HERBERT E. KUBITSCHEK

Introduction to Research with CONTINUOUS CULTURES

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H. E. Kubitschek

Division of Biological and Medical Research
Argonne National Laboratory
Argonne, Illinois

Prentice-Hall, Inc., Englewood Cliffs, N. J.

To Jenny

PRENTICE-HALL INTERNATIONAL, INC., *London*
PRENTICE-HALL OF AUSTRALIA, PTY. LTD., *Sydney*
PRENTICE-HALL OF CANADA, LTD., *Toronto*
PRENTICE-HALL OF INDIA PRIVATE LIMITED, *New Delhi*
PRENTICE-HALL OF JAPAN, INC., *Tokyo*

Current printing (last digit):
10 9 8 7 6 5 4 3 2 1

13-495358-4

Library of Congress Catalog Number 72-140684

Printed in the United States of America

Preface

Up to this time, the transmission of information about continuous culture techniques—apparatus, operation, and potential application—usually has been by personal communication. If one wished to adopt continuous culture techniques he sought the advice of someone who had experience in this field. Of course, reviews have been published and collections of papers delivered at symposiums have appeared, but none of these presents comprehensive information in a fashion that may be assimilated readily by the novice. This first textbook on continuous cultures attempts to satisfy the need for an instruction manual, to provide a general introduction to the theory and concepts that are involved, and to describe applications of continuous culture techniques.

The first three chapters present the history, properties, principles, and techniques of continuous cultures. The remaining chapters describe historically or conceptually important applications of continuous cultures. In these latter chapters, introductions are included because many of the applications are in such specialized areas that not all readers can be expected to be familiar with them, but at the same time, these introductions are kept brief because more comprehensive and balanced reviews are available, and referred to. There is no attempt to treat the subjects in these chapters from the various viewpoints of molecular biology or microbial genetics. Instead, the accent is upon the relationships established with continuous cultures.

Continuous culture techniques are being applied to rapidly developing areas of biological research. Not all results are secure, nor are interpretations held in complete agreement by all investigators. Nevertheless, I have

chosen to describe some present problems, holding the opinion that these mysteries will add interest to the subject, and hoping that some readers of this book will be stimulated to contribute to the solutions.

I wish to thank Dr. Alexander Hollaender, editor of this series, and Dr. M. R. Zelle for suggesting that I write this book. I am indebted to many people for their aid and advice, especially Betty Guttman and Drs. M. L. Freedman, A. L. Koch, R. J. Munson, and R. B. Webb, as well as Dr. R. W. Wolfgang for reading the entire manuscript. Finally, I particularly wish to acknowledge my appreciation to my wife, Jenny, for her constant aid and careful readings of the entire manuscript throughout its several stages of development.

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Introduction
ONE

Continuous cultures of growing and dividing cells have been used for research in biochemistry, genetics, and cell physiology. Although goals differed, all of these studies were concerned with some aspect of cell function. It is here, in fact, that continuous cultures offer a major advantage: they provide an effective method for examining the normal functions of the ultimate unit of independent life as we know it, the cell.

Continuous cultures permit a degree of control and flexibility that was not available with earlier methods of cell culture. These advantages have led to a better understanding of cell growth, metabolic control, and the nature of mutational processes. In the past, the use of continuous culture apparatus in research was sometimes limited by its expense and difficulty in maintenance. Today, simple but reliable equipment is within the reach of any college or commercial laboratory.

A. PROPERTIES AND ADVANTAGES OF CONTINUOUS CULTURES

Nature and Definition

When nutrients are supplied at a constant rate to a growing culture, the cell population reaches a "steady state" of growth and division, in which the total number of cells and the rate of cell division remain constant. To

maintain this steady state the volume of the culture must be kept constant; in other words, the rate at which culture medium is supplied must be equal to the rate at which the culture (cells and depleted medium) is removed.

In practice, it is not possible to obtain an ideally steady state for at least two reasons. First, true steady-state growth requires that all cells in the culture are exposed to the same environment. This condition cannot be attained in continuous cultures because the addition of nutrient produces chemical gradients that disappear only after complete mixing. These gradients arise whenever nutrient is added, whether in a steady stream or, as more frequently occurs, in droplets or other small discrete volumes. Second, mutation and selection occur continually in every culture, causing evolutionary changes in growth and other cell characteristics. Despite these changes the growth of these cultures is often very well described by the mathematical framework of steady-state cultures. Because it is impossible to obtain truly steady-state conditions, however, we shall use the following definition:

> A continuous culture is a flow system in which individual cells are suspended in a (nearly) constant volume, at or near a steady state of growth established by the continual addition of fresh growth medium, and the continual removal of part of the culture.

An alternative definition of continuous cultures (Herbert, 1964) classifies them as "open" when the culture volume remains constant, and as "closed" when there is no provision for washout of used media or when any component of the culture system is continuously recycled. Closed systems are more complex than open systems and have a limited application in research, so for our purposes it is more convenient to use the definition above.

Advantages of Continuous Cultures over Batch Cultures

Classically, the growth of cells was studied chiefly in *batch* cultures, that is, cells were inoculated into a flask of fresh medium and simply allowed to grow. The classical growth pattern of such a culture is shown in Fig. 1-1. At first there is a *lag* period, during which cells increase in volume and mass, but do not divide. The second phase is one of exponential growth; here cell numbers increase exponentially. That is, if N_0 is the number of cells at any time taken as zero, then the number N of cells at any later time t during exponential growth is given by

$$N = N_0 e^{\alpha t}, \tag{1.1}$$

where α is a constant that depends on growth rate. (Because a plot of log N is a straight line during this phase, it is sometimes referred to, incorrectly, as the *logarithmic* growth phase.) Exponential growth is followed by a *sta-*

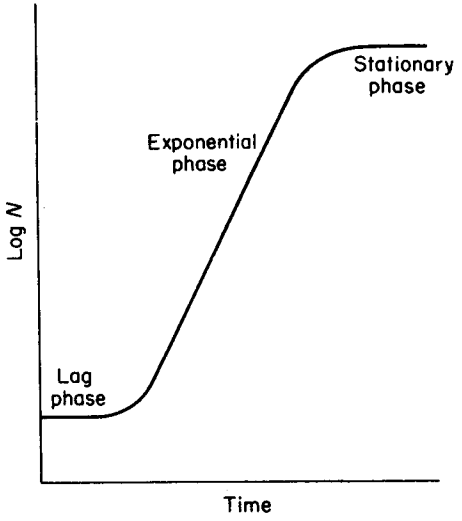

Fig. 1-1. Culture Growth. After inoculation, the number of cells *N* remains constant during the inital, or *lag* phase, then increases exponentially, and finally levels off again during the *stationary* phase.

tionary phase, where growth and division apparently cease. Here, the population has reached a density limited by the lack of nutrients or by other inhibitory conditions.

In batch cultures, cell properties such as size, composition, and functional characteristics vary considerably during the growth of the culture, often making interpretation of results difficult. Average cell properties can remain essentially constant during the exponential growth phase, but this is so usually for only very limited periods. Other advantages of continuous cultures over batch cultures are:

(1) Rates of growth and division are more easily controlled and maintained for long periods.

(2) Cell concentrations can be set and maintained independently of growth rate.

(3) Cells can be grown for longer periods in a constant chemical environment.

(4) Very low levels of critical nutrients, growth factors, mutagens, or toxic agents can be maintained during the growth of the culture.

(5) Cell sizes and biochemical composition are more easily selected and maintained with a given strain, because these cell characteristics depend upon the rate of growth.

Some of these advantages are matters of the degree of control available to the experimenter. Rates of growth and division can be changed in batch cultures by providing a different growth medium for each growth rate, but this approach is cumbersome compared to the fine control possible with some continuous culture techniques. The design and performance of experiments with such continuous cultures is simpler, and the data are easier to obtain and more accurate than those from batch cultures. Consequently, fewer experiments need be done to establish results.

B. CONTROL: INTERNAL AND EXTERNAL

Mode of Control

Rates of cell division in batch cultures depend upon the medium in which the culture is grown. With the bacterium *Escherichia coli*, for example, the average time between successive cell divisions, or *generation time*, at 37°C is about 2 hr in most of the common media consisting of inorganic salts and an acetate as the carbon source. (See Roberts et al., 1955, for a list of frequently used salt solutions.) When acetate is replaced by glucose, division rates are increased about twofold. Even more rapid rates of division are obtained when certain amino acids and other organic molecules which the cells must otherwise synthesize are added. A point is reached, however, where further additions no longer increase cell division rates appreciably. A maximum rate of about 3 generations per hour, corresponding to a generation time of 20 min, is achieved for these bacteria by growing them in nutrient broth at 37°C.

When nutrients are supplied in abundance and cells approach their maximum rate of division, growth is limited by internal processes taking place within the cell itself. This occurs during the exponential phase in batch cultures, even when growth rates are low. Under these conditions where growth rate is limited by an internal cellular reaction, growth is said to be under *internal control*. Alternatively, when growth rate is limited by the availability of a growth factor supplied externally, then growth is said to be under *external control*. If the rate of supply of glucose is continually decreased, for example, rates of cell growth and division will ultimately diminish because this carbon source is no longer sufficiently available. Cell concentration now will depend upon the concentration of glucose, and growth rate upon the rate at which glucose is supplied.

Whether the mode of control in flask cultures is internal or external can be determined by measuring the growth constant α (see Eq. 1.1) at several different concentrations of the critical growth factor at a given temperature. The value of α will remain constant if growth is internally con-

trolled, but α will be directly proportional to the concentration of the critical growth factor if control is external. Alternatively, the mode of control of growth can be determined at constant concentration of the critical substrate by changing the temperature. If growth is under external control, then the generation time will remain constant; if growth is under internal control, the generation time usually can be expected to vary by a factor of two or three over a 10-degree range.

Control of growth also can be either external or internal in continuous cultures. In *turbidostats*, growth is internally controlled, and a photodetector is used to maintain constant turbidity of the culture. Cells grow at or near their maximum rate in the nutrient medium that is provided. In *chemostats*,* growth is controlled externally by limiting the supply of a critical growth factor. In these cultures, growth rates may be varied widely with any growth medium, although the maximum rate cannot be maintained. The dependence of growth rate upon the concentration of a limiting substrate is shown in Fig. 1-2.

The names turbidostat and chemostat should not be taken as indicating that only one of them establishes constant turbidity while the other establishes constant chemical conditions. Both, in fact, provide constant cell concentrations and constant chemical conditions. These instruments are distinguished by the way they control growth. Because the control systems of both are related, as shown in Fig. 1-2, and because both provide steady-state growth, mathematical descriptions of the two devices are quite similar.

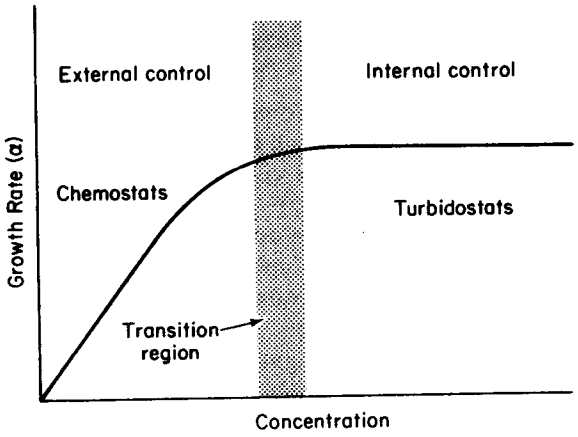

Fig. 1-2. Dependence of growth rate upon concentration of a critical substrate, and the distinctive regions for control of continuous cultures.

*The first syllable is sounded as in *chem*istry, not as in *hem*ostat.

Turbidostats

In turbidostats, cultures are monitored by a photodetector that responds to their turbidity, which is proportional to the cell concentration. As the culture grows, the amount of light scattered into the detector increases (Fig. 1-3). At a chosen (critical) level of light intensity, a relay is triggered, and fresh growth medium is added to the culture. This dilution reduces the turbidity. Some of the culture is washed out through an overflow device used to maintain a constant culture volume.

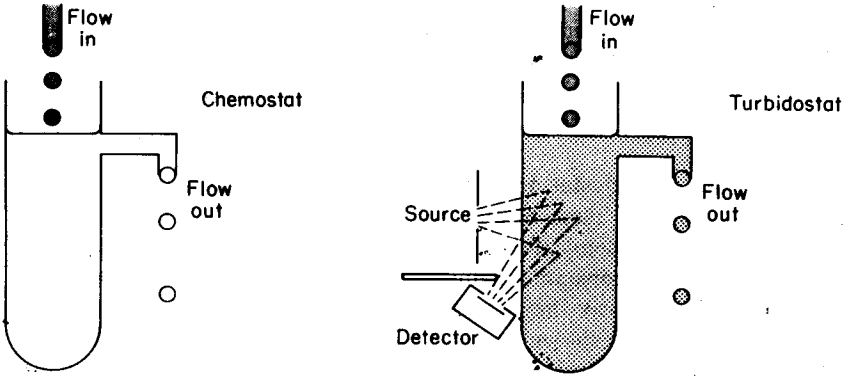

Fig. 1-3. A schematic diagram of a turbidostat and a chemostat. The depth of shading represents the concentration of the critical growth factor. Most of it is consumed in chemostat cultures; little may be consumed in turbidostat cultures.

Cell density can be regulated by optical density rather than turbidity. The light from the source shines through the culture onto the detector. This kind of regulation is limited to more concentrated cultures. Temperature must be kept constant for steady-state growth in turbidostats, because growth rate depends upon temperature as well as upon the growth medium used. Growth rates may also depend upon products released by the cells. At very high cell concentrations, for example, growth rates may be reduced by the production of inhibitory products. The mode of control would nevertheless remain internal, although it might be shifted from the original controlling reaction to a new one involving the inhibitory product.

Chemostats

Chemostat cultures usually are controlled by limiting the amount of a single factor required for growth, such as the carbon source, a required amino acid, or an inorganic ion. In addition, cells capable of photosynthesis can be controlled by light intensity. Other growth factors must be provided

in excess. In chemostats, almost all of the limiting growth factor is utilized by the growing culture, with the result that cell concentration reaches a constant value essentially proportional to the concentration of the limiting growth factor. In Fig. 1-3 the degree of substrate utilization in chemostat cultures is compared with that in turbidostat cultures.

Growth rates, on the other hand, are established quite independently of cell concentration, because they depend solely upon the rate of addition of medium, that is, upon flow rate, as we shall see later. Growth rates are relatively independent of temperature and pH.

C. GROWTH: BALANCED AND STEADY STATE

Cohen and Barner (1954) introduced the concept of *unbalanced growth* to explain the ultimate death of bacteria that continued to grow in the absence of DNA synthesis. Later, Campbell (1957) defined growth as *balanced* when every extensive property of a culture increases by the same factor over a given time interval. An extensive property is simply the total content of any chemical constituent, or any sum of these in the culture. For example, balanced growth occurs in the exponential growth phase, during which total DNA, RNA, protein, and mass of the culture all double at the same, constant rate. Should cell division cease abruptly but all of these extensive properties continue to double at the same rate as that at which cells enlarge, growth will remain balanced. Balanced growth refers to cultures. The term does not apply to individual cells, because different cell components do not increase in the same manner; macromolecules are synthesized at different rates during the cell cycle.

Cultures in the exponential growth phase not only are in balanced growth, but the numbers of cells in these cultures increase at a steady rate. Moreover, cell numbers increase at the *same* average rate as every cell constituent. This is characteristic of the steady state of growth and division, which leads to the following definition:

> A growing culture is in the steady state of growth and division when the average value of every individual cell property remains constant.

Thus, in a steady-state culture, average cellular values of DNA, RNA, protein, mass, volume, lipid content of the membrane, and all other structural and functional characteristics of the cell remain constant, including generation time. Clearly, cells growing under steady-state conditions are also in balanced growth. (The converse is not necessarily true.) In practice, steady-state growth usually is tested by measuring the rate of increase of cell numbers, mass, or some other dependent parameter, such as optical density.

If the mean generation time remains constant for many generations, say 6 to 8, it may be assumed that to good approximation, steady-state growth conditions are established.

For a clearer discrimination between steady state and balanced growth in a particular case it might be necessary to take into account the age distribution of the cells, i.e., the pattern of cell growth during the growth-duplication cycle. An abrupt change might occur in the synthesis of an enzyme, perhaps by induction, at a point during the growth of a culture without any apparent disturbance of cell numbers or division rate. Although the system would appear to be in a steady state of growth if only cell numbers were examined, the change in rate of enzyme synthesis would reveal the departure. Alternatively, averages of other cell properties might *appear* to remain constant during a change in cell division rate. However, any change in the age distribution would ultimately be reflected in the rate of cell production. During a true steady state of growth an invariant age distribution is maintained, which is then reflected in a constancy of cell number and a state of balanced growth in continuous cultures.

D. DEVELOPMENT OF CONTINUOUS CULTURES

Before 1944 "continuous culture" apparatus was designed primarily to give a continuous supply of microorganisms without routine subculturing. Much of this early work was industrial and was limited to increasing the efficiency of utilization of fermentable sugars. There appears to have been little awareness of the value of controlled continuous cultures for purposes of research. Reviews by Novick (1955), Malek (1958), and James (1961) summarize the scientific attempts of this very early period.

Later, many investigators used exponential phase cultures to study and compare the growth of different microorganisms because of the reproducibility that was obtainable with cells growing at their maximum rate. The development of the turbidostat was the result of the search to produce a continuous supply of cells growing at their maximum rate. The first successful prototype of the turbidostat appears to have been that of Myers and Clark (1944), who used a photocell to regulate the growth rate of algae. It was soon followed by many other similar devices that used photodetectors to respond to changes in optical density or turbidity. These instruments differ chiefly in operational details and in the names supplied by their inventors, such as Auxanometer, Breeder, Microgenerator, and Phytostat. Bryson (1952) first called his apparatus a "turbidostatic selector." Later, he shortened this to "turbidostat."

The greatest impetus leading to the modern development of continuous cultures came as the result of the simultaneous studies by Novick and Szilard

(1950*a*,*b*) who developed the "chemostat," and by Monod (1950), who called his apparatus a "bactogen." Their papers formulated the basic concepts of the chemostat, and, along with the subsequent experimental reports by Novick and his collaborators, stimulated the strong interest that led to rapid experimental and theoretical development during the 1950s.

Since then, continuous culture techniques have been used with many kinds of cells, including yeast, algae, protozoa, and mammalian cells. Surveys of studies on algae and of animal cells are given by Retovsky (1966) and Pospisil (1966).

The extent of application of continuous cultures in industry today is difficult to determine because this industrial information is not readily available in print, and because continuous operation is sometimes mistakenly referred to as continuous culture. Nevertheless, the available listing is impressive. Continuous cultures are used in the production of beer, sake, alcohol, and acetic and lactic acids; of vitamins (B_{12}) and drugs (alkaloids); and of antibiotics (penicillin and chlortetracycline). Continuous cultures are also used for steroid conversion and the production of vaccines. Many of these applications are discussed in a paper by Evans (1965).

Future applications appear even more promising, due to the frequent development of greater cell densities in continuous cultures than in simple batch culture, to increased outputs of products, and to the automation that is possible with continuous cultures. Another reason to anticipate greater future application of continuous cultures, as Postgate (1965) suggested, is that continuous cultures of animal cells and of plant cells may someday augment the supply of food now available from conventional animal and vegetable sources. In a limited sense, the continuous culture production of baker's yeast may be considered a beginning in this direction.