SEPARATION AND MICROBIOLOGICAL ASSAY OF AN UNKNOWN GROWTH FACTOR PRESENT IN ANAEROBIC SEWAGE SLUDGE

A Thesis

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INTRODUCTION

The increased urbanization and industrialization of today have caused a much greater need for control of the wastes produced. Anaerobic treatment of sewage waste, one method of control, is widely used by municipal plants in the United States and elsewhere. Despite the extensive employment of the system, little is known about the fundamental nature of the process. Because of this lack of understanding, few control measures are available for correcting or explaining malfunctions of the process.

It is generally assumed that anaerobic digestion of organic wastes proceeds in two stages (Barker, 1956; McCarty, 1964; Smith, 1965; Toerien, 1967). In the first stage, complex compounds are broken down to simple organic materials. Fats, proteins, and carbohydrates are converted, for the most part, to short chain fatty acids. A group of bacteria termed "acid-formers" carry out the primary conversions. These saprophytic bacteria are abundant in sewage, and normally reproduce rapidly during the initial stage of the digestion process with the production of large amounts of volatile acids (McKinney, Langley, and Tomlinson, 1958; Sawyer, Howard, and Pershe, 1954). No waste stabilization occurs during the first stage of the process, but substrates are produced that can be utilized by the bacteria in the second stage.

During the second stage, the organic acids are ultimately converted to methane and carbon dioxide, resulting in waste stabilization. The methane-producing bacteria utilize the volatile acids produced by the acid-formers in a symbiotic relationship. They ferment only very select substrates, and do not utilize carbohydrates and amino acids as do most saprophytes (McCarty, 1964; Smith and Hungate, 1958; Barker, 1956).

Much of the investigation on anaerobic sludge digestion has been concerned with the chemical and biochemical aspects of the problem. Many of the investigations to date have concerned end-product analysis. Some control over the digestion process has been gained by monitoring pH, level of volatile acids, temperature, and trace metals. However, little is known about the organisms which cause initial digestion and secondary stabilization. Very little knowledge exists concerning intermediate compounds that lead to the formation of methane in the fermentation. Due to the importance of methane bacteria in anaerobic digestion, and considering the sporadic digester upsets which do occur, studies in this area would be of great importance. Also, information gained involving factors which could restore normal digestive processes or indicate an impending upset would be of considerable value. The following investigation is an evaluation of an unknown growth factor which could aid in the control of anaerobic digestion.

LITERATURE REVIEW

Although earlier observations of methane bacteria were made, Söhngen (1906) gave one of the first clear descriptions of the forms and character of methane bacteria. He observed the quantitative conversion of lower fatty acids to methane and carbon dioxide. He also observed that a mixture of hydrogen and carbon dioxide could be fermented to methane. Other investigators greatly extended the list of methane-producing bacteria and the list of organic substrates which they decompose. Omelianski (1904, 1916) studied the methane fermentation of cellulose and of ethyl alcohol. Mazé (1915) observed the fermentation of acetone, and Groenewege (1920) found methane to be produced from methyl, ethyl, and butyl alcohols. Bach and Sierp (1924) obtained a methane fermentation of proteinaceous materials. Coolhaas (1928) studied the thermophilic fermentation of various fatty acids (Barker, 1936a, 1936b).

In 1930, Van Niel, prompted by observations of Söhngen's (1906) studies, postulated the "carbon dioxide reduction theory". In his theory, he suggested that carbon dioxide was the ultimate oxidant and was reduced to methane. Also, any oxidizable compound might be regarded as a reductant in place of molecular hydrogen. This theory could be expressed in generalized equations for methane fermentations wherein carbon dioxide was merely an oxidant or terminal electron acceptor for

the oxidation of the variety of alcohols and fatty acids known to be metabolized by the methane-producing bacteria (Barker, 1936a and 1936b; Stadtman, 1967). "A" equals any oxidizable substrate used by methane bacteria.

$$4\ H_2A \longrightarrow 4A + 8H$$

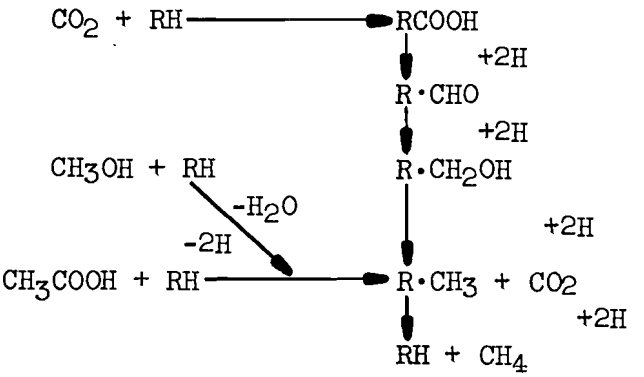
$$CO_2 + 8H \longrightarrow CH_4 + 2H_2O$$

$$\text{Sum:} \quad 4\ H_2A + CO_2 \longrightarrow 4A + CH_4 + 2H_2O$$

Exact physiological or biochemical studies were not possible in the earlier studies on methane bacteria. Until 1936, all attempts to isolate pure cultures or to grow colonies in solid media were unsuccessful (Barker, 1956). In 1936, Barker developed a method for growing highly enriched, but not pure strains, of methanogenic bacteria on solid media. In his methods, he used $Na_2S \cdot 9H_2O$ as a reducing agent and Na_2CO_3 to provide CO_2. Heukelekian and Hienemann (1939) developed a method for enumeration of methane bacteria capable of attacking various substrates in sewage sludge. In 1940, Barker isolated a highly purified strain of Methanobacillus omelianskii. Following this, Schnellen (1947) isolated Methanobacterium formicicum which uses formic acid as its substrate. In 1951, Stadtman and Barker isolated Methanococcus vannielii.

In 1950, Hungate developed methods for working with methane bacteria in solid media. These methods, as modified by Bryant and Robinson (1961) and by Smith (1965), have probably formed a basic part of all the studies involving methane bacteria today. These methods have been devised primarily to exclude the entrance of oxygen into the culture environment. The importance of a low redox potential was stressed by Mylroie and Hungate (1954) while studying Methanobacterium formicicum.

Experimental support of Van Niel's unified concept of methane biosynthesis (Barker, 1936a) was obtained by Barker (1956) in studies on Methanobacillus omelianskii. The carbon dioxide theory was shown to be generally applicable to biological methane formation for organic compounds other than acetate and methanol. The known methanogenic bacteria were studied in relation to the substrates they utilized. The following schematic representation of the possible pathways of carbon in methane formation was presented by Barker (1956).



Smith and Hungate (1958) isolated Methanobacterium ruminantium from rumen fluid. They found it to be a nonmotile, nonsporeforming, gram-positive, encapsulated rod with rounded ends which could utilize either hydrogen or formic acid as oxidizable substrates. In studies on factors affecting the growth of Methanobacterium ruminantium, they found that this bacterium was inhibited in a gas phase containing less than 0.004% oxygen. Methanobacterium ruminantium was shown to occur in large numbers in digesting sludge by Bryant (1961).

To date, only 11 methane microorganisms have been cultured and identified; only 6 of these have been isolated in pure culture (Barker, 1956; Paynter and Hungate, 1968). While there are a number of references to methane-forming bacteria in sewage sludge, only 3 species have been isolated in pure culture and identified, namely Methanobacterium formicicum (Mylroie and Hungate, 1954), Methanobacterium omelianskii (Barker, 1940), and Methanobacterium ruminantium (Smith and Hungate, 1958). Methanobacterium omelianskii has since been shown to have a symbiotic relationship with a methanogenic and a non-methanogenic species (Bryant et al., 1967).

Studies on Methanobacterium ruminantium by Bryant (1966) showed that this bacterium could not be grown in media containing a number of crude ingredients commonly used to grow nutritionally exacting bacteria. Good growth could be obtained, however, in media containing rumen fluid. Bryant showed that the rumen strain required two groups of factors

present in rumen fluid but not present in a complex medium containing yeast extract and trypticase. The factors included those in the volatile acid fraction of rumen fluid and a factor, or factors, not extractable from rumen fluid with ether. The volatile acid factors were found to be replaced by acetate and 2-methylbutyrate, both of which are essential for growth. Further studies on the factor, not extractable with ether, showed it to be of relatively low molecular weight (Sephadex G25, dialyzable) and stable to autoclaving at acid or at neutral pH. However, roughly 50% of the activity was lost during autoclaving in alkali.

Bryant (1966) found that fermentation of rumen fluid with the addition of yeast extract tripled the concentration of the factor or factors. He also surveyed other possible sources of the unknown growth factor. Sewage sludge was found to contain 0.7 units of the unknown growth factor per ml.

The studies conducted by Bryant (1966) on an unknown growth factor required by the rumen strain of M. ruminantium, and the occurrence of this factor in sewage sludge, have prompted the present investigation.

Objectives

The objectives of this study were:

1. To develop an assay method using procedures developed at Midwest Research Institute. To develop proficiency for maintaining cultures of *Methanobacterium ruminantium*.

2. To determine the relative amount of the unknown growth factor in the sewage sludge in this area.

3. To evaluate different methods of treating sewage sludge with a view toward increasing the concentration of the unknown growth factor.

4. To evaluate extraction methods and, if possible, increase the efficiency of extracting the factor from sludge.

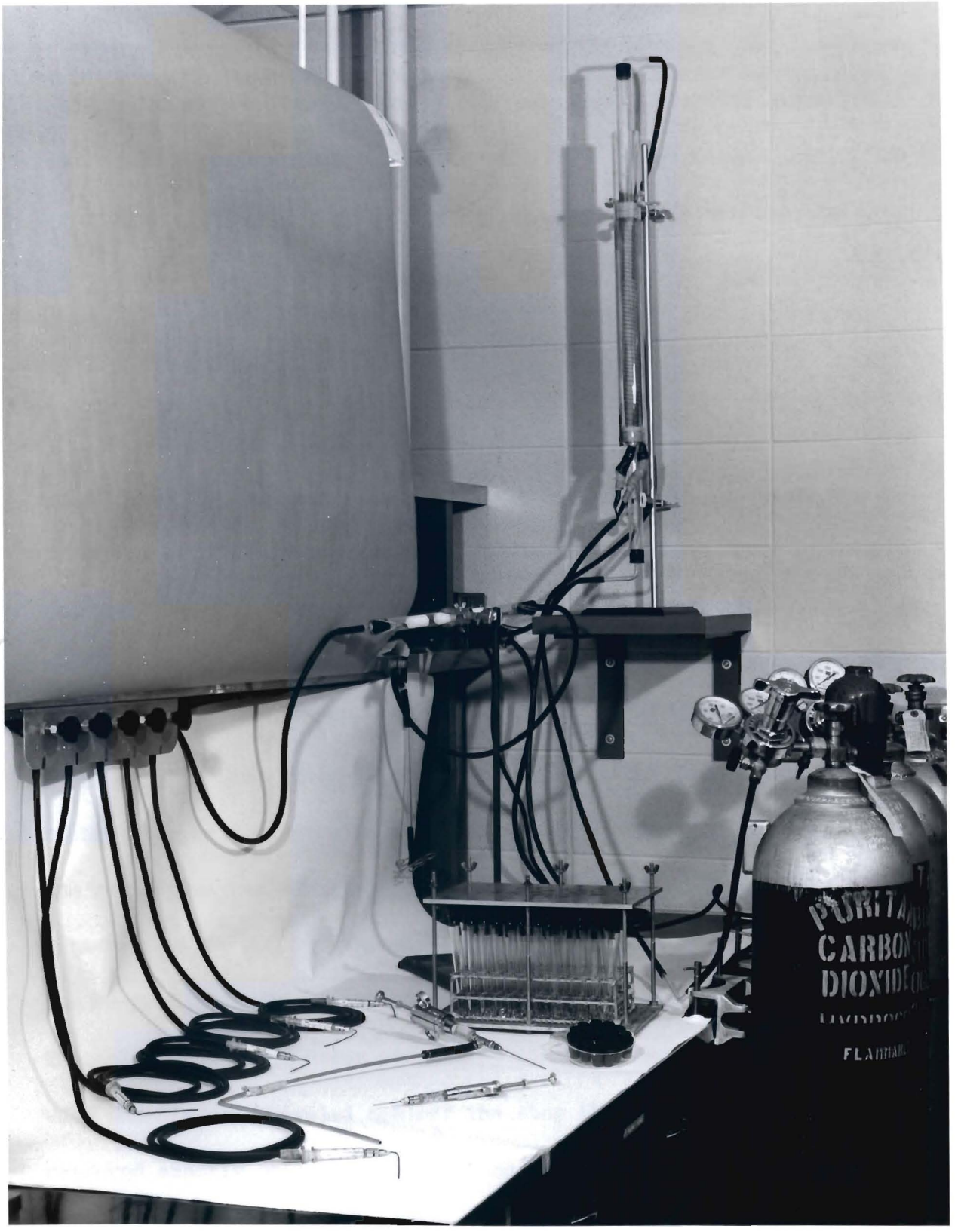
5. To compare digesters in the area that are in different stages of performance.

MATERIALS AND METHODS

Equipment for Anaerobic Procedures

The equipment pictured in Figure 1 consists of a Vycor tube (70 cm x 2.5 cm), packed with reduced cupric oxide wire (copper). The Vycor tube was wrapped with 25 gauge nicrome wire which acted as a heater. Temperature was controlled with a Variac. The copper bed was held at approximately 350 C, which efficiently removed residual oxygen. The outlet from the copper column was connected by rubber tubing to a water trap, to a filter containing approximately 5 inches of sterile cotton, and then to a manifold. The manifold was connected by rubber tubing to 4 inch, 20-gauge syringe needles. The tubes preceding the needles were packed with 2 inches of sterile cotton. The syringe pipetting assembly consisted of a 5 ml Cornwall continuous pipette with a stainless steel tube to serve as a connection between the main reservoir and the continuous pipetting assembly. An air-tight connection of heavy rubber held the stainless steel tube to the continuous pipette. All other methods of passing the media from the main reservoir to the continuous pipette resulted in oxidation of the media. The Cornwall automatic pipetting syringe consisted of a 2 ml Luer-lock syringe and was fitted with a 1.5 inch number 19 needle having a Huber point. Both the continuous pipetting syringe and the automatic pipetting syringe were greased with Fisher brand Cello-Seal (nontoxic) to prevent leakage

Figure 1. Equipment used in anaerobic techniques

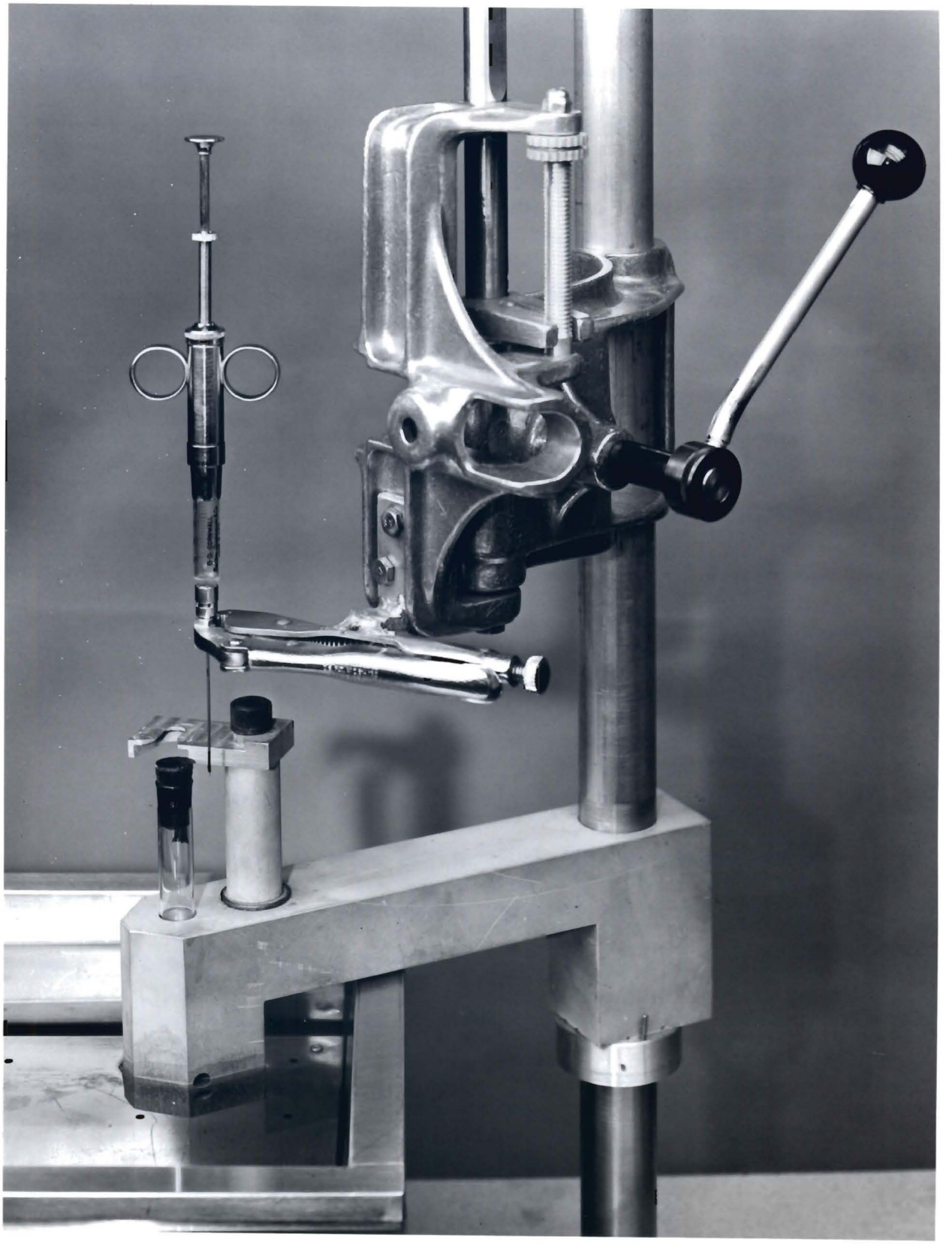

CARBON
DIOXIDE

of oxygen between the barrel and plunger of the syringe. Bellco 16 mm x 150 mm culture tubes for size 0 stoppers were used. The tubes had reinforced lips to reduce the danger of finger lacerations. Butyl rubber stoppers, size 0, were used. The stoppers have a tendency to conform to the shape of the tube and thus form a very effective seal.

Inoculation Press and Roll-Tube Jig

Techniques for inoculation were basically those described by Smith (1965) with the following modifications: The inoculation press and roll-tube jig, built for use in the modified roll-tube technique, are shown in Figure 2. The press consisted of a Dayton Drill Stand (No. 2Z041 for 1/2 inch drill), modified by rigid attachment of a pair of 6 inch vise grips to the spindle. The vise grips were used to hold the hub of a 19 gauge x 1-1/2 inch Huber point needle while it was forced through the rubber stopper during inoculation or transfer. The jig designed to hold the roll tubes was machined from a solid block of aluminum. The jig was attached to the post of the drill press and rested on an adjustable collar so that it could be swiveled out of the way while changing tubes. The collar had a stainless steel stop pin which was adjusted so that when the jig was swiveled against the stop the tube held in the jig was centered exactly under the inoculating needle. A machined cover was attached to the jig on a swivel so that it could be swung over the stopper before the needle was withdrawn (Figure 2). The cover securely

Figure 2. Overall view of inoculating press and roll-tube jig



held the stopper to prevent accidental removal from the tube during withdrawal of the needle (Figure 3). Sufficient clearance was provided so that the cover would not touch the needle or the sterile area of the stopper at any time (Figure 4). The need to sterilize any portion of the press or jig was thus prevented. The jig was designed to be used in a water bath; however, this was not necessary in the procedures used in this study.

All inoculation or transfers were made using a syringe and needle. The syringe holders were of the Cornwall type and contained a 2 ml Luer-lock syringe which had been greased with Fisher brand Cello-Seal (non-toxic) to prevent leakage of oxygen between the barrel and plunger of the syringe. Syringe holders were used so that the volume injected was precisely controlled. The lock nut was removed from all syringe holders so that the adjustment screw could be freely turned. Materials were thus injected by turning the screw rather than by pushing the plunger of the syringe holder. The holders were indexed on the knurled nut and the top of the holder barrel so that as little as 1/2 or one turn could be injected if desired (0.04 ml/turn).

Preparation of Media

Media and dilution solutions were prepared by modification of those described by Hungate (1950) and Smith (1965). All ingredients except cysteine-HCl H_2O, $NaHCO_3$, and $Na_2S \cdot 9H_2O$ were dissolved in a

Figure 3. Overall view of roll-tube jig with needle being depressed through the stopper

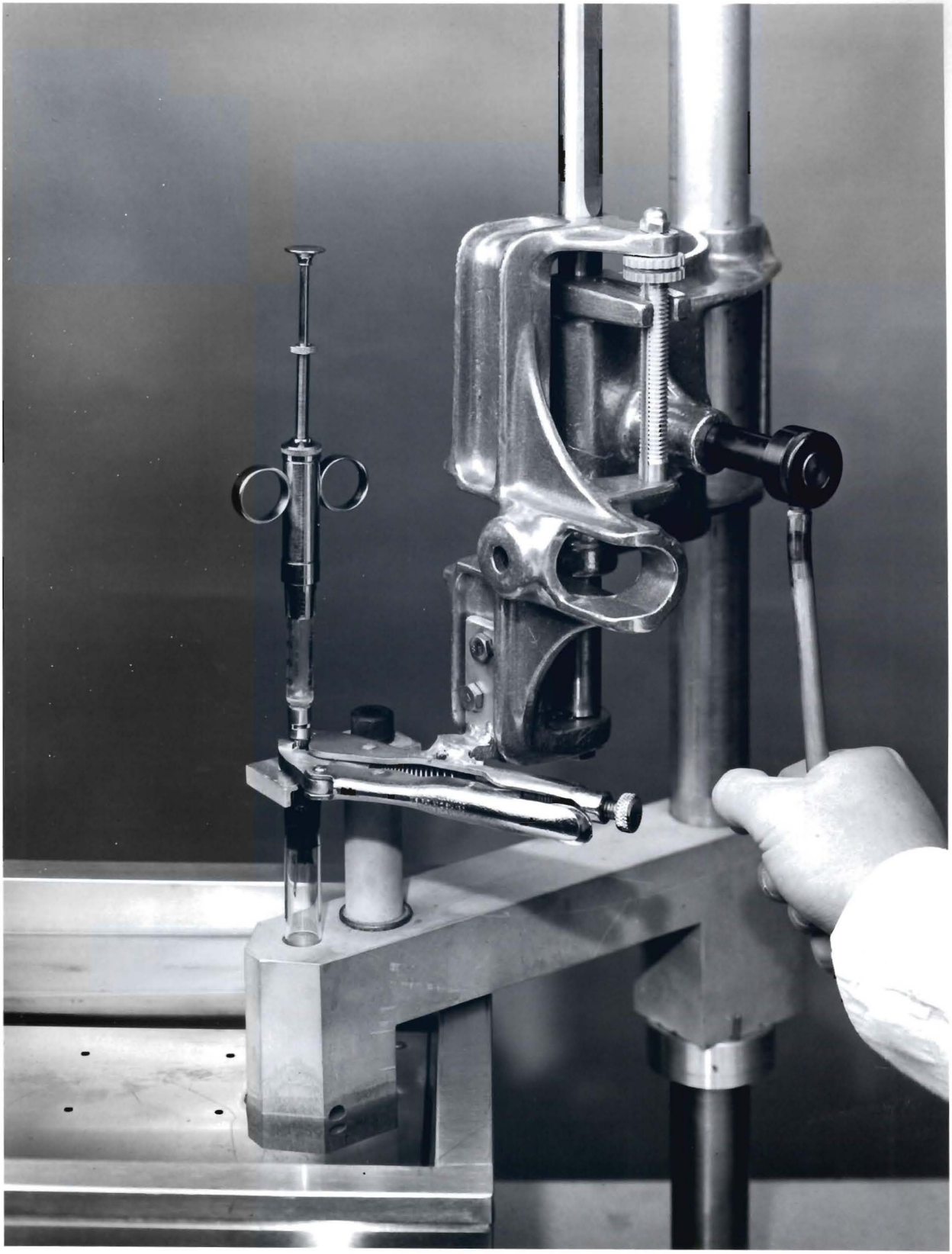
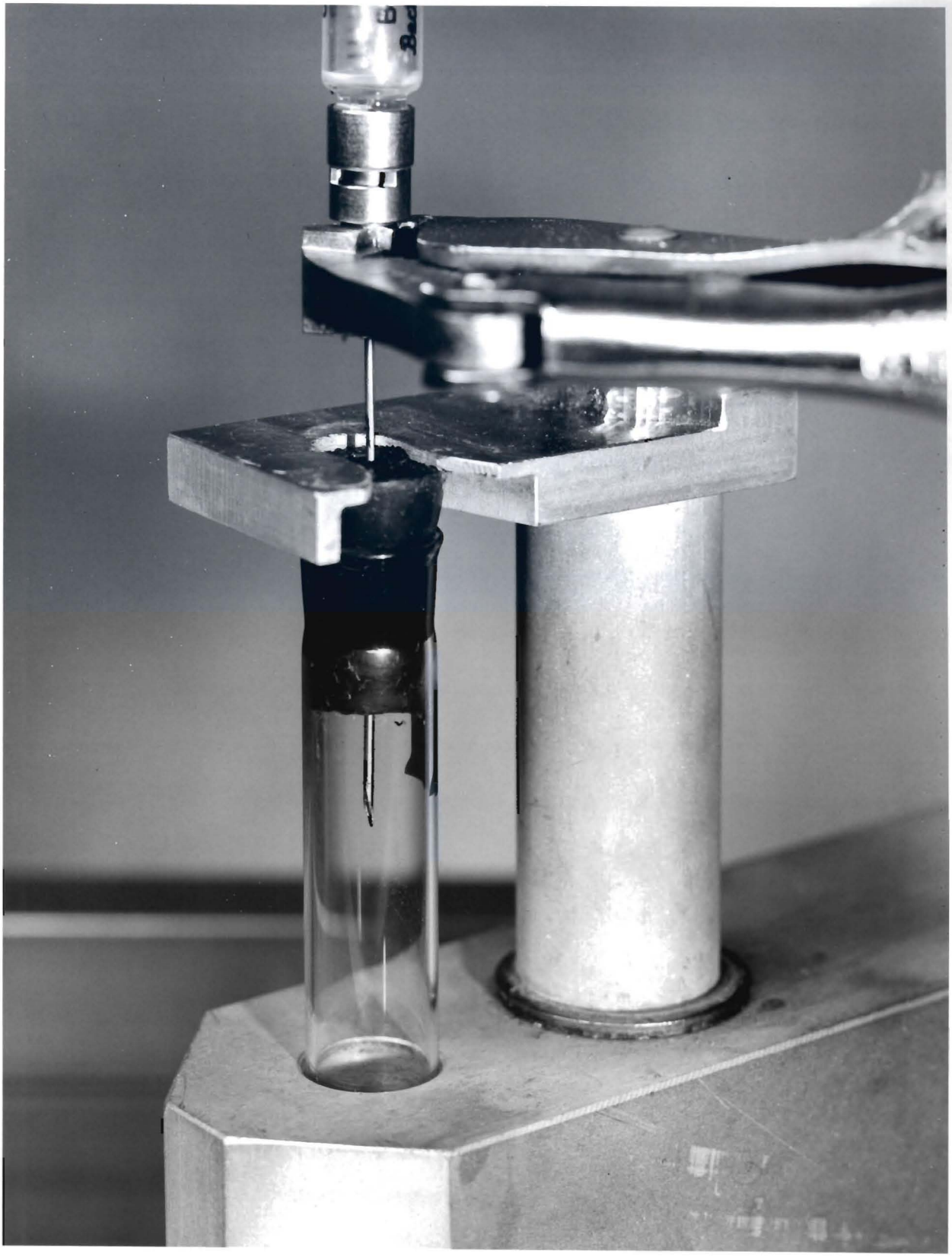


Figure 4. Close-up of roll-tube jig with needle in position



round-bottom flask. $NaHCO_3$ was substituted for Na_2CO_3 because equilibration with CO_2 is much more rapid. Cysteine was added and the mixture was heated while the flask was being flushed with gas until reduction was complete (resazurin; blue $\xrightarrow{\text{red.}}$ red $\underset{\text{ox.}}{\longrightarrow}$ colorless). The flask was cooled (only to 48 C if agar was included), and the proper amount of $NaHCO_3$ was added to buffer the medium at pH 6.8. The amount of $NaHCO_3$ required was 0.9% by weight for a 100% CO_2 atmosphere, and 0.5% for 50-50 CO_2/H_2.

After reduction and gas equilibration were complete, the medium was dispensed directly into tubes by means of 5 ml Cornwall continuous pipette which was washed with several volumes of boiling water to flush oxygen from the system. A few syringefuls of medium or anaerobic dilution solution (ADS) were wasted in washing the syringe to insure proper flushing and oxygen removal. While the main reservoir was being continuously flushed with oxygen-free gas, the medium was tubed in multiples of five, each of which was being simultaneously flushed with gas during filling. A manifold was built for this purpose that employed five hoses and gassing needles each for use in gassing several tubes or flasks simultaneously. After each series of tubes was gassed for a few seconds, a moistened butyl rubber stopper was inserted as the gassing needle was withdrawn. The procedure was continued in multiples of five until a sufficient number of tubes had been prepared. The tubes containing

the medium were then clamped in a jig to provide a final seating and to hold them securely during sterilization and exhausting of the autoclave. Since butyl rubber has an extremely low permeability for oxygen, the medium could be stored in a reduced condition for extended periods of time without oxidation. Sterilization of all media and solutions was accomplished by autoclaving at 15 lb/15 min.

Stock Culture

A stock culture of Methanobacterium ruminantium, strain M-1, was obtained from Dr. Paul Bryant, University of Illinois, Urbana, Illinois. Stock cultures of M. ruminantium were maintained on slants in medium number 1 (Appendix II), containing a H_2/CO_2 (50-50) gas atmosphere. All tubes were continuously flushed with gas when open. Cultures were transferred with a platinum-iridium inoculating needle by stab into the base of the slant. Cultures were monitored for contamination, by Gram stain, and by inoculating carbohydrate agar slants (medium number 2) which were maintained under a CO_2 (100%) gas atmosphere. Medium number 2 is listed in Appendix II. Growth on the carbohydrate agar slants indicated contamination. Methane production was monitored periodically by gas chromatography as a further index for growth of the culture.

Medium for Assay Procedure

The same basal medium as described in Appendix II, medium number 3, was used for an assay medium and for a medium in which cells were grown for inoculum. Ten ml of the 5x basal medium and the desired amount of material to be assayed were placed in a 100 ml round-bottom flask. The volume was then brought up to 50 ml with distilled H_2O. Cysteine-HCl (0.025%) was added and the solution was reduced by boiling. Sodium bicarbonate (0.5%) was added and the medium was tubed in 5 ml amounts anaerobically, as previously described, and autoclaved. Sodium sulfide (0.04 ml/5 ml) reducing solution was added 2-24 hr prior to inoculation. Twenty-percent rumen fluid, or different percentages of AELS (aqueous extract of lyophilized sludge), replaced the assay factor in the broth cultures used to grow and maintain M. ruminantium for inoculum.

Broth cultures containing rumen fluid and broth cultures containing 10% AELS from Indian Creek were examined. Five ml of broth containing 10% rumen fluid was inoculated with cells from a stock slant. When an active broth culture was obtained, 5 ml broth containing 10% rumen fluid and 5 ml broth containing 10% AELS were inoculated (0.2 ml) in triplicate and incubated at 37·C on a shaker. Cultures were compared through three transfers.

Assay Procedure

An actively growing culture was defined as a broth culture with a reading of from 0.3 to 0.4 optical density. Optical density (O.D.) was measured on a Bausch and Lombe Spectronic 20 at 600 nm. Cells from an active culture were centrifuged in an IEC refrigerated centrifuge (Model No. 2) at 4,000 rpm or 2,000 G for 20 min. The supernatant was removed with a 10-ml pipette, which had been previously flushed with H_2/CO_2 (50-50), and discarded. All tubes were continuously flushed with H_2/CO_2 (50-50) when open. The pellet was resuspended in ADS (anaerobic dilution solution) and centrifuged as before. The supernatant was again removed and discarded. The pellet was resuspended in the volume of ADS needed for inoculation of each specific assay. The O.D. of the suspension was read before the washed inoculum was used.

Duplicate or triplicate tubes were inoculated for each level of each fraction to be assayed. The O.D.'s of all assay tubes were measured and the cultures were incubated at 39 C on a shaker. After growth had started (usually one to two days), the O.D.'s were determined and the assay tubes were flushed with H_2/CO_2 (50-50). This procedure was carried out twice daily after the growth in the tubes reached an O.D. of 0.1 at 600 nm. Maximum growth was reached in approximately 5 days.

A unit of activity of the factor was defined as the amount of factor in 50 ml of medium required to allow growth equal to an O.D. of

0.3. Net O.D. was determined by subtracting the amount of growth as O.D. in the control medium from the amount of growth as O.D. in the experimental medium.

A blank containing no factor was used as a control. In the latter assays, a medium containing 2% AELS was used as a standard.

Sampling Stations

Four different plants were selected as sampling stations on the basis of their current and past performance records. Five digesters at various performance levels were sampled to determine if digester condition would be reflected by differences in the amount of factor present.

Indian Creek: This digester had an excellent performance record. Methane production was good, pH had not fluctuated significantly in the past 2-3 years of constant operation, and volatile acids remained within the normal range. It was moderately fed with municipal sludge.

4800 Nall Avenue: This digester was fed only on weekends when the sludge incinerators at this plant were not in operation. The digester received only municipal wastes and laboratory records were not kept on performance. An appreciable amount of methane was produced, and no upsets had occurred in the past 2-3 years.

Kansas City Pollution Control, Kansas City, Kansas: This plant was built to receive only industrial wastes (packing plant and soap manufacturing wastes). In nearly 2 years of operation, normal digestion

had not been achieved. The system had been constantly plagued by excessive foaming, high levels of volatile acids, low pH and low methane production. The plant consisted of 6 digesters of which only number 3 and number 4 were sampled. The two digesters were sampled at different levels of performance.

Digester number 4 was sampled several months before circulation was stopped and after the circulation had been stopped. Samples were taken of the supernatant and of the sediment. A sample was taken from digester number 3 after an effort had been made to reseed the digester. The digester was being circulated at the time of sampling.

Olathe, Kansas: This digester was quite old and had a consistently poor performance record due primarily to overloading. Although pH was not low (volatile acids were not determined), digestion was incomplete and very little methane was produced.

Method of Obtaining and Transporting Samples

Sludge was drawn from the digesters and placed in sterile flasks or carboys. The flasks or carboys had stoppers with bunsen valves. The valves served to let excess gas escape, while keeping outside air from entering the container. The containers were flushed with N_2 (oxygen free) gas prior to departing from our laboratory. A reducing solution of Na_2S (5 ml/800 ml of sample) was placed in the container just prior to drawing the sample. The containers were stoppered and transported to

the laboratory. Thermos bottles were used to transport samples when ambient temperatures were unfavorable. The thermos bottles were also equipped with bunsen valves in their lids.

Extraction of Factor

Extraction of the factor was accomplished using modifications of the procedures employed by Dr. Bryant (1965). For experiments in which crude concentrates of the factor were assayed, the following procedure was carried out (outlined in Figure 5). Fresh gauze-filtered sewage sludge was autoclaved, cooled, and centrifuged or lyophilized and extracted with hot water. The supernatant or extract was treated, batchwise, with Dowex 50, hydrogen form, to lower the pH to about 2.5. The resin and acid precipitates were separated by vacuum filtration and the supernatant was passed through a column of Dowex 50 to remove any remaining positively charged ions. The eluate was extracted with ethylacetate to remove lipids, organic acids, and other relatively nonpolar materials. The aqueous phase, still quite acid, was adsorbed on carbon decolorizing neutral Norit (Fisher Scientific). The Norit was recovered by filtration through a pad of hyflosupercel and the cake was washed twice with hot distilled water. The factor was eluted from Norit with 0.1 M ethanolic NH_4OH and immediately concentrated in a vacuum evaporator to remove NH_3 and concentrate the factor to a small volume. This volume

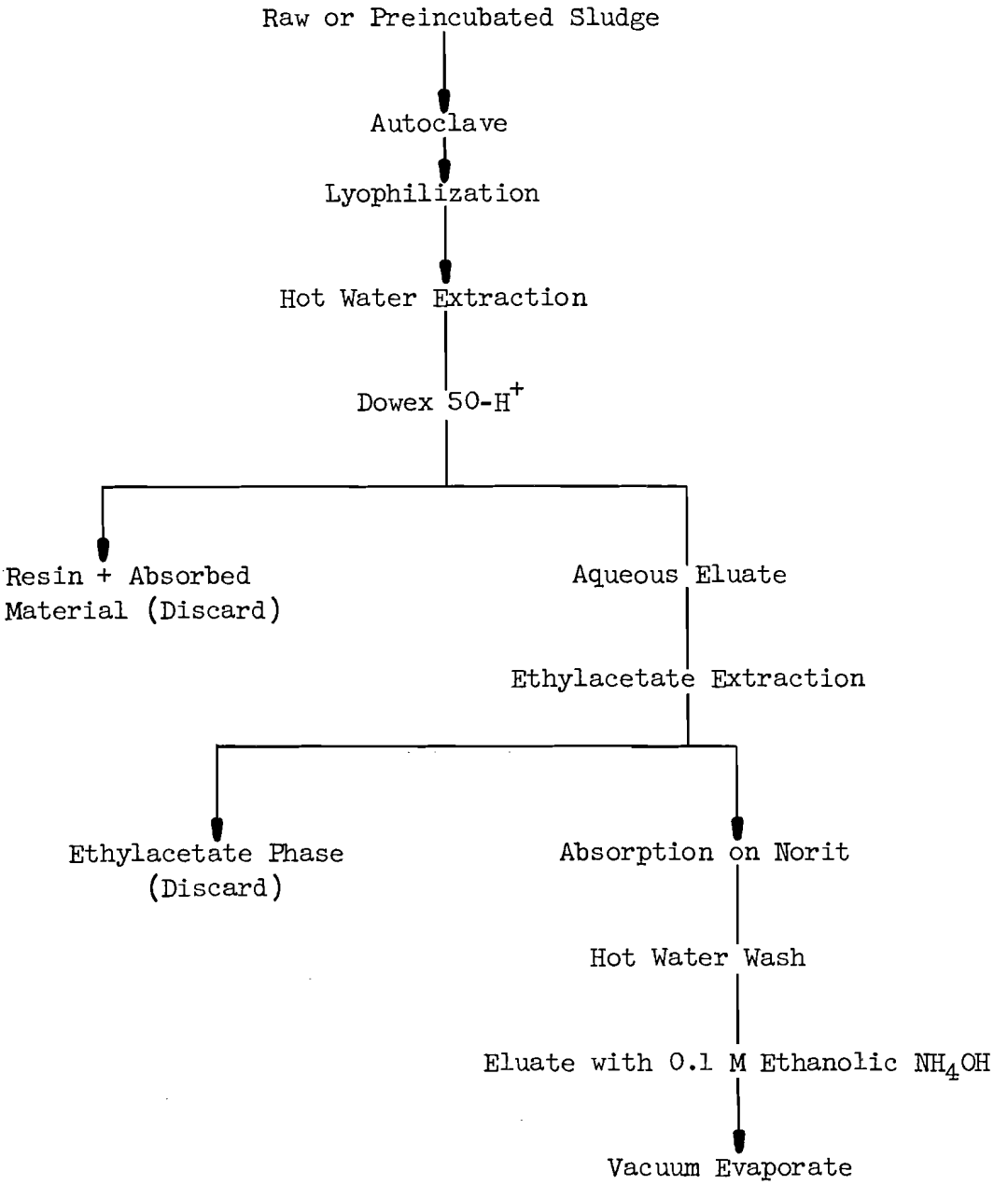
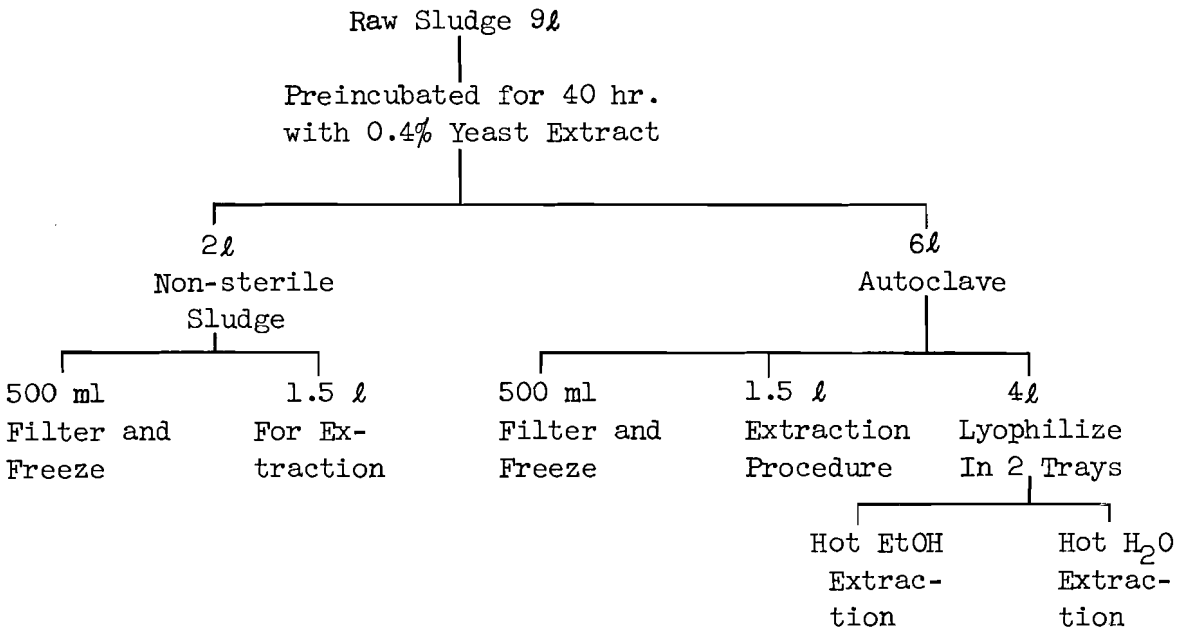


Figure 5. Scheme for purification and concentration of sewage sludge factor

brought back up to 50 ml with distilled water and frozen. Aliquots of this concentrate were diluted for assay.

Treatment of Sludge Samples

Two 9-liter samples of sewage sludge were collected from Indian Creek Sewage Plant and treated as outlined in Figure 6. One 9-liter sample was collected in a sterile carboy containing 0.4% or 36 g of yeast extract and incubated at 37 C for 40 hr. under a nitrogen gas atmosphere. After incubation, 500 ml of the unsterilized yeast extract-treated sludge was filtered through hyflosupercel and the filtrate was frozen and saved for assay. One and one-half liters were filtered through hyflosupercel and carried through the extraction procedure as outlined in Figure 5. The remaining 6 liters were autoclaved. Five-hundred ml were filtered through hyflosupercel and the filtrate was frozen and saved for assay. One and one-half liters were filtered through hyflosupercel and carried through the extraction procedure. Four liters were lyophilized in two trays (2 liters each). Lyophilized sludge (56.5 g) equivalent to 1.5 liters of yeast extract-treated sludge was extracted with a liter of hot 80% ethanol. The extract was then evaporated to near dryness and brought back up to 500 ml with distilled H_2O and carried through the extraction procedure. An equal amount of the lyophilized yeast extract-treated sludge was extracted with hot H_2O and carried through the extraction procedure.



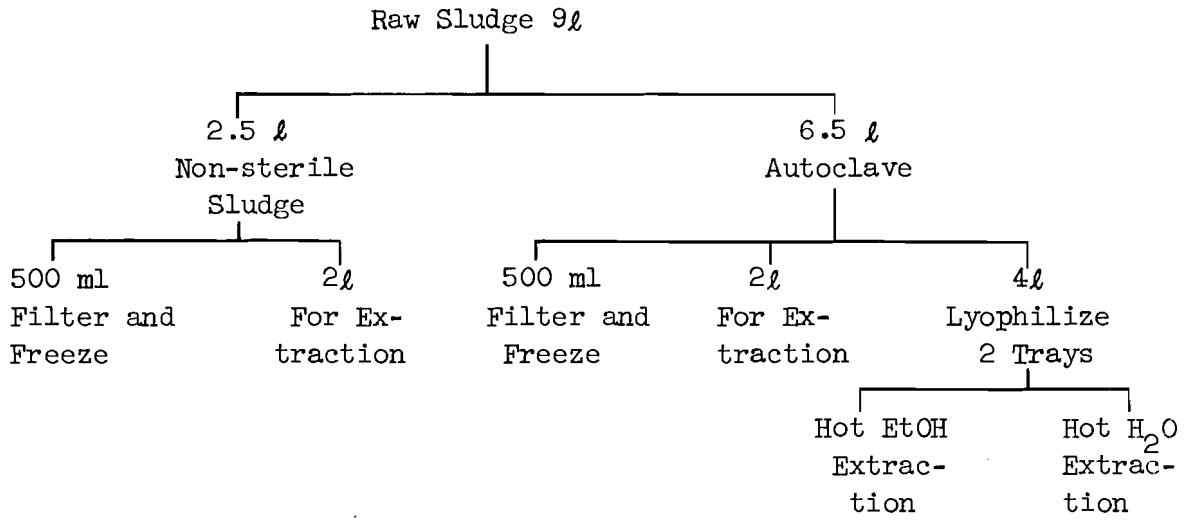


Figure 6. Scheme for treatment of sludge samples

The second 9-liter sample, which was collected from Indian Creek, was treated in the same manner as the yeast extract pre-incubated sludge except for quantities used at various steps in the procedure. This sample was not incubated. Five-hundred ml of the raw unsterilized sludge was filtered through hyflosupercel and the filtrate was frozen and saved for assay. Two liters of raw unsterilized sludge were filtered through hyflosupercel and carried through the extraction procedure as outlined in Figure 5. The remaining 6.5 liters was autoclaved. Five-hundred ml was then filtered through hyflosupercel and the filtrate was frozen and saved for assay. Two liters were filtered through hyflo-supercel and carried through the extraction procedure. Four liters were lyophilized in two trays (2 liters each). Lyophilized sludge (72.8 g) equivalent to 2 liters of sludge was extracted with a liter of hot 80% ethanol. The extract was then evaporated to near dryness and brought back up to 500 ml with distilled H_2O and carried through the extraction procedure. An equal amount (72.8 g) was extracted with hot H_2O and carried through the extraction procedure.

Samples Refluxed in 80% EtOH

Two samples of lyophilized sludge were refluxed in 80% EtOH for 2 hr. These procedures were carried out with the expectation that the pre-extraction would not only result in releasing more factor, but

also the preparation would contain less debris and be more readily extracted. A liter sample of lyophilized sludge was refluxed in 80% EtOH for 2 hr and then carried through the extraction procedure. A second 8-liter sample of lyophilized sludge was refluxed in 80% EtOH for 2 hr, filtered through hyflosupercel and brought up to a liter with distilled H_2O. Five-hundred ml were frozen and saved for assay. The other 500 ml was carried through a modified factor extraction procedure. The sample was treated with Dowex 50 H^+ to lower the pH to about 2.5 and the resin and acid precipitate were separated by vacuum filtration. The filtrate was passed through a column of Dowex 50 H^+ to remove any remaining positively charged ions. Ethyl acetate extraction at this point was omitted. The eluate was treated with Norit. The Norit was recovered by filtration through a pad of hyflosupercel and the cake was washed twice with hot water. The factor was eluted from Norit with 0.1 M ethanolic NH_4OH and immediately concentrated in a vacuum evaporator and brought back up to 500 ml with distilled H_2O. The factor was extracted with ethyl acetate at this time, and treated with Norit. The Norit was recovered by filtration over a pad of hyflosupercel, eluted with ethanolic NH_4OH, concentrated in a vacuum evaporator, brought up to 50 ml with H_2O and frozen.

Treatment of Materials Used in Extraction Procedure

The factor recovery from AELS appeared to be low. To determin at which point the factor was lost and whether the yield could be in creased by treatment of the activated charcoal, the following experi ment was performed (Figure 7). At the stage of the extraction proce in Figure 5, page 27, which involved adsorption into Norit, the extr was split into five samples and equal amounts were treated with Norit and Darco (activated carbon, Atlas Chemical Company) which had been treated as follows: untreated Norit; acid washed Norit; NH_4^+-EtOH, H washed Norit; acid washed Darco; and NH_4^+-EtOH, HCl washed Darco. Ac washing was accomplished by washing the Norit or Darco with a liter 1N HCl at room temperature and then with a liter of 1N hot HCl. NH_4^+ HCl washing was accomplished as follows: washed with distilled H_2O; washed with a liter of 0.1 M NH_4^+-EtOH; rinsed twice in distilled H_2O; washed with a liter of 1N HCl; washed with a liter of hot 1N HCl; and rinsed thoroughly with distilled H_2O. A medium-porosity, sintered- glass funnel was substituted for the pad of hyflosupercel to recover the charcoal-adsorbed factor. Samples from all phases of the extraction procedure were saved for assay.

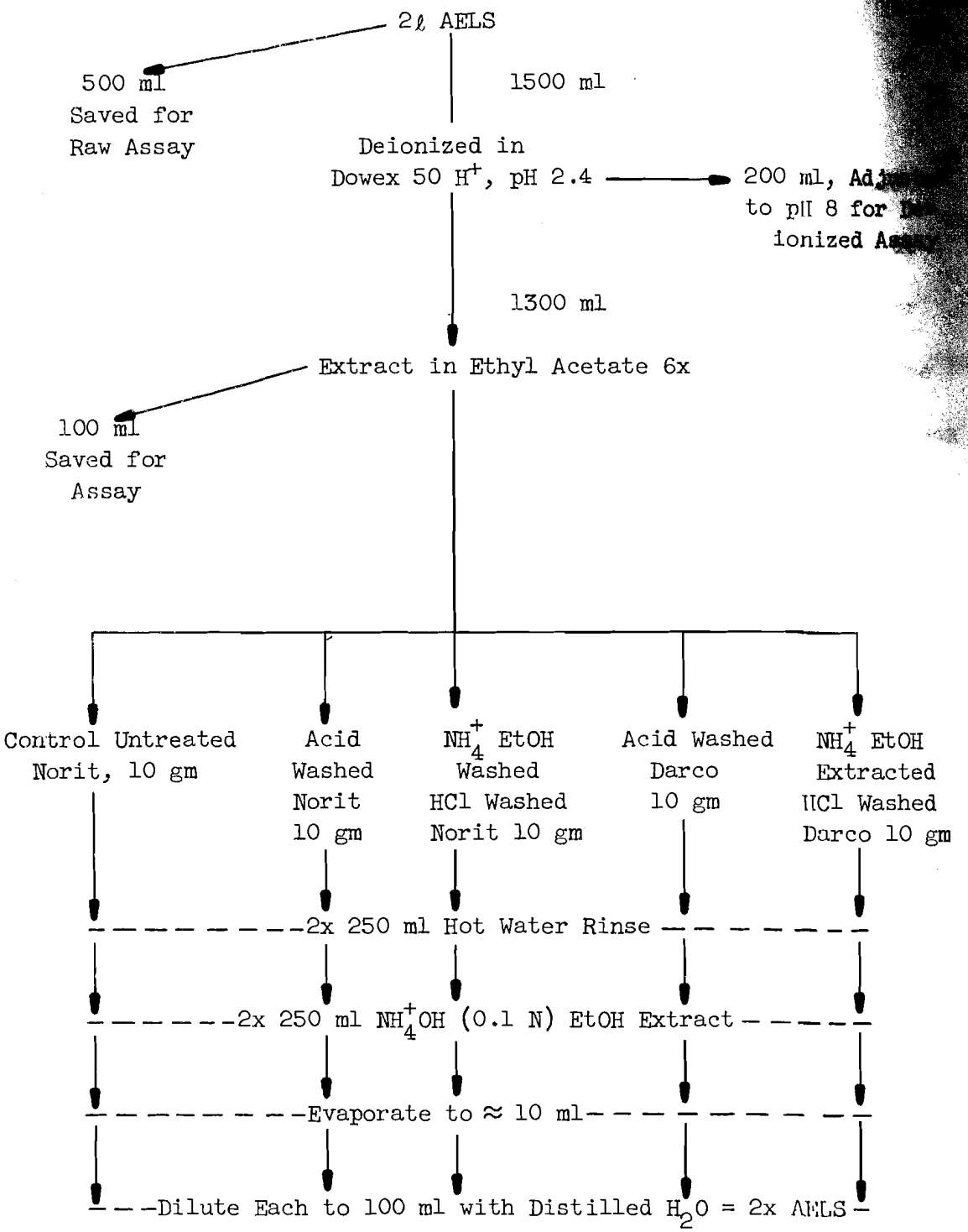


Figure 7. Scheme for altered extraction procedure

Effect of Sludge Treatment and Sterilization on Factor Recovery

Because of results of assays on the yeast extract preincubated sludge in comparison to that of Dr. Bryant on rumen fluid, it was decided to re-examine this portion of the sludge treatment. A 4-liter sample of sludge was obtained from the digester at Indian Creek. The sample was transported in a N_2-flushed, stoppered (stopper had a bunsen valve), 4-liter flask. Sodium sulfide reducing solution was added to the flask just prior to drawing the sample. Four 500-ml aliquots were placed in flasks containing 0%, 0.2%, 0.4%, 0.8% yeast extract flushed with N_2 and incubated at 37 C for 45 hr. After incubation, the samples were autoclaved and filtered through a pad of hyflosupercel and autoclaved. A fifth aliquot was filtered, sterilized through a Seitz filter, and refrigerated. The remaining portion of the sample was autoclaved and filtered through a pad of hyflosupercel and autoclaved again. Samples assayed consisted of the following: raw sterilized filtered sludge; sludge preincubated without yeast extract; sludge samples preincubated with 0.2%, 0.4%, and 0.8% yeast extract; filter-sterilized sludge autoclaved with the media; filter-sterilized sludge added to the media just prior to assay.

Comparison of Different Digesters

Factor levels were to be determined in the sludge supernatants prepared from the digester samples discussed on page 24. Samples of sewage sludge were obtained from four sampling stations. The samples were autoclaved and then filtered through cheese cloth. These filtrates were then filtered through hyflosupercel distributed into Pyrex dilution bottles and autoclaved. Sewage sludge in Digester Number 4 of Kansas City, Kansas was being circulated at the time of sampling but was in a very poor condition. Two sets of assays were run with each sample.

The last samples to be prepared for assay were taken from the Kansas City, Kansas plant several months after those of the preceding paragraph. All digesters had been shut down and the sewage sludge was left to settle in the digesters except in the case of Digester Number 3. An attempt was made by the city to reseed the digester with sludge from an active sewage plant, and the sludge was being circulated. An attempt to start the digester failed and the digester was shut down. Our sample was taken while the sludge was being circulated in the digester. Samples were obtained from the sediment and from the supernatant in Digester Number 4. The samples were weighted and the volume measured before autoclaving. The weights and volumes were as follows: 500 g or 500 ml of Digester Number 3, 500 g or 500 ml of the supernatant from Digester Number 4, and 600 g or 477 ml of the sediment from

Digester Number 4. The samples were autoclaved and lyophilized. The lyophilized material was reconstituted with hot distilled water to equal the original sample before being filtered and sterilized.

RESULTS

Stock Broth Culture

All efforts to maintain a stock broth culture in medium without rumen fluid but with AELS failed. Cultures could not be carried beyond three transfers.

Treatment of Sludge Samples

The effect of different methods of treating sludge samples was studied. The results of these different treatments are listed in Table I. Assays for the factor were repeated 2-3 times per treatment. Samples which were not sterilized prior to filtration revealed very little factor present. In some of the samples, what appeared to be a toxic reaction occurred. With the addition of more sample, the level of factor detected decreased. The only treatments which gave a significant reading were raw sludge which had been sterilized and filtered, raw sludge pre-incubated with 0.4% yeast extract, sterilized and filtered; and aqueous extract of lyophilized sludge. Incubation with yeast extract increased the amount of the factor about two-fold. Extraction procedures did not result in the recovery of a significant amount of factor.

TABLE I. Effect of sludge treatment on factor recovery.

	Units/ml*
Raw unsterilized sludge filtered	0.1**
Factor extracted from raw unsterilized filtered sludge	0.1**
Raw sludge sterilized and filtered	1.1
Factor extracted from raw sterilized and filtered sludge	0.1
Raw sludge pre-incubated with 0.4% yeast extract, unsterilized but filtered	0.2**
Factor extracted from raw unsterilized but filtered, pre-incubated with 0.4% yeast extract	0.1
Raw sludge pre-incubated with 0.4% yeast extract, sterilized and filtered	1.8
Factor extracted from raw sludge pre-incubated with 0.4% yeast extract, sterilized and filtered	0.2
Sludge pre-incubated with 0.4% yeast extract, sterilized, lyophilized sludge extracted with hot 80% EtOH	0.2
Sludge pre-incubated with 0.4% yeast extract, sterilized, lyophilized sludge extracted with hot H_2O	0.2
Factor extracted from lyophilized sterile sludge extracted with hot 80% EtOH	0.4
Factor extracted from lyophilized sterile sludge extracted with hot H_2O	0.2

(continued)

* All values are averages of two to three assays.

** Expressed what appeared to be a toxic reaction, as compared to growth in media containing no factor.

TABLE I. Effect of sludge treatment on factor recovery (continued).

	Units/ml*
Factor extracted from lyophilized sludge refluxed in 80% EtOH	0.3
Factor extracted from refluxed sterile lyophilized sludge with modified extraction procedure	0.1
Sterile lyophilized sludge refluxed in 80% EtOH	0.0**
Aqueous extract of lyophilized sludge	0.8

* All values are averages of two to three assays.

** Expressed what appeared to be a toxic reaction, as compared to growth in media containing no factor.

Refluxed Samples in 80% EtOH

Results from samples which had been refluxed in 80% EtOH are also listed in Table I. These procedures were carried out with the expectation that the pre-extraction would not only result in releasing more factor, but also the preparation would contain less debris and be more readily extracted. The 80% EtOH did not increase factor yield significantly and, in addition, contained much more ethylacetate extractable material which interfered with the extraction procedure.

Treatment of Materials Used in Extraction Procedure

The results shown in Table II indicate the losses at each step in the extraction procedure. Percent recovery was calculated with AELS representing 100% recovery.

Treatment of either Norit or Darco had little effect on total recovery; however, significantly more factor was recovered with Darco than with Norit. The final recoveries were higher than those shown in Table I, even with Norit.

Effect of Sludge Treatment and Sterilization on Factor Recovery

Results from the first series of sample treatments prompted another set of sludge treatment studies. Results from pre-incubation in different percentages of yeast extract and the results of different forms

TABLE II. Factor recovery at various steps in extraction procedure.

Treatment	Units/ml	Percent Recovery
Aqueous extract of lyophilized sludge (AELS)	1.03	--
Deionized AELS	0.86	83
Aqueous layer following ethylacetate extraction	0.78	76
Untreated Norit	0.40	39
Acid washed Norit	0.42	41
NH_4^+-EtOH, HCl washed Norit	0.43	42
Acid washed Darco	0.54	52
NH_4^+-EtOH, HCl washed Darco	0.54	52

of sterilization are shown in Table III. Pre-incubation with 0.4% yeast extract resulted in approximately a two-fold increase in the factor. Addition of more than 0.4% yeast extract did not increase the amount of factor. The type of sterilization used had a pronounced effect on the amount of factor present. Filter sterilization resulted either in the loss of the factor or the inability of the assay procedure to detect it. Sewage sludge which was autoclaved before filtration showed a significant amount of factor present.

Comparison of Factor Levels in Sludge Supernatants from Four Sewage Plants

Results of the assays performed on the sludge supernatant from four different plants are shown in Table IV. The sludge supernatants from "normal" digesters (Indian Creek and 4800 Nall) are much higher than those from unbalanced or upset digesters (Kansas City Pollution Control and Olathe, Kansas).

Comparison of Factor Levels in Sludge from Upset Digesters in Kansas City, Kansas

Table Number V shows the results of samples of two digesters in different stages of performance. Samples from digester number 3 and digester number 4 sediment contained no factor which could be detected. Digester number 4 supernatant showed a greater amount than expected.

TABLE III. Effect of sludge treatment and method of sterilization on factor recovery.

	Units/ml
Aqueous extract of lyophilized sludge	0.7
Raw sludge sterilized and filtered	1.5
Raw sludge pre-incubated, sterilized, and filtered	1.3
Raw sludge pre-incubated with 0.2% yeast extract, sterilized and filtered	1.8
Raw sludge pre-incubated with 0.4% yeast extract, sterilized and filtered	2.4
Raw sludge pre-incubated with 0.8% yeast extract, sterilized and filtered	2.3
Raw sludge filter sterilized and autoclaved with media	0
Raw sludge filter sterilized, no heat	0

TABLE IV. Comparison of factor levels in sludge supernatants from four sewage plants.

Sewage Plant	Supernatant Level (units/ml)
Indian Creek	0.81
4800 Nall	0.68
Kansas City Pollution Control	0.34
Olathe, Kansas	0.17

TABLE V. Comparison of factor levels in sludge from upset digesters in Kansas City, Kansas.

	Units/ml
Sludge from digester number 3	0.0
Sludge from supernatant in digester number 4	0.5
Sludge from sediment in digester number 4	0.0

DISCUSSION

Although assays of the AELS showed the presence of the unknown growth factor described by Bryant (1966), all efforts to maintain a stock broth culture in medium containing AELS failed. A dilution effect was observed. Three transfers were necessary before growth stopped. Although there is no doubt that the factor or factors described by Bryant (1966) are present in digesting sludge, these results suggest the presence of an additional factor in rumen fluid, not present in sewage sludge, which is necessary for the rumen strain of M. ruminantium.

The results shown in Table I indicate that the factor levels found in sludge are comparable to those found in rumen fluid (0.8-1.0 unit/ml) by Bryant in 1966. The factor concentration increased two-fold when the sludge was pre-incubated with yeast extract at 37 C for 40 hr. under nitrogen. Approximately the same two-fold increase was seen when treatment of sludge samples with yeast extract was repeated. This increase is much less than the increase found in rumen fluid (up to eight-fold).

The 0.4 unit/ml recovery from lyophilized sludge, which had been refluxed with 80% EtOH for 2 hr. prior to extraction, was rather disappointing. It had been anticipated that pre-extraction would not only result in releasing more factor, but also the preparation would contain

less debris and be more readily extracted. The 80% EtOH did not increase factor yield significantly and, in addition, contained much more ethylacetate extractable material which interfered with the extraction procedure.

In the first trials for factor recovery from AELS, results appeared to be low (0.2 unit/ml). To determine at which point the factor was lost, and whether the yield could be increased by treatment of the activated charcoal, extraction procedures were studied. Treatment of either Norit or Darco had little effect on total recovery; however, significantly more factor was recovered with Darco than with Norit. The reason for the difference between Norit and Darco may be explained by the fact that Darco has much smaller particles of charcoal. Therefore, more surface area exists for adsorption of the factor. The final recoveries shown in Table III are higher than those shown in Table I, even with Norit. This was probably due to substitution of a medium-porosity, sintered-glass funnel for the pad of hyflosupercel formerly used to recover the charcoal adsorbed factor. It is quite possible that some factor was adsorbed to the hyflosupercel pad and lost when it was discarded.

Because of the results observed during the first studies on the effect of sludge treatment, the effect of different methods of sterilization and the effect of incubation with different percentages of yeast extract were investigated. A marked difference could be seen

between sludge which had been autoclaved and that which had been filter-sterilized. Autoclaving seemed to release the factor in some manner. The factor may be removed by filter sterilization or a toxic material may mask the presence of the factor during assay procedures. A third possibility exists; the factor is present but it is in a form which cannot be used by the bacteria. Pre-incubation with different percentages of yeast extract revealed that approximately 0.4% was optimum. Addition of more yeast extract or less did not improve the concentration of the factor.

Factor levels were determined in the sludge supernatants prepared from the digester samples discussed in Materials and Methods. The results of these comparisons, shown in Table IV, appear to be highly significant in terms of digester performance. As was shown, the sludge supernatants from "normal" digesters (Indian Creek and 4800 Nall) are much higher than those from unbalanced or upset digesters (Kansas City Pollution Control and Olathe, Kansas). While much more study is needed in this area, results indicate that the low factor levels in these digesters could explain why little methane is formed. Studies involving the addition of the unknown growth factor or factors to experimental digesters would provide a method for finding the degree of significance of the factor or factors in anaerobic digesters. Studies should definitely be extended to determine whether factor addition to these sludges would aid in reversing the unbalanced or stuck condition.

As a final set of experiments, two digesters from the Kansas City Pollution Control Plant were compared. As shown in Table V, attempts to reseed digester number 3 did not result in success for the digester or in an increase in the presence of the unknown growth factor. Assay results of samples from digester 4 were somewhat unexpected in that the supernatant did contain 0.5 unit/ml of the factor while the sediment contained none. The amount of factor in the supernatant of digester 4 may have been the result of the settling of material in the digester. The factor may have been concentrated in the upper portion of the digester. The comparison of these digesters again shows the significance of the factor in digester performance.

SUMMARY

Strict anaerobic assay procedures were developed using Methanobacterium ruminantium as the assay organism. Samples of sewage sludge from Indian Creek contained 0.8 units of the unknown growth factor. Pre-incubation of sludge samples with 0.4% yeast extract for 45 hr. increased the amount of the unknown growth factor twofold. Yeast extract (0.4%) was found to be the optimum for pre-incubation of the sludge samples. The unknown growth factor could be detected in samples which had been autoclaved whereas it could not be detected in samples which had been filter sterilized. The efficiency of the extraction procedure was increased by the substitution of a medium-porosity sintered glass for a pad of hyflosupercel and the substitution of Darco activated charcoal for Norit.

A significant difference was found between digesters in different stages of performance. The amount of the unknown growth factor increased with the increased efficiency of the digester.

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APPENDICES

APPENDIX I

APPENDIX I

Solutions for preparation of media.

Mineral Number 1

	%	1 liter
K_2HPO_4	0.6	6 g

Make to 1 liter.

Mineral Number 2

	%	1 liter
K_2HPO_4	0.6	6.0 g
$(NH_4)_2SO_4$	0.6	6.0 g
NaCl	1.2	12.0 g
$MgSO_4 \cdot 7H_2O$	0.245	2.45 g
$CaCl \cdot 2H_2O$	0.159	1.59 g

Dissolve salts in 700-800 ml distilled H_2O in the order given and make to 1 liter. Distribute 100-120 ml amounts into labeled Pyrex screw-cap dilution bottles. Autoclave at 15 lb/15 min.

Appendix I. (cont.)

Mineral Number 3

	1 liter	Final M conc. in medium
KH_2PO_4	18.0 g	6.62×10^{-3}
NaCl	18.0 g	1.54×10^{-4}
$CaCl_2$ ($CaCl_2$-$2H_2O$)	0.4 g	1.80×10^{-4}
$MgCl_2 \cdot 6H_2O$	0.4 g	9.85×10^{-5}
$MnCl_2 \cdot 4H_2O$	0.2 g	5.05×10^{-5}
$CoCl_2 \cdot 6H_2O$	0.02 g	4.20×10^{-5}

Dissolve salts in 700-800 ml distilled H_2O in the order given and make to 1 liter. Distribute 100-120 ml amounts into labeled Pyrex screw-cap dilution bottles. Autoclave at 15 lb/15 min.

Appendix I. (cont.)

Volatile fatty acid mixture

		Molar concentration*
Acetic acid (Glacial)	17 ml	2.9×10^{-2} M
Propionic acid	6 ml	8.0×10^{-3} M
Butyric acid	4 ml	4.3×10^{-3} M
Isobutyric acid	1 ml	1.1×10^{-3} M
n-Valeric acid	1 ml	9×10^{-4} M
Isovaleric acid	1 ml	9×10^{-4} M
DL-α-methylbutyric acid	1 ml	9×10^{-4} M

* Final molarity when added to assay basal medium at level of 3.1 ml/200 ml medium and subsequently diluted 5x for final volume.

Appendix I. (cont.)

Anaerobic dilution solution

	%	300 ml total
Mineral Number 3	5.0	15.0 ml
Resazurin (0.1%)	0.1	0.3 ml
Distilled water		279.0 ml
*$NaHCO_3$	0.5	1.5 g
Cysteine HCl	0.025	0.075 g

Place all ingredients with the exception of $NaHCO_3$ into round-bottom flask and boil under CO_2 (100%). Cool and add *0.5% $NaHCO_3$. Tube in 5 ml amounts under anaerobic conditions in a H_2/CO_2 (50-50) gas atmosphere and autoclave 15 lb/15 min. Add 0.025% NaS (1 turn) 2-24 hr. prior to use.

APPENDIX II

APPENDIX II

Media.

Medium Number 1 - slant medium

	%
RF (rumen fluid)	30.0
Mineral Number 1	3.75
Mineral Number 2	3.75
Resazurin solution	0.1
Na formate	0.2
Trypticase	0.2
Agar (bacto)	1.5
Distilled H_2O to volume	
Cysteine - HCl	0.025

Place all ingredients in a 500 ml round-bottom flask and boil under CO_2 until the resazurin is reduced (colorless). Cool medium in a water bath to 47-48 C and add $NaHCO_3$ (0.5%). Add $Na_2S \cdot 9H_2O$ reducing solution (0.025% final concentration) to the flask just prior to tubing. Tube the medium in 8 ml amounts anaerobically under a H_2/CO_2 (50-50) gas atmosphere. Autoclave 15 lb/15 min and cool in a slant position.

Appendix II. (cont.)

Medium Number 2 - carbohydrate agar slants

	%	300 ml
Resazurin (0.1%)	0.1	0.3 ml
Whole rumen fluid	30.0	90.0 ml
Mineral Number 1	3.75	11.25 ml
Mineral Number 2	3.75	11.25 ml
Glucose	0.05	0.15 g
Cellobiose	0.05	0.15 g
Soluble starch	0.05	0.15 g
Agar (bacto)	1.5	4.5 g
Trypticase	0.5	1.5 g
Distilled water		170.0 ml
Cysteine-HCl	0.025	0.075 g

Place all ingredients in a 500 ml round-bottom flask and boil under CO_2 (100%) until the resazurin is reduced (colorless). Cool medium in a water bath to 47-48 C and add $NaHCO_3$ (0.9%). Add $Na_2S \cdot 9H_2O$ reducing solution (0.025% final concentration) to the flask just prior to tubing. Tube the medium in 8 ml amounts anaerobically under a H_2/CO_2 (50-50) gas atmosphere. Autoclave at 15 lb/15 min and cool in a slant position.

Appendix II. (cont.)

Medium Number 3 - basal medium 5x strength

	%	200 ml
$(NH_4)_2SO_4$	0.05	0.5 g
Na formate	0.2	2.0 g
Yeast extract (Difco)	0.2	2.0 g
Trypticase	0.2	2.0 g
VFA solution	0.31	3.1 ml
$FeSO_4$	0.0002	0.002 g
Resazurin solution	0.1	1.0 ml
Mineral Number 3	5.0	50.0 ml
Hemin	0.15	3.0 ml
Distilled H_2O		97.0 ml

For 200 ml place all ingredients in a 400 ml beaker, adjust to pH 6.5 on a magnetic stirrer with 2.5 N NaOH (about 16 ml) and bring up to 200 ml. Distribute in 100 ml quantities in Pyrex screw-cap bottles. Autoclave at 15 lb/15 min. Store in refrigerator. For use, aseptically place 10 ml in 100 ml round-bottom flask and add indicated volume of material being assayed.