PRODUCTION OF VITAMIN B_{12}-ANTIMETABOLITES BY BACTERIA

by

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I. LITERATURE SURVEY

A. Occurrence of Antimetabolites and Their Role in Nature

An antimetabolite is defined as a substance which interferes with the formation or function of natural metabolites (1). These "metabolites," or more accurately, these "essential metabolites," play decisive roles in metabolism: they may be vitamines, hormones, amino acids, or other compounds which occur in nature and which can be shown to be necessary in some phase of the life process.

The present concept of the antimetabolite has grown slowly with the acquisition of new knowledge of branches of biochemistry. The basic concepts have risen from studies of bacterial nutrition, of animal nutrition, of enzyme action and composition, and of pharmacological antagonisms of drugs. These have shaped the concept as it now is understood.

As long ago as 1910-1914, Michaelis and his collaborators (2) investigated the inhibition of carbohydrate-hydrolyzing enzymes. They observed that some of the inhibitors were similar in chemical structures to the products formed in the reaction. These studies were limited to inhibition caused by a product of the reaction, and, since they do bear structural resemblance to it, these products might be said to be

analogs of the substrates. In 1927, Quastel and Wooldridge (3) reported the competitive inhibition of succinic dehydrogenase by malonic acid, a structural analog of the substrate of the enzyme. This clarified the idea that this structural resemblance was the reason for the inhibition.

From 1920 to 1940, Landsteiner (4) elucidated and described the phenomenon of which relatively simple substances related in structure to antigens would inhibit the formation of a precipitate when those antigens were mixed with their antibodies. The inhibitory substances were called haptens. One of the major features of a hapten was the occurrence with which it interferes. In retrospect, it can be recognized how closely the haptens fit into the concept of the antimetabolites, but it was not realized then.

In the biochemical and nutritional literature from 1920 to 1940, several instances can be found in which, as a result of chance observations, structural analogs of a vitamin, or a hormone, or an amino acid were discovered to antagonize the biological action of the related metabolite. One of the first of such findings made in 1938 (5) was that β-acetylpyridine and pyridine-3-sulfonic acid were toxic to dogs suffering from nicotinic acid deficiency but were harmless to animals receiving a normal diet. The observations were made

during an investigation of various derivatives of nicotinic acid as substitutes for this vitamin, and the possession of toxic rather than beneficial properties in those two analogs were unexpected. After the recognition of the phenomenon of antimetabolites, both of these substances were studied again in a variety of living things and shown to be analogs antagonistic to nicotinic acid.

Other examples such as the appraisal of the antagonism between acetylcholine and (6) structurally similar drugs was published by Clark in 1937. Ethionine, a naturally occurring ethyl analog of methonine, was found to be an effective antimetabolite of this amino acid by Dyer in 1938 (7).

In 1940, the discovery of p-aminobenzoic acid was reported by Woods (8) to be an antagonist of the sulfonamide drugs. Fildes (9) then proposed that useful chemotherapeutic agents against infectious diseases might be produced by altering the structure of some other vitamin or metabolite so as to achieve an antagonistic analog. This concept was immediately tested, especially since at that time the success of sulfonamide drugs raised the possibility of cure of bacterial diseases.

Largely stimulated by Fildes' suggestion, but also partly out of curiosity to see if antagonism between vitamins and their structural relatives was a general phenomenon, many investigators soon produced a variety of

microbial species, and that such effects could be overcome by their related metabolites. Among the earliest studies was that of Fildes in 1940 (10) who showed that indole-acrylic acid antagonized the growth-stimulating properties of tryptophan for the typhoid ~~bacillus~~ bacteria. Also, in 1940 McIlwain (11) found that the pyridine-3-sulfonic acid antagonized the action of nicotinic acid in several bacteria. In 1941, Snell (12) showed that the sulfonic acid analog of pantothenic acid, pantoyltaurine, competed with that vitamin in several bacterial species. Robbins (13) in 1943 and Woolley and White in 1943 (14) found pyrithiamine antagonized the action of thiamine.

After a number of synthetic antimetabolites had been studied, it became increasingly clear that such compounds also existed in nature. The first to be regarded as a naturally occurring antimetabolite was discovered by Link and collaborators (15), 3,3-methylenebis-(4-hydroxy-coumarin), which caused signs of vitamin K deficiency. McIlwain recognized that iodinin, an antibiotic pigment of Chromobacterium iodinum was related in structure to vitamin K and this metabolite would counteract its effect on the growth of bacteria (16). Following this, the antagonism between testosterone and estrone, progesterone and estrogen, pairs of structurally similar porphyrin, adenosine and cytidine, deoxyribonucleic acid and ribonucleic acid, pairs of amino acids, etc. were examined.

In recent years, investigators have been trying to isolate antimetabolites directly from fermentation broths of microorganisms. This search of antimetabolites from natural sources has been quite successful.

In addition to their importance in chemotherapy, antimetabolites have been very useful substances in pharmacology. Although there probably is no single comprehensive explanation of the mode of action of various drugs, the effect of a few classes of pharmacological agents can be understood, at least in part, if they are viewed as antagonist analogs of certain metabolites which show similar pharmacological properties and bear structural resemblance to each other. What is more, the metabolite may antagonize the effect of drugs. In addition, many of the features of the pharmacological effects of the drugs are those which might be anticipated from a lack of the metabolite. These facts are the basis for the opinion that such drugs are metabolites and function primarily in the manner already depicted for such substances. In this way, antimetabolites help to explain the mode of action of some drugs. To many investigators of biochemistry, the antimetabolites have appeared to be rather subtle and useful tools with which to study the mechanism of biological reactions. They have also offered promise, and some success, as aids for the discovery of new metabolites. The way in which they

apparently block specifically one or two reactions has been especially appealing as a means of studying such processes on intact or otherwise normal individuals.

Under these circumstances, antimetabolites of vitamin B_{12} are studied here with two possible uses in mind: (a) they might help to elucidate the modes of action of the vitamin; and (b) they could be of value as chemotherapeutic agents in leukemia or other malignant conditions where vitamin B_{12} plays a role in the diseases.

B. Previous Reports of Antimetabolites in Fermentations

1. Methods of Detecting Antimetabolites

A recent publication, Hanka (17) reported the following technique for detection of potential antimetabolites in the fermentation liquors of such microorganisms as Actinomycetes or fungi: Bacillus subtilis and Escherichia coli are grown in two types of agar: (1) nutrient agar--a complex medium containing 0.3% beef extract, 0.5% peptone and 1.5% agar; and (2) a completely synthetic medium with glucose as the only source of carbon. The composition of the synthetic medium is given in Table I. The nutrient agar for each respective microorganism is adjusted to the same final pH as the synthetic media.

TABLE I

The Composition of Hanka's Synthetic Media

	Bacillus subtilis	Escherichia coli
$Na_2HPO_4 \cdot 7H_2O$	1.5 g	2.2 g
KH_2PO_4	4.3 g	1.0 g
$(NH_4)_2SO_4$	1.0 g	1.0 g
$MgSO_4$	0.1 g	0.1 g
Glucose	2.0 g	2.0 g
Agar	15.0 g	15.0 g
Distilled water	1 liter	1 liter
Metallic ion stock solution	1 ml	
Final pH	6.2	6.7

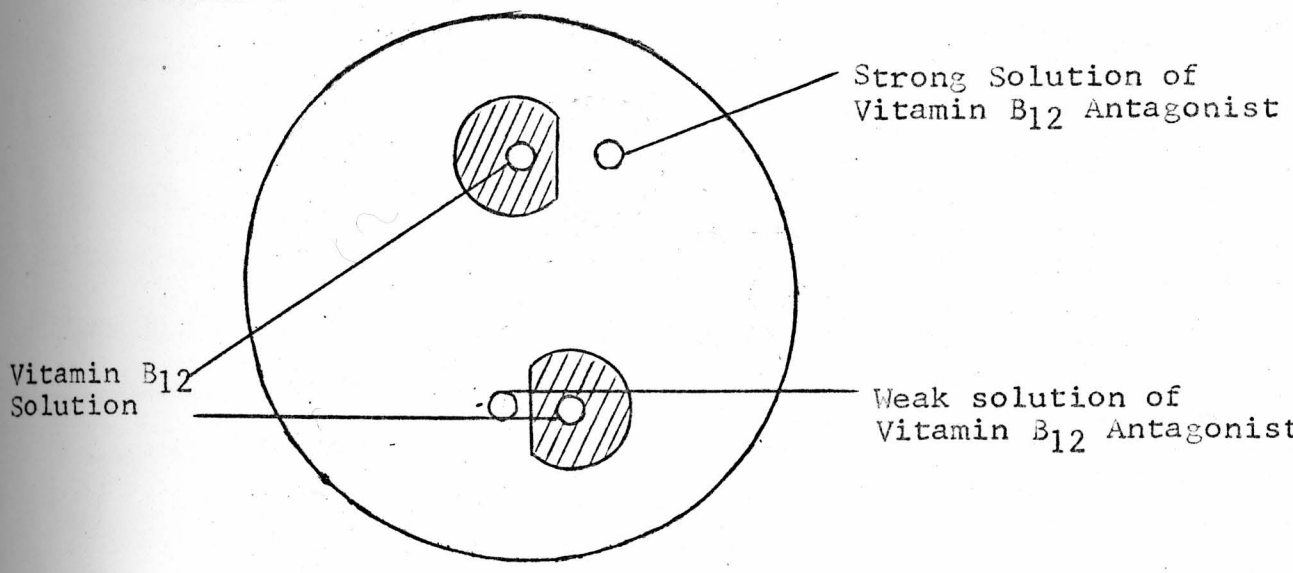
The molten agar is seeded with an overnight culture of the microorganism, poured into a plastic tray, and allowed to solidify. The fermentation samples to be tested are applied on to 13 mm paper discs and these are placed on the surface of the seeded agar. The trays seeded with E. coli are incubated at 32°C and those with B. subtilis at 37°C for 18 hours and then the zones of inhibition of bacterial growth are measured. Fermentation liquors which inhibit one or both of the test organisms in synthetic agar but do not inhibit it (or inhibit much less) in nutrient agar are considered to contain potential antimetabolites. All reversal of the antimicrobial activity against the susceptive test organism by some of

the common metabolites, for example, purines, pyrimidines, amino acids, vitamins and Krebs' cycle intermediates. Each tray containing the test organism in synthetic media supplemented respectively with low concentration of all common metabolites are incubated and the zones are measured. However, even samples that do not show any specific reversal pattern are potentially interesting. The cultures of streptomycetes or fungi which have displayed a typical antimicrobial pattern or an anti-metabolite are then examined to obtain the maximal fermentation titers.

Lester Smith (18) demonstrated competitive inhibition of vitamin B_{12} antimetabolites by a modification of the agar plate method for microbiological assay for vitamin B_{12} with E. coli mutant. Vitamin B_{12} and its antagonist were placed in adjacent cups in the agar plate. After incubating for 18 hours at the right temperature, part of the growth zone around the cup containing B_{12} was cut away in a more or less straight line. If, on the other hand, a non-competitive growth inhibitor like phenol was used, then the clear area cutting away the growth zone took the shape of a circle centered on the inhibitor cup; that is to say, this non-specific antibiotic above a certain concentration prevented growth whatever the vitamin B_{12} concentrations, whereas the relatively straight cut-off marked the locus of approximately constant ratio of

vitamin and inhibitor concentrations. This technique was later modified to yield approximate values for the inhibition indices, based on the simple principle that the index is the ratio of the concentration of inhibitor and vitamin that give a cut-off line exactly midway between two cups.

FIGURE I

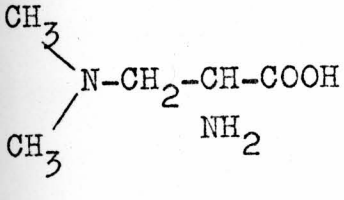


Tube assays, i.e., measuring the turbidity of growing cultures in broth, which are usually more accurate, were often upset by slight growth at high levels of inhibitor. This still occurred when care was taken to remove any residual traces of the vitamin from the antagonist, and was also noted in other test systems.

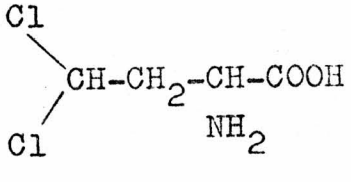
2. Antimetabolites of Amino Acids Found in Fermentations

a. Leucine

In the course of studies related to the production of antibiotics and other secondary metabolites from streptomycetes, Argoudelis, et al. (19) have isolated two new amino acids which have been found to inhibit growth of several microorganisms grown in synthetic media. L-2-amino-3-dimethylaminopropionic acid, the first of these new compounds, has been isolated from fermentations of Streptomyces neocaliberis var. neocaliberis while the other, L-2-amino-4,4-dichlorobutyric acid is produced by Streptomyces armentosus var. armentosus. Preliminary studies indicate that both amino acids act as leucine antagonists. They are structurally related to leucine and their activities against bacteria are most effectively reversed by this amino acid.



L-2-amino-3-dimethylamino-propionic acid



L-2-amino-4,4-dichloro-butyric acid

Korobkova, et al. (20) reported that in the screening program of organisms producing antibiotics with antimetabolite properties, 756 various streptomycetes showing no antibacterial effect on complex media with organic nitrogen sources were studied. The studies revealed 25 streptomycetes producing antibiotics with antimetabolite properties with respect to leucine. The cultures selectively inhibited the growth of E. coli K-13 and had no effect on the other two strains of E. coli strains, B and 335. Additional studies on 113 streptomycetes confirmed connection between the capacity of the cultures for selective inhibition of E. coli K-13 growth and synthesis of substances with antimetabolite properties with respect to leucine.

In another study of antimetabolites from Streptomycetes, Scannell, et al. (21) isolated from the fermentation broth an antimetabolite which was inhibitory to the growth of Bacillus subtilis on a chemically defined minimal medium. The growth inhibition was relieved by addition of D,L-leucine to the medium. The inhibitory substance was isolated by ion exchange techniques and found to be identical with L-2-amino-4-pentynoic acid prepared from synthetic racemic material by enzymatic resolution. The inhibitory effect was also reversed by D,L-methionine.

b. Phenylalanine

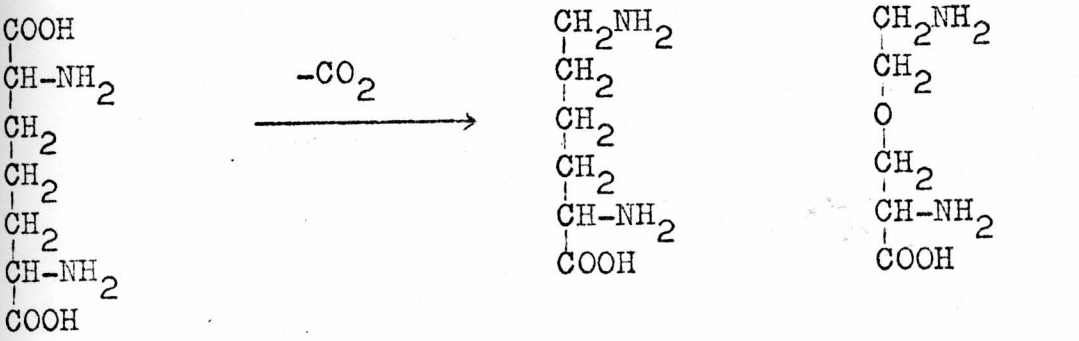
Scannell, et al. (21) also found another antimetabolite from an unidentified streptomycete, which was inhibitory to the growth of B. subtilis and E. coli. The activity was reversed by L-phenylalanine. The antimetabolite isolated by chromatographic methods, was shown to be identical to synthetic L-3-(2,5-dihydrophenyl)alanine.

Yamashita, et al. (22) have also isolated the same antagonist of phenylalanine, (also known as L-1,4-cyclohexadiene-1-alanine), from the culture broth of a strain of designated Streptomyces diastatochromogenes var. sakaii. It is active against plant-pathogenic fungi, and Pseudomonas aeruginosa on synthetic medium. This antimetabolite was chemically synthesized by Snow, et al. (23) in 1957.

c. Lysine

In the course of an investigation of new antimetabolic antibiotic substances, a natural antagonist of lysine and diaminopimelic acid was discovered in the fermentation broth of several streptomycetes. This antibiotic was isolated and chemically characterized as L-4-oxalysine (24). In 1957, McCord, et al. (25) prepared the racemic form of the compound by organic chemical synthesis and described it as a lysine antagonist. In

vitro assay of L-4-oxalysine using *E. coli* grown in chemically defined media, demonstrated the compound's behavior as a competitive antagonist of L-lysine. Diaminopimelic acid, as the immediate precursor of lysine, also reverse the inhibitory effect of L-4-oxalysine. However, in a series of *in vivo* tests, this antibiotic did not protect mice against a lethal infection with either *Salmonella schottmuelleri* or *Staphylococcus aureus*. The fact that diaminopimelic acid is almost as active as L-lysine in reversing the activity of L-oxalysine indicated that the antagonist is competitive with the utilization of lysine rather than with its formation via dicarboxylation of diaminopimelic acid. Since the L-isomer of 4-oxalysine is an effective lysine antagonist and its reversal is stereospecific, they suggested the hypotheses that the 4-oxalysine effect may also be stereospecific and that the D-isomer may not behave as an antimetabolite.



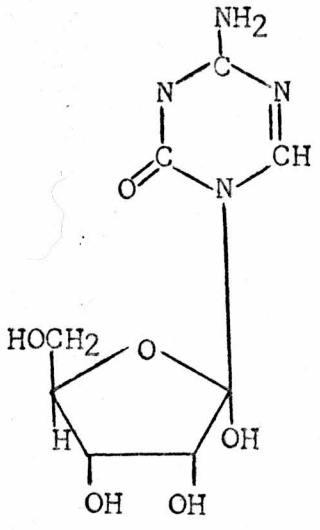
Diaminopimelic acid

Lysine

4-Oxalysine

3. Purine and Pyrimidine Antimetabolites

a. 5-Azacytidine



The antibiotic, 5-azacytidine was isolated from the fermentation liquors of a Streptoverticillium ladakanus (26). Chemical synthesis of this compound was completed in 1964 (27). It was found to inhibit the growth of some Gram-negative bacteria, especially when they were grown in completely synthetic media. The inhibition of E. coli by this compound was reversed by several pyrimidines. The most effective reversing agent was cytidine, followed closely by uridine (27). It has shown in vivo activity against leukemia in mice, but ineffective against E. coli infection in mice.

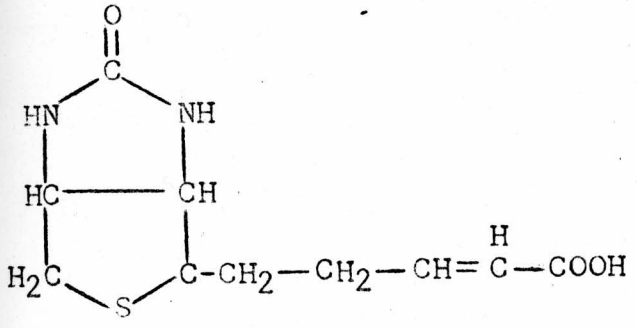
b. Emimycin

During a survey seeking new antibiotics produced by streptomycetes, an antimetabolite, active against E. coli and reversed practically by uracil, was isolated (28). The structure was assigned employing the usual chemical methods as 2-hydroxypyrazine-4-oxide. This compound is actually an antibiotic first isolated in 1960 by Terao, et al. (29) named emimycin.

4. Vitamin Antimetabolites

a. Biotin

(1) α-Dehydrobiotin

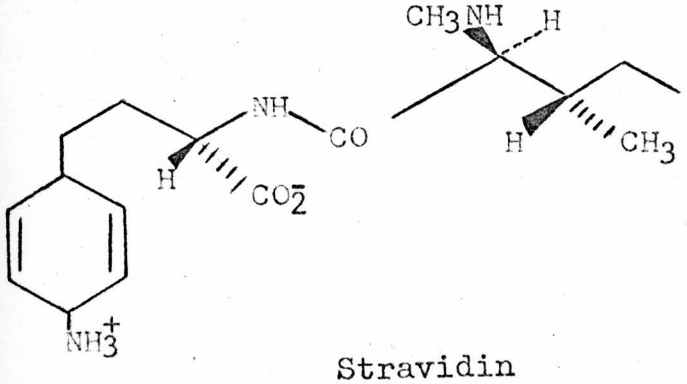


This is an antibiotic produced by Streptomyces lydicus (30). It is active in vitro against a variety of Gram-positive and Gram-negative bacteria and fungi.

Of special interest, however, is its antimetabolite relationship to biotin in that its antibacterial properties are reversed by the presence of biotin in synthetic media.

In order to know whether α-dehydrobiotin was a precursor or a degradative product of biotin and pimelic acid by S. lydicus, an experiment was done using radioactive biotin and pimelic acid. The study (31) showed that α-dehydrobiotin is a product of a biotin catabolism in S. lydicus. α-Dehydrobiotin-^{14}C was isolated from fermentations supplemented with biotin-^{14}C. The addition of pimelic-^{14}C to the growing culture did not react in production of any radioactive α-dehydrobiotin. α-Dehydrobiotin did not substitute for biotin in Lactobacillus plantarum or in Sacchromyces cerevisiae which require biotin for growth. Antimicrobial activity of α-dehydrobiotin was counteracted by avidin. α-Dehydrobiotin appears to be different from several biotin vitamins described in the literature.

(2) Stravidin and Streptavidin (MSD-235) (32)



Stravidin

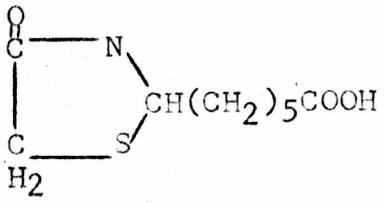
Streptomycetes isolated from soil samples collected from widely separated areas throughout the world found to produce a new synergistic antibiotic complex, antibiotic MSD-235. Several of the cultures were identified as Streptomyces lavendulae, whereas one other isolate was named S. avidinii (33). The antibiotic complex inhibits growth of Gram-negative bacteria, and shows an unusual cross-resistance pattern with unrelated antibiotics. However, some strains of S. lavendulae elaborate, in addition to MSD-235, a narrow-spectrum antibiotic active against Gram-positive bacteria. In such a case, the antibacterial spectrum of the unfractionated broth will not serve as an identifying pattern, because it is made up of two superimposed patterns. MSD-235, produced by Streptomyces avidinii, is derived from a pair of components, MSD-235S and MSD-235L. These compounds have been separated and although each of the pair is inactive, they clearly interact synergistically because when they are combined the antibiotic complex MSD-235 is obtained, which gives in vitro activity and in vivo protection against Gram-negative microorganisms. Streptavidin (MSD-235L) has already been identified as a non-dialyzable material of high molecular weight protein and MSD-235S, having a small molecular weight, includes at least two related compounds ($MSD\text{-}235S_2$ and $MSD\text{-}235S_3$). $MSD\text{-}235S_3$ is now

called Stravidin. Treatment with MSD-235 did not protect mice against infection with Staphylococcus aureus; and did prolong the survival time of mice infected with Shigella gallinarum, Proteus vulgaris or Salmonella typhosa, and protected the animal against S. schott-muelleri.

(3) Thiazolidone (Actithiazic Acid)

Umezawa, et al. (34) reported isolation of an antibiotic, Actithiazic acid, produced by Streptomyces cinnamonensis and effective in vitro against Mycobacterium tuberculosis. Chemical and physical properties indicated that this antibiotic is perhaps identical to the thiazolidone antibiotic isolated by Sobin (35). Degradation and synthesis have shown this compound to be (-)2-(5-carboxypentyl)-4-thiazolidone. Ineffectiveness of the antibiotic in vivo suggests the existence of a substance which counteracts the effect of the antibiotic. From its structural resemblance to desthiobiotin and biotin, the antagonism between biotin and the antibiotic was investigated. It was found that the addition of 0.01 µg per ml of biotin to Kirchner's medium inoculated with M. tuberculosis could eliminate the antibiotic effect of less than 20 µg per ml of the actithiazic acid. Competitive antagonism also was demonstrated by agar diffusion method, using M.

tuberculosis strain 607.



McLamore, et al. (36) studied three types of closely related compounds of 4-thiazolidone: (1) 2-substituted-4-thiazolidones, (2) carboxyl derivatives, and (3) ring substituted analogs of 4-thiazolidones. Microbiological activities of 4-thiazolidones were determined against the assay organism Mycobacterium berolinense using the cup plate method and against Mycobacterium tuberculosis by a turbidometric procedure. Many of the esters and amides of this compound show greater antibacterial activity than the antibiotic itself.

Grundy, et al. (38) of the Abbott Laboratories isolated Actithiazic acid from five strains of Streptomyces virginiae and showed the activity against Mycobacteria. The streak test-screening demonstrated the presence of an antibiotic more active for Mycobacterium species ATCC 607 than for a variety of nonacid-fast organisms. It was shown that the structure of the antibiotic, actithiazic acid, 4-thiazolidone-2-capric acid, is closely related to biotin antimetabolites

known to possess tuberculostatic properties. The effect of the antibiotic *in vitro* was completely removed by the addition of biotin or pimelic acid to the test medium. It appears that actithiazic acid interferes with the synthesis of biotin by *Mycobacteria* since the organisms do not require preformed biotin in the system.

Mycobacidin (39) was named by the investigators of Lederle Laboratory for Actithiazic acid. They isolated the compound from an unusual streptomycete obtained from Venezuelan soil. These streptomycetes have many of the characteristics of *Streptomyces lavendulae*. When tested for competitive inhibition by the broth dilution technique, *Mycobacterium tuberculosis* 607, *Mycobacterium ranae*, and *Mycobacterium phlei* were completely inhibited.

An identical compound was isolated from the culture broth of *Streptomyces acidomyceticus* and named acidomycin by Kawashima, *et al*. (40). It was found that rabbit urine, after the IM injection of acidomycin, gave a double zone during assay procedure by the cylinder plate method using *Mycobacterium tuberculosis* var. *avium*. The double zone was composed of an outside zone owing to inhibitory effect of acidomycin and an inside zone formed by the stimulation of unknown factors. They studied the antagonism between acidomycin and its own chemical degradation products, namely, pimelic acid, thioglycollic acid and ω-acetaminoheptanonic acid, against

L. arabinosus, L. casei and Mycobacterium tuberculosis var. avium in biotin-deficient medium by inhibition analysis. Biotin was used as the control sample. It was found that biotin is the sole antagonist of acidomycin among the substances related to acidomycin. Chromatographic analysis showed that one of the anti-acidomycin factors in rabbit urine might be biotin.

b. Vitamin B_{12}

(1) Descobaltocorrinoids

Toohey (41,42) found some corrinoid compounds containing no metal in Chromatium strain D. Rhodospirillum rubrus and Rhodopseudomonas plustus. Recovery of these compounds was facilitated by passage over Dowex 1 and Dowex 50 (Na) chromatographic columns. One negative, one neutral, and three positive compounds were obtained. These compounds were then tested as antagonists (43) of vitamin B_{12} in some bioassay systems. The cobalt-free corrinoids used were: the neutral compound, believed to be descobaltocobamide (I); the positively charged compound, believed to be descobaltocobinamide (II); and the negatively charged compound, which was not identified (III).

Tests were carried out to determine whether these compounds exhibited any growth promoting activity in the absence of vitamin B_{12} using E. coli 113-3, Lactobacillus leichmannii ATCC 7380, and E. coli B which had been

inhibited from developing by treatment with sulfanilamide. None of the compounds showed significant growth promoting activity in these organisms; the activity was less than 0.01 per cent that of cyanocobalamin. These results are shown in Table II.

TABLE II

Effect of Cobalt-Free Corrinoids on the Cyanocobalamin Growth Response of *E. coli* 113-3 and *L. leichmannii* ATCC 4797

	50% Inhibition Index	
Corrinoid	*E. coli* 113-3*	*L. leichmannii***
I. (neutral)	15	8
II. (positive)	2	19
III. (negative)	40	not tested

*cyanocobalamin concentration = 0.06 ng/ml
**cyanocobalamin concentration = 0.005 ng/ml

It is evident that the cobalt-free corrinoids are strong antagonists of vitamin B_{12}.

Addition of cobalt salts to the medium together with the cobalt-free corrinoids did not bring about the fermentation of active complexes when tested in the *E. coli* 113-3 system. This may have resulted from the failure of the cobalt ions to enter the cells or from the absence of enzymes in this organism for introducing

cobalt into the corrin ring.

The B_{12} antagonism by the cobalt-free corrinoids in the bioassay systems suggested that these compounds block the B_{12} binding sites and that the cobalt atom may not be necessary for binding to these sites.

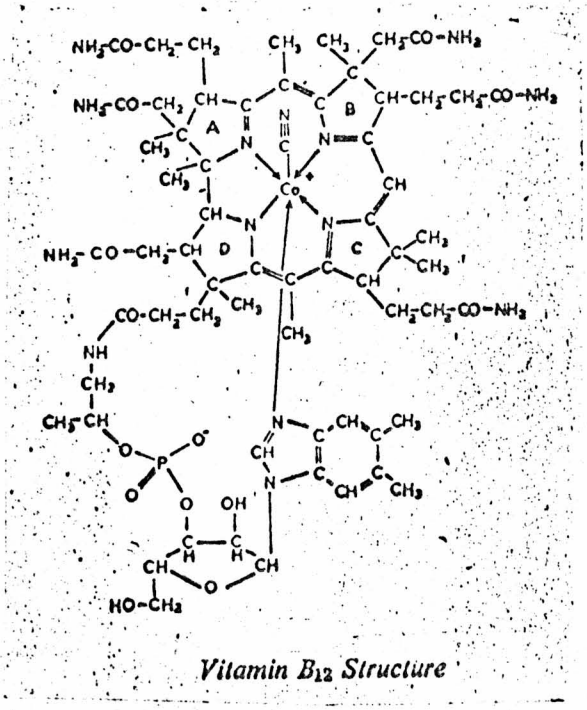
(2) Cobamide-Monocarboxylic Acids

The first naturally occurring vitamin B_{12} antagonist was isolated by Keleman (44) from Propionobacterium shermanii. It was shown to be an acidic material and proved to be homogeneous as determined by paper chromatography and by electrophoresis. Microbiological investigations using the agar plate method showed that the material had a growth-inhibiting effect on E. coli. The inhibition index was found to be 40. Since, on changing the concentrations of vitamin B_{12}, no changes in the inhibition index could be elicited, the antagonism had to be regarded as competitive. Experiments performed with labelled vitamin B_{12} and isolation of vitamin B_{12} at neutral pH showed that the monocarboxylic acid derivative of vitamin B_{12} isolated from Propionobacterium shermanii was not an artifact but was produced during the fermentation process. On fermentation, the quantity of B_{12} monocarboxylic acid showed a gradual decrease. Thus it should be regarded as an intermediate in the vitamin's biosynthesis rather than as a decomposition product. It

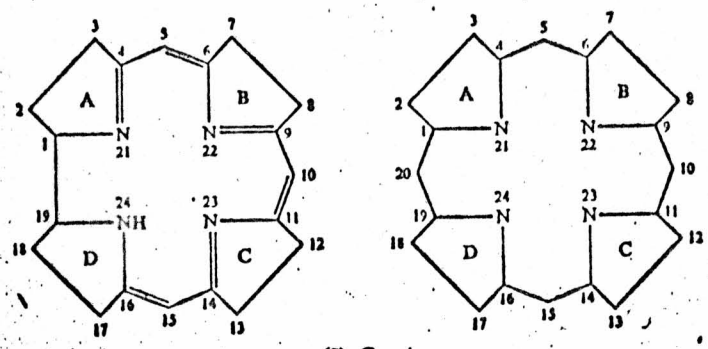
is quite possible, however, that a shift in the equilibrium state of a reversible process is the reason why a decreasing value for monocarboxylic acid derivatives and an increasing value for vitamin B_{12} were evident at different times during fermentation (45).

C. Chemical Modification of Vitamin B_{12} to Form Antagonists

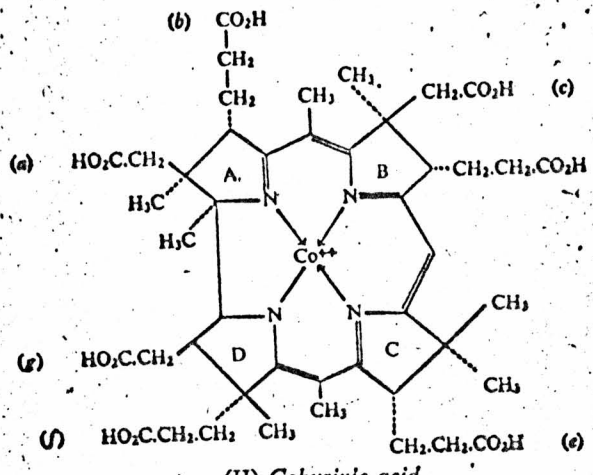
I. Nomenclature



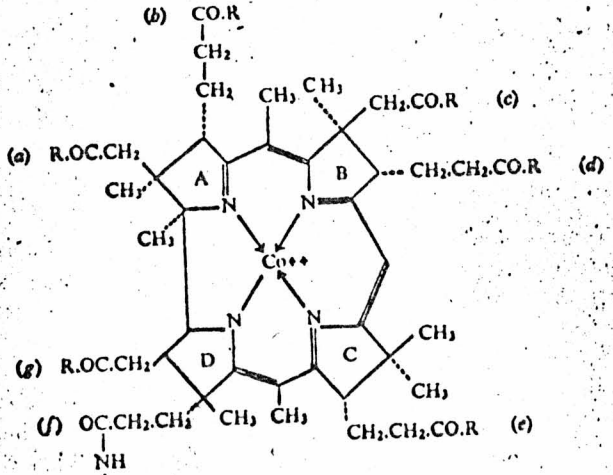
Vitamin B_{12} Structure



(I) *Corrin*



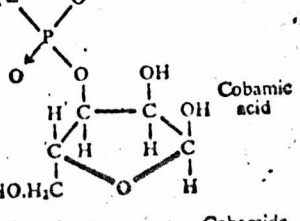
(II) *Cobyrinic acid*



(IIIa): R = OH, X = H Cobinic acid

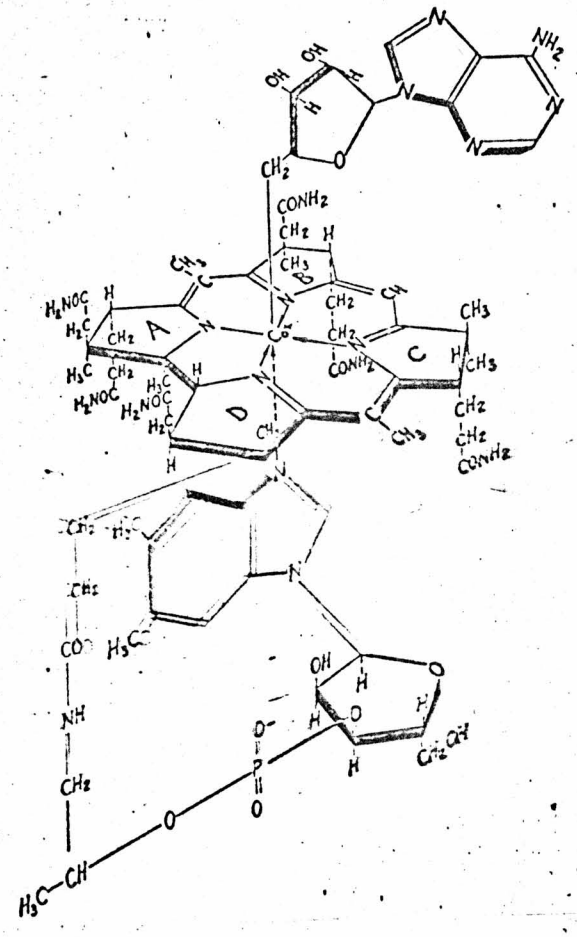
(IIIb): R = NH_2, X = H Cobinamide

(IVa): R = OH, X =



Cobamic acid

(IVb): R = NH_2, X as above Cobamide



Spatial Formula of Coenzyme B_{12}

2. Cobyric Acids

Only a few of the many known ways of modifying B_{12} molecules lead to competitive antagonists. The sensitive points (46) are:

(a) the ribose hydroxyls, on conversion to stable derivatives such as the carbanilide;

(b) the propionamide chains, one in particular, on conversion to substituted amides; and

(c) the alkanolamine bridge, on replacement by related alkanolamines.

Compounds of the second group have been the most intensively studied.

They were first prepared by Lester Smith and colleagues by removing at least one molecule of ammonia from the propionamide group in the vitamin molecule. The resulting acids themselves showed inhibitory activity, but this was increased by converting them into substituted amides of methylamine or some other amine. The necessary acids were made either by deliberate hydrolysis of vitamin B_{12} with cold dilute hydrochloric acid or from by-product fractions arising in the manufacture of vitamin B_{12}. Separation of the mono-, di- and tri-carboxylic acids from each other, and from unchanged vitamin B_{12}, was best effected with columns of

DEAE cellulose or Dowex 1X2 ion-exchange resin, which held the acids and released them successively. The monocarboxylic acid was eluted first by elution with increasing concentrations of sodium chloride. Most of the antagonists tested were prepared from the monocarboxylic acids; separation of three isomers by chromatography was not usually attempted, but in practice one isomer greatly exceeded the others in quantity.

Most of the numerous methods now available for converting carboxylic acids into reactive intermediates had to be rejected because the reagents damaged other parts of the molecule. Usually an alkyl chloroformate was used, at a temperature around -5°C, with triethylamine, in anhydrous dimethylformamide as solvent, to prepare the mixed anhydride with carbonic ester. This was then made to react, without isolation, with the selected amine. Side reactions occurred, leading to brown products and substances lacking nucleotide, so that purification by partition chromatography was necessary before the substituted amide could be isolated in pure crystalline form. The compounds prepared in this way are listed in Table III.

TABLE III

Inhibition Indices of Anti-B_{12}'s for *E. coli* Mutant (46)

Compound	Substituted at: E_1	E_2	Inhibition Index (Plate Test)
Monobasic acid E_2	NH_2	OH	60:80:100
Methylamide of E_2	NH_2	$NH.CH_3$	50:50
Ethylamide of E_2	NH_2	$NH.C_2H_5$	80:100
Butylamide of E_2	NH_2	$NH.C_4H_9$	very high
Amylamide of E_2	NH_2	$NH.C_5H_{11}$	inactive
Diethylamide of E_2	NH_2	$N.(C_2H_5)_2$	---
Anilide of E_2	NH_2	$NH.C_6H_5$	220:250
Monobasic acid E_1	OH	NH_2	400:600
Ethylamide of E_1	$NH.C_2H_5$	NH_2	120
Dibasic acid	OH	OH	very high
Methylamide Monobasic Acid	OH	$NH.CH_3$	1400
Methyl Diamide	$NH.CH_3$	$NH.CH_3$	1000
Ethylamide Monobasic Acid	OH	$NH.C_2H_5$	1800:2000: 2000
Ethyl Diamide	$NH.C_2H_5$	$NH.C_2H_5$	2000: 2000

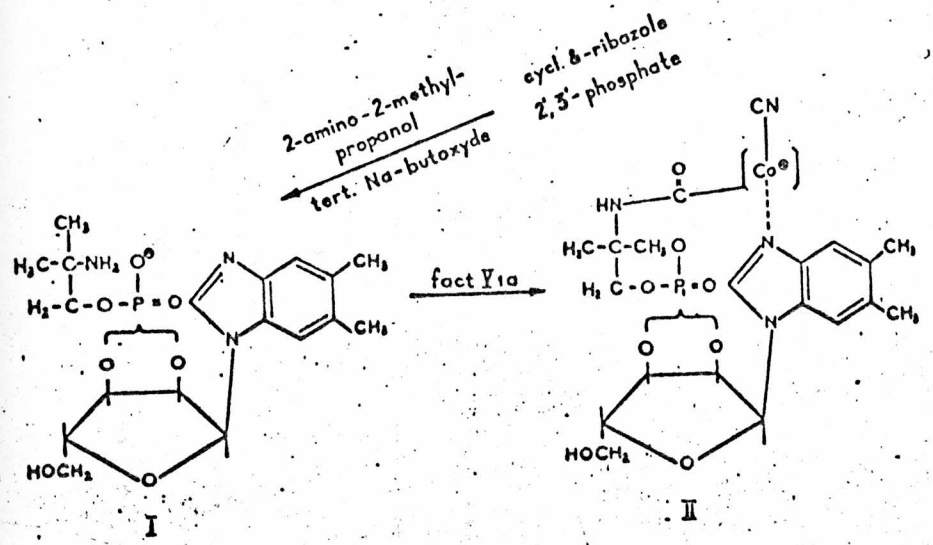
An *E. coli* mutant was used as the test organism here.

Wakin and Weinstein (47) found that two substituted amides competed strongly with radioactive B_{12} for binding sites on plasma and intrinsic factor. These compounds also depressed absorption of oral B_{12} by human subjects and rats. The amide antagonists have shown no toxic effects in various species of animals; prolonged dosing of rats producing barely significant reductions in red cell and haemoglobin levels. Competitive inhibition was demonstrated in rat growth tests by Cuthbertson and

colleagues. More extensive testing has been carried out by Coates and colleagues on chicks and chick embryos (48). Injection of anilide into eggs from B_{12}-deficient hens depressed the already low percentage hatch and the effect could be reversed by B_{12}. Embryos that failed to hatch often had abnormalities such as deformed legs and haemorrhages, as in extreme B_{12} deficiency. In live chicks a similar competitive inhibition of B_{12} was demonstrated by growth rate measurements. In both systems the inhibition index for the anilide was about 250 but the ethylamide and methylamide were less effective, in contrast to their behavior in the E. coli system.

3. Alkanolamine Compounds

Potent antimetabolites have been prepared and tested by Friedrich and Heinrich (49,50), in which the aminopropanol moiety is altered. The C_3'-2-methyl-2-amino-propanol-vitamin-B_{12}-analog (the so-called S102 and biologically the most interesting alkanolamine type analog of vitamin B_{12}), prepared by partial chemical synthetic starting from factor V_{1a} (= cobyrinic acid-abodeg-hexa-amide). The chemical partial synthesis was carried out in two steps:



(1) Condensation of cyclic-&-ribazole-2',3'-phosphate with 2-CH_3-2-NH_2-propanol in the presence of tert sodiumbutylate followed by two chromatographic procedures on amberlite XE 64 and DEAE-cellulose. The product of this condensation in the diester moiety (I), with the phosphoric acid linked to the respective C_3' or C_2' of the ribose.

(2) Condensation of the diester moiety with factor V_{1a} resulting in a mixture of the two "complete" C_3' and C_2' B_{12} compounds (II). The only difference between the C_3'-forms of S102 and vitamin B_{12} is the different alkanolamine.

The separation of this mixture of isomers (II) was done by electrophoresis in 0.5 N CH_3COOH since the C_2'-isomer takes up one proton because of its weak coordinative cobalt-imidazole linkage and thus migrates towards the cathode. The neutral C_3'-isomer of S102 is chromatographically and electrophoretically pure after a second chromatographic separation on cellulose-powder. These compounds were tested and listed in Table IV (49):

TABLE IV

Compound	E. coli 113-3		Ochromonas malhamensis	
	Inhibition Index	Bioactivity	Inhibition Index	Bioactivity
C_3'-D-1-Methyl-2-amino-ethanol	-	100	-	100
C_3'-L-1-Methyl-2-amino-ethanol	-	60	-	100
C_3'-L-2-Methyl-2-amino-ethanol	6:1	0.1	80:1	4
C_3'-2-Methyl-2-amino-propanol	3:1	0.01	60:1	0.01
C_3'-2-amino-ethanol	-	83	-	60

TABLE IV - Cont.

Compound	E. coli 113-3		Ochromonas malhamensis	
	Inhibition Index	Bioactivity	Inhibition Index	Bioactivity
C_3'-3-amino-propanol	12:1	0.01	-	16.
C_3'-DL-1-Phenyl-2-amino-ethanol	17:1	0.01	-	0.01
C_3'-DL-2-Benzyl-amino-ethanol	200:1	0.01	-	0.01
C_3'-N-ethyl-2-ethanol	700:1	0.01	-	0.2

Other alkanolamines gave a less potent inhibitory activity. Among all, the C_3'-2-methyl-2-amino-propanol was found to be the most potent compound that was chemically synthesized.

4. Coenzyme Antagonists

Numerous alkyl and substituted alkyl groups can be attached chemically to the structure of coenzyme B_{12}, and many of them have been tested in biological systems. In the enzyme assay of Abeles and Lee (51), all the analogs tested showed strong competitive inhibition of the coenzyme. Inhibition indices, i.e., the concentration ratios of analog to coenzymes for 50% inhibition, are shown in Table V.

TABLE V

Inhibition Indices of Coenzyme Analogs

Compounds	$\frac{[\text{Inhibition}]}{[\text{Coenzyme}]}$ = Inhibition Index at 50% Inhibition
Methyl analog	13
Ethyl analog	8
Vinyl analog	2
Ethinyl analog	12
Isopropylidin coenzyme	2
Diacetyl coenzyme	5
Uridine analog	1
Mesyl analog	18
Cyanocobalamin	11
Hydroxocobalamin	5

Light-sensitive coenzyme forms of S102 were made by Friedrich (52). The inhibitory effect of the coenzyme-type derivatives of the C_3'-2-methyl-2-amino-propanol-B_{12}-analog on growing cells of E. coli 113-3 was measured under standard conditions. Without exception all the coenzyme forms of the alkanolamin type B_{12}-antivitamin are not as potent vitamin-B_{12} antagonists as the cyano-form of C_3'-2-methyl-2-amino-propanol-B_{12} analog. The activities of these compounds are listed in the following table VI.

TABLE VI

BIOACTIVITY AND INHIBITION INDICES OF S 102-COENZYME FORMS FOR *E. coli*, *Ochromonas* AND DIOL DEHYDRASE

Coenzyme-Type Derivatives of the C_r-2-Methyl-2-amino-propanol-B_{12}-Analogue (≡ S 102)		*Escherichia coli* 113–3		*Ochromonas malhamensis*		Inhibition Index for Diol Dehydrase from *Aerobacter aerogenes* (Propandiol → propionaldehyde)
		Bioactivity*	50% Inhibition Index	Bioactivity*	50% Inhibition Index	
	Co—CN (≡CN—S 102)	<0.01	3:1	<0.1%	30:1	2.5:1†
Methyl-S 102	Co—CH_3	<0.01	5:1	<0.1%	30:1	1:1
Ethyl-S 102	Co—CH_2—CH_3	<0.01	140:1	<0.1%	60:1	0.7:1
Propyl-S 102	Co—CH_2—CH_2—CH_3	<0.01	180:1	<0.1%	250:1	0.7:1
Ethylamine-S 102	Co—CH_2—CH_2—NH_2	<0.01	14:1	<0.1%	580:1	0.4:1
Allyl-S 102	Co—CH_2—CH = CH_2	<0.01	30:1	<0.1%	50:1	1.1:1
Isopropylidene-C_5-deoxyadenosyl-S 102		<0.01	7:1	<0.1%	180:1	—
C_5-deoxyadenosyl-S 102		<0.01	4:1	<0.1%	30:1	1.8:1

* The growth promoting activity of an equimolar amount of cyanocobalamin or DMBC-coenzyme is equal to 100 per cent.
† The 50 per cent inhibition index for cyanocobalamin is 1.9:1.

5. Benziminazoles and Phenylenediamines

Benziminazole moiety is the major portion of the B_{12} molecule. Several examined the actions of the related compounds. Woolley (53) speculated on some compounds such as 1,2-dimethyl-4-amino-5-hydroxybenzene, which caused transient regression of spontaneous mammary tumors in mice (54).

The B_{12} precursor 1,2-dimethyl-4,5-diamino-benzene and also the corresponding 5,6-dimethylbenziminazole are inhibitory towards both Mycobacterium tuberculosis (55) and Lactobacillus lactis. The effect of the latter was nullified by vitamin B_{12}.

Scott, et al. (56) tested many benziminazoles and found that some bearing -SH at position 4 (besides other substituents) weakly inhibited the E. coli mutant. The effect was reversed by vitamin B_{12}. Epstein and Timmis (57) tested some 300 heterocyclic compounds against Euglena gracilis. Only 43 were inhibitory at low B_{12} levels and only 10 showed partial or complete competitive antagonism to B_{12}. The most effective were 6-mercaptopurine and other purines or azapurines. It was suggested that such compounds may act indirectly by interfering with cofactors involved in vitamin B_{12} utilization. Perlman, et al. (58) showed that B_{12} reversed the growth inhibition of E. coli mutant caused

by sulfonamides, 6-mercaptopurine and other purine antagonists. Oginsky and Smith (59) showed that 5,6-dimethylbenziminazole inhibited uptake of radioactive B_{12} by E. coli mutant cells, under certain conditions.

II. OCCURRENCE AND NATURE OF VITAMIN B_{12} ANTAGONISTS IN FERMENTATIONS

A. Materials and Methods

1. Bacteriological Media

H_{26} medium (liquid) - components are given in g/l

K_2HPO_4	7
KH_2PO_4	3
$Na_3C_6H_5O_7 \cdot 2H_2O$	0.5
$MgSO_4 \cdot 7H_2O$	0.1
$(NH_4)_2SO_4$	1

dissolved in distilled water (1 l.)

H_{26} medium (agar)

H_{26} liquid medium	1 l.
Bacto agar	15 g
Glucose (autoclaved separately)	5 g

Media for desired cultures - components are given in g/l

(a) Nutrient agar	13
Glucose, anhydrous	10

dissolved in distilled water (1 l.)

(b) Nutrient broth	8
Glucose, anhydrous	10

dissolved in distilled water (1 l.)

(c) Bacto agar 20
Glycerol 30 ml
Soybean meal 30
dissolved in distilled water (1 l.)

2,3,5-Triphenyl-2H-tetrazolium Chloride

2.5 g of tetrazolium salt was dissolved in 100 ml of distilled water and stored in refrigerator for use.

Vitamin B_{12} Stock Solution

Vitamin B_{12} cyanide (10 mg) was dissolved in 10 ml of distilled water. This solution was stored in the freezer. 0.1 ml of this solution was diluted into 10 ml with distilled water and filtered through Millipore membrane filter. This final sterile solution was used without further treatment.

Glucose Stock Solution

50 g of glucose, anhydrous, was dissolved in 100 ml of distilled water and ready for use after autoclaving.

Medium for Staphylococcus aureus

Antibiotic medium 3 (contains Bacto-beef extract, 1.5 g; Bacto-yeast extract, 1.5 g; Bacto-peptone, 5 g; Bacto-dextrose, 1 g; Dipotassium phosphate, 3.68 g; Monopotassium phosphate, 1.32 g; sodium chloride, 3.5 g) - 17.5 g
Bacto agar - 15 g
dissolved in 1 l. of distilled water

All of these solutions and media except vitamin B_{12} were sterilized by autoclaving at 121°C (15 lbs per square inch) for 20 minutes.

Potassium diphosphate and monophosphate were manufactured by J. T. Baker Chamical Co. Sodium citrate and ammonium sulfate were manufactured by Mallinckrodt Chemical Works. Magnesium sulfate and glucose were made by Allied Chemical. Bacto agar, nutrient agar, nutrient broth and antibiotic medium 3 were produced by Difco Laboratories and 2,3,5-triphenyltetrazolium chloride was produced by Aldrich Chemical Co., Inc.

2. Isolation of Cultures from Natural Sources

The cultures were isolated from soil samples selected in Madison, Wisconsin. One gram of soil was put into 100 ml of distilled water. 0.1 ml of the suspension was diluted to 100 ml of distilled water. 0.3 ml of this final dilution was pipetted into each Petri dish containing 15 ml of nutrient agar. These plates were incubated at 37°C overnight. Isolated colonies were transferred onto agar slants of the nutrient agar and incubated at 37°C overnight. These cultures were then ready for testing.

Various sources besides the soil were used in the process of searching for cultures producing antivitamin B_{12}'s. Cow rumen samples were obtained from the

laboratory of the Dairy Science Department at the University of Wisconsin and was treated in the same way as used for soil samples. Rotted pears and moldy bread were also used for this purpose.

Another agar medium, containing soybean meal, glycerol and bacto agar, was used sometimes in the isolation of cultures. The composition of this medium was listed in the section of "Materials and Methods" (p. 38).

3. Screening of Cultures for Production of Vitamin B_{12} Antagonists

Isolated cultures were inoculated aseptically into each 250 ml Erlenmeyer flask containing 100 ml of nutrient broth (0.5% peptone, 0.3% beef extract per liter) and 1% glucose. They were grown on a rotary shaker at 30°C, 200 rpm for four days. The cells were then removed by centrifugation and the supernatants were used for bioassay against the vitamin B_{12} requiring *E. coli* 113-3.

a. Bioassay Against E. coli 113-3

Davis and Mingioli (60) in 1948 obtained several mutants of *E. coli* that required either methionine or vitamin B_{12} for growth. Their possibilities as assay organisms were quickly appreciated by workers at the Glaxo Laboratories, who developed cup-plate assays with

the mutant 113-3 in Petri dishes or large plates (61). This organism is somewhat less sensitive than the vitamin B_{12} requiring lactobacilli, but it will grow in a much simpler medium and is most dependable for routine assays. This organism does not respond to deoxyribonucleosides. It can utilize methionine as a substitute for vitamin B_{12} but the levels required are much higher and interference by methionine is unlikely to occur with ordinary test samples. This mutant is among the most catholic of all tested organisms in its response to vitamin B_{12} analogs, and for this reason it has been widely used for their assay and bio-autography.

E. coli 113-3 (ATCC 14169) was obtained from the American Type Culture Collection. It was transferred onto H_{26} agar slants containing 10 ml of H_{26} agar medium, 1% glucose, and 0.1 mcg of B_{12} per tube. This culture was incubated at 30°C overnight and inoculated into 250 ml Erlenmeyer flasks containing 100 ml of H_{26} liquid medium, 1% glucose and 1 mcg of B_{12}. They were grown on a rotary shaker at 30°C and 200 rpm for two days. The cells were then collected by centrifugation and washed twice with 10 ml of sterile H_{26} liquid medium. Finally these washed cells were suspended in 10 ml of fresh H_{26} medium and stored in the refrigerator for use.

An agar-diffusion assay was used throughout the whole study. 250 ml of H_{26} agar medium, 2.5 ml of 50%

glucose solution, 1 ml of 25% tetrazolium solution, and 0.8 ml of *E. coli* 113-3 washed cell suspension were mixed and poured into a 35 x 20 x 5 cm Pyrex baking dish. Culture filtrates thought to contain vitamin B_{12} antagonists were applied onto 13 mm paper discs and the discs were placed on the surface of the agar after it had been gelled. A disc of vitamin B_{12} (10 mcg/ml) was placed adjacent to it. Part of the growth zone around the B_{12} disc was inhibited after incubation overnight. This is demonstrated in Figure 2. The diameter of the inhibition zone around the antagonist-disc was measured.

Figure 2

Another technique was used to serve the same purpose: 10 mcg of B_{12} were added to 250 ml of agar medium previous to incubation instead of applying onto paper discs. Clear zones can be seen around the discs of antagonists against the reddish-purple background of the plate which indicated the growth of E. coli 113-3. This is demonstrated in Figure 3. The diameters of the inhibition zones around the antagonists' discs were measured.

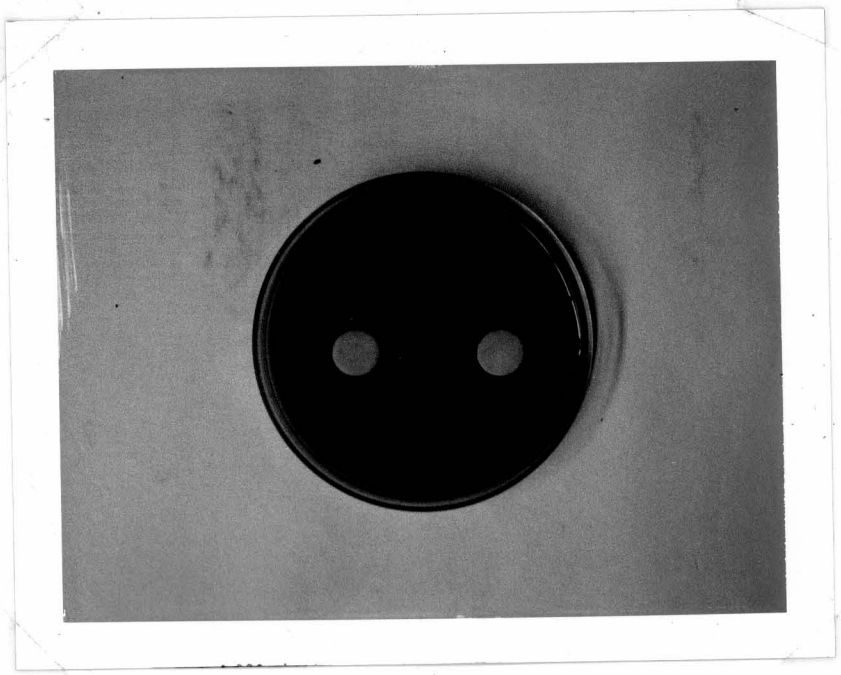


Figure 3

In order to prove that a vitamin B_{12} antagonist is a naturally-occurring antimetabolite instead of an antibiotic, an assay was done using E. coli B as the test organism. E. coli B was grown overnight in a 250 ml Erlenmeyer flask containing 100 ml of nutrient broth (with 1% glucose present) at 37°C. 5 ml of this culture were added to 250 ml of nutrient agar. The same kind of technique was used here as was used in the bioassay against E. coli 113-3.

For the purpose of confirming further that the B_{12} antagonists inhibit only the B_{12} requiring culture-E. coli 113-3, assay was done using Staphylococcus aureus as the test organism. S. aureus was grown in 100 ml of nutrient broth in a 250 ml Erlenmeyer flask at 37°C overnight. 5 ml of this culture were added to 150 ml of antibiotic medium (see page 39). This was poured into a plate already layered with 100 ml of nutrient agar. The antibiotic assay was performed the same way as all those mentioned above.

4. Other Analytical Methods and Preparative Techniques

a. pH Measurement

A Leeds and Northrup direct reading pH meter fitted with Sargent-pH combination electrode (S-30070-10 and S-30072-15, E. H. Sargent & Co. by Jena Glass Works,

Mainz, Western Germany) was used for measuring pH of the solutions.

b. Estimation of Bacterial Growth

Bacterial growth was estimated from measurements of optical density (after dilution of 0.5 ml of the cultures with distilled water to make 5 ml of solution) in a test tube (1.3 cm in diameter) with a Bausch and Lomb Spectronic 20 Colorimeter. The turbidity readings followed Beer's law.

c. Dialysis

A cellophane sack prepared from a 10 cm length of dialysis tubing was filled with 2 ml of the culture filtrate. The sack was tied to a magnetic bar at one end and was placed in a beaker of distilled water. The whole arrangement was placed on a magnetic stirrer in a cold room at a temperature of 9°C for 24 hours. The water was changed three times while the experiment was in process. The filtrates inside the sack were then tested against *E. coli* 113-3 for its anti-vitamin B_{12} activity.

d. Precipitation by Acetone Purification

Acetone (analytic reagent) was added to the crude supernatants to give a concentration of 70%, 80% and

90% (v/v). A yellow amorphous precipitate was collected by centrifugation and removed. The supernatants were evaporated to dryness on a flash evaporator (Buchler Instrument). The concentrate was then dissolved in distilled water to give the original volume. The precipitate was also dissolved in distilled water to give the same volume. Both of the solutions were tested for their inhibitory activity.

e. Absorption on Ion Exchange Resin

A strong acid cation-exchange resin (Dowex-50W, 40% cross linked, dry mesh-100 to 200, obtained from Sigma Chemical Company) was used to absorb the active component from the culture filtrate which inhibited the growth of E. coli 113-3. The supernatant obtained after filtration through glass sintered funnel was adjusted to neutral pH with 1 N ethylmorpholine. The resin was eluted with pyridine (1 N) and the eluate was found to have a pH of 6.0. Both of these solutions were tested for activity against E. coli 113-3. The resins were then eluted with additional pyridine (eluate pH 7.5) and with ethylmorpholine to give an eluate pH around 8.0. The latter eluate was adjusted to around pH 7.0 with 1 M acetic acid. These filtrates were then studied for activity against E. coli 113-3.

f. Characterization by Paper Chromatography and Paper Ionophoresis

Aliquots of the fermentation samples were centrifuged, and a portion of the supernatant solution diluted with acetone as described above. (The untreated supernatant solution was designated as A.) The amorphous precipitate was collected by centrifugation, the supernatant evaporated to near dryness, and then diluted with distilled water (designated as sample B). Both solutions were lyophilized and the solids dissolved in 0.5 ml of distilled water. Aliquots (0.01 to 0.02 ml) were used for descending paper chromatography in a n-butanol:acetic acid:water (4:1:4, by volume) system and paper ionophoresis. A Savant apparatus at 40 volts/cm for 90 min in a mixture of acetic acid and formic acid (20% and 2%, respectively, which gave a pH of 1.8), a pyridine-acetate, pH 4.5, and a pyridine-acetate buffer, pH 6.5, were used for paper ionophoresis.

The position of substances with vitamin B_{12} antimetabolite activity on the chromatograms and ionophorograms was determined by bio-autography as follows: The air-dried paper was washed three times with ethyl ether, the ether evaporated, and then the paper chromatograms were placed in contact with a H_{26} agar plate (35 x 20 x 5 cm) and seeded with *E. coli* 113-3 for 30 minutes. The paper was removed and the plate

incubated overnight at 37°C.

Ninhydrin-positive substances on the paper chromatogram were detected by spraying the paper with a solution containing ninhydrin and 2,4,6-collidine (each 0.16%, w/v) in n-butanol (62).

B. Experimental Observations

1. Selection of Soil Microorganisms for Production of Vitamin B_{12} Antagonists

678 cultures were isolated from various soil samples and other sources. Most of them were found to be Gram-positive and Gram-negative rods and cocci, and some molds were also isolated. Isolates numbered 382, 383, 403, 439, 461, 536, 568, 605, 611, 612, 677 and 678 were found to produce substances inhibitory to the growth of E. coli 113-3 and had no effect against E. coli B, or against Staphylococcus aureus 209 P. Culture #212 had slight inhibitory activity against all three of these organisms. This culture produced a fluorescent compound in agar and had the morphological characteristics of pseudomonad.

Of the 14 cultures, only the first seven were intensively studied. According to the Gram-stain, #212 was identified as a Gram-negative cocci, #382, 383 and 439 were found to be Gram-positive rods, whereas #403, 442 and 461 were Gram-positive cocci.

2. Effect of Fermentation Conditions on the Production of Vitamin B_{12} Antagonists

a. Incubation Temperature

Incubation temperature is known to be a very important factor for bacterial growth. An experiment studying the best temperature for bacterial production of vitamin B_{12} antagonists was carried out by growing these cells at different temperatures including 25°C, 30°C and 37°C. Cells were aseptically transferred from slants into 250 ml Erlenmeyer flasks containing 100 ml of sterile nutrient broth supplemented with 1% glucose. A water-bath shaker was used in this experiment with a shaking speed at 160 rpm. Turbidity measurements (O.D.) were made periodically during the incubation period. pH values were read directly on a pH meter. After sampling the fermentations the cells were removed by centrifugation and the supernatants were assayed for anti-vitamin B_{12} activity against *E. coli* 113-3. The results of this experiment are shown in Table VII.

From the results of this experiment, it was decided that these cells seemed to grow well and produced most anti-vitamin B_{12} activity when incubated at 30°C, and all other experiments were incubated at this temperature.

TABLE VII

Production of Anti-vitamin B_{12} Substances as a Function of Incubation Temperature

A. Culture #212

Hours after incubation	Incubation Temperature								
	25°C			30°C			37°C		
	pH	O.D.*	I.Z.**	pH	O.D.	I.Z.	pH	O.D.	I.Z.
0	7.0	0.03	0	7.0	0.03	0	7.0	0.03	0
2	-	-	-	7.05	0.08	0	-	-	-
4	-	-	-	7.15	0.25	0	-	-	-
6	-	-	-	7.25	0.59	0	7.1	0.3	0
8	-	-	-	7.40	1.15	0	7.3	0.68	0
11	-	-	-	-	-	-	7.5	1.0	0
14	-	-	-	7.65	1.80	0	-	-	-
24	8.0	0.65	16.5	7.7	2.0	0	7.7	1.60	0
28	-	-	-	-	-	-	7.6	1.6	0
30	-	-	-	7.65	2.4	0	-	-	-
48	7.9	2.1	20	-	-	-	7.8	1.8	0
52	-	-	-	7.45	2.7	21	-	-	-
72	7.6	2.8	19	7.95	2.6	22	7.25	1.8	0
96	7.4	2.9	24	-	-	-	-	-	-

*O.D.: optical density read at 660 nm.

**I.Z.: diameter of inhibition zone in mm.

TABLE VII - Cont.

B. Culture #382

Hours after incubation	Incubation Temperature								
	25°C			30°C			37°C		
	pH	O.D.*	I.Z.**	pH	O.D.	I.Z.	pH	O.D.	I.Z.
0	7.0	0.3	0	7.0	0.03	0	7.0	0.03	0
2	-	-	-	7.0	0.04	0	-	-	-
4	-	-	-	6.45	0.17	0	-	-	-
6	-	-	-	5.9	0.32	0	6.10	0.20	0
8	-	-	-	5.8	0.65	0	5.45	0.41	0
11	-	-	-	-	-	-	5.32	0.53	0
14	-	-	-	6.06	1.50	0	-	-	-
24	5.5	1.35	0	7.2	1.85	20.5	5.32	0.59	0
28	-	-	-	-	-	-	5.30	0.56	0
30	-	-	-	7.4	2.95	20.5	-	-	-
48	6.0	1.9	0	-	-	-	5.62	0.55	0
52	-	-	-	6.2	2.65	21	-	-	-
72	6.3	3.1	0	-	-	-	5.10	0.53	19
96	6.2	3.0	0	-	-	-	-	-	-

*O.D.: optical density read at 660 nm.

**I.Z.: diameter of inhibition zone in mm.

TABLE VII - Cont.

C. Culture #383

Hours after incubation	Incubation Temperature								
	25°C			30°C			37°C		
	pH	O.D.*	I.Z.**	pH	O.D.	I.Z.	pH	O.D.	I.Z.
0	7.0	0.03	0	7.0	0.03	0	7.0	0.03	0
2	-	-	-	6.9	0.08	0	-	-	-
4	-	-	-	6.1	0.33	0	-	-	-
6	-	-	-	5.85	0.56	0	6.55	0.09	0
8	-	-	-	5.70	1.20	0	5.45	0.36	0
11	-	-	-	-	-	-	5.25	0.50	0
14	-	-	-	6.25	2.60	0	-	-	-
24	5.5	1.75	0	7.05	3.20	18	5.25	0.59	0
28	-	-	-	-	-	-	5.15	0.56	0
30	-	-	-	6.8	3.10	19	-	-	-
48	6.0	3.9	0	-	-	-	5.45	0.56	0
52	-	-	-	6.25	2.12	19	-	-	-
72	6.25	3.7	0	-	-	-	5.10	0.54	18
96	6.1	3.95	0	-	-	-	-	-	-

*O.D.: optical density read at 660 nm.

**I.Z.: diameter of inhibition zone in mm.

TABLE VII - Cont.

D. Culture #403

Hours after incubation	Incubation Temperature 25°C pH	25°C O.D.*	25°C I.Z.**	30°C pH	30°C O.D.	30°C I.Z.	37°C pH	37°C O.D.	37°C I.Z.
0	7.0	0.03	0	7.0	0.03	0	7.0	0.03	0
2	-	-	-	6.8	0.10	0	-	-	-
4	-	-	-	6.1	0.36	0	-	-	-
6	-	-	-	5.85	0.69	0	5.15	0.42	0
8	-	-	-	5.80	1.40	0	5.10	0.58	0
11	-	-	-	-	-	-	5.10	0.65	0
14	-	-	-	6.55	3.05	0	-	-	-
24	5.5	1.8	0	7.3	3.3	17	5.35	0.63	0
28	-	-	-	-	-	-	5.15	0.60	0
30	-	-	-	7.15	2.82	18	-	-	-
48	6.3	3.25	0	-	-	-	5.40	0.60	20
52	-	-	-	6.85	1.5	19	-	-	-
72	6.4	2.55	0	-	-	-	5.10	0.60	20
96	6.25	2.55	17	-	-	-	-	-	-

*O.D.: optical density read at 660 nm.

**I.Z.: diameter of inhibition zone in mm.

TABLE VII - Cont.

E. Culture #439

Hours after incubation	Incubation Temperature								
	25°C			30°C			37°C		
	pH	O.D.*	I.Z.**	pH	O.D.	I.Z.	pH	O.D.	I.Z.
0	7.0	0.03	0	7.0	0.03	0	7.0	0.03	0
2	-	-	-	6.7	0.13	0	-	-	-
4	-	-	-	5.85	0.39	0	-	-	-
6	-	-	-	6.0	0.69	0	5.25	0.42	0
8	-	-	-	5.90	1.60	0	5.30	0.46	0
11	-	-	-	-	-	-	5.35	0.45	0
14	-	-	-	5.62	3.20	21	-	-	-
24	5.0	0.5	0	6.15	4.0	20.5	5.35	0.46	0
28	-	-	-	-	-	-	5.25	0.46	0
30	-	-	-	6.2	3.95	21	-	-	-
48	6.15	3.25	0	-	-	-	5.60	0.45	0
52	-	-	-	7.35	7.5	-	-	-	-
72	6.30	3.35	21.5	7.9	3.6	-	5.25	0.41	20
96	6.20	2.5	24	-	-	-	-	-	-

*O.D.: optical density read at 660 nm.

**I.Z.: diameter of inhibition zone in mm.

TABLE VII - Cont.

F. Culture #442

Hours after incubation	Incubation Temperature								
	25°C+			30°C			37°C		
	pH	O.D.*	I.Z.**	pH	O.D.	I.Z.	pH	O.D.	I.Z.
0	-	-	-	7.0	0.03	0	7.0	0.03	0
2	-	-	-	6.8	0.105	0	-	-	-
4	-	-	-	6.05	0.42	0	-	-	-
6	-	-	-	5.95	0.66	0	5.30	0.39	0
8	-	-	-	6.0	1.30	0	5.23	0.6	0
11	-	-	-	-	-	-	5.45	0.72	0
14	-	-	-	6.62	2.0	0	-	-	-
24	-	-	-	7.4	2.9	17	6.95	2.0	0
28	-	-	-	-	-	-	7.10	2.0	0
30	-	-	-	7.35	2.20	18	-	-	-
48	-	-	-	-	-	-	7.30	2.5	19
52	-	-	-	7.25	3.30	19	-	-	-
72	-	-	-	-	-	-	7.0	1.30	20
96	-	-	-	-	-	-	-	-	-

+cultures grown at 25°C were contaminated.

*O.D.: optical density read at 660 nm.

**I.Z.: diameter of inhibition zone in mm.

TABLE VII - Cont.

G. Culture #461

Hours after incubation	Incubation Temperature								
	25°C			30°C			37°C		
	pH	O.D.*	I.Z.**	pH	O.D.	I.Z.	pH	O.D.	I.Z.
0	7.0	0.03	0	7.0	0.03	0	7.0	0.03	0
2	-	-	-	6.65	0.13	0	-	-	-
4	-	-	-	5.80	0.50	0	-	-	-
6	-	-	-	5.80	1.12	0	5.50	0.325	0
8	-	-	-	5.80	1.82	0	5.10	0.46	0
11	-	-	-	-	-	-	5.25	0.47	0
14	-	-	-	6.3	3.3	0	-	-	-
24	5.8	1.15	0	7.05	3.9	0	5.25	0.51	20
28	-	-	-	-	-	-	5.20	0.51	20
30	-	-	-	6.75	3.50	18.5	-	-	-
48	6.15	4.1	0	-	-	-	5.52	0.48	20
52	-	-	-	6.85	3.70	19	-	-	-
72	6.20	4.2	0	7.15	3.70	18.5	5.00	0.52	19
96	6.0	3.5	0	-	-	-	-	-	-

*O.D.: optical density read at 660 nm.

**I.Z.: diameter of inhibition zone in mm.

b. Composition of the Fermentation Medium

An experimental study was carried out to determine the effect of medium composition on the production of anti-vitamin B_{12} substances by the three selected bacterial cultures. Bacto peptone, Bacto beef extract, and glucose were the major components of the medium in which anti-vitamin B_{12} substances were being produced. Twelve different kinds of media were prepared by varying the amounts of the components by deleting one component, or by substituting other components instead. (Some of the data collected are summarized in Table VIII.) Production of anti-vitamin B_{12} substances in these media was observed.

From this study, we concluded that both beef extract and peptone, which are usual components of nutrient broth, are required for maximum bacterial growth and for vitamin B_{12} antagonist production. It seemed that the laboratory-prepared nutrient broth (media A, B, C) gave the best results as far as production of vitamin B_{12} antagonists were concerned. However, because of the convenience of preparing media, commercial media from Difco were used for most of the studies.

Also, a level of 10 g/l of glucose seemed to be optimal for the growth of these cultures. Bacteria started to produce vitamin B_{12} antagonists after two days of growth.

TABLE VIII

Effect of Medium Composition on Production of Vitamin B_{12} Antagonists

Culture #382

	Components	g/l	pH				O.D.*				I.Z.**			
			0 hr	18 hr	39 hr	67 hr	0 hr	18 hr	39 hr	67 hr	0 hr	18 hr	39 hr	67 hr
A	glucose beef extract peptone	5 3 5	6.8	6.15	7.35	7.7	0.04	4.10	4.80	-	0	21	20	21
B	glucose beef extract peptone	10 3 5	6.7	6.10	6.2	5.30	0.04	3.70	4.90	-	0	0	20	20
C	glucose beef extract peptone	20 3 5	6.55	6.05	5.95	5.42	0.04	3.10	5.80	-	0	0	20	21
D	glucose peptone	5 5	6.45	7.3	7.6	7.6	0.008	1.22	2.01	1.5	0	0	0	0
E	glucose peptone	10 5	6.3	6.8	6.95	7.10	0.005	1.65	2.0	-	0	0	0	0
F	glucose peptone	20 5	6.10	6.10	7.2	7.1	0.0	1.18	2.2	-	0	0	0	0
G	glucose yeast extract	5 1	6.5	4.85	5.5	4.75	0.0	1.25	2.70	-	0	0	0	0
H	glucose yeast extract	10 1	6.40	4.85	5.10	5.10	0.01	2.35	2.9	-	0	0	0	0
I	glucose yeast extract	20 1	6.0	4.75	4.6	4.25	0.00	0.16	0.23	-	0	0	0	0
J	glucose beef extract	5 3	6.4	6.5	6.35	5.75	0.032	0.45	0.46	-	0	0	0	0
K	glucose beef extract	10 3	6.75	6.5	5.85	5.5	0.03	0.40	0.52	-	0	0	0	-
L	glucose beef extract	20 3	6.60	6.40	6.25	5.75	0.02	0.043	0.32	0.265	0	0	0	-

Antagonists

Culture #439

	Components	g/l	pH 0 hr	pH 18 hr	pH 39 hr	pH 67 hr	O.D.* 0 hr	O.D.* 18 hr	O.D.* 39 hr	O.D.* 67 hr	I.Z.** 0 hr	I.Z.** 18 hr	I.Z.** 39 hr	I.Z.** 67
A	glucose beef extract peptone	5 3 5	6.8	5.48	7.7	8.0	0.04	4.10	5.5	-	0	0	21	2
B	glucose beef extract peptone	10 3 5	6.7	5.48	5.65	6.15	0.04	4.35	6.9	-	0	0	22	2
C	glucose beef extract peptone	20 3 5	6.55	5.40	5.60	5.50	0.04	4.35	6.9	-	0	0	21	2
D	glucose peptone	5 5	6.45	6.7	7.1	7.05	0.008	1.10	1.75	-	0	0	21	1
E	glucose peptone	10 5	6.3	7.0	7.1	7.2	0.005	1.05	1.7	-	0	0	20	2
F	glucose peptone	20 5	6.1	6.7	7.15	7.0	0.0	1.0	1.95	-	0	0	20	2
G	glucose yeast extract	5 1	6.50	5.15	5.0	4.75	0.0	2.22	2.9	-	0	0	0	
H	glucose yeast extract	10 1	6.4	5.15	5.3	5.35	0.01	2.4	3.35	-	0	0	0	
I	glucose yeast extract	20 1	6.0	4.9	4.7	4.5	0.0	0.045	0.125	-	0	0	0	
J	glucose beef extract	5 3	6.9	6.4	5.95	5.7	0.032	0.058	0.58	-	0	0	0	
K	glucose beef extract	10 3	6.75	6.25	5.85	5.55	0.03	0.46	0.47	-	0	0	17	18
L	glucose beef extract	20 3	6.60	6.55	5.60	5.70	0.022	0.63	0.57	0.49	0	0	0	
M	nutrient broth glucose	8 10	6.9	5.6	6.1	5.65	0.02	2.7	5.23	-	0	0	20	21

*O.D.: optical density read at 660

c. Aeration Conditions

Experiments studying the effect of aeration on growth and production of vitamin B_{12} antagonists were carried out as follows: A series of Erlenmeyer flasks containing varying volumes of medium were incubated on a rotary shaker at 30°C and 200 rpm. Another set of flasks were incubated at 30°C without shaking or stirring. Their pH values, O.D. readings and bioassay were used to evaluate the most favorable conditions for the production of antimetabolites. Some data collected in this study are summarized in Table IX.

We concluded from this experiment that the cells required aeration for maximal growth and for producing vitamin B_{12} antagonists. Very little production of vitamin B_{12} antagonistic activity was noted in those flasks which were not shaken even though considerable bacterial growth was noted.

It was found that the flasks containing 25 ml of medium had the best production of anti-vitamin B_{12} substances. This could be ~~explained~~ confirmed by the fact that those flasks had the best aeration condition for growth and for production of anti-vitamin B_{12} substance.

d. pH of Culture

It was noticed that in the process of cell-growth, the pH values changed gradually and we studied the effect

TABLE IX

Effect of Aeration on Antimetabolite Biosynthesis by Culture #439

Values of	Volume of medium (ml per 250 ml Erlenmeyer flask)	Hours after Inoculation			
		Flasks on shaker		Flasks in incubator (static culture)	
		18 hr	42 hr	18 hr	42 hr
pH	25	6.9	5.80	5.6	5.7
	50	6.6	5.95	5.5	5.45
	100	5.85	6.10	5.55	5.25
	150	5.60	6.30	5.55	5.25
O.D. (at 660 nm)	25	3.65	4.95	0.44	0.92
	50	3.5	2.85	0.39	0.58
	100	3.45	3.70	0.205	0.355
	150	2.8	4.50	0.205	0.255
diameter of inhibition zone	25	21	21	0	0
	50	20	21	0	0
	100	20	21	0	0
	150	19	19.5	0	0

*At 0 hours all media had a pH of 7.0 and an O.D. of 0.03.

of adding buffers to the growth medium in order to repress the pH changes. 0.2 M solutions of NaH_2PO_4 and Na_2HPO_4 were used as the buffering reagents for the nutrient broth medium. Media were adjusted and buffered in such a way that the initial and following pH of these media (after autoclaving) were 6.0, 7.0 and 8.0. A control set of flasks of unbuffered nutrient broth medium was part of this experiment (Table X).

The results of this experiment suggested that the medium should not be buffered in any way for the maximal bacterial growth and production of vitamin B_{12} antagonists, although the unbuffered medium fermentations produced the vitamin B_{12} antagonists.

3. Chemical Characterization of the Vitamin B_{12} Antagonaists Isolated from Fermentations

a. Heat, Acid, and Alkaline Stability

Aliquots of supernatant solutions obtained by centrifuging the fermentative samples were adjusted to pH 3.0, 7.0 and 9.0 with HCl and NaOH. These solutions were heated in a boiling water-bath for ten minutes. After cooling, the solutions were all neutralized to pH 7.0 and were then assayed for vitamin B_{12} antagonistic activity using *E. coli* 113-3. An untreated sample was used for the control for the assay. Some of the data collected are summarized in Table XI.

TABLE X

Effect of Addition of Phosphate Buffer to Growth Media on Growth and Production of Vitamin B_{12} Antagonists by Cultures #382, #383, and #439

Culture number	Medium*	pH changes			O.D. values**			Inhibition Zone measurement (mm)		
		0 hr	18 hr	24 hr	0 hr	18 hr	24 hr	0 hr	18 hr	24 hr
439	A	6.1	6.1	6.1	0.03	3.15	3.1	0	20.5	20
	B	7.0	6.95	6.95	0.03	4.5	4.2	0	0	0
	C	8.0	7.3	7.3	0.03	3.4	4.0	0	0	0
	D	7.1	6.05	6.1	0.04	1.38	1.4	0	20.5	20.5
382	A	6.1	6.25	6.1	0.02	4.0	3.6	0	0	0
	B	7.0	6.9	6.9	0.04	3.8	4.0	0	20	21.5
	C	7.9	7.4	7.4	0.028	2.95	3.6	0	0	0
	D	7.1	6.6	6.45	0.04	3.4	3.6	0	19	19
383	A	6.15	6.25	6.15	0.035	3.6	3.9	0	0	0
	B	7.0	7.0	6.9	0.03	3.8	4.0	0	0	0
	C	7.98	7.4	7.4	0.032	3.3	3.0	0	17.5	17.5
	D	7.1	6.15	6.1	0.04	2.2	2.7	0	18	18

*A = medium buffered to pH 6.0
B = medium buffered to pH 7.0
C = medium buffered to pH 8.0
D = medium unbuffered

**O.D.: optical density read at 660 nm.

TABLE XI

Test of Heat, Acid and Alkaline Stability

Culture	Measurements of the Diameter of Inhibition Zones (mm)						
	Before Boiling			After Boiling			
	pH 3	pH 7	pH 9	pH 3	pH 7	pH 9	No Treatment
382	19	18	17.5	17	17,22 (d.z.)*	19	18,21 (d.z.)
383	19,22 (d.z.)	19,22 (d.z.)	22	15	17,22 (d.z.)	22	19,22 (d.z.)
403	18,24 (d.z.)	20,27 (d.z.)	18,22 (d.z.)	20,24 (d.z.)	21,27 (d.z.)	19,21 (d.z.)	18,25 (d.z.)
439	26,28 (d.z.)	25	26	21,26 (d.z.)	25	26	24
442	19	21	21	15	17,21	17,21	18.5
461	19,25 (d.z.)	17,23 (d.z.)	18,24 (d.z.)	23	23	23	18,24 (d.z.)

*d.z.: double zone.

Double zones were often noted on these agar plates: These included an outer zone of clear background indicating the inhibition, and an inner zone of stimulating growth with reddish-purple color on the plate. The inhibition area was measured.

It seemed from this study that the compounds produced by cultures #382, 383, 403, 439, 442, and 461 which antagonized the growth of E. coli 113-3 were stable to heat at neutral pH, while those produced by cultures #383, 439 and 442 were labile at acid pH.

b. Solvent Extraction

Five ml aliquots of the fermentation samples (after removal of the cells) were adjusted to pH 3.0, 7.0 and 9.0 and extracted with equal volumes of chloroform, n-butanol, hexane, and a phenol/benzene mixture (30:70) (a combination commonly used in recovery of vitamin B_{12}). The vitamin B_{12} antagonist activity was determined for both layers using inhibition of E. coli Davis 113-3. No demonstrable antagonist activity could be shown in the chloroform, hexane, or phenol/benzene layers at all pHs, and the small zones noted in the n-butanol was thought to be due to poor separations of the two layers.

From these extraction experiments, we concluded that these compounds produced by the bacterial cultures which could inhibit the growth of E. coli 113-3 had a

very hydrophilic nature since they could not be extracted by any of these organic solvents.

c. Absorption on Charcoal and Ion Exchange Resins

Five ml aliquots of each supernatant of the fermentative samples were adjusted to pH 3.0, 7.0 and 9.0. 0.8 g of Darco G60 was added to each tube. The solutions were mixed well and centrifuged. The supernatants were tested for vitamin B_{12} antagonist activity.

The results of this experiment showed that some of the vitamin B_{12} antagonist compounds were absorbed onto activated carbon. This was especially so for sample #439 at pH 7 and pH 9. No activity was seen in the treated filtrates of these two solutions. The charcoal was then washed with distilled water and resuspended in equal volumes of 95% ethanol. Both the wash and the ethanol eluates showed some inhibitory activity to E. coli 113-3 (Table XII).

Another experiment was done by adding 1 g of charcoal to each of the 50 ml of fermentation broths. These were stirred for 10 minutes and collected by centrifugation. The charcoal was washed with distilled water and collected by centrifugation. Half of the collected charcoal was eluted with methanol and the other half with n-butanol (Table XIII).

TABLE XII

Absorption of Vitamin B_{12} Antagonist Activity for Fermentative Samples on Darco G60

Culture number	pH	Measurements of Inhibition Zones (mm) Untreated	Treated
382	3	19	17.5
	7	18	16
	9	17.5	16
383	3	19.22 (d.z.)*	15.20 (d.z.)
	7	19.23 (d.z.)	18
	9	22	17
403	3	18.24 (d.z.)	22
	7	17.23 (d.z.)	20
	9	18.22 (d.z.)	19
439	3	20	20
	7	19	0
	9	18	0
442	3	19	20
	7	21	18
	9	21	16
461	3	20	20
	7	20	20
	9	19	19

*d.z.: double zone.

TABLE XIII

Extraction with Charcoal and Elution with Methanol and Ethanol

Culture number	Measurements of Inhibition Zones (mm)			
	Untreated	Charcoal Filtrate	MeOH Filtrate	BuOH Filtrate
212	20	0	0	0
382	23	21.5	0	0
383	23	23	0	0
403	24	24	0	0
439	23	23	0	0
442	24.5	23.5	0	0
461	25	25	0	0

5 ml aliquots of fermentation samples were mixed with 1 g of Dowex 50 resin as directed on page 47. The supernatant liquids were poured off into other tubes and the resins were washed with distilled water. (The washes were retained for assay.) One set of resins was eluted with N-pyridine solution and had a pH 6.0; a second with more pyridine had a pH of 7.5; and a third was eluted with N-ethylmorpholine and had a pH 8.0. Bioassay of these solutions, washed, and eluates showed: (a) the vitamin B_{12} antagonist activity was absorbed on the resin; and (b) some activity was eluted with pyridine and with ethylmorpholine.

d. Chromatographic and Ionophoretic Behavior

A concentrate, A, of the antimetabolite-containing supernatant (10 ml) from the fermentation broth of culture 439, and a concentrate, B, from the same culture, prepared by the acetone precipitation method (see Methods and Materials), were subjected to paper electrophoresis and chromatography followed by bio-autography.

Portions (20 µl) of the samples A and B were spotted onto several sheets of Whatman no. 1 paper. Known amino acids were spotted along side for ninhydrin tests. The substances were subjected to the electrophoretic and chromatographic procedures given in Table XIV, and then their positions detected by bio-autography on E. coli 113-3 agar plates.

Bio-autography on E. coli 113-3 of concentrates of culture 439 supernatants were carried out after paper electrophoresis (pH 1.8, 40 v/cm, 90 mins and pH 4.5, 10 v/cm, 100 mins) in the horizontal direction followed by paper chromatography (n-butanol:acetic acid:water, 4:1:4, by volume) in the vertical direction. Two separate inhibition areas on the agar were found for each sample.

TABLE XIV

Chromatographic and Electrophoretic Behavior of Vitamin B_{12} Antagonists from Culture #439

Properties Determined	A	B
Distance moved towards cathode (cm), electrophoresis pH 1.8, 40 v/cm, 90 min	9.2 20.2	4.9 18.9
Distance moved towards cathode (cm, electrophoresis pH 4.5, 10 v/cm, 100 min	1 5	0.7 3.7
Distance moved towards cathode (cm), electrophoresis pH 6.4, 60 v/cm, 90 min	- 0.6, 9.5	1.15 11.7
Distance moved in n-butanol:acetic acid: water (4:1:4) (solvent distance, 56 cm)	4*	2.5*

*Long streaks of inhibition area were seen on the agar plates.

e. Dialysis

A dialysis experiment (see Materials and Methods) was carried out on the supernatant of culture #439. After dialysis for 24 hours at 9°C, it was found that the inhibition activity in the sack had disappeared. This might be evidence that the antagonist is a non-protein molecule having a small molecular weight.

4. Biological Characterization of Vitamin B_{12} Antagonists

a. Reversal of Methionine Growth Stimulation of E. coli Davis

Since Davis and Mingioli (60) reported that methionine can be used as a substitute for vitamin B_{12} in the growth of E. coli 113-3, we tested the antimetabolite solutions against E. coli 113-3. Various concentrations of methionine (100 mcg/ml, 1 mg/ml, 10 mg/ml) were applied onto 13 mm paper discs and these were placed on the surface of an agar plate containing 250 ml of H_{26} medium, 0.5% glucose, 0.01% tetrazolium chloride and seeded with E. coli 113-3 cells. The growth zones around the methionine discs were measured:

Concentration of methionine	Growth zone
100 mcg/ml	20 mm
1 mg/ml	32 mm
10 mg/ml	44 mm

This confirmed the fact that the methionine stimulated the growth of E. coli 113-3.

Another experiment was done to test if the vitamin B_{12} antagonists produced in fermentation broths also inhibit the growth stimulated by methionine. 100 mcg/ml of methionine was applied to the 13 mm paper discs and

placed on the agar plate. Discs containing fermentation broths were placed adjacent to them. It was found that all the supernatants of the seven cultures which were known to inhibit vitamin B_{12}-promoted growth response, but also inhibited the growth of E. coli 113-3 stimulated by methionine.

b. Reversal of Vitamin B_{12} Growth Stimulation of E. coli Davis

10 mcg of vitamin B_{12} were added to H_{26} medium supplemented with glucose, tetrazolium, and seeded with E. coli 113-3. The supernatants removed from the fermentation broths were diluted with distilled water so that their concentrations would be 1/16, 1/8, 1/4, and 1/2 of the original. A sample of non-diluted supernatant was used for comparison in bioassay. Inhibition zones appeared on the agar plate and were measured (Table XV).

TABLE XV

Reversal of Vitamin B_{12} Growth Stimulation of E. coli Davis by Various Concentrations of Fermentation Broths of the Culture

Supernatant concentration in solution / Culture number	Measurements of the Diameter of the Inhibition Zones (mm) 1/16	1/8	1/4	1/2	1
212	0	16	18.5	20	23
382	0	0	15.5	18	17.21 (d.z.)
383	0	0	16	20	23
403	0	15.5	20.5	22	18.23 (d.z.)
439	0	0	16	19	21
442	0	0	0	20	22
461	0	0	18	19.20 (d.z.)	18.24 (d.z.)

In a study of the effect of vitamin B_{12} concentration on the response to the antagonist, different concentrations of vitamin B_{12} (5 mcg, 10 mcg, 15 mcg, and 20 mcg) were added to four assay plates containing E. coli 113-3 and the agar medium required for growth. Paper discs with fermentation broths of selected bacteria were placed on the surface. Inhibition zones were measured after incubation (Table XVI).

TABLE XVI

Effect of Concentration of Vitamin B_{12} on the Inhibition of Vitamin B12 Growth Stimulation of E. coli Davis by Fermentation Broths of Cultures #439, #382, and #383

Culture number	Concentration of vitamin B12	Diameter of Inhibition zone (mm)
439	5	24
	10	24
	15	24
	20	24
382	5	20
	10	21
	15	20
	20	20
383	5	24
	10	25
	15	24
	20	24

The concentrations of vitamin B_{12} did not affect the inhibition zone diameters. This might suggest that the antagonists were non-competitive inhibitors.

III. DISCUSSION

A. Possible Relationship of Antimetabolites to Metabolites

An antimetabolite is a structural analog of an essential metabolite which is able to cause signs of deficiency of the essential metabolite in some living things or in some biological reactions. Not all compounds which cause signs of deficiency of a vitamin are antimetabolites of it. Thus, for example, one can call forth signs of deficiency of thiamine in an animal by treatment of its food with sodium sulfite. The sulfite destroys the thiamine by means of a chemical reaction with it, but it is not an antimetabolite of thiamine. Similarly, diisopropylfluorophosphate (DFP) can antagonize the action of cholinesterase by combining with the active site of the enzyme. This, however, does not make DFP an antimetabolite either of cholinesterase or acetylcholine. In other words, not all blocking agents for a biological reaction are antimetabolites.

The antimetabolite is a substance which is shaped like the substrate in an enzyme system. In other words, it is a structural relative or analog of the substrate. Probably for this reason it also is able to combine with the active center of the enzyme. It occupies the active

site to the exclusion of the substrate. For this reason, an antimetabolite is usually found to display its analogous essential metabolite. In a living animal one observes this by finding that the antimetabolite brings about the excretion of the metabolite of its metabolic degradation products and reduces the content of the metabolite in the tissues.

Although the antimetabolite is able to combine with the enzyme because of its similarity in structure to the metabolite, it may not be similar enough to undergo the rearrangement typical of the normal enzymic reaction. For this reason, products are not formed, and the enzyme is not regenerated. Deficiency of the normal products is produced. However, in many cases the antimetabolite fits the enzyme well enough so that products are formed from it as well as from the essential metabolite. These products are abnormal and may act as antimetabolites in the next step of the metabolic chain.

Many vitamins and other essential metabolites fulfill more than one function. Each is usually a substrate for more than one enzyme. The affinity of an analog of the metabolite for enzyme A may differ from its relative affinity for enzyme B or C. For this reason, it is possible to single out and inhibit only one or two enzymes and to leave the others relatively uninhibited. In this way, only one function of the vitamin may be inhibited without hindrance to the

others, just as it is also possible to inhibit the activities of one vitamin while leaving the activities of the other vitamins untouched. This is one of the powerful characteristics of an antimetabolite (63).

In general, the mechanism of action of an antimetabolite to its relative metabolite can be explained in many ways. The relation between structure and function could hardly be generalized for all systems. Instead, one has learned the impossibility of generalizing. Activities can only be stated in relation to the test system employed, and they differ from one system to the other. Smith postulated three levels at which inhibition may occur (59):

(a) interference with uptake of the metabolite
(b) interference with the conversion to the active form
(c) interference with biochemical functions.

These possibilities may well be applied to many of the relationships of antimetabolite-metabolites.

B. Occurrence of Vitamin B_{12} Antagonists as Microbial Products

From our studies, 14 vitamin B_{12} antagonist-producing cultures were isolated from various soil samples. Seven of them were studied in some detail.

Optimal production of vitamin B_{12} antagonists from the bacterial cultures were found when the cells were grown in the following conditions:

(1) in a medium containing nutrient broth and supplemented with 1% glucose
(2) at a temperature of 30°C
(3) with sufficient aeration.

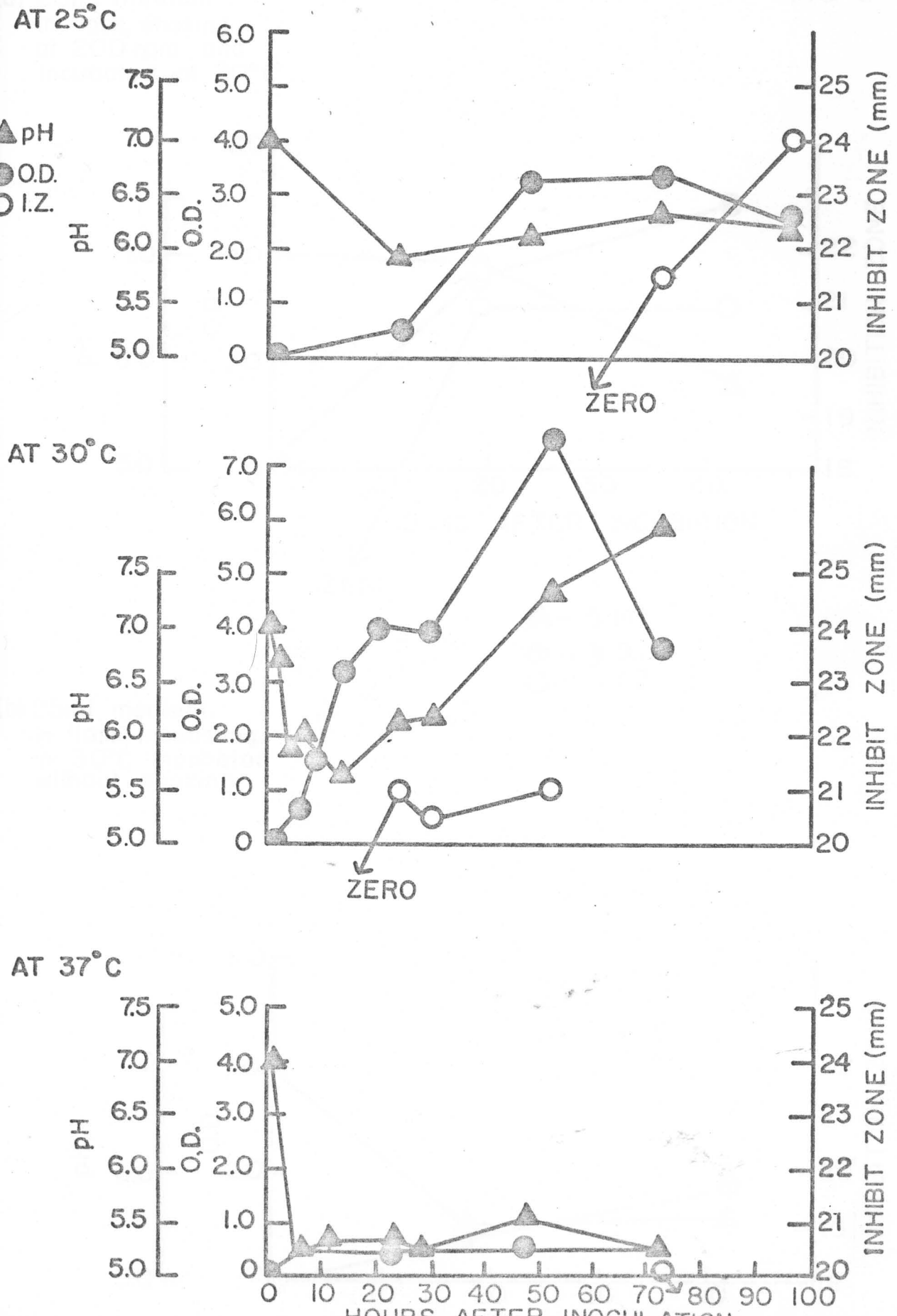
Some of the data supporting these conclusions are summarized in Figures 4 and 5.

It was found that most of these vitamin B_{12} antagonists are stable to heat in neutral pH but labile in acid pH. None are extractable with n-butanol, chloroform, hexane or phenol/benzene. This suggested the hydrophilic characteristics of these compounds.

Activated carbon absorbed some of the active substances that inhibited the growth of vitamin B_{12}-requiring E. coli 113-3. However, nothing yet has been found to elute these compounds from the carbon. The antagonists produced by culture #439 were examined more extensively than the rest. A dialysis experiment showed that the vitamin B_{12} antagonist produced by this culture is a non-protein substance with a small molecular weight. Paper electrophoresis and chromatography followed by bio-autography suggested that the active substance is probably a mixture of several (perhaps two) materials. The direction of the compound moving towards

FIG. 4

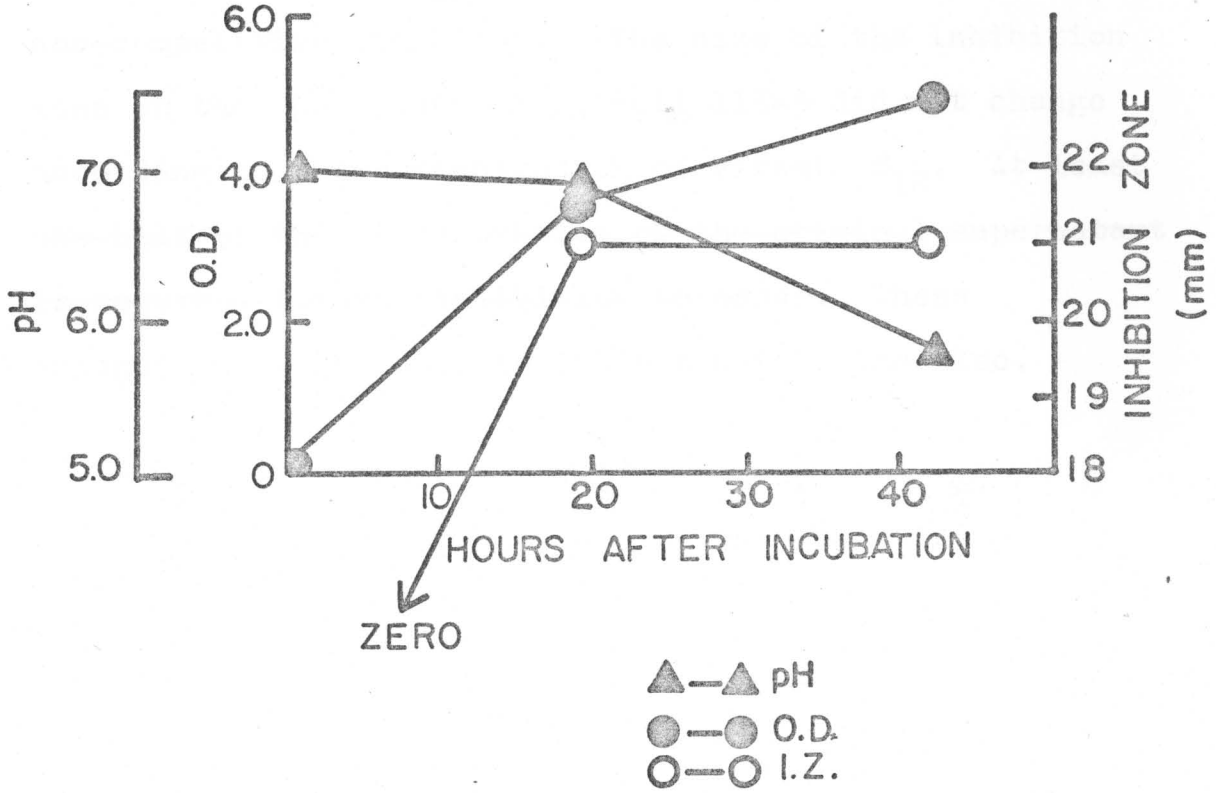
EFFECT OF INCUBATION TEMPERATURE ON CULTURE 439



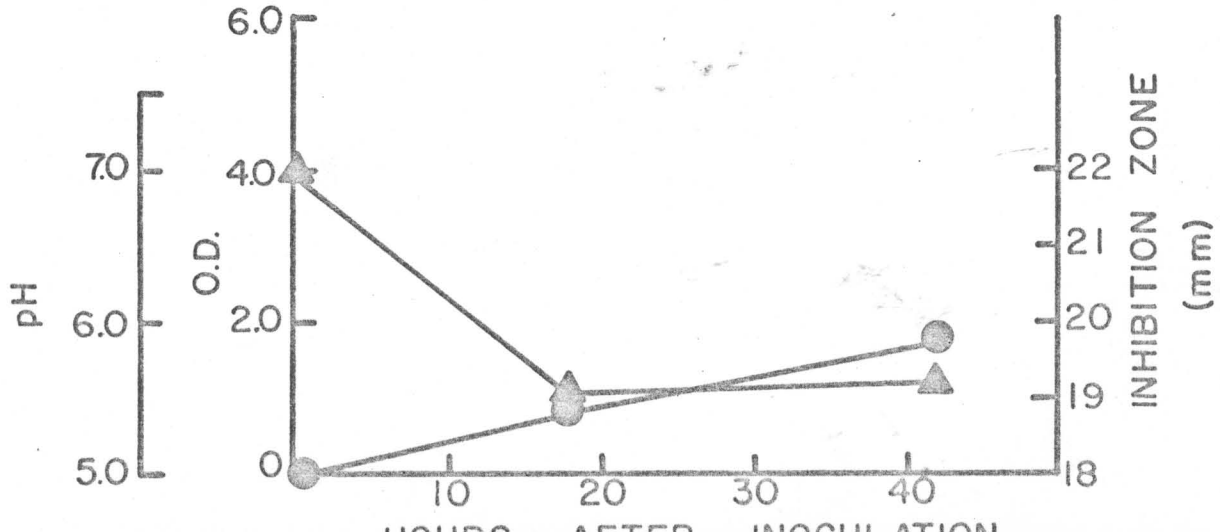
EFFECT OF AERATION CONDITION ON CULTURE 439

FIG. 5

a) 25ml medium in flask, shaking at 200 rpm and incubated at 30°C



(b) 25ml medium in flask, incubating in 30°C incubator without shaking



the cathode suggested that these compounds may have some basic groups attached.

These vitamin B_{12} antagonists were found to be non-competitive inhibitors. The size of the inhibition zone on the agar plate of *E. coli* 113-3 did not change according to the concentration of vitamin B_{12}. ~~At least one-half of the concentration of the original supernatant is required for any inhibition to occur.~~ These antagonists were found to inhibit methionine also.

IV. SUMMARY

A study of the ~~appearance~~ occurence of microorganisms producing substances antagonizing the growth response of Escherichia coli 113-3 to vitamin B_{12} and to methionine showed that 14 out of 678 bacteria examined produced significant activity. Seven of these bacteria were examined in some detail and biosynthesis of the active compounds was shown to depend on the temperature of incubation of the culture, composition of the fermentation medium, and aeration of the culture. Formation of the active materials depend on the growth of bacteria, but good growth is not always accompanied by production of significant inhibition activity. An agar-diffusion assay system was developed to measure the concentration of active materials.

The active factors appeared to be absorbable on ion exchange resins and probably on activated charcoal. They could not be extracted from aqueous solutions at various pHs with chloroform, n-butanol, hexane, or phenol/benzene mixture. The active materials in general appeared to be heat stable at neutral pH but labile at acid pH. Paper chromatographic and ionophoretic examination of the active material produced by culture #439 showed the presence of multiple components which have basic properties.

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