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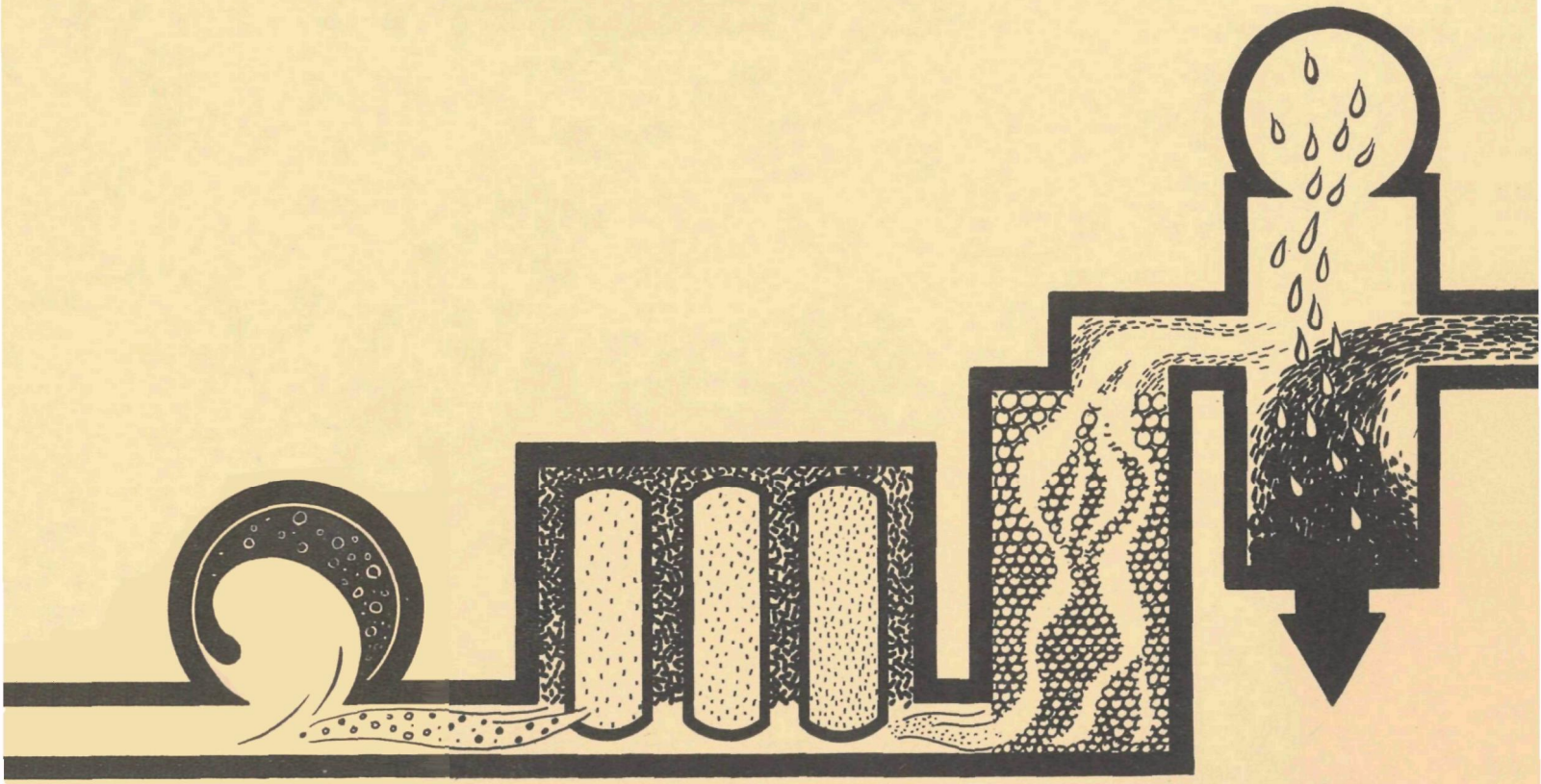
DEVELOPMENT OF TECHNIQUES FOR ESTIMATING THE BACTERIAL POPULATION OF SEWAGE SLUDGE



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DEVELOPMENT OF TECHNIQUES FOR ESTIMATING THE BACTERIAL POPULATION OF SEWAGE SLUDGE

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ABSTRACT

This research program was initiated to develop practical methods for evaluation of the biomass in anaerobic sewage sludge and to determine if predictions could be made concerning digester performance. Sampling and handling methods were improved and standardized to give maximum anaerobic counts. A simplified technique for growing obligate anaerobes that can be safely performed by technicians with minimum training in bacteriology was developed. Anaerobic media were improved to yield as high or higher counts of methanogenic bacteria than heretofore reported. A simple freeze-dry technique was developed for preparation of consistent batches of sludge supernatant used in media as a supplement for growth of obligate sludge anaerobes. The possible relationship between concentration of a growth factor required by Methanobacterium ruminantium (used to evaluate potency of growth factor extracted) and digester efficiency could have important practical implications. Limited data obtained indicated that growth factor concentrations were much higher in "normal" digesters than in unbalanced or "upset" digesters. Practical applications of the methods developed can have considerable impact upon future research and development in anaerobic sludge digestion and could lead to improvements in our ability to predict impending digester failure and control of digester performance.

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CONCLUSIONS

1. Sampling of digester contents was done with a sampler which preserved the anaerobic condition of the sample. Handling methods ensuring continuity of anaerobic conditions were standardized to give maximum total counts by the Hungate procedure.

2. A nutrient medium containing 20% of an aqueous extract of lyophilized sludge supernatant and sodium sulfide plus cysteine-HCl to reduce residual traces of oxygen was used in combination with appropriate gaseous atmospheres (oxygen-free CO_2, H_2, N_2 mixture depending on type of organism to be grown) to maintain anaerobic conditions and low oxidation-reduction potential. Nutrients were added to or deleted from the medium to yield maximum total anaerobic counts.

3. A growth factor present in sludge supernatant was assayed and found to occur at approximately the same concentration in "normal" sludge supernatant as in rumen fluid.

4. The method of preparation of sludge supernatant was improved and simplified by using batch-lyophilized sludge. Aqueous extract of lyophized sludge (AELS) is prepared from this material by placing a weighed quantity on a filter mat of hyflosupercel and washing the mat with sufficient hot water (with suction) to restore the original supernatant volume. Large amounts of sludge can be lyophilized and stored indefinitely to yield a more consistent sludge supernatant as required.

5. The roll-tube technique of Hungate has been simplified and made much safer to use. A permanent butyl rubber stopper is used with a retaining clamp where necessary. All additions to the culture tube are made through syringe needles from a mechanically mounted syringe. Direct colony counts are made in the cylindrical inside agar surface of the tube.

6. Media preparation has been simplified by dispensing gas-flushed, prereduced medium in multiples of five tubes which are simultaneously flushed from a manifold. After sealing with a butyl rubber stopper, the medium is sterilized in clamps. All subsequent operations are performed without removing the stoppers from the tubes. Shortly before use, the medium is reduced further by syringe addition of sodium sulfide--cysteine·HCl reducing solution through the rubber stoppers.

7. A device consisting of a syringe mounted on a modified drill press and an aluminum roll-tube jig in a water bath was used to make all additions, dilutions, and inoculation of roll-tubes. The technique provides a simplified and safer means of manipulating roll-tubes remotely without

the necessity of removing rubber stoppers from tubes. The technique requires much less manual dexterity than the conventional roll-tube technique and can be performed by technicians with little or no prior experience in microbiology.

8. The possible relationship between concentration of growth factor required for growth of Methanobacterium ruminantium (used in this study to evaluate potency of growth factor extracted) and digester efficiency could have important practical implications. Limited data obtained indicated that growth factor concentrations were much higher in "normal" digesters than in unbalanced or "upset" digesters. These trends were observed later in the final phase of the study and more work is needed to validate and establish the significance of these preliminary trends.

RECOMMENDATIONS

On the basis of progress and observations made during the course of this work, several recommendations for further studies can be made:

1. Further experiments should be carried out to microbiologically monitor several digesters over a period of time to determine what changes in types and numbers of microorganisms occur as a digester approaches failure.

2. Media should be modified such that cultural conditions more closely simulate the prevailing condition of a digester being monitored. The modified media should be directly compared to media which closely simulate a "normal" digester. Various modifications should be evaluated for samples taken from poorly functioning digesters--e.g., inclusion of sludge supernatant from each such digester, addition of purified sludge factor to sludge supernatant from the digester in question, and comparison of the supernatant from each digester to normal control supernatant with respect to methanogenesis and types and number of organisms grown.

3. Further studies are needed to identify the unknown sludge factor and to subsequently develop a microbiological or chemical assay which can be quantitatively compared to standard curves, prepared with the known compound, and used to monitor factor levels more accurately.

4. Studies using laboratory digesters are also needed. The digesters could be monitored for total anaerobic counts, aerobic plate counts (for facultatives), methane production, and factor level when the digesters are functioning normally and when induced toward failure. The effect of supplemental addition of purified factor should also be studied, as well as any role for the factor in normal digestion and the possibility of reversing an upset condition with purified factor.

The studies presented herein have laid the groundwork for those studies which now can be undertaken. Through continuation of these studies, a great deal of information could be gained on changes in biomass, factor levels, importance of factor and corrective steps which might be taken to prevent failure of a digester.

INTRODUCTION

The anaerobic treatment of sewage waste 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 process upsets.

The anaerobic digestion of organic wastes generally proceeds in two stages. In the first stage, complex compounds are broken down to simple organic materials. Fats, proteins, and carbohydrates are converted, for the most part, to organic 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.[1,2] 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.[3,4]

The methane bacteria are obligate anaerobes which require very low oxidation-reduction potentials for growth. They cannot be grown by classical anaerobic techniques. Consequently few data, concerning their population in sewage, physiology, and ecology, are available. To date, few species of methane bacteria have been cultured and identified from anaerobic sludge, rumen contents, and anaerobic bottom sediments. Only six species have been isolated in pure culture.[3,5] While there are a number of references to methane-forming bacteria in sewage sludge, only three species have been isolated in pure culture and identified, namely Methanobacterium formicum,[6] Methanobacterium omelianskii,[7] and Methanobacterium ruminantium.[4] Methanobacterium omelianskii has since been shown to be a symbiotic relationship between a methanogenic and a non-methanogenic species.[8]

Much of the investigation on anaerobic sludge digestion has been concerned with the chemical and biochemical aspects of the problem. Considerable data have been obtained on end-products of processes, which resulted in some control over the digestion process by noting certain changes, e.g., pH, volatile acids, temperature, trace metals, etc. However, little is known about the organisms which cause initial digestion

and secondary stabilization. A knowledge of these organisms is essential for proper control and understanding of sludge digestion.

The research described in this report has been designed to simplify the methods and media used in growing sludge (in this report, it should be assumed, unless otherwise stated, that any reference to sludge or sewage sludge is taken to mean anaerobic sewage sludge as opposed to aerobic or "activated" sludge) anaerobes and to obtain useful information concerning the biomass in sewage sludge. The experiments were carried out in four phases, as follows:

Phase I - Development of Techniques for Sampling and Handling of Anaerobic Sewage Sludge.

Phase II - Development of a Simplified Technique for Enumeration of the Obligate Anaerobes in Sewage Sludge.

Phase III - Development of a Culture Medium for Growth of Microorganisms Representative of the Total Biomass in Sewage Sludge.

Phase IV - Determination of Growth Substance(s) in Sewage Sludge that Enhances Growth of Sludge Microorganisms.

Efforts were directed toward development of simple and inexpensive techniques and equipment that are reliable for cultivation and isolation of the microorganisms responsible for anaerobic sludge digestion. Data obtained will be important in developing a more systematic and standardized approach to cultivation of the sewage biomass which is currently lacking. (For a fuller statement of the principal conclusions and recommendations based upon the work in this research program, see page 1-3.)

PHASE I - DEVELOPMENT OF TECHNIQUES FOR SAMPLING AND HANDLING OF ANAEROBIC SEWAGE SLUDGE

The bacteria of importance in anaerobic sludge digestion are obligately anaerobic (particularly the methane bacteria), and require a very low oxidation-reduction potential for growth. Even brief exposure of these organisms to oxygen results in a rapid decrease in numbers of viable organisms present.[9] It is, therefore, mandatory that samples to be studied in the laboratory be collected under anaerobic conditions at a low Eh (oxidation-reduction potential), and be maintained in this condition during transport to the laboratory. The literature reveals that little attention has been given to the collection and handling of sludge samples in the absence of oxygen.

Work during the early stage of this program involved basic laboratory studies to evaluate the sampling and handling procedures for sewage sludge. Since most experiments carried out in this program involved the growth and enumeration of the obligate anaerobes in sewage sludge, it was necessary to initially establish a reliable procedure for collection, suspension, dilution, and counting of samples which would result in a maximum viable count of the obligate anaerobes present in sewage sludge digests.

Preparation of Medium and Roll-Tube Procedure

The medium and roll-tube procedure to be described were used throughout the initial phase of this project (Phase I). The roll-tube procedure described is a modification of that originally developed by Hungate for growth of anaerobic, mesophilic, cellulolytic, rumen bacteria.[10] The medium selected is a rather simple but complete medium designed to contain essential nutrients and gaseous atmosphere for concomitant growth of both methanogenic and non-methanogenic species.

Roll-Tube Medium (100 ml)

Clarified sludge supernatant (CSS)	30.000 ml
Mineral phosphate, S-1	5.000 ml
Phosphate supplement, S-2	5.000 ml
Sodium formate	0.100 gm
Potassium acetate	0.100 gm
Trypticase	0.200 gm
Yeast extract	0.050 gm
Agar	2.000 gm
Cysteine $\cdot HCl \cdot H_2O$	0.025 gm
Resazurin (0.1% solution)	0.100 ml
Distilled water	56.000 ml

Ingredients were placed in a 500-ml round-bottom flask and brought to a boil under oxygen-free CO_2. Oxygen removal was accomplished by passing the gas through a hot copper column. Boiling was continued with gassing until the resazurin became reduced (colorless). The flask was then stoppered and wired to prevent loss of the stopper during sterilization. After autoclaving at 15 lb/15 min, the medium was cooled to 47 to 48°C, in a water bath, and opened aseptically under 50-50 H_2/CO_2. Sterile 8% CO_2 equilibrated Na_2CO_3 was added to give a final pH of 6.8 (2.5 ml/100 ml medium). The medium was tubed in 9 ml amounts by aseptic anaerobic transfer to sterile 18 x 150 mm tubes, and tightly stoppered. (Sterile tubes had been previously gassed with CO_2, stoppered and stored for future use.) Tubes were aseptically opened and gassed with CO_2/H_2 during transfer of medium using a gas-flushed, sterile, 10-ml pipette with a rubber tubing mouthpiece. Gassing was continued for an additional 10 to 15 sec before the gassing needle was removed and the stopper securely seated. The tubed medium was stored for future use.

Final reduction of the medium was accomplished by adding 0.2 ml of sterile $Na_2S \cdot 9H_2O$ (1.25% stored under N_2 in stoppered tubes) 2 to 24 hr prior to use. Tubed medium was melted just before use by placing in a jig which securely held the stoppers in place during melting in an Arnold steamer (5-10 min). Tubes were held in a water bath at 47 to 48°C until used.

Mineral solution S-1 contained the following in g/liter: NaCl, 16.0; $MgSO_4 \cdot 7H_2O$, 1.8; $CaCl_2$, 0.4; $(NH_4)_2SO_4$, 3.0; $MnSO_4 \cdot H_2O$, 0.2; $CaCl_2 \cdot 6H_2O$, 0.02; $NaMoO_4 \cdot 2H_2O$, 0.01; and $FeSO_4 \cdot 7H_2O$, 0.02.

Mineral solution S-2 contained 6.0 g/liter each of K_2HPO_4 and KH_2PO_4. Mineral solutions were dispensed in 100-ml quantities, autoclaved at 15 lb/15 min and stored at room temperature.

Clarified sludge supernatant (CSS), used in preparation of media and anaerobic dilution solution (ADS), was prepared as follows. A 6 to 8 liter sample of sludge was collected and sterilized. Large particles were removed by filtration through a double thickness of cheesecloth in a Buchner funnel under vacuum. Several changes of cheesecloth were necessary, since the sediment had a tendency to cake and impede filtration. The filtrate was clarified by passage through a Sharples continuous centrifuge at approximately 40,000 rpm. Two or more passages were often necesary to achieve final clarification. The clarified sludge supernatant was bottled in 100-ml quantities, sterilized (15 lb/15 min), and stored in the refrigerator. In the initial experiments, CSS was added to media and anaerobic dilution solution (ADS) at an arbitrary concentration of 30% (V/V).

Anaerobic dilution solution used in preparation of serial dilutions for roll-tube inoculation was prepared in the same manner as the roll-tube medium, except that no substrates and agar were included: i.e., no sodium formate, potassium acetate, trypticase or yeast extract. The ADS was tubed aseptically in 8.8-ml amounts. Prior (2-24 hr) to an experiment, 0.2 ml of $Na_2S \cdot 9H_2O$ solution was added to achieve final reduction and give a final volume of 9 ml/tube.

Roll-Tube Procedure

Preparation of roll tubes varied with the number of tubes prepared from each dilution and the number of conditions tested per dilution; however, the basic dilution procedure and method of inoculation did not vary throughout Phase I. Decimal dilutions of the initial 1/10 dilution of sewage sludge were prepared by anaerobic transfer of 1 ml of the dilution to a 9-ml dilution blank. A gas-flushed 1-ml pipette with a rubber tubing mouthpiece attached was used. Each dilution blank was first opened and a gassing needle inserted into the tube to maintain the gaseous atmosphere and prevent entry of oxygen. Following transfer of 1 ml of material, the gassing needle was removed, the stoppers quickly replaced and seated, and the contents of the tube were mixed thoroughly by vigorous shaking. When all dilutions had been prepared in this manner, 0.5-ml aliquots were transferred aseptically and anaerobically from the desired dilutions, in triplicate, to roll-tube medium. The contents of each roll tube were mixed by gentle agitation and the medium was solidified to a uniform layer in the tubes by horizontal rolling in a flat pan containing ice water. Roll tubes thus prepared constitute an oxygen-free, closed system at low oxidation-reduction potential.

Periodic colony counts were performed on the roll tubes by rotating each tube under a Quebec colony counter and carefully counting the colonies which appeared during each successive incubation period. Each colony was marked by a felt pen. A color coding system was established by marking all colonies appearing at different time intervals with a different color. This enabled us to determine when a particular colony type might appear, and eliminated the need to recount all colonies at each counting period.

Evaluation of Sampling Methods

A sampler, shown in Figure 1, was devised and constructed which could be lowered into an anaerobic digester and opened at any desired depth. The sampler is then closed by means of a spring-loaded lid, the sample withdrawn, and a portion removed under anaerobic conditions for transport back to the laboratory. The sample is removed by placing the tubing at the bottom of the sampler to the bottom of the nitrogen-flushed thermos bottle.

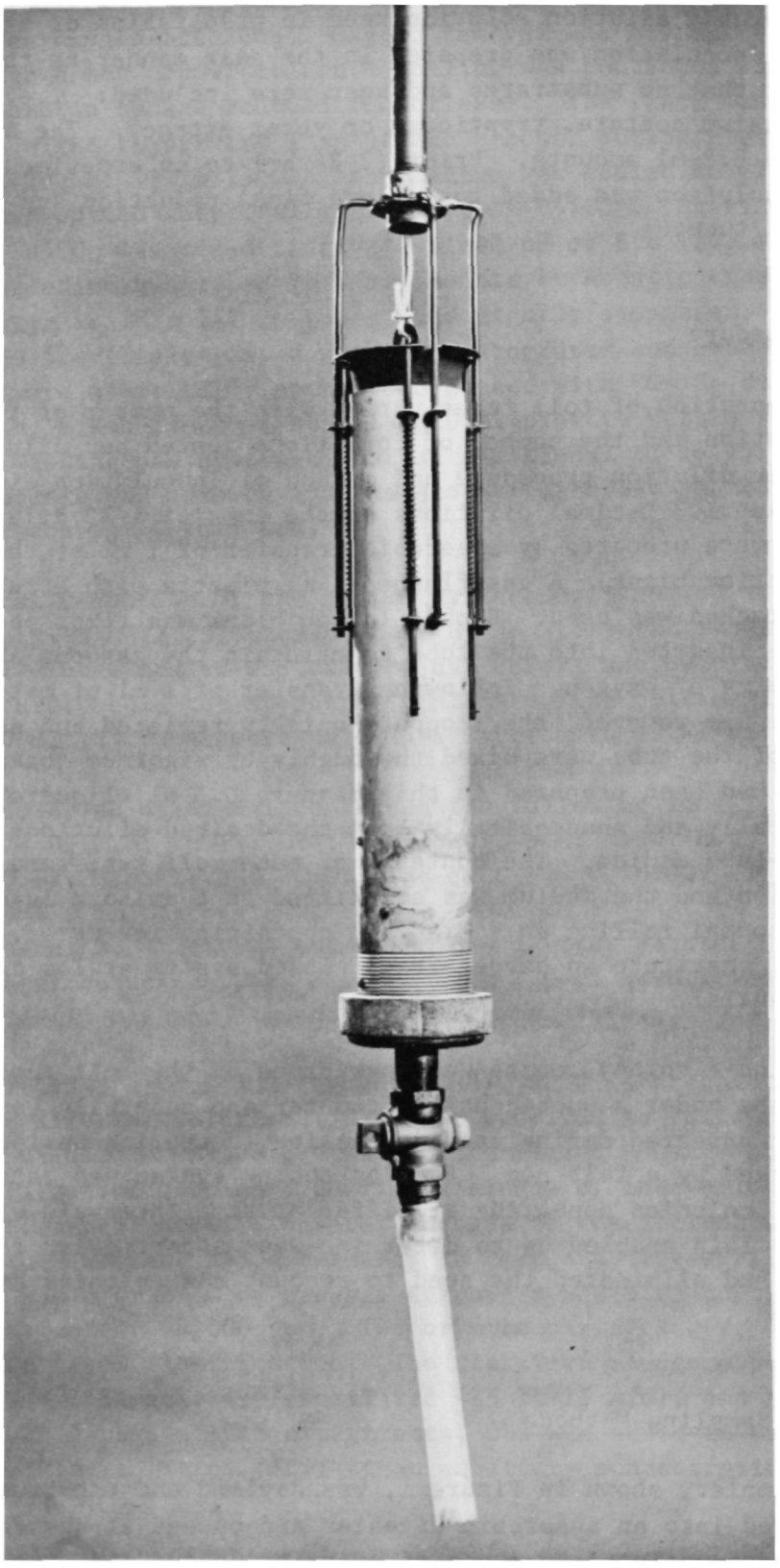

Figure 1 - Anaerobic Sludge Sampler Designed for Collection of Samples Under Anaerobic Conditions

The valve is then opened and the stopper at the top of the sampler is "cracked" to allow flow into the thermos bottle. The uppermost level of material in the sampler was never collected due to the possibility of oxygen diffusion into the upper few inches of sample. The sample thus obtained is homogeneous, free of surface scum and floating debris, and representative of digester contents at the depth sampled. The tubing is flushed with a portion of the sampler contents before the sample itself is taken.

The thermos bottles used for transporting the samples from the sewage plant to the laboratory are nitrogen-flushed to displace air and fitted with gas-tight lids equipped with Bunsen valves to relieve gas pressure while simultaneously excluding entry of oxygen. The thermos bottles were "sanitized" with boiling water for 15 min before flushing with nitrogen.

Sampling was evaluated under the following conditions: (1) sample collection under nitrogen only; (2) sample collection under nitrogen with 0.025% each of cysteine-HCl and Na_2S added at the time of sampling to determine the effect of reducing agents on total counts; and (3) sample collection under nitrogen using 0.25% sodium pyruvate as a protective agent. Pyruvate was added on the assumption that it might protect the obligate anaerobes from oxidative effects by destruction of peroxides which could be present in the samples, or could form as a result of entry of small amounts of oxygen during the sampling process.[11]

Samples transported to the laboratory under the above conditions were diluted 1/10 by transferring 20 ml of sample to a stainless steel Waring blender cup containing 180 ml anaerobic dilution solution consisting of 30% (v/v) clarified sludge supernatant, 10% mineral salts solution, 2.5% CO_2 equilibrated Na_2CO_3 (8% solution), 0.025% cysteine·HCl-Na_2S reducing solution, and 0.1% of a 0.1% solution of resazurin as an O/R indicator. Samples were blended 1 min at high speed while being flushed vigorously with CO_2-H_2 (50-50) to maintain anaerobic conditions. The 10^{-1} dilution was then serially diluted to 10^{-8} in duplicate and triplicate 0.5-ml samples of 10^{-6}, 10^{-7} and 10^{-8} dilutions transferred to roll-tube medium (a total of six roll tubes from each dilution of sample). All tubes were incubated at 37°C and weekly counts made for a total of five weeks. Due to the very slow growth of these organisms, more frequent counting intervals were not necessary.

The results of sampling studies shown in Table 1 are the averages of three experiments for each sampling condition. Each value represents the average of 17 to 18 roll-tube counts which have been evaluated statistically to determine the standard deviation of the means and the significance between the means. In this, and subsequent tables, all counts presented are total anaerobic colony counts per milliliter of orginal sludge sample unless specifically designated otherwise in tables or text. The

TABLE 1

EFFECT OF SAMPLING METHOD ON TOTAL COLONY COUNT

	Days Incubation at 37°C				
	7	14	21	28	35
Sample collected under N_2	6.2×10^7 xy[a] (2.3×10^7) 17[b]	9.8×10^7 x (4.6×10^7) 17	2.5×10^8 x (1.1×10^8) 17	3.6×10^8 x (2.4×10^8) 17	4.5×10^8 x (2.4×10^8) 17
N_2 plus 0.025% Na_2S-$9H_2O$-cysteine-HCl	8.2×10^7 y (2.5×10^7) 18	1.3×10^8 y (0.4×10^8) 18	3.0×10^8 x (1.6×10^8) 18	4.4×10^8 x (2.4×10^8) 18	6.1×10^8 x (2.9×10^8) 17
N_2 plus 0.25% sodium pyruvate	5.4×10^7 x (3.6×10^7) 17	9.7×10^7 x (4.6×10^7) 18	2.4×10^8 x (1.5×10^8) 18	3.9×10^8 x (2.5×10^8) 18	5.1×10^8 x (2.6×10^8) 17

a/ Mean colony counts of replicate tubes. Means followed by one letter: means followed by the same letter are not statistically different (5% level of probability). Other pairs are statistically different. Means followed by two letters: consider that pairs of means followed by one matching letter are not statistically different.

b/ Values in parentheses represent the standard deviation of the mean. Numbers following the parentheses correspond to the number of replicate tubes counted.

colony counts for samples taken under nitrogen and under nitrogen with pyruvate are in good agreement, and no advantage to added pyruvate is indicated. The addition of reducing solution gave consistently higher counts, although the large standard deviation of the mean indicates an insignificant difference. Samples containing reducing solution did, however, result in better handling conditions (less tendency to oxidize during roll-tube preparation), it was decided to include this procedure in subsequent samples.

Evaluation of Handling Methods

The literature contains no methods which are used consistently for the handling of sewage sludge samples. We therefore devoted considerable effort to developing handling techniques which would yield a maximum survival rate during the period required for blending and subsequent dilution of samples to be counted. The method which proved most effective in our hands was adopted for use throughout the remainder of this program. The following parameters were evaluated: (1) composition of anaerobic dilution solution; (2) blending time; (3) use of a surfactant to aid in breaking of clumps and suspension of cells for subsequent dilution and counting; (4) effect of initial mixing; and (5) effect of catalase addition on total colony count.

Anaerobic dilution solution was evaluated by a basal dilution solution consisting of mineral salts solution, 10% (v/v); resazurin (0.1%), 0.1% (v/v); cysteine·HCl·H_2O, 0.025%; Na_2S, 0.025%; Na_2CO_3, 0.2%; and CO_2-H_2 gas phase (50-50). A second solution was compared, which contained basal anaerobic dilution solution plus 30% clarified sludge supernatant (CSS). CSS apparently contains unknown factors that exert a protective effect. The addition of sludge supernatant, however, results in considerable foaming during shaking which can lead to inconsistencies in pipetting. A third comparison was therefore made to determine the value of adding Dow-Corning Antifoam-C to reduce foaming caused by the sludge supernatant.

Data on the effect of the composition of the anaerobic dilution solution are summarized in Table 2. It is apparent that the addition of CSS to the ADS results in consistently higher counts than ADS alone. The addition of Antifoam-C to retard foaming negates the increased count due to sludge supernatant; in fact, the data indicate a lower count than ADS alone and that Antifoam-C is toxic to sludge anaerobes. We found that the foaming resulting from added CSS is of little consequence if the pipette was lowered to the bottom of the dilution tube before solution was allowed to enter. Any foam adhering as the pipette was withdrawn could be eliminated by touching the pipette to the tube during withdrawal. In subsequent experiments, the CSS was included in the ADS with no Antifoam-C added.

TABLE 2

EFFECT OF ANAEROBIC DILUTION SOLUTION COMPOSITION ON TOTAL COLONY COUNT

	Days Incubated at 37°C				
	7	14	21	28	35
Basal anaerobic dilution solution (ADS)	4.7×10^7 x[a/] (3.7×10^7) 11[b/]	7.4×10^7 x (4.1×10^7) 11	8.8×10^7 x (4.2×10^7) 12	1.4×10^8 x (0.9×10^8) 12	1.8×10^8 x (1.0×10^8) 12
ADS plus 30% clarified sludge supernatant (CSS)	1.3×10^8 y (0.5×10^8) 11	1.5×10^8 y (0.5×10^8) 11	1.7×10^8 y (0.7×10^8) 11	2.1×10^8 y (1.1×10^8) 11	2.7×10^8 y (1.0×10^8) 11
ADS + CSS + Antifoam-C	7.1×10^7 x (4.4×10^7) 7	1.0×10^8 x (0.4×10^8) 7	1.1×10^8 x (0.4×10^8) 7	1.2×10^8 x (0.4×10^8) 7	1.4×10^8 x (0.4×10^8) 7

a,b/ See Table 1.

The surfactant Dioctyl sodium sulfosuccinate (DSS) was compared at levels of 0, 0.1% and 0.5% (w/v) in anaerobic dilution solution to determine the effect of reduced surface tension on dispersal of bacterial clumps in the initial 1/10 dilution. Dilutions were prepared to 10^{-8}, and roll tubes were inoculated from the 10^{-6}, 10^{-7} and 10^{-8} dilutions. A control was included in which the original 1/10 dilution was blended without DSS. Antifoam-C was included in the dilution solution, although Table 2 indicates that this compound exhibits toxicity to the sludge bacteria. An overlap of experiments was necessary throughout the program. Because of very slow growth rate of some species; roll tubes in experiments were counted after varying incubation periods up to a maximum of 35 days. Without overlap, only one experiment could be initiated per month. Obviously, it was necessary to begin many experiments without having completed data from concurrent experiments which might directly affect new work.

Table 3 shows the results of addition of surfactant to the ADS. The counts are obviously much lower when DSS is used and it would appear that a significant portion of the anaerobic flora is sensitive to this compound. Dioctyl sodium sulfosuccinate was not used in other experiments.

Bile salts were also tested as a possible aid to suspension of bacteria and/or dispersal of clumps. Anaerobic dilution solution and roll-tube media were prepared with bile salts added at levels of 0, 0.05%, and 0.15%. The raw sludge sample was preblended for 1 min, under anaerobic conditions, without prior dilution to insure a homogeneous inoculum for all subsequent dilutions. Following the preblend, subsamples were diluted 1/10 with ADS containing the indicated levels of bile salts and blended an additional 1 min under anaerobic conditions. Suitable dilutions of each were inoculated into three sets of roll tubes, each containing one level of bile salts.

The results of this experimental set are not shown since growth was obtained only in control sets (no bile salts). The lowest bile salt level tested was almost completely inhibitory at the lowest dilution inoculated (10^{-6}). It appears probable that, unlike the coliforms, the sludge bacteria as a group are rather sensitive to reduced surface tension, and such agents should not be used in preparation of media used for their growth.

The literature reveals no agreement as to blending times or blending conditions used to prepare sludge samples for dilution. Several papers have appeared, however, in which samples were blended as 1/10 dilutions, for no more than 30 sec to 1 min, under anaerobic conditions in a Waring blender.[12-14]

TABLE 3

EFFECT OF DIOCTYL SODIUM SULFOSUCCINATE (DSS) ON TOTAL COLONY COUNT

	Days Incubation at 37°C				
	7	14	21	28	35
"Normal ADS" (ADS with CSS and Anti-foam-C)	5.3×10^7 x[a/] (0.6×10^7) 6[b/]	1.0×10^8 x (0.2×10^8) 6	2.2×10^8 x (0.8×10^8) 6	3.3×10^8 x (0.7×10^8) 6	3.8×10^8 x (0.6×10^8) 6
Normal ADS + 0.1% DSS	1.3×10^7 y (0.4×10^7) 4	4.6×10^7 y (0.6×10^7) 4	5.7×10^7 y (0.4×10^7) 4	6.5×10^7 y (0.1×10^7) 4	7.1×10^7 y (0.7×10^7) 4
Normal ADS + 0.5% DSS	1.2×10^7 y (0.2×10^7) 6	1.9×10^7 y (0.6×10^7) 6	2.7×10^7 y (0.9×10^7) 6	3.6×10^7 y (1.0×10^7) 6	4.0×10^7 y (1.1×10^7) 6

a,b/ See Table 1.

To determine the optimum blending time for anaerobic sludge samples, 20 ml of sludge was blended with 180 ml of anaerobic dilution solution as previously described. Samples were taken for further dilution and roll-tube inoculation after 30 sec, 1, 2, 3, 5, and 10 min blending at high speed in the Waring blender. Blending for periods of greater than 2 min duration resulted in heat generation due to friction. Samples were either blended for no longer than 2-min intervals followed by a 2-min cooling period, or the blender cup was wrapped in a cool towel. Both methods were equally effective in preventing loss of viability due to excessive heat.

The results of these experiments are shown in Table 4. It is apparent that sludge samples can be blended up to 10 min without great loss in viability of sewage anaerobes. There appears to be no advantage, however, to blending longer than 3 min. The 30 sec to 1 min blending period often found in the literature would appear to be too short for maximum colony counts. The most significant blending times appear to be either 2 or 3 min.

The effect of initial mixing on total colony count was tested by comparing 1 min blending time with samples diluted 1/10 before blending, with hand-shaken samples diluted 1/10, and with undiluted samples blended directly.

The results shown in Table 5 indicate that significantly higher counts are obtained when samples are blended undiluted as opposed to blending of the initial 1/10 dilution or shaking by hand. In fact, a 1-min blending of a 1/10 dilution does not give significantly better dispersion than hand shaking of a 1/10 dilution. Apparently blending of a more concentrated sample results in more clumps being broken up per unit time, resulting in a more uniform mixture and better release of cells from clumps. This was further resolved by comparing samples blended for longer periods of time.

The results presented in Table 6 further indicate the need for blending longer than 1 min. In contrast to 1-min blending comparisons, when blending is carried out for 2 min to compare undiluted samples and samples which have been diluted 1/10 before blending, there is not significant difference in total count. There is, nevertheless, a distinct advantage to blending the sample undiluted in that reduced dilution solution is not required in the blender cups. The 1/10 dilution is a rather difficult and time-consuming step which, when eliminated, simplifies the handling procedure considerably. It is still necessary to maintain strict anaerobic conditions, however, which is accomplished by measuring a 200-ml sludge sample (containing 0.025% each of cysteine·HCl·H_2O and $Na_2S \cdot 9H_2O$) into a gas-flushed, sterile graduated cylinder and quickly pouring this, with gassing, into a gas-flushed stainless steel blender cup which is then tightly closed. The sample is blended 2 min at high

TABLE 4

EFFECT OF BLENDING TIME ON TOTAL COLONY COUNT

Blending Time for 1/10 Dilution of Sludge Sample	Days Incubation at 37°C				
	7	14	21	28	35
30 sec	4.8×10^7 x [a/]	6.6×10^7 x	7.6×10^7 x	1.2×10^8 x	1.6×10^8 xy
	(0.6×10^7) 12 [b/]	(0.9×10^7) 12	(0.9×10^7) 12	(0.3×10^8) 12	(0.4×10^8) 12
1 min	6.9×10^7 y [c/]	3.5×10^7 xz	9.6×10^7 xy	1.2×10^8 x	1.6×10^8 x
	(2.6×10^7) 12	(2.8×10^7) 12	(2.5×10^7) 12	(0.3×10^8) 12	(0.4×10^8) 12
2 min	6.9×10^7 y	9.7×10^7 z	1.5×10^8 z	2.0×10^8 z	2.2×10^8 z
	(2.4×10^7) 14	(2.3×10^7) 14	(0.4×10^8) 14	(0.5×10^8) 14	(0.5×10^8) 14
3 min	6.6×10^7 y	8.8×10^7 z	1.2×10^8 y	1.9×10^8 z	2.5×10^8 z
	(2.2×10^7) 17	(2.8×10^7) 17	(0.3×10^8) 17	(0.6×10^8) 17	(0.7×10^8) 17
5 min	5.4×10^7 xy	8.4×10^7 xz	1.1×10^8 y	1.7×10^8 yz	2.1×10^8 yz
	(1.0×10^7) 14	(1.3×10^7) 14	(0.4×10^8) 14	(0.6×10^8) 14	(0.6×10^8) 11
10 min	4.0×10^7 x	5.4×10^7 xy	6.4×10^7 xy	1.3×10^8 xy	1.6×10^8 xy
	(0.8×10^7) 8	(1.4×10^7) 8	(1.9×10^7) 8	(0.5×10^8) 8	(0.4×10^8) 8

a,b/ See Table 1.

c/ Total counts corrected for initial 1/10 dilution.

TABLE 5

EFFECT OF METHOD OF INITIAL MIXING ON TOTAL COLONY COUNT

	Days Incubation at 37°C				
	7	14	21	28	35
1/10 dilution of sample shaken by hand for 1 min	3.9×10^7 x[a/] (1.9×10^7) 16[b/]	6.4×10^7 x[c/] (1.6×10^7) 16	1.5×10^8 x (0.3×10^8) 16	2.2×10^8 x (0.5×10^8) 16	2.8×10^8 x (0.8×10^8) 15
Sample blended undiluted for 1 min	5.3×10^7 y (2.1×10^7) 16	7.3×10^7 x (2.2×10^7) 16	1.8×10^8 xy (0.5×10^8) 16	3.3×10^8 y (1.6×10^8) 16	4.8×10^8 y (2.3×10^8) 16
1/10 dilution blended 1 min	4.5×10^7 xy[c/] (1.3×10^7) 16	6.5×10^7 x (2.0×10^7) 16	1.0×10^8 y (0.3×10^8) 15	1.9×10^8 x (0.9×10^8) 15	2.6×10^8 x (1.1×10^8) 15

a,b/ See Table 1.

c/ Total counts corrected for initial 1/10 dilution.

TABLE 6

COMPARISON OF TOTAL COLONY COUNTS/MILLILITER ON SAMPLES BLENDED UNDILUTED WITH SAMPLES BLENDED AS 1/10 DILUTIONS

	Days Incubation at 37°C				
	7	14	21	28	35
Samples blended undiluted for 2 min	6.1×10^7 x[a/]	9.2×10^7 x	3.1×10^8 x	4.3×10^8 x	4.8×10^8 x
	(0.7×10^7) 20[b/]	(1.2×10^7) 20	(1.1×10^8) 20	(1.2×10^8) 20	(1.3×10^8) 20
Samples blended as 1/10 dilutions for 2 min	6.2×10^7 x[c/]	8.9×10^7 x	2.2×10^8 y	3.5×10^8 y	4.4×10^8 x
	(2.0×10^7) 20	(2.2×10^7) 20	(0.9×10^8) 20	(1.4×10^8) 20	(1.1×10^8) 20

a,b/ See Table 1.

c/ Total counts corrected for initial 1/10 dilution.

speed with gas flushing. Subsequent dilutions are then prepared in dilution tubes. The 2-min blending period is more convenient than a 3-min period since neither a waiting period between blends nor external cooling is required.

Many of the obligate anaerobes lack catalase and are extremely sensitive to hydrogen peroxide which can accumulate in the presence of oxygen. If even trace amounts of oxygen are present in the medium, peroxide toxicity could conceivably result.

To explore the possibility of protection against possible peroxide formation, the effect of adding catalase to the sample container, anaerobic dilution solution, and medium was studied. Duplicate sludge samples were taken in two thermos, each without reductants. One thermos bottle contained 1,000 sigma units of catalase per milliliter of sample. From the sample with catalase, dilutions were prepared in ADS with catalase and medium with and without catalase. Dilutions were prepared from the sample without catalase, and medium with and without catalase was inoculated.

Results of catalase experiments are shown in Table 7. It is apparent that catalase yields a higher count when added to the sample container, ADS, or medium. Although the highest counts were obtained when catalase was added at all stages of roll-tube preparation, it would appear that the greatest benefit is derived only when catalase is added at the time of sample collections--more so than addition to ADS or medium only. Since catalase is heat sensitive, it must be filter-sterilized and added to individual tubes of ADS and/or medium after sterilization. In the interest of simplicity it would perhaps be best used only in the sample container and added at the time of collection.

Because of the broad scope of work and the large number of experiments which were performed throughout this program, no single aspect of a problem could be thoroughly studied. We were thus unable to devote sufficient time to further testing of the effect of catalase in particular, or sampling and handling techniques in general. It would be of particular interest to determine the effect of both reductants and catalase on total colony counts; the effect of catalase on samples taken in open containers without reductants or N_2 atmosphere; and the effect of catalase on viable counts when samples are held for extended periods of time before preparation for roll-tube inoculation.

Recommended Method for Sampling and Handling

On the basis of work accomplished in Phase I, we make the following recommendations regarding the sampling and handling of sewage sludge samples to be used for roll-tube inoculation by the Hungate technique.

TABLE 7

EFFECT OF CATALASE ON TOTAL COLONY COUNT

	Days Incubated at 37°C				
	7	14	21	28	35
Catalase in sample container:					
ADS with catalase, medium without	6.6×10^7 x[a/] (1.1×10^7) 8[b/]	1.0×10^8 x (0.1×10^8) 8	4.5×10^8 y (0.5×10^8) 8	6.3×10^8 x (0.9×10^8) 8	7.0×10^8 x (0.6×10^8) 8
ADS with catalase, medium with	6.6×10^7 x (0.9×10^7) 8	1.1×10^8 x (0.1×10^8) 8	5.7×10^8 x (0.9×10^8) 8	6.7×10^8 x (1.0×10^8) 8	7.4×10^8 x (0.7×10^8) 8
No catalase in sample container:					
ADS without catalase, medium without	4.2×10^7 x (0.9×10^7) 8	8.0×10^7 x (2.0×10^7) 8	3.3×10^8 x (0.7×10^8) 8	4.6×10^8 x (0.9×10^8) 8	5.5×10^8 x (0.8×10^8) 8
ADS without catalase, medium with	6.9×10^7 y (1.3×10^7) 8	1.2×10^8 y (0.2×10^8) 8	4.4×10^8 y (0.8×10^8) 8	5.6×10^8 x (1.0×10^8) 8	6.2×10^8 x (0.8×10^8) 8

a,b/ See Table 1.

It is well established that minute amounts of oxygen are extremely toxic to the obligately anaerobic population of sewage. It is, therefore, mandatory that all precautions possible be taken to exclude oxygen during all phases of sampling and subsequent handling.

Samples should be taken from the digester as directly as possible with minimum exposure to air, and collected in thermos bottles flushed with oxygen-free nitrogen. These containers should have a tight seal which excludes entry of air but provides for simultaneous release of gas (Bunsen valves) which is inevitably produced by any active sludge sample. At the time of sampling, we recommend addition of sufficient reduced Na_2S-cysteine ·HCl solution to give a final concentration 0.025% of each reductant in the sample.

After transport to the laboratory, the thermos bottles should be inverted several times to insure mixing of contents. The sample is then opened, blanketed with oxygen-free CO_2-H_2 (50-50) or N_2 and a 200-ml sample is measured into a sterile stainless steel blender cup which has been freed of oxygen by gassing prior to addition of sample. The sample is blended for a 2-min interval, with gassing. The blended sample is then serially diluted in anaerobic dilution solution containing clarified sludge supernatant, and suitable dilutions are inoculated into anaerobic media for enumeration of obligate anaerobes. The blendor container should be one which can be tightly closed to exclude entry of air, and should have an opening at the top just sufficient to allow entry of the gassing needle. Gas flow during blending should be sufficient to maintain positive pressure in the blender cup and exclude entry of air.

Regardless of improvements in techniques, any method involving enumeration of the obligate anaerobes in sewage sludge will involve sample collection, homogenization, and subsequent dilution of samples for inoculation into media for counting. We therefore believe that effort along the lines of establishing methods which will result in obtaining higher and more consistent total counts has been worthwhile.

PHASE II - DEVELOPMENT OF A SIMPLIFIED TECHNIQUE FOR ENUMERATION OF THE OBLIGATE ANAEROBES IN SEWAGE SLUDGE

The techniques most commonly used for the enumeration of obligate anaerobes generally involve some modification of the anaerobic jar technique, glove box procedures or the roll-tube technique. These techniques all suffer one or more of the following disadvantages: they are slow; they require a high degree of manual dexterity; they require large enclosures in which oxygen-free conditions are difficult and expensive to maintain for long periods of time; the mechanics of sample preparation and plating procedures are difficult, and require long training periods for proficiency; they cannot be handled routinely by unskilled personnel; or the hazard in their use is too great for routine usage.

The anaerobic jar technique involves the growth of anaerobes on agar plates in a closed container (usually a cylindrical jar in which plates are stacked) in which oxygen is removed by alternate evacuation and gas flushing, combustion, or by chemical agents. Although anaerobic conditions can be maintained for long periods of time, once established, the method is only of value if plates to be placed in the jars, and the dilutions used in the plating procedure, are prepared in an oxygen-free atmosphere.[15] Even if this were accomplished, the jars would need to be opened and closed several times to perform plate counts. The plate counts themselves would have to be performed in an inert atmosphere to avoid contact with air during the counting procedure. These limitations make the anaerobic-jar technique of little value in enumerating obligately anaerobic bacteria.

Anaerobic glove box procedures utilize various types and sizes of enclosures, a common characteristic of which is that they contain an oxygen-free environment accessible from the outside by one or more pairs of flexible gloves. All manipulations are performed in the oxygen-free environment, from outside the chamber, by use of these gloves.

Various glove boxes which have been designed specifically for the culture of anaerobes are described in the literature.[16-18] With the exception of the most recent design by Aranki, et al.,[19] these glove boxes suffer from the disadvantages of complexity, expense, elaborate setup procedures, and failure during long-term operations, or are beyond the technical competence of the average bacteriological technician. The glove box described by Aranki is an exception in that it is inexpensive to build, simpler to operate, and can be maintained over long periods of time. This is, however, a design which is best suited to the isolation and study of organisms with generation times sufficiently short that discrete colonies can be detected (a week or less) before the plates evaporate to the point that they will no longer support growth. A decided advantage of the method is that plates do

not become wet, due to condensation in a high humidity, causing the severe swarming encountered with other methods. Although this method should definitely be considered in future isolation and nutritional studies of the obligate anaerobes from sewage sludge, we could not consider use of this technique because of the large number of different experiments which had to be performed, the space required for large numbers of plates which would have to be stored in these chambers for extended periods of time, the necessity of long incubation periods (up to 35 days), and the need to use various gas mixtures without the need for additional sets of equipment for each atmosphere.

The roll-tube technique as originally described by Hungate is the anaerobic equivalent of the "pour plate" method of enumerating bacteria. The name is derived from the method of preparing "plates" by rolling inoculated, liquified agar medium in a rubber-stoppered test tube until solidification occurs on the walls of the tube. Exclusion of air during preparation and tubing of media or transfer of media and specimens is accomplished by introducing a stream of sterile, oxygen-free gas whenever a stopper is removed. The method is simple in principle, but very complex in operation compared to conventional bacteriological procedures. The complexity of the method arises from the necessity of a simultaneous handling of gassing needles, tubes, pipettes, and rubber stoppers during transfer of materials.

The most severe disadvantage to the roll-tube technique is the hazard involved in the numerous manipulations which must be performed by hand in the preparation of media, ADS, and the inoculation of the roll-tubes themselves. From the time of initial sterilization and gassing of the tubes to the final inoculation and rolling of the tube, each tube is opened no less than four times with gassing under aseptic conditions. In addition, each tube of the dilution series is opened and closed a minimum of five times. At each interval, the danger of breakage is encountered. Due to the pressure and twisting motion which must be applied to seat the rubber stopper sufficiently to prevent entry of oxygen and the danger of tube breakage when the gassing needle is withdrawn, there is severe cumulative danger involved in the technique. The hazard is not only very real, but an accident also becomes more likely as one becomes more proficient and at ease with the method.

In spite of this, we adopted the roll-tube method for the initial studies because of its flexibility and proven reliability for growing fastidious anaerobic bacteria. We envisioned that, after completion of Phase I, we would attempt to improve the method to a point that it could be simply and safely handled by personnel with a minimum of bacteriological training. It is unfortunate that these attempts were not begun before an accident occurred involving the roll-tube technique.

The accident, which involved a rather serious laceration of fingers during seating of rubber stoppers, resulted in immediate cessation of use of the technique and initiation of work to eliminate direct handling of tubes.

The method finally adopted is a modification of that developed by Dr. Paul Smith after a similar accident, of a more serious nature, which occurred in his laboratory during the use of the Hungate technique. The method consists of preparation of media in stoppered tubes which are sterilized in clamps to prevent blowing of stoppers when the autoclave is exhausted. All subsequent operations are performed by syringe inoculation through the rubber stopper rather than opening and closing of tubes several times during the course of an experiment. The inoculating press shown in Figures 2 and 3 was assembled and is used to hold the test tubes, while the syringe needle is forced through the stopper by means of mechnical pressure. This technique eliminates the opening and closing of tubes, eliminates the handling of tubes during manipulation, results in greater safety for laboratory personnel without sacrificing accuracy and flexibility, and very significantly simplifies the procedure for media preparations and roll-tube inoculation. A complete description of the method follows.

Modified Roll-Tube Procedures

Media and dilution solutions are prepared in basically the same manner as for the Hungate technique, except that the medium or ADS is tubed directly from the flask before sterilization. All ingredients except cysteine·HCl·H_2O, $NaHCO_3$, and $Na_2S·9H_2O$ are brought into solution in a round-bottom flask. ($NaHCO_3$ was substituted for Na_2CO_3 because equilibration with CO_2 is much more rapid.) Cysteine is added and the mixture is heated with gassing until reduction is complete (resazurin $\longrightarrow$ colorless). The flask is cooled (only to 48°C if agar is included), and the proper amount of $NaHCO_3$ is added to buffer the mediam at pH 6.8. The amount of $NaHCO_3$ required is 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 are complete, the medium is dispensed directly into tubes by means of a 10-ml Cornwall continuous pipette which is washed with several volumes of boiling water to flush oxygen from the system. A few syringefuls of medium or ADS are wasted in washing the syringe to insure proper flushing and oxygen removal. While the main reservoir is being continuously flushed with oxygen-free gas, the medium or ADS is tubed in multiples of five tubes (each of which is being simultaneously flushed during filling). A series of manifolds were built for this purpose containing five hoses and gassing needles each for use in gassing several tubes or flasks simultaneously. After each series of tubes is gassed for a few seconds, a moistened butyl rubber stopper is inserted as the gassing needle is withdrawn. The procedure is continued in multiples of five until a sufficient number of tubes have been prepared. The prepared medium is then clamped in a jig which serves to give the tubes a final seating and holds them securely during sterilization and exhausting of the autoclave. Since butyl rubber has an extremely low permeability for oxygen, the medium

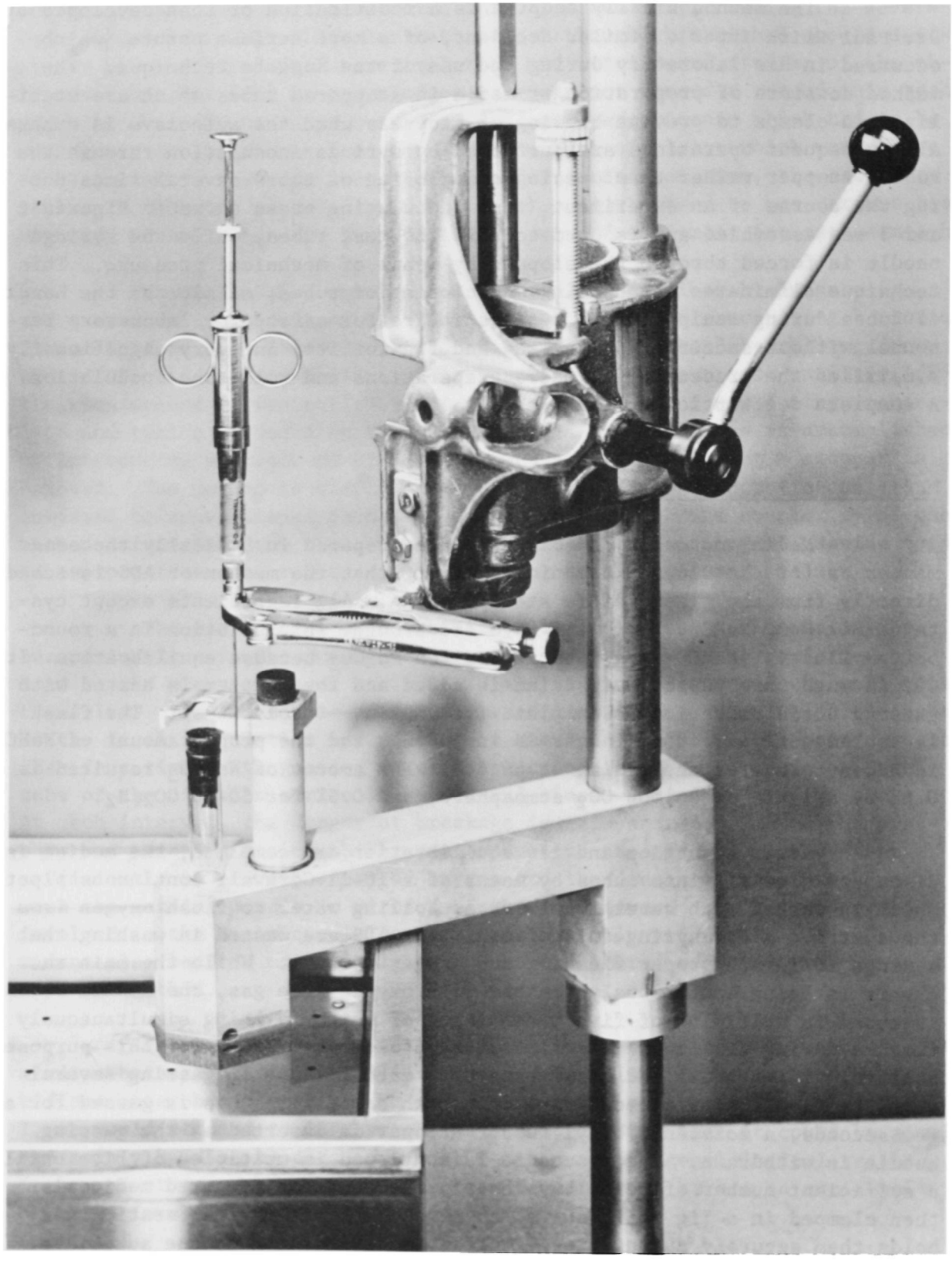

Figure 2 - Overall View of Inoculating Press and Roll-Tube Jig

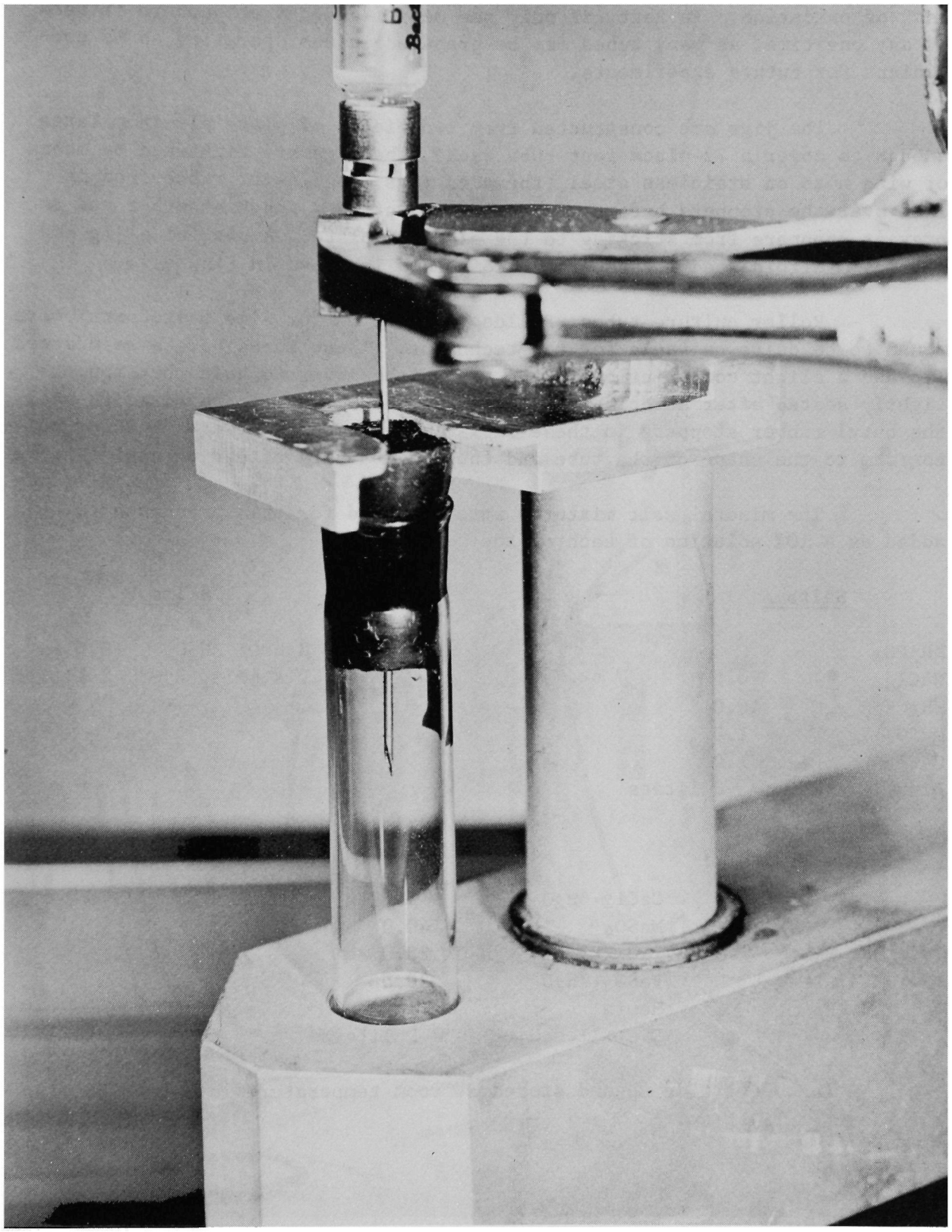

Figure 3 - Closeup of Roll-Tube Jig with Needle in Position for Inoculation

can be stored in a reduced condition for extended periods of time without fear of oxidation. In fact, if only one medium-and-gas atmosphere is used at any one time, as many tubes may be prepared in one operation as is convenient for future experiments.

The jigs are constructed from two pieces of plate aluminum large enough to cover a 72-place test tube rack. The jigs are tightened by means of wing nuts on stainless steel, threaded rods. A 1/4-in. rubber pad is laid over the stoppers before tightening to act as a shock absorber and to prevent stoppers from sticking to the aluminum plate. A view of a jig and gassing manifold used in preparation of media is shown in Figure 4.

Roller culture tubes (Bellco 16 x 150 mm for size 0 stoppers) were found to be quite suitable for the technique. These tubes have a reinforced lip and a slight constriction at the top which serves to hold the stoppers tightly seated after autoclaving. Very little pressure is required to seal the butyl rubber stoppers in these tubes. The stoppers have a tendency to conform to the shape of the tube and thus form a very effective seal (Figure 2).

The mineral salt mixtures were modified for this procedure to be added as a 10% solution of each of the following:

Salts A

KH_2PO_4	10.0 g
NaCl	20.0
NH_4Cl	10.0
$MgSO_4 \cdot 7H_2O$	1.0
$CaCl_2$	1.0
Distilled H_2O	2 liters

Salts B

$K_2HPO_4 \cdot 3H_2O$	10.0 mg
Distilled H_2O	2 liters

Salts C

$CaCl_2 \cdot 6H_2O$	5.0 mg
$MnSO_4$	40.0
$(NH_4)_6Mo_7O_{24} \cdot 4H_2O$	20.0
$FeSO_4 \cdot 7H_2O$	20.0
Hemin	5.0
Distilled H_2O	2 liters

The solutions were made up and stored at room temperature in 500-ml bottles without sterilization.

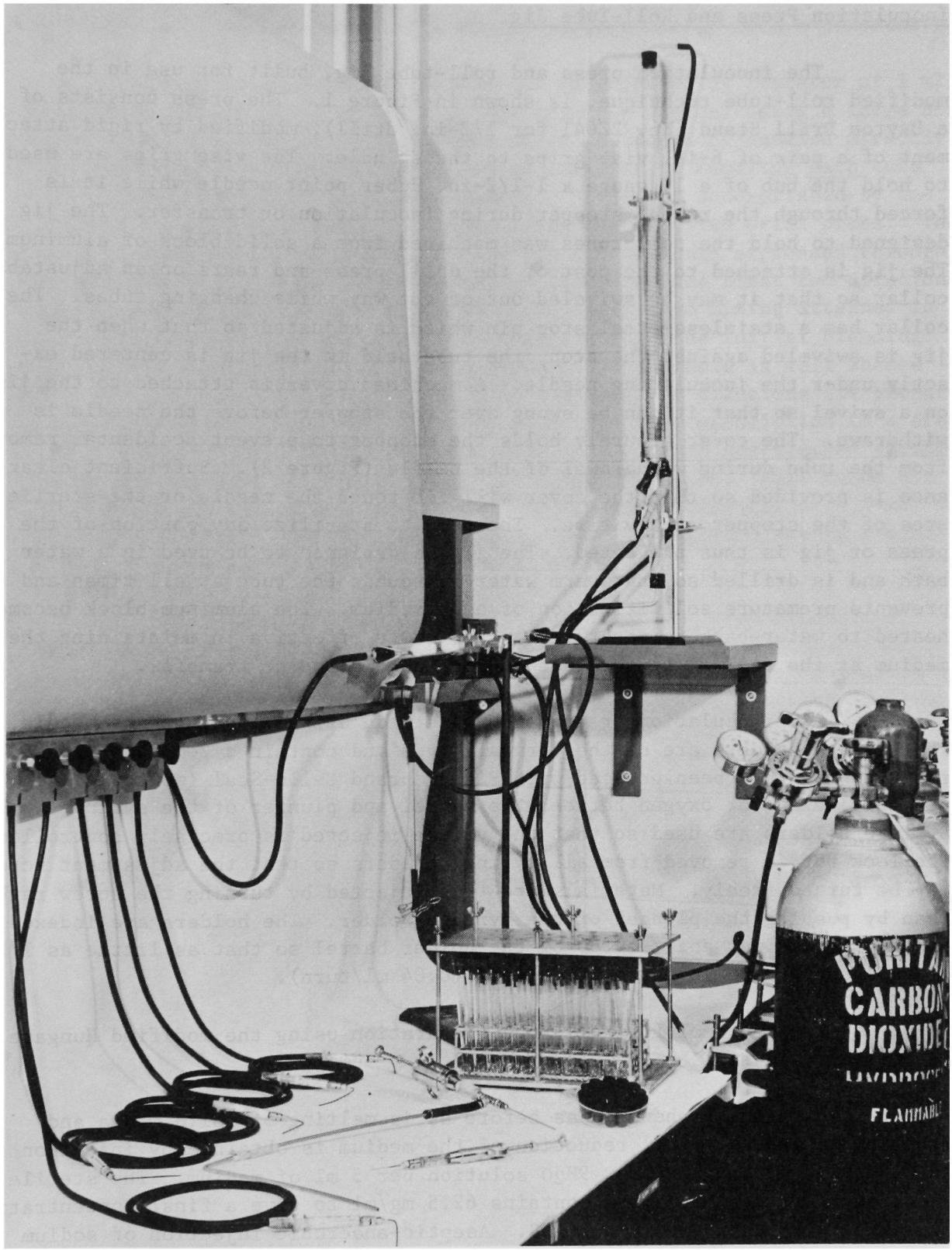

Figure 4 - Clamps, Manifold and Copper Column Used in the Preparation of Media

Inoculation Press and Roll-Tube Jig

The inoculation press and roll-tube jig, built for use in the modified roll-tube technique, is shown in Figure 1. The press consists of a Dayton Drill Stand (No. 2Z041 for 1/2-in. drill), modified by rigid attachment of a pair of 6-in. vise grips to the spindle. The vise grips are used to hold the hub of a 19 gauge x 1-1/2-in. Huber point needle while it is 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 is attached to the post of the drill press and rests on an adjustable collar so that it may be swiveled out of the way while changing tubes. The collar has a stainless-steel stop pin which is adjusted so that when the jig is swiveled against the stop, the tube held in the jig is centered exactly under the inoculating needle. A machined cover is attached to the jig on a swivel so that it can be swung over the stopper before the needle is withdrawn. The cover securely holds the stopper to prevent accidental removal from the tube during withdrawal of the needle (Figure 2). Sufficient clearance is provided so that the cover will not touch the needle or the sterile area of the stopper at any time. The need to sterilize any portion of the press or jig is thus prevented. The jig is designed to be used in a water bath and is drilled so that warm water surrounds the tube at all times and prevents premature solidification of agar medium. The aluminum block becomes heated to water-bath temperature, and is quite effective in maintaining the medium at the desired temperature during inoculation or transfer.

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

The protocol for roll-tube preparation using the modified Hungate technique follows:

Sampling is handled as before as is melting of solid media and labeling of tubes. Final reduction of the medium is obtained by injection of 0.22 ml of a sterile $Na_2S \cdot 9H_2O$ solution per 5 ml of medium. The sterile stock solution of $Na_2S \cdot 9H_2O$ contains 62.5 mg/ml to give a final concentration of 0.025% w/v in the medium or ADS. Aseptic-anaerobic injection of sodium sulfide is accomplished by flushing the syringe several times with sterile, oxygen-free nitrogen by means of a gassing needle in a sterile test tube.

Just prior to injection, the area of the rubber stopper to be contacted by the needle is flamed by means of a CO_2/H_2 gassing needle with a pinpoint flame.

The initial 1/10 dilution in ADS is prepared by transfer of 1 ml of sludge to the first 9 ml blank of the dilution series, using a large bore, gassed pipette. This is the only tube which is opened or handled directly throughout the course of an experiment as compared to handling each tube four or five times by the original method. Mixing is accomplished by inserting a sterile gas-flushed syringe, using the modified drill press. The tube is inverted, the syringe is filled and the contents expressed through the needle several times (a minimum of 10 times for the first two dilutions--five or six for subsequent dilutions). The shear type mixing attained in this manner is surprisingly violent; enough so that the initial blending step can be eliminated by using this method if the sample is well shaken before the initial 1-ml aliquot is taken. Subsequent dilutions are prepared in the same manner by transfering 1 ml from the previous dilution to a fresh blank. Syringes are changed between tubes, and a fresh gas-flushed syringe is used to transfer material to the next higher dilution. Roll tubes are prepared in triplicate from appropriate dilutions by transfer of 0.5 ml. of inocula/tube. Duplicate experiments were usually performed giving a minimun of six roll tubes/dilution/condition tested.

We believe that the technique described is a very significant improvement over that originally used. It is not only much faster (our work output more than doubled with one less technician required), but also is a highly reliable and safe technique requiring very little training for someone with no prior experience with the method. With the exception of the necessity to gas each tube during preparation of media, this technique now compares favorably with the aerobic plate count method with respect to ease of operation and degree of manual skill required.

PHASE III - DEVELOPMENT OF A CULTURE MEDIUM FOR GROWTH OF MICROORGANISMS REPRESENTATIVE OF THE TOTAL BIOMASS IN SEWAGE SLUDGE

All work during this phase was carried out to improve existing media, media supplements, reducing compounds, and gaseous atmospheres such that a maximum total count could be obtained with a simplified medium representative of the sludge environment.

The major problem encountered in development of a medium which will yield a maximum total count of organisms typical of the biomass in sewage sludge is the evaluation of the efficacy of the medium once the medium has been developed. This problem is complicated by the fact that very few organisms have been isolated from anaerobic sludge in pure culture, and the significance of such isolates to the digestion process *per se* has never been established. A true means for evaluation is thus not at hand, and will not be until the medium in question is developed and used to isolate, describe, and assign a role to the major species present in sewage sludge. The solution to this paradox is thus a compromise between the real and the ideal, i.e., to develop a simplified medium which yields a maximum total count and which probably supports the growth of a significant number of predominant species.

Unidentified growth factors are known to exist in rumen fluid which are necessary for the growth of the methane-producing bacteria. Since these factors have never been successfully replaced by other ingredients, rumen fluid is routinely added to media used in the study of rumen microflora. Because of the likelihood that the same, or similar, factor(s) occurs in sewage sludge, sludge supernatant is likewise added to media used in the culture of sludge anaerobes. Various workers do not consistently agree as to whether rumen fluid or sludge supernatant should be used for growth of the obligate anaerobes from sewage sludge. There is also no consistent agreement to the amount of either to be incorporated into media.

The initial experiments in this series were therefore designed to determine which supernatant gives the maximum yield of sludge anaerobes and to determine the optimum amount to be added to culture medium. Experiments were carried out by direct comparison of various percentages of rumen fluid and sludge supernatant using the same basal medium and ADS discussed in Phase I. Rumen fluid or sludge supernatant was added to media to give the following final percentages: 0, 10, 20, 30, and 50.

The results shown in Tables 8 and 9 indicate that there is no significant difference in total count when the optimum concentration of either rumen fluid or sludge supernatant is used. The optimum concentration does, however, vary with 20% indicated as optimum for rumen fluid and 10% for sludge supernatant. These data also confirm the necessity of either

TABLE 8

EFFECT OF SLUDGE SUPERNATANT CONCENTRATION ON TOTAL COLONY COUNT

Percent Clarified Sludge Supernatant in Medium	Days Incubated at 37°C				
	7	14	21	28	35
0	4.4×10^7 x[a/] (0.9×10^7) 8[b/]	9.6×10^7 x (1.1×10^7) 8	2.9×10^8 xy (0.9×10^8) 7	4.2×10^8 x (1.5×10^8) 8	4.7×10^8 x (1.5×10^8) 8
10	5.9×10^7 xy (1.3×10^7) 8	1.2×10^8 y (0.2×10^8) 8	4.2×10^8 y (1.1×10^8) 6	5.7×10^8 x (1.2×10^8) 6	6.5×10^8 z (1.1×10^8) 6
20	6.6×10^7 y (1.0×10^7) 8	1.1×10^8 xy (1.0×10^8) 8	3.0×10^8 xy (1.2×10^8) 8	4.8×10^8 x (1.2×10^8) 8	5.9×10^8 xz (1.1×10^8) 8
30	4.5×10^7 x (0.6×10^7) 8	8.0×10^7 x (0.8×10^7) 8	2.1×10^8 xz (0.7×10^8) 8	4.1×10^8 x (1.4×10^8) 8	4.7×10^8 x (1.6×10^8) 8
50	4.6×10^7 x (2.4×10^7) 8	6.9×10^7 z (2.6×10^7) 8	9.6×10^7 z (3.6×10^7) 8	1.6×10^8 y (0.3×10^8) 8	2.6×10^8 y (0.9×10^8) 8

a,b/ See Table 1.

TABLE 9

EFFECT OF RUMEN FLUID SUPERNATANT CONCENTRATION ON TOTAL COLONY COUNT

Percent Clarified Rumen Fluid Supernatant in Medium	Days Incubated at 37°C				
	7	14	21	28	35
0	2.1×10^7 x[a/]	7.0×10^7 x	2.5×10^8 x	3.9×10^8 xy	4.6×10^8 x
	(1.4×10^7) 8[b/]	(2.8×10^7) 8	(1.2×10^8) 8	(1.4×10^8) 8	(1.2×10^8) 8
10	3.2×10^7 x	7.4×10^7 x	2.9×10^8 x	4.1×10^8 xy	5.1×10^8 xy
	(1.6×10^7) 8	(1.1×10^7) 8	(0.9×10^8) 8	(1.3×10^8) 8	(1.0×10^8) 8
20	2.9×10^7 x	7.0×10^7 x	3.0×10^8 x	5.3×10^8 x	6.4×10^8 y
	(1.8×10^7) 8	(2.8×10^7) 8	(1.9×10^8) 7	(2.1×10^8) 7	(1.8×10^8) 7
30	3.5×10^7 x	6.3×10^7 x	2.0×10^8 x	3.1×10^8 y	4.5×10^8 x
	(1.5×10^8) 8	(1.5×10^7) 8	(1.0×10^8) 8	(1.3×10^8) 8	(1.3×10^8) 8
50	3.1×10^7 x	6.0×10^7 x	2.2×10^8 x	3.7×10^8 xy	4.5×10^8 x
	(1.4×10^8) 8	(1.5×10^7) 8	(0.6×10^8) 8	(1.3×10^8) 8	(1.1×10^8) 7

a,b/ See Table 1.

rumen fluid or sludge supernatant for maximum total counts. Although concentrations over 50% were not used because of the tendency toward excessive foaming of the ADS, it appears that concentrations above the optimum are inhibitory. The significance of this observation remains to be determined.

The method originally used to obtain clarified sludge supernatant, for use as a supplement in media, was an awkward process sometimes requiring up to 3 man-days to process an 8-liter batch. The process involved autoclaving the sludge sample, followed by a filtration step to remove larger particles. Final clarification was achieved by centrifuging in the Sharples until clear. The initial filtration step was often prolonged and messy in that sludge has a tendency to form a solid mat, even on a very porous backing material such as cheesecloth. Several changes of filter were often necessary to filter a batch sufficiently to pass the orifice of the Sharples. In addition, several passes through the Sharples were often necessary to achieve sufficient clarity for use in culture media. In order to improve this procedure, the following method was adopted: freshly collected sludge was autoclaved and a measured quantity was batch-lyophilized (up to 16 liters/batch). The dried product was weighed, mixed thoroughly and stored for future use. As required, small amounts (usually 2-liter batches) of clarified supernatant were prepared by placing a weighed quantity of this material on a filter mat of hyflosupercel and washing the mat with sufficient hot water to restore the original supernatant volume. This supernatant was bottled in 100-ml quantities, autoclaved and stored in the refrigerator for future use. The result is a more consistent CSS which can be prepared, as needed, in a fraction of the time previously required. For convenience and batch consistency, large amounts can be processed at one time and stored in a small space for future use.

Experiments were carried out to compare sludge supernatant prepared as above (aqueous extract of lyophilized sludge, or AELS) with that prepared by filter clarification alone and by filtration plus centrifugation. All supernatants were added to media at the 20% level.

The results of these comparisons are shown in Table 10. It is apparent that both filter-clarified sludge supernatant and AELS give higher counts than "normal" CSS with AELS being significantly higher statistically. It is probable that the difference in the three methods is due to inconsistency in recovery of sludge supernatant by Celite filtration or centrifugation: i.e., the recovery of supplemental ingredients is more complete and consistent when a weighed quantity of well-mixed lyophilized sludge is used to prepare sludge supernatant. This improved method for preparation of sludge supernatant was, therefore, adopted for use in media prepared throughout the remainder of Phase III experiments.

TABLE 10

COMPARISON OF NORMAL SLUDGE SUPERNATANT WITH AQUEOUS EXTRACT OF LYOPHILIZED SLUDGE AND FILTER CLARIFIED SLUDGE SUPERNATANT

	Days Incubated at 37°C				
	7	14	21	28	35
"Normal" CSS	8.7×10^7 x[a/]	1.2×10^8 x	4.6×10^8 x	7.2×10^8 x	8.4×10^8 x
	(3.4×10^7) 8[b/]	(0.2×10^8) 8	(0.8×10^8) 8	(1.8×10^8) 8	(1.3×10^8) 8
Aqueous extract of lyophilized sludge	6.4×10^7 y	2.1×10^8 y	6.9×10^8 y	9.1×10^8 y	9.8×10^8 y
	(2.6×10^7) 8	(0.3×10^8) 8	(1.2×10^8) 8	(1.0×10^8) 8	(0.8×10^8) 8
Filter clarified sludge supernatant	5.6×10^7 y	1.3×10^8 x	5.5×10^8 xy	8.0×10^8 xy	9.0×10^8 xy
	(0.8×10^7) 8	(0.2×10^8) 8	(1.3×10^8) 8	(1.6×10^8) 8	(1.3×10^8) 8

a,b/ See Table 1.

Concurrently with the AELS experiments, tests were run to determine the feasibility of using supernatant fractions or media supplements rather than crude sludge supernatant. The following comparisons were made: medium with no clarified sludge supernatant (CSS); medium with 30% CSS; medium with 30% Norit-treated sludge supernatant; medium with 30% Norit extract and medium with 30% CSS prepared from sludge preincubated with 0.4% yeast extract.

Norit is reported to adsorb the factor(s) present in rumen fluid and can be eluted with 0.1 M ethanolic ammonium hydroxide following a hot water wash (see Figure 3, page 29). The Norit supernatant used in these experiments was prepared by treating CSS with Norit followed by filtration. This filtrate was incorporated directedly into the medium.

Norit extract was prepared by extracting the washed residue with 0.1 M ethanolic ammonium hydroxide. This solution was evaporated to a small volume under vacuum to remove ethanol and ammonia. Following reconstitution to the original CSS volume, the extract was added to the medium at a final concentration of 30%.

Preliminary work by Dr. Bryant indicates that preincubation of rumen fluid with 0.4% yeast extract results in increased rumen fluid factor concentration.

To test this effect with sludge supernatant, sludge was preincubated 40 hr under nitrogen with 0.4% yeast extract. The sludge was then sterilized and CSS was prepared and added to the culture medium to give a final concentration of 30%.

The results of these comparisons are shown in Table 11. Lower colony counts are again indicated for medium without CSS, than for medium containing CSS. Although counts were higher for Norit-treated supernatant than for the control, there is an indication that something has been removed by the Norit. The Norit extract yields counts significantly lower than the control (no CSS). This is rather difficult to explain unless some toxic material was present in the Norit which was eluted with the ethanolic ammonium hydroxide or other nutrients present in the crude supernatant are missing. The latter is a distinct possibility.

Another possible explanation might be that certain species of sludge bacteria are selectively inhibited by the eluted material. The present state of knowledge of the species involved would not allow further clarification of this observation. It is possible that in the future when the sludge factor(s) is identified and the dominant species of sewage sludge bacteria have been classified, it will be possible to design media and select organisms to study such problems further. Further studies of supernatant fractions at this time, however, would be of questionable value.

TABLE 11

EFFECT OF TREATMENT OF CSS ON TOTAL COLONY COUNT

	Days Incubated at 37°C				
	7	14	21	28	35
ADS and Medium with:					
No CSS	3.4×10^7 x[a/]	7.5×10^7 x	3.9×10^8 x	4.7×10^8 x	5.0×10^8 x
	(0.9×10^7) 8[b/]	(0.8×10^7) 8	(1.4×10^8) 8	(1.3×10^8) 8	(1.3×10^8) 8
Filter clarified CSS (30%)	5.2×10^7 y	1.1×10^8 y	4.8×10^8 x	6.1×10^8 x	6.8×10^8 y
	(1.5×10^7) 15	(0.1×10^8) 16	(1.1×10^8) 16	(1.3×10^8) 16	(1.2×10^8) 16
Norit-treated supernatant (30%)	4.1×10^7 xy	8.0×10^7 x	4.2×10^8 x	5.5×10^8 x	6.0×10^8 xy
	(1.3×10^7) 8	(1.6×10^7) 8	(1.3×10^8) 8	(1.2×10^8) 7	(1.0×10^8) 7
Norit extract (30%)	3.9×10^7 xy	6.9×10^7 x	1.3×10^8 y	2.8×10^8 y	3.3×10^8 z
	(1.0×10^7) 8	(1.1×10^7) 7	(0.3×10^8) 7	(0.4×10^8) 8	(0.4×10^8) 8
CSS prepared from sludge (30%) pre-incubated with 0.4% yeast extract	5.2×10^7 y	7.1×10^7 x	1.7×10^8 y	4.5×10^8 x	5.4×10^8 xy
	(1.1×10^7) 8	(0.9×10^7) 8	(0.2×10^8) 8	(1.4×10^8) 8	(1.4×10^8) 8

a,b/ See Table 1.

Development of a Habitat Simulating Medium for Sludge Anaerobes

A series of experiments was carried out to develop and evaluate a medium which would, hopefully, simulate the sludge environment, as closely as possible in solid medium. Such a medium would yield a maximum total count representative of the biomass in sewage sludge. It must, of course, be realized that any medium developed for a colony count cannot completely duplicate the sludge environment for three primary reasons. First, the sludge environment is essentially a continuous liquid culture, whereas the medium used for total count is by necessity a solid medium in which discrete colonies must form. Because of the inability of organisms to migrate throughout the medium, there is much less opportunity for the numerous symbiotic relationships to occur which must surely develop in the natural environment; hence the possibility of not growing some species which require relationships with other species. Second, a medium used for enumeration must be rather transparent and free of particulate matter. This is another strict imposition in that sewage sludge is quite turbid with much more insoluble material available for surface attack and surface adsorption. Finally, the gaseous atmosphere is difficult to simulate in the closed system used for enumeration. Once the system is closed, it cannot be reopened to replenish the gaseous atmosphere; therefore any required gases must be initially present in sufficient quantity to sustain growth. In the case of the methanogens, gaseous hydrogen must be supplied for growth to compensate for metabolic hydrogen which would be scavenged by these organisms in the sludge environment. A higher than normal H_2 concentration is thus obligatory, which could be toxic to certain nonmethanogenic species.[8]

The approach used was to begin with a rather complete medium, then to add or delete ingredients until a medium was found which supported the most growth. As mentioned above, there is a definite limitation to those nutrients which are water soluble so that a transparent medium is obtained without floccules which would interfere with colony counts. Gelatin was selected as a protein source; soluble starch as a source of polysaccharide and cellobiose (repeating unit of cellulose) for cellulose. Sewage sludge is generally high in suspended lipids which would be difficult to simulate under culture conditions. Glycerol and a mixture of short chain, volatile fatty acids (acetate, propionate, butyrate, isobutyrate, valerate, isovalerate, 2-methylbutyrate, and formate) were, therefore, substituted as a soluble "lipid" source. Trypticase and yeast extract was included as a source of peptides, amino acids and vitamins and AELS was included as a source of supplementary nutrients.

The final ingredients selected for the initial habitat, simulating medium, consisted of the following: (designated HSM-1) CSS, 20% v/v; mineral solution, 10% v/v each of salts A, B, and C; gelatin, 0.1% w/v; starch, 0.1%; glycerol, 0.1%; cellobiose, 0.05%; trypticase, 0.05%; yeast extract, 0.05%; volatile fatty acid mixture, 0.32%; 0.025%, cysteine-Na_2S as reductants; and CO_2 equilibrated $NaHCO_3$ as buffer.

For initial comparisons roll tubes were prepared in triplicate using suitable dilutions of anaerobic sludge in the following media: normal (medium previously used in Phase I), HSM, HSM without volatile fatty acids plus acetate and formate, HSM-1 without glycerol, HSM without gelatin, and HSM without starch. Two sets of each medium were prepared--one with 100% CO_2 gas phase and the other with 50-50 CO_2/H_2 gas phase.

Tables 12 and 13 show the results of total colony counts obtained from the initial various modifications of HSM. All experiments were run using "normal" medium as the control. Except for replacement of casamino acids with trypticase and reduction of CSS from 30% to 20%, this medium is the same as that described in Phase I which was used for sampling and handling studies. The counts obtained with the basic HSM were the highest obtained to this time. Of all individual ingredients of this medium tested, starch seems to be most important. Single deletion of VFA (volatile fatty acids), glycerol, and gelatin did not markedly affect the total count. Higher counts were obtained with 100% CO_2 than with CO_2/H_2 mixture--a consistent observation in subsequent experiments.

In addition to testing various nutrients which might affect total colony counts, we also tested essential nonnutrient factors in the medium which might have direct relationship to growth such as reducing agents and buffer systems.

With the exception of oxygen exclusion, perhaps the most important parameter for growth of the obligate anaerobes is a low oxidation-reduction (O-R) potential. It has been shown that an O-R potential of -520 to -530 mv is optimum for anaerobic sludge digestion.[21] It is important to note that methane production decreases significantly and the digestor approaches failure as the O-R potential is increased to -430 mv. Only with extreme care is it possible to obtain an O-R potential, in culture media, of -350 mv or below. It follows that if the O-R potential could be reduced routinely beyond that obtained with reductants currently in use, which exhibit little or no toxicity to sludge anaerobes, colony counts in excess of those previously reported might be obtained. It is also possible that many of the more fastidious anaerobes have never been grown in routine roll-tube cultures and that more highly reduced media would give a more representative picture of the obligate anaerobic flora in sewage sludge.

Four reductants were selected for evaluation: formamidine sulfinic acid, formamidine disulfide dihydrochloride, dithiothreitol, and formaldehyde sulfoxylate.

Preliminary tests showed that formamidine disulfide was unstable to heating and exhibited considerable toxicity to dilute broth cultures of

TABLE 12

COMPARISON OF VARIOUS MODIFICATIONS OF HSM TO CONTROL MEDIUM WITH A 50-50 CO_2/H_2 ATMOSPHERE

	Days Incubated at 37°C				
	7	14	21	28	35
Control	6.5×10^7 x[a/] (3.0×10^7) 11[b/]	1.3×10^8 x (0.5×10^8) 12	3.9×10^8 x (2.6×10^8) 12	7.5×10^8 x (2.2×10^8) 12	9.1×10^8 x (2.1×10^8) 12
HSM	8.8×10^7 xz (0.8×10^7) 6	1.3×10^8 x (0.1×10^8) 6	4.0×10^8 x (2.4×10^8) 6	8.6×10^8 xy (1.5×10^8) 6	1.1×10^9 x (0.1×10^9) 6
HSM with 0.1% acetate and 0.1% formate, without VFA	1.3×10^8 z (0.1×10^8) 3	1.7×10^8 x (0.3×10^8) 3	8.6×10^8 y (1.7×10^8) 3	1.1×10^9 xy (0.2×10^9) 3	1.2×10^9 x (0.1×10^9) 3
HSM with acetate and formate	7.8×10^7 x (1.1×10^7) 3	1.2×10^8 x (0.1×10^8) 3	3.1×10^8 x (0.2×10^8) 3	7.5×10^8 x (1.7×10^8) 3	1.0×10^9 x (0.1×10^9) 3
HSM without glycerol	6.1×10^7 x (4.1×10^7) 6	1.1×10^8 x (0.3×10^8) 6	2.0×10^8 x (0.9×10^8) 6	5.6×10^8 x (5.2×10^8) 6	8.9×10^8 x (4.2×10^8) 5
HSM without gelatin	6.4×10^7 x (4.3×10^7) 6	1.1×10^8 x (0.2×10^8) 6	2.1×10^8 x (0.8×10^8) 6	5.7×10^8 x (4.4×10^8) 6	8.6×10^8 x (4.3×10^8) 6
HSM without starch	4.3×10^7 y (2.7×10^7) 6	1.1×10^8 x (0.5×10^8) 6	1.6×10^8 x (0.6×10^8) 6	2.8×10^8 y (1.4×10^8) 6	4.9×10^8 y (1.3×10^8) 6

a,b/ See Table 1.

TABLE 13

COMPARISON OF VARIOUS MODIFICATIONS OF HSM TO CONTROL MEDIUM WITH A 100% CO_2 ATMOSPHERE

	Days Incubated at 37°C				
	7	14	21	28	35
Control	6.4×10^7 xy[a/] (1.9×10^7) 12[b/]	3.1×10^8 x (2.2×10^8) 12	8.4×10^8 x (2.5×10^8) 12	1.0×10^9 y (0.2×10^9) 12	1.3×10^9 xy (0.2×10^9) 12
HSM	1.0×10^8 x (0.1×10^8) 6	5.0×10^8 x (4.0×10^8) 6	1.3×10^9 z (0.2×10^9) 6	1.6×10^9 x (0.2×10^9) 6	1.7×10^9 z (0.2×10^9) 6
HSM with 0.1% acetate and 0.1% formate, without VFA	1.1×10^8 xz (0.2×10^8) 3	1.1×10^9 y (0.1×10^9) 3	1.4×10^9 z (0.2×10^9) 3	1.5×10^9 x (0.2×10^9) 3	1.5×10^9 xz (0.2×10^9) 3
HSM with acetate and formate	1.2×10^8 z (0.1×10^8) 3	1.5×10^8 x (0.1×10^8) 3	1.2×10^9 xz (0.0×10^9) 3	1.4×10^9 x (0.1×10^9) 3	1.5×10^9 xz (0.1×10^9) 3
HSM without glycerol	5.9×10^7 x (3.8×10^7) 6	3.5×10^8 x (3.4×10^8) 3	1.0×10^9 xz (0.5×10^9) 5	1.4×10^9 x (0.4×10^9) 5	1.5×10^9 xz (0.3×10^9) 5
HSM without gelatin	6.9×10^7 x (3.6×10^7) 6	4.8×10^8 x (4.0×10^8) 6	1.1×10^9 xz (0.4×10^9) 6	1.4×10^9 x (0.2×10^9) 6	1.6×10^9 z (0.2×10^9) 6
HSM without starch	3.9×10^7 y (2.4×10^7) 6	1.3×10^8 x (0.7×10^8) 6	7.0×10^8 x (3.2×10^8) 6	9.9×10^8 y (1.8×10^8) 6	1.1×10^9 y (0.1×10^9) 5

a,b/ See Table 1.

sludge organisms incubated under anaerobic conditions. Formamidine disulfide dihydrochloride was ineffective at physiological pH's. Of the remaining reductants, dithiothreitol (DTT) appeared most promising because of its rapid reaction time, stability to oxygen, and the very low O-R potentials possible with this compound. At concentrations of 0.025% to 0.05% (w/v), the O-R potential obtained in culture media was sufficiently low to easily reduce benzyl viologen (lower than -359 mv). With the addition of 0.025% cysteine and 0.025% to 0.05% DTT, methyl viologen was easily reduced (lower than -446 mv). DTT was evaluated for toxicity by comparison with normal reductants (cysteine-sulfide) using the roll-tube technique. Levels of 0.01, 0.025, and 0.05% DTT alone and 0.01, 0.025, and 0.05% DTT with 0.025% cysteine were tested using the same medium with normal reductants as controls.

The results of evaluation of dithiothreitol as a reductant are shown in Tables 14 and 15. The results indicate that this compound is too toxic to be of value in increasing colony counts by replacement of either or both $Na_2S \cdot 9H_2O$-cysteine·HCl as reductants. This is not to say that organisms requiring a very low O-R potential are not enriched to the exclusion of other species; however, any differences simply are not expressed in terms of increased total counts. Further elucidation would be beyond the scope of this work.

Table 16 shows the effect of sodium formaldehyde sulfoxylate (NaFS) on total anaerobic counts when NaFS is used as the final reductant (as a replacement for $Na_2S \cdot 9H_2O$). This compound was tested because, unlike $Na_2S.9H_2O$ solution, it is stable in air and can be added directly to the medium at the time of preparation. Basal HSM was used in these experiments with basal HSM plus cysteine-sulfide as the control. NaFS was tested under both N_2/CO_2 and H_2/CO_2 atmospheres.

The results indicate that at 0.1% w/v NaFS compares quite favorably with sulfide, but does not yield higher total counts. We, therefore, chose to continue using $Na_2S \cdot 9H_2O$ as the final reductant although NaFS might serve as well. A definite advantage in the use of NaFS (0.1%) would be the elimination of addition of Na_2S after media preparation. When the point is reached that a dehydrated synthetic medium is possible, then NaFS should be considered further as a replacement for sulfide.

Effect of Sodium Deletion on Total Colony Counts

Some workers have indicated that high sodium ion concentration may be toxic to sludge anaerobes. Although effects have been shown using abnormally high concentrations of sodium, we were concerned that the levels of $NaHCO_3$ used to buffer the medium, in addition to the salt added to the mineral salts mixture, might lead to some reduction in total counts. As shown in Table 17, the levels of sodium used do not appear to be a problem in normal medium buffered with $NaHCO_3$.

TABLE 14

EVALUATION OF DITHIOTHREITOL (DTT) AS A REPLACEMENT FOR SODIUM SULFIDE

	Days Incubated at 37°C				
	7	14	21	28	35
Normal medium with 0.025% cysteine, 0.025% $Na_2S \cdot 9H_2O$	4.3×10^6 x[a/]	2.2×10^7 x	4.6×10^7 x	5.3×10^7 xy	1.9×10^8 x
	(3.2×10^6) 6[b/]	(0.9×10^7) 6	(1.2×10^7) 6	(1.3×10^7) 6	(0.4×10^8) 6
Normal medium with 0.025% cysteine, 0.01% DTT	1.2×10^7 y	3.4×10^7 y	5.7×10^7 xy	7.0×10^7 x	1.8×10^8 x
	(0.7×10^7) 6	(1.0×10^7) 6	(1.9×10^7) 6	(1.7×10^7) 6	(0.6×10^8) 6
Normal medium with 0.025% cysteine, 0.025% DTT	5.7×10^6 x	2.1×10^7 x	4.6×10^7 x	5.0×10^7 y	6.2×10^7 y
	(3.2×10^6) 6	(0.5×10^7) 6	(0.5×10^7) 6	(0.7×10^7) 6	(1.5×10^7) 6
Normal medium with 0.025% cysteine, 0.05% DTT	7.7×10^6 xy	3.0×10^7 xy	6.7×10^7 y	7.0×10^7 x	7.8×10^7 y
	(3.7×10^6) 6	(0.6×10^7) 6	(1.4×10^7) 6	(1.4×10^7) 6	(1.9×10^7) 6

a,b/ See Table 1.

TABLE 15

EVALUATION OF DITHIOTHREITOL (DTT) AS A REPLACEMENT FOR SODIUM SULFIDE AND CYSTEINE

	Days Incubated at 37°C				
	7	14	21	28	35
Normal medium with 0.025% cysteine, 0.025% $Na_2S \cdot 9H_2O$	1.8×10^7 x[a/] (0.3×10^7) 6[b/]	4.6×10^7 x (0.4×10^7) 6	6.9×10^7 x (0.5×10^7) 6	9.2×10^7 x (0.7×10^7) 6	2.9×10^8 x (0.2×10^8) 6
Normal medium + 0.01% DTT, no cysteine	2.0×10^7 x (0.6×10^7) 6	6.0×10^7 y (0.5×10^7) 6	7.3×10^7 x (1.0×10^7) 6	8.6×10^7 x (0.8×10^7) 6	1.9×10^8 y (0.4×10^8) 6
Normal medium + 0.025% DTT no cysteine	3.5×10^7 y (0.6×10^7) 6	6.2×10^7 y (0.6×10^7) 6	7.0×10^7 x (0.6×10^7) 6	8.7×10^7 x (0.7×10^7) 6	1.7×10^8 yz (0.2×10^8) 6
Normal medium + 0.05% DTT, no cysteine	3.2×10^7 y (0.6×10^7) 6	6.7×10^7 y (0.8×10^7) 6	7.4×10^7 x (0.7×10^7) 6	8.5×10^7 x (1.4×10^7) 6	9.1×10^7 z (1.4×10^7) 6

a,b/ See Table 1.

TABLE 16

EFFECT OF SODIUM FORMALDEHYDE SULFOXYLATE (NaFS) ON TOTAL ANAEROBIC COLONY COUNT

Gas	Medium	Days Incubation at 37°C: 7	14	21	28	35
H_2/CO_2 50-50	Basal with cysteine-Na_2S	4.4×10^7 x[a/]	7.5×10^7 x	2.8×10^8 x	6.8×10^8 x	8.9×10^8 x
		(1.1×10^7) 6[b/]	(3.7×10^7) 6	(0.9×10^8) 6	(0.7×10^8) 6	(0.8×10^8) 6
Without Na_2S	Basal + 0.01% NaFS	4.2×10^7 xy	6.4×10^7 xy	1.6×10^8 xy	4.1×10^8 x	5.8×10^8 x
		(1.1×10^7) 6	(1.6×10^7) 6	(0.9×10^8) 6	(0.9×10^8) 6	(0.6×10^8) 6
	Basal + 0.02% NaFS	2.5×10^7 yz	4.1×10^7 yz	5.2×10^7 y	6.1×10^7 y	7.2×10^7 y
		(0.9×10^7) 6	(1.7×10^7) 6	(1.7×10^7) 6	(2.0×10^7) 6	(2.2×10^7) 6
	Basal + 0.04% NaFS	1.5×10^7 z	2.9×10^7 z	4.0×10^7 y	5.1×10^7 y	5.5×10^7 y
		(0.7×10^7) 6	(1.2×10^7) 6	(1.5×10^7) 6	(0.4×10^7) 2	(0.9×10^7) 2
N_2/CO_2 50-50	Basal with cyteins-Na_2S	5.7×10^7 x	2.1×10^8 x	8.0×10^8 x	9.5×10^8 x	1.0×10^9 x
		(1.6×10^8) 6	(1.5×10^8) 6	(1.8×10^8) 6	(1.6×10^8) 6	(0.1×10^9) 8
Without Na_2S	Basal + 0.01% NaFS	5.1×10^7 x	1.8×10^8 xy	6.9×10^8 xy	8.6×10^8 x	9.2×10^8 x
		(1.9×10^7) 6	(1.3×10^8) 6	(1.7×10^8) 6	(2.1×10^8) 6	(2.3×10^8) 8
	Basal + 0.02% NaFS	3.8×10^7 xy	7.0×10^7 xy	6.2×10^8 xy	8.3×10^8 x	9.2×10^8 x
		(1.5×10^7) 6	(3.1×10^7) 7	(2.1×10^8) 6	(1.6×10^8) 6	(1.6×10^8) 6
	Basal + 0.04% NaFS	2.7×10^7 y	4.5×10^7 y	5.0×10^8 y	8.5×10^8 x	9.5×10^8 x
		(0.5×10^7) 6	(0.9×10^7) 6	(1.8×10^8) 5	(1.5×10^8) 5	(1.6×10^8) 5

a,b/ See Table 1.

TABLE 17

EFFECT OF SODIUM DELETION ON TOTAL COLONY COUNT

Buffer	Medium	Days Incubated at 37°C				
		7	14	21	28	35
$NaHCO_3$	HSM-1 without minerals	2.6×10^7 x[a/] (1.1×10^7) 6[b/]	5.3×10^7 x (1.2×10^7) 6	6.4×10^7 x (1.8×10^7) 6	1.9×10^8 x (1.3×10^8) 6	4.1×10^8 x (2.1×10^8) 6
	HSM-1 with low sodium	2.2×10^7 x (0.8×10^7) 6	5.8×10^7 x (1.1×10^7) 6	7.7×10^7 x (1.0×10^7) 6	3.8×10^8 y (3.1×10^8) 5	7.2×10^8 y (1.5×10^8) 5
	HSM-1 with normal minerals	2.2×10^7 x (0.5×10^7) 6	4.9×10^7 x (0.6×10^7) 6	6.4×10^7 x (0.8×10^7) 6	4.2×10^8 y (2.4×10^8) 6	7.6×10^8 y (1.8×10^8) 6
$KHCO_3$	HSM-1 without minerals	2.6×10^7 x (0.9×10^7) 6	4.6×10^7 x (0.6×10^7) 6	6.2×10^7 x (1.4×10^7) 6	1.5×10^8 x (0.7×10^8) 6	5.8×10^8 x (1.3×10^8) 6
	HSM-1 with low sodium	2.6×10^7 x (1.1×10^7) 6	5.5×10^7 x (1.8×10^7) 6	9.4×10^7 y (2.1×10^7) 5	3.2×10^8 y (2.2×10^8) 5	7.5×10^8 x (3.2×10^8) 6
	HSM-1 with normal minerals	2.0×10^7 x (1.2×10^7) 6	5.4×10^7 x (2.3×10^7) 6	7.3×10^7 xy (1.9×10^7) 6	2.2×10^8 xy (1.1×10^8) 6	5.9×10^8 x (1.2×10^8) 6

a,b/ See Table 1.

The use of $KHCO_3$ buffer with low sodium indicated no change in counts as compared to the same condition with $NaHCO_3$ buffer. There may, however, be some antagonism due to K^+ if $KHCO_3$ is used to buffer the HSM when normal minerals are used.

Effect of Cellobiose on Total Colony Counts Obtained with HSM

Table 18 shows the effect of deletion of cellobiose and the effect of increased concentration of cellobiose when compared to the normal level (0.05%) in HSM. The results indicate that there is no significant change in total colony counts (a) with or without cellobiose and (b) either a CO_2 or CO_2/H_2 atmosphere. Cellobiose was therefore excluded from media prepared in subsequent testing of HSM.

The effect of deletion of the volatile fatty acid mixture from HSM is shown in Table 19. There appears to be no significant change in colony count for any condition tested for either gaseous atmosphere. For these experiments, the complete HSM, as previously described, was used (page 42).

Tables 20 and 21 show the results of deletion of both yeast extract and trypticase from the complete HSM medium. As indicated, the deletion of yeast extract and trypticase had no significant effect on total colony counts. There was, however, a significant decrease in total colony count, with an H_2/CO_2 atmosphere, when trypticase was deleted and yeast extract concentration was doubled. This effect was not exhibited with a 100% CO_2 atmosphere.

To this point it appeared that with the exception of starch, deletion of various ingredients from HSM had very little effect on the total growth obtained. It thus appears that a rather simple medium containing AELS might yield higher counts for methane bacteria than a complex medium such as complete HSM. In addition to obtaining higher counts for the methanogenic species, it also appeared that a simpler medium might support the growth of nonmethanogens as well as complete HSM. Table 22 shows the results of experiments designed to test this assumption.

Complete HSM plus 0.1% acetate was compared to basal HSM plus acetate with an N_2/CO_2 (50-50) atmosphere and an H_2/CO_2 (50-50) atmosphere. Basal medium consisted of the following: AELS, 20%; soluble starch, 0.1%; trypticase, 0.2%; yeast extract, 0.1%; resazurin, 0.001%; mineral solution, 10% each of salts A, B, and C; agar, 2%; pH adjusted to 6.8 with $NaHCO_3$. Because of the importance indicated for acetate in sludge,[20] it was added to both media at a level of 0.1%. The results presented in Table 21 indicate that basal HSM is as effective as complete HSM for total growth of sludge organisms with a CO_2-containing atmosphere.

TABLE 18

EFFECT OF CELLOBIOSE ON TOTAL COLONY COUNT OBTAINED WITH HSM

Atmosphere	Medium	Days Incubated at 37°C				
		7	14	21	28	35
CO_2/H_2	HSM	3.2×10^7 x[a/] (1.9×10^7) 6[b/]	6.1×10^7 x (2.7×10^7) 6	7.8×10^7 x (3.9×10^7) 6	3.2×10^8 x (2.5×10^8) 6	7.0×10^8 x (2.4×10^8) 6
	HSM without cellobiose	4.6×10^7 x (2.0×10^7) 6	7.6×10^7 x (3.6×10^7) 6	9.1×10^7 x (4.3×10^7) 6	1.8×10^8 x (0.9×10^8) 6	6.7×10^8 x (2.6×10^8) 6
	HSM with 2x (0.1%) cellobiose	3.0×10^7 x (0.9×10^7) 6	7.6×10^7 x (3.6×10^7) 6	8.9×10^7 x (4.5×10^7) 6	1.9×10^8 x (1.3×10^8) 6	4.8×10^8 x (1.7×10^8) 6
CO_2	HSM	4.1×10^7 x (3.5×10^7) 6	7.4×10^7 x (4.4×10^7) 6	5.9×10^8 x (0.9×10^8) 6	9.2×10^8 x (1.3×10^8) 6	9.5×10^9 x (1.0×10^8) 6
	HSM without cellobiose	3.1×10^7 x (2.1×10^7) 6	7.8×10^7 x (4.1×10^7) 6	5.9×10^8 x (2.0×10^8) 6	8.4×10^8 x (2.1×10^8) 6	1.0×10^9 x (0.2×10^9) 6
	HSM with 2x (0.1%) cellobiose	3.1×10^7 x (2.5×10^7) 6	7.5×10^7 x (3.8×10^7) 6	2.7×10^8 y (0.5×10^8) 6	7.4×10^8 x (2.1×10^8) 6	8.6×10^8 x (2.1×10^8) 6

a,b/ See Table 1.

TABLE 19

EFFECT OF VOLATILE FATTY ACID MIXTURE (VFA) AND TRYPTICASE

Atmosphere	Medium	Days Incubated at 37°C				
		7	14	21	28	35
CO_2/H_2	HSM	4.1×10^7 x[a/]	1.2×10^8 x	1.4×10^8 x	2.4×10^8 xy	3.7×10^8 x
		(1.5×10^7) 6[b/]	(0.5×10^8) 6	(0.5×10^8) 6	(0.5×10^8) 6	(1.6×10^8) 6
	HSM without VFA	6.3×10^7 y	1.2×10^8 x	1.4×10^8 x	3.8×10^8 y	5.0×10^8 x
		(1.3×10^7) 6	(0.4×10^8) 6	(0.4×10^8) 6	(2.1×10^8) 6	(3.1×10^8) 6
	HSM without trypticase	4.4×10^7 x	1.0×10^8 x	1.2×10^8 x	1.7×10^8 x	3.0×10^8 x
		(1.4×10^7) 6	(0.3×10^8) 6	(0.2×10^8) 6	(0.1×10^8) 6	(1.1×10^8) 6
	HSM with 0.2% trypticase	2.7×10^7 x	1.5×10^8 x	1.6×10^8 x	2.0×10^8 x	3.2×10^8 x
		(1.9×10^7) 6	(0.3×10^8) 6	(0.4×10^8) 6	(0.3×10^8) 6	(1.5×10^8) 6
CO_2	HSM	4.4×10^7 x	1.2×10^8 x	3.6×10^8 x	7.4×10^8 x	1.2×10^9 x
		(0.8×10^7) 6	(0.3×10^8) 6	(2.7×10^8) 5	(2.4×10^8) 5	(0.1×10^9) 5
	HSM without VFA	3.2×10^7 x	1.2×10^8 x	5.5×10^8 x	9.4×10^8 x	1.2×10^9 x
		(1.5×10^7) 6	(0.3×10^8) 6	(4.1×10^8) 6	(3.4×10^8) 6	(0.1×10^9) 6
	HSM without trypticase	3.0×10^7 x	9.9×10^7 x	2.8×10^8 x	6.5×10^8 x	9.9×10^8 x
		(1.4×10^7) 6	(4.6×10^7) 6	(1.8×10^8) 5	(2.5×10^8) 4	(2.3×10^8) 5
	HSM with 0.2% trypticase	4.0×10^7 x	9.3×10^7 x	4.1×10^8 x	6.3×10^8 x	8.2×10^8 x
		(1.4×10^7) 6	(3.3×10^7) 6	(2.8×10^8) 6	(4.6×10^8 6	(5.3×10^8) 5

a,b/ See Table 1.

TABLE 20

EFFECT OF YEAST EXTRACT AND TRYPTICASE DELETION ON TOTAL COLONY COUNTS WITH HSM USING A 50-50 H_2/CO_2 ATMOSPHERE

Gas	Medium	Days Incubation at 37°C				
		7	14	21	28	35
H_2/CO_2	HSM	3.4×10^7 (0.5 x 10^7)	x[a/] 6[b/]	4.7×10^7 (0.5 x 10^7) x 6	5.3×10^7 (0.6 x 10^7) x 6	2.6×10^8 (1.9 x 10^8) x 6	6.1×10^8 (1.9 x 10^8) x 6
	HSM without yeast extract trypticase	4.0×10^7 (0.7 x 10^7)	x 5	6.2×10^7 (0.8 x 10^7) y 5	6.6×10^7 (0.9 x 10^7) x 5	1.9×10^8 (1.2 x 10^8) x 6	4.8×10^8 (1.2 x 10^8) xy 6
	HSM with 4 x trypticase without yeast extract	3.7×10^7 (0.8 x 10^7)	x 6	4.6×10^7 (0.6 x 10^7) x 6	5.1×10^7 (0.6 x 10^7) x 6	2.1×10^8 (1.6 x 10^8) x 6	5.7×10^8 (1.1 x 10^8) xy 6
	HSM with 2 x yeast extract without trypticase	3.4×10^7 (0.6 x 10^7)	x 6	4.9×10^7 (0.6 x 10^7) x 6	5.5×10^7 (1.0 x 10^7) x 6	1.5×10^8 (1.0 x 10^8) x 6	4.3×10^8 (0.8 x 10^8) y 6

a,b/ See Table 1.

TABLE 21

EFFECT OF YEAST EXTRACT AND TRYPTICASE DELETION ON TOTAL COLONY COUNTS WITH HSM USING A 100% CO_2 ATMOSPHERE

Gas	Medium	Days Incubation at 37°C 7	14	21	28	35
CO_2	HSM	2.4×10^7 x[a/] (1.0×10^7) 6[b/]	4.7×10^7 x (1.3×10^7) 6	5.3×10^8 x (1.1×10^8) 6	8.7×10^8 x (1.7×10^8) 6	9.5×10^8 x (2.0×10^8) 6
	HSM without yeast extract and trypticase	1.8×10^7 x (0.9×10^7) 6	3.9×10^7 x (1.6×10^7) 6	5.0×10^8 x (1.3×10^8) 6	8.5×10^8 x (1.5×10^8) 6	9.4×10^8 x (1.6×10^8) 6
	HSM with 4 x trypticase without yeast extract	2.6×10^7 x (0.7×10^7) 6	5.3×10^7 x (0.9×10^7) 6	3.7×10^8 x (0.9×10^8) 6	7.2×10^8 x (1.3×10^8) 6	7.7×10^8 x (2.1×10^8) 6
	HSM with 2 x yeast extract without trypticase	2.8×10^7 x (0.5×10^7) 6	4.9×10^7 x (1.4×10^7) 6	5.0×10^8 x (1.7×10^8) 6	8.4×10^8 x (3.5×10^8) 6	9.4×10^8 x (2.0×10^8) 6

a,b/ See Table 1.

TABLE 22

COMPARISON OF COMPLETE HSM WITH BASAL HSM

		Days Incubation at 37°C				
		7	14	21	28	35
N_2/CO_2 50-50	HSM + 0.1% acetate	5.3×10^7 x[a/]	9.4×10^7 x	3.2×10^8 x	6.6×10^8 x	7.5×10^8 x
		(0.7×10^7) 6[b/]	(1.3×10^7) 6	(1.9×10^8) 6	(1.9×10^8) 6	(1.9×10^8) 6
	Basal HSM + 0.1% acetate	1.4×10^8 y	1.7×10^8 y	5.0×10^8 y	7.1×10^8 x	7.5×10^8 x
		(0.1×10^8) 6	(0.1×10^8) 6	(0.8×10^8) 6	(1.3×10^8) 6	(1.2×10^8) 6
H_2CO_2 50-50	HSM + 0.1% acetate	5.6×10^7 x	9.0×10^7 x	9.6×10^7 x	1.0×10^8 x	1.4×10^8 x
		(0.8×10^7) 6	(1.2×10^7) 6	(1.2×10^7) 6	(0.1×10^8) 6	(0.2×10^8) 6
	Basal HSM + 0.1% acetate	1.3×10^8 y	1.5×10^8 y	1.5×10^8 y	3.6×10^8 y	5.3×10^8 y
		(0.1×10^8) 6	(0.1×10^8) 6	(0.1×10^8) 6	(0.3×10^8) 6	(0.7×10^8) 6

a,b/ See Table 1.

Further, a simplified medium appears obligatory for maximum total colony count in an H_2/CO_2 atmosphere.

To further test the total growth obtained with basal medium as well as the effect of gaseous atmosphere on total colony count, the following experiments were carried out. Four modifications of basal HSM were compared with each of six gaseous atmospheres. Media tested consisted of: basal HSM, basal HSM + 0.1% acetate, basal HSM + 0.1% formate, and basal HSM + 0.1% each of acetate and formate. The gaseous atmospheres compared with each of these media were: N_2/CO_2, 50-50; N_2, 100%; CO_2, 100%; H_2/CO_2, 50-50; H_2/CO_2, 80-20; and $N_2/CO_2/H_2$, 60-30-10. Triplicate tubes of 10^{-5}, 10^{-6} and 10^{-7} dilutions were inoculated for each combination of gas and medium. Duplicate experiments were run using an arbitrary control of N_2/CO_2. At the end of 35 days' incubation (termination of experiment), all tubes were analyzed for methane. Methane was determined by gas chromatography of high dilution tubes containing significant colonies (20-200). Any tube containing significant amounts of methane was considered to contain at least one methanogenic colony. The highest dilution tube containing methane was therefore used for total estimated methanogens (Example: if the 10^{-6} dilution was positive for methane and the 10^{-7} dilution was negative, then the total number of methanogens per milliliter of original sample would range from 1×10^6 to 9×10^6 and the methanogens per milliliter would thus be reported as $>10^6$).

The results of these experiments are depicted in Tables 23 to 26. Of all combinations tested, highest total colony counts were obtained with basal medium plus 0.1% acetate in an N_2/CO_2 (50-50) atmosphere and with basal medium plus 0.1% formate in an $N_2/CO_2/H_2$ (60-30-10) atmosphere. Of the two combinations, however, the latter would appear to be the combination of choice for maximum total numbers of both methanogens and nonmethanogens, whereas the former most likely yields the maximum number of nonmethanogens with reduced numbers of methanogens. For growth of the maximum numbers of methanogens, basal medium plus formate with an H_2/CO_2 (50-50) atmosphere appears best. It is worthy of note that H_2 appears to be quite toxic to nonmethanogens as reflected by the reduction in total colony and increase in estimated methanogenic counts as H_2 concentration increases.

If H_2 concentration is lowered to 10% and formate is added, a distinct increase in total counts is obtained with a concomitant increase in total methanogens. It would thus appear that addition of formate spares the H_2 requirement for the methanogens and thereby reduces the effect of H_2 toxicity (less H_2 required).

TABLE 23

EFFECT OF GASEOUS ATMOSPHERE ON TOTAL COLONY COUNT WITH BASAL HSM[a/]

Gas	Days Incubation at 37°C					Methanogens/ml
	7	14	21	28	35	
N_2/CO_2 50-50	4.6×10^7 x[b/]	2.4×10^8 x	6.8×10^8 x	7.9×10^8 x	9.0×10^8 x	$> 10^6$
	(1.0×10^7) 30[c/]	(0.8×10^8) 29	(1.6×10^8) 29	(1.5×10^8) 30	(1.6×10^8) 30	
N_2	2.5×10^7 y	3.8×10^8 y	6.8×10^8 x	8.6×10^8 x	9.0×10^8 x	$< 10^5$
	(1.5×10^7) 6	(1.3×10^8) 6	(1.0×10^8) 6	(2.1×10^8) 6	(2.2×10^8) 6	
CO_2	2.9×10^7 y	1.1×10^8 z	6.7×10^8 x	8.2×10^8 x	8.6×10^8 x	$> 10^5$
	(0.6×10^7) 6	(0.2×10^8) 6	(1.0×10^8) 6	(1.8×10^8) 6	(1.9×10^8) 6	
H_2/CO_2 50-50	5.0×10^7 x	7.2×10^7 z	1.4×10^8 y	6.5×10^8 y	8.7×10^8 x	$> 10^6$
	(0.6×10^7) 6	(0.6×10^7) 6	(0.2×10^8) 6	(1.5×10^8) 6	(1.5×10^8) 6	
H_2/CO_2 80-20	3.9×10^7 x	6.3×10^7 z	1.7×10^8 y	5.3×10^8 y	7.4×10^8 y	$> 10^6$
	(0.6×10^7) 6	(0.6×10^7) 6	(0.8×10^8) 6	(1.1×10^8) 6	(0.7×10^8) 6	
$N_2/CO_2/H_2$ 60-30-10	4.5×10^7 x	1.4×10^8 xz	5.5×10^8 x	7.1×10^8 xy	9.0×10^8 x	$> 10^5$
	(1.0×10^7) 5	(0.6×10^8) 5	(0.7×10^8) 6	(0.5×10^8) 6	(0.9×10^8) 6	

a/ Basal medium: AELS 20%; sol. starch 0.1%; trypticase 0.2%; yeast extract 0.1%; resazurin 0.0001%; minerals A,B,C, 30% (10% each); and agar 2%.

b,c/ Same as a,b/ Table 1.

d/ Estimated methanogens per milliliter based upon highest dilution tube which yields significant methane by gas chromatographic analysis.

TABLE 24

EFFECT OF GASEOUS ATMOSPHERE ON TOTAL COLONY COUNT WITH BASAL HSM + 0.1% ACETATE

Gas	Days Incubation at 37°C					Methanogens/ml[c]
	7	14	21	28	35	
N_2/CO_2 80-50	4.5×10^7 x[a]	2.6×10^8 x	7.1×10^8 x	8.4×10^8 x	9.5×10^8 x	$> 10^5$
	(0.7×10^7) 30[b]	(0.7×10^8) 30	(1.7×10^8) 28	(1.4×10^8) 29	(1.5×10^8) 29	
N_2	2.4×10^7 y	4.0×10^8 y	6.9×10^8 x	8.5×10^8 x	8.9×10^8 xy	$> 10^5$
	(0.7×10^7) 6	(1.1×10^8) 6	(1.5×10^8 5	(1.6×10^8) 6	(1.4×10^8) 6	
CO_2	2.8×10^7 y	9.3×10^7 z	5.8×10^8 x	7.1×10^8 x	7.2×10^8 yz	$> 10^5$
	(1.0×10^7) 6	(4.6×10^7) 6	(0.8×10^8) 5	(1.2×10^8) 5	(1.2×10^8) 5	
H_2/CO_2 50-50	4.8×10^7 x	7.3×10^7 z	1.1×10^8 y	3.9×10^8 y	6.1×10^8 z	$> 10^7$
	(0.6×10^7) 6	(1.6×10^7) 6	(0.2×10^8) 6	(0.9×10^8) 6	(1.2×10^8) 6	
H_2/CO_2 80-20	4.7×10^7 x	8.2×10^7 z	1.4×10^8 y	3.1×10^8 y	5.3×10^8 z	$> 10^7$
	(0.4×10^7) 6	(1.0×10^7) 6	(0.3×10^8) 6	(1.5×10^8) 6	(0.7×10^8) 6	
$N_2/CO_2/H_2$ 60-30-10	5.3×10^7 x	1.3×10^8 z	5.0×10^8 x	6.3×10^8 x	8.1×10^8 xy	$> 10^5$
	(0.5×10^7) 6	(0.3×10^8) 6	(0.3×10^8) 6	(1.1×10^8) 6	(0.8×10^8) 6	

a,b/ See Table 1.
c/ See d/, Table 23.

TABLE 25

EFFECT OF GASEOUS ATMOSPHERE ON TOTAL COLONY COUNT WITH BASAL HSM + 0.1% FORMATE

Gas	Days Incubation at 37°C					Methanogens/ml[c/]
	7	14	21	28	35	
N_2/CO_2 50-50	4.4×10^7 x[a/] (1.2×10^7) 30[b/]	2.7×10^8 x (1.3×10^8) 29	6.8×10^8 x (1.2×10^8) 29	8.1×10^8 x (1.1×10^8) 29	9.3×10^8 x (1.3×10^8) 29	$> 10^6$
N_2	2.6×10^7 y (0.8×10^7) 6	2.8×10^8 x (0.5×10^8) 5	6.2×10^8 x (1.1×10^8) 6	6.2×10^8 xy (1.1×10^8) 6	7.3×10^8 xy (1.1×10^8) 6	$< 10^5$
CO_2	2.2×10^7 y (1.1×10^7) 5	1.1×10^8 y (0.2×10^8) 5	6.7×10^8 x (1.7×10^8) 6	6.9×10^8 x (1.9×10^8) 6	7.6×10^8 y (2.0×10^8) 6	$> 10^5$
H_2/CO_2 50-50	4.3×10^7 x (0.3×10^7) 6	6.4×10^7 y (1.1×10^7) 6	1.1×10^8 y (0.2×10^8) 6	4.9×10^8 yz (0.8×10^8) 6	8.2×10^8 xy (2.3×10^8) 6	$> 10^7$
H_2/CO_2 80-20	4.0×10^7 x (0.6×10^7) 6	6.5×10^7 y (1.3×10^7) 6	1.5×10^8 y (0.6×10^8) 6	3.4×10^8 z (2.4×10^8) 6	6.3×10^8 y (1.2×10^8) 6	$> 10^7$
$N_2/CO_2/H_2$ 60-30-10	5.0×10^7 x (0.6×10^7) 6	1.2×10^8 y (0.5×10^8) 6	6.4×10^8 x (1.9×10^8) 6	8.0×10^8 x (2.4×10^8) 6	9.5×10^8 x (2.0×10^8) 6	$> 10^7$

a,b/ See Table 1.
c/ See d/, Table 23.

TABLE 26

EFFECT OF GASEOUS ATMOSPHERE ON TOTAL COLONY COUNT
WITH BASAL HSM + 0.1% ACETATE + 0.1% FORMATE

Gas	Days Incubation at 37°C					Methanogens/ml[c/]
	7	14	21	28	35	
N_2/CO_2 50-50	4.4×10^7 x[a/] (1.1×10^7) 30[b/]	2.3×10^8 x (0.8×10^8) 28	6.3×10^8 x (3.9×10^8) 27	7.1×10^8 x (1.8×10^8) 28	8.9×10^8 x (1.7×10^8) 29	$> 10^6$
N_2	2.9×10^7 y (1.6×10^7) 6	3.3×10^8 x (0.6×10^8) 5	7.4×10^8 x (0.9×10^8) 6	9.1×10^8 x (1.5×10^8) 6	9.4×10^8 x (1.4×10^8) 6	$> 10^5$
CO_2	2.0×10^7 y (1.4×10^7) 6	3.8×10^7 y (2.5×10^7) 6	5.8×10^8 x (0.9×10^8) 6	7.4×10^8 x (1.1×10^8) 6	8.5×10^8 xz (0.7×10^8) 6	$> 10^5$
H_2/CO_2 50-50	4.8×10^7 x (0.5×10^7) 6	7.1×10^7 y (0.8×10^7) 6	9.7×10^7 y (1.4×10^7) 6	3.4×10^8 y (0.7×10^8) 6	6.5×10^8 yz (1.3×10^8) 6	$> 10^7$
H_2/CO_2 80-20	4.1×10^7 x (0.8×10^7) 6	6.9×10^7 y (0.8×10^7) 6	1.3×10^8 y (0.2×10^8) 6	4.0×10^8 y (2.1×10^8) 6	5.8×10^8 y (1.3×10^8) 6	$> 10^6$
$N_2/CO_2/H_2$ 60-30-10	4.2×10^7 x (0.7×10^7) 5	7.2 s 10^7 y (1.8×10^7) 5	5.8×10^8 x (1.0×10^8) 6	7.2×10^8 x (1.7×10^8) 6	8.1×10^8 xz (1.4×10^8) 6	$> 10^6$

a,b/ See Table 1.
c/ See d/, Table 23.

On the basis of the foregoing observations, the following conclusions were reached concerning media and gaseous atmosphere. For isolation of maximum total numbers of mixed methanogens and nonmethanogens, basal medium plus 0.1% formate with an $N_2/CO_2/H_2$ (60-30-10) atmosphere is recommended. For maximum total count of methanogenic species with reduced numbers of nonmethanogens, basal medium plus 0.1% formate in an H_2/CO_2 (50-50) atmosphere is recommended. An N_2/CO_2 (50-50) atmosphere and basal medium plus 0.1% acetate is recommended for maximum total count of nonmethanogens with reduced numbers of methanogens.

Variation in Total Anaerobic Counts in Digesters at Various Performance Levels

As a practical application of the media and techniques developed during the course of this work, four digesters at various performance levels were sampled to determine if digester condition would be reflected by differences in total anaerobic counts. These digesters were selected on the basis of current and past performance records.

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

4800 Nall: This digester is fed only on weekends when the sludge incinerators at this plant are not in operation. The digester receives only municipal wastes and laboratory records are not kept on performance. An appreciable amount of methane is produced, and no upsets have occurred in the past 2 to 3 years.

Kansas City, Kansas: This plant was built to receive only industrial wastes (packing plant and soap manufacturing wastes). In nearly 2 years' operation, normal digestion has not been achieved. The system has been constantly plagued by excessive foaming, high volatile acids, low pH and low methane production.

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

Each of these digesters was sampled twice, and six roll tubes from each sample were inoculated from the 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5}, 10^{-6} and 10^{-7} dilutions. Two media were inoculated; basal HSM plus 0.1% acetate with an N_2/CO_2 atmosphere and basal HSM plus formate with an H_2/CO_2 atmosphere. Aerobic pour plates were also prepared from each sample, using basal

HSM plus 0.1% acetate, to obtain aerobic and/or facultative counts. These plates were incubated at 37°C, and colony counts were made at 24-hr intervals for 4 days. Roll tubes were counted after 4, 7, 14, 21, 28 and 35 days' incubation at 37°C. Methane was determined at each counting interval. Low dilution uncountable tubes were used so that high dilution tubes could be maintained for subsequent anaerobic counts. At the termination of the experiment, high dilution tubes became available and were used for the final methanogen estimates.

Tables 27 and 28 show the resultant total anaerobic counts for N_2/CO_2 and H_2/CO_2 atmospheres, respectively. With the exception of the samples taken from the Olathe digester, there would appear to be no significant difference in total anaerobic counts. In this case, a significant difference is apparent from the initial to the final count. These counts were initially the highest (significantly so), but showed very little increase in total count over the 35-day counting period. This is partially clarified by the aerobic plate counts (Table 29). The facultative anaerobic count (aerobic plates) from the Olathe sludge was much higher than the other three digesters; hence, the initially high total anaerobic count.

Tables 30 and 31 show the numbers of methanogenic colonies recorded at each counting interval. These results are surprising in that high numbers of methanogenic bacteria are indicated for the digesters known to produce very little methane (Kansas City and Olathe). This, obviously, is not an expression of actual digester condition. It must be emphasized that the AELS used to supplement all media came from sludge taken from a digester (Indian Creek sewage disposal plant) which was normal with respect to methane production. It is then quite possible that either growth of methanogens and methanogenesis or methanogenesis, per se, is inhibited in the unbalanced or upset digesters. Methanogenesis is obviously restored when the organisms from the upset digesters are placed in a more favorable environment, such as that present in the culture medium supplemented with AELS from a digester functioning normally. It appears that the methanogens are present in equal or greater numbers in the upset digesters as in "normal" digesters but that methanogenesis is being inhibited. As will be subsequently explained, we feel that the lack of methanogenesis in the upset digester is due to the lack of a sludge factor, required for methanogenesis, rather than due to the inhibition of growth of the methanogenic bacteria. The presence of this factor in the AELS used to prepare all media would thus allow methanogenesis to occur in the roll tube but not in the digester.

Further studies need to be performed in which media containing AELS are compared to media supplemented with clarified sludge supernatant taken from the digester in question at the time of sampling and compared for total anaerobic counts, aerobic and/or facultative counts and methanogenesis. The experiments which could now be performed with the information and techniques provided in the current study would yield new information pertaining to digester function, and could quite possibly lead to better means for digester control.

TABLE 27

COMPARISON OF TOTAL COLONY COUNTS OF SLUDGE SAMPLES FROM FOUR DIFFERENT DIGESTERS WITH AN N_2/CO_2 ATMOSPHERE

	Days Incubation at 37°C											
	4		7		14		21		28		35	
Indian Creek basal + 0.1% acetate	3.1×10^7 (1.7×10^7)	x[a/] 12[b/]	8.8×10^7 (1.5×10^7)	x 12	2.0×10^8 (0.4×10^8)	x 12	8.6×10^8 (2.8×10^8)	x 12	1.0×10^9 (0.2×10^9)	x 12	1.2×10^9 (0.3×10^9)	x 12
4800 Nall basal + 0.1% acetate	2.0×10^7 (1.8×10^7)	x 12	3.9×10^7 (2.6×10^7)	z 12	2.2×10^8 (1.7×10^8)	x 12	7.9×10^8 (2.7×10^8)	xy 12	1.0×10^9 (0.2×10^9)	x 12	1.1×10^9 (0.3×10^9)	x 12
Kansas City, Kansas, basal + 0.1% acetate	1.8×10^7 (1.6×10^7)	x 12	4.3×10^7 (2.2×10^7)	z 12	1.2×10^8 (0.4×10^9)	x 12	9.3×10^8 (1.7×10^8)	x 12	1.1×10^9 (0.2×10^9)	x 12	1.2×10^9 (0.3×10^9)	x 12
Olathe, Kansas, basal + 0.1% acetate	3.3×10^8 (1.8×10^8)	y 12	3.7×10^8 (2.1×10^8)	y 12	4.8×10^8 (2.7×10^8)	y 12	5.7×10^8 (3.5×10^8)	y 12	6.4×10^8 (3.3×10^8)	y 12	6.6×10^8 (3.2×10^8)	y 12

a,b/ See Table 1.

TABLE 28

COMPARISON OF TOTAL COLONY COUNTS OF SLUDGE SAMPLES FROM FOUR DIFFERENT DIGESTERS WITH AN H_2/CO_2 ATMOSPHERE

	Days Incubation at 37°C											
	4		7		14		21		28		35	
Indian Creek basal + 0.1% formate	3.8×10^7 (1.2×10^7)	x[a] 12[b]	7.3×10^7 (1.9×10^7)	x 12	9.1×10^7 (1.8×10^7)	x 12	2.2×10^8 (1.2×10^8)	x 12	5.0×10^8 (1.5×10^8)	x 12	7.5×10^8 (0.9×10^8)	x 12
4800 Nall basal + 0.1% formate	2.0×10^7 (1.7×10^7)	x 12	3.1×10^7 (1.8×10^7)	x 12	4.1×10^7 (2.3×10^7)	x 12	1.4×10^8 (0.4×10^8)	x 12	5.6×10^8 (1.7×10^8)	x 12	7.2×10^8 (2.7×10^8)	x 12
Kansas City, Kansas, basal + 0.1% formate	1.7×10^7 (1.5×10^7)	x 12	3.7×10^7 (2.5×10^7)	x 12	1.0×10^8 (0.2×10^8)	x 11	4.0×10^8 (0.9×10^8)	y 12	1.0×10^9 (0.1×10^9)	y 12	1.1×10^9 (0.2×10^9)	y 12
Olathe, Kansas, basal + 0.1% formate	1.7×10^8 (0.2×10^8)	y 12	2.0×10^8 (0.3×10^8)	y 12	2.4×10^8 (0.2×10^8)	y 12	3.5×10^8 (0.8×10^8)	y 12	3.8×10^8 (0.9×10^8)	x 12	4.0×10^8 (1.0×10^8)	z 11

a,b/ See Table 1.

TABLE 29

RESULTS OF AEROBIC PLATE COUNTS FOR ANAEROBIC SLUDGE FROM FOUR DIFFERENT DIGESTERS

	Days Incubation at 37°C			
	1	2	3	4
Indian Creek	1.5×10^6 [a]	3.3×10^6	-	6.8×10^6
4800 Nall	7.5×10^4	9.1×10^5		1.2×10^6
Kansas City, Kansas	2.2×10^5	3.3×10^5	4.4×10^5	4.8×10^5
Olathe, Kansas	6.2×10^6	1.3×10^7	2.6×10^7	4.1×10^7

a/ Total count per milliliter.

TABLE 30

TOTAL ESTIMATED METHANOGENS BASED UPON METHANE DETERMINATIONS ON ROLL TUBES WITH AN N_2/CO_2 ATMOSPHERE

	Days Incubation at 37°C					
	4	7	14	21	28	35
Indian Creek	$> 10^2$ [a]	$> 10^2$	$> 10^3$	$> 10^3$	$> 10^4$	$> 10^5$
4800 Nall	$> 10^2$	$> 10^2$	$> 10^3$	$> 10^3$	$> 10^4$	$> 10^5$
Kansas City, Kansas	$> 10^2$	$> 10^3$	$> 10^4$	$> 10^4$	$> 10^5$	$> 10^5$
Olathe, Kansas	$< 10^2$	$> 10^2$	$> 10^3$	$> 10^3$	$> 10^4$	$> 10^4$

a/ Methanogens per milliliter, see d/, Table 23.

TABLE 31

TOTAL ESTIMATED METHANOGENS BASED UPON METHANE DETERMINATIONS ON ROLL TUBES WITH AN H_2/CO_2 ATMOSPHERE

	Days Incubation at 37°C					
	4	7	14	21	28	35
Indian Creek	$> 10^2$ [a]	$> 10^3$	$> 10^4$	$> 10^5$	$> 10^5$	$> 10^6$
4800 Nall	$> 10^2$	$> 10^3$	$> 10^4$	$> 10^4$	$> 10^4$	$> 10^5$
Kansas City, Kansas	$> 10^2$	$> 10^3$	$> 10^4$	$> 10^5$	$> 10^6$	$> 10^6$
Olathe, Kansas	$> 10^2$	$> 10^3$	$> 10^4$	$> 10^5$	$> 10^5$	$> 10^6$

a/ Methanogens per milliliter, see d/, Table 23.

PHASE IV - DETERMINATION OF GROWTH SUBSTANCE(S) IN SEWAGE SLUDGE THAT ENHANCES GROWTH OF SLUDGE MICROORGANISMS

An unknown factor was found in rumen fluid which is essential to the growth of Methanobacterium ruminantium and possibly other species of methanogenic bacteria. An extraction procedure and a microbiological assay were developed for the factor, the presence of which was determined in both rumen fluid and sludge supernatant. Subsequent studies on the factor, not extractable with ether, showed it to be of relatively low molecular weight (Syphadex G25, dialyzable) and stable to autoclaving at acid or at neutral pH, but roughly 50% of the activity was lost during autoclaving in alkali.22/

The factor has not been identified nor has a metabolic role been assigned to it. The requirement has never been satisfactorily replaced by known vitamins, amino acids, purines, pyrimidines, etc.

Since the factor is present in sludge supernatant and has been shown to be necessary to the growth of some methanogenic species, it is quite probable that the factor plays an important role in sludge digestion.

Experiments were carried out during this phase of work to determine the levels of factor which occur in sewage sludge, and to determine whether factor concentration might vary as a function of digester performance. A description of the assay procedure and the method of factor extraction follows.

Methods and Media for Sludge Factor Assay

Stock cultures of M. ruminantium were maintained on slants in a H_2/CO_2 (50-50) gas atmosphere. Cultures were transferred with a platinum-indium loop by stab into the base of the slant.

Slant Medium

RF (rumen fluid)	30.0 %
Mineral No. 1[1/]	3.75%
Mineral No. 2[2/]	3.75%
Resazurin solution	0.1 %
No formate	0.2 %
Trypticase	0.2 %
Agar	1.5 %
Distilled H_2O to volume	
Cysteine-HCl	0.025%

1/ Mineral No. 1: K_2HPO_4 0.6% 6.0 g; make to 1 liter.

2/ Mineral No. 2:

KH_2PO_4	0.6%	6 g	1 liter
$(NH_4)2SO_4$	0.6%	6 g	
NaCl	1.2%	12 g	
$MgSO_4 \cdot 7H_2O$	0.245%	2.45 g	
$CaCl \cdot 2H_2O$	0.159%	1.59 g	

All ingredients were placed in a 500-ml round-bottom flask and boiled under CO_2 until the resazurin was reduced (colorless). The medium was cooled in a water bath to 47 to 48°C and $NaHCO_3$ to yield a concentration of 0.5% was added. $Na_2S \cdot 9H_2O$ reducing solution (0.025% final concentration) was added to the flask just prior to tubing. The medium was tubed in 8-ml amounts as described in Phase II.

The tubes were then autoclaved as previously described (15 lb for 15 min) and cooled in a slant position.

Salts were dissolved in order given in 700 to 800 ml distilled water and made to 1 liter. The solution was distributed (100-120 ml) into labeled, screwcap dilution bottles (Pyrex) and autoclaved 15 psi for 15 min.

Contamination was checked microscopically and by inoculating carbohydrate agar slants which were maintained under a CO_2 (100%) gas atmosphere. Growth on the carbohydrate agar slants indicated contamination. Ingredients are listed below.

Carbohydrate Agar Slants

CO_2 gas phase used to check sterility of assay organism.

Resazurin (0.1% solution)	0.1%
Whole rumen fluid	30.0%
Mineral No. 1	3.75%
Mineral No. 2	3.75%
Glucose	0.05%
Cellobiose	0.05%
Soluble starch	0.005%
Agar	1.5%
Trypticase	0.5%
Distilled H_2O to volume	
Cysteine-HCl	0.025%
$NaHCO_3$	0.9% added after medium cooled to 47-48°C

Na_2S solution (0.25% final volume) added just prior to tubing (0.04 ml/5 ml medium)

The same basal medium was used for assay medium and for medium in which cells were grown for inoculum.

Basal Medium (for 200 ml; 5x concentration)

$(NH_4)_2SO_4$	0.5 g
Na formate	2.0 g
Yeast extract	2.0 g
Trypticase	2.0 g
VFA solution	3.1 g
$FeSO_4 \cdot 7H_2O$	0.002 g
Resazurin	1.0 ml
Mineral No. 3	5.0 ml
Hemin (1 mg/ml)	1.5 ml
Distilled water	97.0 ml

Ingredients were placed in a 400-ml beaker, on a magnetic stirrer and the pH was adjusted to 6.5 with 3N NaOH. The medium was then brought up to 200 ml with distilled H_2O. One hundred milliliter amounts were transferred to Pyrex screwcap bottles; autoclaved 15 psi/15 min, and stored in the refrigerator. For use, 10 ml was placed in a 100-ml round-bottom flask and the indicated volume of material to be assayed was added. Cysteine-HCl (0.025%) was added, the solution was reduced, and the total volume brought to 50 ml.

$NaHCO_3$ (0.5%) was added and the medium was tubed in 5-ml amounts anaerobically and autoclaved. Na_2S (0.04 ml/5 ml) reducing solution was added 2 to 24 hr prior to inoculation. Twenty percent rumen fluid was used in the medium in which the inoculum was grown and maintained.

Mineral No. 3

	1 Liter	Final Min Med
KH_2PO_4	18.0 g	6.62×10^{-3}
NaCl	18.0 g	1.54×10^{-2}
$CaCl_2$ ($CaCl_2$-$2H_2O$)	0.4 g	1.80×10^{-4}
$MgCl_2 \cdot CH_2O$	0.4 g	9.85×10^{-5}
$MnCl_2 \cdot 4H_2O$	0.2 g	5.05×10^{-5}
$CaCl_2 \cdot CH_2O$	0.02 g	4.20×10^{-5}

Salts were dissolved in 700-800 ml distilled water and made up to a liter. Mineral solution was distributed in Pyrex bottles and autoclaved 15 psi/15 min (0.0066 M PO_4 final) (5% v/v added to media).

VFA (Volatile Fatty Acid) Mixture

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

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

Assay Procedure

Cells for inoculum were grown in basal medium plus 20% rumen fluid. Cells from an actively-growing culture (OD 0.3 to 0.4) were centrifuged (4,000 rpm, 2,000 g's for 20 min) and the pellet was washed in ADS under aseptic and anaerobic conditions. The cells were resuspended in ADS for inoculation of assay media.

ADS (Anaerobic Dilution Solution)

Mineral No. 3	5.000%
Resazurin	0.1 %
Distilled water	
$NaHCO_3$	5.000%
Cysteine-HCl	0.025%

Na_2S reducing solution added before use (0.04 ml/5 ml medium)

The O.D. of the cell suspension was measured at 600 mμ. Duplicate or triplicate tubes were inoculated for each level of each fraction to be assayed. Cultures were incubated at 39°C on a shaker. After the first day, the tubes were flushed twice daily with H_2/CO_2 (50-50) gas. Growth was estimated as O.D. at 600 mμ. Readings were taken prior to flushing the tubes. Maximum growth was reached in approximately 5 days.

A unit of activity of the factor is defined as the amount in 50 ml of medium required to allow growth equal to O.D. of 0.3. Net O.D. was determined by subtracting the amount of growth (O.D.) in the control medium with no factor addition from the O.D. of the experimental medium (containing various concentrations of factor).

Extraction of Factor

For experiments in which crude concentrates of the factor were assayed, the following procedure was carried out as 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 precipitate was separated by vacuum filtration and the supernatant was passed through a column of Dowex 50 to remove any remaining positively-charged ions remaining. 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 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 to remove NH_3 and concentrate the factor to a small volume. Aliquots of this concentrate were diluted for assay.

The effect of sludge treatment on factor recovery was studied using the following samples: aqueous extract of lyophilized sludge; raw sludge, filtered and sterilized; raw sludge preincubated with 0.4% yeast extract, sterilized and filtered; factor extracted from hot 80% alcohol extract of sterile lyophilized sludge; factor extracted from AELS.

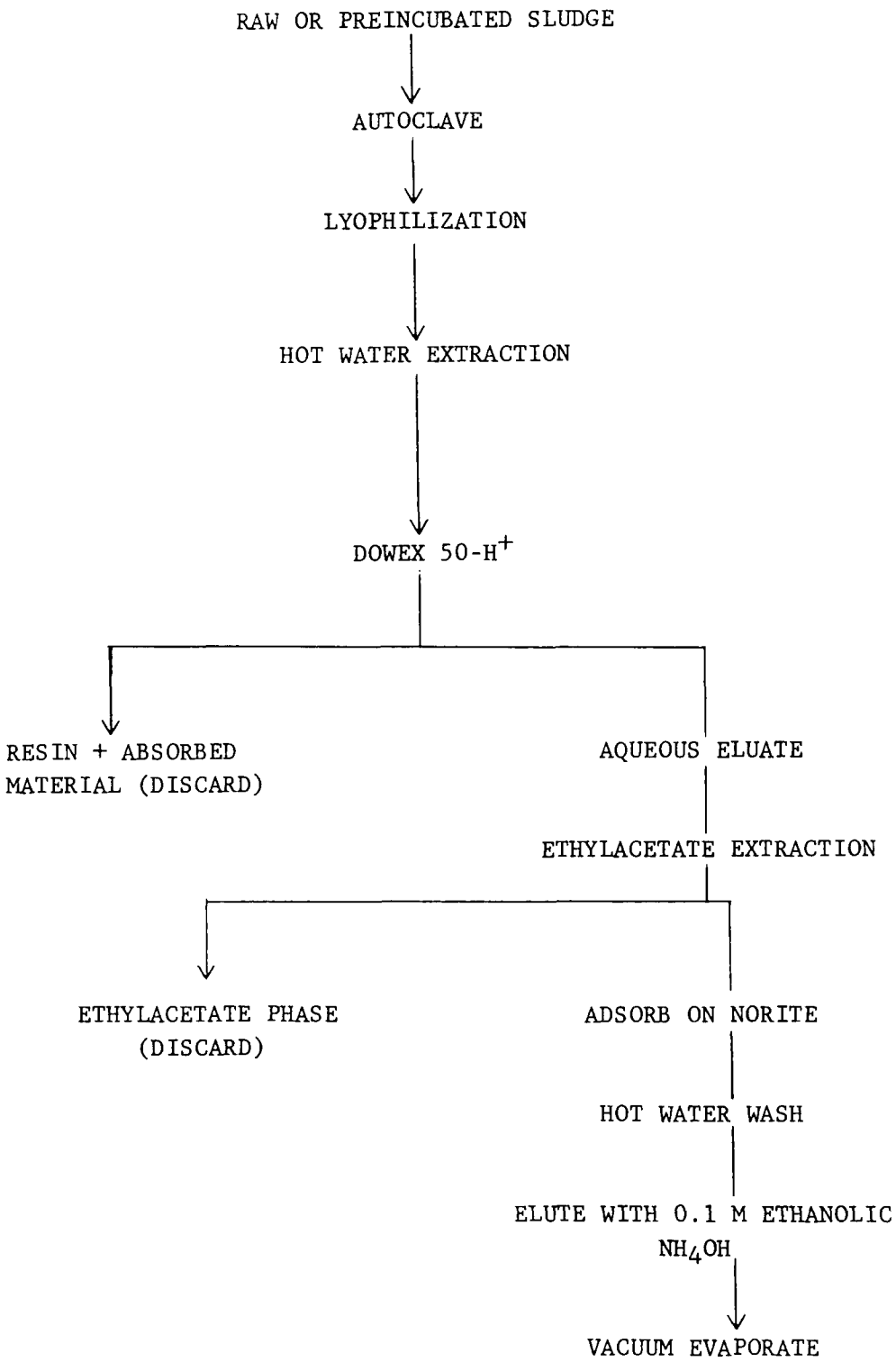

Figure 5 - Scheme for Purification and Concentration of Sewage Sludge Factor

The results shown in Table 32 indicate that the factor levels found in sludge are comparable to those found in rumen fluid (0.8-1.0 unit/ml by Bryant). The factor concentration increased twofold when the sludge was preincubated at 37°C for 40 hr under nitrogen. This increase is much less than the increase found in rumen fluid (up to eightfold). The 0.3 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 preextraction 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.

The factor recovery from AELS (0.2 unit/ml) appeared to be low. To determine at which point the factor was lost and whether the yield could be increased by treatment of the activated charcoal, the following experiment was performed. A 2-liter batch of freshly prepared AELS was taken through the extraction procedure. At the Norit stage, the extract was split into five samples and equal amounts were treated with Norit and Darco which had been treated as follows: untreated Norit; acid washed Norit; NH_4^+-EtOH, HCl washed Norit; acid washed Darco; and NH_4^+-EtOH, HCl washed Darco. Samples from all phases of the extraction procedure (except the wash water) were assayed using the M-1 strain of Methanobacterium ruminantium. The results shown in Table 33 indicate the losses at each step in the extraction procedure. 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 are higher than those shown in Table 32, even with Norit. This is 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.

Although time would not permit, the status of the factor extraction procedure is such that large batches could be extracted and used to determine the actual significance of the factor in sludge digestion. With further purification and mass spectrometry, gas chromatography methodology which exists at MRI, we are also in a position to further characterize and identify the factor.

As the final experiment carried out in this work, factor levels were determined in the sludge supernatants prepared from the digester samples discussed in Phase III. The results of these comparisons, shown in Table 34, appear to be highly significant in terms of digester performance. As will be noted, 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).

TABLE 32

EFFECT OF SLUDGE TREATMENT ON FACTOR RECOVERY

Material	Units/ml
Aqueous extract of lyophilized sludge (AELS)	0.8
Raw sludge sterilized and filtered	1.0
Raw sludge pre-incubated with 0.4% yeast extract, sterilized and filtered	2.0
Factor extracted from hot 80% alcohol extract of sterile lyophilized sludge	0.3
Factor extracted from AELS	0.2

TABLE 33

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

TABLE 34

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

Although this work could not be followed up, there are definite indications that the low factor levels in these digesters could explain why little methane is formed in the digesters while high levels are formed in cultures grown in media containing AELS from a digester which is functioning normally (Indian Creek sewage plant). This work should definitely be pursued further to determine whether factor addition to these sludges would aid in reversing the unbalanced or stuck condition.

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Anaerobic - The absence of free-molecular oxygen.

Anaerobic Dilution Solution - An oxygen-free, reduced, buffered, salt solution used to make serial dilutions under anaerobic conditions.

Anaerobic Sludge Digestion - The anaerobic process, generally considered to be biphasic, during which digestable complex organic substrates are converted to short-chained fatty acids and subsequently to carbon dioxide and methane.

Aseptic Technique - Generally referred to in bacteriological nomenclature as manipulations involving the transfer of organisms, preparation of dilutions, etc., during which no extraneous or unwanted organisms are introduced; i.e., sterile technique.

Biomass - The total amount or number of living organisms in a particular area or volume.

Bunsen Valve - As used in this report, refers to a short piece of butyl rubber tubing closed to the atmosphere but containing a very small slit which allows the relief of gas pressure while preventing the entry of air.

Clarified Sludge Supernatant (CSS) - The opalescent aqueous phase of anaerobic sewage sludge prepared by filtration to remove large solids followed by high-speed centrifugation to remove suspended solids.

Inhibitor - An agent which slows down or interferes with the growth of bacteria.

Methanogenic - Refers to the generation of methane by bacteria.

Microbiological Assay - The use of a microorganism with an obligatory requirement for a material to measure the concentration of that material. The growth rate or some measurable physiological activity of the test organism must be directly proportional to the concentration of that material throughout the limiting range of concentration.

Nonmethanogen - For this report, refers to obligate anaerobic species of anaerobic sludge which do not form methane.

Obligate Anaerobe - A bacterial species to which free-molecular oxygen is lethal.

Plate Count - A method of enumerating viable bacteria in a sample by counting the total number of colonies produced on an agar surface when a suitable dilution of material is spread on the surface of the agar medium or mixed with the medium before solidification and allowed to incubate until visible colonies appear.

Reducing Agents - Materials added to the culture medium which reduce the oxidation-reduction potential to a point suitable for growth of obligate anaerobes.

Roll Tube - Refers to the process of rolling a tube of melted agar medium in a horizontal position until the agar solidifies uniformly on the walls of the tube.

Saprophytic - Living or growing on dead or decaying organic matter.

Sludge Factor - An unknown factor or factors present in sewage sludge and rumen fluid, necessary for the growth of certain methanogenic species of bacteria.

"Stuck" Digester - A digester unbalanced to the point that methane production ceases. This condition is generally irreversible and requires shutdown drainage, and reseeding of the digester.

Symbiosis - A condition occurring when two or more organisms growing together, produce a reaction or end-product that none of the individual species can produce when grown in pure culture.

Total Colony Count - The total number of colonies counted on a plate or in a roll tube multiplied by the reciprocal of the dilution of that plate or roll tube.

Viability - The ability to grow and multiply in a given environment.

SELECTED WATER RESOURCES ABSTRACTS

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16. *Abstract* This research program was initiated to develop practical methods for evaluation of the biomass in anaerobic sewage sludge and to determine if predictions could be made concerning digester performance. Sampling and handling methods were improved and standardized to give maximum anaerobic counts. A simplified technique for growing obligate anaerobes that can be safely performed by technicians with minimum training in bacteriology was developed. Anaerobic media were improved to yield as high or higher counts of methanogenic bacteria than heretofore reported. A simple freeze-dry technique was developed for preparation of consistent batches of sludge supernatant used in media as a supplement for growth of obligate sludge anaerobes. The possible relationship between concentration of a growth factor required by Methanobacterium ruminantium (used to evaluate potency of growth factor extracted) and digester efficiency could have important practical implications. Limited data obtained indicated that growth factor concentrations were much higher in "normal" digesters than in unbalanced or "upset" digesters. Practical applications of the methods developed can have considerable impact upon future research and development in anaerobic sludge digestion and could lead to improvements in our ability to predict impending digester failure and control of digester performance.

17a. *Descriptors*
* Anaerobic Digestion, * Methane Bacteria, * Anaerobic Bacteria, Sewage Sludge, Sludge Digestion, Waste Treatment, Water Pollution Control, Biomass

17b. *Identifiers*
* Anaerobic Techniques, * Roll-Tube Procedures, * Anaerobic Sludge Extracts, * Sludge Factor Assay, Sludge Factors, Obligate Anaerobes, Anaerobic Culture Media, Digester Failure, Sludge Supernatant

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