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SOME FACTORS INFLUENCING THE GROWTH
OF A METHANOBACTERIUM SPECIES

Thesis for the Degree of Ph. D.
MICHIGAN STATE UNIVERSITY
Charles Woodbury Fifield III

This is to certify that the

thesis entitled

Some Factors Influencing the Growth
of a Methanobacterium Species

presented by

Charles Woodbury Fifield III

has been accepted towards fulfillment
of the requirements for

Ph.D. degree in Microbiology and
Public Health


Major professor

Date May 15, 1956

**SOME FACTORS INFLUENCING THE GROWTH
OF A METHANOBACTERIUM SPECIES**

By

Charles Woodbury Fifield III

AN ABSTRACT

**Submitted to the School for Advanced Graduate Studies of
Michigan State University of Agriculture and Applied Science
in partial fulfillment of the requirements
for the degree of**

DOCTOR OF PHILOSOPHY

Department of Microbiology and Public Health

1956

Approved by



A methane bacterium identical in most respects to Methanobacterium omelianskii was isolated by enrichment culture and partially purified. A modification of a medium suggested by Barker (1940) gave excellent results. Chlorides were substituted for the sulfates and cysteine replaced sodium sulfide as a reducing agent.

The presence of inert solids in the methane fermentation were advantageous. Asbestos proved to be the most satisfactory substance of those studied in respect to sub-culturing and staining of the organisms, as well as to the enhancement of gas production.

The measurement of oxidation-reduction potentials proved to be of value in determining the role of the inert solid. An apparatus was constructed for the simultaneous measurements of the potentials in several cultures or solutions or in the upper and lower portions of a medium.

The presence of asbestos in the culture medium prevented diffusion into the lower limits. Evidence points to the conclusion that the role of the asbestos is to supply an environment where the oxidation-reduction potential may be lowered and maintained even the presence of oxygen and where enzyme systems and hence energy may be concentrated for the use by the slow metabolizing methane bacteria.

Oxygen is not lethal to the Methanobacterium omelianskii type organism. In this respect it does not differ greatly from other anaerobes.

Ethyl violet had little influence on the growth of the organism but sodium azide and penicillin exhibited a marked inhibitory effect.

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To Anne

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INTRODUCTION

The presence of flammable gas produced in swamps, mines and marshlands is commonly known. Perhaps the first to venture a logical explanation for the origin of the gas, which consisted mainly of methane, was Volta (1776) who concluded that the gas arose from rotting vegetative matter. That the formation of methane was directly associated with microbial processes in the decomposition of cellulose in mud and in the intestine of the ox was suggested by Popoff (1875), Hoppe-Seyler (1886) and Tappenheimer (1884). Omelianski (1904, 1916) studied the methane fermentation of cellulose and ethyl alcohol. It is now believed however, that the production of methane from cellulose, in Omelianski's experiment was the result of impure cultures. The important work of Sohngen (1910) gave the first clear insight into the origin of methane and the morphology of the methane bacteria. Sohngen experienced the same difficulties in isolation that have plagued subsequent workers in the field. Sohngen obtained enrichment cultures by several transfers into media containing the substrate under investigation as the sole source of carbon, thereby practically eliminating all extraneous organisms. He showed that the calcium salts of various fatty acids could be decomposed quantitatively to carbon

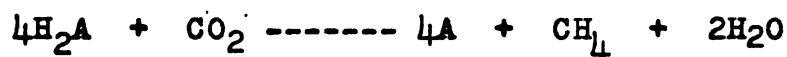
dioxide and methane and that formate decomposing cultures could synthesize methane from CO_2 and hydrogen.

The number of other organic substrates which could be decomposed with the formation of methane was discovered by Maze (1915) who investigated the fermentation of acetone; by Groenwege (1920) who observed that methane could be produced from methyl, ethyl and butyl alcohols and by Coolhaas (1928) who investigated the thermophilic breakdown of several fatty acids. Buswell and Neave (1950) and Tarvin and Buswell (1934), by the use of enrichment cultures in studies on sewage purification, found that almost every type of organic compound could be decomposed with methane as an end product. Barker (1936a) suggested in this connection, however, that Buswell's work provided no certainty that methane arose by direct conversion of the organic substrates. It was probable that the simple organic compounds such as the lower fatty acids, alcohols, and ketones, which were known not to be attacked by other bacteria under conditions of anaerobiosis except in the presence of sulphates or nitrates, would be directly fermented by the methane bacteria. In all probability the more complex compounds including cellulose would be attacked first by the common saprophytic anaerobes thereby producing the lower type compounds. Barker (1936a) stated that the most remarkable conclusion to be drawn from the investigations up to this time was that no matter what organic compound was fermented, the only hydrocarbon formed was methane.

Thayer (1931) hypothesized that the methane arose from a decarboxylation of acetic acid and investigated the fermentation of propionic and butyric acids assuming that the hydrocarbon produced would be ethane and propane respectively. Methane, however, was the only hydrocarbon found in both fermentations.

Stephenson and Strickland (1933) isolated an unidentified organism which produced methane from formate and which also could utilize H_2 for the reduction of various one-carbon compounds.

The turning point in the research on the methane bacteria probably began with the presentation of two papers by Barker (1936a, 1936b). He mentioned the deficiency in the knowledge of the mechanism of methane production, attributing it to the exceedingly difficult task of isolating the organisms. Barker (1936a) tested a hypothesis suggested personally to him by C. B. Van Niel, based on Sohngen's earlier observations of the reduction of carbon dioxide by gaseous hydrogen. Van Niel postulated that methane was derived in all instances from the reduction of carbon dioxide at the expense of any compounds, organic or inorganic, which were capable of donating a hydrogen by simple dehydrogenation without the loss of carbon dioxide according to the following equation



where H_2A represents any compound that can be activated by the methane bacteria. Barker quantitatively showed that in the ethyl alcohol fermentation the alcohol was dehydrogenated to acetic acid and that there was a decrease in carbon dioxide equivalent to the methane formed. He again confirmed Van Niel's theory by showing that butyl alcohol, in the presence of the methane organism, was dehydrogenated to butyric acid and then to acetic acid with a simultaneous reduction of CO_2 and production of methane occurring in both reactions. Barker suggested a similar type of reaction for the fermentation of acetic acid, however, earlier Buswell and Neave (1930) advanced the theory that methane originated primarily from a simple decarboxylation of acetic acid. Barker (1936a) criticized Buswell's theory on the basis that it did not explain why methane was the only hydrocarbon produced. An examination of the data of Symons and Buswell (1933) showed that only 3.5 liters of methane was formed over a period of 100 days when hydrogen and carbon dioxide were circulated through 2 liters of culture but that such a culture fermenting acetic acid would produce 75 liters of methane. It would appear, then, that the mechanism of the fermentation of acetic acid is a special case not related to the fermentation of higher fatty acids and alcohols by the methane bacteria. Buswell and Sollo (1948) used C^{14} labeled carbon dioxide in an investigation of the anaerobic fermentation of acetic acid and found only a small amount of the labeled carbon present in the methane

produced. Later Stadtman and Barker (1949), with a partially purified culture of a Methanococcus species, confirmed the results of Buswell and Sollo and in addition showed that methane was derived, for the most part, from the methyl group of the acetic acid and that the carbon dioxide was derived from the carboxyl group. More recent results by Stadtman and Barker (1951b) using an acetate fermentation by a Methanoscarcina also vindicated the previous investigation with isotopes.

Barker (1936b) summarized the morphological and physiological characteristics of the methane bacteria and divided them into six types. He described the organisms as being obligate, non-spore forming, anaerobes which fermented a great variety of compounds with subsequent methane production and which seemed to be extremely sensitive to oxygen. Their rate of development was slow and carbon dioxide was necessary for their metabolism. Barker described methods for partial isolation of the methane bacteria taking advantage of the fact that these organisms have exceedingly simple requirements. He succeeded in producing colonies in agar shake tubes, however, transfers from the colonies yielded partially purified but none-the-less impure cultures. Failure to grow colonies on the surface of agar plates was attributed to the killing effect of even a brief exposure to oxygen.

In 1937 Barker described a fermentation of ethyl alcohol by Methanobacterium omelianskii and an associated spore former,

in which the alcohol was converted to acetic, butyric, and caproic acids. The mechanism was a condensation of a C_2 compound.

The first to isolate a methane organism in pure culture was Barker (1940), who succeeded in obtaining Mb. omelianskii in pure culture by the agar shake method. The organism was described as a thin, unbranched, gram variable rod. Motility, if present, was sluggish. In contradiction to earlier statements, spores of low heat resistance were observed and their possible function as a means of survival in the presence of oxygen was given. Within the medium discussed, the growth of the organism was influenced by such factors as hydrogen sulfide concentration, pH, phosphate and carbonate concentrations. Ammonia served as a nitrogen source but organic nitrogen compounds did not stimulate growth. Techniques of isolation as well as a suitable medium were described.

Barker (1941) described some of the biochemical activities of Mb. omelianskii. The organism oxidized simple, primary and secondary alcohols to the corresponding fatty acids and ketones. Oxygen and nitrate were found to be toxic. A linear relationship existed between the amount of ethanol oxidized to acetic acid and the amount of cell material formed.

Kluyver and Schnellen (1947) described the anaerobic conversion of carbon monoxide by a pure culture of Methanosarcina barkerii.

Studies on the influence of carbon dioxide reduction by molecular hydrogen in the presence of cells of Mb. omelianskii were reported by Barker (1943). The shape of the curve was noted to be similar to that of other enzymatic processes.

Data gathered by Stadtman and Barker (1949) clearly indicated that 82 to 100 percent of the methane produced during the fermentation of ethyl alcohol in the presence of C^{14} -labeled carbon dioxide by Mb. omelianskii could be attributed to the reduction of carbon dioxide. These two investigators (1951a) described two new species of methane bacteria which incompletely oxidized fatty acids. Methanobacterium suboxydans converted valeric acid to propionic and acetic acids by a beta-oxidation. Likewise butyric and caproic acids were oxidized quantitatively to acetate. The fermentation of propionic acid by Methanobacterium propionicum resulted in an oxidation such that the carboxyl carbon gave rise to carbon dioxide and the alpha and beta carbons yielded acetate. In the fermentation of methanol by a species of Methanosarcina, Stadtman and Barker (1951b) showed that 98 percent of the methane originated with the alcohol. This reaction is, of course, similar to the fermentation of acetate.

Buswell, Fina, Mueller and Yahiro (1951) labeled carbons 1, 2 and 3 respectively of several propionates and concluded that the gases evolved during fermentation by enrichment cultures arose from all three carbons and that carbon dioxide

was a precursor, in part, of the methane formed. Likewise in the fermentation of butyric acid by enrichment cultures, methane was partly derived from the reduction of carbon dioxide.

Stadtman and Barker (1951c) isolated a new species, Methanococcus vannieli, which converted formate to methane, carbon dioxide and hydrogen and appeared to be unable to use any other organic compound as an energy source. Clark and Fina (1952) reported on the anaerobic decomposition of benzoic acid during the methane fermentation. A comprehensive review of the mechanism of methane fermentation was presented by Buswell and Mueller in 1952.

Perhaps the first to develop a technique for total counts of the methane bacteria in agar dilution culture directly from the natural source without the use of enrichment cultures were Mylroie and Hungate (1954). Tubes containing gaseous hydrogen and carbon dioxide as the energy source were employed. Tubes positive for methane always showed the presence of colonies of a formate oxidizing organism Methanobacterium formicicum, which according to the authors indicated the specie's importance in sewage fermentation.

Pine and Barker (1954) discovered that Mb. omelianskii was able to fix atmospheric nitrogen. Fixation was enhanced by low levels of NH_3 and inhibited by higher concentrations.

Little work has been reported on the effect of inhibiting agents on the methane bacteria, per se. In this connection Noordam-Goedenagen, Manten, and Muller (1949) indicated that 0.01 percent Na_2SO_3 produced inhibition of the methane fermentation of sodium and calcium acetates. McNary, Walford and Patton (1951) reported that the anaerobic fermentation of citrus fruit wastes was inhibited by traces of peel oil or D-limonene. This substance was later found to inhibit gas production from methane cultures when a substrate of acetic acid was used.

The use of inert solids in liquid bacteriological media and in industrial processes involving fermentations by microorganisms has been in effect for some time. The commonest uses for such solids are to supply a mechanical support for the organisms such as in the vinegar fermentation, trickling filters, etc. and to promote the growth of strict anaerobes in solutions exposed to the air (Buswell and Neave, 1930; Robinson, 1922).

The presence of inert solids in the methane fermentation where the liquid is not always exposed to air appears to be highly advantageous. Sterile swamp mud similar to that found in the organisms natural habitat has been used by many investigators. Buswell and Neave (1930) emphasized the value of such solids where "in two parallel series of 80 cultures each, only two failed to produce gas from acetic and propionic acids when digested sludge was used for the

inoculation, but only three showed gas formation when the supernatant liquor from this sludge was used". Experiments by Breden and Buswell (1933) using shredded asbestos as an inert solid and acetate and overflow liquor from sludge digestion tanks as the medium and inoculum suggested that the function of the solid was to furnish a physical environment for the organisms rather than to furnish a heavy inoculum. These investigators also reported that the bacterial cells seemed to cling to the asbestos fibers but that an increase in gas production when shredded asbestos was used was not due to a protective action against oxygen. When sand was substituted for asbestos their experiments failed. Fischer, Lieske and Winger (1932) used precipitated FeS or $\text{Al}(\text{OH})_3$ in a medium and unless the colloidal suspension was present acetic acid collected, but little or no methane was produced. They referred to the finely divided material as a catalyst for the production of carbon dioxide and methane from acetic acid.

Barker (1940) derides the idea that large quantities of inert sediments are essential for the growth of the methane bacteria and that excellent growth in his experiments invariably occurred in media which contained small precipitates of calcium phosphate and iron sulfide but which were devoid of such special sediment as asbestos or calcium carbonate. However, a personal communication (1955) from an investigator from Barker's laboratory revealed that he recently

found celite, a filter aid, to work advantageously in respect to the methane fermentation.

Thus in the last 15 years the knowledge of the growth of the methane bacteria and of the mechanisms involved in the methane fermentation has been greatly extended. The methane group of bacteria has already achieved importance both from the academic and economical standpoints. It is the purpose of this thesis to add to the present day information concerning the effect of some factors on the growth and gas production of a Methanobacterium species.

EXPERIMENTAL

Isolation Procedures

Approximately 50 ml of sewage sludge was placed in a 100 ml glass-stoppered reagent bottle. Two ml of ethyl alcohol and enough tap water to fill the bottle was added leaving a small air space to take care of frothing. This method permitted the escape of the gases by the slight raising and re-seating of the stopper. No other anaerobic precautions needed to be observed with the primary enrichment culture since the sludge was in a highly reduced state. At the end of 24 hours a vigorous fermentation was observed. Five ml of the sediment was inoculated into a medium formulated as follows:

| | |
|--------------------------|---------|
| NH_4Cl | 5g |
| K_2HPO_4 | 1g |
| KH_2PO_4 | 1.5g |
| MgCl_2 | 0.1g |
| Tap Water | 1000 ml |

Immediately after autoclaving, 1.5 volume percent $\text{C}_2\text{H}_5\text{OH}$, 1 to 2 ml of a 1 percent $\text{Na}_2\text{S} \cdot 9\text{H}_2\text{O}$ solution; and 8 ml of a 5 percent Na_2CO_3 solution were added. The sulfide and carbonate solutions were autoclaved separately to remove dissolved oxygen. A glass stoppered bottle with a small amount

of asbestos covering the bottom was used. The volume was adjusted so that after inoculation the liquid would fill the neck. The glass stopper was then replaced with a slight spinning motion to remove excess liquid and to prevent the formation of an air bubble. As soon as gas bubbles were noticed after incubation at 37°C, a few ml of the sediment was transferred to a fresh medium until four such enrichment cultures were obtained.

Colonies were isolated by the agar shake culture method. Five-tenths ml of the sediment from the fourth enrichment culture was seeded into a tube of an agar medium. Seven serial tube dilutions were made. Transfers from one tube to another (.5 ml) were made with 2 ml pipettes which had been flushed with CO₂ immediately before using. Since it was more convenient to add the carbonate-sulfide solution to one flask of medium rather than to a number of tubes, all the sterile tubes which were subsequently to be used for the shake cultures also were flushed with CO₂ before being filled with the medium. After solidification of the agar, the cottonplugs were pushed into the tubes just above the surface of the medium and a few crystals of pyrogallol were added. A few ml of a 10 percent sodium carbonate solution were added to the crystals. The tubes were immediately sealed with rubber stoppers, inverted, and incubated at 37 C. Tests with several anaerobic indicators showed this to be a suitable method of achieving and maintaining anaerobiosis.

The medium employed for the isolation of the colonies was suggested by Barker (1940) for Mb. omelianskii

| | |
|------------|-------|
| K_2HPO_4 | 0.6% |
| KH_2PO_4 | 0.9% |
| NH_4SO_4 | 0.03% |
| $MgSO_4$ | 0.01% |
| Tap Water | |

Agar was added in the concentration of 1.5 percent. The carbonate-sulfide solution as well as the ethanol was added after autoclaving as previously described.

After twenty-four hours incubation the agar in the first tube was cracked by gas and after 13 days the fourth dilution showed several well isolated, lens shaped, brownish yellow, colonies. The tube was broken open and the colonies were transferred by capillary pipettes, filled with the reduced medium, to another series of agar shake tubes. Of four colonies originally picked, one showed evidence of growth and in seven days several colonies from the second dilution were picked and transferred to small, 6 ml, screw-cap vials containing a small amount of asbestos and the liquid medium. Two of the cultures produced gas in four days. One of the cultures was chosen for these studies.

To assure gas production in a reasonable length of time, a rather large inoculum was necessary. A two to five percent inoculum always gave good results. Smaller amounts could

have been used depending on the activity of the culture and the type of sediment, if any, transferred.

Stock cultures were maintained in 200 ml of medium in 250 ml flat-bottom flasks with about one-half inch of shredded asbestos on the bottom. Anaerobic conditions were maintained by sealing tightly with rubber stoppers. No other anaerobic precautions such as flushing the flask with nitrogen were found to be necessary since the head space over the liquid was small. A stock culture of this volume served to inoculate several series of flasks. It was of the utmost importance to release the gas pressure periodically during an incubation of over 24 hours. As an added precaution the flasks were incubated in a strong cardboard or wooden box.

The cultures were often refrigerated for long periods of time without any loss of activity.

Extent of Culture Impurity

Until Barker isolated Mb. omelianskii in pure culture in 1940, all previous work on the methane fermentation was carried out with impure enrichment cultures. Indeed, much of the work today is done with partially purified cultures because of the difficulty and time involved in their purification. Thus such circumstances need not invalidate the conclusions drawn from cultural and biochemical experiments with the organism particularly if certain precautions are

observed. The methane bacteria grow abundantly and show an active gas production in a medium consisting of a few minerals and a simple organic substrate. The simplicity of the medium itself tends to eliminate most, if not all other anaerobic organisms from attaining numbers large enough to enter into the reactions. The study of certain reactions, however, particularly those involving special enzymes could not be carried out in other than pure culture.

Despite the number of enrichment cultures, agar shake tubes, and the extreme precautions used in the procedures, the isolated culture was found to contain two different types of organisms other than the methane group. Both types were isolated in pure culture and were found to be obligate, heterotrophic anaerobes. One was a gram variable diplobacillus often occurring in short chains. The other belonged to the sulfate reducing group. It was gram negative and consisted of slightly curved rods. The diplobacilli were isolated in Brewer's Thioglycollate agar. The sulfate reducer was isolated in an ordinary anaerobic medium to which 0.5 percent sodium sulfate and 0.001 percent ferric sulfate had been added.

In an experiment to determine the activity of the contaminants, day to day counts from the methane medium showed that their numbers were relatively small and did not increase during the period of active methane fermentation, in fact a decrease was often noticed. A very slight increase after six or seven days could be attributed to the contribution of

protein nutrients by dead methane bacteria. No further attempts to classify the contaminants was made nor did time permit further purification of the methane culture. Frequent microscopic examinations of the cultures revealed but a few cells of the contaminants per field. To eliminate the possibility that either of the contaminating organisms were responsible for gas production, each was inoculated into the medium used for the methane culture. After two weeks of incubation at 37 C both organisms failed to produce gas.

Identification of the Methane Organism

The methane species used in these studies was similar to the organism described by Barker (1940, 1941) as Mb. omelianskii. The cells were thin, straight or slightly bent, unbranched gram variable rods. The commonest length was 3.8 to 4.0 microns; the average width was 0.35 to 0.40 microns.

The fermentation reactions of the culture were checked and of those substances tested, ethanol, propanol, butanol and pentanol were oxidized while methanol, formic acid, valeric acid and glucose were not attacked. Identical fermentation reactions were reported by Barker (1941) for Mb. omelianskii. The cells were slightly smaller than those described by Barker; however, as is well known, the size of a cell within certain limits is not always a reliable point of identification. Barker (1936a) described an alcohol fermenting rod-shaped bacterium which agreed rather closely with

the alcohol fermenting bacterium of Omelianski although it appeared somewhat smaller. Without doubt the culture described in this thesis is of the Mb. omelianskii type if not Mb. omelianskii itself.

Periodically the gases produced by the culture were analyzed by means of a Burrell gas analyzer. One hundred ml gas samples were used. CO_2 was determined by absorption in KOH , O_2 was absorbed in alkaline pyrogallol while any H_2 was converted to H_2O when the gas was passed through a copper oxide heater. Methane was oxidized to CO_2 when passed through a catalyst heater and measured by absorption in KOH . On the average the analyses showed 80 percent methane.

Culture Medium

Barker (1940) suggested a satisfactory medium for the growth of Mb. omelianskii which consisted of 0.4 volume percent $\text{C}_2\text{H}_5\text{OH}$, 0.6 percent K_2HPO_4 , 0.9 percent KH_2PO_4 , 0.03 percent $(\text{NH}_4)_2\text{SO}_4$, 0.01 percent $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.001 percent $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ and 1 percent of a saturated solution of CaSO_4 . Distilled water could be used but in general, according to Barker, tap water gave better results. With the latter the CaSO_4 and FeSO_4 could often be eliminated. After autoclaving, 1 to 2 ml of a 1 percent $\text{Na}_2\text{S} \cdot 9\text{H}_2\text{O}$ solution and 5 to 8 ml of a 5 percent Na_2CO_3 solution were added.

Sulfate reducing bacteria are often present in small numbers when the enrichment technique is used to obtain certain species of the methane bacteria group. (Barker, 1936a, 1940; Stadtman and Barker, 1951; and the present investigation). Since the sulfate group readily reduce inorganic sulfates, sulfites and sulfides, it seemed desirable to slightly modify the above medium. NH_4Cl and MgCl_2 were substituted for $(\text{NH}_4)_2\text{SO}_4$ and $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$. Cysteine hydrochloride at a concentration suggested by Bryant and Burkey (1953) replaced sodium sulfide as a reducing agent. Ethanol was added after sterilization to prevent volatilization loss. The cysteine and carbonate solutions were added together and autoclaved separately from the medium.

The complete medium used in the investigation that follows is given below. It will be subsequently referred to as medium C. It proved to be extremely satisfactory and supported active growth of the methane bacteria.

| | |
|--------------------------|-------|
| K_2HPO_4 | 0.6% |
| KH_2PO_4 | 0.9% |
| NH_4Cl | 0.03% |
| MgCl_2 | 0.01% |
| Tap Water | |

After autoclaving at 121 C for 15 minutes, the following were added

| | |
|------|------|
| ETOH | 1.5% |
|------|------|

Cysteine Hydrochloride 3.3 ml of a 1%
soln./200 ml medium

Na_2CO_3 16 ml of a 5% soln./200 ml medium

The pH of the final medium was approximately 7.2 to 7.4

Inert Solids

A. Studies on the Effect of Inert Solids on the Methane Fermentation

The presence of conflicting opinions in the literature as to the value of an inert sediment to the growth of methane bacteria indicated the need for further investigation in this area. Several materials were compared and evaluated. They were: shredded asbestos; vermiculite, an insulation material also known as exploded mica, Zonalite or Micafil; celite, a fine particled filter aid; coarse sea sand and fine sea sand (ground from the coarse). Unless the materials were obtained in a purified form, they were washed with dilute HCl and distilled water before being used.

A 5/8 inch layer of the material to be tested was placed in the bottom of a 250 ml flat-bottom flask and 200 ml. of medium C was added. All flasks were sterilized for 15 minutes at 121 C. Immediately after autoclaving, the ethanol and cysteine-carbonate solutions were added. Each flask was inoculated with 10 ml of culture and attached to a fermentometer designed to measure gas production. The fermentometer consisted of an electrically heated and controlled water bath

with a rack to hold the flasks such that only the necks protruded above the surface of the water. The flasks, in turn, were connected by rubber stoppers and tygon tubing to 250 ml burettes which were attached to the outside of the water bath and were equipped with leveling bulbs. The displacing fluid was 5 percent H_2SO_4 and 20 percent Na_2SO_4 . A small amount of methyl orange was added to facilitate reading. The entire system was flushed with nitrogen and the fluid leveled at zero. The volume of gas was recorded at 24-hour intervals. The results of a five day incubation period are given in Figure 1.

There is no doubt but what the addition of the inert solid enhanced the growth and gas production. Asbestos seemed to be noticeably better than the other materials.

B. Staining

Several hundred smears were made, during the course of these experiments, of the various cultures and were stained either by the gram stain method or with erythrosine. Smears made from the supernatant were very unsatisfactory owing to the lack of cells suspended in the liquid phase. If the sediment or inert substance was included, better results were obtained. The asbestos containing cultures were far superior in this respect, both from the standpoint of ease of handling and the number of cells present.

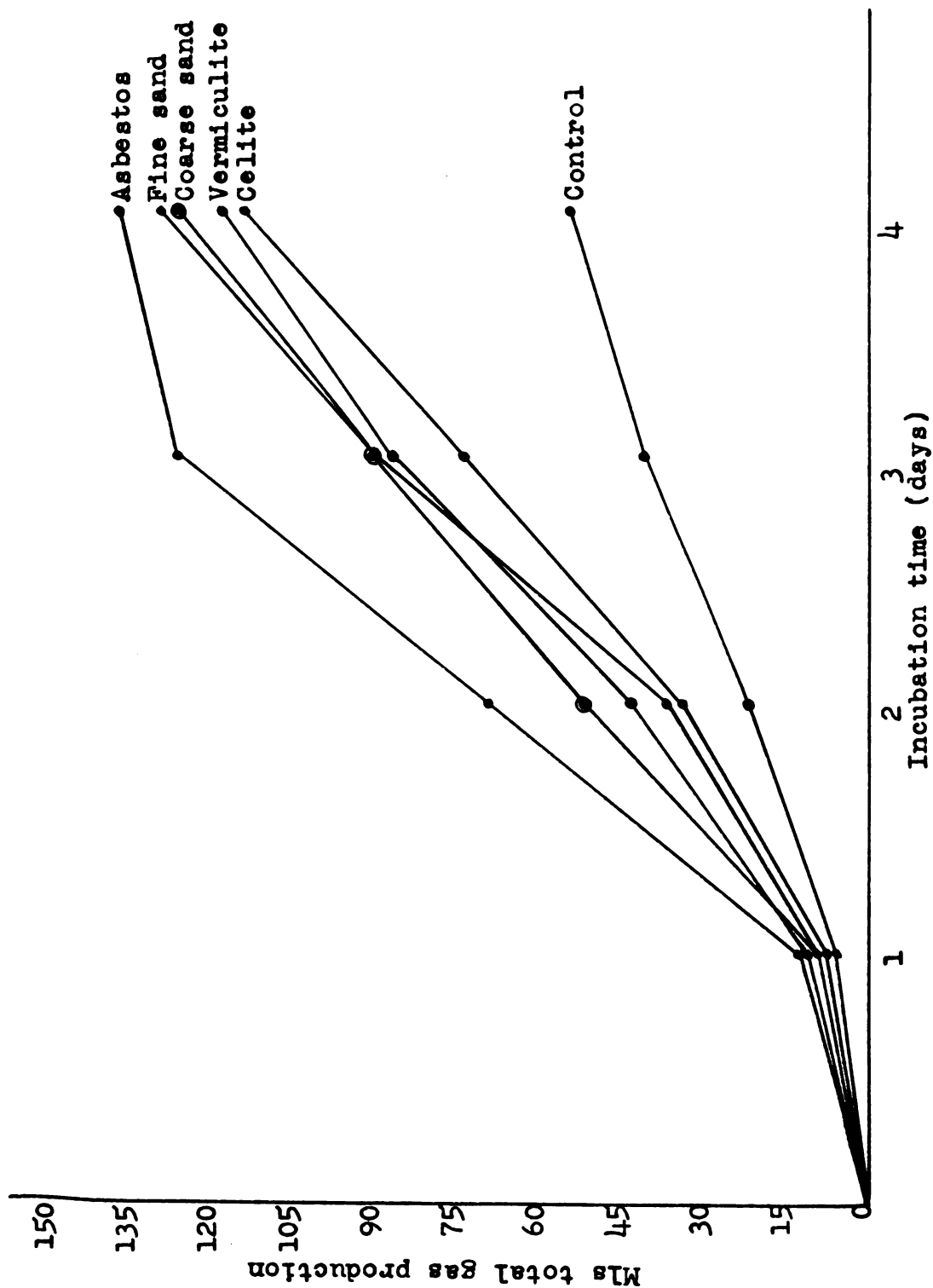


Figure 1. Gas production from the Methanobacterium culture when incubated in the presence of various inert solids at 36 C.

C. Inoculation

Based on the results obtained from the above mentioned staining experiments and on the work of Breden and Buswell (1953) it was concluded that more cells could be transferred per volume of inoculum if some of the inert material were included and that possibly a more rapid fermentation would result. The hypothesis was tested by inoculating one flask of medium C with a 10 ml inoculum from an asbestos culture whereby some of the asbestos was included, and a second flask with 10 ml of liquid alone from a 6 day coarse sand culture. The sand culture was agitated previous to pipetting to liberate all cells possible and the inoculum was taken from directly above the sand level. The experiment was repeated using various inert solids in the inoculated flasks.

The results shown in Table 1 indicate that the carry-over of asbestos with the inoculum increases gas production regardless of what particulate matter is used in the inoculated culture flask.

D. Adsorption of Acetic Acid by Inert Solids

Two criteria were used to ascertain growth of the methane cultures; one was a measure of gas production; the other was the determination of the volatile acid (acetic) which was produced by the oxidation of the alcohol during the fermentation. The procedure for the measurement of acetic acid was to take 100 ml from the 200 ml culture, acidify with H_2SO_4

TABLE 1

GAS PRODUCTION AFTER 2 DAYS INCUBATION AT 36 C FROM
 CULTURES CONTAINING THE VARIOUS INERT SUBSTANCES
 STARTED WITH AN INOCULUM CONTAINING ASBESTOS
 AND WITH AN ASBESTOS FREE INOCULUM

| Inert Substance
Present in
Culture Flask | Mls Total Gas Production | |
|--|---------------------------------|---------------------------|
| | Inoculum Containing
Asbestos | Asbestos Free
Inoculum |
| None | 43.0 | 1.0 |
| Coarse sand | 94.5 | 10.0 |
| Fine sand | 64.0 | 8.0 |
| Asbestos | 115.5 | 29.0 |
| Vermiculite | 68.5 | 18.0 |
| Celite | 59.5 | 5.0 |

and by means of a steam distillation distill over approximately three times the original volume. The distillate was then titrated with NaOH, 0.05 N. Little if any inert solid was transferred to the distillation flask. On several occasions less acid was present in the vermiculite culture when measured by steam distillation and titration than in any of the other cultures, yet a comparable amount of gas was produced. This phenomenon suggested an adsorption or ion exchange reaction by the vermiculite. To test the hypothesis a controlled experiment was run. A layer, approximately $3/4$ inches thick, of the various inert solids was added to 250 ml flasks and 100 ml distilled water then added. Two ml of a 0.125 N acetic acid solution was pipetted in each of the flasks. The flasks were tightly stoppered and were shaken for ten seconds. After being allowed to stand for 45 minutes, 50 ml of the clear liquid was removed and titrated with 0.05 N NaOH. The results given in Table 2 indicate some type of reaction between the acid and the vermiculite but a negligible adsorption or reaction in the cases of the other substances.

Oxidation Reduction Studies

A. Apparatus

All oxidation-reduction potentials were measured electrometrically. The apparatus was a modification of a design

TABLE 2

ADSORPTION OF ACETIC ACID BY VARIOUS INERT SOLIDS AS
SHOWN BY TITRATION (0.05 N NaOH) OF 50 ML
OF THE CLEAR SUPERNATANT FROM THE FLASKS

| Inert Material | Ml 0.05N NaOH Used |
|----------------|--------------------|
| None | 2.50 |
| Vermiculite | 1.75 |
| Asbestos | 2.47 |
| Celite | 2.50 |
| Sand | 2.35 |

suggested by Hewitt (1950). Eight cultures or solutions could be measured simultaneously without removing the electrodes or otherwise disturbing the cultures in the incubator. Since the electrodes were in equilibrium with the solution at all times during the measuring period, slight changes in oxidation reduction potential could be rapidly and accurately noted.

The apparatus consisted of a board $3\frac{1}{4}$ " x 10" x 10" in the center of which a hole $2\frac{1}{2}$ inches in diameter and $\frac{1}{2}$ inch deep was drilled. Eight smaller holes, $1\frac{1}{16}$ " x $\frac{1}{2}$ " were drilled around the larger hole. A specimen jar about 6 cm in diameter, which acted as a KCl reservoir, was placed in the center hole. The jar was fitted with a screwcap through whose center a hole large enough to accommodate a 6-inch calomel electrode was drilled. Eight smaller holes for the agar bridges surrounded the center. Screw-cap vials (7.0 cm x 2.5 cm) were used to hold about 20 ml of the culture or solution to be measured. The vials were set in the holes surrounding the KCl reservoir. The entire set-up could be easily placed in a small table incubator.

The electrodes were of platinum wire and sealed into a 7.5 cm lime glass tube (6 mm o.d.) with lime sealing glass. The platinum wire was approximately 1.8 cm in length but was coiled into a spiral. Mercury was dropped into the tubes so that contact with the platinum could easily and quickly be

made by inserting the lead from a switch box into the tube. Each culture vial contained two electrodes, one as a check on the other. The electrodes were inserted through a no. 2 rubber stopper along with the agar bridge and a small vent tube which could be plugged with plasticene or cotton or connected to a tube of mercury if anaerobiosis was to be established in the presence of gas producing organisms (Figure 2). Leads from the calomel cell and the electrodes ran to the outside of the incubator through a vent hole in the top and were connected to a series of single-pole double throw knife switches mounted on a chassis (Figure 3). Each electrode could be switched into the circuit in turn. The center terminals of the switches were joined to a double-pole double throw knife switch which enabled the connections to the electronic potentiometer to be reversed. The electronic potentiometer and indicating meter were a Beckman model H pH-Eh meter. The KCl reservoir was filled with a saturated KCl solution. KCl saturated agar bridges joined each culture vial to the calomel cell through the reservoir. The completed set-up in reverse was as follows: indicating meter (through jack on Beckman unit), calomel cell, KCl reservoir, agar bridge, culture, platinum electrode, single-pole switch, double pole switch, electronic potentiometer (through jack on Beckman unit), ground.

The electrode-rubber stopper unit could be sterilized in the autoclave if mounted in the vial containing a drop of water.

Figure 2. Diagram of the electrode assemblies used for measuring oxidation-reduction potentials.

- A - 250 ml flask
- B - 20 ml vial
- C - rubber stopper
- D - agar bridge
- E - vent
- F - platinum wire
- G - mercury
- H - shallow electrodes
- I - deep electrodes
- J - electrodes

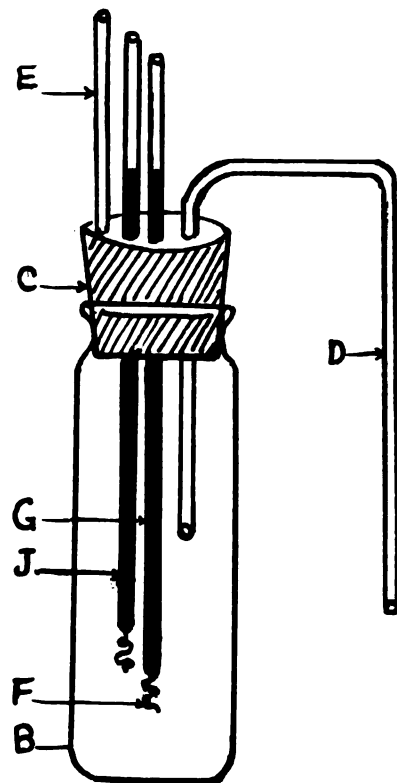
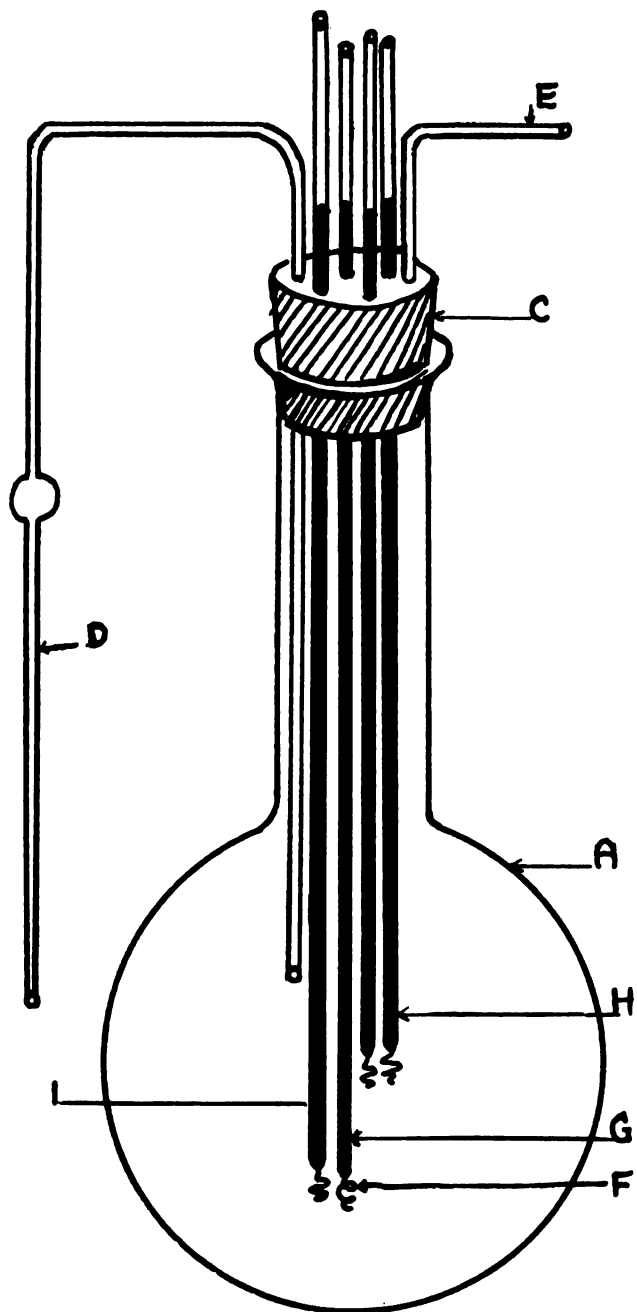
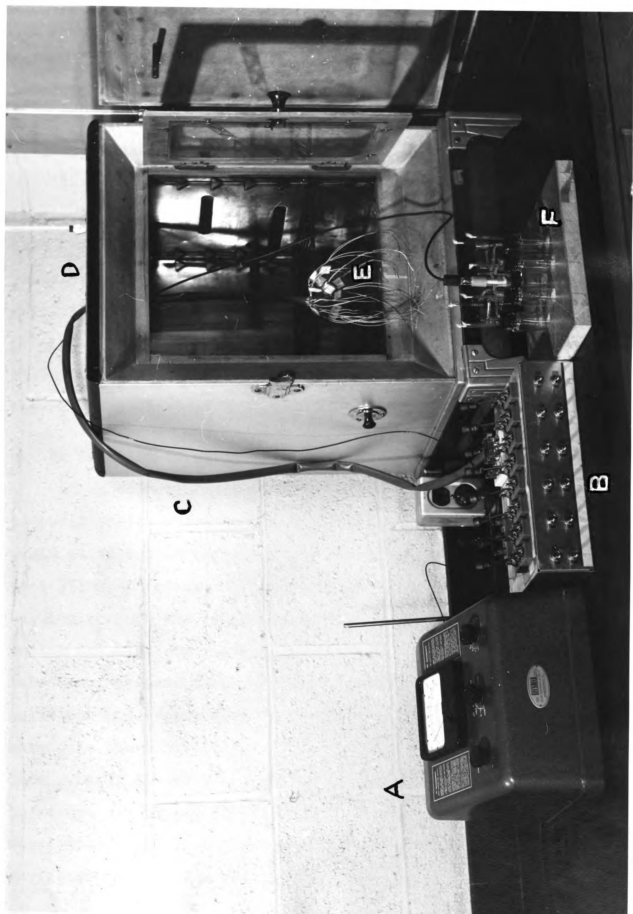


Figure 3. Apparatus for measuring oxidation-reduction potentials.

- A - electrometric potentiometer
- B - switch box
- C - plastic tubing housing leads
- D - incubator
- E - labeled leads from switch box
(inserted into electrode tube
for use)
- F - vials, electrodes, agar bridges,
HCl reservoir on portable
platform, and calomel cell.



The agar bridge had to be removed but was dipped into a hypochlorite solution and then immediately rinsed with sterile, distilled water.

The platinum electrodes were cleaned by electrolysis in dilute HCl. The electrode was connected to a negative pole of a 22 volt dry cell battery so that hydrogen was rapidly evolved. Ten to 15 seconds exposure was usually a sufficient cleaning period.

All readings are referred to the normal hydrogen electrode. To obtain these readings 240 mv were added to the readings obtained with the calomel cell.

B. Eh Capacity of Medium C

It is common practice when working with liquid cultures of strict anaerobes or with their culture media to avoid all possible opportunities for oxygen to come in contact with the medium. Often sterile, oxygen free, gas is bubbled through the medium to prevent oxygen contamination during the time the flask is opened even though a reducing substance may have been previously added. After the initial isolation procedures such precautions were not always observed in these investigations nor were "oxsorbent" or alkaline pyrogallol saturated cotton plugs used above the medium to remove any oxygen present, yet the activity of the culture was not impaired to any noticeable extent. This observation suggested an experiment to determine the oxidation-reduction

capacity of the uninoculated medium. Twenty ml of medium C was autoclaved in the screw-cap vials and the ethanol and cysteine-carbonate solution were added. The screw cap was replaced with the electrode assembly. A thirty minute period was allowed for the electrodes to come to equilibrium with the medium. Readings were taken every 10 minutes. After a 60 minute period air was vigorously bubbled into the medium through a capillary inserted through the vent opening. Readings were taken at minute intervals during aeration.

Table 3 shows that the medium can maintain a low potential in the presence of oxygen, indicating a good capacity or poisoning effect.

C. Extent of Oxygen Diffusion into Asbestos Layer

The exact role of the inert solid in the methane fermentation is by no means clear. The hypothesis that they act as a support for the organisms (Breden and Buswell, 1933) would seem to have some merit. Precursory observations during these studies suggested that Breden and Buswell's conclusion that the action of the asbestos was not to protect the organism from oxygen might not hold true in all cases particularly if the medium were exposed to air.

To determine the protective properties of asbestos against oxygen, an experiment was designed whereby the Eh within the layer of asbestos and in the clear liquid above the asbestos could be determined at frequent intervals as

TABLE 3
INFLUENCE OF BUBBLING AIR THROUGH UNINOCULATED MEDIUM C
ON THE OXIDATION-REDUCTION POTENTIAL

| Time in Minutes After
Cysteine Added | Eh (millivolts) |
|---|-----------------|
| 30 | -158 |
| 40 | -170 |
| 50 | -205 |
| 60 | -254 |
| Air Bubbled Through | |
| 1 | -140 |
| 2 | -118 |
| 3 | -115 |
| 4 | -113 |
| 5 | -110 |
| 10 | -108 |
| 15 | -105 |
| 20 | -104 |

air was passed through the liquid layer. A 250 ml flat bottom flask was $\frac{1}{3}$ filled with shredded asbestos and 200 ml of medium C was added. The flask was autoclaved and reduced as if it were to be inoculated.

Four electrodes, prepared as previously described but of greater length (18 cm) were inserted through a no. 4 rubber stopper along with a vent tube and an agar bridge* (Figure 2). One set of electrodes extended down into the asbestos layer while the other set remained about $\frac{5}{8}$ inches below the surface of the liquid. After the electrodes had reached equilibrium, air was bubbled through the liquid with a capillary pipette just above the asbestos layer. Air was allowed to bubble for 30 minutes and potential readings were taken during this time and for 90 minutes afterwards. The results recorded in Figure 4 demonstrate dramatically that the asbestos layer could act as a protective agent for the organisms particularly if the cells had not already established a low oxidation-reduction potential within the asbestos layer.

D. Investigation as to Catalytic Effect of Inert Solid

The possibility that the inert substances might act as catalytic agents in lowering the oxidation-reduction potential to a more suitable level for the growth of the methane organisms was suggested.

*A bubble was blown in the glass tube of the agar bridge to prevent slippage of the agar under conditions of slight temperature-pressure changes within the flask.

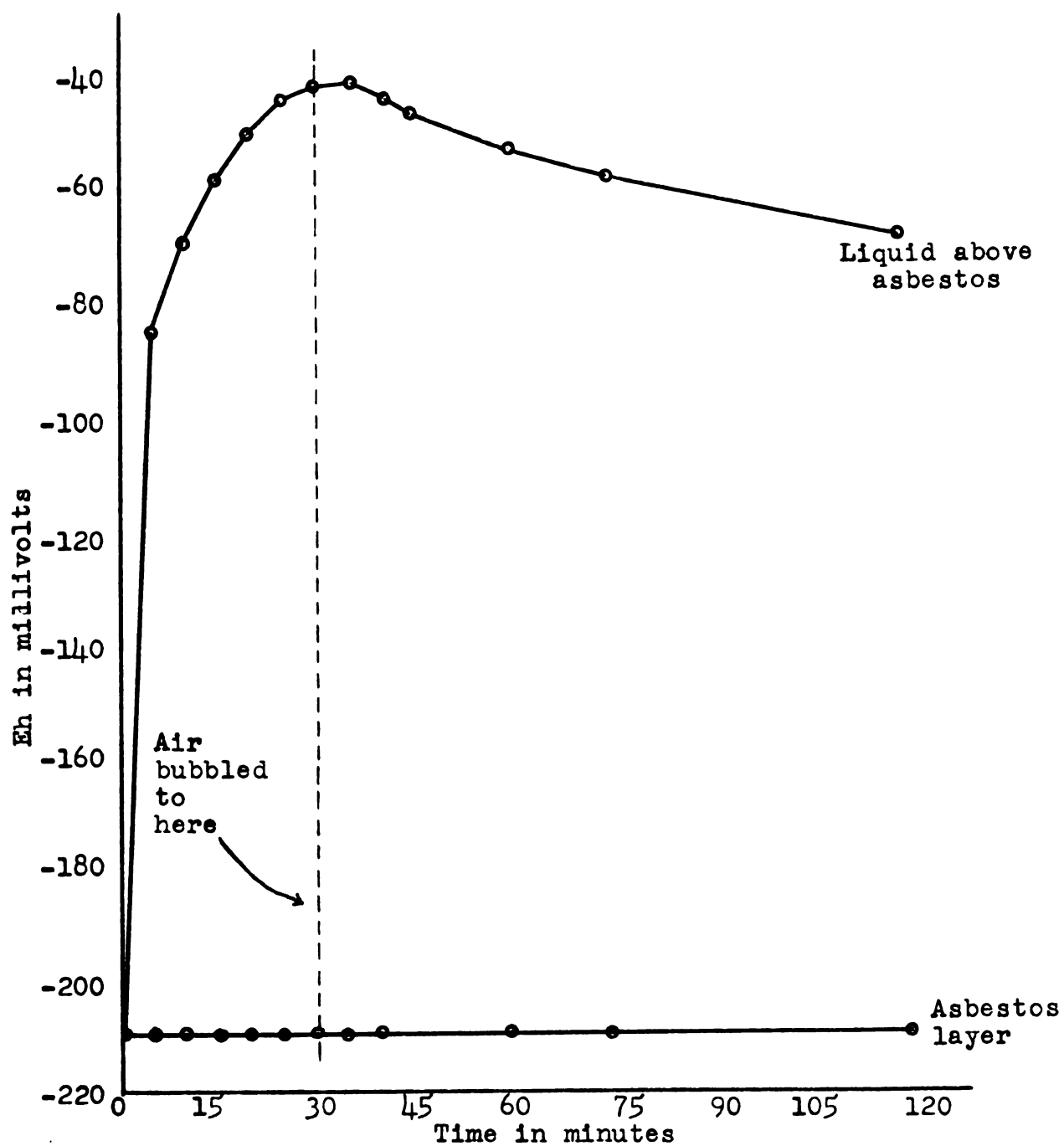


Figure 4. Eh readings in the asbestos sediment and in the clear liquid above the sediment during and after the period air was bubbled through medium C. Not inoculated.

Approximately $3/8$ inch of the inert substance to be tested was added to the bottom of the 7.0 cm x 2.5 cm vials. Twenty ml of medium C was added and the vials were autoclaved with the screw caps loosely screwed down. Ethanol, carbonate and cysteine were added after autoclaving. The double electrode assembly replaced the screw caps on each vial. Each vent tube had been previously plugged with plasticene. After equilibrium had been reached, readings were recorded at frequent intervals up to 120 minutes and a final reading at 16 hours. The data given in Table 4 reveal that all solutions had about the same potential at the time of the final reading with the exception of vermiculite, which was consistently high throughout this and repeated experiments. The medium without the inert substance was slower to reach the low potential but eventually did so. In other experiments the potential was comparable to that in the other media.

E. Studies on the Culture's Sensitivity to Oxygen

It is the opinion of many investigators that the methane bacteria are more sensitive to oxygen than are other anaerobic groups (Barker, 1936b), in fact, oxygen has been described as extremely toxic and even lethal to them.

The question arose as to whether this was actually the case or whether it was simply the inability of a few cells to acquire the proper conditions of growth in the presence of O_2 .

TABLE 4
OXIDATION-REDUCTION POTENTIAL (MILLIVOLTS) OF NON-INOCULATED
MEDIUM CONTAINING VARIOUS INERT SUBSTANCES

| Time in
Minutes | Inert Materials | | | | | |
|--------------------|-----------------|----------------|--------------|-------------|--------|------|
| | Asbestos | Coarse
Sand | Fine
Sand | Vermiculite | Celite | None |
| 30 | -103 | -165 | -160 | -014 | -170 | -010 |
| 45 | -125 | -172 | -175 | -016 | -190 | -020 |
| 60 | -170 | -183 | -182 | -023 | -187 | -028 |
| 70 | -190 | -188 | -186 | -025 | -190 | -030 |
| 130 | -214 | -200 | -205 | -060 | -207 | -110 |
| 190 | -240 | -212 | -221 | -086 | -224 | -190 |
| 330 | -239 | -212 | -229 | -140 | -232 | -220 |
| 960 | -230 | -226 | -221 | -165 | -232 | -235 |

Experience pointed to the fact that on isolation, where only a relatively few cells were involved, oxygen had to be excluded. It seemed desirable, however, to learn of the effect oxygen had on an active inoculum and at the same time to acquire additional information as to the role of the asbestos when used in the culture medium.

Varying environmental conditions were established as are shown in Table 5. Two hundred ml of medium C were used in 250 ml flat bottom flasks. In most cases a duplicate flask containing no asbestos was employed. Dissolved oxygen was removed at the time of autoclaving. In those flasks where dissolved oxygen was present at the time of inoculation, the medium was autoclaved, cooled and agitated on a shaker for 15 minutes. A cotton plug replaced the usual rubber stopper. In those cases where the medium was exposed to air during incubation, cotton plugs were also used. The description of the treatment given each flask (Table 5) is in the order in which it occurred in the actual experiment. To eliminate the slight possibility that the cysteine might protect the cells during exposure to oxygen, it was not added to flasks 4, 5, 6 and 7 (Table 5) until after inoculation but was added just prior to incubation. The cells, then, were inoculated into a medium already saturated with oxygen and thus remained in its presence for 45 minutes before the reducing agent was added. The inoculum was 5 ml of an active culture.

The Eh was measured, immediately before incubation, at the top and bottom of the liquid in each flask by means of the long electrode assembly previously described. The cultures were incubated at 37 C for 24 hours and the Eh was again recorded. The results shown in Table 5 represent a typical experiment. Growth was indicated by the presence of gas from the methane fermentation. No attempt was made to compare quantitatively the amount of gas produced under the various circumstances. The gas was, however, frequently analyzed for methane.

Several facts seem evident from the experiment. Oxygen had little or no effect on the gas production under the conditions of the experiment, if a potential suitable for anaerobic growth had been established. The Eh in the lower portions of the medium in all flasks containing asbestos was considerably lower than the top portion especially in those cases where no reducing agent was present.

The Effect of Ethyl Violet,^{*} Sodium Azide and Penicillin on Gas Production

There is an increasing interest in the methane bacteria especially in the fields of dairy nutrition and ruminology and among those concerned with the decomposition of organic wastes. It was not the purpose of this thesis to ascertain

^{*}Lot no. 12522 National Aniline Co.

TABLE 5

THE INFLUENCE OF VARIOUS EXPOSURES TO OXYGEN AND THE EFFECT OF ASBESTOS UNDER
THESE CONDITIONS ON THE EH AND GAS PRODUCTION OF THE
METHANOBACTERIUM CULTURE

| Flask
No. | Treatment | Eh Before
Incubation | | Eh After 24 hrs.
Incubation | | Difference
Top and Bottom
After Incub. | Gas |
|--------------|--|-------------------------|--------|--------------------------------|--------|--|-----|
| | | Top | Bottom | Top | Bottom | | |
| 1 | Asbestos, free O ₂ removed, cysteine added, inoculated, not exposed to air during incubation | -195 | -170 | -110 | -164 | 54 | + |
| 2 | Asbestos, cysteine added, inoculated, medium shaken in air 45 min., exposed to air during incub. | +002 | 000 | -035 | -185 | 150 | + |
| 3 | No asbestos, otherwise same as above | +005 | +005 | -005 | -042 | 37 | + |
| 4 | Asbestos, medium shaken in air 15 min., inoculated, shaken in air 45 min., cysteine added, not exposed to air during incubation | +013 | +016 | -110 | -170 | 60 | + |
| 5 | Same as above but exposed to air during incubation | 000 | -002 | -105 | -170 | 65 | + |
| 6 | No asbestos, medium shaken in air 15 min., inoculated, shaken in air 45 min., cysteine added, not exposed to air during incubation | 001 | 000 | -068 | -080 | 12 | + |
| 7 | Same as above but exposed to air during incubation | 000 | +003 | -046 | -081 | 35 | + |
| 8 | Asbestos, free O ₂ removed, inoculated, no cysteine, not exposed to air during incubation | +125 | -012 | -050 | -169 | 119 | + |

TABLE 5 (Cont.)

| Flask
No. | Treatment | Eh Before
Incubation | | Eh After 24 hrs.
Incubation | | Difference
Top and Bottom
After Incub. |
|--------------|---|-------------------------|--------|--------------------------------|--------|--|
| | | Top | Bottom | Top | Bottom | |
| 9 | Same as above but exposed to air during incubation | +188 | +170 | -033 | -160 | 126 |
| 10 | No asbestos, free O ₂ removed, no cysteine, not exposed to air during incubation | +198 | +200 | -055 | -070 | 15 |
| 11 | Asbestos, medium shaken in air 15 min., no cysteine, inoculated, not exposed to air during incubation | +200 | +199 | -020 | -135 | 115 |

the effects of a large number of chemicals and antibiotics on the Mb. omelianskii type organism, however, it was deemed of interest to test a few compounds.

Two hundred ml of Medium C was employed in 250 ml flasks for the tests. Asbestos was not used when ethyl violet was investigated because of the noticeable adsorption of the colored portion of the dye on the fibers. Asbestos was present in the tests which dealt with sodium azide and penicillin. The various concentrations of penicillin were added after the medium was sterilized and cooled. A 5 ml inoculum was used in each flask and the gas was measured by the previously described fermentometer. Incubation temperature was 36 C. Figures 5, 6 and 7 show that of the three substances tested, ethyl violet exerted the least effect while the action of sodium azide and penicillin were most pronounced.

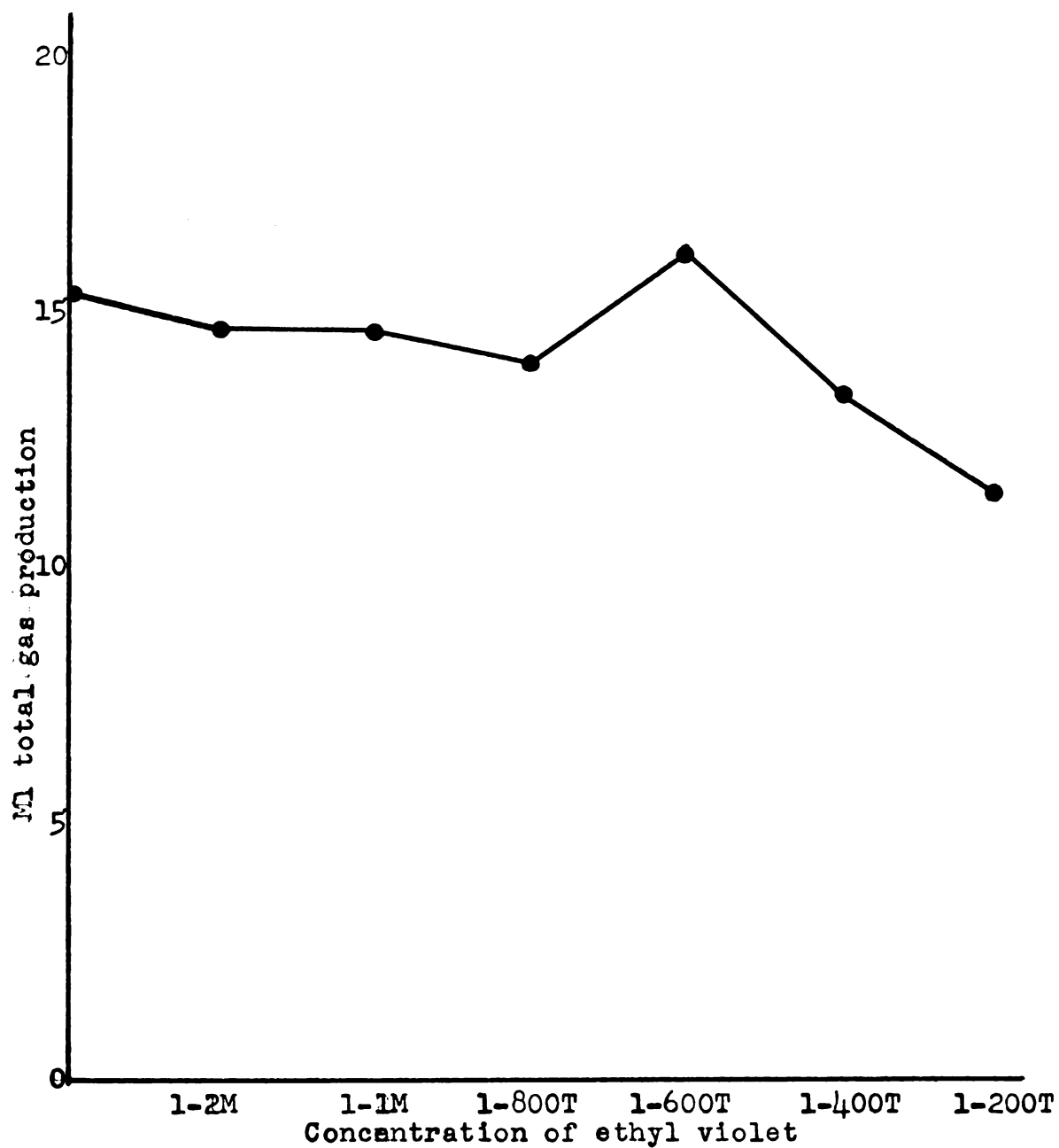


Figure 5. The effect of various concentrations of ethyl violet on the gas production from a Methanobacterium culture after 2 days incubation at 36 C.

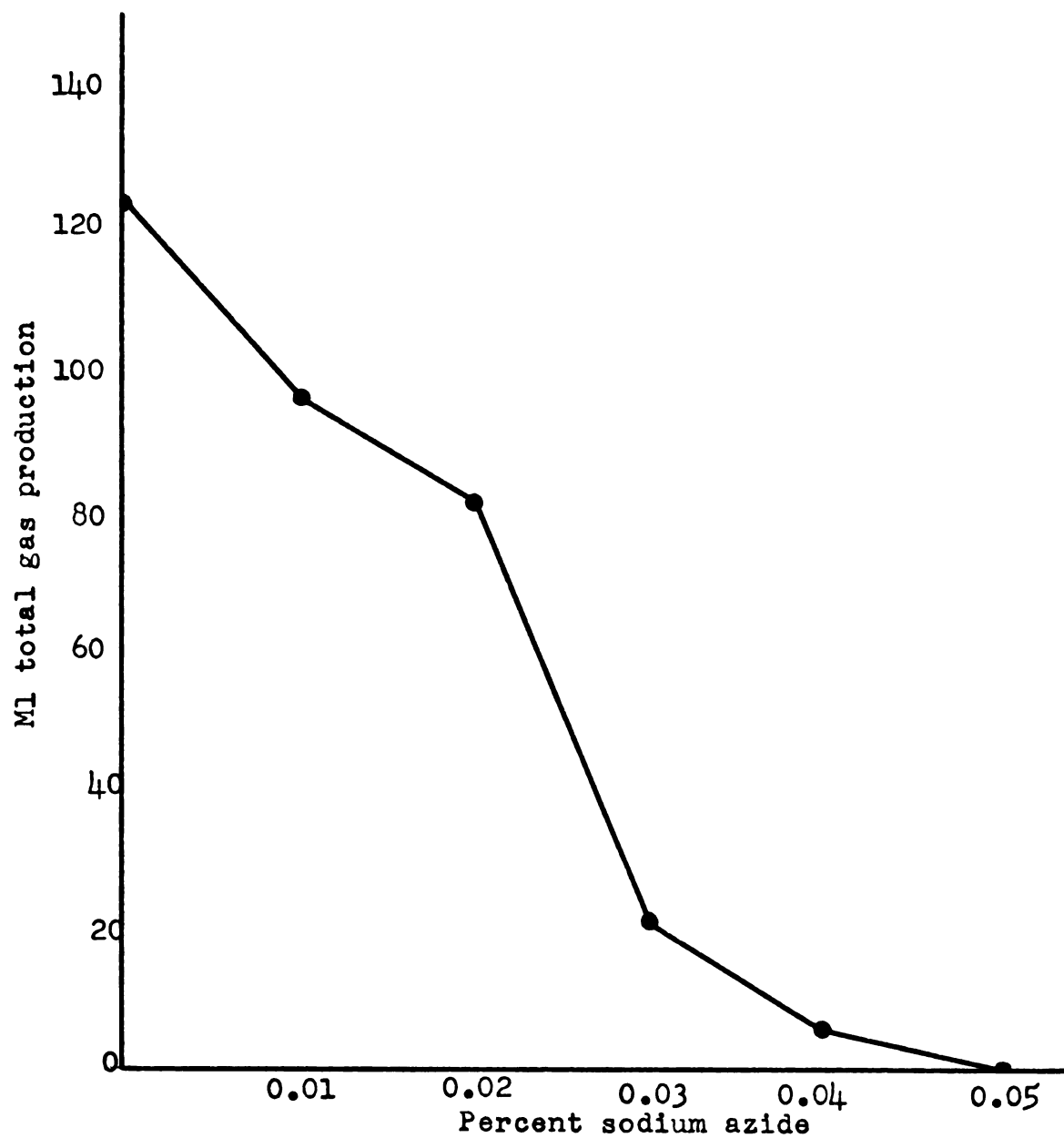


Figure 6. The effect of various concentrations of sodium azide on the gas production from a Methanobacterium culture after 3 days incubation at 36 C.

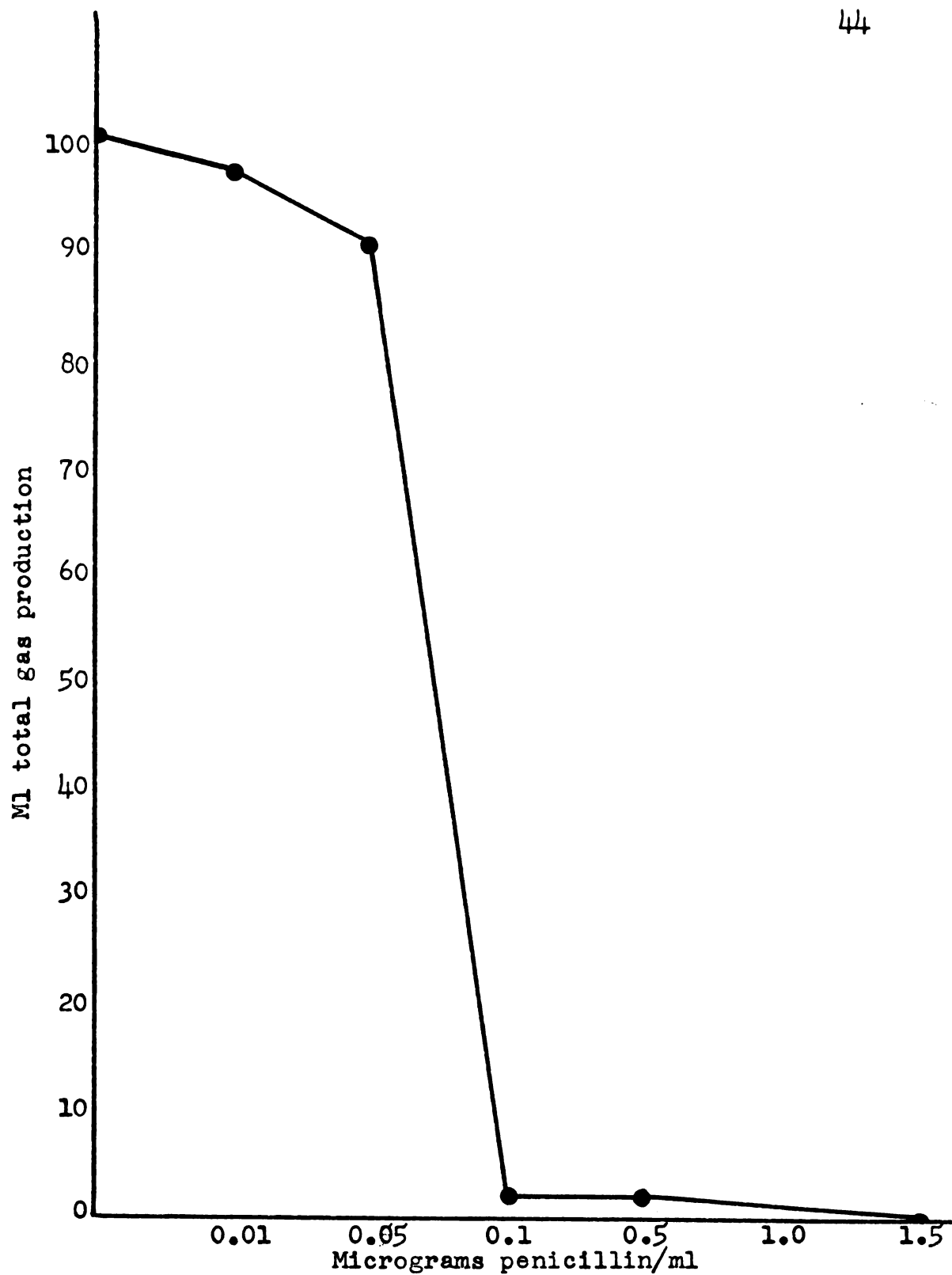


Figure 7. The effect of various concentrations of penicillin on the gas production from a Methanobacterium culture after 2 days incubation at 36 C.

DISCUSSION

The simple requirements of the methane bacteria usually work to advantage in excluding the growth of other organisms in enrichment cultures yet they may be detrimental when isolation attempts are made in agar shake tubes. The development of a well isolated methane bacteria colony, particularly in the lower dilutions, is no assurance that the cells of other anaerobic genera, unable to grow because of lack of nutrients, are not present in the immediate area. The difficulties in isolating the methane group in pure culture may be partly attributed to this.

Excellent results were obtained with a modification of a medium described by Barker (1940). The substitution of the sulfates by chlorides noticeably lessened the sulfate reducing bacteria. Cysteine hydrochloride was substituted for $\text{Na}_2\text{S} \cdot 9\text{H}_2\text{O}$ on the basis of the work reported by Ishimoto, Koyama, Omura and Nagai (1954) who found that washed cells of Desulfovibrio desulfuricans failed to catalyze the production of H_2S from organic sulfur compounds. On the other hand, Grossman and Postgate (1953) claimed that both sodium sulfide and cysteine satisfied the reduction requirements of Desulfovibrio.

The cysteine was autoclaved separately from the medium to avoid any toxicity effects such as were found by Konowalchak, Clunie, Hinton and Reed (1954) for Mycobacterium tuberculosis when cysteine was autoclaved together with the ferric salts in the medium.

A fault of existing media for the methane bacteria is the frequent use of tap water. It is common knowledge that the composition of tap water varies in different localities and it therefore is reasonable to assume that results obtained by some experiments in a hard water area might not be reproducible in a soft water area.

Barker (1940) reported that a high percentage of phosphate is valuable in the medium. He commented on the surprising fact that an organism such as Mb. omelianskii, which produces an organic acid, should be sensitive to small pH changes but explained that in nature other organisms were always present to remove the accumulation of acid.

The data in Table 3 show that the uninoculated medium in the presence of cysteine hydrochloride had a rather high high oxidation-reduction capacity. The addition of oxygen failed to raise it above -104 mv, a potential low enough, according to Table 5, to initiate the growth of an active inoculum of the methane bacteria.

An examination of the data represented in Figure 1 leaves no doubt as to the value of an inert solid in the

methane fermentation by the Methanobacterium species. The gas production curves fall roughly into three groups: (1) asbestos, (2) other solids, and (3) no solid. Growth of the culture in the presence of asbestos was consistently ahead of the other cultures all through the growth phase. Likewise the culture which had no solid lagged considerably behind. Eventually all curves except the no-solid control reached a common point. Lack of time prevented an attempt to determine whether or not the control would ever reach a comparable maximum.

It seems unlikely that surface area alone was responsible for the enhancing action of the solid. Were this the case, the curves representing the gas produced from cultures with coarse and fine sand should greatly differ.

A careful microscopic examination revealed myriads of cells clinging to the asbestos fibers thus confirming the observation of Breden and Buswell (1933). Very few cells were present in the supernatant. This probably accounts for the vast differences in Table 1.

As previously mentioned the microscopic examination of the stained culture was greatly facilitated when the asbestos fibers were allowed to remain on the slide. The fibers themselves were only weakly stained. The other solids, celite, vermiculite and sand were not applicable to the staining method.

Table 2 indicates that acetic acid formed by the oxidation of ethyl alcohol by the methane culture could be, in part, adsorbed by the vermiculite, if this substance were used. The mechanism of the depletion of the acid could be an ion exchange reaction but no attempt was made to confirm the hypothesis. The experiments suggest that certain precautions must be observed if volatile acidity is to be measured in the presence of the vermiculite.

The use of oxidation-reduction measurements proved of value in establishing an insight into the role of the asbestos or inert solid in the methane fermentation. The results given in Table 4 indicate that a catalytic action involving the lowering of the potential was not present since the control assumed nearly the same potential as that in the vials with inert solids. The vermiculite medium was consistently higher in potential yet the rate of gas production from a culture containing vermiculite was comparable to that of cultures with coarse sand, fine sand and celite. The slight difference in potential, then, had no noticeable effect on the actual growth as measured by the production of gas (Figure 1).

There were instances which lead to the belief that the function of the asbestos might be to protect the cells from oxygen, if oxygen was present. Its action might be that of hindering the diffusion of oxygen through the medium. It is

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not necessarily implied here, however, that the oxygen is toxic, but without its presence a low oxidation-reduction potential could more easily be established by the culture. Small amounts of agar are often used for such a purpose in some anaerobic media.

The striking results shown in Figure 4 tend to substantiate the inference that the asbestos slows the oxygen diffusion into the asbestos layer. Air was vigorously bubbled through the liquid above the settled asbestos for 30 minutes. The electrodes submerged within the asbestos layer recorded no change in Eh but at the same time those in the liquid portion indicated an immediate and continuous rise.

If we assume for the moment that oxygen has a decided effect on the methane organisms, then in such experiments where oxygen was carefully excluded and a reducing agent was added the oxygen effect would be negligible. Figure 1 shows that under conditions of an oxygen free environment asbestos is most desirable. The function of the asbestos in such cases must be other than a protective mechanism. On the other hand, if oxygen was present, as shown in Table 5, (all flasks but 1, 8 and 10), there was a considerable difference in Eh between the top and bottom of the medium after incubation in those flasks which contained asbestos. The Eh difference was not as pronounced in those flasks which did not contain asbestos but which were exposed to air

1. The first step in the process of identifying a problem is to recognize that a problem exists. This is often done by comparing current performance with a desired state or goal. If there is a discrepancy, a problem is identified.
 2. The second step is to define the problem. This involves identifying the specific aspects of the problem that need to be addressed. It is important to be clear and concise in the definition of the problem.
 3. The third step is to analyze the problem. This involves identifying the causes of the problem and the factors that contribute to it. This step is often the most difficult, as it requires a deep understanding of the problem and the ability to identify the underlying causes.
 4. The fourth step is to develop a solution. This involves identifying the actions that need to be taken to address the problem. It is important to consider the feasibility of the solution and the potential consequences of each action.
 5. The fifth step is to implement the solution. This involves putting the solution into action and monitoring the results. It is important to be flexible and willing to make adjustments as needed.
 6. The sixth step is to evaluate the results. This involves comparing the results of the solution with the desired state or goal. If the results are not satisfactory, the process may need to be repeated.
- The process of identifying a problem is a continuous one. As new information is gathered, the problem may be redefined or the solution may be refined. It is important to remain open to new ideas and to be willing to change course when necessary.
- There are many factors that can contribute to the identification of a problem. These include changes in the environment, new information, or a change in goals. It is important to be aware of these factors and to be prepared to respond to them.
- The process of identifying a problem is a critical one. It is the first step in the process of solving a problem, and it is essential to get it right. By following the steps outlined above, you can ensure that you are identifying the problem correctly and that you are developing a solution that is effective and sustainable.
- There are many examples of problems that have been identified and solved. These range from simple problems, such as a broken light bulb, to complex problems, such as a global climate crisis. The process of identifying a problem is the same in all cases, and it is a process that can be applied to any problem.
- It is important to remember that the process of identifying a problem is not a one-time event. It is a continuous process that requires ongoing attention and effort. By following the steps outlined above, you can ensure that you are identifying the problem correctly and that you are developing a solution that is effective and sustainable.
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(flasks 3, 6, 7). Flask 8 was not exposed to oxygen but the starting potentials were high because of the absence of cysteine. The top and bottom difference in Eh, however, was large after incubation. This was not the case in the culture in flask 10 which was grown under identical conditions but in the absence of asbestos.

The nature of the role of asbestos now becomes more clear. If fast diffusion of oxygen into the asbestos layer can be prevented then it is reasonable to assume that other systems, once established within the asbestos slurry would not rapidly diffuse out. The methane bacteria are slow growers. The energy yield from reactions involving autotrophic, anaerobic bacteria is low. The yield is much higher from aerobic reactions involving the transfer of hydrogen to oxygen with the consequent formation of H_2O . In the methane fermentation energy is derived from the dehydrogenation of ethyl alcohol but is partially spent in the reduction of carbon dioxide to methane. The carbon dioxide serves as a source of oxygen. The hydrogen from the substrate reduces CO_2 to CH_4 and H_2O . Thus, the oxidation of the hydrogen to form H_2O also supplies some energy to the system. The over all reaction may be stated as



In a low energy-yielding reaction such as this, energy for the establishment of an optimal environment becomes a precious item.

It would seem logical that it would be advantageous to the cells to be clustered together in a small optimum area, contributing to a common end rather than to be scattered throughout a large volume of medium. In part, this is substantiated by the fact that a large inoculum is required if growth is to be initiated in a reasonable time. It has been observed that the active methane fermentation takes place mainly in the sediment phase. If asbestos was present the cells tended to cluster around the fibers. The presence of asbestos greatly increased gas production (Figure 1).

The asbestos in effect, supplies an environment where the oxidation-reduction potential may be quickly lowered and maintained even in the presence of oxygen in the medium and where enzyme systems and hence energy may be concentrated. The question might arise as to how fresh medium could gain entrance rapidly enough to supply the needs of an actively fermenting culture. The diffusion in and out of the asbestos area is not, of course, entirely halted; also as gas is formed the rising bubbles cause a certain amount of agitation. The question as to why asbestos is more suitable than the other solids studied cannot be answered at this time. Perhaps its physical characteristics serve to answer the needs to a greater extent.

It has been generally accepted that methane bacteria are more susceptible to oxygen than are other anaerobes.

The lethal exposure to small amounts of oxygen has been supplied as the reason for the lack of development of methane colonies on the surface of anaerobic agar plates. On the other hand, Barker (1940) observed that cultured in his laboratory remained viable if left standing exposed to air for a month or more. He reasoned that the function of the spores was to maintain survival in the presence of oxygen rather than to offer protection against heat and that the persistence of viability could be explained in this manner.

It was often observed during the course of these experiments that techniques demanded for strict anaerobiosis were often abused without ill effects. The theory that oxygen was extremely toxic to the cells of the methane bacteria seemed worthy of challenge. It is possible that this dogma grew from unsuccessful isolation attempts where a relatively few cells were unable to cope with even small amounts of O_2 contamination. Table 5 shows the results of an experiment undertaken to test the lethal-oxygen theory. The cells were subjected to various oxygen exposures and in all cases gas produced. The most severe treatment was given flask no. 11, yet gas was produced, although it was slower to occur than in the other flasks.

There is no doubt that the cells were subjected to oxygen under the conditions of the experiment for considerable periods. An examination of Table 5 shows that the initial Eh

at the start of incubation was somewhat lower than would be expected of air saturated media. In those flasks that did not contain a reducing agent the lower potential was caused by the inoculum. Had the potential been allowed to remain at a high level growth would not have occurred.

It can be justifiably reasoned that cells which had to develop from spores would take a greater length of time to produce gas than already active vegetative cells. In this respect these results do not agree with Barker's hypothesis (1940) since gas was produced in most flasks at a time comparable with the positive control (flask 1).

Thus it would appear that the methane species studied here does not differ greatly from other anaerobes in respect to oxygen sensitivity. The typical anaerobe is unable to reduce ordinary aerobic media, but if the medium is subjected to a partial reduction the anaerobe can continue reduction to its optimum. Evidence presented in this thesis points to the fact that the oxidation-reduction potential is the important factor regardless whether the potential is established in the presence or absence of oxygen.

The influence of ethyl violet, sodium azide and penicillin on the Methanobacterium species were studied (Figures 5, 6 and 7). Ethyl violet, an aniline dye, is generally inhibitory for the gram positive group of bacteria and has less toxicity for gram negatives, according to Litsky, Mallmann and Fifield (1951), than such dyes as crystal violet and

brilliant green. Ethyl violet had very little inhibitory action against the Methanobacterium species studied, in fact, Figure 5 shows a slight stimulatory effect within the range of 1:600,000 to 1:800,000 ethyl violet concentration. At an approximate dilution of 1:1,000,000, ethyl violet will inhibit the majority of gram positive organisms (unpublished data. Litsky, Mallmann and Fifield) while at a dilution of 1:200,000 E. coli is affected but slightly. Ethyl violet's action with the methane culture would seem analogous to its action against the gram negative group.

Sodium azide, a slow oxidizing agent, is specific in its action against the gram negative bacteria and against those bacteria whose systems contain a cytochrome group. Sodium azide, as shown in Figure 6, exhibited a marked influence on the methane culture. A concentration of 0.04 percent completely prevented gas production while 0.005 percent caused some stasis. The results obtained with these two agents would tend to indicate that the Methanobacterium had a similar makeup to the gram negative organisms and if on this strength a prediction of the effect of penicillin were made it might be concluded that the antibiotic would have little or no effect. This however, was not the case. Figure 7 shows a decided inhibitory action with concentrations greater than 0.05 micrograms per ml. Penicillin with a few exceptions directs its antibacterial powers against gram positive

organisms, regardless whether these organisms are aerobic or anaerobic (Herrell, 1945).

It is interesting to note that in its response to ethyl violet and sodium azide the Methanobacterium culture appeared to be grouped with the gram negative bacteria but its susceptibility to penicillin would tend to classify it with the gram positive group. The antibiotic's selectivity for the gram positive spectrum is not, however, a hard and fast rule for some important gram negative microorganisms are not resistant.

The casual reader may wonder at the exclusion of streptomycin from these tests. Streptomycin is known to be specific for a number of gram negative species but its action is completely nullified in the presence of sulfhydryl groups (Waksman, 1949). Cysteine was used as a reducing agent in the medium; therefore, any results obtained might be erroneous.

Some thought was given to the method to be used for the determination of the effects of the above agents. The measurement of gas production was chosen rather than a determination of the acetic acid produced for the possibility existed that although the production of acetic acid from the ethanol might have proceeded normally, those systems involved in the reduction of CO_2 to methane could have been blocked. Volatile acidity was measured, however, in the

penicillin test and the curves obtained were parallel to those obtained by gas measurements.

SUMMARY

1. A methane producing bacterium identical in most respects to Methanobacterium omelianskii was isolated by enrichment culture and partially purified.
2. A modification of a medium suggested by Barker (1940) gave excellent results. Chlorides were substituted for the sulfates and cysteine replaced sodium sulfide as a reducing agent. The uninoculated medium maintained a low, stable, oxidation-reduction potential in the presence of oxygen.
3. An apparatus was constructed for the simultaneous measurement of the oxidation-reduction potentials in several cultures or solutions or in the upper and lower portions of a medium.
4. The presence of inert solids in the methane fermentation were extremely valuable. Asbestos proved to be the most satisfactory substance of all of the solids studied in respect to sub-culturing, and staining as well as gas production. The presence of asbestos prevented the diffusion of oxygen into the lower limits of the medium. Evidence points to the conclusion that the role of the asbestos is to supply an environment where the oxidation-reduction potential may be lowered and maintained even in

the presence of oxygen and where enzyme systems and hence energy may be concentrated for the advantageous use of the slow metabolizing methane bacteria.

5. Oxygen is not lethal to the Methanobacterium omelianskii type organism. In this respect it does not differ greatly from other anaerobes. in its sensitivity to oxygen.
6. Ethyl violet had little influence on the growth of the organism but sodium azide and penicillin exhibited a marked effect.

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