboxylation of pyruvic acid with a concomitant reduction of the rates of synthetic processes dependent upon pyruvic acid.

The addition of sodium pyruvate to the medium, however, did not seem to alter the inhibitory effect of thiamine. Nevertheless sodium pyruvate is probably highly ionized at pH 7.0 so the absorption of pyruvic acid by E. coli 113-3 might be extremely limited and the lack of a reversing action of added pyruvate may be due to non-absorption.

McLaughlan et al (34) suggested that possibly thiamine inhibited aerobic growth due to mixed disulphide formation with compounds such as cysteine and homocysteine that seem to be essential for methionine syntheses. The aerobic conditions of the shaken assay appear favourable for disulphide formation and experiments reported here indicated that the inhibitory effect of thiamine was related to the degree of aerobiosis. Matsukawa and Yurugi (32) reported that cystine reacted with the thiol thiamine to produce a mixed disulphide. During growth added cystine might therefore react with thiamine synthesized by E. coli 113-3, thereby depriving the organism of thiamine necessary for growth; added thiamine appeared partially to reverse the cystine inhibition and this finding seemed to strengthen the conception that added thiamine and cystine inhibited growth due to disulphide formation. Although thiamine seemed to reverse the inhibition only slightly, it now appears probable that two or more different inhibitions occur when the medium contains autoclaved cystine.

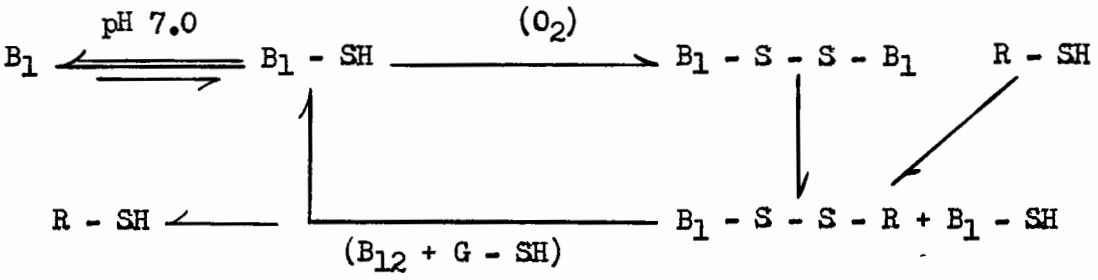
Dubnoff (9, 10) and Dubnoff and Bartron (11, 12) have postulated that vitamin B_{12} is related either directly or indirectly to the maintenance of the reduced forms of sulphydryl compounds such as homocystine and enzyme proteins containing sulphydryl groups;

glutathione also seemed to be required for the reduction of the oxidized sulphydryl compounds. It is possible, therefore, that vitamin B_{12} reduces mixed disulphides of thiamine but presumably this would increase the organism's requirement for vitamin B_{12}. Certain of the experimental findings appear to be compatible with such a conception; these are (a) thiamine had no apparent effect on the growth of E. coli 113-3 when the medium contained an optimal concentration of vitamin B_{12} and (b) there did not appear to be any experimental evidence for the existence of a "pool" of inactive disulphides within the organism.

Thiamine did not inhibit growth of E. coli 113-3 when the medium contained either optimal or sub-optimal amounts of methionine. Cohn et al (7) reported that E. coli grown in a medium with methionine contained little or no "methionine synthase" enzyme(s); this should spare the requirement for cysteine and homocysteine. Possibly cells grown with methionine have sufficient amounts of sulphydryl compounds so that thiamine-disulphide formation is unimportant and thiamine might therefore have no apparent effect on the growth of E. coli 113-3 when the medium contains methionine.

Neither thiamine nor cystine inhibited growth in the stationary assay but homocystine appeared to inhibit growth of E. coli 113-3 in both the shaken and stationary assays. Dubnoff (9, 10) reported that in the absence of vitamin B_{12} E. coli 113-3 rapidly reduced cystine but not homocystine unless reducing agents such as cysteine or glutathione were added to the medium; possibly the different inhibitory effects observed with these compounds is related to the ability of the organism to reduce these compounds in semi-aerobic conditions.

It is known (15) that only a small fraction of the thiamine in solution at pH 7.0 is in the thiol form and that most is in the thiazole form. It is only the thiol form of thiamine, however, that is oxidized to the disulphide, so it would appear that if thiamine disulphides are formed <u>in vivo</u> and are reduced by vitamin B_{12} and glutathione, then thiamine functions catalytically in the oxidation of sulphydryl compounds. This conception may be outlined as follows:



B_1 - SH represents the thiol form of thiamine.

B_1 - S - S - B_1 represents the disulphide form of thiamine.

R - SH represents sulphydryl compounds.

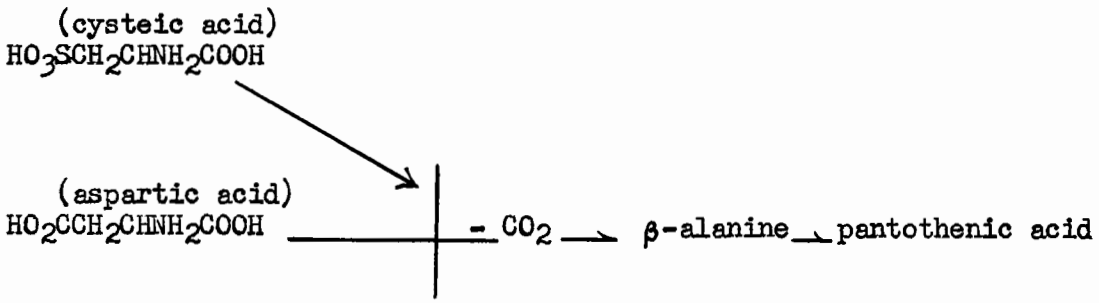
G - SH represents glutathione.

These reactions are essentially the same as those suggested by Matsukawa and Yurugi (32) except that vitamin B_{12} and glutathione reduce the disulphides and thiamine catalyses the oxidation of sulphydryl compounds. The operation of this cycle in aerobic conditions should increase the requirement for vitamin B_{12} and possibly for glutathione. Although there is little direct evidence, the only logical explanation at present seems to be that thiamine disulphide oxidizes essential sulphydryl compounds. The evidence for this conceeption is: (a) the conditions that seemed most favourable for disulphide formation, at pH 7.0,

also appeared to produce the greatest inhibitory effect with thiamine and (b) cystine appeared to inhibit aerobic growth and the inhibition seemed to be partially reversed by thiamine. Although the other experimental findings did not strengthen this idea, at least they were compatible with this conception.

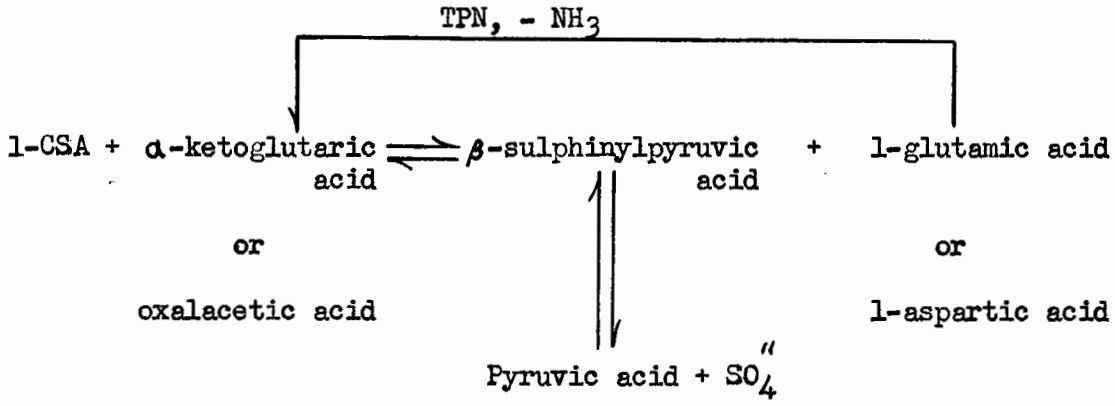
Thiamine reversed the cystine inhibition only slightly but it appears that the main inhibitory effect of autoclaved cystine is due to interference with pantothenic acid synthesis. Cystine, when added aseptically to the medium, appeared to stimulate the growth of E. coli 113-3 but inhibited growth when autoclaved with the medium. It appeared therefore that a toxic product was formed when cystine was autoclaved with the medium. Other experiments indicated that the toxicity of autoclaved cystine was reversed to a considerable extent by pantothenic acid and therefore the inhibition resembled Ravel and Shive's (37) cysteic acid inhibition. Tests with cysteic acid, however, indicated that 100 μg of cysteic acid per tube did not inhibit the growth of E. coli 113-3. This finding appeared to eliminate the possibility that cysteic acid is the toxic compound produced during autoclaving of cystine solutions.

Schuhardt et al (44) suggested that cysteine sulphinic acid (CSA) is formed during autoclaving of cystine solutions. CSA resembles aspartic acid and might inhibit growth of E. coli 113-3 in a manner similar to Ravel and Shive's postulated mechanism for the cysteic acid inhibition for E. coli; the postulated mechanism is outlined as:



According to Ravel and Shive, cysteic acid inhibited competitively the decarboxylation of aspartic acid to produce β-alanine, a precursor of pantothenic acid. They found that the inhibition was reversed competitively by aspartic acid and non-competitively by either β-alanine or pantothenic acid. The formula for cysteine sulphinic acid is $HO_2SCH_2CHNH_2COOH$; it appears to resemble aspartic acid more closely than does cysteic acid and might therefore be a more efficient competitive inhibitor than cysteic acid. Preliminary tests with CSA indicated that this compound inhibited growth quite markedly at concentrations as low as 5 μg per tube and the inhibition seemed to be reversed by pantothenic acid and aspartic acid. Since Schuhardt et al reported that CSA is probably formed during autoclaving of cystine solutions, it appears that the toxicity of autoclaved cystine solutions for E. coli 113-3 is due mainly to the formation of CSA during autoclaving and that CSA inhibits growth in a manner similar to the cysteic acid inhibition reported by Ravel and Shive.

Singer and Kearney (47) have shown that CSA is rapidly metabolized aerobically by Proteus vulgaris to pyruvic acid, NH_3, and SO_4''. They found that this process required either α-ketoglutaric acid or oxalacetic acid. They suggested that the following reactions represent the transamination reaction between CSA and the keto-acids.



Cobey and Handler (6) found that cell-free extracts of E. coli also appeared to metabolize CSA by the same reactions. Although Singer and Kearney designated the transamination as being reversible, Cobey and Handler stated that the desulphination of β-sulphinyl-pyruvic acid is an exergonic reaction and reversal of this process appears unlikely. Singer and Kearney reported that even with crude extracts the "turnover" in the α-ketoglutaric acid-CSA reaction exceeds the rate of any other known transamination reaction; with excess α-ketoglutaric acid or oxalacetic acid present and Mn^{++} to ensure continuous removal of β-sulphinyl-pyruvic acid the reaction goes to completion.

The studies of the metabolism of CSA suggest another possible mechanism for the apparent inhibitory effect of CSA upon the growth of E. coli 113-3. If the series of reactions producing pyruvic acid, NH_3 and SO_4'' from CSA may be considered as being spontaneous then most of the available aspartic acid may be involved in the aspartic acid-oxalacetic acid cycle deaminating CSA so that insufficient aspartic acid is available for pantothenic acid synthesis. Such an inhibition

might be reversed by pantothenic acid or β-alanine or aspartic acid or glutamic acid and experiments indicated that pantothenic acid and aspartic acid reversed the CSA inhibition.

The inhibition with autoclaved cystine did not appear to be completely reversed by pantothenic acid or a combination of compounds that Ravel and Shive found effective in reversing the cysteic acid inhibition. The shape of the growth-response curves when cystine was added aseptically or when the toxicity of autoclaved cystine was reversed by pantothenic acid and other pantothenic acid precursors, seemed to indicate that some other factor limited the rate of growth at the high dose of B_{12}. Although the "residual" inhibitory effect may be due to colloidal sulphur or metal sulphides (24, 25, 44, 52), thiamine appeared to have an additive effect with pantothenic acid in reversing the cystine inhibition. The "residual" inhibition might therefore be due to oxidation of sulphydryl compounds or disulphide formation with thiamine as mentioned near the beginning of this discussion.

SUMMARY

1. Thiamine decreased the amount of aerobic growth of E. coli 113-3 in Davis and Mingioli's medium.
2. It was found that thiamine does not destroy vitamin B_{12} in the aerobic conditions of the shaken assay for vitamin B_{12} with E. coli 113-3 and thiamine did not seem to convert vitamin B_{12} to vitamin B_{12b}.
3. It appeared that the thiamine effect did not result from the formation of toxic degradation products of thiamine.
4. E. coli 113-3 removed relatively large amounts of thiamine from the medium during growth.
5. Several amino acids and certain compounds that might be related to thiamine metabolism were tested but none reversed the thiamine inhibition.
6. It appeared that, within certain limits, the more aerobic the conditions for growth, the greater was the inhibitory effect of thiamine.
7. It is suggested that thiamine inhibits growth as a result of oxidation, of, or disulphide formation with, essential sulphydryl compounds.
8. Cystine, cysteine and homocystine, when autoclaved with media appeared to inhibit the aerobic growth of E. coli 113-3, but cystine seemed to stimulate growth in semi-anaerobic conditions. Cystine seemed to stimulate aerobic growth also when it was sterilized by Seitz filtration and added aseptically to media.
9. It appeared that pantothenic acid or β-alanine almost completely reversed the cystine inhibition and that thiamine also partially reversed the inhibition.

10. It is suggested that cystine inhibits aerobic growth, to a limited extent, due to disulphide formation with thiamine synthesized by the organism.

11. The cystine inhibition closely resembled the cysteic acid inhibition reported by other workers, but cysteic acid did not seem to inhibit the growth of E. coli 113-3 in aerobic conditions.

12. Cysteine sulphinic acid appeared to inhibit growth of E. coli 113-3 markedly and the inhibition seemed to be completely reversed by pantothenic acid and aspartic acid. Since cysteine sulphinic acid is probably formed during autoclaving of cystine solutions it appears that the cystine inhibition is mainly caused by cysteine sulphinic acid formed during autoclaving of media. It is suggested that cysteine sulphinic acid interferes with pantothenic acid synthesis.

ACKNOWLEDGEMENTS

The experimental studies reported in this thesis were done in the laboratories of the Food and Drug Directorate, Department of National Health and Welfare, Ottawa.

The author wishes to express his sincere thanks to Dr. C. A. Morrell, Dr. L. I. Pugsley, and Dr. J. A. Campbell of the Food and Drug Directorate for arranging educational leave; to Dr. J. A. Campbell and Dr. R. H. Wallace for their many suggestions, interest and encouragement, and critical reviews of this thesis.

The author is indebted also to Mr. C. G. Rogers of the Food and Drug Directorate for helpful suggestions and to Dr. T. P. Singer of the Edsel B. Ford Institute for Medical Research, Detroit, Michigan, for a generous gift of cysteine sulphinic acid.

REFERENCES

1. Berger, M., and Lardy, H.A.,

Growth inhibition of a biotinless mutant of Escherichia coli by purines and pyrimidines.
Proc. Soc. Exptl. Biol. Med. 85: 89-93. 1954.

2. Brown, G. M., and Snell, E. E.,

Pantothenic acid conjugates and growth of Acetobacter suboxydans.
J. Bacteriol. 67 : 465-471. 1954.

3. Burkholder, P.R.,

Determination of vitamin B_{12} with a mutant strain of Escherichia coli.
Science. 114 : 459-460. 1951.

4. Campbell, J. A., McLaughlan, J. M., Clark, J. A., and Dunnett, C.W.,

The six-point design in the U.S.P. microbiological assay of vitamin B_{12}.
J. Am. Pharm. Assoc. Sci. Ed. 42 : 276-283. 1953.

5. Chiao, J. S., and Peterson, W. H.,

Microbiological assay of vitamin B_{12} with a mutant strain of Escherichia coli.
Appl. Microbiol. 1 : 42-46. 1953.

6. Cobey, F. A., and Handler, P.,

Sulphite metabolism in E. coli.
Biochem. et Biophys. Acta. 19 : 324-327. 1956.

7. Cohn, M., Cohen, G. N., and Monod, J.,

The specific inhibiting effect of methionine in the formation of methionine synthase in Escherichia coli.
Compt. rend. 236 : 746-748. 1953.

8. Davis, B. D., and Mingioli, E. S.,

Mutants of Escherichia coli requiring methionine or vitamin B_{12}.
J. Bacteriol. 60 : 17-28. 1950.

9. Dubnoff, J. W.,

The role of B_{12} in methionine synthesis in E. coli.
Arch. Biochem. and Biophys. 37 : 37-45. 1952.

10. Dubnoff, J. W.,

The growth requirements of variants of E. coli mutant 113-3.
Arch. Biochem. and Biophys. 52 : 151-156. 1954.

11. Dubnoff, J. W., and Bartron, E.,

The effect of B_{12} on enzyme activity in E. coli mutant 113-3.
Arch. Biochem. and Biophys. 61 : 99-110. 1956.

12. Dubnoff, J. W., and Bartron, E.,

The activation of protein sulphydryl groups by vitamin B_{12}.
Arch. Biochem. and Biophys. 62 : 86-90. 1956.

13. Fling, M., and Horowitz, N. H.,

Threonine and homoserine in extracts of a methionineless mutant of Neurospora.
J. Biol. Chem. 190 : 277-285. 1951.

14. Gots, J. S., and Koh, W. Y.,

Methionine synthesis in Escherichia coli.
Bact. Proc. 134-135. 1950.

15. Gunsalus, I. C.,

Personal communication.

16. Harrison, E., Lees, K. A., and Wood, F.,

The assay of vitamin B_{12}. VI. Microbiological estimation with a mutant of Escherichia coli by the plate method.
Analyst, 76 : 696-705. 1951.

17. Heathcote, J. G.,

The toxicity of small concentrations of cystine to acid-producing bacteria.
J. Gen. Microbiol. 3 : 392-394. 1949.

18. Helleiner, C. W., and Woods, D. D.,

Cobalamin and the synthesis of methionine by cell-free extracts of Escherichia coli.
Biochem. J. 63 : 26P. 1956.

19. Jakobovits, J., Wiggins, E. H., and Harrison, J. S.,

The effect of optically active forms of some amino acids on the response of yeast to β-alanine and pantothenic acid.
J. Gen. Microbiol. 5 : 648-656. 1951.

20. Johansson, K. R.,

Response to and assay of vitamin B_{12} by a mutant of Escherichia coli.
Proc. Soc. Exptl. Biol. Med. 83 : 448-453. 1953.

21. Johnson, B. C.,

Water-soluble vitamins, Part III.
Ann. Rev. Biochem. 24 : 419-464. 1955.

22. Kalan, E. B., and Ceithaml, J.,

Methionine biosynthesis in Escherichia coli.
J. Bacteriol. 68 : 293-298. 1954.

23. King, T. E., and Cheldelin, V. H.,

Pantothenic acid studies IV. Propionic acid and -alanine utilization.
J. Biol. Chem. 174 : 273-279. 1948.

24. Konowalchuk, J., Hinton, N. A., and Reed, G. B.,

Antibacterial action of a reaction product of cysteine and iron I. Development of the substance in media for Mycobacterium tuberculosis.
Can. J. Microbiol. 1 : 175-181. 1954.

25. Konowalchuk, J., Clunie, J. C., Hinton, N. A., and Reed, G. B.,

Antibacterial action of a reaction product of cysteine and iron II. Preparation and properties of the substance.
Can. J. Microbiol. 1 : 182-189. 1954.

26. Korkes , S., del Campillo, A., Gunsalus, I. C., and Ochoa, S.,

Enzymatic synthesis of citric acid IV. Pyruvate as acetyl donor.
J. Biol. Chem., 193 : 721-735. 1951.

27. Lampen, J. O., Roepke, R. R., and Jones, M. J.,

Studies on the sulfur metabolism of Escherichia coli III. Mutant strains of Escherichia coli unable to utilize sulfate for their complete sulfur requirements.
Arch. Biochem. 13 : 55-56. 1947.

28. Lang, C. A., and Chow, B. F.,

Inactivation of microbiological activity of crystalline vitamin B_{12} by reducing agents.
Proc. Soc. Exptl. Biol. Med. 75 : 39-41, 1950.

29. Maas, W. K., and Davis, B. D.,

Pantothenate studies I. Interference by D-serine and L-aspartic acid with pantothenate synthesis in Escherichia coli.
J. Bacteriol. 60 : 733-745. 1950.

30. Maas, W. K.,

Pantothenate studies II. Evidence from mutants for interference by salicylate with pantoate synthesis.
J. Bacteriol. 63 : 227-232. 1952.

31. Maas, W. K., and Vogel, H. J.,

α-Ketoisovaleric acid, a precursor of pantothenic acid in Escherichia coli.
J. Bacteriol. 65 : 388-393. 1953.

32. Matsukawa, T., and Yurugi, S.,

A derivative of thiamine with cysteine.
Science. 118 : 109-111. 1953.

33. McLaughlan, J. M., Clark, J. A., and Campbell, J. A.,

The effect of cyanide on the apparent vitamin B_{12} content of liver extracts in E. coli plate assays.
Arch. Biochem. and Biophys. 46 : 244-246. 1953.

34. McLaughlan, J. M., Rogers, C. G., and Campbell, J. A.,

The influence of thiamine on the microbiological assay for vitamin B_{12} with Escherichia coli 113-3.
J. Am. Pharm. Assoc. Sci. Ed. 44 : 594-598. 1955.

35. Methods of vitamin assay.

Published for the Association of Vitamin Chemists, Inc., by Interscience Publishers, Inc., New York, U.S.A.

36. Prince, H. N., and Cleverdon, R. C.,

The flavobacteria II. Utilization of nitrogen compounds.
J. Bacteriol. 69 : 307-309. 1955.

37. Ravel, J. M., and Shive, W.,

Biochemical transformations as determined by competitive analogue-metabolite growth inhibitions IV. Prevention of pantothenic acid synthesis by cysteic acid.
J. Biol. Chem. 166 : 407-415. 1946.

38. Reed, L. J.,

Metabolic functions of thiamine and lipoic acid.
Phys. Rev. 33 : 544-559. 1953.

39. Rose, C. S., Feldbaum, E. P., Norris, R. F., and György, P.,

Toxicity of autoclaved cystine for Lactobacillus bifidus.
Proc. Soc. Exptl. Biol. Med. 81 : 709-712. 1952.

40. Rosenberg, H. R.,

Chemistry and Physiology of the Vitamins.
Interscience Pub. Inc., New York, N.Y. 1945.

41. Rowley, D.,

Interrelationships between amino-acids in the growth of coliform organisms.
J. Gen. Microbiol. 9 : 37-43. 1953.

42. Sahashi, Y., Funahashi, S., Yamashita, K., and Akatsuka, T.,

Biochemical studies on intermediate metabolism of vitamins I. Interaction of vitamin B_1 and sulfhydryl groups in protein molecules.
J. Biochem. (Japan) 41 : 463-467. 1954.
Cited from Chem. Abstr. 48 : 13739g. 1954.

43. Saxena, K. C., Ghatak, S., and Agarwala, S. C.,

Inhibition of B_1 synthesis by B_{12} in wild strains of E. coli.
Experientia. 10 : 488-489. 1954.

44. Schuhardt, V. T., Rode, L. J., Oglesby, G., and Lankford, C. E.,

Toxicity of elemental sulfur for brucellae.
J. Bacteriol. 63 : 123-128. 1952.

45. Shive, W., and Macow, J.

Biochemical transformations as determined by competitive analogue-metabolite growth inhibitions. I. Some transformations involving aspartic acid.
J. Biol. Chem. 162 : 451-462. 1946.

46. Simmonds, S.,

Utilization of sulfur-containing amino acids by mutant strains of Escherichia coli.
J. Biol. Chem. 174 : 717-722. 1948.

47. Singer, T. P., and Kearney, E. B.,

Enzymatic pathways in the degradation of sulfur-containing amino acids.
in "Amino Acid Metabolism" (McElroy, W. D., and Glass, B., eds.) p. 558. The John Hopkins Press, Baltimore, 1955.

48. Stekol, J. A.,

Synthetic pathways of methionine, cysteine and threonine.
in "Amino Acid Metabolism" (McElroy, W. D., and Glass, B., eds.).
p. 509. The Johns Hopkins Press, Baltimore, 1955.

49. Teas, H. J., Horowitz, N. H., and Fling, M.

Homoserine as a precursor of threonine and methionine in Neurospora.
J. Biol. Chem. 172 : 651-658. 1948.

50. Vitamin B_{12} Activity Assay.

United States Pharmacopoeia, 14th rev., Third Supplement,
Mack Publishing Co., Easton, Pa., 1952.

51. Williams, W. L., Esposito, R. G., and Pierce, J. V.,

A rapid simplified microbiological assay method for
vitamin B_{12} and other vitamins.
Federation. Proc. 11, No. 1 March, 1952.

52. Woiwod, A. J.,

The inhibition of bacterial growth by colloidal heavy-metal
sulphides and by colloidal sulphur.
J. Gen. Microbiol. 10 : 509-520. 1954.

53. Wood, E. C., and Finney, D. J.,

The design and statistical analysis of microbiological assays.
Quart. J. Pharm. and Pharmacol. 19 : 112-127. 1946.

54. Wright, L. D., and Skeggs, H. R.,

Reversal of sodium propionate inhibition of Escherichia coli
with β-alanine.
Arch. Biochem. 10 : 383-386. 1946.

55. Zima, O., and Williams, R. R.,

An antineuritically active oxidation product of aneurine.
Ber. deut. chem. Ges. 73 : 941-949. 1940.
Cited in ref. 40.

56. Zima, O., Ritsert, K., and Moll, T.,

Aneurine disulfide.
Hoppe-Seyler's Z. physiol. Chem.
267 : 210-227. 1941. Cited in ref. 40.