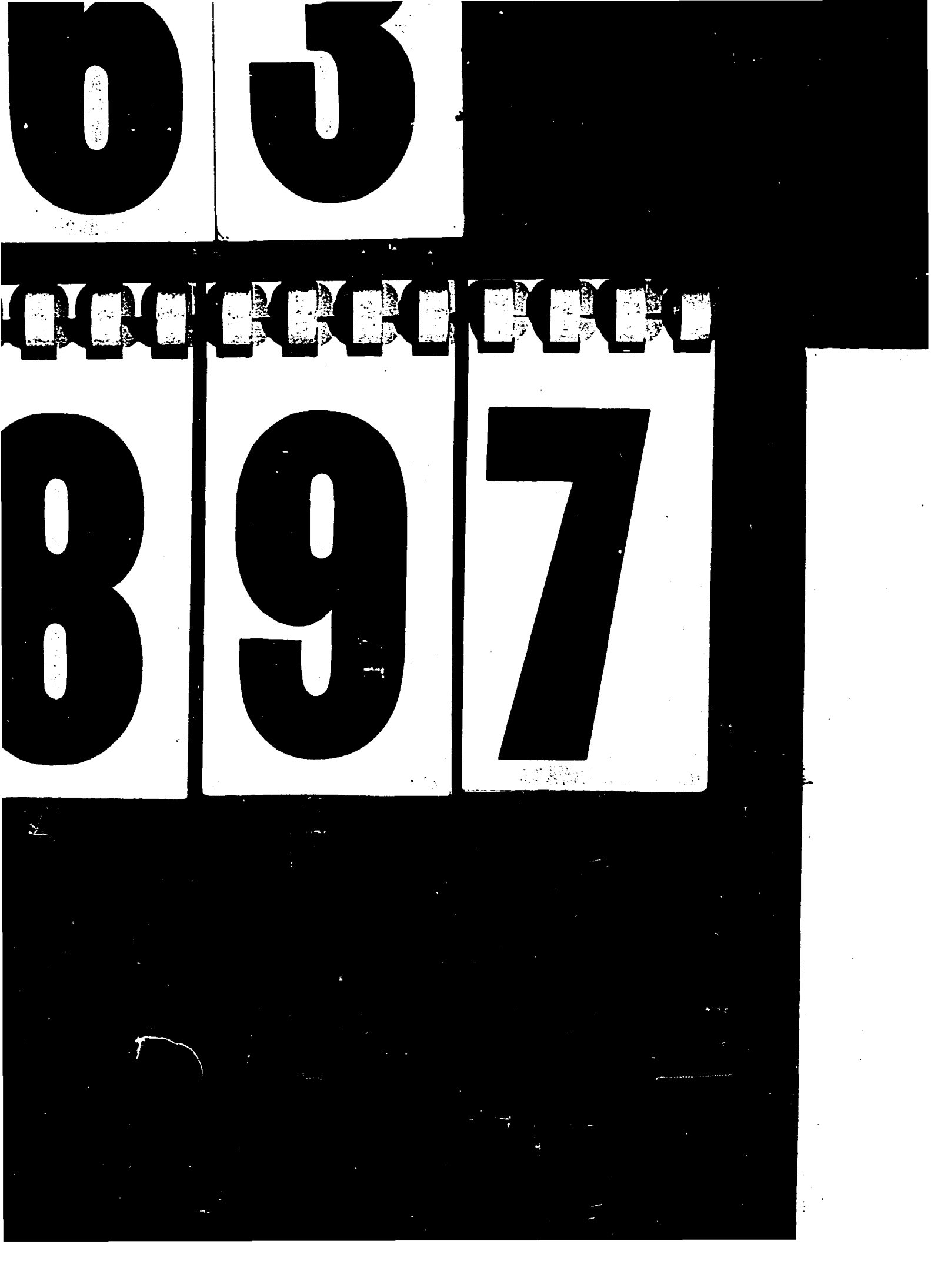
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GRADUATE COLLEGE

COMPARISON OF RESPIRATION AND METABOLISM OF BIOLOGICAL SLIMES USING RADIO-PHOSPHORUS

A DISSERTATION

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COMPARISON OF RESPIRATION AND METABOLISM OF BIOLOGICAL SLIMES USING RADIO-PHOSPHORUS

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When to the sessions of sweet silent thought
I summon up remembrance of things past,
I sigh the lack of many a thing I sought,
And with old woes new wail my dear time's waste:
Then can I drown an eye, unused to flow,---

Shakespeare

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CHAPTER I

INTRODUCTION

General

Water, although an invaluable natural resource, has the objectionable feature of being a common vehicle of many communicable diseases. Probably the major cause of this is the contamination of streams with pathogenic bacteria and viruses by the indiscriminate and discriminate mixing of sewage with potable water. Additional pollution of streams also occurs whenever sewage contributes various undesirable oxygen-demanding materials. Consequently, sewage is frequently treated to avoid gross pollution of streams; with the degree of treatment depending in large part upon dilution water available and other qualities of the receiving stream.

The treatment of sewage can be divided into two phases: (a) primary or hydraulic; and (b) biological. The biological phase is by far the more important and the most complex of the two. As slimes are responsible for the biological reduction of organic wastes an increase in knowledge of biological slime

behavior is necessary. For this reason, the present investigation limited itself to this subject with the ultimate objective of providing a tool to more properly evaluate biological waste treatment processes and to develop new treatment methods. Past evaluation has been based on slime respiration studies, the tool herein is based on metabolism in essence, the study resorts itself into a comparison of respiration and metabolism of sewage slimes.

Slime-Formation

To better understand the biological processes involved in sewage treatment plants, it is essential to keep in mind the fundamentals of biochemical changes that take place. For, in contrast to treated water, raw sewage always contains comparatively large amounts of organic matter and greater numbers of micro-organisms. Under optimum conditions the development of microbial flora is rapid; and soon the little oxygen normally present in sewage is used up by respiration. In other words, oxygen dissolved in water can be used by bacteria in oxidizing and utilizing the organic matter present in the sewage. However, as the initial content of dissolved oxygen in sewage is so small the whole amount is normally consumed in a matter of a few minutes.

Nevertheless micro-organisms are capable of stabilizing the organic matter into stable end products, provided favorable conditions are maintained for a sufficient period of time. All biological methods involved in the treatment of waste water,

either in a sewage treatment plant or in natural bodies of water, are based upon the development and activities of micro-organisms. A film, or matrix, formed by certain micro-organisms, together with the organisms themselves, constitute the essential element of the aerobic biological treatment system. The film formed is of a viscous, jelly-like nature, and high in microbial population. This film is usually called a zoogleal mass and is commonly referred to as biological or sewage slime.

Such slimes are capable of adsorbing to themselves the substances that are dissolved or suspended in the carrying water. These can be metabolized by slimes to produce energy and its end products. Sanitary engineers, still in quest of a better definition, refer to this process as "biological oxidation," or "biochemical oxidation," with the strength of the sewage in terms of this process being called the Biochemical Oxygen Demand or B.O.D.

Slimes will form whenever there are environmental conditions providing the elements for their growth; namely food, water, and air. Slimes grow in trickling filters, and activated sludge units in attached or free floating conditions.

Many bacteria are responsible for the development of slimes upon surfaces. Since one species may dominate in a given environment, the composition of any natural slime will be determined by the biochemical and physical environments in which it grows. Many bacteria can grow attached to surfaces

submerged in either pure or polluted waters. Such attachment may be either by means of secretion of mucilaginous material from the cell or by use of stalks and holdfasts. If favorable environmental conditions prevail, the slimes will have a tendency to build up. As the thickness of the slime layer increases, the lower layers tend to receive less food and insufficient oxygen. The result is that the lower layers enter into an anaerobic endogenous metabolism. Once a slime film is formed its rate of growth increases, at a rate directly proportional to the B.O.D. of the substrate, except under conditions of very large B.O.D. concentration where the growth rate is constant.

Slimes are primarily responsible for the metabolism of organic matter into stable end products in a sewage treatment plant. The organic substrate, or food, within the slime bacteria, undergoes biological oxidation, wherein various types of enzymes catalyze the transfer of electrons in a series of biochemical reactions. The oxidizable substrates are thus converted to stable end products.

The supply of organic material in the incoming sewage is sufficient to provide a continuous source of the food required for the bacterial growth. Inorganic material used as food by some of the bacteria is also present in adequate quantities in the sewage. Aside from the lack of food, potentially limiting growth factors are pH, temperature, oxygen supply, and osmotic pressure. Of these limiting factors, it appears that only pH and the oxygen supply can be controlled economically.

The pH will vary according to the type of sewage. The pH of domestic sewage is rarely so acidic or alkaline as to cause extensive destruction in the bacterial growth. However, some industries do produce an effluent which may be very low or very high in pH value. In such cases the pH must be controlled by dilution or by neutralization. A sufficient supply of oxygen is essential in order to maintain a proper growth of aerobic bacteria responsible for the formation of slime. The temperature of sewage will vary depending largely upon the ambient temperature. Usually it will lag a few degrees behind atmospheric. Very little is known about the effects of osmotic pressure and no further reference will be made to it.

Reaction Rate Constant

It is customary to measure the rate of depletion of organic material in terms of the slope of the first order depletion curve as

$$\frac{dy}{dt} = -K(L-y) \qquad (1)$$

where L is the total depletable amount, y is the depletable amount at any time t, and the proportionality factor "K" is called the reaction rate constant. This rate, $\frac{dy}{dt}$, can be expressed in terms of uptake of food, oxygen, or phosphorus, or in terms of bacterial growth.

Zooglea ramigera is the major organism responsible for sewage purification in trickling filters and activated sludge units. The existing method for determining the "K" value,

which reflects the rate of purification of the waste water, was developed by Theriault and Hammon (65). This method is time consuming, cumbersome, and demands special technical abilities on the part of the investigator. It is based on the first order reaction, which states that the rate of biochemical oxidation of organic matter is proportional to the remaining concentration of oxidizable material. The rate of utilization of oxygen due to the aerobic micro-organisms, both bacteria and protozoa, is determined in sealed bottles containing known amounts of oxygen to which has been added predetermined amount of the polluted water under study. This procedure is commonly referred to as the B.O.D. Test. This is for a fixed time and temperature-usually five days at 20^{o} C.

Logically, the first order reaction implies a constant rate of reaction. If plotted on a semi-log paper with the logarithm of per cent remaining as ordinate and time as abscissa, the resulting curve will be a straight line, and the slope of the curve will be proportional to "K".

Various investigators (7, 8, 9, 11, 13, 14, 21, 23, 34, 39, 61) have commented on the applicability of the first order reaction occuring in the heterogenous microbial population feeding on widely varied composition of organic material. Most of these investigators have attempted to evaluate "K" values through oxygen uptake studies.

Tracers

Reid (51) developed a technique using P^{32} to determine the value of "K", and measuring basically metabolism as opposed

to the respiration measurements usually determined by oxygen uptake studies. The present study is concerned with the tracer technique measure of phosphorus uptake with the direct measurement of respiration.

When existing elaborate and time-consuming methods fail to produce reliable results, it is frequently desirable for the investigators to attempt a more rapid approach through the introduction of a tracer. A tracer may be defined as a non-injurious agent possessing easily recognizable physical or chemical properties capable of tracing the path of the investigation. In sanitary engineering practice different types of tracers have been used and proven to be satisfactory.

These tracers may be chemical salts like sodium chloride, or organic dyes like fluorescein. Even bacterial cultures of Serratia marcescens have been used with satisfactory results. However with recent developments of radiochemistry, the attention of sanitary engineers has been largely devoted to the use of radioactive tracers. Sanitary engineers have been using such tracers for many years. Various investigators have described the use of radioactive phosphorus, P^{32}, in their studies (27, 46, 60, 51, 53). Indications were given that, when present in concentrations at or below 10 millicuries per liter, P^{32} exerts no significant effect on the biological oxidation of the sewage (27). In addition, it was reported that P^{32} inhibits the biochemical reaction in sewage when the activity level ranges from 10 to 80 millicuries per liter (60). In

studies of slimes found in sinks and traps, P^{32} was used as a tracer. Other slime studies have shown that P^{32} is metabolized by slimes and that the plots of uptake of P^{32} vs time are similar to the plots obtained in B.O.D. studies (51, 53).

The tracer used in the present study was P^{32} because it does not have any adverse effect on the rate of biochemical reaction and it is a moderate energy β (beta) emitter. The P^{32} can be handled easily and does not require much health precautions when used in low concentration, in addition to having a convenient half-life of 14.7 days. Further, the phosphorus, a necessary nutrient in cell growth, fulfills the requirement that the tracer should accurately reflect the rate of the reaction measured.

Summary

As oxygen uptake measures respiration and phosphorus uptake measures metabolism, it is necessary to know the correlation under varying conditions between these two processes in order to be able to use the latter for "K" determination rather than the generally employed oxygen uptake procedures. A direct result of this study was the development of this correlation and the explanation of such deviations as may occur in terms of the processes involved. If there is a direct or an indirect correlation, the P^{32} uptake technique may be substituted for the existing method with obvious advantages. If there is a substantial deviation, the investigator will have not one but two tools to study the reaction rate constant.

CHAPTER II

SLIMES: RATE CONSTANTS AND P^{32} UPTAKE

Background

For almost one hundred years slime-forming bacteria have been studied by various investigators. In 1914 Johnson (37) was perhaps the first person to observe the importance of the bacteria in the activated sludge process. However, zooglea bacteria were mentioned by Flugge (25) in his book in 1886. Kruse's chapter in this book states that Itzigshon in 1867 was the first person to observe the bacteria. In 1897, Stutzer and Hartleb (64) described them as spore formers capable of oxidizing nitrogenous compounds. Buswell and Long (9) while presenting a theory on the microbiology of the activated sludge process contended that the zooglea, assisted by protozoa, are responsible for rapid purification of waste water.

Extensive research on this subject did not start until 1935, when Butterfield (10) isolated the bacteria from activated sludge and described some of its morphological characteristics. Heukelekian and Schulhoff (32) purified a sterile solution of sewage by introducing a small quantity of the organism. Later, Wattie (68), McKinney and Horwood (42) conducted

extensive investigation on the ability of Zooglea ramigera to stabilize organic substrate in activated sludge. Butterfield (10), Ruchoft and Watkins (55), Breedsley (6), Hall (31) have studied those slimes which are commonly found in sewage treatment plants while Starkey (63), Sanborn (56), and Beckwith (4) have studied slimes encountered in raw waters, particularly those associated with the coliforms and aerobacteria.

Investigations to date have shown that Zooglea ramigera has the ability to grow in floc or in colonies in a liquid medium in quiescent state, and also in liquid subjected to slow agitation produced by aeration sufficient to maintain aerobic conditions. The isolation of the pure culture of the bacteria has been described by McKinney and Weichlein (43) as well as by Dugan and Lundgren (19). However, slimes for the present study were selected by environmental conditions rather than by pure culture techniques.

In the biological sewage treatment process, the organic material is synthesized into its stable end products principally by aerobic bacteria. Zooglea ramigera is chiefly responsible for the stabilization of the organic material in the sewage. Zooglea bacteria form slimes, which may be described as the gelatinous matrix or film formed on submerged water surfaces constituted both by living and associated non-living matter. The bacteria, at times, use for food the gelatinous mass which binds the bacteria together. The thickness and life of the film will depend upon various physical, chemical, and biological environmental factors.

Slimes usually appear in polluted streams or rivers and in sewage treatment plants; their growth is especially luxuriant in sewage containing large concentrations of organic matter. They are particularly sensitive to changes in temperature and pH. The oxygen supply, the supply of nutrients, and capacity to support the mass of growth without sinking are also important for proper growth.

Since various other types of bacteria are found associated with Zooglea ramigera, the resulting slimes may sometimes appear grayish-white in neutral and alkaline water. In acidic water the slimes may appear rust-colored when covered by other bacteria. Even traces of organic matter will provide the necessary food for their growth in the sea and in otherwise unpolluted streams. Zobell and his associates (72, 73, 74) have described many fundamental aspects of attached growths in salt water media.

Rate Constants and Growth

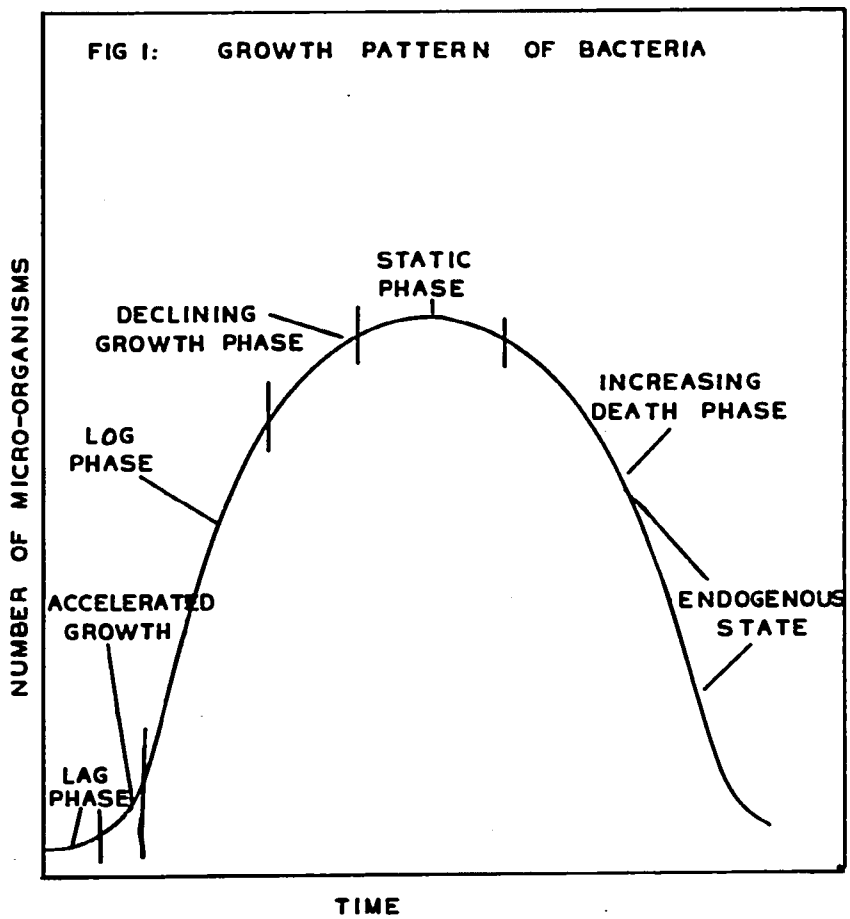
The present standard B.O.D. test (62) by the dilution method determines "K" values by calculating the amount of oxygen consumed by a particular sample of the waste. It is an indirect method which involves the determination of the values of L and y in equation (1),, $\frac{dy}{dt} = -K(L-y)$. The "K" value will determine the rate of slime growth, upon which depends the rate of sewage purification.

The quantitative study concerned with the rate at which the reactions occur, is called reaction kinetics. Reaction

kinetics provide a convenient way to classify the reactions according to their molecularity, (i.e., the number of atoms or molecules taking part in the chemical reaction). Determination of the reaction rate constant, "K", in a purely chemical reaction poses no problem, and can be determined rather easily. The rate of chemical reaction varies from an exceedingly large value to essentially zero depending upon the composition of the mixture, the temperature, and the presence or absence of a catalyst. Accurate knowledge of these variables affecting the rate is very important.

Chemical reactions brought about in a biological substrate are termed biochemical reactions and are more complicated than ordinary chemical reactions. If other parameters, such as pH and temperature, are held constant, the rate of biochemical reaction will increase with the rate of increase in the population of the organisms and the food supply.

The population of the organisms increases at varying rates in time from lag phase to accelerated growth, and thence to a logarithmic phase of growth (see Figure 1). Thereafter a static phase is reached where only minor variations occur in the population. Or to paraphrase, initially where a population of the micro-organisms is small, the "K" value will be small. The value of "K" will increase slowly and steadily as the population of bacteria increases. It would be practically constant during the static phase of microbial growth. The final stage would have a negative "K". This is the endogenous stage in



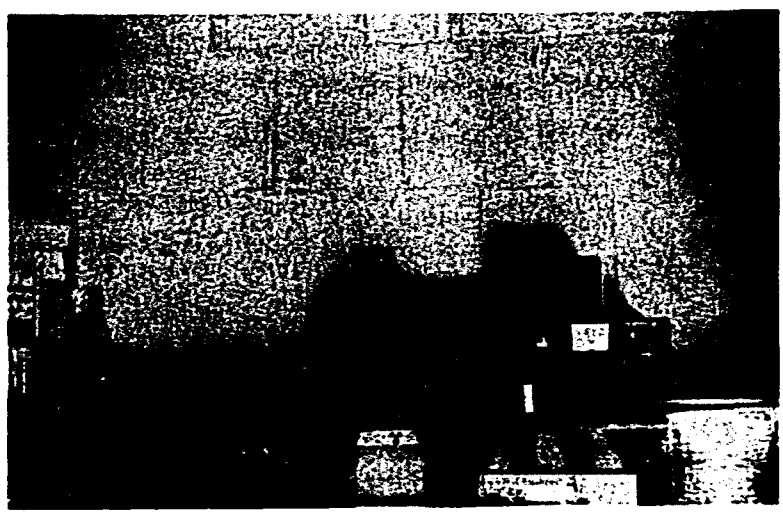


Figure 2 A Photograph of Rotating Drum Apparatus

which the bacteria "live off of" themselves. In the typical bacterial growth curve, the area of principle interest is the logarithmic phase, because in this phase the population of bacteria is increasing at the maximum rate and the food utilization is high. The relation between food utilization and bacterial growth here is linear, the rate being logarithmic.

Zooglea ramigera exhibits an S-shaped or logistic type growth curve typical of many bacteria and is apparently more of an autocatalytic than monomolecular type. The rate of growth, rather than the absolute count determines the rate of oxidation. Inadequate supply of food, decomposition of attached portions, abrupt changes in temperatures, and scouring velocities will cause detachment sloughing of the attached living or non-living slimes.

The aerobic bacteria will grow in a free supply of oxygen, with some species even being capable of drawing limited amounts of oxygen from their food substances. As long as the concentration of oxygen does not fall below the critical limit, the growth of bacteria is independent of the amount of oxygen available for their respiration.

Bacteria grow best in a limited optimum temperature range, which varies from species to species. The growth will decrease or increase if the temperature is varied much on either side of the optimum range. The bacteria are also susceptible to small changes in pH, with the upper and lower limits of the pH being 4.0 and 10.0 respectively. For the optimum

bacteria growth, Bergey's Manual (5) recommends a temperature of 20^{o} to $30^{o}C$, and a pH value of 7.0 to 7.4.

When the bacterial population reaches its maximum, a condition of equilibrium occurs. This condition would be somewhere near the point of maximum growth. At this point, there is every possibility that a slight depletion in the concentration of food will shift the balance to initiate the declining phase of the bacterial growth curve. Naturally, sanitary engineers would like to maintain the bacterial population at somewhere close to the maximum rate of multiplication for then the rate of utilization of organic food, (i.e., "K" value) would be a maximum.

The organisms themselves break the complex substances into simpler forms and use them as a source of energy in their metabolism. In doing so, they ultimately break down the nutrients into stable end products. The end products are not putrescible and do not pollute a natural body of water. As in the case of all other organisms, the living processes of bacteria are dependent upon the activity of specific enzyme systems.

Since enzymes serve essentially as catalysts, accelerating or retarding specific biochemical reactions, they have much influence on the reaction velocity constants. Enzymes are in no way associated with the end products, nor are they rapidly used up in the reactions catalyzed by them. The effect of enzymes may be shown by

$$E + S \rightleftharpoons ES \longrightarrow E + \text{end products} \qquad (2)$$

where E represents an enzyme, S a substrate, and ES, a substrate complex.

It is assumed that a change of concentration of food per unit time is proportional to "K" multiplied by the concentration, C, of oxidizable organic matter remaining in solution. This is expressed as

$$-\frac{dy}{dt} \propto KC \qquad (3)$$

It is further assumed that optimum temperature and pH conditions prevail. The position of this with respect to time will then be a direct function of the food supply, L, as previously shown in

$$-\frac{dy}{dt} = K\,(L-y) \qquad (1)$$

where, L, may be measured in terms of: (1) oxygen uptake; (2) carbon dioxide production; (3) the concentration of the nutrients; (4) total bacterial growth; (5) increase in the slime weight; or (6) the uptake of P^{32}.

In their studies Monod (46) and Hinshelwood (35) suggest that the factors limiting the bacterial growth are: (a) depletion of nutrient material; and (b) presence of toxic material. When depletion of nutrient material is the major factor, the "K" value becomes a function of concentration of the nutrient remaining in solution. The presence of toxic materials will greatly affect the rate of growth of bacterial population. When the toxic substances are present in high concentrations,

the micro-organisms may die, thus stopping their metabolism. If such conditions arise, it is necessary to reduce the toxicity by dilution.

In the biological treatment of sewage or industrial waste, when organic matter is brought in contact with the micro-organisms, the substrate is broken down and converted to cellular constituents of the organisms. Stoichiometrically, the two stage process can be illustrated by the following:

$$\text{Organic matter} + NH_3 + P + O_2 \longrightarrow \text{Bacteria Cell Material} + CO_2 + H_2O \quad (4)$$

$$\text{Cell Material} + O_2 \longrightarrow CO_2 + H_2O + NH_3 \quad (5)$$

In the first stage, the micro-organisms process the organic matter primarily for the purpose of creating new cells. Thus, the micro-organisms continuously remove organic matter from the liquid substrate by synthesizing new protoplasm for use in building new cells and rebuilding old cells. Also, the chemical composition of protoplasm varies not only for the various groups of micro-organisms, but may vary for a simple species, depending upon the physical and chemical environments.

Porges, et al (49) suggested that an empirical composition of the cells may be established by direct analysis of oxygen, carbon, hydrogen, nitrogen, and ash. When the concentration of nutrients decreases and is not sufficient to propagate the building of new cellular masses then the endogenous oxidation starts. In this part of the reaction, shown by Equation 5, the cell material is oxidized to its stable end products.

Respiration and Metabolism

The bacteria like any other living form respire and consume food. The bacteria first take the organic matter into their cells and then process it for energy and synthesis. Unlike higher forms, bacteria are not capable of consuming solid food. So the complex organic matter is broken into simple soluble food by exoenzymes before the bacteria can consume it. Solubility of food is very important, because all the food must diffuse through the cytoplasmic membrane before it is available in the form of energy for bacteria. During the process of growth, aerobic bacteria consume oxygen freely.

For many years, scientists have been trying to correlate the uptake of oxygen, with the rate of utilization of the organic matter from the substrate. Sir Edward Frankland in 1870 (26) was perhaps the first to use oxygen as the yardstick for the measurement of pollution. Independently and about the same time Geradin (28), Dupre (20), tried to establish that the amount of oxygen consumed during an oxidation process may give a quantitative index of the amount of organic matter oxidized. After the publication of the Eighth Report of the Royal Commission on Sewage Disposal, the American Public Health Association (A.P.H.A.) appointed a subcommittee to make a thorough report on the method proposed by the British Commission. Later Theriault and Hammon (65) published their classical paper. Their procedure for measurement of oxygen uptake in a polluted water became a standard test, and is referred to as the B.O.D. Test.

Respiration was originally defined as being strictly the uptake of gaseous oxygen. The current school of thought advocates that, since oxidation could occur by removal of hydrogen or removal of electrons (without employing gaseous oxygen), the term respiration therefore includes all reactions by which the bacterial cells obtain energy, whether gaseous oxygen is involved or not. Since the latter definition does not distinguish fermentation (anerobic digestion) from aerobic digestion - and this distinction is very important in the sanitary engineering field - it is desirable to define these terms as they are used in this report:

RESPIRATION - The uptake of gaseous oxygen. It includes oxygen used by synthesis and endogenous respiration of the cell.

FERMENTATION - The transformation which occurs in a living cell in absence of gaseous oxygen.

Since this study is devoted to respiration only, no further reference will be made to fermentation. The uptake of oxygen may be measured by: (1) various types of manometric techniques; (2) respirometer; (3) B.O.D. techniques, or (4) by measuring production of CO_2.

The biochemical changes involved in microbial respiration are very complex. Micro-organisms require food for growth and construction of new protoplasm, as well as for repairing old and worn out protoplasm, and for supplying energy in carrying out endothermic reactions. The stabilization of organic

food occurs in a continuous chain of reaction, involving oxygen as a necessary constituent. Investigators have often believed that the volume of millions of micro-organisms is very small in comparison to their surface area. As a result their respiratory activity is much greater than that of their food uptake. Apparently the respiratory activity of the micro-organisms is more or less related to the surface area used; for this reason, all research to date on sewage treatment processes has been done on respiration rather than on metabolism.

Metabolism involves the intake, digestion, assimilation of food into tissues, and transformation of potential energy of food into kinetic energy. With this transformation the organisms accomplish work and the elimination of waste products. Metabolism may be endothermic or exothermic, depending whether energy is absorbed or liberated during the reaction.

The organic constituents encountered in a sewage plant are very complex molecules of unknown composition. Although the decomposition of simple organic material will be accomplished much more readily than that of complex food substrate molecules, complexity is not the only criterion of resistance to attack.

The stabilization of organic matter in the waste water will also depend upon the rate of permeability. It is known that hydrophilic hydroxyl, carboxyl, and amino groups will diffuse much more readily than hydrophobic groups. However, if the carbon chain to which the hydrophilic groups are attached

exceeds twelve atoms, the rate of diffusion decreases with an increase in the number of carbon atoms (42). The hydrolysis of complex organic matter is brought about by extracellular enzymes, and recent studies (22) have shown that extracellular enzymes may be located in the cell wall or cytoplasmic membrane.

Placak and Ruchhoft (48), Heukelekian, et al (34), and Helmer et al (32) found that there is a direct relation between the removal of B.O.D. and the growth of the sludge solids. However, in the opinion of the author, this relationship is not constant for all types of waste material and the conversion factor will change for different types of food.

As has been indicated earlier, "K" values can be determined by using either O_2 consumed or CO_2 generated. In such studies, O_2 and CO_2 for a system should essentially be equal to each other. The ratio of carbon dioxide evoled to oxygen uptake is called the respiratory quotient (R.Q.) and may be calculated theoretically from a chemical equation, which may be expressed as:

$$\text{Soluble food} + \text{oxygen} + \text{bacteria} \longrightarrow \text{Less soluble food} + \text{more bacteria} + \text{carbon dioxide} + \text{water} \qquad (6)$$

Food has been shown in the soluble form, because bacteria generally cannot consume solid food. The above equation may also be written into a general chemical form

$$AC_aH_bO_cN_d \cdot XH_2O + YO_2 \longrightarrow C_sH_tO_uN_v \cdot MH_2O + NCO_2 + BH_2O \qquad (7)$$

The small letters represent constants for a given organic food.

Although the above equation represents the general course of the reaction process, the chemical equations for the oxidation of sugar may be written as follows:

(a) Considering synthesis only:

$$C_6H_{12}O_6 + O_2 + NH_3 \longrightarrow C_5H_7NO_2 + CO_2 + 4H_2O \quad (8)$$

R. Q. = 1/1 = 1.0

(b) Considering respiration only:

$$C_6H_{12}O_6 + (1\text{-}5)\ O_2 \longrightarrow (5\text{-}1)\ CH_2O + (1\text{-}5)\ CO_2 + (1\text{-}5)\ H_2O \quad (9)$$

$$\text{R. Q.} = \frac{(1 \text{ to } 5)}{(1 \text{ to } 5)} = 1.0$$

(c) Combination of respiration and synthesis:

$$8C_6H_{12}O_6 + 18O_2 + 4\ NH_3 \longrightarrow 10CH_2O + 4C_5H_7NO_2 + 18\ CO_2 + 30H_2O \quad (10)$$

$$\text{R. Q.} = \frac{18}{18} = 1.0$$

From stoichiometry it appears that the R.Q. factor for the oxidation of sugar is 1.0. Investigators in the sanitary engineering field have often accepted this as holding true for all cases. However, an arbitrary assumption of R.Q. as 1.0 may often be erroneous as demonstrated by Siegle and Clifton (57, 58). Results of their studies in Table 1 show that R.Q. varies from 0.70 to 1.95. Therefore it must be emphasized that the experimentally derived R.Q. factor may or may not equal 1.0. This is particularly true when the substrate is of a complex nature, and the oxidation reactions yield intermediate products in the synthesis process. The stoichiometry

TABLE 1

COMPARATIVE CARBON BALANCES IN THE OXIDATION ASSIMILATION OF SEVERAL SUBSTRATES DURING THE GROWTH OF *ESCHERICHIA COLI*[a] (DURATION OF EXPERIMENT 4.5 HOURS)

Substrate, mg.c	Succinate	Fumarate	Lactate	Pyruvate	Glycerol
Initial substrate.C	5.51	3.14	3.31	4.45	5.40
Cell-C-after assimilation	0.55	0.44	0.37	0.51	0.72
Cell-C-before assimilation	0.35	0.21	0.29	0.20	0.51
C Store	0.20	0.23	0.08	0.31	0.21
Supernatant-C, at end of experiment	5.04	2.57	3.19	3.76	5.15
CO_2-C	0.29	0.30	0.12	0.39	0.10
Total recovered	5.53	3.10	3.40	4.46	5.46
Total recovered, Percent	100.3	98.7	102.7	100.2	101.1
µl CO_2 produced	536	534	214	724	182
µl O_2 consumed	380	274	222	394	266
R. Q. observed	1.41	1.95	.97	1.84	0.70

[a]From Siegel and Clifton (57).

does not always account for the intermediate products. Because of this it is believed that a direct study of metabolism will not only alleviate the shortcoming of the respiration test, but also provide a research tool for further investigations of metabolic activity.

Theory of Uptake of P^{32}

When P^{32} is added to the substrate it remains in solution. Bacteria during their growth remove P^{32} as well as organic matter from the solution. This uptake of radiophosphorus by bacteria is not in any way different than the uptake of non-radioactive phosphorus, as bacteria cannot differentiate between the two. The slimes will pick up P^{32} from solution by (a) adsorption initially removed on the surface, and then (b) assimilated by the bacteria. Thus, when P^{32} is used, the process of consumption can be readily detected with a Geiger tube.

The total surface area of the bacterial slime is very high compared to its volume, and the adsorption rate is strictly dependent on the surface area of the slime. The slimes will first adsorb P^{32} and then slowly assimilate it during their growth. For short periods of exposure, the rate of adsorption of P^{32} will be more pronounced. As time progresses both the bacteria population and the rate of assimilated P^{32} will increase simultaneously. This implies that adsorption occurs very rapidly, and is most important during the initial phase of the experiment. The mechanism of uptake of P^{32} may be shown as:

$$\text{Solution } P^{32} \underset{\text{Sloughing}}{\rightleftharpoons} \text{Adsorbed } P^{32} \qquad (11)$$

$$\text{Adsorbed } P^{32} \longrightarrow \text{Assimilated } P^{32} \qquad (12)$$

where the rate of growth of slimes is represented by assimilated P^{32} instead of total uptake of P^{32}.

In order to make a distinction between the adsorbed P^{32} and assimilated P^{32}, Reid (51) has shown that slime growth can be inhibited by a chemical agent. Such a chemical agent should have two properties: (a) it should have sufficient power to inhibit growth, and (b) it should not accelerate the rate of sloughing. For example, Reid found that a buffer solution at a pH of 4.0 arrested growth but led to rapid sloughing. A merthiolate solution (1:2000 or 1:4000) was found to be successful for the measurement of adsorption by inhibiting the growth without affecting the rate of sloughing. Sloughing of attached slimes from the surface is important because the manometric technique developed for attached slimes depends on surface contact. As the slimes grow and age, the important factors producing sloughing are the forces discussed in the following:

Sloughing is a common phenomenon in trickling filters. Studies of attached slimes on a rotating drum indicate that slime layers will build up at a rate depending upon the amount of food available and its temperature (54). In order to better understand causes of sloughing, it is necessary to know the forces acting on the slime and the influence of the surface they are attached to. These forces are (a) the cohesive force

between the slimes and the rotating drum surface; (b) cohesive force between two adjacent layers of slime; and (c) the tractive force between the medium and the slime. The tractive forces remain constant unless the speed of rotation is changed. The cohesive force between the primary layer and the surface apparently does not change, unless endogenous conditions develop in deep layers. As layers feed from adjacent ones, thin layers will to a certain extent prevent sloughing. The primary layer invariably remains attached to the drum while secondary layers may slough off, either gradually or abruptly. However the cohesive force between two layers may be reduced due to the increase in the death rate of the bacteria, or may in some cases be overcome by excessive growth of the secondary layer. Therefore, changes in the forces (a) and (b) described above, will cause slime sloughing.

From the proceeding it can be concluded that:

1. In aerobic biochemical reaction, the respiration (uptake of oxygen) results in the transformation of the organic matter into its end products which are carbon dioxide, sulfates, nitrates, and water;

2. The organic matter is consumed by the bacteria through diffusion (partly oxidized and partly converted into building cells and cytoplasm);

3. Only stabilized portions can be considered to be oxidized;

4. The oxygen uptake will only show the oxidized portions of the food material, and will not represent the organic

material remaining behind. Usually the quantity of oxygen consumed is correlated with the consumption of food. It is doubtful if the quantity of oxygen consumed is in any way directly related with the complexity of food; and

5. It would be much more convenient to calculate the rate of stabilization of organic matter with the use of radio-phosphorus-32.

CHAPTER III

PRELIMINARY STUDIES OF ATTACHED SLIMES

Experimental Apparatus

The apparatus used in this study was designed to study the proper evaluation of the following factors that affect the rate of slime growth and thus its direct influence on the uptake of P^{32}:

1. Separation of adsorption and assimilation;
2. Concentration of food;
3. Type of food;
4. Temperature.

The apparatus consisted of three glass drums 3 3/4 inches in diameter and 2 inches wide, mounted on a copper rod. (See Figures 2A and 2B.) These drums were rotated approximately half submerged in individual stainless steel troughs containing synthetic sewage. The outer surface of the rotating drums were roughened by sand blasting to facilitate adherence of the bacteria. The drums were allowed to rotate at a constant speed of 2 rpm until a satisfactory growth appeared.

Experimental Procedure

Once the desired growth was established, a known quantity of P^{32} was injected with a one cc Tuberculin-hypodermic

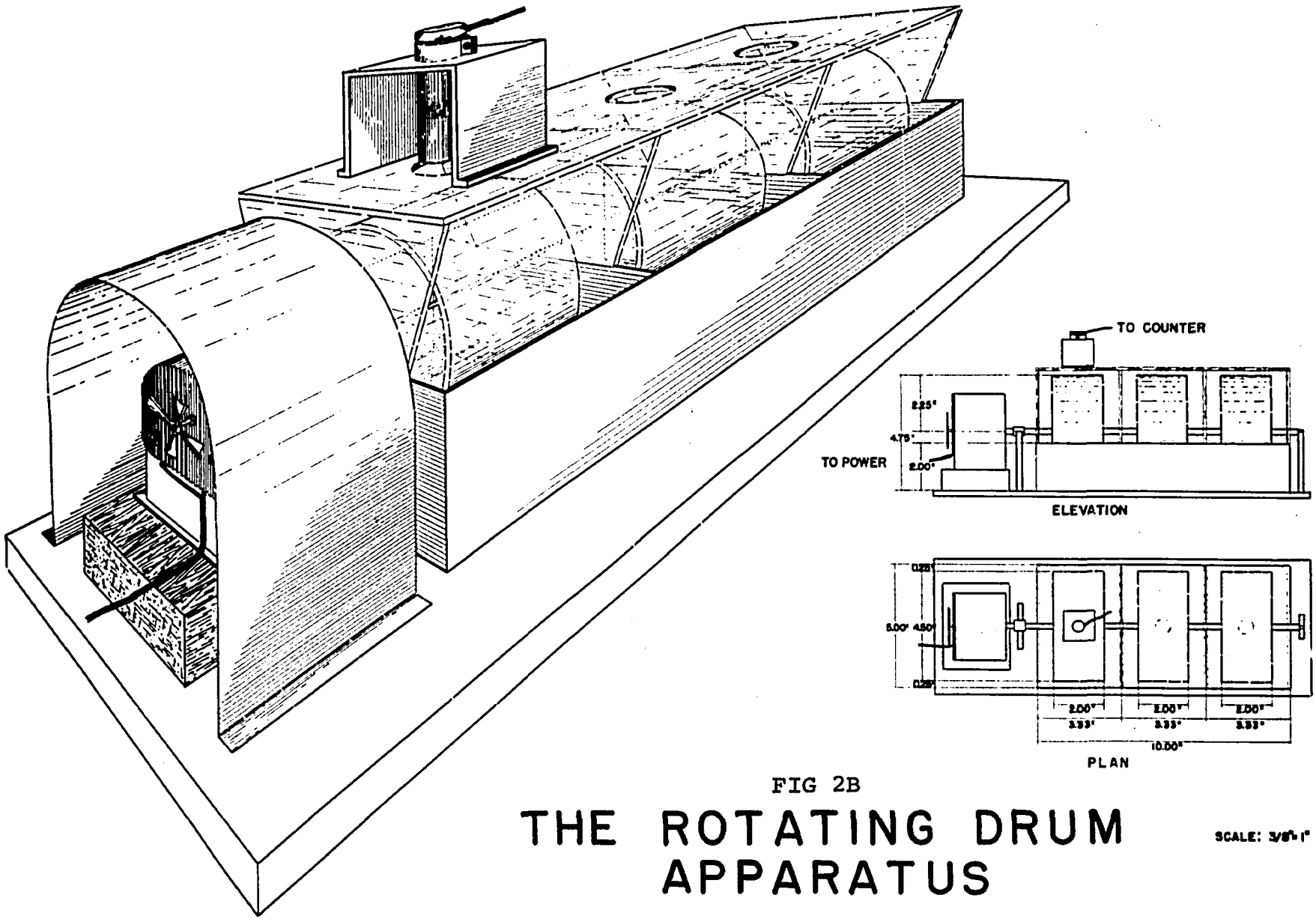


FIG 2B

THE ROTATING DRUM APPARATUS

SCALE: 3/8"=1"

syringe into each trough. Pulses were counted every hour with a Geiger-Mueller (G.M.) detector tube, for fifteen hours; one additional reading was made at the end of 25 or 26 hours. All of the P^{32} readings were corrected for decay and background.

The growth rate of Zooglea ramigera expressed in terms of oxygen uptake essentially measures respiration and in terms of P^{32} uptake measures metabolism. However the experimental technique involves the study of the simultaneous uptake of oxygen and P^{32}; both for the attached and free floating slimes. After considerable experimentation, three general approaches were used for this research. The first included preliminary studies of the attached slimes on the rotating drums, primarily to evaluate various variables, described later. The second was to place the entire rotating drum apparatus inside of a manometric device - a procedure whereby the uptake of oxygen was measured along with the uptake of P^{32} in an air-tight system. Finally in a third series of experiments, a procedure was developed where free floating slimes were grown in enclosed environments. This last technique included the use of the standard B.O.D. test along with a P^{32} tracer. These studies are described in detail in the following sections.

All of the experiments used dehydrated lactose broth as the source of synthetic sewage. One gram/liter of lactose broth has a 5 day 20°C B.O.D. of 400 mg/liter. In a few experiments, Bacto-peptone was added to lactose broth. The growth in the synthetic sewage was initiated by seeding with

raw sewage from the Norman, Oklahoma Sewage Treatment Plant. Merthiolate, 1:2000, was used to inhibit the growth when it was necessary to separate adsorption from total uptake of P^{32} and thus obtain the desired data on assimilated P^{32}.

Preliminary Studies

In the first phase of the investigation, several tests were made in which P^{32} was added initially to the synthetic sewage. The rate of uptake of P^{32} was observed periodically at various stages of slime growth. No attempt was made to inhibit slime growth and as they grew, the uptake of P^{32} increased. Typical curves of the total uptake of P^{32} are shown in Figures 3 and 4. These are characteristic of bacterial growth curves. Figure 3 shows the relation of counts per minute vs time, while Figure 4 represents the per cent uptake of P^{32} vs time. For purposes of calculation, the highest reading of counts per minute of P^{32} was assumed to be equal to 100 per cent utilization.

The rate of growth increases from a lag phase to a logarithmic growth and slowly reaches a peak although the slimes may remain for long periods at a maximum population or may decline due to lack of food. The curves reached a population peak at about 86 hours. Thereafter they declined due to the insufficiency of food, as evidenced in these experiments by the sloughing of the slime. In the logarithmic growth phase, there is always an excess of food around the micro-organisms. As the slimes grow, they utilize the food by removing the

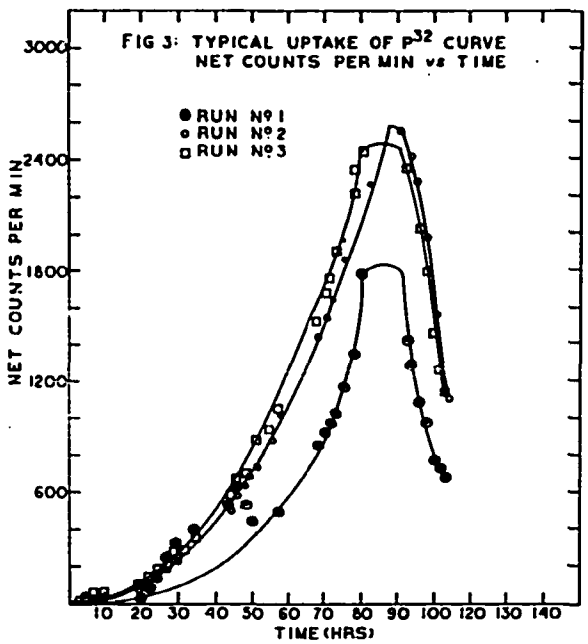


FIG 3: TYPICAL UPTAKE OF P32 CURVE
NET COUNTS PER MIN vs TIME
● RUN Nº 1
○ RUN Nº 2
□ RUN Nº 3
NET COUNTS PER MIN
3000
2400
1800
1200
600
10 20 30 40 50 60 70 80 90 100 120 130 140
TIME (HRS)

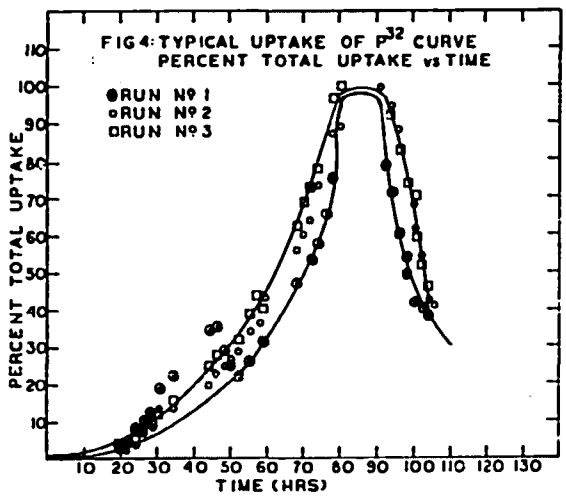


FIG 4: TYPICAL UPTAKE OF P32 CURVE
PERCENT TOTAL UPTAKE vs TIME
●RUN Nº 1
○RUN Nº 2
□RUN Nº 3
PERCENT TOTAL UPTAKE
110
100
90
80
70
60
50
40
30
20
10
10 20 30 40 50 60 70 80 90 100 110 120 130
TIME (HRS)

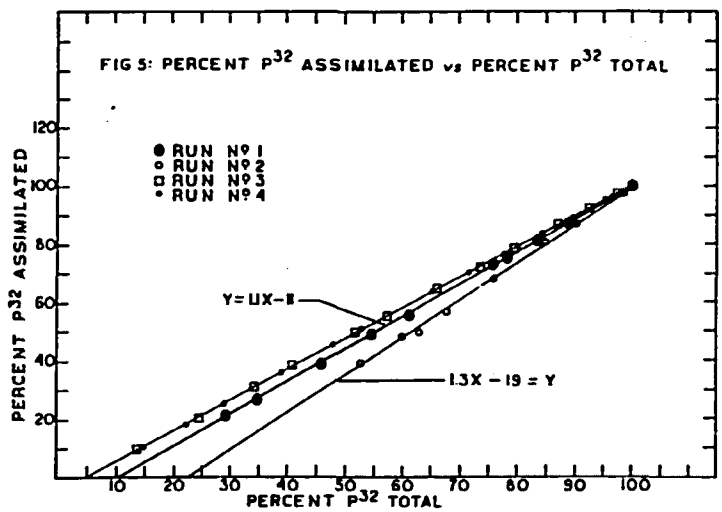


FIG 5: PERCENT P32 ASSIMILATED vs PERCENT P32 TOTAL
● RUN Nº 1
○ RUN Nº 2
□ RUN Nº 3
• RUN Nº 4
Y = 1.1X - 8
1.3X - 19 = Y
PERCENT P32 ASSIMILATED
120
100
80
60
40
20
10 20 30 40 50 60 70 80 90 100
PERCENT P32 TOTAL

organic matter from the solution. When the organic matter is removed, it is converted into bacterial protoplasm, which is eventually reduced to stable end products.

There appears to be a linear relationship between the effective mass of slimes and food concentration. When there is a continuous depletion in the concentration of the available food, the bacterial growth reaches its maximum population and thereafter decreases, as shown in Figures 3 and 4. The maximum population will continuously vary in response to the concentration of available food. When the depletion of food is negligible, P^{32} will increase or remain at a constant value for a long period. But when food is depleting continuously, the uptake of P^{32} will reach a peak value and will thereafter decline, indicating a decrease in the growth rate.

The following conclusions were made from these preliminary studies:

1. The "K"-values for trickling filters or aeration tanks may be maintained at a desired level throughout the period of their operation, but under ordinary conditions the "K" values in a stream will vary depending upon the concentration of available food, which cannot be controlled.

2. The sloughing of slimes is an important factor to consider when comparing data of P^{32} uptake with respiration, because the sloughed-off mass may include living and growing bacteria which may be consuming food and oxygen, yet are not included in the counting of P^{32} on the rotating drum. This

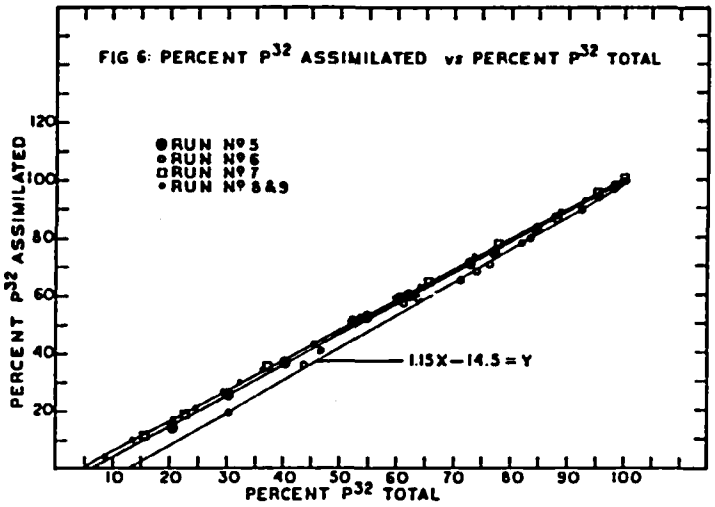
error in counting the P^{32} needs correction, since there is a possibility that due to excessive sloughing, the P^{32} count may decrease consistently while the uptake of oxygen may continue to increase.

3. There is a definite relationship between the number of bacteria and metabolism of P^{32}. Hence P^{32} uptake rate can be used for the studies of oxidation rates.

Adsorbed and Assimilated P^{32}

In order to measure the adsorbed P^{32}, it was necessary to record the uptake of P^{32} by inhibiting the bacterial slime growth. Thus the measured uptake of P^{32} would predominately be due to adsorption only. To inhibit growth of the slime, the bacteria were grown in the synthetic sewage as before, until significant growth appeared on the drums. Then an inhibiting agent was introduced. A known quantity of P^{32} was injected and readings were taken every hour. Since the slime growth may be inhibited with a very acidic, or basic buffer solution one of pH 4.00 was tried. However, the rate of sloughing was increased and a sufficient amount of sloughing was detected in less than two hours. A merthiolate solution in concentration of 1:2000 and 1:4000 was used next and proved to be an excellent agent for inhibiting growth without affecting the sloughing rate. As a result a merthiolate solution 1:2000 was selected as the inhibiting agent.

The results of the adsorption measurements are shown in Figures 5 and 6. They show that the adsorption rate is



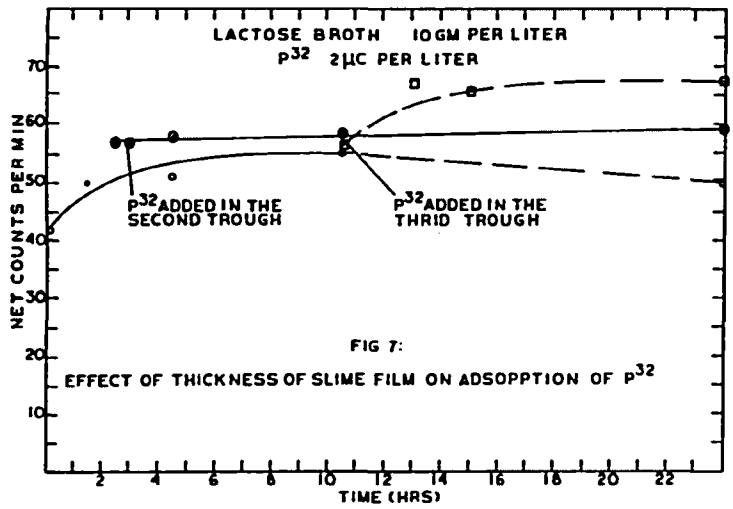
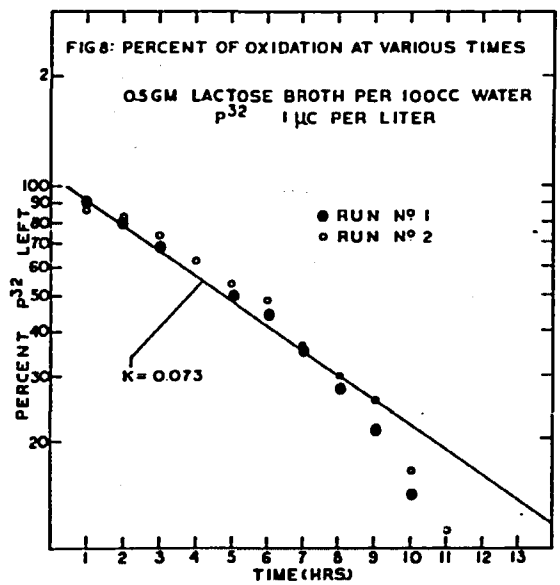
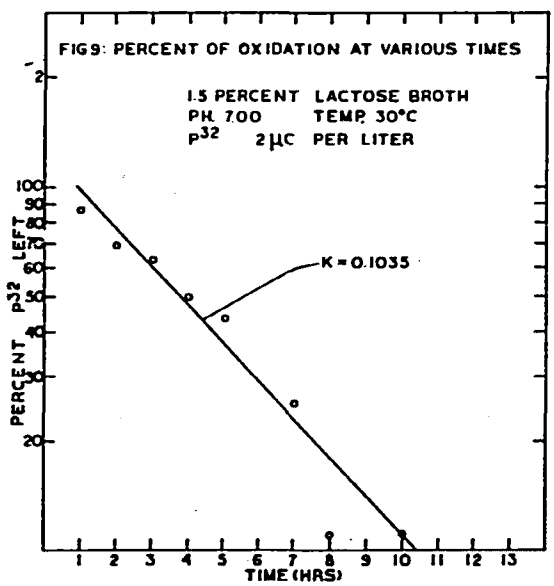


FIG 7:
EFFECT OF THICKNESS OF SLIME FILM ON ADSOPPTION OF P^{32}





more pronounced in the first two hours with a relatively constant rate thereafter. The assimilated P^{32}, however, continues to increase so long as the bacterial population increases. When P^{32} is added to a substrate containing bacteria, almost instantaneously a small quantity of P^{32} is adsorbed by the bacteria in the substrate. As the bacteria grow and multiply, adsorbed P^{32} is assimilated by the bacterial cells. In the process of assimilation of the food the adsorbed P^{32} diffuses through the cell nucleus and becomes a part of the cytoplasm. In the first two hours, the P^{32} measured was mostly due to adsorption. From the second to the sixth hour it was present in both the adsorbed and assimilated states. After about six hours the rate of assimilation apparently exceeds the rate of adsorption and the P^{32} measured thereafter is primarily assimilated P^{32}.

Adsorption of P^{32} is necessarily a function of surface area and depends upon the surface properties of bacteria. Plots of per cent of assimilated P^{32} vs per cent total P^{32} are shown in Figures 5 and 6, and represent the results of experiments conducted at different concentrations of food. The findings appear to be in generally good agreement, within the limits of experimental error. The result is a straight line, which can be represented by:

$$\text{Per cent } P^{32} \text{ metabolized} = 1.15\ (\text{Per cent total uptake}) - 14.5 \qquad (13)$$

The plots show that, when no metabolization has occurred,

adsorption of 3 to 20 per cent has taken place. This suggests that adsorption occurs rapidly and is most important in the initial phases of growth. The linearity of the plots seems to indicate a high degree of direct correlation. These results are in agreement with the postulated hypothesis.

Additional experiments were conducted to evaluate the effect of slime thickness on P^{32} adsorption. Slimes were grown in the rotating drum apparatus and when a uniform thickness was established, the synthetic sewage was inhibited with the merthiolate solution and P^{32} was injected. No inhibition was used in the other two identical troughs and the slimes in these continued to grow. After two and one half hours, metabolism in the second trough was arrested by merthiolate solution. No changes were made in the third trough at this time; but at the end of 10 1/2 hours the sewage in the third troughs was replaced by merthiolate to arrest the slime growth. This arrangement made it possible to perform the experiment under conditions of different slime thicknesses. Results are shown in Figure 7. Apparently the thickness of film has little or no effect on the adsorption of P^{32}. From this the conclusion was drawn that adsorption is a function of surface area only.

Plotting of "K"-Value

The uptake of P^{32} by the slime at various time intervals and at varying food concentration is shown in Figure 14. The plots show a gradual increase in the uptake of P^{32}, up to maximum. For the purpose of calculations, this maximum value

was assumed to be equal to 100 per cent utilization. The net counts obtained in each test per minute, were changed into percentages of maximum counts. These percentages were then subtracted from 100 per cent remaining. The percentage remaining plot vs time (hours) on a semilog paper is shown in Figures 8, 9, 10, 11, 12 and 14. The plots are straight line and the "K"-values have been summarized in Table 2. Table 2 indicates clearly that the "K"-value varies with differences in pH and temperature and the effect of pH is more pronounced at higher than at lower temperatures.

The curves for determining "K" by using P^{32} as a tracer are similar to oxidation plots obtained by measuring B.O.D. To prove the applicability of this method for determining "K" it is necessary to compare the findings of these studies with those of comparable respiration studies. This was done and the findings are presented in the subsequent chapters.

Food Substrate Effects

Dextrose, lactose, and d-levulose sugars were used as the source of nutrients for the growth of slimes in some of the experiments. Not only the concentration, but also the composition of the food was found to decidedly effect the growth rate. The sloughing of the slimes occurred more readily at the lower concentrations of nutrients as shown in Table 3. It is evident from the results that growth in d-levulose and dextrose sugars was very poor. In lactose sugar the growth was better.

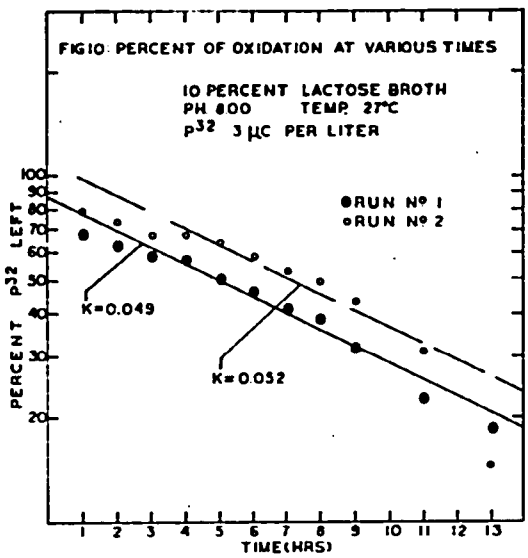


FIG 10: PERCENT OF OXIDATION AT VARIOUS TIMES
10 PERCENT LACTOSE BROTH
PH 8.00 TEMP. 27°C
P32 3 µC PER LITER
●RUN Nº 1
○RUN Nº 2
K=0.049
K=0.032
PERCENT P32 LEFT
TIME(HRS)

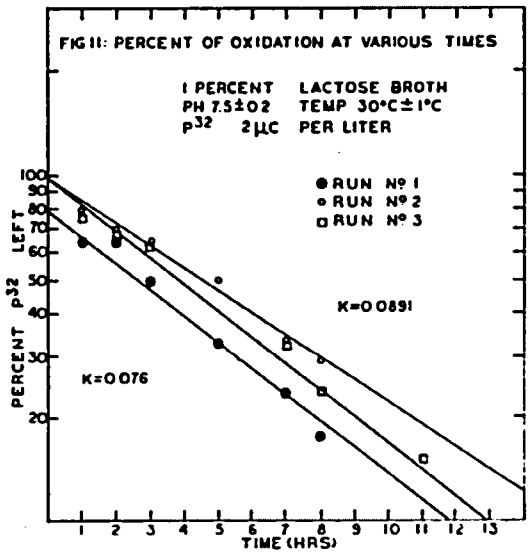


FIG 11: PERCENT OF OXIDATION AT VARIOUS TIMES
1 PERCENT LACTOSE BROTH
PH 7.5±0.2 TEMP 30°C±1°C
P32 2µC PER LITER
●RUN Nº 1
○RUN Nº 2
□RUN Nº 3
K=0.0891
K=0.076
PERCENT P32 LEFT
TIME (HRS)

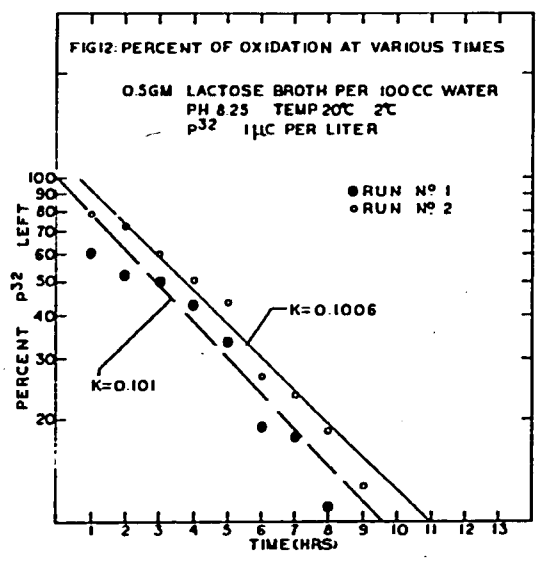


FIG 12: PERCENT OF OXIDATION AT VARIOUS TIMES
0.5GM LACTOSE BROTH PER 100CC WATER
PH 8.25 TEMP 20°C 2°C
P32 1µC PER LITER
●RUN Nº 1
○RUN Nº 2
K=0.1006
K=0.101
PERCENT P32 LEFT
TIME(HRS)

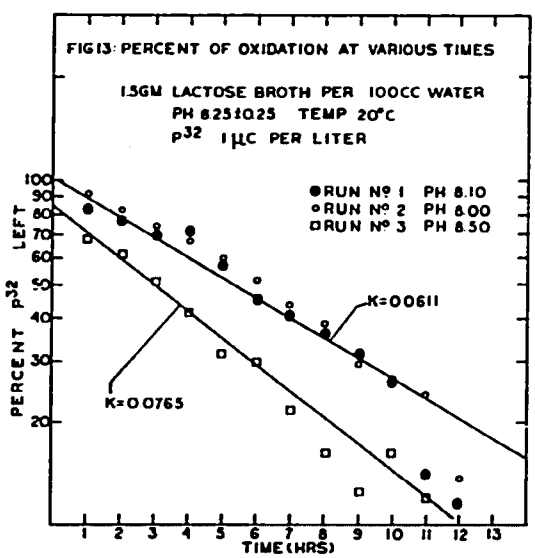


FIG 13: PERCENT OF OXIDATION AT VARIOUS TIMES
1.5GM LACTOSE BROTH PER 100CC WATER
PH 8.25±0.25 TEMP 20°C
P32 1µC PER LITER
●RUN Nº 1 PH 8.10
○RUN Nº 2 PH 8.00
□RUN Nº 3 PH 8.50
K=0.0611
K=0.0765
PERCENT P32 LEFT
TIME(HRS)

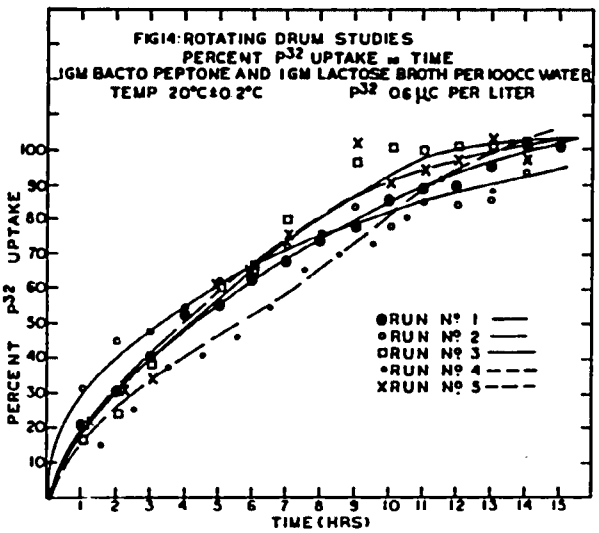


FIG14: ROTATING DRUM STUDIES
PERCENT P32 UPTAKE vs TIME
IGM BACTO PEPTONE AND IGM LACTOSE BROTH PER 100CC WATER
TEMP 20°C±0.2°C P32 0.6 µC PER LITER
PERCENT P32 UPTAKE
TIME (HRS)
●RUN Nº 1
○RUN Nº 2
□RUN Nº 3
•RUN Nº 4
XRUN Nº 5

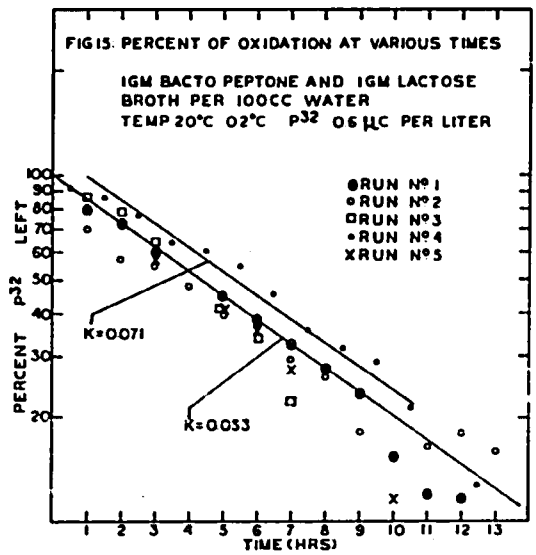


FIG 15. PERCENT OF OXIDATION AT VARIOUS TIMES
IGM BACTO PEPTONE AND IGM LACTOSE
BROTH PER 100CC WATER
TEMP 20°C 0.2°C P32 0.6 µC PER LITER
PERCENT P32 LEFT
TIME (HRS)
●RUN Nº 1
○RUN Nº 2
□RUN Nº 3
•RUN Nº 4
XRUN Nº 5
K=0.071
K=0.033

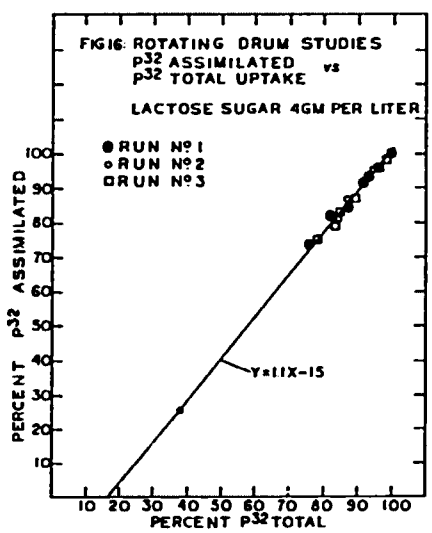


FIG 16: ROTATING DRUM STUDIES
P32 ASSIMILATED vs
P32 TOTAL UPTAKE
LACTOSE SUGAR 4GM PER LITER
●RUN Nº 1
○RUN Nº 2
□RUN Nº 3
PERCENT P32 ASSIMILATED
PERCENT P32 TOTAL
Y=1.1X-1.5

TABLE 2

"K" VALUES OF BIOLOGICAL SLIME AS DETERMINED BY P^{32} UPTAKE TECHNIQUE

Synthetic Sewage Nutrients per 100 cc.	pH	Temp °C ± 2°	K	Remarks
1 gm. Lactose Broth + 1 gm. Peptone	8.2	20°	0.071-0.053	Ave. of 5 runs
1.5 gm. Lactose Broth	8.0	20°	0.0611	Ave. of 2 runs
1.5 gm. Lactose Broth	8.5	20°	0.0765	
	8.25	20°	0.1006	
	8.25	20°	0.101	
1.0 gm. Lactose Broth	7.5-7.7	30°	0.076-0.089	
1.0 gm. Lactose Broth	8.0	27°	0.052-0.049	Ave. of 2 runs
1.5 gm. Lactose Broth	7.0	30°	0.1035	
0.5 gm. Lactose Broth	---	20°	0.073	

TABLE 3

ATTACHED SLIME STUDIES - UPTAKE OF P^{32} IN DIFFERENT SUBSTRATES AT VARIOUS TIME INTERVALS
P^{32} 2 MICRO CURIES PER LITER

	d-Levulose		Lactose Sugar		Dextrose Sugar	
Time Hours	Net Counts Per Min.	% Total	Net Counts Per Min.	% Total	Net Counts Per Min.	% Total
1	32	50.0	777	59.4	29	37.6
2	37	57.8	1051	80.4	40	51.9
3	42	65.6	1163	88.9	49	63.6
4	43	67.2	1299	99.3	43	55.8
6	43	67.2	1286	98.4	56	72.6
7	52	81.2	1276	97.6	67	86.9
9	64	100.0	1276	97.6	62	80.5
10	--	---	1306	99.9	77	99.9
	P^{32} 3 Micro Curies Per Liter					
1	227	60.4	249	77.6	20	19.4
2	283	75.3	267	83.3	26	25.2
3	300	79.8	260	81.1	30	29.1
4	310	82.4	275	85.8	39	37.8
5	323	85.9	262	81.7	40	38.8
6	336	89.3	269	83.9	41	39.7
8	335	89.1	284	88.6	48	46.5
9	320	85.0	285	88.9	51	49.4
11	336	89.3	288	89.8	61	59.2
12	348	92.5	307	95.7	72	69.8
14	369	98.1	309	96.4	82	79.5
15	376	99.9	320	99.9	103	99.9

Figure 14 shows the relation of per cent P^{32} uptake to per cent assimilated P^{32} when 4 mg/100ml lactose sugar was used as a synthetic sewage. The results are shown in Table 4. The plots obtained in this study do not differ greatly from those of the previous studies presented in Figures 8 through 15. In Figure 16, most of the plotted points lie on the upper part of the curve. Only one point is on the lower portion of the curve. This again shows that the rate of growth of slimes in lactose broth is faster than in lactose sugar.

TABLE 4

ATTACHED SLIME STUDIES - P^{32} UPTAKE IN 4 PERCENT d-LEVULOSE SOLUTION

Time (Hours)	Total Counts Per Minute	% Total Uptake	Assimilated P^{32}	Percent P^{32} Assimilated
1	400	85.0	324	83.2
2	409	87.0	333	85.6
3	447	95.0	339	87.1
5	444	94.6	364	93.5
6	452	96.0	372	95.6
7	436	92.9	356	91.5
8	436	92.9	356	91.5
10	432	92.1	352	90.5
11	4.69	99.9	389	99.9

CHAPTER IV

MANOMETRIC RESPIRATION STUDIES OF ATTACHED SLIMES

Manometric Theory

In general the oxidation reactions may be described as the uptake of oxygen and the liberation of carbon dioxide by the living organism. It is customary to measure the respiration of organisms by determining the rate at which they absorb oxygen in a sealed chamber. As the concentration of the oxygen in the liquid suspension and surrounding atmosphere decreases below the critical limit the respiration rate diminishes. If the concentration of oxygen continues to decrease, organisms may enter into endogenous respiration and eventually a stage is reached where metabolism in the micro-organisms ceases, and they die.

The development of manometric equipment for the determination of gaseous exchange in biological metabolism has provided an excellent technique for the measurement of respiration. The metabolism of urea was measured manometrically in 1932 by Krebs and Henseleit (38). Archibald (2) successfully employed the more recently developed colorimetric technique for measurements of urea metabolism. In many cases the choice

between manometric and colorimetric is optional with the investigator, but in some metabolic studies the manometric system is wholly satisfactory, and the easiest to use, even with the recent development of micro-colorimetric methods.

Manometric instruments, designed for measurement of gases either absorbed or evolved, depend upon exchanges between the gas phase and the liquid phase. Manometric techniques provide a rapid method for estimating the rate of absorption of oxygen by the substrate. However, when more than one gas is evolved the situation becomes complex, although it can be resolved in some situations.

When bacteria are suspended in a liquid, they use up oxygen both from the liquid and the surrounding atmosphere if the supply of nutrient remains adequate. This results in the release of carbon dioxide. If only CO_2 and O_2 are involved in the process, one can easily measure the uptake of oxygen by absorbing liberated CO_2 in an alkaline solution. The carbon dioxide gas in the presence of an alkaline solution produces little or no measurable pressure. Therefore, any change in manometric pressure will be directly related to the uptake of oxygen.

Carbon dioxide is less soluble in water than oxygen, but the errors involved in the manometric method are negligible. When dissolved in water, a small part of the CO_2 combines with water to form carbonic acid in a manner which may be expressed as

$$CO_2 + H_2O \rightleftharpoons H_2CO_3 \rightleftharpoons H^+ + HC\bar{O}_3 \qquad (14)$$

However, nearly all of the CO_2 in solution is dissolved CO_2 with less than one percent existing as H_2CO_3. The exact concentration will depend upon the pH of the solution. Manometric methods may be designed according to:

1. Change in pressure at constant volume;
2. Change in volume at constant pressure;
3. Change in both pressure and volume;

although this investigation was limited to method number one.

Manometric Background

The investigator is able to choose from the variety of manometric methods available, depending upon his own personal experience, the type and amount of equipment available, and the accuracy desired.

Barcroft and Halden (3), in 1902, were perhaps the first to devise the "blood gas" manometer. In 1900, Adeney (1) attempted to measure the oxygen demand of sewage by use of a direct adsorption method. In 1909 Rideal and Burgess (53) devised several forms of manometric apparatus, but these proved unsatisfactory due to leakage. These earlier manometric methods required shaking during the course of an experiment.

Sierp (59), in 1928, developed an apparatus wherein sewage could be maintained in contact with pure oxygen and its rate of absorption measured in a calibrated eudiometer tube. His method was much less cumbersome than the dilution method. In 1926, Warburg (66) developed another type of respirometer

for measuring uptake of oxygen. In the tenth edition of Standard Methods (62) the Warburg apparatus, after some modifications, has been accepted as a tentative standard procedure for measuring dissolved oxygen in waste water. In 1930, Dixon and Elliot (17) repeated the work of Barcroft (3) and showed that the rate of respiration is independent of the rate of shaking. Later the manometric method became very popular in the field of respiration studies.

Wooldridge and Standfast (70, 71) studied the effect of temperature on the activity of bacterial metabolism, and the effect of pH on oxygen uptake using the Barcroft respirometer in their studies. When the oxygen uptake values of sewage as determined by the Barcroft respirometer and B.O.D. dilution methods were compared, the manometric method always showed higher values. Their investigations failed to derive any constant ratio between the two values. However, they did conclude that the oxygen uptake in the first few hours (10 to 20) was more rapid than later stages.

Dixon (16) has presented nomographs for use in manometric studies. Falk and Rudolf (23) studied the various factors affecting the rate of oxygen uptake, and recommended that temperature variations should be kept at a minimum. They further showed that the respirometer's direct utilization method gives results which are comparable to standard dilution method for measuring B.O.D., in contrast to the findings of Wooldridge and Standfast (70, 71).

In 1948, Caldwell and Langelier (11) increased the volume of the respiration flask in the Warburg apparatus from 15 ml to 125 ml in order to get more accurate determinations. Their results were more closely reproducible than those of previous investigators. They were also successful in demonstrating the usefulness of the manometric method in studies of sanitary engineering problems, and were able to derive the following general relationship between weight of the oxygen consumed and height of the manometer,

$$W = h \frac{Vm}{V_L} C \qquad (15)$$

where,

W = oxygen uptake mg/liter

h = manometer reading (cm water)

V_m = total volume of gas (ml)

V_L = total volume of the fluid in flask (ml)

C = constant

The value of C varies with the density of the manometer fluid used; with a manometer fluid density of 1.00, C is 1.27.

Lee and Oswald (39) obtained five per cent accuracy in duplicated tests. They concluded that the results of manometric techniques are not strictly comparable to those obtained by the standard dilution method, and that the Warburg test when applied to sterile waste is particularly sensitive to seed and nutrient relationships.

Ludwig, Oswald, and Gotass, (40) did extensive work at the University of California on the application of manometric

techniques in determining B.O.D., with encouraging results. They used the formula derived by Caldwell and Langelier (11) for calculation of B.O.D. Using Brodie's fluid in their manometer they derived the following:

$$W = 1.3\ h\ \frac{V_m}{V_L} \qquad (16)$$

It may be noted that their formula is identical to that of Caldwell and Langelier, except for the value of C.

Dawson and Jenkins (13, 14) investigated the oxygen requirement of oxygen uptake for activated sludge. They studied the effect of shock load of both organic and inorganic substances on the oxygen uptake of the sludge. In 1958 Wasserman, Hopkins, and Porge (67) using the Warburg apparatus studied the oxygen uptake of the yeast, Saccharomyces fragilis in whey. They found that the rate of oxygen uptake in the presence of either lactose or galactose was rapid in the first hour and later decreased correspondingly with time. In 1959, Snaddon and Harkness (61) successfully showed that respiration of a sewage sample receiving regular additions of oxygen does not differ from that of an identical sample which receives a large single dose of oxygen.

Experimental Technique

The apparatus shown in Figures 17 and 18 was designed by the author to study uptake of P^{32} by slimes. The respiration chamber encloses the entire respiration system in a small box (6 1/2" wide x 7 1/2" long x 3" high) except for the manometer and the carbon dioxide absorption tubes.

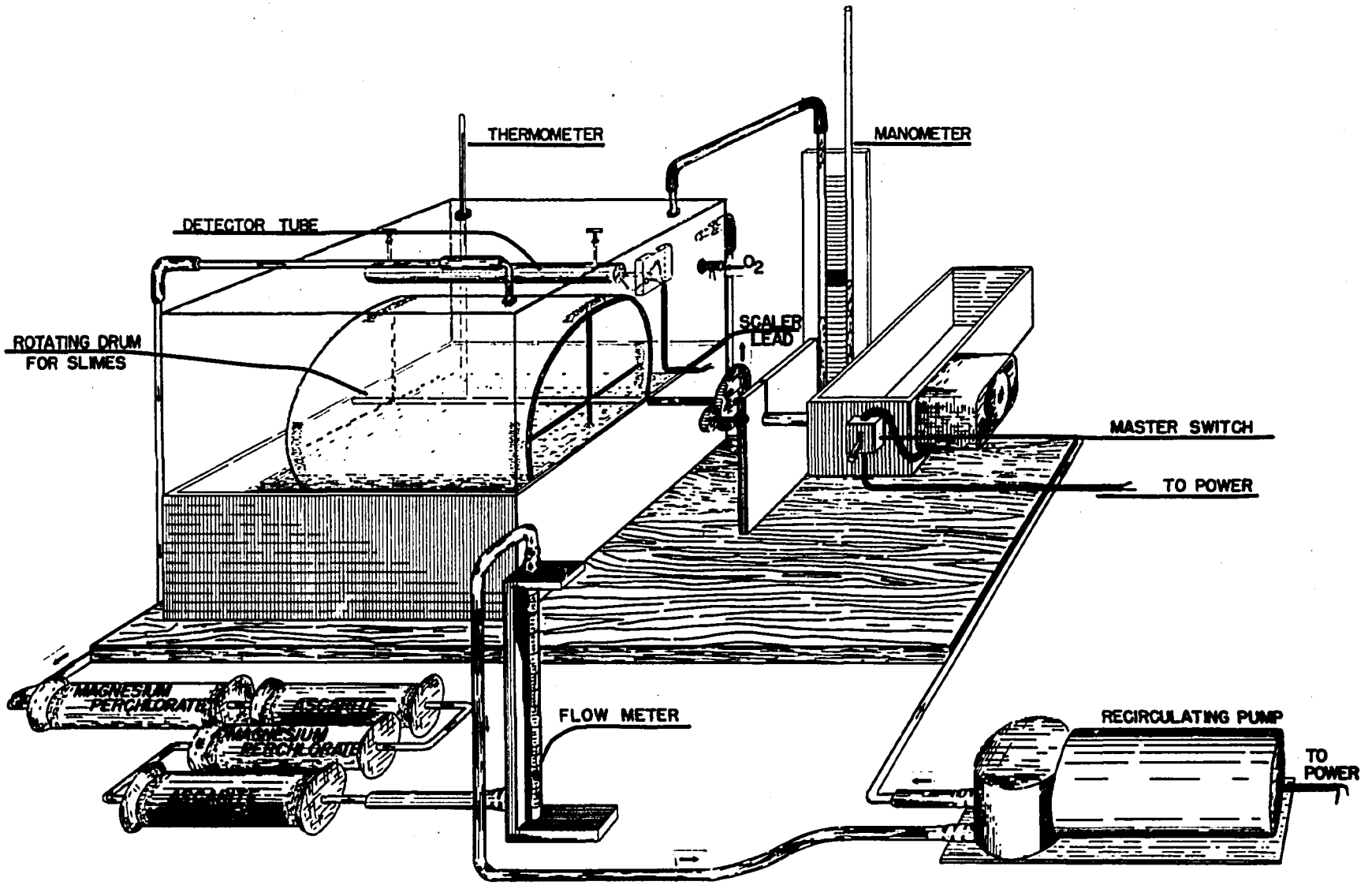


FIG 17 SCHEMATIC DIAGRAM OF MANOMETRIC-ATTACHED SLIME APPARATUS

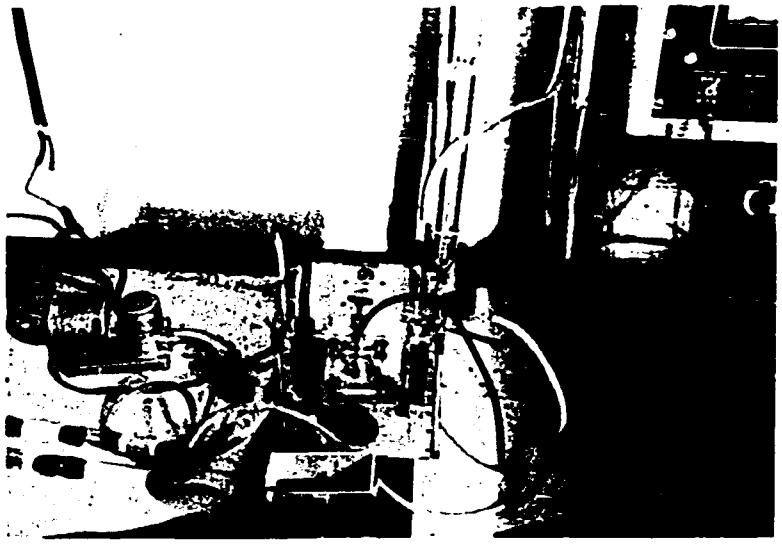


FIG 18: PHOTOGRAPH OF RESPIRATION STUDIES OF ATTACHED SLIMES

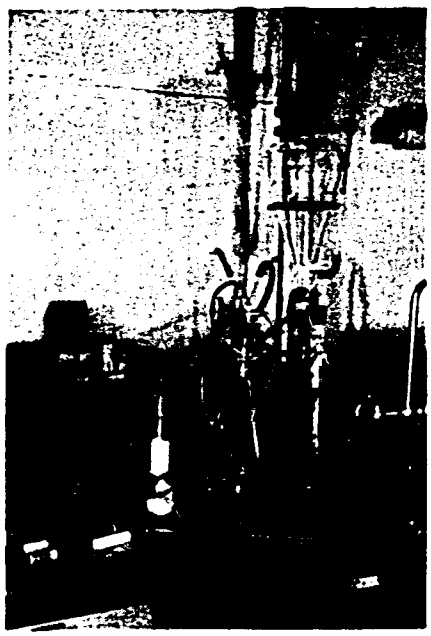


FIG 19: PHOTOGRAPH OF FREE FLOATING SLIME STUDIES APPARTUS

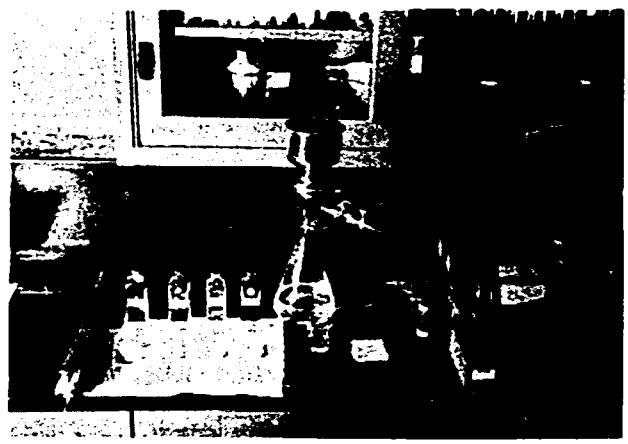


FIG 20: PHOTOGRAPH OF MILIPHORE FILTER APPARATUS FOR BACTERIOLOGICAL AND UPTAKE OF P^{32} STUDIES

The lower portion of the box was made of two thin sheets of stainless steel. The annular space between the two sheets was packed with felt to prevent radiation of heat. Temperature changes of the liquid in the box were negligible as a consequence. A 3 1/2" diameter glass drum, driven by a motor mounted outside the box on a stand, rotated in the box. The outer surface of the rotating drum was made rough by sand blasting in order to facilitate attachment of the slimes. The entire assembly was mounted on a wooden platform. The upper part of the box was made of plexiglass, and could be screwed to the lower portions for an air-tight fit. A Gieger Muller tube was permanently fixed in the box to record uptake of P^{32} by the slimes. The box had two connections: one to the manometer for pressure measurements; the other to a train containing Ascarite for carbon dioxide absorption and magnesium perchlorate for moisture absorption.

Mercury was used as the manometric fluid to avoid problems associated with solubility of carbon dioxide in water. The mercury was replaced by light motor oil in the latter part of the investigation. This provided better measurement in the changes of the pressure in the manometric tube. In the absorption train, Ascarite, a composition of sodium hydroxide and asbestos, was used because of its superiority to alkaline fluids in CO_2 absorbing qualities. Ascarite permits the quantity of the gas liberated by the metabolic action of the organisms to be accurately calculated by the difference in weight of the

Ascarite before and after absorption of the gas. The Ascarite should be changed when half of the contents have changed color. Magnesium perchlorate was used as a dehydrant as it is porous and easy to handle, and is able to absorb up to 60 per cent of its weight of water without reducing its efficiency.

The gases other than carbon dioxide and oxygen are not significant factors in the manometric method. The possibility of liberation of any gases other than CO_2 and O_2 is remote, particularly when pure oxygen is used in the experiments as it was in the present studies. Porter, (50) has shown that some carbon dioxide must be available to the micro-organisms for their proper growth. Hence the small quantity of the carbon dioxide gas liberated in the experiment had no adverse influence on the bacterial growth.

Experimental Procedure

Twelve hundred (1200) cc of synthetic sewage was put in the lower portion of the apparatus. The drum was allowed to rotate until a satisfactory growth appeared. The background counts were obtained by placing the upper portion of the box on the lower portion. Then a calculated quantity of P^{32} was injected with a hypodermic syringe and the whole apparatus was made air-tight. The box was flushed with oxygen by connecting the apparatus to an oxygen reservoir. The apparatus was filled with oxygen, flushed, and refilled several times, to ensure that the gas in the respiration chamber was all oxygen.

A 50 cc hypodermic syringe was used to introduce a measured amount of oxygen to the chamber through a side opening to bring the manometer to a designated pressure. The drum was allowed to rotate for an hour. This was sufficient time for the slimes to utilize an appreciable amount of oxygen, thus evolving a corresponding amount of CO_2. The gases in the chamber were then circulated through the Ascarite absorption train, with the aid of a small recirculating pump. The pump was operated for eight minutes, a period sufficient to permit recirculation of the gases approximately ten times. After the CO_2 was absorbed by the Ascarite, measured amounts of O_2 were added to the chamber to bring the manometric pressure up to the initial pressure. The volume necessary for this purpose is the volume of oxygen used through respiration. The uptake of P^{32} was measured concurrently with that of the oxygen.

Discussion of Results

The P^{32} readings were corrected for half-life decay and slime sloughing error. An attempt was made to formulate a mathematical equation to predict the sloughing of slimes on the rotating drums. Several experiments were performed, but the results were unreliable and statistically uncorrelatable. However, the uptake of P^{32} vs time is an exponential curve, which means that if there is no sloughing and the bacteria grow in the logarithmic phase, a plot of $(P^{32})^2$ vs time would be a straight line. Therefore a correction for sloughing was made by plotting $(P^{32})^2$ vs time and the best fitting straight line was drawn.

The highest reading of P^{32} uptake thus obtained was considered to be 100 per cent uptake. The accumulative quantities of oxygen uptake were reduced to per cent uptake total and were plotted as per cent total P^{32} uptake vs per cent oxygen uptake. The intercept at the Y axis, corresponding to the zero consumption of oxygen, was taken as the initial adsorption. Corrections for adsorption were made as previously indicated. The final plotting of per cent P^{32} assimilated and per cent uptake of oxygen is a straight line of approximately 0.9 slope with a maximum error of about 8 per cent. This error may be due to the experimental technique. One experiment (results are shown in Figure 23) was continued for 57 hours. These results with an error less than 5 per cent show that for a long period of time, approximately 50 hours, the effect of adsorption is negligible.

Experiments were performed using lactose sugar as the source of food and the resulting plots of per cent P^{32} vs per cent oxygen uptake were straight lines having a slope of 0.8 (see Figure 28). Invariably it was observed that the bacteria were acid producing; even the phosphate buffer which initially held the solution of a pH of 7.2 was overcome by the acid produced. Generally speaking, Zooglea ramigera is not an acid producer, this lowering in pH was due to acid-producing bacteria present in the mixed culture used.

Similar experiments were performed at a pH of 8.6. The solution was brought to the required pH by adding sufficient

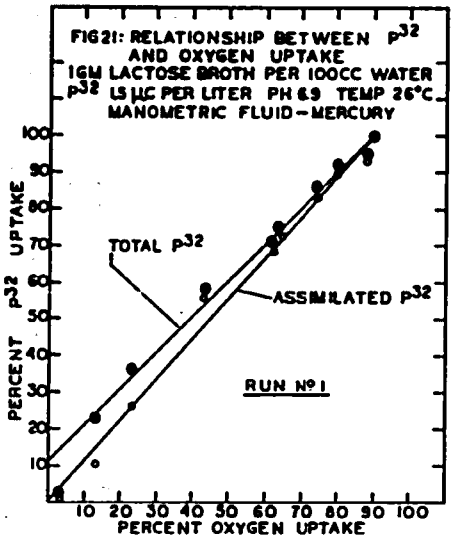


FIG 21: RELATIONSHIP BETWEEN P32 AND OXYGEN UPTAKE
1GM LACTOSE BROTH PER 100CC WATER
P32 1.5 µC PER LITER PH 6.9 TEMP 26°C
MANOMETRIC FLUID—MERCURY
TOTAL P32
ASSIMILATED P32
RUN Nº 1
PERCENT P32 UPTAKE
PERCENT OXYGEN UPTAKE

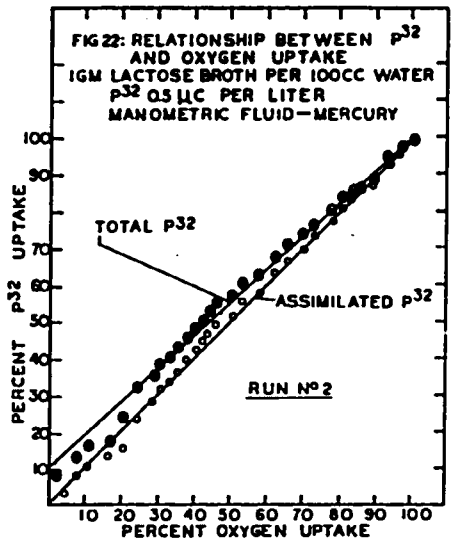


FIG 22: RELATIONSHIP BETWEEN P32 AND OXYGEN UPTAKE
1GM LACTOSE BROTH PER 100CC WATER
P32 0.5 µC PER LITER
MANOMETRIC FLUID—MERCURY
TOTAL P32
ASSIMILATED P32
RUN Nº 2
PERCENT P32 UPTAKE
PERCENT OXYGEN UPTAKE

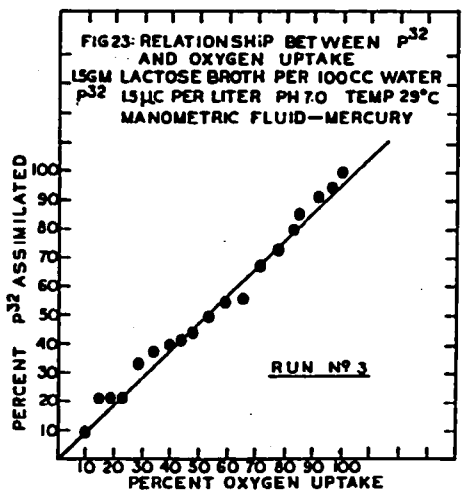


FIG 23: RELATIONSHIP BETWEEN P32 AND OXYGEN UPTAKE
1.5GM LACTOSE BROTH PER 100CC WATER
P32 1.5 µC PER LITER PH 7.0 TEMP 29°C
MANOMETRIC FLUID—MERCURY
RUN Nº 3
PERCENT P32 ASSIMILATED
PERCENT OXYGEN UPTAKE

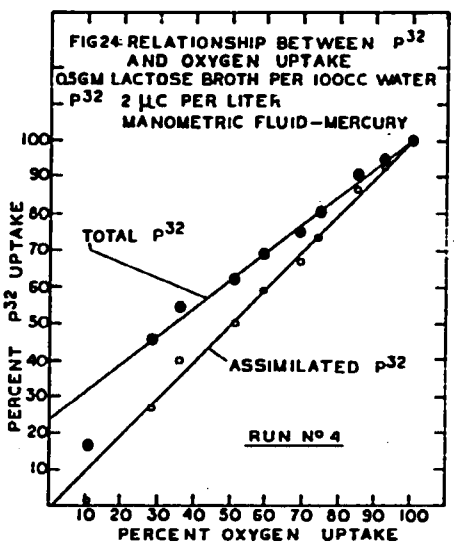


FIG 24: RELATIONSHIP BETWEEN P32 AND OXYGEN UPTAKE
0.5GM LACTOSE BROTH PER 100CC WATER
P32 2 µC PER LITER
MANOMETRIC FLUID—MERCURY
TOTAL P32
ASSIMILATED P32
RUN Nº 4
PERCENT P32 UPTAKE
PERCENT OXYGEN UPTAKE

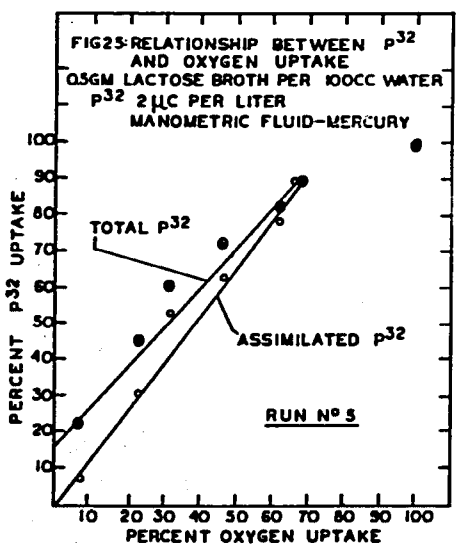


FIG 25: RELATIONSHIP BETWEEN P32 AND OXYGEN UPTAKE
0.5GM LACTOSE BROTH PER 100CC WATER
P32 2 µC PER LITER
MANOMETRIC FLUID—MERCURY
TOTAL P32
ASSIMILATED P32
RUN Nº 5
PERCENT P32 UPTAKE
PERCENT OXYGEN UPTAKE

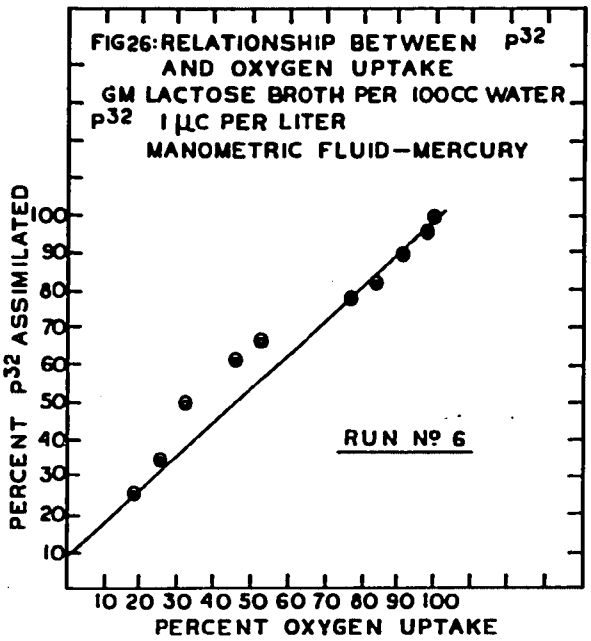


FIG26: RELATIONSHIP BETWEEN P32 AND OXYGEN UPTAKE
GM LACTOSE BROTH PER 100CC WATER
P32 1 µC PER LITER
MANOMETRIC FLUID—MERCURY
PERCENT P32 ASSIMILATED
RUN Nº 6
10 20 30 40 50 60 70 80 90 100
PERCENT OXYGEN UPTAKE

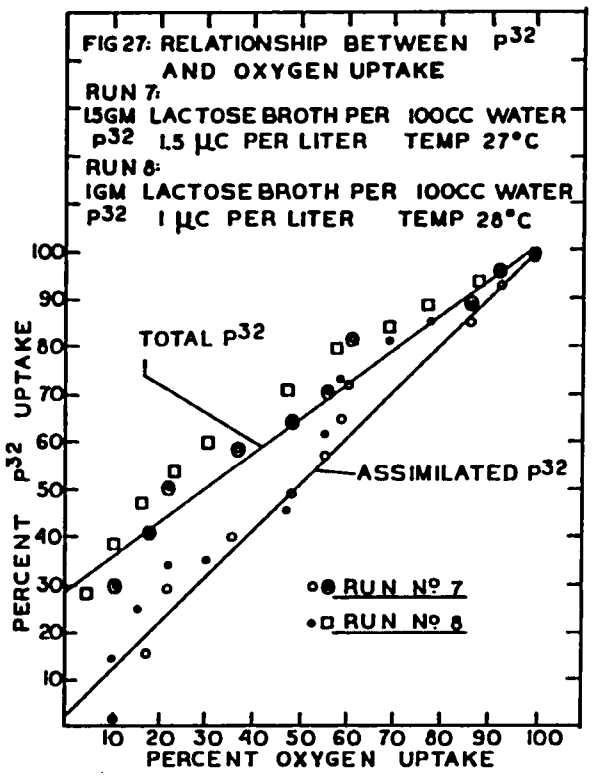


FIG 27: RELATIONSHIP BETWEEN P32 AND OXYGEN UPTAKE
RUN 7:
1.5GM LACTOSE BROTH PER 100CC WATER
P32 1.5 µC PER LITER TEMP 27°C
RUN 8:
1GM LACTOSE BROTH PER 100CC WATER
P32 1 µC PER LITER TEMP 28°C
PERCENT P32 UPTAKE
TOTAL P32
ASSIMILATED P32
RUN Nº 7
RUN Nº 8
10 20 30 40 50 60 70 80 90 100
PERCENT OXYGEN UPTAKE

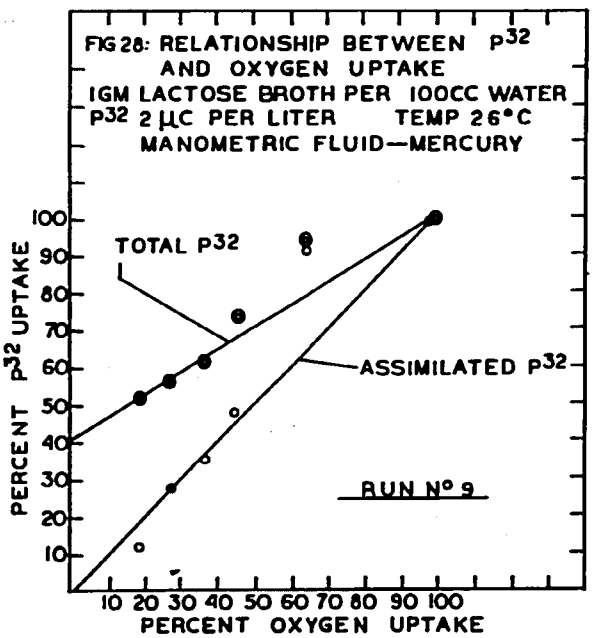


FIG 28: RELATIONSHIP BETWEEN P32 AND OXYGEN UPTAKE
IGM LACTOSE BROTH PER 100CC WATER
P32 2 µC PER LITER TEMP 26°C
MANOMETRIC FLUID—MERCURY
PERCENT P32 UPTAKE
TOTAL P32
ASSIMILATED P32
RUN Nº 9
10 20 30 40 50 60 70 80 90 100
PERCENT OXYGEN UPTAKE

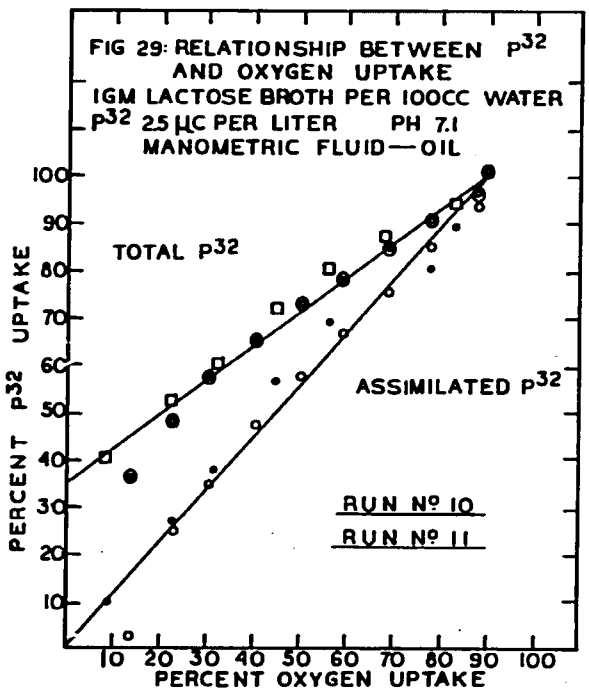


FIG 29: RELATIONSHIP BETWEEN P32 AND OXYGEN UPTAKE
IGM LACTOSE BROTH PER 100CC WATER
P32 2.5 µC PER LITER PH 7.1
MANOMETRIC FLUID—OIL
PERCENT P32 UPTAKE
TOTAL P32
ASSIMILATED P32
RUN Nº 10
RUN Nº 11
10 20 30 40 50 60 70 80 90 100
PERCENT OXYGEN UPTAKE

buffer solution of the type described in Diehl and Harvey (15). The result of a typical experimental run is shown in Figure 34. The rate of oxygen uptake was slow in the initial few hours, and thereafter gradually increased until sloughing started. A concentrated solution of lactose broth was added periodically by a 5 cc hypodermic syringe to maintain an excess of nutrient. Once the sloughing started, it could not be stopped. Apparently the sloughing is accelerated by the production of intoxicants during metabolism. At the end of the experiment, the pH was down to 7.5, as a consequence of bacterial acid production.

Experiments were also performed at a pH of 5.0. The oxygen consumption was very small throughout these experiments, being almost negligible in the first few hours. The low pH of the substrate solution might have inhibited the bacterial respiration to such an extent that utilization of oxygen could not be detected manometrically. The small uptake of oxygen in the first few hours indicates a low respiration rate, not that respiration was chemically stopped, because in the latter part of the experiment, the rate of oxygen uptake increased considerably.

In studying pressure change effect on P^{32} uptake, mercury was replaced by light motor oil as the manometric fluid; because of the distinct advantage in sensitivity in detecting significantly small changes in volume. Figures 29, 30, 31, 32, 33, and 34 show the findings of this set of experiments. The relation between the rate of P^{32} uptake and that of oxygen is

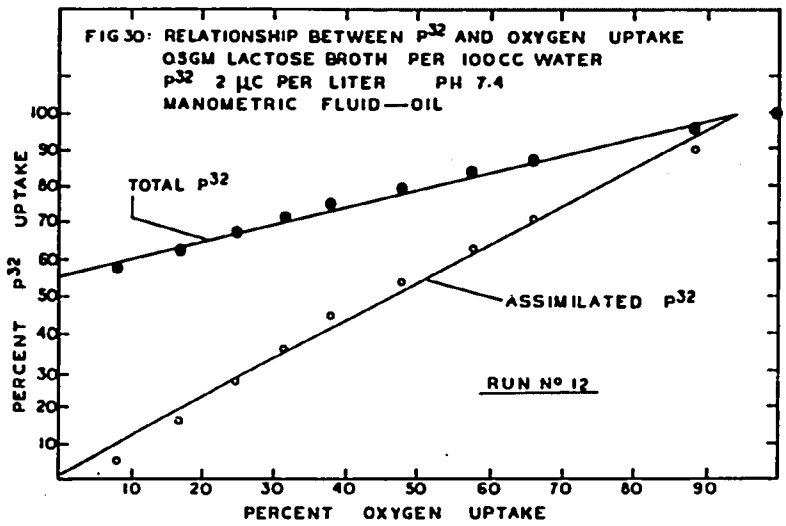


FIG 30: RELATIONSHIP BETWEEN P32 AND OXYGEN UPTAKE
0.5GM LACTOSE BROTH PER 100CC WATER
P32 2 µC PER LITER PH 7.4
MANOMETRIC FLUID—OIL
TOTAL P32
ASSIMILATED P32
RUN Nº 12
PERCENT P32 UPTAKE
PERCENT OXYGEN UPTAKE

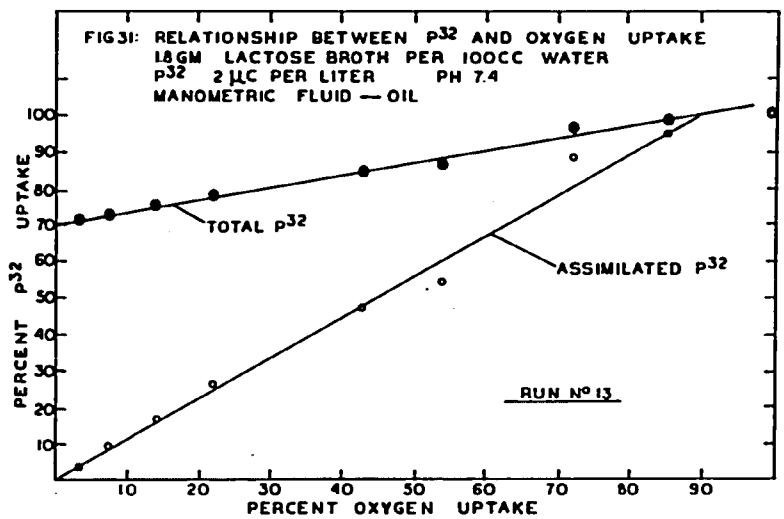


FIG 31: RELATIONSHIP BETWEEN P32 AND OXYGEN UPTAKE
1.8GM LACTOSE BROTH PER 100CC WATER
P32 2 µC PER LITER PH 7.4
MANOMETRIC FLUID — OIL
TOTAL P32
ASSIMILATED P32
RUN Nº 13
PERCENT P32 UPTAKE
PERCENT OXYGEN UPTAKE

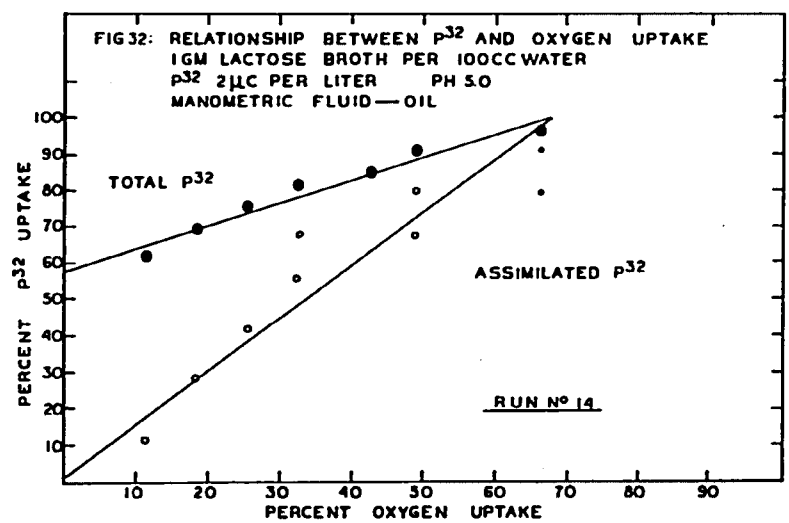


FIG 32: RELATIONSHIP BETWEEN P32 AND OXYGEN UPTAKE
1 GM LACTOSE BROTH PER 100CC WATER
P32 2 µC PER LITER PH 5.0
MANOMETRIC FLUID—OIL
TOTAL P32
ASSIMILATED P32
RUN Nº 14
PERCENT P32 UPTAKE
PERCENT OXYGEN UPTAKE

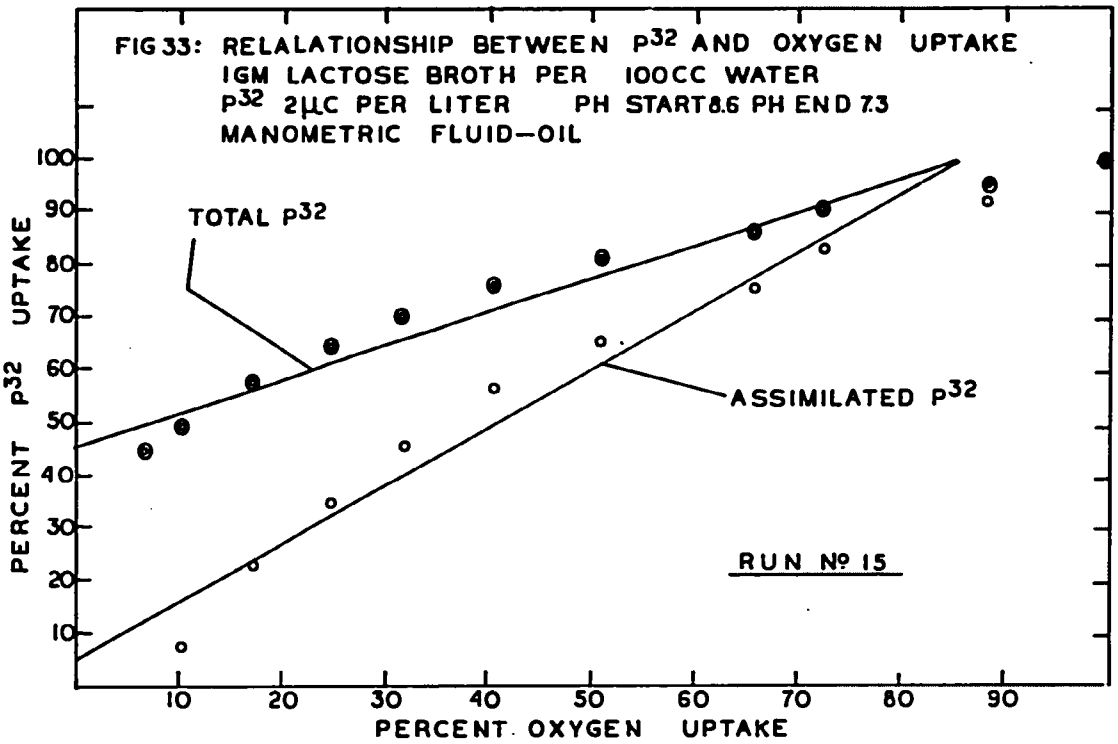


FIG 33: RELALATIONSHIP BETWEEN P32 AND OXYGEN UPTAKE
1GM LACTOSE BROTH PER 100CC WATER
P32 2μC PER LITER PH START 8.6 PH END 7.3
MANOMETRIC FLUID—OIL
TOTAL P32
ASSIMILATED P32
RUN Nº 15
PERCENT P32 UPTAKE
PERCENT OXYGEN UPTAKE
10 20 30 40 50 60 70 80 90 100

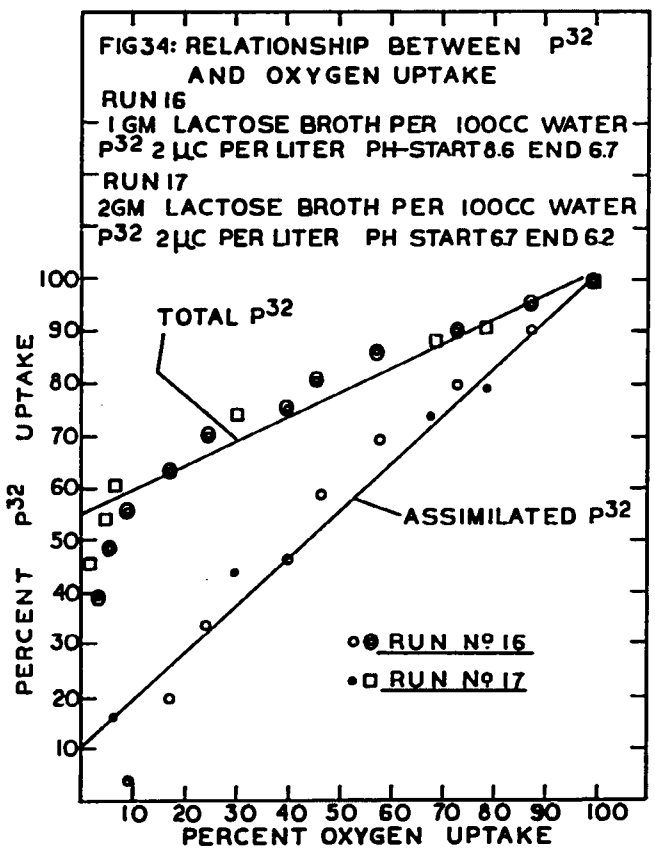


FIG 34: RELATIONSHIP BETWEEN P32 AND OXYGEN UPTAKE
RUN 16
1 GM LACTOSE BROTH PER 100CC WATER
P32 2 μC PER LITER PH-START 8.6 END 6.7
RUN 17
2GM LACTOSE BROTH PER 100CC WATER
P32 2μC PER LITER PH START 6.7 END 6.2
TOTAL P32
ASSIMILATED P32
RUN Nº 16
RUN Nº 17
PERCENT P32 UPTAKE
PERCENT OXYGEN UPTAKE
10 20 30 40 50 60 70 80 90 100

linear. It can be concluded from these results that the rate of P^{32} uptake is not affected by pressure changes in the manometer.

Stoichiometrically, it would seem that for every mole of oxygen used, one mole of CO_2 is produced and hence the measurements of either oxygen of carbon dioxide concentration should be equally good in measuring bacterial activity. However, the ratio of moles of carbon dioxide produced to the moles of oxygen consumed was usually less than one in the initial part of the experiments. And in the latter part of most of the experiments the ratio was more than one. The respiratory quotients for the various tests are listed in Table 5. In Experiments 14, 16, and 17 with pH values of 5.0, 8.6, and 6.20 respectively, the R.Q. was greater than one. Apparently whenever the pH deviated significantly, on either side of neutral pH (7.0), the respiration rate was affected and the consumption of oxygen could not be measured accurately. This behavior is of fundamental importance, and it should be emphasized that if this relationship is verified, much can be done to clarify the behavior of slimes.

A statistical analysis was made of the data obtained from two sets of experiments and the results are presented in Table 6. Sample calculations for one set are given in the Appendix.

The general equation is:

$$\text{Percent total } P^{32} = K\ (\text{Percent } O_2) + C \qquad (17)$$

Where K = Slope of the line
C = y intercept

TABLE 5

MANOMETRIC STUDIES OF ATTACHED SLIMES - RESPIRATORY QUOTIENT OF VARIOUS RUNS AT VARIOUS TIME INTERVALS
(R. Q. = ΣCO_2 MOLES/ ΣO_2 MOLES)

Time (Hours)	R. Q.	Time (Hours)	R. Q.	Time (Hours)	R. Q.
Run No. 1		Run No. 2 Cont'd.		Run No. 2 Cont'd.	
6	0.348	30	0.750	65	0.768
12	0.669	31	0.822	67	0.762
15	0.863	32	0.869	69	0.756
20	1.000	33	0.918	Run No. 3	
22	1.05	34	0.934	5	0.266
23	1.16	35	0.983	12	0.331
25	1.16	36	0.995	16	0.671
27	1.24	37	0.982	19	0.728
29	1.26	38	0.960	21	0.798
31	1.25	40	0.939	23	0.985
Run No. 2		42	0.862	25	1.059
4	0.069	44	0.840	27	1.036
8	0.111	46	0.828	29	1.047
15	0.283	48	0.814	31	1.086
18	0.330	50	0.815	33	1.104
20	0.370	53	0.797	35	1.104
24	0.453	56	0.794	38	1.092
26	0.565	58	0.789	40	1.068
28	0.670	59	0.784	42	1.056
29	0.716	62	0.773	43	1.053

TABLE 5--Continued

Time (Hours)	R. Q.	Time (Hours)	R. Q.	Time (Hours)	R. Q.
Run No. 3 Cont'd.		Run No. 5 Cont'd.		Run No. 7 Cont'd.	
45	1.045	10	1.304	7	1.272
47	1.036	12	1.408	8	1.395
49	1.035	14	1.249	10	1.152
52	1.010	Run No. 6		11	1.229
55	1.003	2	0.000	12	1.242
57	1.016	4	2.126	Run No. 8	
Run No. 4		5	2.081	1	1.354
1	0.91	7	2.203	2	1.187
3	1.04	9	1.943	3	1.152
4	1.24	10	1.887	4	1.223
5	1.69	12	1.525	5	1.274
6	1.70	13	1.570	7	1.074
7	1.82	15	1.667	8	1.071
8	1.81	16	1.603	9	1.113
10	1.76	17	1.608	10	1.072
11	1.73	Run No. 7		11	1.095
12	1.71	1	0.812	12	1.092
Run No. 5		2	0.913	14	1.102
2	1.129	3	1.259	Run No. 9	
4	1.196	4	0.833	1	0.00
6	1.306	5	0.955	2	7.07
8	1.169	6	1.042	3	7.86

TABLE 5--Continued

Time (Hours)	R. Q.	Time (Hours)	R. Q.	Time (Hours)	R. Q.
Run No. 9 Cont'd.		Run No. 11 Cont'd.		Run No. 13 Cont'd.	
4	8.46	5	0.799	22	1.14
6	8.35	6	0.765	24	1.11
7	9.31	7	0.703	25	1.13
9	12.57	8	0.656	Run No. 14	
13	9.56	Run No. 12		1	5.71
15	6.25	1	0.944	2	7.75
Run No. 10		2	0.983	3	8.70
1	2.11	3	0.981	4	8.57
2	1.49	4	1.039	5	9.68
3	1.26	5	1.08	6	6.48
4	1.08	6	1.02	7	5.11
5	0.997	7	0.998	Run No. 15	
6	0.939	8	0.964	1	0.791
7	0.870	10-1/2	0.902	2	0.960
8	0.843	12	0.896	3	1.067
9	0.821	Run No. 13		4	1.118
10	0.771	1	1.66	5	1.22
Run No. 11		2	1.74	6	1.19
1	1.41	4	1.68	7	1.16
2	0.916	6	1.60	8	1.14
3	0.932	11	1.34	9	1.15
4	0.820	13	1.30	10	1.11

TABLE 5--Continued

Time (Hours)	R. Q.
Run No. 15 Cont'd.	
11	1.08
Run No. 16	
1	7.04
2	6.70
3	8.35
4	6.72
5	5.78
6	4.23
7	4.08
8	3.56
9	3.14
10	2.82
11	2.63
Run No. 17	
2	24.6
3	14.9
4	17.3
6	4.48
8-1/2	2.94
9	2.92
11	3.23

TABLE 6

SUMMARY OF THE STATISTICAL RESULTS OF ATTACHED SLIME STUDIES

	Experiments	
	Set 1	Set 2
Experimental coefficient of correlation "r"	0.91	0.86
Theoretical coefficient of correlation "r"	0.319	0.348
Slope of the line	0.69	0.9
y intercept, i.e. per cent P^{32} adsorbed	21	17.9

The validity of the correlation coefficient (r) was checked with the tables provided by Fisher and Yates (51). The "r" value according to the table for 52 degrees of freedom at the 0.01 probability level is 0.319. This means that when the value of "r" is more than or equal to 0.319, we can say, with 99 per cent certainty, that there is a significant correlation between the two variables. The experimental value of "r" is 0.91, which is much higher than the theoretical value of 0.314. This gives more evidence to the validity of the postulation - "that the rate of assimilated P^{32} is equal to the rate of uptake oxygen."

Conclusions and Recommendations

The findings of the experiments discussed above, have led to the following conclusions:

1. The rate of uptake of assimilated P^{32} is the same as the rate of uptake of oxygen.

2. The relation between the rate of uptake of oxygen and that of uptake of assimilated P^{32} is not affected by the type or concentration of the food.

3. The relation between the rate of uptake of oxygen and the uptake of assimilated P^{32} is not affected by manometer pressure.

4. Similar experiments should be performed using a complex food source.

5. More work should be performed to further study the R. Q.

CHAPTER V

FREE FLOATING SLIMES

General

Since World War II, the chemical industry has expanded enormously and has created manifold new and serious problems in the area of waste water treatment. The industry discharges organic materials which are foreign to those ordinarily encountered in domestic sewage. These new waste materials have increased the B.O.D. of the sewage. By experience, sanitary engineers have found the use of activated sludge particularly suited for the treatment of waste water with a high oxygen demand. In this process the mixed liquor is agitated by the passage of forced air. Flocs of slimes remain floating in the tank while vast numbers of varied bacterial species utilize the organic compounds in the liquor as their source of food, oxidizing these into stable end products.

A bench scale experiment was set up wherein free floating slimes were grown in an enclosed environment in an attempt to compare their metabolism and respiration with that of the attached slimes. P^{32} was injected and samples were measured at intervals for P^{32} uptake. Air was passed through the mixed liquid, and the CO_2 was removed on an Ascarite train.

Apparatus and Procedure

The apparatus shown in Figure 35 was used for growing the slimes. This consisted of a four-liter flask, flow meter, and an air regulator with a pressure gauge. The flask had three orifices; one served for introduction of air, another as an outlet vent for gases, and a third as an entry for collecting samples of the liquid. The height of the sampling tube could be adjusted allowing samples to be collected at different levels. Since gases from the flask were saturated with water, the excess water was removed in an ice and salt bath. One end of the condensing coil in the bath was connected to the vent tube and the other to the vacuum flask. The vent gases were then passed through an Ascarite train.

This method provided an excellent way for measuring the production of CO_2. However, as oxygen uptake could not be measured accurately, a revised procedure similar to that used in the studies of attached slime was substituted, and is shown in Figures 19 and 36. Almost all of the experimental work was performed by this modified apparatus, which permitted accurate measurements.

The four-liter flask used as the aeration chamber was retained without change. The condensing bath, flow meter, and pressure gauge were removed, and were replaced by another four-liter flask. This was used as an oxygen reservoir tank. The stopper in this flask was provided with three holes, each served with glass tubing. One tube with a clamp was used for

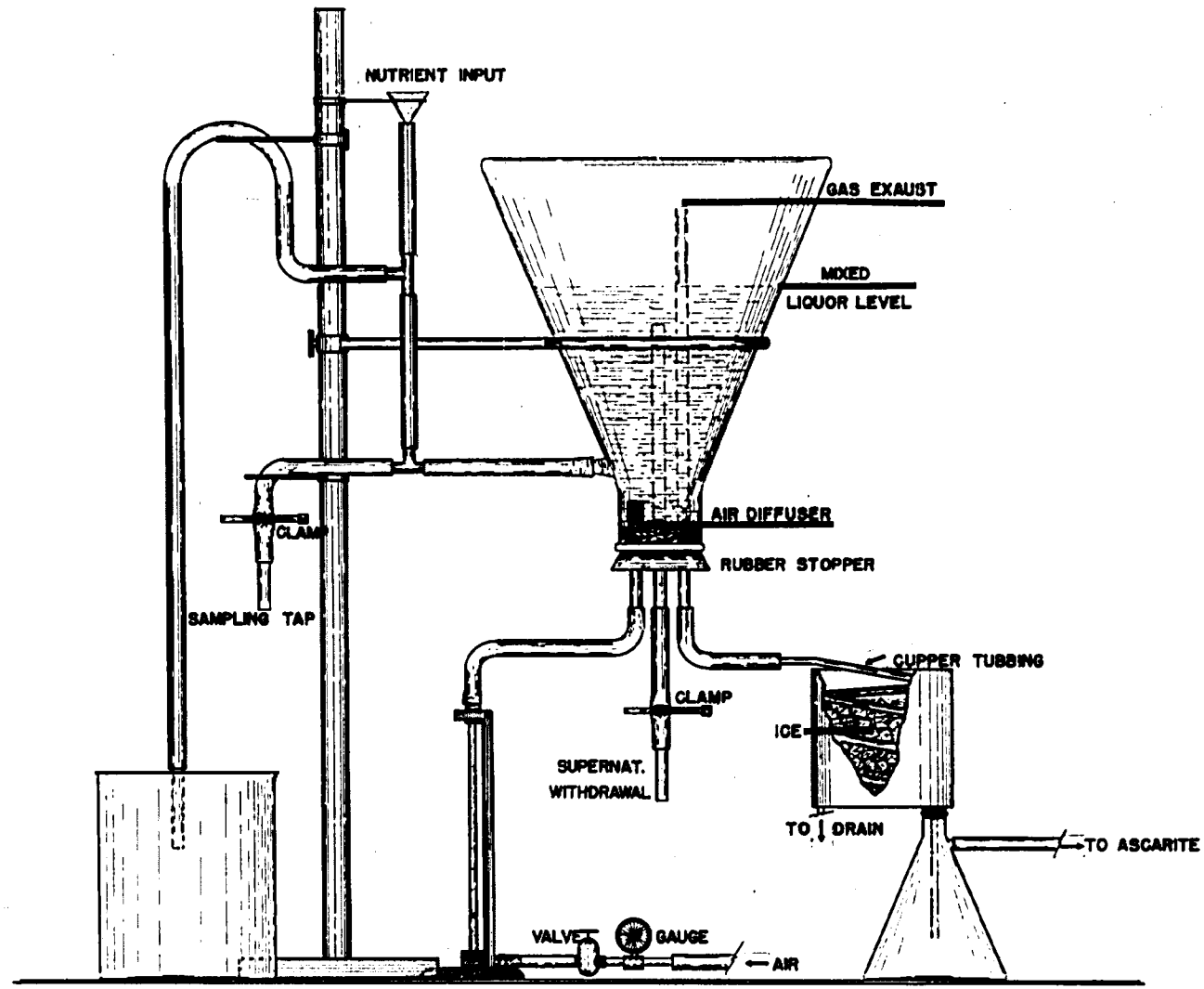


FIG 35 SCHEMATIC DIAGRAM FREE FLOATING SLIME STUDIES

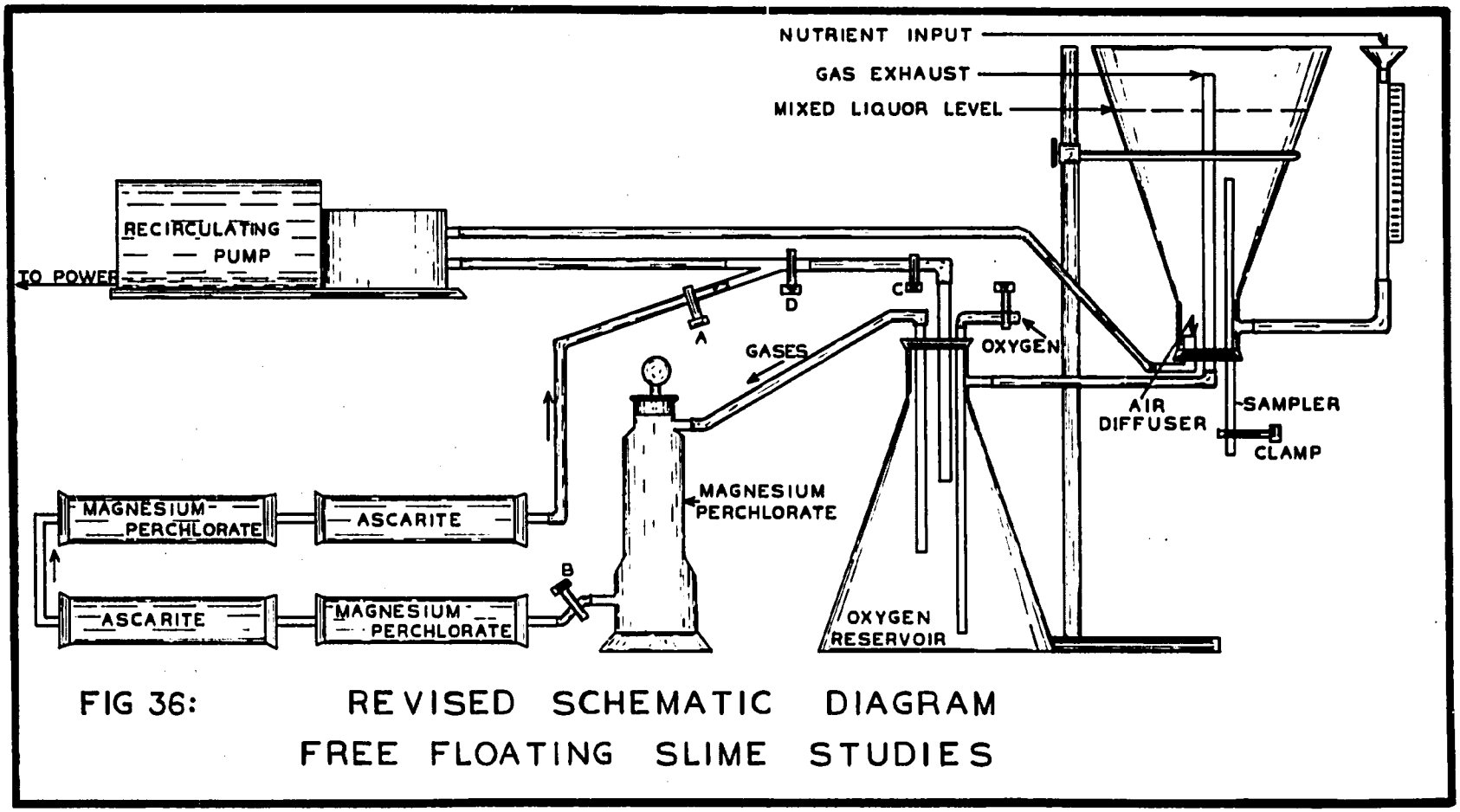


FIG 36: REVISED SCHEMATIC DIAGRAM
FREE FLOATING SLIME STUDIES

injecting pure oxygen by means of hypodermic syringe. Another tube was connected to a magnesium perchlorate tower leading to an Ascarite train.

Raw sludge was obtained from the Norman Sewage Treatment Plant and was mixed with synthetic sewage in the ratio of 1:2 by volume. After the mixture had been aerated for several minutes, 50 cc mixed-liquor samples were separately collected at the top, center, and bottom of the aeration flask. Solids were determined by centrifuging the samples for ten minutes. Samples from all three locations had the same per cent of solids, showing that the mixture had been well aerated and agitated. The synthetic sewage was replaced every twelve hours to promote maximum growth without disturbing the sludge. This was done by momentarily stopping the supply of air, decanting the top liquid and replacing it with equal quantities of fresh synthetic sewage.

After sufficient slimes were grown, a measured amount of P^{32} was added to the mixed liquor. At selected intervals a 50 cc sample was collected from the aeration flask into a dilution bottle. One cc of this sample was filtered through a 0.8 micron, Type AA Millipore membrane filter. The filter was dried under an infra-red light and the beta emmission was counted with a G.M. detector tube. The filter was then washed with a 2 per cent saline water, dried and recounted. This was done to separate the adsorbed P^{32} from the assimilated. Three samples were filtered and counted with the average taken as the activity of the P^{32} uptake.

The gases were circulated through the Ascarite train throughout the experiment except for periodic weighings of accumulated CO_2. During these weighings, the gases in the aeration chamber were by-passed and recirculated by clamping at "A" and "B" and leaving clamps "C" and "D" open. The amount of carbon dioxide was determined by weighing both the Ascarite and magnesium perchlorate tubes. Oxygen was added with a 50 cc hypodermic syringe and the level of water in the manometric tube was brought back to the initial height.

Discussion of Results

Experiments were performed using different concentrations of suspended solids at different pH values. The plots of per cent P^{32} uptake vs per cent oxygen uptake are shown in Figures 37, 39, 41, 42, 43, 44, 45, 46, and 47. From Figure 37 it is evident that the rate of P^{32} uptake has a linear correlation with the rate of oxygen uptake. When the P^{32} uptake is 30 per cent the oxygen uptake is zero. The latter situation is a measurement of the adsorbed P^{32} on the membrane filter which could not be removed by washing.

Figure 39 shows the results of another experiment in which the solids concentration was 7680 mg/l. In the first 205 minutes of this test, the P^{32} uptake increased linearly with the oxygen uptake. After this time the rate of P^{32} uptake decreased as compared to that of oxygen. This may be due to an increase in death rate of the bacteria caused by lack of available food, or the bacteria in the sludge may have undergone

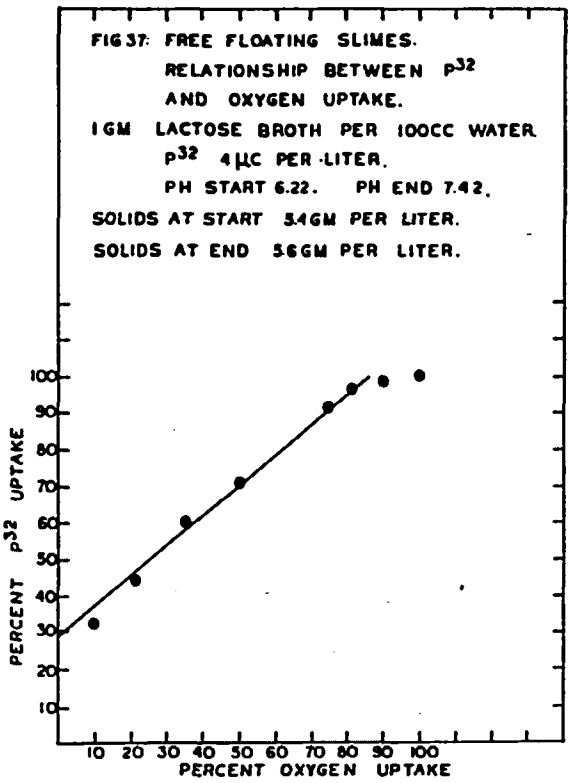


FIG 37: FREE FLOATING SLIMES.
RELATIONSHIP BETWEEN P^{32}
AND OXYGEN UPTAKE.
1 GM LACTOSE BROTH PER 100CC WATER.
P^{32} 4 μC PER LITER.
PH START 6.22. PH END 7.42.
SOLIDS AT START 5.4GM PER LITER.
SOLIDS AT END 5.6GM PER LITER.
PERCENT P^{32} UPTAKE
PERCENT OXYGEN UPTAKE

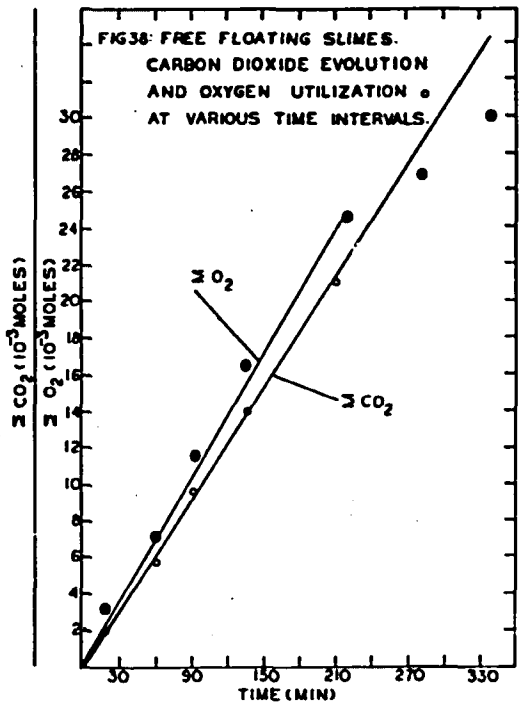


FIG 38: FREE FLOATING SLIMES.
CARBON DIOXIDE EVOLUTION
AND OXYGEN UTILIZATION
AT VARIOUS TIME INTERVALS.
ΣO_2
ΣCO_2
ΣCO_2 (10^{-3} MOLES)
ΣO_2 (10^{-3} MOLES)
TIME (MIN)

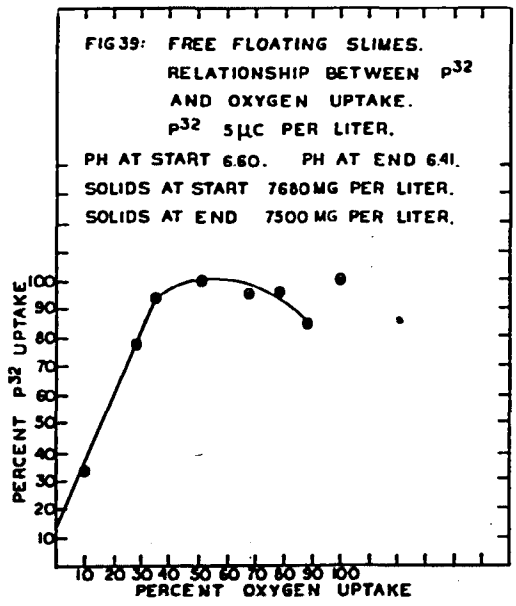


FIG 39: FREE FLOATING SLIMES.
RELATIONSHIP BETWEEN P^{32}
AND OXYGEN UPTAKE.
P^{32} 5 μC PER LITER.
PH AT START 6.60. PH AT END 6.41.
SOLIDS AT START 7680MG PER LITER.
SOLIDS AT END 7500 MG PER LITER.
PERCENT P^{32} UPTAKE
PERCENT OXYGEN UPTAKE

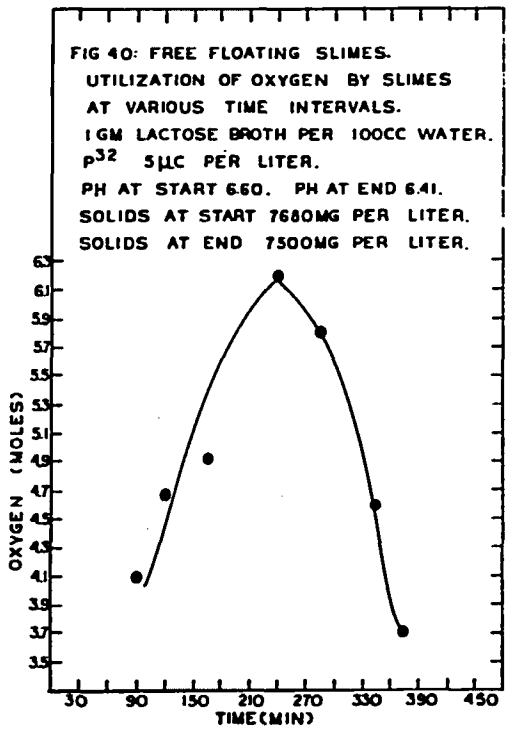


FIG 40: FREE FLOATING SLIMES.
UTILIZATION OF OXYGEN BY SLIMES
AT VARIOUS TIME INTERVALS.
1 GM LACTOSE BROTH PER 100CC WATER.
P^{32} 5 μC PER LITER.
PH AT START 6.60. PH AT END 6.41.
SOLIDS AT START 7680MG PER LITER.
SOLIDS AT END 7500MG PER LITER.
OXYGEN (MOLES)
TIME (MIN)

FIG 41: FREE FLOATING SLIMES
RELATIONSHIP BETWEEN P^{32}
AND OXYGEN UPTAKE
P^{32} 5μC PER LITER
1 GM LACTOSE BROTH PER 100CC WATER
3GM LACTOSE ADDED AT START
3GM LACTOSE ADDITIONAL ADDED AT 5.75 HRS
PH AT START 6.89 PH AT END 7.78 TEMP 29.8°
SOLIDS AT START 7140MG PER LITER
SOLIDS AT END 6780 MG PER LITER

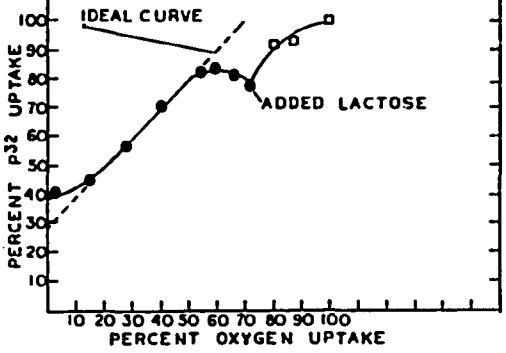


FIG 42: FREE FLOATING SLIMES.
RELATIONSHIP BETWEEN P^{32}
AND OXYGEN UPTAKE.
P^{32} 5μC PER LITER.
4GM LATOSE BROTH ADDED AT THE
TIME OF INJECTING P^{32}.
PH AT START 8.87. PH AT END 8.40.
SOLIDS AT START 3480MG PER LITER.
SOLIDS AT END 3240MG PER LITER.

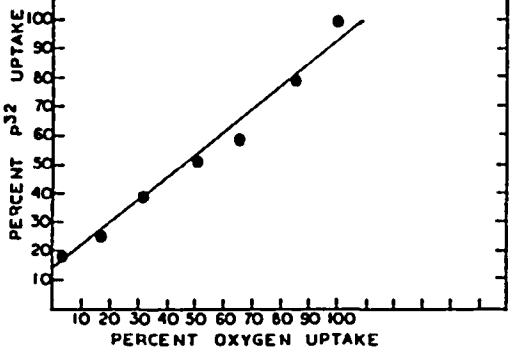


FIG 43: FREE FLOATING SLIMES.
RELATIONSHIP BETWEEN P^{32}
AND OXYGEN UPTAKE.
5 μC P^{32} ADDED.
4GM LACTOSE BROTH ADDED AT START.
PH AT START 8.74. PH AT END 8.12.
SOLIDS AT START 1560MG PER LITER.
SOLIDS AT END 1740MG PER LITER.

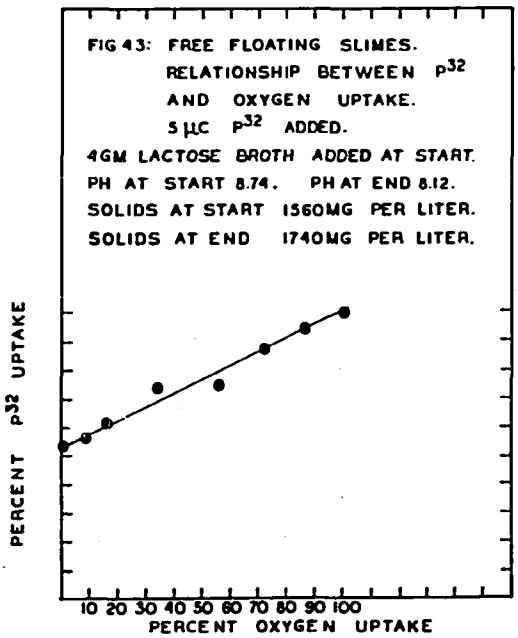
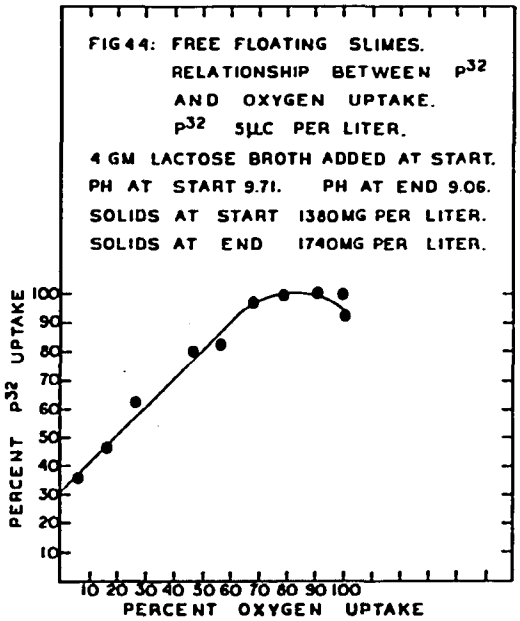


FIG 44: FREE FLOATING SLIMES.
RELATIONSHIP BETWEEN P^{32}
AND OXYGEN UPTAKE.
P^{32} 5μC PER LITER.
4 GM LACTOSE BROTH ADDED AT START.
PH AT START 9.71. PH AT END 9.06.
SOLIDS AT START 1380MG PER LITER.
SOLIDS AT END 1740MG PER LITER.



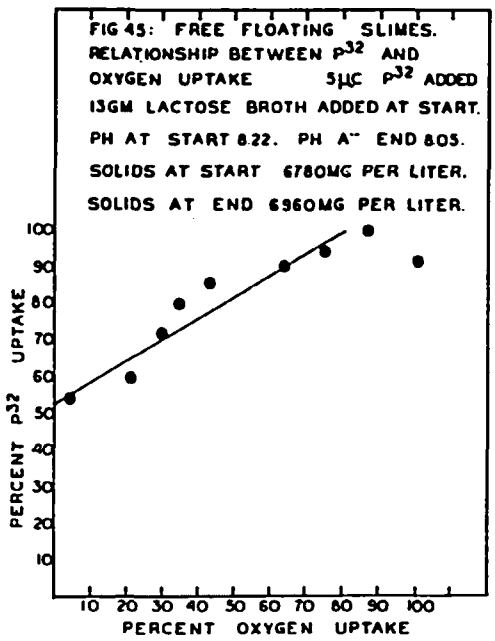


FIG 45: FREE FLOATING SLIMES.
RELATIONSHIP BETWEEN P32 AND
OXYGEN UPTAKE 5μC P32 ADDED
13GM LACTOSE BROTH ADDED AT START.
PH AT START 8.22. PH AT END 8.05.
SOLIDS AT START 6780MG PER LITER.
SOLIDS AT END 6960MG PER LITER.
PERCENT P32 UPTAKE
PERCENT OXYGEN UPTAKE

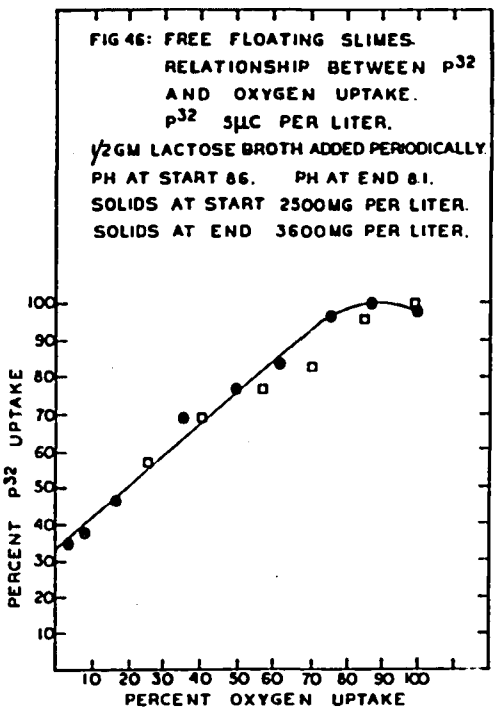


FIG 46: FREE FLOATING SLIMES.
RELATIONSHIP BETWEEN P32
AND OXYGEN UPTAKE.
P32 5μC PER LITER.
½GM LACTOSE BROTH ADDED PERIODICALLY.
PH AT START 8.6. PH AT END 8.1.
SOLIDS AT START 2500MG PER LITER.
SOLIDS AT END 3600MG PER LITER.
PERCENT P32 UPTAKE
PERCENT OXYGEN UPTAKE

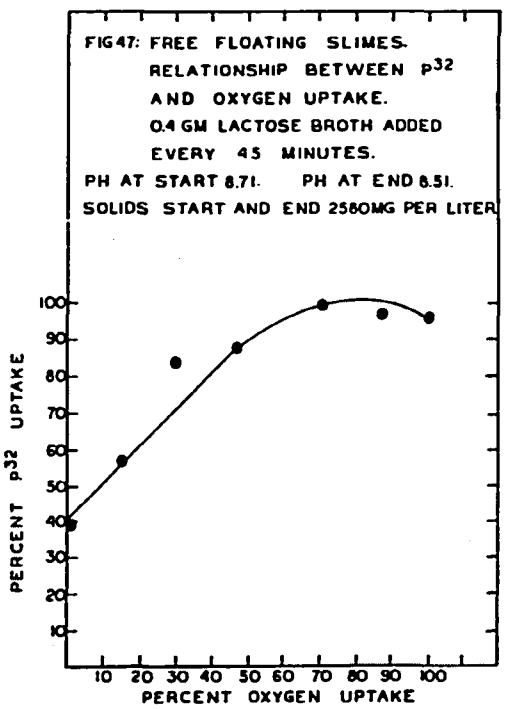


FIG 47: FREE FLOATING SLIMES.
RELATIONSHIP BETWEEN P32
AND OXYGEN UPTAKE.
0.4 GM LACTOSE BROTH ADDED
EVERY 45 MINUTES.
PH AT START 8.71. PH AT END 8.51.
SOLIDS START AND END 2580MG PER LITER.
PERCENT P32 UPTAKE
PERCENT OXYGEN UPTAKE

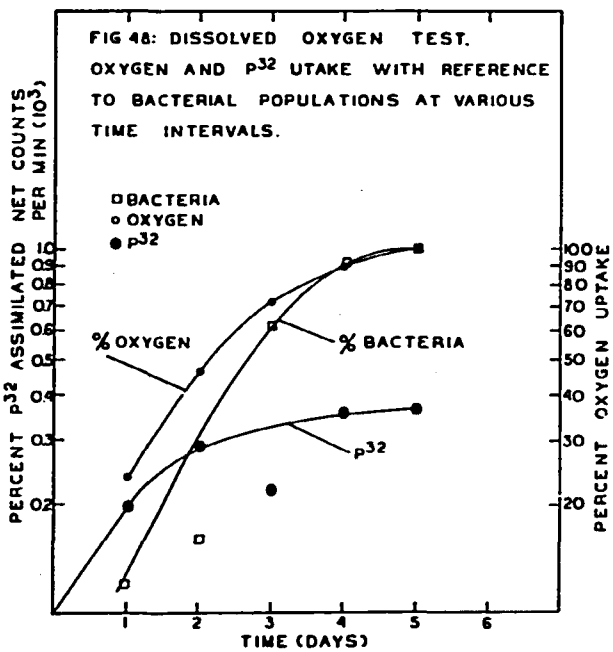


FIG 48: DISSOLVED OXYGEN TEST.
OXYGEN AND P32 UTAKE WITH REFERENCE
TO BACTERIAL POPULATIONS AT VARIOUS
TIME INTERVALS.
BACTERIA
OXYGEN
P32
% OXYGEN
% BACTERIA
P32
PERCENT P32 ASSIMILATED NET COUNTS PER MIN (10³)
PERCENT OXYGEN UPTAKE
TIME (DAYS)

oxidation of their own tissues (endogenous respiration). Endogenous respiration customarily occurs in the absence of available food, whereupon the cells oxidize their own tissues to obtain the necessary energy for maintenance. Eckenfelder (23) contends that endogenous respiration may even take place concurrently with synthesis. This was probably one of the causes of sludge reduction in the mixed liquor which is discussed below.

Stabilization of the organic material is accomplished both by oxidation and synthesis. So long as conditions are favorable, synthesis will exceed oxidation, and during this interval the rate of B.O.D. removal is both high and linear. Up to this point, concurrent with B.O.D. reduction, there is an increase in the sludge mass. As long as the slimes are increasing, the membrane filter will retain increasing quantities of solids each time a sample is filtered. One would expect that the P^{32} uptake would increase correspondingly, but the moment the rate of oxidation exceeds the rate of synthesis, both the growth curve and the B.O.D. removal curve abruptly slope downward. Under these conditions the mass of solids is decreasing, and as a result the membrane filter will show a decrease in P^{32} uptake.

In contrast, the accumulative oxygen uptake will continue to increase. Figure 40 shows the curve between moles of oxygen uptake vs time. After reaching its peak the curve abruptly slopes downward. This shows that, although the accumulative value of oxygen uptake continued to increase, its rate

per unit time decreased. A significant hypothesis can be proposed here: that if sufficient food is added, the curve in Figure 39 may again turn upward. This hypothesis was tested and the experimental results are shown in Figure 41. The experiment was similar to the preceding one, with one exception; after five hours and twenty minutes, two grams of lactose broth were added. The curve thereafter resumed its upward trend. This proves that the P^{32} uptake rate is linear to the oxygen uptake rate, but only when the rate of synthesis exceeds the oxidation rate.

In other experiments (see Figures 46 and 47), a food source in the form of synthetic sewage was added periodically to maintain the logarithmic stage of growth of the bacteria in the sludge. However, it became evident that the flocculant sludge bacteria could not be maintained in a growth stage indefinitely, because after a few hours the bacteria started to die. This may have been due to the toxic effect of metabolic by-products produced in the oxidation process. This situation may be avoided in a continuous system where the sludge is constantly recirculated, and an inoculum of growing bacteria is added continuously in the form of raw settled sewage. This is the case in a properly operated sewage treatment plant. If this is done, an equilibrium stage may be reached where extremely large populations of bacterial organisms may be kept alive by providing a constant excess of food. Here, the rate of synthesis would be sufficient to counterbalance the loss of

bacteria due to death. This condition can be maintained easily in a sewage treatment plant.

The R.Q.'s of the experiments discussed above are shown in Table 7. They varied from 0.637 to 2.25, but most of the time were close to 0.8.

Conclusions and Recommendations

From the above studies it can be concluded that when the supply of nutrient was abundant, the P^{32} uptake had a linear correlation with the uptake of oxygen. If all of the adsorbed P^{32} could have been washed from the filter membrane, the resulting line would pass through the origin with a slope of 1.0. In addition, the following findings were made:

1. Reaction rate coefficient of free floating biological slimes can be measured by using P^{32}, and results are comparable to respiration studies.

2. The proposed method of measuring the reaction rate coefficient is applicable only when the rate of synthesis exceeds the rate of oxidation.

3. Similar experiments should be carried out using complex food sources.

4. Chemical analysis of slimes should be made in order to study their stoichiometry and respiratory quotients.

TABLE 7

FREE FLOATING SLIME STUDIES - RESPIRATORY QUOTIENT OF VARIOUS RUNS

Time (Minutes)	R. Q.	Time (Minutes)	R. Q.	Time (Minutes)	R. Q.
Run No. 1		Run No. 3 Cont'd.		Run No. 5 Cont'd.	
20	0.637	85	1.68	100	1.08
60	0.805	125	1.69	140	0.81
95	0.830	185	1.75	205	0.82
135	0.843	230	1.79	250	0.89
220	0.850	290	1.84	295	0.86
280	1.163	330	1.81	325	0.82
340	1.135	370	1.82	Run No. 6	
380	1.078	390	1.76	5	1.00
Run No. 2		440	1.76	50	1.33
125	0.661	Run No. 4		90	0.85
165	1.11	10	0.120	125	0.71
205	1.07	50	0.430	170	0.71
270	1.10	90	0.663	210	0.73
325	1.06	130	0.710	250	0.72
385	1.04	170	0.800	285	0.73
415	1.03	230	0.870	325	0.74
465	0.99	230	0.890	365	0.76
Run No. 3		Run No. 5		Run No. 7	
10	0.107	20	0.93	10	1.29
50	1.57	60	1.16	45	1.90

TABLE 7--Continued

Time (Minutes)	R. Q.	Time (Minutes)	R. Q.
Run No. 7 Cont'd.		Run No. 9	
70	2.25	15	0.296
100	2.19	60	1.21
130	2.48	105	1.21
175	1.99	150	1.30
205	1.81	195	1.14
235	1.66	240	1.15
270	1.53	285	1.22
Run No. 8			
25	1.175		
70	0.903		
105	0.905		
160	0.900		
195	0.866		
235	0.868		
270	0.850		
305	0.851		
330	0.859		

CHAPTER VI

DISSOLVED OXYGEN TEST

General

The amount of oxidizable material present in water is expressed quantitatively as the amount of oxygen required by the bacteria in the water to oxidize the organic compounds present into stable end products. This quantity of oxygen required for stabilization is dependent on temperature, time, and other factors. Stream pollution may also be due to physical or chemical characteristics of wastes such as oil or dye liquors. Although compounds like phenol may add to undesirable odors and certain toxic materials may kill fish, they do not contribute to the B.O.D. pollution load. Pollution from various types of organic solids is, however, a prime consideration in abating stream pollution.

Much credit for the development of the Standard B.O.D. Test as described in the Standard Methods (62) goes to Theriault and Hammond (65). This test has been used for many years to evaluate the effectiveness of sewage treatment plants. In addition, by virtue of its professional acceptance, the Standard B.O.D. Test has also been used for many years as an effective

criterion for measuring stream pollution caused by the discharge of excessive organic wastes, and thereby abating stream pollution.

This section discusses experiments that were performed to correlate the P^{32} uptake with oxygen uptake in B.O.D. Test. In these experiments, both the uptake of oxygen and that of P^{32} versus time, were determined under similar conditions and at controlled temperatures. It was anticipated that the P^{32} would be altered, in its position in the sample, by changes occurring in the bacterial population in the solids. Samples were, therefore, analyzed not only for oxygen and P^{32} uptake, but also for the number of bacteria existing in the sample.

As the Standard Test (62) for the bacteriological examination of water is cumbersome and takes five to six days to obtain results, all bacteria counts for the experiments described below were made using the relatively new membrane filter technique. This technique was developed in Germany during World War II (29) and its use has been studied extensively in this country since 1946. It requires approximately sixteen hours for the full development of the colonies at incubation temperatures of 37°C. The membrane technique, the results of which are comparable to those of the Standard Test, has been accepted as a standard method for the bacteriological examination of water (62).

Experimental Procedure

The Standard B.O.D. Test by dilution was used to determine the oxygen uptake. Measurements were made with a Baush

and Lomb Spectronic 20, as recommended by Oulman and Baumann (47). Two sets of standard B.O.D. bottles were set up with one set acting as a control and the other tagged with P^{32}. At intervals, the tagged samples were analyzed for P^{32} uptake while the control samples were analyzed for oxygen uptake. The essential equipment used for this test is shown in Figure 20.

The contents of the tagged B.O.D. bottles were passed through a 0.45 micron membrane filter. The filter was then washed with 2.5 per cent saline water to remove adsorbed P^{32}. One cc of the filtrate was transferred to a planchet, which was dried on a rotating table under an infra-red lamp and then counted for remaining P^{32}. The counts were multiplied by 300 to obtain estimated total counts in the 300 cc of the filtrate in the B.O.D. bottle. Each set of measurements was corrected for half-life decay. To check consistency of results, the number of counts on the planchet and in the filtrate were summarized. These values should be constant if corrected for decay, and should total to that of the original radioactivity. The results showed a statistical fluctuation and therefore the mean value was used in computing the uptake.

Bacterial Test Procedure

In the initial experiments, no attempt was made to study the growth of bacteria in the B.O.D. bottles. After it was observed that the P^{32} uptake was different than the oxygen uptake, records were kept of each day's bacterial counts. Separate bottles were used for this phase of the investigation.

The apparatus used for making the bacterial counts consisted of a stainless steel funnel clamped to a removable base for supporting a membrane filter and is illustrated in Figure 20. After sterilizing this equipment, a sterile membrane filter was placed on the base and the funnel clamped over it. The sample containing the bacteria to be counted was measured, poured into the funnel, and filtered under 12-15 p.s.i. of vacuum. The funnel was then removed and the vacuum was released.

The membrane filter was removed with sterile forceps and placed on an absorbent pad which was saturated with a selected media in a sterile ointment tin.

The selection of culture media depends upon the type and purpose of analysis (e.g., for total count, or for a particular organism or group). For these tests M. Enrichment broth (12) was used for total bacterial count, and M-HD Endo broth was used for coliform count (30). The ointment tin was closed and inverted for incubation so that the nutrient would diffuse downward through the membrane. Duplicate samples were tested and all of the plates were incubated at 35^{o}-37^{o}C (29) for a period of sixteen hours. At the end of this period counts were made on those filters that showed thirty to three hundred colonies.

Discussion of Results

Results of the several trials are shown in Figures 48 through 58. It is evident from the plottings that P^{32} uptake

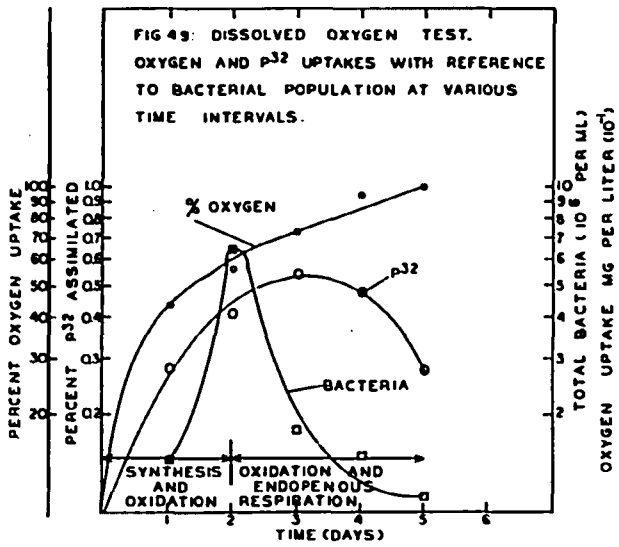


FIG 49: DISSOLVED OXYGEN TEST.
OXYGEN AND P32 UPTAKES WITH REFERENCE TO BACTERIAL POPULATION AT VARIOUS TIME INTERVALS.
% OXYGEN
P32
BACTERIA
SYNTHESIS AND OXIDATION
OXIDATION AND ENDOPENOUS RESPIRATION
PERCENT OXYGEN UPTAKE
PERCENT P32 ASSIMILATED
TOTAL BACTERIA (10⁸ PER ML)
OXYGEN UPTAKE MG PER LITER (10⁻¹)
TIME (DAYS)

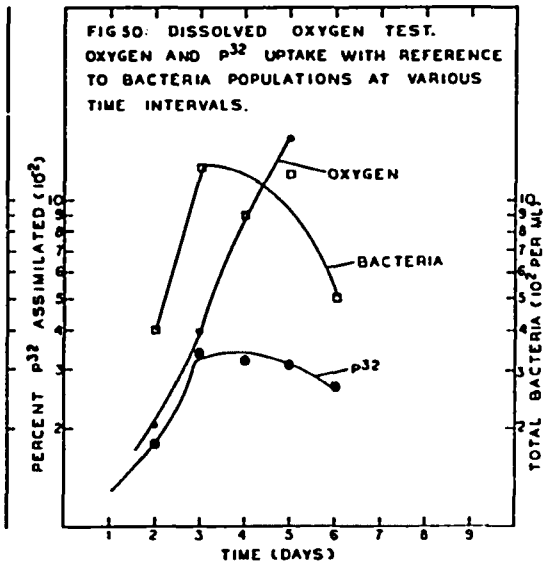


FIG 50: DISSOLVED OXYGEN TEST.
OXYGEN AND P32 UPTAKE WITH REFERENCE TO BACTERIA POPULATIONS AT VARIOUS TIME INTERVALS.
OXYGEN
BACTERIA
P32
PERCENT P32 ASSIMILATED (10²)
TOTAL BACTERIA (10⁸ PER ML)
TIME (DAYS)

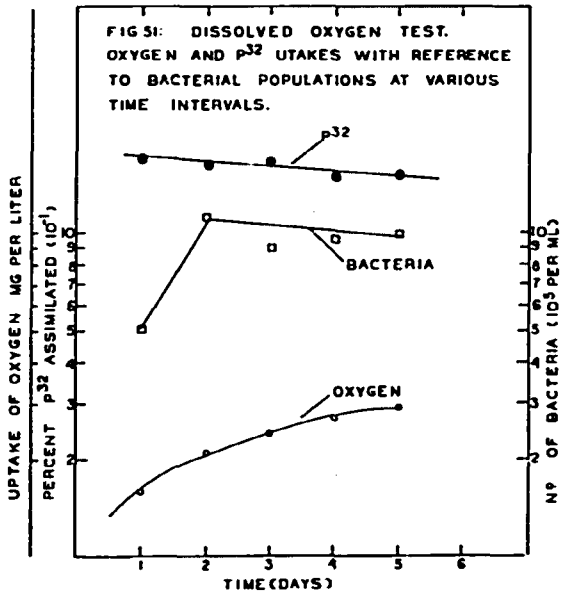


FIG 51: DISSOLVED OXYGEN TEST.
OXYGEN AND P32 UTAKES WITH REFERENCE TO BACTERIAL POPULATIONS AT VARIOUS TIME INTERVALS.
P32
BACTERIA
OXYGEN
UPTAKE OF OXYGEN MG PER LITER
PERCENT P32 ASSIMILATED (10⁻¹)
Nº OF BACTERIA (10⁵ PER ML)
TIME (DAYS)

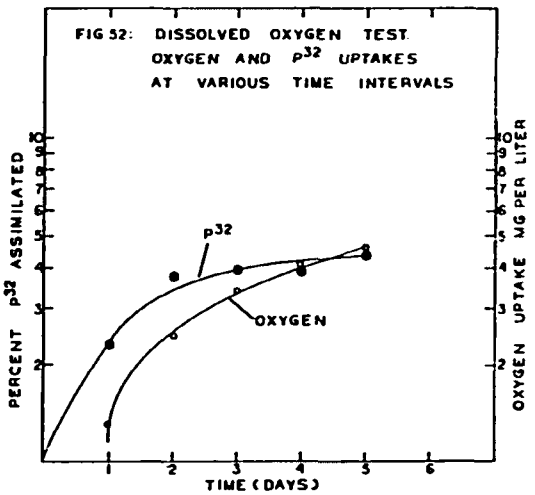


FIG 52: DISSOLVED OXYGEN TEST.
OXYGEN AND P32 UPTAKES AT VARIOUS TIME INTERVALS
P32
OXYGEN
PERCENT P32 ASSIMILATED
OXYGEN UPTAKE MG PER LITER
TIME (DAYS)

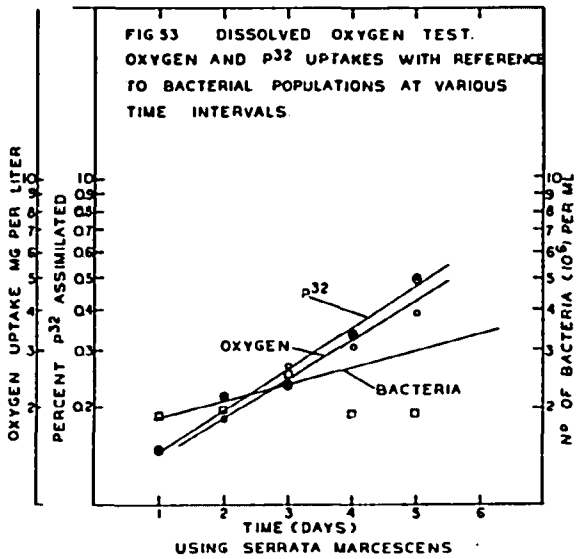


FIG 53 DISSOLVED OXYGEN TEST.
OXYGEN AND P32 UPTAKES WITH REFERENCE TO BACTERIAL POPULATIONS AT VARIOUS TIME INTERVALS.
OXYGEN UPTAKE MG PER LITER
PERCENT P32 ASSIMILATED
Nº OF BACTERIA (10⁶) PER ML
P32
OXYGEN
BACTERIA
TIME (DAYS)
USING SERRATA MARCESCENS

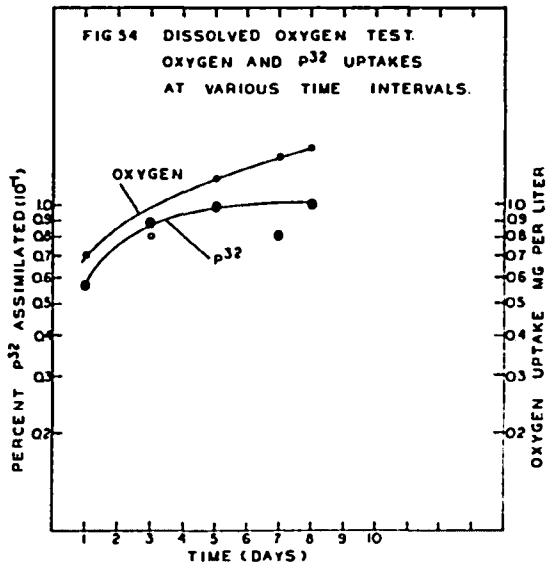


FIG 54 DISSOLVED OXYGEN TEST.
OXYGEN AND P32 UPTAKES AT VARIOUS TIME INTERVALS.
PERCENT P32 ASSIMILATED (10⁻¹)
OXYGEN UPTAKE MG PER LITER
OXYGEN
P32
TIME (DAYS)

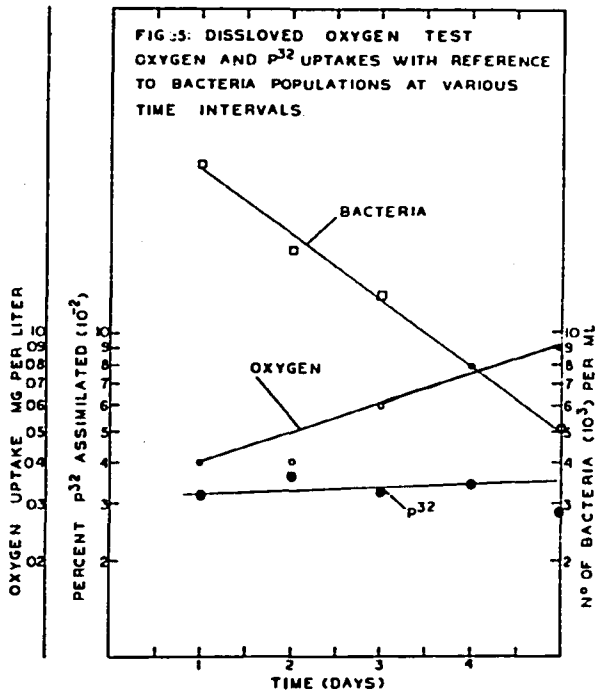


FIG 55: DISSLOVED OXYGEN TEST
OXYGEN AND P32 UPTAKES WITH REFERENCE TO BACTERIA POPULATIONS AT VARIOUS TIME INTERVALS.
OXYGEN UPTAKE MG PER LITER
PERCENT P32 ASSIMILATED (10⁻²)
Nº OF BACTERIA (10³) PER ML
BACTERIA
OXYGEN
P32
TIME (DAYS)

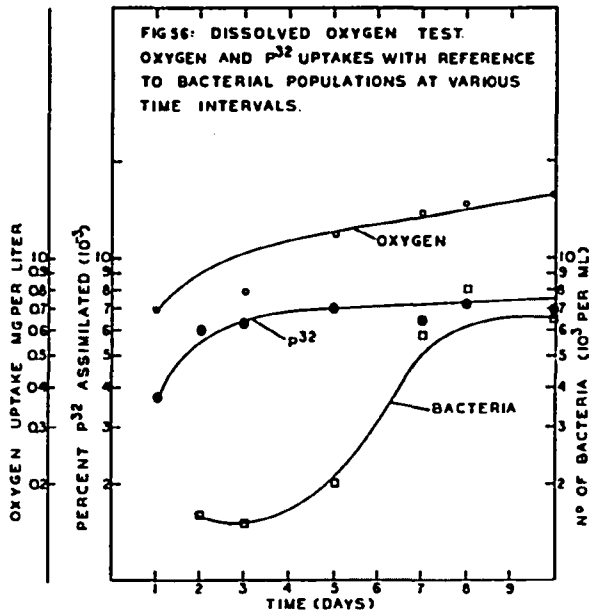


FIG 36: DISSOLVED OXYGEN TEST. OXYGEN AND P^{32} UPTAKES WITH REFERENCE TO BACTERIAL POPULATIONS AT VARIOUS TIME INTERVALS.

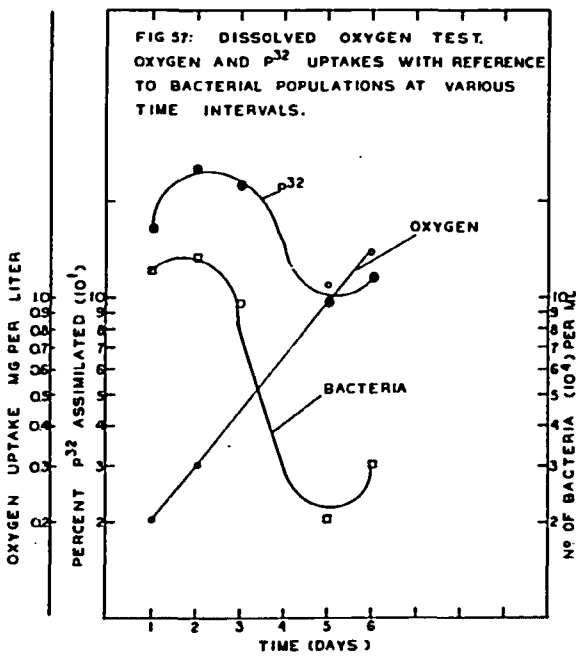


FIG 37: DISSOLVED OXYGEN TEST. OXYGEN AND P^{32} UPTAKES WITH REFERENCE TO BACTERIAL POPULATIONS AT VARIOUS TIME INTERVALS.

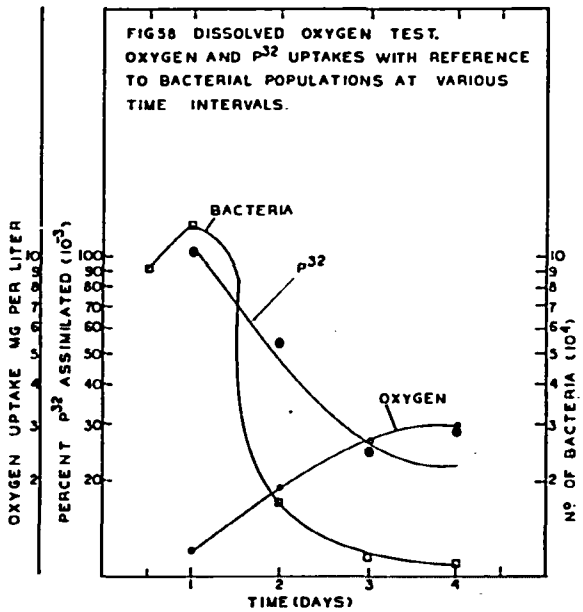


FIG 38 DISSOLVED OXYGEN TEST. OXYGEN AND P^{32} UPTAKES WITH REFERENCE TO BACTERIAL POPULATIONS AT VARIOUS TIME INTERVALS.

and bacterial count consistently follow the same pattern, while the oxygen uptake does not. The curve representing the cumulative oxygen uptake consistently increases, whereas the P^{32} uptake and total bacterial count curves decline after a time. During the first twenty-four hours of the study, represented by Figure 49, the bacterial population was approximately 1.42 x 10^3 per milliliter of sample. Assimilated P^{32} showed 128 counts per minute, and the bacteria consumed 2.3 mg/liter of oxygen. During the next twenty-four hours the bacteria attained a peak population of 6.5 x 10^3 bacteria per milliliter. The assimilated P^{32} was 189 counts per minute, and bacteria exerted an oxygen demand of 0.7 mg/liter.

On the third day the population of bacteria decreased, most probably because of depletion of the available food. At this stage, the bacteria were in a state of endogenous metabolism. Uptake of P^{32} increased, but the bacterial population decreased to 1.7 x 10^3 bacteria per milliliter. The oxygen demand was 0.8 mg/liter. 'On the fourth day both bacterial population and the uptake of P^{32} decreased.

On the fifth day, the most important day of the test, a significant decrease in bacterial population was observed. The bacterial population was 1.1 x 10^3 bacteria per milliliter, and the uptake of P^{32} decreased considerably. The oxygen uptake was only 0.32 mg/liter. For the five day period of incubation, the total uptake of oxygen was 5.28 mg/liter; the result of the respiration of bacteria. Total consumption of oxygen is due to both active and endogenous metabolism.

A large portion of available food was consumed in the first two days, after which the number of bacteria started decreasing. Investigators have often indicated, although there is little supportive evidence in the literature, that at the end of five days of incubation at 20° C about 90 per cent of the food is consumed.

Similar results were indicated by additional trials as shown in Figures 48, 50 through 58. Of particular significance are the data plotted in Figures 57 and 58. For these two experiments, domestic sewage from the Norman sewage treatment plant was used instead of synthetic sewage. The number of bacteria and uptake of P^{32} were shown to decrease consistently after the first day, while the cumulative oxygen steadily increased as in the previous tests.

Since the bacteria used in these experiments were a mixture of aerobic and facultative species it was considered desirable to run parallel experiments with only aerobic species of Serratia marcescens. This particular strain of bacteria has a remarkable property of growing into large pink colonies at room temperature in only 48 hours. A pure strain of the bacteria was obtained from the Department of Microbiology of the University of Oklahoma, and the bottles were set up by the method previously described, except that dilution water after aeration was sterilized by passing it through a membrane filter. Results are shown in Figure 53. In this case both P^{32} and oxygen uptake followed the same trends, although a good correlation

between the two could not be established. Since the results were not correlatable, in order to simplify the line of research it was decided to perform short term B.O.D. studies.

Short Term Studies

It is customary to incubate the B.O.D. bottles at 20°C for five days, but in recent years investigations have been made of B.O.D. tests using shorter incubation periods and temperatures of 30°C or more. It has been shown (7, 8) that shorter incubation periods have also yielded reproducable and accurate results. Therefore several short term B.O.D. studies were performed at 30°C to compare the suitability of this method with the standard five-day B.O.D.

Bacterial populations have an ability to adapt to the prevailing environment and it is often possible to grow bacteria abundantly after a period of conditioning in environmental conditions which are usually toxic for them. Mills and Stack (44) have shown that even secondary and tertiary amines, which are not usually oxidized by micro-organisms in domestic sewage, may be oxidized by acclimatized micro-organisms. The B.O.D. of industrial wastes always yields a higher value if acclimatized seeding is used (45). The adaptability of bacterial populations may be temporary, or they may even undergo changes of permanent character which involve a change in, or loss of a particular gene. Whether the adaptation results from changes in species composition, a selective process among individuals of a species, or even a permanent change in individual cells, is not yet known.

In the experiments represented in Figures 59 and 60, acclimatized seeding was used at incubation temperatures of 30°C. The seeding was prepared in the manner described by Mills and Stacks (44). From the results, it is quite evident that the rate of oxygen uptake was much higher at 30°C than at 20°C, as was expected. Although the plots of the P^{32} uptake and oxygen uptake are fairly straight lines, a good correlation between the two could not be established.

It appeared from the results of the preceding trials that no useful conclusions could be drawn in these particular phases of study. Because of this it was necessary to survey the literature in an attempt to locate the reasons for these erratic results.

Numerous investigators have felt that the Standard B.O.D. Test is limited in the supply of total oxygen available for the bacteria to grow. Because of this the quantity of sewage sample that can be inoculated into the B.O.D. bottle is also limited. This second factor puts a strict limit on the concentration of food available for the bacterial growth. Consequently, after two days of incubation at 20°C, the food supply decreases drastically and the bacterial population starts to die. At this point the rate of oxidation exceeds rate of synthesis. As explained previously, under such circumstances the curve representing P^{32} uptake slopes downwards. In almost all of the present studies the maximum available oxygen in the dilution water was 7.5 mg/liter, i.e., 0.703×10^{4} moles of

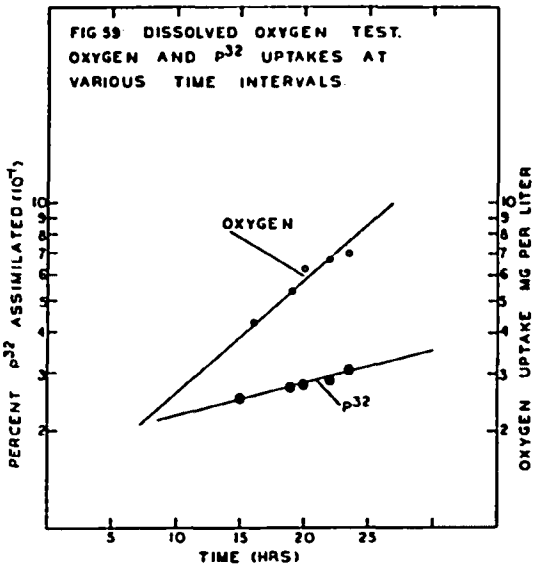


FIG 59 DISSOLVED OXYGEN TEST.
OXYGEN AND P32 UPTAKES AT
VARIOUS TIME INTERVALS
OXYGEN
P32
PERCENT P32 ASSIMILATED (10-4)
OXYGEN UPTAKE MG PER LITER
TIME (HRS)

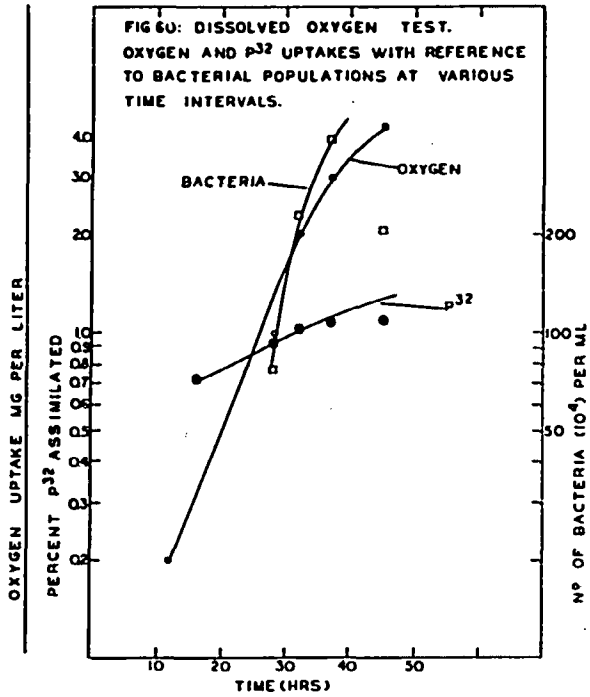


FIG 60: DISSOLVED OXYGEN TEST.
OXYGEN AND P32 UPTAKES WITH REFERENCE
TO BACTERIAL POPULATIONS AT VARIOUS
TIME INTERVALS.
BACTERIA
OXYGEN
P32
OXYGEN UPTAKE MG PER LITER
PERCENT P32 ASSIMILATED
Nº OF BACTERIA (10⁴) PER ML
TIME (HRS)

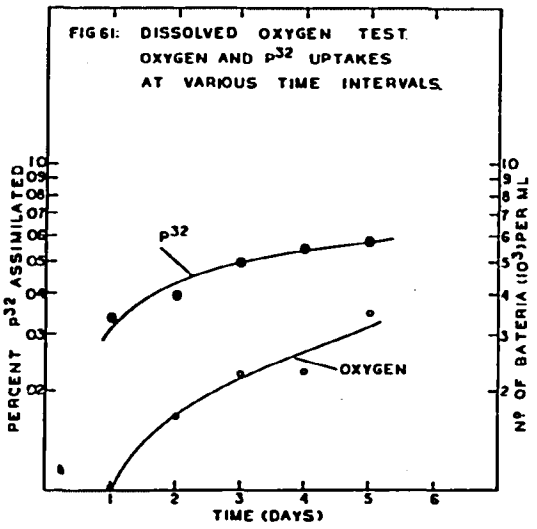


FIG 61: DISSOLVED OXYGEN TEST.
OXYGEN AND P32 UPTAKES
AT VARIOUS TIME INTERVALS.
P32
OXYGEN
PERCENT P32 ASSIMILATED
Nº OF BATERIA (10³) PER ML
TIME (DAYS)

oxygen per B.O.D. bottle. This quantity is little less than 2 cc of oxygen at standard temperature and pressure. It appears that, with such a small available supply of oxygen, the proposed method of measuring the reaction rate coefficient is not adequate.

Conclusions

From the above studies it can be concluded that:

1. The rate of uptake of P^{32} and oxygen are directly correlatable, so far as the supply of oxygen and food is sufficient, and that B.O.D. is removed mainly by synthesis.

2. In early stages of the B.O.D. test, the rate of uptake of food is fast and directly proportional to the biological growth which is evident from the bacterial growth curves. Probably this B.O.D. is stored in the cell as a reserve food.

3. When a major portion of the food is stored in the cell, the oxidation of cellular material starts through endogenous respiration. During the endogenous respiration the plotting of uptake of P^{32}, and bacterial growth decline down, and do not bear a direct correlation with cummulative uptake of oxygen.

4. Cellular material is oxidized to its end products by endogenous respiration when the organic matter (B.O.D.) is insufficient to support active bacterial growth.

CHAPTER VII

CONCLUSIONS

In this age of rapid evolution in research techniques in so many of the disciplines, scientists must be ever careful that they do not cling to the old techniques useful as they may have been and thereby delaying the quest for new explanation of the natural phenomenon. In the field of sanitary engineering the conventional B.O.D. test and manometric techniques have served and still are serving as excellent tools of research. However there are indications as this report has attempted to prove that there are methods somewhat different from the existing methods, which by attacking the problem from a different approach will yield similar results as indicated by the manometric or the standard B.O.D. tests.

The present assignment concerned itself to develop a correlation between respiration and metabolism studies of the biological slimes using P^{32}. The results have been compared in three different ways, which have been discussed in detail in the preceeding pages. However a summary of the conclusions is given below. The results indicate a high degree of correlation in the metabolic and the respiration activities of slime growth. This verifies the validity of using P^{32} uptake techniques.

1. The rate of uptake of assimilated P^{32} is the same as the rate of uptake of oxygen.

2. The relation between the rate of uptake of oxygen and that of uptake of assimilated P^{32} is not affected by the type or concentration of the food.

3. The relation between the rate of uptake of oxygen and uptake of assimilated P^{32} is not affected by manometric pressure.

4. Reaction rate constant of biological slimes can be measured accurately by using radiophosphorus.

5. The proposed method is only applicable and comparable to respiration. So far rate of synthesis exceeds the rate of oxidation.

6. B.O.D. although is a quantitative measure for the concentration of the organic substrate oxidized by micro-organism, it is not a true representation of metabolic processes in a sewage treatment plant. The growth of micro-organisms is restricted in a B.O.D. test due to the limited supply of oxygen and food.

BIBLIOGRAPHY

1. Adeney, W. E., The course and nature of fermentative changes in natural and polluted waters and artificial solutions as indicated by composition of dissolved gases. Report of the Royal Commission of Sewage Disposal, London, England, 5th Report, 1908.

2. Archibald, Reginald M., Colorimetric determination of urea, Biol. Chem., 157:507-518. 1945.

3. Barcroft, J., and Halden, J. S., A method of estimating oxygen and carbonic acid in small quantities of blood. Jour. Physiology 28:232-401. 1902.

4. Beckwith, T. P., Bacteriology of pulp slime. Jour. Bact. 22:15-22. 1931.

5. Breed, Robert S., Murray, E. G. D., and Smith, Nathan R., Bergey's Manual of Determinative Bacteriology. Seventh Edition. The Williams and Wilkins Company. Baltimore 2, Maryland. 1957.

6. Breedsley, C. W., Suppression of sewer slimes. Sew. Works Jour. 21:1-13. 1949.

7. Busch, A. W., An improved short-term B.O.D. test. Water and Sewage Works Jour. 108:255-260. 1961.

8. Busch, A. W., and Myrick, N., Aerobic bacterial degradation of glucose. Jour. Water Pollution Control Federation. 33:897-905. 1961.

9. Buswell, A. M., and Long, H. L., Microbiology and theory of activated sludge, Am. Water Works Assoc. Jour. 10:309-321. 1923.

10. Butterfield, C. T., A zooglea-forming bacterium isolated from activated sludge. Public Health Reports, 50: 671-683. 1935.

11. Caldwell, D. H., and Langelier, W. F., Manometric measurement of the B.O.D. of the sewage. Sewage Works Journal 20:202-217. 1947.

12. Clark, Harold F., Geldrich, Edwin E., Jeter, Harold L., and Kabler, Paul W., Membrane filter in sanitary bacteriology. Pub. Health Report 66:951-977. 1951.

13. Dawson, P. S. S., and Jenkins, S. H., The oxygen requirements of activated sludge determined by manometric method. I. Sewage Works Journal 21:643-658. 1948.

14. Dawson, P. S. S., and Jenkins, S. H., The oxygen requirements of activated sludge determined by manometric method II. Chemical Factors Affecting Oxygen Uptake. Sewage Industrial Wastes 22:490-570. 1950.

15. Diehl, Harvey, and Smith, G. Frederick, Quantitative Analysis. John Wiley and Sons, Inc. New York. 1957.

16. Dixon, M., Nomograms for manometer constants, Cambridge University Press, Reprinted from Biochem. Journal 39:427-437. 1945.

17. Dixon, M., and Elliot, K. A. C., The use of Barcroft apparatus for the measurement of tissue respiration. Biochem. Journal 24:820-833. 1930.

18. Drown, T. M., Effect of the aeration of natural waters. Twenty-third Annual Report of the State Board of Health of Massachusetts. 1892.

19. Dugan, P. R., and Lundgren, D G., Isolation of the floc-forming organism zooglea ramigera and its culture in complex and synthetic media. Applied Microbiology. 8:357-361. 1960.

20. Dupre, A., Report on changes in the aeration water as indicating the nature of the impurities present in it. Fourteenth Annual Report of the Local Government Board. 1883-84.

21. Eckenfelder, W. Wesley, Studies on the oxidation kinetics of biological sludge. Sewage and Ind. Waste Journal. 28:983-990. 1956.

22. Elsden, S. R., and Peel, J. L., Metabolism of Carbohydrate and related compounds. Annual Review of Microbiology. 12: 1958.

23. Falk, L. L., and Rudolfs, W., Factors affecting the direct oxygen utilization method. Sewage Works Journal 19: 1000-1006. 1947.

24. Fisher, R. A., and Yates, Frank. Statistical tables for biological, agricultural, and medical research. Oliver and Boyd Ltd. Edinburgh & London; and Hafner Pub. Co. New York. 1953.

25. Flugge, C., Microorganisms - Eliology of the infectious diseases. Gottingen.

26. Frankland, E., First report the River Pollution Commission of 1868. pp. 18-50. 1870.

27. Gaune, Werner N., and Eliassen, Rolf, Studies on the effect of radio active phosphorous on the biochemical oxidation of sewage. Sew. Works Jour. 23:141-154. 1951.

28. Geradin, A., Altivations de la Sein Aux de Paris depuis. November, 1874, jusque Mai, 1875. Compt. Rend., 80: 1326-1328. 1875.

29. Goetz, A., and Tsuneishe, N., Application of molecular filter membranes to bacteriological analysis of water. Jour. Am. Water Works Assoc. 43:943-968. 1951.

30. Hajana, A. A., and Damon, S. R., California detection in water by a single step technique using membrane filter. Pub. Health Reports 69:58-60. 1954.

31. Hall, J. W., Fungus growth in sanitary sewers. Water Works Jour. 66:177. 1927.

32. Helmer, E. N., Frame, J. D., Greenberg, A. E., and Sawyer, C. N., Nutritional requirement in the biological stabilization of industrial wastes II. Treatment with domestic sewage. Sewage and Ind. Waste Jour. 23:884-899. 1951.

33. Heukelekian, H., and Schulhoff, H. B., Studies on the clarification of activated sludge process IV. Preliminary notes on the clarifying organisms in activated sludge. Sewage Works Jour. 10:43-48. 1938.

34. Heukelekian, H., Oxford, H. E., and Manganelli, R., Factors affecting the quality of sludge production in the activated sludge process. Sew. and Ind. Waste Jour. 23:945-958. 1951.

35. Hinshelwood, C. N., The chemical kinetics of the bacterial cell. Oxford: Claredon Press. 1947. United Kingdom.

36. Hoover, C. B., A method for determining the parts per million of dissolved oxygen consumed by sewage and sewage effluents. Eng. News. 65:311-312. 1911.

37. Johnson, J. W., and Haith, A. A Contribution to the biology of the sewage disposal. Jour. Biol. 9:105-127. 1914.

38. Kreb, Hans A., and Henseleit, K., Urea formation in the animal body. Zeitschrift Physiol. Chem. 210:33-66. 1932.

39. Lee, E. W., and Oswald, W. J., Comparative studies of dilution and Warburg method for determining B.O.D. Sewage and Industrial Waste Journal 26:1097-1108. 1954.

40. Ludwig, W. H., Oswald, W. J., and Gottas, H. B., Manometric technique for measurement of B.O.D., Issue No. 2, Series No. 44. University of California, Institute of Engineering Research, Berkeley, California.

41. McKinney, Ross E., Biological oxidation of organic matter. Presented at Conference on Biological Waste Treatment. Manhattan College. New York. April, 1960.

42. McKinney, Ross E., and Horwood, Murray P., Fundamental Approach to the activated sludge process. Sew. Works Jour. 24:117-123. 1952.

43. McKinney, R. D., and Weichlein, R. G., Isolation of floc producing bacteria from activated sludge. Appl. Microbiol. 1:259:261. 1953.

44. Mills, E. J., and Stack, T. Vernon, Jr., Acclimation of micro-organisms for the oxidation of pure chemicals. Proceedings of Ninth Industrial Waste Conference. Purdue University, Lafayette, Indiana. 1954.

45. Mills, E. J., and Stack, T. Vernon, Jr., Biological oxidation parameter applied to industrial wastes. Indst. Engineering Chemistry. 48:260-262. 1956.

46. Monod, Jaques, Recherches Sur La Croissance Des Cultures Bacteriennes. Paris: Hermann and Cie. 1942.

47. Oulman, Charles S., and Bauman, E. R., A colorimetric method for determining dissolved oxygen. Sewage and Ind. Waste Journal. 28:1461-1465. 1956.

48. Placak, O. R., and Ruchhoft, C. C., Studies of sewage purification XVII. The utilization of organic substrates by activated sludge. Sewage Works Jour. 19: 423-440. 1947.

49. Porges, Nandor; Jasewicz, Lenore, and Hoover, Sam R., Principal of Biological Oxidation in Biological Treatment of Sewage and Industrial Waste. Vol. I, by McCabe, Brother Joseph, and Eckenfelder, W. W. Reinhold Publishing Corporation, New York, New York, 1955.

50. Porter, J. R., Bacterial Chemistry and Physiology. John Wiley and Sons. New York. 1946.

51. Reid, G. W., Adsorption and assimilation of P^{32} by bacterial slimes. Tech. Inf. Div., O. R. L. Oak Ridge, Tenn. 1949.

52. Reid, George W., and Bizzell, Oscar M., Planning a small radioisotope program. Indus. Med. and Surgery 19:594-553. 1950.

53. Rideal, S., and Burgess, W. T., The new standards for sewage effluents. Analyst 34:193-205. 1909.

54. Rorschach, Richard, and Reid, George W., A Study of biological sewage oxidation using radiophosphorous as a tracer. Sewage and Ind. Waste Jour. 24:1229-1237. 1952.

55. Ruchoft, C. C., and Watkins, J. H., Bacteriological isolation and study of the filamentous organisms in activated sludge of Des Plaines River sewage treatment works. Sew. Works Jour. 1:52-58. 1928.

56. Sanborn, J. R., Slime producing coliform and coliform-like bacteria. J. Bact. 48:211-217. 1944.

57. Siegel, B. V., and Clifton, C. E., Energy relationship in carbohydrate assimilation Eschericia coli. J. Bacteriol. 60:573-583. 1950.

58. Siegel, B. V., and Clifton, C. E., Energetics and assimilation in the combustion of carbon compound by Escherichia coli. J. Bacteriol. 60:585-593. 1950.

59. Sierp, F., A new method for determining biochemical oxygen demand. Translation by Neave, S. L., of the original paper. Industrial Engineering Chemistry. 20:247. 1928.

60. Skrinde, R. T., and Sawyer, C. N., Application of the Warburg respirometer to industrial waste analysis. Special studies on radioactive wastes. Seventh Ind. Waste Conf. Bull. 1952.

61. Snaddon, X. V. M., and Harkness, N., A manometric apparatus for the measurement of oxygen uptake of activated sludge. Sewage Effluent and Trade Wastes. Water and Waste Treatment Journal 7:250-253. 1959.

62. Standard Method for the Examination of Water and Sewage. Tenth Edition. Am. Pub. Health Assoc., Inc. 1790 Broadway, New York, 19, New York.

63. Starkey, Robert L., Transformation of iron by bacteria in water. A. W. W. A. Jour. 37:963-984. 1945.

64. Stutzer, A., and Hartleb, R., De Salpeter Pilze Centabl f. Bakt u. Parasitenk Abt. II.

65. Theriault, E. J., and Hammon, H. B., Determination of biochemical oxygen demand of industrial waste and sewage. Pub. Health Bull. No. 97:71-86. 1918.

66. Warburg, O., Uber den Stoffwechsel der Tumoren. Springer. Verlag, Berlin. 1926.

67. Wasserman, A. E., Hopkins, W. J., and Porges, N., Whey utilization growth conditions for saccharomyces fagilis. Sewage Industrial Wastes. 30:913. 1950.

68. Wattie, E., Culture characteristics of zoogleal-forming bacteria isolated from activated sludge and trickling filter. Sewage Works Jour. 15:476-490. 1943.

69. Wooldridge, W. R., and Standfast, A. F. B., The use of Barcroft differential manometer in the estimation of oxygen absorption of sewage. Biochem. Journal 30:141-147. 1936.

70. Wooldridge, W. R., and Standfast, A. F. B., Some experiments on the oxidation of sludge and sludge sewage systems. Biochem. Journal 30:149-155. 1936.

71. Wooldridge, W. R., and Standfast A. F. B., Certain factors that influence the rate of activated sludge and sewage oxidation. Biochem. Journal 30:156-162. 1936.

72. Zobell, C. E., The effect of solids surfaces upon bacterial activity. Jour. Bact. 46:39. 1943.

73. Zobell, C. E., and Allen, Esther C., Significance of marine bacteria in the fouling of submerged surfaces. Jour. Bact. 29:239. 1935.

74. Zobell, C. E., and Anderson, D. O., Observations on the multiplication of bacteria in different volumes of sea water and the influence of oxygen tension and solid surfaces. Biol. Bull. 71:324. 1936.

APPENDIX

Statistical Analysis

In almost all areas of scientific studies, statistics is playing an increasingly important role in the evaluation of the research data. The techniques of analysis may vary from one science to another, but the basic philosophy is generally common to all. Statistical analysis provides an excellent device for testing a formulated hypothesis, or acquiring an adequate amount of knowledge from which conclusions may be drawn with a certain degree of confidence. Often a research worker has to establish a relationship between two or more variables, or parameters. It is frequently desirable to express this relationship by a mathematical equation. In determining the equation it is customary to plot the points on a suitable graph paper, such as rectangular, semilog, or log. From these points it is possible to draw a curve, which will describe a relationship between the variables. A linear relationship described by a straight line, or an approximately straight line, provides a simple mathematical equation which may be of the form

$$Y = C + mX$$

where m is the slope of the line and represents the change in Y divided by the corresponding change in X; and "C" is a constant, and is the value of Y when X = 0 and is called the Y intercept. If all the points lie on a perfect straight line, then there is perfect linear correlation between X and Y. But invariably, the points are scattered, and the investigator is tempted to determine "r" the coefficient of correlation.

$$r = \pm \sqrt{\frac{\text{explained variation}}{\text{total variation}}}$$

The quantity "r" is a measure for the usefulness of the regression line for estimating purposes. "r" is a dimensionless quantity and its value may vary from 0 to $\pm$ 1. If the value is close to zero the line is incapable of predicting, and a perfect prediction is envisioned if the value is $\pm$ 1. The sign of plus or minus before "r" merely tells if the value of Y increases or decreases as X increases. It is the magnitude of "r" that is important and not its sign.

Analysis of the Data

In order to establish a correlation between the percent uptake of P^{32} and the percent uptake of oxygen, the data collected on the various experiments in the attached slime studies were analyzed statistically. Percent uptake of P^{32} was plotted on the Y axis. The data were analyzed in two different sets for a total of nine experiments. The calculations of one set are shown in Table 8.

TABLE 8

ATTACHED SLIME STUDIES - CALCULATIONS OF COEFFICIENT OF CORRELATION

Run No.	% O_2	% P^{32}	$O_2-\bar{O}_2$	$P^{32}-\bar{P}^{32}$	$(O_2-\bar{O}_2)^2$	$(P^{32}-\bar{P}^{32})^2$	$(O_2-\bar{O}_2) \times (P^{32}-\bar{P}^{32})$
7	10.1	29.2	-35.2	-35.9	1239.0	1288.8	1263.7
	17.7	40.6	-27.6	-24.5	761.7	600.2	676.2
	21.5	50.1	-23.8	-15.0	566.4	225.0	357.0
	36.6	58.0	- 8.7	- 7.1	75.7	50.4	61.7
	48.0	64.7	+ 2.7	- 0.4	7.3	0.2	- 1.1
	55.6	70.6	+10.3	+ 5.5	106.1	30.2	56.6
	58.1	76.8	+12.8	+11.7	163.8	136.9	149.7
	60.6	81.8	+15.3	+16.7	234.1	278.9	255.5
	85.9	89.7	+40.6	+24.6	1648.4	605.2	998.7
	92.2	95.9	+46.9	+30.8	2199.7	948.6	1444.5
	99.9	99.9	+54.6	+34.8	2981.2	1211.0	1900.1
8	3.7	27.5	-41.6	-37.6	1730.5	1413.7	1564.2
	9.8	38.5	-35.5	-26.6	1260.2	691.2	944.3
	15.9	47.0	-29.4	-18.1	864.4	327.6	532.1
	22.7	54.0	-22.6	-11.1	510.7	123.2	250.8
	30.0	60.0	-15.3	- 5.1	234.1	26.0	78.0
	47.2	71.0	+ 1.9	+ 5.9	3.6	34.8	11.2
	54.6	72.5	+ 9.3	+ 7.4	86.5	54.8	69.5
	58.3	80.0	+13.0	+14.9	169.0	222.0	193.7
	69.3	84.5	+24.0	+19.4	576.0	376.4	465.6

TABLE 8--Continued

Run No.	% O_2	% P^{32}	$O_2-\bar{O}_2$	$P^{32}-\bar{P}^{32}$	$(O_2-\bar{O}_2)^2$	$(P^{32}-\bar{P}^{32})^2$	$(O_2-\bar{O}_2) \times (P^{32}-\bar{P}^{32})$
	76.7	89.0	+31.4	+23.9	986.0	571.2	750.4
	87.7	93.0	+42.4	+27.9	1797.7	778.4	1182.9
	99.9	100.0	+54.6	+34.9	2981.2	1218.0	1905.5
9	0	47.6	-45.3	-17.5	2052.1	306.2	792.7
	18.2	52.2	-27.1	-12.9	734.1	166.4	349.6
	27.2	57.0	-18.1	- 8.1	327.6	65.6	146.6
	36.4	62.0	- 8.9	- 3.1	79.2	9.6	27.6
	45.4	70.0	+ 0.1	+ 4.9	.01	24.0	0.5
	45.4	74.2	+ 0.1	+ 9.1	.01	82.8	0.9
	45.4	.2	+ 0.1	+16.1	.01	259.2	1.6
	63.6	94.6	+18.3	+29.5	334.9	870.2	539.8
	99.9	100.0	+54.6	+34.9	2981.2	1218.0	1905.5
10	13.7	35.6	-31.6	-29.5	998.6	870.2	932.2
	22.6	47.5	-22.7	-17.6	515.3	309.8	399.5
	30.5	56.8	-14.8	- 8.3	219.0	68.9	122.8
	40.8	64.9	- 4.5	- 0.2	20.2	0.04	0.9
	50.0	72.0	+ 4.7	+ 6.9	22.1	47.61	32.4
	58.8	78.2	+13.5	+13.1	47.2	171.6	176.8
	69.0	84.2	+23.7	+19.1	561.7	364.8	452.6
	78.3	89.7	+33.0	+24.6	1089.0	605.2	811.8
	87.5	95.0	+42.2	+29.9	1780.8	894.0	1261.8
	99.9	99.9	+54.6	+34.8	2981.2	1211.0	1000.1

TABLE 8--Continued

Run No.	% O_2	% P^{32}	$O_2-\bar{O}_2$	$P^{32}-\bar{P}^{32}$	$(O_2-\bar{O}_2)^2$	$(P^{32}-\bar{P}^{32})^2$	$(O_2-\bar{O}_2) \times (P^{32}-\bar{P}^{32})$
11	7.8	39.6	-37.5	-25.5	1406.3	650.2	956.2
	22.5	51.6	-22.8	-13.5	519.8	182.2	307.8
	32.6	59.6	-12.7	- 5.5	161.3	30.2	69.8
	44.4	71.6	- 0.9	+ 6.5	0.81	42.2	- 5.8
	56.1	79.9	+10.8	+14.8	16.6	219.0	159.8
	68.2	86.6	+22.9	+21.5	524.4	462.3	492.3
	83.2	93.3	+37.9	+28.2	1436.4	795.2	1068.8
	100.0	99.9	+54.7	+34.8	2992.1	1211.0	1903.5
Σ	2446.1	3518.3			42,985.2	22,350.1	29,918.9

$N = 54$

$\bar{O}_2 = 45.3$

$\bar{P}^{32} = 65.1$

$$O_2 = \sqrt{\frac{42985.2}{54}} = \sqrt{796} = 28.3$$

$$P^{32} = \sqrt{\frac{22350.1}{54}} = \sqrt{413.8} = 20.4$$

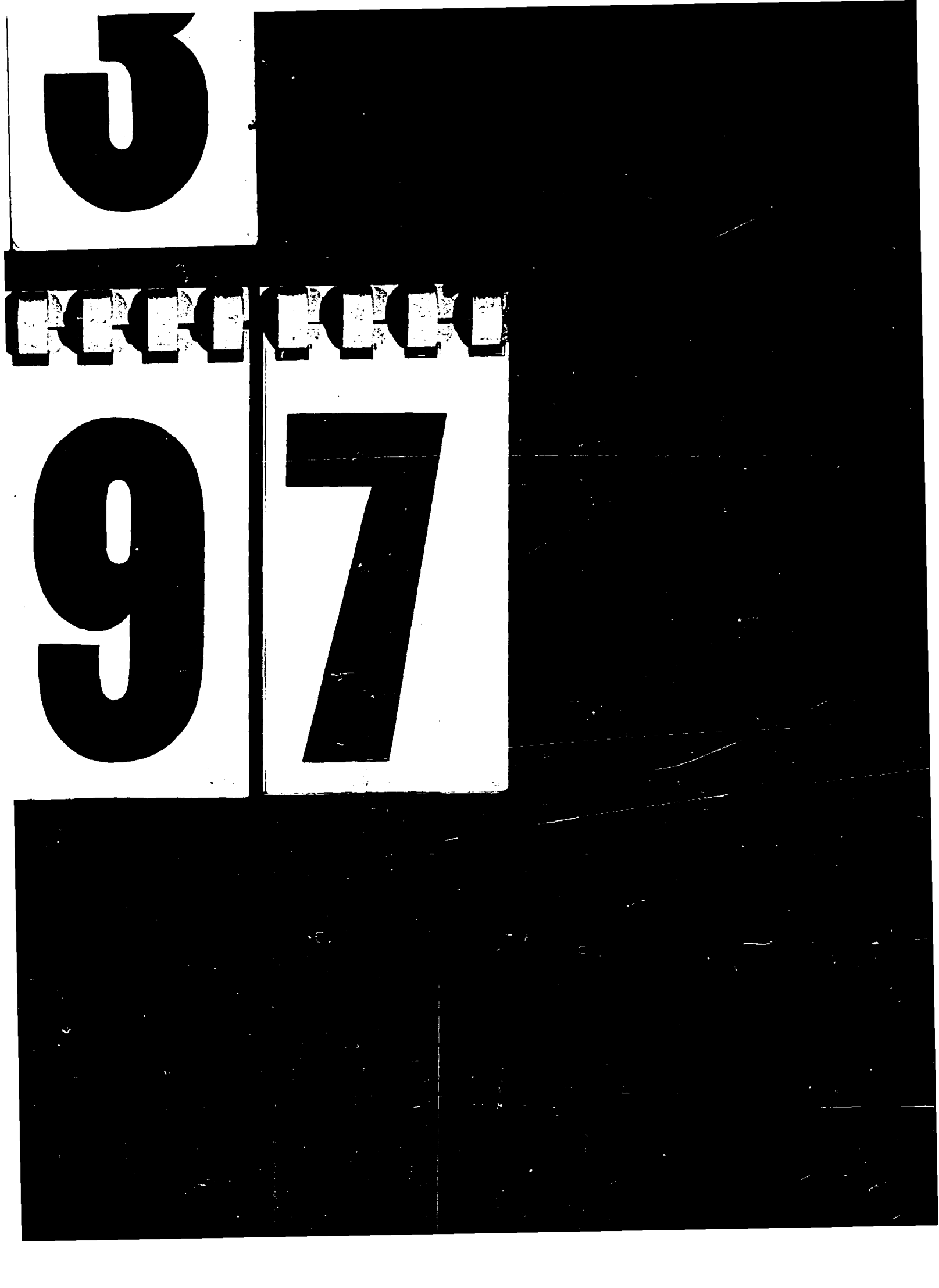
$$r = \frac{29918.9}{(54)(28.3)(20.4)} = 0.959$$

$$\text{Slope} = (0.959)\left(\frac{20.4}{28.3}\right) = 0.691$$

$$P^{32} - 65.1 = \left[0.959 \times \frac{20.4}{28.3}\right]\left[O_2 - 45.3\right]$$

$$= (0.619\ O_2 - 31.3)$$

$$P^{32} = 0.691\ O_2 + 21.8$$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