THE RELATIONSHIP BETWEEN SUBSTRATE CONCENTRATION, RESPIRATION RATE, AND GROWTH RATE OF ESCHERICHIA COLI IN CONTINUOUS FLOW CULTURE

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ABSTRACT

THE RELATIONSHIP BETWEEN SUBSTRATE CONCENTRATION, RESPIRATION RATE, AND GROWTH RATE OF ESCHERICHIA COLI IN CONTINUOUS FLOW CULTURE

By Robert S. Lipe

A continuous flow unit was constructed and satisfactorily operated at dilution rates ranging from 0.01 to 0.85. Steady-state conditions existed between dilution rate values of D equal to 0.02 and 0.69. A non-steady-state condition existed at D values below 0.02 and above 0.69 which resulted in a wash-out of organisms from the unit at a rate faster than replacement by growth. The actual wash-out rate was found to be less than the wash-out rate predicted by theoretical equations.

A maximum growth rate of k_m of 0.69 was established. This value was obtained by both batch culture and continuous flow procedures. In addition k_m was calculated by the method of Reed and Theriault from a series of experimental data. The value of 0.69 obtained by this method was in full agreement with the experimentally obtained maximum growth rate.

The relationship between substrate concentration and the specific growth rate k during the steady-state was established, not as a linear relationship, but as a curve

that reaches a maximum asymptotically. Theoretical equations proposed by Mondo, Novick and Szilard, and Herbert et al. did not agree with the experimental relationship obtained between substrate concentration and k. Furthermore, values for k_m and S_a obtained by a Lineweaver-Burke plot did not agree with experimental data. In contrast the results obtained from the use of a monomolecular type of equation, such as proposed by Teissier, indicated that this type of equation more correctly expresses the relationship between substrate concentration and k. Data were presented which indicated that at a substrate concentration of 1 mg/l or below k approaches zero. The data also indicate that the specific growth rate k becomes independent of substrate concentration at glucose concentrations above 180 mg/l.

A linear relationship was established between k and the respiration rate of the organism. The maximum oxygen uptake rate was obtained at k_m and did not increase further at D values greater than k_m. The data indicate that under these conditions approximately 37 percent of the assimilated substrate was oxidized independently of k.

The economic coefficient of the organism was found, not to be constant, but to increase from 44 percent at the lower k values to 55 percent at the higher k values.

The theoretical steady-state equations, proposed by Monod and Herbert *et al*. were found to be inadequate in expressing the experimentally obtained relationships.

THE RELATIONSHIP BETWEEN SUBSTRATE CONCENTRATION, RESPIRATION RATE, AND GROWTH RATE OF ESCHERICHIA COLI IN CONTINUOUS FLOW CULTURE

By

Robert S. Lipe

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TABLE OF CONTENTS

LIST OF TABLES

Table | Page

LIST OF FIGURES

I. INTRODUCTION

In the past few years a great deal of interest has been stimulated in the possibility of conducting continuous flow operations. By definition, continuous flow entails the maintenance of constant operating conditions, including organism concentration, in one or a series of vessels while fixed volumes of nutrients and continuous flow reactor contents are respectively added and removed at the same continuous rate.

From an industrial standpoint, prolonging the useful time of fermentation such a system would result in fewer unproductive periods, more uniform products, and reduced labor costs. A continuous production would also be more adaptable to instrumental control, and the effect obtained by varying experimental conditions could be evaluated more readily and accurately. In addition the yield might be increased by better control of pH and other substrate variables.

On the other hand, continuous flow operation involves certain disadvantages, such as complexity, relatively high initial cost and the need for stringent control. In addition, the benefits of continuous operation might easily be offset by mechanical failure, contamination, or selective growth of undesirable strains. However, it is possible that proper design of equipment might minimize mechanical failures and

sources of contamination, and that the selective growth of undesirable strains might be prevented by nutrient adjustment to favor the growth of the parental type. Moreover, the problem of strain degeneration might turn out to be of little importance, since the constant environment maintained by a continuous operation might tend to reduce the risk of degeneration that may occur, for example, during serial transfers between batch growth periods.

The essential feature of this technique is that microbial growth in a continuous culture takes place under steady-state conditions; that is, growth occurs at a constant rate and in a constant environment. Such factors as pH, concentrations of nutrients, metabolic products, and oxygen may be independently controlled by the experimenter. These features of the continuous culture technique make it a valuable research tool. In spite of this the technique has so far been comparatively little used. The reasons for this lack of use are, in general, twofold: first, the lack of a generally accepted theoretical background and second, the widespread belief that the technique is impracticable for industrial application.

The theoretical considerations, which are actually the basic factors that determine the relative advantages of continuous flow operation, have been studied in some detail

and are now sufficiently well advanced to provide a good foundation for experimental studies.

The foregoing considerations led to the design and assembly of a small laboratory continuous flow unit; the main purpose of which was to provide experimental data to test mathematical equations which have been developed to explain the kinetics of continuous flow operation. Of particular interest to this work was the relationship between substrate concentration inside the continuous flow unit and the specific growth rate k. Very little data are to be found in the literature on this relationship, especially at the very low k values. There is also a need for data concerning the relationship between k and respiration rate during the steady-state operation of a continuous flow unit.

II. LITERATURE REVIEW

A. Introduction

The continuous culture of microorganisms is a technique that is of increasing importance in microbiology both from the standpoint of its use as a research tool and its enormous potential in industrial application. Although it has been only in the past twenty years that this technique has been subjected to a more detailed investigation the technique itself has been used in limited areas for hundreds of years. The first continuous generators for vinegar production have reportedly been in operation since 1670 (Mitchell, 1926). Yeast has been produced using continuous culture techniques since about 1879 (Rainier, 1879) and sewage has been so treated since before 1890 (Massachusetts State Board of Health, 1890). In fact, these processes have been developed to such a high degree over the years that future developments will probably consist merely of advancements based on newer technology and improved equipment.

In other areas, such as the continuous propagation of fungi for producing antibiotic drugs, and the continuous propagation of algae for food and fodder, the possibilities are only just beginning to be realized. In the past ten years research emphasis has shifted to more difficult processes, which in turn involve greater susceptibility to contamination,

instability of the organisms, and biochemical complexity. The increasing urgency of the "population explosion" has led to the initiation of a number of projects for the continuous propagation of aglae and yeast for food (Brunol et al., 1950; Burlew, 1953; Tamiya, 1957).

B. Application of Continuous Culture Techniques as a Research Tool

The potential application of the continuous culture technique for research purposes is almost endless. It is fair to state that the limits of such application exist only in the imagination of the investigator. Continuous culture techniques of the single unit type, with some exceptions (Cekan, 1939; Dyr and Protiva, 1958; Malek , 1952a, b, 1955; Malek et al., 1953a, b, 1957, 1958; Protiva and Dyr, 1958), have become established in research laboratories as a means of studying microbial physiology and metabolism (Anderson, 1953, 1956; Andreev, 1955; Barnes and Dewey, 1947; Castor and Stier, 1947; Cekan, 1939; Cleary et al., 1935; Cohn and Torriani, 1953; Contois, 1959; Davies, 1956; Drobnica, 1959; DeHaan and Winkler, 1954a, b; Dyr and Protiva, 1958; Gorini, 1958; Harris-Smith et al., 1958; Heden et al., 1955a, b; Holme, 1957, 1958, 1959; Horodko et al., 1958; Hotchkiss, 1954;

Jacob, 1953; Jordon and Jacobs, 1944, 194 , 1948; Kalyuzhniyi, 1955, 1957; Kalyuzhniyi et al., 1955b; Kautsky and Kautsky, 1951; Kuska, 1958; Lark and Maaloe, 1954; Maaloe et al., 1958; Macura and Kotkova, 1953; Malek, 1950, 1952a, b, 1953, 1956, 1958; Malek et al., 1953a, b, 1957, 1958; Myers and Clark, 1944; Novick and Weiner, 1957; Ogburn et al., 1958; Oswald et al., 1953b; Perret, 1953; Pirt, 1957; Protiva and Dyr, 1958; Raper and Alexander, 1945; Rosenberger, 1958; Rosenberger and Kogut, 1958; Rotman, 1958; Savage and Florey, 1950; Schulze, 1956; Sevcik, 1952; Svachulova and Rushka, 1956; Utenkov, 1941, 1944; Vavra, 1958; Wean and Young, 1939; Yerusalimsky, 1958 a, b, c; Zubrzycki and Spaulding, 1957, 1958) and genetics (Bryson 1952, 1959; Cocito and Bryson, 1958; Cocito and Vogel, 1958; Fox and Szilard, 1955; Graziosi, 1959; Labrum, 1953; Lee, 1953; Malek, 1955; Moser, 1957a, b, 1958; Northrop, 1954; Northrop and Kknitz, 1957; Novick, 1955, 1958; Novick and Szilard, 1950a, 1951, 1952, 1953; Saenko, 1950; Scherbaum and Zeuthen, 1954; Verbina, 1955).

In 1924 Felton and Dougherty (1924) reported the continuous cultivation of Pneumococcus. Since that time this technique has been used in cultivating a number of organisms. Representative genera of the organisms that have been cultivated by continuous flow techniques are as follows:

Algae - Ankistrodesmus (Kautsky and Kautsky, 1951),

Chlorella (Myers, 1946; Myers and Clark, 1944; Myers and Johnston, 1949), Euglena (Logotkin, 1937; Vavra, 1958), Nitzchia (Ketchum and Redfield, 1938).

Bacteria - Aerobacter (Elsworth and Meakin, 1954; Herbert et al., 1956; Pirt, 1957; Pirt and Callow, 1958), Azotobacter (Macura and Kotkova, 1953; Malek, 1952a, b, 1956; Owen and Johnson, 1955; Rotman, 1956), Bacillus (Harris-Smith et al., 1958; Malek et al., 1953a; Monod, 1950; Northrop, 1954, 1957; Savage and Florey, 1950; Sevcik, 1952), Brevibacterium (Finn and Wilson, 1954), Brucella (Gerhardt, 1946; Sterne, 1958), Chlorobiom (Butlin, 1958), Chromatium (Butlin, 1958), Clostridium (Malek, 1955; Dyr and Protiva, 1958; Dyr et al., 1958; Protiva and Dyr, 1958; Yerusalimsky, 1958a, b, c), Corynebacterium (Savage and Florey, 1950), Desulfovibrio (Butlin, 1958; Maxon, 1955), Escherichia (Anderson, 1953, 1956; Clearly et al., 1935; Fox, 1955; Fox and Szilard, 1955; Gorini and Maas, 1957; Gorini, 1958; Holme, 1958, 1959; Jacobs, 1953; Jordan and Jacobs, 1944, 1947, 1948; Marcura and Malek, 1958; Malek, 1950; Malek et al., 1953b; Novick, 1958; Novick and Szilard, 1950b, 1951, 1952; Novick and Weiner, 1957; Rogers and Whittier, 1930; Rotman, 1955, 1956, 1958; Savage and Florey, 1950), Mycobacterium (Duche and Neu, 1950, Kuska, 1958; Savage and Florey, 1950; Svachulova and Rushka, 1956), Penumococcus (Felton and Dougherty, 1924; Hotchkiss, 1954;

Pirt and Callow, 1958), Propionibacterium (Jerusalimsky, 1958a, b, c), Proteus (Savage and Florey, 1950), Pseudomonas (Finn and Wilson, 1954; Rosenberg and Kogut, 1958), Salmonella (Formal et al., 1956; Horodko et al., 1958; Lark and Maaloe, 1954; Maaloe et al., 1958; Malek et al., 1953b), Serratia (Guenther, 1957; Smith, 1954), Shigella (Barnes and Dewey, 1947), Staphylococcus (Rogers, 1957), Streptococcus (Karush et al., 1956; Ogborn et al., 1958; Rogers and Whittier, 1930; Rosenberger, 1958).

Fungi - Actinomyces (Kolesnikova, 1959), Ophiostoma (Von Hofsten et al., 1953), Penicillium (Bartlett, 1958; Bartlett and Gerhardt, 1958; Gerhardt, 1959; Duche and Neu, 1950; Lewis and Lucas, 1945; Moor, 1945; Soltero and Johnson, 1954), Trichophyton (Duche and Neu, 1950, Schulze, 1951).

Protozoa - Tetrahymena (Scherbaum and Zeuthen, 1954; Vavra, 1958).

Yeast - Monilia (Malek et al., 1957), Rhodotorula (Kautsky and Kautsky, 1951), Saccharomyces (Beran, 1958; Davies, 1956; Finn and Wilson, 1954; Malek et al., 1958; Maxon and Johnson, 1953; Moor, 1945; Novick and Weiner, 1957; Plevako et al., 1958), Torula (Feustel and Humfeld, 1946), Zygosaccharomycetes (Harris and Hajny, 1959; Peterson et al., 1958).

In more recent years some very interesting applications of the principles of continuous culture have appeared. Zubrzycki

and Spaulding (1957) have used a modified chemostat to study norman human fecal microflora. The microflora composition was found under certain conditions to be analogous to that present in vivo. Macura and Malek (1958) used the continuous method to study microbiological processes in the soil. By their method nitrification and transformation of glucose in soil samples could be studied.

An especially interesting application of the principles of continuous culture has been in the area of continuous cultivation of animal cells. Bryson (1959) and Henderson (1959) point out that the utmost care must be taken in continuous cultivation of neoplastic cells as the most able cells are therein selected which need not be equivalent to the inoculum and the results are therefore of limited significance for normal biological systems. They also point out that cells in continuous culture can change very rapidly. Cooper et al., (1958) used continuous culture techniques to study the critical effect of oxygen tension on the growth rate of animal cells. Greig (1958) grew kidney cells continuously from bovine embryos for 45 passages without any changes in morphology. Epithelial tissue from tonsils was grown in a continuous culture by Evans (1957). The spontaneous occurrence of intranuclear inclusion bodies was studied in continuous cultures of renal cells by Larin (1958).

The possibility of applying continuous cultivation techniques to cream souring during the production of butter is suggested by Masek (1959) and Wilkovske and Fronte (1958).

In the field of waste water treatment Ware and Evans (1959) carried out experiments with the treatment of phenol waste waters in simple aerated vessels by continuous culture techniques. Yeast waste waters were treated by using sulphate-reducing bacteria in a continuous anaerobic fermentation on a technical scale by Barta and Gregr (1956). A similar process of sulphur fermentation of waste waters was described by Butlin et al., (1956), Butlin (1958) and Burgess et al., (1958).

The wide range of published papers is an indication that the continuous flow method is acquiring a more sound position in theoretical research wherever investigators begin to understand its value as a means for solving a number of problems of microbial biochemistry, physiology and genetics which could hardly be solved by any other method. It is still true, however, that the majority of papers in this field is concerned with the direct application of this method. It should be stated in this connection that in the practical application of the method success is ensured only by a sound knowledge of the theoretical basis of continuous culture and that the most valuable contribution of the last few years consists in the fact that the empirical approach to the solution

of practical problems popular in the past, has been abandoned.

Rogers and Whittier (1930), followed by Cleary et al., (1935), were the first to discover that the density of organisms grown under continuous culture conditions could be determined by the concentration of added nutrients. With this information it soon became apparent that, in most, if not all, of these cultures there existed actually only two different types of experimental conditions. Novick (1955) has referred to these types as being either internally or externally controlled.

The internally controlled continuous flow units may be defined as those in which the growth rate of the organism depends on the rate of some process occurring within the cell in the presence of an excess of nutrients. In such systems constant conditions are usually maintained by regulating the nutrient feed rate to maintain a given turbidity of the culture as measured by a photocell (Anderson, 1953, 1956; Bryson, 1952, 1953; Myers, 1946; Myers and Clark, 1944; Myers and Johnston, 1949). Northrop (1954) has described a continuous culture device that substitutes a commercial colorimeter for the photo-cell system. These techniques are very useful for studying the rate of formation of a product of bacterial metabolism and have also been adapted to large-scale processes. The devices that have been used for experimental work are basically similar and have been given various names

such as "Auxanometer" (Anderson, 1953, 1956), "Turbidostat" or "Turbidostatic selector" (Bryson, 1952, 1953) and "Breeder" (Fox, 1955; Fox and Szilard, 1955). Characteristic features of these various types of apparatus include the use of scrapers or glass beads to prevent growth on the sides of the culture tube (Anderson, 1956; Northrop, 1954, 1957, 1958; Northrop and Murphy, 1956), and the intermittent rather than continuous measurement of cell density (Fox, 1955, Fox and Szilard, 1955).

The externally controlled continuous flow units are those in which the growth rate is dependent on the concentration of some nutrient in the medium. Among the controlling nutrients that have been employed are a required amino acid such as tryptophan (Novick and Szilard, 1950b), arginine, proline and histidine (Novick and Szilard, 1954); an energy source such as lactate (Novick and Szilard, 1951), glucose (Monod, 1950), maltose (Cohon and Torriani, 1953), glycerol (Herbert et al., 1956); the nitrogen source such as ammonia (Novick and Szilard, 1950b); and phosphate (Novick and Szilard, 1951; Novick, 1955). This principle has been especially valuable in studying mutation rates and other problems in genetics. Monod's "Bactogen" (Monod, 1942, 1949, 1950) and Novick and Szilard's "Chemostat" (Novick and Szilard, 1950a, b) are two of the best known examples of equipment designed to provide external control. The "Bactogen" probably gives better aeration but the "Chemostat" is simpler to set up and operate, less

likely to become contaminated, and more accurately defined in volume. The "Chemostat" has become more widely used than the "Bactogen" and has undergone more modification (Browning and Lockinger, 1953; Duche and Neu, 1950; Fox and Szilard, 1955; Kubitschek, 1954; Novick and Szilard, 1950b; Rotman, 1955; Zubrzycki and Spalding, 1957); however, the "Bactogen" also has been applied and redesigned (Harrison, 1958; Heden et al., 1955a; Rogers, 1957; Von Hofsten et al., 1953).

It may be stated that, in general, the vast number of devices that have been reported are but modifications of the "Chemostat" and the "Bactogen." The basic equipment is represented by a continuous flow reactor vessel which is complemented by a specific apparatus for continuous cultivation, such as the continuous preparation and sterilization of media (Begma et al., 1956; Fremel, 1955; Fukimbara, 1956; Gallagher et al., 1942; Malchenko, 1947; Malchenko et al., 1947; Petkov, 1957; Pfeiffer and Vognovich, 1952; Stark et al., 1943; Watanabe, 1956), accurate dosing of medium volumes, an apparatus for keeping the volume constant and another for continuous treatment of the product. The apparatus has further been equipped by a device for continuous measurement and regulation of pH (Deindoerfer and Wilker, 1957; Nilsson, 1958), of the redox potential (Squires and Hosler, 1958), of dissolved oxygen (Sawyer et al., 1959), of turbidity and of gas composition

(Telling et al., 1958).

In the extensive literature dealing with equipment for continuous cultivation a marked development may be observed toward more perfect and frequently more complicated apparatus. The more complicated the device the more difficult it is to operate. This has been expressed very fittingly by Novick (Malek and Hospodka, 1960) who said: "In my country I had considerable difficulty because people began to use the apparatus without properly understanding it and published papers and made a bad name for the apparatus. The second kind of person (I do not like) is a man who published his paper describing a new continuous flow apparatus with which he does no experiments because the apparatus is so complicated that it requires five engineers in constant attendance to keep it in operation. These people are what we call in English 'gadgeteers' and I think that such people should be scolded."

C. Industrial Application of Continuous Culture

The numerous attempts at the practical application of continuous culture data back to 1670 (Mitchell, 1926), but have constantly encountered difficulties which may be ascribed to the empirical appraoch to the problems as well as to inadequate technical level. Only after a definite theory of homogeneous

continuous cultivations had been developed was it realized that each application requires a certain suitable continuous fermentation system. It is also important to realize that continuous cultivation processes require specifically designed apparatus of a new type, in which a great deal of our knowledge of microbiology and chemical engineering is taken into account. The most attractive feature of continuous cultivation in practical application is the possibility of a considerable increase in productivity as compared to the batch process (Herbert et al., 1956). The greatest drawback of the method is the high probability of contamination during long-term cultivations on an industrial scale. This problem will likely be solved by further technical development.

The many applications of continuous cultivation all have certain points of similarity by which they may be grouped together. DeBecze and Rosenblatt (1943) have suggested a classification according to basic design of the processes that have been used in industry. Three types of design may be recognized: single-stage, modified single-stage and multiple-stage.

The single-stage continuous fermentations may be defined as those in which the entire process is completed in one vessel, nutrients being added and cells and products being removed at the same rate. This type of apparatus is simple and has found

wide application, especially in the yeast industry. Before 1940 most of the information on its use in growing yeast, usually either Torula or Saccharomyces, was in patent records although a few references did appear in scientific and technical journals (Bilford et al., 1942; Illes, 1938a, b; Unger et al., 1942). Since the fall of Germany a number of reports were published on the significant advances made in that country during the war, principally on the use of single-stage Waldhof fermenters, with sulfite waste liquor as a starting raw material (Schulze, 1956). It has been reported (Saeman et al., 1945) that almost all of the sulfite liquor in Germany had been used to produce yeast or alcohol. Since that time a number of other papers have appeared (Harris et al., 1948a, b, c; Hidalgo Fernandeq-Cano and Cid, 1952a, b; Imhoff and Fair, 1956; Krauss and Thomas, 1954; Leopold and Fencl, 1955; Machenko, 1947; Negre, 1949; Nomura, 1956; Ruf et al., 1948; Shichiji, 1956).

Another large-scale, industrially important, single-stage continuous fermentation in operation at the present time is the trickling filter used for treating sewage (Babbitt, 1953; Halvorson et al., 1936; Imhoff and Fair, 1956; McCabe et al., 1956). This process is one in which sewage is applied to a bed of rocks, on which a mixed culture of microorganisms is maintained. Substances other than stone have also been used

as a support for the microorganisms (Schulze, 1957, 1960). The trickling filter was first used in this country in 1890 and is still the method of choice for municipalities with a population of less than 10,000 people.

Continuous fermentations of the single stage type have also been developed for the large scale production of algae, such as Chlorella, Euglena or Scenedesmus, using shallow troughs or tubes rather than the usual vertical fermenters, in order to give better illumination of the cultures (Bryson, 1952; Burlew, 1953; Cook, 1951; Krauss, 1955; Krauss and Thomas, 1954). Algae have also been used as a source of oxygen supply in the continuous treatment of sewage (Gotaas et al., 1954; Ludwig et al., 1951; Oswald et al., 1953a, b).

Other applications of single-stage continuous fermentation include the continuous propagation of an Aspergillus for use as a medium supplement (Ruf et al., 1948) and a lactic acid process, using Lactobacillus (Whittier and Rogers, 1931). This process was abandoned because side reactions could be more easily avoided in a batch process (Olive, 1949).

The modified single-stage process is generally used where a product other than the microorganisms themselves is desired. Many single-stage fermenters have been designed to permit the reuse of the cells by recirculation or by constructing the fermenters so that most of the cells are retained

in the reactor vessel. The activated sludge process, discovered in 1914 (Ardern and Lockett, 1914), is an example of this type (Babbitt, 1953; Imhoff and Fair, 1956; McCabe et al., 1956; Metcalf and Eddy, 1935). In this process, a biomass consisting of a mixed culture of microorganisms, is recirculated from the final settling tanks back to the aeration tanks to serve as a massive inoculum for the incoming sewage. Such treatment lowers the organic content of the sewage and there is some indication that it may be an economical way of reducing the sulfates, which are present, to sulfides, which would be of value in manufacturing sulfuric acid (Butlin, 1958; Butlin et al., 1956). Yeast processes might also be conducted in a similar way, with a protion of the yeast being continually recirculated (Kalyuzhniyi et al., 1955b).

Another example of the modified single-stage process is the vinegar generator (Allgeier et al., 1952, 1953, 1954) which reused the acetic acid-producing bacteria, except that in this case the Acetobacter organisms remain fixed to the packing, while the medium, partially acidified by recirculating some of the vinegar, flows continuously through the generator. This operation permits the re-use of the microorganisms at optimum conditions for vinegar formation rather than at optimum conditions for growth. The "Fesselhefe" method of making alcohol from sulfite waste liquor (DeBecze and Rosenblatt, 1943) as well as a few other systems (Powell, 1958a; Sarkov,

1950), uses a packed bed to which yeast is bound. A pulp suspension has also been described which is used for binding the yeast in much the same way as a packed bed (Andreev, 1955; Kalyuzhniyi, 1955, 1957). Northrop et al., (1919) described acetone-ethyl alcohol fermentations in which a Bacillus used as the fermenting organism was bound to a bed of wood shavings. A fungus product, itaconic acid, has been made in much the same way, with an intermittent feed replacing half the fermenter contents with fresh medium at the conclusion of each fermentation (Pfeiffer et al., 1952).

In addition there are a number of processes which, though based on the principles of the batch process, approach the continuous process in actual operation. The Boinot alcohol process (DeBecze and Rosenblatt, 1943) recovered all the yeast left after fermentation and used it to initiate a new alcoholic fermentation. Gluconic acid fermentations, in which the fungi removed from the beer by contrifuging or filtering are used to start a new fermentation, have also been described (Moyer et al., 1940; Porges et al., 1940, 1941).

A number of processes have been described in which the fermentation is initiated in one vessel and finished in others. For purposes of this discussion these processes have been grouped together under the heading of multiple-stage processes. This method generally results in either more

efficient use of the nutrient or better yield of product, by permitting different conditions in each of the fermenters. Many yeast fermentation processes have been reported. (Altsheler et al., 1947; Andreev and Bolondz, 1955; Asai et al., 1952; Ashkinuzi et al., 1953; Berenshtein, 1954; Cekan, 1939; DeBecze and Rosenblatt, 1943; Dyr et al., 1958; Ericcson, 1947; Gladhii, 1946; Harris et al., 1948a; International Yeast Co., Ltd., 1926; Invention and Daranyi, 1932; Kazumov, 1957; Keussler, 1943; Kuffner and Kuffner, 1933; Lebedev, 1936; Logotkin, 1937, 1939, Malchenko and Christyakov, 1949; Malek et al., 1957, 1953b; Olsen, 1927; Roseira de Mattos, 1951; Savchenko, 1957; Smidrkal and Nejedley, 1956; Van Riju et al., 1906; Yarovenko et al., 1957; Zak and Dedkov, 1957), which are designed to produce either yeast, alcohol, or glycerol, and may use as many as eight fermenters in series. In many of the small and medium-sized European distilleries, this system is reported to be quite popular (DeBecze and Rosenblatt, 1943).

A complex study of the continuous fermentation of starch raw materials to alcohol was taken up by Yarovenko et al., (1957). In this case the situation was complicated by the subsequent hyrolysis of dextrins to sugars during fermentation. A Japanese worker (Ueda, 1956) described the conditions of continuous fermentation of sugar and starch substrates from a theoretical and practical point of view. The optimum conditions

for yeast growth in a multi-stage system of tanks according to the utilization of nitrogen were discussed by Konovalov (1959). The problem of utilization of poorly assimilable sugars and of adaptation to these sugars and toxic substances in sulphite liquors was studied by Fencl and Burger (1958).

Other papers dealing with continuous multi-stage fermentation describe a system for the production of baker's yeast (Rost, 1957); the pilot-plant production of glycerol by using Zygosaccharomyces (Harris and Hajny, 1959); the culture of Saccharomyces rouxii (Dawson, 1959); the production of fruit wines (Krawczyk, 1958); the continuous production of acetone-butanol (Yerusalimsky, 1958a, b, c); the long-term vegetative transfer and continuous culture of Clostridium acetobutylicum (Dyr and Protiva, 1958; Dyr et al., 1958) and the production of 2,3-butylenglycol by bacteria (Pirt and Callow, 1958). Beer and yeast yield was studied in relation to the rate of dilution, temperature and number of fermenters by Hough and Rudin (1958).

The continuous production of antibiotics is potentially the single most important application of continuous culture techniques. Although a number of commercial organizations are known to have carried steady-state fermentations for antibiotics production through varying degrees of development, the published literature shows very little indication of the progress in

this area. In 1950 and 1951 patents were awarded to the Distillers Co., Ltd. for single-stage continuous fermentation processes to be used in making streptomycin (Distillers Co., Ltd., 1951, 1953). A fermenter with a 30 liter operating capacity has also been described (Kroll, et al., 1956) which was used for continuous fermentation of unspecified antibiotics. Subtilin has been made by a modified single-stage fermentation where a small portion of the preceding run is left in the fermenter as inoculum for the next run (Garibaldi, 1949). With the exception of these brief references, penicillin and chloramphenicol are the only antibiotics which the literature indicates as being produced by continuous fermentation (Abrahm, 1941; Clifton, 1943; Ehrlich et al., 1948; Gerhardt, 1959; Lewis and Lucas, 1945; Moor, 1945; Stice and Pratt, 1946).

III. THEORETICAL CONSIDERATIONS

The various types of processes used for the continuous cultivation of microorganisms described in Section II of this dissertation are governed by certain mathematical principles which are common to all. Monod (1942, 1949, 1950) was the first to develop in a satisfactory manner the fundamental equations which define the behavior of a continuous culture. These fundamental concepts were later elaborated upon by Novick and Szilard (1958, 1954, 1951). Since the appearance of these papers a number of other authors (Contois, 1959; Drobnica, 1959; Elsworth et al., 1957; Herbert, 1958a, b, 1959a; Herbert et al., 1956; Ludwig et al., 1951; Luedeking and Piret, 1958a, b; Maaloe et al., 1958; Maxon, 1955; Moser, 1957a, 1958; Northam, 1958; Pasynskii, 1957; Powell, 1954, 1955, 1956, 1958; Rosenberger, 1958; Spicer, 1955; Ueda, 1956; Yerusalimsky, 1958a, b, c, 1959a, b) have published further information concerning the theory of continuous cultivation of microorganisms.

The theory of continuous culture, as well as the application of the method, has been the subject of several reviews (Bartlett, 1958; Herbert, 1958b; Malek and Hospodka, 1960; Maxon, 1955; Monod, 1950; Novick, 1955; Serfontein and Weyland, 1959). Malek and Hospodka (1960) list a number of symposia (Beran, 1958; Tunevall, 1959; Yerusalimsky, 1959a;

Symposia, 1954, 1958a, b, 1959) concerning the theory of continuous cultivation of microorganisms.

Continuous flow systems generally consist of a reactor into which nutrients flow at a steady rate and from which products emerge. The factors governing the operation of such a continuous flow system are (1) the way in which materials pass through the reactor and (2) the kinetics of the reaction taking place in the reactor. As Danckwerts (1954) pointed out the first may be characterized by the distribution of residence-times of molecules or minute particles passing through the system.

In general two types of reactors are listed in the literature. These are the completely-mixed tank and the ideal tubular type with piston flow and no mixing. In the ideal piston flow type all of the particles have the same residence-time, equal to the mean residence-time while complete mixing generally produces a wide spread of residence-times about the mean. The piston flow reactor will be the more efficient for chemical reactions whose rates fall off as the reaction proceeds, but the completely-mixed reactor will be more efficient for reactions of the "autocatalytic" type whose rates increase with time (Danckwerts, 1954). Since bacterial growth is an auto-catalytic process, the completely-mixed reactor should be the most efficient type for continuous bacterial culture

(Herbert et al., 1956) and will be the only type considered in this discussion.

The reactor used in the experiments described later in this thesis consisted of a one or three liter culture vessel in which the organism could be grown under controlled conditions. Sterile growth medium was fed into the vessel at a controlled flow rate and culture emerged from it at the same rate. The volume of the liquid in the reactor remained constant. The contents of the reactor were sufficiently well stirred, via the aeration system, to approximate the ideal of complete mixing and the entering growth medium was instantaneously and uniformly dispersed throughout the vessel.

The assumption of "perfect mixing"--of instantaneous and homogeneous dispersal of the ingoing medium--greatly simplifies the theoretical analysis of continuous cultures. It is obvious that for purposes of study, at least, the degree of mixing should be well defined in some sense, but it is not at all obvious that an adequate approximation to "perfect mixing" is practically attainable (Powell, 1956). A continuous culture is particularly sensitive to lack of homogeneity, because of the steep fall in output near the critical dilution rate. Moreover, the growth rate is not strictly equal to the dilution rate unless the mixing is perfect.

Average residence-times in a vessle, such as used in

these experiments, will be determined not by the absolute values of the flow-rate and culture volume but by their ratio which may be called the dilution rate, D, defined as the number of complete volume-changes per unit time. Expressed mathematically D is equal to f/v where f is the flow rate into the culture vessel and v is the volume of the vessel. The mean residence-time of a particle in the culture vessel is equal to 1/D (Herbert et al., 1956).

Herbert et al., (1956) developed equations which define the behavior of a continuous culture. These equations are essentially identical to those developed by Monod (1942, 1949, 1950) although their derivation is different and somewhat simpler than Monods. The derivation of the fundamental mathematical relationships of continuous culture presented here follows closely that given by Herbert et al., (1956). The symbols used in this derivation are as follows:

x = cell concentration, mg cell dry wt/liter,

$\bar{x}$ = steady-state value of x,

x_o = beginning cell concentration,

Δx = increase in cell concentration,

t = time, hours,

$\bar{t}$ = mean residence time, hours,

t_d = doubling time, i.e., the time required for the concentration of organisms to double, hours,

Δt = change in time,

s = substrate concentration inside the reactor, mg/l,

$\bar{s}$ = steady-state value of s,

s_R = concentration of substrate entering reactor, mg/l,

S_a = constant equal to the substrate concentration at which $k = 1/2\ k_m$,

D = dilution rate = f/v = number of complete volume changes/hour,

D_o = critical value of the dilution rate D above which complete "wash out" occurs,

f = rate of flow of feed solution into reactor, ml/hr,

v = volume of reactor, ml,

Y = yield constant which is equal to weight of bacteria formed/weight of substrate used,

k = growth constant or the specific growth rate = rate of increase/unit of organism concentration which is equal to $1/x \cdot dx/dt$,

k_m = maximum rate of growth = the maximum value of k at saturation levels of substrate.

A. Kinetics of Bacterial Growth

1. Derivation of the specific growth rate k--The specific growth rate k may be derived starting with the familiar exponential

growth formula

$$x = x_o e^{kt} \tag{1}$$

upon differentiation equation (1) beomes

$$\frac{\Delta x}{x} = k\Delta t \tag{2}$$

equation (2) may be expressed as

$$\frac{dx}{x} = kdt \tag{3}$$

solving equation (3) for k

$$k = \frac{dx}{dtx} = \frac{1}{x} \frac{dx}{dt} \tag{4}$$

The expression $1/x \cdot dx/dt$ is termed the specific growth rate and defines the rate of increase in cell concentration per unit of cell concentration per unit time. The actual rate of increase of concentration of organisms, dx/dt, is sometimes also called the growth rate but is obviously not a constant. It has also been shown (Herbert et al., 1956) that the expression $1/x \cdot dx/dt$ is equal to $\log_e 2/td$ where t_d represents the doubling time, i.e., the time required for the concentration of organisms to double. k then may be expressed as

$$k = \frac{1}{x} \frac{dx}{dt} = \frac{\log_e 2}{t_d} \tag{5}$$

In equation (5) k and t_d are generally assumed to be constants. It should be pointed out that this assumption is correct only when all substrates necessary for growth are present in excess (Herbert et al., 1956).

2. Relationship between the specific growth rate and the concentration of an essential growth substrate--Monod (1942) was the first to show the relationship between the specific growth rate, k, and the concentration of an essential growth substrate. k is nearly proportional to the substrate concentration when the substrate concentration is low but reaches a maximum value at high substrate concentrations. To formulate these conditions mathematically Monod proposed the following equation:

$$k = k_m \left(\frac{s}{S_{a+s}}\right). \tag{6}$$

It follows from this equation that exponential growth can occur at specific growth rates having any value between zero and k_m, provided that the substrate concentration can be held at the appropriate value (Monod, 1950; Novick and Szilard, 1950).

Monod (1942) was also the first to show that there is a simple relationship between growth and utilization of substrate. This may be demonstrated in a growth media containing a single organic substrate; under these conditions the growth rate is a constant fraction, Y, of the substrate utilization rate:

$$\frac{dx}{dt} = -Y \frac{ds}{dt} \tag{7}$$

where Y is known as the yield constant. Thus over any finite

period of growth

$$Y = \frac{\text{weight of bacteria formed}}{\text{weight of substrate used}}. \qquad (8)$$

If the values of the three constants k_m, S_a, and Y are known equations (5), (6), and (7) provide a complete quantitative description of the growth cycle of a batch culture (Monod, 1942). These same equations and constants are equally applicable to the theoretical treatment of continuous culture.

B. Kinetics of Growth in Continuous Culture

In the development of a theoretical basis for growth in a continuous culture certain assumptions are usually made. It is generally assumed that the bacteria are growing under complete mixing; that the inflowing medium contains a single organic substrate, such as glucose, at concentration S_R; that all other substrates are present in excess; and that the culture vessel is so efficiently aerated that the oxygen supply is always adequate. Under these conditions the supply of organic substrate is the sole growth-limiting factor. The variables within the control of the experimenter are then the substrate concentration and flow rate of the incoming culture medium. A complete theory must describe how variation of these factors affects the growth rate and concentrations of organisms and of substrate in the growth vessel.

1. Expression of the wash-out rate--If one makes the assumption that the bacteria in the reactor are not growing or dividing then every organism in the vessel having a residence-time greater than t is e^{-Dt}. The wash-out rate (the rate at which organisms initially present in the vessel would be washed out if growth ceased but flow continued) may be expressed as

$$-\frac{dx}{dt} = Dx \qquad (9)$$

2. Changes in concentration of organisms--The organisms in a continuous culture vessel are growing at a rate described by equation (5) and at the same time are being washed out of the vessel at a rate determined by equation (9). The net rate of increase of concentration of organisms is given by the simple balance equation (the individual terms refer to rates in each case):

$$\text{increase} = \text{growth} - \text{output}$$

or

$$\frac{dx}{dt} = kx - Dx \qquad (10)$$

It may be seen from equation (10) that if k is greater than D, dx/dt is positive and the concentration of organisms will increase, while if D is greater than k, dx/dt is negative and the concentration of organisms will decrease, eventually to zero. When k equals D, dx/dt equals zero and x is constant then a steady-state exists in which the concentration of organisms

does not change with time. Under these steady-state conditions, the specific growth rate, k, of the organisms in the culture vessel is exactly equal to the dilution rate D. During the steady-state condition k equals D.

3. Changes in substrate concentration--In the continuous culture vessel, substrate is entering at a concentration s_R, being consumed by the organisms and flowing out at a concentration s. The net rate of change of substrate concentration is obtained from the balance equation (the individual terms refer to rates);

$$\text{increase} = \text{input} - \text{output} - \text{consumption}$$

or

$$\text{increase} = \text{input} - \text{output} - \frac{\text{growth}}{\text{yield constant}}$$

or

$$\frac{ds}{dt} = Ds_R - Ds - \frac{kx}{Y} \qquad (11)$$

4. Fundamental equations of continuous culture--Equations (10) and (11) both contain k, which is itself a function of s (see equation 6). By substituting (6) in equations (10) and (11) the following equations are obtained:

from (10)

$$\frac{dx}{dt} = kx - Dx$$

or

$$\frac{dx}{dt} = x\left(km \frac{s}{S_a + s}\right) - Dx \qquad (12)$$

and from (11)

$$\frac{ds}{dt} = Ds_R - Ds - \frac{kx}{Y}$$

or

$$\frac{ds}{dt} = Ds_R - Ds - \frac{x\left[km\left(\frac{s}{s_a+s}\right)\right]}{Y}$$

or

$$\frac{ds}{dt} = D(s_R - s) - \frac{k_m x}{Y}\left(\frac{s}{S_a+s}\right) \qquad (13)$$

Equations (12) and (13) define completely the behavior of a continuous culture in which the fundamental growth relations are given by equations (5), (6) and (7) (Herbert et al., 1956). These equations are virtually identical to the equations developed by Monod (1950), although their derivation is different from and much simpler than Monods.

5. The steady-state system--Considering equations (12) and (13) it is apparent that if s_R and D are held at constant values and D does not exceed a certain critical value D_o then steady-state values exist for x and s for which both dx/dt and ds/dt are zero. Solving (12) and (13) for dx/dt and ds/dt equal to zero these steady-state values of x and s may be given as

$$\bar{s} = S_a\left(\frac{D}{k_m - D}\right) \qquad (14)$$

and

$$\bar{x} = Y(s_R - \bar{s}) = Y\left[s_R - S_a\left(\frac{D}{k_m - D}\right)\right] \qquad (15)$$

From these equations the steady-state concentrations of bacteria and substrate in the culture vessel can be predicted for any value of the dilution rate and concentration of inflowing substrate, provided the values of the growth constants k_m, S_a, and Y are known. These equations were also first derived by Monod (1950).

The equations describe accurately the situation existing once a steady-state has been established but there is no evidence that starting from non-steady state conditions, a steady-state must inevitably be reached. The proof of this was provided by Powell (1956) who has shown that, starting from any initial values of x and s, the system inevitably adjusts itself to the steady-state defined by equations (14) and (15), and that this is the only stable state of the system. For example, consider a system which has just been inoculated, when x is very small, s is nearly equal to s_R and k is greater than D. The concentration of organisms will increase but owing to the resulting fall in substrate concentration the specific growth rate will decrease, until eventually k becomes equal to D. At this point the combined rates of substrate consumption and loss just balance the rate of substrate addition and the system shows no further tendency to change. The system is stable in the sense that small accidental fluctuations from the steady-state values will set up opposing reactions which

will restore the status quo. It is this automatic self-adjusting property of the system that makes continuous culture such a valuable research tool.

As already stated, in the steady-state the specific growth rate is equal to the dilution rate

$$k = \frac{\log_e 2}{t_d} = k_m \left(\frac{\bar{s}}{S_a + \bar{s}}\right) = D \qquad (16)$$

The doubling time t_d is therefore equal to 0.693/D; i.e., if one volume per hour is flowing through the culture vessel, the mass of organisms will be doubling every 42 minutes.

It is evident from equations (14) and (15) that the steady-state values of the concentrations of organisms and substrate depend solely on the values of s_R and D (since k_m, S_a, and Y are constant for a given organism and growth medium). By varying s_R and D an infinite number of steady-states can be obtained.

A somewhat different mathematical approach than the one just given to the fundamental concepts of continuous culture has been presented by at least three authors (Finn and Wilson, 1954; Golle, 1953; Northrop, 1954). Their tehoretical treatments are quite different from that of Herbert et al., (1956) and very little experimental data are presented in support of their theoretical conclusions.

C. Effect of Contamination

The possibility of contamination is one of the major objections generally raised (Warner et al., 1954a, b) to the use of continuous flow techniques on a large scale. This objection has been answered by Dawson and Pirt (1954) as based solely on conjecture. It will have to be admitted however that contamination is potentially a serious problem in continuous culture work since the long operating periods make them particularly liable to the occasional introduction of undesirable organisms. Mathematical equations (Golle, 1953; Bartlett, 1958) have been developed which show that the mere entry of a foreign organism into a continuous culture does not necessarily mean that the process will fail.

Assume that a continuous culture unit has been contaminated by a foreign organism. There are three possibilities with respect to the growth rate of the foreign organisms in the contaminated unit. Their growth rate can be greater than, equal to, or less than the dilution rate. If the growth rate is greater than the dilution rate then the concentration of the contaminant will increase exponentially with time until the steady-state concentration of the limiting substrate is reduced to the point where the growth rate equals the dilution rate. Under these conditions, the growth rate of the original

organism will be less than the dilution rate and its concentration decrease to zero exponentially. An infection by an organism with this type of growth rate will cause a complete failure of the process.

If, on the other hand, the growth rate of the contaminant is less than the dilution rate then its concentration will approach a limit under the point where the growth rate equals the dilution rate. A contamination by such an organism will become serious only if its rate of entry is extremely high and its growth rate only slightly less or equal to that of the desired organism.

D. Effect of Mutation

When mutation occurs in a continuous culture unit in operation it is governed by the same principles that have been applied in the discussion of contamination. By the same line of reasoning that was applied in the treatment of contamination, if the growth rate of the mutant, is greater than the dilution rate, the entire culture will be replaced by the mutant form. If the growth rate of the mutant is equal to the dilution rate, the concentration of the mutant will increase linearly and if the growth rate of the mutant is less than the dilution rate, the concentration of the mutant

will approach a limit under the point where the growth rate equals the dilution rate.

Two general conclusions may be drawn with respect to mutation. Regardless of the growth rate of the mutant, the continuous culture unit cannot be entirely free of variant organisms, and if the growth rate of the mutant is greater than the dilution rate a steadily increasing number of mutants will be encountered. This was used to advantage by Novick and Szilard (1950, 1951) to study mutation rates and to obtain large populations of a desirable mutant.

In the case where the growth rate of the mutant equals the dilution rate, the concentration of the mutant would probably increase very slowly. However, if selective growth in favor of an undesired type should occur in this case the continuous culture unit would have to be stopped. The only condition that will permit uninterrupted operation requires that the growth rate constant of the undesired variant be less than that of the parent organism.

IV. EXPERIMENTAL APPARATUS, CULTURE AND METHODS

A. Description of Apparatus

An experimental apparatus was developed which consisted of a continuous flow reaction chamber in which a constant volume of a growing pure culture of bacteria was maintained. Techniques were developed for the continuous feeding of medium to this culture, continuous aeration of the culture, sampling of the culture and continuous discharge of the culture from the reaction chamber at a constant rate. This apparatus is shown in Figure 1.

1. Reaction Chamber--Due to the wide range of k values investigated it was necessary to use two separate reaction chambers. In one of these a constant culture volume of one liter was maintained and in the other a constant culture volume of three liters was maintained. The three liter reaction chamber was used for the very small k values while the one liter reaction chamber was used for the higher k values.

The reaction chambers were identical except for the position of the discharge opening. The discharge opening was positioned so that the contents of the chamber would flow out when the volume reached one liter in one reaction chamber and

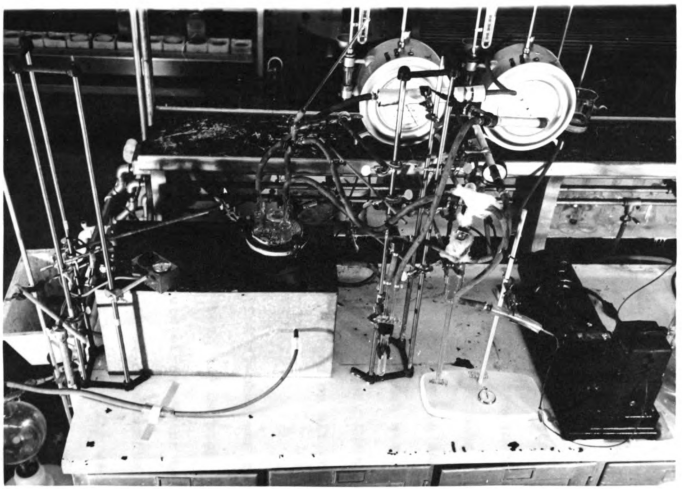

Figure 1. Continuous flow apparatus.

chamber and three liters in the other. The one liter reaction chamber is shown in Figure 2.

Each reaction chamber consisted of a pyrex glass container 12.5 cm wide and 32 cm deep. The top of the reaction chamber was also of pyrex glass with three 2.5 cm openings positioned around a 3.5 cm center opening. The top was fastened to the reaction chamber by means of a metal ring with three spring clamps. In order to prevent contamination at the union between the top and the reaction chamber Lubriseal (Arthur H. Thomas Co.) was placed between the two and the union was in turn wrapped tightly with one inch masking tape. This arrangement proved to be very satisfactory throughout these experiments. Lubriseal was also used on all the glass fittings and stopcocks of this apparatus.

The temperature of the reaction chamber was regulated by immersing the chamber in a water bath (30 cm high, 30 cm deep and 60 cm long). The temperature of the water bath was maintained at 30C $\pm$ 0.5C throughout these experiments by means of a Fenwal thermoregulator and heater. The water in the bath was kept in constant agitation by means of a metal stirring rod attached to a small motor.

2. System for inoculation and sampling of the reaction chamber--A schematic diagram of the system used for sampling and inoculation of the reaction chamber is shown in Figure 3.

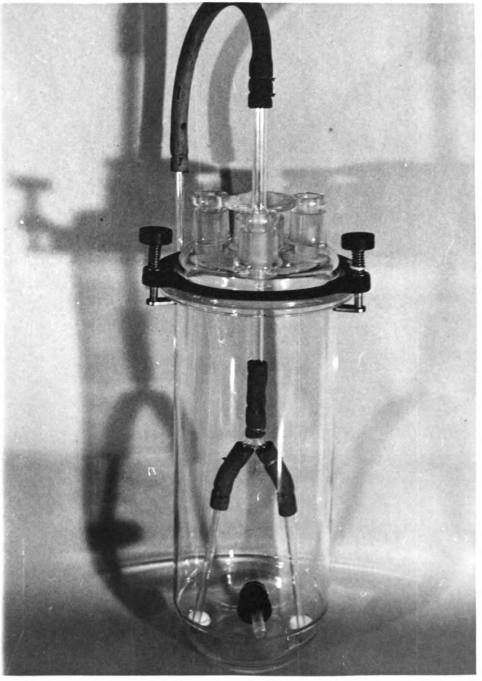

Figure 2. One Liter Reaction Chamber.

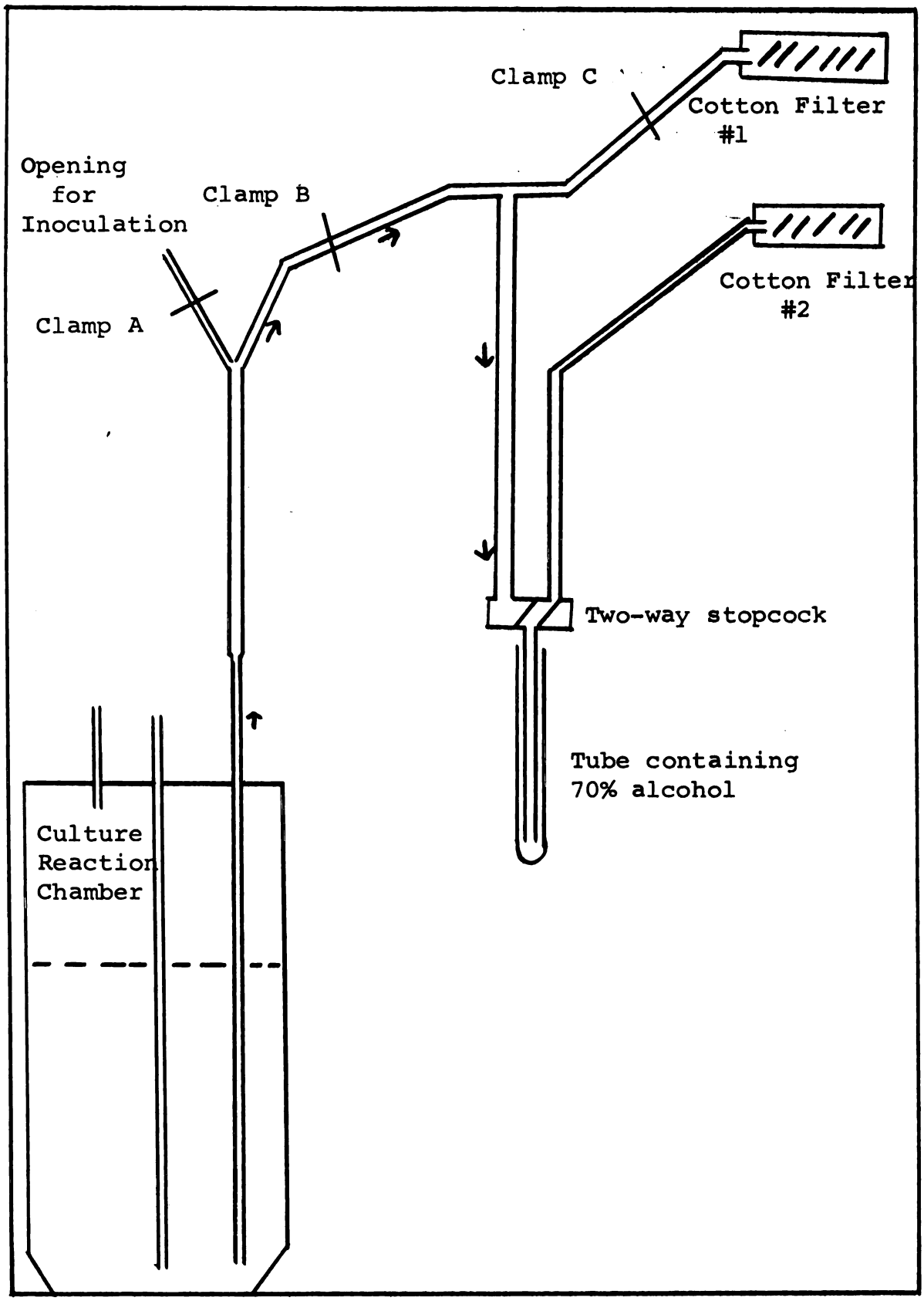

Figure 3. System for Sampling and Inoculation.

The procedure used for inoculation was as follows: A pipette containing five ml of a 48 hour culture of E. coli was inserted in the opening for inoculation in the system. Clamp B was closed and Clamp A was opened. Clamps B and C were then opened and the inoculum entered the reaction chamber. Clamp A was then closed for the remainder of the experiment.

The tubing used in this sytem, as with the aeration and pumping systems, was a thick walled rubber tubing (I D - 0.40 cm, O D - 1.50 cm). An attempt was made to use plastic tubing for the connections in this apparatus but this proved to be very unsuccessful due to the fact that the tubing would expand during autoclaving and would not contract upon cooling. Numerous attempts to run the unit with plastic tubing failed due to contamination at the union of the plastic tubing with the glass tubing. The use of thick walled rubber tubing, along with metal screw type clamps at the union of the rubber tubing with the glass tubing, was found to be very satisfactory in the prevention of contamination.

3. System for the sampling of culture in the reaction chamber--Referring again to Figure 3, the technique for the removal of samples for analytical purposes from the reaction chamber was as follows: The tube containing 70 percent ethyl alcohol (this tube was used to prevent contamination while the sampling system was not in use) was removed. The two-way

stopcock was then adjusted so as to open the system containing cotton filter II. This allowed the alcohol to drain from the end of the sampling system. Clamp C was then closed and Clamp B was opened. The two-way stopcock was adjusted so as to allow liquid to flow from the reaction chamber. Suction was then applied to the end of the sampling tube and an appropriate amount (usually 50 ml) of sample was drawn into a flask.

After the desired amount of sample was taken the two-way stopcock was closed. Clamp C was then opened thus allowing the sample in the sampling system between the reaction chamber and the T to re-enter the reaction chamber. Clamp B was then closed again sealing off the reaction chamber. The two-way stopcock was again adjusted allowing the rest of the sample in the sampling system to drain into the sample flask. The two-way stopcock was closed and the tube containing 70 percent alcohol was then placed over the end of the sampling system. The two-way stopcock was then adjusted so as to open the system containing cotton Filter II. This allowed the alcohol to fill the end of the sampling tube from the two-way stopcock down.

The cotton filters used in this system consisted of glass tubes 25 cm long and 1.5 cm in diameter which were packed with absorbent cotton. This size filter proved to be very

satisfactory throughout the experiments. These filters were removed every two or three weeks and new filters put in their place.

4. System for the aeration of the culture in the reaction chamber--A schematic diagram of the system used for the aeration of the culture in the reaction chamber is given in Figure 4. Due to the fact that the pressure of the laboratory air supply was extremely variable (between 80 and 120 psi) a system was developed to insure a steady and reproducible air supply. The air from the compressed air line was passed first through a pressure reducing valve which reduced the air pressure from 80 - 120 psi to 1 - 15 psi. From the pressure reducing valve the air passed through a pressure gauge and then through a needle valve. The air flow was controlled by setting the needle valve at a constant position and adjusting the pressure reducing valve to the desired pressure. As long as the needle valve setting remained constant a given reading on the pressure gauge would always give the same rate of air flow. This system was found to be very reliable and varied from day to day by only a very small amount.

Next the air passed through a Wet test meter (Precision Scientific Co.). Daily readings from this meter gave information on the rate of air flow to the reaction chamber. The Wet test meter was followed by a large sterile cotton filter

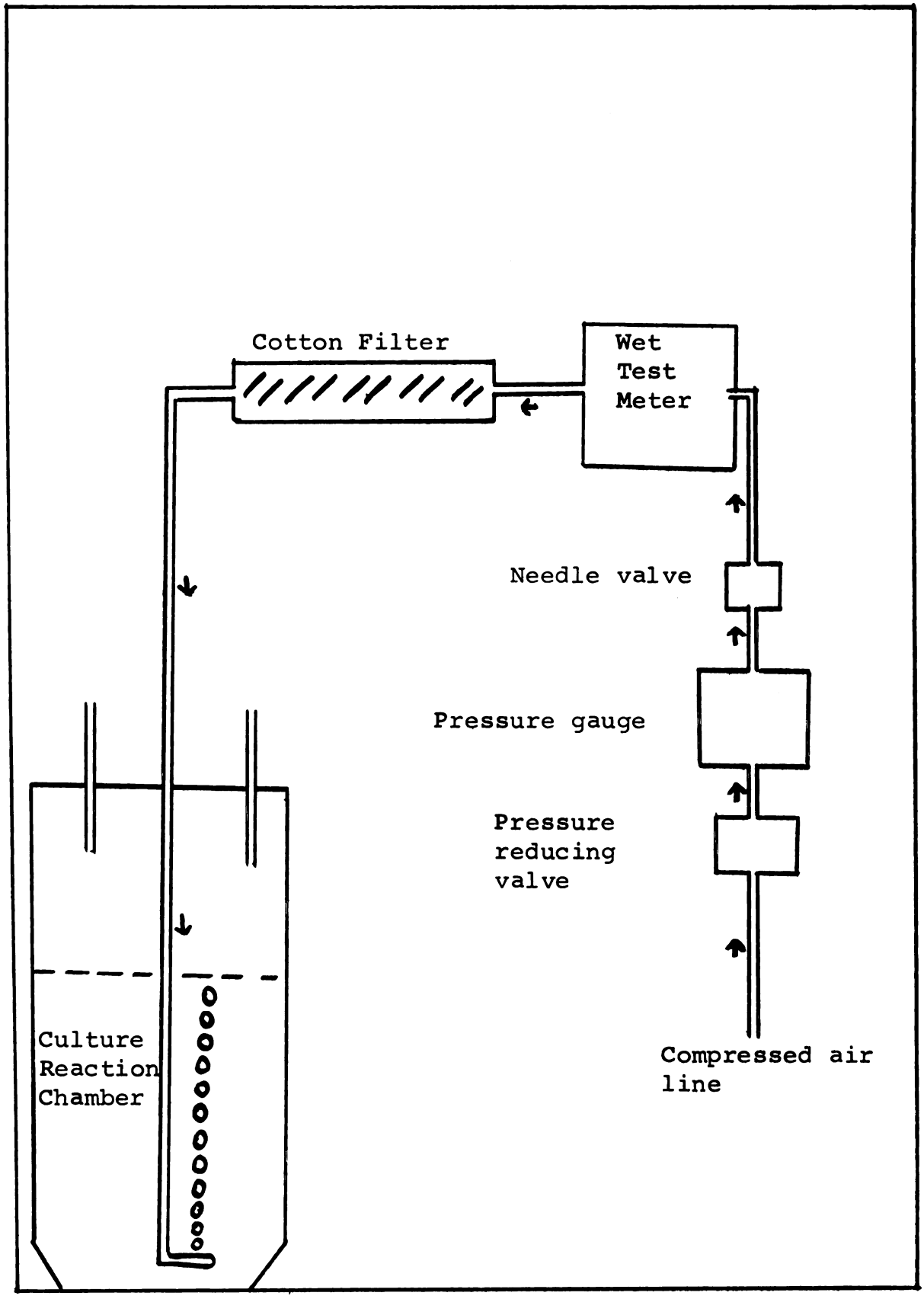

Figure 4. System for Aeration of Culture in Reaction Chamber.

(26 cm long and 3.5 cm in diameter). This filter was replaced at least once a week by another sterile filter of the same size.

The sterile air then passed into the reaction chamber and was dispersed into small bubbles by fritted glass dispersion tubes. The three liter reaction chamber contained one large fritted glass dispersion tube while the one liter chamber contained two smaller glass dispersion tubes. Besides supplying the needed oxygen to the culture this air supply also served another purpose. The rate of air passing through the unit was always high enough to keep the culture in a state of constant agitation. This helped to keep the culture in a uniform state and to prevent the bacterial cells from settling to the bottom of the reaction changer and as a mixing device to thoroughly mix the incoming substrate supply with that already present in the reaction chamber.

The air left the reaction chamber by way of the substrate overflow line. This constant flow of air out the substrate overflow line helped to prevent a back-up of contamination into the reaction chamber through this line.

5. System for providing the reaction chamber with a constant supply of substrate--A schematic diagram of the system used for the constant feeding of substrate to the reaction chamber is shown in Figure 5. The substrate used in these experiments

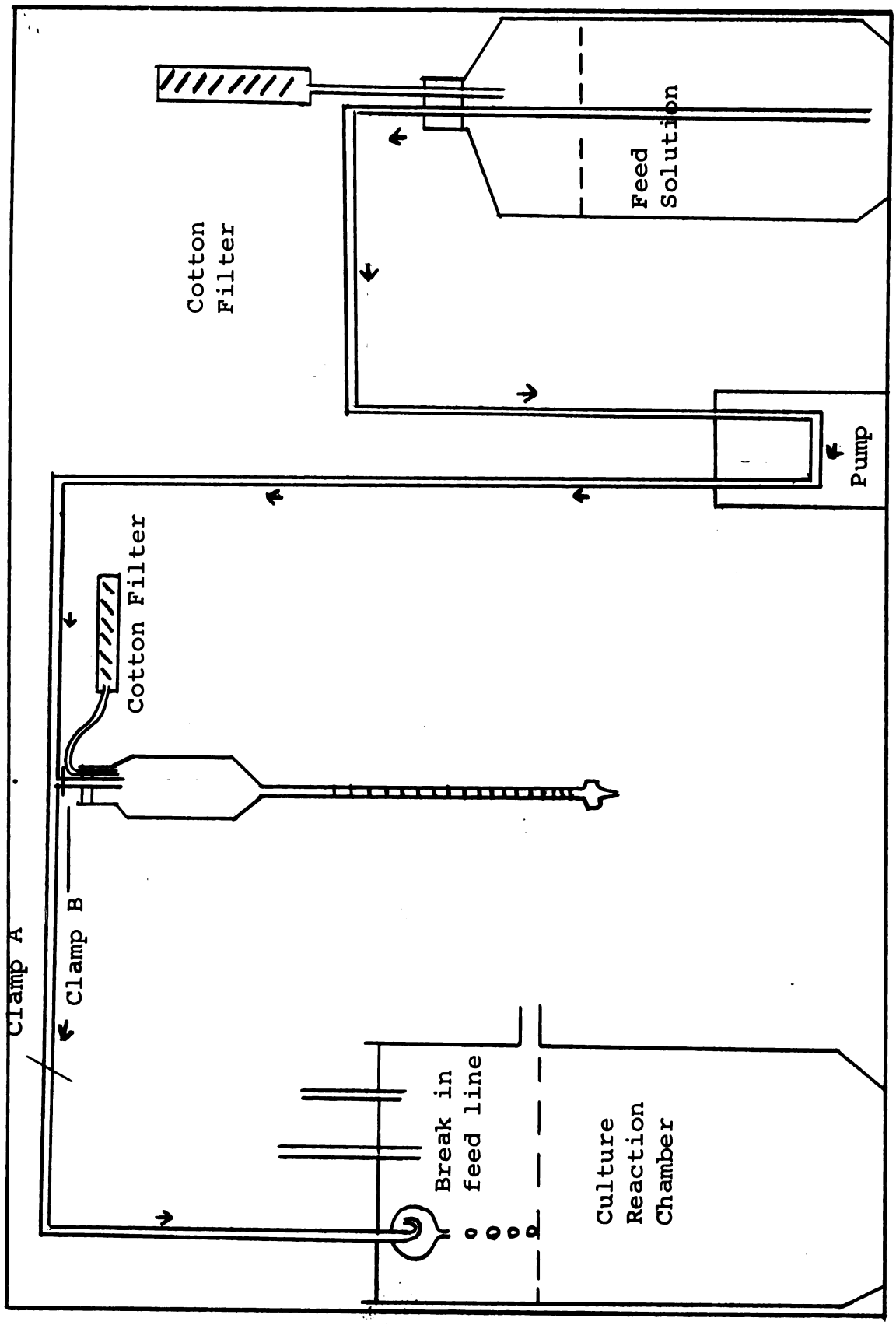

Figure 5. System for Feeding Substrate to Reaction Chamber.

was made up in batches of twenty liters. The container was a twenty liter pyrex bottle and was protected from contamination by a rubber stopper, covered at all times by a thick layer of cotton, and a cotton filter placed in the air intake line.

The feed solution was pumped from the twenty liter bottle to the reaction chamber by means of a Sigmamotor Model T65 pump. With this pump the flow rate could be adjusted by two methods; first, by varying the rpm of the motor and second, by varying the size of the rubber tubing passing through the pump. Both methods were used in these experiments. This pump proved to be entirely satisfactory and was far superior to roller action pumps or to a gravitational feed system.

It was found necessary to provide a break in the feed line inside the reaction chamber in order to prevent growth of the E. coli from backing up into the feed line. The device shown in Figure 5 was found to be very satisfactory in preventing the aerosol, which formed as a result of the aeration of the culture, from contaminating the feed line. Once the feed line was contaminated with the culture from the reaction chamber it was not possible to prevent contamination of the twenty liter bottle of feed solution.

A long stem graduated separatory funnel was inserted in the feed line for the purpose of determining the feed rate of the substrate entering the reaction chamber. As may be seen

in Figure 5 this feed rate determination was made as follows: Clamp A (normally open) was closed and Clamp B (normally closed) was opened. This directed the flow of feed solution down into the graduated separatory funnel and the feed rate was determined as ml/min with the aid of a stopwatch. A small air line connected to a cotton filter was inserted in this system in order to prevent a build-up of air inside the closed graduated separatory funnel.

6. System for the discharge of effluent from the reaction chamber--A schematic diagram of the system used for the discharge of culture from the reaction chamber is given in Figure 6. This system served two purposes. First, it acted as the culture overflow system (thereby maintaining a constant volume in the reaction chamber) and second, it served as the air exit line.

As with the substrate supply system it was found necessary to have a break in the overflow line in order to prevent contamination from growing back up the line. Contamination of various types was always present in the twenty liter collection bottle at the end of the system but at no time did the contamination grow past the break in the overflow line. Two factors helped in preventing a back-up of contamination into the reaction chamber. First, there was a constant flow of air out this line and second, the walls of the flask were

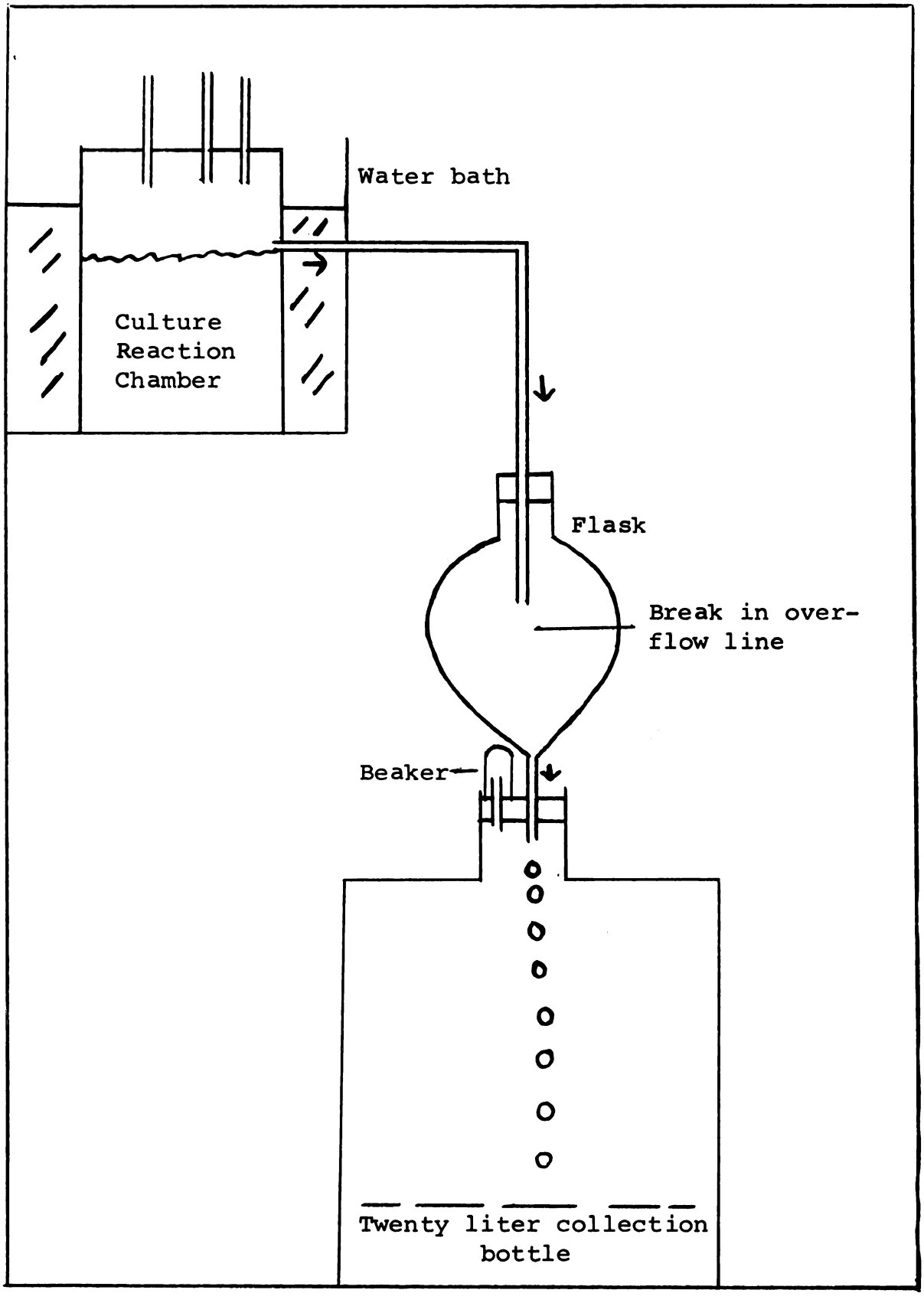

Figure 6. System for the Discharge of Culture from the Reaction Chamber.

dry at all times. All attempts to run the apparatus without this break in the line failed due to the back-up of contamination in the overflow line.

7. Sterilization of the apparatus--All parts of this apparatus were sterilized in a gas autoclave at 15 lbs pressure and 125 C for at least 30 minutes. The reaction chamber, aeration system, sampling system, and feed system (without the twenty liter bottle) were sterilized together. The other parts of the apparatus were sterilized separately and connected to the rest of the system by means of three inch pieces of sterile, heavy rubber tubing.

B. Medium

The medium used for an aerobic continuous flow system must meet at least three requirements. First, it must supply all of the essential growth requirements for the organism used. Second, all constituents, except the one being studied, must be present in excess to insure only one growth limiting substance in the media. Third, the growth of the organism in the medium must not equal or surpass the available oxygen supply and thereby making oxygen a growth limiting factor.

1. Composition--The medium used in these experiments was a modification of that used by Garrett and Sawyer (1952). This

medium had the following composition:

Substance	gm/liter
Glucose	1.0
Urea	0.5
KH_2PO_4	0.14
$MgSO_4$	0.03
$FeSO_4$	0.005
$CaCl_2$	0.01
Yeast extract	0.01

In addition to the above constituents 100 ml of 0.106 N sulfuric acid was added to each 20 liters of the medium for the initial pH adjustment.

The amount of each constituent in the medium is such that the amounts of nitrogen, phosphorus, magnesium, iron, calcium and nutriles (supplied by the yeast extract) are present in excess. The amount of growth of the E. coli in this medium was limited only by the amount of glucose present.

During preliminary experiments, using the above medium containing 2 grams of glucose per liter, the efficiency of the aeration system was found to be a limiting factor in the amount of growth produced. Under these conditions the amount of dissolved oxygen in the reaction chamber soon reached zero and remained there. No such difficulty was found with the medium containing a glucose concentration of 1 gram per liter

as dissolved oxygen was always present in the reaction chamber.

2. Preparation--The medium was prepared in batches of 20 liters. In order to prevent caramelization of the glucose during sterilization the glucose was sterilized in 100 ml of 0.106 N sulfuric acid. This was then added to the rest of the medium after the sterilization was complete. Each batch of medium was sterilized in a gas autoclave for at least one hour at 15 lbs pressure.

C. Culture

A number of different organisms were tried in the continuous flow unit before a suitable one was found. Many of the organisms tested were unsuitable for use in the system because of clumping, sticking to the sides of the reaction chamber, inconsistent growth or a tendency to settle to the bottom of the reaction chamber. An accurate determination of growth was not possible using an organism with any of these characteristics.

The organism selected for these studies was a strain of Escherichia coli (obtained from Dr. E. D. Devereux, Department of Microbiology and Public Health, Michigan State University). After a short adaptation period this organism was found to have the ideal characteristics for use in the

continuous flow system. Its growth was evenly dispersed with no tendency to clump or to stick to the sides of the reaction chamber.

The physiological characteristics of this strain were tested both before and after its prolonged use in the continuous flow system. With the exception of sucrose fermentation the physiological characteristics of the organism at the end of its use in the continuous flow system were identical to those found before its adaptation to this system. This strain of E. coli apparently lost its ability to ferment sucrose during its use in the continuous flow system.

The physiological and morphological characteristics of this strain were as follows:

Characteristic	Strain of E. coli used
Morphology	Small rod, usually occurring singly, a few pairs always present, non-motile, non-encapsulated, non-spore forming, gram negative.
Fermentation reactions	Glucose - acid with gas Lactose - acid with gas Maltose - acid with gas Mannitol - acid with gas Sucrose - acid with gas before use in the continuous flow system; No acid or gas after use in the continuous flow system.
Hydrogen sulfide production	Negative

Characteristic	Strain of *E. coli* used
Citric acid or salts utilized	Negative
Methyl red test	Positive
Voges-Proskauer test	Negative
Indol production	Positive
Fecal odor	Negative
Gelatin liquefaction	Negative

D. Analytical Techniques

1. Glucose determination--The procedure used for the determination of glucose was a modification of the method used by Nelson (1944). Nelson's procedure consists of a photometric adaptation of the Somogyi method (Somogyi, 1937) for the determination of glucose. This method is based on the autoreduction of copper in the presence of glucose followed by the development of a blue color with a arsenomolybdate reagent. The optical density of the color developed is proportional to the glucose concentration and is stable over long periods of time (Woods and Mellon, 1941). The composition of the reagents used in this test is listed in the appendix.

The optical density was determined using a Bausch and

Lomb Spectronic 20 colorimeter connected to a Raytheon Voltage Stabilizer. The optical density for this procedure was determined at the following wave lengths: 350, 400, 450, 500, 550, 600, and 650 μ. The widest optical density spread was obtained at a wave length of 650 μ and this was used for all determinations.

The procedure used for the determination of glucose was as follows:

(1) To 20 ml of the solution containing the glucose to be determined,

(2) Add 1 ml of $Ba(OH)_2$ and 1 ml of $ZnSO_4$ solutions to precipitate the bacterial cells. Several different volumes (from 0.5 to 10 ml) of these two reagents were tried. The volume found necessary to precipitate the bacterial cells in 20 ml of suspension from the reaction chamber was 1 ml.

(3) Filter.

(4) To 10 ml of the filtrate add 5 ml of copper reagent. This was made up daily by adding 1 part of copper solution B to 25 parts of copper solution A.

(5) Place the tubes in boiling water for 20 minutes.

(6) Place tubes in cold water to cool.

(7) Add 5 ml Arsenomolybdate color reagent.

(8) Mix thoroughly until all carbon dioxide has evolved.

(9) Read optical density in Spectronic 20 at 650 μ.

Several different ratios of glucose filtrate to the copper reagents and the Arsenomolybdate were treid to find which would give the best results in the very low (1-10 ppm) glucose concentration range. The best results were obtained with the ratios given above.

Determination of standard glucose curve--Eight different concentrations of glucose were made up by dilution from a solution containing 1000 mg/l glucose. The optical density of the color formed by the arsenomolybdate color reagent was determined on each of these solutions. The results obtained were as follows:

Tube No	Glucose con mg/l	Optical density
Blank	--	0
1	50	2
2	40	2
3	30	2
4	20	1.49
5	10	0.71
6	5	0.29
7	3	0.26
8	1	0.04

By using the method of least squares a line was obtained from these data with a slope of 0.073. The equation for the line was $y = 0.073x$. The standard deviation of the slope

was ± 0.0159. The graphic relationship between glucose concentration and optical density is given in Figure 7.

2. Dry weight determinations--The relationship between optical density and mg/l cell dry weight was determined by using a Bausch and Lomb Spectronic 20 colorimeter connected to a Raytheon Voltage Stabilizer. The optical density for this procedure was determined at the following wave lengths: 350, 400, 450, 500, 550, 600, and 650 μ. The widest optical density spread was obtained at a wave length of 350 μ. This wave length was used for all cell dry weight determinations.

The mg/l cell dry weight was determined by passing a known volume of cell suspension through a millipore filter which had been previously weighed. The filter and cells were then dried at 103 C for 24 hours. The mg/l cell dry weight was obtained from the difference in these two weights. A weight determination on the millipore filter indicated no appreciable loss of weight of the filter due to the heating at 103 C for 24 hours.

Determination of the standard density curve--A flask containing 1000 ml of nutrient medium was inoculated with E. coli and aerated for 48 hours. Three 25 ml samples of the resulting suspension were passed through previously weighed millipore filters. The cell dry weights obtained from these samples were 8.8, 8.7, and 8.7 mg. This corresponds to 349.2

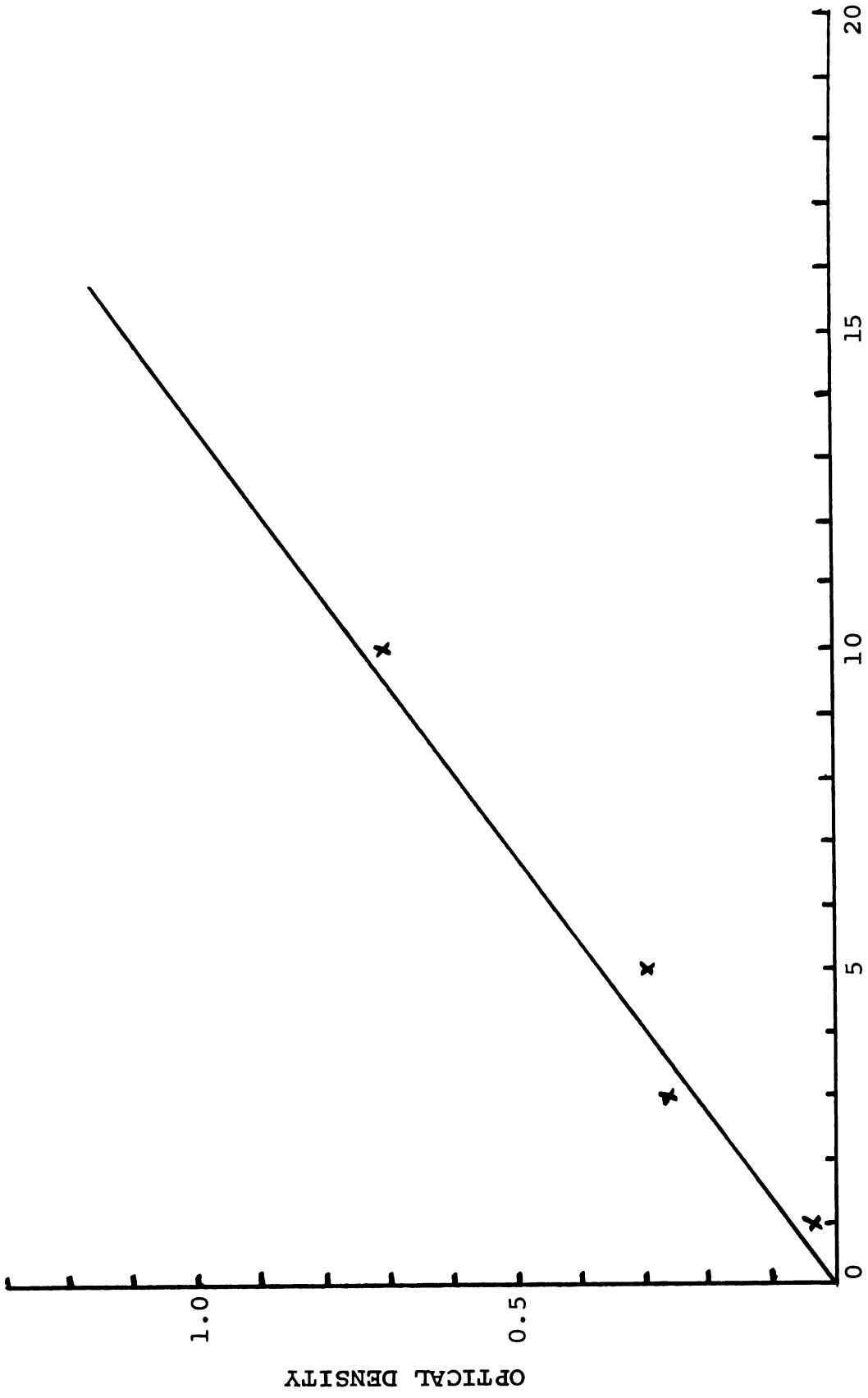

Figure 7. Standard Glucose Curve.

mg/l of E. coli suspension. Optical density determinations were made on the suspension containing 349.2 mg/l cell dry weight and dilutions of this suspension. The following table shows the results of these determinations:

Tube No.	mg/l cell dry wt.	Optical density
1	349.2	0.64
2	232.8	0.44
3	155.2	0.28
4	103.5	0.15
5	69.0	0.065

By using the method of least squares a line was obtained from these data with a slope of 0.0018. The equation for the line was $y = 0.018x$. The standard deviation of the slope was $\pm$ 0.00036. The graphic relationship between mg/l cell dry weight and optical density is shown in Figure 8.

3. Dissolved oxygen determinations--The procedure used for the determination of dissolved oxygen in the reaction chamber was a modification of the standard Winkler method (Standard Methods for the Examination of Water, Sewage and Industrial Wastes, 1955). This method is based on the oxidation of manganous hydroxide in a highly alkaline solution. The solution was then acidified in the presence of an iodide and free iodine was liberated in an amount equivalent to the oxygen

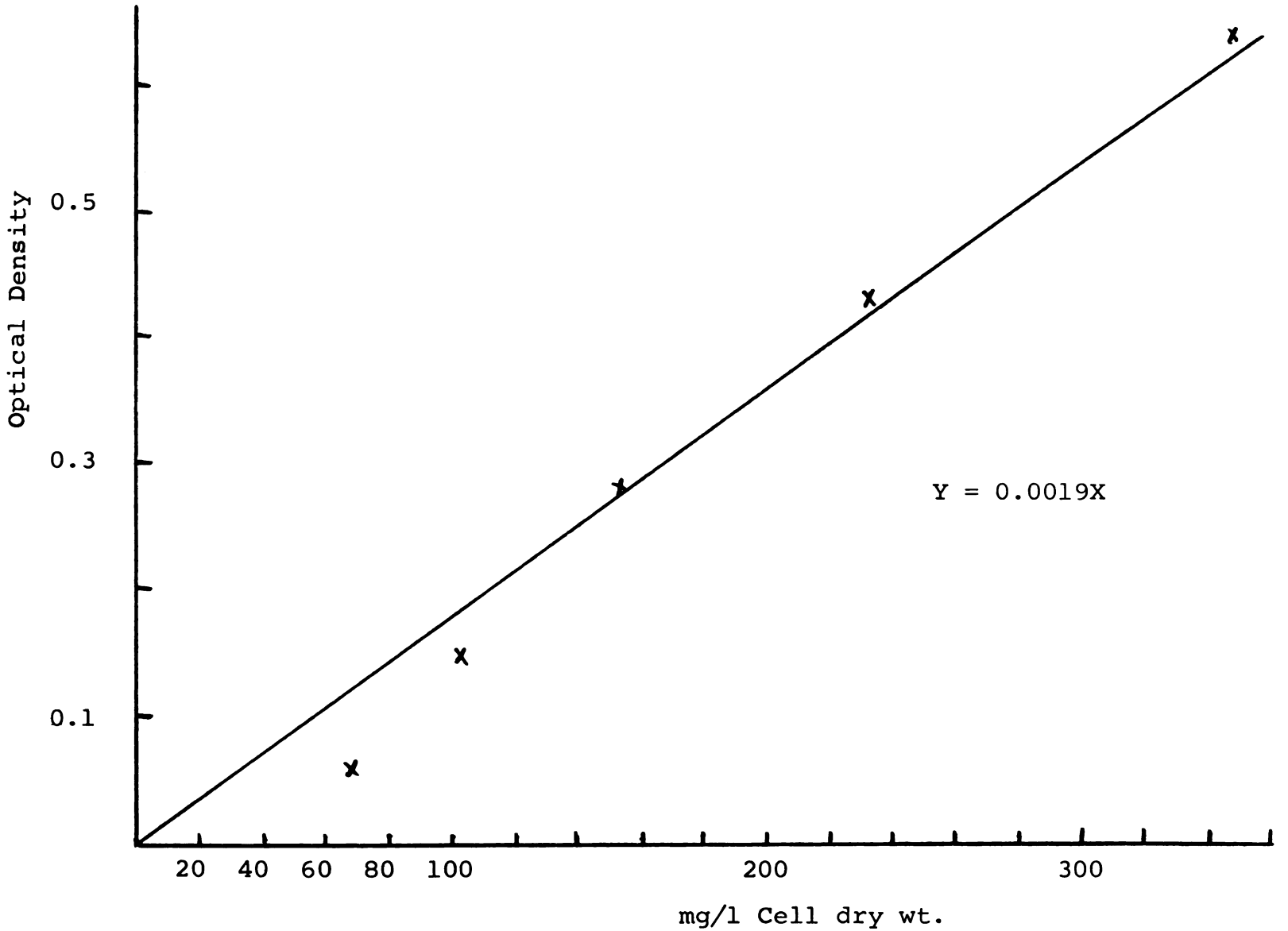

Figure 8. Standard cell dry wt. curve.

originally dissovled in the sample. The free iodine was then titrated with a sodium thiosulfate solution, using starch as an indicator after most of the iodine had been reduced.

The composition of the reagents used in this test is given in the appendix. The procedure used for this test was as follows:

(1) To 50 ml of the sample from the reaction chamber,

(2) Add 0.5 ml of manganous sulfate solution and 0.5 ml of alkaline-iodide reagent,

(3) Shake and allow to stand for 1 minute,

(4) Add 0.5 ml concentrated sulfuric acid, shake and allow to stand for five minutes,

(5) Titrate with 0.025 N sodium thiosulfate solution using starch as an internal indicator after most of the iodine had been reduced.

The above test was carried out in a tube specially made for this purpose. This tube had a total volume of 51.0 ml in order to prevent aeration of the sample during shaking.

4. Oxygen uptake determinations--Determination of the osygen uptake rates, at the different k values studied, were conducted in a circular 20 unit Warburg constant volume respirometer. The procedure used in the determination of the respiration rates was as follows:

Five ml of E. coli suspension taken from the reaction

chamber was placed in each of six Warburg flasks. A 2 cm square piece of folded filter paper was placed in the center well of each flask in addition to 0.2 ml of a ten percent solution of potassium hydroxide to absorb the carbon dioxide that was produced. The flasks were then attached to their respective manometers and immersed in a water bath. The flasks were then allowed to equilibrate, with shaking, for ten to fifteen minutes before the manometer stopcocks were closed and readings started. The shaking rate used in all of the experiments was 116 strokes per minute.

The time interval between manometer readings varied during the experiments. At the very small k values it was necessary to use a time interval of at least 1 hour between each reading. At the higher k values manometer readings could be taken every five or ten minutes. Care was taken not to use any data in which there was a significant decrease in the respiration rate between manometer readings.

Most of the oxygen uptake rates reported represent an average of at least 18 separate determinations for each k value studied. These 18 determinations represent six separate determinations each day at least three days for each k value. All oxygen uptake data were calculated in terms of $\mu l\, O_2$/mg cell dry weight/hour.

The following two equations, as given by Umbreit *et al.*,

(1957), were used in calculating the oxygen uptake rates:

$$(1)\ \text{Flask constant } K = \frac{V_g \frac{273}{T} + V_f\ \sigma}{P_o}$$

where V_g = Volume of gas phase in flask,

T = absolute temperature,

V_f = volume of fluid in flask,

σ = Bunsed Coefficient = 0.0260 (ml oxygen in solution per ml liquid at 1 atm pressure at 30 C,

P_o = 10,035 (standard pressure of mercury in terms of manometer fluid).

(2) Amount of gas exchanged $X = h \times K$,

where h = alteration in reading on open arm of manometer,

k = flask constant.

The composition of the Brodie's solution used in the manometers is given in the appendix.

5. pH determination of control--pH determinations were made three or four times daily using a Beckman Model H-2 pH meter. Twenty ml of sample was taken from the reaction chamber, placed in a 50 ml beaker, and the pH determined at room temperature.

The pH of the feed solution was adjusted so that after sterilization the pH was between 7.8 and 8.2. Due to the production of acid by the organisms the pH of the material

inside the reaction chamber would drop to 5.2-5.6 if allowed to go unadjusted. For this reason a 500 ml container of sterile 0.1 N sodium hydroxide was inserted in the system. By addition of sodium hydroxide three or four times daily the pH of the material in the reaction chamber was maintained between 6.4 and 7.0. The average pH value obtained during the experiments was 6.7. If the unit was running at very low k values only small amounts of sodium hydroxide were needed but at higher k values it was necessary to add as much as 10 to 30 ml of the sodium hydroxide solution three or four times daily in order to maintain the pH at approximately 6.7.

6. Microscopic examination--Daily samples were taken from the reaction chamber and examined under the oil objective lens of the microscope. Both wet mounts and gram stained preparations were routinely examined. When contamination did occur it was usually detected by the microscopic examinations. In such cases the unit was shut down, cleaned, sterilized, and inoculated again with a culture known to be free of contamination.

7. Nitrogen determination--Total nitrogen determinations made on organisms taken from the reaction chamber served two purposes. First, to characterize the strain of E. coli used and second, to determine if nitrogen was present in the

medium in excess so that it did not constitute a growth limiting factor.

The procedure used for the total nitrogen determinations was essentially the same as that listed by the A.O.A.C Official Methods of Ananysis (1950). This procedure was as follows:

(1) Six one gram samples of dried organisms were placed in Kjeldahl flasks,

(2) Two grams of copper sulfate, 4 grams of potassium sulfate along with 30 ml of concentrated sulfuric acid were added to the flasks,

(3) The mixture was digested for 1.5 hours and then allowed to cool,

(4) 200 ml of amonia free distilled water was added to each flask along with two or three pieces of granulated zinc (to decrease bumping) and 60 ml of sodium hydroxide solution (450 gm sodium hydroxide/l water) to make the mixture alkaline,

(5) The mixture was again heated and approximately 150 ml was distilled into 25 ml of 0.106 N sulfuric acid,

(6) The amount of acid used up was determined by titration with 0.115 N sodium hydroxide using methyl red as an indicator,

(7) The percent nitrogen in the sample was calculated as follows:

$$\text{Percent nitrogen} = \frac{1.4 \times \text{N acid}}{\text{gms of sample}} \text{ X ml acid used by } NH_3$$

$$\text{Ml acid used by } NH_3 = (\text{ml acid used} - \frac{\text{N base}}{\text{N acid}} \text{ x ml base})$$

8. Ash determination--Ash determinations were made on samples of E. coli taken from the reaction chamber. The procedure used for these determinations was as follows:

(1) One gram of dry cell material was placed in an electric muffle furnace heated to a low red heat (650 C),

(2) After at least three hours in the furnace the sample was removed, allowed to cool in a desiccator and weighed,

(3) The percent ash was then calculated using the following formula:

$$\text{Percent ash } \frac{\text{wt of ash}}{\text{wt of sample}} \text{ x } 100$$

9. Viable cell count--In order to obtain an estimate of the relationship between mg/l cell dry weight and the number of viable cells present per liter in the reaction chamber a technique similar to the drop plate technique first reported by Miles and Misra (1938) and later modified by Tomales-Lebron and Fernandos (1952) and Mallmann and Broitman (1956) was used. This procedure is predicated on the premise that colonies will appear on a suitable medium from each single culturable cell placed on the medium. The procedure used was as follows:

(1) Medium was prepared consisting of the same composition as the feed solution and containing two percent agar. The medium was poured into standard size plastic Petri dishes and allowed to stand in an inverted position over night at room temperature in order to allow the surface of the plates to become dry,

(2) Six equidistant circles approximately 2 cm in diameter were drawn on the bottom of each Petri dish,

(3) Appropriate dilutions were made from samples taken from the reaction chamber. Using a 0.2 ml pipette, 0.1 ml of each dilution was delivered to each of the six spots. Duplicate plates were set up giving a total of 12 counts for each dilution,

(4) To prevent the drops from running together the plates were not moved for at least one hour,

(5) After the drop had been completely absorbed by the agar the plates were incubated at 37 C for 48 hours and then counted.

V. RESULTS

The data presented in this section were taken daily from the continuous flow unit during three separate experiments. The first experiment lasted for a period of 64 days. The purpose of this experiment was to establish the relationship between substrate concentration in the unit and k and to obtain the approximate value for the maximum growth rate k_m. Oxygen uptake data were also taken in order to determine the relationship between k and the respiration rate. The first experiment covered a D range of 0.059 to 0.85.

The second experiment lasted for a period of 33 days. The main purpose of this experiment was to evaluate k_m as close as possible and also to confirm the data from the first experiment. The D range of the second experiment was 0.061 to 0.717.

The third experiment lasted for a period of 37 days and its purpose was to establish the relationship between k and substrate concentration in the unit at extremely low k values. The D range of this experiment was between 0.011 and 0.081.

At the beginning of each of these three experiments the unit was sterilized and filled with medium. The unit was then allowed to stand for a period of three or four days in order to determine if any contamination were present. If the

medium remained clear five ml of a 48 hour culture of E. coli was added for inoculation. The unit was then allowed to stand for another period of two or three days until heavy growth was visible in the unit. The flow of nutrient solution was then started and maintained for at least seven days at a low flow rate (1 ml/min) in order to allow the unit to stabilize itself.

Data were obtained daily from the continuous flow unit as follows:

1. Glucose determinations--Two glucose determinations were made on the influent medium and two more on samples taken from the unit,

2. Dry weight--mg/l cell dry weight was determined at least once each day,

3. Dissolved oxygen--Dissolved oxygen measurements were made once daily during the investigation of the low D values and 3 to 5 times daily while the unit was operated at the higher D values. The dissolved oxygen range obtained during the three experiments was 0.5 to 4.8 ppm, in other words, the dissolved oxygen concentration did not decrease below 0.5 mg/l at any time. The rate of air supply, as measured by a Wet-test Meter, varied from 1.0 l per min at small D values to 3.1 l per min at the higher D values. The dissolved oxygen figures indicate that at no time during the operation

of the unit was dissolved oxygen a limiting factor.

4. Oxygen uptake--Six Warburg flasks were set up each day in order to determine the respiration rate,

5. pH--pH determinations were made at least once a day during the investigation of the low D values and 4 to 8 times daily while the unit was running at the higher D values,

6. Microscopic examinations--At least one wet mount and one gram stain preparation were examined each day.

A. Experiment No. 1

The one liter continuous flow unit was operated at the following D values for a period of three consecutive days for each D value: 0.059, 0.091, 0.124, 0.178, 0.240, 0.301, 0.360, 0.425, 0.485, 0.546, 0.610, 0.660, and 0.730. In addition to these values the unit was also operated for two days at a D value of 0.794 and one day at a D value of 0.847. After data were obtained over a three day period for one of the D values listed, the flow rate of medium into the unit was changed, thus adjusting the unit to a new D value. The unit was then allowed to run at the new D value for at least 24 hours before any additional data were taken. When the D value at which the unit was being operated reached 0.730 the cell concentration in the unit dropped below the steady-state average.

The data obtained from the continuous flow unit during experiment No. 1 are given in Tables 1-15. Averages of these data, along with the averages of data obtained from experiment No. 2 are given in Table 17.

B. Experiment No. 2

In order to confirm the data from experiment No. 1 and to pinpoint more exactly the value of k_m the one liter continuous flow unit was operated for a period of 33 days at the following D values: 0.061, 0.119, 0.238, 0.307, 0.427, 0.529, 0.599, 0.658, 0.662, 0.668, 0.658, and 0.725 for a period of two days; one day at D values ranging from 0.689 to 0.717; and 4 days at a D value of 0.694. The same procedure was used as in experiment No. 1. The data obtained from the unit during this experiment are given in Table 16. Averages of these data, along with the averages of the data obtained from experiment No. 1 are given in Table 17.

C. Experiment No. 3

In order to establish the relationship between k and substrate concentration at low concentration values the three liter unit was operated for a period of 37 days at the following

D values: 0.081, 0.062, 0.052, 0.020, and 0.011. The larger unit capacity was necessary in order to obtain the very small D values. The data obtained during this experiment are given in Table 18.

D. Establishment of the Steady-State

The relationship between cell concentration in the unit and D is shown in Figure 9. The data used for this plot were taken from Table 17. Each of the points on this figure represent an average of three days data (with the exception of the D values above 0.694). As may be seen from Figure 1 and Table 19 the cell concentration remained at a constant value between D values of 0.059 and 0.694. As the unit was operated at D values above 0.694 the concentration of cells in the unit decreased. This indicates that steady-state conditions existed between D values of 0.059 and 0.694. Since, by definition, D is equal to k during the steady-state, the D values occurring within the steady-state will be referred to as k values from this point on. The average cell concentration during this steady-state condition was found to be 427 mg dry cell wt/l with a standard deviation of 9 mg/l.

The points above a D value of 0.69, shown in Figure 1 and Table 19, represent a combination of data taken from

experiments Nos. 1 and 2. Points 1-7 were obtained from experiment No. 1 in the following manner: Prior to point 1 the unit had been running for two days at a D value of 0.69 and a cell concentration of 426 mg/l. The unit was then adjusted to a D value of 0.73 for a period of 32 hours. As shown by points 1, 2, and 3 cell concentration values of 400, 394, and 394 mg/l were obtained at 7, 21, and 32 hours respectively. The unit was then adjusted to a D value of 0.79 and allowed to run at this value for 20 hours. The cell concentration at the end of this 20 hour period was 311 mg/l indicating a wash-out of cells from the unit (point 4, Figure 9). The unit was then adjusted to a D value of 0.85 and allowed to run at this rate for 23 hours. Cell concentrations of 202, 176, and 156 mg/l were obtained at 12, 16 and 23 hours respectively (points 5, 6, and 7, Figure 9). This indicates that at a D value of 0.85 cells are washed out of the unit faster than they are replaced by growth, i.e., k_m must be below 0.85. Finally the unit was adjusted to a D value of 0.69 and the cell concentration rose to the steady-state range value of 417 mg/l.

Points 8-11, Figure 9, were plotted from data obtained from experiment No. 2 and were obtained in the following manner: Prior to point 8 the unit had been running for two days at a D value of 0.66 with a cell concentration of 422 mg/l. The unit was then adjusted to a D value of 0.73 and allowed to

run at this rate for 2 days. At the end of this period the cell concentration dropped to 370 again indicating wash-out of cells from the unit (point 8, Figure 9). The D value of the unit was then adjusted to 0.66 and allowed to run at this rate for 6 days. At the end of this 6 day period the cell concentration was 429 mg/l. Following this 6 day run the unit was then operated for another 6 days at a D value of 0.69. The cell concentration at the end of this period was 430 mg/l. The D value of the unit was then adjusted to 0.71 and allowed to run at this rate for 3 days. The daily cell concentrations were 400, 390 and 360 mg/l (points 9, 10, 11, Figure 9). This represents a slow but continuous decrease over the 3 day period indicating that a D value of 0.71 is slightly above k_m. Finally the unit was adjusted to a D value of 0.69 and allowed to run at this rate for 4 days. The cell concentration again rose to a steady-state range value of 422 mg/l.

As mentioned before, from the experimental data presented in Figure 9 and Table 19, it can be seen that the cell concentration in the unit decreased, indicating non-steady-state, as the D value increased beyond 0.69. As illustrated by points 1-3, 5-7, and 9-11 (Figure 9), this decrease in cell concentration was a function of time; i.e., as the unit was operated at a given D value above 0.69 the

unit did not adjust itself to a new steady-state, instead the cell concentration continued to decrease even though the D value was kept constant. This may also be seen from Table 19 which shows a summation of the data taken when the unit was operated at D values above 0.69. This decrease in cell concentration, at a particular D value above 0.69, indicates that cells are washed out from the unit faster than they are replaced.

Herbert et al., (1956) gave the following formula to express the wash-out rate from a continuous flow unit:

$$-\frac{dx}{dt} = Dx \qquad (9)$$

This equation would express the wash-out rate under the condition that k is equal to zero. It may be assumed that under conditions where D is greater than k_m the organisms are still growing at a rate equal to k_m. Equation (9) would then have to be modified as follows:

$$-\frac{dx}{dt} = D - k_m)\ x \qquad (17)$$

A similar equation has been proposed by Finn and Wilson (1954).

The theoretical concentration of cells in the unit after the unit has been running at a D value above k_m for a given period of time may then be calculated from the relationship

$$y = xe^{-(D - k_m)} \qquad (18)$$

where y equals the cell concentration after time t and x is

equal to the initial cell concentration. The theoretical cell concentration was calculated using the data obtained at a D value of 0.85. The cell concentration at the time the unit was adjusted to this D value was 311 mg/l. After operating at this D value for 23 hours eht cell concentration was 156 mg/l. The theoretical cell concentration for the unit running at a D value of 0.85 for 23 hours was calculated to be 7.78 mg/l. This relationship between actual cell concentration and calculated cell concentration is shown in Figure 10. As may be seen from this figure the actual time for complete wash-out at a D value of 0.85 would be approximately 40 hours while the complete wash-out period based on the calculated figures would be approximately 25 hours. In other words the actual wash-out rate from the unit was less than the theoretical wash-out rate. One possible reason for this is that the equations are based on the assumption of complete and instaneous mixing. Actually the unit may not have had complete mixing, therefore the difference. k was also assumed to be constant under these conditions but there was no way to prove this experimentally.

The relationship between D and substrate concentration over a D range of 0.059 to 0.855 is shown in Figure 11. The data used for this figure were taken from Table 17. Figure 11 shows that as D is increased the substrate concentration

also increases. It was shown in Figure 9 and Table 19 that the non-steady-state begins at D values higher than 0.69. Since k_m (0.69) corresponds to a substrate concentration of 180 mg/l it can be deducted that above 180 mg/l substrate concentration k is independent of the substrate concentration. Below 180 mg/l substrate concentration k is related to substrate concentration in the manner shown by the curve in Figure 13.

E. Establishment of k_m

k_m may be defined as the maximum rate of growth of a culture or the maximum value of k at saturation levels of nutrients. The value of k_m for this strain of E. coli grown under these experimental conditions was determined by two methods. The results obtained by these two methods were as follows:

1. Continuous flow determination of k_m--As already indicated steady-state conditions existed between k values of 0.059 and 0.694. When the unit was operated at D values above 0.694 the concentration of cells in the unit decreased. The higher the D value above 0.694 the faster the cell concentration decreased. The data shown in Figure 9 therefore indicates that the k_m value for this strain of E. coli, grown under these experimental conditions, was between 0.69 and 0.71.

2. Batch culture determination of k_m--In order to determine k_m by the use of batch culture procedures and also to determine if selective mutation occurred for different growth rates, two batch culture determinations of k_m were made. The first of these (listed as Flask A) was made before the culture of E. coli was introduced into the continuous flow unit. The second batch culture determination of k_m (listed as Flask B) was made using organisms taken from the continuous flow unit after the unit had been running for three months. For each of these determinations a flask containing 1 liter of liquid medium was inoculated with a heavy inoculum of the culture. Optical density determinations were made every 30 minutes on each flask for a period of seven hours. The results of these determinations are shown in Figure 12. The following table also shows a summation of the data obtained from these two batch culture determinations.

	Flask A	Flask B
Slope by method of least squares (k_m)	0.665	0.686
Slope by method of averages	0.66 $\pm$ 0.11	0.69 $\pm$ 0.076
Generation time (min)	62.4	60.6
pH range	6.5-6.8	6.4-7.0

The t test, according to the procedure given by Youden (1951), gives a t value of 3.008 on a comparison of the two slopes.

The 1 percent critical value for t with 8 degrees of freedom (n + n - 4) is 3.355. This indicates that there was no significant difference in the slopes of the two lines and therefore no significant difference in the growth rates of the organisms in the two samples.

F. Relationship between Substrate Concentration, Experimental k and Calculated k values

The data pertaining to the relationship between substrate concentration and k obtained from the continuous flow unit are given in Table 20 and Figure 13. From Figure 13 it can be seen that, during the steady-state condition, as the substrate concentration in the unit increases the k value also increases.

Also given in Table 20 and Figure 13 are the k values that have been calculated by two different methods. The first method was by using the relationship

$$k = k_m \left(\frac{s}{S_a + s}\right) \qquad (6)$$

as given by Monod (1942) and Herbert et al., (1956). The values of k_m and S_a used in this calculation were obtained from the experimental data and were 0.69 and 40 respectively. S_a is defined as that substrate concentration at which k is equal to $1/2k_m$ and may be obtained, along with the value for k_m from

the data presented in either Table 17 or Figure 13. As may be seen in Figure 13 the k values calculated by this method follow the experimental values closely up to a k value of about 0.4 and then fall considerably below the experimental values at the higher s values.

Another method of obtaining the constants k_m and S_a would be to employ the Lineweaver-Burke plot (Dixon and Webb, 1958), which is based on a modification of equation (6):

$$\frac{1}{k} = \frac{1}{k_m} + \frac{s}{k_m} \times \frac{1}{s} \qquad (19)$$

When 1/k is plotted on the y axis and 1/x on the x axis, the y intercept is equal to $1/k_m$ and the x intercept is equal to $1/S_a$. The results obtained by plotting the experimental data from experiment No. 1 in this manner are shown in Figure 14. The values of k_m and S_a were calculated to be 1.20 and 120.5 respectively by the method of least squares. This indicates that the experimental relationship between k, k_m, and s does not follow this expression as the values for k_m and S_a are much higher than the experimentally obtained values of 0.69 and 40 respectively.

The general shape of the experimental curve suggests that the relationship between substrate concentration and k may follow a unimolecular expression of the type given by Teissier (1936).

$$k = k_m (1 - e^{-cs}) \qquad (20)$$

c in this case may be defined as a reaction velocity constant. Here too there is a problem of calculating the values for k_m and c. Reed and Theriault (1931) devised a method whereby k_m and c can be caluclated from a series of experimental data by three simulteneous equations:

$$\Sigma f_1 Y - A\Sigma f_1^2 - B\Sigma f_1 f_2 - C\Sigma f_1 f_3 = o$$

$$\Sigma f_2 Y - A\Sigma f_1 f_2 - B\Sigma f_2^2 - C\Sigma f_2 f_3 = o$$

$$\Sigma f_3 Y - A\Sigma f_1 f_3 - B\Sigma f_2 f_3 - C\Sigma f_3^2 = o$$

where

$$f_1 = 1 - e^{-c^1 s}$$

$$f_2 = se^{-c^1 s}$$

$$f_3 = e^{-c^1 s}$$

$$A = k_m$$

$$Y = k$$

$$-B = c''$$

$$c' = 2.302585c''$$

$$c = c' xh$$

$$h = \frac{B}{k_m}$$

Using the data presented in Table 17 (only the data obtained during the steady-state condition were used) in the solution of these three equations, k_m was calculated to be 0.69.

This is in full agreement with the experimental data. c was calcualted to be 0.012 from these equations. The results from the use of this method indicate that the equation for the relationship between substrate concentration and k would be

$$k = 0.69\ (1-e^{-0.012s}) \qquad (21)$$

k values calculated according to this equation are given in Table 20 and are shown graphically in Figure 13. Although the calculated values are all lower than the experimental values it can be seen that the general shape of the curve shows good agreement with that obtained from the experimental values.

G. Relationship between D and Substrate Concentration at Low D Values

The relationship between D and substrate concentration at low D values was investigated in experiment No. 3. The results of this experiment are given in Table 18. As may be seen from this table, as the D value drops the substrate concentration in the unit also drops. The relationship between D and substrate concentration obtained during experiment No. 3 is shown graphically in Figure 15. These data indicate that steady-state conditions existed at D values as low as 0.02. These figures also indicate that the k range at which this

strain of E. coli may be kept in a steady-state under these experimental conditions is 0.02-0.69. When the unit was operated at a D value below 0.02 the cell concentration in the unit decreased. At this point the substrate concentration in the effluent or inside the unit was 1 mg/l or below and too low to be measured. This indicates that at D values below 0.02 and at substrate concentrations below 1 mg/l k appraoches zero so that the cells are washed out according to equation (9).

H. Relationship between k and Respiration Rate During Steady-State Conditions

Respiration rate determinations were made during experiment No. 1. The summation of the oxygen uptake data is presented in Table 17. The relationship between respiration rate and k during the steady-state operation of the continuous flow unit is presented in Figure 16. An examination of this figure indicates that there is a direct linear relationship between respiration rate and k during the steady-state operation. A linear relationship was also obtained by Herbert (1959b) measuring the oxygen uptake at different k values of Aerobacter aerogenes grown in continuous culture. The oxygen uptake data presented in Table 17 also indicate that the respiration rate increases with k until a maximum rate is reached. The maximum respiration rate corresponds to the maximum k value

(k_m) at which the unit can be operated under steady-state conditions. An increase in D above 0.69, the value corresponding to k_m does not increase the respiration rate of the organisms.

The equation for the line shown in Figure 16, as calculated by the method of least squares is

$$Q_{O_2} = 9.43 + 543.6\ k$$

where Q_{O_2} is the respiration rate in μ l/mg cell dry wt/hr. From this equation the respiration rate for any given k value can be calculated. It is suggested that the value for the constant a (9.43), is given in this equation, represents the endogenous respiration rate of this organism under these experimental conditions.

The average respiration rate obtained at a D value of 0.69 and higher was 347 μ 10_2/mg cell dry wt/hr. This value is higher than the respiration rate of 272 μ 10_2/mg cell dry wt/hr at 32 C given by Spector (1956) for E. coli. In fact such a respiration rate would correspond to a k value of 0.49 under the experimental conditions described here.

A linear relationship was obtained between the actual amount of oxygen consumed and the theoretical amount of oxygen needed for complete oxidation of the sugar used by the organisms. The data used in the calculation of this relationship are given in Table 21 and are shown graphically in Figure 17. As may be seen from Table 21 only 33.8 to 38.9 percent of the

theoretical oxygen needed for complete oxidation of the amount of glucose assimilated was actually consumed. The equation for this relationship, as shown in Figure 21, was calculated by the method of least squares and found to be

$$Y = 0.37X$$

Where Y is equal to the actual amount of oxygen consumed in terms of μ 10_2/mg cell dry wt/hr and X is equal to the theoretical amount of oxygen needed for the complete oxidation of the sugar assimilated. These data indicate that during the steady-state operation of the unit, approximately 37 percent of the assimilated substrate was oxidized.

The relationship between percent of theoretical oxygen utilized and k during the steady-state operation of the unit is given in Figure 18. The data shown in this figure also demonstrate that regardless of the value of k 37 percent of the theoretical oxygen needed are consumed.

I. Calculation of the Economic Coefficient During Steady-State Conditions

The economic coefficient was calculated for each of the k values used during the steady-state operation of the unit. The following formula was used for these calculations:

$$\text{Economic coefficient} = \frac{\text{weight of Bacteria formed/unit time}}{\text{weight of Substrate used/unit time}}$$

The data obtained from these calculations are given in Table 22. The graphic presentation of the relationship between the economic coefficient and k may be seen in Figure 19. These data indicate that the economic coefficient of the organism during the steady-state operation of the unit was between 44.3 and 54.7 percent. These values agree well with a value of 53.0 percent reported by Herbert et al., (1956) for the steady-state growth of Aerobacter cloacae utilizing glycerol as a carbon source. These data also indicate that there may be a gradual rise in economic coefficient as the k value increases. This rise in economic coefficient appears to be of the order of ten percent, i.e., the organisms were converting substrate into cell material at a ten percent higher efficiency at the maximum k value than at the lowest k value. This may be related to the problem of endogenous respiration and will be discussed later in Section VI of this report.

J. Application of Experimental Data to Theoretical Steady-State Equations

The following relationship between $\bar{s}$, S_a, D and k_m has been proposed by Monod (1950) and Herbert et al., (1956):

$$\bar{s} = S_a \left(\frac{D}{k_m - D}\right) \qquad (14)$$

The results obtained from the application of the experimental data during the steady-state condition to this equation are presented in Table 23 and Figure 20. The values of S_a and k_m used in these calculations were 40 and 0.69 respectively. As may be seen from Table 23 and Figure 20 the calculated $\bar{s}$ values follow the experimental $\bar{s}$ values closely until a k value of 0.4 is reached. From this point on the calculated $\bar{s}$ values are much higher than the experimental $\bar{s}$ values. It will be recalled that a similar breaking point occurred when the experimental data were applied to equation (6). There appears to be no relationship between these calculated $\bar{s}$ values and reality since for a k value of 0.69 the calculated $\bar{s}$ value is higher than that for S_R, the substrate concentration entering the unit.

Monod (1950) and Herbert et al., (1956) give the following equation to show the relationship between $\bar{x}$, Y, S_R, k_m, S_a, and D:

$$\bar{x} = Y (S_R - \bar{s}) = Y \left[S_R - S_a \left(\frac{D}{k_m - D}\right)\right] \qquad (15)$$

The experimental values obtained for Y, S_R and S_a were applied to the first part of this relationship

$$\bar{x} = Y (S_R - \bar{s})$$

The results obtained for the calculation of the steady-state cell concentrations are presented in Table 24. As may be seen from this table the calculated steady-state cell concentrations are in full agreement with the experimentally obtained values.

This was to be expected since Y has been calculated previously in the same manner.

An entirely different picture was obtained when the steady-state cell concentrations were calculated using the last part of this relationship

$$\bar{x} = Y\left[S_R - S_a\left(\frac{D}{k_m - D}\right)\right]$$

The results obtained from the application of the experimental data during the steady-state condition to this equation are presented in Table 25. The results were calculated using both an average Y value of 47 percent and the actual experimental Y values obtained during the operation of the unit. An average value of 964 was used for S_R and a value of 40 was used for S_a. As may be seen from Table 25 the $\bar{x}$ values calculated using the average Y value of 47 percent are higher than the experimental values at the low k values and are much lower than the experimental $\bar{x}$ values at the high k values. The $\bar{x}$ values calculated using the actual Y values are in close agreement with the experimental $\bar{x}$ values until a k value of 0.4 was reached. From this point the calculated $\bar{x}$ values indicate that wash-out is occurring from the unit while the experimental $\bar{x}$ values indicate that the steady-state condition still exists. This breaking point again occurs at about the same k value as in equations (6) and (14).

K. Nitrogen, Ash, and Moisture Determinations

Samples were taken from the unit while it was running at a k value of 0.062. The cells from these samples were obtained by high speed centrifugation. Nitrogen, ash, and moisture determinations were made as described in Section IV. The results of these determinations are summarized in the following table:

Nitrogen % dry wt	Moisture %	Ash %
12.6	72.5	8.5
12.0	72.0	8.3
11.9	72.8	8.3
12.7	72.6	8.7
11.4	72.9	9.7*
11.7	70.6*	8.5

* not used in calculating average

The average values of 12.1 percent nitrogen, 72.6 percent moisture and 8.5 percent ash are in agreement with the values of 73 percent moisture, 8.6 percent ash and 9-13 percent given by Spector (1956) for *E. coli*.

L. Culturable Cell Count

Culturable cell counts were made by the method described in Section IV. The following data were obtained at a cell concentration of 422 mg cell dry wt/l during steady-state

operation of the unit:

Count x 10^8 cells/ml

5.4
7.3
6.4
8.1
7.2
7.5

The average of these data is 7.0 x 10^8. Each figure given represents an average of 12 counts; i.e., two plates each containing six counts. These figures indicate that 1 mg cell dry wt represents approximately 16.6 x 10^8 viable cells and that during steady-state conditions the unit was operated at a concentration of approximately 7.0 x 10^8 cells/ml.

Table 1. Data obtained from the continuous flow unit at a D value of 0.059 during experiment No. 1.

Day	Liquid flow ml/min	Detention time-hrs	pH	Cell con mg/l	Substrate mg/l Influent	Substrate mg/l Effluent	Oxygen uptake $\mu l O_2$/mg/hr
1	0.96	17.36	6.4	427	960	4.9	35
					965	5.3	27
							28
							37
							33
							32
2	0.99	16.84	6.7	422	950	4.7	14*
					945	5.5	44
							30
							21*
							38
							36
3	0.98	17.01	6.6	417	980	5.2	39
					950	5.2	39
							30
							33
							39
							30
Average	0.98	17.00	6.5	422	958	5.1	34

*Data not used in calculations

Table 2. Data obtained from the continuous flow unit at a D value of 0.091 during experiment No. 1.

Day	Liquid flow ml/min	Detention time-hrs	pH	Cell con mg/l	Substrate mg/l Influent	Substrate mg/l Effluent	Oxygen uptake $\mu l O_2$/mg/hr
1	1.53	10.89	6.7	422	960	7.9	50
					960	8.2	52
							56
							48
							27*
							59
2	1.50	11.11	6.5	427	980	8.6	52
					945	8.2	14*
							59
							51
							19*
							56
3	1.51	11.04	6.5	438	950	8.2	55
					970	8.4	47
							50
							43
							59
							43
Average	1.51	11.04	6.6	429	961	8.3	52

*Data not used in calculations

Table 3. Data obtained from the continuous flow unit at a D value of 0.124 during experiment No. 1.

Day	Liquid flow ml/min	Detention time-hrs	pH	Cell con mg/l	Substrate mg/l Influent	Substrate mg/l Effluent	Oxygen uptake $\mu l 0_2$/gm/hr
1	2.07	8.05	6.7	427	945	13.7	59
					950	12.2	64
							58
							75
							59
							69
2	2.10	7.94	6.5	483	830*	16.4	72
					975	11.6	84
							67
							79
							75
							8*
3	2.03	8.21	6.5	427	980	13.4	63
					950	12.6	72
							81
							61
							70
							79
Average	2.07	8.05	6.6	431	960	13.3	70

*Data not used in calculations

Table 4. Data obtained from the continuous flow unit at a D value of 0.178 during experiment No. 1.

Day	Liquid flow ml/min	Detention time-hrs	pH	Cell con mg/l	Substrate mg/l Influent	Substrate mg/l Effluent	Oxygen uptake $\mu l O_2$/mg/hr
1	2.96	5.63	6.4	433	985	19.2	103
					950	20.8	94
							106
							110
							93
							101
2	2.94	5.67	6.6	417	965	21.6	109
					820*	20.2	116
							127
							96
							112
							104
3	2.99	5.57	6.6	433	975	20.2	93
					980	19.8	62*
							115
							104
							97
							112
Average	2.96	5.63	6.5	428	971	20.3	105

*Data not used in calculations

Table 5. Data obtained from the continuous flow unit at a D value of 0.240 during experiment No. 1.

Day	Liquid flow ml/min	Detention time-hrs	pH	Cell con mg/l	Substrate mg/l Influent	Substrate mg/l Effluent	Oxygen uptake $\mu l 0_2$/mg/hr
1	4.02	4.15	6.5	417	985	32.0	145
					960	28.8	155
							137
							139
							140
							133
2	3.9'	4.20	6.7	428	950	31.6	144
					985	38.4*	136
							137
							139
							140
							118*
3	4.03	4.14	6.5	417	975	30.2	154
					960	29.6	146
							140
							145
							138
							132
Average	4.01	4.16	6.6	421	969	30.4	141

*Data not used in calculations

Table 6. Data obtained from the continuous flow unit at a D value of 0.301 during experiment No. 1.

Day	Liquid flow ml/min	Detention time-hrs	pH	Cell con mg/l	Substrate mg/l Influent	Substrate mg/l Effluent	Oxygen uptake $\mu l O_2$/mg/hr
1	4.95	3.37	6.5	433	945	36	180
					965	37	174
							107*
							167
							187
							193
2	5.04	3.31	6.5	438	975	38	181
					960	37	212
							207
							176
							197
							194
3	5.09	3.27	6.8	427	960	37	180
					960	37	93*
							194
							187
							202
							172
Average	5.03	3.32	6.6	433	961	37	188

*Data not used in calculations

Table 7. Data obtained from the continuous flow unit at a D value of 0.36 during experiment No. 1.

Day	Liquid flow ml/min	Detention time-hrs	pH	Cell con mg/l	Substrate mg/l Influent	Substrate mg/l Effluent	Oxygen uptake µl/mg/hr
1	6.04	2.76	6.6	422	965	41.9	200
					960*	43.1	237
							222
							241
							219
							227
2	5.95	2.80	6.4	405	995	42.3	136*
					960	41.1	241
							227
							239
							238
							220
3	5.91	2.82	6.5	433	975	42.6	217
					950	41.4	88*
							207
							234
							210
							129*
Average	5.97	2.79	6.5	420	969	42.1	225

*Data not used in calculations

Table 8. Data obtained from the continuous flow unit at a D value of 0.425 during experiment No. 1.

Day	Liquid flow ml/min	Detention time-hrs	pH	Cell con mg/ml	Substrate mg/l Influent	Substrate mg/l Effluent	Oxygen uptake µl/mg/hr
1	7.05	2.36	6.6	422	975	56	259
					950	60	250
							238
							263
							242
							255
2	7.10	2.35	6.8	422	985	55	263
					960	61	271
							257
							264
							242
							250
3	7.10	2.35	6.7	417	975	58	243
					635*	61	230
							256
							249
							227
							237
Average	7.08	2.35	6.7	420	969	58	249

*Data not used in calculations

Table 9. Data obtained from the continuous flow unit at a D value of 0.485 during experiment No. 1.

Day	Liquid flow ml/min	Detention time-hrs	pH	Cell con mg/ml	Substrate mg/l Influent	Substrate mg/l Effluent	Oxygen uptake µl/mg/hr
1	7.96	2.09	6.8	417	960	72.0	254
					960	74.5	261
							247
							270
							264
							268
2	8.17	2.04	6.6	406	975	75.5	174*
					865*	76.5	264
							281
							259
							271
							269
3	8.10	2.06	6.8	417	965	72.5	249
					950	76.0	272
							257
							263
							284
							279
Average	8.08	2.06	6.7	413	962	74.5	265

*Data not used in calculations

Table 10. Data obtained from the continuous flow unit at a D value of 0.546 during experiment No. 1.

Day	Liquid flow ml/min	Detention time-hrs	pH	Cell con mg/ml	Substrate mg/ml Influent	Substrate mg/ml Effluent	Oxygen uptake µl/mg/hr
1	9.2	1.81	6.5	427	945	93	328
					970	99	312
							320
							306
							337
							334
2	9.05	1.84	6.7	438	955	97	317
					980	95	336
							321
							284
							317
							299
3	9.10	1.83	6.4	433	980	100	305
					950	95	312
							287
							323
							291
							319
Average	9.12	1.83	6.5	433	963	96.5	314

Table 11. Data obtained from the continuous flow unit at a D value of 0.610 during experiment No. 1.

Day	Liquid flow ml/min	Detention time-hrs	pH	Cell con mg/l	Substrate mg/ml Influent	Substrate mg/ml Effluent	Oxygen uptake µl/mg/hr
1	10.2	1.63	6.5	428	960	108	323
					980	112	336
							341
							348
							329
							315
2	10.1	1.65	6.7	433	945	114	301
					975	111	329
							348
							340
							311
							323
3	10.2	1.63	6.6	417	955	115	324
					685*	109	344
							376
							345
							338
							351
Average	10.17	1.64	6.6	426	963	112	335

*Data not used in calculations

Table 12. Data obtained from the continuous flow unit at a D value of 0.66 during experiment No. 1.

Day	Liquid flow ml/min	Detention time-hrs	pH	Cell con mg/ml	Substrate mg/ml Influent	Substrate mg/ml Effluent	Oxygen uptake µl/mg/hr
1	10.90	1.53	6.5	438	990	158	341
					950	163	337
							366
							354
							360
							358
2	11.15	1.50	6.7	422	980	162	361
					895*	164	340
							335
							356
							361
							264*
3	11.10	1.50	6.4	444	955	159	361
					955	203*	358
							364
							340
							361
							344
Average	11.05	1.51	6.5	434	966	161	353

*Data not used in calculations

Table 13. Data obtained from the continuous flow unit at a D value of 0.73 during experiment No. 1.

Day	Liquid flow ml/min	Detention time-hrs	pH	Cell con mg/ml	Substrate mg/ml Influent	Substrate mg/ml Effluent	Oxygen uptake µl/mg/hr
1	12.20	1.40	6.7	400	960	193	333
					950	197	339
							361
							330
							359
							341
2	11.90	1.36	6.5	394	965	190	340
					940	199	327
							328
							336
							320
							332
3	13.10	1.38	6.8	394	980	200	352
					945	189	348
							361
							335
							359
							337
Average	12.07	1.38	6.6	396	957	195	341

Table 14. Data obtained from the continuous flow unit at a D value of 0.794 during experiment No. 1.

Day	Liquid flow ml/min	Detention time-hrs	pH	Cell con mg/ml	Substrate mg/ml Influent	Substrate mg/ml Effluent	Oxygen uptake µl/mg/hr
1	13.2	1.26	6.8	333	965	246	360
					990	230	349
							342
							352
							357
							331
							348
2	13.2	1.26	6.5	311	950	252	343
					985	266	362
							337
							367
							351
							354
							352
Average	13.2	1.26	6.6	---	---	---	---

Table 15. Data obtained from the continuous flow unit at a D value of 0.850 during experiment No. 1.

Day	Liquid flow ml/min	Detention time-hrs	pH	Cell con mg/ml	Substrate mg/ml Influent	Substrate mg/ml Effluent	Oxygen uptake µl/mg/hr
1(2 hr)	14.15	1.18	6.4	202	820*	304	362
					975	336	330
							321
							347
							207*
							342
							340
1(7 hr)	14.21	1.17	6.7	176	945	372	321
					980	384	347
							352
							336
							341
							359
							343
1(12 hr)	14.17	1.18	6.5	156	960	386	337
					985	378	344
							362
							369
							351
							367
							355
Average	14.18	1.18	6.6	---	---	---	---

*Data not used in calculations

Table 16. Data obtained from the continuous flow unit during experiment No. 2.

D	pH	Substrate con mg/l Influent	Effluent	Cell con mg/l
0.061	6.5	965	5.6	427
		950	4.3	
0.119	6.8	980	13.0	434
		965	13.9	
0.238	6.9	960	34.6	417
		830*	31.0	
0.307	6.4	975	42	438
		960	38	
0.427	6.0	960	68	422
		950	61	
0.529	6.5	955	98	427
		980	106	
0.599	6.7	945	124	434
		975	120	
0.658	6.3	980	159	422
		900*	150	
0.658	6.5	990	157	427
		960	148	
0.662	6.5	960	140	438
		985	155	
0.668	6.8	970	153	422
		834*	148	
0.689	6.7	980	175	434
		950	168	
0.689	6.4	630*	170	422
		680*	165	
0.694	6.5	975	178	434
		960	171	
0.694	6.7	965	176	422
		970	179	
0.694	6.4	980	168	434
		955	164	
0.709	6.4	965	210	400
		960	196	
0.714	6.8	990	217	390
		970	224	
0.717	6.5	965	237	360
		840*	143*	
0.725	6.8	960	200	352
		970	220	

*Data not used in calculations

Table 17. Summary of averages of data taken during experiments 1 and 2.

D	Cell con mg/l	Substrate con mg/l Influent	Substrate con mg/l Effluent	Oxygen uptake l/mg/hr
0.059	422	958	5.1	34
0.061	427	957	5.0	---
0.091	429	961	8.3	52
0.119	434	973	13.5	---
0.124	431	960	13.3	70
0.178	428	971	20.3	105
0.238	417	960	32.8	---
0.240	421	969	30.4	141
0.301	433	961	37.0	188
0.307	438	968	40.0	---
0.360	420	969	42.1	225
0.425	420	969	58.0	249
0.427	422	955	65.0	---
0.485	413	962	74.4	265
0.529	427	968	102.0	---
0.546	433	963	96.5	314
0.599	434	960	122.0	---
0.610	426	963	112.0	335
0.658	422	980	155.0	---
0.660	434	966	161.0	353
0.662	438	973	148.0	---
0.668	422	970	151.0	---
0.689	434	965	172.0	---
0.689	422	---	168.0	---
0.694	434	968	175.0	---
0.694	422	968	178.0	---
0.694	434	967	166.0	---
0.709	400	963	203.0	---
0.714	390	980	221.0	---
0.717	360	965	237.0	---
0.725	352	965	210.0	---
0.730	396	957	195.0	341
0.794	311	968	259.0	352
0.794	333	977	238.0	348
0.847	202	975	320.0	340
0.847	156	972	382.0	355
0.855	176	963	378.0	343

Table 18. Data obtained from the continuous flow unit during experiment No. 3.

Day	D	Cell conc mg/l	Substrate con mg/l Influent	Effluent	pH
1	0.081	438	950	6.3	6.5
			975	6.0	
3	0.062	422	960	5.4	6.4
			840*	4.7	
5	0.052	442	965	4.3	6.7
			980	3.9	
7	0.020	422	975	0-1	6.4
			975	0-1	
12	0.020	434	960	0-1	6.6
			985	0-1	
17	0.011	380	970	0-1	6.9
			951	0-1	
22	0.011	355	965	0-1	6.6
			974	0-1	
31	0.011	314	960	0-1	6.4
			770*	0-1	
37	0.011	293	965	0-1	6.7
			950	0-1	

*Data not used in calculations

Table 19. Concentration of organisms at D values greater than 0.694.

Experiment	D	Cell con mg/l	Time unit running at D value-hrs	Remarks
2	0.66	422	48	Steady-state
1	0.69	426	48	Steady-state
2	0.71	400	24	Non-steady-state
2	0.71	390	48	Non-steady-state
2	0.71	360	72	Non-steady-state
1	0.73	400	7	Non-steady-state
1	0.73	394	21	Non-steady-state
1	0.73	394	32	Non-steady-state
2	0.73	370	48	Non-steady-state
1	0.79	311	20	Non-steady-state
1	0.85	202	12	Non-steady-state
1	0.85	176	16	Non-steady-state
1	0.85	156	23	Non-steady-state

Table 20. Relationship between substrate concentration and experimental and calculated k values.

Substrate con mg/l	Experimental k	Calculated k $k = k_m \left(\frac{s}{S_a+s}\right)$	Calculated k $k = k_m (1-e^{-et})$
5.1	0.059	0.078	0.040
5.0	0.061	0.077	0.040
8.3	0.091	0.119	0.066
13.5	0.119	0.174	0.102
13.3	0.124	0.173	0.102
20.3	0.178	0.233	0.153
32.8	0.238	0.311	0.228
30.3	0.240	0.298	0.213
37.0	0.301	0.332	0.250
40.0	0.307	0.345	0.267
42.1	0.360	0.354	0.276
58.0	0.425	0.408	0.347
65.0	0.427	0.427	0.377
74.5	0.485	0.449	0.409
102.0	0.529	0.495	0.490
96.5	0.546	0.489	0.476
122.0	0.599	0.519	0.533
112.0	0.610	0.509	0.513
155.0	0.658	0.549	0.586
161.0	0.660	0.553	0.592
148.0	0.662	0.543	0.576
151.0	0.668	0.546	0.579
172.0	0.689	0.565	0.605
168.0	0.689	0.558	0.600
175.0	0.694	0.562	0.608
178.0	0.694	0.564	0.610

Table 21. Comparison of the actual amount of oxygen used with the theoretical amount of oxygen required for complete oxidation of sugar used during steady-state.

k	Sugar in mg/hr	Sugar out mg/hr	Sugar used mg/hr	µl oxygen needed for complete oxidation µl/mg/hr	Actual O_2 used µl/mg/hr	% Theoretical
0.059	56.7	0.299	56.4	98.7	34	34.4
0.091	87.3	0.752	86.6	154.0	52	33.8
0.124	119.7	1.652	118.1	208.2	70	33.8
0.178	171.2	3.605	167.6	293.3	105	35.8
0.240	231.9	7.314	224.6	393.4	141	35.8
0.301	290.9	11.167	279.7	490.7	188	38.3
0.360	345.3	15.080	330.2	578.9	225	38.9
0.425	409.5	24.638	384.9	675.5	249	36.9
0.485	465.3	36.118	431.2	756.0	265	35.1
0.546	527.5	52.805	474.7	832.3	314	37.7
0.610	588.2	68.342	519.9	911.4	335	36.8
0.660	639.1	106.743	532.4	933.8	353	37.8

Table 22. Relationship between economic coefficient and k during the steady-state.

k	Cell conc mg/l	Substrate used mg/l	Economic coefficient
0.059	422	953	44.3
0.061	427	952	44.9
0.091	429	953	45.0
0.119	434	960	45.2
0.124	431	947	45.5
0.178	428	951	45.0
0.238	417	927	45.0
0.240	421	939	44.8
0.301	433	924	46.9
0.307	438	928	47.2
0.360	420	927	45.3
0.425	420	911	46.1
0.427	422	890	47.4
0.485	413	888	46.5
0.529	427	866	49.3
0.546	433	867	50.0
0.599	434	838	51.7
0.610	426	851	50.1
0.658	422	825	51.1
0.660	434	805	53.9
0.662	438	825	53.1
0.668	422	819	51.5
0.689	434	793	54.7
0.694	434	793	54.7
0.694	422	790	53.4
0.694	434	801	54.2

Table 23. Comparison of experimental and calculated $\bar{s}$ values.

k	Experimental $\bar{s}$	Calculated $\bar{s}$
0.059	5.1	3.8
0.061	5.0	3.9
0.091	8.3	6.0
0.119	13.5	8.3
0.124	13.3	8.8
0.178	20.3	13.9
0.238	32.8	21.1
0.240	30.4	21.3
0.301	37.0	31.0
0.307	40.0	32.1
0.360	42.1	43.6
0.425	58.0	64.2
0.427	65.0	65.0
0.485	74.5	94.4
0.529	102.0	131.4
0.546	96.5	151.7
0.599	122.0	263.0
0.610	112.0	305.0
0.658	155.0	822.5
0.660	161.0	880.0
0.662	148.0	945.7
0.668	151.0	1214.6

Table 24. Comparison of experimental $\bar{x}$ and $\bar{x}$ calculated using the expression $\bar{x} = Y (S_R - \bar{s})$

Experimental $\bar{x}$	Calculated $\bar{x}$*
422	419
429	428
431	418
428	428
421	422
433	434
420	417
420	419
413	414
433	433
426	426
434	434

*Calculated using experimental Y values.

Table 25. Comparison of experimental $\bar{x}$ and $\bar{x}$ calculated using the expression $\bar{x} = Y\left[S_R - S_a\left(\frac{D}{k_m - D}\right)\right]$

k	Experimental $\bar{x}$	Calculated $\bar{x}$*	Calculated $\bar{x}$**
0.059	422	449	425
0.061	427	449	---
0.091	429	450	431
0.119	434	449	---
0.124	431	449	434
0.178	428	446	428
0.238	417	443	---
0.240	421	443	422
0.301	433	438	438
0.307	438	438	---
0.360	420	432	417
0.425	420	423	415
0.427	422	423	---
0.485	413	409	405
0.529	427	391	---
0.546	433	382	405
0.599	434	329	---
0.610	426	309	327
0.658	422	67	---
0.660	434	---	42

*Calculated using average Y value.
**Calculated using experimental Y values.

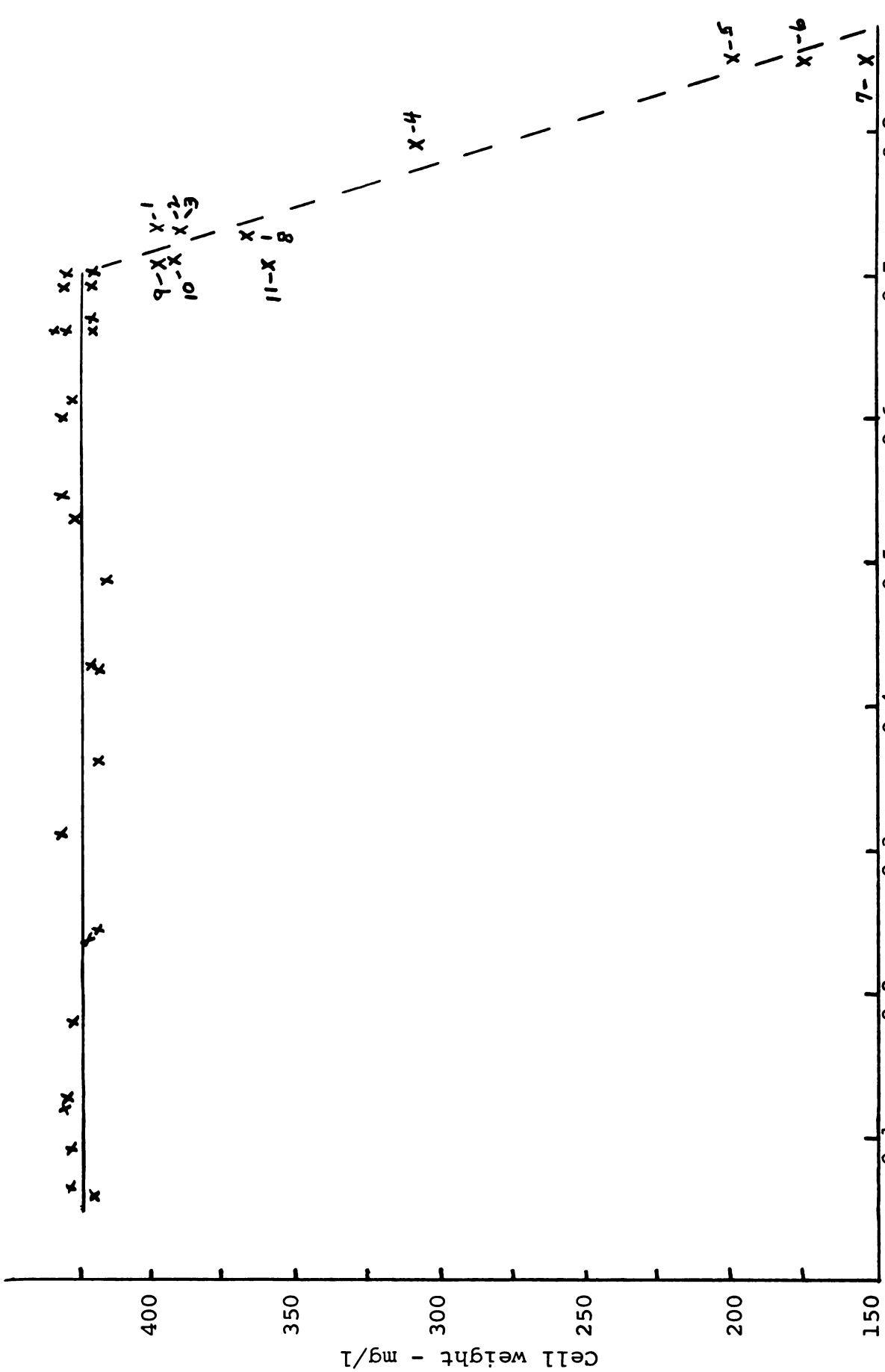

Figure 9. Relationship between Cell Concentration and D.

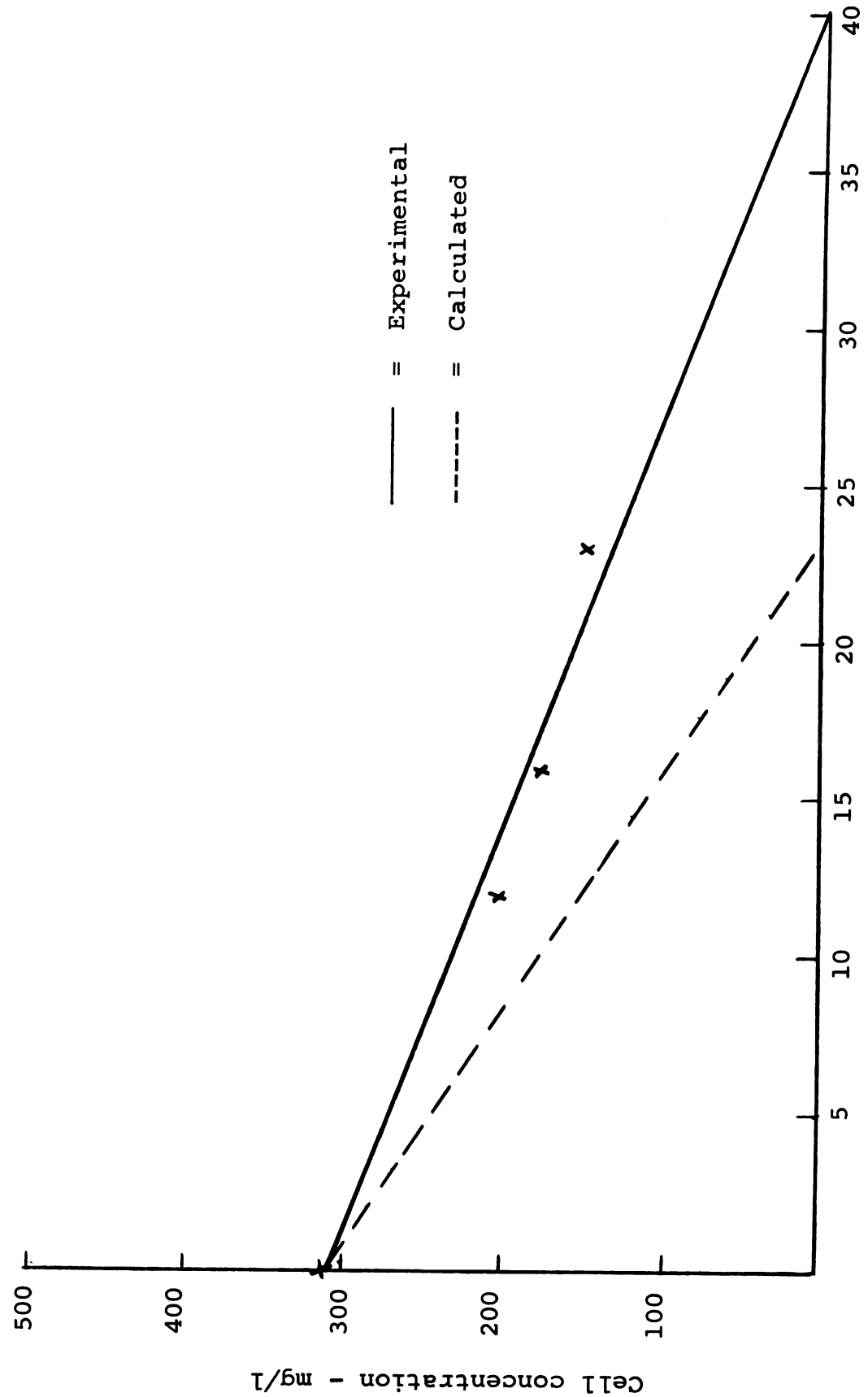

Figure 10. Wash-out Rate from the Unit.

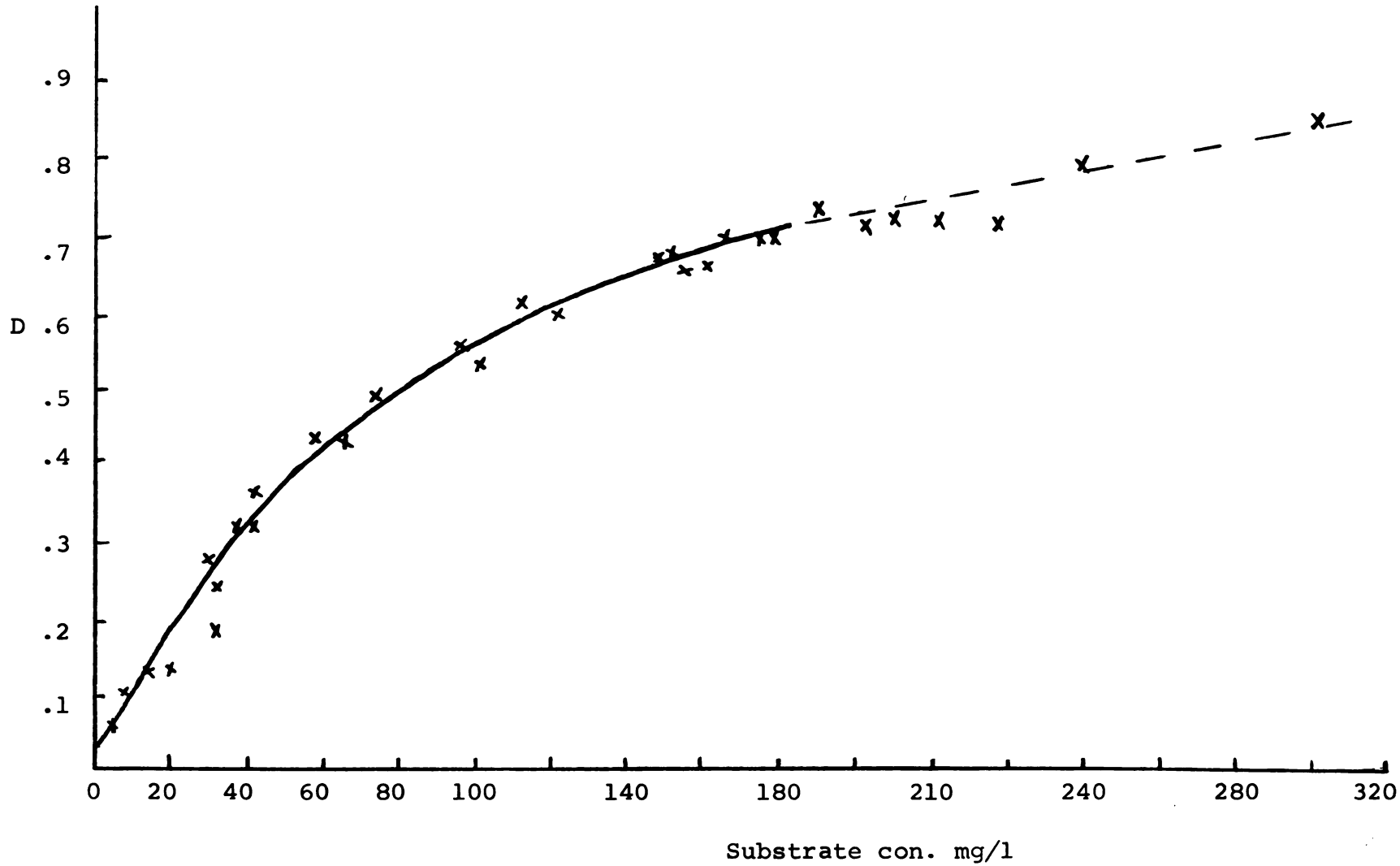

Figure 11. Relationship between D and Substrate Concentration over a D range of 0.059 to 0.855.

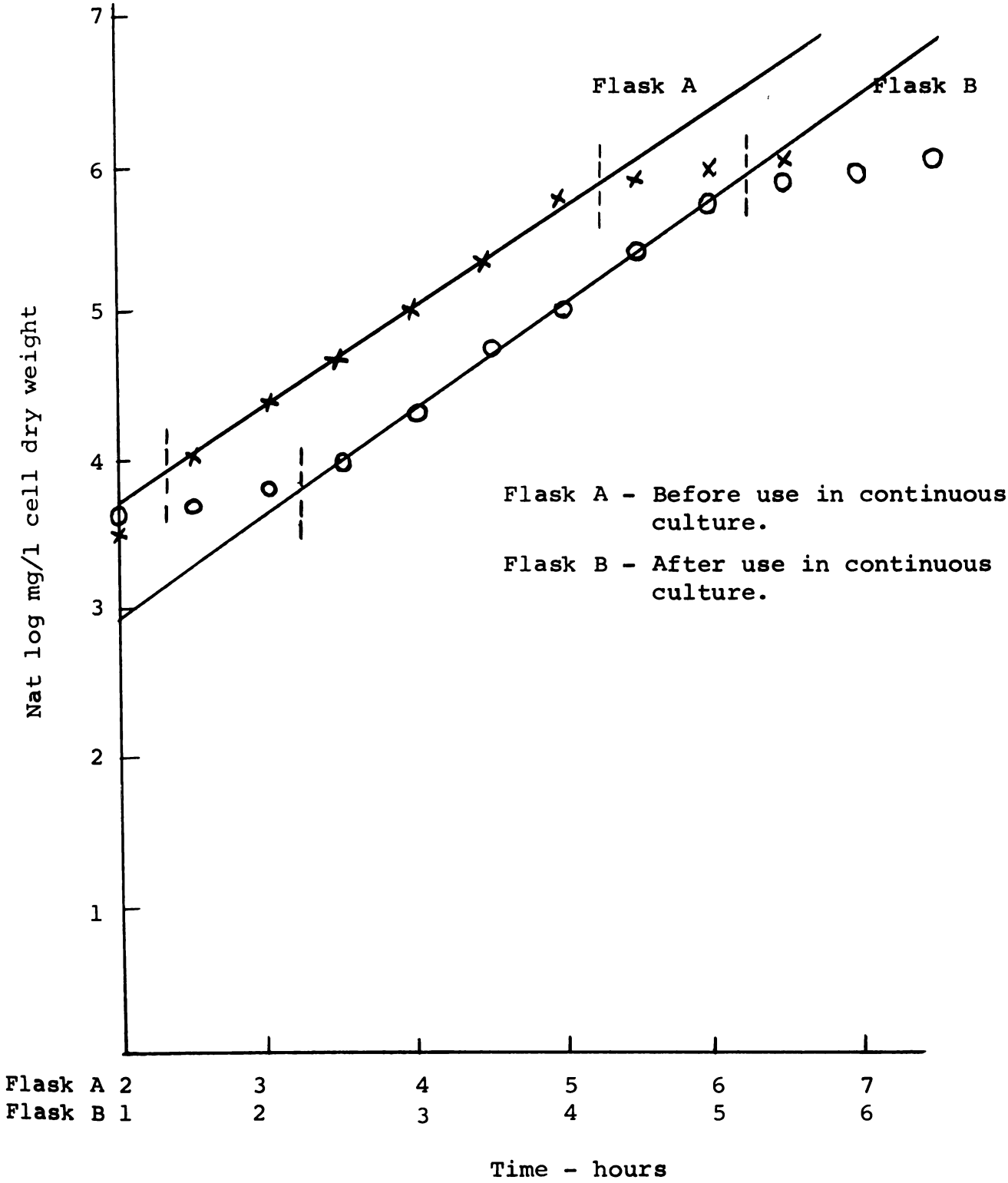

Figure 12. Batch Culture Determination of k before and after growth in the continuous flow unit.

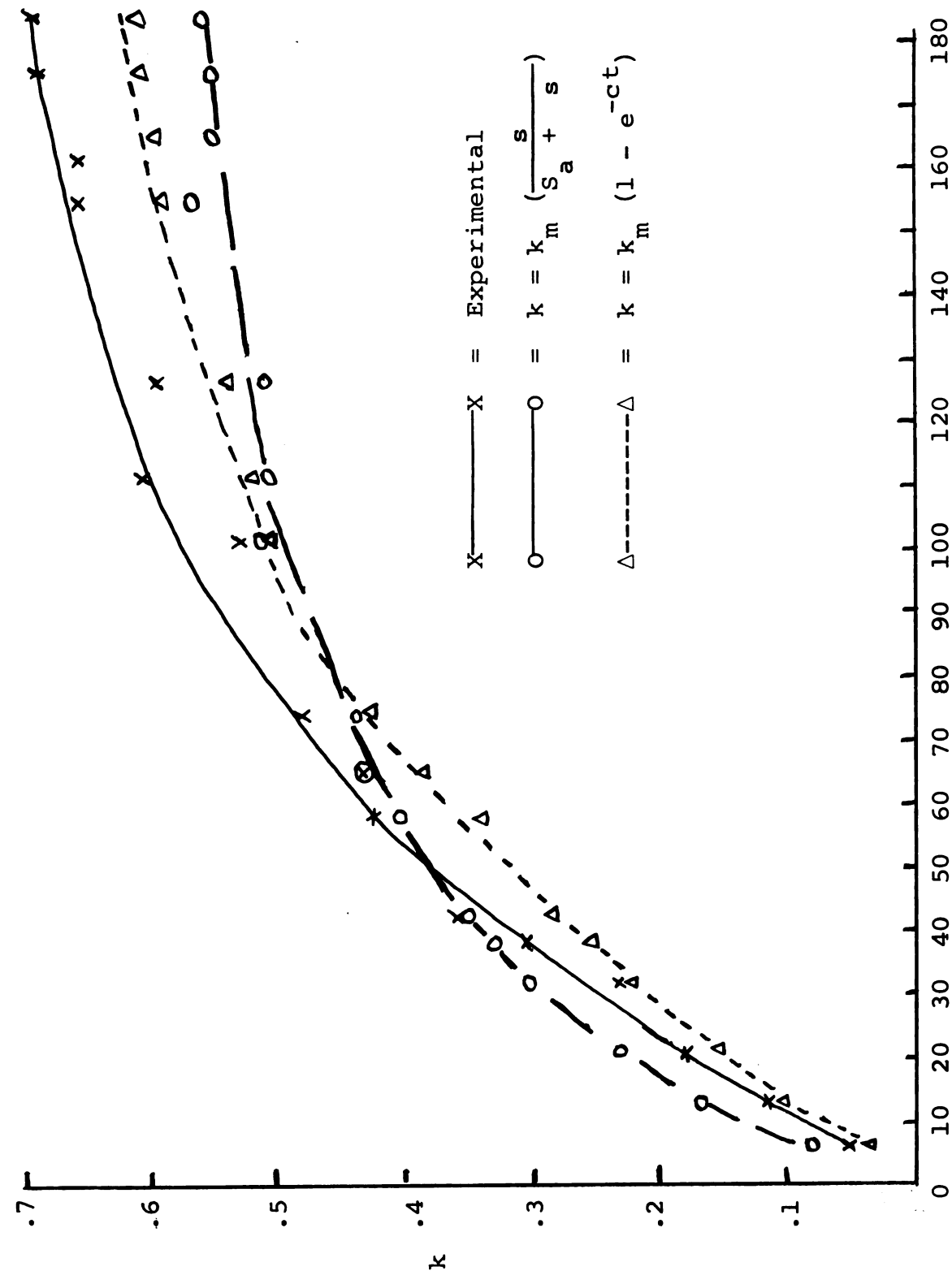

Figure 13. Relationship between Substrate Concentration, Experimental and Calculated k values.

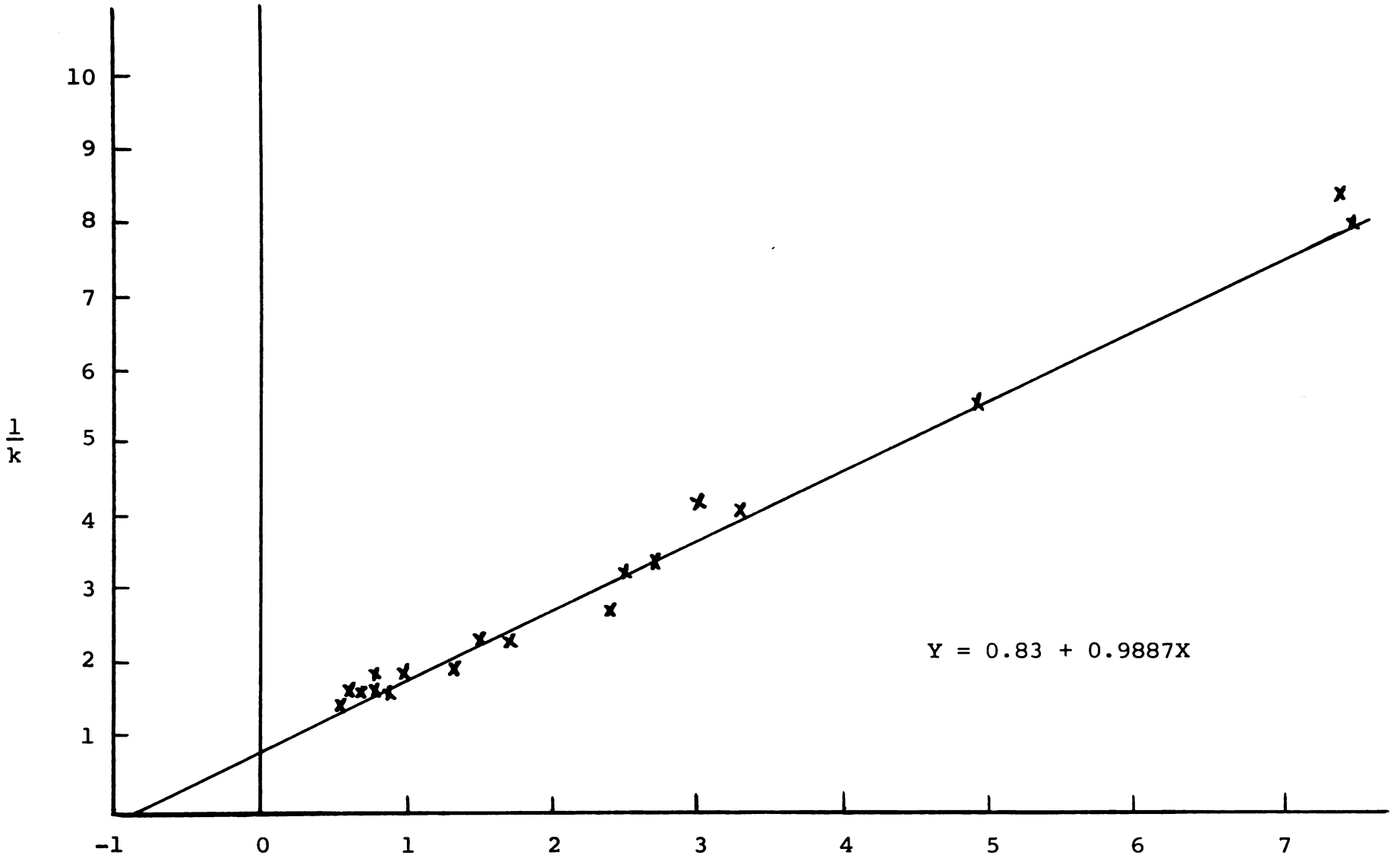

Figure 14. Lineweaver-Burke Plot of Steady-state Data.

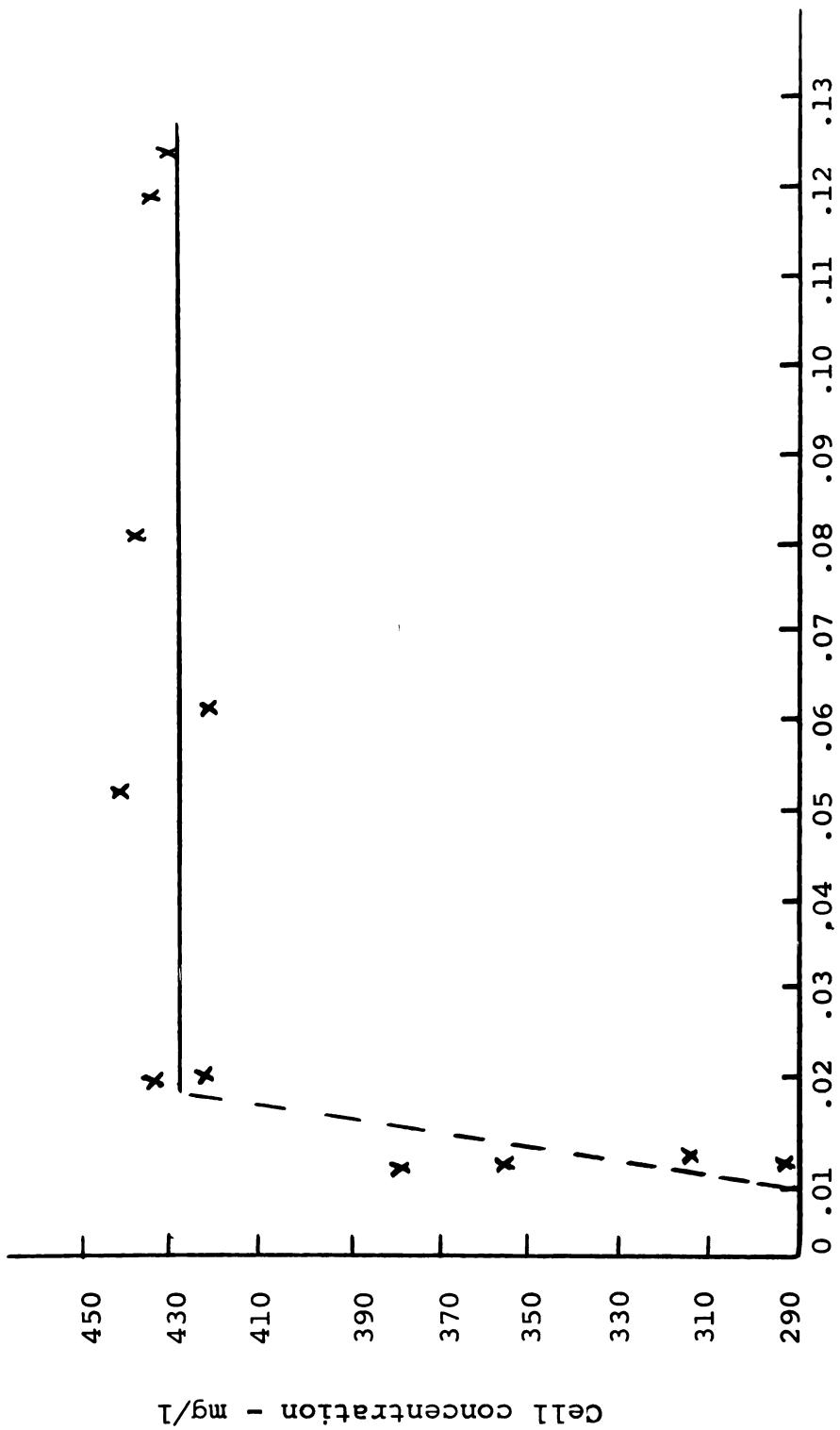

Figure 15. Relationship between Cell Concentration and D at Low D values.

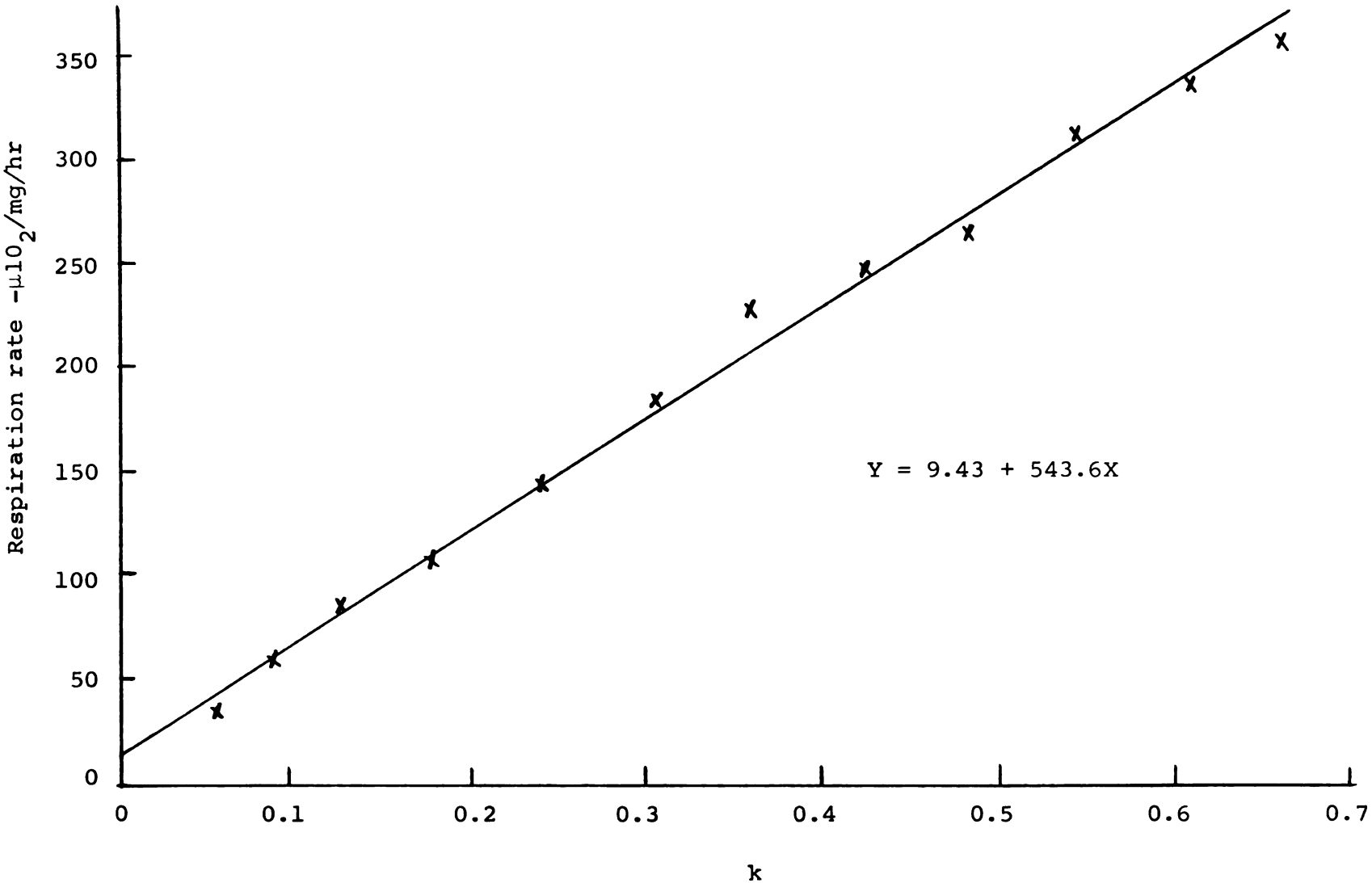

Figure 16. Relationship between Respiration Rate and k during the Steady-state.

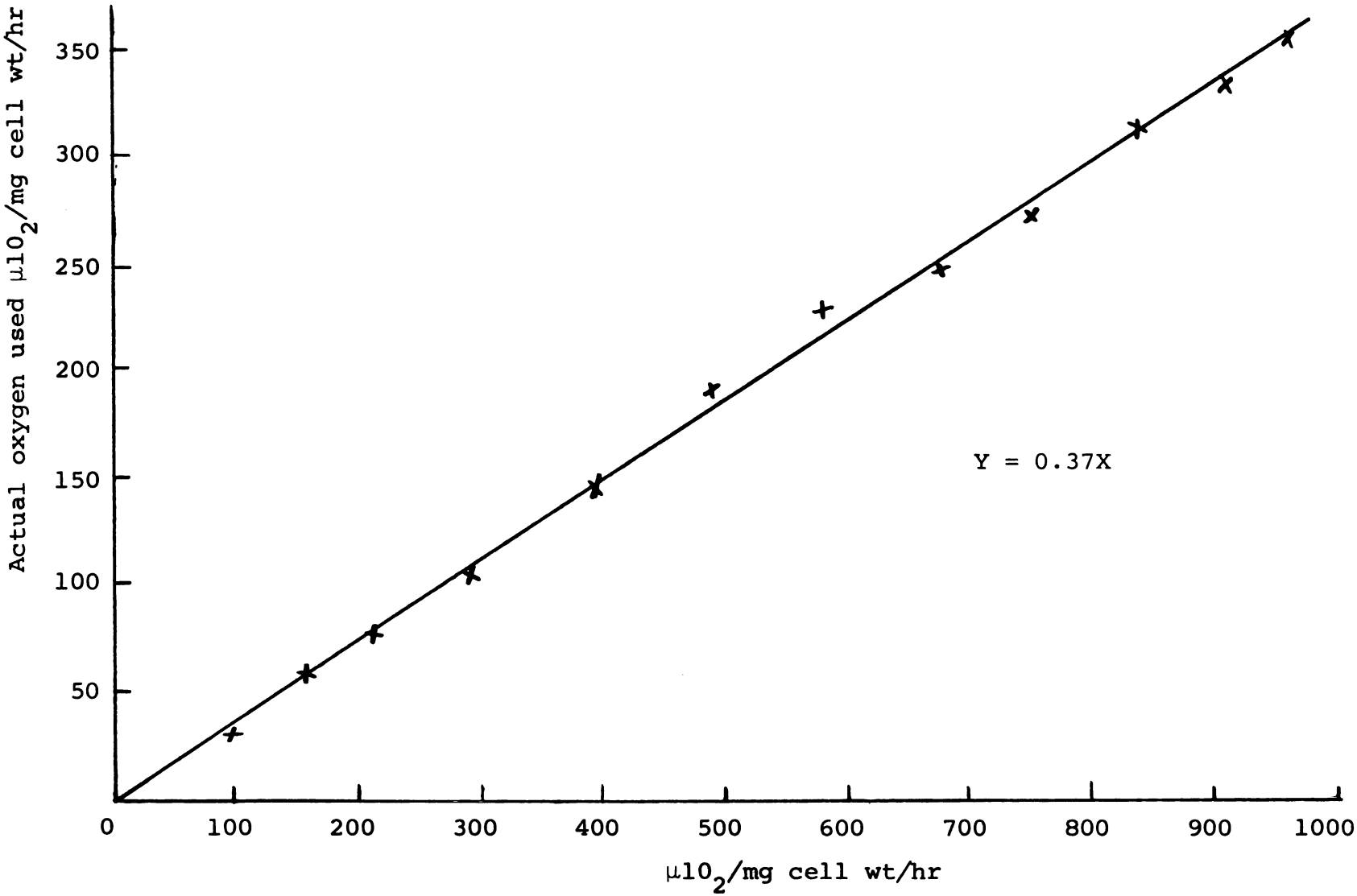

Figure 17. Actual µl oxygen used vs theoretical oxygen needed for complete oxidation of sugar consumed during the steady-state.

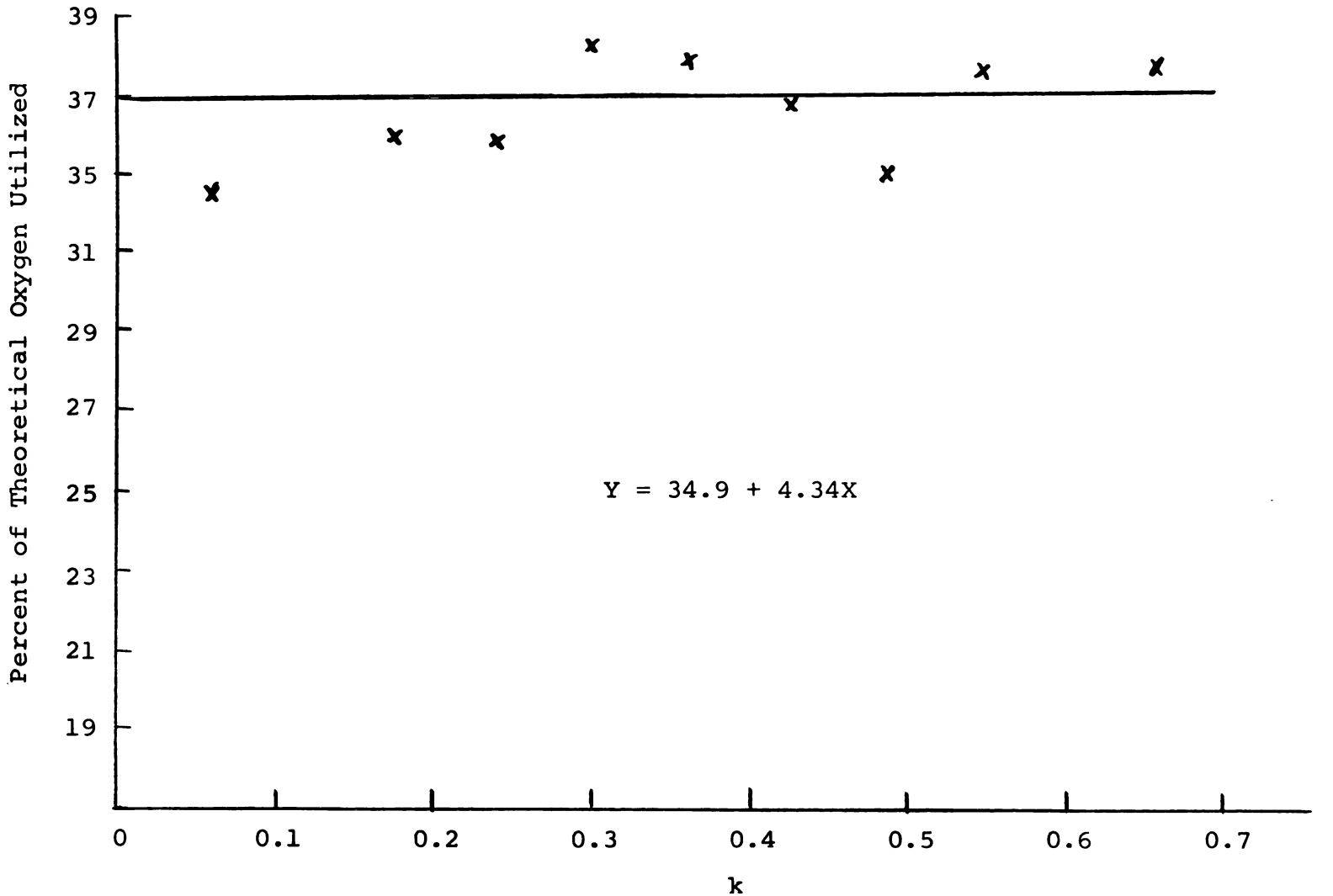

Figure 18. Percent of Theoretical Oxygen Utilized vs k during the Steady-state.

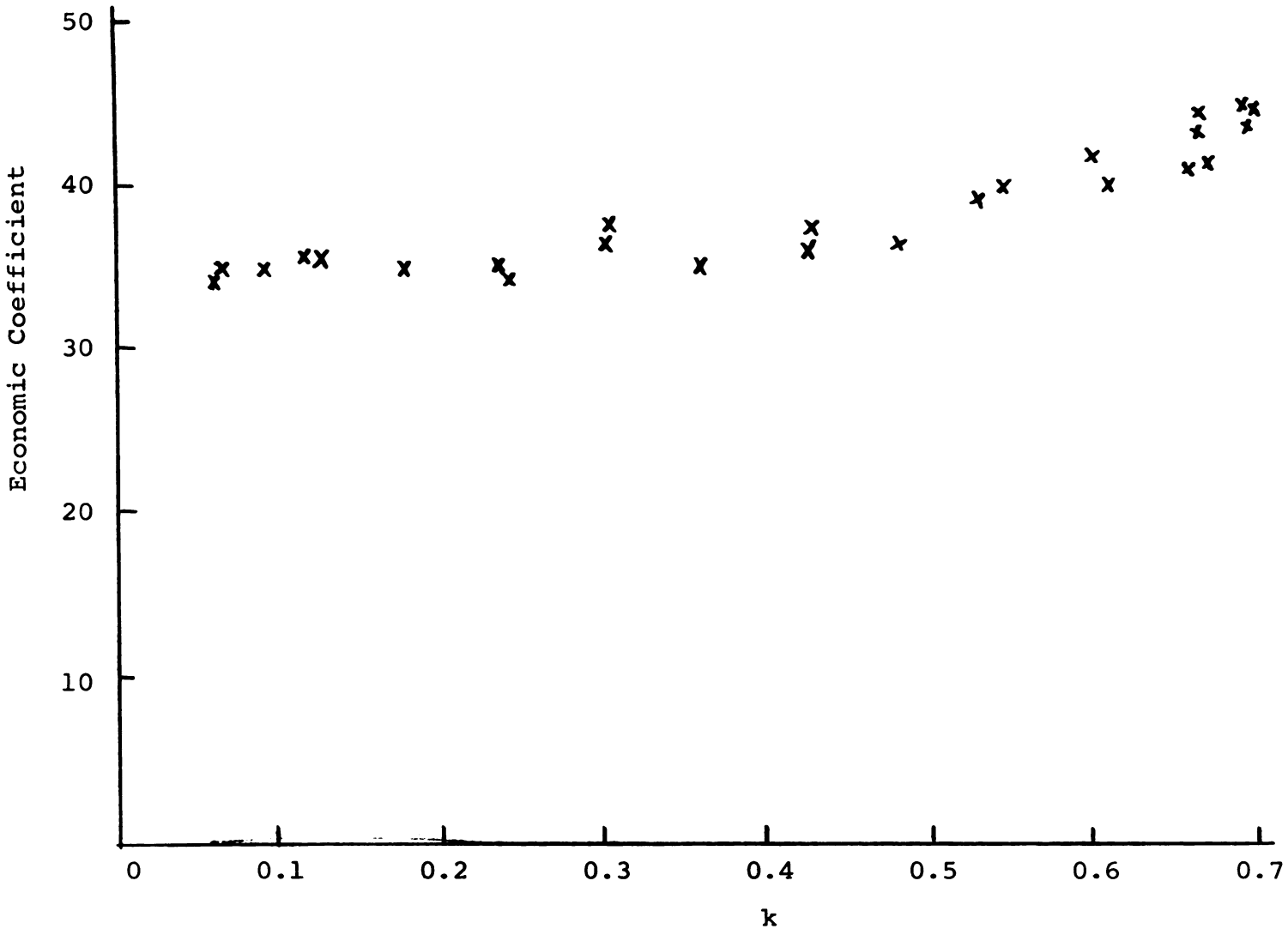

Figure 19. Relationship between Economic Coefficient and k during the Steady-state.

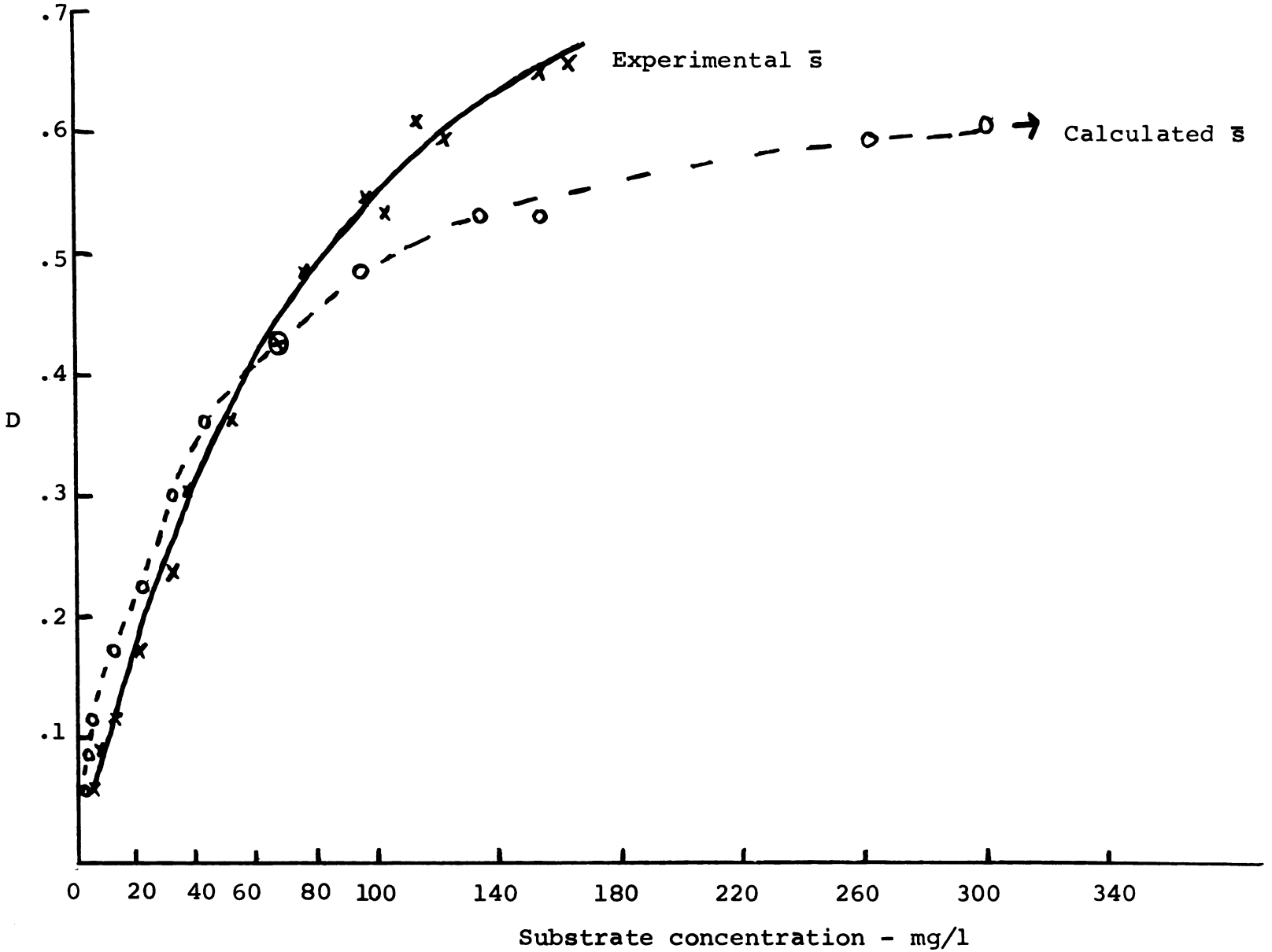

Figure 20. Comparison of experimental and calculated $\bar{s}$ values

VI. DISCUSSION

The primary object of this dissertation was to compare the results obtained during the operation of a continuous flow unit with those predicted by the theory originated by Monod (1950) and elaborated on by Novick and Szilard (1950b) and Herbert et al., (1956). It is also of interest to compare the experimental results with the results that might be predicted by the theories of other workers.

A feature that is common to most theories of continuous flow culture is that the net growth must be the resultant of exponential growth minus exponential wash-out. This may be expressed mathematically by re-writing equation (10) as follows:

$$\frac{d\ (\log_e x)}{dt} = k - D \qquad (22)$$

It follows from this equation that when a steady-state ($d(\log_e x)/dt = 0$) exists, then k is equal to D, i.e., the specific growth rate must be equal to the dilution rate.

An apparent error that has been made by a number of authors in mathematical discussions of continuous culture is to confuse, in equation (22), the specific growth rate k (which varies with the substrate concentration) and the maximum growth rate k_m. If, in the above equation k equals k_m equals a constant, it must follow that a steady-state is possible only at one particular

flow rate, i.e., when D is equal to k_m. This is the assumption that has been made by Golle (1953) and Finn and Wilson (1954). The same idea appears to be implicit also in the writings of Adams and Hungate (1950) and Northrop (1954). This is in obvious disagreement with the results presented in Section V. The experimental data show very conclusively that logarithmic growth is possible at any k value below k_m and that a continuous culture is an inherently stable system that will adjust itself automatically to changes in dilution rate at dilution rates below $D = k_m$. The data presented in Figure 9 and Tables 17 and 18 show that any number of steady-states can be obtained at different dilution rates as the theories of Monod (1950), Novick and Szilard (1950b) and Herbert et al., (1956) predict.

The theories of Monod (1950) and Novick and Szilard (1950b) predict that steady-states can be obtained at different dilution rates anywhere between zero and k_m. Herbert et al., (1956) also stress this point. The data presented in Figures 9 and 15 indicate that any number of steady-states may be obtained at dilution rates varying from 0.02 to 0.69. As may be seen in Figure 15 there is a decrease in cell concentration below dilution rates of 0.02. This indicates that in the operation of a continuous flow unit there is a minimal dilution rate below which the steady-state no longer exists. The probable reason for this minimal dilution rate may be seen

in the data presented in Table 18. As the dilution rate reached values of 0.02 and lower the substrate concentration in the unit reached values of 1 mg/l or lower. It is possible that at a substrate concentration of 1 mg/l or below k becomes zero.

The essential feature of the theories given by Monod (1950), Novick and Szilard (1950b) and Herbert et al., (1956) is that they take into account the observed facts that bacteria can grow only at the expense of the substrate utilized, and that their specific growth rate is a function of the substrate concentration. It then becomes obvious that a continuous culture unit is a device for controlling growth through control of the substrate concentration; each dilution rate fixes the substrate concentration at that value which makes k equal to D. This important role of the substrate is not considered in the mathematical papers of Golle (1953), Finn and Wilson (1954), Adams and Hungate (1950) and Northrop (1954).

Herbert et al., (1956) state the following on the relationship between substrate concentration and dilution rate: "over the useful range of flow rates in a continuous culture, the substrate is nearly completely utilized and the issuing medium virtually exhausted; hence negligible growth could occur in any subsequent culture vessels in series with the first. Our experimental results confirm that almost complete

utilization of substrate does in fact occur." As may be seen from the data presented in Figure 11 the amount of substrate leaving the continuous flow unit over the useful range of dilution rates cannot be considered negligible. It is probable that this is relative and may depend on the type of organism and type of media used. The substrate concentration in the effluent increased with increasing D values and at D values approaching k_m reached concentrations as high as 180 mg/l. This represents approximately 18 percent of the substrate put into the unit and cannot be considered as "almost complete utilization of substrate" as stated by Herbert (1959b).

In considering the relationship between cell concentration and D, as presented in Figure 9, a number of quantitative divergences from the theory of Monod (1950) have been summarized by Herbert (1959b). These are presented graphically in Figure 21.

In Figure 21, curve (a) is the usual theoretical plot of cell concentration against dilution rate. One type of divergence is illustrated by curve (b); here instead of the cell concentration falling sharply to zero at a wash-out point slightly higher than k_m the wash-out rate of organisms from the unit is lower than theoretical considerations predict. As may be seen from the data presented in Figure 9 this is the type of curve that was obtained in these experiments. This

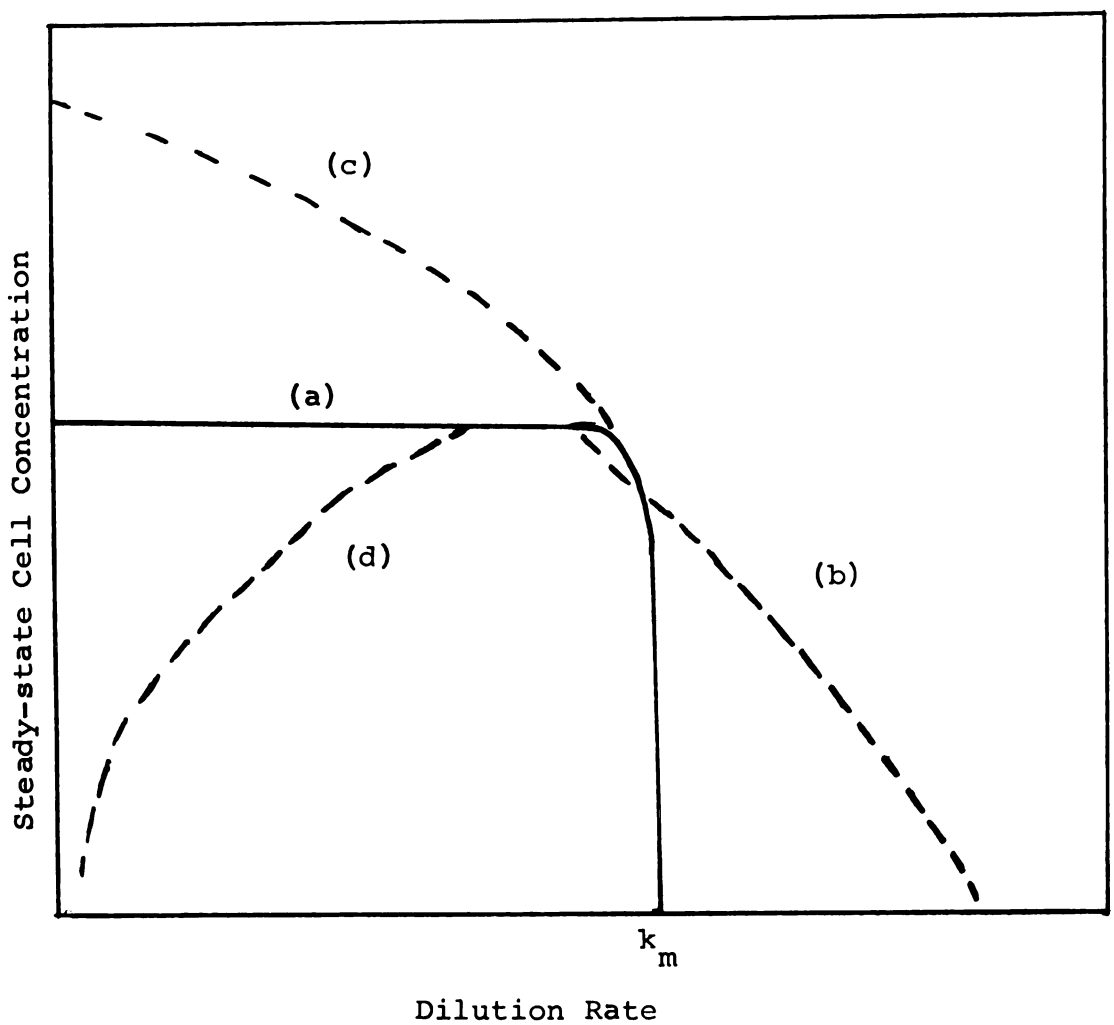

Figure 21. Quantitative Divergences from the Theory of Monod.

type of curve was explained by Herbert (1959b) as an artifact due to imperfect mixing of the inflowing culture medium with the contents of the continuous flow unit. It would appear that under conditions where D is greater than k_m the organisms present in the unit would continue to grow at a rate equal to k_m. Equation 17 was presented in Section V to take into account this continued growth. The actual wash-out rate obtained from the use of this equation was still less than the predicted rated. Herbert et al., (1956) and Herbert (1959b) also found the actual wash-out rate to be less than the predicted theoretical value.

A second type of deviation from the theoretical is shown by curve (c) in Figure 21. Herbert (1959b) explains this type of curve as being due to non-consistency of the yield coefficient Y. In this case Y is said to increase as the growth-rate decreases. This type of behavior was first observed by Holme (1957) with E. coli growing in glucose-NH_3 medium with NH_3 as the growth limiting substrate, and was shown to be due to the accumulation of intracellular glycogen at low growth rates. Herbert (1959b) also found similar results with Torula utilis growing in an ammonia limiting medium. In this case also the increased cell yield at low growth rates can largely be accounted for by the increase in cell carbohydrate.

The opposite type of behaviour is illustrated by curve (d) in Figure 21; here the yield coefficient Y decreases at low growth rates. Herbert (1959b) found this type of curve with Torula utilis growing on a glucose-NH_3 medium with the carbon source as the growth limiting substrate and also with A. aerogenes growing on a glycerol-NH_3 medium with glycerol as the growth limiting substrate.

Similar results, to a limited degree, have been obtained in these experiments. From the data shown in Figure 19 it may be seen that as the growth rate increases the economic coefficient increases from 44 to 54 percent. This would indicate that the organisms are converting substrate into cell material at a ten percent higher efficiency at the high k values than at the lower k values.

The most probably explanation of these results is that in addition to anabolic metabolism of the organisms they also have a constant endogenous metabolism, by which cell-substance is oxidized to carbon dioxide. This has been expressed mathematically by Herbert (1959b) by the following equation:

$$\frac{dx}{dt} = (k - E)\, x \qquad (23)$$

where E is a constant term representing the endogeneous metabolism.

More direct evidence for the existence of this constant

endogenous metabolism comes from the studies of Herbert (1959b) on cell respiration at different growth rates. In this experiment, which was done with A. aerogenes, the respiratory carbon dioxide output was found to be a linear function of the growth rate. However, the straight line did not pass through the origin but extrapolated back to a finite value when the growth rate was zero. These results were explained by assuming the total respiration to be the sum of an anabolic respiration which is proportional to the growth rate and an endogenous respiration independent of the growth rate. Further evidence comes from carbon balance studies (Herbert, 1959b) which have shown that, as the growth rate is reduced, a higher proportion of the substrate carbon used is converted to carbon dioxide and a lower proportion to cell carbon.

The results obtained from these experiments give additional evidence for the existence of an independent endogenous respiration rate. The data given in Figure 16 inidcate a linear relationship between the respiration rate of the organism and k during the steady-state operation of the unit. The equation for this linear relationship between respiration rate and k was calculated by the method of least squares and found to be

$$Q_{O_2} = 9.43 + 5436\ k$$

The value 9.43 may be considered to represent the respiration rate of the organism when k is equal to zero under these experimental conditions.

Whether or not one should correct total observed respiration values (exogenous values) by subtracting endogenous rates has long been a debatable problem in various studies of bacterial metabolism. With bacteria having low endogenous and high exogenous rates of respiration, correction may not markedly influence the values obtained. Wilner and Clifton (1954) have reviewed earlier studies dealing with this problem as it relates to oxidative assimilation. They found with B. subtilis, a species exhibiting relatively high rates of endogenous respiration and only several-fold increases in rates of oxygen consumption in the presence of utilizable substrates, that the percentages of assimilation varied markedly with the concentration of substrate employed. On correction for observed endogenous values the percentages of assimilation agreed quite closely. They interpreted this as indicating that endogenous respiration continues at about the same rate, in either the presence or absence of an oxidizable foodstuff. Santer and Ajl (1954) came to the same conclusion in studies with Pasteurella pestis. They reported that washed cells grown in the presence of labeled substrate evolved labeled carbon dioxide at about the same rate irrespective of the

presence or absence of substrate.

The observations that endogenous respiration does continue in many species, or in some may even be stimulated, lend further support to the concept of the necessity for a pool of intracellular organic matter needed by the organism, not only for the maintenance of the status quo, but also for use during growth.

To this writer's knowledge there are no published figures on the percentage of oxidative assimilation of glucose by E. coli during the steady-state operation of a continuous flow unit. As was noted in Section V, a linear relationship was obtained between the actual amount of oxygen consumed and the theoretical amount of oxygen needed for complete oxidation of the sugar used by the organisms. Data pertaining to this relationship are presented in Table 21 and are shown graphically in Figure 17. These data indicate that 33.8 to 38.9 percent of the theoretical oxygen needed for complete oxidation of the amount of glucose assimilated was actually consumed. An equation for the linear relationship between the actual amount of oxygen consumed and the theoretical amount of oxygen needed for complete oxidation of the sugar used by the organisms was given in Section V as

$$Y = 0.37X$$

where Y is equal to the actual amount of oxygen consumed in

terms of $\mu l O_2$/mg cell dry wt/hr and X is equal to the theoretical amount of oxygen needed for the complete oxidation of the sugar assimilated. This equation indicates that during the steady-state operation of the unit, approximately 37 percent of the assimilated substrate was oxidized. This value is further substantiated by the relation between percent of theoretical oxygen utilized and k during the steady-state operation of the unit that is shown in Figure 18. The data shown in this figure also indicate that, regardless of the value of k, 37 percent of the theoretical oxygen needed are consumed.

The only data available, for comparative purposes, were obtained using batch culture techniques and washed suspended cells. The values given for the percentage of glucose assimilated that was oxidized ranged from 50 percent (Cook and Stephenson, 1928; Clifton and Logan, 1939; and Siegel and Clifton, 1950) to 70 percent (Krebs, 1937). The latter value of 70 percent was obtained at a temperature of 40 C.

The data obtained during these experiments indicate that the efficiency of conversion of substrate into cell material increased approximately 10 percent as the k values, during the steady-state operation of the unit, increased and that at the same time the percentage of the glucose assimilated that is oxidized remains at a constant value of 37 percent.

This would indicate that 44-54 percent of the substrate was converted into cell material; 37 percent converted into carbon dioxide and water; and 9-19 percent that might be accounted for by the production of by-products.

It was noted in Section V that the dissolved oxygen concentration in the unit varied between 0.5 and 4.8 ppm during the steady-state operation of the unit. No investigation of the effects of this varied dissolved oxygen concentration on the activity of the organisms was made because it was felt that sufficient evidence has been presented by Winzler (1941) and Longmuir (1954) to indicate that the effect, if any, would be negligible. The amount of available oxygen in a submerged culture depends upon the rate of oxygen transfer from the gas to the liquid phase and also on the rate of oxygen transfer from the liquid to the cell. This later process, as has been shown for yeast by Winzler (1941) and for bacteria by Longmuir (1954), is independent of the concentration of dissolved oxygen unless this is extremely small as compared with the saturation concentration of oxygen.

The data from these experiments indicate that, especially at the low k values, there was not enough sugar present in the Warburg flasks to maintain the observed respiration rate. For example, at a k value of 0.059 and a substrate concentration of 5.1 mg/l each Warburg flask contained 0.026 mg of glucose.

In order to maintain a respiration rate of 34 $\mu l O_2$/mg cell dry wt/hr for one hour the cells in the flask would require 0.11 mg glucose, assuming complete oxidation. At a k value of 0.69 and a substrate concentration of 180 mg/l each Warburg flask contained 0.9 mg of glucose. In order to maintain a respiration rate of 350 $\mu l O_2$/mg cell dry wt/hr for one hour the cells in the flask would require 1.1 mg of glucose. It is suggested that, especially at the low k values, the cells are utilizing stored substrate material in order to maintain the observed respiration rates.

The single most important relationship to be considered in the operation of a continuous flow unit is the relationship between substrate concentration, s, and the growth rate k. The key to the mode of action of a continuous flow unit lies in the way in which the growth-rate k depends on the concentration of a limiting growth substrate in the culture medium. A number of different mathematical equations have been proposed for the relationship between substrate concentration and k. The concepts put forth by Golle (1953), Fin and Wilson (1954), Adams and Hungate (1950), and Northrop (1954) were discussed at the beginning of this section and require no further discussion.

Garrett and Sawyer (1952) proposed a linear relationship between substrate concentration and k. Working with mixed

cultures obtained from activated sludge they state "the results that have been obtained indicate that the rate of growth is directly proportional to the concentration of food remaining up to a critical concentration above which it is constant and independent of the concentration of food." An examination of their experimental data reveals that the k range of their experiments was 0.03 to 0.16 and the substrate determinations were made at only 3 k values (0.05, 0.10, and 0.16). Comparing this with the data presented in Figure 11 it may be seen that their data cover only a very small portion of the total k range that might be expected. It is doubtful that the results which they present are sufficient to state "that the rate of growth is directly proportional to the concentration of food" over a full range of k values. The data presented in Figure 11 clearly show that the relationship between substrate concentration and k is not a linear relationship but is instead a curve that reaches a maximum asymptotically.

The most widely accepted mathematical equation to express the relationship between substrate concentration and k is the one proposed by Monod (1950) (Equation 6). It should be pointed out that, in Section III, the theoretical treatment of continuous culture is based on a minimum number of simplified postulates. It might well be objected that even under ideal conditions the

growth behavior of bacteria cannot be completely represented by such equations as (5), (6), and (7). Such was found to be the case in this series of experiments. As may be seen in Figure 13 Monod's equation provides only partial fit to the experimental data. In the comparison between the experimental curve and that which is predicted by the equation agreement exists only up to a k value of 0.4; from this point on, the theoretical line is considerably lower than the experimental line. One possible explanation for this lack of agreement between experimental and predicted data may be found in one of the basic assumptions made in the development of the theoretical equation. The relationship between growth of bacteria and utilization of substrate is expressed as Y in Equation 7. The assumption was made by Monod that the yield constant Y is actually a constant and this was confirmed to his satisfaction in batch culture studies (Monod, 1942). The data presented in this study indicate that the yield constant Y may not be constant under these conditions. From the data presented in Figure 19 it can be seen that the yield constant Y increased from 44 to 54 percent. Data presented by Herbert (1959b) also indicate that the yield constant Y may be variable.

The experimental data obtained in these experiments were plotted according to the Lineweaver-Burke plot which is based on a modification of Equation (6). The results of this

plot are given in Figure 14. The values of k_m and S_a were calculated from this plot to be 1.20 and 120.5 respectively by the method of least squares. The values are clearly out of line with the experimentally obtained values of 0.69 and 40 and indicate that the relationship between substrate concentration and k does not follow an expression such as Equation (19).

Powell (1959) in a summation of mathematical forms which have been proposed for the relationship between substrate concentration and k lists three additional expressions. The first of these is the monomolecular type of equation used by Teisseir (1936).

$$k = k_m (1 - e^{-cs})$$

The second of these, listed as the Moser equation is the expression

$$k = k_m \left(\frac{s^r}{S_a + s^r}\right)$$

where r represents a constant. This expression is clearly a modification of the original equation used by Monod.

As an additional modification of Monod's original education Powell (1959) rewrites Monod's equation in terms of s as a function of k to give the following form

$$s = \frac{S_a k}{k_m - k}$$

Powell then makes the hypothesis that Monod's original equation

applies to the concentration of substrate inside the organism, but that the access of substrate to the interior is impeded by the cell membrane and by diffusion. He then proposes that s, the concentration outside the cell is given by the relationship

$$S = \frac{S_a k}{k_m - k} + A \frac{k}{k_m}$$

Here the new constant A depends on diffusion coefficients inside and outside the organism, on the permeability of the membrane, and on the shape and surface:volume ration of the organism.

The experimental data obtained in these experiments were applied to the monomolecular type of equation. The method of Reed and Theriault (1931) was used to calculate the constants k_m and c from the exerpimental data. k_m was calculated to be 0.69. This is in complete agreement with k_m values obtained by both continuous flow and batch culture procedures. The value for c was calculated to be 0.012. The resulting equation (Equation 21) was then plotted and compared with the experimental values. This comparison is given in Figure 13. The values obtained by this equation are somewhat lower than the values obtained from the experimental data but the shapes of the two curves are similar. As may be seen from Figure 13 the results obtained by use of the monomolecular type of equation are in better agreement with the experimentally

obtained results than the values calculated by the use of Monod's equation. An almost perfect fit of the experimental curve may be obtained by changing the constant, c, from 0.012 to 0.016 in the momomolecular type of equation.

The values for the steady-state substrate concentrations were calculated using equation 14. The results from these calculations are presented in Table 23 and Figure 20 along with the experimentally obtained values. As may be seen in Figure 20 the calculated values follow the experimentally obtained values closely for k values up to 0.4 then there is a marked deviation between the two. There appears to be no relationship between these very high calculated substrate values and reality as in the case of a k value of 0.688 the calculated substrate value indicates that there is more substrate leaving the unit than is being put into it. It will be recalled that a similar breaking point occurred when the experimental data were applied to Monod's equation (Equation 6). As equation (14) is based, in part, on the relationship given by equation (6) this break at the same point is to be expected.

The values for the steady-state organism concentrations were calculated using equation 15. As may be seen in Table 25 the calculated values are in close agreement with the experimental values for k values up to 0.4. From this point

on the calculated organism concentrations indicate that wash-out is occurring from the unit while the experimentally determined organism concentrations indicate that the steady-state condition still exists. This breaking point again occurs at about the same k value as in Equations (6) and (15). This is again to be expected since equation (15) is based on the fact that equation (6) represents the true relationship between substrate concentration and k. As has already been pointed out, equation (6) does not represent the true relationship between the substrate concentration and k obtained in these experiments.

It should also be pointed out that equations (14) and (15) are based further on the assumption that Y is a constant value and in addition that equation (9) represents the wash-out of organisms from the unit. It has been shown that neither of these assumptions was found to be true in these experiments.

The problem of mutation is one that must always be given consideration in experiments of this type. If a mutation occurred that affected the growth rate of the experimental organism it would seriously affect the results of these experiments. With this possibility in mind the growth rates of the organism were determined by batch culture determinations both before and after its use in the continuous flow unit. The results of these batch culture experiments

are shown in Figure 12. It may be seen from this figure that the maximum growth rate, k_m, of the organism did not change during the experiments. As was pointed out in Section III, mutation would only become a serious problem if the growth rate of the mutant were equal to or higher than the experimental organism.

VII. CONCLUSIONS

A continuous flow unit was constructed and satisfactorily operated over a D range of 0.01 to 0.85. Steady-state conditions existed between D values of 0.02 and 0.69. A non-steady-state condition existed at D values below 0.02 and above 0.69 which resulted in a wash-out of organisms from the unit at a rate faster than they could be replaced by growth. The actual wash-out rate was found to be less than the wash-out rate predicted by theoretical equations.

A maximum growth rate of k_m of 0.69 was established under these experimental conditions. This value was obtained by both batch culture and continuous flow procedures. In addition k_m was calculated by the method of Reed and Theriault (1931) from a series of experimental data. The value of 0.69 obtained by this method was in full agreement with the experimentally obtained maximum growth rate.

The relationship between substrate concentration and the specific growth rate k during the steady-state was established, not as a linear relationship, but as a curve that reaches a maximum asymptotically. Theoretical equations proposed by Monod (1950), Novick and Szilard (1950), and Herbert *et al.*, (1956) did not agree with the experimental relationship obtained between substrate concentration and k.

Furthermore, values for k_m and S_a obtained by a Lineweaver-Burke plot did not agree with experimental data. In contrast the results obtained from the use of a monomolecular type of equation, such as proposed by Teissier (1936), indicated that this type of equation more correctly expresses the relationship between substrate concentration and k. Data were presented which indicated that at a substrate concentration of 1 mg/l or below k approaches zero. The data also indicate that the specific growth rate k becomes independent of substrate concentration at glucose concentrations above 180 mg/l.

A linear relationship was established between k and the respiration rate of the organism. The following equation was found to express this relationship.

$$Q_{O_2} = 9.43 + 43.6k$$

The maximum oxygen uptake rate was obtained at k_m and did not further increase at D values greater than k_m. A value of 9.43 $\mu l O_2$/mg cell dry wt/hr appears to be the endogenous respiration rate of the organisms under these experimental conditions. The data further indicated that under these conditions approximately 37 percent of the assimilated substrate was oxidized independently of k.

The economic coefficient of the organism was found, not to be constant, but to increase from 44 percent at the lower k values to 55 percent at the higher k values.

The theoretical steady-state equations, proposed by Monod (1950) and Herbert et al., (1956) for the relationship between $\bar{s}$, $\bar{x}$, Y, S_R, S_a, D and k_m were found to be inadequate in expressing the experimentally obtained relationships.

VIII. APPENDIX

A. Composition of Reagents used in Glucose Determinations

(1) Copper Reagent A

25 gm anhydrous sodium carbonate

25 gm Rochelle salt

20 gm Sodium bicarbonate

200 gm anhydrous sodium sulfate

Dissolve reagents in 800 ml water and dilute to 1 liter.

(2) Copper Reagent B

15% $CuSO_4 \cdot 5H_2O$) containing one or two drops of concentrated sulfuric acid per 100 ml.

(3) Arsenomolybdate color reagent

Dissolve 25 gm of ammonium molybdate in 450 ml water

Add 21 ml of concentrated sulfuric acid

Add 3 gm of $NaHAsO_4 \cdot 7H_2O$ dissolved in 25 ml water

Place in an incubator at 37 C for 24 to 48 hours.

(4) 5% $ZnSO_4 \cdot 6H_2O$.

(5) Approximately 0.3 N barium hydroxide. The zinc and barium solutions should be adjusted so that 5 ml of zinc require between 4.7 and 4.8 ml of barium to produce a definite pink to phenolphthalein.

B. Composition of Reagents used in the Dissolved Oxygen Determinations

(1) Manganous sulfate solution

480 gm $MnSO_4 \cdot 4H_2O$ dissolved in 1 liter water

(2) Alkaline-iodide reagent

500 gm sodium hydroxide

135 gm sodium iodide

Dissolve in 1 liter water

(3) Starch solution

5 to 6 gms of soluble starch dissolved in 1 liter boiling water

(4) Sodium thiosulfate solution

6.205 gm sodium thiosulfate dissolved in 1 liter freshly boiled water. This solution was standardized against a standard potassium bi-iodate solution.

C. Composition of Brodie's Solution

23 gm Sodium chloride

5 gm Sodium choleate

5 gm Dreft

500 ml distilled water

Colored with Safrain dye.

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