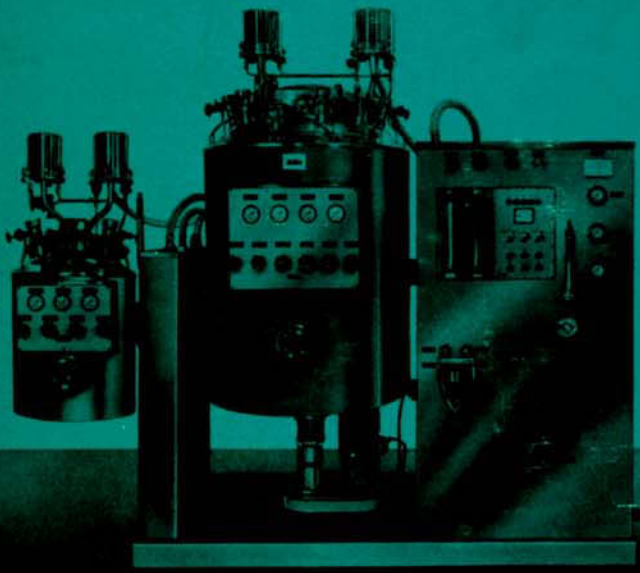
METHODS IN MICROBIOLOGY



VOLUME 2

Edited by
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Edited by

J. R. NORRIS
Milstead Laboratory of Chemical Enzymology, Sittingbourne, Kent, England

D. W. RIBBONS
Department of Biochemistry, University of Miami School of Medicine, and Howard Hughes Medical Institute, Miami, Florida, U.S.A.

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LIST OF CONTRIBUTORS

D. E. BROWN, *Biochemical Engineering Group, Department of Chemical Engineering, University of Manchester Institute of Science and Technology, Manchester, England*

J. BRYANT, *Glaxo Laboratories, Ltd, Ulverston, England*

N. G. CARR, *Department of Biochemistry, University of Liverpool, Liverpool, England*

R. ELSWORTH, 25 *Potters Way, Laverstock, Salisbury, Wilts., England*

C. G. T. EVANS, *Microbiological Research Establishment, Porton, Salisbury, Wilts., England*

W. H. FOSTER, *The Wellcome Research Laboratories, Langley Court, Beckenham, Kent, England*

D. FREEDMAN, *New Brunswick Scientific Co., Inc., New Brunswick, N.J., U.S.A.*

D. HERBERT, *Microbiological Research Establishment, Porton, Salisbury, Wilts., England*

H.-E. JACOB, *Institut für Mikrobiologie und Experimentelle Therapie, Jena, D.D.R.*

D. G. MACLENNAN, *I.C.I. Ltd, Agricultural Division, Billingham, Teesside, England*

RICHARD Y. MORITA, *Departments of Microbiology and Oceanography, Oregon State University, Corvallis, Oregon, U.S.A.*

A. L. S. MUNRO, *Marine Laboratory, Aberdeen, Scotland*

R. J. MUNSON, *Medical Research Council Radiobiological Research Unit, Harwell, Didcot, Berks., England*

J. W. PATCHING, *School of Biological Sciences, Bath University of Technology, Bath, Somerset, England*

G. A. PLATON, *G. A. Platon Ltd, Basingstoke, Hants., England*

J. ŘIČICA, *Institute of Microbiology, Czechoslovak Academy of Science, Prague, Czechoslovakia*

A. H. ROSE, *School of Biological Sciences, Bath University of Technology, Bath, Somerset, England*

D. W. TEMPEST, *Microbiological Research Establishment, Porton, Salisbury, Wilts., England*

R. O. THOMSON, *The Wellcome Research Laboratories, Langley Court, Beckenham, Kent, England*

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PREFACE

Traditional methods of cultivation of micro-organisms provide little control over the ever-changing physical and chemical environments to which growing populations are exposed. The need for a variety of highly standardized conditions of growth and the selection of several new parameters to measure growth or cultural progress has been clearly recognized. This Volume describes the measurement and control of the physical and chemical factors which affect or indicate microbial activities, usually in homogeneous liquid culture. An introduction to the general principles of chemical and physical measurements, and to the automatic recording and control of them, is given first. Individual Chapters then provide more detailed information about the various parameters. The last four Chapters of this Volume are devoted to the theoretical and practical aspects of techniques of continuous cultivation in the laboratory with indications of its application to research problems.

As with all the Volumes in this Series, the choice of material presented has been the prerogative of the individual authors, and we have only suggested additions to, or deletions from, manuscripts in the interests of providing a more complete but not repetitive work. We again thank all our contributors for their co-operation. It has been a pleasure to obtain such enthusiasm and understanding from them. We are also indebted to the staff of Academic Press for the processing and the close scrutiny of manuscripts and proofs.

J. R. NORRIS

D. W. RIBBONS

November, 1969

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CHAPTER I

Principles of Automatic Measurement and Control of Fermentation Growth Parameters

D. G. MacLennan*

Imperial Chemical Industries Ltd., Pharmaceuticals Division, Alderley Park, Cheshire, England

I. INTRODUCTION

One of the most fundamental characteristics of micro-organisms is their ability to adapt to environmental conditions. The response of a culture to changed environment may involve the induction and repression of enzymes or the selection of mutants more suited to the new conditions than the parent culture. Irrespective of the mechanism(s) involved, it has become clear that to study the growth, metabolism or kinetics of a culture most effectively, it is important that the environment be controlled to as great an extent as possible. Thus the last decade has seen the introduction of automatic measurement-record-control techniques into microbiological work, and it is apparent that the use of these techniques will increase rapidly. As the concepts upon which such techniques are based are not generally

* Present address: I.C.I. Ltd., Agricultural Division, Billingham, Teesside, England.

covered in formal microbiological courses, it is the purpose of this Chapter to provide the microbiologist with sufficient background knowledge to enable him to select the correct automatic recording and control instruments for his purpose. The factors to be treated are general and will apply equally well to pH, temperature, dissolved oxygen tension (DOT), redox potential, etc.

The selection of automatic record-control equipment for particular processes is the domain of the specialist, and a great many books have been written on both the theoretical and practical aspects of it. It is not proposed to present here a detailed theoretical analysis of the problem, but rather to provide a simplified version for practical use by the microbiologist. The more important principles governing the selection of equipment will be discussed; however the treatment will be mainly directed towards the conditions encountered in fermentation work. For those who wish to pursue the subject further a more general treatment can be found in the technical bulletin, "Fundamentals of Industrial Instrumentation", published by Minneapolis-Honeywell Regulator Company, Philadelphia, Pa., U.S.A. More comprehensive treatises on automatic process control are given by Porter and Considine (1950) and Young (1960).

Expense

Before examining the more technical aspects of selecting automatic record-control equipment, it is appropriate to discuss the most predominant factor in the mind of the microbiologist when considering such apparatus—the expense. First and foremost the microbiologist must be convinced of the necessity for automatic control of particular growth parameters, and in the following Chapters the principal growth parameters are discussed with this in mind. The problem is then to choose the most economical equipment which will do the job *satisfactorily*. The emphasis here must be on satisfactory performance, and this brings out the first fundamental in the selection of equipment, that quality and reliability are in direct proportion to cost. It is false economy to buy a cheap instrument where failure could involve considerable expense. This is not to suggest that only expensive equipment should be bought, but that a critical appraisal of the necessity for reliability must be made before equipment is selected. For example, the purchase of reliable and thus expensive record-control equipment is generally advisable for research continuous culture equipment to be used for long experiments involving accurate kinetic work. Similarly, reliable equipment would be essential to control the pH in a production fermenter as failure could involve large financial loss. On the other hand less exacting equipment is often adequate for use in laboratory scale batch experiments

lasting only a few days, for example where the main requirement is for a few litres of culture for enzyme extraction.

Thus the quality and reliability of equipment, and hence its cost, is determined directly by the particular purpose for which the equipment is required.

II. SELECTION OF RECORDERS

The principal factors to be considered in the assessment of measuring and recording instruments are accuracy, reproducibility, long term stability, reliability and robustness. Considerable insight can be obtained into all these factors by examining the principle on which the instrument operates. There are two main categories of instruments with electrical inputs: those operating on a "direct-measurement" principle, and those on a "null-point" principle.

A. Direct-measurement instruments

Instruments operating on a direct-measurement principle rely on the input signal (or an amplification or modification of it) to cause the appropriate movement of the indicating device. An example of a direct-measurement instrument is the common ammeter, the principle of which is illustrated diagramatically in Fig. 1.

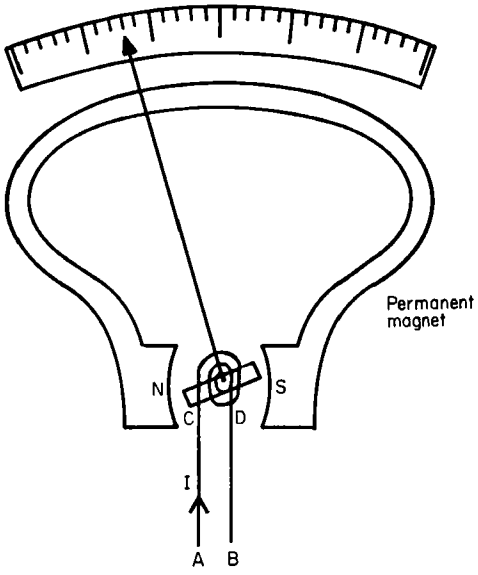

FIG. 1. Principle of a simple ammeter.

When current (I) flows through the wire AB an electromagnetic field is formed about the coil CD. This field causes the coil to orientate itself in the field of the permanent magnet by rotation about a pivot. The degree of rotation of the coil, which is dependent on the magnitude of the current flowing, is indicated by an attached pointer. The reliance on the input signal for deflection of the pointer necessarily involves current being drawn at the expense of the input signal. This is undesirable, principally because it may distort the signal. To draw as little current as possible the internal resistance of the meter must be large. However, this characteristic renders the instrument insufficiently sensitive for accurately measuring small electrical signals (e.g., the output from a DOT electrode, which is typically 10–100 μA).

Although direct-measurement instruments do not all operate in the same way as the ammeter they all have one characteristic in common: to effect measurement, power is used at the expense of the input signal. In addition to this disadvantage, direct-measurement instruments often lack long-term stability. The main attraction of such instruments is their simplicity and hence cheapness.

B. Null-point instruments

The principle of null-point measurement is to balance the value of the parameter being measured against a known value (or ratio) of the parameter in such a way that when the true value is indicated no current flows through the measuring device. Because no current is drawn by the measuring device, null-point instruments are capable of very accurate measurement of small input signals. Typical examples of such instruments are self-balancing potentiometric and Wheatstone bridge recorders.

1. *Self-balancing potentiometric recorders*

Potentiometric circuits are used for the accurate measurement of potential difference. They can also be used to determine current by measuring the potential drop produced by the current when flowing through a known resistance. Potentiometric recorders are used extensively both in the laboratory and plant because the output from electrodes, thermocouples, measuring instruments etc., is usually in one of the above forms. Potentiometric recorders are generally calibrated to measure millivolts D.C.

The principle of potentiometric measurement is to balance the unknown potential difference against an equal and opposite known potential difference such that no current is drawn from the unknown source. The basic potentiometer circuit is shown in Fig. 2.

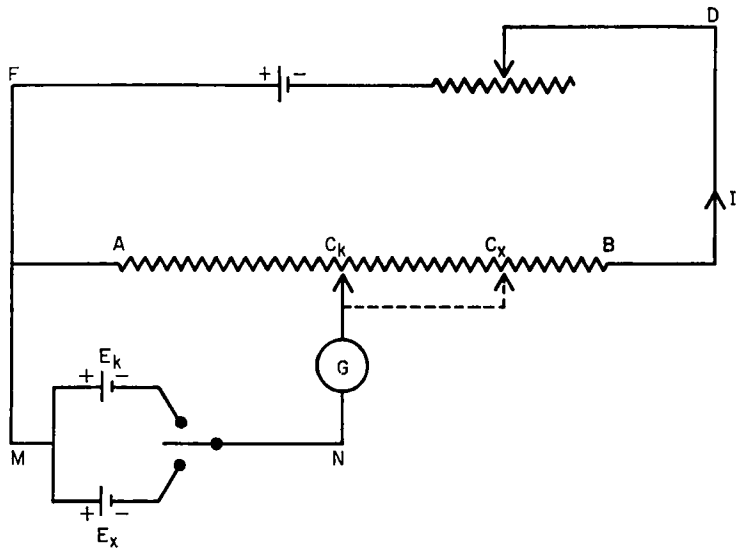

FIG. 2. Basic potentiometer circuit.

Before measuring the unknown potential difference (E_x) the potentiometer must be standardized. This is done by switching the known potential difference (E_k) into the circuit and moving the sliding contact along the slide wire resistance AB until it reaches a position C_k where no current flows through the galvanometer (G). At this point the potential across AC_k is balanced equally and oppositely by the known potential difference E_k or—

$$E_k = (\text{Resistance } AC_k).\ I \tag{1}$$

where I is the current flowing in the loop ABDF. The unknown potential difference (E_x) is then substituted for E_k and the sliding contact again adjusted so that no current flows through the galvanometer, (point C_x). At this point—

$$E_x = (\text{Resistance } AC_x).\ I \tag{2}$$

From equations (1) and (2)—

$$E_x = \frac{(\text{Resistance } AC_x)}{(\text{Resistance } AC_k)} \cdot E_k$$

As the right hand side of the equation can be evaluated, the true value of the unknown potential difference can be determined under conditions where no current is drawn by the measuring device (galvanometer).

In a self-balancing potentiometric recorder a continuous balance is maintained automatically. This is done by substituting for the galvanometer a self-balancing system which operates in the following way. If the value of the measured potential difference changes, the balance will be disturbed and current will flow through the arm $AMNC_x$. This "out of balance" current is amplified and used to drive a balancing motor which returns the sliding contact (attached to the instrument indicator) to a position where the circuit is balanced and once again no current flows. Self-balancing potentiometric recorders designed for continuous use are equipped with an automatic standardization mechanism to ensure long term stability.

2. *Self-balancing Wheatstone bridge recorders*

Wheatstone bridge circuits are used for the accurate measurement of resistance (e.g., temperature measurement with a resistance thermometer). The basic circuit is shown in Fig. 3.

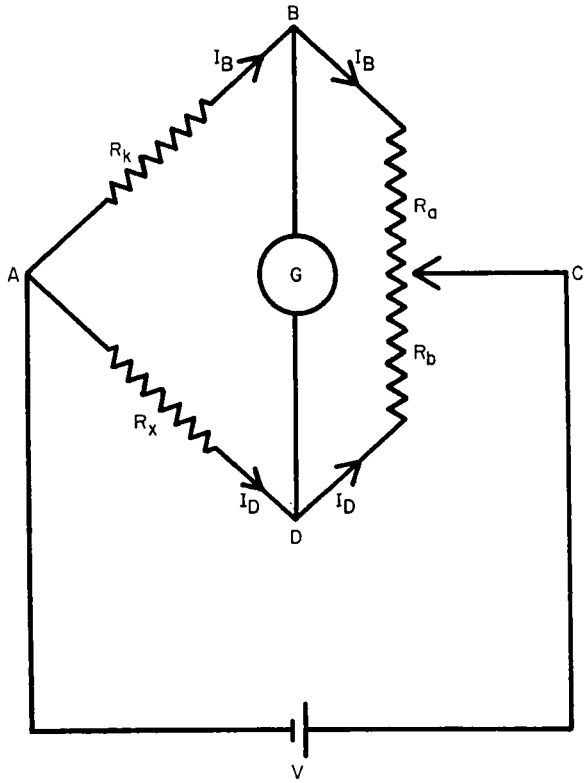

FIG. 3. Basic Wheatstone bridge circuit.

R_x is the unknown resistance, R_k is a known resistance, and R_a, R_b are resistances whose ratio can be accurately determined. To balance the bridge the sliding contact C is adjusted until no current flows through the galvanometer (G). At this point the potential at B is equal to that at D. Thus—

$$\text{potential drop across AB, } (\text{pd}_{AB}) = \text{pd}_{AD} \quad (3)$$

$$\text{and} \quad \text{pd}_{BC} = \text{pd}_{DC} \quad (4)$$

Applying Ohm's Law to equations (3) and (4) respectively—

$$R_k . I_B = R_x . I_D \quad (5)$$

$$\text{and } R_a . I_B = R_b . I_D \quad (6)$$

where I_B and I_D are the currents flowing in the arms ABC and ADC respectively.

Dividing equation (5) by equation (6)—

$$\frac{R_k}{R_a} = \frac{R_x}{R_b}$$

Hence,

$$R_x = R_k . \frac{R_b}{R_a}$$

As the right-hand side of the equation can be evaluated, the true value of the unknown resistance can be determined under conditions where no current is drawn by the measuring device (galvanometer). The servomechanism of a self-balancing Wheatstone bridge recorder operates in the same way as the self-balancing potentiometric recorder.

Self-balancing null-point instruments are characterized by a high degree of accuracy and reproducibility, and when designed for continuous industrial use they are stable over indefinite periods of time and are robust and reliable. They are initially expensive; however in the long run they are often the most economic proposition. Instruments of this type are supplied by most leading firms manufacturing record-control equipment, such as (in the U.K.) Leeds and Northrup Ltd., Birmingham; Honeywell Controls Ltd., Middlesex; Kent Industrial Instruments Ltd., Bedfordshire; Foxboro-Yoxall Ltd., Surrey; and others.

III. SELECTION OF AUTOMATIC CONTROL EQUIPMENT

Automatic control of a variable in a process is most commonly achieved using a "closed loop" control system. The principle of closed loop control is illustrated in Fig. 4, which shows a system for the control of pH in a stirred fermenter. The pH (the controlled variable*) of the culture (the

* Defined terms, listed by Porter and Considine (1950).

controlled medium*) is measured by pH electrodes (the primary element*) and recorded on a recorder–controller. Depending on the deviation of the recorded value of the controlled variable from the set point, the controller transmits a signal to the pump (the final control element*) which pumps alkali (the control agent*) into the fermenter. A closed loop control system can be divided into three parts: the *measuring means**, which includes the primary element, the system for transmission of the signal and the measuring

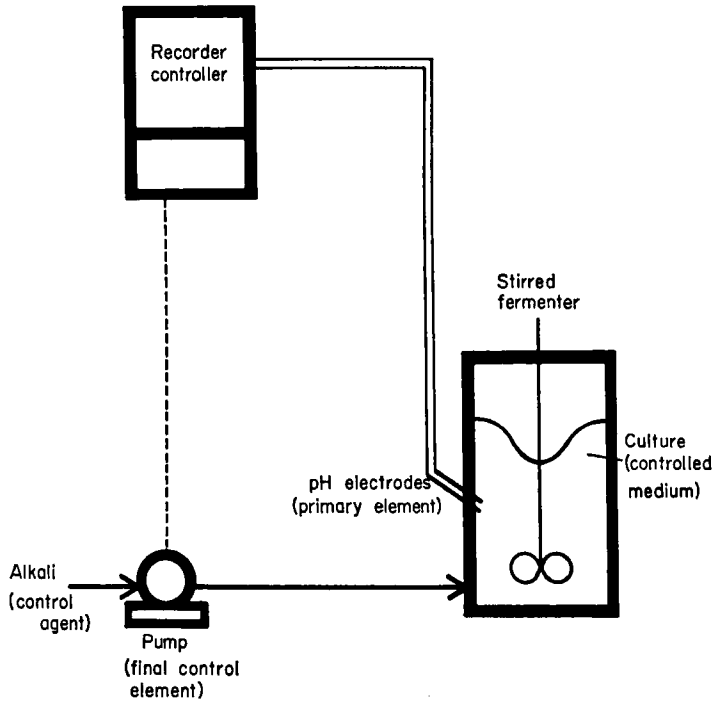

FIG. 4. A "closed loop" control system for the automatic control of pH in a stirred fermenter.

mechanism of the instrument; the *controlling means**, which includes the controlling mechanism of the instrument and the final control element; and the *process**, which is defined as "the collective functions performed in and by the equipment in which a variable is to be controlled".

When choosing automatic control equipment for a particular process it is first necessary to consider the basic characteristics both of the measuring means and of the process. The most suitable method of control can then be selected by considering the characteristics of the various types of controllers in terms of the above factors. In this section the basic characteristics of

measuring means, of processes, and of various types of controllers will be discussed, and the suitability of particular types of controller for different fermentation processes will be considered.

A. Basic characteristics of measuring means

There are two main factors in the evaluation of the measuring means: the speed of response (or lag) and the accuracy.

1. *Speed of response*

Complete response by a measuring means to a change in the measured variable is never instantaneous. The response may start immediately but takes time to complete its effect. This time element is called "lag". For example, consider the measurement of temperature by a resistance thermometer inserted into a pocket in a stirred fermenter. When a temperature change occurs in the broth, heat must be transmitted through the wall of the pocket, across the space between the pocket wall and the thermometer wall, and through the thermometer wall to the resistance element. A signal related to the resulting change in resistance must then be transmitted to the instrument and measured. Thus lag in a measuring means can occur in the primary element system, the transmission system and the measuring mechanism of the instrument.

In fermentation processes by far the greatest lag in the measuring means is due to the response of the primary element. For dissolved oxygen electrodes, for example, Johnson *et al.* (1964) have reported lags of about 1 min for 90% response and about 3 min for 99% response at 30°C. The responses of pH electrodes and bare temperature sensing elements (i.e., not inserted into a pocket) are much faster than dissolved oxygen electrodes, generally being below about 10 sec.

A very important factor influencing the speed of response of a primary element is the turbulence of the fluid surrounding the element. In microbial cultures lag can be reduced considerably by maintaining a high velocity of broth past the sensing probes. This can be achieved by siting probes either in a continuous flow system and pumping broth past them, or in a well stirred fermenter. The high velocity and resulting turbulence achieved in either of these ways will reduce the stagnant film of broth around the probe, thus speeding the detection of any change of the variable in the bulk of the medium. A high turbulence around the probe is of paramount importance when measuring DOT with a membrane-protected electrode, as the factor actually being measured is the rate of oxygen transfer across the membrane. For the rate of oxygen transfer to be proportional to the DOT in the culture

fluid it is essential that inadequate turbulence is not the rate-limiting factor. A high turbulence also helps to prevent cells from adhering to, and subsequently growing on probes.

Any form of lag in the measuring means is obviously detrimental for automatic control because it constitutes a delay between the time a change in the controlled variable occurs and the time when it is registered by the measuring instrument. Even small lags in the measuring means (in the order of tens of seconds) can, under some circumstances, result in considerable difficulties in automatic control systems.

2. *Accuracy*

The accuracy of a measuring means can be assessed in terms of four main characteristics: static error, reproducibility, dead zone, and dynamic error.

(a) *Static error.* The static error is the difference between the true value of a *static* variable and the value indicated by the measuring means. Large static error is undesirable. However for automatic control, where it is often more important that the variable be held constant rather than at an exact value, relatively small static error is often acceptable. The accuracy of the measuring means is normally expressed in terms of static error, as a percentage of the instrument range.

(b) *Reproducibility.* Reproducibility is the closeness with which the same value of a variable can be measured at different times. Reproducibility is most important in automatic control particularly when, as mentioned above, it is more desirable that the variable be maintained constant than exact.

(c) *Dead zone.* The dead zone is the largest range through which the variable can change without the change being registered by the measuring instrument. By creating an initial delay it reduces the speed of response of the instrument.

(d) *Dynamic error.* When the measured variable is *changing*, any lag in the measuring means will result in a difference between the true value of the variable at any instant and the value indicated by the measuring instrument. This difference is termed the "dynamic error". Dynamic error is important because in an automatically controlled process the actual value of the controlled variable cycles (to some extent) around the set point due to control action. Under these circumstances any dynamic error will result in the indicated value oscillating about the set point with a smaller amplitude than the actual value in the process. This is because the actual value of the variable reaches one extreme of its cyclic swing and starts in the other direction (due to control action) before the indicated value catches up with it. Thus, the measuring instrument indicates better control precision than is actually being obtained.

B. Basic process characteristics

There are two important characteristics of the process which should be taken into account before automatic control equipment is selected: load changes and process lag.

1. *Load changes*

The process load is the total amount of control agent required by a process at any one time to maintain a balanced condition. Thus process load is directly related to the setting of the final control element: any change in the process load requires a change in the rate of supply of control agent to keep the controlled variable at the set point. Changes in process load due to changing conditions in the process are called load changes. As will be discussed in Part C of this Section, load changes present a relatively difficult problem to the controller with which the simpler types of controller cannot cope. Both the magnitude and rate of load changes in a process are important. Typical examples of load changes in fermentation processes are the changes in pH and DOT which occur during batch growth. In the early stages of growth, where cell mass is relatively small, the change in these parameters may be small and slow; however towards the end of exponential growth the changes may be both large and rapid. Load changes are thus an inherent characteristic of batch cultures. By definition, load changes due to microbial growth do not occur in steady state continuous culture, although they could occur during the establishment of steady states.

2. *Process lag*

The delay before a process variable reaches a new value when a load change occurs is called the process lag. This lag is caused by one or more of the process characteristics of capacitance, resistance and dead time.

(a) *Capacitance.* The capacitance of a process is a measure of its ability to hold a quantity of energy or material per unit quantity of some reference variable. For example, a process with a high thermal capacitance would require more calories to raise the temperature 1°C than would a process with a lower thermal capacitance. In this case the thermal capacitance with respect to temperature could be expressed in calories per °C. The pH capacitance of a process per unit quantity of a particular acid or base is analagous to that of temperature, although it cannot be expressed so simply due to the logarithmic nature of the pH scale, and also as it would be expected to vary with the pH of the controlled medium. It can, however, be thought of in terms of the buffering action of the controlled medium at the pH concerned.

A large capacitance relative to the flow of control agent is usually favourable for automatic control in that it tends to keep the controlled variable constant in spite of load changes. It has the disadvantages, however, of making it more difficult to change the variable to a new value and also of introducing a lag between the time a change is made in the rate of supply of control agent and the time the controlled variable reflects the change. The importance of process capacitance is well illustrated by considering the automatic control of DOT in a vortex aerated fermenter, by varying the oxygen partial pressure in the aerating gas. The very low "demand side" oxygen capacitance, due to the low solubility of oxygen in aqueous media, is a distinct disadvantage because it renders the system extremely sensitive to even small load changes which may be reflected by large changes in DOT. This situation is aggravated when the "supply side" oxygen capacitance (that of the gas volume above the medium) is appreciable, because after corrective action by the controller has ceased, oxygen will continue to be transferred to the culture until the oxygen tensions in the gas phase and in the medium are the same. The overall result of these conditions is that overshoot is extremely difficult to avoid, and to achieve adequate control highly developed equipment is necessary. The volume of medium in the fermenter is a most important factor in assessing the thermal, pH and dissolved oxygen capacitance of a fermentation process, and this is one of the main reasons why a working volume of 1·5–3·0 litres is usually chosen as a minimum for laboratory scale fermenters, where good control over the above parameters is required. Good control can be obtained at lower volumes although usually with some difficulty.

(b) *Resistance.* The second basic type of process lag is resistance, defined as opposition to flow. The influence of resistance can also be illustrated by reference to the DOT control system mentioned above. To effect a given change in DOT the necessary oxygen enrichment in the aerating gas will depend on the rate of oxygen transfer to the medium. If there is a high resistance to oxygen transfer (i.e., low oxygen transfer coefficient) more oxygen (a higher oxygen partial pressure) will be required to change the DOT than in a process with a low resistance (high oxygen transfer coefficient).

(c) *Dead time.* Dead time can be defined as any definite delay period between two related actions. Dead time often occurs in processes where the primary element is situated away from the process, for example pH electrodes sited in a fermenter side-arm through which culture is continuously pumped. The detection of any pH change in the fermenter would be delayed by the time required for the culture to reach the electrode system, and control action would be delayed that length of time. This type of dead time is referred to as distance–velocity lag. Any corrective action by the controller would also have this same lag before it would be reflected by the primary

element. In general dead time introduces more difficulty in automatic control than does a lag at any other point in the control system. Distance–velocity lag can best be minimized by inserting all primary elements directly into the fermenter.

It should be stressed that adequate mixing is essential for effective automatic control, as it ensures that the response of the primary element is rapid and representative of the entire culture, and also ensures immediate dispersion of control agent.

C. Characteristics of automatic controllers and their suitability for fermentation processes

Automatic control systems can be divided into three main types, two-position controllers, floating controllers, and proportional controllers (the last of which includes two refining modes of control, "reset" and "rate" actions).

1. *Two-position control*

The simplest type of control system, the two-position controller, is one in which the final control element moves to one or other of two extreme positions. It includes, as a special case, on–off controllers in which the final control element is either fully opened or is completely shut off.

The two-position controller is simple and relatively cheap. It generally functions satisfactorily if the process has a fairly large demand side capacitance and minimum dead time or transfer lag. To achieve good control the extreme positions of the final control element must be set so that one permits a flow of control agent just slightly above the normal requirement of the process, and the other a flow slightly below. However, these requirements for good control render the two-position controller extremely vulnerable to load changes, since significant changes in load could move the control agent requirement outside the carefully set extreme limits. Thus, when setting the extreme positions of the final control element a compromise must be reached between setting them widely apart to cope with load changes and closely together to achieve good control. In general the compromise is such that the control system can accommodate only small and slow load changes. Two-position (in particular on–off) control is suitable for controlling the temperature in both batch and continuous fermenters and is used extensively for this purpose. On–off control is also adequate for controlling the pH in continuous fermenters, however it is not ideally suited for batch cultures because of the large load changes which can occur during batch cultivation. To control pH in a batch culture satisfactorily either a floating or proportional type controller is necessary. Unfortunately these latter types of controller are more complicated and expensive than on–off con-

trollers, and in many instances, particularly for small laboratory fermenters, it is more convenient to use on–off controllers and to readjust the maximum capacity of the final control element periodically, than it is to use the correct type of controller. A modification of on–off pH control, which is often used for both batch and continuous processes to overcome difficulties caused by lags in the system, is the insertion of a period timer between the controller and the final control element. The function of the timer is to interrupt periodically any signal to the final control element. Thus control agent is added to the culture in distinct "doses", the duration of each dose and the period between doses being determined by the timer settings. This arrangement ensures that there is time for each dose of control agent to disperse completely and for its effect to be monitored by the electrodes before a further dose is added.

On–off control is not satisfactory for controlling DOT on either batch or continuous processes, as a combination of large and sometimes rapid load changes with the low demand side oxygen capacitance of fermentation medium would give rise to excessive cycling.

2. *Floating control*

To return the controlled variable to the set point a floating controller moves the setting of the final control element (e.g., a proportioning valve) at a *constant speed* in either direction. While the value of the controlled variable lies within a "neutral zone" no control action occurs. However once the value of the controlled variable is outside the neutral zone the valve moves in the appropriate direction and continues to do so until the value of the controlled variable returns to the neutral zone, or the valve reaches one or other of its extreme positions.

The principal advantage of floating control over two-position control is that it can cope with gradual load changes. However, it cannot be used where there is any significant lag or rapid load changes since cycling, which is an inherent characteristic of both two-position and floating control, would become excessive.

Floating control can be used to control pH in batch fermentations, and instruments for this purpose are available commercially; however proportional type controllers are more common.

3. *Proportional type control*

The simple proportional controller moves the final control element (e.g., a proportioning valve) proportionally to the deviation of the controlled variable from the set point. It moves the valve to a definite position for each value of the controlled variable within the proportional band.

The proportional band is the range of values of the controlled variable

over which there is proportional control action. The width of the proportional band determines the sensitivity of control action, and it is adjustable. With a narrow proportional band the control valve will move from fully closed to fully open over a narrow range of the controlled variable. Thus a small change in the controlled variable will instigate a relatively large change in the setting of the control valve.

Proportional action provides a much smoother control than two-position controllers, because it can move the control valve to intermediate positions.

(a) *Reset action.* A simple proportional controller, however, is unable to cope with any load change, because when a load change occurs a different valve position from that initially fixed (by the proportional band setting) will be required to maintain the controlled variable at the set point. As the simple proportional controller cannot change the fixed relationship between the valve position and the controlled variable, it will begin to control about a new value, thus producing "offset". Offset can be eliminated by the introduction of a second form of control called "reset" action, which shifts the proportional band about the set point so as to maintain control at the set point. Reset action can be either manual or automatic.

Proportional-plus-automatic reset action provides good control even when the process capacitance is small, the process reaction rate is fast, and when load changes are large. This type of control is ideally suited for pH and DOT, both for batch or continuous fermentation processes on either laboratory or plant scale.

(b) *Rate action.* There is another refinement which can be used with proportional type controllers, referred to as "rate" action. Rate action applies a correction to the movement of the control valve according to the *rate* of change of the controlled variable. It greatly speeds return to the set point by instigating a large initial over-correction, then begins to remove this effect leaving only the proportional action to determine the final position of the valve. Rate action is used in conjunction with proportional or proportional-plus-reset control for processes with large lags or dead time. With the possible exception of DOT control, rate action is seldom required in fermentation processes. However, if the purchase of a proportional-plus-reset action controller for control of DOT is being considered, the inclusion of rate action adds little to the price (in the order of £10) but provides added versatility which is always an asset, particularly in research work.

(c) *Final control element.* The final control element used with proportional type controllers is often very different from that used with on–off controllers, and some comment on the subject is appropriate at this point. The output from proportional type controllers is generally in one of four forms, a continuous electrical signal (mA or mV), a continuous pneumatic signal (p.s.i. air pressure), a continuous mechanical output (i.e., movement of a

lever or arm), or a pulsed electrical signal. The first three types are designed to actuate the final control element by the continuous readjustment of its setting, whereas with the pulsed electrical output the position of the final control element is either on or off, and it is the *ratio* of the "on time" to the "off time" which is proportional to the value of the controlled variable. The length of the complete cycle (i.e., on plus off time) is constant. Controllers with pulsed electrical outputs are known as "duration adjusting" or "time proportioning" controllers.

For larger scale operations (pilot or plant scale) the most common final control element is the pneumatic proportioning valve, continuously actuated either from a controller with a pneumatic output or from a controller with an electrical output via an electro-pneumatic converter. These systems can also be used for laboratory scale work using miniature pneumatic control valves which are available down to exceedingly low capacities. MacLennan and Pirt (1966) have described a system for the automatic control of DOT based on this type of equipment.

Electrically operated final control elements of the proportioning type are also available, such as motorized valves or pumps. Alternatively the speed of a pump could be varied by a mechanical output from a proportional controller by regulation of the position of a speed control lever.

As the "pulsed electrical output" type of control system operates on an on–off basis, the final control element can be identical to that used for two-position controllers, e.g., a solenoid valve or a normal pump. This type of control system is particularly useful for laboratory scale pH control where the flow rates of control agent are generally very low. It has all the advantages of adding the control agent in distinct doses and also ensures that when the valve is in the off position no leakage of control agent occurs—a fault sometimes occurring with incorrectly selected proportioning control valves. A disadvantage of the pulsed type of control is that the flow of control agent is discontinuous and thus is not easy to measure as a rate of flow (i.e., on a flowmeter).

IV. MULTIPOINT RECORDING AND CONTROL

In the past automatic control equipment has generally been selected on the basis of one instrument per control point. However, recent advances in instrument technology together with greater usage of control systems and the resulting demand for more economic instrumentation have caused an increasing interest in multipoint control.

The principal advantages of multipoint control are that the cost per point can be very much lower than with single controllers and also that a great deal less space is required to accommodate control equipment. Multipoint

control at this stage is restricted to two-position control and has the disadvantage that if the recorder fails then all control points are affected. However this disadvantage can be largely overcome by purchasing a first quality instrument and keeping it properly maintained. Another limitation on the use of multipoint controllers arises from the way in which the control system functions. In general, at the time the recorder prints the value of a particular point, the final control element for that point is either activated or deactivated. The final control element is then held in this position until the instrument has monitored all of the other points and has returned to the point in question. If, for example, a six-point recorder-controller takes five seconds to record each point, thirty seconds will elapse before the point in question is again monitored. To avoid excessive overshoot due to this thirty seconds "dead time" it is very important that the rate of supply of control agent be carefully adjusted. Because of this requirement multipoint control cannot be used where appreciable load changes occur (see Section III, Part C: Two-position control). However, where load changes are not significant, difficulties due to dead time can be minimized by choosing a recorder with a fast printing time (i.e., time per point). In general the more control points per instrument the faster the printing time necessary to achieve good control. Recorders with printing times as short as about one second per point are commercially available. A minor inconvenience with multipoint control systems is that when control is required at the same value for more than one point, superimposition of the printout may prevent a clear record of each point. Inconveniences of this type, however, are usually of little consequence, particularly for research and development work.

Multipoint control is suitable for controlling temperature in both batch and continuous fermentations and for controlling pH in continuous fermentations. However, it would not be suitable for controlling pH in batch cultures because of load changes and the resulting difficulty in adjusting the rate of supply of final control agent to the required precision. Also, at the present stage of development multipoint control would probably not be the wisest choice for plant use, as large amounts of money are usually involved and instrument failure, involving several fermenters, could be ruinous (unless suitable safety devices or alternative control systems were also installed). Nevertheless multipoint control could be particularly attractive to universities and research institutions, where finance for capital expenditure is often a limiting factor. In such institutions being *able* to achieve precise control of pH or temperature on several fermenters far outweighs the minor inconveniences involved.

Equipment suitable for multipoint recording and control is manufactured by, amongst others, the four industrial instrument companies mentioned previously.

(a) *Multipoint temperature control system.* A typical example of a multipoint temperature control system is that installed for laboratory scale fermenters at the Commonwealth Serum Laboratories, Melbourne, Australia. The instrument used is a 4-point, 20–45°C, Leeds and Northrup "Speedomax G" Recorder (Wheatstone bridge type) fitted with our on–off control mechanisms. Each point is capable of controlling the temperature in an individual fermenter (heating only). The instrument has a 4 second printing rate and thus each point is monitored every 16 seconds. The measuring element is a resistance thermometer inserted into a protecting well made from $\frac{1}{4}$ in. diameter 24 gauge stainless steel tube. The final control element consists of two infrared lamps (total capacity 300 watts) whose beam falls directly on the fermenter wall (glass cylinder 20 in. long × 3 in. diameter × 1/8 in. wall thickness; fermenter working volume 2 litres). The intensity of the heat falling on the fermenter wall is set initially by adjusting the distance of the lamp assembly from the fermenter. With this system temperature is controlled at 37°C with a maximum tolerance of $\pm 0{\cdot}1$°C. The system has been operating satisfactorily for several years.

(b) *Multipoint-multifunction record-control system.* In an attempt to reduce the cost of automatic recording and control equipment for a continuous fermentation assembly to an absolute minimum, a multipoint-multifunction record-control system has recently been installed at I.C.I. Pharmaceuticals Division, Cheshire. The system was designed to provide for automatic recording and control of pH and temperature (heating and cooling), and recording of DOT for each of three continuous fermenters, and is shown diagramatically in Fig. 5.

The basic recording and control equipment involves one multipoint recorder–controller, three pH indicator–controllers and one thermocouple cold point reference junction unit, all of which are specified in Table I. The recording instrument has three roles: measurement of the outputs from both the pH indicator–controllers (mA) and the DOT electrodes (μA), and measurement of temperature. A potentiometric recorder would perform all tasks satisfactorily if thermocouples were used for temperature measurement. The range of the instrument was determined by the range of temperatures required to be measured. A range of 0–40°C was chosen which, with chromel–alumel thermocouples, corresponded to an instrument range of 0–1·61 mV when the reference junction temperature was 0°C (provided by the Frigistor unit, see Table I). This range is quite satisfactory for recording the outputs from both the pH indicator–controllers and the DOT electrodes.

A 12-point instrument is used, 6 points of which are fitted with on–off controllers. The control points are all for temperature (control points 1 to 3 for cooling and 7 to 9 for heating). Points 4 to 6 are used for pH recording

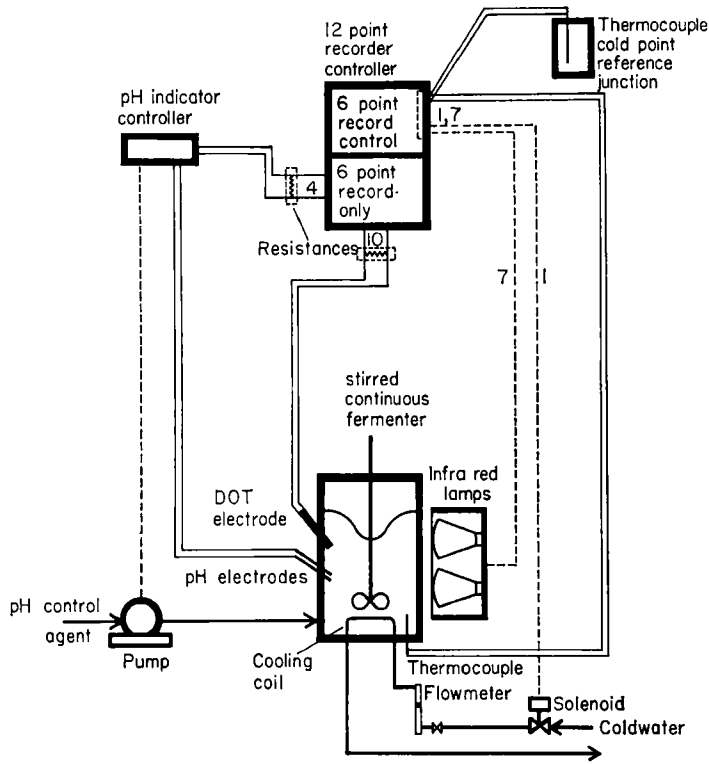

FIG. 5. Schematic diagram of multipoint-multifunction record-control system for continuous fermenters, in use at I.C.I. Pharmaceuticals Division, Cheshire. Although only one fermenter is shown, the system is capable of automatic recording and control of pH and temperature and recording of DOT, in each of three fermenters. Recorder point numbers used for fermenter 1 are shown by the leads from the devices to which they refer. See Table I for equipment specifications.

and points 10 to 12 for DOT recording; the range of both being determined solely by the choice of suitable resistances. Control of temperature over the range 20–40°C can be obtained to within ±0·1°C with fermenters of both 3 litre and 12 litre capacities.

Table I shows the cost of the multipoint-multifunction record-control equipment described, and Table II the approximate cost if less expensive *single* point instruments are used exclusively. (The costs shown in the Tables are for the instruments only and do not include the costs of primary elements or installation.) It can be seen from the Tables that for a three fermenter assembly the saving in using multipoint-multifunction record-control equipment would be in the order of £360, or about £120 per fermenter. Thus the multipoint-multifunction system described enables the use of

TABLE I

Specifications of multipoint-multifunction instrumentation to record and control both pH and temperature and to record DOT in each of three continuous fermenters.†

Instrument specification	Number	Approx. cost‡
Leeds & Northrup "Speedomax H" self-balancing potentiometric recorder, 0–1·61 mV, 12 points, with a printing rate of 3 sec per point. Six points equipped with on–off control switches and corresponding "lock-in" relays. All control settings independently adjustable. Range 0–1·61 mV corresponds to 0°–40°C with chromel-alumel thermocouples and a 0°C cold point reference junction. Recorder accuracy, ±0·3% of range.	1	£506
De La Rue "Frigistor" ice point thermocouple reference chamber, Model 136. Reference temperature 0°C; accuracy, 0·00 to +0·05°C; stability ±0·01°C.	1	£98
Electronic Industries Ltd. pH indicator–controller, Model 91B (on–off control). Indicating meter accuracy, better than ±0·1 pH; stability in continuous use, better than ±0·1 pH in 24 h.	3	£474
TOTAL COST		£1078
COST PER FERMENTER		£360

† As installed at I.C.I. Pharmaceuticals Division, Cheshire.

‡ Instrument cost only, not including costs of primary elements or installation.

TABLE II

Approximate cost of less expensive† *single* point instrumentation to record and control both pH and temperature and to record DOT in each of three continuous fermenters

Instrument specification	Number	Approx. cost‡
Temperature Recorder–Controller	3	£340
pH Indicator–Controller (EIL Model 91B)	3	£474
pH Recorder	3	£260
DOT Recorder, potentiometric type	3	£370
TOTAL COST		£1444
COST PER FERMENTER		£480
APPROXIMATE SAVING PER FERMENTER USING MULTIPOINT-MULTIFUNCTION RECORD-CONTROL EQUIPMENT		£120

† As compared with equipment described in Table I.

‡ Instrument cost only, not including costs of primary elements or installation.

first quality equipment at a *lower* cost than less expensive single point equipment and consequently is a very attractive proposition. It is stressed, however, that the success of multipoint-multifunction record-control equipment depends both on the purchase of first quality robust industrial equipment, and on adequate maintenance. Most firms marketing such equipment also offer a scheduled "preventative" maintenance scheme. These schemes usually involve 3, 6 or 12 monthly inspections by specially trained technicians who are sufficiently familiar with the instrument to be able to spot and rectify a potential breakdown before it occurs. Such maintenance procedures are well worthwhile for most precision equipment but are essential for multipoint-multifunction record-control equipment.

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CHAPTER II

The Effects and Control of Temperature

J. W. PATCHING AND A. H. ROSE

School of Biological Sciences, Bath University of Technology, England

I. EFFECTS OF TEMPERATURE ON MICRO-ORGANISMS

A. Effect on growth

The pioneer microbiologists were quick to realize that the rate of growth of a microbe is affected by the environmental temperature, and several studies on the effect of temperature on growth of pure cultures of micro-organisms were made during the late nineteenth century. The need for water in the liquid phase restricts growth of microbes largely, though not exclusively, to temperatures in the range $-2°$ to 100°C, a range usually referred to as the *biokinetic zone*. In a given medium, a microbe grows most rapidly at a particular temperature (or over a small range of temperatures) and this is referred to as the *optimum temperature* for growth (Farrell and Rose, 1967a). In most studies, the optimum temperature for growth is based upon the rate of growth, although it is occasionally based instead on the size of the crop of micro-organisms produced. When based on this latter criterion, the optimum temperature for aerobic micro-organisms is usually found to be several degrees below that based on growth rate. The reason for this discrepancy was shown by Sinclair and Stokes (1963) to be due to the greater solubility, and therefore availability to the organisms, of oxygen at lower temperatures. These workers obtained equally large

crops of micro-organisms at higher temperatures when batch cultures were vigorously aerated.

At temperatures above the optimum there is usually a very rapid decline in growth rate (Fig. 1), and the *maximum temperature* for growth is often only a few degrees (3°–5°C) above the optimum. The maximum temperature for growth is defined as the highest temperature above the optimum at

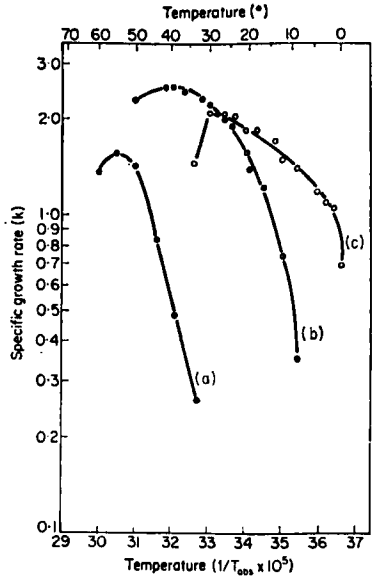

FIG. 1. Arrhenius plots of the specific growth rates of (a) a thermophilic strain of *Bacillus circulans*, (b) a mesophilic strain of *Escherichia coli*, and (c) a psychrophilic pseudomonad. Data for the psychrophile and the mesophile were replotted from those of Ingraham (1958). The thermophile data are from Allen (1953).

which growth just takes place. By contrast, at temperatures below the optimum, the rate of growth declines much more slowly. The *minimum temperature* for growth, which is defined as the lowest temperature below the optimum at which growth just takes place, can be as much as 30°C below the optimum.

While the optimum, maximum and minimum temperatures for growth are considered to be cardinal by most microbiologists, it is as well to remember that the values for these temperatures, for any one organism, may vary depending upon the chemical and physical properties of the environment.

Despite these reservations concerning the cardinal temperatures for growth, microbiologists have continued to use them to separate microbes into the three categories known as *psychrophiles*, *mesophiles* and *thermophiles*. The data in Fig. 1 show Arrhenius plots for rates of growth of a typical

psychrophile, mesophile and thermophile. These are useful divisions of the microbial world if, as Ingraham (1962) has pointed out, one asks no more of them than that they refer to microbes that grow most rapidly at low temperatures (psychrophiles), intermediate temperatures (mesophiles) or high temperatures (thermophiles). Further information on the different physiological characteristics of these groups of micro-organisms can be found in Farrell and Rose (1967a,b).

B. Effect on metabolic activities

When considering the effects of temperature on microbes, most microbiologists are concerned solely with the effects on growth, and very few studies have so far been reported dealing primarily with the effects on individual microbial metabolic activities. Indeed, it is frequently assumed that there is a tight coupling of growth with individual metabolic activities and that, for example, the optimum temperature for pigment production or rate of synthesis of an enzyme is the same as the optimum for growth. The literature shows that this is frequently not so, although these data are often contained in papers dealing with other aspects of microbial metabolism. The optimum temperature for pigment production in micro-organisms is often below that for growth (Williams *et al.*, 1965; Uffen and Canale-Parola, 1966). This also applies to polysaccharide production by many micro-organisms (Neely, 1960; Brown and Rose, 1969a). These examples emphasize that it is necessary to distinguish between effects of temperature on growth and on individual metabolic activities of micro-organisms.

II. CONTROL OF TEMPERATURE IN MICROBIOLOGICAL STUDIES

A. Maintenance of stock cultures

It is common practice to store stock cultures of micro-organisms on, or in, solidified nutrient media at a temperature near or below the minimum for growth of the organisms on that medium. Under these conditions, further growth of the organisms is prevented or severely restricted, thereby ensuring that metabolic waste products do not accumulate in high concentrations in the culture. Cultures stored under these conditions contain a large proportion of viable organisms. Mesophilic micro-organisms are customarily stored in a laboratory refrigerator (3°–5°C). Storage at room temperature (18°–22°C) is also used in some laboratories although, since many mesophiles can grow quite rapidly at these temperatures, the cultures need to be transferred at fairly frequent intervals. Storage at room temperature is also commonly used for stock cultures of thermophiles, since these organisms

do not usually grow below about 30°C. Unfortunately, nothing has been reported on the effects of these temperatures on the metabolism of thermophilic micro-organisms, and the practice of storing stock cultures of thermophiles at room temperature must therefore be accepted with some reservations.

In our experience, stock cultures of psychrophiles are best stored in a refrigerator. The temperature of most laboratory refrigerators (3°–5°C) is above the minimum for growth of psychrophiles, and the organisms in stock cultures may grow quite extensively in a relatively short time. It is advisable, therefore, to transfer stock cultures of psychrophiles fairly frequently. Some obligate psychrophiles have a maximum temperature for growth of 20°C or even lower (Stanley and Rose, 1967) and, if stock cultures of these organisms are maintained at laboratory temperatures even for a brief period, there can be a dramatic fall in viability. In general, when working with psychrophilic micro-organisms, it is advisable to ensure that stock cultures are never exposed for long periods to temperatures much above 5°C.

B. Incubation of experimental cultures

1. *Static cultures*

It is common laboratory practice to incubate static cultures of micro-organisms in incubator cabinets, hot rooms or walk-in incubators. Although a few laboratories still use water-jacketed and gas-operated incubators, anhydric incubators fitted with electrically operated thermostatic devices are the general rule. Information on anhydric incubators can be found in the account by Collins (1967). The larger models usually contain a fan which, by circulating air in the cabinet, minimizes problems that arise from the formation of temperature gradients.

Most laboratory anhydric incubators can be operated only at temperatures between ambient and about 50°C. Constant temperatures in the range from −2°C to ambient or above can be obtained with the use of suitable low-temperature incubators which are now manufactured by several suppliers. These incubators are essentially domestic refrigerators fitted with a heating element and a thermostatic device. Probably the cheapest way of obtaining these incubators is to commission a refrigeration engineer to modify domestic refrigerators. Cultures have usually to be incubated at low temperatures for long periods, often weeks. Particularly when using plate cultures, it is necessary that suitable precautions be taken to ensure that condensation water does not enter the plates. This is usually done by incubating plates upside-down in a metal canister or plastic bag.

Constant temperatures in the range 50°–90°C, which are needed for growing static cultures of thermophiles, are usually obtained by the use

of laboratory hot-air ovens fitted with a fan or blower. The main problem encountered when using these ovens is the loss of water from cultures. For this reason, use of high-temperature incubators is often restricted to cultures on solidified media which are placed in the incubator in a suitable plastic bag (e.g. Saran, Dow Chemical Co., Michigan, U.S.A.). Thermophilic microbes grow rapidly at high temperatures, so that problems that arise from the prolonged incubation of cultures are largely avoided.

Anhydric incubators satisfy the requirements of most microbiological laboratories when the need is often only to incubate static cultures under conditions where variations in temperature are not critical. However, for more rigorous studies, the use of these incubators raises two main problems. Firstly, except when a fan or blower is installed in the cabinet, there can be considerable variations in temperature, variations that are exacerbated when the door of the incubator is frequently opened. These variations can however be minimized by increasing the capacity of the heater. Anhydric incubators usually have a mercury thermometer inserted through the roof, and temperatures recorded by this thermometer can be very different from those at the corners of the cabinet, a fact easily verified by placing thermometers in tubes of oil at the corners, or better still by the use of thermistors coupled to a suitable recorder. With this type of cabinet, it is impossible to make accurate measurements of the effect of temperature on the rate of growth of micro-organisms. A second problem which often arises in laboratories when attempts are made to study the effect of temperature on some activity is that not enough incubators are available to make a sufficiently large number of observations.

The first of these problems can be overcome by incubating liquid cultures statically in constant-temperature water or kerosene baths. In many laboratory water baths, the liquid is stirred or agitated with a paddle, thereby minimizing the formation of temperature gradients in the bath. The extent of the temperature variation in the bath can be ascertained by immersing thermometers or thermistors at different places in the liquid. For routine temperature measurements of this nature, there are many types of small thermistors available (e.g., Rustrak thermistor probe, Rustrak Instrument Co. Inc., Manchester, New Hampshire, U.SA.), and it is possible to couple several of these to one recorder and so monitor the temperature over a period of several hours. The Rustrak miniaturized automatic chart recorder is well suited for this purpose.

The second problem can be overcome, to some extent at least, by the use of temperature-gradient incubators. Several different designs have been made, but all temperature-gradient incubators consist basically of bars of heavy-gauge metal at one end of which heat is applied. This heat is dissipated along the length of the bar so that a temperature gradient is formed.

The magnitude of the gradient can be regulated by controlling the amount of heat applied, by varying the nature of the insulating material surrounding the bar, and by controlling the ambient temperature. Culture tubes are inserted in holes along the length of the bar. Nakae (1966) has constructed an apparatus in which a temperature gradient is maintained by conduction in a liquid.

Temperature-gradient incubators have been used to study the effect of temperature on growth of bacteria (Oppenheimer and Drost-Hansen, 1962; Landman *et al.*, 1962; Elliott and Heiniger, 1965; Dimmick, 1965), yeasts (Battley, 1964; Fries, 1963) and algae (Halldahl and French, 1958). With some types of temperature-gradient incubator, it is possible to incubate cultures at temperatures that differ by as little as 0·2°C. Using such an apparatus, Battley (1964) found that the maximum temperature for growth of a strain of *Saccharomyces cerevisiae* is $40{\cdot}9 \pm 0{\cdot}4$°C when the yeast is grown aerobically, and $40{\cdot}3 \pm 0{\cdot}6$°C when grown anaerobically. The corresponding minimum temperatures for growth were $9{\cdot}6 \pm 0{\cdot}9$°C and $9{\cdot}7 \pm 0{\cdot}3$°C. Although most temperature-gradient incubators can accommodate only cultures on solidified media, modifications have been made to some instruments so that they can be used with static-liquid cultures (Landman *et al.*, 1962). Okami and Sasaki (1967) have described a temperature-gradient incubator for use with shake cultures.

2. *Agitated cultures*

Most laboratories grow small volumes (50–1000 ml) of stirred culture in reciprocating or rotary shaker-incubators, which are marketed by several suppliers. Most of these incubators operate at temperatures above ambient and up to about 45°C. Models are also now available in which the air in the cabinet is cooled, and these instruments can be used to grow cultures down to about 10°C. Low temperatures can also be obtained by operating a shaker-incubator in a cold room. Shaker-incubators which operate at temperatures above 50°–60°C are not available commercially, but these temperatures can be obtained by inserting an immersion heater element in a shaker-incubator cabinet (T. D. Brock, personal communication).

Shaker-incubators of the type referred to above contain temperature gradients and, if rigorous studies are to be made on the effect of temperature on the rate of growth or some aspect of metabolism of micro-organisms in shake culture, it is essential to use cultures immersed in constant-temperature water baths. The most convenient method is to immerse a culture vessel in a water bath (or a bath of kerosene if temperatures below about 2°C are required) and to agitate the culture by shaking or by sparging with sterile air or oxygen. Almost any type of culture vessel can be used in this way. One of the most useful vessels for this type of work is a cylindrical glass tube,

4–10 cm in diameter and 25–75 cm long, and about two-thirds filled with medium. By immersing this tube in a bath at an angle of about 30° from the vertical and inserting the sparger the full length of the tube, an efficient aeration can be obtained. This type of apparatus has been used extensively in Ingraham's laboratory (Ng *et al.*, 1962).

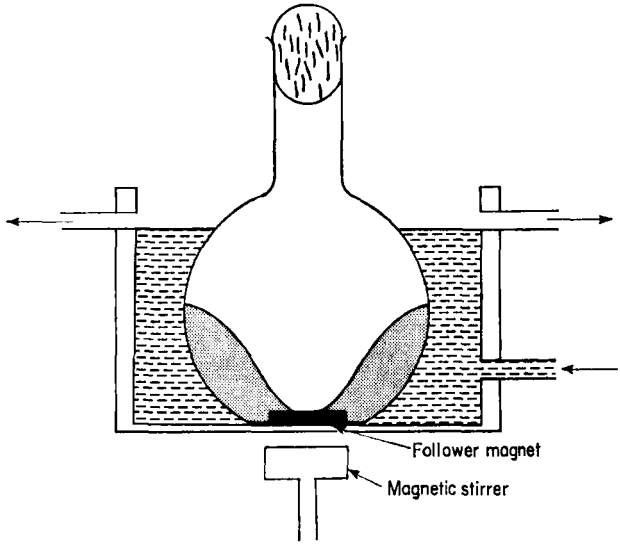

FIG. 2. Diagram (side view) of an apparatus for incubating batch cultures (2–5 litres) in round flat-bottomed flasks at different temperatures. For incubating 2 litre flasks, the vessel consists of a section (5 in. long) of "Transpalite" tubing (8 in. dia.; $\frac{1}{2}$ in. wall thickness) with a sheet of Perspex ($8\frac{1}{2}$ in. × $8\frac{1}{2}$ in.) on the bottom and Perspex tubing ($\frac{1}{2}$ in. dia.) for entry and exit ports. For incubating 5 litre flasks, the tubing section is 6 in. long ($\frac{1}{2}$ in. wall thickness) and $9\frac{3}{4}$ in. in diameter; the base plate measures 12 in. × 12 in.

If the oxygen-transfer rate obtained with cylindrical tubes is inadequate, a more efficient transfer can be obtained using an apparatus first constructed in our laboratory by Dr. S. O. Stanley. This consists of a section of cylindrical plastic tubing about 8 in. in diameter ("Transpalite", Stanley Plastics Ltd., Chichester, Sussex, England) with a square sheet of Perspex to form the base, and entry and exit ports to allow water to be circulated in the bath (Fig. 2). Round flat-bottomed flasks (2–5 litres) can be placed in these baths, and the contents of the flasks maintained at any required temperature by circulating water (or kerosene) from a constant-temperature bath. The contents of the vessel are agitated by including a follower magnet (4–6 cm long) in the flask and installing a magnetic stirrer beneath the bath. Cultures can be incubated anaerobically by fitting the neck of the flask with a rubber

stopper and sparging the culture with sterile nitrogen. Our experience has been that this type of apparatus is easily constructed and very adaptable. "Transpalite" tubing of the required size is expensive, but it represents a valuable investment. The main problems result from mechanical failure of the stirrer motors.

Larger volumes of culture (more than 5 litres) are usually grown in conventional laboratory fermenters (see this Series, Volumes 1 and 2). Temperature control in these fermenters can be obtained by operating in a temperature-controlled room, although this is not recommended if accurate temperature control is considered to be critical. Many models of laboratory fermenter are fitted with a temperature-sensing element which operates a thermostat that supplies heat to the culture in the form of a heating element. The temperature-sensing element may be a thermocouple, thermistor or resistance thermometer, with preference being given to a resistance thermometer because of its ruggedness and stability. A platinum resistance thermometer suitable for use with fermenters is manufactured by Sangamo Weston Ltd., London, England (Model S110G, Form 4). This thermometer is enclosed in a stainless steel sheath, $\frac{1}{4}$ in. in diameter and 6–12 in. long. It stands repeated autoclaving. The resistance thermometer is used in conjunction with an electronic temperature controller (Type TCB2, Fielden Electronics Ltd., Manchester, England) which controls the supply of heat (and/or cooling medium) to the contents of the vessel. Laboratory fermenters are usually fitted with cooling coils, through which cold water or other liquid (eg., kerosene) can be circulated. Maintenance of a temperature below ambient can usually be obtained in a fermenter using only these cooling coils. It is also advisable to circulate water through the coils when operating at a temperature above ambient in order to avoid overshoot of temperature.

3. *Continuous cultures*

Regulation of temperature in continuous cultures is best obtained in the same general way as described for agitated cultures (see p. 28). Many laboratory chemostats are small (about 100 ml working volume) and, because of the difficulty of introducing a large number of ports into the lids of these vessels, it is common practice to control the temperature of the culture either by operating the chemostat in a constant-temperature room or using an external infrared lamp (about 150 W) controlled by a signal from a resistance thermometer immersed in the vessel (Herbert *et al.*, 1965). The latter method is certainly preferable to operating the vessel in a constant-temperature room. When chemostats are operated at high cell densities, it is also advisable to cool the culture, which cannot be done satisfactorily in a constant-temperature room.

Control of temperature in larger chemostats is best accomplished using a thermostat and a cooling coil, as with agitated batch cultures. When the chemostat is operated at temperatures below ambient, an efficient temperature regulation can be obtained simply by circulating cold water from a low-temperature water or kerosene bath (Brown and Rose, 1969a,b).

Heating and cooling may be achieved by circulating steam (or hot water) and cold water, respectively, through the same coil. The temperature differential recorded in 10 litre stirred vessels by simple on–off control of solenoid valves connected to a common immersion coil was better than 0·2°C in the range 29°–45°C.

C. Harvesting and handling of suspensions

1. *Harvesting of suspensions*

It is common practice, particularly in studies on microbial biochemistry and physiology, to harvest and wash crops of micro-organisms in a refrigerated centrifuge, usually at a temperature around 0°C. At this temperature, the metabolic activities of the organisms are arrested or severely depressed. Rapid chilling of micro-organisms may, however, bring about certain changes in the activities of cold-labile enzymes such as ATPase as well as in the behaviour of lipids (Chapman and Wallach, 1968). In critical studies on the effect of temperature on microbial activities, it is therefore desirable to wash the organisms at the temperature at which they have been grown. Fortunately, many of the recent models of refrigerated centrifuge can be operated over a fairly wide range of temperatures ($-10°$ to 40°C), although it is as well to realize that temperature regulation in these centrifuges is often not very accurate.

For harvesting and washing thermophilic micro-organisms, it may be advisable to work at temperatures as high as 60°C. Unfortunately, as far as we are aware, no commercial centrifuge is fitted with a thermostat that will allow it to be operated at this temperature.

2. *Handling of suspensions*

Suspensions of micro-organisms in buffer or medium are frequently chilled in ice baths before they are used, mainly in order to minimize any loss in activity or viability. Storage at higher temperatures may be required in some studies, but it invariably leads to a loss of enzyme activity and to a release of endogenous low-molecular-weight compounds.

Storage of suspensions of micro-organisms at near-zero temperatures can sometimes lead to a rapid loss in viability. This effect is particularly pronounced when dilute suspensions of Gram-negative bacteria in water or dilute buffer are rapidly chilled to near-zero temperatures, an operation which is not uncommon among laboratory workers who wish to keep a

suspension for later use. This particular phenomenon, which has been termed *cold shock*, was first reported by Sherman and Albus (1923) with suspensions of *Escherichia coli*. The effect has since been demonstrated with many other Gram-negative bacteria (Meynell, 1958; Gorrill and McNeil, 1960; Strange and Dark, 1962). It is shown only with exponential-phase bacteria, and is more pronounced with bacteria grown in chemically defined media than with organisms grown in complex media (Strange, 1964). A drop in viability, which is the main manifestation of cold shock, is accompanied by a release of endogenous low-molecular-weight compounds (Strange and Dark, 1962). It would appear, therefore, that cold shock results from low temperature-induced changes in the bacterial cytoplasmic membrane. That these changes may be associated with a solidification of lipid components in the membrane is suggested by the finding (Farrell and Rose, 1968) that cold shock is not shown by pseudomonads which are grown at 10°C and which, as a result, contain membrane lipids that are more unsaturated and therefore have a lower melting point.

D. Methods used to study metabolic activities

1. *Spectrophotometric methods*

Several spectrophotometric techniques used in microbiology require the temperature of the sample to be accurately controlled. Studies on enzyme kinetics or on the action of lytic agents on whole cells, for example, usually call for a constant temperature in the range 0°–60°C. Several constant temperatures in this range are required when temperature coefficient (Q_{10}) values are to be determined. Another important use for the constant-temperature cuvette is in the determination of T_m values or melting temperatures of double-stranded DNA extracted from micro-organisms. These values, which are calculated from the change in extinction at around 260 nm when the temperature of the DNA solution is raised, provide a measure of the guanine + cytosine content of the DNA (Szybalski, 1967), and have proved extremely valuable in microbial taxonomy (Marmur *et al.*, 1963). The requirements for this technique are a spectrophotometer that will provide constant temperatures in the range 50°–100°C and, preferably, a system that will stabilize rapidly to avoid prolonged waiting between measurements.

The commonest form of constant-temperature attachment for spectrophotometers is the hollow cuvette holder. This has spaces for two or more cuvettes, and is generally constructed of brass. Water or other liquid from a constant-temperature bath is circulated through the hollow interior. The pump should be placed on the outlet side of the cuvette holder, or immersed in the bath, to avoid transmitting additional heat to the cuvettes. For

temperatures below ambient, a refrigerated bath must be used. It is also necessary to pass dry air through the cuvette housing to prevent water condensing on the surfaces of the cuvettes. For temperatures near or just below 0°C, the use of odourless kerosene is recommended.

Marmur and Doty (1962) were among the first to determine T_m values using a spectrophotometer cuvette housing fitted with "thermal spacers", through which water at the required temperature was passed. These spacers were on either side of the cuvettes rather than forming the cuvette holder. An additional spacer was installed on the photocell side of the chamber, and a slow stream of water at ambient temperature was passed through this to provide protection for the photocells. Marmur and Doty (1962) state that this system may be used for temperatures up to 100°C providing that ethylene glycol is mixed with the circulating water, and that the cuvettes are covered to prevent losses by evaporation.

One of the advantages of circulating systems is their simplicity. Constant-temperature baths are standard equipment in most laboratories. The large thermal capacity of the contents of the bath stabilizes the system against temperature fluctuations due to changes in ambient temperature or to the cutting in and out of the heating element. Providing the contents of the bath are efficiently mixed and the bath is fitted with an accurate thermostat, the temperature of solutions in the spectrophotometer cuvette should not vary by more than $\pm 0{\cdot}2$°C.

However, the large thermal capacity of the system also means that the system is usually slow to stabilize when the operating temperature is changed. When working in the range 0°–10°C, as much as two hours must be allowed for each degree change in temperature. Decreasing the capacity of the bath, or increasing the power of the heater, can shorten the time interval required to obtain a constant temperature, but this will be achieved at the expense of stability, and may cause an overshoot of temperature and an oscillation about the set temperature. Most commercial baths are designed to avoid these contingencies. Nel (1968) gives some useful practical details on the problem of temperature control in water baths.

Because of the difficulty of setting a water bath to a required temperature, and of heat losses in the circulating system, it is essential to monitor the temperature of the cuvette contents. Marmur and Doty (1962) used a mercury thermometer for this purpose, but this can be done only when the cuvette is not in the light path, as the thermometer blocks this path and introduces stray light. One way of continuously monitoring the temperature of the cuvette contents is to take the average of the temperatures at the input and the output of the water circulating through the cuvette holder. A more elegant and accurate method is to introduce a needle-like temperature-sensing element into the cuvette along one side. A thermistor is ideally

suited for this purpose, although a small resistance thermometer or thermocouple can be used. In this laboratory, a Rustrak thermistor probe coupled to a Rustrak automatic chart recorder has proved very useful for this purpose. Price (1966a) gives practical details of another system.

Mention must also be made of four other methods for temperature control in spectrophotometer cuvettes. One commercial firm (Beckman Instruments Inc., Fullerton, California, U.S.A.) markets a constant-temperature cuvette holder which is heated directly by two 50 W heaters controlled by a thermoregulator embedded in the holder. A copper cooling coil is also embedded in the block to permit operation at temperatures below ambient. It is claimed that this unit enables measurements to be made at any temperature in the range 0°–100°C with a fluctuation of less than 1·0°C; sub-ambient temperatures can be obtained by circulating a suitable fluid in the coils.

Paris and Damme (1965) describe a method utilizing the Peltier effect in which a current passed through a pair of thermocouples cools one and heats the other. This method was originally designed for operation at sub-ambient temperatures. The cold junction withdraws heat from the cuvette by means of a hollow brass cold-finger, with the same dimensions as the cuvette and with windows in the path of the light beam. The hot junction is placed in a cold heat sink; melting ice or, for lower temperatures, liquid nitrogen may be used. The temperature in the cuvette is inversely proportional to the current passed, and the authors claim an accuracy of $\pm 0{\cdot}5$°C. By reversing the current and using a hot heat sink (e.g., boiling water), temperatures above ambient may be obtained. Under these circumstances, the temperature is directly proportional to the current passed.

An apparatus described by Deutsch (1962) incorporates several novel features. The apparatus is designed for maintaining cuvette contents at temperatures above ambient. Heat is produced by a coil of platinum wire placed in the cuvette, and this is so constructed that it surrounds the optical path. The coil also acts as a temperature-sensing device by forming one arm of a Wheatstone bridge. Deviation from the set temperature causes an increase in the voltage applied to the coil by means of a motor-driven variac. The contents of the cuvette are stirred by a minature magnetic follower. The system is claimed to be accurate to ± 1°C, and is capable of adjusting the cuvette contents to any temperature in the range 20°–100°C at the rate of about 5°C/sec. There may be some electrolysis around the coil, but this can be avoided, at the cost of a slight decrease in performance, by using insulated wire.

Finally, Ribbons, Hewett and Smith (1968) describe what seems to be an elegant form of constant-temperature cuvette which incorporates a Clark oxygen electrode, so that simultaneous spectrophotometric and

polarographic measurements may be taken. Heat is supplied from a nichrome heating coil incorporated into a stainless-steel sidewall of the cuvette. The temperature control, which utilizes the temperature effect of a transistor to control the power supplied to the heater, is claimed to give an accuracy of $\pm 0{\cdot}2$°C over the range 22–38°C.

2. *Manometric methods*

Umbreit *et al.* (1964) stress the need for an accurate and uniform operating temperature in manometric studies. With most instruments, the flasks are maintained at a constant temperature by immersion in a constant-temperature water bath. It should be emphasized that this arrangement is possible only when the volume of the flask is very much greater than the volume of the manometer. This is the most common situation, and it is then possible to ignore the fact that the temperature of the gas in the arms of the manometer is not at the same temperature as the gas in the reaction flask. Instruments are marketed which use very small reaction flasks, and in these the entire assembly of flask and manometer is immersed in the constant-temperature bath. In each experiment in manometry, a thermobarometer, consisting of a manometer and reaction flask containing only water, is set up in addition to the experimental flasks. Any increase or decrease in the reading on the thermobarometer is recorded and used to apply a correction for changes in bath temperature or atmospheric pressure that occurred during the experiment. However, the thermobarometer readings do not take into account the change in the flask constant caused by any deviation in the experimental temperature from the temperature used to calculate the constant. Umbreit *et al.* (1964) point out that a rise in temperature of one degree from the set value at 28°C could cause an error of 0·3%. It is also important that the temperature of the bath be uniform. A flask that is at a temperature one degree higher than another in the bath will indicate a pressure corresponding to about 33 μl of gas in a total gas volume of 10 ml. A maximum permissible variation of $\pm 0{\cdot}05$°C has been suggested (Umbriet *et al.*, 1964). This would correspond to an excess pressure of 1·7 μl in a total gas volume of 10 ml (see also Beechey and Ribbons, this Series, Volume 6).

Lardy *et al.* (1948) first described a circular bath for use in manometric studies, and this has advantages over the earlier types of apparatus. The circular design has since been widely adopted. Water is circulated in the bath, either by a centrifugal pump or a stirrer motor, and because of the shape of the bath this ensures that the temperature variation in the bath is small.

A contact thermometer and electronic relay are now almost universally used for control of bath temperature in apparatus used for manometric work. The introduction of the thyristor has now made this a very reliable

piece of equipment. Heating is provided by an immersion heater. One manufacturer (W. Braun, Melsungen, West Germany) heats the water in the bath by passing a current directly through the water; a small quantity of salt must be dissolved in the water. This system is illegal in the United Kingdom unless an isolating transformer is fitted. The heater may have a provision for running at higher power so that the water may be heated rapidly up to the desired temperature. The power is then cut down so that the control system can maintain the temperature without oscillation. For operating at temperatures below ambient, a cooling coil can be fitted in the bath through which cold water or kerosene can be circulated.

3. *Microscopical methods*

The temperature-regulated microscope stage was originally a tool of the physical chemist. Later, these stages were adapted and used in the microscopical examination of biological material, especially protozoa. More recently, the need for a constant-temperature stage has become important in time-lapse cinematography (see also Quesnel, this Series, Volume 5).

Many difficulties must be overcome in the design of a constant-temperature stage for biological work. The area that is maintained at a constant temperature must not contain anything that might interfere with passage of the light beam. Also, heat must pass to the specimen through the glass microscope slide which has a low heat conductivity and a small cross-sectional area. The slide, however, has a large surface area and a relatively low thermal capacity, and these factors make it susceptible to irregular temperature changes caused by convection heat losses. Some provision must also be made against evaporation of water from the specimen. Finally, the whole assembly must not be so bulky that it interferes with the efficient operation of the microscope. Very few of the commercially available assemblies come near to solving all of these problems.

Heating is generally applied directly to the stage by electrical elements. These may be controlled by a thermistor or a resistance thermometer. One manufacturer (Carl Zeiss, Jena, D.D.R.) uses a regulating resistance for rough adjustment of the temperature, while the fine adjustment is made by means of a miniature contact thermometer. The problem of temperature variations due to convection is solved by providing a Plexiglass cover for the stage and slide; this cover has an aperture through which the nosepiece of the objective protrudes. It is claimed that this stage can be operated in the range 30°–60°C with a maximum deviation from the set value of $\pm 0{\cdot}5$°C at 40°C and $\pm 1{\cdot}5$°C at 60°C. After 15 min stabilization, the temperature of the stage remains constant to within $\pm 0{\cdot}2$°C. Several microscope stages are also available fitted with cooling chambers through which cold water can be circulated. Using these stages, material can be examined at tempera-

tures below ambient. (See Quesnel, this Series, Volume 1, for full details of microscopical culture techniques.)

Probably the most satisfactory solution to the problem of temperature-controlled microscopic examination is to use a small incubator constructed of transparent plastic material, into which the entire microscope is placed. Such a device is described by Price (1966b). Extension shafts are added to the focusing controls so that they may be operated from outside the incubator. The eyepiece projects through an aperture at the top of the incubator. A door is provided at the front to give access to the microscope stage, and the temperature in the incubator is controlled by electrical heaters and a thermistor mounted at stage height. A more refined temperature-control mechanism described by Buchsbaum and Kuntz (1954) places the heaters in a separate chamber from which hot air is mechanically blown into the incubator.

E. Temperature measurement in natural environments

There is often a need, particularly in ecological studies, for the temperatures of soils, lakes and oceans to be monitored over fairly long periods of time. Sometimes the need is to record only the maximum and minimum temperatures encountered in a particular environment, and these data can easily be obtained by immersing a maximum-and-minimum thermometer in the soil or water. More often, the need is for a more or less continuous log of the temperature changes in the environment. Over short periods of time, such as a few days, these data can be obtained by immersing thermocouples or thermistors in the soil or water and recording changes on a suitable recorder. The Rustrak automatic chart recorder is well suited for this work. Macfadyen (1956, 1967) has listed other instruments which can be used. In recent years, the periodical recording of temperatures at several points in soil has been made possible using a battery-operated waterproof "logger". This instrument can be buried under the ground, and the readings collected as infrequently as once a year. Such an apparatus is the d-Mac Limpett Logger (d-Mac Ltd., Glasgow, Scotland).

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CHAPTER III

Measurement and Control of pH Values

A. L. S. MUNRO

Marine Laboratory, Aberdeen, Scotland

I. FUNDAMENTAL PRINCIPLES AND THE pH SCALE

A. Acid–base relationships

Brønsted (1923) and Lowry (1923) have defined an acid as a compound with a tendency to lose a proton and a base as a compound with a tendency to gain a proton—

$$\text{acid} \rightleftharpoons \text{base} + H^+ \qquad (1)$$

where H^+ represents a proton or a hydrogen ion. In aqueous solution hydrogen ions are associated with water of hydration.

$$H^+ + H_2O \rightleftharpoons H_3O^+$$

However in this text the proton will be referred to as H^+ or the hydrogen ion. Sørensen (1909) first suggested the use of the negative logarithm of the

hydrogen ion activity to express the concentration of the hydrogen ion in a solution. This he called the pH value in which p stands for potzen (German, power) that is, the index or logarithm—

$$\mathrm{pH} = -\log_{10} a_{H^+} = \log \frac{1}{a_{H^+}} \tag{2}$$

where a_{H^+} is the activity of the hydrogen ion and may also be represented as the product of the concentration c_{H^+}, in gram ions or moles/litre, and the activity coefficient f_{H^+}.

$$a_{H^+} = c_{H^+} \times f_{H^+} \tag{3}$$

Only at great dilution in ionic solutions does the activity coefficient approach unity and only then is the hydrogen ion concentration equivalent to the hydrogen ion activity. For strong electrolytes at concentrations other than very dilute, the activity coefficient differs considerably from unity and also varies with the concentration. At intermediate concentrations Lewis and Randall (1921) found that the activity coefficient depended on (1) the valency of the given ion and (2) the ionic strength (I) of the solution. The ionic strength is given by the equation—

$$I = \tfrac{1}{2} \Sigma m_i z_i^2 \tag{4}$$

where m_i is the molarity of the ion and z_i its valency, I is obtained by summing the individual $m_i z_i^2$ of each ion present in the solution, e.g., the ionic strength of a medium containing 0·01 M sodium chloride and 0·005 M magnesium chloride is calculated as follows—

$$\begin{aligned} I = \tfrac{1}{2}[&(0{\cdot}01 \times 1^2) \quad + (0{\cdot}01 \times 1^2) \\ &(0{\cdot}005 \times 2^2) \quad + 2\,(0{\cdot}005 \times 1^2)] \\ &= 0{\cdot}025 \end{aligned}$$

Potassium ion, Chloride ion, Magnesium ion, Chloride ion

Debye and Hückel (1923) showed that the variation of the activity coefficient of a given ionic species in dilute solutions ($I < 0{\cdot}1$ M) of all electrolytes is represented by the approximate equation—

$$-\log f = \frac{A z_i^2 \sqrt{I}}{1 + \sqrt{I}} \tag{5}$$

For water at 25°C, A has the value 0·509.

B. The dissociation constant

In a solution of an electrolyte there exist in equilibrium, free ions and undissociated molecules. Writing the equilibrium as—

$$HA \rightleftharpoons H^+ + A^-$$

the equilibrium constant, called the dissociation constant is given by—

$$K = \frac{a_{H^+} \times a_{A^-}}{a_{HA}} = \frac{c_{H^+} \times c_{A^-}}{c_{HA}} \times \frac{f_{H^+} \times f_{A^-}}{f_{HA}} \quad (6)$$

where a represents the activity, c the concentration and f the activity coefficient of the species. Some compounds undergo almost complete dissociation and are known as strong electrolytes whereas compounds which dissociate only to a limited extent are known as weak electrolytes.

If α is the degree of dissociation of the electrolyte, i.e., the fraction of the electrolyte present in the form of free ions, and c is the total concentration in moles/litre, then c_{H^+} and c_{A^-} are each equal to αc while c_{HA} is equal to $(1-\alpha)c$. It follows therefore that—

$$K = \frac{\alpha c \times \alpha c}{(1-\alpha)c} \cdot \frac{f_{H^+} \times f_{A^-}}{f_{HA}}$$

$$= \frac{\alpha^2 c}{1-\alpha} \cdot \frac{f_{H^+} \times f_{A^-}}{f_{HA}} \quad (7)$$

For weak electrolytes the ionic concentrations are low and the solutions do not deviate appreciably from ideal behaviour, therefore the activity coefficients do not deviate greatly from unity.

C. Ionic product of water

Before explaining the use of the pH scale it is necessary to understand that water behaves as both acid and base in that it is capable of gaining or losing a proton. The equilibrium is expressed—

$$H_2O = H^+ + OH^- \quad (8)$$

and as before

$$H^+ + H_2O = H_3O^+$$

The equilibrium constant for the dissociation of water is given by—

$$K_w = \frac{c_{H^+} \times c_{OH^-}}{c_{H_2O}} \quad (9)$$

Experimental evidence has shown that in pure water—

$$K_w = 1{\cdot}00 \times 10^{-14} \text{ at } 25°C$$

and since both ions are present in equal amounts—

$$c_{H^+} = c_{OH^-} = 1 \times 10^{-7} \text{ gram ion/litre} \quad (10)$$

Equation (9) means that in any moderately dilute aqueous solution, the product of the concentrations of the hydrogen and hydroxyl ions is constant. Consider a 0·001 N solution of a strong acid, the resulting hydrogen ion concentration being 10^{-3} gram ion/litre. The ionic product of water is

10^{-14}, hence the hydroxyl ion concentration is deduced as follows if the activities are taken as unity—

$$c_{OH^-} \times c_{H^+} = 10^{-14} \text{ and } c_{H^+} = 10^{-3}$$

taking logarithms to base 10 we have —

$$\log c_{OH^-} + \log c_{H^+} = 14 \text{ and } \log c_{H^+} = 3$$
$$\therefore \log c_{OH^-} = 11 \text{ and } c_{OH^-} = 10^{-11}$$

Similarly, in a 0·001 N solution of NaOH the hydrogen ion concentration is 10^{-11}

D. Use of the pH scale

We have seen from equation (2) that—

$$pH = -\log a_{H^+}$$

Similarly the logarithmic method of representation can be extended to other agents, e.g., pOH is used for hydroxyl ion activities so that—

$$pOH = -\log a_{OH^-}$$

Dissociation constants of acids and bases and the ionic product of water can also be represented in a logarithmic manner, thus—

$$pK_a = -\log K_a \text{ and } pK_w = -\log K_w$$

where pK_a and pK_w are called the dissociation exponents of the acid and of water respectively. By taking logarithms of equation (9) and changing the sign we have—

$$-\log c_{H^+} - \log c_{OH^-} = -\log K_w$$
$$pH + pOH = pK_w = 14 \text{ at } 25°C \qquad (11)$$

Therefore the sum of pH and pOH is equal to 14 in water or any other *dilute* aqueous solution. A pure solution of water at 25°C where $c_{H^+} = c_{OH^-} = 10^{-7}$, and therefore pH and pOH are both 7, is known as a neutral solution. The relationships between pH and pOH are shown in Table I.

TABLE I

Interrelationships between acid and alkaline solutions

	Acid solutions			Neutral solution	Alkaline solutions		
	N	N/10	N/100		N/100	N/10	N
c_{H^+}	1	10^{-1}	10^{-2}----	10^{-7}----	10^{-12}	10^{-13}	10^{-14}
pH	0	1	2-----	7-----	12	13	14
c_{OH^+}	10^{-14}	10^{-13}	10^{-12}---	10^{-7}----	10^{-2}	10^{-1}	1
pOH	14	13	12-----	7------	2	1	0

In making calculations of fractions of a pH unit it is necessary to remember that the values of the mantissae given in logarithm tables are always positive. For example, when the hydrogen ion concentration is 4×10^{-6}, pH $= -\log (4 \times 10^{-6}) = -[\log 4 + \log 10^{-6}] = -[0{\cdot}6021 - 6] = 5{\cdot}3979 \approx 5{\cdot}40$.

To convert a fractional pH value such as 6·35 to the corresponding hydrogen ion concentration, it is necessary to find the number whose negative logarithm is 6·35, i.e., characteristic -7, mantissa $+ 0{\cdot}65$. The hydrogen ion concentration in this case is $4{\cdot}5 \times 10^{-7}$.

To find the pH value of a weak electrolyte such as 0·001 N acetic acid, let α be the degree of dissociation and V the volume in litres containing 1 gram equivalent of the acid. For a 0·001 N solution $V = 1000$ litres. Expressing the dissociation as—

$$CH_3COOH \rightleftharpoons CH_3COO^- + H^+$$

and the fraction contributed by each molecule as—

$$\frac{1-\alpha}{V} \quad \frac{\alpha}{V} \quad \frac{\alpha}{V}$$

and

$$K_{\text{acetic acid}} = \frac{(a_{CH_3COO^-})\,(a_{H^+})}{a_{CH_3COOH}} = \frac{\alpha^2}{V(1-\alpha)}$$

As the degree of dissociation is small we can equate $1-\alpha$ to 1 and therefore

$$K_{\text{acetic acid}} = \frac{\alpha^2}{V} = \frac{\alpha^2}{1000}$$

$$\therefore \alpha = \sqrt{1{\cdot}8 \times 10^{-2}} = 0{\cdot}134$$

From values of dissociation constants in Table XI—

$$pK_{\text{acetic acid}} = 4{\cdot}756 \text{ at } 25°C$$

and

$$\begin{aligned} K_{\text{acetic acid}} &= -\text{antilog } 4{\cdot}756 = \text{antilog } (-5 + 0{\cdot}244) \\ &= 1{\cdot}8 \times 10^5. \end{aligned}$$

This means that in 1000 litres there are 0·134 g ions H^+ and per litre $1{\cdot}34 \times 10^{-4}$. If the activities are taken as unity in this dilute solution then—

$$\begin{aligned} \text{pH} = -\log c_{H^+} &= -\log (1{\cdot}34 \times 10^{-4}) \\ &= -\bar{4}{\cdot}1271 = 3{\cdot}87 \end{aligned}$$

There are two points to bear in mind when using the pH scale, the first is that it is a reciprocal scale and therefore the pH number decreases as the hydrogen ion concentration increases. Secondly it uses a logarithmic scale with 10 as its base which means that each change of one pH unit corresponds to a tenfold change in hydrogen ion concentration. Also since the scale

represents logarithmic values the difference in acidity between, for example, pH 5 and 5·3 is greater than between pH 7 and 7·3 as is shown by calculating the hydrogen ion concentration.

At pH 5 c_{H^+} is 1×10^{-5}	At pH 7 c_{H^+} is 1×10^{-7}
pH 5·3 c_{H^+} is $0{\cdot}5 \times 10^{-5}$	pH 7·3 c_{H^+} is $0{\cdot}5 \times 10^{-7}$

Therefore a difference of 0·3 pH units represents a c_{H^+} of $0{\cdot}5 \times 10^{-5}$ g ion per litre in one case and $0{\cdot}5 \times 10^{-7}$ g ion per litre in another. This example also illustrates another feature of the pH scale that is often not understood, namely that at pH 5 or at 7 the hydrogen ion concentration is twice that at pH 5·3 and 7·3 respectively.

II. THE EFFECTS OF pH ON THE CELL

Many micro-organisms have an optimum pH for growth around 7 with most favouring the pH range 5 to 8. However, there are many exceptions including the acetic acid bacteria oxidizing ethanol to acetic acid, the thiobacilli which oxidize sulphur to sulphuric acid and, at the other extreme, urea-decomposing bacilli many of which cannot grow below pH 8 and numerous algae living in natural waters above pH 10. The apparent indifference of these micro-organisms to pH is due to the impermeable nature of the cytoplasmic membrane to H^+ and OH^- ions.

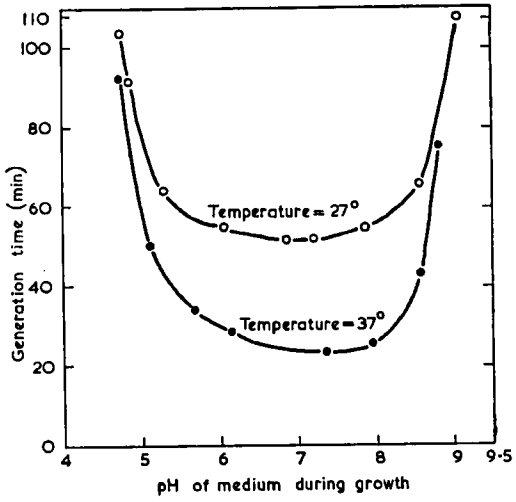

FIG. 1. Variation of generation time with growth pH in *Escherichia coli*. Data of Gale and Epps (1942).

Growth is one criterion that can be used as a measure of the effects of pH as Gale and Epps (1942) demonstrated (Fig. 1) for *Escherichia coli*. However, although growth occurred between pH 4·5 and 9, the growth pH had a marked influence on the enzymic content, the enzymes most influenced being the deaminases and decarboxylases, the latter being strictly adaptive. Deaminase production occurred between pH 7 and 8 and decarboxylase

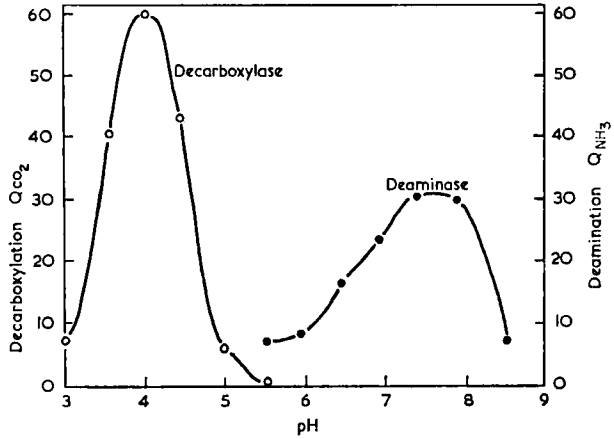

FIG. 2. Variation with pH of the activities of the enzymes of *Escherichia coli* which attack L-glutamic acid. Data of Gale (1940).

TABLE II

Mixed acid fermentation of *Escherichia coli*. Data of Blackwood *et al.* (1956)

Products	mmoles per 100 mmoles glucose fermented	
	pH 6·2	pH 7·8
2, 3-Butanediol	0·3	0·26
Acetoin	0·059	0·190
Glycerol	1·42	0·32
Ethanol	49·8	50·5
Formic acid	2·43	86·0
Acetic acid	36·5	38·7
Lactic acid	79·5	70·0
Succinic acid	10·7	14·8
Carbon dioxide	88·0	1·75
Hydrogen	75·0	0·26
Carbon recovered, %	91·2	94·7

production between pH 6 and 4·5. Fig. 2 (Gale, 1940) illustrates that enzymes have pH optima for maximum activity and that, for *E. coli*, decarboxylase production is optimal at about one pH unit higher than the optimal pH for enzyme activity. Fermentation studies by Blackwood *et al.* (1956) using automatic pH control demonstrate another distinct effect of pH on the enzymic composition of *E. coli* (Table II); at pH 6·2 carbon dioxide and hydrogen are produced from glucose fermentation whereas at pH 7·8 the production of these gases is inhibited due to the inactivation of formic hydrogen lyase and an equivalent amount of formic acid is produced instead. The formation of at least part of the hydrogen lyase enzyme system is suppressed by high pH. An examination of the products of ethanol fermentation from glucose (Table III) shows how different pH values affect the metabolic activities of yeast (Neish and Blackwood, 1956). A more detailed analysis of growth at a sub-optimal pH value is shown in Table IV (Hernandez and Johnson, 1967) where, because of the pH induced change in the fermentation, there is a net reduction in the amount of ATP produced per mole of glucose fermented, calculated on the basis of the energy steps in the fermentation pathways of the products. This decrease accounts for the drop in yield of cells and the generation time is also doubled as a result of the imposed 2 unit decrease in pH.

TABLE III

Ethanol fermentation by yeast. Data of Neish and Blackwood (1956)

Product	mmoles/100 mmoles of glucose fermented				
	pH 3·0	pH 6·0	pH 6·0[a]	pH 7·6	pH 7·6[a]
2,3-Butanediol	0·75	0·53	0·39	0·68	0·33
Acetoin	Nil	Nil	Nil	0·19	0·01
Ethanol	171·5	160·5	165·9	129·9	148·0
Glycerol	6·16	16·2	10·4	32·3	25·1
Butyric acid	0·13	0·36	0·39	0·21	0·35
Acetic acid	0·52	4·03	4·27	15·1	9·16
Formic acid	0·36	0·82	0·46	0·49	0·43
Succinic acid	0·53	0·49	1·14	0·68	0·43
Lactic acid	0·82	1·63	1·73	1·37	0·87
Carbon dioxide	180·8	177·0	178·0	148·5	167·8
Glucose carbon assimilated	12·4	12·4	—	—	—
Fermentation time, h	29·0	15·5	16·0	25·0	32·0
Glucose fermented, %	98·5	98·0	98·5	60·3	98·1
Carbon recovered, %	93·8	96·4	94·0	91·3	94·1

[a]Automatic pH control

TABLE IV

Aerobacter cloacae **grown anaerobically on glucose. Data of Hernandez and Johnson (1967)**

pH	Generation time h	Total yield of cells (*g*)	mmoles of products/100 mmoles of glucose fermented							
			Ethanol	Acetic acid	2 : 3-butanediol	Lactic acid	Succinic acid	Acetoin	ATP mmoles	Y_{ATP}[a]
5·0±0·1	4	1·77	61·8	5	38·8	Nil	3·3	Nil	153	11·6
7·2±0·1	2	2·58	67·5	69·5	2·3	3·6	6·4	1·5	224	11·5

[a]Grammes of cells per mole of ATP.

Other cellular mechanisms sited in or sufficiently near the cell surface can be influenced by the pH of the environment. Pirt and Callow (1959), using a continuous culture apparatus to study the effect of pH on the morphology of *Penicillium chrysogenum*, found that hyphal length decreased when pH exceeded 6 while at pH 7 and above, an increasing number of hyphae showed swelling. From this result it was concluded that cell wall structure and composition were dependent on the pH of the environment and, in the swollen cells, the change of structure resulted in loss of cell wall rigidity and consequent inability to resist internal osmotic pressures. The terminal cytochrome oxidases are another class of enzyme influenced by pH due to the fact that their ability to reduce terminal electron acceptors is a function of redox potential which, in turn, is markedly influenced by pH (see Jacob, this Volume, p. 91).

Toxic effects of hydrogen ion concentration can arise indirectly through the penetration of the cell by molecules undissociated in the environment which, on entry to the near neutral interior, dissociate thereby changing the conditions inside the cell. Some of the commonly used organic acid buffers are known to be inhibitory because of this phenomenon.

III. CHANGES OF pH AS A RESULT OF MICROBIAL ACTIVITY

The nature of the activities of micro-organisms is such that the pH of the environment of a metabolizing culture will not remain constant for long. These changes of pH are associated with the uptake of certain anions and cations, the degradation of proteins and other nitrogenous compounds yielding ammonia or other alkaline products and the metabolism of carbohydrates and hydrocarbons yielding organic acids.

If nitrogen is supplied as an ammonium salt, then utilized ammonia leaves in the medium a corresponding amount of free acid. When an organism is grown aerobically on a limiting carbon source such as carbohydrate then ammonia uptake is likely to be the major contributor of hydrogen ions to the medium. An example of an alkaline change is the uptake of CO_2 from HCO_3^-. Similarly, the metabolism of either ion or the undissociated molecule of an organic buffer will lead to changes in pH of a culture medium by upsetting the existing ratios of acid and salt molecules.

The aerobic metabolism of a single or of a mixture of amino-acids that are also the major or sole carbon source, will lead to the production of ammonia in excess of the cells' nitrogen requirements. Assuming that the uptake of amino-acids does not disturb the existing buffer equilibrium, then the ammonia production will result in OH^- ions and an increase in pH. The anaerobic metabolism of amino-acids is more complex and

partly pH dependent. Decarboxylase activity will result in alkaline amine products whereas deaminase activity will give both acid and alkaline products.

During exponential growth under aerobic conditions, many organisms produce partially metabolized compounds such as acetic and pyruvic acids in the presence of excess sugar. Hanson *et al.* (1963) found that in batch cultures of *Bacillus cereus* strain T grown on glucose, considerable amounts of acetic acid were produced during growth although in the subsequent stationary phase the acetic acid was largely metabolized. Continuous culture studies by Pirt (1957) and Wilkinson and Munro (1967) using automatic pH control demonstrated that, with glucose limiting growth, both *Aerobacter aerogenes* and *Bacillus megaterium* respectively produced quite large amounts of acetic acid as the growth rate approached the maximum. Similarly Wilkinson and Munro (1967) found that in the continuous culture of *B. megaterium* appreciable amounts of acetic and other acid products were formed when glucose and oxygen were in excess and growth was limited by the different mineral nutrients, nitrogen, potassium or sulphate

The anaerobic metabolism of carbohydrates results in a complexity of acid and neutral products, much depending on the organism and the conditions of culture. One example which is the concern of diagnostic bacteriologists is the mixed acid fermentation of the Enterobacteriaceae. This family can be divided into two groups, the first containing organisms resembling *E. coli* which produce volatile acids (Table II) and no butanediol, and the second, typified by *A. aerogenes*, which produce the neutral compound, 2,3-butanediol and correspondingly less acid.

The products of the first group, the *E. coli* fermentation, are—

$$\text{glucose} \rightarrow \text{lactate} + \text{acetate} + \text{ethanol} + CO_2 + H_2.$$

This reaction is strongly acid-forming, providing the basis of the methyl red test. The test relies on the production of sufficient acid to turn methyl red indicator red, which occurs at pH values of 5·1 and below. The test should always be carried out in unbuffered medium with excess (1% or more) glucose and the minimum of peptone or broth to reduce the formation of alkaline products.

The second type of mixed acid fermentation produces the neutral compound, 2,3-butanediol at the expense of acid.

$$\text{glucose} \rightarrow \text{2,3-butanediol} + \text{acetate} + \text{formate} + \text{ethanol} + CO_2$$

Small quantities of acetoin (acetylmethylcarbinol), a precursor of 2,3-butanediol that is always present, are easily detected by the Voges-Proskauer reaction and, consequently, this test affords a ready means of detecting 2,3-butanediol production. The diversion of some intermediates to

2,3-butanediol, limits acid production to such an extent that a negative methyl red reaction results. The pH of the medium has a marked effect on the 2,3-butanediol fermentation (Table IV). Above pH 6·3, acetic and formic acids are produced at the expense of CO_2, acetoin and 2,3-butanediol whereas below pH 6·3, 2,3-butanediol production is large and acetic acid is converted to acetoin and 2,3-butanediol. Therefore in a fermentation of this type, starting at pH 7–7·4 in unbuffered media, the pH will drop to 5·5–6·0 in 24–36 h as the result of acid production, and will remain thereafter at this level until the glucose is exhausted, when acetic acid will be converted to 2,3-butanediol and the pH will rise to 6·3–6·5 after 72 h.

IV. CONTROL OF pH BY BUFFER ACTION

A. Theory of buffer action

Aqueous solutions of salts which have the property of resisting change of pH when acid or alkali is added, display the property of buffer action. A buffer solution is therefore one which is resistant to change of pH upon the addition of an acid or a base. For example, solutions of potassium nitrate and ammonium acetate have a pH of 7 but on adding 0·1 ml of 1 N nitric acid to 1 litre of each of these solutions the pH of the former changes to 4 while the latter is hardly affected. Similarly the addition of 0·1 ml of 1 N potassium hydroxide would change the pH of potassium nitrate solution from 7 to 10 while causing little change of pH of the ammonium acetate solution. Thus ammonium acetate, the salt of a weak acid and a weak base, displays buffer action while potassium nitrate, the salt of a strong acid and a strong base, has no buffer action.

The buffer action of a solution of a weak acid, HA, and its highly ionized salt, KA, which contributes A^- ions can be explained by the neutralization of added hydrogen ions by the anions, A^-, acting as a base. As HA is a weak acid, much of it will exist in the undissociated form—

$$H^+ + A^- \rightarrow HA$$

Addition of OH^- ions promotes—

$$OH^- + HA \rightarrow H_2O + A^-$$

Similarly in the case of a buffer containing a weak base B and its salt providing BH^+ ions—

$$H^+ + B = BH^+ \text{ and } OH^- + BH^+ = H_2O + B$$

From equation (6), where the activities have been replaced by the product of the concentrations and the activity coefficients, the activity coefficient f_{HA} of the undissociated molecules, the buffer in this case, may be taken as

unity. This is only true provided the ionic strength is not too high and therefore equation (6) becomes—

$$a_{H^+} = K_a \frac{c_{HA}}{c_{A^-}} \times \frac{1}{f_{A^-}}$$

To simplify matters if f_{A^-} is taken as unity and the activity term a_{H^+} replaced by the corresponding concentration of hydrogen ions—

$$c_{H^+} = k_a \frac{c_{HA}}{c_{A^-}} \tag{12}$$

To indicate that the equation is not exact, the dissociation constant K_a is replaced by k_a.

As the weak buffer acid is only ionized to a small extent, especially in the presence of the salt, the quantity c_{HA}, representing the concentration of undissociated molecules, can be taken as being equal to the total concentration of acid—

c_{HA} = total concentration of weak buffer acid = [acid]

and, as the salt of the buffer acid is supposed to be completely ionized—

c_{A^-} = concentration of completely ionized salt = [salt]

Therefore equation (12) can be expressed

$$c_{H^+} = k_a \frac{[\text{acid}]}{[\text{salt}]} \tag{13}$$

On taking logarithms and changing the sign on both sides, we have—

$$-\log c_{H^+} = -\log k_a + \log \frac{[\text{salt}]}{[\text{acid}]}$$

$$\therefore \text{pH} = \text{p}k_a + \log \frac{[\text{salt}]}{[\text{acid}]} \tag{14}$$

where equation (14) is known as the Henderson equation. From this equation, the pH of a buffer solution of known concentration can be calculated with fair approximation in the pH range of 4 to 10. Additionally, it may be used to prepare buffers of a definite pH value, a fact which is of great use in the construction of buffer solutions. From equation (14) the pH of a solution of a given acid and its salt is given by the logarithm of the ratio of the salt concentration to that of the acid as $\text{p}k_a$ is a constant. The addition of a strong acid will convert some of the salt to the buffer acid so that the ratio [salt]/[acid] changes. This change should be small if the buffer is chosen correctly. Maximum buffer capacity is realised when [salt]/[acid] equals unity, i.e., when the concentration of salt and acid are equal.

For accurate work the pH of a buffer solution should be checked with a glass electrode because in the foregoing equations the activity coefficients

have been neglected and the dissociation constant is given its value at infinite dilution. If the ionic strength is not too great the required corrections can be computed from the Debye-Hückel equation.

If we consider the addition of 0·2 mole of acid to a buffer solution containing 1 mole of a weak acid and 1 mole of its salt then the change in ratio is from 1 to 0·8/1·2 as 0·2 moles of the base has been converted to 1·2 moles of acid. Therefore, before addition of acid—

$$\text{pH} = \text{pk}_a + \log 1 = \text{pk}_a$$

After addition of 0·2 mole acid—

$$\text{pH} = \text{pk}_a + \log \frac{0{\cdot}8}{1{\cdot}2} = \text{pk}_a - 0{\cdot}17$$

Therefore, the change in pH is 0·17. Similarly, if 0·2 moles of acid are added to a buffer solution containing 1·6 moles of weak acid and 0·4 moles of its salt then the change in the ratio of acid to base is from 0·250 to 0·111.

TABLE V

Change of pH as a result of salt effect in three buffer solutions

Buffer solution	Molarity of added salt		
	0·01	0·02	0·05
Potassium hydrogen phthalate, 0·05 M (added KCl)	−0·008	−0·019	−0·044
Potassium dihdrogen phosphate, 0·025 M: disodium hydrogen phosphate, 0·025 M (added NaCl)	−0·012	−0·022	−0·051
Borax 0·01 M (added NaCl)	−0·007	−0·014	−0·035

The resulting pH change is 0·35. These two examples show that the greatest resistance to change of pH in a buffer solution is when the ratio of [salt]/[acid] is nearest unity. A rough guide to assessing the usefulness of buffer capacity suggests that when the ratio of salt to acid is greater than 10 to 1 or 1 to 10 the buffer has reached the limit of its capacity. When the ratio of [salt]/[acid] is unity the pH of the buffer solution is pk_a but if the ratio [salt]/[acid] is 10 then the pH is $\text{pk}_a + 1$, and if the ratio is 0·1 then the pH is $\text{pk}_a - 1$. On this basis the range of a simple monoacidic or monobasic buffer is $\text{pk}_a - 1$ to $\text{pk}_a + 1$.

B. Salt effects

The primary effect of adding a neutral salt to a dilute buffer solution is to lower the activity coefficients of the ions present. Activity coefficients of uncharged species remain largely unchanged. The pH of solutions of strong acids and of weak base salt buffer mixtures is raised whereas that of solutions of strong bases, weak acid–salt buffer mixtures, and solu ions of acid salts is lowered (Table V).

C. Choice of a suitable buffer

Whether the buffer is for a growth medium, an enzyme assay or an intracellular preparation, it should be considered with as many of the following criteria as is relevant to the experiment.

(1). The pK should be close to the desired experimental pH. An exception of this general rule is where an automatic pH controller is employed, allowing the use of a partially dissociated salt over the whole of its buffering range.

(2). The buffer should be very soluble in water and non-volatile.

(3). The medium concentration, temperature and ionic composition should have a minimum effect on the dissociation of the buffer.

(4). If possible the buffer should not complex with cations and if it does they should be soluble complexes. The binding constants of such complexes should be known.

(5). The buffer should not absorb light in the visible or ultraviolet regions of the spectrum. Absorption at wave lengths greater than 230–240 nm may interfere with spectrophotometric assays.

(6). Ionic buffers (for examples see Tables XIII–XXVI), especially with increasing concentration, produce salt effects which adversely affect many systems.

(7). Buffers exist as two different molecular species, protonated and non-protonated, either of which species may be toxic or have unwanted side effects.

(8). The buffer should pass through biological membranes with the utmost difficulty to minimize the effects detailed under (7) and (9).

(9). The buffer should be non-metabolizable, resist enzymic degradation, and should not act as an analogue inhibitor.

(10) The cost of the buffer materials should always be considered.

At the end of this chapter there are tables of buffers prefaced by a table containing pK values at different temperatures of each of the buffer acids and other acids of general interest. In some of the buffer tables a column appears under the heading β whose values represent the buffering capacity in mmoles of base required to change the pH value of the buffer in question

from 0·1 unit below the stated value to 0·1 unit above it. Recently a new range of zwitterion buffers has been described by Good *et al.* (1966) which are suitable for biochemical assays with cell-free preparations and for tissue culture (Williamson and Cox, 1968), although they may have limited use for bacterial growth systems owing to their susceptibility to bacterial attack. These buffers cover a pK range from 6 to 8, are very soluble, have low binding capacities for divalent ions, are stable, and where tested have no known cytotoxic effects. An organic buffer, range pH $7 \pm 0{\cdot}8$ units, for use in nutritional studies where the absence or control of nitrogen, phosphate or sulphate is required, has been described by Mallette (1967). Another buffer, often favoured in tissue culture, is the sodium bicarbonate – 5% CO_2 system. If the partial pressure of CO_2 in the gas phase is fixed, then as bicarbonate concentration in the medium increases so does the pH value. One remedy to counteract acid production and maintain near neutrality in liquid cultures which has been used almost since the start of microbiology as a science is the addition of calcium carbonate, in its cheapest form as chalk.

TABLE VI

Variation of pH, pOH and pK_w with temperature

Temperature	pH	pOH	pHOH (log K_w)
0	7·472	7·472	14·943
5	7·367	7·367	14·734
10	7·268	7·268	14·535
15	7·173	7·173	14·346
20	7·084	7·084	14·167
25	6·998	6·998	13·996
30	6·917	6·917	13·833
35	6·840	6·840	13·680
40	6·768	6·768	13·535
45	6·698	6·698	13·396
50	6·631	6·631	13·262
55	6·569	6·569	13·137
60	6·509	6·509	13·017

D. Buffers and temperature variation

Finally the very significant effects of temperature on the pK_a values of most buffers shown in Table XI cannot be over-emphasized. Thus a Tris buffer adjusted to pH 7·8 at room temperature will have a pH of 8·4 in the cold room where it may be used for an extraction procedure and yet the

same buffer will have a pH of 7·4 at 37°C when the extract is assayed. It should be remembered that although pure water has a pH of 7 at 25°C, a neutral solution at any temperature is one which has the same pH as pure water at the same temperature (Table VI).

V. MEASUREMENT OF pH

A. Indicator dyes

These substances are organic compounds which possess different colours according to the pH of the solution in which they are dissolved. The useful characteristic of these dye-stuffs is that they have a predominantly "acid" colour and a predominantly "alkaline" colour; however, the change between the two colours *usually* extends over 1·5–2 pH units. Indicator dyes are weak acids or bases and their colour in any hydrogen ion concentration is determined by the ratio of the concentrations of acid and alkaline forms of the dye.

$$\text{Colour at a given pH} = \text{pK}_{\text{indicator dye}} + \log \frac{[\text{Form with alkaline colour}]}{[\text{Form with acid colour}]}$$

Both forms are present in any hydrogen ion concentration but the human eye has a limited ability to detect the subsidiary colour when the other predominates. Experience shows that the dye solution will have the acid colour when the ratio of acid to alkaline form is approximately 10 and an alkaline colour when the ratio alkaline to acid form is 10. The acid colour is given by $\text{pH} = \text{pK}_{\text{indicator}-1}$ and the alkaline colour by $\text{pH} = \text{pK}_{\text{indicator}+1}$. The colour change interval of the indicator dye is therefore $\text{pH} = \text{pK}_{\text{indicator}\pm1}$. However, when one or other of the two forms of the dye is colourless or of a much weaker colour than the other form, then the dominant colour will probably be apparent at the pK value of the indicator. Table VII shows the colour changes and pH ranges of some common indicator dyes. Universal pH indicators are a mixture of several indicator dyes giving a wide range of colour change with pH, and are most useful in determining the approximate pH of an unknown solution.

1. *Methods of Using Indicator Dyes*

There are many ways of using indicator dyes to determine the pH of solutions. The commonest is to add a small amount of the indicator to the solution or medium in question. A second is the use of commercially available pH indicator paper which is either dipped in the solution to be tested or the solution is dropped on to the paper from a loop or Pasteur pipette. The manufacturers supply a colour chart showing the colour changes over

TABLE VII

Colour changes and pH ranges of some indicator dyes

Indicator	pH range	Colour in acid solution	Colour in alkaline solution	$pK_{indicator}$
Thymol blue† (acid)	1·2–2·8	Red	Yellow	1·7
Bromophenol blue†	2·8–4·6	Yellow	Blue	4·0
Bromocresol green†	3·8–5·4	Yellow	Blue	4·7
Methyl red	4·2–6·3	Red	Yellow	5·1
Bromocresol purple†	5·2–6·8	Yellow	Purple	6·3
Bromothymol blue†	6·0–7·6	Yellow	Blue	7·0
Neutral red	6·8–8·0	Red	Orange	—
Phenol red†	6·8–8·4	Yellow	Red	7·9
Cresol red (base)†	7·2–8·8	Yellow	Red	8·3
α-Naphtholphthalein†	7·3–8·7	Yellow	Blue	8·4
Thymol blue (base)†	8·0–9·6	Yellow	Blue	8·9
Phenolphthalein†	8·3–10·0	Colourless	Red	9·6
Thymolphthalein†	8·3–10·5	Colourless	Blue	9·2

† Denotes a derivative of a sulphonaphthalein or phthalein.

the useful range of the indicator. Such methods cannot be relied upon to better than ± one pH unit.

A cheap and reliable method makes use of a visual comparator. A diagram of the Lovibond "1000" Comparator is shown in Fig. 3. The comparator

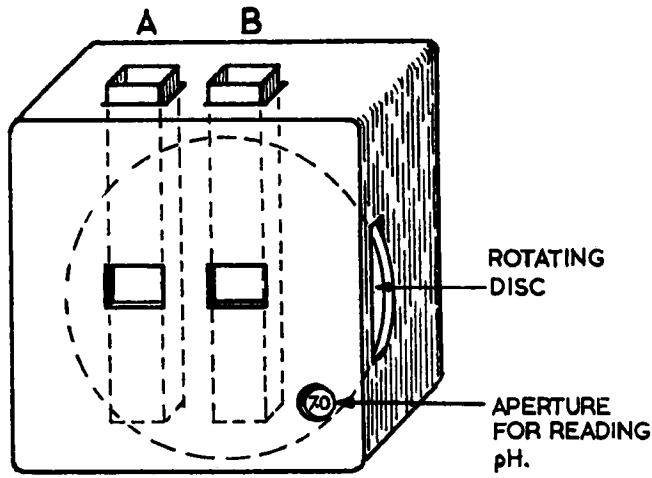

FIG. 3. Lovibond "1000" Comparator. Tubes A and B viewed by prism attachment which brings both fields of view together for easier comparison.

holds two colourless glass tubes of standard bore. Tube A contains the solution of unknown pH and tube B the same solution containing a measured amount of indicator dye for which the comparator is calibrated. The appropriate rotatable disc containing a series of standard coloured glasses is moved until the best match is obtained between aperture A and B. An accuracy of $\pm 0{\cdot}2$ pH units is usually obtainable unless the unknown solution is strongly coloured.

When only small volumes of material are available, such as from a fermentation tube, then the "BDH Capillator Outfit" (British Drug Houses Ltd.) method offers a series of indicator dyes and calibration cards ranging from pH 1·2–11·0. Initially it is necessary to measure the approximate pH of the unknown solution in order to choose the indicator dye capillary card containing an indicator dye whose pK value is closest to that of the unknown. This first step is best done with a universal indicator solution or universal indicator paper. The pH is then determined more accurately using the capillator with the chosen indicator. The apparatus itself is simple and consists of a series of capillary tubes filled with buffered solutions and indicates the colours corresponding to the whole range of the indicator, approximately $pK \pm 1$. The capillator set contains a series of calibration cards, an indicator solution for each card, spare capillary tubes, rubber teat for withdrawing solutions into the capillary tubes and a watch glass. The set is used as follows—

(1). A capillator tube is filled with the double strength indicator solution and added to the watch glass.

(2). A capillator tube is filled with the solution or culture of unknown pH and then added to the watch glass. If the culture is to remain uncontaminated then a sterile capillator tube must be used.

(3). The contents of the watch glass are mixed by sucking in and out of the capillator tube and finally the tube is filled and compared with the colours in the Standard card.

Care should be taken with pathogenic cultures to ensure that the capillator tubes and watch glass are immersed in a bactericidal solution after use.

2. *Preparation of indicator solutions*

As a rule the stock solutions of indicators contain 0·5–1 g of indicator/litre of solvent. If the substance is a sodium salt, water is the usual solvent; in most other cases 50–90% alcohol is employed. The final concentration used will depend on circumstances, e.g., bromocresol purple is used at a final concentration of 0·005% in sugar fermentation tests. Similarly, phenol red is used in Christensen's urease test medium (fuller details can be found in Medical Microbiology by Cruickshank) at a concentration of 0·005% to detect an increase of pH due to ammonia production, bromothymol blue

at 0·0025% in Hugh and Leifson's medium to distinguish between aerobic and anaerobic breakdown of carbohydrates.

B. Electronic systems

The hydrogen electrode is the primary pH indicating electrode, being the basis of the pH scale and the reference to which glass electrode performances are compared. The oxidation potential of such an electrode in a solution of hydrogen ions at activity a_{H^+} is given by—

$$E_{\text{Hydrogen}} = E^{\circ}_{\text{Hydrogen}} - 2{\cdot}303\,\frac{RT}{F}\log a_{H^+} \qquad (15)$$

where R is the gas constant (8·314 joules/deg./mole), F is a constant (96,494 coulombs), T is the absolute temperature and $E^{\circ}_{\text{Hydrogen}}$ is the standard potential of the hydrogen electrode with gas pressure 1 atm and by definition is zero at all temperatures. Taking the temperature as 25°C equation (15) reduces to—

$$\begin{aligned} E_{\text{Hydrogen}} &= -0{\cdot}05915\log a_{H^+} \\ &= \quad 0{\cdot}05915\ \text{pH} \end{aligned}$$

The hydrogen electrode represents a half cell and must be combined with another half cell whose potential is constant (called the reference cell) before the hydrogen electrode can be used to measure pH. The equation for the combined cells is given by the sum of the potential of the two half cells, $E_{\text{Hydrogen}} + E_{\text{Reference}}$ hence—

$$E = E_{\text{Hydrogen}} + E_{\text{Reference}} = 0{\cdot}05915\ \text{pH} + E_{\text{Reference}}$$

$$\therefore\ \text{pH} = \frac{E - E_{\text{Reference}}}{0{\cdot}05915}\ \text{at } 25^{\circ}\text{C}$$

In practice, the hydrogen electrode has many disadvantages and for practical uses in microbiological work the glass electrode is invariably used.

1. *Glass electrode*

Some glasses have the property of behaving as hydrogen electrodes when placed in aqueous solution because they give rise to potentials at the glass liquid interface that follow the equation—

$$E_G = E^{\circ}_G + 2{\cdot}3026\frac{RT}{F}\,\text{pH}$$

where E_G is the e.m.f. or potential of the glass electrode under the conditions of test and E°_G is the standard oxidation potential of the electrode defined by comparison with the standard oxidation potential of the reversible hydrogen electrode. As the potential of an electrode cannot be measured

in absolute terms the potential of the hydrogen electrode is agreed by convention to be zero at all temperatures and all other electrodes are compared to it.

In practice the electrode is made by fusing a membrane of pH responsive

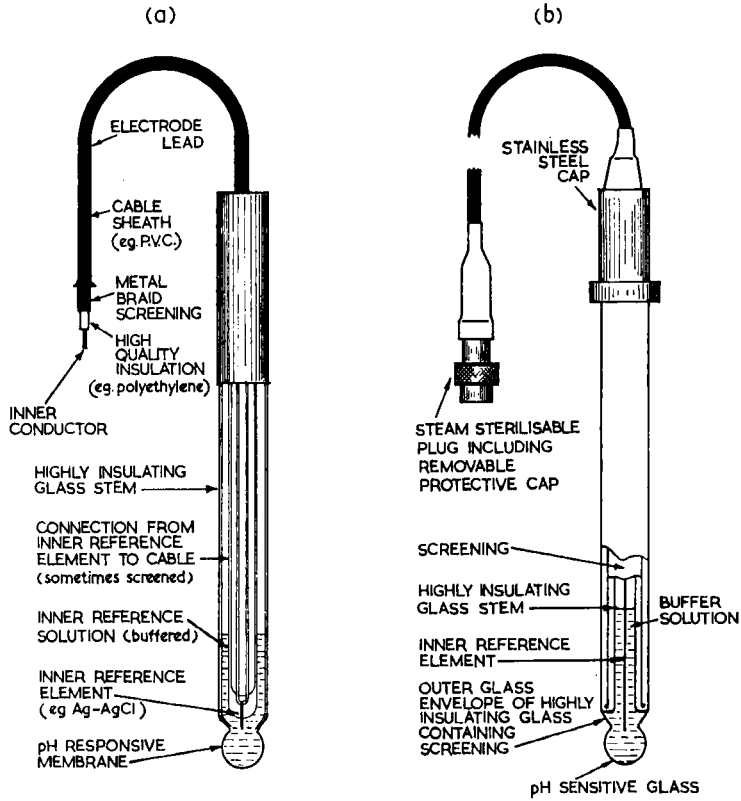

FIG. 4. (a) Typical construction of a glass electrode and lead; (b) construction of a high temperature steam sterilizable glass electrode and lead (Activion Glass Ltd., No. SS12SY/6).

glass across the end of a glass tube. The inner cell is usually a silver–silver chloride or calomel electrode in HCl or a buffered chloride solution. A typical arrangement is shown in Fig. 4a. The potential of the glass electrode relative to the external reference electrode and the change of this potential with temperature are determined, amongst other factors, by the type of the inner electrode and solution. The type of inner cell is chosen so that its

temperature coefficient opposes the effect of temperature changes on the reference cell. Commercial electrodes are usually designed so that the potential between the glass and reference cells will have an e.m.f. of zero at a particular pH, usually 2, 4 or 7. Similarly pH meters have a point on their pH scale where zero input e.m.f. corresponds to 2, 4 or 7 pH units. It is always advisable to use an electrode pair whose zero e.m.f. is at the pH corresponding to zero input e.m.f. on the meter. Inability to standardize an electrode system in buffer solutions is indicative of cases where this is not so. Another important feature of construction of the glass electrode is

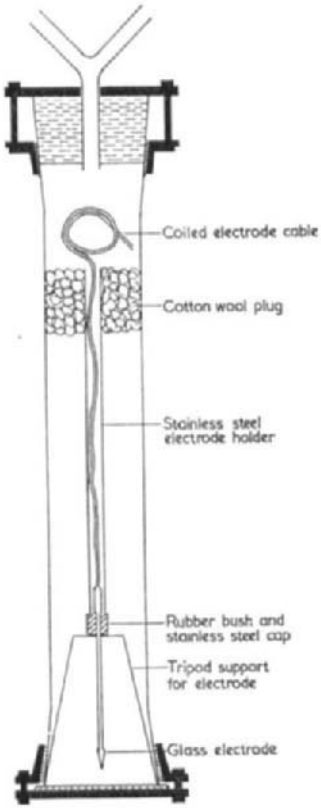

FIG. 5. Apparatus for sterilizing glass electrode by ethylene oxide gas.

the provision of sufficient electrical insulation between the two sides of the pH responsive membrane. For a 0·1% accuracy the insulation must be 1000 times the resistance of the glass membrane which itself may be of the order of 100 MΩ.

(a) *Care of glass electrodes.* New electrodes or those that have been stored dry may require to be conditioned by soaking for 1–2 h or even overnight in water or phosphate buffer pH7. Inadequately conditioned electrodes will require frequent standardization. Care should be taken to ensure that the pH sensitive membrane is not scratched or cracked from contact with hard or sharp surfaces. Standardization of the pH cell with two buffer

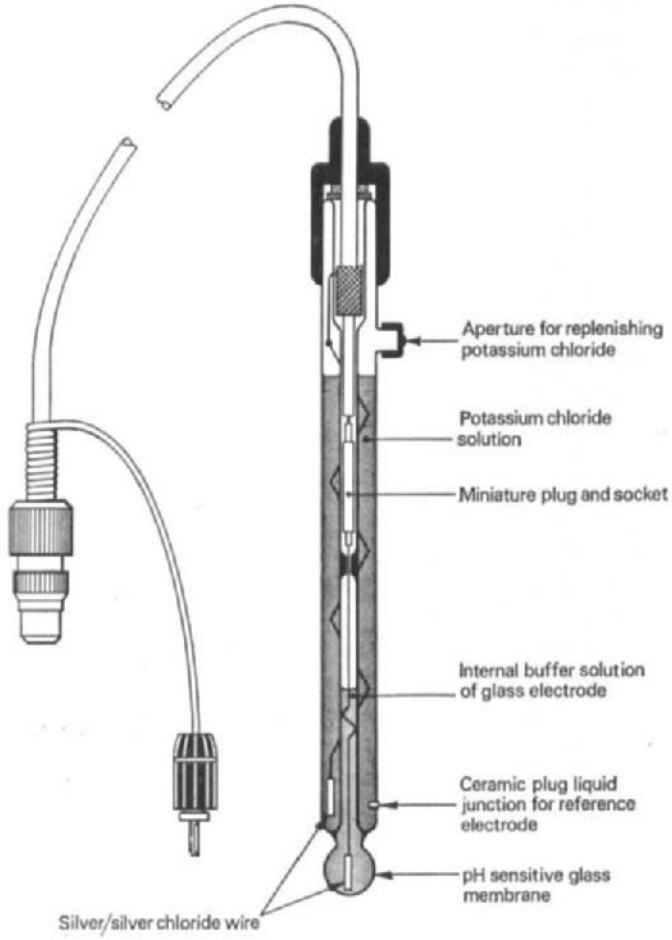

FIG. 6. Construction of a Pye Type 401 combined glass and reference electrode.

solutions of different pH should reveal the imperfect pH response of a cracked electrode. Immersion of the electrode in strong dehydratory agents should be avoided if possible. Cleaning can be performed by washing in 6 N HCl followed by thorough rinsing in distilled water.

(b) *Sterilization of glass electrodes. Chemical methods.* Callow and Pirt (1956) have described a method for the sterilization of glass electrodes using ethylene oxide. The apparatus used is shown in Fig. 5. The clean electrode is placed in the chamber, the vessel evacuated by means of a water pump and ethylene oxide vapour allowed to fill the chamber. A contact time with ethylene oxide of four hours was found sufficient. After sterilization the electrode is removed from the chamber by asceptic technique directly into its position in the fermenter vessel. A simpler technique using ethylene oxide has been described by Tempest (1965). The glass electrode complete with its rubber bung or O ring and boss cap (see Fig. 7a) is clamped inside a clean 500 ml measuring cylinder. Liquid ethylene oxide is introduced into the cylinder and a loose plug of cotton wool wrapped above the boss cap to seal the cylinder. The present author has satisfactorily used this method with a Pye Ingold Type 401 (Fig. 6) or 405 combined glass and reference electrode on many occasions. British Drug Houses supply sealed ampoules of ethylene oxide. However, caution should be exercised as ethylene oxide forms an explosive mixture with air. The whole operation should therefore be carried out in a fume cupboard until the asceptic transfer to the fermenter vessel. A contact time of 2–3 h is sufficient.

Chemical sterilization by immersing the electrode in quaternary ammonium compounds has been described by Deindoerfer and Wilker (1957).

Steam sterilization. With the availability of steam sterilizable electrodes in recent years there should be little need to resort to the somewhat arduous practices of chemical sterilization especially as such electrodes cost little more than conventional ones and save considerable time and labour. Electrodes capable of withstanding 130°C have been available for some time but only recently has there been available cable and plugs to retain electrical resistance after wet steam treatment at 121°C in the normal autoclaving cycle. The extremely high resistance of the glass electrode (10–1000 MΩ) makes it imperative that the resistance between inner cable (which is in direct contact with glass half cell) and outer screening cable approaches two orders of magnitude more than the resistance of the glass electrode. Conductors such as water, graphite and even dust break down this resistance. The new steam sterilizable electrodes are fitted with Teflon (polytetrafluoroethylene) insulated cable to withstand elevated temperatures. In addition they require a special coupler or junction box to join them to the standard coaxial cable. During autoclaving, this special coupler is tightly sealed with a plug to prevent steam entering the cable and condensing there after the autoclaving cycle. After sterilizing, the plug is removed and connection made by means of a special fitting to the standard coaxial cable. Fig. 4b shows the construction of a typical commercial, glass only, steam sterilizable electrode (Activion Glass Ltd., No. SS1257/6). Combined glass

and reference steam sterilizable electrodes are available from Activion Glass Ltd., W. G. Pye Ltd., New Brunswick (U.S.) and W. Ingold (Switzerland).

The mounting of glass and reference electordes in fermentation vessels presents few problems. Typical arrangements for the mounting of electrodes are shown in Fig. 7a–c. Tempest (1965) has described mountings 7a and b whilst a commercial variation of Fig. 7a using an O ring instead of a bung is available from W. G. Pye Ltd. (Cat. No. 761–35B and 764–31B). The mounting shown in Fig. 7c (Baker, 1968) is a standard screw cap to cone joint adapter (Quickfit and Quartz). All mountings in Fig. 7 can be used with

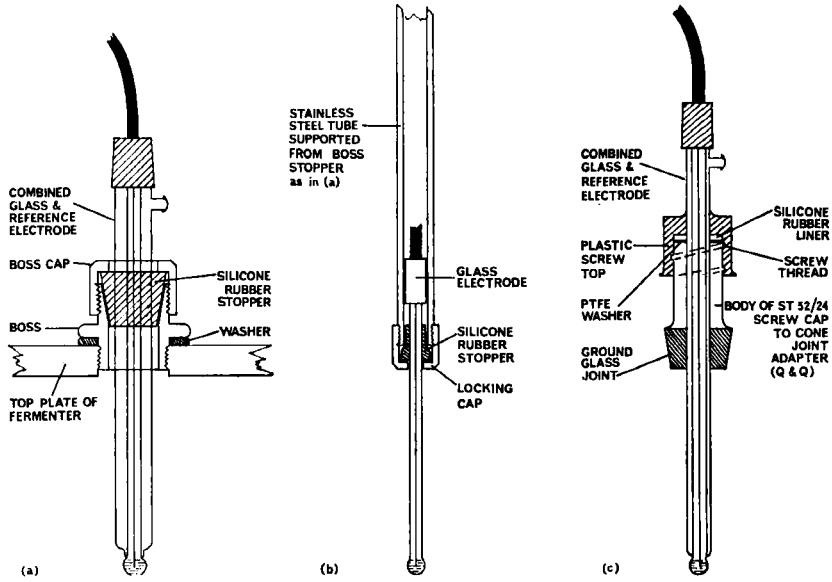

FIG. 7. Methods of top mounting pH electrodes in fermentation vessels.

standard electrodes at inclined angles of up to approximately 45° but whilst the designs shown can be used for mounting on the bottom of fermentation vessels, special non-standard electrodes must be used for upside down mounting.

(c) *Asymmetry potential.* Both sides of a glass membrane do not behave exactly alike, and as a result a small and variable potential difference results. This potential apparently arises out of asymmetrical or non-uniform features in the glass membrane. For example buffer standarization, followed by heat sterilization and cooling, may lead to significant asymmetry potentials which can be of interest to the microbiologist. Such a temperature cycle on a commercial electrode constructed of one of the newer glasses may not

require any change in the original calibration setting within an error of 0·1 pH unit, although awareness of its existence is desirable. Asymmetry potentials also cause drift in the course of protracted use. Over a few hours this is unlikely to be significant but after periods of use of days or weeks in a continuous culture apparatus, drifts of 0·1–0·2 units occur (Elsworth, 1960). Again the microbiologist should be aware of this phenomenon and devise systems to check the performance of an electrode in continuous use. For fuller details on the glass electrode the reader should consult other texts (Mattock and Bates, 1962; Bates, 1954).

2. *Reference electrode*

The essential requirements for pH measurement are an electrode whose potential is pH dependent (the glass electrode) and to complete the cell a reference electrode whose potential is invariant with pH. The reference electrode is chosen to provide as near constant voltage as possible in relation to temperature changes and is always separated from the test solution by a

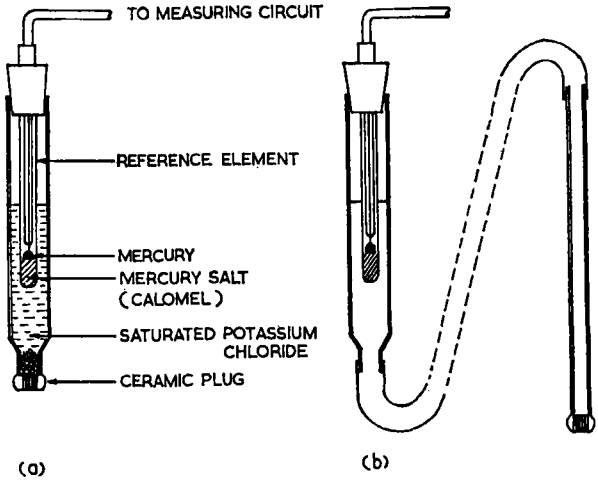

FIG. 8. Calomel electrode assemblies: (a) Typical construction; (b) arrangement suitable for steam sterilization.

salt "bridge". The commonest commercial electrodes are mercury mercurous chloride (calomel) used with a solution of potassium chloride (usually saturated) to form the "bridge". A typical commercial assembly is shown in Fig. 8a. It is important that there should always be a flow of electrolyte, no matter how small, through the liquid junction and therefore the electrode should always be placed so that a positive head exists between the KCl reservoir and the solution being measured. Where a flow of K^+ ions or Cl^-

ions is disadvantageous an alternative electrode of mercury–mercurous sulphate, using sodium sulphate as electrolyte, is available.

(a) *Liquid junction potential.* The liquid boundary which exists in the reference cell at the salt bridge has a certain and variable potential associated with the heteroion boundary. This liquid junction potential is affected by pH, ionic strength and species and temperature. However, these factors are only likely to be important to a discrimination of 0·02 pH units. Large junction errors approaching 1 pH unit can occur when the medium composition changes from a dilute solution of ions in water. The presence of organic solvents such as acetone and alcohol is one example, also the presence of large charged molecular species such as proteins in blood serum or the presence of other phases as in an emulsion or suspension. The presence of these materials allows no interpretation of measured pH values in terms of the hydrogen ion activity.

(b) *Sterilization.* This is readily achieved using the electrode system shown in Fig. 8b where the electrode is contained in a reservoir of saturated KCl connected by flexible tubing to a liquid junction tube (Electronic Instruments Ltd., RSM 23). The liquid junction tube must be first checked to determine that bacteria cannot pass through the ceramic plug, as this is not guaranteed by the manufacturer. Only the liquid junction tube need be heat sterilized although care should be taken to ensure that water is removed from inside the tube after autoclaving, to prevent dilution of the saturated KCl solution when it is added, or alternatively the tube can contain saturated KCl solution during autoclaving.

If a virus or phage has been cultured prior to sterilization then further precautions will be necessary. The liquid junction tube, flexible tubing and reservoir (if heat resistant) will require autoclaving and the electrode, which should be high temperature stable, must be disinfected by continuous steaming for 20 minutes.

3. *pH meters*

pH meters can be divided into two types of instrument, null point and direct reading. In modern commercial pH meters both types require that the signal from the glass electrode be amplified electronically to ensure that the minimum of current is drawn from this electrode to prevent depression of its potential. A simplified circuit illustrating the differences between the two types is shown in Fig. 9 where a triode valve is used as a means of signal amplification.

In the null point or potentiometric system, R is adjusted to provide a null point of balance on the galvanometer, the position of R being calibrated in millivolts or pH units, whereas in the direct reading system R is merely

used to bring an ammeter reading onto a scale calibrated in pH units by compensating for the reference electrode potential. The null point method is inherently capable of greater accuracy but the commercial development of the direct reading instrument has led to very accurate pH measurement

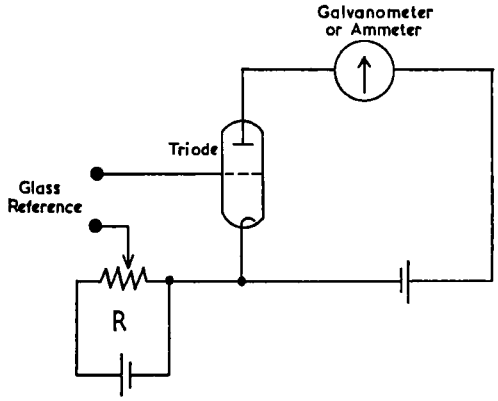

FIG. 9. *pH Meter Amplifiers*: In Potentiometric Systems "*R*" is adjusted to provide a null-point balance on the Galvanometer, the position of "*R*" being calibrated in mV or pH units. In direct-reading systems "*R*" is only used to bring the meter, calibrated in mV or pH units, on scale by compensation for the reference electrode potential.

and the widespread use of the direct reading instruments in most biological applications.

Direct reading instruments can be divided into two types, the direct-current (D.C.) amplifier instruments with the valves connected by resistances receiving a constant e.m.f. from the glass electrode and, secondly, alternating current (A.C.) amplifier instruments where the valves are connected by condenser or transformer coupling and the constant e.m.f. signal of the glass electrode is converted to alternating current. The use of D.C. amplifiers has two main disadvantages, zero instability due to drift in the amplifier valve, and amplifier valve grid current instability due to variation in voltage supply. Numerous devices are employed in commercial pH meters to provide additional stability to combat these effects but *at best* a stability of 0·02 pH units per day can be expected after allowing for valve warm-up and making use of fully stablized power supplies. In general, however, a value of 0·1 pH unit per day is more realistic, rendering the D.C. amplifier instrument suitable for routine pH measurement of buffers and media but imposing limitations on its use for continuous pH monitoring or automatic control where better precision is required. In contrast, A.C. amplifier instruments are more stable because they are free from zero shift error and are also relatively insensitive

to variation of voltage supply. The conversion of the constant e.m.f. signal is achieved using either a synchronous chopper or a vibrating capacitor. The data quoted for the Pye Dynacap pH meter illustrate the capabilities of the vibrating capacity range; a mains voltage change of 15% gives a resultant pH shift of 0·005 pH unit while a change in the frequency of 2 c.p.s. gives a shift of less than 0·02 pH. Therefore A.C. amplifier instruments are suitable for continuous pH monitoring or automatic control as well as for routine duties.

Many pH meters incorporate scale expansion facilities enabling the scale zero to be set at intervals of, usually, 2 pH units. This device allows discrimination to about +0·02 pH units and is useful in both pH measurement and continuous automatic pH control. Some of the principal manufacturers of pH equipment are listed in Table XXVII.

TABLE VIII

Values of $2 \cdot 30259RT/F$ from 0° to 100°

t, °C	$2 \cdot 30259RT/F$ abs. v.
0	0·054196
5	0·055188
10	0·056180
15	0·057172
20	0·058164
25	0·059156
30	0·060148
35	0·061140
40	0·062132
45	0·063124
50	0·064116
55	0·065108
60	0·066100
65	0·067092
70	0·068084
75	0·069076
80	0·070068
85	0·071060
90	0·072052
95	0·073044
100	0·074036

$R = 8 \cdot 31439$ joules/deg./mole; $F = 96{,}493 \cdot 1$ coulombs/equiv.; $T = 273 \cdot 160$ at 0°C.

4. *Temperature effects in pH cells*

Measurement of pH is based on the equation—

$$E = E^\circ + \left(2{\cdot}303\frac{RT}{F}\right) \text{pH} \tag{16}$$

in which three temperature dependent terms occur.

(a) The term 2·303 RT/F is known as the slope factor.
(b) The E° or zero term is the summation of the potentials of the two half cells, E°_{glass} and $E^\circ_{\text{reference}}$. The temperature coefficient of E° is generally never zero.
(c) The true pH of the solution being measured.

(a) *Slope factor.* The pH/e.m.f. curve is quite sensitive to temperature. The value of the slope factor in volts is given for different temperatures in Table VIII where a difference of 1 pH unit at 25° corresponds to 59 mV and 40° to 62 mV. Commercial pH meters contain a compensation for this effect which allows the meter to be calibrated at any *one* temperature by incorporation of an adjustment of the electrical circuit. It cannot be stressed too often that the calibration of a pH meter only holds good for one temperature. Most modern pH meters allow this to be carried out either manually or automatically when a resistance thermometer or thermistor is incorporated in the circuit.

(b) *Zero shift.* If we consider measuring the pH of a solution at a temperature different from that of the standard buffer solution the term E° is different at the temperature of the standard buffer and test solution and will not cancel out in the difference equation—

$$\begin{aligned} E^\circ_{\text{test}} - E^\circ_{\text{buffer}} = {} & [E_{\text{test}} - 0{\cdot}1984 \times 10^{-3} T_{\text{test}}\,\text{pH}] \\ & - [E_{\text{buffer}} - 0{\cdot}1984 \times 10^{-3} T_{\text{buffer}}\,\text{pH}] \end{aligned} \tag{17}$$

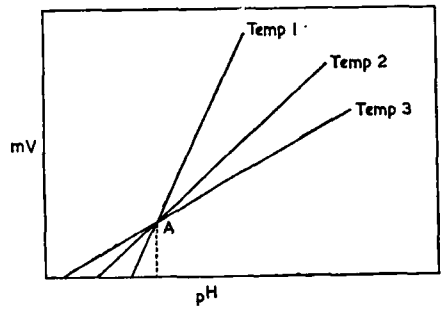

FIG. 10. Variation of pH e.m.f. curve with temperature for an electrode pair. Point A is the isopotential point of the electrode pair.

The difference, $\Delta E°$, is the zero shift error based on the buffer measurement. To express the zero shift error in pH units the isopotential concept was introduced by Jackson (1948). Point A in Fig. 10 is known as the isopotential point (pH_i) and is the pH value for an electrode pair at which the e.m.f. is approximately constant over a range of temperatures of about 20°C. Therefore equation (17) reduces to—

$$E = E_0° + 0{\cdot}1984 \times 10^{-3} T\,(pH - pH_i) \qquad (18)$$

where pH_i is the isopotential pH, and $E_0°$ is constant at all temperatures. If the glass and reference electrodes are at exactly the same temperature then their pH_i will be different from that of a glass electrode held in a test solution with only the tip of the reference electrode being immersed, such as is practised in many laboratory observations. Table IX shows some pH_i values for these two situations where it can be seen that they are not completely independent of temperature. The remote junction reference electrode used in many culture systems will be at the temperature of the reservoir, not the temperature of the fermentation. Temperature fluctuation in standardize the pH meter against a solution of known pH at the temperature coefficient of the saturated potassium chloride mercury calomel electrode is only 0·2 mV/°C, or less than 0·004 pH units/°C.

The only sure and certain method of eliminating zero shift error is to standardize the pH meter against a solution of known pH at the temperature of the unknown solution. Zero shift compensation is not common on most commercial pH meters and when available, only holds for the pH_i characteristics of one particular electrode pair.

(c) *True pH change.* Solutions may often show large pH temperature coefficients. Acid solutions usually have very small or negligible positive coefficients but alkaline solutions may have large negative coefficients ranging from $-0{\cdot}01$ to $-0{\cdot}04$ pH units/°C rise (see pK values in Table XI).

Of course the pH only measures differences between a standard and an unknown, both of which must be at the same temperature. This arises because the standard potential of the hydrogen electrode is defined as zero at all temperatures which means that strict comparison of hydrogen ion activity at different temperatures is not possible. This means we have a different pH scale *at each temperature.*

(d) *Electrical resistance of glass electrode.* This is another factor outside equation (16) and can be important if a temperature difference exists between the standard and unknown. It is particularly pronounced at low temperatures where differences of 20°C between standards and unknown solutions cause errors of as much as 1 pH/unit. However, it is easily eliminated by having the standard and unknown at the same temperature. The error is caused

TABLE IX

Isopotential pH value for some glass electrode cells

Type of inner reference electrode of glass electrode	External reference electrode	Glass and external reference electrodes both at solution temperature Solution temperature 0 – 20	20 – 40	40 – 60	External reference electrode at constant temperature, glass electrode at solution temperature Solution temperature 0 – 20	20 – 40	40 – 60
Ag : AgCl/0·1 M HCl	Saturated KCl Calomel	2·7	2·3	2·0			
	or 3·8 M KCl Calomel	1·4	1·1	0·8	3·7	3·3	3·0
Ag : AgCl/0·1 M HCl + 0·15 M CH_3CO_2Na	Saturated KCl Calomel		5·5	5·6		6·5	6·6
	or 3·8 M KCl Calomel		4·3	4·4			

by the electrical resistance of the glass electrode increasing considerably as the temperature is lowered.

5. *Determination of pH values using the pH meter*

To determine the pH of an unknown solution at least two determinations must be carried out, first a standardization of the pH meter with a solution of known pH (see Table XII) and then the unknown solution is measured. Ideally the pH of the standard should be close to that of the unknown and a second standard which brackets the unknown should be employed to check the linearity of response of the meter and electrode. The temperature of the standard should be within 2° of the test solution.

The determination can be carried out as follows—

(1). If the temperature of the electrode system is much different from that of the standard, immerse it deeply into the solution.

(2). Set the temperature compensation switch to the temperature of the solution or, if a resistance thermometer is used, set to automatic.

(3). Switch to read pH and set the pH meter to the stated pH of the standard solution at the temperature of measurement by adjusting the set buffer control. A repeat pH determination on the standard should agree within 0·02 pH units.

(4). Remove the electrodes and wash with distilled water, replace in the second standard. Duplicate readings should agree within 0·02 pH units and should be within 0·05 units of the stated pH of the standard.

(5). Remove the electrodes from the second standard solution, wash with distilled water, and immerse in the unknown solution. If the pH meter is switched off recalibration will be necessary. When the pH meter is left on it will depend on the pH meter and the glass electrode how often recalibration is necessary.

6. *Measured pH values*

An operational definition of the measured pH which has received wide acceptance is formulated.

$$\mathrm{pH} = \mathrm{pH}_s + \frac{(E - E_s)F}{2{\cdot}3026\,RT} \tag{19}$$

where pH_s is the pH of the standard and E and E_s are the values of the e.m.f. of a pH cell with electrodes immersed in the unknown fluid and the standard. For this condition to be realized the liquid to liquid junction potentials should remain unchanged when the standard solution is replaced by the unknown. Many test solutions do not meet this requirement and the measured pH cannot then be regarded as an approximate measure of the hydrogen ion activity.

VI. AUTOMATIC pH CONTROL

The control of pH in batch or continuous cultures is achieved by supplementing the electrode system and pH meter with some form of electronic control equipment which, on receipt of a signal, will operate the controlled addition of acid or alkali to the culture. The controller may be a separate unit receiving a signal from the pH meter or the pH meter may be part of a controller indicator itself. A record of the success of the control system is usually an integral part of any automatic pH controller (Herbert *et al.*, 1965).

The commonest choice is to use a pH meter/controller. Such instruments are made by all the leading manufacturers and can be easily combined with cheap miniature "slave" recorders such as the Rustrak (West Instruments) or the Elmes 12 (Swiss Instruments Ltd.). The control unit can be preset to hold the pH of any system at a fixed value which should be within $\pm 0{\cdot}1$ pH units or better in adequately mixed vessels. The compensating response of the pH controller can vary from simple opening of a fixed flow valve to complex addition systems which allow for the logarithmic nature of the pH scale and the rate of the pH deviation. The two simplest systems are two-position and proportional position control. In two-position control, in which on-off is the commonest in small laboratory fermenters, the control unit is in one of two positions. In proportional control the position of the control unit can assume any intermediate position from closed to open and the actual position at any time is a value of the pH at that time. Proportional control only assumes importance in large scale fermentation systems and it is not discussed further.

In small laboratory growth vessels the method of addition of acid or alkali by the controller is through activation of a solenoid valve or tubing pump although in industrial systems pneumatic valves are also used. Pinchcock solenoid valves (W. G. Pye and Co. Ltd. No. 11610) which restrict tubing, are relatively inexpensive but do not always give reliable closure or opening if the tubing has been under pressure from the solenoid spring for any length of time. Flow rates can be adjusted by constricting the line but continued attention is necessary. Tubing pumps, which can be either peristaltic (Sigmamotor Inc.) or rotary (Kinetic Clamp A2, V. A. Howe and Co. Ltd.) types, give precise addition of fluids, reliable shut off and different flow rates if the drive speed is altered. Timing devices (Londex Ltd.) which limit the duration of the addition can, in some instances, add precision and safety from overshoot when used with solenoid valves and tubing pumps.

The acid and/or alkali to be added is piped to the fermenter via the chosen addition device by a surgical grade of silicone rubber tubing (Esco Rubber

Ltd.) of the correct wall thickness for the addition device. Thick-walled tubes can result in incomplete closure, thin-walled tubes can permanently collapse or leak after sufficient wear in pinchcock valves or peristaltic pumps. The acid and alkali solutions should be heat sterilized separately from the silicone rubber tubing. Silicone rubber tubing has a limited life after contact with concentrated alkali and therefore it is recommended only to be used once.

In the simplest system the controller only governs the addition of one reagent, say alkali; however, it is often the practice for commercial controllers to be capable of adding alkali if the pH drops below the preset level or acid if above the present level. Therefore in the case of an acid-producing fermentation, alkali is normally added but, in the event of an overdose of alkali, the controller will correct this by adding acid.

Two methods of siting the pH detecting element have been reported. In the first the elements are immersed directly in the vessel (Callow and Pirt, 1956). This method has the advantage that the response time is minimized; at best it is equal to the response time of the electrode system, but in the event of electrode failure, repair or replacement means stopping the culture. The second method, called the continuous flow method, requires medium to be pumped around a loop circuit from the bottom of the vessel to the top (Denison *et al.*, 1958). This loop circuit has a chamber in which the sensing elements are housed. In the event of electrode failure the loop circuit can be sealed off and repairs effected without shut-down. It will be obvious that unless the circulating volume in the loop outside the fermenter is minimized a longer response time will result and increase the chance of overshoot. The addition of the acid or alkali below the medium surface was found important by Callow and Pirt (1956), especially under foaming conditions if rapid response times and no overshoot were to be achieved.

When setting up an automatic pH control system whether it be two position on/off control or a more complex system the following points should be considered in the light of the known circumstances of the fermentation—

(1). Rate of production of acid (or alkali) by the culture will determine the amount of neutralizing addition necessary.

(2). Choice and strength of buffer. The experimenter can choose any buffer whose pK need only be within 1–1·5 units of the chosen pH value. The buffer molarity and hence the capacity of the system determines the ability to neutralize the control reagent without great change in the pH. However, the buffer will probably only be incorporated into the medium to control the pH value and the less of it present the less likely will be any undesirable effects. Therefore the buffer concentration should be minimal, sufficient to balance the amount of the correcting reagent such that a change

of at poorest 0·2 units occurs, or, if the sensing system is sufficiently sensitive, at best 0·04 units.

(3). The molarity of the reagent and consequently the volume added, as well as the rate of addition are important. With a concentrated solution the mixing time of the fermenter and the response time of the apparatus may be too slow resulting in "overshoot" and large pH oscillations. In a continuous culture system a weak solution may result in large volume additions adding significantly to the dilution rate.

The following hypothetical example of a continuous culture illustrates (Table X): (a) the varying degrees of success which can be achieved when using different concentrations of buffer and correcting reagent, (b) how the correcting reagent affects the dilution rate and (c) how many additions per hour the controller and its adding mechanism will be asked to provide. Suppose an anaerobic continuous fermentation is carried out at a pH of $6{\cdot}6 \pm 0{\cdot}1$ in phosphate buffer in a 1 litre fermenter at a dilution rate of $0{\cdot}2\ h^{-1}$. The limiting nutrient is glucose at 20 g/litre, of which 90% (18 g/litre = 0·1 M) is converted to 1 mole fraction each of acetic acid, lactic acid and carbon dioxide. Therefore, after fermentation, each mole of glucose is converted to 2 moles of acid, or, for each 200 ml of medium consumed/h, 40 mmoles of acid have to be neutralized. From buffer Table XIV the β value (milli equivalents of alkali required to change the pH from 6·5 to 6·7) of buffers of different molarity is known.

TABLE X

Buffer molarity and the required strength and number of alkali additions/hour during the course of a theoretical anaerobic continuous fermentation of glucose.†

Buffer molarity	mmole equivalents of alkali required to change pH from 6·5–6·7	Number of additions/hour of strong alkali		
		1N	2N	4N
0·01	0·6	65	33	16
0·025	1·5	27	14	7
0·05	3	13	6	3
0·1	6·1	6·5	3·5	1·7
0·2	12·2	3·2	1·6	0·8
Volume of alkali added		40 ml	20 ml	10 ml
Volume of alkali added as % of dilution rate		20	10	5

† See text for fuller details.

By drawing up a table such as that shown (Table X) the investigator can decide what conditions of buffer and reagent molarity are compatible with the chosen dilution rate. In the example in Table X when 4N alkali is used the dilution rate will be 5% more than is evident by measuring the medium flow rate only. As long as the experimenter can accurately measure the volume of addition of reagents he will be able to calculate successfully the dilution rate. Additions of reagents whose volume is less than 1–2% of the medium flow rate can probably be neglected in most applications especially as the accuracy of measuring the medium flow rate may be no greater than 1–2%. If 4N alkali is used then the addition device will be asked to provide, in the case of a 0·01 M buffer, 16 additions/hour of 0·63 ml and, for a 0·2 M buffer, 1 addition every 75 minutes of 12·5 ml. A flow rate of 1 ml/min would suffice for both situations.

The very large amounts of fully dissociated salts which would be produced in a fermentation of this type would make the measured pH difficult to interpret in terms of the hydrogen ion activity. However the pH value during the continuous culture would at least be constant even if a direct comparison of the fermentation pH with that of a standard pH solution was not feasible. In the case of a fermentation of similar type, but carried out in batch conditions, the ionic strength of the fermentation liquor would continually change; even with automatic pH control it is certain that the cells in the culture would experience a continuously changing hydrogen ion activity due to changing ionic strength.

TABLE XI

Thermodynamic dissociation constants of some weak acids

Acid	pK_0^0	pK_{25}^0	pK_{37}^0
Acetic	4·781	4·756	4·765
Ammonium ion	10·084	9·245	
Boric	9·500	9·235	9·147
Isobutyric	4·824	4·848	4·877
n-Butyric	4·804	4·820	4·856
Carbonic K_1	6·576	6·352	6·303
K_2	10·626	10·329	10·238
Citric K_1	3·222	3·128	3·105
K_2	4·837	4·761	4·751
K_3	6·397	6·396	6·429
Formic	3·785	3·752	3·760
Lactic	3·885	3·860	3·870
Phosphoric K_1	2·057	2·148	2·206
K_2	7·312	7·200	7·182
Propionic	4·895	4·874	4·885
Pyruvic	—	2·490	2·420
Succinic K_1	4·284	4·267	4·191
K_2	5·675	5·638	5·650
Tris (hydroxymethyl)-aminomethane	8·663(5°)	8·079	7·805(35°)

TABLE XII

pH Standards

T°C	0·05 M KH-phthalate	0·025 M KH_2PO_4 + 0·025 M Na_2HPO_4	0·01 M Borax
0	4·01	6·98	9·46
5	4·01	6·95	9·39
10	4·00	6·92	9·33
15	4·00	6·90	9·27
20	4·00	6·88	9·22
25	4·01	6·86	9·18
30	4·01	6·85	9·14
35	4·02	6·84	9·10
37	4·02	6·84	9·09
40	4·03	6·84	9·07
45	4·04	6·83	9·04
50	4·06	6·83	9·01
55	4·07	6·84	8·99
60	4·09	6·84	8·96

0·05 M KHphthalate 10·21 g: $KHC_8H_4O_6$/litre of buffer. The salt should be dried at 100°–110° for 1–2 h. before use.

0·025 M KH_2PO_4 + 0·025 M Na_2HPO_4: 3·40 g KH_2PO_4 and 3·55 g Na_2HPO_4/litre of buffer. Both salts should be dried at 110° for 1–2h.

0·01 M Borax. 3·81 g $Na_2B_4O_7.10H_2O$/litre of buffer. The salt should not be heated and CO_2 free distilled water should be used in the preparation of this buffer.

TABLE XIII

Acetic acid–sodium acetate stock solutions†

pH	$I = 0{\cdot}05$ A ml M acetic acid + 50 ml M NaOH				$I = 0{\cdot}1$ A ml M acetic acid + 100 ml M NaOH				$I = 0{\cdot}2$ A ml M acetic acid + 200 ml M NaOH			
	0°		25°		0°		25°		0°		25°	
	A	β	A	β	A	β	A	β	A	β	A	β
3·6	702	21	650	21	—	—	—	—	—	—	—	—
3·8	460	20	428	20	896	41	828	41	—	—	—	—
4·0	309	19	288	19	602	38	559	38	—	—	—	—
4·2	213	18	200	17	417	35	389	34	—	—	760	67
4·4	153	15	145	15	300	31	283	30	587	60	553	58
4·6	115	13	110	13	226	25	215	25	444	50	423	48
4·8	90·9	10	87·7	10	180	18	173	19	354	40	341	38
5·0	75·8	7·8	73·8	7·5	150	15	146	14	297	30	289	28
5·2	66·3	5·6	65·0	5·3	132	11	129	10	261	22	256	20
5·4	60·3	3·9	59·5	3·6	120	7·6	118	7·0	239	15	235	14
5·6	56·5	2·6	56·0	2·4	113	5·1	112	4·8	224	10	222	9·1
5·8	54·1	1·7	53·8	1·6	107·9	3·4	107·3	3·1	215·4	6·6	—	—

A : M solution of acetic acid (57·5 ml pure glacial acetic acid to 1 litre).

B : M solution of sodium hydroxide.

A ml M acetic acid + B ml M NaOH diluted to 1 litre (β = buffer value which represents the buffering capacity in mmoles of base required to change the pH value of the buffer from 0·1 unit below the stated value to 0·1 unit above it).

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TABLE XIV

Potassium dihydrogen phosphate–disodium hydrogen phosphate stock solutions†

pH	$I = 0{\cdot}05$ 0°			$I = 0{\cdot}1$ 0°			$I = 0{\cdot}2$ 0°		
	A	B	β	A	B	β	A	B	β
5·8	—	—	—	—	—	—	322	26·0	5·7
6·0	79·2	6·92	1·5	152	15·9	3·4	289	37·0	7·7
6·2	70·6	9·78	2·0	134	22·0	4·4	249	50·4	9·7
6·4	60·4	13·2	2·5	112	29·2	5·4	204	65·4	10·1
6·6	49·0	17·0	2·9	89·2	37·0	6·0	158	80·6	12·3
6·8	37·8	20·8	3·0	67·4	44·2	6·1	117	94·4	12·0
7·0	27·6	24·2	3·0	48·6	50·4	5·7	82·8	106	10·7
7·2	19·4	26·8	2·6	33·6	55·4	4·8	56·6	114	8·7
7·4	13·2	29·0	2·1	22·6	59·2	3·8	37·6	121	6·6
7·6	8·76	30·4	1·6	14·9	61·6	2·8	24·6	125	4·7
7·8	5·70	31·4	1·1	9·66	63·4	1·9	15·9	128	3·2
8·0	3·68	32·2	0·77	6·22	64·6	1·3	10·2	130	2·2

TABLE XIV (*continued*)

pH	$I = 0{\cdot}05$ 25°			$I = 0{\cdot}1$ 25°			$I = 0{\cdot}2$ 25°		
	A	B	β	A	B	β	A	B	β
5·6	—	—	—	—	—	—	333	22·4	4·9
5·8	—	—	—	159	13·8	3·0	303	32·4	6·9
6·0	74·2	8·58	1·8	142	19·5	4·0	265	44·8	8·9
6·2	64·6	11·8	2·3	121	26·4	5·0	222	59·4	10·8
6·4	53·4	15·5	2·8	98·2	34·0	5·8	176	74·6	12·1
6·6	42·0	19·3	3·0	75·6	41·4	6·1	133	89·2	12·2
6·8	31·4	22·8	3·0	55·4	48·2	5·9	95·2	102	11·3
7·0	22·4	25·8	2·7	39·0	53·6	5·2	65·8	111	9·5
7·2	15·4	28·2	2·3	26·4	57·8	4·2	44·2	119	7·4
7·4	10·3	30·0	1·8	17·6	60·8	3·1	29·2	124	5·4
7·6	6·74	31·0	1·3	11·5	62·8	2·2	18·9	127	3·8
7·8	4·36	31·8	0·90	7·38	64·2	1·5	12·1	129	2·6
8·0	2·80	32·4	0·61	—	—	—	—	—	—

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A : 0·5 M potassium dihydrogen phosphate (68·05 g of dry salt to 1 litre).

B : 0·5 M disodium hydrogen phosphate (70·99 g of dried salt to 1 litre of CO_2 free water).

A ml 0·5 M KH_2PO_4 + B ml 0·5 M Na_2HPO_4 diluted to 1 litre (β = buffer value which represents the buffering capacity in mmoles of base required to change the pH value of the buffer from 0·1 unit below the stated value to 0·1 unit above it).

TABLE XV

***Tris*(hydroxymethyl)aminomethane hydrochloride–*tris*(hydroxymethyl)aminomethane stock solutions‡**

pH	$I = 0{\cdot}05$ A (= 50) ml M HCl + B ml M tris				$I = 0{\cdot}1$ A (= 100) ml M HCl + B ml M tris				$I = 0{\cdot}2$ A (= 200) ml M HCl + B ml M tris			
	0°		25°		0°		25°		0°		25°	
	B	β	B	β	B	β	B	β	B	β	B	β
7·0	—	—	53·6	1·6	—	—	107·2	3·0	—	—	214·0	6·1
7·2	—	—	55·7	2·4	—	—	111·3	4·5	—	—	222·0	9·2
7·4	—	—	59·1	3·5	—	—	117·8	6·8	—	—	235·3	14
7·6	—	—	64·4	5·1	—	—	127·5	9·9	—	—	257	20
7·8	54·0	1·7	72·9	7·2	107·3	3·2	144	14	213·1	5·7	290	28
8·0	56·4	2·6	86·1	9·6	111·6	4·9	169	19	220·7	8·7	342	38
8·2	60·1	3·9	107	11	118·4	7·2	208	24	233	13	421	48
8·4	66·0	5·6	141	15	129	10	270	29	252	19	550	58
8·6	75·4	7·7	194	17	146	15	367	33	282	27	738	66
8·8	90	10	279	19	173	19	524	37	331	36	507†	70
9·0	114	13	414	20	216	25	761	39	407	47	—	—
9·2	151	15	627	22	284	30	574†	41	528	57	—	—
9·4	210	18	—	—	392	34	—	—	720	66	—	—
9·6	304	19	—	—	562	38	—	—	512†	74	—	—
9·8	452	20	—	—	833	41	—	—	—	—	—	—
10·0	687	22	—	—	631†	43	—	—	—	—	—	—

† For these values use 2M tris.

A : M hydrochloric acid. (Use 90 ml of 36% HCl to 1 litre).

B : M Tris (121·14 g recrystallized salts to 1 litre CO_2-free water).

A ml M HCl + B ml M tris (hydroxymethyl)aminomethane diluted to 1litre (β = buffer value which represents the buffering capacity in mmoles of base required to change the pH value of the buffer from 0·1 unit below the stated value to 0·1 unit above it).

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TABLE XVI

Sodium bicarbonate–sodium carbonate stock solutions†

pH	$I = 0{\cdot}05$ 0°			$I = 0{\cdot}1$ 0°			$I = 0{\cdot}2$ 0°		
	A	B	β	A	B	β	A	B	β
9·2	41·6	2·79	1·2	80·9	6·36	2·7	155·8	14·7	6·3
9·4	37·9	4·03	1·7	72·8	9·07	3·7	138·0	20·7	8·3
9·6	33·2	5·60	2·2	62·8	12·4	4·8	117·0	27·8	10
9·8	27·7	7·42	2·7	51·6	16·1	5·7	93·9	35·4	12
10·0	22·0	9·33	3·0	40·2	19·9	6·1	71·7	42·8	12
10·2	16·6	11·1	3·0	29·8	23·4	6·0	52·1	49·3	12
10·4	11·9	12·7	2·8	21·1	26·3	5·4	36·3	54·6	10
10·6	8·18	13·9	2·4	14·4	28·5	4·4	24·5	58·5	7·9
10·8	5·44	14·8	1·8	9·52	30·1	3·3	16·2	61·3	5·9
11·0	3·49	15·5	1·3	6·11	31·2	2·4	10·4	63·2	4·2
11·2	2·12	15·9	0·94	3·81	32·0	1·7	6·54	64·4	2·8
11·4	1·15	16·2	0·64	2·22	32·5	1·1	3·97	65·2	1·9

TABLE XVI (*continued*)

pH	$I = 0{\cdot}05$ 25°			$I = 0{\cdot}1$ 25°			$I = 0{\cdot}2$ 25°		
	A	B	β	A	B	β	A	B	β
9·0	39·8	3·41	1·5	76·8	7·74	3·3	147	17·8	7·3
9·2	35·5	4·83	2·0	67·6	10·8	4·3	127	24·4	9·4
9·4	30·3	6·55	2·5	56·8	14·4	5·3	104·4	31·9	11
9·6	24·6	8·44	2·9	45·3	18·2	6·0	81·5	39·5	12
9·8	18·9	10·3	3·0	34·3	21·9	6·1	60·5	46·5	12
10·0	13·8	12·0	2·9	24·6	25·1	5·7	42·9	52·3	11
10·2	9·58	13·4	2·6	17·0	27·6	4·8	29·2	56·9	8·9
10·4	6·29	14·5	2·1	11·2	29·5	3·8	19·3	60·1	6·8
10·6	3·86	15·2	1·5	7·06	30·8	2·8	12·3	62·4	4·9
10·8	2·01	15·7	1·1	4·07	31·7	2·0	7·41	63·9	3·3
11·0	—	—	—	1·80	32·3	1·3	3·92	64·9	2·2

A : M Sodium bicarbonate (84 g in CO_2-free water to 1 litre. Store below 20°C).

B : M Sodium carbonate (106 g in CO_2-free water to 1 litre).

A ml M $NaHCO_3$ + B ml M Na_2CO_3 diluted to 1 litre (β = buffer value which represents the buffering capacity in mmoles of base required to change the pH value of the buffer from 0·1 unit below the stated value of 0·1 unit above it).

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TABLE XVII

Citrate buffer stock solutions

x	*y*	pH
46·5	3·5	3·0
43·7	6·3	3·2
40·0	10·0	3·4
37·0	13·0	3·6
35·0	15·0	3·8
33·0	17·0	4·0
31·5	18·5	4·2
28·0	22·0	4·4
25·5	24·5	4·6
23·0	27·0	4·8
20·5	29·5	5·0
18·0	32·0	5·2
16·0	34·0	5·4
13·7	36·3	5·6
11·8	38·2	5·8
9·5	41·5	6·0
7·2	42·8	6·2

A: 0·1 M solution of citric acid (21·01 g in 1000 ml).

B : 0·1 M solution of sodium citrate (29·41 g $C_6H_5O_7Na_3.2H_2O$ in 1000 ml; the use of the salt with $5\frac{1}{2}$ H_2O is not recommended).

x ml of A + *y* ml of B, diluted to a total of 100 ml. Data of Lillie (1948).

TABLE XVIII

Citrate–phosphate buffer stock solutions

x	*y*	pH
44·6	5·4	2·6
42·2	7·8	2·8
39·8	10·2	3·0
37·7	12·3	3·2
35·9	14·1	3·4
33·9	16·1	3·6
32·3	17·7	3·8
30·7	19·3	4·0
29·4	20·6	4·2
27·8	22·2	4·4
26·7	23·3	4·6
25·2	24·8	4·8
24·3	25·7	5·0
23·3	26·7	5·2
22·2	27·8	5·4
21·0	29·0	5·6
19·7	30·3	5·8
17·9	32·1	6·0
16·9	33·1	6·2
15·4	34·6	6·4
13·6	36·4	6·6
9·1	40·9	6·8
6·5	43·6	7·0

A : 0·1 M solution of citric acid (19·21 g in 1000 ml).

B : 0·2 M solution of dibasic sodium phosphate (53·65 g of $Na_2HPO_4.7H_2O$ or 71·7 g of $Na_2HPO_4.12H_2O$ in 1000 ml).

x ml of A + *y* ml of B, diluted to a total of 100 ml. Data of McIlvaine (1921).

TABLE XIX

Succinate buffer stock solutions

x	pH	*x*	pH
7·5	3·8	26·7	5·0
10·0	4·0	30·3	5·2
13·3	4·2	34·2	5·4
16·7	4·4	37·5	5·6
20·0	4·6	40·7	5·8
23·5	4·8	43·5	6·0

A : 0·2 M solution of succinic acid (23·6 g in 1000 ml).

B : 0·2 M NaOH.

25 ml of A + *x* ml of B, diluted to a total of 100 ml. Data of Gomori (unpublished).

TABLE XX

Maleate buffer stock solutions

x	pH	x	pH
7·2	5·2	33·0	6·2
10·5	5·4	38·0	6·4
15·3	5·6	41·6	6·6
20·8	5·8	44·4	6·8
26·9	6·0	—	—

A : 0·2 M solution of acid sodium maleate (8 g of NaOH + 23·2 g of maleic acid or 19·6 g of maleic anhydride in 1000 ml).

B : 0·2 M NaOH.

50 ml of A + x ml of B, diluted to a total of 200 ml. Data of Temple (1929).

TABLE XXI

Cacodylate buffer stock solutions

x	pH	x	pH
2·7	7·4	29·6	6·0
4·2	7·2	34·8	5·8
6·3	7·0	39·2	5·6
9·3	6·8	43·0	5·4
13·3	6·6	45·0	5·2
18·3	6·4	47·0	5·0
23·8	6·2	—	—

A : 0·2 M solution of sodium cacodylate (42·8 g of $Na(CH_3)_2AsO_2.3H_2O$ in 1000 ml).

B : 0·2 M HCl.

50 ml of A + x ml of B, diluted to a total of 200 ml. Data of Plumel (1940).

TABLE XXII

Phosphate buffer stock solutions

x	*y*	pH	*x*	*y*	pH
93·5	6·5	5·7	45·0	55·0	6·9
92·0	8·0	5·8	39·0	61·0	7·0
90·0	10·0	5·9	33·0	67·0	7·1
87·7	12·3	6·0	28·0	72·0	7·2
85·0	15·0	6·1	23·0	77·0	7·3
81·5	18·5	6·2	19·0	81·0	7·4
77·5	22·5	6·3	16·0	84·0	7·5
73·5	26·5	6·4	13·0	87·0	7·6
68·5	31·5	6·5	10·5	90·5	7·7
62·5	37·5	6·6	8·5	91·5	7·8
56·5	43·5	6·7	7·0	93·0	7·9
51·0	49·0	6·8	5·3	94·7	8·0

A : 0·2 M solution of monobasic sodium phosphate (27·8 g in 1000 ml).

B : 0·2 M solution of dibasic sodium phosphate (53·65 g of $Na_2HPO_4.7H_2O$ or 71·7 g of $Na_2HPO_4.12H_2O$ in 1000 ml).

x ml of A + *y* ml of B, diluted to a total of 200 ml. Data of Sørensen (1909).

TABLE XXIII

Barbital buffer stock solutions

x	pH
1·5	9·2
2·5	9·0
4·0	8·8
6·0	8·6
9·0	8·4
12·7	8·2
17·5	8·0
22·5	7·8
27·5	7·6
32·5	7·4
39·0	7·2
43·0	7·0
45·0	6·8

Solutions more concentrated than 0·05 M may crystallize on standing, especially in the cold.

A : 0·2 M solution of sodium barbital (veronal) (41·2 g in 1000 ml).

B : 0·2 M HCl.

50 ml of A + *x* ml of B, diluted to a total of 200 ml. Data of Michaelis (1930).

TABLE XXIV

Boric acid–borax buffer stock solution

x	pH	x	pH
2·0	7·6	22·5	8·7
3·1	7·8	30·0	8·8
4·9	8·0	42·5	8·9
7·3	8·2	59·0	9·0
11·5	8·4	83·0	9·1
17·5	8·6	115·0	9·2

A : 0·2 M solution of boric acid (12·4 g in 1000 ml).

B : 0·05 M solution of borax (19·05 g in 1000 ml; 0·2 M in terms of sodium borate).

50 ml of A + x ml of B, diluted to a total of 200 ml. Data of Holmes (1943).

TABLE XXV

Glycine–NaOH buffer stock solutions

x	pH	x	pH
4·0	8·6	22·4	9·6
6·0	8·8	27·2	9·8
8·8	9·0	32·0	10·0
12·0	9·2	38·6	10·4
16·8	9·4	45·5	10·6

A : 0·2 M solution of glycine (15·01 g in 100 ml).

B : 0·2 M NaOH.

50 ml of A + x ml of B, diluted to a total of 200 ml. Data of Sørensen (1909).

TABLE XXVI

Borax–NaOH buffer stock solutions

x	pH
0·0	9·28
7·0	9·35
11·0	9·4
17·6	9·5
23·0	9·6
29·0	9·7
34·0	9·8
38·6	9·9
43·0	10·0
46·0	10·1

A : 0·05 M solution of borax (19·05 g in 1000 ml; 0·02 M in terms of sodium borate).

B : 0·2 M NaOH.

50 ml of A + x ml of B, diluted to a total of 200 ml. Data of Clark and Lubs (1917).

TABLE XXVII

Major commercial suppliers of pH equipment

North America
- Applied Physics Corporation
- Beckman Instruments Inc.
- Cambridge Instrument Co.
- Coleman Co.
- Corning Glass Co.
- Leeds and Northrup Co.
- Metrohm AG

Continent of Europe
- Beckman Instruments Inc. (W. Germany)
- Cambridge Instrument Co.
- Hartmann and Brown (Germany)
- Ludvig Siebold (Austria)
- Metrohm AG (Switzerland)
- Phillips (Holland)
- Radiometer (Denmark)

United Kingdom
- Analytical Measurements Ltd.
- Beckman Instruments Ltd.
- Electronic Instruments Ltd.
- Fielden Instruments
- W. G. Pye & Co. Ltd.
- V. A. Howe Ltd. (agents for Radiometer).

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CHAPTER IV

Redox Potential

H.-E. JACOB

Institut fur Mikrobiologie und Experimentelle Therapie, Jena, D.D.R.

LIST OF ABBREVIATIONS

E_H Potential of the standard hydrogen electrode (0 mV).

E_h Potential measured in a solution, based on the standard hydrogen electrode (pH at which measurements are carried out must be given).

E_{cal} Potential measured in a solution, based on the calomel reference electrode (pH at which measurements are carried out and concentration of KCl in the reference electrode must be given).

E_o Standard redox potential of a 50% reduced substance, based on the standard hydrogen electrode.

E_o' Standard redox potential of a 50% reduced substance at pH 7, based on the standard hydrogen electrode.

rH The rH value is the negative logarithm of the partial pressure of the gaseous hydrogen (pH_2), see Section IIB.4.

With all the measurements of redox potentials referred to in this section, the saturated calomel electrode was used as a reference unless otherwise stated.

I. HISTORICAL DEVELOPMENT

Helmholtz (1883) was the first to describe the decolorization of litmus in a medium containing decaying protein. This was a reductive process, since on shaking or on passing air into the solution, the original colour could again be obtained. Ehrlich (1885) injected redox dyes into living animals, killed the animals and investigated the redox state of the dyes in the organs. He attributed the varying state of reduction to the oxygen uptake of the organs. Other investigations also showed the decolorization of dyes by reducing substances. These dyes could therefore be used as indicators for particular reducing conditions. Potter (1910) carried out the first electrometric measurements of reducing conditions in bacterial cultures. He detected with a platinum electrode that the bacterial culture had a more negative potential than the uninoculated nutrient medium. Gillespie (1920) followed the development of bacterial cultures and showed that strongly negative potentials became more positive when air was passed into the culture. Gillespie also first applied the physical-chemical term "redox potential".†

Clark and his school made important contributions to the study of redox potential (Clark and Cohen, 1923). Other authors also carried out a great number of investigations into redox potential in biological systems, covering isolated substances, cell suspensions and individual cells. The results are contained in the monographs of Hewitt (1950) and Rabotnowa (1963); short descriptions include those of Hill (1956), Cohen (1957), Slater (1960), and Wurmser and Banerjee (1964).

At this point oxidation and reduction should be defined. Oxidation is a process in which a substance gives up electrons. Reduction is defined as the reverse of this process. Whenever in a system one substance is oxidized (i.e., loss of electrons), another substance must be reduced (i.e., gain of electrons). The relation between reduction and oxidation may be written as—

$$\text{reduced form} \rightleftharpoons \text{oxidized form} + \text{electron(s)}$$

Redox potential may be determined as voltage (in volts or millivolts), when an inert noble metal electrode is connected to a reference electrode and both are placed in a solution containing a reversible redox system. This value gives information about—

(i) The formation of redox potential in cultures of micro-organisms.
(ii) The order of redox substances in a redox chain (e.g., arrangement of enzymes in the respiration chain).

† The terms redox potential, reduction–oxidation potential, electrode potential and reduction potential are used synonymously by various authors.

(iii) The free enthalpy of redox substances, making it possible to describe chemical reactions.

The potential difference between the aerated nutrient solution and the metabolically active bacterial culture can be used to perform work and obtain energy. Potter (1911) was the first to show this. The theoretical aspects of this type of biochemical fuel cell were given by Gray Young *et al.* (1966).

II. MEASUREMENT OF REDOX POTENTIALS

A. Redox-potential determination with indicator dyes

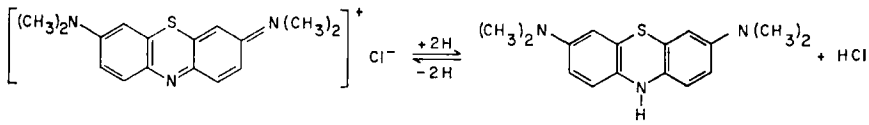
1. *Redox dyes*

Redox dyes can be used in two ways in the characterization of micro-organisms and their metabolic efficiency—

(i) Determination of redox state and redox properties of cultures of micro-organisms. The redox dyes may indicate the redox-potential values existing in cultures which can also be estimated electrometrically. The redox properties can only be established by titration.

(ii) Determination of rate of reduction of dyes by micro-organisms. Information about enzyme activities can be obtained from observation of decolorization caused by the micro-organisms or their enzymes. The methods for this range from the visual determination of decolorization time in the Thunberg tube to their automatic assessment by modern physicochemical measurements (e.g., spectroscopy, polarography). Enzyme activities can be calculated from these values.

Redox dyes are mostly indophenols or indigo derivatives with reversible quinoid structure. In general they are coloured in the oxidized form and colourless in the reduced form. Transfer of hydrogen is necessary for the reduction. The following formula shows the reduction for methylene blue—



Methylene blue (oxidized form)

Leucomethylene blue (reduced form)

Obviously dyes which alter in colour during changes in pH are not suitable for redox indicators. This is true of neutral red (red in oxidized form up to

pH 7, orange-yellow pH 7–8, reduced form yellow), reazurine† (blue at pH 6·5, red at pH 5·3), thymol indophenol (red below pH 9, blue above pH 9) and 2, 6-dichlorophenol indophenol (reddish below pH 6, blue above pH 6). Janus green also gives two different reactions: an irreversible reduction of blue-green to red and a reversible reduction of red to colourless. Resazurin causes similar difficulties: the first reduction step to resorufin is irreversible and results, as with resazurin, in a colouration dependent on the pH value (pink at pH 6·5, yellow at pH 4·8); the second reduction step to colourless dihydroresorufin is reversible. In the irreversible cases (Janus green blue-green to red; resazurin to resorufin) these are not redox dyes. Individual redox dyes are reduced at different potentials. In Table I are given the standard redox-potential values for dyes in a 50% oxidized, 50% reduced state.

TABLE I

Standard redox potentials (E_0') of 50% reduced redox indicator redox dyes at pH 7

Dye	E_0' (mV)[a]
Standard oxygen electrode	810
Phenol-*m*-sulphonate indo-2,6-dibromophenol	273
m-Chlorophenol indo-2, 6-dichlorophenol	254
Phenol-*m*-sulphonate indophenol	250
m-Bromophenol indophenol	248
Phenol-*o*-sulphonate indo-2,6-dibromophenol	242
o-Chlorophenol indophenol	233
o-Bromophenol indophenol	230
Phenol indophenol	227
Bindschedler's green	224
Phenol blue	224
2,6-Dichlorophenol indo-*o*-chlorophenol	219
Phenol indo-2,6-dibromophenol	218
2,6-Dichlorophenol indophenol	217
m-Cresol indophenol	208
o-Cresol indophenol	191
2,6-Dichlorophenol indo-*o*-cresol	181
Thymol indophenol	174
2,6-Dibromophenol indoguaiacol	159
m-Toluylene diamine indophenol	125
1-Naphthol-2-sulphonate indophenol	123
1-Naphthol-2-sulphonate indo-2,6-dichlorophenol	119
Toluylene blue	115
Thionine	62
Prune	56
Cresyl blue	47
Gallocyanine	21
Azure I	11

TABLE I—(*continued*)

Dye	E_o' (mV)[a]
Methylene blue	11
Toluidine blue	−11
Janus green (blue-green to red, irreversible)	−35
Ciba scarlet sulphonate	−36
Indigo tetrasulphonate	−46
Resorufin	−51
Methyl capri blue	−60
Ethyl capri blue (nitrate)	−72
Indigo trisulphonate	−81
Indigo disulphonate	−125
Gallophenine	−142[b]
Nile blue	−142
Indigo monosulphonate	−160
Cresyl violet	−167
Brilliant alizarin blue	−173[b]
2-Methyl-3-hydroxy-1,4-naphthoquinone	−180
Neutral blue	−192
1,5-Anthraquinone sulphate	−200
β-Anthraquinone sulphate	−250
Phenosafranine	−252
Tetraethylphenosafranine	−254
Janus green (red-colourless)	−258
Dimethylphenosafranine	−260
Tetramethylphenosafranine	−273
Rosinduline G 2	−281
Safranine-T	−289
Induline scarlet	−299
Neutral red	−325
Neutral violet	−340
Benzyl viologens	−359
Rosindone sulphonate	−385
Standard hydrogen electrode	−421

[a] E_o' according to Clark (various publications) and Wurmser (1940).
[b] Except for these values, which are at 25°C, all values are at 30°C.

As Table II shows, the potential range for the conversion of 99% oxidized to 99% reduced dye by taking up two electrons ($n = 2$) is 120 mV (for taking up one electron it is 240 mV), and the conversion can be followed electrometrically and spectrophotometrically.‡ Thus the redox dyes can only be used in a very narrow region for potential determinations, but the range can be extended by using suitable combinations of several dyes.

† This dye is irreversibly reduced by a redox potential ~60 mV more positive than methylene blue and is often used in milk testing.

‡ If the values are plotted graphically, one obtains a curve similar to that of the redox titration where the change in potential of, for example, an oxidized dye is plotted against the admixture of a reducing substance (cf. Fig. 5b).

TABLE II

Variations of potential of a redox dye with varying oxidation at constant pH, 30°C, $n = 2$ (two electron change) (Hewitt, 1950)

%-oxidized	Voltage (mV)	Colour
99	60	almost full colour
98	51	
95	38	
90	29	
80	18	
70	11	
60	5	
50	0	half colour
40	−5	
30	−11	
20	−18	
10	−29	
5	−38	
2	−51	
1	−60	almost colourless

The value of the redox potential also varies with pH. This is illustrated by the three dyes given in Table III.

TABLE III

Dependence of redox potential (E_h in mV) on pH at 30°C (Rabotnowa, 1963)

	pH									
	1	2	3	4	5	6	7	8	9	10
Methylene-blue				170	101	47	11	−20	−50	−82
Indigo mono-sulphonate	210	135	80	25	−50	−100	−160	−210	−240	−270
Neutral red					−205	−279	−340	−392	−440	

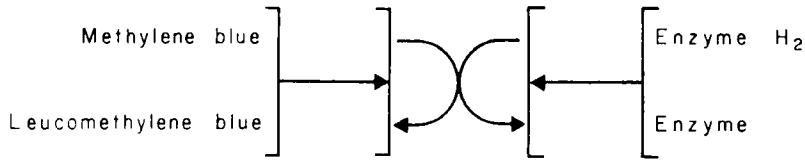
Dyes of importance in testing the reducing properties are the water-soluble, virtually colourless tetrazolium salts. As they can be easily reduced to formazans of generally intensive red or blue colour, the tetrazolium salts are frequently used in biology, medicine and chemistry (Jambor, 1960). Another reason for their popularity is that the dehydration of the formazans to tetrazolium will only take place when a strong oxidant is used. In practice, the reduction of the tetrazolium to formazan is an irreversible reaction and an oxidation of the kind experienced with redox dyes, e.g., leucomethylene blue, in contact with the oxygen of the air is virtually impossible. When

using tetrazolium salts, it must be remembered that the reduction proneness increases with increasing pH; with some of these salts, formazan will be formed spontaneously even at a pH of 10. As the formazans are light sensitive, the tests should be carried out in the dark. The best-known tetrazolium salt is triphenyl tetrazolium chloride (TTC). If TTC is added to a cell suspension, it is reduced by the cells to formazan, which is a red substance practically insoluble in water. The suspension is centrifuged and, from the sediment, formazan can be extracted in organic solvents (glacial acetic acid, ethyl acetate, toluol and others) and the quantity determined, most conveniently by colorimetric methods.

Potassium tellurite can be used to mark sites of reduction in micro-organisms. This is reduced by the enzymes of micro-organisms to black, amorphous tellurium. Nermut (1960) used this reaction to carry out investigations into the localization of oxido-reductases. Electron-microscope photographs (Iterson and Leene, 1964) showed that the tellurium was deposited close to the cytoplasmic membrane.

2. *Mechanism of reduction of dyes by the living cell*

The hydrogen-carrying dehydrogenases (e.g., succinate-dehydrogenase, now named succinate β (acceptor) oxidoreductase 1 . 3 . 99 . 1) are responsible for the reduction of dyes by living cells (Thunberg, 1920). This can be represented schematically—



3. *Uses of redox dyes*

Dyes can be used in the oxidized or reduced states. The smallest possible quantity of redox dye should always be used, because their use is limited by several factors—

(a) The dyes can influence the reactions in various ways and alter equilibria, since they—

(i) Act as electron acceptors or donors and so take part in associated redox reactions.
(ii) Act as intermediate carriers and catalyze biological oxidations.
(iii) Inhibit reactions.

(b) The dyes can also poison the micro-organisms.

Methylene blue can be used as an example of the effect of a redox dye on reactions: in the presence of methylene blue an acceleration of oxygen

uptake is noticed for various types of cells and cell components (bacteria, yeasts, ascites cells, erythrocytes, mitochondria).

The results of investigations with dyes must be considered with great care, especially when using coloured media (e.g., broths) or combinations of dyes, when it is seldom possible to detect a clear change in colour.

In a culture solution only those dyes whose redox potential is not more negative than that measured electrometrically can be present in the reduced form. For example, at a measured redox potential (E_h, pH 7) of -100 mV, a dye with a standard redox potential (E_o') of about -200 mV will not be detected in the reduced form; a dye with a standard redox potential of 50 mV will be reduced.

An idea of the capacity of reducing systems can be obtained by the use of indicator dyes. Their use is also recommended to clarify reduction behaviour. A survey of the redox-potential values in the culture solution can also be obtained. For exact measurement, however, electrometric methods must be used.

B. Electrometric redox-potential determinations

1. *Theoretical basis of measurement*

If an inert electrode is dipped into a solution containing a redox system, it can either give up electrons to the solution or take electrons from it. The oxidized form of the substance must be ready to accept electrons (reduction), causing "electron suction" from the electrode, which is equivalent to the potential of the electrode becoming more positive. The reduced form must be capable of electron donation (oxidation), causing "electron pressure" on the electrode, which is equivalent to the potential of the electrode becoming more negative. The electrode potential resulting from the two processes is a measure of the difference between the electron suction of the electron acceptor and the electron pressure of the electron donor. The potential of the measuring electrode is compared with the constant potential of a reference electrode. Nernst investigated the dependence of redox potential of redox substances on the ratio of oxidized to reduced forms. He found that—

$$E_h = E_o + \frac{RT}{nF} \ln \frac{(\text{Ox.})}{(\text{Red.})}$$

where R is the gas constant (1986 cal/(°C) (mole)); T, the temperature in °K; n, the number of electrons taking part in the process; F, Faraday's constant (96,500 coulombs or 23 kcal); (Ox.) the concentration of the oxidized state; and (Red.), the concentration of the reduced state. (E_h and E_o must be considered for the same pH value.) If measurements are carried

out at 30°C and instead of the natural logarithms we use logarithms to the base 10 (ln 2·303 = log), this formula is simplified to—

$$E_h = E_o + \frac{0{\cdot}06}{n} \log \frac{(\text{Ox.})}{(\text{Red.})}.$$

2. *Electrodes used for measurement and their calibration*

(a) *The measuring electrode.* The indicator electrode must be inert, i.e., it must not take part in the reaction of the redox system, but only act as an inert conductor of electrons to or from the system when the circuit is connected. The electrode is therefore prepared from non-corrodable noble metals: platinum, gold or iridium. The measuring electrode can be produced by a skilled glassblower. First of all, the electric outlet, mostly consisting of copper wire, is welded to the noble metal. During the welding of the noble metal (wire or sheet metal) in glass, it is necessary to ensure that the melting point is free from blows. But the measuring electrode can also be purchased from competent firms (Radiometer, Beckman, and others). The measuring electrode is connected as cathode in the circuit. Usually platinum is used for the measurement. Platinized and smooth electrodes must be differentiated. Platinized platinum electrodes are used, for example, in the standard hydrogen and standard oxygen electrodes. The preparation of a flawless electrode is difficult and, in addition, platinum black can catalyze biochemical processes in a culture. Since the purification of the surface is very difficult, platinized platinum electrodes cannot be recommended for measurements in microbial cultures. The manipulation of smooth platinum electrodes is considerably simpler, and they are used much more frequently. The measured potential is independent of the size of the electrode, as some comparative investigations have shown. It is assumed for exact measurements that the electrode surface is flawless. In order to obtain an even surface, the electrode is polished after removal of unevenness, by using ultra-fine sandpaper. For polishing, use is made of a metal oxide such as ceroxide (Jacob, 1967). The best way of polishing is to use wire electrodes with a diameter not less than 1·5 mm because these, due to their rigidity, are sure not to bend and thus to damage the sealing point.

In this way the measured values are quite substantially improved compared to those obtained with electrodes prepared by methods published by other authors. This hold for the absolute values (Table IV) and for the time-dependent variations of the electrode (Fig. 1a).

The electrode also adjusts very quickly to the redox potential of the culture solution after transfer from nutrient solution into a culture (see Fig. 1b).

(b) *The reference electrode.* Nowadays, the potential ascribed in electrochemistry to the standard hydrogen electrodes (electrode of the first type) is zero ($E_H = 0$). The electrode consists of platinized platinum sheet

TABLE IV

Redox-potential variation in *Proteus vulgaris* SG2 using different electrodes (satd. calomel electrode at 37°C as reference, pH 7)

	Initial redox potential	Most negative redox potential	Total redox-potential change
Non-polished electrode	85 mV	−310 mV	395 mV
Polished electrode	115 mV	−595 mV	750 mV
pH value	7·35	7·10	

about half of which is immersed in a 1N hydrogen ion solution (most convenient is H_2SO_4) at a hydrogen pressure of 1 atm; the upper part is located in the hydrogen atmosphere. With this electrode, hydrogen gas is continually introduced into the solution. This electrode is rarely used in routine investigations.

The reference electrodes most frequently used are those of the second type. These are characterized by a constant potential. This is due to the use of salts of low solubility so that the electrodes cannot be polarized. The

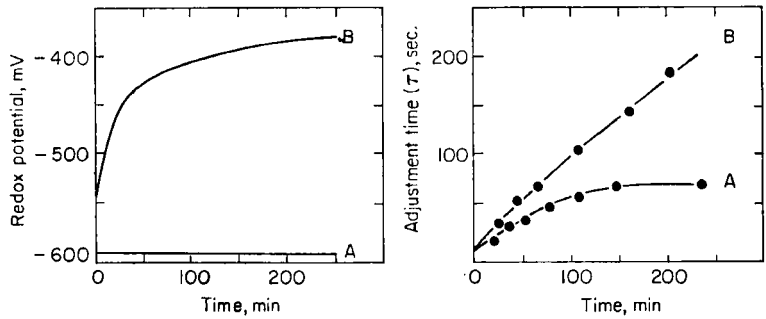


FIG. 1. (a) The most negative redox-potential values (E_{cat}, pH 7) measured in cultures of *Proteus vulgaris* OX19 SG 2 by means of (A) a polished electrode and (B) a non-polished electrode (cleaned in nitric acid). The pre-treated electrodes were repeatedly transferred from oxygenated meat broth–peptone solution into the culture. Readings were recorded 180 sec after transfer. The time axis represents the time in minutes which had elapsed since the pre-treatment of the electrode. The polished electrode remained stable and sensitive for the duration of the experiment whilst the non-polished electrode rapidly declined in sensitivity. (b) The time taken (adjustment time, τ) for the electrodes to record 90% of the final redox-potential value. The time axis represents the time in minutes which had elapsed since the pre-treatment of the electrode. Even the polished electrode (A) takes longer to adjust as the period since pre-treatment increases but the final values recorded by this electrode were identical throughout the course of the experiment (see Fig. 1a). The non-polished electrode (B) showed a much increased adjustment time as the experiment proceeded and the final values achieved rose progressively (see Fig. 1a).

calomel electrode is the one most frequently used in practical measurements. Its structure can be described as follows—

Pt (smooth)/Hg/Hg_2Cl_2/KCl–solution/measuring solution.

The potential of the electrode is determined by the concentration of Cl^- ions. Various concentrations of conducting salt are possible; usually saturated, normal or N/10 KCl solutions are applied. A reference electrode with more positive potential (see Table V) is the sulphate electrode—

Pt (smooth)/Hg/Hg_2SO_4/K_2SO_4–solution/measuring solution.

The concentration of SO_4^- ions determines the potential of the electrode. Various concentrations of conducting salts are also used. The silver chloride electrode consists of a silver wire which has a thin cover of silver chloride—

Ag (smooth)/AgCl/KCl–solution/measuring solution.

The potential of the silver chloride electrode is, like the calomel electrode, determined by the concentration of Cl^- ions. The potentials of the electrodes described are given in Table V, in which the potential of the standard hydrogen electrode is set at 0 mV.

TABLE V

Potential values of various reference electrodes at 25°C (Vetter, 1961; Kortüm, 1962; Brdicka-Durselen, 1965)

Reference electrode	Concentration of conducting salt	Value of constant potential (mV)
Standard hydrogen electrode		0
Silver chloride electrode	normal	235
Calomel electrode	saturated	241
	normal	280
	N/10	338
Sulphate electrode	normal	615

It must again be noted that the potentials of the reference electrodes are temperature dependent. By using the table, it is possible to convert a potential obtained with one reference electrode into one based on another standard. For example, E_h of 190 mV corresponds to E_{cal} of -90 mV. Further details of reference electrodes are found in Ives and Janz (1961).

(c) *Calibration of electrodes.* This is necessary in order to be sure that the electrodes are flawless. Testing of reference electrodes is carried out most conveniently with the polarograph. The thallium wave is recorded with a dropping mercury electrode against the reference electrode under test. The half-wave potential of thallium is 496 mV when used with the normal

calomel electrode (25°C). If the measured value differs from this, then the reference electrode is not correct and must be changed or renewed.

In the latter case, the electrode must be refilled. In the process, the part with the platinum wire is filled with mercury. The mercury surface is coated with the paste, produced by grinding mercury and calomel with a drop of KCl solution. This is followed by introducing the KCl solution.

Testing the measuring electrode presents problems. If the solution used by Michaelis (1929) consisting of 3·3 mM $K_3Fe(CN)_6$, 3·3 mM $K_4Fe(CN)_6$, 0·1 M KCl is used for calibration, all electrodes give the same values independent of their preparation, and without any delay. They also give the same result with 1% $FeCl_3$ solution. Kordatzki (1953) suggested the use of solutions of redox dyes, which are 50% reduced, for the testing of electrodes. This procedure has two disadvantages: first of all, it is difficult to produce a solution of redox dyes where the dye is continuously, for a period of time, present in reduced form at exactly 50%; second, because of the necessary concentration, all the electrodes will be set for the same values irrespective of their surface condition. The measured values in all three cases are independent of the conditions of the surface. It follows that these solutions are not suitable for calibration of the electrode. This is certainly due to the high concentration of the redox system. In bacterial cultures on the other hand the concentrations of the redox partners are very low; thus addition of small amounts of oxygen causes large variations in redox potential. It has been shown already that the state of the surface during the measurements in microbial cultures has a marked effect on the measured values.

We have found it most convenient to test the electrodes in a phosphate solution (0·066 M, pH 7) saturated with air at 760 torr and in a bacterial culture solution (*Proteus vulgaris* SG 2 is very suitable). The redox potential of phosphate buffer solution (E_{cal}) is +350 mV and that of the bacterial culture solution is −590 mV. Another criterion of serviceability is the rate of adjustment of the potential on transfer into the culture medium. The adjustment time of electrodes with a polished surface is very much shorter than that of electrodes with a poor surface (cf. Fig. 1b). If the potential becomes slightly more positive after attaining the most negative value, this is due to alteration in the electrode surface. Moreover, the differences measured by electrodes with poor surfaces are not as great as those measured with electrodes with polished surfaces (cf. Fig. 1a).

3. *Principle of various measuring instruments and the question of polarizability of the electrodes*

(a) *Low-resistance instruments.* Measurements involving the compensation method on the Wheatstone bridge are simple and cheap. However,

because of the low resistance, there is a danger of polarization of the electrodes. If the correct value is not selected right at the beginning of the measurements, a current flows (i.e., electrolysis occurs) and the potential produced on the electrode by the solution immediately alters. If this type of apparatus is selected, for greater accuracy several electrodes should be used, the first for rough estimation, the next for closer testing and the third for exact measurement. Another disadvantage is that a well-trained worker is needed to carry out the measurement. Also the potential cannot be measured continuously. The use of the compensation method therefore cannot be recommended, especially today when more suitable instruments are available in nearly every microbiological laboratory (e.g., pH meters).

(b) *High-resistance instruments.* The best-known instrument of this type is the pH meter. In addition to the scale for pH measurement, there is one for the measurement of voltage. Unlike the low-resistance instruments, practically no current flows when sensitive valve amplifiers of high input resistance (of the order of 10^{12} ohm) are used. Thus no polarization of the electrodes occurs. Another important advantage is the continuous connection of the electrodes to the instrument. This makes possible the continuous measurement of the potential of the solution and if necessary the continuous recording of the values. When high resistance instruments are used, the electrodes should be connected with screened cables.

4. *Dependence of redox potential on pH—the rH value*

The potential values measured are dependent on pH, so that, in each case, measurements of redox potential should be accompanied by a statement of the pH value at which they were taken. In general a pH variation of one unit (e.g., from pH 7·0 to pH 6·0) causes the potential to become more positive by 57·7 mV. Clark and Cohen (1923) introduced the concept of rH in order to eliminate this pH dependence of the potential by calculation. The rH value is the negative logarithm of the partial pressure of gaseous hydrogen (pH_2): an rH of 0 corresponds to a pH_2 of 1 atm, an rH of 10 corresponds to a pH_2 of 10^{-10} atm, etc. Later it was seen that the assumptions made were not correct in every case. Firstly the value taken as a basis by Clark varied if the reduced phase dissociated to yield hydrogen ions on alteration of pH. An example of this is methylene blue (HCl formation at reduction, see formula p. 93). Variation of pH from 5 to 6 causes the potential to become more negative by 54 mV, but from 6 to 7 only by 36 mV (see Table III). Secondly, the system being measured contains redox systems for which the relation between E_h and pH is not known, so that calculation with 57·7 mV/pH leads to false results. According to Michaelis, the E_h variation for each pH unit can amount to 120 mV in certain cases.

Therefore Clark himself has demanded that the term rH should no longer be used. Dixon (1949) has stressed that it has advantages in biochemical investigations. Hewitt (1950) on the other hand considered it confusing and suggested that it should not be used. We agree with this. However, in order to compare results with those in the literature using rH, we shall briefly consider the conversion. The measured redox potentials obtained at known pH with a selected reference electrode are referred to the pH-dependent hydrogen electrode (E_h) with the help of Table V. The rH value can be calculated from the following formula—

$$\mathrm{rH} = \frac{E_h \text{ (in volts)}}{0{\cdot}03} + 2 \times \mathrm{pH}.$$

An example illustrates this. A potential of -530 mV ($-0{\cdot}53$ V) is measured at pH 7·0, using a calomel electrode. Therefore—

$$E_h = -530 \text{ mV} + 280 \text{ mV} = -250 \text{ mV}$$

$$\mathrm{rH} = \frac{-0{\cdot}250}{0{\cdot}03} + 2 \times 7 = -8{\cdot}3 + 14 = 5{\cdot}7.$$

Obviously E_h can also be calculated from reported rH values. The above formula is converted to—

$$E_h = 0{\cdot}03\,(\mathrm{rH} - 2 \times \mathrm{pH}).$$

Table VI lists the E_h values corresponding to rH at pH 7.

It is clear from the above that in publications of redox-potential values, the pH value at which they were measured should always be given. This is also true of the reference electrode. To make comparisons possible, more details of the measuring electrode should also be given.

5. *Practical details for the measurement of redox potential*

Oxygen plays an important role in these measurements. Therefore in general it is not recommended that redox potentials be determined on withdrawn samples, because the danger arises that during the sampling oxygen may enter the liquid sample. Moreover the electrode requires a definite time, depending upon the measured object, to attain the redox potential value. Again during this time, the proportions in the test can alter. Therefore continuous measurement in the culture solution is preferred. Most conveniently, the electrode is placed in the nutrient solution before inoculation. Either it is sterilized with the nutrient solution in the autoclave or it is introduced after chemical sterilization. A good sterilizing agent is H_2O_2. The electrode is kept for 5 min in a 5% H_2O_2 solution; it is then rinsed

TABLE VI

E_h Values corresponding to rH at pH 7

rH	E_h (mV)	rH	E_h (mV)
41	810	20	180
40	780	15	30
35	630	10	−120
30	480	5	−270
25	330	0	−421

in a special apparatus with sterile distilled water and placed in the measuring cell. Chemical sterilization can also be performed with ethylene oxide.

The latter processes have the advantage that they avoid any alteration of the electrode surface due to heating in the medium.

In addition to the measuring electrode connected as cathode, the communication for the reference electrode must also be immersed in the nutrient solution. A glass tube fitted at the bottom with a glass sinter (grain size G 3, Schott & Gen., Jena, pore width 15–40 μm) is most convenient for this. This glass tube containing KCl/agar can be autoclaved together with the nutrient solution without difficulty. Before the investigation is begun the agar is covered with a layer of saturated KCl solution and the saturated calomel electrode introduced.

Measurement of the potential of definite substances must be carried out in the absence of oxygen. The oxygen is removed by degassing with nitrogen or argon, from which oxygen has been removed. There are various methods for this (e.g., nitrogen is passed over red-hot copper which has been reduced with hydrogen).

III. REDOX-POTENTIAL VARIATIONS DURING CULTIVATION OF MICRO-ORGANISMS

A. General redox-potential variations

If the potential of a nutrient solution is measured and this solution then inoculated, a change of the redox potential to a more negative value indicates the start of growth. Oxygen determination carried out at the same time shows a decrease in oxygen pressure. The rate and extent to which the potential becomes more negative are dependent on the growth rate and on the physiological type of bacteria. In this way aerobic bacteria are differentiated clearly from facultative anaerobes in a stirred culture solution, in which oxygen is introduced by diffusion from the liquid/gas phase boundary. If the measurements are carried out for long enough, the

potential becomes more positive, due to diminished metabolism resulting from the beginning of lysis.

B. Redox potential of different physiological types of bacteria

1. *Redox potential of aerobic bacteria*

Fig. 2 gives the curve for *Bacillus subtilis* UT6. The measured potential (E_{cal}; pH 7) varies within 24 h from +135 mV to −280 mV under the culture conditions described above. The total change is 415 mV to a more negative potential. The most negative value is first measured at the end of this time. As Fig. 2 clearly shows, the growth rate decreased at more negative redox-potential values. Essentially the same curve was also obtained for other aerobic bacteria. *Staphylococcus aureus* SG 511 occupies a position intermediate between typically aerobic organisms and facultative anaerobes (Fig 3). The redox-potential variation in 11 h amounts to about 400 mV and the rate of reproduction is not affected as the potential becomes more negative (i.e., lower oxygen pressure). This type is classified as aerobic, facultatively anaerobic in Bergey (1957). It is also characteristic of the aerobic bacteria that the electrode adjusts very slowly to the final value when it is transferred from nutrient to culture solutions.

2. *Redox potential of facultative anaerobes*

The redox-potential curve for *Proteus vulgaris* SG 2 is given in Fig. 4. In the first part of the curve, there is no difference when compared to the curve for aerobic types. In the region from about 30 mV, the rate at which the potential becomes negative is slower; this is also found with aerobic bacteria. In contrast to the aerobic types, in which this rate continually decreases, the potential of facultative anaerobes becomes very rapidly more negative from about −40 mV and the most negative value is very soon reached. It seems as though a buffered region has been passed. The facts that the curves of aerobic and anaerobic bacteria are completely identical up to the end of this "buffer region" and that aerobic bacteria attain more negative potentials are contrary to this. Therefore, it is very probable that in this region the metabolism of the facultative anaerobes adapts to the new oxygen content of the medium. The results of aerating a culture solution support this interpretation of the "buffer region". The redox potential becomes, by aeration, more positive than that observed in the buffer region. In the subsequent change of potential to more negative values, which are caused by oxygen consumption by the bacteria, this buffer region does not appear (Fig. 5). These values are measured for a long time with an electrode, whose surface is flawless. At pH 7, the potential range is between +160 mV and −590 mV (E_{cal}), the total change to a more negative value being about

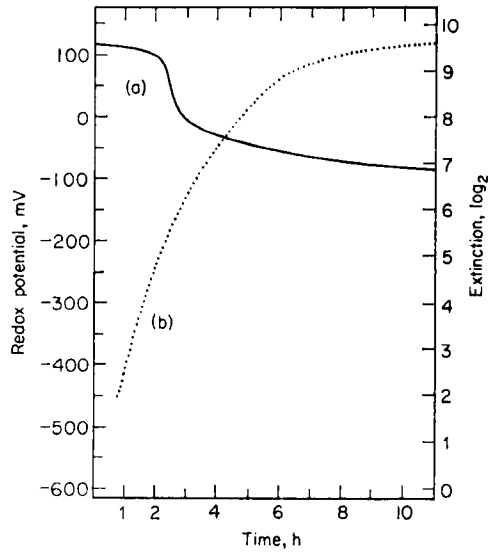


FIG. 2. Results of continuous measurement in a culture of *Bacillus subtilis* UT6. (a) Redox potential with polished electrode (E_{cal}, pH 7); (b) turbidity (Extinction, log).

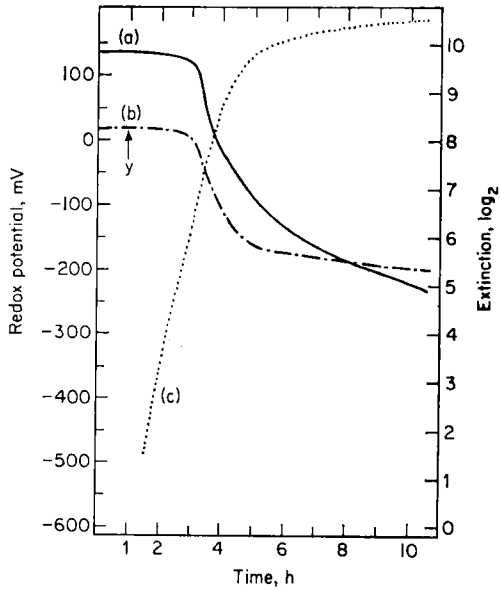


FIG. 3. Results of continuous measurement in a culture of *Staphylococcus aureus* SG 511. (a) Redox potential with polished electrode (E_{cal}, pH 7); (b) redox potential with non-polished electrode (E_{cal}, pH 7); (c) turbidity; y, inoculation.

750 mV. Essentially the same curve is obtained with *Escherichia coli* and other facultative anaerobes. The adjustment time of electrodes is much more rapid for these anaerobic bacteria than for aerobes.

3. *Redox potential of anaerobic bacteria*

In normal cases, oxygen must be exhaustively removed from anaerobic media before inoculation. This can be carried out by boiling or by passing in oxygen-free nitrogen. The potential becomes more negative on de-aeration with gases and this can be clearly recorded (Fig. 6). Suitable redox conditions are also obtained by addition of suitable chemical substances. The reducing substances given in Section IV may be used and in each case we are able to measure the loss of oxygen from the solution by polarographic pO_2-measurement and by the change of the potential to more negative values. Details of the cultivation of anaerobic bacteria are described in this Series, Vol. 3B. If the cultivation is in liver broth (medium for some anaerobic types), then the removal of oxygen is not necessary for large inocula. The

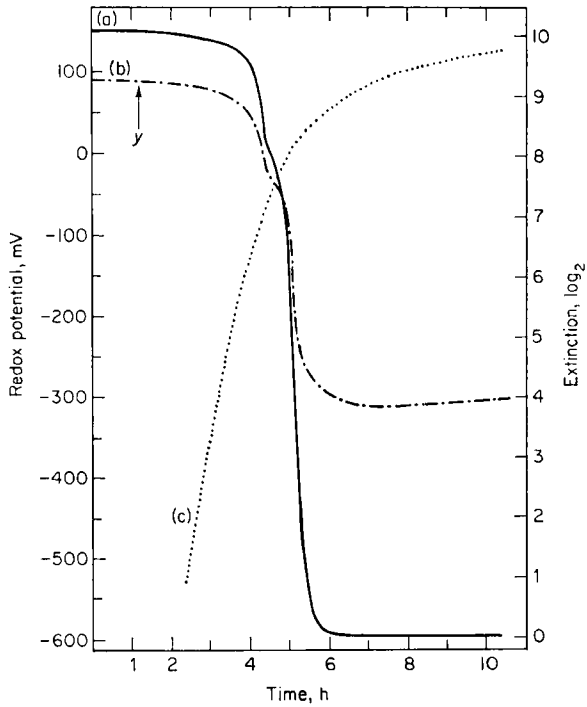


FIG. 4. Results of continuous measurement in a culture of *Proteus vulgaris* SG 2. (a) Redox potential with polished electrode (E_{cal}, pH 7); (b) redox potential with non-polished electrode (E_{cal}, pH 7); (c) turbidity; y, inoculation.

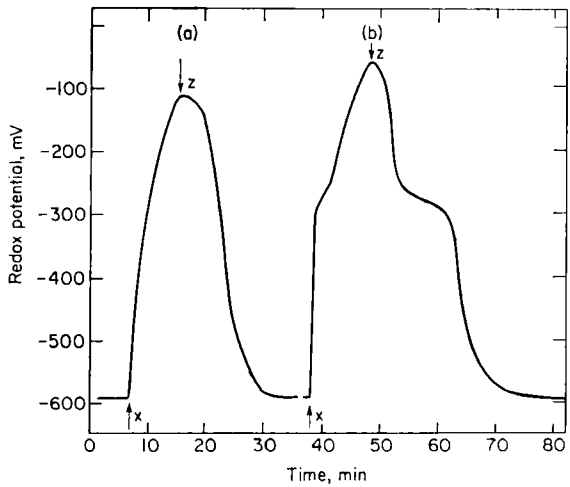


FIG. 5. Redox-potential variation in a culture solution of *Proteus vulgaris* SG 2 (E_{cal}, pH 7) during aeration and the subsequent uptake of oxygen by the bacteria in the absence and presence of dye. (a) No dye in the culture solution; (b) methylene blue (2×10^{-5} M) in the culture solution; x, aeration starts; z, aeration ends.

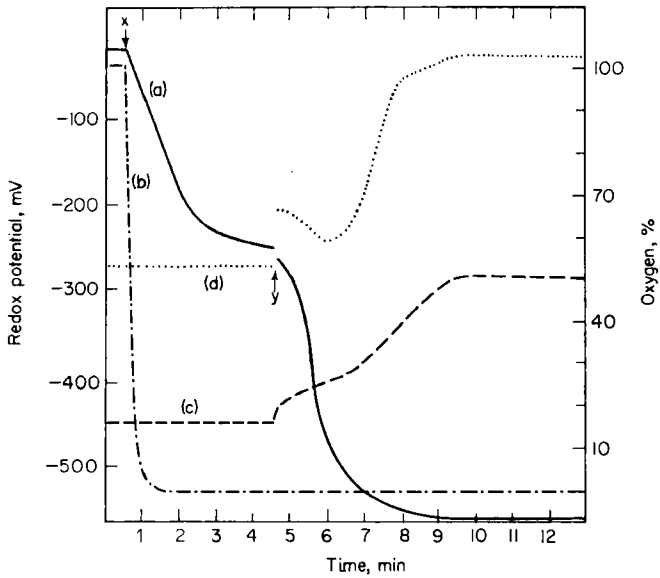


FIG. 6. Redox-potential variations in a nutrient solution during removal of oxygen with nitrogen (x to y) and the redox-potential variations after inoculation (y) with *Clostridium paraputrificum* H2. (a) redox potential (E_{cal}); (b) oxygen pressure; (c) pH variation (from 7·15 to 5·95); (d) turbidity (Extinction, $\log_2$).

oxygen present is used up by the anaerobic bacteria, as is also shown by measurements using the Warburg technique. Fig. 7 shows the curve of redox potential during cultivation of *Clostridium paraputrificum* H2.

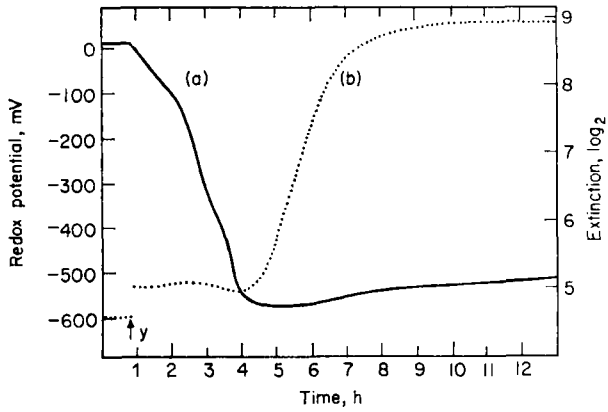


FIG. 7. Results of continuous measurement in a culture of *Clostridium paraputrificum* H2 (in liver broth). (a) redox potential (E_{cal}); (b) turbidity; y, inoculation.

It is noticeable that the decrease of redox potential is more or less uniform; the buffer region found with the facultative anaerobes is not present. This is indirect evidence that the apparent redox-potential buffering in these bacteria is due to a change in metabolism. Reproduction of the bacteria introduced by inoculation only begins when the redox potential of the culture solution has become considerably more negative. In a culture of *Cl. paraputrificum*, the redox potential varies from ± 0 mV to -665 mV (E_{cal}; pH 7). The total variation of 665 mV is smaller than that found with facultative anaerobes. This is due mainly to the more negative redox potential of the nutrient solution (about 150 mV more negative than meat broth–peptone medium; a similar large difference occurs between meat broth-peptone medium and phosphate buffer solution). The final potential, however, is more negative by 70 mV than that of the facultative anaerobes (the end value nearly corresponds to the potential of the hydrogen electrode).

IV. REDOX BUFFERING AND CONTROL OF REDOX POTENTIAL

A. Buffering of redox potential

The buffering action of a redox substance is dependent on its concentration in the solution. The capacity term for redox potential is the "poising effect", which characterizes the capacity of a system to counteract a variation

in potential. An example is the presence of methylene blue (MB) in a culture solution. If the concentration of MB in the experiment illustrated in Fig. 5 is doubled, then the length of time for the reduction is also doubled. Redox dyes (whose potential is independent of concentration) show a buffering effect during their reduction in culture solutions (see Fig. 5). In this experiment, the culture solution was first aerated and the change of potential to more negative values due to the uptake of oxygen by the bacteria registered. If now MB is added to the solution and it is aerated, then the potential becomes more positive more rapidly than in the absence of methylene blue. The change to more positive values then becomes slower at about 50 mV below the redox-potential value of MB, passes through this value with the lowest rate, and subsequently becomes quicker again. After the end of the aeration the potential again becomes more negative. The reverse effect is now seen in the potential range of MB. In principle, the trend of the curve within the MB reduction range corresponds to that of the redox titration; as reducing agents, the bacteria come into their own. If the values tabulated in Table II are plotted graphically and if "% oxidized" is replaced by the corresponding quantity of reducing agent, one obtains the result of a redox titration. In this case, it is clearly apparent that, at the equivalence point, a large quantity of reducing agent is needed in order to obtain a shift of potential. The greater the distance from the point of inflexion of the curve the greater become the changes in the potential while the quantity of reducing agent is held constant. The greatest buffering action occurs when the oxidized and reduced forms are present in the solution in about equal amounts, since substances can only buffer when they are present in partly oxidized (or partly reduced) forms. Fully oxidized and fully reduced substances have no buffering action and also no influence on the potential values of the solution.

The measuring electrode itself can show a buffering action, as Hoare (1962) found on immersing the platinum electrode into an arsenic solution. Such influences should be removed by suitable preparation of the electrode.

B. Continuous control of redox potential

Continuous measurement of redox potential and suitable regulating apparatus are needed to carry out continuous control of redox potential. Essentially the redox proportions in a culture solution can only alter in two directions: in the oxidized or reduced directions. As we already know, the redox potential becomes more negative during the cultivation of microorganisms. In order to counteract this and to hold the redox potential constant in one region, which is more positive than the most negative value attained by the culture, oxidant must be added. The equipment used

by us for regulating purposes consists of a multi-channel compensation recorder with position adjuster (produced by Messgerätewerk Erich Weinert, Magdeburg), which is able to regulate six measuring points. If the freely adjustable regulating range is exceeded, the regulator releases a signal which can be used for opening a valve, operating a pump, etc. In this way, it is possible to add controlled quantities of different substances. When the regulating range has been regained, the cessation of the signal will terminate the admixture. In using this equipment for the regulation of redox potential, the simplest procedure is to control admixture of oxygen. The redox potential can be held constant by the introduction of air into the solution (Fig. 8). Aeration improved the oxygen supply of the bacteria in this experiment. This led to a greater rate of reproduction, as is shown in the turbidity curve. Because of the higher oxygen uptake of the culture caused by increased growth rate and a higher population, the rate of oxygen solution may be limiting. In this case the potential cannot be controlled at the desired value.

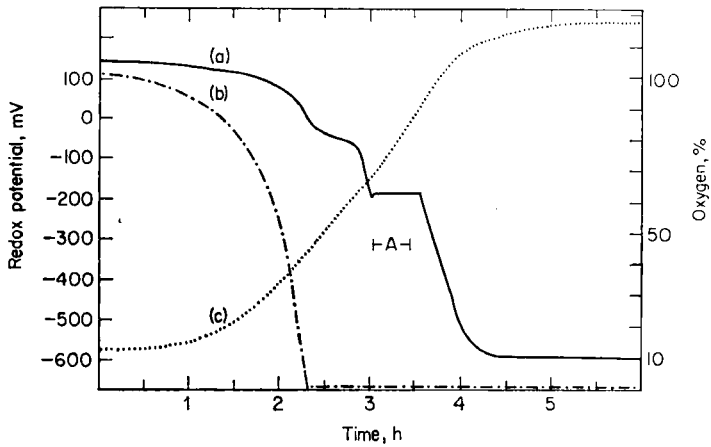


FIG. 8. Control of redox potential in a culture of *Proteus vulgaris* SG 2 with oxygen. A, control time; (a) redox potential (E_{cal}, pH 7); (b) oxygen pressure; (c) turbidity (Extinction, log).

On the other hand the oxygen content can be controlled by the redox potential. Lengyel and Nyiri (1962) made use of this to control oxygen introduction during the production of antibiotics. If it is necessary to counteract the oxidation of a culture medium or reduce the potential of a nutrient solution, this can be carried out by the controlled introduction of inert gases (e.g., nitrogen, argon) or the measured addition of reducing substances (e.g., dithionite, sodium thioglycolate, ascorbic acid). The control impulses can also be used for the control of electrolytic oxygen or hydrogen development. For this purpose, special electrodes must be used. However, some

pilot tests carried out by us showed that this would give rise to a number of problems. It would, for instance, be necessary to clarify the influence of the strong currents which flow into the solution during the electrolysis. For the time being, a regulation through the production of electrolytic oxygen or hydrogen will therefore presumably have to be ruled out for critical work. The effect of control can be clearly demonstrated when carried out in the redox-potential range of a dye. The control process is then clearly seen in the colour variation.

V. EVALUATION AND INTERPRETATION OF REDOX-POTENTIAL MEASUREMENTS

A. Consideration of pH variation in evaluation

If pH variation occurs during cultivation, then it must be taken into account in the redox-potential evaluation and the measured values must be corrected. Measurement of pH dependence of redox potential in the nutrient and culture solutions must establish whether the often valid relation of one pH unit causing a potential variation of 57·7 mV holds. Control experiments should also be carried out in buffered nutrient solution. The use of buffered media is strongly recommended because this avoids pH influence on redox potential. (See Section IIB.4 for rH presentation.) It is clear from this that it is advantageous to study redox potential and pH together.

B. Intracellular redox potential of micro-organisms

Up to now, an electrometric measurement of the intracellular redox potential of micro-organisms has not been possible. However, redox dyes are able to provide indications of the magnitude of the potential and to contribute to the clarification of the main question of whether measurements of redox potential in bacterial culture solutions permit conclusions regarding the intracellular redox potential.

To clarify this question, it is first of all necessary to examine the results of studies where, due to the size of the object, the redox potential can be determined directly in the cell. With the redox dyes used, the penetration capacity of the dye into the cell will of course play a decisive role, and it is obvious that the pH value of the cell is also important, depending on whether acid or basic dyes are used. It is also important that the dye is used in concentrations which permit its recognition in the cell. On the other hand, the indicator must not be used in such quantities that the reduction capacity of the cells is not sufficient to reduce the dye. Dyes reduced by the cell can be re-oxidized by $K_3[Fe(CN)_6]$. The question of toxicity must also be kept in mind. Problems connected with the use of redox dyes have been discussed by Drawert (1956).

The investigations carried out by Cohen and others (1928) on *Amoeba dubia* provide valuable information. These authors, using a pH of 7, found an intracellular redox potential (E_h) under aerobic conditions of -60 mV; under anaerobic conditions, the redox potential was more negative, -148 mV. The intracellular redox potential was thus dependent on the aeration conditions in the medium and it can be concluded that the redox potential in the interior of the cells cannot be strongly buffered. In this connection, it should also be mentioned that according to Machlis and Green (1933), the redox potential of the cells is composed of the negative redox potential of the dehydrogenase systems and the positive redox potential of the oxidases. The potential is said to be dependent on the equilibrium conditions of these systems so that it has dynamic character.

Investigations into the intracellular redox potential of micro-organisms are best carried out with yeast cells which are large enough to permit microscopic observations as well. Baumberger (1939) compared the trend of redox potential in yeast suspensions with the absorption spectrum of the cytochromes. It was possible to show the existence of clear correlations between the redox potential of the medium and the condition of the cytrochromes in the interiors of the cells: changes in the redox potential of the outer solutions were reflected in the redox condition of the cytochromes in the interior of the cells. Rabotnowa (1963, p. 60) has this to say in connection with the problem of the measurement of the intracellular redox potential in the outer medium: "It is noteworthy in this connection that the existence of reductive substances is registered by an electrode which is placed outside the cell. The redox systems are thus also effective outside the cell . . . the redox systems thus extend outwards both from the bacteria and from the yeast. So far, however, the nature of the substances responsible is not known either with yeasts or with bacteria." Particularly significant for our purposes are the results obtained by Leman (1965) who carried out investigations into the intra- and extracellular redox potentials of fermenting yeast suspensions. The redox dye used for this purpose was nile blue ($E_o' = -142$ mV) because its redox potential is somewhat more positive than the constant potential of the yeast suspension (*Saccharomyces cerevisiae*). The measurements were carried out colorimetrically for the intracellular redox potential and electrometrically for the extracellular redox potential. The results obtained by both methods were largely in agreement; major differences were only observed during the adjustment time of the redox potential for the more or less constant final values (during this time, the intracellular redox potential was much more negative than the extracellular). After reaching the constant potential, the colorimetrically measured redox potential was only slightly more negative than that determined electrometrically. These results are also, in principle, in agreement with those published by Meisel and

Pomoschnikowa (1952), who observed that neutral red ($E_0' = -325$ mV) is reduced under anaerobic conditions in yeast cells (*Endomyces magnusii*); the dye was also partially reduced in the external medium.

We ascribe the results quoted in this section to the fact that, in the interior of the cells, due to the high oxygen consumption, the oxygen pressure is lower, so that one measures a more negative redox potential than in the surrounding medium. Moreover, for the same reason, the concentration of oxygen decreases from the solution towards the cells, and it is highly probable that the intracellular redox potential of micro-organisms is always slightly more negative than the extracellular redox potential; there are no indications that the redox potential of the medium is more negative than the intracellular redox potential

From these results it is seen that oxygen plays an important role even at these relatively negative potentials. These are the traces which are undetected by the usual methods of oxygen measurement.

If the examples given are generally applicable, then the redox potential values of culture liquid and cell contents must be nearly the same in a culture. The conditions in a culture solution directly after inoculation with a micro-organism can only be guessed at. If it is assumed that a low oxygen pressure is produced in the cell during metabolism, then there must be a large potential difference between culture solution and cell at the beginning of cultivation. This difference is subsequently almost removed by oxygen consumption in aerobic bacteria and facultative anaerobes. In anaerobic micro-organisms, high oxygen pressure causes irreversible damage and it is necessary to create suitable conditions for growth. If the anaerobes can find suitable conditions for themselves in existing environmental niches (e.g., in kieselguhr or liver) the oxygen is consumed and favourable breeding conditions arise (see Fig. 7). In other cases oxygen must be removed (see Fig. 6). With these micro-organisms also, the most negative redox potential values of culture solution and cell contents are similar.

C. Interpretation of redox potential

The significance of the redox potentials measured during the cultivation of micro-organisms can be discussed in various ways. They could be caused by metabolic products with redox character present in the solution or by the activity of different enzymes (oxidases, dehydrogenases) in the cell. Alternatively they could be explained by variations of oxygen pressure, resulting from oxygen uptake by the micro-organisms. We will not consider the first explanation further, since it does not give a satisfactory explanation of the characteristic redox potentials, and it is not possible to describe the substances which are involved. The fact that the redox potential of a culture solution can be controlled by measured introduction of oxygen, that after

aeration of a culture of facultative anaerobes no buffered regions appear when the redox potential becomes more negative (from this it follows that either no potential-determining substances are present or that these substances have very nearly adjoining potentials and must be present in more or less equal concentrations) and that even a very small addition of oxygen to the culture solution causes the potential to become more positive immediately (it follows that potential-determining redox substances can be present in only very low concentration, otherwise they would buffer the potential value) shows very clearly the importance of oxygen in redox-potential measurements. Many experimental results can be explained if oxygen pressure is responsible for the redox potential of micro-organism cultures.† The first part of the potential change to more negative values can be correlated with the decrease in the pressure of dissolved oxygen, caused by the respiration of the bacteria and determined polarographically. For technical reasons, however, classical polarography can only be used for measuring the pO_2 for two orders of magnitude (in a solution open to the air, the pO_2 value is about 150 mm; in which case, the measuring limit would lie around 1·5 mm). It follows that the correlation between redox potential and the pressure of the dissolved oxygen can only be shown for a small range of the redox potential. Squires and Hosler (1958) carried out comparative measurements of redox potential and pO_2 in nutrient solutions and found that the redox potential is proportional to the logarithm of the oxygen pressure (log pO_2). Matkovics *et al.* (1959) and Tengerdy (1962) also established this. We have therefore carried out experiments in phosphate buffer solution and tested whether the above relation holds in this solution which contains no redox substances. In this experiment also, a delay was noticed in the oxygen detection of the redox electrode and this led to the development of technique for electrode preparation (see Section IIB.3). With polished electrodes, the relationship of both values could be clearly demonstrated in the buffer solution (Fig. 9).

We have tested the oxygen dependence of redox potentials in culture solutions by the use of special platinum electrodes. These electrodes were covered with a membrane permeable to gases. We used two methods of preparation—

(i) The measuring electrode is covered with a membrane by dip-coating. For this purpose, the electrode was dipped into a solution of polyacrylnitrile in chloroform and was then dried.

(ii) The measuring electrodes are placed in an oxygen-saturated buffer

† For the interpretation of the redox potential of biological systems, mention must also be made of the work carried out by Grosz and Farmer (1967). The authors found that the redox potential of blood is a function of the partial pressure of oxygen.

solution separated from the culture solution by a polyethylene membrane permeable to gases (this membrane material, thickness 15 μm, is used for polarographic measurements of pO_2 with membrane-covered electrodes) (Fig. 10).

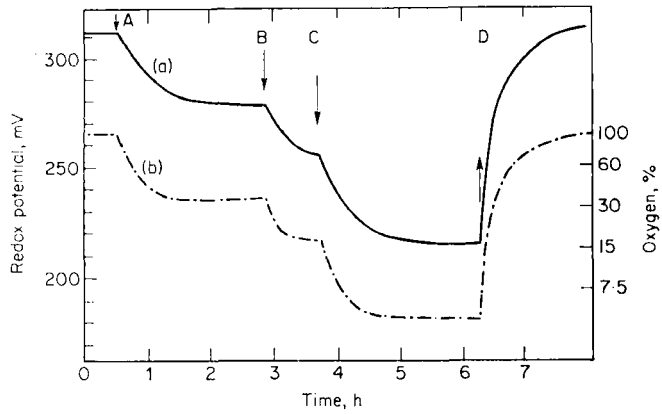


FIG. 9. Redox potential (a) and dissolved oxygen pressure (b) in phosphate buffer during various periods of vigorous deaeration with pure nitrogen (E_{cal}, pH 7). A, B, C, Stages of deaeration; D, stage of oxygenation.

In the first case, the same value was measured for the electrode with the dip-coating as for the uncovered electrode. In the second case we must give a little explanation. During growth the oxygen pressure in the culture solution decreased. This gives rise to a pO_2 difference between the culture solution and the buffer solution. As a result, oxygen diffuses from the buffer solution into the culture solution. This leads to a reduction of the pO_2 in the buffer solution and causes the redox potential measured there to become negative. The potential value follows that in the culture solution after a certain delay, as was expected (see Fig. 11). After some time the electrodes showed the same values as those in the culture solution. The electrode placed directly on the membrane attained this value first.

For a discussion of the influence of oxygen on the potential of redox electrodes, the results obtained by Schuldiner *et al.* (1966) are of great importance. These authors were able to demonstrate, with electrochemical measurements in a gas-tight system with a negligible oxygen leak, the dependence of the potential of their platinum electrodes on the pO_2 up to oxygen pressures of 10^{-9} atm; in this connection, reduction of pO_2 by one order of magnitude caused a negative shift of about 60 mV.

If the potential on a polished platinum electrode is used as an indication of

oxygen pressure in measurements of bacterial cultures, the relation to the standard oxygen electrode must be clarified. Clark gives an illustration on p. 349 of his monograph (1960), from which it can be seen that oxygen pressures of 10^{-42} to 10^{-60} atm are associated with the range for aerobic cells (at pH 7 and the standard hydrogen electrode between + 200 and − 100 mV).

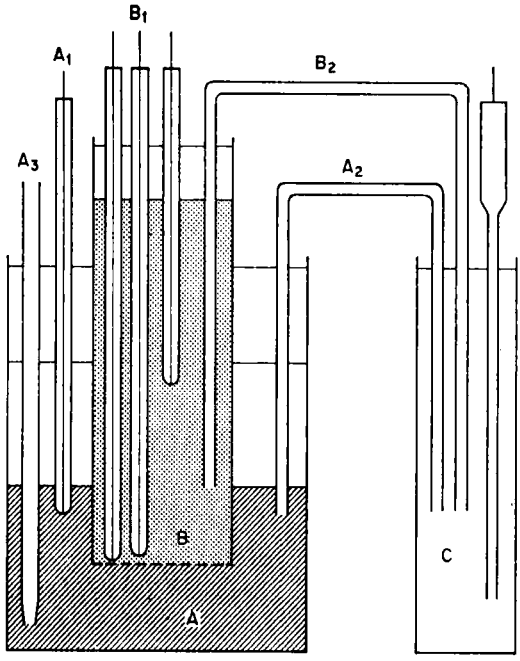


FIG. 10. Illustration of construction of measuring cell. A, Culture vessel with culture solution (c. s.); A_1, platinum electrode; A_2, KCl agar bridge; A_3, tube for aeration of c.s.; B, vessel containing buffer solution separated from c.s. by membrane; B_1, platinum electrodes; B_2, KCl agar bridge; C, vessel containing KCl solution and calomel reference electrode.

In these calculations of the thermodynamic equilibrium, the oxygen pressure emanates from the standard oxygen electrode (with this, 60 mV reduction corresponds to a pO_2 variation of the order of magnitude of 6). Calculations based on the theoretical oxygen standard electrode do not agree with any hitherto published redox-potential determination for microorganisms. The redox potential with oxygen saturation of a medium (pO_2, approx. 0·2 atm) are much more negative than the values calculated on the basis of the theoretical standard oxygen electrode (Squires and Hosler, 1958; and others). Our results show that in a meat broth–peptone medium

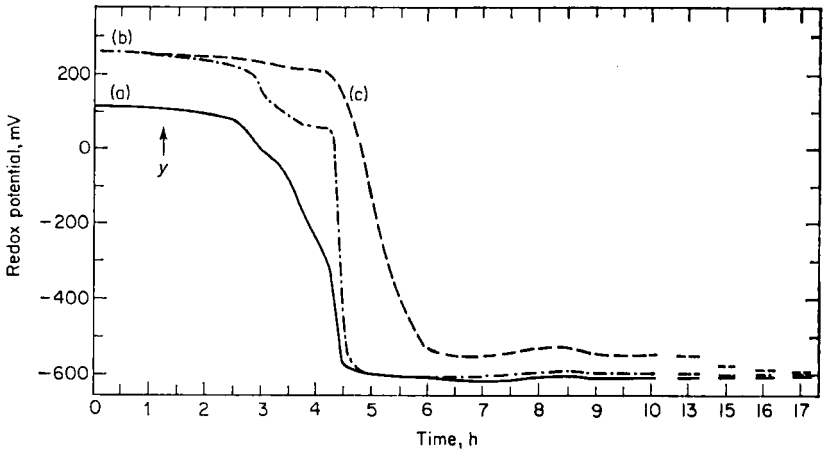


FIG. 11. Results of redox-potential measurement (E_{cal}, pH 7) with the measuring cell illustrated in Fig. 10. (a) Electrode in culture solution; (b) electrode in buffer solution (at the membrane); (c) electrode in buffer solution (approx. 1 mm away from the membrane); y, addition of a large inoculum.

(pH 7) with a pO_2 of 0·2 atm† a polished platinum electrode measured an E_h of about 400 mV. The redox potential of the standard oxygen electrode (a platinized platinum electrode) is 810 mV. A reduction of pressure to $0{\cdot}2 \times 10^{-1}$ atm caused the potential on the polished platinum electrode to become more negative by about 60 mV; further pressure reduction to $0{\cdot}2 \times 10^{-2}$ atm caused an additional reduction of potential of about 60 mV. In this way the polished platinum electrode can be standardized for this range, in which the oxygen can be measured polarographically. If, after the cessation of the polarographic measurability of the oxygen, the correlation thus found continues to be valid (and this seems to be corroborated by the results obtained by Squires and Hoseler as well as by Schuldiner and others), the oxygen pressure extant in the culture solution can be determined by extrapolation. From our results, the oxygen pressure in a culture solution of aerobic bacteria is about 10^{-8} atm, in a culture solution of faculative anaerobes, about 10^{-12} atm. The lower reduction in potential (i.e., higher pO_2) of aerobic bacteria can be explained because they can only carry out their metabolism at higher oxygen pressures. They are not able to remove the oxygen as exhaustively as the faculative anaerobes because of their physiological requirements. The difference between our values and those of Clark must be due to the nature of the measuring electrodes. The polished platinum electrode does not behave in the same way towards oxygen as does

† 6·36 cm^3 (9·05 mg) oxygen dissolved in 1 litre H_2O at 20°C and 760 mm air pressure (D'Ans and Lax, 1949) = $3{\cdot}5 \times 10^{-4}$M = 9 ppm.

the platinized electrode. The platinized electrode should give the potential of the standard oxygen electrode in a correspondingly aerated nutrient solution. If this is not so, it is certainly due to the influence of the nutrient medium on the surface. The variation of 60 mV in redox potential which we have noted with the polished platinum electrode during decrease of oxygen pressure from 150 to 15 mm also does not agree with the values calculated by Clark on the theoretical standard oxygen electrode (with this, 60 mV reduction corresponds to a pO_2 variation of the order of magnitude of 6). It is therefore clear that these calculations cannot be used for the interpretation of redox potentials measured in micro-organism cultures; suffice to refer to results obtained by Schuldiner *et al.* (1966). Further investigation into these problems are necessary. Among other problems, the influence of the composition of the nutrient medium on the potential value during saturation with air must be explained.

D. Some results of redox potential, turbidity and oxygen-pressure measurements in bacterial cultures influenced by inhibitory substances

In earlier sections we referred to the dependence of redox-potential values on oxygen pressure and it is therefore advantageous to measure the pH variation and oxygen pressure in addition to the redox potential. If, in addition to these measurements, a continuous measurement of turbidity (as a measure of cell reproduction) and a determination of CO_2 pressure are carried out, an apparatus is required such as we have had in operation since 1963. There is not only the advantage of simultaneous investigation of the factors with this combination. When the apparatus is used to measure the effect of a definite substance, the effect is frequently shown more clearly by one of the factors than by others. In this way, the sensitivity of polarographic oxygen measurements provides a good complementation to that of redox-potential determination (Jacob and Horn, 1965).

If the influence of penicillin on *Staphylococcus aureus* is investigated solely with redox potential (Kramli, *et al.*, 1955), then all that is noticed is that the potential ceases to become more negative and later becomes more positive. If in addition turbidity and oxygen pressure are recorded, then greater insight into the process is obtained; cell lysis is noted from turbidity measurements, and decrease of oxygen consumption can be seen first from the redox potential. This leads, later, to the enrichment of the culture solution with oxygen—a phenomenon which, when the oxygen concentration reaches the polarographic measuring limit, can also be registered with the pO_2 electrode (Jacob and Horn, 1966). Measurements of the effect of actinomycin and oxytetracyclin on the corresponding test organisms clearly reveal the different action mechanisms: immediately after the admixture of

actinomycin, oxygen consumption is reduced for a short time; later, the growth rate declines. Oxytetracyclin, on the other hand, has an immediate inhibiting effect on respiration and growth. With suitable antibiotic concentrations, respiration and growth increase again and one attains, albeit slowly, the same numbers of cells as in the control tests. In the study of the influence of penicillin on *Proteus vulgaris* SG 2, the oxygen tension in the culture solution gives the clearest information. Almost complete lysis of the culture, formation of sphaeroplasts from the non-lysed cells, extensive lysis even of these sphaeroplasts and, after destruction of the penicillin by the freed penicillinase, an increase of normal bacteria are all clearly discernible. Because of the high oxygen pressure in the first part of the experiment, little information was obtained on redox potential. On the other hand, formation of the potential due to growth of the re-formed normal bacteria showed the typical curve of *Proteus vulgaris*. If lysis caused by phages is studied by means of the three factors, then the most useful are the variation in turbidity and the development of redox potential. In this experiment the oxygen pressure is so low that it cannot be measured by classical polarography. The combined measurement of several factors can be used also in the investigation of the influence of the cell wall on the natural resistance of *Proteus* to Actinomycin C and macrolid antibiotics (Magnamycin, Erythromycin u.a.) (Taubeneck and Jacob, 1967).

These considerations show very clearly that redox-potential investigations can provide valuable information about the processes in a microbial culture.

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CHAPTER V

Aeration in the Submerged Culture of Micro-organisms

D. E. BROWN

Biochemical Engineering Group, Department of Chemical Engineering, University of Manchester Institute of Science and Technology, Manchester, England

NOMENCLATURE

a	Specific area of gas–liquid interface.
A	Oxygen required to convert 1 g substrate to CO_2 and H_2O.
a'	Specific area of cells.
a_a'	Specific area of solid–liquid interface.
a_m'	Volume averaged mean area for transfer in pellet material.
B	Oxygen required to convert 1 g of cells to CO_2 and H_2O.
c	Concentration.
c_{crit}	Critical oxygen concentration.
c_E	Equilibrium concentration of oxygen in liquid.
c_{elect}	Concentration of oxygen at electrode surface.
c_{Em}	Mean equilibrium concentration of oxygen in fermenter liquid.
c_l	Concentration of oxygen inside membrane electrode cell.
c_{int}	Concentration of oxygen inside cell.
c_L	Concentration of oxygen in liquid.

c_L'	Concentration of oxygen at cell–liquid interface.
c_{Lo}	Concentration of oxygen in liquid at $t = 0$.
c_m	Concentration of oxygen liquid at membrane–liquid interface.
c_m'	Volume averaged mean concentration of oxygen in pellet.
d	Diameter of cell.
d_B	Gas-bubble diameter.
D_L	Diffusivity in the liquid phase.
D_P	Diffusivity of oxygen in pellet mass.
D_w	Cell-wall oxygen permeability.
G	Production capacity, or gas flow rate.
H	Henry's law constant.
H_i	Henry's law constant for liquid inside membrane electrode cell.
H_{int}	Henry's law constant for cell contents.
K	Rate constant in equation (37).
k'	Rate constant in equation (35).
$k_1, k_2, k_3, k_4, k_5, k_6, k_7$	Constants.
k_A	Constant in equation (20).
k_a'	Pellet mass-transfer coefficient.
k_d	Mass-transfer coefficient for diffusion layer at cathode.
k_g, k_L	Gas and liquid-film mass-transfer coefficients.
K_g, K_L	Overall mass-transfer coefficients based on gas and liquid driving forces.
k_L'	Liquid-film mass-transfer coefficient for the solid–liquid interface.
k_m	Mass-transfer coefficient for electrode membrane.
K_s	Michaelis–Menton constant.
k_o	Overall mass-transfer coefficient for oxygen electrode.
K_o	Michaelis–Menton constant for oxygen.
K_{oi}	Cell internal oxygen Michaelis–Menton constant.
k_w	Cell-wall mass-transfer coefficient.
k_w'	Cell-wall mass-transfer coefficient with driving force in terms of oxygen tension.
l	Length of fungal cell.
N	Number of cells in culture.
N_T	Total rate of oxygen transfer.
p	Partial pressure of oxygen in the gas phase.
p_E	Partial pressure of oxygen in equilibrium with c_L.
p_m	Mean gas-phase composition.
q	Oxygen uptake rate per cell.
Q	Specific oxygen uptake rate.
Q'	Oxygen uptake rate per unit volume $= xQ$.
q_m	Maximum oxygen uptake rate per cell.
Q_m	Maximum specific oxygen uptake rate.
r	Radius of a fungal pellet.
R	Average rate of transfer per unit area.
r'	Oxygen uptake rate recovery constant.
s	Concentration of limiting nutrient.
S	Rate of surface renewal.

t	Time.
T	Oxygen tension.
t_2	Doubling time.
T_{ac}	Apparent critical oxygen tension.
T_i	Oxygen tension in membrane electrode cell.
T_{int}	Oxygen tension in microbial cell.
T_m	Oxygen tension at membrane–liquid interface.
T_m'	Volume averaged oxygen tension in cell pellet.
T_{tc}	True critical oxygen tension.
V	Volume of culture.
v_B	Bubble rise velocity.
x	Distance, or concentration of cells.
x_L	Liquid film thickness.
x_0	Concentration of cells at $t = 0$.
x_w	Cell-wall thickness.
Y	Yield of dry cells.
Z	Oxygen consumed per unit weight of cells produced.
μ	Specific growth rate.
μA	Microamperes.
μ_m	Maximum specific growth rate.
ρ_P	Density of pellet.
θ	Exposure time.
ATP	Adenosine triphosphate.
OAR	Oxygen availability rate.
OTR	Oxygen transfer rate.
OUR	Oxygen uptake rate.

I. INTRODUCTION

The processes involved in the life cycle of a micro-organism require a supply of organic materials from which to synthesize new protoplasm. Suitable conversion of some of this material supplies the energy for this synthesis. Oxidation conversions are necessary in most microbial systems. A limited amount of oxidation is possible in some micro-organisms without the requirement of molecular oxygen. This anaerobic metabolism is inefficient and very often incomplete, sometimes giving rise to toxic by-products. The inclusion of molecular oxygen as a necessary requirement in the oxidative processes of many micro-organisms gives rise to the much more efficient aerobic metabolism.

In order to achieve some particular desired rate of aerobic metabolism it is necessary to supply the appropriate quantity of molecular oxygen to each organism. If the production of cell material is the desired end-product for a given aerobic industrial fermentation, then the limitation in the productivity of the particular plant may well lie in the supply of oxygen. However, some products of industrial interest (e.g., alcohol) arise as a result of oxygen

starvation at some phase in the life-cycle of the micro-organism. From an industrial point of view it is therefore necessary to establish the particular, possibly changing, oxygen requirements which will give rise to the maximum production of the desired product.

The supply of a specified oxygen requirement per unit mass of cells becomes then the engineering problem. Design of suitable equipment is based on a knowledge of the quantitative ability of aeration devices to supply this oxygen to the desired weight of cells. The ultimate choice of design and process rate then results from the additional application of economic factors.

The study of oxygen requirements in a particular aerobic micro-organism can lead to much improvement in the efficiency of the fermentation. This may involve, for example, such aspects as the selection of new strains of organism, modification of nutrients and careful control of the supply of oxygen. From such investigations a greater understanding of the mechanism of oxygen transfer to respiring organisms enables improved designs of equipment to be contemplated. The application of new designs on an industrial scale requires that accurate performance data is able to be obtained for existing fermentation systems. A continual development of methods of design, investigational procedures and control techniques is thus the engineering contribution to the overall improvement of industrial micro-biological processing.

II. OXYGEN REQUIREMENTS

Requirements for oxygen differ between species of organisms and change during the life cycle of the cell. The supply of oxygen to the cells depends on its availability in the environment and the mechanism of transfer. Cell morphology considerably affects the characteristics of the culture and thus the oxygen transfer rate.

A. Biology and kinetics of growth of industrial micro-organisms

General discussions of the biology of micro-organisms appear in Butlin (1967) and Aiba *et al.* (1965), and more detailed accounts can be found in Frobisher (1957) and Hawker *et al.* (1960).

The growth of micro-organisms usually refers to the growth of the whole culture rather than to any change in the size of an individual cell. During the life cycle of a bacterium the volume of the cell will approximately double in value. In the case of a spherical cell this will only mean a change in the diameter by $\sqrt[3]{2} = 1{\cdot}26$. When an adequate supply of all nutrients is available then the bacterium will produce a new generation by dividing into

two new cells. Each new cell will contain a representative half sample of the original cell. Cell material from a previous generation is thus part of the new generation and only a very small amount of debris accumulates.

The length of time that a bacterium takes to pass through its full life cycle and ultimately become two cells is known as the doubling time, t_2. This doubling time has a unique value for a particular strain of bacterium and a given set of environmental conditions (e.g., temperature, pH, types of nutrient). In practice the doubling time is determined from measurements of the growth of a culture of the bacterium (see for example this Series, Vol. 1). Tempest (this Vol., p. 259) describes the kinetics of microbial growth.

Some species of bacteria remain in contact with each other due in part to the presence of the slime layer which surrounds them. The actual number of cells formed together in this way is used as a method of identification for certain species. On a much larger scale the mixed population of organisms present in activated sludge aggregate together to form floc particles of diameter from about 200 to 1000 μm (Downing and Bayley, 1961). These clumps are mainly of bacteria together with organic debris and higher organisms such as fungi and protozoa.

When the nutrients necessary for growth have been consumed some microbes form dormant but viable spores which are resistant to a wide range of environments.

Growth of fungal cells only occurs at the tips of the hyphae, as it would seem (Mandels, 1965) that the cell wall, soon after formation, loses its ability to expand. The rate of growth at the tip is quite independent of the length of the hyphae (Smith, 1923). Nutrient material streams along the hyphae cells supplying the developing tip. The growth of a fungal culture on the surface of a solid medium produces a circular mat. Measurement of this type of growth indicates that the radius of the mat increases linearly with time, evidence for the linear growth at the hyphal tips. Mandels (1965) points out that because the fungal hyphae grow by extension at their tips, the unlimited or vegetative growth phase is no longer described by autocatalytic kinetics. Hyphal-growth kinetics are thus given by—

$$dl/dt = k_1 \tag{1}$$

If the fungal cell is assumed to be a cylinder of diameter d and length l, then an expression for the cell concentration can be obtained for linear growth in submerged culture—

$$x = \frac{N}{\bar{V}} \rho \frac{\pi d^2 l}{4} \tag{2}$$

where N is the number of cells, V the volume of culture, and ρ the cell density. For a constant number of cells we can write equation (1) as—

$$dx/dt = k_2 \tag{3}$$

Linear growth at fungal cell tips accompanied by considerable branching of the hyphae, which has been observed to occur in submerged culture (Pirt and Callow, 1960; Borrow *et al.*, 1964), results in autocatalytic cell-growth kinetics. The growth process can thus be described by—

$$dx/dt = k_3 x \tag{4}$$

and Pirt (1966) quotes a typical value of $k_3 = 0{\cdot}18\ \mathrm{h}^{-1}$ for fungal cultures of this type, corresponding to a doubling time $t_2 = 230$ min.

The effect of agitation in association with other factors such as the strain of organism, types of nutrients and form of inoculum results, in several cases of fungal culture, in the formation of rigid spherical mycelial pellets. Several reports of this phenomenon are available (Emerson, 1950; Phillips, 1966). Emerson (1950) has shown that the rate of increase in the radius of such pellets is constant. A typical stirred fungal culture will most probably consist of a mixture of filamentous branched hyphae and a size range of spherical pellets (Pirt, 1966) which can reach 1 mm in diameter (Phillips, 1966). Fungal cultures which form only pellets can be described mathematically by—

$$dr/dt = k_4 \tag{5}$$

where r is the radius of the pellet. Again the concentration of cells can be expressed by—

$$x = \frac{N}{V}\rho\frac{4}{3}\pi r^3 \tag{6}$$

An initial inoculum of spores which proceeded to form a constant number of pellets would grow in submerged culture in a manner described by—

$$dx/dt = k_5 x^{2/3} \tag{7}$$

Alternatively, a plot of the cube root of cell volume versus time should produce a straight line, as confirmed by several workers (Mandels, 1965).

The non-vegetative reproduction of fungi can take a variety of forms. As with the previous two groups, a spore condition is very common. The spores are contained in capsules produced at the ends of special hyphae (conidia) and their individual characteristics (Butlin, 1967) assist in the identification of the organism. Other methods of reproduction (Hawker *et al.*, 1969)

include thick-walled cells, buds and the breaking off of hyphal cells at cross-walls.

B. Biochemistry of oxidation

The intake of nutrient by a microbial cell is required to accommodate the two basic processes of biosynthesis and energy production. As the biosynthesis of protoplasm is a constructive process, the reactions involved require energy. Many reactions and intermediates are common to both biosynthesis and energy formation, so that the two processes cannot be considered as separate activities (Sistrom, 1962). There are energy requirements additional to those for biosynthesis, which become more extensive the more complex is the organism. Pirt (1965) has discussed "maintenance energy" in some detail and points out (Pirt, 1968) that failure in this basic energy need results in autolysis of the cell.

Solar radiation and chemical oxidation are the only two sources of energy which can be made available to micro-organisms, but only oxidation is considered here.

Shortage of molecular oxygen, which is the final electron acceptor in aerobic organisms, can have serious consequences on overall metabolism. The main concern of this Chapter is the problems associated with cultures whose energy requirements are supplied by aerobic respiration. It is important to appreciate that many aerobic micro-organisms can metabolize anaerobically. The onset of anaerobic conditions in an otherwise aerobically grown cell culture will cause many changes in the biochemistry of the cell. Some of these changes may be permanent and detrimental to the process in question.

The detailed manner in which molecular oxygen is involved in the oxidation processes of a cell has been discussed by Mason (1957). Three main mechanisms are described. The first type involves the direct incorporation of oxygen into substrates catalysed by oxygenases. Mixed-function oxidases or mono-oxygenases, in which one atom of oxygen is included in a substrate and the other acts as an electron acceptor forming water, are a second type. The third type, usually quantitatively more important and the only type known to be energy-linked with formation of ATP, are the electron-transfer oxidases. These reduce oxygen to water or hydrogen peroxide.

C. Molecular oxygen and the microbial cell

Adequate transfer of oxygen from the surrounding environment (liquid nutrient in the case of submerged culture, and nitrogen–oxygen gaseous mixtures in the case of surface culture), and its subsequent unhindered involvement with the enzyme systems will be necessary for its adequate

utilization. Many factors must be considered when an attempt is made to analyse this complex process.

1. *The source of molecular oxygen*

Oxygen is readily available from the air which has an approximate volumetric composition of 21% oxygen and 79% nitrogen. At atmospheric pressure, say 760 mm Hg, the partial pressure of oxygen by Dalton's law is 150 mm Hg. This forms an alternative way of expressing the gas composition. If air is doubled in pressure, the volumetric ratio remains the same, so that although the partial pressure of oxygen will now be 300 mm Hg, unfortunately the partial pressure of nitrogen will also have doubled. A supply of this compressed air will result in an inefficient system in which 79% of the gas being compressed is of no use to the organism. Clearly, the economics of compression must be compared very carefully with any advantages obtained from the increased oxygen partial pressure, particularly in large-scale operation. Also, the exposure of microbial cells to total pressures greater than atmospheric may have general effects on their morphology. An increase in the partial pressure of oxygen can alternatively be achieved, at atmospheric pressure, by mixing oxygen and nitrogen supplied from two pure sources of the gases.

For cells growing in submerged culture the oxygen must be made available predominantly as a dissolved gas in the liquid nutrient, although the possibility of gas/cell oxygen transfer may also operate (Bennett and Kempe, 1964). Oxygen dissolves in water to an extent which is dictated by an equilibrium with an oxygen-containing gas phase. Montgomery *et al.* (1964) have reported detailed measurements of oxygen solubility in pure and saline waters. An empirical equation expresses the results for pure water in equilibrium with air at a pressure of 1 atm, in the temperature range 4°–33°C is—

$$c_E = \frac{468}{31{\cdot}6+t} \tag{8}$$

where t is the temperature in °C and c_E is the oxygen concentration in the water in p.p.m. (or mg/litre). At a temperature of 20°C equation (8) gives a value of $c_E = 9{\cdot}08$ mg/litre. As equation (8) indicates, an increase in the temperature reduces the solubility, as does the presence of dissolved salts. A value of 7·40 mg/litre was obtained for water of 35% salinity in equilibrium with air at 1 atm and 20°C. Nutrient solutions for submerged culture contain dissolved sugars and salts. Thus the value of the oxygen concentration will be less than that for pure water. A value of 5·88 mg/litre has been reported by Phillips and Johnson (1961b) as the corresponding liquid equilibrium value for air in contact with a nutrient solution at 30°C containing 5% glucose and several dissolved salts. The relationship between

the gas composition (p atm) and the liquid-phase composition of oxygen (c_E) is found to be linear, i.e., it obeys Henry's law—

$$p = Hc_E \tag{9}$$

where H is the Henry's law constant, specific to a given gas component and liquid phase, and also temperature. Henry's law may be variously expressed, depending on the choice of the units. Thus the constant H will possibly have the units of (litre-atm)/mg. Using the value quoted above for the oxygen concentration at 20°C and 1 atm pressure, when in equilibrium with air containing 21% oxygen, a value of $H = 0{\cdot}21/9{\cdot}08 = 0{\cdot}0231$ (litre-atm)/mg is obtained. The highest concentration of dissolved oxygen would, by Henry's law, for this given temperature and pressure, occur when pure oxygen was used as the gas phase. Thus $p = 1$ atm and $c_E = 1/0{\cdot}0231 = 43$ mg/litre. The units of oxygen solubility are also differently quoted such that 43 mg/litre = 1·34 mmole/litre, a value very similar to that of 1·38 mmole/litre quoted by Finn (1967). These figures serve as an order of magnitude indication but it is recommended that the actual value specific to a particular system be determined experimentally when required. Methods for doing this determination are discussed in a later section of this chapter. The composition of the cytoplasm differs from that of pure water. Many materials such as proteins, lipids, carbohydrates and mineral salts will be present in the water. Organic materials will be present in a colloidal form. The number of molecules of oxygen per unit volume of such a solution will be much less than that in even the extra cellular nutrient liquor, when in equilibrium with a particular gas.

2. *Transfer of oxygen across a membrane*

Henry's law describes the equilibrium compositions of oxygen in air and a liquid with which it is in contact. The constant H is unique to the particular liquid and temperature. Consider the two liquid systems pure water 1 and a salt solution 2, then the equilibrium constants will be H_1 and H_2. If each system is now separately equilibrated with air containing oxygen of composition p atm, then the concentrations of oxygen in the solutions will be $c_{E1} = p/H_1$ and $c_{E2} = p/H_2$. It has been shown above that $c_{E1} > c_{E2}$.

In a situation where these two liquids are separated by a membrane which is permeable to molecular oxygen, and only the pure water is exposed to the gas of oxygen concentration p atm, then ultimately the liquids on both sides of the membrane will reach equilibrium and have concentrations of c_{E1} and c_{E2}. Thus, although there will be a concentration difference across the membrane, no further transfer of oxygen will occur. The concentrations of oxygen in the gas phase with which these two concentrations

in the liquid phase are in equilibrium will be the single value of p atm. The driving force for transfer of oxygen across a membrane is thus the difference between the partial pressures of oxygen in gas phases which would be in equilibrium with the particular concentrations in the liquid phases. In order to deal with this situation more easily the term "oxygen tension" (T) is employed. The oxygen tension is thus related to the concentration of oxygen in solution by—

$$T = Hc_L \tag{10}$$

As this equation has the same form as Henry's law, the units are the same, so that T is expressed in atm or mm of Hg. When pure water is equilibrated with air of oxygen concentration p atm, then $c_L = c_E$ and T will have the same value as p.

It is possible to extend further the situation above of two liquids separated by a membrane by introducing some oxygen-consumption mechanism. This could be achieved by having a small flow of either solution through its appropriate chamber. For the case of consumption in the salt system and gas feed to the pure water $c_{L1} = c_{E1} = p/H_1$, and $T_1 = p$. In the salt system $c_{L2} \neq c_{E2}$ and $T_2 = H_2c_{L2}$, and the flow of oxygen will be caused by the difference in oxygen tension between T_1 and T_2. Consumption in the water system will result in $c_{L1} < c_{E1}$ and $T_1 < p$, then the salt system will equilibrate such that $T_2 = T_1$ and $c_{L2} = T_1H_2$.

3. *Oxygen in the microbial cell*

The cytoplasm of micro-organisms is contained within a membrane supported by a rigid outer cell wall. The transfer of molecules across this membrane is controlled by various mechanisms (Rothstein, 1965) which can result in higher concentrations of some materials inside the cell than would be expected from chemical or electrochemical equilibrium. The transfer of molecular oxygen into the cell, however, is most probably a straightforward diffusion process through the membrane. The driving force for transfer is the difference in oxygen tension between the nutrient liquid and the cytoplasm. Oxygen cannot be stored in the cell in the same way that the accumulation of more complex materials can occur, so that a failure in the supply affects the cell immediately.

When oxygen is the final electron acceptor for the energy production reactions, it is catalysed by cytochrome oxidase. The cytochrome system in fungi and mature yeast cells is located in the special mitochondrial bodies which possess an outer membrane wall. The respiratory enzyme chain is probably incorporated into the mitochondrial membrane *in vivo* (Haggis, 1964). A clear diagrammatic representation of the system is presented by Lehninger (1961). Thus molecules of oxygen must further pass from the

cytoplasm into the mitochondria before they can be utilized. The number of mitochondria in a cell, as well as the supply of both substrate and oxygen to these bodies, will determine, to some extent, the rate of oxygen consumption. Provided that the substrates and other components are present in unlimiting quantities, and oxygen is similarly available, then respiration will proceed at a maximal rate governed by the overall kinetics. At low concentrations of oxygen this rate will be affected by the random activity of oxygen molecules in their ability to locate with available active enzyme sites. This process will be proportional to the oxygen concentration and gives first order relationship of respiration rate with oxygen concentration, i.e., Michaelis–Menten kinetics (Fig. 1).

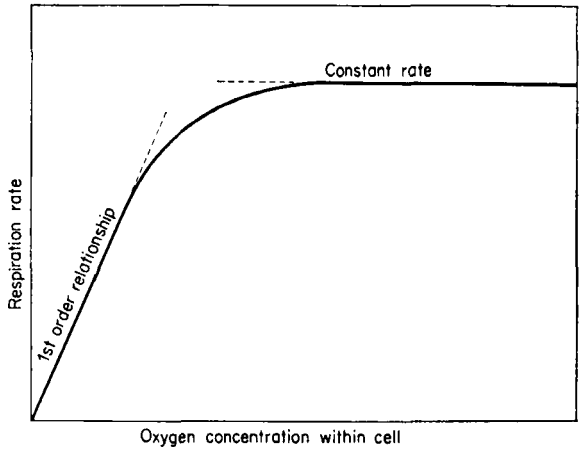

FIG. 1. Diagrammatic representation of the effect of oxygen concentration within a bacterial cell on its respiration rate.

4. *Oxygen demand and usage by cultures of cells*

Much of the above discussion has been presented in the context of the single cell. However, the determination of quantitative data for design purposes can only be obtained from the study of cells in large numbers. The growth of cultures of various organisms with unlimited supply of nutrient in the simplest case of exponential growth can be expressed—

$$\mu = \frac{1}{x}\frac{dx}{dt} \tag{11}$$

where μ is the specific growth rate of the culture (see Tempest, this Vol., p. 259). The relationship between specific growth rate and concentration of the limiting nutrient in submerged culture is similar to that between

enzymic activity and substrate concentration. Monod (1942, 1949) first pointed out the similarity and described the shape of the curve (Fig. 2) with the equation—

$$\mu = \mu_m \frac{s}{K_s + s} \tag{12}$$

where s is the concentration of limiting nutrient and K_s is a constant (similar to the Michaelis–Menton constant). The constant is determined from experimental data as the value of s at which the specific growth rate of the culture is half that of maximum. The several curves labelled s_1, s_2 and s_3 in Fig. 2 represent the effect of the limitation of growth by different substrates. Although all the curves have the same maximum for a particular organisms, nutrient components and temperature, different substrates will be characterized by different values of K_s (see Tempest, this Vol., p. 259).

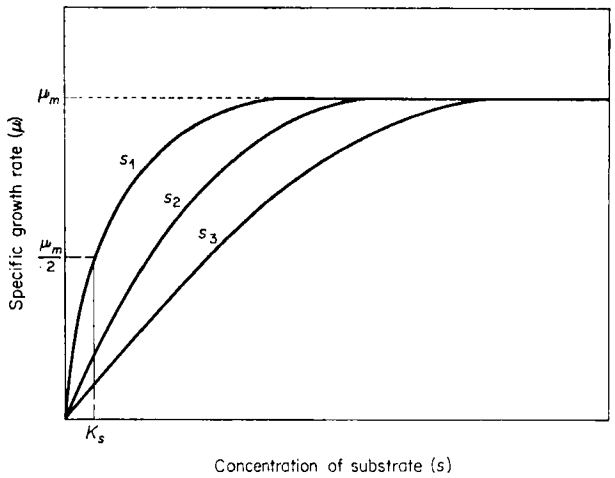

FIG. 2. Relationship between specific growth rate and substrate concentration.

In aerobic cultures, a curve of this type is obtained when the limiting substrate is dissolved oxygen, then—

$$\mu = \mu_m \frac{c_L}{K_o + c_L} \tag{13}$$

where K_o is the Michaelis–Menton constant for oxygen in the particular system. A specific oxygen uptake rate Q (volume of oxygen per unit dry wt of cells per unit time) can be defined for a culture with an aerobic metabolism. It is reasonable to suppose (Hospodka, 1966) that the specific growth rate

of such a culture is related to the specific oxygen uptake rate. If endogenous respiration is ignored then a form can be proposed—

$$\mu = k_6 Q \tag{14}$$

and substitution of equation (14) into (13) gives—

$$Q = Q_m \frac{c_L}{K_o + c_L} \tag{15}$$

The general shape of this relationship will thus be the same as the curves of Fig. 2, plotting Q in place of μ and c_L in place of s. The maximum specific oxygen uptake rate Q_m is often referred to as the specific oxygen demand of a culture, and will have a constant value for a given organism in a defined environment. Some examples extracted from the literature are recorded in Table I.

TABLE I

Specific oxygen demand

Organism	Q_m (mmole O_2/g cells per h)	Reference
Aspergillus niger	3·0	Phillips & Johnson (1961)
Streptomyces griseus	3·0	Bartholomew *et al.* (1950)
Penicillium chrysogenum	3·9	Phillips & Johnson (1961)
Klebsiella aerogenes	4·0	Harrison & Pirt (1967)
Saccharomyces cerevisiae	8·0	Maxon & Johnson (1953)
Escherichia coli	10·8	Phillips & Johnson (1961)

The most practical way to obtain the value of the specific oxygen demand for a particular culture is to measure the oxygen uptake rates and cell concentrations of batch cultures when no nutrient component is limiting. Cells in a culture in which some substrate has become limiting will not consume oxygen at the maximum specific uptake rate. However, it is customary to determine a maximum value for cells by re-suspending a sample in the original unlimited nutrient and following the respiration in a Warburg apparatus (Umbreit *et al.*, 1964). This equipment consists of a small sealed glass flask to which is connected a manometer. The flask can be continuously shaken in a constant temperature bath. A small inner partition contains filter paper saturated with caustic soda. During a respiration-rate measurement the carbon dioxide produced by the culture sample is absorbed by the caustic soda and oxygen, initially charged to the system, passes into the culture and is consumed by the micro-organisms. The oxygen uptake is noted as a change in the liquid level in the manometer. This aspect of the

device introduces a limitation in its operation because as the oxygen is consumed and the liquid level of the manometer changes then a reduction in pressure occurs in the flask. It is possible for the partial pressure of oxygen to fall to such a value that the respiration rate becomes controlled by the supply of oxygen from the gas to the liquid phase. The respiration rate recorded may not then be that of maximum uptake rate. Respirometers have been devised (Abson *et al.*, 1967) which automatically maintain the partial pressure of the oxygen at a constant value and thus overcome the difficulties associated with the Warburg apparatus.

The product of the specific oxygen uptake rate and the cell concentration is used by many workers to represent the total oxygen uptake rate per unit volume of culture $Q' = Ox$. Typical units will be mmole O_2/litre of culture per hour. During the early stages of a batch culture $Q = Q_m$ and the increase in Q' with time will be due to the increase in the cell concentration. As nutrient limitation develops, then the value of Q decreases, although the cell concentration may still continue to increase. Eventually, particularly when oxygen limitation has developed, the decrease in Q will become more rapid. Also, the cell concentration will reach a maximum and may even decrease due to autolysis. The overall effect will be that Q' passes through a maximum value. Such peak oxygen demands per unit volume of culture (Q'_m) are commonly reported in the literature. However, the actual values which they assume have little general application because they may embody the capacity of the individual aeration equipment, in that this especially contributes to the ultimate rapid reduction of the specific oxygen uptake rate due to its inability to maintain an unlimiting value of dissolved oxygen at high cell concentrations. This aspect is demonstrated typically by the results of Virgilio *et al.* (1964) for the rifamycin fermentation, who found that in a given fermenter at a power input of 3·1 W/litre, Q'_m = 130 ml O_2/100 mls culture per hour compared with Q'_m = 100 ml O_2/100 mls culture per hour at a power input of 0·9 W/litre.

If a particular organism is only supplied with a single carbon-containing substrate as its oxidizable material, then its consumption together with the resultant yield of cells will be a measure of the respiration rate. A relationship has been advanced by Maxon and Johnson (1953) and more recently generalized by Elsworth *et al.* (1968) for the oxidation of a single substrate to carbon dioxide, water and ammonia (if it contains nitrogen)—

$$Z = \frac{A}{Y} - B \tag{16}$$

where Z is the mmole O_2/g cells produced, A is the mmole O_2 required for the complete conversion of 1 g of the substrate to CO_2, H_2O (and NH_3), Y is the yield of dry cells in g/g of substrate and B is the mmole O_2 required

to convert 1 g of the cells to CO_2, H_2O (and NH_3). The analysis of the cells will be required in order that B can be calculated. For the very simple case of glucose being converted into yeast cells and carbon dioxide, Maxon and Johnson (1953) thus used the expression—

$$Q' = \left[\frac{33{\cdot}3}{Y} - 41{\cdot}3\right] G \tag{17}$$

where Q' is in mmole O_2/litre per hour, and G is the yeast production capacity in g yeast/litre per hour. This equation is developed from a consideration of the stoichiometry of the process, and its interpretation must infer the idea that $Q' = Q_m x$, whereas in practice $Q' = Qx$, with Q changing. Thus the value of the cell concentration predicted by equation (17) which could be supported by an aeration system of a given potential capacity Q' is always greater than the actual measured quantity. The assessment of the oxygen requirements for the complete oxidation of a complex mixed-component substrate may be treated similarly. This is the special problem associated with biological effluent-treatment processes. A sample of the mixed nutrient solution is diluted with oxygenated water, inoculated with a suitable culture of organisms and allowed to incubate for 5 days under standard conditions. The analysis of the oxygen uptake over the 5 days is then interpreted as a measure of the oxidizable material in the nutrient solution. Details of the 5 day Biochemical Oxygen Demand (BOD) test can be obtained in the more appropriate literature (Abson and Todhunter, 1967).

5. *Oxygen limitation*

Modern measurements of respiration rates (Longmuir, 1954) involve the monitoring of the decrease of dissolved oxygen with time in a culture contained in a special sealed polarograph cell. Alternatively, a similar measure can be obtained on the whole culture in a fermenter by stopping the air flow and recording the subsequent decrease in dissolved oxygen (Bennett and Kemp, 1964). Fig. 3 is a typical plot of such an experiment for cells which are initially growing in unlimited supplies of nutrient. For this system the uptake per unit volume is given by—

$$Q' = Q_m x = \frac{-dc_L}{dt} = \frac{Q_m x\, c_L}{K_o + c_L} \tag{18}$$

As the cell concentration will remain constant over the short duration of the test, equation (18) can be integrated—

$$\frac{c_{Lo} - c_L}{t} = Q_m x + \frac{K_o}{t} \ln \frac{c_L}{c_{Lo}} \tag{19}$$

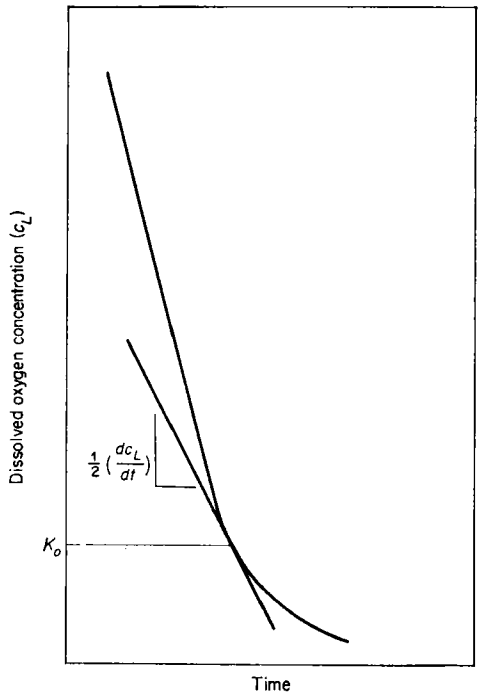

FIG. 3. Oxygen uptake and the determination of K_o.

A suitable linear plot of the data can thus yield both K_o and Q_m. The results of such experiments can also be converted into a graph of oxygen uptake rate versus dissolved oxygen concentration. An example of this is reported by Longmuir (1957) for respiring rat-liver cells, and confirms the use of equation (15) as a mathematical formulation of the relationship. The limitation of much of such data is that the rate of oxygen uptake is not quoted in specific terms but commonly has the units of mmole O_2/litre per hour. However, as the cell concentration is constant, these units will be proportional to those of Q (mmole O_2/g cells per hour). It is also customary to indicate the magnitude of the respiratory activity by quoting a value of K_o, although this approach can only have comparative application. K_o can be very simply determined from experimental data of the form shown in Fig. 3, without using a plot of equation (19), by determining the dissolved oxygen concentration at which the slope of the tangent is half that of the linear portion.

The value at which the specific oxygen uptake rate ceases to be at the maximum Q_m in an otherwise unlimited nutrient is termed the critical oxygen concentration (c_{crit}). Examples of approximate critical oxygen

concentrations are listed in Table II. If a value of 8 mg O_2/litre is assumed for the concentration of oxygen in a nutrient solution in equilibrium with air then the figures in Table II represent between 5% and 25% of saturation. In terms of oxygen tension the critical value will thus fall between 7·5 and 37·5 mm Hg. As equation (15) represents the respiration process it is evident that K_o is almost directly proportional to c_{crit}.

TABLE II

Critical oxygen concentration

Organism	c_{crit} (mg/litre)	References
Escherichia coli	0·26	Kempner (1937)
Penicillium chrysogenum	0·40	Phillips & Johnson (1961)
Saccharomyces cerevisiae	0·60	Hixson & Goden (1950)
Pseudomonas oralis	1·10	Bennett & Kempe (1964)
Torulopsis utilis	2·0	Button & Garver (1966)

The possible kinetics of respiration within bacterial cells which do not contain mitochondria have already been discussed and typical results indicated in Fig. 1. Measurements were made by Longmuir (1954) on disintegrated cell preparations of different sized bacteria, and he found a substantially constant value for K_o. The conclusion from this fact is that all cytochrome material is identical in respiratory activity per unit quantity regardless of the size of bacterium in which it exists. It can thus be assumed from this that a single concentration of oxygen within the cell will be the critical value for all sizes of bacteria. Alternatively, all sized bacterial cells will have the same internal oxygen Michaelis–Menton constant K_{oi}. As absolute respiration rates for these cell preparations were not reported, the relationship between oxygen uptake rate per cell and cell size is not known.

Very small whole bacterial cells gave a K_o of the same order of magnitude as the disintegrated cells. However, as the cell diameter (d) increased, so also did the value of K_o. For cells ranging in size from 0·4 μm to 6 μm it was reported (Longmuir, 1954) that $K_o \propto d^{2\cdot6}$. Protoplasts were found to be capable of respiring at the same rate as the bacteria from which they were prepared. However, K_o values were always less than those for the whole bacterium (Longmuir *et al.*, 1960). These differences were related to varying degrees of removal of the cell wall.

If the respiratory enzyme system in a bacterial cell can be considered

to be proportional to the cell surface area, then the maximum rate of consumption of oxygen per cell (g) will be given by—

$$q_m = k_A d^2 \tag{20}$$

In limited concentrations of oxygen, assuming a 1st order process in oxygen concentration, the rate will be—

$$q = k_A d^2 c_{int} \tag{21}$$

where c_{int} is the oxygen concentration inside the cell. At half the maximum respiration rate we can define the internal Michaelis–Menton constant K_{oi} by—

$$q = q_m/2 = k_A d^2 K_{oi} \tag{22}$$

and K_{oi} will be assumed constant for all cell sizes. At steady state the rate of oxygen consumption will be the same as the rate of transfer to the cell. If it is assumed that oxygen transfer to the cell is caused by a concentration difference between the external nutrient and the cell contents, then the steady state will be described at the rate equal to half that of maximum by—

$$k_A d^2 K_{oi} = k_w \pi d^2 (K_o - K_{oi}) \tag{23}$$

where $k_w = D_w/x_w$ is the cell-wall transfer coefficient, composed of the oxygen permeability (D_w) and the wall thickness (x_w). From equation (23) it is evident that K_o is a constant regardless of the cell size, which is a very different result from the experimental evidence of Longmuir (1954). The modification of the above model by the suggestion that the rate of consumption of oxygen per cell is proportional to the volume of the cell, rather than its surface area will only change the result such that $K_o \propto d$. A possible explanation of the power of 2·6 on the cell diameter measured experimentally is that considerable differences exist in the cell wall for larger bacteria, thus affecting k_w. As the cell size increases, the thickness of the wall increases and possibly the permeability decreases.

A possible limitation in this polarographic technique of respiration measurements has been pointed out by Button and Garver (1966). A curve of growth rate versus dissolved oxygen was prepared from steady state oxygen limited continuous culture runs with *Torulopsis utilis*. The K_o value measured from this graph was very much higher than the figures quoted by Longmuir (1954). It is possible that adaptation occurs in steady state oxygen limited cultures, whereas the rapid polarographic method records the instantaneous response of a given culture to its changing environment. High values of K_o on the basis of previous discussions might indicate a modification in the cell-wall structure for steady state oxygen limited continuous cultures.

A special situation of oxygen limitation arises in the case of cultures which tend to form large mycelial pellets. The critical oxygen concentration for such cultures is very much higher than for non-pelleting cultures of the same organism. Phillips (1966) studied the effect of pellet formation on the oxygen uptake rates for *Penicillium chrysogenum*. By assuming that at steady state the rate of diffusion of oxygen into the pellet was equal to the rate of consumption in the volume and that the specific oxygen uptake rate per unit volume of pellet ($Q\rho_p$, where ρ_p is the density of the pellet) was not dependent on the dissolved oxygen concentration, it was shown theoretically that the apparent critical oxygen tension T_{ac} should be given for a pellet of radius r by—

$$T_{ac} = \frac{r^2 Q\rho_p}{6\, D_p H} + T_{tc} \tag{24}$$

where T_{tc} is the true critical oxygen tension for pelleting mycelium, D_p is the diffusion coefficient of oxygen in the pellet mass and H is Henry's law constant. Very good agreement between experimental measurements and calculated results from equation (24) was obtained, verifying the postulation that oxygen supply to the interior of a pellet is by diffusion.

III. MEASUREMENT OF AERATION

Quantitative values for process-rate coefficients are required for both design and investigation purposes. The effects of various parameters on the process rate can only be utilized if their influences are fully understood. Suitable simple models allow for the interpretation of the process in mathematical terms and thus indicate the precise measurements required for a quantitative description. Since several methods are now available for the determination of oxygen tension in nutrient solutions the detailed study of the performance of aeration equipment is possible.

A. Oxygen transfer models and rate coefficients

Models for the transfer of oxygen from a gas phase to the inside of a microbial cell have been described. There are two broad classes of system which can be considered. Arnold and Steel (1958) and Finn (1967) describe the various possible steps which may develop resistance to oxygen transfer for the case where the cell is completely submerged in the culture medium. The alternative situation of a gas bubble in direct contact with a cell agglomerate (Bennett and Kempe, 1964) or the similar case of a gas in contact with a cell slime adhering to a solid surface (Atkinson *et al.*, 1967) can be considered also.

1. *Culture models*

Transfer of oxygen from a gas bubble to a cell is caused by a gradient of activity or tension. There are several steps involved and each affects the driving force for transfer to a different extent. A diagrammatic representation of the process is shown in Fig. 4. Oxygen is dissolved into the liquid phase from the gas bubbles in what is commonly referred to as the "supply" part of the transfer. The liquid phase then contains oxygen as a nutrient for metabolism which must be removed from solution by the cells. This part of the transfer is then sometimes referred to as the "demand" side.

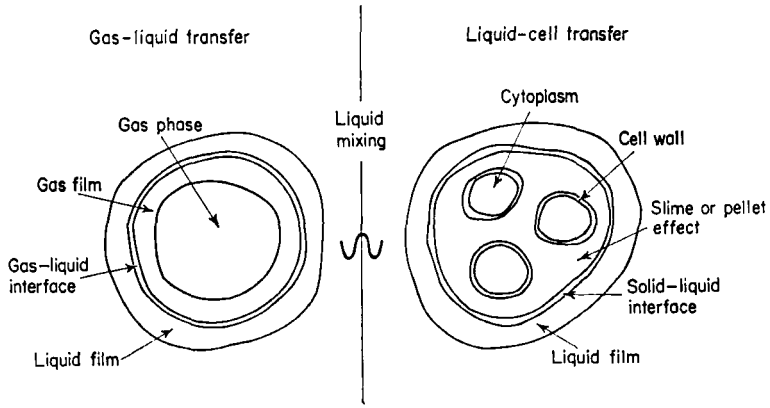

FIG. 4. Submerged culture model.

In considering the transfer of a component from a gas to a liquid it has been postulated that the difference in activity between the two phases can be assumed to occur within two stagnant zones on each side of the gas–liquid interface (Lewis and Whitman, 1924). Also, the presence of surface-active materials at the gas–liquid interface may create a resistance to transfer and therefore a gradient of activity. A constant concentration of oxygen might be expected to exist for a perfectly mixed system. However, in practice this is probably not a true assumption (Phillips and Johnson, 1961b), especially when the cell–liquid mixture exhibits viscous non-Newtonian characteristics. Hydrodynamic considerations justify the presence of a concentration gradient in some liquid film at the solid–liquid interface. The details of the liquid film are not relevant at this point in the discussion but could be assumed to be stagnant (Noyes and Whitney, 1897) or have a laminar-boundary-layer characteristic (Bird *et al.*, 1960).

As in the case of the gas–liquid interface, the possibility exists of solid–liquid interface resistance due to the presence of adsorbed molecules of

surface-active material. The resistance to oxygen transfer within an aggregate or pellet of cells has already been discussed in a previous Section. The effect of cell-wall permeability and thickness has also been considered in the discussion of the transfer of oxygen to a cell. Each of the steps mentioned here in the model have involved diffusional transfer (assisted in some cases by bulk flow due to velocity gradients).

The final aspect of the overall rate of usage of oxygen is the kinetic coefficient for the oxidation process within the cell. The overall rate of usage of oxygen will thus result from the combined effect of each individual step. It is important to be able to assess which individual step is contributing the greatest resistance to transfer in order to be able to improve or modify the overall rate.

A slightly different model is indicated in Fig. 5 corresponding to the type

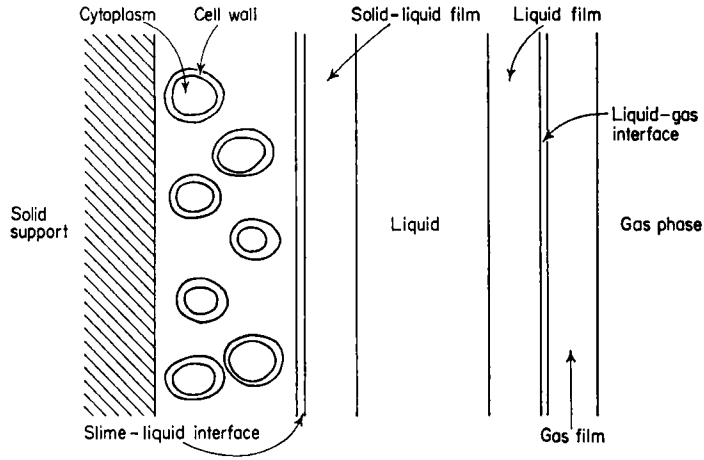

FIG. 5. Surface-film culture model.

of culture which might be expected to occur in a trickle fed packed bed effluent treatment unit. A slime material containing microbial cells is adhered to a solid surface, and over this slime layer the liquid falls in a smooth film with gas supply available from the surrounding atmosphere. Similar resistances to transfer exist in this case, as in the previous model and can be listed as due to:

(i) The gas film.
(ii) The gas–liquid interface.
(iii) The liquid film at the gas–liquid interface.
(iv) Non-homogeneous mixing of the liquid.
(v) The liquid film at the solid–liquid interface.

(vi) Solid–liquid interface.
(vii) Pelleting or agglomeration.
(viii) The cell wall.
(ix) Kinetic rate of oxidation.

2. *Definition of rate coefficients*

Consider the case of a submerged culture of micro-organisms in a fermenter consuming oxygen at a steady rate. In theory this will require the system to be a steady state continuous culture process but in practice will be relevant to a batch process during a short period of time. The total rate of consumption of oxygen can be determined from measurements of the flow rate and composition of the inlet and outlet gas streams (Elsworth, this Series, Vol. 1). This value is referred to as the total "oxygen uptake rate" (OUR) and is commonly expressed per unit volume (Q'). Other terms used for the same quantity are "oxygen transfer rate" (OTR) and "oxygen availability rate" (OAR). Although numerically the same as the other quantities, OAR infers a measurement made under conditions of maximum ability for oxygen transfer by a particular experimental system in steady state, Q' = rate of transfer of oxygen from the gas to the liquid = rate of transfer of oxygen from the liquid to the cytoplasm = kinetic rate of oxidation in the cell. Each of these steps can be considered separately.

(a) *Gas-liquid system.* The gas–liquid transfer process can be described by—

$$\begin{bmatrix}\text{Rate of transfer}\\ \text{of oxygen per}\\ \text{unit volume}\end{bmatrix} \propto \begin{bmatrix}\text{Specific surface}\\ \text{of gas–liquid}\\ \text{interface}\end{bmatrix} \times \begin{bmatrix}\text{Concentration}\\ \text{difference}\\ \hline \text{Distance}\end{bmatrix}$$

or

$$Q' = \text{Constant} \times a \times \text{Concentration difference} \tag{25}$$

where a is the specific area of the gas–liquid interface (cm^2/cm^3). The constant embodies both the distance across which the concentration difference exists and also any unknown effects of hydrodynamics on the basic diffusion process. If equation (25) is compared with the general definition for a transport process, i.e., flux = potential difference/resistance, it can be seen that the resistance to transfer is the reciprocal of the rate constant. The development of the Two Film theory described above allows the concentration difference to be expressed either in terms of gas or liquid-phase compositions such that—

$$Q' = K_g a\,(p - p_E) = K_L a\,(c_E - c_L) \tag{26}$$

where K_g and K_L are overall gas and liquid film mass-transfer coefficients, p is the partial pressure of oxygen in the gas phase, c_L the concentration of oxygen in the liquid phase and c_E and p_E are the hypothetical compositions

which would exist in equilibrium with p and c_L. The overall coefficients can be related to the individual coefficients for the gas film (k_g) and the liquid film (k_L) if Henry's law is assumed for the equilibrium relationship, and if no resistance exists at the interface and thus gas and liquid compositions are in equilibrium at that point. From these relationships it can be shown (Norman, 1961) that for the case of a sparingly soluble gas, such as oxygen, negligible resistance exists in the gas phase and $K_L = k_L$ so that—

$$Q' = k_L a\,(c_E - c_L) \tag{27}$$

When the composition has the units of mmole O_2/litre then the units of the mass-transfer coefficient (k_L) will be cm/h.

(b) *Solid–liquid system.* By reference to Fig. 4 it can be seen that the solid–liquid system is slightly more complex than the gas–liquid system. The oxygen composition will fall from the bulk liquid to the cell cytoplasm. A mass-transfer coefficient can be defined for the liquid film (k_L') such that—

$$Q' = k_L' a_a'\,(c_L - c_L') \tag{28}$$

where a_a' is the area of the solid–liquid interface per unit volume of culture and may be the total cell surface if no pelleting or agglomeration occurs, and c_L' is the concentration of oxygen at the solid–liquid interface.

A concentration gradient of oxygen within a pellet has been demonstrated (Phillips, 1966). This will mean that cell structures at different depths into the mass will be exposed to different values of concentration. A similar effect will occur for cells embedded in a slime structure. If this complex system is to have a rate coefficient defined for the oxygen transfer, then an average concentration must be defined for the extracellular slime. Thus—

$$Q' = k_a' a_m'\,(c_L' - c_m') \tag{29}$$

where k_a' is the agglomerate mass-transfer coefficient, c_m' is the volume averaged mean concentration in the extra-cellular material, and a_m' is a mean area for the agglomerate cross section.

Transfer across the cell wall is by diffusion through the membrane and support structure. A coefficient has already been defined and this will be included in the transfer-rate equation as—

$$Q' = k_w a'\,(c_m - c_{int}) \tag{30}$$

where a' is the cell interfacial area per unit volume of culture. This latter equation is better defined in terms of the oxygen tension in view of the presence of the membrane structure such that—

$$Q' = k_w' a'\,(T_m' - T_{int}) \tag{31}$$

where k_w' has the units of mmole O_2/(h)(cm^2)(atm), for oxygen tension expressed in atm.

(c) *Kinetics of oxidation.* The rate of oxygen transfer to the culture equates, in the steady-state condition, to the rate of consumption by oxidation within the cells. For the case where growth is proportional to the usage of oxygen and all nutrients are present in unlimiting concentrations then—

$$Q' = Qx = Q_m x = \text{constant} \tag{32}$$

In general, for limiting concentrations of oxygen, a slight modification to equation (15) will describe the kinetics—

$$Q' = \frac{Q_m x c_{int}}{K_{oi} + c_{int}} \tag{33}$$

Equation (33) asymptotes to equation (32) for high values of c_{int}, and for low values of c_{int}—

$$Q' = \frac{Q_m x}{K_{oi}} c_{int} = k_7 c_{int} \tag{34}$$

(d) *Rate controlling step.* The relative magnitude of each mass-transfer coefficient and the kinetic rate coefficient will determine the particular step which represents the controlling influence on the overall rate of the process. A knowledge of this fact is most important in the selection of design procedures. For the comparatively simple case of separate yeast cells there will be no aggregate resistance and $c_m' = c_L' = c_L$. Also if it is assumed that no resistance to transfer occurs across the cell wall, then $c_{int} = Hc_L/H_{int}$, and $K_{oi} = H\,K_o/H_{int}$, so that the rate equation (34) becomes—

$$Q' = \frac{Q_m x}{K_o} c_L = k' c_L \tag{35}$$

Equating this with the transfer equation from the gas to the liquid gives—

$$Q' = k_L a\,(c_E - c_L) = k' c_L \tag{36}$$

or as Van de Vusse (1958) has shown—

$$Q' = k_L a\; c_E\; \frac{k'}{k' + k_L a} = K\, c_E \tag{37}$$

Because of the 1st order kinetics described by equation (35) the final result of equation (37) is also first order in the equilibrium concentration so that it does not indicate immediately whether the control is due to the gas–liquid transfer or the kinetics of the oxidation. The magnitude of the coefficients in equation (37) are most important. Thus for $k_L a \ll k'$ control is in the gas–liquid interface, $c_L \to 0$ and $Q' = k_L a c_E$. Alternatively, for $k_L a \gg k'$ then $c_L \to c_E$, and the kinetics of the oxidation controls the process rate which is given by $Q' = k' c_L$. For values of the coefficients which are of the same

order of magnitude then both contribute to the overall rate and this is given by equation (37).

If a batch-culture process is considered, then the kinetic rate coefficient defined by equation (35) will change with time as the cell concentration changes. It is possible for the rate control to start as kinetic and pass to physical transfer as the value of k' changes. The use of actual values from a yeast culture will demonstrate this fact. From Table I a value of Q_m = 8·0 mmole O_2/g cells per hour is obtained. If K_o is assumed to be approximately half the value of c_{crit} then from Table II K_o = 0·3 mg/litre. A value of c_E can be taken of 8 mg/litre and k_La = 3·5 min^{-1} is reported by Bandyopadhyay *et al.* (1967) for this fermentation. The results of calculating Q' from equation (37) are listed in Table III and indicate that the growth process is initially controlled by the kinetics but that as the cell concentration passes through a value of 2 gm/litre there is a change to asymptotic approach to physical transfer control given by $Q' = k_La c_E$. In an actual practical situation the increase in cell concentration will probably lower the value of k_La so that the change to physical transfer control occurs earlier than would be predicted.

TABLE III

Effect of changes in the value of the kinetic rate coefficient on the rate-controlling step calculated from equation (37)

x g/litre	k_La(h^{-1})	k'(h^{-1})	Q' mmole O_2/ litre per hour
0·1	210	85	15·0
0·5	210	425	35·0
1·0	210	850	42·0
2·0	210	1700	47·0
5·0	210	4250	50·0
10·0	210	8500	51·3
∞	210	∞	52·5

3. *Fundamental aspects of the mass-transfer coefficients*

The previous analysis conveniently included all unknown aspects of diffusivity and the effect of hydrodynamics on the transfer processes into the mass-transfer coefficients. Calculations of rate of oxygen usage on the basis of equations (27) or (28) can only be made if actual measured values of these proportionality constants are available. A greater understanding of each transfer step is possible from more fundamental models of the

various systems. The ultimate goal is thus the description of the processes in absolute terms rather than experimentally determined coefficients.

(a) *Gas–liquid interface.* It has already been indicated that, for the case of a sparingly soluble gas, the resistance to transfer lies in the liquid phase. The equation of continuity of mass transfer without chemical reaction in a single (x) direction and through a stagnant film (no velocity term required) can be expressed (Bird *et al.*, 1960) by—

$$\frac{dc}{dt} = D_L \frac{d^2c}{dx^2} \tag{38}$$

For the established steady state of the Two Film theory $dc/dt = 0$ and equation (38) can be solved for the boundary conditions—

$$x = 0 \qquad c = c_E$$
$$x = x_L \qquad c = c_L$$

where x_L is the thickness of the liquid film, to give—

$$\frac{dc}{dx} = \frac{c_L - c_E}{x_L} \tag{39}$$

Fick's first law of diffusion states that—

$$\text{Average rate of transfer per unit area } (R) = -D_L \frac{dc}{dx} = \frac{D_L}{x_L}(c_E - c_L) \tag{40}$$

Comparison of equation (40) with equation (27) shows that $k_L = D_L/x_L$, that is, the coefficient is directly proportional to the diffusivity of the gas in the liquid (D_L).

Examination of experimental results of mass transfer in gas–liquid contact devices does not confirm this relationship. Another explanation has emerged on the basis that when a gas bubble contacts a liquid then the liquid film is not present for a long enough period of time to allow steady state to be reached. Thus Higbie (1935) solved the unsteady-state form of the continuity equation, equation (38) with the boundary conditions—

$$t = 0 \qquad x > 0 \qquad c = c_L$$
$$t > 0 \qquad x = 0 \qquad c = c_E$$
$$t > 0 \qquad x = \infty \qquad c = c_L$$

By application of Fick's first law the instantaneous rate of transfer per unit area (R) is given by—

$$R = \sqrt{\frac{D_L}{\pi t}}(c_E - c_L) \tag{41}$$

A further postulation is that each interface is exposed for the same length of time θ, so that the average rate of transfer per unit area for this time is given by—

$$R = \frac{1}{\theta}\int_0^{\theta} \sqrt{\frac{\overline{D_L}}{\pi t}}\,(c_E - c_L)\,dt = 2\sqrt{\frac{\overline{D_L}}{\pi\theta}}\,(c_E - c_L) \qquad (42)$$

The mass-transfer coefficient is thus $k_L = 2\sqrt{\frac{\overline{D_L}}{\pi\theta}}$. It is not possible to have a value for θ although Higbie suggested that for a bubble rising in a tube then $\theta = d_B/v_B$ where d_B is the bubble diameter and v_B is its velocity of rise. The important difference in this result is that $k_L \propto D_L^{1/2}$ and this is confirmed by experiments (Norman, 1961).

A further extension to this approach was proposed by Danckwert (1951) who argued that the idea of a constant time for the unsteady-state process should be replaced by a random time distribution of the form $\emptyset(t) = Se^{-st}$, where S is a proportionality constant, sometimes referred to as the rate of surface-renewal. This analysis gives $k_L = \sqrt{D_L S}$, but as S is not known for a practical situation, the main significance is its similarity with Higbie's concept in that $k_L \propto D_L^{1/2}$.

A considerable amount of experimental work on the measurement of mass transfer coefficients has been carried out and reviewed by Calderbank (1967). Results (Calderbank and Moo-Young, 1961) for pure liquids aerated in mixing vessels and giving rise to average bubble sizes greater than 2·5 mm were correlated by—

$$k_L\left[\frac{\mu}{\rho D_L}\right]^{1/2} = 0{\cdot}42\left[\frac{\Delta\rho\mu g}{\rho^2}\right]^{1/3} \qquad (43)$$

For smaller bubbles the results fit—

$$k_L\left[\frac{\mu}{\rho D_L}\right]^{2/3} = 0{\cdot}31\left[\frac{\Delta\rho\mu g}{\rho^2}\right]^{1/3} \qquad (44)$$

Thus the liquid film coefficient for the gas–liquid transfer process is unaffected by impeller power input and does not depend on either the bubble size or the relative velocity of the bubble and liquid. A power of $\frac{1}{2}$ on the diffusivity in equation (43) confirms the Higbie treatment, but in the case of small bubbles the power of $\frac{2}{3}$ infers some relative velocity and thus a boundary-layer treatment of the model (Bird *et al.*, 1960).

(b) *Solid–liquid interface.* The application of the diffusion equation to radial molecular diffusion from a sphere into a stagnant system to infinity gives the result that $k_L = 2D_L/D$ where D is the diameter of the sphere. With D replaced by the diameter of a cell this value will be the lowest expected for the solid–liquid mass transfer coefficient. Any movement of

the surrounding liquid relative to the solid surface will increase the value of the coefficient. However, for the case of fungal mycelial floc it is most likely that, as the specific gravity difference between the solid material and the liquid is small, no relative motion will occur. The presence of fungal floc in a liquid creates a pseudoplastic non-Newtonian fluid characteristic (Diendoerfer and West, 1960) to the overall mass, which has the ability to damp out severe velocity fluctuations in a very short distance. Thus it is most probable that for such a system the solid–liquid mass-transfer coefficient is unaffected by changes in the overall hydrodynamic conditions.

Relatively large pellet structures free to move in a low viscosity Newtonian fluid may, however, be affected by turbulent velocity fluctuations. Measurements of mass-transfer coefficients for many different solid–liquid systems have been correlated (Calderbank and Moo-Young, 1961) by—

$$k_L = 0{\cdot}13 \left(\frac{\rho D_L}{\mu}\right)^{2/3} \left(\frac{(P/V)\,\mu}{\rho^2}\right)^{1/4} \tag{45}$$

where (P/V) is the energy dissipation per unit volume of the system. The $\frac{2}{3}$ power on the diffusivity indicates a boundary-layer type of solution of the continuity equation, which is appropriate for a model which includes a velocity gradient at the solid–liquid surface.

Small cells may well have a size of the same order of magnitude as the smallest velocity fluctuations and thus may not be affected by them. For the case when the small-scale fluctuations might be described as homogeneous and isotropic (Keey, 1967) some effect may be present and the coefficient given by a film theory result (Kolar, 1959).

(c) *Solid phase.* Mass transfer in the solid phase is by molecular diffusion. Changes in the mass-transfer coefficients will be achieved only by changes in the size of agglomerates and cell-wall thickness. The possibility of changes in the permeability of oxygen through the cell wall might also be encountered.

B. Measurement of gas–liquid rate coefficients in submerged culture

For aerobic fermentations in which kinetic control does not affect the rate of process and in which little solid-phase resistance is encountered, the main resistance to oxygen transfer lies in the liquid film of the gas–liquid transfer step. As a result, much effort has been expended on the measurement of this liquid-film rate coefficient.

1. *The absorption coefficient*

On the basis of the whole fermenter contents the oxygen uptake rate per unit volume is given by—

$$Q' = k_L a\,(c_{Em} - c_L) \tag{46}$$

where c_{Em} is a fermenter contents mean equilibrium interface concentration. Inspection of equation (46) reveals that, in order to determine a value for the mass-transfer coefficient for a given system, values of four quantities must be measurable.

The value of the oxygen transfer rate per unit volume Q' has already been mentioned to be given by the product of the air flow rate and the difference in oxygen concentration between the inlet and outlet gas streams. This oxygen transfer rate (OTR) or oxygen uptake rate (OUR) has been used sometimes (Steel and Maxon, 1966; Bennett and Kempe, 1964) to describe the performance characteristics of fermentation apparatus. As equation (46) indicates that OUR involves four unknown possible variables each of which could vary with parameters such as stirrer speed, reported values are specific to individual equipment and do not contribute to the fundamental understanding of the transfer process. Another terminology mentioned in this context was that of "oxygen availability rate" (OAR). This quantity has the particular connotation of a limitation by the aeration equipment in its ability to supply oxygen to the liquid. Steel and Maxon (1962) define the OAR as the OUR of the culture when it is limited in its respiration rate by oxygen supply. Under these conditions then, an increase in stirrer speed ought to give an increase in OUR. Very approximately it can be stated that OAR is the OUR when the dissolved oxygen value is zero, or OAR = $k_L a c_{Em}$ (Finn, 1967).

The gas–liquid interfacial area per unit volume, a, has created considerable difficulty in its measurement. Methods have been applied to this measurement in the ideal condition of pure liquids in bubble contact in stirred vessels, and correlations are available for both bubble size and gas hold-up ratio (the ratio of gas volume to gas + liquid volume), from which a is derived (Calderbank, 1967; Valentin, 1967). No such values of interfacial area have been possible for submerged culture fermentation liquors. It is thus the practice to associate the area with the mass-transfer coefficient in a so-called "absorption coefficient" ($k_L a$).

In order to determine an absorption coefficient, a mean equilibrium concentration must be defined. The mean interface concentration in equation (46) will be related to the gas-phase composition by $p_m = Hc_{Em}$ as defined in equation (9). However, the air introduced at the base of a fermenter loses oxygen as it proceeds through the system. One procedure has been to use a log mean partial pressure of oxygen in the gas phase between the inlet and outlet—

$$p_{lm} = \frac{p_{in} - p_{out}}{\ln (p_{in}/p_{out})} \tag{47}$$

and then $c_{Em} = p_{lm}/H$. Alternatively this technique is expressed as a log

mean driving force such that—

$$\Delta c_{lm} = \frac{(c_{Ein} - c_L) - (c_{Eout} - c_L)}{\ln \left[\frac{c_{Ein} - c_L}{c_{Eout} - c_L} \right]} \tag{48}$$

with $c_{Ein} = p_{in}/H$ and $c_{Eout} = p_{out}/H$, when—

$$Q' = k_L a \, \Delta c_{lm} \tag{49}$$

Recent work on the residence time distribution of the gas phase of a gas–liquid stirred vessel (Hanhart *et al.*, 1963) has indicated that considerable back-mixing of the gas phase occurs. The result of this is that the mean concentration of the oxygen in the gas bubbles is equal to the oxygen concentration in the outlet, i.e., $p_m = p_{out}$. Thus the rate equation becomes

$$Q' = k_L a \, (c_{Eout} - c_L) \tag{50}$$

Although equations (49) and (50) offer alternative ways of determining the absorption coefficient the most meaningful information will be obtained from equation (46) using an actual measured value for c_{Em} for the fermentation liquor. The method for determining c_{Em} for a fermenter is described below.

Methods for determining the value for the dissolved oxygen concentration in the bulk of liquid have interested workers for a very long time. The various techniques are so numerous that they have been assigned a separate treatment below. At this point it will be assumed that a value can be obtained.

2. *Steady-state measurements*

For a system at steady state, or a point reading in a batch process, the application of equation (46) will give a value of $k_L a$ corresponding to the appropriate system geometry, physical properties, gas flow rate and stirrer speed. In this case measurement of the oxygen uptake rate and dissolved oxygen value together with an estimation of the mean equilibrium concentration through equation (47) are required.

Hospodka (1966) describes a control system for the continuous culture of yeast cells. Dissolved oxygen is linked with the sugar feed so that a control of the oxygen uptake rate can be achieved due to the interaction between sugar concentration and respiration rate for this particular micro-organism. At a particular system condition and steady state it is thus possible to obtain a series of Q' values corresponding to different c_L values. From equation (46) a plot of Q' versus c_L gives a straight line of slope $(-k_L a)$. Of considerable additional importance is the facility of this method to produce a value for the most realistic mean equilibrium concentration c_{Em} from the intercept on the c_L axis where $Q' = 0$. Also, a check can be obtained from the intercept on the Q' axis of $k_L a c_{Em}$.

This method is not restricted to systems controlled only in the manner described by Hospodka (1966) but is suitable for any system in which control of the oxygen uptake rate occurs only as a result of control of the dissolved oxygen value.

3. *Unsteady state technique*

The ability to measure and record the dissolved oxygen in a fermentation process liquor forms the basis of a further method for the determination of the absorption coefficient. If the air supply to a fermenter is stopped, then the value of the dissolved oxygen concentration falls linearly with time. When the air supply is started again, the dissolved oxygen concentration returns to its original value. Fig. 6 is a representation of the record of the transient described above.

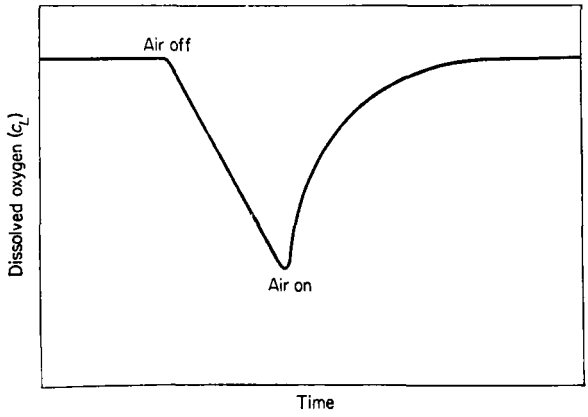

FIG. 6. Idealized transient record for unsteady-state technique.

A mass balance on the oxygen can be written—

$$\begin{bmatrix}\text{Rate of change of}\\ \text{oxygen concentration}\end{bmatrix} = \begin{bmatrix}\text{Rate of}\\ \text{oxygen in}\end{bmatrix} - \begin{bmatrix}\text{Rate of oxygen out} + \text{Rate of}\\ \text{consumption of oxygen}\end{bmatrix}$$

or

$$V dc_L = G_{in}\, p_{in} - G_{out}\, p_{out} - VxQ \tag{51}$$

and as

$$G_{in}\, p_{in} - G_{out}\, p_{out} = N_T = V(k_L a)\,(c_E - c_L)m$$

then on the basis of unit volume, and as $xQ = Q'$, equation (51) becomes—

$$\frac{dc_L}{dt} = k_L a\,(c_E - c_L)_m - Q' \tag{52}$$

When the air supply is stopped then $N_T = 0$ and $Q' = -dc_L/dt$, as in the case of a sample of culture in a respirometer.

Equation (52) can be re-written in the form—

$$c_L = (-1/k_La)\frac{dc_L}{dt} + (c_{Em} - Q'/k_La) \tag{53}$$

so that a plot of c_L versus dc_L/dt (obtained from the recorded dissolved oxygen concentration values as they change after the "air on" point) gives a straight line of slope $(-1/k_La)$. A value for the mean equilibrium concentration (c_{Em}) can be obtained from this method as Q' is available from the linear decrease portion of the dissolved oxygen concentration transient.

This technique has been investigated by Bandyopadhyay *et al.* (1967) on cultures of *Saccharomyces cerevisiae*. They found that certain precautions were necessary in the practical situation in order to obtain satisfactory results from the technique. The gas phase did not immediately leave the system when the air was shut off. Transfer of oxygen thus continued to occur for a period. The linear portion of the record did not thus start at the moment that the air supply was stopped. Further, particularly during the second part of the transient, transfer of oxygen through the surface of the liquid caused a distortion in the shape of the record. A supply of nitrogen to the space above the fermentation liquor was found to minimize this effect. Of additional concern was the possibility that the value of the specific oxygen uptake rate (Q) might be changed when the culture was exposed to low levels of oxygen concentration. By exposing cultures to low oxygen concentrations for a range of time values it was found that the effect could be handled by the replacement of Q in equation (51) by $Q(1-e^{-r't})$, where r' is an oxygen uptake rate recovery constant whose value is dependent on the length of time of oxygen starvation.

C. Sulphite oxidation

In the absence of a method for the determination of the dissolved oxygen concentration in a fermentation process liquor it is not possible to obtain a value for the absorption coefficient. The development of efficient designs of fermenter equipment for submerged culture required that some quantitative assessment of this type of gas–liquid transfer process might be available. A system for use as a simulator of a stirred fermentation process must be capable of consuming oxygen. Also, in order that the value of the oxygen concentration in the liquid might be known, it must have a fixed value for all situations and most conveniently be equal to zero. A most suitable system for this purpose was found in the catalysed reaction between oxygen and sodium sulphite in water.

It was intuitively believed that the rate of transfer of oxygen from the gas bubbles to the sodium sulphite solution is controlled by the liquid-film resistance and thus this reaction has been used by many workers (e.g.,

Cooper *et al.*, 1944; Westerterp *et al.*, 1963) for assessing the performance of gas–liquid contactors. However, much confusion still exists concerning the exact mechanism of this oxidation reaction. As Srivastava *et al.* (1968) have indicated, the reaction appears to depend on the type of equipment, the purity of the sodium sulphite solution, the catalyst, pH of the solution and the concentration of oxygen and sodium sulphite. Cupric ions were chosen by Cooper *et al.* (1944), who noted that the rate of absorption was independent of sulphite ion concentration over a wide range of values. Pirt *et al.* (1957) compared cobalt with copper ions as the catalyst and found, as did Roxburgh (1962), that much higher rates of reaction were obtained using cobalt rather than copper. Roxburgh also examined the effect of iron and nickel as catalysts and obtained rather confusing and unreproducible results. Until greater knowledge is available much care must be taken, when using this reaction to obtain absorption coefficients, to reproduce the system conditions each time, when some degree of relative assessment will be possible.

The mechanics of a typical determination of an absorption coefficient by sulphite oxidation are straightforward. A solution of approximately 0·01 M sodium sulphite and 0·001 M cupric sulphate is placed in the fermenter. The air rate, stirrer speed and temperature are adjusted to the desired levels and samples of the solution are then removed at various time intervals and stored in filled stoppered bottles. Sulphite ion concentrations are determined by reaction with a standard iodine solution followed by a titration to excess iodine with standard sodium thiosulphate solution using starch as the indicator. The analysis of this gas absorption process assuming an irreversible first order chemical reaction to be taking place (Bird *et al.*, 1960) has shown that the rate of sulphite oxidation per unit volume is given by $k_L a c_{Em}$.

The rate of sulphite oxidation expressed in mmole O_2/litre per hour has been used by many workers to describe the oxygen transfer efficiency of fermenters. Although considerable limitation exists in the extrapolation of rate coefficients, measured for a system which is essentially water, to the case of viscous non-Newtonian solid–liquid mixtures typical of many fermentation liquors, some success has been achieved in the correlation of fermentation productivity with sulphite oxidation rates (Strohm and Dale, 1961).

The use of sodium sulphite has also been implemented (Mancy and Westgarth, 1962) in the removal of oxygen from a system in a method similar to the unsteady-state technique described above. Following the removal of oxygen but with the continuation of stirring and aeration, the concentration of dissolved oxygen eventually will return from zero to the equilibrium value in a transient similar to the second phase in Fig. 6. A record of this changing

concentration can be analysed using equation (53), setting $Q' = 0$ when micro-organisms are not present.

D. Determination of dissolved oxygen concentration

The measurement of dissolved oxygen concentration (or activity) does not present a problem of great difficulty in itself. Well-developed chemical analytical techniques are available. These methods do require a degree of purity which is not typical of the liquids for which this information must be obtained. As a check for other types of measurements it will, however, remain a necessary background to the subject.

From an application of polarographic techniques of analysis has emerged a quite different electrochemical electrode system for dissolved oxygen studies. Contamination by fluids of industrial interest has required that the electrodes be contained within a protective cell and separated from the fluid by an oxygen permeable membrane. The rigorous requirements of some industrial applications has further stimulated the development of the oxygen electrode unit which is now taking its place as a standard monitor on most submerged culture processes. Some additional measurement techniques and applications are described elsewhere (Beechey and Ribbons, this Series, Vol. 6).

1. *Chemical analysis*

Methods of determining dissolved oxygen in pure water are described in most relevant texts of analytical procedures (Furman, 1962). In general, they involve the addition of excess quantities of a standard solution of a reduced ion, such as ferrous or manganous, followed by a back titration of the excess with a standard oxidizing agent. Considerable effort has been expended into the investigation of the effects of various likely contaminants on the main reaction (D.S.I.R., 1953). A microtechnique has been developed for use on samples from submerged cultures (Calam, 1963) but requires considerable dexterity and skill in its application.

2. *Dropping mercury polarographic electrode*

When a potential is applied between two electrodes submerged in a liquid, then a current will flow. The quantity of current will be determined by the resistance of the cell to the flow of electrons. At characteristic potentials the flow of current increases markedly due to reduction reactions occurring at the cathode. Reaction products can easily contaminate the electrode and this has been avoided by using mercury drops as a continuously renewed cathode. The mercury-drop cathode is formed by allowing

a slow flow of mercury through the capillary tube. The effect of oscillating values of the current as each drop breaks can be damped by adjusting the rate of drop formation with respect to the response of the recorder. A typical plot of current (usually in microamperes) versus applied potential in volts is shown in Fig. 7 for the reduction of oxygen which occurs at two levels—

$$O_2 + 2\,H_2O + 2\epsilon \rightarrow H_2O_2 + 2\,OH^-$$
$$O_2 + 2\,H_2O + 4\epsilon \rightarrow 4O\,H^-$$

The second and most clear reduction occurs at a potential of 0·75 V with respect to zero. At zero applied potential there is usually a small residual current. This is probably due to a natural potential difference arising from polarization and the presence of contamination at the anode.

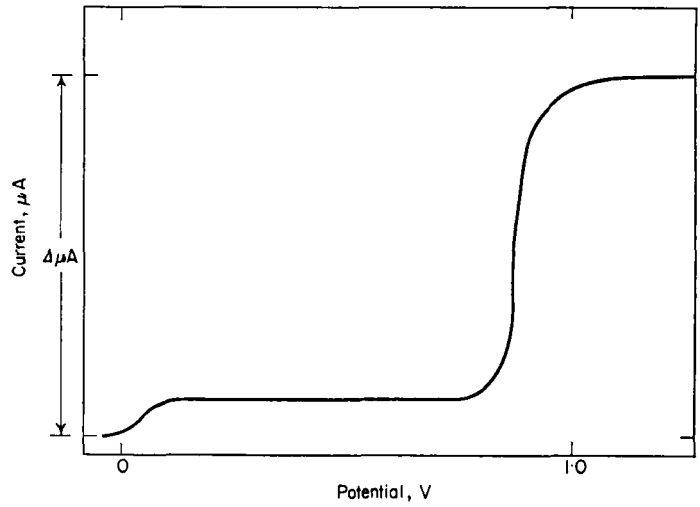

FIG. 7. Typical record of oxygen polarograph.

An interpretation of current readings from the polarograph in terms of dissolved oxygen concentration requires a consideration of the mass-transfer process involved. The quantity of current difference ($\Delta\mu A$) between zero potential and some predetermined value, such as 0·8 V, is a measure of the number of molecules of oxygen which are reduced at the cathode per unit time, i.e., the rate of oxygen transfer to the electrode. It is assumed that the drop of mercury is surrounded by a stagnant film of fluid and that oxygen

is transferred by molecular diffusion through this film. A rate equation can be written—

$$\begin{aligned}\text{Rate of flow of electrons} &= \text{Rate of transfer of oxygen by diffusion}\\ &= \text{Constant} \times \text{concentration gradient}\\ &\quad \times \text{diffusion coefficient} \times \text{area}\end{aligned}$$

or

$$\Delta\mu\text{A} = \text{constant}\left(\frac{c_L - c_{elect}}{x_L}\right) D_L A \tag{54}$$

where x_L is the distance across which the concentration difference exists, and c_{elect} is the oxygen concentration at the mercury drop interface. This concentration is in fact zero because the oxygen molecules are removed, by reduction at the interface, from the system at the drop surface. Confirmation of this is found in the fact that no change in $\Delta\mu$A occurs with increase in applied potential. For this fixed system geometry and by equation (10)—

$$\Delta\mu\text{A} = \text{constant}\, D_L\, C_L = \text{constant}\, \frac{D_L}{H}\, T \tag{55}$$

If the diffusion coefficient of oxygen can be considered constant then a plot of $\Delta\mu$A for different values of dissolved oxygen concentration is linear and the calibration constant can be obtained from the slope.

The dropping mercury electrode measures the actual dissolved oxygen present in a liquid. If solutions of different concentrations of sodium chloride in water are saturated with oxygen then a range of dissolved oxygen concentrations result, due to the change in the value of the Henry's law constant, equation (9). Strohm and Dale (1961) have investigated this situation and obtained a linear relationship between $\Delta\mu$A and salt concentration (or dissolved oxygen concentration).

Some useful data has been obtained using this technique for filtered samples removed from a fermenter (Hixson and Gaden, 1950) and a successful attempt to install a shielded dropping mercury electrode is reported by Bartholomew *et al.* (1950).

3. *Rotating brush electrode*

The difficulties associated with the use of the dropping mercury electrode in a fermenter led to the development by Gualandi *et al.* (1959) of a silver amalgam disc electrode, supplied with a slow feed of mercury and brushed continuously with a rotating velvet pad. Although its design is complex and the overall dimensions restrict its satisfactory application to vessels greater than 50 litres, it has proved to be steam sterilizable and robust. Further advantages included insensitivity to local hydrodynamic conditions in the liquid coupled with a quick response to changes in dissolved

oxygen concentration. The main problems associated with the use of this electrode arise from physical failures in the multitude of parts which are involved in its construction.

4. *Membrane electrodes*

A further step in the attempts to employ the polarograph approach for the measurement of dissolved oxygen has involved the isolation of the electrodes within a cell and protection from the fluid by an oxygen permeable membrane. This approach has allowed the use of a variety of materials for the electrodes. Silver has been in general found satisfactory for the anode but cathode materials have varied somewhat. Clark (1956), in one of the earliest published reports of electrodes of this type employed platinum for the cathode, as also did Pittman (1962) and Strohm and Dale (1961). Phillips and Johnson (1961a) constructed a cell containing a silver cathode, and a gold cathode was used by Kinsey and Bottomley (1962). The fluid contents of the cell is most commonly an aqueous solution of potassium chloride and as was shown from the dropping mercury electrode data of Fig. 7, an applied potential of 0·6–0·8 V is the usual range of operation.

An alternative form of membrane electrode has been developed which does not require the application of a potential between the electrodes. The replacement of the noble metal anode by a material of differing natural electrode potential from the cathode results in a natural potential difference between the electrodes. A microammeter in the circuit between the two electrodes is thus all that is required in order to make measurements with this type of instrument. Mancy and Westgarth (1962), Mackereth (1964), and Borowski and Johnson (1967) have reported galvanic electrode designs with silver as the cathode and lead as the anode, and a cell composed of an aluminium anode and a platinum cathode has been described by Gobe and Phillips (1964). Improved modifications to the Mackereth electrode are reported by Flynn *et al.* (1967) and Brookman and Owen (1968).

Whether the electrode is of the applied potential or the galvanic type does not influence its basic mode of operation. Differences in the geometry of the cell and electrodes do, however, result in a range of performance characteristics. Also, the presence of the membrane has resulted in considerable controversy over the interpretation of the measurements.

(a) *Principles of operation.* A generalized design for a membrane covered polarographic electrode is shown in Fig. 8. If the electrode is placed in a turbulent fluid at a dissolved oxygen bulk concentration c_L, then a boundary-layer velocity and concentration profile will form at the membrane interface. The thickness of the boundary layer will be caused by the particular hydrodynamic conditions. Within the cell, the internal fluid is completely stagnant and unaffected by the outside fluid motion.

As in the case of the dropping-mercury electrode, the instrument is essentially a device for measuring a rate of oxygen transfer. Within the cell a diffusion layer will be present at the cathode and the oxygen concentration will fall from that in the cell contents to zero at the electrode surface. This concentration of oxygen in the cell contents will depend on the drop across the membrane and the boundary layer from the bulk value in the turbulent steam. It has already been explained (Section IIC.2) that when a membrane separates two different fluid systems transfer of oxygen across the membrane is caused by a driving force of oxygen tension, rather than concentration.

In order to be able to assess the potential influence of a given electrode characteristic on its overall performance it is convenient to treat the system in a similar way to the model of oxygen transfer to a cell discussed in Section IIIA.1. The profile of oxygen concentration or tension is also indicated in Fig. 8. Several situations can be considered.

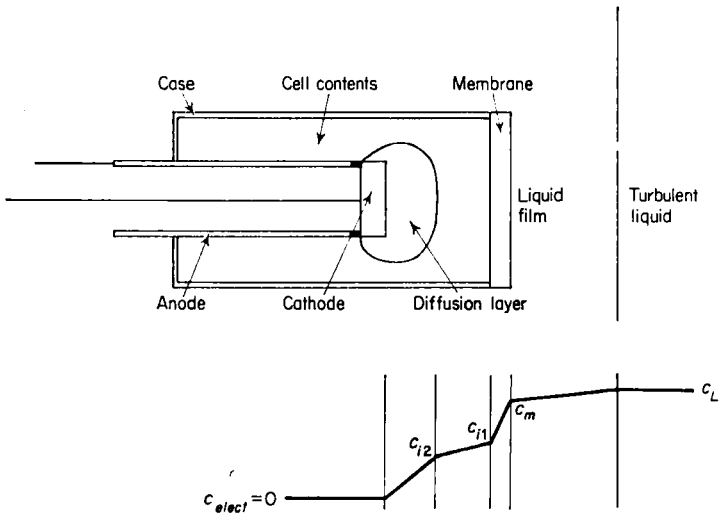

FIG. 8. Schematic diagram of membrane electrode.

(i) The first design geometry to be considered is described by the diagram. A small cathode area relative to the size of the cell will result in transfer of oxygen into the cell at a rate which cannot be handled by the electrode process. This will occur most easily if, in addition, the cell is constructed with a large thin membrane of high permeability. The overall effect is that the cell contents will equilibrate with the outside liquid oxygen composition, i.e., $T_i = T$, where T_i is the oxygen tension inside the cell and T the oxygen tension outside the cell. At the cathode the current flow will be related to

the concentration of oxygen in the cell c_i (equation 55). For a fixed cell contents, the Henry's law constant H_i will be constant, thus—

$$\Delta\mu A = \text{constant}\,\frac{T_i}{H_i} = \text{constant}\,T = \text{constant}\,HC_L \qquad (56)$$

Such an extreme design of electrode would have a very low response characteristic in that changes in oxygen tension in the outside fluid would take a long time to be noticed within the cell. However, this would render the device almost completely insensitive to hydrodynamic effects. A design of this type may be most suitable for monitoring a mean value for oxygen tension in a river where many changes in hydrodynamics may occur but where oxygen tension transients may be very slow.

(ii) If the cell space is reduced in size then $c_{i1} \rightarrow c_{i2} = c_i$ and further, an increase in cathode area together, possibly, with a reduction in membrane area will result in zero concentration of oxygen within the cell. Resistance to oxygen transfer will thus lie in the membrane and in the boundary layer.

When an electrode of this design is placed in a stagnant fluid or if the membrane is constructed of some very thin and highly permeable material then most of the resistance tends to exist in the liquid boundary layer. The transfer of oxygen is due to the concentration gradient across the liquid boundary layer and if $c_m \rightarrow 0$, then the reading of the current is proportional to the dissolved oxygen concentration. Such an oxygen electrode will be highly sensitive to changes in the hydrodynamics of the fluid system but has a very quick response to changes in the oxygen concentration.

In the presence of turbulent motion in the fluid system the resistance to oxygen transfer will lie in the membrane, especially if this is relatively thick for robust service. Transfer of oxygen is then due to the difference in oxygen tension across the membrane. If the oxygen tension on the liquid side of the membrane approaches that of the bulk $T_m \rightarrow T$ and on the inside of the membrane equals zero, then the current measurement is proportional to the oxygen tension in the bulk of the liquid. Provided that agitation is present at the electrode then this type of device is essentially independent of the degree of agitation. The response will largely depend on the thickness and permeability of the membrane material.

It has already been pointed out by Kinsey and Bottomley (1963) that when both the membrane and the boundary layer have similar effects on the overall resistance to transfer then the electrode measures something between oxygen tension and concentration. This kind of situation would be avoided in the design and application of a dissolved oxygen electrode.

(iii) Some electrodes are assembled with a tissue between the cathode and the membrane in order to contain the electrolyte for the inside of the cell. The effect of this addition will be to augment the membrane resistance,

thus reducing the response characteristic and the sensitivity of the electrode to agitation. A reading of oxygen activity will continue to be obtained.

The presence of a deposit of antifoam oil on the outside of the membrane creates a more complex situation. Even when the agitation is sufficient to reduce the boundary layer resistance to a relatively insignificant value, a composition gradient will remain in the oil layer. This will mean that the reading of current will be due to a combination of oxygen tension through the membrane and oxygen concentration across the oil layer. A reduction in sensitivity to agitation will be accompanied by a loss in response.

(b) *Additional factors affecting the performance of electrodes.* The complex requirements of industrial applications of the membrane electrodes requires the consideration of some additional features.

(i) Temperature: Most antibiotic fermentation units are sterilized using live steam at a pressure of 15 psig giving a temperature of approximately 120°C. Membrane materials which will withstand this type of treatment are restricted to Teflon (Phillips and Johnson, 1961a) and some silicone rubber materials, although the latter tend to be too permeable to substances other than oxygen. The materials chosen for the other parts of the electrode must also be able to survive repeated cycles of this kind of heat treatment without cracking. A further problem is associated with the electrolyte which may possibly boil off if the internal part of the device is not equilibrated in pressure with the outside. An alternative technique which has been used most successfully (Gobe and Phillips, 1964) is to prepare the electrolyte as a saturated solution of potassium chloride in ethylene glycol containing 5% of water. No loss of this electrolyte solution occurs during sterilization as the boiling point at atmospheric pressure is greater than 120°C.

Changes in the operating temperature will affect the rate of mass transfer. A simple solution to this problem is to calibrate the electrode at the temperature at which it is intended to operate. This approach is satisfactory for such systems as antibiotic fermentations for which careful temperature control is required. However, for the case of an electrode located in a river the temperature is quite likely to fluctuate depending on the climatic conditions and the period of the day or night.

Some indication of the extent to which temperature may affect the reading can be obtained by a consideration of the basic design of the device. The galvanic electrode of Mancy and Westgarth (1962) was shown to have essentially all the resistance to the transfer of oxygen in the membrane. A high-temperature effect was ascribed to change in the permeability of the membrane.

An automatic compensation of temperature can be achieved by incorporating the response from a thermistor temperature sensor into the

electrode circuit. A particularly simple compensation circuit of this type is described by Morisi and Gualandi (1965).

(ii) Pressure: The effect of pressure on physical aspects of the electrode will depend on the particular design of the probe. Movement of an unsupported membrane is one way in which pressure might change the calibration. Application of pressure to the electrode of Kinsey and Bottomley (1963) was examined and found to have no effect.

Some confusion may result from readings obtained in systems operating at pressures greater than one atmosphere if the electrode has been calibrated in percentage saturation at one atmosphere. A calibration in terms of mm Hg or atmospheres is a more realistic approach so that the measurement in a solution saturated by air at 2 atm should be approximately 0·4 atm or 300 mm Hg (Section IIC.1).

(c) *Practical aspects of membrane electrode measurement techniques.* In the development of the membrane oxygen electrode the search has been to obtain a low-priced, robust sterilizable electrode of small dimensions with a significant sensivity (μA/unit of dissolved oxygen) and a good response unaffected by hydrodynamic conditions. Commercial versions of original designs have been marketed and in Table IV a number of British suppliers of electrodes are listed. The price of the electrode cannot be considered in isolation from its associated instrumentation. Galvanic electrodes, requiring only a microammeter for their measurement instrument, should be available at a lower price than the applied potential versions. However, low outputs may require amplification before being suitable for recorder and/or controller systems, so that prices tend to be comparable between types.

For industrial purposes the sterilization of the electrode *in situ* with live steam is to be favoured. A completely reliable electrode in such circumstances is not available at the present time. On a small scale it is possible to sterilize the fermenter system with ethylene oxide or β-propiolactone (Holme, 1965) when the oxygen electrode can be present without its performance characteristics being affected in any way. Where aseptic transfer techniques can be used for the installation of the electrode then it can be sterilized by conventional chemical means using ethanol, formaldehyde, ethylene oxide, or β-propiolactone solutions. Care must be taken to wash the electrode thoroughly with sterilized distilled water to prevent any of the agent from being transferred to the fermenter.

It is advisable when making or purchasing an electrode to obtain an understanding of its mechanism by carrying out controlled tests on known systems. The effect of hydrodynamic conditions on the electrode could be considerable and it is thus important to determine a reproducible environment for all subsequent measurements. A check on the linearity of the output

TABLE IV

Oxygen electrode manufacturers

Type	Maker	Address	Cathode	Anode	Basis of Design
Galvanic	Electronic Instruments Ltd.	Lower Mortlake Road, Richmond, Surrey.	Silver	Lead	Mackereth (1964)
	Scientific Techniques Ltd.	10 Dover Street, London W.1.	Silver	Lead	Mancy & Westgarth (1962)
Applied potential	D. A. Pitman Ltd.	91 Heath Road, Weybridge, Surrey.	Gold or Carbon	Silver	
	Shandon Scientific Company Ltd.	65, Pound Lane, Willesden, London N.W.10	Gold	Silver	Clark (1956)
	Beckmann Instruments Ltd.	Glenrothes, Fife, Scotland	Gold	Silver	
Redox	Union Carbide U.K. Limited	P.O. Box 2LR, 8 Grafton Street, London W.1.	Gold	Thallium	Capuano (1965)

with changes in the oxygen tension can be carried out by measuring the reading in a stirred beaker of water sparged with a series of oxygen and nitrogen mixtures.

Absolute output current values of different designs of electrodes will differ considerably. However, ancillary instrumentation can usually be obtained which will enable any magnitude of signal to be utilized. A choice of electrode should not thus be made only on this aspect of design.

The membrane oxygen electrode has proved to be a considerable advance in the facilities available to fermentation experimenters and present developments in its design will ultimately establish its all-purpose reliability.

5. *Tubing method*

A quite different technique from the membrane electrode but involving the use of an oxygen permeable membrane has been developed by Phillips and Johnson (1961a). Several feet of Teflon tubing are installed in the fermenter and through this tube is passed a stream of nitrogen. Gas analysis of the outlet stream coupled with an accurate control of the nitrogen flow rate allows the determination of the oxygen tension in the fermenter liquor. The amount of oxygen present in the nitrogen effluent will depend on the length, diameter and wall thickness of the tubing as well as the nitrogen flow rate. Reports of satisfactory measurements using this method are made by Steel and Maxon (1966) and Roberts and Shepherd (1968).

6. *Redox potential measurements*

A further technique for measuring the quantity of dissolved oxygen in a culture medium is that derived from redox potential values. Squires and Hosler (1958) reported such measurements made in a streptomycin fermentation. The use of a platinum electrode together with a saturated calomel electrode, both submerged in the liquid, was reported by Tengerdy (1961a). Two values were determined, one of the electrode potential in the presence of oxygen, E_{O_2}, and one when the system was flushed with nitrogen E_{N_2}. This latter value represented the redox potential for the nutrient liquor. It was found that a linear relationship existed between $(E_{O_2} - E_{N_2})$ and the $\log_e c_L$. Some limitation exists at low values of c_L, particularly as E_{N_2} is likely to change due to alterations in the basic oxidation–reduction characteristics of the liquid.

Tengerdy (1961b) employed this device in the study of the formation of 2-keto-L-gulonic acid by *Pseudomonas*. Also a successful control system is described by Lengyel and Nyiri (1965) in which the calomel electrode is located outside the vessel and linked to the vessel contents through a saturated KCl–3% agar–agar bridge and sintered-glass plug.

IV. CONTROL OF AERATION

In the consideration of the control of aeration it will be assumed that a submerged culture fermenter is supplied with air and the gas–liquid mixture is stirred with an impeller. The mechanics of operation of the instrumentation of control systems is discussed elsewhere by MacLennan (this Vol., p. 1). The general design and analysis of control systems is beyond the scope of this chapter. Reference is made to more relevant works on this subject (Buckley, 1964). This section will therefore be limited to an account of the several techniques which have been successfully employed for aeration control.

A. The monitoring system

In all control systems the value of the parameter is measured by a sensor and then compared with a set position; any deviation is used through amplifiers and converters to drive some unit which will maintain the desired value. Hospodka (1966) has pointed out that the control of aeration is essentially concerned with the control of the rate of oxygen uptake. This requires that the rate of oxygen uptake be monitored. Many practical difficulties are involved in the instantaneous determination of this parameter. Gas outlet oxygen concentrations (see Elsworth, this Volume, p. 213) have been monitored as an approximation to the more complex situation and used successfully in a control problem (Jensen *et al.*, 1966).

It can be shown that in most circumstances the aeration rate is a simple function of the dissolved oxygen concentration or tension. Thus many control systems for aeration are basically control systems for dissolved oxygen. The requirements for such a dissolved oxygen sensor are that it has a good sensitivity (change of output signal with change in the measured parameter) and good response characteristics. In addition, it must have some compensation of temperature effects and be insensitive to changes in the hydrodynamics of the system. For continuous, long-term service the instrument must have a robust construction. Reference to Section IIID.2 on membrane-type oxygen electrodes will indicate that the design which will fit these requirements must employ a galvanic cell principle, have a small cell capacity, relatively large cathode and membrane area but relatively thick and robust membrane (e.g., E.I.L. Model).

B. Types of fermenter aeration control

Several aspects of fermenter design and operation have been shown to affect the rate of aeration. Because of these various parameters which are associated with oxygen transfer, it is possible to design control systems based on a selection of different manipulated variables. A selection of laboratory

installations are listed in Table V. Extension of most of these systems to large-scale installations might not be practically feasible.

Application of control by changing the impeller speed will require a continuously variable speed drive unit. On a small scale this does not impose too difficult a problem but on a large scale it might cause considerable expense. Most industrial stirrer units in existence at present operate at a fixed speed. The inclusion into the drive of either a fluid or magnetic coupling could however solve the problem of continuous variability. Hydraulic drive motors operated from some remote prime mover would facilitate the application of variable stirrer speed aeration control.

Any control system which alters either the gas flow rate or the position of the gas inlet pipe will involve not only a change in gas hold-up and therefore aeration rate, but will also thus cause changes in the mixing characteristics. Such changes could cause the poor supply of other important nutrients to the cells. However, these techniques do offer the most promising simple form of aeration control suitable for the application to large-scale fermenters. In particular, a modification of the variable geometry arrangement could be applied in which the controller caused air to be supplied alternatively to two different air inlets of widely differing aeration efficiency.

Although the use of pure nitrogen and oxygen gases allows very convenient techniques for aeration control on a laboratory scale, it is not a feasible approach for industrial processes. The economics of obtaining or manufacturing large quantities of the separate gases would be prohibitive.

Of particular importance is the type of aeration control system described by Hospodka (1966) involving the manipulation of a nutrient feed. A special optimization problem arises in the continuous manufacture of food yeast. Sugar feed is converted directly by aerobic metabolism into cell mass provided that the supply of air is not limited. It is desirable therefore to operate such a fermenter at the maximum capability of the machine to transfer oxygen or at its highest k_La value. With this as a fixed constraint it is then necessary to maintain an economic balance between rate of formation of cells and the cost of unconsumed sugar which passes from the outlet.

The steady-state situation can be described by equation (46)—

$$k_La\,(c_E - c_L)_m = xQ \tag{46}$$

and yeast cells possess the particular facility to be able to momentarily change their specific oxygen uptake rate in the presence of changed quantities of sugar (Pasteur effect) without a measurable change in the cell concentration. Thus it can be seen from equation (46) that if Q changes then c_L will also change.

Hospodka (1966) represents this system as a plot of specific oxygen uptake as a function of both sugar and dissolved oxygen concentration on the same

TABLE V

Aeration control systems

Manipulated variable	Monitor	Scale of operation (litres)	System	Reference
Stirrer speed	Gas outlet oxygen concentration	13	Positioning motor on variac controlled DC motor	Shu (1956)
	Dissolved oxygen	8	Electropneumatic transducer to piston actuator to Zero-max continuously variable gear	Moss & Bush (1967)
Air flow rate	Redox electrode	8	Proportional valve on air inlet	Lengyel & Nyiri (1965)
Gas composition	Dissolved oxygen	7	Mixing valve supplied with oxygen and nitrogen	Siegell & Gaden (1962)
		2	Nitrogen steady; electropneumatic converter to proportional valve on air	MacLennan & Pirt (1966)
Geometry	Dissolved oxygen	5	Nitrogen steady; air steady through inlet moved with respect to impeller by rack and pinion	Flynn & Lilly (1967)
Nutrient feed	Dissolved oxygen	3·5	On–off valve on nutrient feed line	Hospodka (1966)

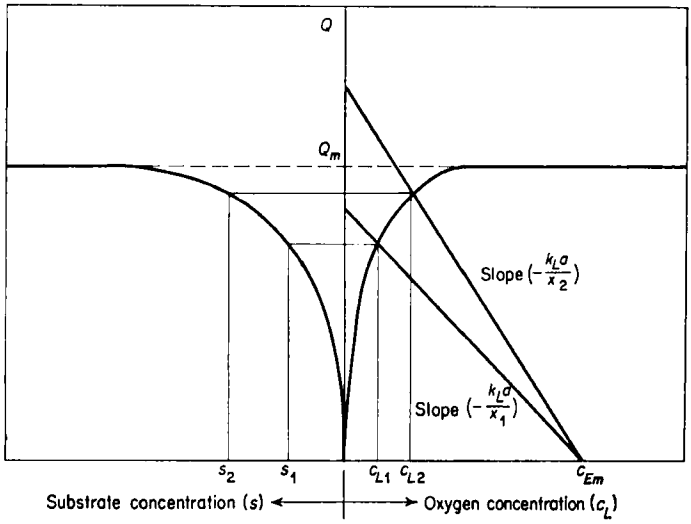

FIG. 9. Nutrient feed control system.

graph as shown in Fig. 9. The line of equation (46) can also be included on this figure for a given k_La and x_1. The control system is arranged so that as the oxygen concentration rises from a set value of c_{L1} then the nutrient feed valve is opened, Q increases and the dissolved oxygen concentration returns to the set value. The selection of a new value of set position c_{L2} results in a new steady-state sugar concentration s_2 and eventually cell concentration x_2, when the line of equation (46) will have a new position. The economic optimum productivity can thus be established at a given setting of dissolved oxygen. It is quite feasible that as the application of aeration theory is increased, particularly on large-scale installations, this type of optimization of productivity by aeration control could well become a commonplace activity.

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CHAPTER VI

The Shaker in Bioengineering

D. FREEDMAN

New Brunswick Scientific Co., Inc., New Brunswick, N.J., U.S.A.

I. INTRODUCTION

The shaking machine is one of the most common pieces of hardware in biological research and industrial laboratories. In microbiological research shakers have been used for washing organisms, to increase rates of gas exchange, to increase gas solubility rates, to maximize cell contact with substrates and to prevent cell clumping. Although its applications are extensive and the demands on its performance are great, relatively little attention has been paid to this important tool by scientists. Many who purchase shakers are unaware of the sophistication and precision required in their design and manufacture. Equally they are often unaware of the performances obtainable from them. It is with this latter topic that this Chapter is largely concerned.

Since oxygen is a rather insoluble gas, many aerobic organisms have a need for oxygen or air to be supplied during growth. There have been many published reports on oxygen absorption rates as determined both by polarographic methods and by measurement of sulphite oxidation rates. Chain and Gualandi (1954) have probably provided the most comprehensive study of this area. Finn (1954) has tabulated oxygen transfer rates in shakers as well as other types of equipment used for culturing micro-organisms. Smith and Johnson (1954) Gaden (1962) and McDaniel (1965) have provided worthwhile information showing a comparison of baffled and unbaffled flasks of different types.

A. Reciprocating shaking

Because of the many variables that affect the shaking of a flask, it is difficult to present a concise picture of reciprocating shakers. However, some fairly good oxygen absorption rates obtained in reciprocating shakers are shown in Table I. The type of reciprocating motion achieved in machines manufactured by various companies is frequently different and results in further variation. In some, the mounting platform or structure describes an arc as a result of being suspended from four points. Others describe an opposite arc as a result of being supported on four vertical arms. In these machines, the deviation from a pure planar motion of the shaken platform has a strong influence on the fluid patterns within the flasks, as does the reversing backlash that is always present to some degree. During reciprocating shaking, fluid surges are inconsistent, the effects of viscosity are substantial, and evaporation of the media will frequently affect the motion of the fluid being shaken. As well as this, the fluid activity that starts with a surge will usually develop into a return curl effect, then to a swirl and, under various conditions, will produce geyser eruptions that are unpredictable and difficult to control. During start-up the motion of the liquid always differs from that which is experienced while normal shaking is taking place. Depending on the start-up time of the machine, this motion will frequently contribute to a geyser effect which may wet the flask's stopper and contaminate the culture being grown. The changes that take place within the flask from the return curl effect to the swirl are also unpredictable. Where reciprocating shakers are used, the operators will frequently initiate a swirling motion in the liquid in larger flasks to aid in establishing a more consistent pattern of fluid activity.

B. Rotary shaking

A basic difference between reciprocating and rotary shaking appears in the consistent swirl pattern set up in rotary shaking. Compared with the reciprocating shaker, the swirl pattern set up in the rotary shaker is not nearly as affected by media, viscosity, machine start-up or flask volume. This is undoubtedly the reason that the rotary shaker is in such common use in the culture of micro-organisms.

Probably the most widely used current design for rotary shakers is the vertical, multi-shaft system. This system incorporates three or more eccentric shafts, all independent of one another. This mechanism transmits a positive, uniform motion to all flasks, with either three, four or five eccentric shafts. Successful implementation of this design depends on highly accurate location of the various eccentric shafts in relation to each other, as well as extreme precision and uniformity in the eccentricity of the individual shafts. Apart from the technical advantage of producing exactly uniform

TABLE I

Oxygen absorption rates on a reciprocal shaker as a function of stroke and speed (Cooper *et al.*, 1944)†

Agitation speed (rpm) and stroke (in.)		Variation in maximum fluid height from 0 to 0·5 min time		Sol. $Na_2S_2O_3$ 0·1 N used (ml)	Oxygen absorption rate		% of best performance value (9·6 ml = 100%)
					mmole O_2/litre per hr	mmole O_2/litre per min	
A (stroke)	¼	60	60	1·0	15	0·25	10·3
	½	200	80	1·2	18	0·30	12·4
	¾	320	100	2·4	36	0·59	24·8
	1	300	150	2·6	39	0·65	26·8
	1¼	375	220	3·0	45	0·75	31·0
	1½	500	420	3·4	51	0·83	35·0
	1¾	520	370	3·8	57	0·94	39·0
	2	Max	400	4·6	69	1·15	47·8
	2¼	500	425	5·0	75	1·25	52·0
	2½	Max	450	5·4	81·5	1·35	56·4
	2¾	520	460	5·8	87·5	1·45	60·0
	3	510	420	6·4	96	1·58	66·2
	3¼	520	460	7·2	108	1·80	75·0
	3½	510	480	8·2	123	2·05	85·0
	3¾	Top	480	9·0	135	2·24	93·0
	4	Top	500	9·6	145	2·41	100·0
B (speed)	60	150	110	0·8	12	0·20	16 (5·0 ml
	100	300	300	2·8	42	0·68	56 =100%)
	160	420	380	4·0	60	1·00	80
	200	500	380	4·6	69	1·15	91
(max)	240	520	420	5·0	75	1·25	100

† Experimental conditions: 500 ml Erlenmeyer flask used at 25°C with a liquid volume of 50 ml. The flask was vertical and time of test condition was 20 min. (*A*) Speed = 200 rpm; (*B*) stroke = 2 in.

agitation in every culture flask, the major mechanical advantage of this system is that it permits excellent application of ball bearings for handling the thrust and radial loads imparted during shaking. The disadvantage is that the bearing locations must be maintained within tolerances of 0·0005 in., in order to achieve rotation within a perfect orbit. Similarly, the eccentric shafts also require the same degree of precision for parallelism, as well as eccentricity. The materials require heat treatment and stress relieving of the steel and castings to prevent grain growth and distortion.

An extremely important consideration in the design and application of shaking machines in the fermentation field is the very intensive and rigorous use to which this type of machine is subjected. A shaker frequently carries a heavy, non-uniform load which usually produces considerable vibration and uneven bearing loading. The machine is normally in operation 24 h a day, and continues in use for long periods without interruption, therefore a good lubrication system for the shaker bearings is essential.

It is evident that balancing and balancing concepts are of critical importance in a shaking machine. An attempt has been made in almost all of the shakers in use today to provide some means of balancing to reduce the wear and vibration problems. What has been most commonly attempted was a compromise of a static balance of the load, but in static balancing at the speeds and strokes required, a variety of undesirable conditions have resulted. Relatively high maintenance costs have also been a major concern with this equipment. Of primary concern, however, has been the limitation of oxygen absorption rates within culture flasks due to the difficulties in achieving the speed of rotation necessary to increase these rates. This factor has been imposed by mechanical balance limitations. It has been evident for some time that there is a need for dynamic balancing, rather than static balancing for flask shaking machines. The oxygen absorption rate determinations which follow were achieved on a dynamically balanced rotary shaker permitting speeds up to 800 rpm.

II. OXYGEN ABSORPTION RATES IN SHAKEN FLASKS

A. Sulphite values

A total of 148 experiments were conducted in triplicate. The reported values are an average of these. Oxygen absorption rates were determined by the method of Cooper *et al* (1944) with 0·5 N sodium sulphite and 0·001 M cupric sulphate solution as a catalyst. After 20 min of agitation under the selected test conditions, 5 ml of solution were withdrawn to determine the extent of oxidation. This sample was transferred into 50 ml of 0·1 N iodine reagent and back-titrated with standard 0·1 N sodium thiosulphate.

B. Apparatus

A new experimental dynamically balanced gyrotory shaker (GS), New Brunswick Scientific Co. (NBS) Model G-10 (modified) was used. The machine has a speed range of 0–800 rpm, producing a uniform, rotary motion describing a 1 in. (25·4 mm) dia. circle. Other apparatus used were: a standard NBS G-77 GS with 0·5 in. (12·7 mm) total stroke; NBS G-33 GS with 0·75 in. (19 mm) total stroke; NBS G-52 GS with 2 in. (50·8 mm) total stroke; NBS R-8 reciprocating shaker with 0–4 in. total stroke and 50–240 reciprocal cycles/min (cycle = one complete back and forth motion); NBS three-finger vertical flask clamps; NBS 15, 30 and 45 deg ErlAngle flask clamps; Pyrex Erlenmeyer flasks; and baffled flasks; a Stroboscope to assist viewing liquid heights on various shakers; and 14 litre fermenter for correlation of temperature relationship to oxygen absorption rates.

C. Results

In the primary oxygen absorption rate (OAR) studies carried out at NBS tests were performed at 25°C at speeds ranging from 100–800 rpm, at 1 in. rotary stroke, on an experimental, dynamically balanced gyrotory shaker. The tests were each of 20 min duration.

Fig. 1 shows the OAR at speeds from 100–800 rpm, under what would normally be sterile conditions. Table II shows the relationship of OAR to temperature. Fig. 1 indicates that the higher the speed of rotary shaking, the higher is the OAR. Furthermore, there is no indication that the limit

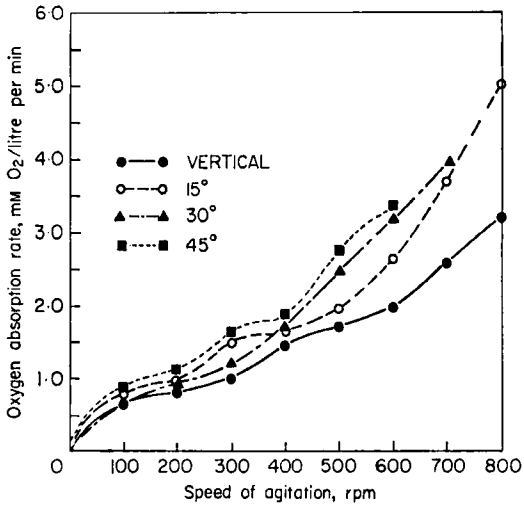

FIG. 1. Oxygen absorption rates related to agitational speed and flask angles.

or OAR has been reached at the speeds attainable in the experiments. Generally, the best performances were obtained at speeds above 300 rpm.

TABLE II

Oxygen absorption rates as a function of temperature. Sulphite oxidation procedure in a 14 litre fermenter (Cooper *et al.*, 1944)†

Temperature (°C)	Sol. $Na_2S_2O_3$ 0·1 N used (ml)	Oxygen absorption rate (mmole O_2/ litre per min)	% difference of temperature increase 19·6 = 100%
25	19·6	4·9	100
30	20·8	5·2	106
37	22·0	5·5	112

† Experimental conditions: Volume = 10 litres; time = 20 min; air pressure = 10 psig; air volume 16 litre/min; sample volume = 5 ml; agitation speed = 1000 rpm; impellers, two standard 6-blade turbines without discs; and partial pressure O_2 = 0·3 atm.

In comparing flasks held vertically, at 15 deg from the vertical axis, at 30 and at 45 deg, the upper speed limit at which all flasks had dry plugs was found to be 600 rpm. (At speeds in excess of 600 rpm, the 45 deg flasks had wet plugs in every instance.) The OAR level at 600 rpm and at every lower speed level was found to be higher in flasks held at 45 deg angle while shaking. Under these conditions, the uniformity appears to be substantially better than that obtained with baffled flasks.

Tests were run at 400 rpm in 500 ml Erlenmeyer flasks containing varying volumes of liquid from 25–300 ml. The relationship of OAR to these working volumes and the effect of varying the inclination of the flask is shown in Fig. 2. The best sulphite value is achieved at 25 ml in a 45 deg angled flask. Limitations of working volume in this position are obvious.

Further studies were then carried out in various size flasks from 10 ml to 6 litres, including 2800 ml Fernbach flasks. These tests were performed at a shaker speed of 400 rpm with 10% of the total flask volume in vessels held in a vertical position. It was surprising to find that the smaller flasks provided the highest efficiency, and that the most commonly used flask today provides the poorest OAR (Table III). It has been a common misconception that the smaller the flask, the poorer the oxygen transfer rate. However, these studies indicate that the reverse is true. Fig. 3 shows the higher OAR values in small flasks with the lowest value being obtained in 250 ml flasks. It is interesting and important to note that the 250 ml flask, which is the most

TABLE III

Oxygen absorption rates as a function of flask size. Sulphite oxidation procedure, experimental gyrotory shaker†

Flask size (ml)	Solution volume (ml)	Base of flask		Sol. $Na_2S_2O_3$ 0·1 N used (ml)	Oxygen absorption rate		% of best performance value (9 ml = 100%)
		Diameter (cm)	Surface (cm^2)		mmole O_2/ litre per h	mmole O_2/ litre per min	
10	1·0	3·0	7·1	9·0	135	2·25	100·0
25	2·5	4·0	12·6	7·6	114	1·90	84·0
50	5·0	4·8	18·1	6·2	94	1·56	68·5
125	12·5	6·4	32·0	4·0	60	1·00	44·0
250	25·0	8·0	50·5	2·8	42	0·70	30·8
500	50·0	10·0	78·5	3·4	51	0·85	37·5
1000	100·0	13·0	134·0	3·2	48	0·80	35·5
2000	200·0	16·0	203·0	3·0	45	0·74	33·3
2800	280·0	20·4	330·0	5·6	84	1·38	62·0
3000	300·0	18·0	255·0	2·8	42	0·70	30·8
6000	600·0	22·5	400·0	2·8	42	0·70	30·8

† Experimental conditions: Speed agitation = 400 rpm; cotton plug was dry; temperature = 25°C; agitation total stroke, 1 in or 25·4 mm; vertical flask; liquid volume = 10% of total flask volume; and time of test condition = 20 min.

commonly used flask today throughout the world, gives the poorest results. Four rotary shakers were used to establish the OAR as a function of stroke. This test was run at 300 rpm, with 50 ml liquid in a 500 ml vertical Erlenmeyer flask. Table IV demonstrates the differences in values reached by this stroke variable. OAR measurements were then made using three different types of baffled flasks at speeds between 100 and 500 rpm. Fig. 4 indicates the different values reached with baffled flasks. The OAR values reached were as high with the side-baffled flask as the MF-114 fermenter running at 1000 rpm and 1·6 volume of air per volume of medium/min using two six-blade impellers (Table II).

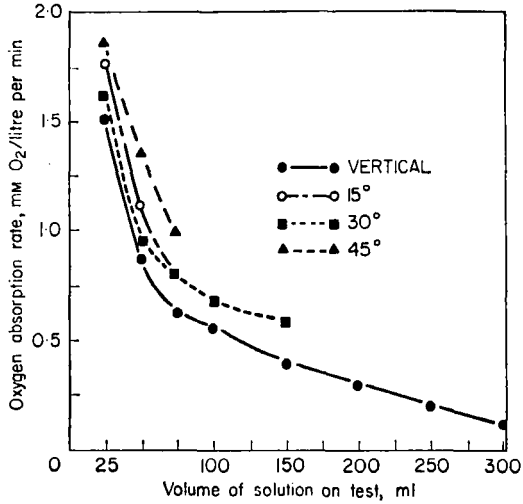

FIG. 2. Oxygen absorption rates and inclination of flask related to test solution volume (in 500 ml Erlenmeyer flasks).

Experiments with bottom corner- and side-baffled flasks at 400 rpm using different volumes, showed that the best values were obtained with 50 ml fluid volume. It is believed that the use of the 300 ml flask will affect the results by no more than 10%.

Table I provides OAR's on reciprocating shakers with varying strokes from 0·25–4 in. in 0·25 in. increments. In this series of tests, the agitation speed was 200 rpm; the Erlenmeyer flask used was 500 ml; and volume was 50 ml. Note the difference in fluid height at the start of agitation as compared with the height during operation. Fluid movements make the difference between a contaminated culture and a sterile culture. Table I also indicates the different OAR's at a constant stroke and varying speeds.

The previous OAR's reported on rotary shakers were accomplished

with cotton plugs and under open conditions, Fig. 5 demonstrates the values reached at speeds between 100 and 600 rpm with open and plugged flasks.

III. CONCLUSIONS

It is now possible to achieve oxygen absorption rates comparable to those achieved in conventional stirred culture vessels. This, of course, makes a shaker an even more valuable tool in scale-up procedures. From a practical point of view, these experiments clearly demonstrate the significant value

TABLE IV

Oxygen absorption rates on rotary shakers as a function of total stroke†

Total stroke (in)	Sol. $Na_2S_2O_3$ 0·1 N used (ml)	Oxygen absorption rate		% of best performance value (3·8 ml 100%)
		mmole O_2/ litre per h	mmole O_2/ litre per min	
½	1·4	21	0·35	37
¾	2·6	39	0·65	68
1	3·2	48	0·80	84
2	3·8	57	0·95	100

† Experimental conditions: Dry cotton plug; temperature = 25°C; 500 ml Erlenmeyer flask; liquid volume of test = 50 ml; speed agitation = 300 rpm; vertical flask; and 20 min test time.

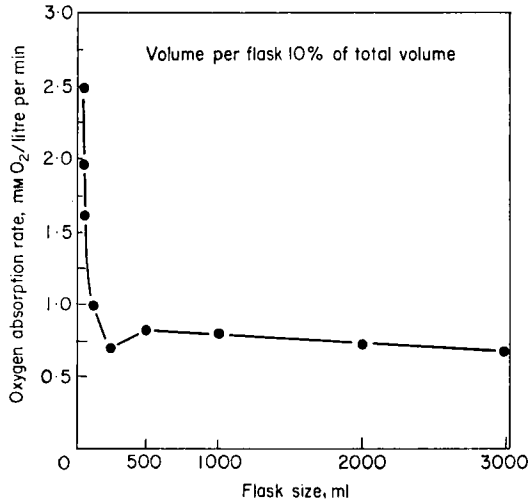

FIG. 3. Effect of flask size on oxygen absorption rates.

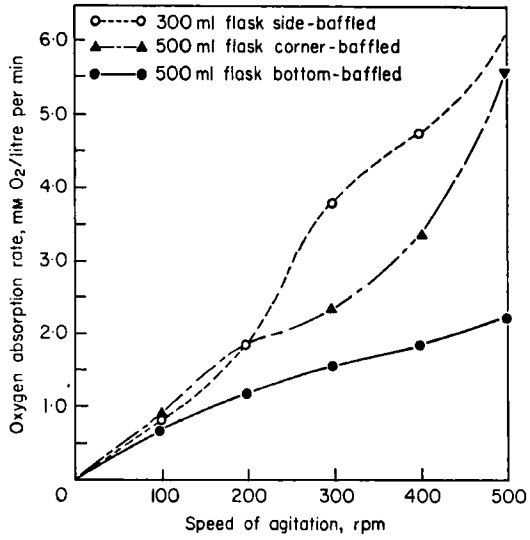

FIG. 4. Relationship between agitation speed and baffles on oxygen absorption rate.

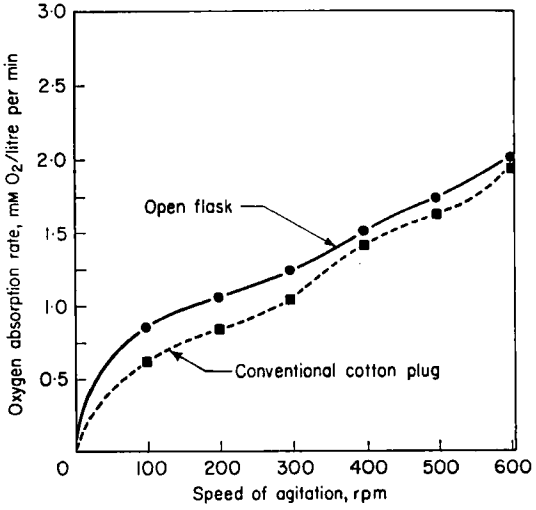

FIG. 5. Comparison of open and plugged flasks using 500 ml vertical Erlenmeyer flasks at a stroke of 1 in. and containing 25 ml of fluid.

of rotary shaking in comparison to reciprocating shaking, especially in angled flasks.

Speeds above 300 rpm are found to be more effective than the lower speed ranges, and OAR values increase in almost direct relation to the increase in shaking speed. Observations indicate that the use of baffled flasks, although providing higher OAR values in most instances, mitigate against uniformity among culture flasks. It can be concluded, therefore, that for scale-up purposes, where uniform results among large numbers of flasks are necessary, standard (i.e. unbaffled) flasks should be employed. In situations where higher culture population is the primary object, regardless of uniformity among flasks, the side-baffled flasks seem to produce the best results.

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CHAPTER VII

Anti-Foam Agents

J. BRYANT

Glaxo Laboratories, Ltd, Ulverston, England

I. INTRODUCTION

The object of this Chapter is to describe methods of foam prevention and destruction in fermenters, with particular reference to the use of chemical anti-foam agents. An attempt will be made to answer four questions—

(i) What is foam?
(ii) How is foam formed?
(iii) How may foam formation be prevented?
(iv) How may foam be destroyed?

For practical purposes, of course, the answers to the third and fourth questions are the most important, and form the bulk of this Chapter. However, a review of the mechanism of foam formation is a necessary introduction. It serves to indicate the different types of action that may be taken to prevent foaming or to destroy foam once it has been formed, and so provides a rationale for answers to the last two questions.

Many fermentations require high rates of aeration and vigorous stirring if they are to yield their best. These conditions, imposed on the types of nutrient solutions commonly employed, provide an ideal situation for foam formation. The most obvious problem created by foaming is that the effective volume of the batch in the fermenter increases. This increase can be such that unless some control is exercised there will be a loss of culture

liquid and micro-organism from the fermenter through the air exhaust line. This loss is intolerable commercially, as well as in the laboratory or pilot plant. When the contents of a fermenter foam to this extent, there can be seepage into stirrer bearings and other attachments. Also the micro-organisms may be deposited on the lid or walls where they are no longer useful. In continuous culture, foam may cause another kind of problem owing to its high surface area. If the overflow, or off-take line, is situated in a layer of foam, the liquid so removed may have an enhanced concentration of micro-organisms, and it may not therefore be representative of the bulk of the culture liquid. Excessive foaming of some cultures may even result in the loss of more than half the population by flotation, and its discharge outside the fermenter. The same remarks apply to any surface active agents, as these will be preferentially removed in such a system.

Even if the foam does not expand to such an extent as to lead to loss from the fermenter, its presence is still undesirable. The reasons for this are that the conditions of aeration and agitation will in general then be different from those in a non-foaming system. This aspect of the problem will be considered in more detail in Section VII. Foam production is sometimes almost auto-catalytic in its generation. Small amounts of foam can create conditions in cultures that promote lysis of some of the cells and this leads in turn to heavier foam production.

The classical definition of a foam is that it is a dispersion of gas in liquid, with the liquid in the form of thin films separating gas bubbles. In some bacterial fermentations and tissue cultures foam of this nature can be seen on the surface of the liquid. There is a clear division between the foam and the aerated liquid underneath it. However, in some thick mycelial fermentations no such clear distinction exists: there is rather a gradual increase in the proportion of gas dispersed in the liquid, increasing from the bottom of the fermenter upwards. It is only near the top that the proportion of gas to liquid is such that the dispersion is recognized as foam.

Although true foam, i.e., a predominantly gaseous dispersion, is an obvious nuisance, the enhanced hold-up of gas in some fermentations is also undesirable. Like true foam, this mainly liquid dispersion affects the conditions of aeration and agitation, and of course reduces the effective charge that may be used in a fermenter by increasing the apparent volume. Dawson (1961) calls this dispersion with a high proportion of liquid a fluid foam. Fluid foam may also constitute a problem by raising the level of the foam on the surface of the batch so that it becomes a nuisance.

So far, foam has been considered as a product of dispersion. It can also be formed by a process of agglomeration into bubbles of gases formed by reaction in the liquid.

Although these two ways of generating foam are quite different, the

problems created are similar, and similar methods of destruction may be employed.

The emphasis of this Chapter is on the general principles of foam destruction, and the general properties of anti-foam agents. It is hoped that this generality may be more useful than cataloguing the different anti-foams that have been used in specific fermentations. In a way the latter is symptomatic of the largely empirical approach to foam destruction during fermentation which is necessary because of the complicated nature of the problem.

II. MECHANISM OF FOAM FORMATION

The interest in foam formed during a fermentation is purely negative: the main concern is to discover how to prevent its appearance, or, if this is impossible or impracticable, how to destroy it. In this context, where foam is a nuisance to be avoided, there is little stimulus to study the mechanism of its formation.

The presence of foam, and more particularly its extent, represents a balance between the processes creating it and the forces causing its destruction. The high specific surface areas of foams make them thermodynamically unstable, as in the separated state the gas and liquid have a lower surface energy. Two kinds of foam exist: there are unstable foams that continuously approach the equilibrium state, and metastable foams in which the progress to equilibrium is arrested.

It is generally accepted that pure liquids do not foam. The property which distinguishes a foaming solution from a non-foaming solution is its ability to resist local thinning of a liquid film whilst allowing general thinning to proceed. In a foaming solution the liquid films must possess elasticity. Several theories have been proposed to account for this property; they are not mutually exclusive and several of the different mechanisms may act simultaneously (Kitchener and Cooper, 1959).

If a film of liquid containing a surface-active agent is locally stretched the surface tension of that part increases because the solute is positively absorbed in the surface. The stretching leads to a decrease of solute concentration within the film, and therefore, a rise in equilibrium surface tension. This force tends to resist local thinning and to restore the thin area. This is the basis of Gibb's theory of surface elasticity. It is mainly significant with thin films where the amount of solute absorbed on the surface is comparable with that contained within the film.

Marangoni's theory differs from Gibb's only in that it concerns dynamic rather than static systems. According to Marangoni the difference in surface tension caused by thinning is greater in a dynamic system, where equilibrium values are not reached. The effect can operate in thin films, and is therefore a direct aid to foaming. It acts too on any expanding surface with

an adsorbed layer, whatever the depth of liquid, and is therefore an indirect aid to foam formation in that it tends to reduce surface ripples and to smooth the process of drainage.

The mechanism of a surface tension difference in the surface layers as a cause of film stability has been extended in the surface-transport theory. In addition to the surface layer moving to restore local thinning, it is suggested that this movement drags the bulk liquid underneath it, and so enhances the purely surface effect.

Plateau's theory of foaming involves surface viscosity; this viscosity is similar to conventional viscosity except that it is in two dimensions only. The main function of surface viscosity is to reduce the rate of loss of liquid from films as they thin due to drainage. This effect is most strong in thin films, but rapid drainage is possible in relatively thick films where surface viscosity has less effect. Some protein solutions have high surface viscosity which can almost amount to surface rigidity. Rigidity itself does not necessarily favour foaming, because a rigid structure may be brittle and lack elasticity.

The theories so far mentioned can account for the formation of unstable foams. The occurrence of metastable structures is explained by electrical double-layer repulsion. Thin films derive their stability from the repulsion between ionic double layers formed by adsorption on the two sides of the film. This effect is particularly important in black films, that is, the thinnest possible films obtainable with a given solution.

The mechanisms by which liquid films may possess elasticity have been discussed, and they represent those forces tending to increase the life of films, and hence foam. Breakdown of foam may be caused by drainage of liquid from the film, by gravity, and also by suction exerted by the plateau borders. This drainage reduces the volume of underlying solution available to provide elasticity, and so reduces the magnitude of the Marangoni effect.

It has been shown that well-drained films are less able to withstand mechanical shock (Bikerman, 1952). Mechanical shocks, or vibrations, can come from the fermenter itself, and also from the foam; this latter source is caused by diffusion between bubbles, which changes the size distribution and causes mechanical rearrangement to take place. The pressure inside a bubble is inversely proportional to its radius, and so in a dispersion of bubbles of different sizes there is diffusion from the smaller bubbles to the larger, and consequent shock upon rearrangement.

Although it is probably not a significant factor in a fermenter, increased temperature shortens the life of a foam by reducing the viscosity. Similarly evaporation losses increase the rate of thinning. The presence of solids tends to stabilize liquid films if the solids are wetted, as is the case with microorganisms.

III. CRITERIA OF FOAMING CAPACITY

The measure most frequently required is an indication of how much a particular broth will foam, or expand, under the conditions of aeration and agitation within an actual fermenter. In addition to the magnitude of the expansion, other measures often needed are those defining the effectiveness of chemical anti-foam agents. For this purpose, it is necessary to know how fast the agent acts in breaking foam, for how long it will prevent foaming and the concentration required.

Although all these properties depend on the physical and chemical conditions of the whole system it is not possible to describe foaming capacity and anti-foam action entirely in these terms. In general the properties of the foam itself are not often of interest. The main exceptions are foam density and foam viscosity, which values are needed for the design of nozzle-type foam breakers.

This discussion suggests that an empirical method should be used, such as that described by Ghosh and Pirt (1954). The only reliable measures can come from experience with the actual fermenter concerned during a typical fermentation. This system of measurement is often impracticable and the best alternative is to use bench-scale stirred vessels, whose aeration and agitation characteristics are similar to those of the real fermenter.

Ghosh and Pirt (1954) describe two such vessels, one with a vortex system of aeration, and the other employing the conventional baffled stirrer and sparger. The bodies of the vessels should be made of glass or some other transparent material so that one can easily observe and measure foam depth above the level reached by a non-foaming system.

The principal measurements made in such apparatus consist of noting the height to which foam rises before any anti-foam agent is added, the rate at which the foam collapses when addition is made, and the duration of the action of the addition. Generally the agent should act as quickly as possible, and should prevent the recurrence of foaming for as long as possible.

The liquid used in the test apparatus can be either a sample of broth from a fermentation, or a standard foam-forming solution. Ghosh and Pirt (1954) used a solution of corn steep liquor (about 3·5% solids) and 3% lactose adjusted to pH 7 with sodium hydroxide. If fermentation broth is used it must be remembered that its properties will change with time depending upon when the sample is taken during the progress of the fermentation, and also during the period of test. This second source of variation is particularly important if the real fermenter is continuously supplied with an essential nutrient. It should be recognized that the presence of bacteria tends to stabilize foam (Pirt and Callow, 1958), and so results obtained with a standard foaming solution should be treated with some

caution. Similar caution is needed if the anti-foam agent is metabolized by the micro-organism, as the action of the agent would be expected to last longer in a non-living system.

This form of test is, however, very useful for the preliminary selection of agents on a qualitative basis before examining the best in a more quantitative way.

Foam viscosity can be measured in a conventional rotational viscometer, and its density found by weighing a known volume of foam (see Bickerman, 1953).

IV. FOAM BREAKING

The general principles of foam breaking by mechanical or chemical means will be discussed here. Although foam can be destroyed by passing it through heated grids, or by irradiation with α particles (Kato and Kono, 1953), neither method is very suitable for use in fermentation.

A. Mechanical foam breaking

One advantage of mechanical foam destruction is that no extraneous agents need be added to the system. Mechanical foam breakers may be divided into those types in which the foam to be broken is contained within the fermenter, and those in which the foam and exhaust air are separated outside the fermenter, the liquid being returned.

The simplest form of internal foam breaker consists of rotating paddles or vanes attached to the stirrer shaft. Their action is most effective when the foam is well drained and has lost its self-healing properties. Such a device is described by Steel *et al.* (1955) to supplement the action of chemical anti-foam agents.

Ultrasonic whistles have also been used to contain foam within a fermenter, whilst allowing the air to exhaust freely. Boucher and Weiner (1963) attribute the effectiveness of ultrasonic foam breaking to four factors. These are the acoustic pressure, the unidirectional radiation pressure, the induced resonant vibrations in the bubbles, and the turbulence produced by the sonic wind. These factors are described in detail, and their relative values as causes of breakdown discussed.

Dorsey (1959) describes the use of a Hartmann whistle to control the foam produced during growth of *Serratia marcescens*. The generator consisted of a jet 0·78 in. in diameter and a cavity of the same diameter and depth. The minimum air pressure needed to operate the whistle was 40 p.s.i.g., and the consumption of air was between 2 and 4 ft^3/min free air. The whistle was tested at frequencies of 26, 29 and 34 kc/s, and found to be effective at all three frequencies. Separate experiments showed that the

use of ultrasonic energy caused no reduction in cell count. Haas and Johnson (1965) used a sonic whistle at 12 kc/s to control dodecylbenzenesulphonate foam, and found that its capacity was 0·05 ft^3 foam per standard ft^3 air fed to the whistle. This was lower than the expected value of 0·2 ft^3/standard ft^3 air, and was reduced still further if a diaphragm was interposed between the whistle and the foam. The conclusion is that although sonic and ultrasonic energy are technically satisfactory as foam breakers, power costs are too high.

Foam may also be broken by accelerating it through a nozzle. Phillips *et al* (1960) describe a nozzle-type foam breaker installed on a 200 U.S. gal fermenter. The air and entrained foam were exhausted through a convergant nozzle where the velocity reached 100–300 ft/sec; this sudden acceleration broke the foam. The nozzle discharged against a deflector place, the broken foam fell down to be pumped back to the fermenter, and the air was allowed to pass to exhaust. A positive displacement pump was used, connected to the deflector plate so that it operated only when the batch was actively foaming. A set of nozzles of different diameters was provided to accommodate different air-flow rates. During operation the head pressure in the fermenter did not exceed 5 p.s.i.

A similar device has been described by Haas and Johnson (1965), who observed that foam destruction occurred in the nozzle itself and that subsequent impingement was not necessary to destroy foam. Some form of deflector plate is still necessary in order to separate the flow paths of the condensed foam and gas, and it was found that the small volume of foam aerated by impingement could be reduced if glass were replaced by a Teflon surface. As with the rotating type of foam breaker, nozzles are more effective the more the foam has drained.

Haas and Johnson (1965) worked with a sodium salt of dodecylbenzenesulphonate as the foaming agent, and found that the capacity of the nozzle and pressure drop were related by the equation—

$$q = 6000\, D^2 \sqrt{\frac{\Delta P}{E}}$$

where q is the total foam flow rate (cm^3/min); D, orifice diameter (cm); ΔP, pressure drop (cmHg); E, volume fraction of liquid in foam. It should be noted that this equation indicates slightly higher flow rates than would be expected for a gas having the same density as foam.

Nozzle-type foam breakers make use of part of the energy of the sparged air to accelerate foam and break it. In centrifugal foam breakers the energy is supplied by an external drive to a suitable bowl or basket.

Haas and Johnson (1965) used a perforated bowl centrifuge lined with 80–100 mesh screen. Lower speeds gave smaller proportions of uncondensed foam, and the capacity increased with speed. As with the

nozzle-type breaker it was noted that the material used for the stationary wall affected the amount of refoaming on impingement.

Ebner *et al* (1967) describe a centrifugal foam breaker suitable for use during, for example, the vinegar fermentation or the production of baker's yeast. The device consists of a horizontal shaft fitted with vanes, and rotating at 1450–1500 rev/min. The exhaust gas and entrained foam pass through the vanes and are separated there by centrifugal action. The condensed foam and any unbroken foam flow back to the fermenter by gravity; the exhaust air is allowed to escape freely. The power consumption, which depends on the nature of the foam, is in the range 0·4–1·6 hp h/1000 ft^3 foam.

Another centrifugal foam separator designed specifically for fermenters has been patented (N.R.D.C., 1949). Centrifuges similar in principle are described in other patents (Denhard, 1949, and Sharples, 1949).

A different form of external foam breaker is described in a Russian plant (Loginov, 1947). Bulk liquid is pumped out of the fermenter and sprayed onto the surface of the foam. In effect this is simple mechanical rupture of the liquid films forming the foam and, again, it would be expected to be more effective the drier the foam (Bickerman, 1952).

Rapid shearing has also been used to break foam. Goldberg and Rubin (1967) describe a device, not specifically suitable for fermenters, in which foam to be destroyed is directed onto the surface of a rapidly rotating disc.

The disadvantages of mechanical foam breakers are that they are often of complicated design and expensive to operate. This latter aspect is particularly true of ultrasonic foam breakers, as not only do they need relatively large volumes of air for their whistles, but the pressure required is probably higher than the normal supply pressure of the air used for sparging. Simple rotating vanes are cheap to fit and cost little to operate, and they may help to conserve chemical anti-foam agents, although they are unlikely to control foam by themselves.

B. Chemical foam breaking

The use of chemical agents to control foam during fermentation is well established. Their action is fundamentally different from that of mechanical foam breakers in that they can prevent foam formation as well as destroy existing foam; mechanical devices only destroy foam after it has been formed.

The properties of an anti-foam agent used in a fermentation fall into three classes. There are those properties needed for it to function as an anti-foam agent, those dictated by the fact that it is to be added to a living organism, and finally that it must not interfere with other analytical systems such as pH probes or dissolved-oxygen concentration detectors.

Because the agent is to be added to the fermentation it must neither be

toxic nor inhibit the process of the fermentation. Toxicity tests are conveniently carried out in shake flasks, not only at the effective concentration of a single addition of the agent, but also at the concentration likely to have accumulated by the end of the fermentation.

The presence of any anti-foam agent should not interfere with any subsequent processing of the broth during extraction. For instance, it should not gum up ion-exchange-resin particles, and it should not promote emulsion formation during solvent extraction or broth concentration.

As one must add the agent in a sterile manner, the act of sterilization by steam or dry heat must not worsen its properties. It is also worth finding out if the agent is corrosive at sterilization temperatures, or if it forms corrosive products.

Anti-foam agent is usually added to a fermenter on demand, that is, when the batch is foaming heavily. In this case the first requirement is that it should act quickly and suppress the foam. Other desirable properties of the agent are that it should be effective in low concentrations, and that its action should last for as long as possible.

The factors involved in the final choice of agent are complicated by economic considerations. The cost of labour or automatic equipment to add anti-foam agents to the fermenter must be determined, and of course the cost of the agent itself.

In Section II it was shown that solutions foam only if they possess surface elasticity; therefore, any chemical anti-foam agents must eliminate surface elasticity (Kitchener and Cooper, 1959). The anti-foam should produce a surface having a constant surface tension when subjected to expansion or compression. In order to displace the foaming agent from the surface an anti-foam agent must have a low surface tension in the pure state, and be able to spread over the interface. It must also be present in a sufficient quantity to maintain a high surface concentration even under the dynamic conditions found in a fermenter, and so a low solubility is advantageous.

Metastable foams and their films derive stability from electrical double-layer repulsion. Films of such nature can be destroyed, or made less stable, by the addition of certain indifferent electrolytes to the system. The addition of an agent, itself capable of foaming, can destroy an existing foam if it is of opposite charge (Sebba, 1963). It is common practice to emulsify the agent with a carrier to increase its ability to spread. The ability of an agent to spread is given by the spreading coefficient S—

$$S = \gamma_F - \gamma_A - \gamma_{AF}$$

where γ_F and γ_A are the surface tensions of the foaming liquid and the anti-foam agent, and γ_{AF} is the interfacial tension. The greater the value of S the greater is the spreading power of the agent.

The main function of an anti-foam agent is to eliminate surface elasticity, but it can also act by reducing surface viscosity and thus promoting more rapid drainage.

In view of the importance of chemical anti-foam agents a separate Section is devoted to a discussion of the different types.

V. CHEMICAL ANTI-FOAM AGENTS

In this Section an attempt will be made to describe some of the more common types of chemical compounds which have been used as anti-foam agents. From the literature, e.g., Pattle (1950) and Currie (1953), it can be seen that the choice of agents is indeed wide. Currie classifies chemical anti-foam agents, suitable for use in fermentations, under the following headings—

(i) Alcohols.
(ii) Esters.
(iii) Fatty acids.
(iv) Fatty acid derivatives.
(v) Silicones.
(vi) Sulphites and sulphonates.
(vii) Miscellaneous.

In addition to this wide range of chemical types there are two further factors to consider. The first is the possible use of a carrier, and secondly there is the problem of synergistic action. A carrier is generally an inert liquid, with respect to anti-foam action, in which the anti-foam agent is dissolved or with which it is emulsified. The simplest view of the action of a carrier is that it serves as a reservoir from which the active anti-foam agent is slowly released. However, this does not fully explain its action as it has been shown that the use of a carrier accelerates and prolongs the effectiveness of the action of the anti-foam agent. Solomons (1967), too, shows that the simple reservoir theory is insufficient to explain the carrier's action. He gives an example of a silicone oil; the slow addition of neat oil was less effective in controlling foam than was the addition of a similar amount of oil dispersed in a carrier. Quite apart from this incompletely understood function a carrier may bring a further practical advantage by reducing the viscosity of the agent and so make it easier to add to a fermenter.

The synergistic effect may be loosely described as the mutual enhancement of action when two different agents are mixed. Their effectiveness when combined is greater than the effectiveness of either one used separately. This effect may be exploited in two ways. The first is to mix the two agents prior to addition to the fermenter. The second is to use two different anti-foam agents alternatively during the course of a fermentation.

Vegetable oils have long been used in fermentation to suppress foam formation, and in addition they often serve to control pH because they are metabolized. Examples are cotton seed, linseed, rape-seed, soybean and olive oils. Sulphonated oils, such as castor oil and rape-seed oil, have been found to be more effective than the soaps of tall oil in controlling foam during the yeast and alcoholic fermentations. In general the vegetable oils prove to be effective anti-foam agents in a wide variety of fermentations.

The alcohols are another group of compounds finding wide use as anti-foam agents. Octadecanol is perhaps the most frequently used, either alone or with the addition of a carrier. It has been used with cold-pressed lard oil to control foam during the penicillin fermentation, while 3% alcohol in mineral oil has proved to be very effective for the citric acid fermentation. Bungay (1960) notes that those alcohols derived from fatty acids are the most effective anti-foam agents.

Alcohols and vegetable oils may be considered as anti-foam agents whose use has been traditional and empirically established. There are, however, compounds now available specifically designed to destroy foam, the most notable class being the silicone oils. There are two forms of silicone fluids available, the pure polysiloxanes in a carrier, or aqueous emulsions containing about 10% silicone. Methyl polysiloxane is commonly used with paraffin oil as a carrier, although Solomons and Perkin (1958) found castor oil to be more effective. The disadvantage of this form of anti-foam is that it is difficult to handle, as the silicone fluid is itself rather viscous, as is castor oil. Autoclaving may cause the silicone fluid to separate from the carrier; paraffin oil showing less tendency to do so than does castor oil (Solomons, 1967).

A more useful form of silicone anti-foam is as an aqueous emulsion. It is less viscous and does not require the addition of a carrier. Solomons (1967) reports that in general silicone anti-foams are more effective in bacterial fermentations at alkaline pH's. They are less effective in mould fermentations, where the pH may be around 5, but since they are good in yeast fermentations which are slightly acidic mycelium may have some hindering effect. Bungay (1960) notes that careful checks should be made to ensure that the agent is in no way inhibitory. Some silicone emulsions are liable to microbial decomposition on storage. In spite of these reservations silicone emulsions are very effective agents, and, if added to a medium at batching, may prevent the appearance of foam during the entire course of the fermentation.

Polyglycols have similarly long action and are particularly suitable for mould fermentations. With a molecular weight of about 2000 the glycol is a clear slightly viscous fluid, and no carrier is required.

Of the other compounds specially produced as anti-foam agents there are

the alkonylated fatty bases and alkylene glycols supplied by Glovers (Chemicals) Ltd. Of these Texofor D10 is the best for fermentation applications since it is effective in the presence of high electrolyte concentrations such as are found in culture media (Solomons, 1967).

Glyceride oils and fatty acids are available from the Hodag Chemical Corporation. Those recommended for use in fermentation are Kg-1, M-8, M-9 and GN-31 (Solomons, 1967). They are oily liquids and although they may be used alone they are more effective with paraffin oil as a carrier. A great advantage they possess for fermentation work is that they are stable to heat sterilization.

Alkaterge C, a fatty acid derivative is widely used in fermentation work. For example, Deindorfer and Gaden (1955) used 3% in lard oil, whereas Pirt and Callow (1958) used 30% v/v in liquid paraffin.

Enough has been written to show that the choice of anti-foam agents is extremely diverse. Because of this diversity of chemical types it is not possible to state that any one agent is ideal for a particular fermentation. The aim has been to show the choice, and below are listed the addresses of some of the larger manufacturers. The "Buyers Guide" published annually by the "Society for Chemical Industry" gives a full list of anti-foam manufacturers in this country, but it must be remembered that not all supply agents suitable for use in fermenters.

Main suppliers of anti-foams in the U.K. are—

Berk Limited,
Berk House,
P.O. Box 1 BL,
8 Baker Street,
London, W.1.

Glovers (Chemicals) Ltd.,
Wortley Low Mills,
Leeds 12.

Imperial Chemical Industries Limited,
Nobel Division,
Silicones Group,
Nobel House,
Stevenston,
Ayrshire.

Midland Silicones Ltd.,
Reading Bridge House,
Reading,
Berks.

Union Carbide U.K. Limited,
Union Carbide House,
High Street,
Rickmansworth,
Herts.

VI. HANDLING ANTI-FOAM AGENTS

The factors affecting the choice of anti-foam agents, and the different types of agents available, have been discussed in previous Sections. Here the problem of adding the agents to a fermenter will be considered.

The most common form of addition is on demand, when the batch is foaming heavily and some corrective action is needed immediately. In its simplest form the addition of agent is controlled by the operator, and its success depends entirely on the operator's vigilance and care. The disadvantage of this form of addition is that it is a non-routine job, and it is apt to lead to loss of control simultaneously in many tanks and clash with routine jobs.

All automatic methods of adding anti-foam agents on demand are basically the same. The fermenter is fitted with a high-level probe near its lid, and when foam reaches it a circuit is closed, allowing anti-foam to enter the fermenter. In its crudest form the closing of the probe circuit opens a solenoid valve, and anti-foam flows in to the fermenter until the foam collapses and the probe circuit is opened again. As with the manual addition technique this system wastes anti-foam agent. Another disadvantage is that agent is admitted when the probe is splashed or touched by transient foam.

In order to prevent waste it is usual to fit some delay into the electrical circuit. Bartholomew and Kozlow (1957) describe an automatic addition kit for bench-scale fermenters. When the probe circuit is closed a shot of anti-foam agent is added, and, if the foam persists, there is a delay of 1 min before another addition is made. The circuit they used was controlled by a timer making one revolution in 60 sec. There was a 0–5 sec control of the "on" cycle for opening the solenoid valve to admit anti-foam. The period during which the valve was opened could be varied to allow different volumes of anti-foam agent to be added. Bartholomew and Kozlow used a period of 0·5 sec, corresponding to a volume of 1–2 drops. After one opening of the valve the 60 sec timer ensured that a further addition could not be made until 1 min later. Although this is a simple device, it proved to be reliable and used less anti-foam than was found to be necessary when manual addition was used.

Similar circuits have been described by Dworschack, Lagoda, and Jackson (1954), Nelson, Maxon, and Elferdink (1956), and Fuld and Dunn (1958). The presence of foam is detected by a simple grid bias circuit in which the grid of the control valve becomes negative with respect to the cathode when foam touches the probe: the anode current through the relay decreases abruptly and the solenoid valve is actuated. A timer is included in the circuit and is adjusted to allow the solenoid valve to open for 2 sec every 30 sec. The

volume of anti-foam agent admitted during this 2 sec period is adjusted by means of the throttle valve. A switch may be included in the circuit to enable one to add anti-foam even if the foam does not reach the probe.

A common form of probe consists of an insulated support in the lid of the fermenter, through which a stainless steel rod passes. The length of the rod can be varied to determine the height to which foam can rise before anti-foam agent is added.

The use of a high-level probe near the lid implies that the anti-foam agent should act quickly, and this requires the agent to be able to spread rapidly. In these circumstances the manner in which the agent is introduced to the fermenter is not critical. A single line directing agent onto the surface of the foam is simple and generally satisfactory. More complete coverage of the surface of the foam can be obtained if the agent is discharged onto a rotating disc fitted to the stirrer shaft (Nelson *et al.*, 1956). Stefaniak *et al.* (1946) introduced anti-foam agent into the main air flow being sparged into the fermenter. A spray nozzle has been used but it has been suggested that because of entrainment of fine droplets in the exhaust air anti-foam was wasted (Bungay *et al.*, 1960).

An important factor in the design, construction and maintenance of all anti-foam addition kits is reliability. It is essential because such kits cannot fail safe: too much anti-foam harms the fermentation and subsequent extraction or treatment of the broth, and too little anti-foam or none at all is equally harmful.

In the control devices so far described no addition is made until foam reaches a detector near the lid of the fermenter. This form of control allows large changes in the apparent volume of the batch between additions, and results in a wide range of conditions of aeration and agitation. More stable conditions are obtained if the height to which the foam rises before anti-foam is added is reduced. Bungay *et al.* (1960) describe a device where the horizontal section of the fermenter is fitted with wicking material supported on a wire mesh. Foam rides just below the level of the wicks, and anti-foam is drawn into the foam whenever it touches the mesh. The advantage of this method of control is that it can minimize excessive anti-foam being consumed by metabolism, and reduce total usage: 25–30% reductions were observed.

The ultimate form of control is to add anti-foam to prevent the appearance of foam, rather than to allow foam to form before additions are made. Pirt and Callow (1958) used this technique, and added anti-foam at regular intervals and so prevented the formation of foam. Besides reducing the quantity of anti-foam used, periods of uncontrollable foaming were eliminated; these periods had occurred when anti-foam was added on demand.

As anti-foam must be added to a fermenter in a sterile condition, the

factors affecting the design of piping and choice of valves are the same as those for any nutrient solution. Stainless-steel lines are to be preferred, and Saunders-type valves with the sleeve-packed cock should be used. Elsworth and Stockwell (1958) discuss the merits of valves and cocks and besides the Saunders or diaphragm type of valve, recommend seatless piston valves and the more recently introduced spherical plug cock.

Natural oils may tend to decompose to form carbonaceous or greasy sediments during sterilization and this fact must be recognized in the layout of the piping. Without the addition of carrier many anti-foam agents are viscous liquids and some form of heating may be necessary to enable them to be handled more easily.

VII. EFFECTS OF ANTI-FOAM AGENTS ON AERATION/AGITATION

The addition of anti-foam agent to a fermenter changes the conditions within the system in two ways. The first change results from the breakdown of the foam layer on the surface of the aerated liquid. If surface aeration is used, as in a Waldhof fermenter for instance, aeration will be improved by the disappearance of this barrier between the bulk liquid and the atmosphere. In conventional stirred and baffled fermenters the surface foam can cause a delay between the addition of, and response of a batch to, nutrient or pH control solution if addition is made through the lid of the fermenter. There is then the possibility of overshoot and poor control.

The more important way in which anti-foam agent affects aeration and agitation is by reducing or eliminating the fluid foam in the bulk of the batch. Fluid foam, a fine dispersion of gas bubbles, is commonly experienced in mycelial fermentations. An impeller can rotate in this dispersion without being able to cause any mixing of the medium (Chain *et al.*, 1966). The bubbles in the dispersion stay in the batch for very much longer than the average residence time of the sparged air and therefore become rich in carbon dioxide and depleted of oxygen. The elimination of fluid foam is therefore beneficial.

The net effect of the addition of anti-foam agent is difficult to predict, as it depends on the type of fermenter being used and the particular fermentation. To be more specific, it depends on the limiting stage in the oxygen supply process. This process may be divided into three stages. The first involves transfer across the gas/liquid interface, and is followed by transport of the dissolved oxygen through the liquid. The third stage is the transfer across the liquid boundary layer from bulk liquid to the surface of the micro-organism.

Anti-foam reduces the gas/liquid interfacial area, by eliminating the fluid foam, and by reducing the volume of hold-up. If the first stage, i.e., the oxygen solution rate, is limiting, then the addition of anti-foam agent will reduce the overall rate of aeration. It will do this by reducing the interfacial area available for mass transfer, and possibly by increasing the interfacial resistance. There will also be a reduction of the time of contact (Solomons, 1966). This net effect has been demonstrated in baffled fermenters (Phillips *et al.*, 1960; Deindorfer and Gaden, 1955).

Stirrers in baffled fermenters are designed to operate in the turbulent region, where, for a fixed speed and geometry, power is directly proportional to fluid density. The reduced hold-up of gas resulting from the addition of anti-foam increases the density of the fluid surrounding the stirrer, and so increases the mechanical power drawn by the system. Power increases of 20% have been reported (Bungay *et al.*, 1960). If transport within the culture liquid is limiting then this increased power will improve aeration by improving the mixing. This improvement is enhanced by the changed rheology of the broth. Fluid foams, in common with foams, have a high viscosity amounting in some cases almost to rigidity. The removal of fluid foam then reduces the effective viscosity to the fluid to be stirred. In general it would be expected that improved agitation would increase the rate of the final stage of the oxygen supply process. However, if anti-foam accumulates on the surface of the micro-organism, the net change may be to reduce the rate. Whether or not this final rate affects the overall rate depends on the limiting stage in the overall supply process.

Most bacterial fermentation broths have a low viscosity, and so the net effect of anti-foam is to reduce the rate of aeration, as the limiting stage is probably the rate of solution of oxygen. With thicker broths this same effect may be masked by an improvement in liquid mixing.

VIII. CONCLUSIONS

The aim throughout this Chapter has been to describe the general principles of foam destruction and prevention, rather than particular applications. The reason for this is that foam control, like so much else in fermentation technology, is an empirical art. The best method established in one plant for a particular fermentation is not necessarily the best for the same fermentation carried out in a different factory or site. The reasons for this are that natural products are often used in the preparation of fermentation media, and their properties are not necessarily constant or reproducible. Similarly the design of a fermenter, and its operation, may affect the properties of the foam formed. The economic factors governing the choice of anti-foam agent vary from plant to plant, so no generally applicable rules may be defined.

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CHAPTER VIII

Production and Measurement of Photosynthetically Useable Light

N. G. CARR

Department of Biochemistry, University of Liverpool, England

I. UNITS AND MEASUREMENT

Relatively little attention has been given to growth of photosynthetic micro-organisms in light of precisely defined wavelength or intensity, and most of our knowledge of the production and measurement of such light comes from plant physiology (see Rabinowitch, 1951; Withrow and Withrow, 1956; Altman and Dittmer, 1966).

Any radiant energy may be described in terms of its wave motion by either wavelength or frequency; wavelength is the difference between equal points of a wave motion, and frequency is the number of vibrations at a given point per second. Frequency is equal to the velocity of the wave form divided by the wavelength. In biological literature, wavelength is expressed as nanometers† (10^{-9} metres; 1 nm = 10 Ångstroms). Wavenumber is the number of waves in unit time and is the reciprocal of wavelength. Radiant energy is related to the frequency of the wave motion and the quantum energy of light is dependent upon its wavelength. As frequency increases

† The expressions micron and millimicron are no longer used and have been replaced by micrometre (μm) and nanometer (nm) respectively.

(and wavelength decreases) so does the energy per quanta of radiation. The relationship between these characteristics of radiant energy are summarized, for the visible spectrum, in Table I.

TABLE I

Radiant energy in the visible region.

Wavelength, nm	Wavenumber, cm^{-1}	Frequency, sec^{-1} ($\times 10^{12}$)	ergs/quanta ($\times 10^{-12}$)	Standard luminosity coefficient
400	25,000	750	4·96	0·00040
450	22,200	667	4·41	0·03800
500	20,000	600	3·97	0·32300
550	18,200	545	3·61	0·99500
600	16,700	500	3·31	0·63100
650	15,400	462	3·06	0·10700
700	14,300	429	2·84	0·00410
750	13,300	400	2·65	0·00012

Luminous flux may be expressed in absolute terms of energy, as ergs/sq. cm/sec or as joules/sec, but a separate system of measurement based on the illumination provided by a standard source has been developed, the units of which are lumens (lm)/sq. ft (or foot candelas), lux (lm/sq. m) and the phot (lm/sq. cm). The original standard source was a candle of known composition, but a black-body radiator at the freezing point of platinum (2042°K) has been finally adopted as the most reproducible and reliable source. The standard unit from this is the foot candelas (ft cd), which is slightly greater than the original foot candle. The relationship between some of the photometric units employed are listed below. The quoted conversion factor between ergs/sq. cm/sec and the lumen group of units applies to light of 555 nm wavelength; this factor will change with wavelength and outside the range 500–600 nm an error of approximately 50% would be introduced—

$$
\begin{aligned}
\text{lm/sq. m}^{-2} &= 1{\cdot}470 \times 10^{-3}\ \text{W/sq. m} \\
&= 1{\cdot}470\ \text{ergs/sq. cm/sec} \\
&= 9{\cdot}28 \times 10^{-2}\ \text{lm/sq. ft (ft cd)} \\
\text{lm/sq. ft} &= 1\ \text{ft cd or } 0{\cdot}98\ \text{ft candles} \\
&= 10{\cdot}76\ \text{lm/sq. m} \\
&= 15{\cdot}78\ \text{ergs/sq. cm/sec} \\
\text{egs/sq. cm/sec} &= 10^{-3}\ \text{W/sq. m} \\
&= 0{\cdot}682\ \text{lm/sq. m} \\
&= 0{\cdot}0633\ \text{lm/sq. ft}
\end{aligned}
$$

The most accurate means of measuring illumination is to measure the energy (as ergs/sq. cm/sec) by means of a heat detector (thermopile or bolometer), and this method should always be employed when monochromatic light is used. When estimating visible radiation, these instruments must be screened from infrared radiation; the thermopile may be used to measure "light" in the far-red region that is invisible to the eye, yet is used by photosynthetic microbes. Units of illumination, as distinct from energy, are originally related to the sensitivity of the eye, that is, to "visible" light. This has been described as the "standard luminosity coefficient" for different wavelengths, and these values represent the relative capacity of radiant energy to produce visual sensations (Table I). Visible radiation may be measured using conventional light meters, which are calibrated for a particular spectral emission (often tungsten lamps or sunlight) and appropriate correction factors can be applied when other sources are used. Such a meter would however be relatively insensitive to light of wavelength greater than 650 nm, although such radiation is most important to many photosynthetic micro-organisms. There are however photocells whose relative sensitivity is shifted to either the red or violet side of "white" light (see Phototubes and Photocells, 1963). The relative sensitivities of two photocells are described in Fig. 1. For ideal measurement of polychromatic light in photosynthetic studies, the photocell used (with any necessary filters) should have a relative spectral response that is the same as the *in vivo* absorption spectra of the photosynthetic organism. This is not the case with

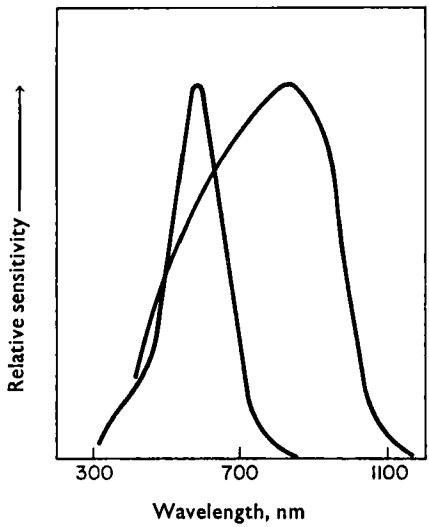

FIG. 1. Two examples of photocell wavelength sensitivity. (From Phototubes and Photocells, 1963.)

standard commercial photocells, and hence relative, rather than absolute, degrees of illumination are recorded.

II. LIGHT SOURCES

The natural source for all photosynthetic growth, the sun, has considerable irradiance; at a solar angle of 90° at sea level in the temperate zone a maximum visible intensity of 10,000 ft cd may be recorded. Most of this is absorbed or reflected by air mass and clouds, and for an average cloudy day at noon this drops to 500–1500 ft cd (see Withrow and Withrow, 1956). At sea level about 40% of the irradiation from the sun lies in the region below 700 nm, and maximal intensity is around 500 nm.

A. Incandescent lamps

Tungsten bulbs have broad emission spectra with considerable irradiance in the near infrared (800–1500 nm) and maximum emission around 900 nm (Fig. 2). This maximum depends upon the temperature of the lamp, which

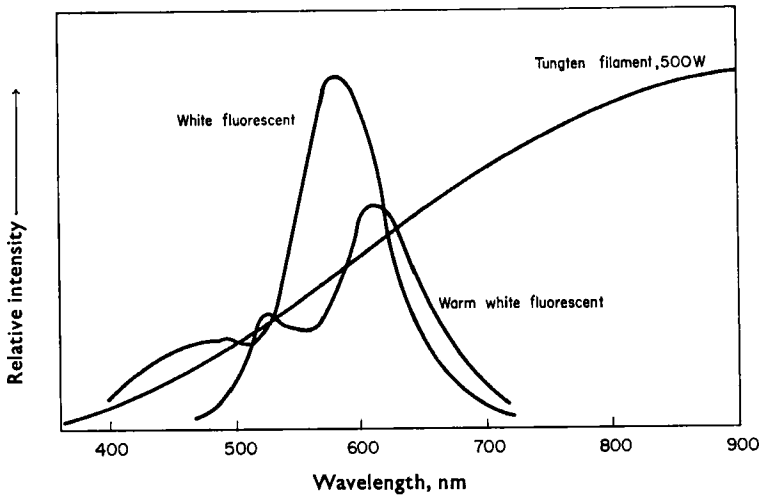

FIG. 2. Emission spectra of tungsten and fluorescent lamps. (Courtesy, General Electric Co. Ltd.)

in turn is a function of the operating voltage. Thus the light obtained from a 500 W bulb is of a different character from that from a 50 W; at higher wattages (and operating temperature) the emission spectrum is shifted (10–30 nm) toward the ultraviolet. The visible flux of a tungsten lamp decreases continually with time until the filament burns out; it is therefore necessary to check regularly the illumination actually supplied from such a

light source. Frosted bulbs fitted in a reflector made of steel or aluminium are generally satisfactory, but for high intensity over a larger area, bulbs fitted with an internal reflector are to be preferred. A disadvantage of the tungsten bulb is the fact that over 80% of the input energy is irradiated as heat and it is always necessary to dissipate this, or absorb it by a water filter, in constructing growth chambers.

B. Fluorescent lamps

In these bulbs, ultraviolet light emitted by a low-pressure mercury discharge lamp is absorbed by phosphors within the tube that fluoresce at longer wavelengths. Phosphors are usually inorganic salts and oxides, and by varying their proportion, visible light of different emission characteristics may be obtained. More than one type of "white" light lamp is available in which relative emission spectra are altered. The "warm white" fluorescent lamp has an emission spectrum shifted toward the longer wavelengths in comparison with the "white" fluorescent lamp (Fig. 2). Superimposed upon the continuous spectra of fluorescent lamps is a line spectrum from the mercury source. A marked feature of fluorescent lamps is the absence of infrared radiation. In contrast to the tungsten bulb, most of the energy input is dissipated by convection and conduction. Thus the fluorescent lamp is cooler to the touch, and provided that it is unenclosed, it does not provide the heating problems associated with tungsten bulbs. Of course in an entirely enclosed unit the same heat per electrical input will be obtained with either type of light source.

C. Discharge lamps

There are wide ranges of discharge lamps operating at high and low pressures, with electrodes of different materials yielding ultraviolet, visible and infrared emission spectra, which may be continuous or sharplined. The use of such lamps, sometimes in conjunction with filters, permit the production of narrow bandwidth light.

The line emission of a sodium arc is a close doublet at 589·0 and 589·6 nm, and since the major part of the energy lies in the doublet, this lamp provides a convenient and efficient source of monochromatic light. There is a minor absorption peak of bacteriochlorophyll around 600 nm, whereas the major absorption by blue-green algae of the sodium lines would be by phycocyanin and not chlorophyll.

The use of a Kodak Wratten No. 98 and a low-pressure mercury arc produces radiation in two spectral lines between 400 and 450 nm, suitable for absorption by carotenoids. These two examples illustrate the possibilities of specific discharge lamps, the characteristics of other discharges are to be found in physical texts.

III. PRODUCTION OF DEFINED-WAVELENGTH LIGHT

The most adaptable method involves the use of the grating monochromators available from several manufacturers. These consist, in essence, of a light source and collimator system for producing parallel light, which is focused on to a grating (or dispersal system). This bends the incoming light according to its wavelength, and light of very small bandwidth may be isolated by slits. Although this procedure has been extensively used in photosynthetic studies, its use as a source of monochromatic light for growth work is limited by the very small intensities of defined light that may be produced. Of much more practical application has been the use, singly or in combination, of various types of filters.

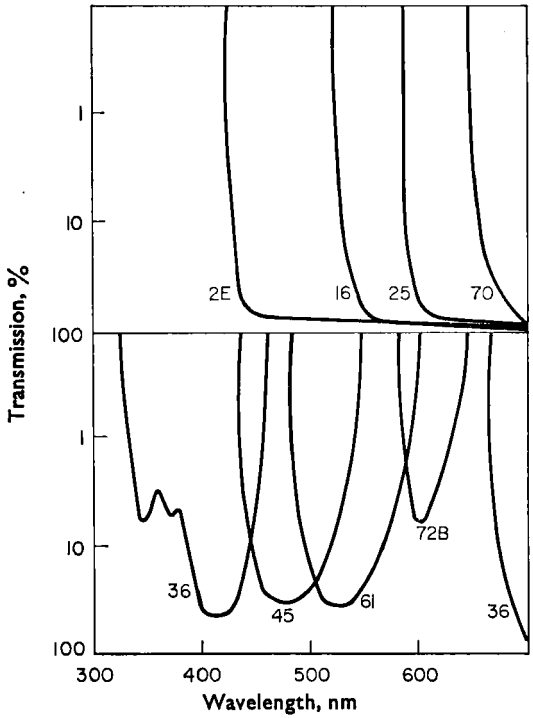

FIG. 3. Spectral transmission of examples of self-absorption filters (Kodak Wratten Filter numbers are indicated in the Figure).

A. Self-absorption filters

The earlier filters were all of the self-absorption type and were frequently simple chemical solutions; thus a copper sulphate solution will transmit virtually all emission between 390–460 nm, but has the disadvantage of wide "tails" of transmission out of this range. Occasional use has been

made of various natural products as filters, and for short-term experiments certain photosynthetic pigments are sufficiently stable to act as a chemical filter between the culture and light source. The more usual self-absorption filters consist of water-soluble dyes suspended in a thin film of gelatin (transparent to visible and ultraviolet light above 300 nm), which in turn may be sealed between glass plates. The absorption characteristics of several such filters are shown in Fig. 3, which presents data for a few examples of the "Wratten" range produced by Kodak Ltd. Both cut-off filters that omit all light below a certain wavelength and filters that transmit only light from a defined area of the spectrum may be obtained. Combination of these filters and the use of various light sources permits the production of light of distinct wavelengths. Thus Wratten No. 29 used with a tungsten lamp would transmit only light of wavelengths greater than 600 nm, absorbed by only the bacteriochlorophyll, and not the carotenoids, of photosynthetic bacteria. Self-absorption filters have two disadvantages: they possess a relatively wide bandwidth, and they tend to overheat owing to the absorbed light energy that is degraded to heat.

B. Interference filters

Interference filters (optical filters) operate by selective interference and scattering of unwanted light and permit the desired wavelength to be transmitted. This can result in a much narrower bandwidth and no overheating. However a collimated light beam is required for interference filters, and there is greater "background" transmission throughout the whole spectrum. Examples of interference filters are shown in Fig. 4. The combination of

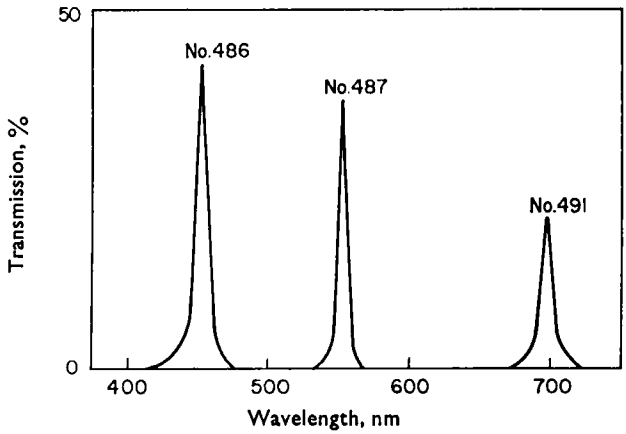

FIG. 4. Interference filters: examples of the range manufactured by Balzers AG, Lichtenschien.

absorption and interference filters to produce band light with ten different maxima between 450–750 nm has been described by Larsen (1953).

IV. CONTROL OF LIGHT INTENSITY

The total light supplied to a growing culture of a micro-organism may be altered in various ways, the simplest being to alter the number of lamps at the source. If this is impractical, the voltage supplied may be varied, but in the case of tungsten lamps this would alter the emission spectra and, unless monochromatic light was selected by filters, would be undesirable. Providing that reflection from the object is eliminated, the application of the inverse-square law allows useful alteration in luminous flux supplied. To satisfy this law, source and object of light must be small compared with their distance apart, and the surface of the object must be normal to the light path from the source. Perhaps the most convenient means of variation in intensity of radiant energy is by inserting neutral filters between the lamp and the object. These may be purchased as partially darkened photographic plates or constructed out of fine wire mesh or woven metal cloth.

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CHAPTER IX

The Measurement of Oxygen Absorption and Carbon Dioxide Evolution in Stirred Deep Cultures

R. ELSWORTH*

Microbiological Research Establishment, Porton, Salisbury, Wilts., England

I. THE PURPOSE OF THE MEASUREMENTS

Oxygen consumption and CO_2 evolution as features of microbial metabolism have been discussed by Mallette (this Series, Vol. 1).

The measurement of these two factors and their relationship, in the first place, to dissolved O_2 tension and consequently to such phenomena as the creation of microbial substance, substrate utilization or product formation, is of importance in understanding and controlling microbial processes.

The CO_2 and O_2 concentration in the effluent air stream are each a

* Present address: 25 Potters Way, Laverstock, Salisbury, Wiltshire, England.

measure of the metabolic rate of the process, and therefore continuous analysis is of great value as a supervisory tool. We have found that CO_2 measurement is invaluable in detecting disturbances in a culture. These may be due to failure to control factors, such as temperature, pH or foaming, or they may be the result of variations in air flow rate, medium pumping rate or even failure to observe that the stirrer motor has stopped. A positive use of the ability to detect changes immediately they occur is in the control of deep cultures of bacteriophage. Thus the time for inoculation with the phage is signalled when the $\%CO_2$ in the effluent air shows that the host cells have reached the required concentration. Likewise, the time for harvesting the phage is shown by a marked fall in CO_2 evolution.

II. OBSERVATIONS INVOLVED

As well as analysing the effluent gas from aerobes for CO_2 and O_2 and that from anaerobes for CO_2, it is also necessary to know the inlet air rate in aerobic cultures and the effluent gas rate in aerobic and in anaerobic cultures. Effluent gas rate in aerobic cultures can be calculated if the inlet air rate and the analysis of the effluent gas are known (see Section V). In anaerobic cultures the effluent rate must be measured directly. For up to 2 litre scale anaerobic cultures, a simple gas jar and pneumatic trough, or its equivalent, is convenient. For a larger scale an integrating rotary wet gas meter is suitable. In the U.K., Parkinson Cowan supply a range of these meters.

The values of CO_2 and O_2 concentration likely to be met with in cultures aerated at 1 volume of air/volume of culture/min are 0–5% CO_2 and 16–21% O_2. With anaerobes, the CO_2 concentration may exceed 5%, depending on whether or not the culture is fed with a bleed of N_2. In either event, in instrumental analysis the sample stream from an anaerobe can be diluted with a known proportion of air to bring the mixture within the 0–5% range. In this way one CO_2 meter will cope with both types of culture.

III. THE ORSAT APPARATUS FOR CHEMICAL ANALYSIS OF CO_2 AND O_2

A. Reasons for choice. Accuracy of the method

The trend of all analysis is away from methods involving chemical reactions and towards the measurement of a physical property. Nevertheless all so-called direct physical methods are, in fact, secondary. They have to be calibrated by means of a series of analysed samples or a series of synthetic mixtures of pure components. Hence it is advisable for a laboratory to be equipped for chemical gas analysis either for occasional use, or in order to challenge a physical instrument that is giving dubious results. It may also

be that a chemical method is best in the preliminary stages of an investigation, if only to confirm and justify the need for a physical instrument which is inevitably expensive in cost and supervisory skill.

Classical methods of measuring CO_2 and O_2 in air include passage of a measured stream of the gas through absorbants and end in gravimetric or volumetric measurements. The Haldane apparatus which will measure CO_2, O_2, CO, H_2 and CH_4, though comparatively new, is of classical status. All these methods call for an experienced analyst and will not be discussed here. On the other hand, the Orsat method, which has a long history of use in industrial chemistry, is simple, and therefore is to be recommended.

The Orsat apparatus is described fully (along with the Haldane apparatus mentioned above) in a British Standard (British Standards Institution, 1952) that deals with the analysis of flue gases. It is cited as "the best known apparatus . . . for the determination of CO_2, O_2 and CO where a high degree of accuracy is not required". The minimum size of sample to allow for sweeping out the apparatus is 150–200 ml. The accuracy of the determination is given as $\pm 0{\cdot}1\%$ for CO_2 and $\pm 0{\cdot}2\%$ for O_2. A critical account of the Orsat method has been given by Strouts *et al.* (1955). The complete unit, or individual components can be purchased from most suppliers of laboratory equipment. An average laboratory workshop should be able to construct the complete apparatus apart from the gas-measuring burette. The apparatus and a suitable set of sample bottles are shown in Fig. 1.

B. Sampling the air

The sampling bottles consist of a 250 ml aspirator fitted with a Green and Frederick tap and connected by about 3 ft of $\frac{1}{4}$ in. rubber tube to a 500 ml aspirator. A satisfactory confining liquid is 2M H_2SO_4 coloured with methyl orange, in which CO_2 is less soluble than in water. The small aspirator is filled with liquid by raising the larger one and opening and then closing the tap. The sample point on the effluent line should be located downstream of the exit filter. It is connected with rubber tube to the straight leg of the sample bottle cock. Air is then purged out of the bent tube, to sweep out the line. The cock is then turned in order to fill the sample bottle with sample. If there is not much pressure in the sample line it may be necessary to lower the larger aspirator. On first use, or if the sample to be taken differs radically in CO_2 content from the previous one, the bottle should be filled completely with the sample air and then allowed to bubble out through the larger aspirator. This ensures that the confining liquid is saturated with CO_2 at the partial pressure of the gas sample. In this way there is a reduced risk of transfer of CO_2 to or from the actual gas sample. On completely filling the bottle in order to reduce the area of liquid interface, through which CO_2 can diffuse from the sample, the bottle cock is closed and the sample is

retained under a slight head by raising the reservoir bottle. Samples should be analysed within a few minutes to minimize loss of CO_2.

C. Operation of the apparatus

The absorption reagents and method are fully described in the Standard (1952). Briefly 100 ml of the sample is introduced into the measuring burette E_1 (Fig. 1). The gas is then passed repeatedly into and out of absorption

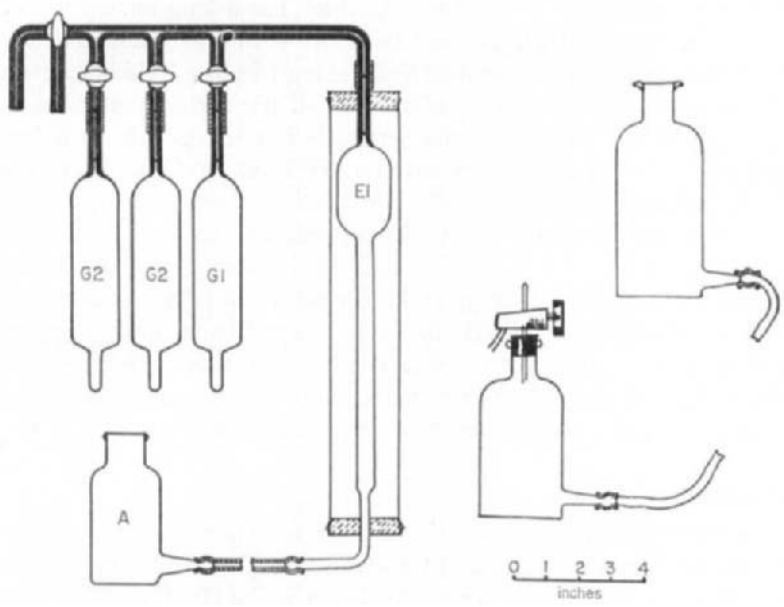

FIG. 1. Orsat apparatus: A, levelling bottle; E1, measuring burette; G1, G2, absorption vessels. Sampling bottles are shown to the right.

vessel G_1 by manoeuvring the levelling bottle A. The absorption vessel G_1 contains KOH, which absorbs CO_2. When absorption is complete the sample is returned to the measuring burette. The contraction in volume, which represents $\%CO_2$, is then measured. The residual gas is then treated in absorption vessel G_2. This contains alkaline pyrogallol, which absorbs O_2. Percentage O_2 is determined in the same way as CO_2, by measuring the contraction in volume. Note that the pyrogallol reagent will also absorb CO_2. Thus CO_2 absorption in alkali must always precede O_2 absorption in pyrogallol. In making and using pyrogallol solution the ingress of atmospheric oxygen should be avoided. Either a layer of paraffin or a rubber bag should be fitted to the open leg of the absorption vessel. An analysis for CO_2 and O_2 need not take more than 3 min, and an analysis every 15 min is not

impossible. The British Standard (1952) should be consulted for full operational details.

IV. INSTRUMENTAL ANALYSIS

A. Continuous CO_2 analysis

There are two methods to be recommended. First there is the non-dispersive analyser, which is specific for CO_2 in fermentation gases, providing the sample is dried. There is also the thermal conductivity method, which employs a katharometer. This method is of satisfactory specificity for mixtures of CO_2 and air. Calibration is affected by variable amounts of H_2, so the method is not suitable for anaerobic cultures. The infrared machine is expensive, requires skill and some dedication if it is to be used to greatest advantage. Its use is obligatory if the intention if to carry out accurate material balances for carbon. The relative cheapness, ruggedness and high stability make the katharometer the best choice as a monitor in process control.

1. *Infrared analysis*

(a) *General.* Jones (1956) has briefly described two commercial instruments. Telling *et al.* (1958) dealt with the principles of the method and its use and performance in examining effluent air from bacterial cultures. The principle of the method is as follows. When analysing for a single component in a gas stream, a so-called non-dispersive analyser is used. This examines absorption in the wavelength range 3–15μm. The light beam after passing through the absorption cell, through which the sample gas is flowing, is scanned by a detector that is selective for the wavelength at which the component being analysed absorbs. The intensity of the transmitted light is compared with the intensity of the incident light by using a "blank" or reference cell filled with CO_2 free gas. The difference between the two signals is a measure of the CO_2 and may be measured directly on an indicator–recorder. In a null-balance instrument this difference is used to actuate a servo system. This adjusts a shutter situated in the light path of the reference cell, until the sample and reference radiations are matched. The shutter position is a measure of the CO_2 concentration and is displayed by means of a mechanical link on a recorder scale.

The instrument on which Telling *et al.* (1958) reported was a null-balance type made by the Infra Red Development Co. Ltd (now Hilger–I.R.D. Ltd, London N.W.1.). It had three scales, the sample cell being in three sections, the scale ranges being: A, 0–0·05%; B, 0–0·5%; C, 0–5·0%. The sampling rate was 1 litre/min. They reported that the reproducibility of the results was about $\pm 1\%$ of full scale. Stability was constant over a

6 day test. Over a 45 day period there was a significant gradual deterioration. This was inferred to be due to leakage of gas from the detector. The major breakdown rate of a group of three analysers was about once each six months per instrument. Water vapour in the sample gas results in significant error, but the effect of ethanol vapour can, for most purposes, be neglected. The practical examples of the use of the instrument were measurement of generation time in a batch culture, measurement of growth rate in continuous culture and use as a monitor in batch sorbose fermentations.

(b) *Optical paths.* Figure 2 is a diagram of the instrument. The light source, A, is divided into two similar beams that are converted into pulses by the

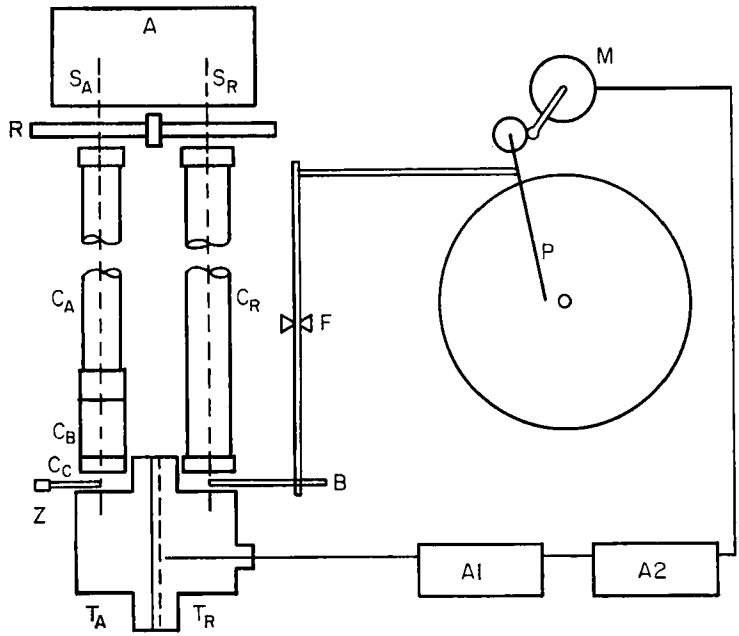

FIG. 2. The type SB/H analyser: A, light source; S_A and S_R, analysis and reference beams; R, rotating shutter; C_A, C_B and C_C, analysis cells; C_R reference cell ; T_A and T_R, detector compartments; Z, zero shutter; B, balancing shutter; F, pivot point of balancing shutter operating arm; M, servomotor; A1, A2, amplifiers; P, pen arm.

rotating shutter, R. The analysis beam, S_A, passes, in turn, through the analysis cells, C_A, C_B and C_C, and past the zero shutter, Z, which is used to equalize the effective intensity of the two incident beams. The analysis beam then enters compartment T_A of the selective detector, where the appropriate radiation energy is converted to heat. The reference beam, S_R,

passes through the reference cell, C_R, passes the balancing shutter, B, and enters compartment T_R of the detector, the appropriate radiations there being converted into heat.

(c) *The detector.* The division between the two compartments T_A and T_R is made by a flexible diaphragm containing a capillary, which allows the gas pressure on both sides of the diaphragm to equilibrate. The detecting element consists of a condenser, one plate of which is the flexible diaphragm and the other is a stationary metal grid placed near the diaphragm on the reference side of the detector. Another grid placed on the analysis side of the detector balances out the continuous electrostatic attraction between the two plates of the detecting element. The detector is charged to a fixed total pressure with a mixture of 5% CO_2 in argon to give a partial pressure of CO_2 of about 40 mm Hg. A satisfactory total pressure is 79 cm Hg. This ensures that even at the maximum barometric pressure encountered in the laboratory the detector pressure is always more than atmospheric—a practical aid when the detector needs re-charging. Because infrared absorption varies with temperature, the detector and the optical cells are each maintained by a thermostatically controlled heater at a constant temperature, which is higher than the maximum ambient temperature.

(d) *The flushing circuit.* The gas to be analysed is led into the required analysis cell through a 3-way tap. The same tap arranges that the two analysis cells not in use are connected in series with the reference cell. The flushing system is operated by a pump that draws air from the optical compartment and delivers through the purifier, through the reference cell and then through the two spare analysis cells and back into the optical compartment. In this way the optical paths inside the compartment are kept in similar condition to those in the reference and spare analysis cells.

(e) *Working principle.* This can be understood by considering the effect of an increase in the CO_2 concentration of the gas being analysed. Immediately before the increase in concentration, the radiation energy of the two beams entering the detector is equal, the balancing shutter having been adjusted by the servo motor until this is so. As a result, the pressure produced in each compartment during each short pulse is the same and the diaphragm is undeflected. No change is occurring in the capacitance of the condenser, so the servo motor and the pen are stationary. Immediately the concentration increases, the radiations are unequal—that reaching the reference side is greatest. Since the thermal capacity of the detector gas is small, the pressure produced in the reference compartment during each pulse will be greater than that in the analysis side. In consequence the diaphragm will vibrate. This will be shown as a repeated change in the capacitance of the condenser, which is used as a signal to start the servo motor, M. The direction of

rotation is such that the movement of the balancing shutter reduces the amount of effective light in the reference beam until it is equal to the new value of the intensity of the analysis beam. As a result the signal from the condenser is extinguished and the pen arm, P, has taken up a new stable position corresponding to the new concentration of CO_2.

The scale reading is correct at normal atmospheric pressure (P_s = 760 mm Hg). Infrared absorption is proportional to the mass of absorbing molecules in the light path and is therefore proportional to the absolute pressure of the gas in the analysis cell. Thus if, for an observed reading of Q_{obs} $\%CO_2$ the barometer is P_o mm Hg, then the corrected reading (Q_{act} $\%CO_2$) is equal to $Q_{obs} \times P_s/P_o$ $\%CO_2$.

(f) *Purification of gas in the flushing system.* If it is decided to re-standardize whenever a change of scale is made, soda lime (10–16 mesh) may be used by itself as absorbent. The bed is 5 in. long × 1 in. dia and is retained between cotton-wool plugs to prevent solids being carried into the cells. The absorbent weighs about 40 g, and is changed every third day when about 60% exhausted.

When scale changing without having to re-standardize is desired, the soda lime tube should be followed by a 12 in. length of 1 in. dia Pyrex pipe line containing 80 g of activated alumina (4–8 mesh, Peter Spence & Sons Ltd, Widnes, U.K.) with a layer of indicating silica gel at the exit, and retaining plugs of cotton wool at each end.

(g) *Drying and metering the sample gas.* The sample gas is passed at a controlled rate of 1 litre/min through an air-cooled condenser made from about 12 ft of $\frac{1}{4}$ in. bore copper tube of $\frac{3}{8}$ in. o.d. wound in a spiral, followed by a catchpot of about 1 litre capacity. The cooled gas is then dried by passing through a train consisting of—

(a) Two 12 in. lengths of 1 in. Pyrex pipe line, each holding 80 g of activated alumina retained between cotton wool plugs.
(b) One 6 in. length of 1 in. Pyrex pipe line holding 30 g of indicating silica gel also held between cotton-wool plugs.
(c) A filter to remove bacteria.
(d) A rotameter (range, 0·2–2·0 litres/min) followed by a T piece to which a water manometer (0–6 in. gauge) is connected.
(e) A sintered glass filter (10 μm particle size) connected to the analyser inlet by a well washed polythene tube in preference to natural rubber.

The manometer is used to ensure that the supply pressure never exceeds 2 in. of water, and is normally isolated with a screw clip. The final connection of polythene tube is to minimize the chance of solids entering the analysis cells. The drying tubes need renewing every 6–12 h.

Activated alumina and silica gel were chosen as drying agents highly suitable for routine use. In the dry state, both desiccants adsorb CO_2 to a slight extent and give it up when wet. When high accuracy is required the degree of adsorption should be investigated and, if necessary, an absorbent such as $Mg\,ClO_4$ used instead.

(h) *Standardization.* If it is desired to make measurements of an increasing magnitude (e.g., effluent air from a batch culture) then to get the greatest accuracy it is desirable to be able to make continuous measurements on successive scales during the course of a single experiment. If a drier is inserted in the flushing circuit this can be done, because then re-standardization is not necessary at a scale change. In other instances, observations on only one scale may be needed. In such cases soda lime alone is a satisfactory flushing circuit purifier.

Having ensured that the absorbent in the flushing section is in good order and that the detector is correctly charged, the meter should be switched on for at least 1 h, and preferably overnight, to become stable. It is best to adjust for zero on the most sensitive range (scale A) by passing CO_2 free nitrogen, dried as described, into the corresponding analysis cell. If the instrument is otherwise in order, the pen arm is then adjusted to read zero by moving the zero shutter, which controls the intensity of radiation in the analysis beam.

Standard gases are analysed by other methods (e.g., chemical absorption). Thus the analytical error on a standard gas suitable for use on scale C will be less, in terms of percentage of full-scale instrument reading, than on the other two scales. Hence scale C should be chosen for adjustment of scale reading, to obtain the best accuracy of standardization.

A gas containing Q_{act} $\%CO_2$ corresponding to 60–70% of full-scale reading is passed through the analysis cell (C_C). When a steady reading is obtained, if the observed reading Q_{obs} differs from $Q_{act} \times P_0/P_S$, then the pivot point, F (Fig. 2), of the arm operating the balancing shutter, B, is adjusted until the reading is correct. As this involves opening the case, the internal temperature will change and so time must be allowed to regain stability before taking further readings.

Standardization for use of one scale only is carried out in a similar manner by adjusting zero and scale reading on the range it is proposed to use.

(i) *Routine calibration.* The meter should be calibrated about twice weekly with the standardizing gas. The error is plotted on a quality-control chart on which the control limits are set at $\pm 1\%$ of full scale. This value is based on that of the reproductibility, which is, for practical purposes, $\pm 1{\cdot}0\%$ of full scale at the 95% confidence level. At these limits it can be expected that by chance about five in every hundred samples will fall outside the

limits. When correcting action is deemed necessary, before adjusting the instrument other more likely causes, such as a defective drying train on the sample line, or a breakdown of the flushing circuit purifier (which will also produce a zero drift) should first be examined. Failing a solution from these causes, instrument faults may then be considered, after first ensuring that the thermostat controlling the detector heater is working properly.

2. *CO_2 analysis by katharometer*

(a) *Principle.* Jones (1956) has also described various types of katharometer. Whitaker (1966) reported on the use of a katharometer supplied by the Cambridge Instrument Co. Ltd for the analysis of culture effluent gas. The instrument consists of a thermal conductivity meter (katharometer) mounted in a heat-insulated case. The temperature of the katharometer block is thermostatically controlled. The operation of the meter depends on the measurement of the thermal conductivity of the gas. The meter contains four small cells, each containing a glass-coated platinum wire identical with the others. The four wires form the arms of a Wheatstone bridge (Fig. 3). Two of the cells (B, D) are exposed to a reference gas. The other two (A, C) are exposed to the sample gas. The gas flows through the meter, but enters the cells by diffusion.

As explained in Cambridge Instrument Co. Ltd pamphlet (List 144/A), when the bridge current is constant and all four cells are exposed to the same gas, each wire will attain the same temperature and resistance. Under these conditions the bridge is balanced and no current flows through galvanometer G.

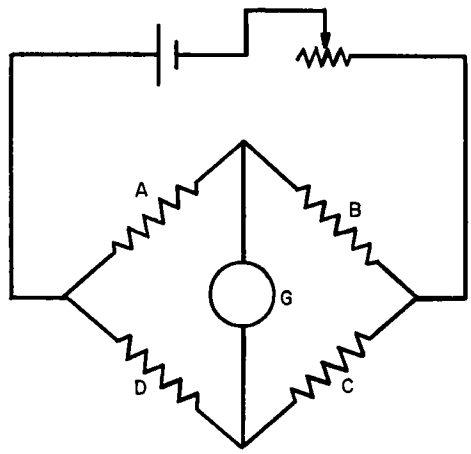

FIG. 3. Simplified katharometer bridge circuit: A and C, cells for sample gas; B and D, cells for reference gas; G, galvanometer (or recorder).

If two gas mixtures with different thermal conductivities, such as air and a CO_2–air mixture are introduced to the cells—air into cells containing wires B and D, and the CO_2–air mixture into the other two, containing wires A and C—then wires B and D will lose more heat to their cell walls than wires A and C. This is because the thermal conductivity of air is greater than that of CO_2. The consequent change in electrical resistance of the wires will unbalance the bridge and cause a deflection of galvanometer G, the size of the deflection depending on the difference between the thermal conductivity of the two mixtures. The galvanometer can, in this instance, be calibrated to show directly the percentage of CO_2.

(b) *Description.* In the equipment used by Whitaker, cells A and C are exposed to dry sample gas and cells B and D to dry sample gas from which CO_2 has been removed. Instead of using a galvanometer, the CO_2 content of the sample gas is displayed on a recorder with a scale graduated 0–5% CO_2.

The temperature of the meter block is normally controlled to $45° \pm 1°C$. The bridge circuit is supplied with a source of constant current and is adjusted at the factory to give correct output and constant sensitivity, both of which should remain constant during use. The zero, which is also set at the factory, may be subject to drift. It should not need checking at less than weekly intervals unless high accuracy is required. Zero is checked by replacing the *entire* CO_2 removal tube (which contains soda asbestos) with a tube containing $CaCl_2$, as is used on the gas supply to the reference cells. Thus when air is passed through the meter the gas in both sets of cells has had the same treatment. Having allowed an elapse of time, to obtain a stable reading, zero is adjusted by a rheostat.

(c) *The sample gas.* Water vapour in either gas stream can affect the reading so, as a safeguard, the sample gas from the culture vessel is passed through a water-cooled condenser ($T \leqslant 17°C$) fitted with catchpot. The cooled gas then passes through a bacterial filter, which also removes dust. This is followed by two 12×1 in. dia tubes containing activated alumina and indicating silica gel (see Section IVA.1(g) above), as used on the infrared analysers. By this means the gas is dried before it reaches the instrument panel and the life of the soda asbestos and $CaCl_2$ in the instrument absorption chambers is increased.

The analyser requires a total flow of about 300 ml/min of sample gas to maintain each of the two flowmeters at its correct level. The gas flow is not critical provided it is not more than a total of 500 ml/min. At the instrument panel, the gas is divided into two flow paths each controlled by a needle valve and measured on the flowmeters situated on the meter exit. After passing the needle valve, one gas stream passes through $CaCl_2$ to remove

water vapour. This is the sample stream. The other stream passes through soda asbestos, which removes CO_2 and also dries the gas. This is the reference stream. The difference in thermal conductivity of the two streams thus gives a measure of the CO_2 content which is displayed on the recorder.

(d) *Performance.* Testing the meter over its full range on batch cultures, Whitaker found satisfactory agreement with Orsat analysis. In a 5 day stability test the meter was operated on air, except that twice each day a calibrated supply of 3% CO_2 was substituted. The reading obtained for the test supply agreed within $\pm 0{\cdot}1\%$ CO_2 of the expected value (i.e., $\pm 2\%$ of full scale). There has been no major breakdown in $1\frac{1}{2}$ years' service.

B. Continuous O_2 analysis

1. *Principle of method*

There are two methods for the continuous analysis of O_2 in gases, both of which depend on the paramagnetic properties of O_2 gas. In fermentation work, the magnetic wind instrument (Medlock, 1952) appears to be less in favour than the Pauling type (Hoover *et al.*, 1954; Jones, 1956; Ent, 1961). In the Pauling type, a dumb-bell formed by two hollow glass spheres is suspended on a taut fibre in a magnetic field. It is contained in a test chamber, held at constant temperature, into which the sample gas is introduced. When the sample gas contains no O_2 the dumb-bell takes up a reference position where the torque on the fibre is balanced by the magnetic field. When a gas containing O_2 is introduced the magnetic force is changed. The spheres are repulsed and rotate to a new position until the torque on the fibre again balances the magnetic field. The degree of rotation is a measure of the O_2 concentration. There are two classes of meter: (1), an indicating meter that reads the displacement of the dumb-bell directly; (2) a null-balance meter. In the automatic version of the latter, a photocell senses the dumb-bell position and actuates a servo system, which in one example applies current to a coil wound on the dumb-bell, and supplies a rotating torque to return the dumb-bell to its reference position. The current in the coil is a measure of O_2 concentration and is displayed on a recorder. In a simpler and therefore cheaper indicating version, the null-balance is set manually each time a reading is taken. According to the makers' claims, for a scale range 16–21% O_2 the indicating direct-reading types measure to an accuracy of $\pm 0{\cdot}2\%$ O_2. The manual and automatic null-balance types, which are more stable than the direct-reading instrument, will both measure to an accuracy better than $\pm 0{\cdot}1\%$ O_2.

2. *The Pauling type*

There is only a restricted number of makes of the Pauling types of O_2 analyser. In the U.K., Servomex Controls Ltd produce a range of meters.

A similar range, the design of which apparently originates in the U.S.A., is made by Beckman Instruments Ltd.

(a) *General.* The instruments are obtainable as single-scale units with various spans and as multi-scale units. A scale range of 16–21% O_2 is suitable for examining effluent air from a culture vessel. There is usually provision for adjustment of scale span, which needs to be undertaken infrequently. The electrical output is linear with respect to O_2 concentration. This makes it possible to calibrate the instrument by checking at two points only, one of which may be provided by atmospheric air. At the lower end (16%) a cylinder of an air–N_2 mixture, analysed chemically, could be used.

(b) *The sample gas.* In analysing culture effluent air, no special precautions need be taken in treating the sample gas. It should be freed from dust by filtration, and should be unsaturated with respect to water vapour, but not necessarily dry. Magnetic susceptibility is a function of temperature. Hence the temperature of the detector unit is controlled thermostatically, but the temperature of sample gas at entry may vary from as low as $-10°C$ to 40°C.

(c) *Operating pressure.* Like the infrared analyser the instrument measures partial pressure. Pressure variations such as may result from changes in flow rate should be avoided. The permitted range for the sample rate is wide—say 50–250 ml/min—but note that changes in sample rate will affect response time. For maximum accuracy, either a pressure controller should be fitted, or the barometer should be read simultaneously, and the meter reading corrected accordingly (see Section IVA.1(e)).

3. *Applications*

There are more references to culture equipment which is equipped for analysis of the effluent gas than there are actual examples of the application of the technique to throw light on a given problem. Seigell and Gaden (1962) described a system in which the dissolved O_2 in a yeast culture is measured and automatically controlled. There are O_2 analysers on the inlet and effluent air streams which measure the depletion of O_2 by the culture. There is a flow controller on the inlet air stream. From these measurements they give results for O_2 uptake rates. In their calculations they appear to assume that the effluent gas rate is the same as the inlet air rate. This implies that when O_2 is absorbed, it is replaced by an equivalent volume of CO_2. This is not necessarily so, and if quantitative work is the aim then in practice, as will be shown in Section V, the effluent air must be analysed for CO_2, O_2 and N_2. The possibility of CO_2 accumulation in the medium may also bear examination.

C. Combined CO_2 and O_2 analysis

In the study of aerobic yeast cultures Phillips (1963) recognized the need for analysing for both CO_2 and O_2. The effluent gas was analysed for O_2, but periodically a scrubber that removed CO_2 was interposed in the stream before the O_2 analyser. In this way, from the increase in O_2 concentration in the scrubbed gas, he was able to estimate the CO_2 concentration in the effluent gas and so provide data from which O_2 absorption could be calculated.

V. CALCULATION OF RESULTS

A. Aerobic cultures

To assess O_2 consumption and CO_2 evolution in an aerobic culture the experimental information required is the inlet air rate, CO_2 and O_2 concentration in the effluent air and the effluent gas rate. If the inlet air is measured, and the N_2 concentration in the effluent air is known, then the effluent air rate can be calculated. In fully aerobic cultures it can be assumed that the effluent gas contains only CO_2, O_2 and N_2, so that $\%\ N_2 = 100 - \%CO_2 - \%O_2$. In the case of some facultative aerobes, at low or limiting conditions of aeration, there is a chance that the effluent gas may contain other components (e.g., H_2, volatile organic compounds). In this case a complete gas analysis (Strouts *et al.*, 1955) would be required in order to estimate N_2. However in such a case it might be easier to fit a meter and measure the effluent gas directly. The following calculations assume that the effluent gas contains only CO_2, O_2 and N_2.

Given that inlet air rate, expressed as dry gas at STP is A litres/min and that the concentration of O_2 is $o\%$ and of CO_2 is $c\%$, then since the analysis of air is 20·87% O_2, 0·03% CO_2 and 79·1% N_2 the supply of O_2 and CO_2 to the vessel is given by —

$$o_i = 20{\cdot}87\,A/100 \text{ litres/min of } O_2$$
$$c_i = 0{\cdot}03\,A/100 \text{ litres/min of } CO_2$$

Considering the effluent air, by assuming that it contains only CO_2, O_2 and N_2, then the N_2 content is $100–o–c = n\%$. Since N_2 is assumed inert, the rate of N_2 leaving the vessel is the same as that going in (0·791 A litres/min). If the effluent gas rate is E litres/min, then—

$$nE/100 = 0{\cdot}791\,A$$
$$\therefore E = 79{\cdot}1\,A/n$$

The O_2 and CO_2 leaving the vessel are given by—

$$o_e = oE/100 \text{ litres/min of } O_2$$
$$c_e = cE/100 \text{ litres/min of } CO_2$$
$$\therefore O_2 \text{ absorption} = (o_i - o_e) \text{ litres/min}$$
$$CO_2 \text{ evolution} = (c_e - c_i) \text{ litres/min}$$

If c_e is large, the correction for c_i may be neglected, but note the calculation has not taken into account the possibility of CO_2 accumulation in the culture medium.

B. Anaerobic cultures

Given successive meter readings for effluent gas, the rate at the time of gas sampling is calculated and corrected to dry gas at STP. If this is E litres/min and the CO_2 analysis is $c\%$, then CO_2 evolution is $Ec/100$ litres/min. Note again that account has not been taken of accumulation of CO_2 in the medium.

VI. CONCLUSIONS

Techniques are available for continuous analysis of effluent gas streams for CO_2 and O_2, but there are not many references to their use, and little or no serious quantitative work has been published. One reason is perhaps the expensive nature of the equipment. The other, and probably the more important, is that the measurements by themselves are not highly informative, except in producing material balances. Most deep cultures present a complex situation in which concepts like respiratory and metabolic quotients are not always amenable to direct measurement. An aerobic culture generally starts off consuming the carbon source substantially by assimilation in order to produce cells. Given an ammonium nitrogen source there are simple relations between CO_2 evolution and O_2 absorption. If a protein hydrolysate is the nitrogen source, the situation is less simple. In any phase of product formation that follows the cell-production stage, the process will most likely be largely dissimilative and accompanied by changes in O_2 requirement. For proper interpretation of effluent gas analyses, other factors need to be considered. These include, besides measurement of the product being sought, dissolved O_2 concentration or redox potential, or both. Both techniques are, for most workers in deep culture, still in the development stage. When these measurements are matters of routine, the use of CO_2 and O_2 analysis will be stimulated.

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CHAPTER X

Flow Measurement and Control

G. A. PLATON

G. A. Platon Ltd, Basingstoke, Hants., England

I. INTRODUCTION

The parameter "rate-of-flow" is vital in all continuous processes—physiological, preparative, and analytical. Its much deeper significance derives from the facility of its modulation, which allows the secondary control of many other parameters important to the biochemist, such as pH, temperature, and dissolved gas concentration. Fluids are infinitely varied. This article attempts to cover gases, gas mixtures, and true liquids, but touches only lightly on non-Newtonian liquids containing cells and excludes foams, slurries and fluidized solids. Instantaneous rate-of-flow instruments are given prior consideration, but references are made to volume measurements and to flow-inducing devices, particularly in those circumstances where the accuracy of inferential measurement is inadequate. It

must be understood that instantaneous flow-rate can only be measured in practical fashion by inferential methods and that accuracy of the order of $\pm 1\%$ is extraordinarily good; reference to the British Calibration Service Survey 6001 will show the paucity of rigs and standards available, even for the commonest of fluids. Practicality also confines this study to fluids in closed pipes, to the exclusion of open-channel flows and current rates, which are of marginal interest to the microbiologist and the biochemist.

II. TYPES OF FLOWMETER

Group A flowmeters depend on conversion of fluid kinetic energy and require some modification of flow pattern. They can be classified as—

(i) Differential-pressure.

(ii) Variable-area.

(iii) Target (or impact).

(iv) Turbine.

Group B flowmeters utilize fluid properties that can be sensed outside the pipe and do not necessarily disturb the flow pattern. They can be classified as—

(i) Electrical (e.g., conductivity, ionization cross-section).

(ii) Thermal.

(iii) Sonic.

These two lists cover all types in common use, but many others exist and the number grows rapidly as research expands. The choice of flowmeter may depend on any of a large number of specialized requirements—sterility, high response speed, uncommon construction material, suitability for operation in extreme cold or high vacuum, minute size—so that no single type is universally acceptable. The only logical approach is the historical one followed in the above classification.

A. Group A flowmeters

Group A flowmeters are of earlier origin and their calibration depends only on the density and viscosity of the fluid, providing it is Newtonian. The equation of Bernoulli governs the flowmeter shown in Fig. 1 since they all utilize an element restricting the fluid flow.

These instruments are all affected by the viscosity of the fluid according to the geometric design, and the many proprietary designs marketed all have different calibration formulae.

Only the differential-pressure flowmeter (Fig. 1a) has reached any degree

of standardization, the design and formulae for various orifice plates, venturis and nozzles being embodied in B.S. 1042/64. The limitations are nevertheless serious, the designs being suitable only for pipes 1″ dia. and larger, and for Reynolds number 250 upwards. Furthermore the design of the read-out device is not given and the mercury manometer, to which reference is made, is not acceptable in many laboratories. Dry differential-pressure indicators have not been developed to the state where lasting accuracy and reliable resistance to overloads can be expected. Proprietary differential-pressure transmitters (d-p cells) are now the most commonly used elements, for the application of this type of flowmeter is predominantly on full-scale plant justifying the cost of automatic controls systems. In the laboratory the d-p flowmeter is invariably home-made by the glass-worker and calibrated by experiment on the fluid it is required to measure. The dead-water pockets at the pressure tappings often present problems for liquid measurements and the instrument is more commonly used for small gas flows. However made, the d-p direct indicating flowmeter suffers the disadvantage of "square-root" scale shape, which limits the range owing to cramped readings below 40% of full-scale reading. Elimination of this characteristic can be achieved mechanically or electrically in transducers with appreciable increase in cost.

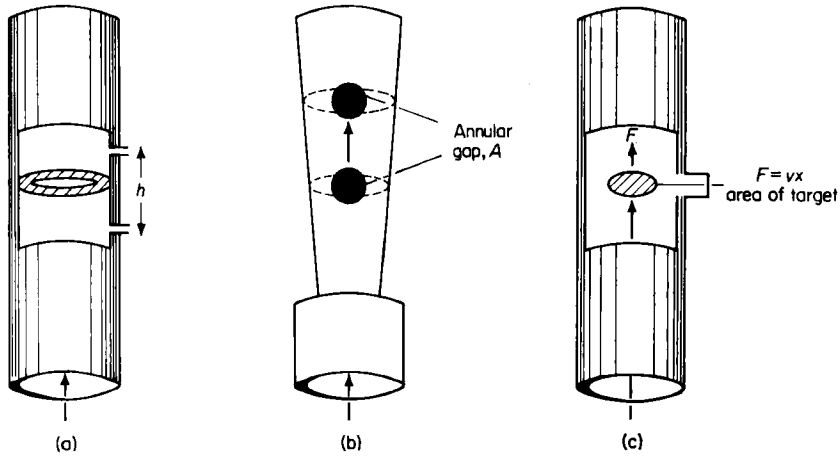

FIG. 1. Group A flowmeters: (a) differential-pressure, $h \propto Q^2$; (b) variable-area, $A \propto Q$ (h is constant); (c) impact, $F \propto Q^2$.

The variable-area flowmeter (Fig. 1b) is produced in far larger quantities than any other, being of simple, inexpensive construction, and enjoying the advantages of low and constant pressure drop together with linear

scale shape. The most common construction utilizes a vacuum-formed, precision-tapered Pyrex glass tube containing a freely moving indicator float; placed in the pipe with largest bore uppermost and with the fluid flowing upwards, the height to which the float is lifted is the measure of the flow rate. The two metering components are easily made of inert, sterilizable materials and the operation is frictionless so that lasting accuracy results.

Brand names of variable-area flowmeters include Flowrator, Gapmeter and Rotameter—the latter being derived from the flutes commonly cut in floats to impart a self-centring rotation. Floats are made in many shapes, e.g., simple spheres in hard materials, stream-lined for high-capacity and with sharp edges to minimize viscous drag.

The variable-area flowmeter is suitable for measuring very small flow-rates, i.e., down to a few ml/min of gas, but when scaled-up to large sizes the glass tubes become expensive and somewhat hazardous. A common read-out characteristic can, however, be achieved as illustrated in Fig. 2. The

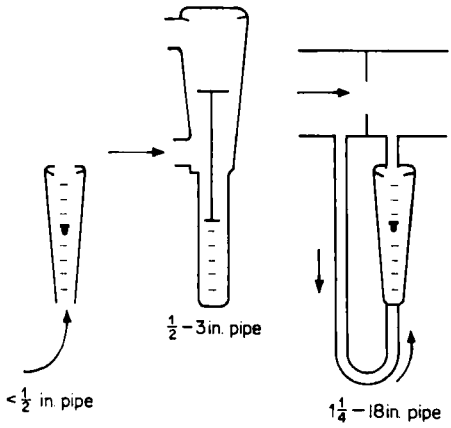

FIG. 2. Variable-area flowmeters with common read-out characteristic.

small size uses a tapered glass tube, the medium size has a glass extension pocket to a tapered tube in metal or plastic, and the large size uses a tapered tube in the "shunt" line around an orifice plate. Such meters are normally direct indicating and have rapid response speed; they are, however, readily fitted with standardized signalling devices to operate alarms or remote recording and control equipment. "Sterile" designs and laboratory kits are commonly available.

The target or impact flowmeter (Fig. 1c) is a speciality, suitable for slurries and liquids (however black) and available in a sterile design that is rapidly

demountable from pipelines to BS 1864. The force on the target is measured either with a strain gauge or a pneumatic force-balance device. It functions only with fluids moving at quite high velocity and allowing significant energy dissipation.

The turbine flowmeter was introduced for the measurement of liquid fuel supply to rocket motors primarily for high response speed and tolerance to shock flows. Such success has been achieved with the design of self-cleaning bearings in a variety of materials that the accuracy of measurement of gases as well as liquids has reached a very high level, and the instrument has become widely popular. The turbine flowmeter consists of a free-running rotor mounted axially in a piece of pipe and virtually filling its cross section; with current meters and anemometers the piece of pipe is portable. The speed of rotation imparted by the flowing fluid is a linear function of flow rate over a wide range after a rather high threshold (say 3 ft/sec). Electrical output signals, analogue or digital, are conveniently obtained from such devices at modest cost, and are much preferred to mechanical tachometers for accuracy. The smallest size of turbine is likely to be for $\frac{1}{2}$ in. pipe.

B. Group B flowmeters

Group B flowmeters are more recent innovations based either on inherent properties of the measured fluid or the modulation of some property by external means. They have the advantage that no fluid kinetic energy is lost, and can therefore be applied to very small or very weak flow conditions.

Electrical flowmeters include both sorts. The "magnetic" flowmeter normally consists of windings outside the pipe energized to create a field so that the movement of a conducting fluid through the pipe induces a measurable e.m.f. between two electrodes in the non-conducting pipe wall. This meter is limited to the measurement of liquids possessing a conductivity value exceeding a significant threshold value. In practice the magnetic flowmeter is extensively applied to aqueous liquids, for it can be made in very small and very large sizes in a wide range of resistant materials and because it is unaffected by entrained gases or solids (it was originally designed for sandy water in dredging operations).

The meter function with liquids containing cells is to measure the total volume flow rate, and while it is used in the construction described for *in vitro* blood flow measurement modified designs have been developed for *in vivo* use.

The ionization flowmeter typically uses a radioactive source to ionize a gas, which, flowing along the pipe, deposits a charge on an electrode forming part of the pipe wall, the charge being proportional to gas flow rate.

Again, in practice, the ionization flowmeter is limited to measurement of pure gases with known ionization cross section, so that the calibration is a difficult problem.

The thermal flowmeter is used for small gas flows, particularly at high pressure or with radioactive properties. Two symmetrical coils are wound on the pipe and wired in a bridge circuit so that the flow rate is read out as a voltage change. A significant time lag is inherent, but can usually be tolerated for such difficult applications.

The sonic flowmeter depends on the doppler effect on a signal transmitted through the pipe, but the practical difficulties make it expensive. It has been refined for such difficult flow measurements as those of gas borne solids.

III. TYPES OF FLOW CONTROLLER

A. Flow inducers

Where measurement of flow-rate is difficult or insufficiently accurate due to variations in, or uncertainty of, fluid properties, positive displacement devices are used such as metering pumps, motorized syringes or peristaltic pumps, according to the rates required. The peristaltic pump (Fig. 3) consists of a flexible pipe through which the liquid or gas is squeezed by the action of rollers driven by a variable-speed electric motor, and designs have been refined to the point of producing entirely smooth (non-pulsatile) flow. The flexible pipe is cheap and disposable in the interests of accuracy and sterility. The flow range of the instrument is very broad and it has achieved great popularity for applications where the controlled fluid has low potential energy. The metering pump is more expensive, being made of rigid materials, and often has pulsatile output which can be disadvantageous, particularly when the flow-rate adjustment depends on a change of piston stroke. The motorized syringe is particularly suitable for extremely small liquid flow rates.

B. Hand valves

Manual control of flow rate is used where the supply and back pressure conditions are stable, and the choice of valve depends on the application. For best accuracy the valve should be incorrodible, of rigid materials and designed to obviate accumulation of silt or gas bubbles. Glass stopcocks, stainless steel needle valves, ball valves and countless others all have their uses, but it should be remembered that sleeve valves and diaphragm valves with restrictive devices of flexible materials need constant monitoring.

The flow performance of any valve is a function of its size and "characteristic". The size of a valve is determined by its maximum flow-channel area

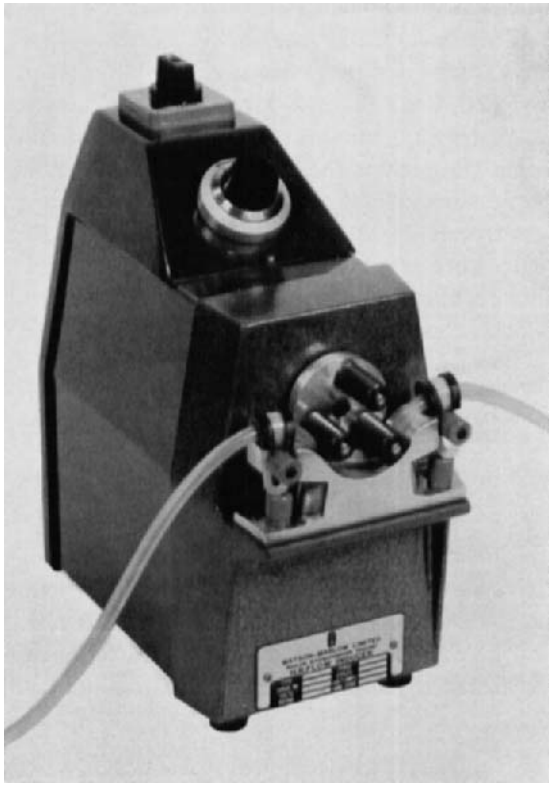

FIG. 3. Peristaltic pump. (By permission of Watson Marlow Ltd.)

(not its pipe connection) and should be chosen to suit the maximum flow required at the minimum pressure drop envisaged. Many manufacturers are strangely reluctant to provide adequate performance data on their products, but the most helpful define their valves by capacity (*Cv*). The unit *Cv* defines a fully open valve passing 1 g/min water at 60°F with pressure drop 1 psi. Typical formulae for calculating valve capacities are given below, but the units used are *not* universal and manufacturers' literature must be carefully scrutinized (e.g. Imperial or U.S. gallons). For liquids—

$$Cv = \text{Gal/min} \sqrt{\frac{\text{Specific gravity}}{\text{Pressure drop, psi}}}$$

For gases—

$$Cv = \frac{\text{Standard cu ft/h}}{1360} \sqrt{\frac{\text{Specific gravity} \times (460 + °\text{F})}{\text{Pressure drop, psi} \times \text{Downstream pressure, psia}}}$$

The characteristic of a valve relates the travel of its control element to the flow rate achieved. A *linear* valve has travel directly proportional to flow, an *equal percentage* valve gives more sensitive flow adjustment as the closed position is approached, whilst a *quick-opening* valve is most suited to on–off control. Very few valves are suitable for modulation over flow range greater than 20 : 1, so that the greater the range required the more carefully the valve must be sized. The use of a supply pressure regulator is a great aid to stable flow control of a gas and further provides the useful facility of selecting a valve pressure drop suited to the valve size available. The cheaper forms of pressure regulator are progressively less precise as the flow rate is reduced and precision regulators with a bleed to atmosphere must be used at very low flows.

C. Automatic controls

These are listed below in order of increasing sophistication.

1. *On–off control*

On–off control, most commonly by electric signal to a solenoid valve, is used for protective interlocks and for those systems where cycling of the controlled value is tolerated (e.g., thermostat operated heating or cooling circuit). The flow alarm switch is of growing importance in the protection of plant and processes from failure of supply of a constituent fluid or ancillary.

2. *Open-loop control*

Open-loop control is used where the control action is exactly predictable, e.g., the transmitting flowmeter in stream A gives a signal that controls the motor speed of a peristaltic pump in additive B so that A and B remain in constant ratio. Calibrated valves relating input signal to flow rate are few and far between, and the only reliable types include a measuring element.

3. *Self-acting flow control valves*

Self-acting flow control valves are simple closed-loop controllers using some energy of the controlled fluid rather than an external power supply (Fig. 4). The "Flostat" principle is clearly seen from the diagram to embody a balanced control valve which is positioned by a diaphragm sensitive to the pressure difference created at a flow measuring orifice. Increasing load $P_1 - P_3$, causes increased differential $P_1 - P_2$ and moves the valve towards the closed position so that the preselected flowrate is restored. With a liquid this device gives constant flow rate despite changes in either upstream or downstream pressures, provided the pressure drop exceeds a threshold value chosen by the designer; since this threshold may be anything between 10″ water gauge and 25 psig the selection of the controller must be made with care. With a gas the constant flow rate depends on the pressure at the meter-

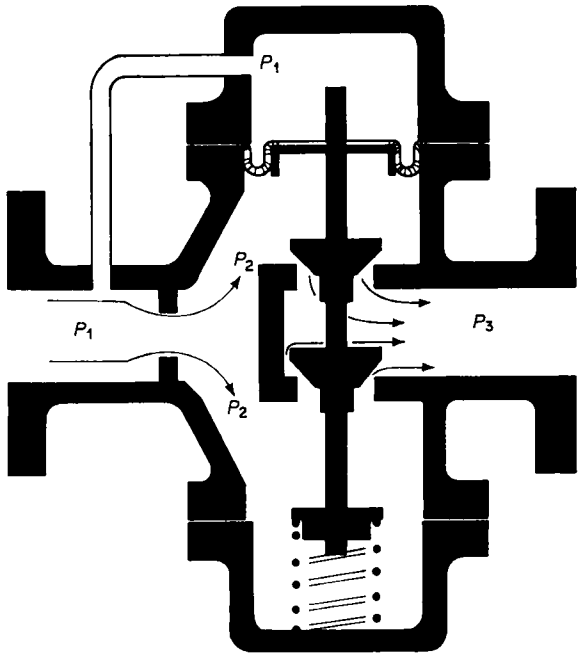

FIG. 4. Self-acting flow control valve.

ing orifice and two models are made, one with orifice upstream of the valve and the second with downstream orifice. The first model is used where the gas supply is at regulated pressure, and the second for those applications where the supply pressure fluctuates but the back pressure is constant (e.g., atmospheric). The metering orifice in a self-acting flow controller can be factory set for one particular flow rate, but is more often manually adjustable and provided with a flow scale. Some makers also fit actuators accepting pneumatic or electric signals so that the controlled flow can be linearly related to another metered variable (see Section IIIC.2 above).

4. *Closed-loop or feedback systems*

Closed loop or feedback systems involve the use of a controlling comparator to compare the signal transmitted by a flowmeter in the slave line to the desired value (set by the signal transmitted by the master parameter) and to send a correcting signal to the control valve in the slave line. These systems are of the high capital cost originally tolerable only in oil refineries and similar large-scale plant, and their installation and maintenance used to require highly-trained personnel. Tremendous progress has, in recent

years, been made towards simplification and cost reduction, both with electronic and pneumatic controllers. Whilst electronic controllers would be preferred by the majority of laboratories, it must be remembered that there is still no satisfactory alternative to air operation of the final control valve.

5. *Flow programme systems*

Self-acting flow control valves and the servo-operated valves used in feed-back control can be fitted with programme devices to vary flow-rate according to a predetermined time-base.

IV. FLOW MEASUREMENT PROBLEMS

A. Calibration

Simple, direct-reading flowmeters of the v-a type are the most flexible tools for investigating processes and phenomena in the laboratory, and they are cheap because of large-scale production. However, the smaller the flows being measured the greater are the calibration problems and users must be prepared to carry out experimental calibrations when uncommon and non-Newtonian fluids are being handled. An important virtue of the v-a meter is that its precision is of a high order; this means that once a calibration has been made the repetition of readings can be expected to fall within 0·5% of indicated flow rate. The v-a meter is sensitive to fluid density and viscosity. The volumetric flow capacity of the instrument varies inversely as the square root of the density of gases, but with liquids the bouyancy of the float is involved and the variation is inverse to the square root of the liquid density minus float density. The viscosity effect is not straightforward, being greater at the lower readings in every size of meter, and becoming dominant in very small instruments; e.g., at 1 ml/min water flow the effect of 1°C temperature change is inaccuracy of approx. 1%. Fortunately, as predicated calibrations become less reliable at low flows, the apparatus for experimental calibration becomes easier to handle.

Using a stopwatch and balance it is a simple matter to carry out a liquid calibration, but temperature control and cleanliness are vital elements in the accuracy achieved. Gas calibrations are equally straightforward when the "Volumeter" is used. This apparatus comprises a precision-bore glass cylinder, volumetrically calibrated and fitted with a lightweight piston floating on mercury "O" seals. It is infinitely easier to use than the earlier soap-film burette, suffers none of the problems of gas solubility found with "wet" gas meters (and soap films) and escapes the mechanical shortcomings of the "dry" gas meter. Particular care must, however, be paid to barometric pressure measurement and subsequent flow readings must be

corrected by applying a multiplying factor—

$$\times \sqrt{\frac{\text{Observed absolute pressure}}{\text{Calibration absolute pressure}}}$$

Larger v-a meter calibrations can be predicted by the makers with high precision and conversion scales are readily obtainable from their computers. High-viscosity and non-Newtonian liquids still demand experimental calibrations and the user must turn to the most convenient positive displacement meter available. The dynamic-weighing apparatus used by meter manufacturers, which requires only a small volume of liquid to secure a high flow-rate calibration, is expensive to buy but can sometimes be hired.

B. Installation hazards

Providing the v-a meter is truly vertical and the float is rotating, the flow reading will be accurate. This statement assumes that the fluid is in a pure state and care must be taken to avoid contaminating solids or gases in liquid or condensing vapour in a gas. The float weight in a liquid will be disturbed by deposited sediment or the adhesion of gas bubbles, and the annulus area will be reduced by condensation of vapour or deposition of algae on the wall of the metering tube.

V. FLOW CONTROL APPLICATIONS

These have been selected to exemplify the use of various metering and control instruments.

A. pH control

This parameter is vital in all microbial culture techniques and particularly the suspended culture of tissue cells, where control within at most 0·1 unit is required. The pH meter is provided with limit switches to give on–off action of solenoid valves in the flow lines of acidic and/or alkaline additives. For maximum precision the differential of the pH switching must be small, and the action of the corrective agent must be gentle. Gases are more easily and precisely controlled than liquids at low flow rates and FIC sets (flow indicating-controller) comprising v-a flowmeter and self-acting controller are used for CO_2 gas to control pH (Smith and Burrows, 1966).

B. Dissolved oxygen control

Since this parameter affects both the growth rate and metabolism of micro-organisms its control in fermentation is critical, and manual methods have been superseded by automatic. The dissolved oxygen recorder is used to provide either on–off or modulated signal to a correcting device

which is most sensitively the composition of the aerating gas. The on–off signal can operate a valve to add air or oxygen to nitrogen (MacLennan and Pirt, 1966) both lines being fitted with FIC sets, whereas the modulated signal can be used to adjust an oxygen–nitrogen mixing valve (Siegall and Gaden, 1962), the total flow being kept constant by a self-acting flow controller.

C. Gas blending

The production of accurate gas mixtures in the laboratory is essential to studies of microbial growth and activity. The Flostat automatic gas blender (Fig. 5) operates on the synchronous delivery of any number of constituent

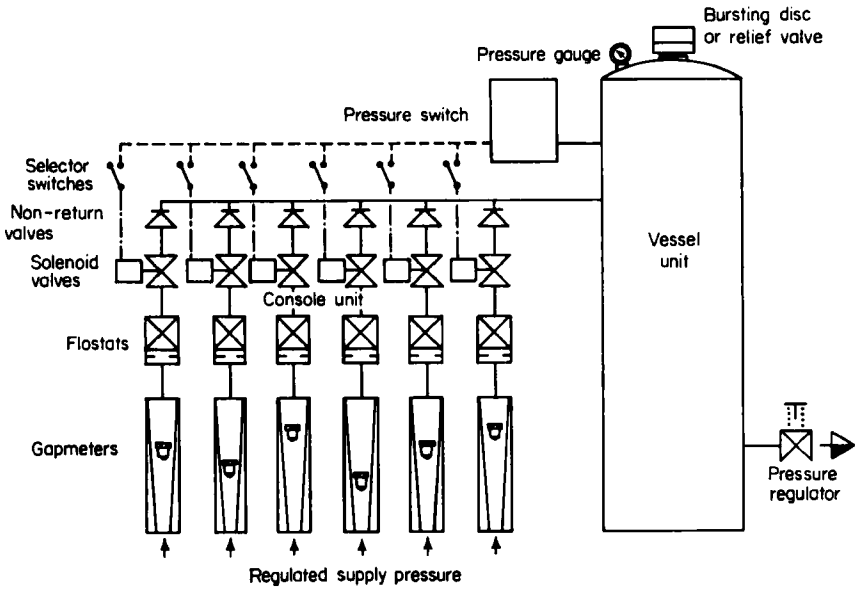

FIG. 5. Schematic diagram of Flostat automatic gas blender.

gases to storage so that an accurate blend is achieved despite variable and intermittent demand. The vital elements determining the accuracy of the blend are the self-acting flow controllers which maintain the pre-selected flow rates despite changes in vessel pressure.

VI. LIST OF MANUFACTURERS

The following is a list of manufacturers specializing in control equipment for small flows—

A.E.I. Ltd., Instrumentation Division,
Barton Dock Road, Urmston, Manchester.

Brooks Instrument Ltd.,
Brooksmeter House,
Cross Lane, Marple, Stockport, Cheshire.

Crouzet England Ltd.,
Thanet House,
High Street, Brentford, Middlesex.

Fischer and Porter Ltd.,
Salterbeck Trading Estate, Workington, Cumberland

A. Gallenkamp & Co. Ltd.,
Technico House,
6 Christopher Street, London, E.C.2.

Jencons (Scientific) Ltd.,
Mark Road, Hemel Hempstead, Herts.

Meter-Flow Ltd.,
North Feltham Trading Estate, Feltham, Middlesex.

G. A. Platon Ltd.,
Wella Road, Basingstoke, Hants.

Pressure Control Ltd.,
Davis Road, Chessington, Surrey.

Rotameter Manufacturing Co. Ltd.,
330 Purley Way, Croydon, Surrey.

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Smith, H. M., and Burrows, T. M. (1966). *Lab. Practice*, **15**, 864–866.
MacLennan, D. G., and Pirt, S. J. (1966). *J. gen. Microbiol.*, **45**, 289.
Siegell, S. D., and Gaden, E. L. (1962). *Biotechnol. Bioengng*, **6**, 345.

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CHAPTER XI

Application of Hydrostatic Pressure to Microbial Cultures

RICHARD Y. MORITA

Departments of Microbiology and Oceanography, Oregon State University, Corvallis, Oregon, U.S.A.

I. INTRODUCTION

Hydrostatic pressure is generally not employed by investigators working with organisms that normally live at 1 atm. However, in the aquatic environment the Ideal Gas Law must be taken into consideration, since all organisms living below the surface of the water live under various degrees of hydrostatic pressure. In the ocean, the rough rule of thumb is to apply an increase of 1 atm for every 10 metres in depth. Recognizing that the deepest oceanic environment is greater than 10,000 metres, 1100 atm is roughly the highest pressure at which organisms exist in the biosphere. However, the application of pressure can be an important variable in the study of biological processes not involving organisms that live in the abyssal portions of the oceans. Applications of pressure have been used in the study of hydrophobic bonding of proteins (Kettman *et al.*, 1965), sol–gel reactions in cells involved in cytoplasmic movement (Marsland and Brown, 1936; Landau *et al.*, 1954; Landau, 1959), pH change in sea water (Buch and Gripenberg, 1932, Distèche, 1959, 1962, 1964; Pytokowicz and Conners, 1964), responses of marine animals to pressure (Knight-Jones and Morgan, 1966) and the germination of seeds (Vidaver and Lue-Kim, 1967).

Hydrostatic pressure as it affects micro-organisms is reviewed by Johnson

et al. (1954), Johnson (1957), Hedén (1964), ZoBell (1964) and Morita (1967), and its influence on marine organisms by Knight-Jones and Morgan (1966). Hydrostatic pressure equipment designed by various investigators is listed by Morita (1967), who includes such items as pressure viscometers, optical rotation cells and electrochemical measurements under pressures. It should be remembered that no manufacturer produces pressure equipment, and most investigators have employed their own design or modified other designs for their own special use.

Since micro-organisms as well as the various sub-units of micro-organisms are bathed in an aqueous menstruum, the investigator should always

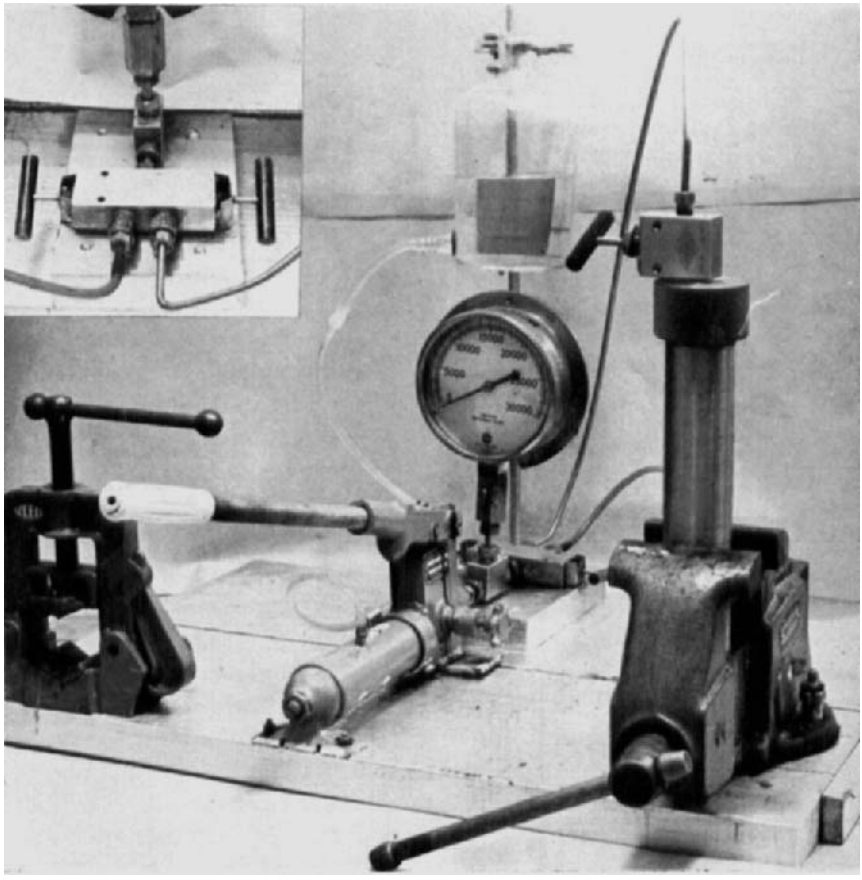


FIG. 1. Pressure pump assembly with pressure cylinder; insert, rear of the assembly.

take into consideration the effect of hydrostatic pressure on the physical and chemical environment. Some of the factors that the investigator should be cognizant of are the pressure effects on ionization of water (Owen and Brinkley, 1941; Hamann, 1963), viscosity of fluids (Horne and Johnson, 1966), pH (Buch and Gripenberg, 1932; Distèche, 1959; Pytokowicz and Conners, 1964), chemical reaction rates (Hamann, 1964), ionization of various substances (Owen and Brinkley, 1941; Hamann and Strauss, 1955; Ewald and Hamann, 1956; Hamann, 1963; Distèche and Distèche, 1965), hydrophobic bonding of proteins (Kettman *et al.*, 1965) and viscosity of water (Horne and Johnson, 1966, 1967).

II. HIGH-PRESSURE APPARATUS

A. Pressure-pump assembly

Various types of pressure pumps have been employed to generate the needed hydrostatic pressure. The most widely used type in the U.S. for biological research is a modification of a high-pressure hydraulic truck jack (Enerpac Model P 228, Blackhawk Industrial Products, Butler, Wisconsin), in which a substitution of a mixture of glycerine and water (1 : 1) for the hydraulic fluid is employed (Fig. 1). This substitution is deemed necessary since the hydraulic fluid normally used in this pump is a hydraulic oil that would render the material inside the pressure cylinder very unworkable. Glycerine and water can be washed off very readily with water. The use of glycerine and water necessitates the overhauling of the pump periodically owing to the corrosion that takes place. The use of glycerine helps retard the corrosive action of water.

In the prefabrication of the pump assembly, super-pressure fittings and tubing are employed throughout. The pump is generally the weakest link in the entire assembly as far as pressure tolerance is concerned. These items can be purchased from various manufacturers specializing in super-pressure equipment. Figure 2 gives a schematic drawing of the parts necessary to assemble the unit with each part in its proper place.

The truck jack is modified so that the reservoir hydraulic oil supply (see Fig. 1) can be continuously fed by the glycerine–water mixture through a port. This port is made by drilling a hole into the main body of the reservoir hydraulic oil supply (see A of Fig. 2) and welding a piece of tubing to it. A piece of Tygon tubing is then connected to the welded tubing and connected to an aspirator bottle filled with the glycerine–water mixture. The original hydraulic fluid that comes with the hydraulic pump should be discarded and the internal portions of the pump cleaned to rid the system of the oil.

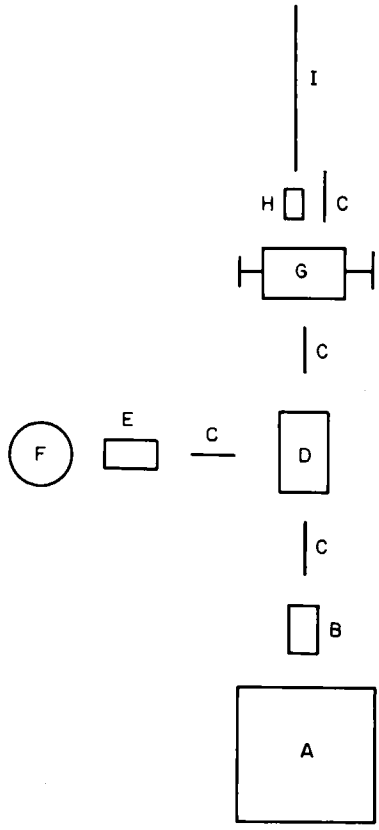


FIG. 2. Diagramatic assembly of parts for the pressure pump: A, hydrostatic pump; B, male pip-thread adapter (45–16508); C, nipple, $\frac{3}{8}$ in. o.d., $\frac{1}{8}$ in. i.d., 3 in. length (45–11163); D, tee (45–14321); E, gauge connector (45–17222); F, dial pressure gauge, Bourdon type (47–18330); G, combined inlet and bleeder valve (44–13190); H, reducer bushing (45–11483); I, superpressure tubing, desired length, 4–6 ft (45–11012). The numbers in the brackets are the catalogue numbers in the Superpressure Catalog 466A, Aminco Instrument Co, Inc., 8030 Georgia Avenue, Silver Springs, Maryland 20910. Sufficient glands, nuts and sleeves are supplied with the above.

Since it is not desirable for the glycerine–water mixture to return to the pump, the pressure-pump assembly is made so that the pressure gauge is before the combined inlet and bleeder valve (item G in Fig. 2). The position of the combined inlet and bleeder valve is important, since any small piece of material may result in the malfunction of the pump, gauge or needle valves in the combined inlet and bleed valve. In operating the pump

assembly, the needle valve of the combined inlet and bleeder valve that connects to the pressure tubing (left-hand valve of item G in Fig. 2) is always left open. The valve should be only slightly turned open from the closed position. The right-hand valve of the combined inlet and bleeder valve is used instead of the pressure-pump valve (B in Fig. 2) since this valve is not generally made of durable material and wears out very readily. As a result it is always left in the open position.

Other pressure-pump assemblies can be prefabricated according to the investigator's wish. Recently, stainless-steel hydraulic pumps that can use water have been developed, but their initial cost is much greater than converting a hydraulic truck jack. The truck jack mentioned previously has a rating of 40,000 psi.

B. Pressure cylinder

The most commonly employed pressure cylinder (Fig. 3) is one developed by ZoBell and Oppenheimer (1950), which is a modification of the pressure cylinder employed by Johnson and Lewin (1946). The pressure cylinder may be machined from stainless steel (types 303, 304 or 316). This cylinder can tolerate pressures up to 2000 atm at room temperature with a wide margin of safety. The pressure cylinder is machined so as to fit a two-way through valve (Aminco Super-pressure catalog 466A, No. 44–13106). This valve permits the pressure to be sealed in after pressure has been applied to the system, and should be slightly open when the unit is pressurized. If the valve is wide open when the pressure is applied, the closing of the valve will increase the pressure, since the compressibility of water is small. Pressure cylinders as shown in Fig. 3 have been machined by Bair Machine Co. (Lincoln, Nebraska).

C. Containers for use in pressure cylinders

The pressure cylinder described above provides a high-pressure environment for most materials that need pressurization. Generally, a small test tube (10×75 mm) is employed which is fitted with a No. 000 stopper—preferably a soft white neoprene rubber stopper or white non-toxic rubber No. 124 (West Co., Phoenixville, Pa.). We have found that the Pyrex brand of test tubes are superior to others, since the lips have been fire polished, thereby reducing any strain in the glass, since fire polishing aids the annealing process of glass. If hard neoprene stoppers are employed, the culture tube will generally shatter owing to the force created by the neoprene stopper as it enters the test tube and acts as a neutral piston. If this test tube is filled to capacity, a small piece of nichrome wire (22 s.w.g.) is inserted in the test tube before the rubber stopper is secured. The insertion of the

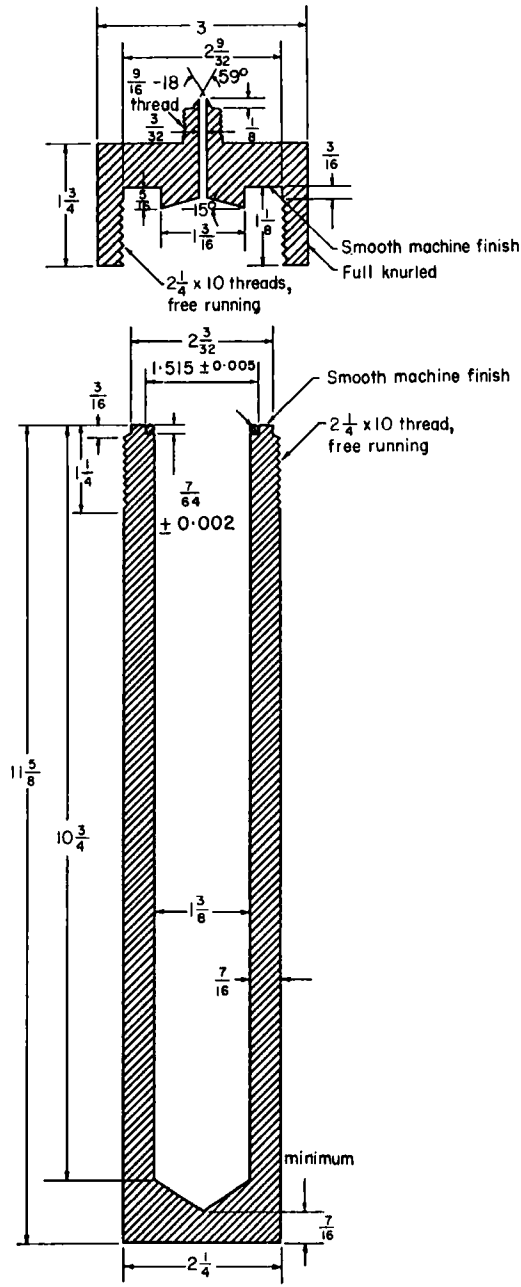


FIG. 3. Cross-section of pressure cylinder; all dimensions are in inches. (From ZoBell and Oppenheimer, 1950.)

small diameter nichrome wire produces a channel in the neoprene stopper so that excess of fluid can leave the tube and the stopper can be inserted sufficiently far enough that it will not come out. This wire is then pulled out leaving no air bubbles in the stoppered test tube.

When cultures are to be grown under hydrostatic pressure, the test tubes and stoppers are sterilized separately. The medium to be employed is inoculated with the desired organism and then pipetted aseptically into the test tubes. For aerobic growth the inoculated medium is added to within 1·25 cm from the top of the test tube before the sterile stopper is inserted. The volume of inoculated medium can be measured beforehand to ensure uniform amounts in each test tube. If too much air space is left in the test tube after the rubber stopper has been inserted, the stopper under pressure will be forced deeply into the tube thereby making it difficult to pull out the rubber stopper when the experiment is completed as well as putting an undue stress on the glass, which might result in breakage of the test tube.

For anaerobic growth of micro-organisms, the test tube is filled to the top. A piece of nichrome wire (about 6 cm long) is bent in the middle to form an inverted U or V. One side of the bent nichrome wire is heat sterilized and the heated end is placed into the inoculated test tube. Since the wire is bent in an inverted U or V shape, the wire will stay in the proper place in the test tube, leaving a hand free to insert the rubber stopper. The wire is then pulled out.

Several test tubes can be grouped together with a rubber band, so that the chamber of the pressure cylinder can be used efficiently. In addition, it is best to insert the test tubes upside down so that the rubber stopper, instead of the glass test tube, will fall against the bottom of the cylinder, thereby preventing breakage. It also ensures that the port on the pressure cylinder cap is not clogged with rubber when the pressure is released from the cylinder.

During pressurization, the test tubes do not break, since the rubber stopper acts as a neutral piston, thereby making the pressure on the inside of the chamber equal to that inside of the test tube.

Other variations on the container to fit into the chamber of the pressure cylinder have been made, such as employing a large plastic tube with rubber stoppers at both ends.

D. General procedure

The general procedure for pressurization of various materials is as follows—

1. Fill the pressure cylinder with water (iodine can be added in order to have a sterile hydraulic fluid) and equilibrate it to the temperature for the experiment.

2. Place the material to be pressurized in small test tubes (or other suitable receptacles) and then fit it with the proper size neoprene stopper. If need be, the test tubes and stoppers can be sterilized beforehand and the nichrome wire used.
3. Insert the stoppered test tube into the chamber of the pressure cylinder, place a neoprene O ring in the O ring seat and secure the pressure cylinder cap by hand.
4. Connect the pressure line from the pump assembly with the use of a wrench, and open the two-way through valve of the pressure cylinder slightly. (Sometimes it is necessary to pump the glycerine–water mixture through the pump until it comes out the pressure tubing so that there will be no air bubbles in the system.)
5. Close the right-hand valve of the combined inlet and bleeder valve (G of Fig. 2) and apply pressure through the pump. When the desired pressure is reached as indicated by the pressure gauge, secure the two-way through pressure valve on the pressure cylinder.
6. Open the right-hand valve of the combined inlet and bleeder valve. This takes the pressure off the entire system, leaving the pressure intact inside the pressure cylinder chamber.
7. Disconnect the pressure tubing from the pressure cylinder and then incubate the pressure cylinder as desired.
8. After the incubation period, again attach the pressure cylinder to the pressure tubing with a wrench. Close the right-hand valve of the combined inlet and bleeder valve and elevate the pressure again to the pressure previously applied. When the correct pressure is reached, open the two-way through valve of the pressure cylinder. If the needle of the pressure gauge deflects downwards (decrease in pressure), then a leak in the pressure cylinder has taken place. This is a check on the ability of the pressure cylinder to maintain its pressure. If a leak has developed, discard the experiment. If everything is properly done, leaks very seldom occur.
9. Open the right-valve of the combined inlet and bleeder valve slowly to release the pressure. After the pressure is released, disconnect the pressure tubing from the two-way through pressure valve and remove the pressure cylinder cap. The contents of the chamber are easily removed by tipping the cylinder upside down or with the use of long tweezers. If the pressure cylinder cap does not come off manually, then it is suggested that it be put in a pipe vice and a 24 in. pipe wrench employed to loosen the cap. Ways to prevent this situation from occurring include making certain that the O ring is always properly placed in the pressure cylinder before securing the cap, employing grease on the threads of the cylinder, and making certain that high temperatures

(ca. 80°C or higher) are not used for incubation. In the latter situation, O rings have a tendency to extrude, owing to the pressure and temperature, and thereby penetrate into the threads.

There is a small increase in heat due to the sudden compression of the cylinders (ZoBell, 1959), but owing to the large mass of the pressure cylinder and internal contents this heat dissipates readily.

III. OTHER TYPES OF PRESSURE CYLINDERS

Where small amounts of fluids are involved, the type of pressure cylinder described by Landau and Peabody (1963) can be employed (Fig. 4). One of the main disadvantages to this system is the lack of a means by which to keep the hydraulic fluid from the reaction mixtures.

In situations that call for the reaction mixture or substance to be mixed

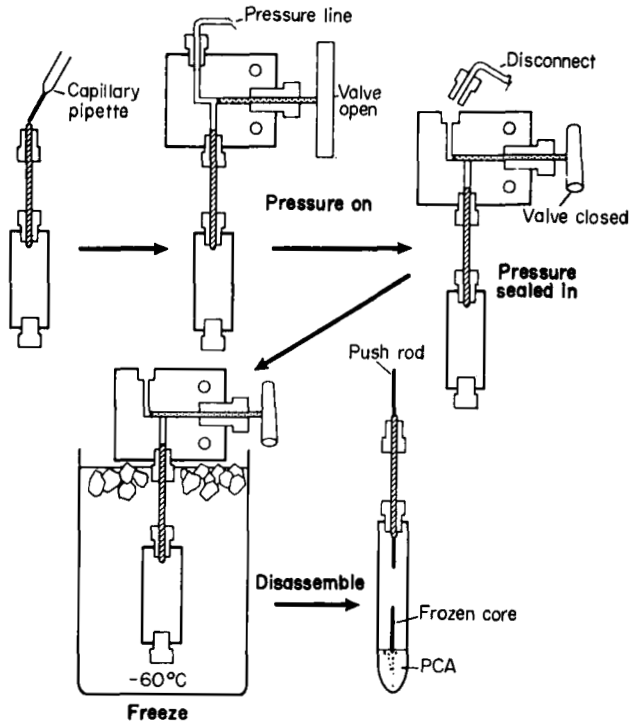


FIG. 4. A diagrammatic representation of the experimental technique employed by Landau and Peabody (1963), which can be adapted for use when small volumes of material are pressurized. The cross-hatched tube is a piece of high-pressure tubing. (From Landau and Peabody, 1963.)

under pressure (fixation of cells held under pressure for electron microscope work, etc.) a chamber devised by Landau and Thibodeau (1962) appears to offer the best solution. In this case, the contents to be mixed are separated into two stainless-steel chambers in which the fluids are separated by a coverslip (Fig. 5). Rubber diaphragms on the ends of the two stainless-steel chambers equalize the pressure applied to the system when placed in a pressure cylinder as shown in Fig. 5. A stainless-steel ball is placed in one of the steel chambers and the entire unit when under pressure is inverted so that the steel ball breaks the glass coverslip and thereby the two solutions can be mixed under pressure.

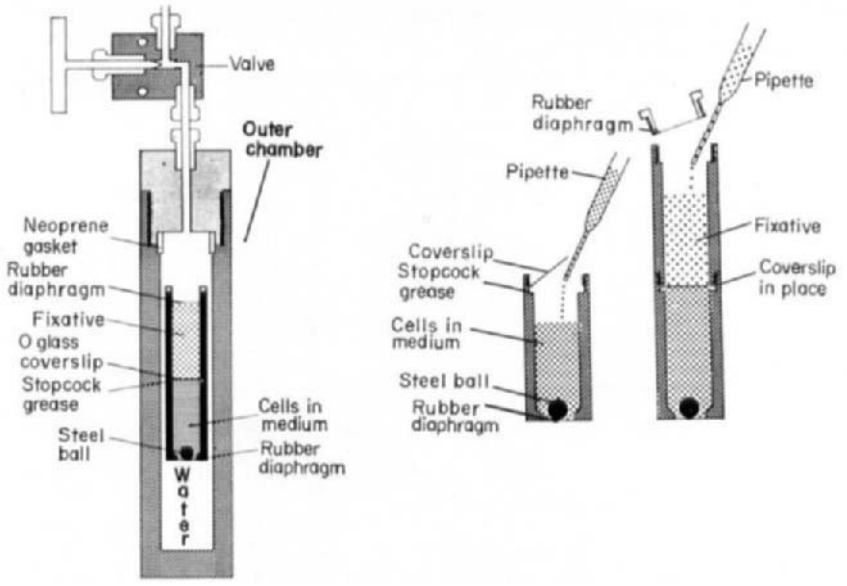


FIG. 5. Schematic diagram of an inner mixing chamber (on right) and its position in a pressure cylinder (on left). (From Landau and Thibodeau, 1962.)

High-pressure cylinders have been described (see Morita, 1957) that enable spectrophotometric measurements to be made while various materials are under hydrostatic pressure, especially enzyme reaction mixtures. White sapphire windows (Union Carbide Corp., Linde Division, Crystal Products Division or Insaco Inc.) may be employed and O ring seals are employed to make certain that leaks do not develop. The optical qualities of synthetic white sapphire are superior to quartz, but quartz lenses are used on pressure cells sold commercially (American Instrument Co., Superpressure Catalog 466, No. 41–11551 or 41–11541). When the shorter wavelengths of light are not required, lucite may be substituted.

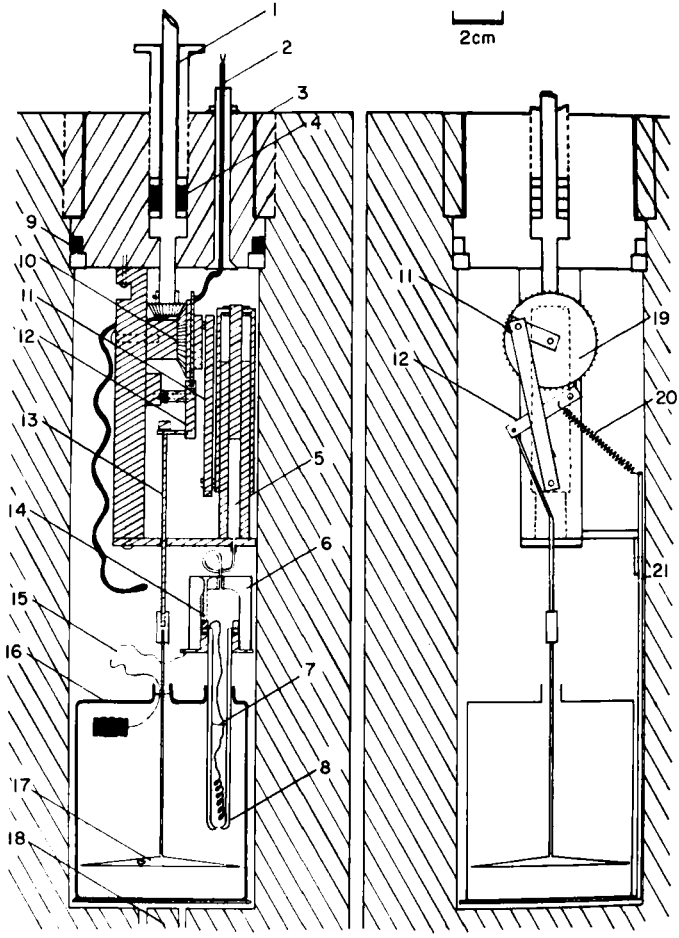
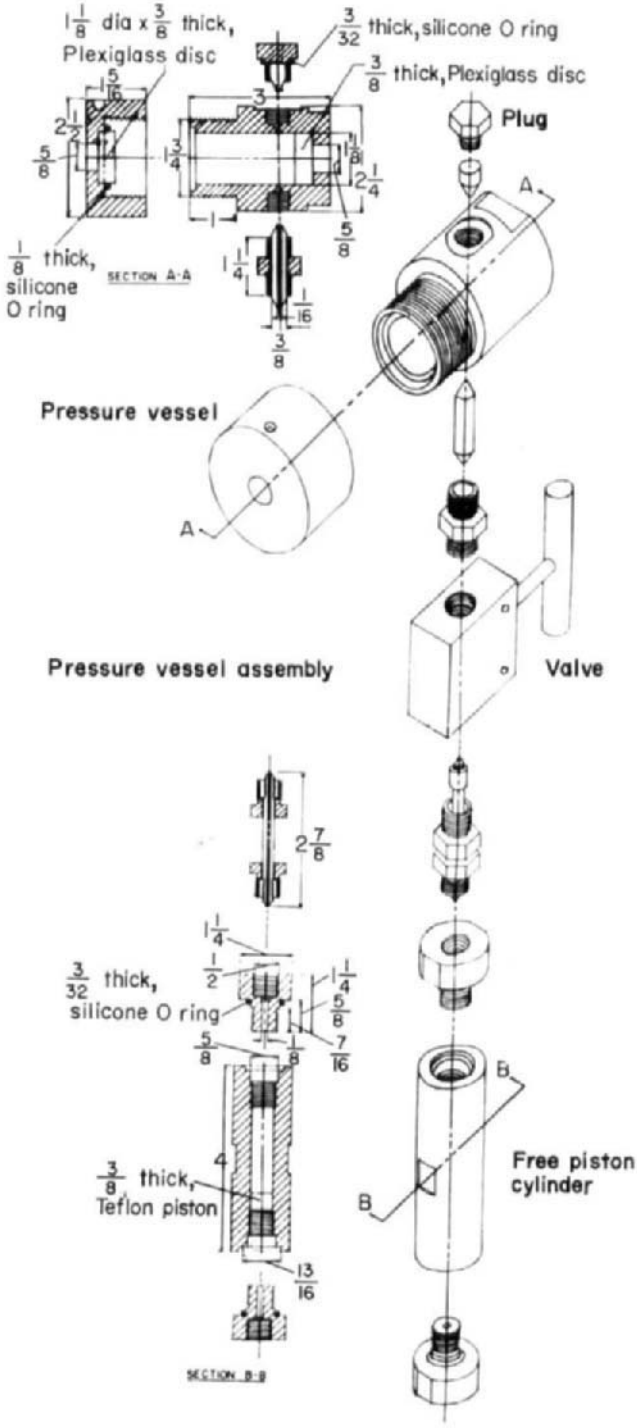


FIG. 6. Diagram of the counting apparatus mounted inside the pressure vessel. Left is a sectional diagram, right is a view obtained at right angles to the former. 1, drive shaft; 2, thermocouple wires; 3, retaining collar; 4, rotary seal; 5, piston, connected to the Perspex cap, 6, which fits over the orifice tube, 8; 7, oil–water interface inside the orifice tube; 9, packing ring; 10, bevel gear; 11, push rod; 12, lever that actuates the plunger-shaft, 13; 14, clamping screw that holds the electrode against the brass collar fitted to the rim of the Perspex cap, 6; 15, wires from electrodes; 16, glass vessel containing the cell suspension; 17, plunger; 18, inlet and outlet pipes, connected to the high-pressure pump and drain cock, respectively; 19, wheel-bearing cams; 20, spring; 21, support from the platform that holds the growth vessel, 16. (From Macdonald, 1967.)


1 1/8 dia x 3/8 thick, Plexiglass disc
3/32 thick, silicone O ring
3/8 thick, Plexiglass disc
Plug
1/8 thick, silicone O ring
SECTION A-A
Pressure vessel
Pressure vessel assembly
Valve
3/32 thick, silicone O ring
3/8 thick, Teflon piston
SECTION B-B
Free piston cylinder

In the optical high-pressure cylinder described in more detail by Morita (1957), a neutral piston is employed, so that the hydraulic fluid does not mix with the liquid fluid (enzyme reaction mixture, etc.) under investigation. This neutral piston is fitted with an $\frac{8}{32}$ in. self-seal screw with one side cut away to allow for the escape of gas or excess fluid in the optical high- pressure cell. After the excess of fluid and/or gas has been allowed to escape, the self-seal screw is secured. Such optical high-pressure cells have been adapted to fit the Cary and Beckman DU spectrophotometers. Although commercial ones are available (American Instrument Co., Silver Springs, Maryland), it should be recognized that no neutral piston is employed or built into the cell. Other methods to fit lenses in pressure apparatus are described by Robertson (1963).

In high-pressure systems where temperatures near 100°C or above are employed, the use of O rings is not advised, since it is very easy to develop leaks (see Morita and Haight, 1962; Morita and Mathemeier, 1964). Since O rings are not designed to take both high pressure and high temperature, it is suggested that a metal-to-metal seal be employed. In this situation, a union-type connection is employed, where the male portion of the cylinder cap has a 59° conical seating surface that fits into a corresponding 60° female conical seat of the pressure cylinder. When such seals are used, a good deal of force is necessary to make a pressure-tight seal. To help develop the necessary force to make this seal, a 24 in. pipe wrench is used.

A method for counting cells of *Tetrahymena* as well as to measure growth was devised by Macdonald (1967), in which a modified sampling device connected to a Model A Coulter Counter was used (Fig. 6). This method could be adapted for determining the number of bacteria resulting from growth under pressure.

A rather unique method for conducting photobiological experiments has been designed by W. E. Vidaver (personal communication) that permits a pressure vessel to be operated up to 1200 atm at controlled light intensity and quality, temperature, and sterility for a period up to several days (Fig. 7). Such a pressure vessel can be employed in a spectrophotometer. In

FIG. 7. Optical photobiological cell: all dimensions are in inches. The windows of the screw cap and the body of the optical pressure vessel (A–A) are in Plexiglass, so as to permit illumination. The Plexiglass window in the cap fits tightly in the recessed bottom of the cap. When the cap is in place, the Plexiglass window protrudes with a close fit into the body of the vessel. A silicone rubber O ring placed over the Plexiglass of the cap forms a seal between the stainless-steel bearing surfaces when pressure is applied. A needle valve (Autoclave Engineers No. 3CVM–Y071) attached to the vessel maintains pressure. A cylinder (B–B), which is fitted with a Teflon piston is attached to the other end of the valve to allow sterile medium to be pumped into the vessel as pressure is applied, permitting the use of sterile preparations.

order to operate this optical pressure vessel, it is filled with the appropriate liquid medium and closed with the screw cap (see Fig. 7). The plug and valve are then screwed to the body of the vessel. The Teflon piston in the cylinder is pushed to one end and attached to the other side of the valve. To prevent contamination, the assembled apparatus is then heated in a water bath at 70°C for 1 h. Because of the thermal properties of the Plexi glass, higher temperatures are not employed. After cooling, the plug is removed and the vessel can be inoculated with algal cells, gases or other material and then the plug replaced. The cylinder (BB in Fig. 7) acts as a neutral piston so that the hydraulic fluid does not contaminate the material in the vessel.

Other types of pressure equipment useful to microbiologists have been designed by Ferling (1957) (use of low hydrostatic pressures), Hedén and Malmborg (1961) (for growing cells under hyperbaric conditions), Distèche (1959) (for measuring pH under pressure), Rifkind and Applequist (1964) (for optical rotatory measurements), Horne and Johnson (1966) (viscometric measurement), Gill and Rummel (1961) (for optical absorption measurements) and Vidaver (1964) (pressurized polarographic cell and optical pressure cuvette).

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The author wishes to express his appreciation to Dr W. Vidaver and his colleagues, Messrs H. Lue-Kim and Frank Wick, for making available the design of the optical photobiological cell.

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CHAPTER XII

The Continuous Cultivation of Micro-organisms

1. Theory of the Chemostat

D. W. TEMPEST

Microbiological Research Establishment, Porton, Salisbury, Wilts., England

Much has been written on the theory of microbial growth in a nutrient-limited "continuous" culture since the first principles were expounded by Monod (1950) and by Novick and Szilard (1950a). However, it is not the purpose of this Chapter to provide an exhaustive review of the subject, but to concentrate on those facts that must be taken into account when designing a single-stage chemostat (the subject of the next Chapter) and when operating this both as a tool for research and for producing microbes and microbial products. For greater detail the reader is referred to the

proceedings of three symposia on continuous culture (Málek, 1958; Málek *et al.*, 1964; Powell *et al.*, 1967), the textbook of Málek and Fencl (1966), and the fundamental papers of Monod (1950), Novick and Szilard (1950a), Herbert *et al.* (1956), Herbert (1958, 1961a, 1964), Moser (1958), Gaden (1959), Fencl (1966) and Powell (1958, 1965, 1967).

I. GROWTH OF MICRO-ORGANISMS IN A "BATCH" CULTURE

When microbial cells are inoculated into a nutrient medium and incubated at a suitable temperature, a characteristic sequence of changes occurs. After a variable "lag" period, the organisms increase in mass and then divide. As growth proceeds (the so-called "exponential growth phase") nutrients are taken up from the medium and end products of metabolism excreted into it. Metabolism may, and usually does, cause the pH value to change and, with cultures of aerobic organisms, the oxygen solution rate ultimately may be insufficient to keep the culture adequately aerated. Thus, the processes of growth cause the environment to change and eventually it becomes so changed that it is unable to support further growth; at this time the culture enters the so-called "stationary phase". The sequence of changes ("lag" to "exponential" to "stationary" phases) have been analysed in detail (Monod, 1942, 1949) and collectively are referred to as the "growth cycle". But clearly, this progression of events is not an inherent property of the organisms, but a consequence of their interaction with the environment.

During the exponential growth phase the culture biomass doubles at a relatively constant rate. Thus, if the initial concentration of organisms is x, then—

$$\frac{1}{x}\frac{\mathrm{d}x}{\mathrm{d}t} = \mu \left(= \frac{\mathrm{d}(\log_e x)}{\mathrm{d}t} = \frac{\log_e 2}{t_d} \right) \tag{1}$$

where t_d is the culture doubling time and μ is the specific growth-rate constant (Fencl, 1963). Generally both μ and t_d are constant, but they are influenced markedly by the environment, particularly by the concentration of various essential nutrients. If the concentration of one such nutrient in the culture medium is decreased to a low level, then the specific growth rate is lowered correspondingly. The dependence of μ on substrate concentration (s) was shown by Monod (1942, 1950) to be of a form that could be represented by a Michaelis–Menten type function, i.e.—

$$\mu = \mu_{\max} \left(\frac{s}{K_s + s} \right) \tag{2}$$

where μ_{max} is the maximum value of μ (i.e., when s is no longer growth limiting) and K_s is a saturation constant (numerically equal to the growth-limiting substrate concentration at 0·5 μ_{max}). However, in a batch culture all nutrients usually are present initially in concentrations sufficient not to be limiting for the growth rate so that, during the exponential growth phase, the growth rate generally is equal to μ_{max}.

Monod (1942) also observed a constant relationship between the rates of growth and of substrate consumption—

$$\frac{dx}{dt} = -Y\frac{ds}{dt} \tag{3}$$

Y is termed the "yield factor" and, over any finite period of time during the exponential growth phase—

$$Y = \frac{\text{Weight of bacteria formed}}{\text{Weight of substrate consumed}}$$

Thus, if the values of μ_{max}, K_s and Y are known, a completely quantitative description of the events occurring during the growth cycle of a batch culture can be made (Monod, 1942; Herbert *et al.*, 1956).

II. GROWTH OF MICRO-ORGANISMS IN A "CONTINUOUS" CULTURE

All continuous cultures start their existence as batch cultures, in that medium, contained in a growth vessel, is inoculated with organisms that then proceed to grow and divide as described earlier. However, if during the exponential growth phase fresh medium is added to the culture at a rate sufficient to maintain the culture population density at a fixed, sub-maximal, value, then growth should not ultimately cease (as in a batch culture) but continue indefinitely. Clearly, the medium input rate and culture volume would have to increase exponentially, with biomass, unless provision was made for the continuous removal of culture at a rate equal to that of the medium flow. The maintenance of a constant volume of culture at a constant microbial population density (in practice, a constant culture absorbance) is essentially the operating principle of one type of continuous culture apparatus, namely the "turbidostat" (Bryson, 1952).

But organisms can be cultured continuously, at specific growth rates less than μ_{max}, in a second type of apparatus, the "chemostat" (Novick and Szilard, 1950b). The chemostat is similar to the turbidostat in that a culture of fixed volume (V) is contained in a suitably constructed vessel to which medium is pumped at a constant rate (f) (see Fig. 1). The culture in

the chemostat differs from that in the turbidostat, however, in that the microbial population density is not controlled directly. Instead, the medium is compounded in a way such that all substances essential for the growth of the organisms, except one, are present in the culture at concentrations that are in excess of the microbial growth requirement. The one "growth-limiting" nutrient is present in the medium at a concentration sufficient to support only a limited amount of growth; thus, if the culture was allowed to develop as a batch culture, this nutrient would become depleted first, causing growth to cease.

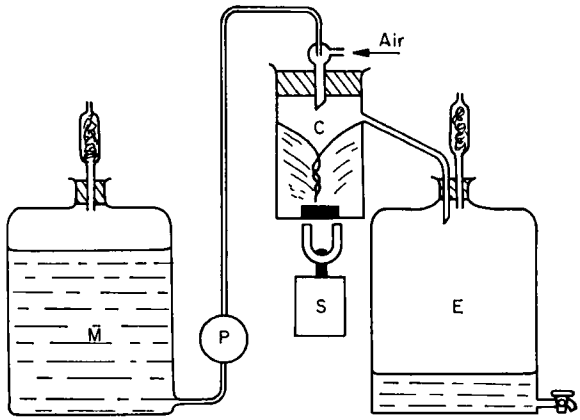

FIG. 1. Essential features of a chemostat. Sterile medium (M), contained in a reservoir, is added to the culture (C) at a pre-determined rate by means of a metering pump (P). The culture is agitated with a magnetically coupled stirrer (S) and aerated. The culture volume is maintained constant by a constant level overflow device and effluent culture (E) is collected in a receiver bottle.

Now let us suppose that the culture in the chemostat is allowed to grow up "batchwise" until the growth-limiting nutrient becomes depleted. If, at this time, fresh medium is pumped to the culture at a low rate, growth will continue. But clearly, it can do so only at a rate proportional to the medium flow rate; that is, at a rate proportional to the rate of supply of growth-limiting nutrient to the culture.

With the experimental arrangement depicted in Fig. 1, in which medium flows through a culture of fixed volume, the growth rate of organisms will depend not merely on the medium flow rate but on the dilution rate (D), i.e., the number of culture volumes of medium passing through the growth vessel per unit time (f/V); the dimensions being reciprocal time (usually h^{-1}). Thus, other things being equal, two chemostat cultures of differing size will contain organisms growing at identical rates if their dilution rates

are equal; but their medium flow rates will be different. Similarly, organisms in a growth vessel of 1 litre capacity, supplied with medium at a rate of 1 litre/h will be growing ten times as fast as organisms in a second vessel of 10 litres capacity, also supplied with medium at a rate of 1 litre/h (see equation 5).

A. Dilution rate and the growth rate of micro-organisms

In order to appreciate fully the kinetics of bacterial growth in a chemostat culture, it is essential to understand the exact relationship between the dilution rate and the growth rate of organisms in the culture. In the culture vessel, organisms are growing; they are also being simultaneously washed from the fermenter. The net change in concentration of organisms (x) with time therefore will be determined by the relative rates of each process. Thus—

$$\text{Increase} = \text{Growth} - \text{Output}$$

$$\frac{dx}{dt} = \mu x - Dx$$

$$\frac{dx}{dt} = x(\mu - D) \tag{4}$$

It follows that if $\mu > D$, dx/dt will be positive and the concentration of organisms in the culture will increase with time. On the other hand, if $\mu < D$, then dx/dt will have a negative value, i.e., the cell concentration will diminish with time as the culture "washes-out" from the growth vessel. Only when μ is equal to D will dx/dt be zero and the concentration of organisms in the culture remain constant with time, i.e., the culture will be in a steady state. The continuous culture of organisms in a chemostat depends on providing conditions in which the specific growth rate (μ) and dilution rate (D) can be equal and invariant with time, i.e., in which—

$$D = \mu = \mu_{\max}\left(\frac{s}{K_s + s}\right) = \frac{\log_e 2}{t_d} \tag{5}$$

This is not difficult to achieve since the growth rate of organisms in the culture, being limited by the rate of supply of growth-limiting nutrient to the culture, must be proportional to the dilution rate; provided that the dilution rate is maintained constant the system is inherently self balancing (see later, p. 265).

Because the growth rate of organisms in the chemostat is controlled by the dilution rate, the growth rate of the organisms in the culture can be adjusted, within certain limits, to any value desired. However, the specific growth rate (μ) cannot be made to exceed $\mu_{\max}$ (equation 2) and therefore

steady-state conditions cannot be obtained at dilution rates above a critical value (D_c) nearly equal to μ_{max}. If the dilution rate is set to a value greater than D_c, the culture will be progressively washed out from the fermenter. At the other extreme, the existence of a minimum growth rate value is speculative. However, for most organisms growing at their optimum temperature and pH value, it is likely to be less than 0·05 μ_{max} (Tempest *et al.*, 1967b).

B. Influence of dilution rate on the concentration of organisms and growth-limiting substrate in the culture

For a completely quantitative statement of the behaviour of microbial cultures in a chemostat, one must consider also the effect of dilution rate on the concentration of growth-limiting substrate (s) and of organisms (x) in the culture. Substrate enters the growth vessel, from the reservoir, at a concentration S_r; it is consumed by the organisms and emerges, in the overflow culture, at a concentration s. Therefore, the net change in substrate concentration resulting from passage through the growth-vessel is:

$$\text{Change} = \text{Input} - \text{Output} - \text{Consumption}$$

$$\frac{ds}{dt} = DS_r - Ds - \frac{\text{Growth}}{\text{Yield}}$$

$$= DS_r - Ds - \frac{\mu x}{Y}$$

Re-arranging, and substituting for μ (equation 2)—

$$\frac{ds}{dt} = D(S_r - s) - \frac{\mu_{max} x}{Y}\left(\frac{s}{K_s + s}\right) \tag{6}$$

Similarly, one can substitute for μ in equation (4), whence—

$$\frac{dx}{dt} = \mu_{max} x \left(\frac{s}{K_s + s}\right) - Dx$$

or—

$$\frac{dx}{dt} = x\left[\mu_{max}\left(\frac{s}{K_s + s}\right) - D\right] \tag{7}$$

Equations (5), (6) and (7) (see Herbert *et al.*, 1956) define quantitatively the behaviour of the culture in the chemostat. Furthermore, they show that, irrespective of the initial state of the culture, a steady state should ultimately be established. For example, in the case of equation (7) consider the situation prevailing shortly after inoculating the medium in the fermenter with the appropriate organisms: then, x is small and s is large (almost equal

to S_r); assuming $S_r \gg K_s$ and the organisms respond instantaneously, then the specific growth rate will be near to its maximum value and—

$$\frac{dx}{dt} \simeq x(\mu_{max} - D)$$

If the dilution rate is set at a value which is low, relative to μ_{max}, the concentration of organisms in the chemostat culture will increase rapidly and the concentration of growth-limiting substrate fall correspondingly. As s decreases, so μ must decrease (equation 2). Ultimately μ will approach the value of D and at this stage the rate of substrate consumption (plus loss in the overflow culture) will be balanced exactly by the rate of substrate entry to the culture in the inflowing medium. Any fluctuations that may occur in the steady state (for example, due to apparatus malfunction) will set up opposing reactions that will tend to restore the steady state. It is this inherent self-balancing property of the chemostat culture that makes this form of continuous culture easy to operate and stable over a long period of time.

It can be deduced from equations (6) and (7) that if S_r and D are maintained constant, and D is less than D_c, that when the system is in a steady state (i.e., $dx/dt = 0$ and $ds/dt = 0$) unique values exist for both the culture microbial concentration and growth-limiting substrate concentration; these values are generally designated $\bar{x}$ and $\bar{s}$ (Herbert *et al.*, 1956). Thus, taking into account that at equilibrium—

$$\mu = D = \mu_{max}\left(\frac{s}{K_s + s}\right)$$

from equation (6)—

$$\bar{x} = Y(S_r - \bar{s}) \tag{8}$$

and from equation (7)—

$$\bar{s} = K_s\left(\frac{D}{\mu_{max} - D}\right) \tag{9}$$

Substituting $\bar{s}$ (equation 9) in equation (8), it follows that—

$$\bar{x} = Y\left[S_r - K_s\left(\frac{D}{\mu_{max} - D}\right)\right] \tag{10}$$

Thus, since μ_{max}, K_s and Y are constants, the principal effect of varying the dilution rate is to change the growth-limiting substrate concentration in the culture (equation 9), thereby effecting a change in the specific growth rate and doubling time of the organisms (equation 5). Assuming the yield value (Y) to be independent of dilution rate, and K_s to be small relative to S_r, it follows from equations (5), (8) and (10) that varying the dilution rate should produce changes in the steady-state microbial concentration and

growth-limiting substrate concentration as represented in Fig. 2a. However, if K_s is large relative to S_r, then the pattern of change in $\bar{x}$ and $\bar{s}$ with dilution rate should be more like that represented in Fig. 2b.

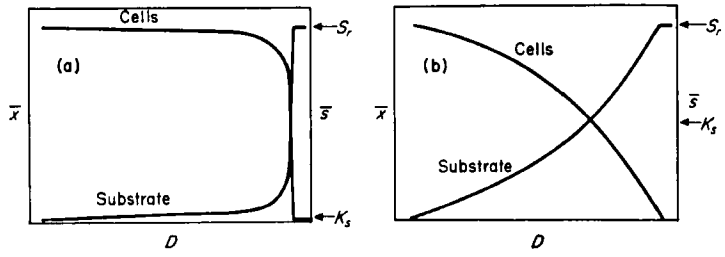

FIG. 2. Influence of dilution rate (D) on the steady-state concentration of organisms ($\bar{x}$) and growth-limiting substrate ($\bar{s}$) in a chemostat culture: (a) when K_s (the bacterial saturation constant for growth-limiting substrate) is small relative to S_r (the growth-limiting substrate concentration in the feed medium), and (b) where K_s is large relative to S_r.

C. Dilution rate, growth-limiting substrate concentration and the output of culture

Since the growth-limiting substrate concentration in the culture ($\bar{s}$) is independent of its concentration in the inflowing medium (equation 9), the sole effect of varying S_r should be to change the steady-state microbial concentration (see, for example, Herbert, 1958; Tempest, *et al.*, 1965).

An important practical consideration in operating a chemostat is the rate of output of microbial cells and extracellular substances. The output of cells per unit volume of culture is the product of the cell concentration and the dilution rate ($D\bar{x}$). Thus, from equation (10)—

$$\text{Output} = D\bar{x} = DY\left[S_r - K_s\left(\frac{D}{\mu_{max} - D}\right)\right] \qquad (11)$$

Usually K_s is small relative to S_r; therefore, at dilution rates less than the critical value (D_c), the output of cells is approximately equal to DYS_r. But at near critical dilution rates, where $D \simeq \mu_{max}$, the expression—

$$K_s\left(\frac{D}{\mu_{max} - D}\right)$$

approaches infinity and the output ($D\bar{x}$) falls to zero. These changes in output with dilution rate are shown graphically in Fig. 3.

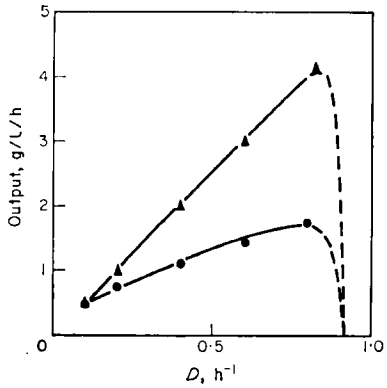

FIG. 3. Effect of dilution rate on the output of *Aerobacter aerogenes* organisms from glycerol-limited (▲) and Mg^{2+}-limited (●) chemostat cultures. (Data from Tempest *et al.*, 1965.)

D. Transitional stages between steady states

Although the macromolecular composition and metabolic activity of organisms in a chemostat culture remain relatively constant when the system is in a steady state, they vary considerably with growth condition (Herbert, 1961b; Neidhardt, 1963; Tempest and Herbert, 1965; Tempest and Hunter, 1965). Therefore, when the steady-state growth condition is suddenly changed, a period of time is required for re-adjustment to occur in the physiological properties of the organisms in the culture. During this transition period, organisms continue to be lost from the culture in the growth vessel (through the overflow) and new organisms are synthesized. But the rate of change in any particular property (e.g., RNA, enzyme content), between one steady-state value and the other equilibrium value, will depend on whether adaptation to the new environment involved newly synthesized organisms only or changes in the organisms present in the culture at the time of change-over. Thus, supposing the organisms expressed a property p (e.g., an enzyme) that was present in high concentration in the initial population, but almost absent from the steady-state population following a change in the growth condition, then if at time t_0 the cells ceased to produce this substance (but did not destroy that already present in the culture) its concentration in the culture would diminish at a rate such that—

$$p_t/p_0 = \exp(-Dt)$$

or—

$$\log_e(p_t/p_0) = -Dt \qquad (12)$$

where p_0 is the concentration of the substance at time t_0, and p_t its concentration after time t. Similarly, if change over occurred in the reverse direction

and there was instant synthesis of the new property by newly formed cells only, then its concentration would increase at a rate such that—

$$p_t/p_s = 1-\exp(-Dt)$$

or—

$$\log_e(1-p_t/p_s) = -Dt \qquad (13)$$

where p_s represents the final steady-state concentration of the property. A rate of change substantially different from this predicted rate would indicate that expression of the new property (or disappearance of the old property) was not associated solely with the synthesis of new organisms, i.e., it was also synthesized (or degraded, as the case may be) by those organisms present in the culture at the time of change-over (see Fig. 4).

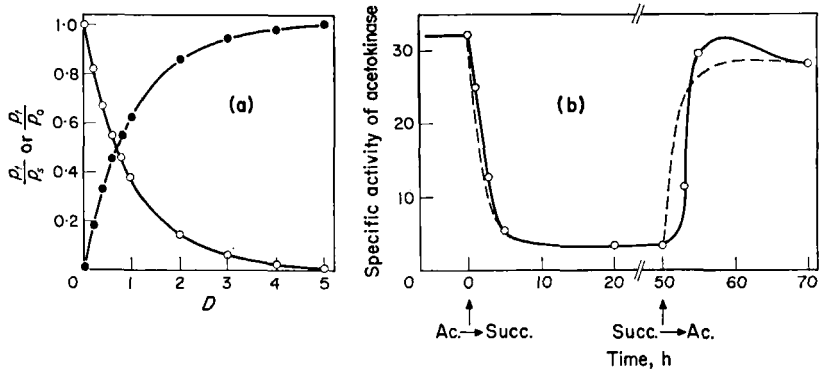

FIG. 4. (a) Theoretical plots of the "wash-out" (○) and "build-up" (●) of a property (p) following a change in the growth condition and assuming acquisition of the new property to be associated solely with the synthesis of new organisms. (b) Plot of the changes of acetokinase content of *Pseudomonas ovalis* Chester following change-over from acetate limitation to succinate limitation, and *vice versa* (unpublished observation). The broken line indicates the theoretical plot assuming the conditions stated in Fig. 4(a).

E. Maximum growth rate and "wash-out"

Equation (12) may be applied without modification to changes in microbial-cell concentration following a change in the dilution rate and, within limits, can be used as the basis of a method for determining the maximum growth rate of organisms in a particular chemostat environment. Thus, if in a chemostat culture the organisms ceased growing at time t_0, their concentration in the culture vessel would diminish at a rate such that—

$$\log_e(x_t/x_0) = -Dt$$

where x_0 is the concentration of organisms at time t_0, and x_t is the concentration after time t (h). However, if the organisms did not stop growing completely, but the growth rate was suddenly lowered to a fixed value (β) that was less than D (e.g., following a change in temperature), then the change in concentration of organisms over any period of time (t_2-t_1) would be such that—

$$\log_e(x_2/x_1) = (\beta-D)(t_2-t_1)$$

A sudden decrease in growth rate, independent of a change in dilution rate, seldom occurs in practice. However, one can envisage circumstances in which the dilution rate may be increased suddenly but the growth rate cannot increase correspondingly. This happens, for example, when the dilution rate is set at a value greater than the critical rate (D_c); then growth rate increases to μ_{max}, but no further, and thereafter "wash-out" occurs at a rate such that—

$$\log_e(x_2/x_1) = (\mu_{max}-D)(t_2-t_1) \qquad (14)$$

Thus, measuring the rate of change of bacterial concentration in a chemostat culture, following an increase in dilution rate to a known value greater than D_c, should provide data from which μ_{max} may be calculated easily. But since an increase in D is seldom, if ever, accompanied by an instantaneous increase in μ, precautions must be taken when using this technique to determine the exact value of μ_{max}. The physiological properties of organisms vary with growth rate and require time to adjust to changes in growth rate; also there may be corresponding changes in the yield value (Tempest and Dicks, 1967). Therefore the determined value of μ_{max} would be accurate only if, before "step-up", the steady-state growth rate had been close to μ_{max}, thus requiring little change in the properties of the organisms for them to assume their maximum rate of growth. Even then account must be taken of the fact that some changes in the properties of the cells will occur during the initial stages of "wash-out". This adjustment period can be minimized by selecting a dilution rate that is substantially greater than D_c, thereby ensuring that the limiting substrate concentration reaches a cell-saturating level rapidly.

F. Kinetics of growth of a mixed microbial population in a chemostat culture: fate of a mutant or contaminant organism

A mixed microbial population may occur unintentionally in a chemostat culture through either entry of a contaminant organism into the growth chamber or mutation of an organism in the culture. In either case the fate of the foreign organism can be predicted from the theory of the chemostat outlined previously (particularly from those considerations implicit in equations (2), (6) and (7); see also Powell, 1958).

The fate of the contaminant organism will be influenced by many factors (e.g., culture pH value, temperature, nutritional requirements of the contaminant and composition of the medium), but assuming that (a) the dilution rate is constant, (b) the culture is in a steady state at the moment of contamination, (c) growth rate is limited by the deficiency of a single nutrient which is essential for growth of the contaminant organisms also, (d) there is no interaction between the organisms other than competition for the growth-limiting nutrient, (e) the culture is perfectly mixed and (f) growth rates of the organisms adjust themselves to changes in substrate concentration without appreciable lag, then theory predicts that the contaminant must either be washed from the growth vessel or completely replace the species originally in the chemostat culture.

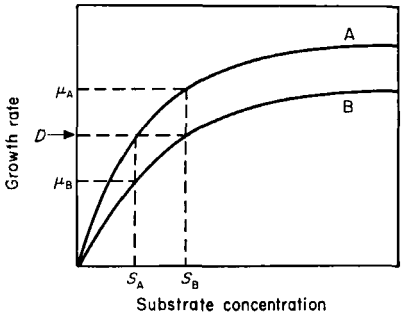

FIG. 5. Theoretical saturation curves for two organisms (A and B) growing in separate chemostats in identical media. At a particular dilution rate (D), the growth-limiting substrate concentration would be s_A for organism A and s_B for organism B. If the two organisms were present in the same chemostat culture, operated at the same dilution rate, then organism A would outgrow B since at the growth-limiting substrate concentration s_A, organism B could only grow at the rate μ_B which, being less than D, would be insufficient to prevent it from being washed out of the culture (see equation 4).

This situation can be appreciated most readily by reference to Fig. 5, which depicts the relationship between growth rate and the growth-limiting substrate concentration in the culture (see equation 2) for two different organisms (A and B). Let us assume that initially the chemostat contains a pure culture of bacterium B (growing at a dilution rate of $D\ h^{-1}$) and that this becomes contaminated with organism A. The growth-limiting substrate concentration in the culture at the time of contamination would be s_B, and at this concentration the contaminant organism would start growing at a rate equal to μ_A. Since this growth rate is greater than the dilution rate (D), the concentration of contaminant organisms in the culture must increase

(equation 4); but this will cause the growth-limiting substrate concentration to decrease correspondingly until μ_A equals D. At this time, the substrate concentration in the culture will have diminished to a value equal to s_A which will permit organism B to grow only at a rate of μ_B. But since μ_B is less than the fixed dilution rate (D) the concentration of B type organisms in the chemostat culture must diminish (ultimately to zero) as they are progressively washed from the growth vessel. On the other hand, had the saturation curves for the two types of organisms borne the opposite relationship, then the contaminant organisms would never become established in the chemostat culture irrespective of the size of inoculum or frequency of contamination.

It must be emphasized that Fig. 5 represents an "ideal" situation in which the two saturation curves are well separated (i.e., the maximum growth rate of organism A is greater than that of organism B, and so is its saturation constant [K_s in equation 2]). It is possible that the two saturation curves may cross; in this case the fate of the culture would depend on the dilution rate.

III. MODIFICATIONS TO THE BASIC THEORY

The simple theory of microbial growth in a nutrient-limited continuous culture, described above, is not only easy to understand but accounts quite well for the observed behaviour of many real chemostat cultures. There are some exceptions, however, and these necessitate modifications to be made to a few of the basic equations; these modifications are detailed below.

A. Variations in the yield values

Initially it was assumed that the yield value was independent of the growth rate (Monod, 1950; Herbert *et al.*, 1956), but subsequent experimentation has shown this assumption to be generally, if not invariably, invalid. A constant yield value would imply that the cellular content of growth-limiting substance did not vary with the growth condition. However, the magnesium, potassium and phosphorus contents of micro-organisms each are known to vary with growth rate (Wade, 1952; Wade and Morgan, 1957; Tempest and Strange, 1966; Tempest *et al.*, 1966; Dicks and Tempest, 1966); therefore the yield may be expected to vary with dilution rate when the availability of any one of these substances is made to limit growth. Also yield may vary as a result of the synthesis of storage-type compounds (glycogen, polyhydroxybutyric acid, polyphosphate) under appropriate conditions—principally NH_4^+ limitation (Holme, 1957; Wilkinson and Munro, 1967). Frequently the cellular content of a storage-type compound

has been found to vary with growth rate, thus causing the yield to vary accordingly.

Although the carbon content of organisms does not seem to vary greatly with growth rate (D. Herbert, personal communication), when the carbon source is made the growth-limiting component of the medium the yield still is not constant. This is because the carbon source provides both material for assimilation into cell substance and oxidizable substance required to provide the energy for biosynthesis. The ratio of substrate oxidized to that assimilated seemingly depends upon the rate of cell synthesis (i.e., on the growth rate) and this variation in the efficiency of carbon-substrate assimilation causes the yield to vary considerably, particularly at low growth rates (i.e., $<0{\cdot}1\ \mu_{max}$).

The variation in yield of carbon-limited cultures, with growth rate, has been taken as an indication of a cellular "maintenance energy" requirement (Herbert, 1958; Marr *et al.*, 1963; Dawes and Ribbons, 1964); this may be independent of growth rate (Pirt, 1965) but is not invariably so (Tempest and Herbert, 1965). Thus, one can distinguish between the "observed yield" (Y) and "true growth yield" (Y_G) as follows—

$$Y = Y_G \left(\frac{\mu}{\mu + \mu_e} \right) \tag{15}$$

where μ_e is the maintenance energy rate and μ is the specific growth rate (see Powell, 1967). However, this distinction is meaningful only with regard to carbon assimilation or, presumably, to other situations in which the growth-limiting substance provides both energy and essential cell substance.

B. Variation in the steady-state concentration of growth-limiting nutrient with parameters other than the dilution rate

The simple theory of continuous culture states that the concentration of growth-limiting substrate in the culture extracellular fluid is independent of its concentration in the inflowing medium, being solely dependent on the dilution rate (equation 9). But Contois (1959) reported that with NH_4^+-limited cultures of *Aerobacter aerogenes*, growing in a chemically defined medium at a fixed dilution rate, the concentration of extracellular ammonia varied with the culture population density; this observation was confirmed (qualitatively but not quantitatively) by D. Herbert, P. J. Phipps and D. W. Tempest (unpublished data), who concluded that the probable cause was an inability of the apparatus used to provide perfect mixing (i.e., the instantaneous and homogeneous dispersion of the inflowing medium through the culture). Thus, when the efficiency of mixing was deliberately impeded, the effect of S_r (and hence population density) on

the steady-state culture ammonia concentration was exaggerated (unpublished observation).

For different reasons, the population density was found to influence the steady state extracellular Mg^{2+} concentration in Mg^{2+}-limited chemostat cultures of *Bacillus subtilis* (Tempest *et al.*, 1967a; Meers and Tempest, 1968). Here, an increase in population density was associated with a more efficient uptake of Mg^{2+}, in contrast to the uptake of NH_4^+ by *A. aerogenes* described above. The increased efficiency of Mg^{2+} uptake by the *B. subtilis* culture resulted from the secretion by the organisms of substance(s) that stimulated growth and the uptake of Mg^{2+}; thus, the greater the population density the greater the concentration of growth-promoting substance(s) in the culture, and the greater the efficiency of Mg^{2+} assimilation.

One can suppose that whenever organisms produce substances that either stimulate or suppress assimilation of the growth-limiting nutrient, chemostat cultures of these organisms will show a population effect with respect to the relationship between the steady-state growth-limiting substrate concentration in the culture and the dilution rate (i.e., specific growth rate). Where the effect is on the maximum growth rate of the organisms, equation (9) may be modified as suggested by Meers and Tempest (1968), as follows—

$$\bar{s} = K_s \left[\frac{D}{\mu_{\max} \left(\frac{1+\lambda p}{1+p} \right) - D} \right] \tag{16}$$

where p represents the concentration of growth-affecting substance and λ is a constant; $\mu_{\max}$ is now defined as the maximum growth rate when $p = 0$. At cell-saturating concentrations of substance p, the maximum growth rate approaches the value $\lambda\mu_{\max}$.

C. Influence of non-limiting nutrients on the growth of microorganisms in a chemostat

As stated above, whenever organisms produce substances that influence assimilation of the growth-limiting nutrient, cultures of these organisms will show a population effect with regard to the steady-state concentration of this nutrient (equation 16). Similarly, if the medium contains a nutrient that antagonizes assimilation of the growth-limiting nutrient, then the steady-state growth-limiting substrate concentration will vary with the concentration of this non-limiting nutrient in the environment. Thus, if the antagonist is assimilated to some extent by the organisms, its concentration will vary with the microbial population density (and metabolic activity) and so therefore will its effect on the growth of the organisms in the culture.

Some nutrient antagonisms may not influence growth directly, but nevertheless alter the yield value by affecting the synthesis of particular

macromolecular components of the cell. Thus the K^+/NH_4^+ ratios in various simple salts media determined the intracellular glycogen content of *A. aerogenes* organisms in chemostat cultures (Dicks and Tempest, 1967).

In some instances the presence of a particular nutrient in the growth environment may repress competitively the synthesis of enzyme(s) required for the metabolism of the growth-limiting substance. In this case a steady state may be unattainable and undamped oscillations occur in the culture bacterial concentration and cellular content of substrate-metabolizing enzyme(s) (see Boddy *et al.*, 1967).

D. Growth of organisms on potentially toxic substances

Phenol is toxic to most organisms, but some phenol-sensitive microbes are capable of assimilating low concentrations of phenol (Evans and Kite, 1961; Rieche *et al.*, 1964). Continuous cultures of organisms growing on, or in the presence of, phenol obviously are potentially unstable. But steady-state conditions can usually be obtained by arranging for the phenol to be the growth-limiting substrate, thereby ensuring that its concentration is maintained at the lowest practical level. Since the steady-state concentration of phenol in the culture will increase with growth rate and thereby adversely affect growth, the maximum rate of growth attainable will depend to some extent on the phenol content of the input medium.

E. Modification of theory for steady-state cultures of low viability

In deriving equations for the growth of organisms in a chemostat, no account was taken of the culture viability, nor was this necessary. It is possible, indeed probable, that a proportion of the steady-state population in a chemostat culture may be non-viable, but if so the viable organisms in the culture clearly must grow at a rate greater than the dilution rate, since they must continuously replenish both the viable and non-viable organisms that are lost from the growth vessel in the effluent culture.

Thus, if the culture doubling time (t_d) is related to the dilution rate by equation 5 ($t_d = \log_e 2/D$) then the doubling time of viable organisms (t_d^*) will be related (according to E. O. Powell, personal communication) as follows—

$$t_d^* = \frac{\log_e 2\alpha}{D} \tag{17}$$

where α is the "index of viability" (i.e., the probability that an organism will divide into two viable progeny) and is equal to $(V+1)/2$, V being the fraction of viable organisms in the culture.

When the culture is in a steady state, the specific growth rate (μ) is equal to the dilution rate (Section IIA), but in a culture of low viability the

rate of growth of the viable organisms in the population (μ^*) must be greater than the specific growth rate of the culture. Thus—

$$\mu^* = \frac{\log_e 2}{t_d^*} = \frac{D \log_e 2}{\log_e 2\alpha} \tag{18}$$

It was found by Tempest *et al.* (1967b) that the steady-state viability of a glycerol-limited culture of *A. aerogenes* was less than 40% when grown at a dilution rate of 0·004 h^{-1} (35°C, pH 6·5). At this dilution rate, the culture doubling time was 173 h, but the doubling time of the viable portion of the population was about 80 h.

In a culture of low viability, cell-bound growth-limiting nutrient may be released from dead organisms, thus enhancing the yield value by facilitating "cryptic" growth. A detailed analysis of the growth kinetics of such a heterogeneous population is exceedingly difficult, and quite beyond the author's capability.

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CHAPTER XIII

The Continuous Cultivation of Micro-organisms

2. Construction of a Chemostat

C. G. T. Evans, D. Herbert and D. W. Tempest

Microbiological Research Establishment, Porton, Salisbury, Wilts., England

I. INTRODUCTION

The theory of microbial growth in a nutrient-limited environment, developed in the previous Chapter (Tempest, p. 259), prescribes the essential requirements for chemostat design. Thus, all continuous-culture devices must fulfil five or six basic conditions, *viz*, (i) that the culture be enclosed in a container (growth vessel) in which it is protected from contaminating organisms; (ii) that a flow of fresh sterile medium to the culture be maintained at a constant, pre-determined, but variable, rate; (iii) that the culture volume be maintained constant; (iv) that the culture be sufficiently agitated to permit near-perfect mixing—that is, the instantaneous and homogeneous dispersion of the inflowing medium throughout the culture; (v) that if the organisms require oxygen, the aeration and agitation of the culture be sufficient to maintain the dissolved oxygen at a level above that which would limit growth—unless oxygen is intended to be the growth-limiting component of the medium; (vi) that if the ambient temperature fluctuates greatly, or the organisms are to be grown at temperatures different from ambient, some form of temperature regulation be provided. These basic requirements are met in the type of apparatus shown in Fig. 1.

Many attempts to improvise continuous culture apparatus from standard pieces of laboratory equipment must have been made, and there is little doubt that where the cultural requirements were modest, and long-term reliability was not aimed at, many of these chemostats proved satisfactory.

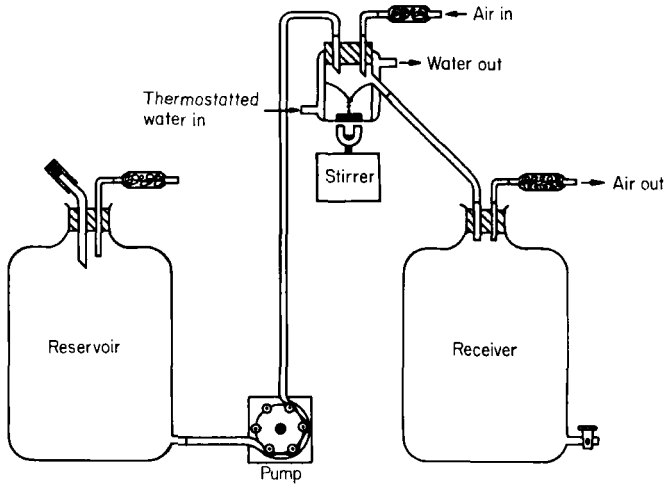


FIG. 1. Continuous culture apparatus (schematic), the essential components being a medium reservoir, metering pump, growth vessel (in which the culture is maintained at a constant temperature, aerated and continuously agitated) and a receiver for the effluent culture.

However, if it is desired to grow volumes of culture larger than 0·5 litre at microbial concentrations greater than 10^9 bacteria/ml (or > 1 mg equivalent dry weight organisms/ml) and to maintain steady-state conditions over extended periods of time (i.e., > 1 week) without fear of mechanical breakdown, then more sophisticated and carefully designed equipment is necessary. Such an apparatus still can be relatively inexpensive and simple to construct, although considerable instrumentation is required for the chemostat to be made sufficiently versatile to exploit extensively its main asset—the provision of a fully controlled growth environment.

The main purpose of this Chapter is to provide a detailed description of the design and construction of a 1 litre "Porton-type" chemostat, but in doing this we feel that it may be of added value to discuss briefly those considerations influencing the choice of individual components, or systems, and to indicate possible alternative arrangements. We believe that the apparatus described herein will meet the varied demands of research workers for an operationally versatile apparatus that is nevertheless easy to operate and service, reliable, and not too demanding of space, medium supply and manpower. A similar apparatus, but of 0·5 litre capacity, has been described previously (Herbert *et al.*, 1965).

II. DESCRIPTION OF A "PORTON-TYPE" CHEMOSTAT OF ONE LITRE CAPACITY

A. General design

The chemostat is constructed in two separable parts, the "fermenter unit" and the "control unit", which are connected by a multi-core cable through multi-point plugs and sockets. Separation of the control instruments from the fermenter unit facilitates servicing and allows one set of controls (which are the most expensive part of the apparatus) to be used with several fermenter units of differing capacities. Furthermore, it is safer both for the operator and for the instruments, since spillage of water and titrant can occur occasionally onto the fermenter unit.

The fermenter unit (Fig. 2) houses the growth vessel and stirrer power unit, titrant reservoirs and associated solenoid-operated valves or pumps, antifoam reservoir and pump, medium reservoir and metering pump, and receiver. Also located on this unit is a reservoir of saturated KCl solution (containing the calomel reference electrode) and the electrode connectors. All the instruments required for the automatic control of temperature, pH value, foaming and air flow rate, with their associated switches and taps, are built into the control unit (Fig. 3); a schematic wiring diagram is given in Fig. 4.

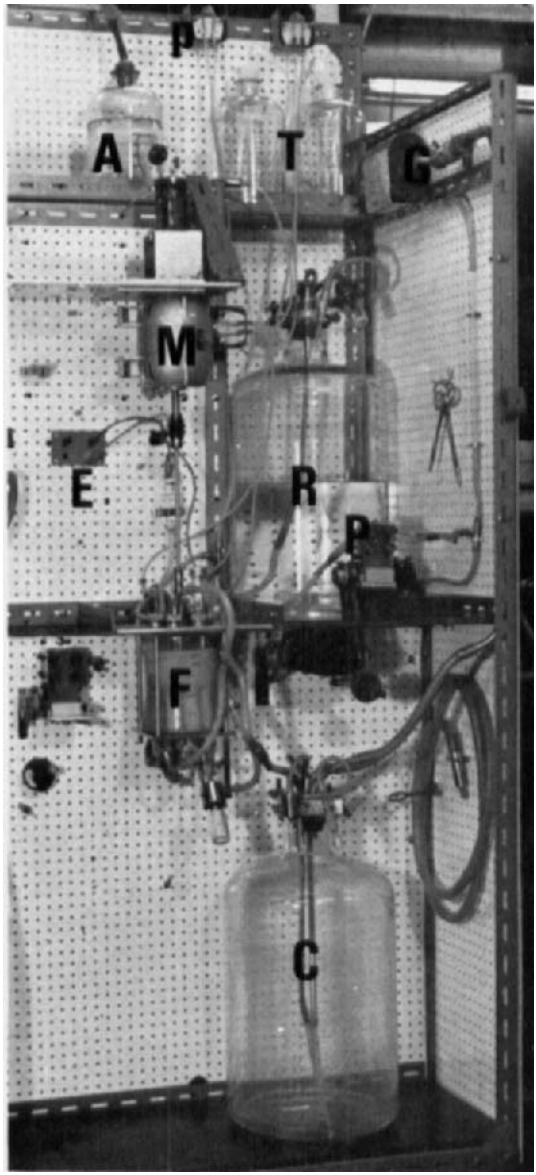


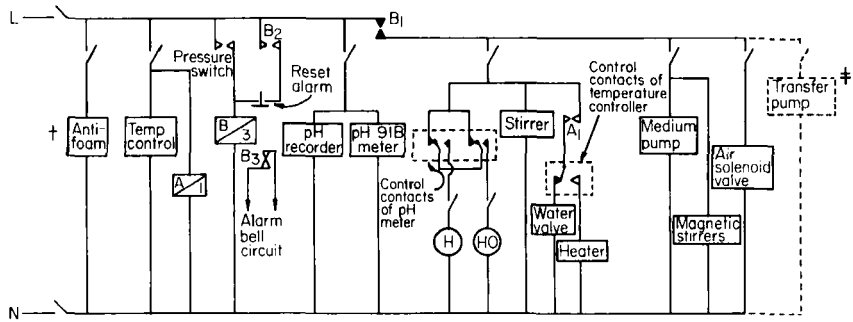
FIG. 2. Fermenter unit, showing position of the culture vessel (F), reservoir (R) and medium pump (P), receiver (C), antifoam (A) and titrant (T) bottles and pumps (P), stirrer motor (M) and pH electrode connector block (E).

FIG. 3. Control box. The upper third of the front panel houses the instruments for automatic pH control (an EIL Model 91A pH Indicator-Controller and an Elmes 12 Cyclogram Recorder, with associated switches); the lower third houses the antifoam timer (left) and temperature controller (right); the centre area locates the air-flow controllers (each side), air-pressure regulator (right) and Variac auto-transformer (left). A main fuse, indicator light and series of switches are positioned along the bottom of the panel.

B. Materials

The framework of the fermenter unit is constructed of slotted steel angle and panelled with "peg-board" (Tufnol is a good alternative material). Since "peg-board" is not resistant to acids and alkalis, it should be coated with a film of polyurethane varnish. Alternatively, Laconite peg-board can be used; this has a resistant enamel finish and needs no further treatment. The unit stands on four castors, which allow easy movement for cleaning and servicing. The controls may be built into standard electronic racks; this facilitates servicing and the easy exchange of faulty components. The control box is made of aluminium sheet, coated with an acid-resistant hard-crackle paint; the framework is of aluminium angle.

All the materials coming in contact with the culture, or with the solutions feeding into the culture (e.g., medium, antifoam, titrants), must be corrosion resistant, non-toxic, sterilizable (preferably by autoclaving), unbreakable and, if possible, transparent. Pyrex glass fulfils most of these requirements, but an all-glass stirred fermenter is impractical, if not impossible, to construct. A good compromise is provided by a glass-walled vessel with stainless-steel top and bottom plates (Fig. 5). Chrome–nickel steels to British Standard Specification 971 (En series) are suitable for this purpose (e.g., En 58B), though the more resistant grade En 58J would be preferred if cultures were to be grown persistently at very low pH values (see Brookes, this Series, Vol. 1). Slight dissolution of the steel is inevitable, irrespective of the culture pH value, and if the iron content of the medium must for any reason be kept within prescribed limits then the metal parts in contact with the various solutions should either be coated with a suitable varnish or replaced by parts machined from inert metals, such as titanium or tantalum.



† For circuit see text and Fig 23.
‡ For 2 stage apparatus only

FIG. 4. Basic wiring diagram (schematic) for a single-stage continuous culture apparatus; see text for detailed description. A circuit diagram for the control of antifoam addition (†) is given in Fig. 23; a control switch for the transfer pump would be added where indicated (‡) with a multistage chemostat control box.

Hose connections can be of natural or synthetic rubber, but silicone rubber tubing of the "medical" or "blood-transfusion" grade (e.g., Esco (Rubber) Ltd tubing, grade TC156) is preferable, since it is completely non-toxic, can withstand repeated cycles of heat sterilization and is resistant to 5M acids and alkalis; also it is translucent. Silicone rubbers are permeable to oxygen and therefore are not suitable for use when growing anaerobic organisms (see later Section); also they are attacked by some silicone antifoaming agents (e.g., "Alkaterge") and oils. In both cases, neoprene is a suitable alternative material, though opaque and somewhat less flexible.

Hard plastics, such as nylon and polytetrafluoroethylene, can be used instead of metals or other materials for some components (e.g., sampling ports). Both plastics are non-toxic, heat stable and corrosion resistant, but do tend to undergo small dimensional changes on autoclaving. Tufnol has been used instead of stainless steel for the construction of the fermenter end plates (G. C. Ware, personal communication).

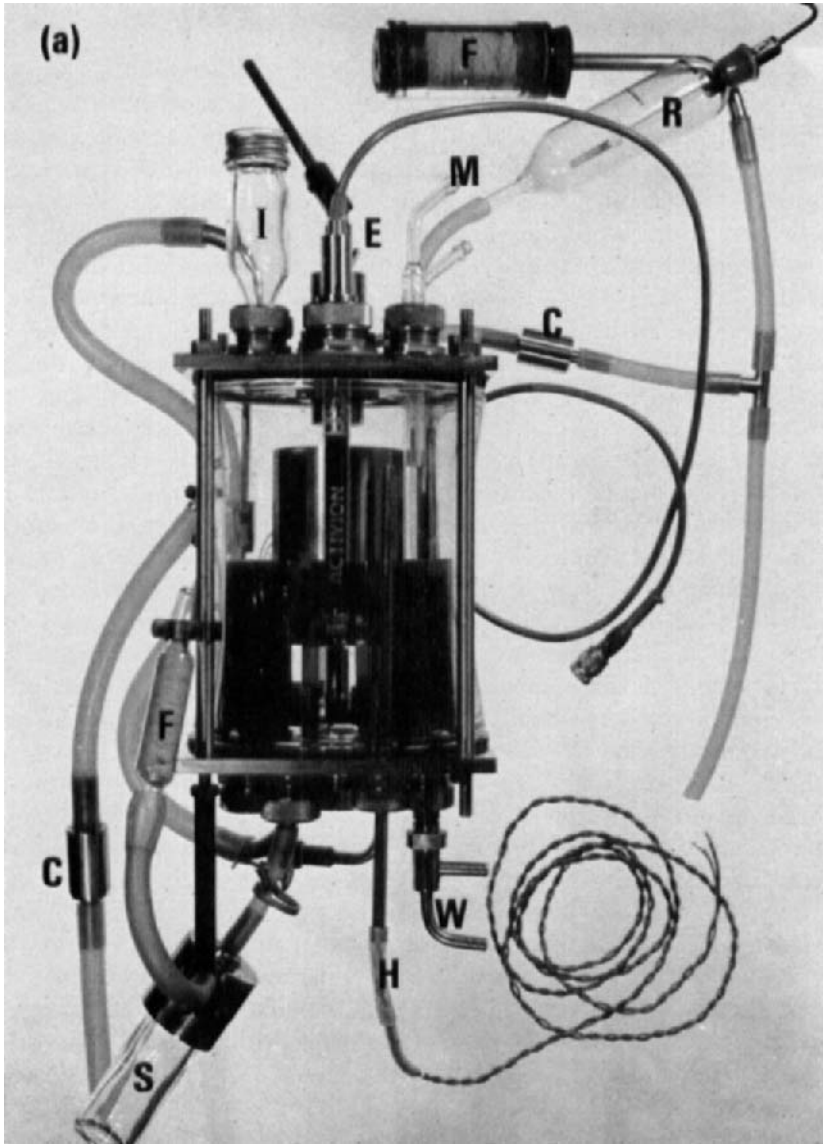
C. Wiring of the control box

A schematic wiring diagram for the single-stage chemostat is shown in Fig. 4; here it will be seen that the circuit is in two parts, separated by a pair of normally closed contacts (B_1). In the first part are the indicators and recorders of temperature and pH value, the antifoam timer and the protecting relays (A_1 and B_3). Relay A_1 is wired in parallel with the temperature controller, and relay B_3 in series with the normally open contacts of a pressure switch; this switch is activated by the pressure in the culture aeration line. Relay B_3 has one pair of normally closed contacts (B_1) and two pairs of normally open contacts (B_2 and B_3). The contacts (B_3) are used to control an alarm bell, or other warning device, to indicate that the pressure in the culture vessel has risen above some pre-set value, the contacts (B_2) ensuring that the relay holds "IN" even after the pressure has fallen and the contacts in the pressure switch have reopened. The contacts (B_1) opening when the relay is energized isolate the rest of the circuit to ensure that stirring, temperature and pH control, medium and air supplies all are interrupted. It is necessary to hold the relay (B_3) in, because if, for example, the exit air filter becomes wetted the pressure will fall gradually after the air and medium supplies cease, and the pressure switch therefore would alternatively "make" and "break" as the pressure rose and fell. A small "push to break" single-pole switch (e.g., Radiospares) is incorporated in the self-holding circuit of B_2 to allow the relay to be reset after remedial action has been taken by the operator.

In the antifoam circuit, any manual addition (i.e., bypassing the control circuit) should be through a "biased OFF" switch. Thus, the switch cannot inadvertently be left on thereby emptying the contents of the antifoam reservoir into the growth vessel.

It will be seen that the stirrer switch also supplies the pH meter control contacts and the temperature control contacts; this arrangement prevents spurious corrections being made to the temperature and culture pH value when the stirrer is off and the contents of the culture vessel are not being adequately mixed. The connection of the pH control contacts, as shown, is suitable when either the EIL 91A or 91B model pH indicator-controllers (Electronic Instruments Ltd, Richmond, Surrey) are used, even though the 91B embodies its own circuit to prevent simultaneous addition of acid

and alkali. This situation can arise with the more sensitive model 91A, especially when a bulb in one of the photocell circuits burns out. A manual switch is fitted in the electrical supply line to both titrant pumps, and as only one pump is in use when the culture is in a steady state, the other can safely be switched off.



In order to ensure that the heating and cooling elements are capable of being energized only when the temperature controller and stirrer are both on, the temperature-control contacts are supplied through the normally open contacts of relay A_1. Depending on the type of controller employed, it may be necessary to use an intermediate "slave" relay to operate the heater and cooling valve as the current handling capacity of the contacts in the controller may be too small for the inductive load of the cooling water

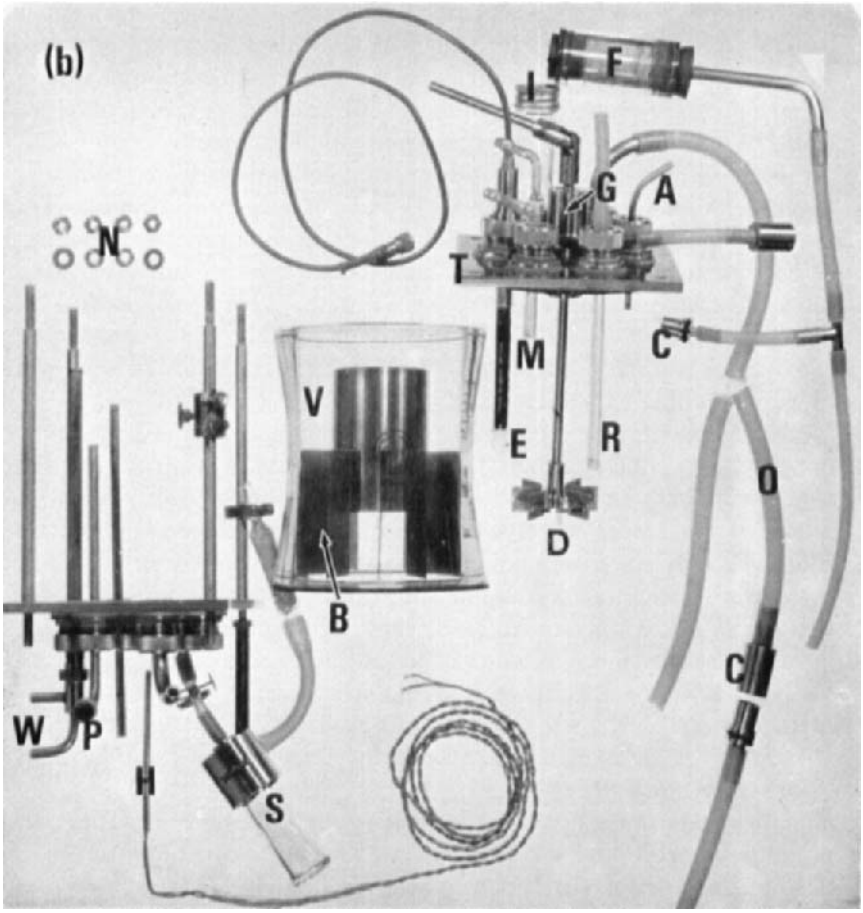


FIG. 5. (a & b). Assembled and "exploded" views of the 1 litre fermenter vessel showing the position of: A, antifoam inlet tube; B, down-draught tube and baffle system; C, connectors; D, impeller; E, glass electrode; F, air filter; G, stirrer gland; H, heating element; I, inoculation port; M, medium inlet; N, locking nuts and washers; O, overflow tube; P, platinum resistance thermometer; R, reference electrode bridge tube; S, sampling port; T, top plate assembly; U, lower plate and tie-rods; V, glass vessel; and W, cold-water finger.

valve (in some controllers the contacts are rated as low as 150 mA). It is good practice to fit a "slave" relay irrespective of the current-carrying capacity of the controller contacts, since, when necessary, the "slave" relay can be changed much more readily than the contacts in the controller.

The air-control solenoid is fitted in the main air supply line when, as is usual, the pressure in this is less than 35 psi; when the main air supply is at a pressure greater than this value, a reduction valve (e.g., Fisher Governor model 67P Small Volume Regulator) must be fitted in the line before the pressure switch. Similar considerations apply to the cooling-water solenoid; a suitable pressure reducer for this is the Williams & James type R6/2, which can be screwed to an ordinary cold-water tap with a Simplifix union. Fitting a pressure-reduction valve allows ordinary rubber tubing to be used, instead of pressure hosing, for connections to the apparatus.

It is advisable to fit lights to indicate that corrections are being made to the temperature (heating and cooling) and pH value, for example. Their frequency of switching can be a useful guide to the proper functioning of the chemostat since, when steady-state conditions prevail, the rates of change of each parameter should be constant. Thorn AEI mains neon signal lamps (type SGF 20/220/Neon) are suitable and available in a variety of colours; it is usual practice to use red indicator lamps for warning lights or for circuits that should come on only occasionally.

It is convenient, where possible, to use a common type of plug-in relay throughout, regardless of the total number of contacts required, since this practice simplifies the problem of replacement should one of them fail. Keyswitch type MK3P relays (with three change-over contacts) are suitable.

Each piece of electrical equipment should be separately fused with an appropriately rated fuse in the line supply only. For this purpose, Rowan FCA switches are convenient, since they incorporate both a fuse and a neon light. Furthermore, since they are only 1 in. in diameter they can be positioned close together thereby saving space on the control box display panel. Unfortunately these switches are available from the U.S.A. only, but even so are cheaper than the combined price of a switch, fuse holder and neon indicator light.

In a two-stage apparatus it is advisable to supply, and control, the transfer pump from the first chemostat control panel; this is indicated by the dotted line on the schematic wiring diagram (Fig. 4).

D. Growth vessel

Essentially, this is a 6 in. length of 4 in. i.d. Pyrex pipe-line (with butt ends ground flat) each end sealed with a $\frac{1}{4}$ in. thick stainless-steel plate separated from the pipe-line by a $\frac{3}{16}$ in. gasket cut circular from a standard neoprene gasket (Fig. 5). The base plate, which is $5\frac{3}{8}$ in. square, has four

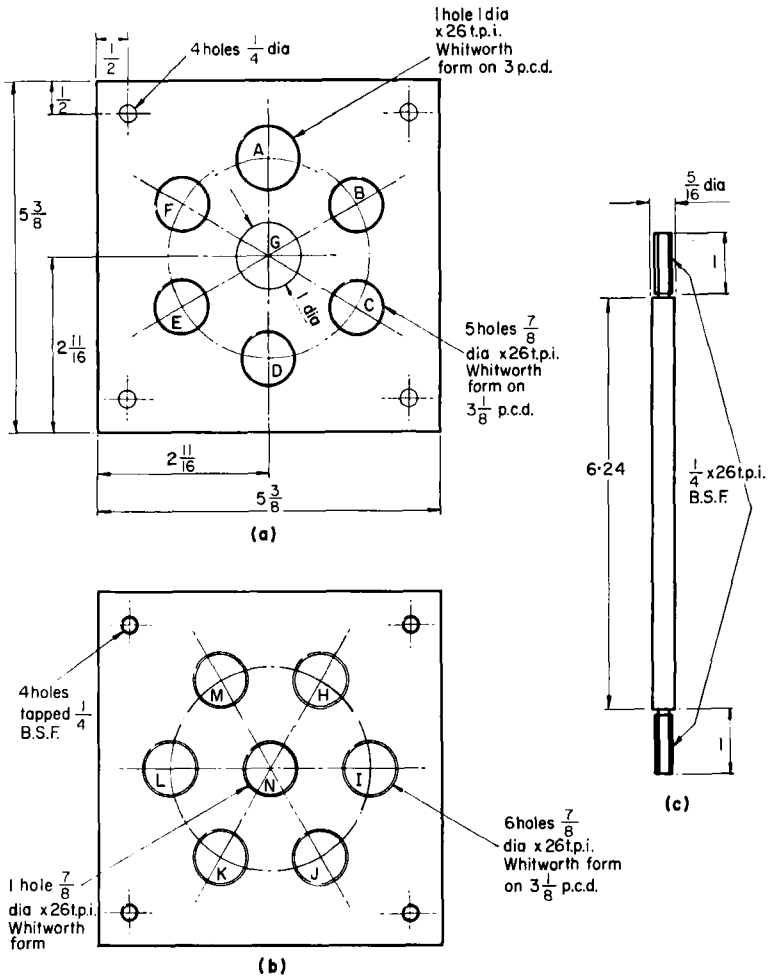


FIG. 6. Drawing of the 1 litre fermenter vessel end-plate assembly (dimensions in inches). The holes in the two plates are drilled to accept bosses for the following: A, oxygen electrode (antifoam inlet when not required for an oxygen electrode); B, liquid junction tube (connected to reservoir of KCl in which dips the calomel reference electrode); C, medium inlet tube; D, pH glass electrode; E, inoculation port; F and L, overflow tube arrangement; G, stirrer gland; H, Sangamo-Weston platinum resistance thermometer; I, cooling finger; J, heating element; K, sampling port; M, titrant inlet tube; N, spare (antifoam inlet when oxygen electrode occupies boss A).

stainless-steel tie rods $\frac{5}{16}$ in. in diameter (Fig. 6) screwed $\frac{1}{2}$ in. from the corners with $\frac{1}{4}$ in. B.S.F. threads at each end. The other end of the tie rods locate with, and pass through, 4 clearance holes in the top plate, the whole

assembly being held together by 4 nuts on the tops of the rods. The tie rods are stepped to prevent over tightening and to ensure equal tightening all round.

Both plates are tapped ($\frac{7}{8}$ in. × 26 t.p.i. Whitworth form) to accept six equispaced bosses on a radius of $1\frac{1}{2}$ in. from the centre (Fig. 6). The base plate can accept a seventh boss in the centre and it is possible to substitute a 1 in. dia. boss in the top plate for a $\frac{7}{8}$ in. boss if the hole is placed on a $1\frac{9}{16}$ in. radius. This substitution makes possible the use of an oxygen electrode (EIL type). A Dowty bonded seal PP45E for $\frac{7}{8}$ in. or PP45F for 1 in. boss or stirrer gland is used between each boss or the stirrer gland and the culture vessel end plate. The seal is replaced every time the boss is removed. The bosses (Fig. 7) have been redesigned to obviate the dead space that

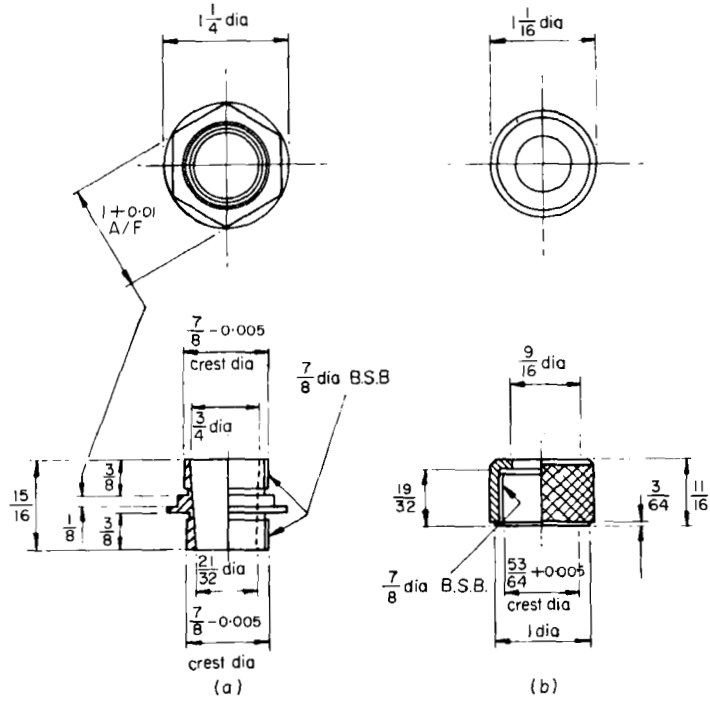


FIG. 7. Drawing of (a) reactor port (stainless steel) and (b) cap (brass), the dimensions being in inches.

used to be present at the bottom of the closing bung in the older types of boss (Herbert *et al.*, 1965); suitable silicone bungs for the new types are Esco 15A for the 1 in. boss and Esco E 17 for the $\frac{7}{8}$ in. boss. The centre of the top plate is drilled with a 1 in. clearance hole for the stirrer gland (Fig. 8).

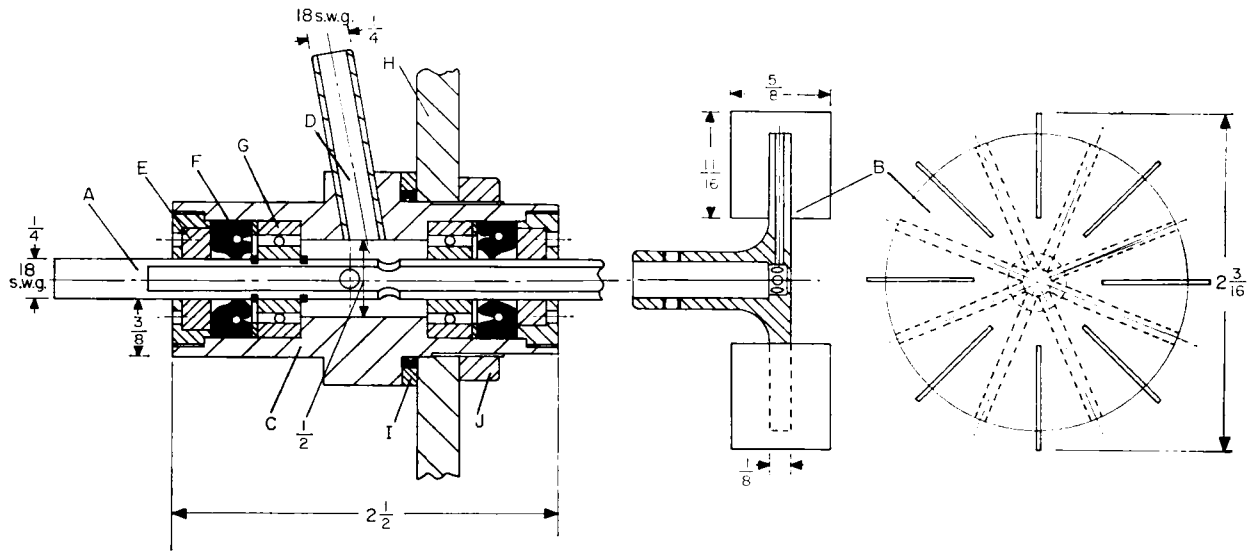


FIG. 8. Drawing of the stirrer and gland assembly (dimensions in inches) showing the position of: A, stirrer shaft; B, impeller; C, gland body; D, air inlet tube; E, felt washers (impregnated with a high-temperature silicone grease); F, Gaco oil seals; and G, stainless-steel ball races. The positions of the fermenter top plate (H), Dowty seal (I) and gland nut (J) also are shown.

Those bosses that pass through the top plate locate the medium-inlet tube (Fig. 9), titrant and antifoam inlets, inoculation port (Fig. 10), KCl bridge tube, glass electrode and air outlet tube; the main air inlet is through the stirrer shaft. The bosses through the base plate accept the overflow tube arrangement (Fig. 11a), sampling port (Fig. 12), cooling coil or finger, heater pocket and platinum resistance thermometer. Since both the top and bottom plates must be easily accessible, the growth vessel is suspended (in a cradle) by the top plate; locking the top plate in a fixed position also facilitates alignment of the impeller and stirrer-motor shafts.

Larger growth vessels may be made from other stock lengths of Pyrex pipeline (QVF Ltd); thus, a 9 in. length of 6 in. dia pipeline would be suitable

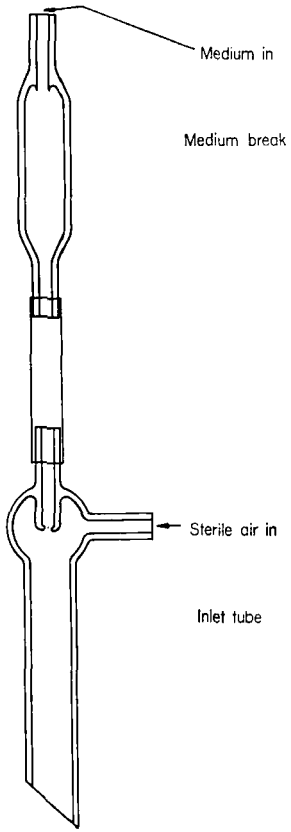


FIG. 9. Medium inlet tube assembly. A slow flow of sterile air is maintained over the medium inlet point in order to prevent aerosolized organisms in the culture vessel from contaminating the medium in the feed line. The "break" tube provides a second barrier to the growth of organisms in the feed line. Both the inlet tube and break tube are made of Pyrex glass.

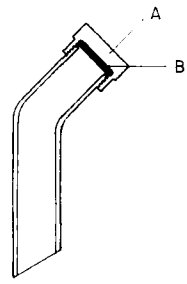


FIG. 10. Stainless-steel inoculation port showing (A) cap, (B) Teflon washer Alternatively the inoculation port can be made from a modified 1 oz. Universal glass container (see Figs. 5 and 29).

for a culture volume of 3 litres, and a 15 in. length of 9 in. dia. pipeline would provide a vessel holding 12 litres of culture. It is even possible to construct a growth vessel of 150 litres capacity by using a 36 in. length of 18 in. dia. pipeline.

E. Mixing and aeration

Agitation of the culture must be sufficient to promote near-perfect mixing and, in the case of aerobic cultures, to allow oxygen to dissolve in the culture at a rate greater than the maximum at which it is used by the growing organisms. For volumes of culture less than 0·5 litre, containing concentrations of organisms less than 1×10^9 bacteria/ml, adequate stirring may be provided by a magnetically coupled rotating small iron bar (coated with PTFE or silicone rubber to prevent corrosion); for larger and more dense cultures, however, more powerful stirring is essential. For the 1 litre

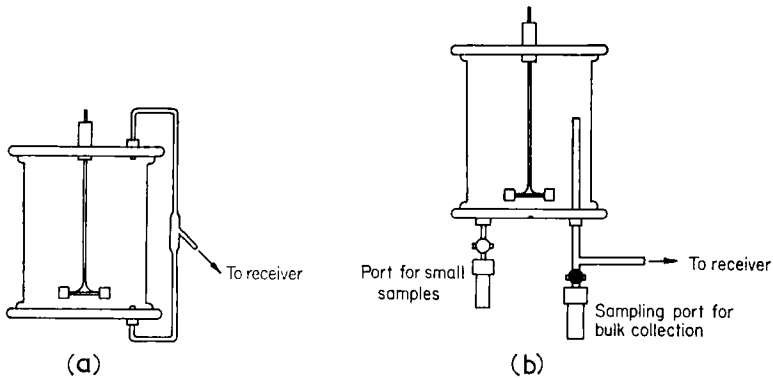


FIG. 11. Arrangement for (a) an external overflow assembly, and (b) an internal overflow tube.

fermenter, described here, the impeller is driven by a 1/30th horse-power a.c. induction motor turning at 1350 rpm. The motor drive is connected to the impeller shaft through universal-joint couplers (Essex or Mollart types). The impeller shaft must pass into the growth vessel through a bacteria-proof gland; the design shown in Fig. 8 has proved most satisfactory in this respect. The shaft ($\frac{1}{4}$ in. dia.) runs in two stainless-steel ball races that are lubricated with a silicone grease (preferably a high-temperature lubricant, such as Midland Silicones Ltd grease, type MS 44). An air-tight seal is maintained by two standard "GACO" oil-seals (Geo. Angus & Co. Ltd), and again these are lubricated with silicone grease. Further lubrication

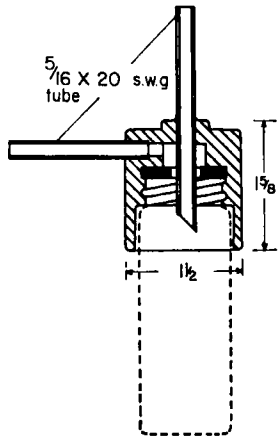


FIG. 12. Stainless-steel hooded sampling port designed to accept a 1 oz Universal glass container (dimensions in inches). The thread form is to B.S. 1918, shallow continuous thread (R 3/2), size 28.

is provided by two grease-packed felt washers and the whole assembly is held in position by two locking nuts. In the centre of the gland, between the ball races, is an air space that connects with the main air-inlet tube. Sterile air flows into this space and is maintained at a pressure slightly above that of the atmosphere. This sterile air passes down the centre of the stirrer shaft and into the culture through holes in the impeller body (Fig. 8). The slightly elevated pressure in the gland tends to prevent both extraneous matter contaminating the sterile air through the upper seal and culture from passing into the gland through the lower seal.

It must be emphasized that the life of the gland depends, to a large extent, upon the quality and finish of the stirrer shaft, and it is recommended that centre-less ground rod be used for at least that portion of the shaft passing through the gland. Any roughness on the shaft will result in the GACO

seals failing early in a run. Though by no means essential, it is good practice to change the seals after every run; these seals are relatively cheap, whereas failure of the glands can be costly in terms of time and materials invested in the experiment.

Mixing is facilitated by the presence of a down-draught tube with baffles (Fig. 13). The impeller is sited so that its blades are close to the bottom

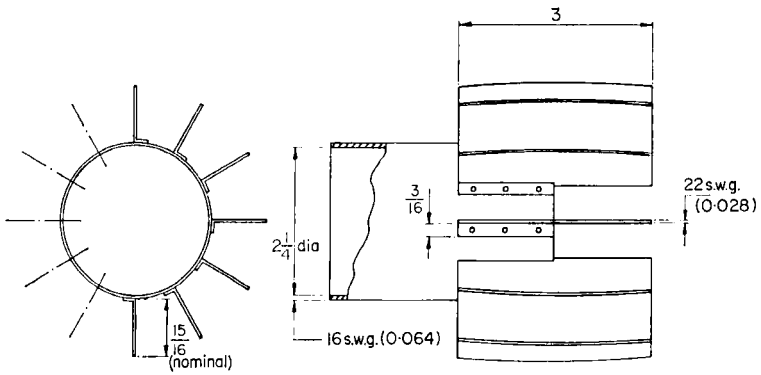


FIG. 13. Down-draught tube and baffle system for the 1 litre growth vessel (dimensions in inches), fabricated of stainless steel.

of the growth vessel; when turning, the impeller causes the culture to circulate up around, and down through, the tube, This arrangement, besides promoting good mixing and aeration, damps oscillations in the culture surface level and thus allows the culture volume to be regulated by means of an internal overflow tube (Fig. 11b), if desired. If the down-draught tube is omitted, an internal overflow tube cannot be used, since the surface level of culture in the vessel fluctuates greatly; this is overcome by using the external overflow arrangement shown in Fig. 11a. With this, the rapid fluctuations in the culture surface level are damped, and a reasonably constant culture volume therefore is maintained in the growth vessel. A disadvantage of this latter method resides in the fact that the culture emerging from the overflow line has spent a period of time (depending on the dilution rate) in the non-aerated side limb where some physiological changes in the organisms may have occurred; these effluent organisms therefore are not representative of those in the growing culture. However, it should be emphasized that this objection is only serious with cultures of small volume, or those being grown at a low dilution rate (i.e., where the rate of flow of culture through the overflow limb is small). But when this apparatus is being used as a research tool (as opposed to a production tool) generally only the organisms in the growing culture are of interest; these are sampled by

direct removal of culture through the specially designed sampling port (Fig. 12).

Larger fermenters may have stirring devices very similar to that detailed in Fig. 8, but the shafts usually are solid. In such cases air is sparged into the culture through a tube whose outlet is located beneath the impeller. A 3 litre fermenter would require a $\frac{3}{8}$ in. dia. shaft and a $\frac{1}{2}$ in. dia. shaft would be necessary for a vessel holding 10 litres of culture; in both cases the gland size would be proportionately bigger than that detailed in Fig. 8.

In some circumstances it may be desirable to vary the rate of stirring. Reducing the speed of a motor by reducing the voltage applied to it will result in a loss of torque and the speed will fluctuate with load variations; moreover, the effective speed range is generally small. However, if a d.c. shunt-wound motor is used, the speed can be varied by altering the voltage applied to the armature. This is quite conveniently done by using two separate rectifier bridges supplied from a.c. mains. One of the bridges supplies the field windings of the motor; the other, which is supplied through a variable transformer, supplies the armature. This arrangement gives a wide range of speeds at fairly constant torque (e.g., Berco Series SR speed regulator). As d.c. motors have brushes that need occasional replacement (often before an experiment is concluded) it may be preferable to use an a.c. slip motor that has an acceptable voltage/torque curve, but again speed will vary with load.

If long-term constant speed is required, there must be a feed-back circuit from the motor to the controller, which then makes the appropriate correction when necessary. In practice this means that the motor must be coupled to a small dynamo whose output voltage is proportional to speed. Linear tachogenerators designed for this purpose are available, but they are very expensive and generally unnecessary for all but the most critical control; a small permanent-magnet d.c. motor will generally suffice. The most precise control is obtained with a d.c. motor driven from a d.c. ampilfier whose output is controlled by the tachogenerator. If rather less precise speed control is acceptable, a much cheaper system is provided by the use of an a.c. slip motor controlled in a similar manner. The advent of the "thyristor" (silicon-controlled rectifier) has enabled simpler switching controllers to be used in place of the amplifier, and although the control is less precise, full torque can be obtained from the motor at very low speeds. The tachogenerator can, of course, be used to give simultaneous visual indication of motor speed on a suitably calibrated meter or recorder (Fig. 14).

To maintain a constant rate of aeration it is more necessary to keep constant the stirrer speed than the rate of air flow. However, some degree of air flow control is necessary, and probably the most widely used system is a flow meter (e.g., Rotameter) supplied through a constant differential-type

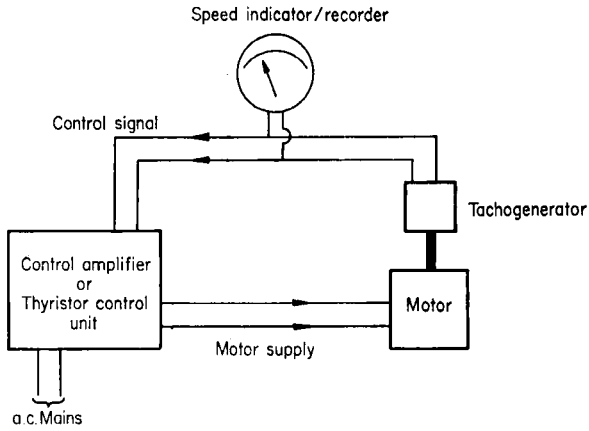


FIG. 14. Schematic drawing of a circuit for varying the speed of an induction motor (stirrer motor) without loss of torque.

flow controller (e.g., AEI Sunvic Model 63BD). The controller maintains a constant flow of air (or gas) through the needle valve of the rotameter by maintaining a constant pressure drop within itself; this is effected by means of a feed-back loop, in the air-supply line, to a spring-loaded valve in the controller (see Fig. 15).

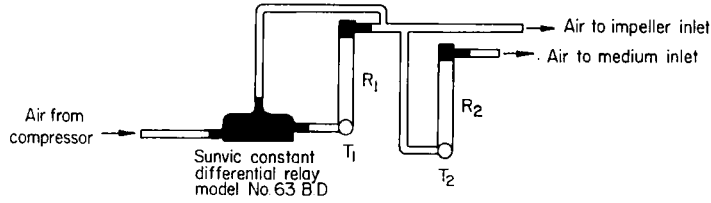


FIG. 15. Arrangement for the control of air flow to the culture vessel. R_1 and R_2 are rotameters with their associated needle valves (T_1 and T_2).

F. Control of medium flow rate

To achieve steady-state conditions in a chemostat, fresh sterile medium must be added continuously to the culture at a pre-set, unvarying, rate. Therefore, the essential requirements of the medium flow control system are that (*a*) the pumps must be capable of delivering medium over a wide range of flow rates (at least 20 : 1), but that at a particular setting the rate of flow must not fluctuate by more than a few percent from the required value, (*b*) all parts of the system that come in contact with the medium must be sterilizable, preferably by autoclaving, (*c*) all parts coming in contact

with the medium must be non-corrodible and non-toxic and (*d*) the pump should be capable of operating continuously for extended periods of time (i.e., several months) without servicing.

Two different types of pumps fulfil these conditions: piston pumps, such as the DCL "Micropump", and peristaltic pumps, such as the Sigmamotor pump. With the Micropump, the reciprocating plunger is driven at a constant speed and alteration of the medium flow rate is effected by varying the

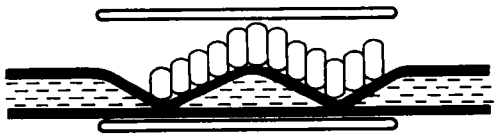


FIG. 16. Peristaltic pump—reciprocating-finger type (e.g., Sigmamotor pump).

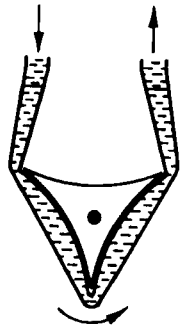


FIG. 17. Peristaltic pump—roller type (e.g., Watson Marlow Delta pump).

plunger stroke. The pump head and plunger are of stainless steel and can be detached easily from the pumping mechanism for sterilization. By suitable choice of plunger size and motor speed, flow-rate ranges between 10 and 1500 ml/h can be attained; the rate with any particular combination of motor and plunger can be adjusted over a range of about 25 : 1.

Several different types of peristaltic pumps are marketed; these may be of the "roller" or "finger" variety (Figs. 16 and 17). The Sigmamotor pump, model TS 6, is a finger-type, in which the flow rate is varied either by means of a timed "on–off" cycling of the power supply to the motor (e.g., with an Elcontrol Ltd timer, model CTRC) or by changing the rate of peristalsis. While the use of the timer is theoretically objectionable, it has been found acceptable in practice provided that the on and off times are kept as nearly equal as possible; but frequent small additions are preferable to occasional large ones. The rate of peristalsis can be changed either by varying the motor

speed or by interposing a variable speed gear-box (e.g., Revco Zeromax) between the drive motor and the pump head to vary the pumping speed. A more compact assembly, which is also more versatile, is provided by the Watson Marlow MHRE variable speed flow inducer, which is a pump of the roller-type. The motor speed is controlled by the setting of a 10 turn helipot resistance; this gives a range of flow rates from 100 to 1; by using tubing of different diameters, a whole series of flow-rate ranges are possible; different sizes of tubing from $\frac{1}{16}$ to $\frac{3}{8}$ in. bore may be used enabling the maximum flow rate to be varied from 0·48 to 54·0 litres/h. Silicone-rubber tubing is excellent for use with these pumps, being much more durable than the natural rubbers.

With all these pumps, the variations in flow rate at a fixed setting should be insufficient to require adjustment of the pumping rate more than once every 24 h. However, it is advisable to check the medium flow rate more frequently (never less than daily) and this is easily done by means of the burette arrangement shown in Fig. 18; the medium is drawn into the burette from the reservoir and then allowed to flow through the metering pump to the culture. The time required to pump a measured volume of medium is determined, and from this the medium flow rate calculated. A slightly

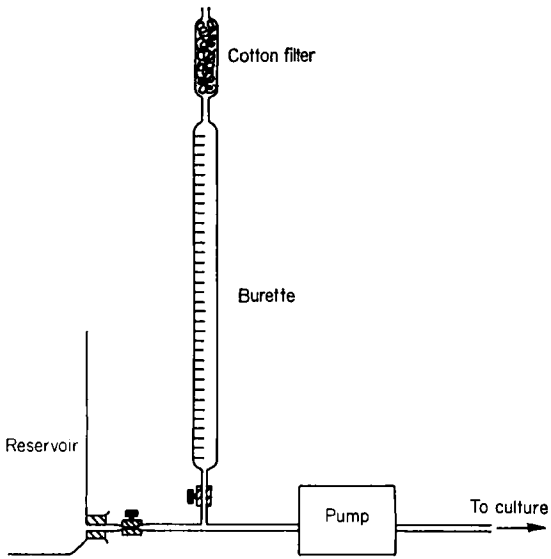


FIG. 18. Arrangement for determining the medium flow rate. The burette is filled from the reservoir, which is then closed off; the time taken for a given volume of medium to be pumped from the burette is measured, and from this the medium flow rate calculated. With this arrangement the flow of medium to the culture is at no time interrupted.

more accurate method with peristaltic pumps is to use the burette to determine the volume delivered by each cycle of the pump (i.e., by measuring the volume delivered by, say, 10 pump cycles) and then measure the pump rate with a counter (e.g., Veeder-Root series 1341) activated by a cam on the pump. Continuously counting the pump revolutions allows also an overall check to be kept on the daily flow rate. In practice it is found that the delivery per cycle alters very little unless new tubing is used; in this case changes do occur during the first few days due to "bedding in" of the tubing.

G. Control of culture temperature

Several methods of controlling the culture temperature are possible, e.g., the apparatus can be located in a constant-temperature room or incubator, or the growth vessel can be placed in a water bath or jacket. However, with vigorously stirred cultures containing dense populations of organisms, these methods are either unreliable or impractical. Furthermore, both these methods are indirect, in that it is not the culture temperature that is being regulated but the fermenter surroundings; the differential between the temperature of the culture and its surroundings may be considerable (i.e. $> 10°C$ with a vigorously stirred dense culture of organisms in a 1 litre glass fermenter). It is better, both in principle and in practice, to control directly the temperature of the culture. This requires a temperature-sensing element within the culture, heat source, cooling system and controller. With the 1 litre chemostat described here, we recommend using a gas-filled platinum resistance thermometer as the sensing element (e.g., Sangamo Weston Ltd, Model S110G, form 4) in conjunction with a precision temperature controller (e.g., Fielden Electronics Ltd, Model TcB2); this system is capable

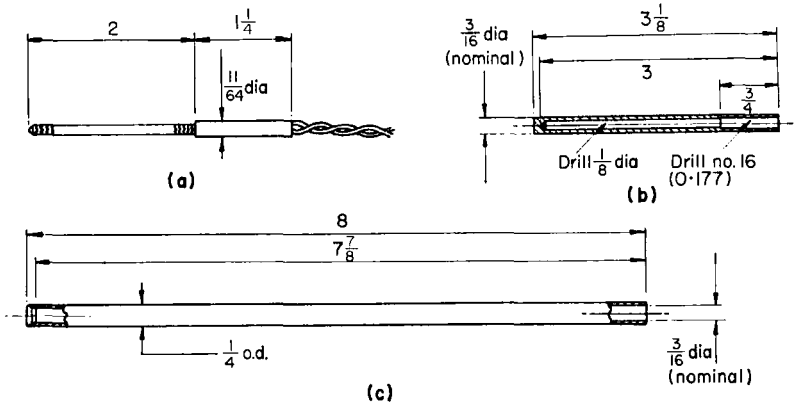


FIG. 19. Construction of a 25 W immersion heater from an Antex soldering iron element. The dimensions are in inches.

operating with a differential of less than 0·2°C. If dissolved oxygen measurements are to be made, a TcB9 controller, which has a differential of 0·05°C should be used. Since the thermometer is mounted in a stainless-steel jacket, it can be inserted directly into the culture through a boss in the bottom plate; the sensitivity of the thermometer is not damaged by heat sterilization and it can therefore be autoclaved with the growth vessel providing the end plug is covered.

The recommended source of heating is a 20 W immersion heater fabricated from a soldering iron element (e.g., Antex E 240E; Fig. 19). As this is

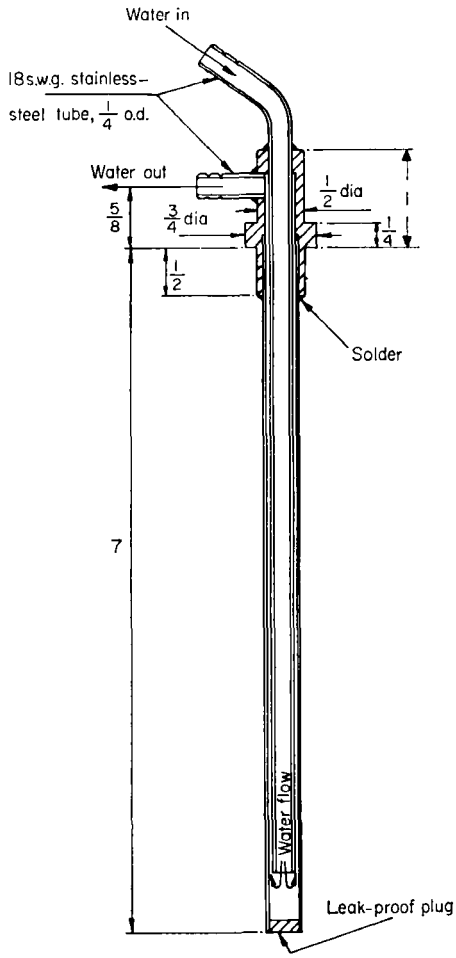


FIG. 20. Drawing of a stainless-steel cooling finger, hose connections not shown. Dimensions in inches.

rather fragile, it is placed in a brass sleeve and the electrical connections are embedded in Araldite. The assembly is made a sliding fit into a pocket made from thin walled $\frac{1}{4}$ in. o.d. polished stainless-steel tubing. In use, these heaters have been found to have a life of several thousand hours. Alternatively, heating can be provided by an infrared lamp (though this has rather a limited life and may burn out during a "run") or by steam or hot water (circulating through a stainless-steel coil or "finger") or by a thermoelectric cooling device (see later). Positive cooling is best provided by a regulated supply of cold water through a coil or "finger" (Fig. 20) placed within the culture. Control of the water supply is effected by a solenoid operated valve (e.g., Asco valve, LM826213). This type of valve could be used also to regulate the flow of hot water or steam through the coil, if the culture is heated by one of these means.

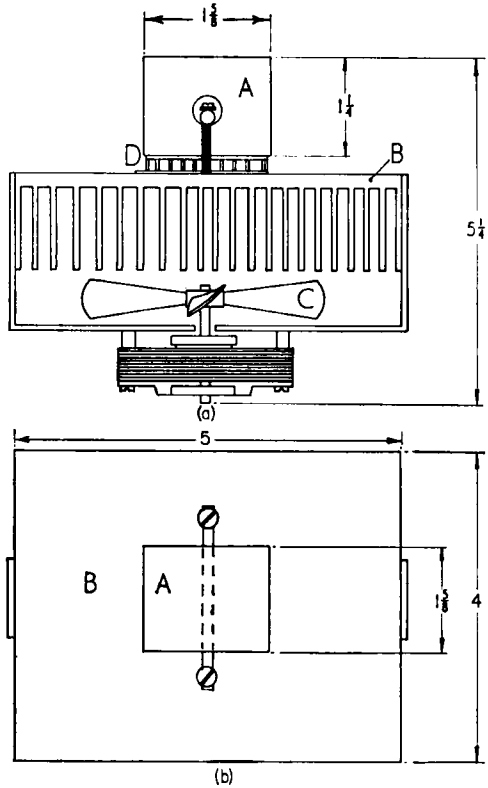


FIG. 21. Drawing of a thermoelectric devices heat pump (type HP 3215), which can be used both as a heat source and cooling source for a 1 litre chemostat; (a), side view; (b), plan view of this unit. A, heat transfer block; B, aluminium finning; C, fan; D, thermoelectric device. Dimensions in inches.

In certain circumstances (e.g., when growing pathogens) it may not be desirable to use cooling water. It is then worth considering the use of some thermoelectric cooling device, such as the "heat pump" (Thermo-Electric Devices). This utilizes the Peltier effect, viz, the adsorption of heat at the junction of two dissimilar conductors. When direct current is passed, one side of the device (depending on polarity) becomes cold and the other side hot; thus, by arranging for the temperature controller to operate a relay to reverse the direction of current flow, one such module can be used for both heating and cooling. The heating efficiency of such a module is greater than that of a resistance heater and it is necessary to dissipate heat pumped to the module face that is not in contact with the base of the culture vessel; this is done by a small fan blowing air over aluminium finning attached to that face (Fig. 21).

The cost of this device—which is used in conjunction with a low-voltage, high-current power pack—is such as to make it worthwhile only in special circumstances. A possible disadvantage is that when fitted, the culture vessel base plate has room for two bosses only; but as three bosses (two for the cooling coil and one for the heater pocket) are no longer required, only the temperature probe needs re-siting in the top plate.

As a precautionary measure the heater and the cooling-water valve preferably should be supplied through the stirrer switch; thus spurious temperature corrections are avoided when the stirrer is switched off to check the culture volume, for example.

H. Control of culture pH value

The culture pH value may change as a result of acid production or assimilation of either anion (e.g., acetate, phosphate) or cation (NH_4^+, K^+) by the growing organisms. Small changes can be suppressed by adequate buffering of the culture medium, but large changes, which almost invariably occur with dense cultures (e.g., 10 mg bacteria/ml contain the equivalent of 0·1 M NH_4^+), require controlling by regular addition of titrant to the culture.

The basic requirements for automatic pH control have been described by Callow and Pirt (1956) and are (*a*) a pair of suitable electrodes, (*b*) a pH meter of the direct-reading type (i.e., the type which is essentially an electronic millivoltmeter having an exceptionally high input impedance) and (*c*) a controller to regulate the flow of titrant to the culture. A continuous recording of the culture pH value is also desirable. Since the glass electrode dips into the culture it must be sterilized. Glass electrodes of any type may be sterilized chemically (e.g., with ethylene oxide) though autoclavable small glass electrodes are now available (e.g., Activion, sterilizable type). In practice it is not necessary to sterilize the reference electrode because it

is located outside the growth vessel and connected with the culture through a KCl "bridge" (an NaCl bridge is used with some reference electrodes). The KCl solution also need not be sterilized providing junction with the culture is effected through a ceramic plug of sufficiently fine porosity to prevent the passage of contaminant organisms (e.g., the liquid junction tube type LZ 28 supplied by EIL has a porosity of less than 1 μm; however they must be tested with *Serratia marcescens* before use).

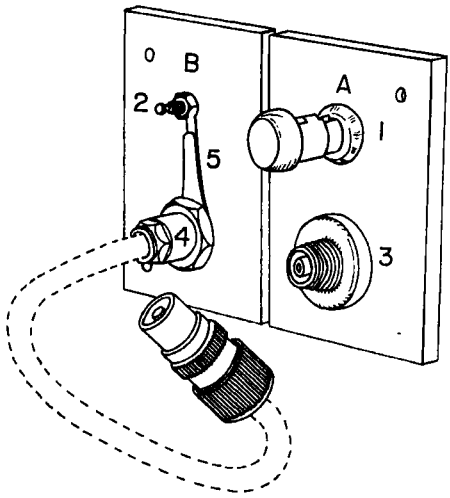


FIG. 22. Drawing of (A) front and (B) rear views of connector block for PH electrode leads. (1) & (2) Painton terminal, (3) & (4) PET connector, (5) linking of terminal to connector screen.

Control of the culture pH value may be effected either with a direct-reading pH meter, whose output is proportional to the pH value, actuating a recorder–controller, or better with a pH indicator–controller (preferably feeding a "slave" recorder). A suitable pH indicator–controller is the EIL Model 91B; this meter is very compact and, since it is fully transistorized, has a very short "warm-up" time (< 5 sec). It can be coupled with an Elmes 12 "Cyclogram" recorder and titrant flow controller as seen in Fig. 3; a wiring diagram is given in Fig. 4. It seems to be insufficiently realized that there is no need for temperature compensation of pH electrodes in a temperature-controlled culture vessel; this greatly simplifies the connection of the electrodes to the meter on the instrument panel and ordinary television coaxial sockets are quite adequate. Alternatively, bulkhead sockets (e.g., Belling and Lee, or Precision Electrical Terminations, which are to be found in the EIL 91B meters) can be used. They should, however,

be mounted upon a good insulating material, such as a paper- or fabric-reinforced phenolic resin, e.g., Paxoline or Tufnol; a very convenient arrangement, which allows easy replacement of the reference electrode, is shown in Fig. 22.

The Model 91B pH indicator–controller has a simple 3-position on–off control with adjustable "High" and "Low" contacts switching the acid and alkali delivery units, respectively, when the culture pH value drifts beyond the set values. There is an adjustable "neutral zone" between the two settings in which titrant flow is not called for; this zone should be small, but not so small that the meter "hunts" between the High and Low contacts. Alternatively, one contact can be switched off since the pH drift is unidirectional when the culture is in a steady state. To avoid overshoot, and to minimize the pH differential, the titrants (usually 2–5M HCl and either 2M NaOH or 2M NH_4OH) should be added to the culture at a slow rate. Where titrant is added by gravity feed through a solenoid-operated pinch-valve (e.g., Pye delivery unit) the rate of flow can be regulated effectively either by a variable on–off timer (Wright, 1960) or, more simply but less reliably, by constriction of the titrant line with a thumb-clip. Recently, small cheap peristaltic pumps (rotary type) have become available (e.g., Delta and pumps) which are ideal for titrant delivery, since the flow rate is small (< 25 ml/h). If Delta pumps are used to control the pH it is advisable to mount them higher than the titrant reservoirs and the culture vessel to ensure that if the tubing splits titrant will not run out of the reservoir into the pump and over the apparatus. We have never experienced failure of the tubing, but on one occasion it was pulled from the pump nipple. With this control system a differential of less than 0·05 pH is possible even with poorly buffered cultures; development of a more complicated proportional control system is therefore unnecessary, even when very strong titrant solutions (5M) are used.

The titrant feed line and inlet ports must be of materials resistant to acids and alkalis. Silicone-rubber tubing is satisfactory for the feed line, and the inlet ports can be fabricated from either tantalum or glass tubing.

Although control of the culture pH value is easy to effect, it is more susceptible to failure than the other control systems. Coating of the electrodes with organisms is the most frequent source of trouble, and, in extreme cases, the electrodes must be removed from the growth vessel and either cleaned or changed. Coating can be minimized by locating the membrane of the glass electrode in the zone of maximum turbulence. Another problem accompanies the use of valve-operated pH meters in the control system; these have a long warm-up period (during which the wrong pH value is indicated) and are troublesome in areas subject to power failures (see later Section). It is good practice to arrange for the electrical supply to the titrant

valves or pumps to be through the stirrer switch; this avoids spurious corrections to the culture pH value when the stirrer switch is turned off.

I. Control of culture volume; suppression of foaming

The culture volume must be maintained constant by removing liquid from the growth vessel at a rate equal to the sum of the rates of addition of medium, titrant, etc. The simplest and most effective way of doing this is by means of a "weir", which may be situated either within the culture vessel (a simple overflow tube; Fig. 11b) or outside it (Fig. 11a). However, when the culture is aerated and vigorously agitated, much air is entrapped in the liquid, thereby causing culture to be displaced from the growth vessel. The external weir is much less sensitive to changes in the foam content of the culture than is the internal overflow tube and may be preferred for this reason. Furthermore, with the external overflow arrangement it is easy to change the culture volume (even to drain all the culture from the vessel) by varying the position of the side-limb.

In order to maintain precise control of the culture liquid volume by the above means, it is necessary to control foam formation. Foaming generally occurs when cultures containing more than the equivalent of 2 mg dry weight bacteria/ml are aerated and vigorously agitated. But the tendency of any particular culture to foam will depend on many other factors, including the surface properties of the organisms and the composition of the medium. Excessive foaming can be controlled by adding suitable defoaming agents to the culture. However, of the many substances with marked antifoaming properties that are known, only a few are of practical value, in that they are non-toxic, non-corrosive, non-metabolizable and sterilizable. One such substance is Polyglycol PPG 2000 (a polypropylene glycol supplied by Shell Chemical Co. Ltd); another is Silicone Antifoam RD (Hopkins & Williams Ltd), but this is an emulsion requiring some care in heat sterilization (see later Section).

One method of adding antifoam to the culture is to incorporate it in the medium before sterilization. The difficulty here lies in keeping the antifoam uniformly dispersed; some complex media appear to contain substances that act as emulsifying agents and assist dispersion, but the method is not satisfactory with simple chemically defined media. A second method is to incorporate in the fermenter a "foam probe" consisting of an insulated electrode connected to an electronic relay; rising foam contacts the electrode and activates the relay, which switches on a pump or opens a solenoid valve to admit antifoam to the culture. In our experience, such probes usually fail after a few days, owing to bridging of the insulation by a moisture film. In any case, it is better to prevent the onset of foaming rather than attempt to quench it once it has occurred, since organisms tend to

lyse in the foam and the associated release of protein markedly increases foaming and the antifoam requirement (Pirt and Callow, 1958).

The preferred method is to add antifoam at a constant rate (determined by trial) just sufficient to suppress foaming; this method is actually more economical of antifoam. Ideally the antifoam addition should be continuous; this is quite feasible on the plant scale, where it is the recommended method, but is difficult on the laboratory scale because of the low flow rates involved (ca. 0·1–0·2 ml antifoam/h). It is simpler and quite satisfactory to add small "shots" of antifoam (e.g., 0·05 ml) at regular intervals determined by an adjustable process timer. This may be done by causing the timer to switch on a small antifoam pump for a brief period determined by a time-delay relay; a suitable circuit is shown schematically in Fig. 23.

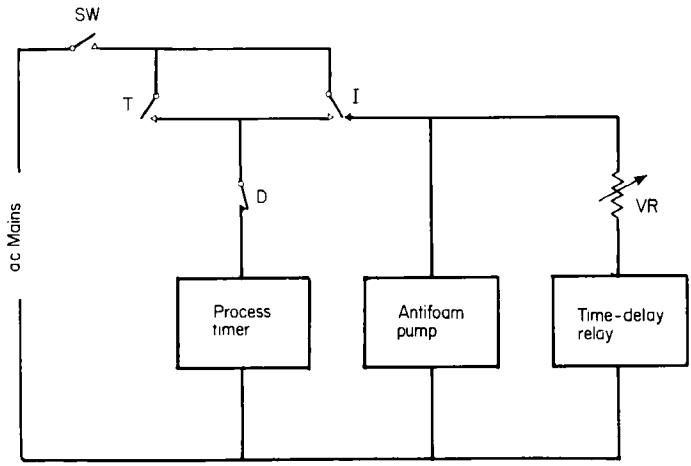


FIG. 23. Circuit diagram of a timer unit that provides for a variable frequency, variable dose addition of antifoam to the chemostat culture. SW, mains on–off switch; I, "instantaneous" and D, "delay" contacts of timer; T, contact of relay; VR, variable resistor; see text.

The auto-resetting process timer, which determines the time between antifoam additions, is a synchronous motor type and may be set to operate repetitively at any time interval from 1 to 60 min. It is fitted with "instantaneous" contacts, I, which operate at the beginning of the timing cycle, and "delay" contacts, D, which operate at the end of the cycle. The time-delay relay is an electronic type, with a delay time adjustable from 0·2 to 60 sec by the variable resistor VR. The antifoam pump and the time-delay relay are wired in parallel, are connected to the mains supply through the normally closed contacts of I, and are energized together when the mains

switch SW is closed. The antifoam pump then operates for a period determined by the delay time of the relay, which is adjusted by VR to give an antifoam "shot" of the required size. When the time-delay relay operates, its contact, T, closes and energizes the process timer through D, causing the timer to start its cycle and contact I to change over. This de-energizes both antifoam pump and time-delay relay; the latter re-sets and contact D re-opens, but the process timer remains energized through I until the end of its timing cycle is reached. Contact D then opens and de-energizes the process timer, causing I to return to the "off" position; the entire cycle then repeats indefinitely.

This arrangement has been found to be a very flexible one. Many suitable process timers are available, e.g., the Type IMP timer. The time-delay relay is an inexpensive electronic type (Type PT-900A-C) giving a timing range of 0·2–60 sec with a variable resistor VR of 500 kΩ. Alternatively, a thermal relay can be used, with a value of VR about equal to the resistance of the heater winding; some thermal relays have a mechanical adjustment of time delay, in which case VR may be omitted. A pneumatic relay is another possibility, but these, like thermal relays, usually have a smaller range of time adjustment than electronic types. Many types of antifoam pump are available, a suitable one being the Delta pump; a solenoid valve may be used instead to admit antifoam by gravity from an overhead reservoir, but a pump allows more precise metering of the antifoam.

J. Continuous measurement of culture absorbance

The continuous measurement of culture absorbance is an essential requirement for operating a turbidostat; this is dealt with extensively by Munson, this Volume, p. 349. However, the 1 litre chemostat described here can easily be modified to allow a continuous measurement of the culture absorbance. With the arrangement shown in Fig. 24, culture is circulated rapidly from the growth vessel through an external "flow-through" photometer which continuously monitors the optical density (cf. Cook, 1951; Moss 1956; Herbert *et al.*, 1965). A peristaltic pump is used for circulation and the outlet tube from the culture is made to descend vertically in order that air bubbles in the culture have time to coalesce and leave the tube before being carried into the photometer. After passage through the photometer the culture is returned to the growth vessel through the top plate.

Design of the photometer is not critical. Almost any commercially available photoelectric absorptiometer capable of being fitted with a flow-through cell could be used; we have found a very simple unit using a 1 W lamp and CdS photoconductive cell (cf. Řičica, 1966) very satisfactory. Whatever type of photometer is used, the main technical problem arises from adherence of organisms to the walls of the flow-through cell, giving

falsely high absorbance readings. This is overcome in the arrangement shown in Fig. 24, which allows the flow-through cell to be periodically flushed through with 5M NaOH, followed by sterile distilled water; if this operation (which takes only a few minutes) is carried out once or twice a day, the flow-through cell can be kept optically clean for many weeks.

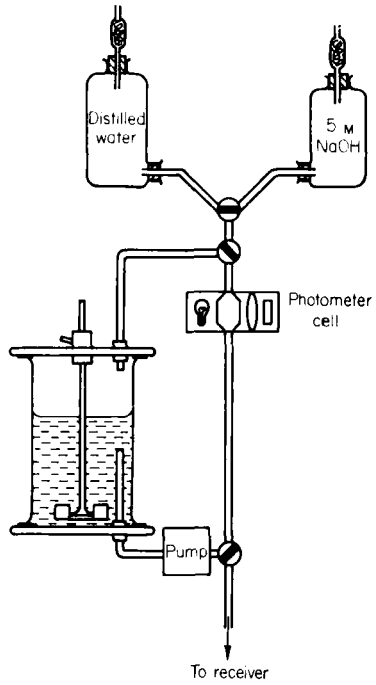


FIG. 24. Arrangement for the continuous measurement of culture absorbance.

The output from the photometer may be coupled to a recorder for continuous monitoring of absorbance. If a recorder–controller is used, it is easy to arrange for this to switch on a pump that adds fresh medium to the fermenter whenever the absorbance exceeds a pre-set level; the arrangement then becomes a turbidostat.

K. Measurement and control of culture dissolved oxygen concentration

Since the metabolism of aerobic organisms is greatly influenced by the level of oxygen dissolved in the environment, it is desirable to measure this parameter in steady-state nutrient-limited cultures to ascertain that it is

not limiting growth. Furthermore, if techniques could be devised for the rapid and continuous measurement of the dissolved oxygen concentration in a growing culture, it should be easy to develop methods for its regulation. Although several such methods have been suggested (Herbert *et al.*, 1965, MacLennan and Pirt, 1967), an oxygen electrode of adequate reliability and long-term stability is still lacking. The electrode described by Mackereth (1964) fulfils most of the essential requirements and, further, has the desirable properties of a very high oxygen current and very low residual current. But it is too large for a 1 litre chemostat and although a miniaturized model (EIL A–15A) is available, it cannot be sterilized by autoclaving and has a rather short life (< 7 days) at 37°C.

The Mackereth electrode is of the galvanic-cell type and generates its own EMF so that no external stabilized d.c. power supply is required; all that is necessary for continuous dissolved-oxygen measurement is connection of

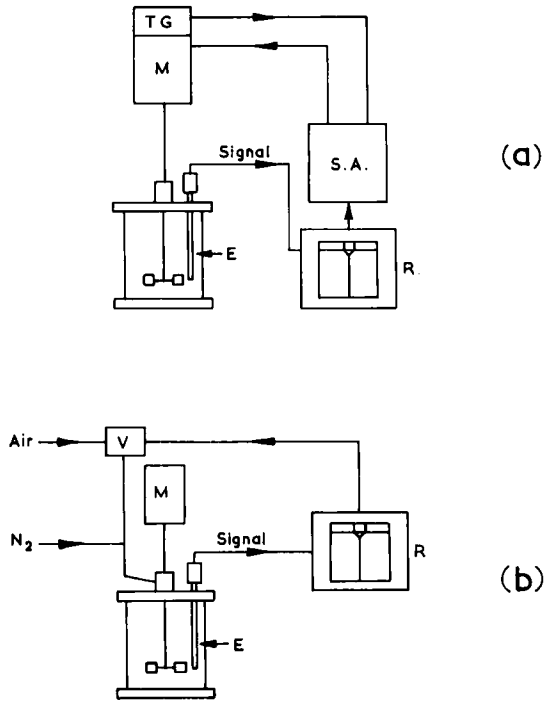


FIG. 25. Alternative arrangements for the measurement and control of culture dissolved oxygen concentration. The oxygen solution rate is varied in (a) by varying the stirring speed, and in (b) by varying the oxygen content of the gassing mixture. E, oxygen electrode; M, stirrer motor; TG, tachogenerator; V, valve; R, recorder; S.A., servo amplifier.

the output signal to a suitable recorder. If very low oxygen tensions are to be measured, a sensitive potentiometric recorder is needed; the Kipps and Zonen "Micrograph" recorder (50 μV full-scale deflection) is satisfactory. Temperature control must be very efficient, since oxygen electrodes generally are very temperature sensitive; a differential of $\pm 0{\cdot}05°C$ has a detectable effect on the output from a Mackereth electrode and is the maximum acceptable when the oxygen solution rate is to be controlled.

Since the concentration of dissolved oxygen in a culture, at any instant, depends on the rate of oxygen consumption by the organisms and the rate of solution of oxygen from the gaseous phase, control of the culture dissolved oxygen concentration at a fixed value can be effected only through controlling the oxygen solution rate. Preliminary experiments (D. Herbert and P. J. Phipps, unpublished work) have shown that reasonably good control can be achieved over a wide range of dissolved oxygen levels by servo control of the stirrer speed, the air "flow-through" rate being kept constant (Fig. 25). Alternatively, servo control of the oxygen "flow-through" rate could be effected (MacLennan and Pirt, 1967). Neither method is wholly satisfactory; changing the stirrer speed inevitably influences the mixing efficiency (but permits a continuous measurement of oxygen uptake to be made), whereas varying the gassing rate, or oxygen content of the gassing mixture, does not affect mixing but complicates measurement of oxygen uptake by the growing organisms.

III. OPERATIONAL CONSIDERATIONS

A. Sterilization of the apparatus

A 1 litre chemostat is sufficiently small to be sterilized in an autoclave (20 min with steam at 20 psi). To facilitate packing into the autoclave and subsequent re-assembly, stainless-steel connectors (Fig. 26) are inserted in the various hoses at appropriate places.

The growth vessel is sterilized with the glass electrode removed and the port plugged with a silicone-rubber bung (if the electrode is of the non-autoclavable type), the immersion heater withdrawn from its jacket and the hoses uncoupled. Hoses to the cooling finger and KCl liquid-junction tube are disconnected and that to the air supply uncoupled with the filter attached to the culture vessel. The connectors, filters and sample ports are wrapped in aluminium foil and the vessel (with titrant reservoirs) and attachments carefully arranged on a tray. The reservoir, receiver and antifoam bottles (and hose connections) are packed separately.

After sterilization, the whole apparatus is re-assembled aseptically. If a standard glass electrode is used it must be sterilized chemically before being

transferred aseptically to the growth vessel. Ethylene oxide vapour is probably the most convenient and frequently used sterilizing agent, but it is toxic and, when mixed with 3–90% air, explosive. It should therefore be handled with care and perferably be used with the addition of CO_2—generally 10% ethylene oxide in 90% CO_2. A damp cotton-wool pad in the bottom of the electrode container raises the relative humidity to above 40%, increasing the efficiency of sterilization.

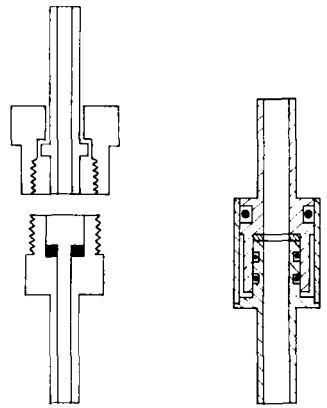


FIG. 26. Two types of stainless-steel connectors: (a), screwed connector; (b), push connector.

After assembly, the titrant reservoirs are filled with 2–5 M acid and alkali, respectively, and the medium reservoir with sterile medium. The growth vessel is then filled with medium from the reservoir and the stirrer motor switched on. The pH value, temperature and aeration rate can then be adjusted to the required values. Ideally, the chemostat should be left running for a period of at least 24 h before inoculation, in order to ensure that (*a*) the medium is not contaminated with micro-organisms and (*b*) all control systems are functioning adequately.

B. Preparation of media

Since, in a chemostat culture, the population density should be dependent on the concentration of a single "growth-limiting" nutrient (see previous Chapter), any particular growth medium must be compounded in a way such that all other nutrients are present in excess of requirement. But it is difficult to prescribe the degree of excess, since some compounds may be

toxic to the organisms when present in high concentration. As a "rule of thumb" one might suggest an amount equivalent to 2–3 times that present in the growing organisms. However, this would be only a crude approximation, since the nutrient content of cells may be either difficult to ascertain or extremely variable (see Powell, 1965; Tempest and Dicks, 1967).

With each organism, and with each medium, it is preferable to establish experimentally that the expected growth-limiting nutrient is, in fact, limiting growth, and that all other nutrients are present in adequate excess. To do this, all that is necessary is to show that at a fixed dilution rate, and within limits, the microbial population density is directly proportional to the growth-limiting substrate concentration in the reservoir (Fig. 27). If the

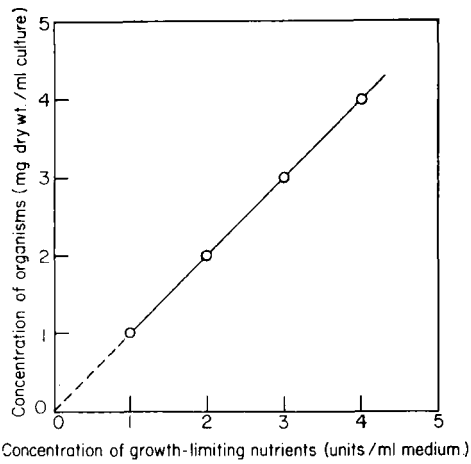


FIG. 27. Theoretical plot of the relationship between the medium growth-limiting substrate content and the steady-state bacterial concentration in a chemostat culture growing at a fixed dilution rate, temperature and pH value.

organisms have an absolute requirement for the growth-limiting nutrient, then the extrapolated plot should pass through the origin (as, for example, with the Mg^{2+}-limited growth of *Aerobacter aerogenes*—see Tempest *et al.*, 1965).

The growth-limiting component of the medium can be any compound or element essential for the growth of the organisms being cultured. For a heterotroph that is capable of growing in a simple salts medium, the growth-limiting component can be either the carbon source, or the source of nitrogen, sulphur, phosphorus, magnesium or potassium. Growth-limitation with regards to "trace" elements is difficult to arrange, since these are needed only in small amounts and are frequently present in adequate concentrations

as contaminants of other medium constituents; also they may be present in materials, such as stainless-steel, used to fabricate the growth vessel. Some trace elements may not be essential for growth, but nevertheless affect the physiology of the organisms when excluded from the medium. Therefore all trace elements known to be present in micro-organisms are added routinely to all media. It is convenient to add them together as a concentrated solution, as detailed in Table I.

TABLE I

Stock solutions for simple-salts media

Solution	Concentration			Concentration, g/10 litres
1. Phosphorus source	2M $NaH_2PO_4.2H_2O$			3120
2. Nitrogen source	4M NH_4Cl			2140
3. Potassium source	2M KCl			1490
4. Sulphur source	M $Na_2SO_4.10H_2O$			3220
5. Chelating agent	M Citric acid			2100
6. Magnesium source	0·25M $MgCl_2$			238
7. Calcium source	0·02M $CaCl_2$			22
8. Trace metals				
	Conc. HCl	50 ml	in 5 litres	
	ZnO	2·04 g		
	$FeCl_3.6H_2O$	27·0 g		
	$MnCl_2.4H_2O$	10·0 g		
	$CuCl_2.2H_2O$	0·85 g		
	$CoCl_2.6H_2O$	2·38 g		
	H_3BO_4	0·31 g		
9. Molybdenum source	0·001M Na_2MoO_4			2

The actual concentration of "limiting" nutrient necessary to support the growth of organisms at the desired concentration can be calculated from the yield coefficient (i.e., g organisms formed/g growth-limiting nutrient consumed). Since the yield usually varies with the dilution rate (as well as with temperature and pH value) a mean value must be used.

Media which support the growth of *A. aerogenes* in a chemostat (D = 0·2h^{-1}, 35°C, pH 6·5) at a concentration of about 4 mg equiv. dry weight bacteria/ml are detailed in Table II. The individual constituents can be kept, and most conveniently dispensed, as stock solutions whose concentrations depend on solubilities. Some form of chelating agent also must be added to prevent the formation of insoluble salts. EDTA is most effective as a chelating agent, but toxic to many species; citric acid is less toxic but less effective in preventing the formation of ammonium magnesium

phosphate. If, however, the medium pH value is kept below 6, precipitation of the magnesium salt does not readily occur. Furthermore, since automatic pH control is essential where organisms are to be grown to a high population density, the storage of media at low pH is not inconvenient and is to be strongly recommended.

TABLE II

Composition of simple-salts media limited with respect of various essential nutrients

Solution	Concentration of limiting solution, ml/litre					
	P	N	K	S	Mg	C
1. (phosphate)	1·0	5·0	5·0	5·0	5·0	5·0
2. (nitrogen)	25·0	7·5	25·0	25·0	25·0	25·0
3. (potassium)	5·0	5·0	1·0	5·0	5·0	5·0
4. (sulphate)	2·0	2·0	2·0	0·4	2·0	2·0
5. (chelate)	2·0	2·0	2·0	2·0	1·0	2·0
6. (magnesium)	5·0	5·0	5·0	5·0	1·0	5·0
7. (calcium)	1·0	1·0	1·0	1·0	1·0	1·0
8. (trace elements)	5·0	5·0	5·0	5·0	5·0	5·0
9. (molybdate)	0·1	0·1	0·1	0·1	0·1	0·1
Glucose or other carbon source (g)	30	30	30	30	30	10

(*a*) All the chemicals used are of analytical reagent quality. The water is purified by distillation in a glass apparatus followed by treatment with a mixed-bed ion-exchange resin.

(*b*) These media will support the growth of organisms such as *Aerobacter aerogenes*, *Bacillus subtilis* and *Torula utilis* at a concentration of about 4 mg equiv. dry wt organisms/ml culture. However, since the yield value may vary considerably both with the nature of the species being cultured and the dilution rate, adjustments will be necessary in the concentration of some of the media growth constituents. Nevertheless the above Table (and that of Tempest, 1965) should provide a good guide for the growth of many species of organism. When it is necessary to supplement these media with complex nutrients (e.g., casamino acids and yeast extract) account should be taken of the fact that these may contain much magnesium, potassium and phosphate, as well as carbon, nitrogen and sulphur.

C. Sterilization of media

In order to maintain an adequate supply of medium to a 1 litre chemostat, the reservoir should hold not less than 20 litres of the medium. Since replacing the reservoir bottle, when empty, constitutes a contamination

hazard (due to breaking a wet line) we recommend that supplies of fresh medium be prepared in a separate bottle and aseptically transferred to the reservoir.

The medium can be conveniently prepared in a 20 litre Pyrex bottle and sterilized by heat treatment (30 min with steam at 20 psi). Should this procedure cause decomposition of some of the medium constituents, then either the medium must be sterilized by other means (e.g., filtration or chemical sterilization) or the heat-labile component(s) sterilized separately and then added to the bulk sterile medium. We generally sterilize glucose separately (concentrated and slightly acidified) at 20 psi for 20 min, using a Buchner flask assembled in a way that facilitates aseptic transfer (Fig. 28). Both glycerol and mannitol can be added to the medium before sterilization since they do not decompose at 121°C.

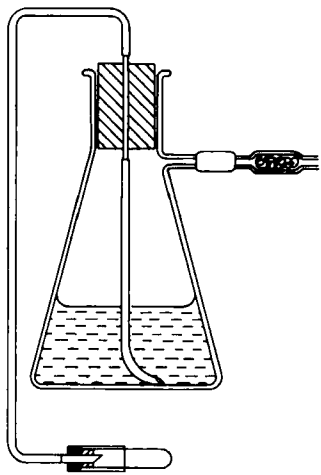


FIG. 28. Modification of a Buchner flask to provide a vessel for the growth of an inoculum culture and a system for its aseptic transfer to the culture vessel. This arrangement also is suitable for the separate treatment of medium consitituents and their aseptic transfer to the reservoir bottle.

D. Sterilization of antifoams

Polyglycol PPG 2000 and silicone aqueous emulsion RD can both be sterilized by autoclaving (20 min with steam at 20 psi for 1 litre volumes in Pyrex bottles). However, care is needed with the latter in order to prevent "cracking" of the emulsion during cooling. We have found that a slow rate of cooling combined with continuous agitation (using a magnetic stirrer) is generally effective in preserving the emulsion.

E. Sterilization of the titrants

The titrants that we use routinely (2–5 M HCl and either 2–3 M NaOH or NH_3) are sterile as prepared and are not treated further.

F. Air sterilization

Air flowing through a 1 litre chemostat culture at the recommended rate (1 litre/min) can be effectively sterilized by being passed through a cotton-wool filter. Alternatively a fibre-glass filter may be used (Elsworth, 1960); this can consist of a 3 in. length of 1 in. dia Pyrex pipeline fitted with a bung at each end and filled with 15 discs (each 1 in. dia) of resin-coated glass fibre (Fibre Glass Ltd) punched from a sheet of this material.

It might be pointed out here that compressed air from a piston-type compressor usually contains few viable organisms. On the other hand, the air emerging from the culture vessel is heavily contaminated with an aerosol of organisms which must be removed before it is discharged into the room atmosphere; this is done most conveniently by filtration. Since the air leaving the growth vessel generally is at a temperature above ambient and saturated with water vapour, condensation may occur in the filter which would thereby lose its efficiency. Therefore a condenser is inserted in the effluent air line.

G. Growth of anaerobic organisms

Few changes in the apparatus are necessary in order to grow anaerobic organisms in the chemostat. Vigorous stirring is still required to provide efficient mixing and a slow flow of oxygen-free nitrogen or argon through the culture is needed to flush out CO_2 and other fermentation gases.

With really strict anaerobes, the relatively high rate of diffusion of oxygen through silicone rubber renders this material unsuitable for the medium feed line and gassing lines (see Hobson, this Series, Vol. 3B). Neoprene tubing is a suitable substitute but is opaque. Some grades of transparent PVC tubing are available that can, with care, be autoclaved (e.g., Escoplastic tubing from Esco (Rubber) Ltd); however, these contain stabilizers that are aliphatic derivatives of metals, such as cadmium, barium or tin, and may prove toxic or may discolour due to reaction with products, such as H_2S, in the fermentation gases.

Maintenance of anaerobiosis may be assisted by adding non-toxic reducing agents, such as ascorbate or thioglycollate, to the culture medium.

H. Growth of pathogenic organisms

As described, the 1 litre chemostat is not suitable for culturing potentially dangerous organisms. The culture is maintained at a pressure slightly above

atmospheric, so that any leakage would be from the culture vessel rather than into it. This arrangement is very effective in excluding contaminant organisms from the culture, but is equally ineffective in containing aerosolized organisms within the growth chamber, particularly when the stirrer gland and other seals become slightly worn. Drawing air through the culture, rather than blowing it into the growth vessel, reduces the risk of organisms escaping from the chemostat, but increases greatly the chances of contamination of the culture; also it increases the difficulties of sampling and harvesting safely. Sampling and harvesting are potentially hazardous operations, since no matter how they are done an aerosol will be generated. Bearing in mind these facts and the dangers resulting from culture vessel breakage or tubing rupture, it would seem that the only genuinely safe way to grow pathogens continuously is in a chemostat totally enclosed in a ventilated box maintained at a pressure less than atmospheric, the pressure in the culture vessel still being kept positive with respect to that in the cabinet. Such an apparatus has been designed and built (Evans and Harris-Smith, 1966) and will be described in full elsewhere; a brief description is given here in order to illustrate some of the precautions that are necessary when culturing pathogens in stirred fermenters. A photograph of the apparatus is shown as Fig. 29.

Essentially, the culture apparatus is similar to that described earlier, but some minor modifications are necessary. Thus, thermoelectric devices must be used to provide cooling (and heating) in order to eliminate the need for water circulation (see the Section on temperature regulation). Furthermore, when an experiment is concluded, it is essential to sterilize the apparatus before it is disassembled and cleaned; therefore the reservoirs, culture vessel and receiver are attached to one another in a way that permits them to be handled as a single unit. When required, this unit can be removed from the cabinet through a side port provided with a seal. In most other respects the culture vessel is similar to that described previously.

The cabinet in which the apparatus is enclosed is formed from resin-bonded fibre glass. It is divided into three sections: the process chamber; the transfer chamber; and the instrument compartment.

1. *The process chamber*

This contains the culture apparatus, working surface and spill well. The apparatus is operated through gloves fitted to oval glove-ports set in the Perspex window. Articles can be passed into the process chamber through an air lock angled downwards to help to prevent its use as an exit; articles to be removed from the process chamber must be removed through a lock containing a sterilizing liquid, such as formalin.

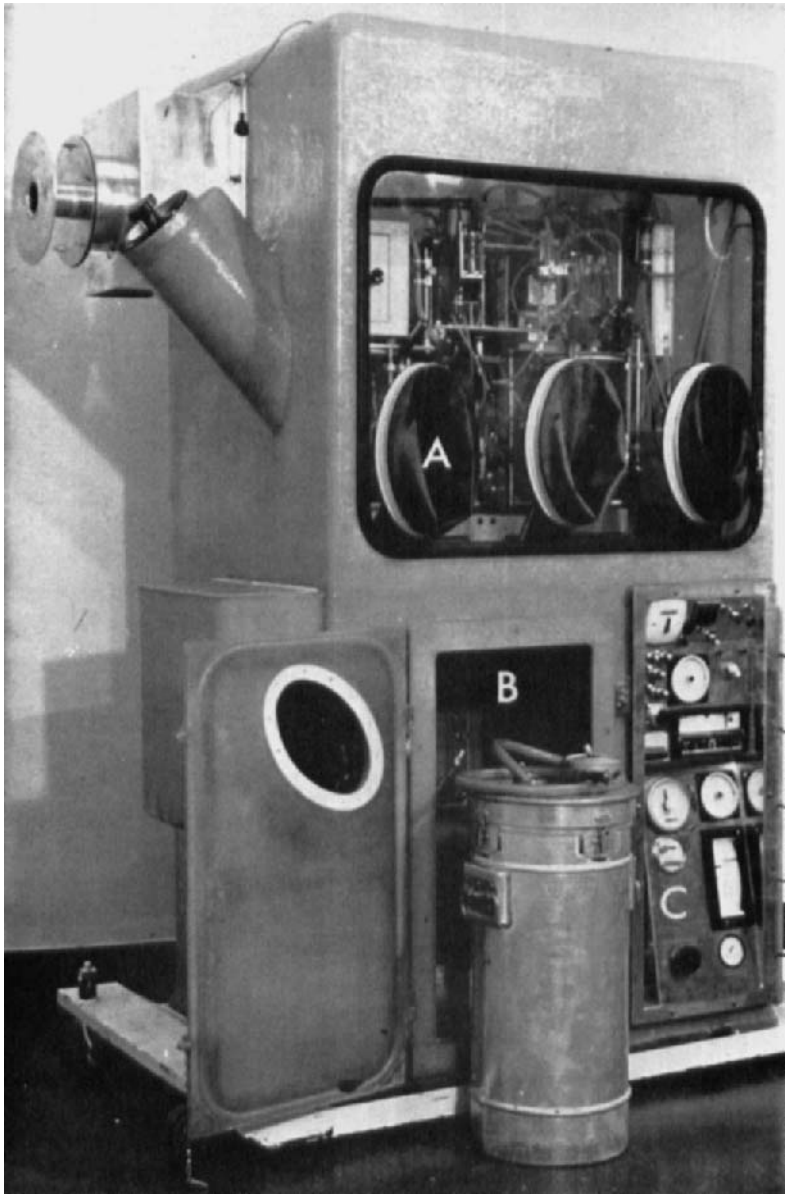


FIG. 29. Photograph of a portable 1 litre continuous-culture apparatus suitable for growing pathogenic organisms. Shown in this picture are the positions of the process chamber (A), transfer chamber (B) and instrument compartment (C). The fermenter assembly is visible, within the process chamber, and the transfer bottle (enclosed in a protective container) stands in front of the transfer chamber.

2. *The transfer chamber*

Provision is made for transferring the culture from the culture receiver to a 20 litre bottle enclosed in a metal container. This transfer is done by gravity within the safety of the ventilated cabinet; the container is in a separate compartment (the transfer chamber) which is immediately below the process chamber. The container can be removed in complete safety from this compartment, through a sealable doorway, for the processing or destruction of its contents. The conjunction of the transfer bottle and the culture receiver is effected in the process chamber; a rubber tube is passed through a side tube that projects from the side of the liquid lock up into the transfer chamber. The side tube is itself sealed by the liquid disinfectant. The medium reservoir can be replenished by a reversal of this procedure, thereby obviating the danger from an unsuspectedly contaminated medium reservoir.

3. *The instrument compartment*

This is situated alongside the transfer chamber and houses all the power supplies and instruments for the culture apparatus. The switches, instruments and culture air supply regulators are mounted on a panel that can be covered with a lockable transparent door to make it tamper-proof. Various alarm and protecting devices are also incorporated in the circuits to give warning of potentially dangerous situations as they arise (see below).

In order to ventilate the cabinet, room air is drawn through a pre-filter in the wall of the instrument compartment and thence into the transfer chamber through a high-efficiency filter. From the transfer chamber it is made to pass into the process chamber through a second high efficiency filter and then, sweeping up past the culture apparatus, it is drawn in series through two larger high efficiency filters by fans mounted on the outside wall of the cabinet. The size difference of the filters ensures that the cabinet is maintained at a pressure of about $\frac{1}{2}$ in. water gauge below atmospheric. The transfer chamber is kept sterile (until the door is opened) by being swept with sterile air at a rate of at least one volume/min; the second filter in the transfer chamber ensures that the process chamber is protected against the entry of contaminating organisms from the outside atmosphere. In the event of failure of the fans, the process chamber remains isolated from the outer atmosphere by the two exit filters and by at least one of the two inlet filters. If the differential pressure in the cabinet should become less than some pre-set value, the stirrer, temperature and pH control, and supply of medium and air are all switched off automatically and an alarm bell rings. If the cause is the failure of the fans a message indicator marked FAN is illuminated, but if the cause is other than the fans, a similar message

indicator marked CABINET is illuminated. Similar action occurs if the culture air outlet filter becomes wet and the pressure builds up in the culture vessel. In all cases (except of course when the fans have failed) the fan remains on to maintain some degree of negative pressure in the cabinet even if the main switch on the instrument panel is off.

Since the air entering and leaving the cabinet is filtered, the process chamber can be used as a safety cabinet and provision is made for routine sterility checks (by plating out the culture) and bacterial counts to be done inside it. The inclusion of a small incubator enables the samples to be taken, plated, incubated and examined within the cabinet. Afterwards, they can be placed in safety containers and passed out through the formalin lock for autoclaving.

By a slight re-arrangement of the ventilating system it is possible to keep a positive pressure in the cabinet; this would make it most suitable for the continuous production of tissue cells or other non-pathogenic, but easily contaminated, organisms.

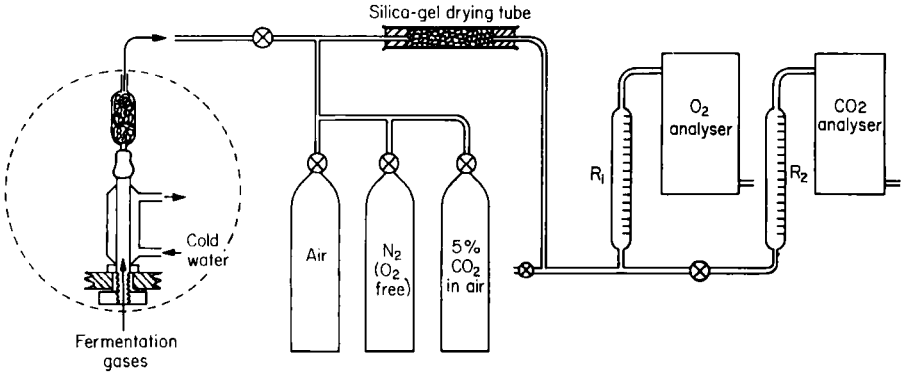


FIG. 30. Arrangement for the analysis of effluent gases from the culture vessel. Water vapour is removed from the gases by passage through a water-cooled stainless-steel condenser and a drying tube filled with silica gel crystals. R_1 and R_2 are appropriate rotameters.

I. Measurement of respiration

The oxygen and CO_2 contents of the culture effluent gas provide one of the clearest indications of microbial growth and of the constancy of the steady state. Although any method of gas analysis may be used, rapid and continuous readings can be obtained only with automatic gas analysers. Paramagnetic oxygen analysers (e.g., Beckmann Model E2) and infrared CO_2 analysers (e.g., Mines Safety Appliances LIRA model) are rapid, accurate and reliable, but expensive relative to the rest of the equipment.

Normally, the effluent gas from the growth vessel emerges via the receiver,

having passed through the overflow tube. The composition of this gas will be substantially different from that in equilibrium with the culture, since the receiver will contain an amount of respiring culture; analysis of the effluent gas will therefore not provide a measurement of culture respiration rate. Consequently we suggest that a small volume of effluent gas be removed from the air spaces above the culture in the growth vessel. Since this air is saturated with water (at the culture temperature) it should be first passed through a water-cooled condenser, and then through a silica-gel drying tube before being analysed. The arrangement shown in Fig. 30 is recommended.

J. Multi-stage chemostats

No details of multistage chemostats need be given here since these are dealt with extensively elsewhere (Řičica, this Volume, p. 329). It should be mentioned in passing, however, that two or more chemostats of the type described in this Chapter may be coupled in series to provide a multistage system. A pump would be required to transfer culture from one vessel to the next, as indicated in Fig. 4, in addition to those needed for medium supply to each chemostat.

IV. OPERATIONAL DIFFICULTIES

A. Failure of the inoculum to grow

Because the medium in the growth vessel is highly aerated and lacks CO_2, or because it may be substantially different from that on which the inoculum cells were grown, low concentrations of organisms frequently fail to grow (or start growing only after a considerable lag period) when added to the medium in the chemostat. Cells that have been adapted by growth in a shake-flask culture containing the chemostat medium tend to grow up after a shorter lag period, but again difficulty may be experienced in getting growth to commence in the inoculum culture.

Theoretically, and in practice, there is no good reason why adaptation of the organisms to growth in the chemostat medium should not take place in the chemostat; the problem is to prepare a good inoculum of rapidly growing organisms. Therefore we recommend the following procedure. Wash organisms from the surface of a nutrient agar slope culture into about 100 ml of nutrient broth containing 1 % glucose (or other "rich" medium). Incubate for about 6–8 h (or until the organisms are growing rapidly) and then aseptically transfer the whole culture to the growth vessel containing about 900 ml of the appropriate "limiting" medium. Switch on the medium pumps and set the flow rate to give a dilution rate equivalent of 0·5 D_c (i.e., a growth rate of about 0·5 μ_{max}). After six volumes through-put (about 20 h at a dilution rate of 0·3 h) the complex medium constituents will have been

almost completely washed out of the growth vessel and less than 0·1% of the initial population will be still present in the culture. The cells will be fully adapted to their new environment and the experiment can be started.

B. Growth of organisms on the walls of the culture vessel

This is the adhesion, and subsequent growth, of organisms on the walls of the growth vessel, on the baffle plates and on other appendages in the culture chamber. It results in the organisms being heterogeneously distributed in the culture. Wall growth usually represents a small proportion of the total growth, but occasionally it may amount to a significant proportion of the whole (Larsen and Dimmick, 1964). The problem is particularly serious with turbidostats, where coating of the measuring cell completely unstabilizes the system.

The extent of wall growth depends largely on the nature of the organisms, and on the particular growth environment; frequently it varies with dilution rate, temperature and pH value (unpublished observations). Since it is rarely practicable to alter the growth conditions, physical means must be used to clean the walls periodically. We suggest an internal bar magnet (coated with silicone rubber to prevent corrosion) held against the side of the growth vessel by coupling to an external magnet. When necessary, the walls can be "scrubbed" by moving the coupling magnet over the surface of the growth vessel. It is prudent to switch off the stirrer motor while doing this since the "scrubber" magnet may become uncoupled, fall onto the impeller and be thrown against the more delicate instruments suspended in the culture. After removing the wall growth, the culture must be left to equilibrate for several hours.

C. Growth of organisms in the medium feedline

The air space above the culture contains a high concentration of aerosolized organisms. Therefore precautions must be taken to prevent contamination of the medium inlet tube by these organisms, otherwise growth back along the medium feed line may occur—particularly if the rate of flow of medium is slow. This hazard can be reduced by careful design of the medium inlet port (see Section on medium supply), but should it fail, the medium inlet must be either changed or sterilized *in situ*. Two devices can be introduced into the medium feed line to facilitate these procedures: (i) positioning stainless-steel connectors (Fig. 26) at appropriate places to permit easy exchange of various sections of the line, and (ii) placing a "break" tube (Fig. 9) in the medium line about 3 in. above the inlet tube. This provides a temporary barrier to the back-growth of organisms in the feed line and the tubing between this and the inlet can be easily raised to a high temperature

with infrared radiation. In practice we have found this latter procedure most effective in eliminating back-growth.

If the medium line and the main air supply line are joined at some point between the medium pump and the culture vessel, then the air will blow the medium down the hollow stirrer shaft and into the culture. With this arrangement we have never experienced trouble from back-growth. If a solid shaft is used, then air and medium lines can be joined at a glass growth breaker similar to the medium inlet tube (Fig. 9) which should be mounted higher than the culture vessel; the air–medium line can then enter the culture vessel through the base plate immediately under the stirrer.

D. Bacterial contamination of the culture

This may arise from either inefficient sterilization of the apparatus or entry of contaminant organisms into the growth vessel after sterilization. Both these causes can be avoided by good routine aseptic technique and by minimizing certain hazards associated with assembly and operation of the equipment. Thus, the medium, titrant and antifoam reservoirs should be refilled when empty, rather than replaced, so that the risk of bacterial contamination associated with breaking a wet line is avoided.

Any mutant or variant organism that arises in the culture also must be regarded as a contaminant. At present no effective means for controlling mutant selection can be suggested; all one can do is to check frequently the homogeneity of the culture (e.g., by plating out samples from the culture on nutrient agar) and discontinue the experiment when the concentration of variant organisms reaches an intolerable level. It might be emphasized, however, that in our experience serious trouble from this cause is most infrequent—possibly because our experiments have tended to last less than 8 weeks each.

Sometimes it is possible to reduce the numbers of contaminant organisms in a culture by radically changing the growth condition (e.g., the growth rate or the nature of the growth limitation). However, it is generally better in practice, and a saving in time, to clean out the apparatus and re-start the experiment.

E. Component failure

Most of the control devices used in the design of the chemostat are inherently "fail safe"; that is, they fail in a manner that does not result in destruction of the culture. Component failure generally produces an alteration in the steady-state conditions, and since it may be important to know the precise time that the failure occurred, a continuous recording of at least one of the parameters should be made. The small regular fluctuations in the

culture pH value provide a very sensitive indicator of the steady state, and a continuous recording of the output from the pH indicator–controller is easy to arrange. A continuous record of the rate of oxygen uptake, or CO_2 output, also would be most valuable.

F. Electrical power failure

Chemostat cultures usually can withstand frequent and prolonged periods of power failure without serious deterioration, since most of the control devices are fail safe. Obviously power failure disturbs the steady state and some time is required for the culture to re-equilibrate after such a disturbance.

A problem does arise if valve-amplified pH indicators and controllers are used, since these have a considerable warm-up period. Where these are used, a time-delay device must be installed in the power line leading to the titrant solenoid valves so that these remain closed until the meter has reached equilibrium. With an EIL Model 91A or B indicator–controller the warm-up period is less than 5 secs (since it incorporates transistor amplifier circuits) so that time-delay devices become unnecessary.

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APPENDIX

List of manufacturers and/or distributors of components used in the construction of a 1 litre chemostat

Item	Catalogue No. or Part No.	Supplier	Approximate cost each, £
Growth vessel			
Complete with top and bottom plates, stirrer gland and bosses, etc.	..	Taylor Rustless Fittings Co. Ltd, Ring Road, Lower Wortley, Leeds 12	230
Pyrex pipeline for 1 litre vessel	PS4/6	QVF Ltd, Sales Dept., Stoke-on-Trent	2
Bonded seals for bosses and stirrer gland	PP45E and PP45F	Dowty Seals Ltd, Ashchurch, Gloucester	0·05
Silicone-rubber bungs for inlet ports	Grade TC156 Sizes 15A & E17	Esco (Rubber) Ltd, Walsingham House, Seething Lane, London E.C.3	0·15 to 0·35
Mixing			
Stirrer speed controller	Series SR	British Electrical Resistance Co., Queensway, Enfield, Herts	16
Variable transformer—2 amp	42AN	British Electrical Resistance Co., Queensway, Enfield	8
d.c. Shunt-wound motor	SH 336/B	Fractional Horse Power Motors Ltd, West Heath Works, Rookery Way, Hendon, London N.W.9	6
d.c. Tachogenerator	FFIA/20D	Evershed & Vignoles Ltd, Devonshire Works, Dukes Ave, Chiswick, London W.4	22
Slip motor (2800 rev/min)	SD13 Slip	Parvalux Electric Motors Ltd, Wallisdown Rd, Bournemouth	9
Magnetic Stirrer	MS 1	Grant Instruments, Barrington, Cambridge	4
PTFE-covered bar magnets	..	Fisons Scientific Apparatus Ltd, Loughborough, Leics.	0·5
Micromotor, 6 V, industrial type	..	Sleyride Ltd, Bishopstoke Rd, Eastleigh, Hants	0·5
Oil seals for stirrer shaft	MIS 04 or MIS 06 ($\frac{1}{4}$ & $\frac{1}{2}$ in., resp.)	George Angus & Co. Ltd, Oilseal Division, Coast Rd, Walls End, Northumberland	0·05
Essex couplings	XX02039 (for $\frac{1}{4}$ in. shaft)	Motor Gear & Engineering Co, Essex Works, Chadwell Heath, Romford, Essex	1
Mollart couplings	1 L (for $\frac{1}{2}$ in. shaft)	Mollart Engineering Co. Ltd, Kingston Bypass, Surbiton, Surrey	2

APPENDIX—*Continued*

Item	Catalogue No. or Part No.	Supplier	Approximate cost each, £
Temperature control			
Electronic controller, with platinum resistance thermometer	TcB9 (thermometer type S110G form 4)	Fielden Electronics Ltd, 51 Bradford St, Walsall, Staffs.	53
Heater	E 240E element	Antex, Grosvenor House, Croydon, Surrey	1
Valve for cooling water	LM826213 (Asco–Dewrance)	Dewrance & Co. Ltd, Gt Dover St, London S.E.1	4
Water-presure reducing valve	R6/2	Williams & James Ltd, Chequers Bridge, Gloucester	3·5
Standard brass OD fittings	..	Simplifix Couplings Ltd, Hargrave Rd, Maidenhead, Berks	0·1
Heat pump (with power pack)	HP3215	Thermo-Electric Devices, P.O. Box No. 10, Reading, Berks	40
Plug-in relay	MK 3P	Keyswitch Relays Ltd, 120–132 Cricklewood Lane, London N.W.2	1
pH control			
pH indicator–controller	91B	Electronic Instrument Ltd, Lower Mortlake Rd, Richmond, Surrey	150
Liquid-junction tube	LZ 28	Electronic Instruments Ltd, Lower Mortlake Rd, Richmond, Surrey	1
Calomel reference electrode	RJ 23	Electronic Instruments Ltd, Lower Mortlake Rd, Richmond, Surrey	3
Glass electrode (not autoclavable)	GCR 23/B (9 in. long)	Electronic Instruments Ltd, Lower Mortlake Rd, Richmond, Surrey	4·5
Glass electrode (autoclavable)	..	Activion Glass Ltd, Mitchell Hall, Kinglassie, Fife	5
pH recorder	Elmes Cyclogram	Swiss Instruments & Components Ltd, 54 Cheam Rd, Worcester Park, Surrey	39
Coxial sockets and plugs	L 604/S & L 781/P	Belling & Lee, Great Cambridge Rd, Enfield	0·05 0·05
Titrant delivery unit	..	W. G. Pye & Co. Ltd, Granton Works, Cambridge	18
Titrant (Delta) pump	Delta/B SA 15S	Watson Marlow Ltd, Marlow, Bucks	10

APPENDIX—*Continued*

Item	Catalogue No. or Part No.	Supplier	Approximate cost each, £
Spring-loaded terminal	311147	Painton & Co. Ltd, Bembridge Drive, Kingsthorpe, Northampton	0·1
Coxial sockets and plugs	PET 122 & PET 101	Precision Electrical Terminations Ltd, St. Johns Hill, Sevenoaks, Kent	0·5 0·3
Air (flow and pressure) control			
Air-flow meter	Type 825—V-frame with 300 mm tube	Rotameter Manufacturing Co. Ltd, Purley Way, Croydon, Surrey	33
Pressure differential relay	63 BD	AEI Sunvic Controls Ltd, 10 Essex St, Strand, London W.C.2	6·5
Air flow control valve	LM 826213 (Asco–Dewrance)	Dewrance & Co. Ltd, Gt Dover St, London S.E.1.	4
Pressure-reducing valve	67 P (small volume regulator)	Fisher Govenor Co. Ltd, Airport Works, Rochester, Kent	7·2
Air-filtration medium	..	Fibre Glass Ltd, St. Helens, Lancs.	
Pressure switch	PS/LB 1	Londex Ltd, Annerley Works, London S.E.10	10
Medium flow control			
DCL Micropump	..	F. A. Hughes & Co. Ltd, Blenheim Rd, Longmead Epsom, Surrey	42
Alternative electric motor for above pump	SD 14 (118 rev/min.)	Parvalux Electric Motors Ltd, Wallisdown Rd, Bournemouth, Hants	10
Peristaltic pump	Sigmamotor TS 6	V. & A. Howe Ltd, 46 Pembridge Rd, London W.11	40
Variable speed drive (for above)	Zeromax type 20J400	V. & A. Howe Ltd, 46 Pembridge Rd, London, W.11	35
Variable on–off timer (for above)	Model CTRC	Elcontrol Ltd, Wilbury Way, Hitchin, Herts	35
Re-set ratchet counter	Series 1341	Veeder–Root Ltd, King Henry's Drive, New Addington, Surrey	2
Pump	Flowinducer MHRE & MHRK	Watson Marlow Ltd, Marlow, Bucks	92 & 35
Tubing for pumps (minimum wall thickness of 1·5 mm)	..	Esco (Rubber) Ltd, Walsingham House, Seething Lane, London E.C.3	Depends on size and quality

APPENDIX—*continued*

Item	Catalogue No. or Part No.	Supplier	Approximate cost each, £
Antifoam flow control			
Pump	Delta/B SA15S	Watson Marlow Ltd, Marlow, Bucks	10
Process timer	IMP	Londex Ltd, Annerley Works, Annerley Rd, London S.E.10	12·5
Transistorized timing unit for use with process timer	PT-900A	Solid State Controls Ltd, 30–40 Dalling Rd, London W.6	3·25
Electrical sundries			
Relays	MM2 and MK3P (with 11 pin socket)	Keyswitch Relays Ltd, 120–132 Cricklewood Lane, London N.W.2	1·1
Switches	Miniature push button type	Radiospares Ltd, 4–8 Maple St, London W.1	0·3
Signal lamps	SGF 20/220/ neon/(colour)	Thorn Electrical Industries Ltd, Special Products Division, Gt. Cambridge Rd, Enfield, Herts	0·2
Indicating and fused switches	FCA	Rowan Controller Co., 30 Bridge Ave, Red Bank, New Jersey, U.S.A.	0·6
Sundry constructional items			
Steel angle	$1\frac{1}{2} \times 1\frac{1}{2}$ in. 14 gauge	Handy Angle Ltd, Nine Locks, Brierley Hill, Staffs	7 (100 ft.)
Peg-board	..	Laconite Ltd, Half-way Green, Walton-on-Thames, Surrey	0·15 per sq. ft.
Bungs and tubing	Grade TC156	Esco (Rubber) Ltd, Walsingham House, Seething Lane, London, E.C.3	various
Gas analysis			
Oxygen analysis	Model E2	Arnold O. Beckman Inc., Queensway, Glenrothes, Fife	425
Dissolved oxygen electrode	A 15A	Electronic Instruments Ltd, Lower Mortlake Rd, Richmond, Surrey	35
High sensitivity potentiometric recorder	Kipps & Zonen Micrograph	Shandon Scientific Co, 65 Pound Lane, London N.W.10	470
CO_2 analyser	Lira model 300	Mines Safety Appliances, Queenslie Industrial Estate, New Edinburgh Rd, Glasgow E.3	556

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CHAPTER XIV

Multi-stage Systems

J. Ričica

Institute of Microbiology, Czechoslovak Academy of Science, Prague, Czechoslovakia

I. BASIC THEORETICAL ANALYSIS

In a batch culture, individual growth phases are a result of sequential physiological states which dynamically change in time, but are independent of it. Each physiological state is dependent on those preceding it, i.e., each point of the growth curve has its own developmental history.

On the other hand, in a single-state chemostat or turbidostat the population is compelled to multiply under constant environmental conditions in a steady state. The physiological state is no longer dependent on the preceding ones and becomes dependent on the dilution rate; thus the culture has no history. If the cells cannot pass through a certain historical physiological phase controlling the subsequent biosynthesis of a certain product, differences in the yield occur between batch and continuous cultures although the growth rates, at a chosen point on the batch curve and in a simple chemostat may be identical. Consequently, a cultivation system should be employed which will enable the population, even under continuous conditions, to pass through stages of development which would influence optimally its final state. This temporal distribution of stages may be imitated by spatial distribution in the form of an ideal tubular plug or piston flow reactor with feed-back or permanently seeded from a homogeneous reactor (Herbert, 1960).

Owing to the physiological changes, the growth reaction order usually varies from autocatalytic first to ordinary first order during the course of growth (Grieves *et al.*, 1964). The kinetics of product formation may be of the same or of a different order from the growth kinetics. By applying the findings from chemical reactions to microbiological processes (Danckwerts, 1954), we can show that if the reaction rate increases with concentration, the optimal output per unit volume of completely mixed reactor is higher than that from a tubular type. If the rate of a particular reaction decreases with concentration, the ideal piston flow reactor is superior to a homogeneous one, operated for the same period. With zero-order reactions, the effectiveness of both types is equal. Therefore, for most microbial processes, the combination of a homogeneous reactor with a heterogeneous tubular plug flow reactor is optimal, and gives the lowest total holding time needed to achieve a desired stage of conversion (Bischoff, 1966).

However, such a system is difficult to operate, and with aerobic processes it is almost impossible, to realize an ideal tubular plug flow reaction. It can be approximated by a chain of homogeneous completely mixed reactors with short individual holding times (Powell and Lowe, 1964).

Theoretical analyses both of the growth of a population and of product formation in multi-stage continuous cultivations were elaborated by Herbert (1960, 1964), Fredrickson and Tschuchiya (1963), Humphrey (1965) and Fencl (1966). Their work analyses this problem in more detail than this Chapter and even takes into consideration particular cases. In the following text, some ideas, opinions and equations necessary for explaining the basic principles of the multi-stage systems are employed following the descriptions of single-stage chemostats in Chapter XII, pp. 259 of this Volume, by D. W. Tempest.

A. Simple chain of chemostats (single-stream)

1. *Cell mass balance*

Let us assume that a multi-stage continuous system consists of a chain of chemostats, i.e., of homogeneous, perfectly mixed reactors (Fig. 1) of either equal or unequal volumes, V_1, $V_2 \ldots V_n$. The number of reactors may be 2 or n. The volume, F, of a single medium flows in to the first stage (reactor), and the whole volume is passed on to the next and so on, so that the actual flow is the same through all stages—

$$F = D_1V_1 = D_2V_2 = \ldots D_nV_n \tag{1}$$

The dilution rate, D, in different stages (reactors) depends then on the volume of the culture, $D = F/V$ (h^{-1}). The reciprocal value of the dilution rate is the holding time, $1/D = \theta$ (h). For simplicity, take the example

of a two-stage system in which the concentrations of the cells and of the limiting substrate are x_1, x_2 and s_1, s_2, respectively. Cell mass and substrate balance equations for the second stage can be employed for any following nth stage. The first stage in the chain is identical in behaviour with a single-stage chemostat (see Tempest, this Volume, page 259).

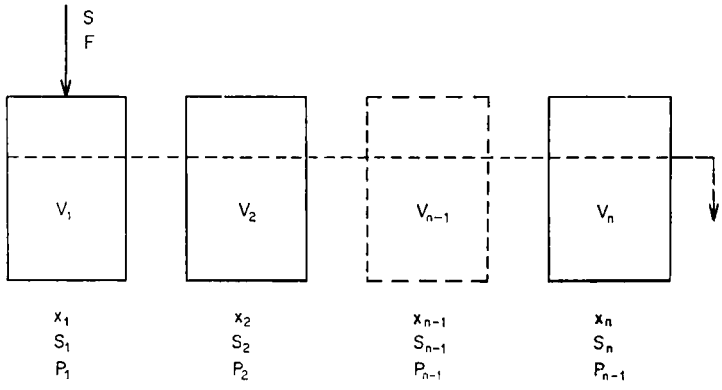

FIG. 1. Single-stream multi-stage culture system.

Since both the cells and the unutilized substrate from the first stage flow into the second stage, the cell balance equation under steady state in the second stage is as follows—

$$\text{inflow} + \text{growth} = \text{outflow}$$

$$D_2x_1 + \mu_2x_2 = D_2x_2 \tag{2}$$

and the substrate balance equation reads—

$$\text{inflow} - \text{consumption} = \text{outflow}$$

$$D_2s_1 - \frac{\mu_2x_2}{Y_2} = D_2s_2 \tag{3}$$

The specific growth rate, μ_2, is dependent on the constant K_s (substrate concentration at which $\mu = \frac{1}{2}\mu_{max}$) and on the concentration of the limiting substrate, s—

$$\mu_2 = \mu_{max}\frac{s_2}{K_s + s_2} = \frac{D_2(x_2 - x_1)}{x_2} = \frac{D_2Y_2(s_1 - s_2)}{x_2} \tag{4}$$

The amount of organisms in the second stage is dependent on the yield coefficient, Y_2, expressing the effectiveness of substrate utilization for cell mass production—

$$x_2 - x_1 = Y_2(s_1 - s_2) \tag{5}$$

The yield coefficient is usually constant for a considerable part of the growth curve, but owing to the decreasing concentration of the limiting

substrate and to the accumulation of metabolites the metabolism is changed. These changes affect a decrease in effectiveness and rate of substrate utilization and cause a lowering of Y_n, μ_n and x_n. The extent to which Y is constant and the value of Y to be substituted into the equations may be derived from the experimental batch data, $Y = -\Delta x/\Delta s$, corresponding to the pertinent part of the growth curve or from continuous culture data; in the first stage $Y_1 = x_1/(s-s_1)$, in the nth stage $Y_n = (x_n-x_{n-1})/(s_{n-1}-s_n)$. The value of Y may change with different metabolism and cause the dependence of μ on s to change. Then $\mu \neq \mu_{max}s/(K_s+s)$. In such cases, the above mentioned equations may be modified (Contois, 1959; Schulze and Lipe, 1964; Pirt, 1965; Powell, 1967).

2. *A graphical solution of design of a multi-stage single-stream culture system*

A useful approach to multi-stage single stream systems is to attempt graphical solutions to the problems of design. Although there is not as yet extensive experimental support for this particular approach, there is much current interest in the general concept of graphical solutions. Predictions of the performance of continuous cultures from batch experimental data by graphical methods is often more rewarding than analytical approaches. Since the specific growth rate is a complex function of many variables and

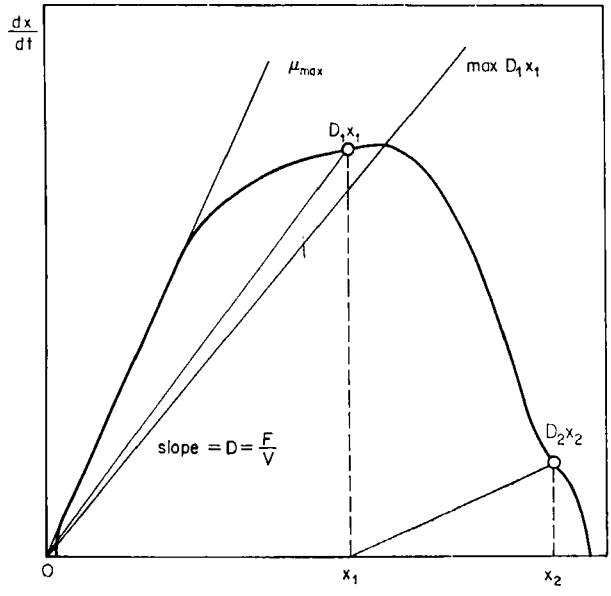

FIG. 2. Growth curve of *E. coli* B. Example of graphical solution for a two-stage unequal volume continuous culture (method of Luedeking and Piret, 1959).

can be considered as an indicator of the physiological state of the culture, it was employed for such graphical solutions by Luedeking and Piret (1959). If the growth of a population is given by the equation $dx/dt = \mu x$, then by plotting dx/dt against x we obtain a curve representing the output, $\mu x = Dx$ (Fig. 2). The values of dx/dt can be obtained from a batch growth curve if, by means of a plane mirror, lines are drawn normal to the curve at the desired points and the corresponding tangents are then constructed as perpendiculars to the normals (Adams and Hungate, 1950). The slope of the tangents represents the growth rate dx/dt or μx at the cell concentration selected. Assuming that in a particular short time interval the value of μ is constant, then the calculation can be made on the basis of the difference of measured values of $\Delta x/\Delta t = \mu x_a$, where x_a is the average cell concentration in time interval Δt. As the batch data are obtained under changing conditions, the results of the graphical solution are not necessarily identical with those obtained in continuous culture.

If in the first stage the population is growing in the steady state, then—

$$\text{increase} = 0 = \text{growth} - \text{output}$$

$$\frac{dx_1}{dt} = 0 = \left(\frac{dx_1}{dt}\right) - D_1 F_1 \tag{6}$$

and from this—

$$x_1 = \frac{1}{D_1} \cdot \frac{dx}{dt} \tag{7}$$

Since the entering cell concentration in the first stage is zero ($x_0 = 0$), a straight line passing through the origin and a certain point on the growth curve gives at this point the output ($D_1 x_1$) for the corresponding value x_1 (on the abscissa axis). The slope of this curve is equal to D_1 (Fig. 2). In this way the dilution rate, D, for the desired concentration of micro-organisms may be found and vice versa.

Under steady state conditions in the second stage the balance equation is—

$$\text{increase} = 0 = \text{growth} - \text{output}$$

$$\frac{dx_2}{dt} = 0 = \left(\frac{dx_2}{dt}\right) - D_2 (x_2 - x_1) \tag{8}$$

$$x_2 - x_1 = \frac{dx_2}{D_2} \tag{9}$$

As the entering concentration of organisms in the second stage is x_1, then the beginning on the abscissa shifts to this point. Again the slope of a straight line through the point x_1 and a chosen point $D_2 x_2$ gives the value of D_2 for the desired x_2. Similarly, the value of D_n for x_n in the nth reactor may be derived.

Using the mathematical analysis of the graphical method, Bischoff (1966) elaborated a method to optimalize a two-stage system. He employed the shortest holding time, $\theta = 1/D$, necessary for achieving a desired conversion throughout the growth which he considered to be autocatalyctic. To use his equations for practical calculation, they need to be modified for the physiological state (dependent on the holding time), which is to be achieved in individual reactors of the multi-stage continuous culture. Nevertheless, the graphical method worked out by Bischoff (1966) illustrates the situation in a multi-stage system.

The growth rate, r_x, and the substrate consumption rate, r_s, are functions of both the organism (x) and substrate (s) concentrations. For the homogeneous chemostat the cell and substrate balances are—

$$\text{flow in} + \text{growth} = \text{flow out}$$

$$Fx_1 + Vr_x(x_2, s_2) = Fx_2 \tag{10}$$

$$Fs_1 + Vr_s(x_2, s_2) = Fs_2 \tag{11}$$

where the subscripts 1 and 2 indicate the conditions entering and leaving the chemostat, respectively. In the first stage $x_1 = 0$. By rearranging these equations the mean holding time may be expressed—

$$\theta = \frac{x_2 - x_1}{r_x(x_2, s_2)} = \frac{s_1 - s_2}{r_s(x_2, s_2)} \tag{12}$$

Bischoff (1966) also developed the cell and substrate balance equations for the differential element of volume in the plug flow tubular reactor. Readers are referred to Bischoffs' paper for details of his interesting theoretical approach which is outside the scope of the present Chapter.

3. *Product formation*

As with cells and substrate, the mass balance equation for product, P, in the nth reactor in a series may be expressed—

$$D_n P_{n-1} + k_n x_n = D_n P_n \tag{13}$$

where k_n is the specific rate of product formation which is usually derived for the pertinent x from batch culture,

$$k = \frac{\mathrm{d}P}{\mathrm{d}t}\frac{1}{x} = Y_P \mu \tag{14}$$

where Y_P is the yield coefficient of product based on cell mass. The coefficient, k, is a complex function of many factors obtained under variable conditions and in a continuous process, operating under steady state, its value can differ, both in the positive and negative senses.

For graphical illustration of product formation in the nth reactor equation (13) may be modified as follows—

$$\text{input} + \text{production} = \text{output}$$

$$D_n P_{n-1} + \left(\frac{dP_n}{dt}\right) = D_n P_n \tag{15}$$

If we employ the solution by Bischoff (1966) then in the nth reactor the nominal holding time, necessary for achieving the concentration of product P_n, is—

$$\theta = \frac{P_n - P_{n-1}}{r_P(x_n, s_n, \ldots)} \tag{16}$$

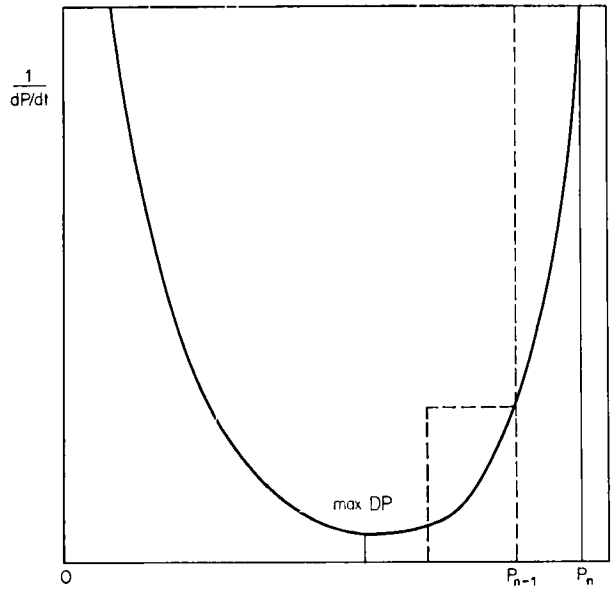

FIG. 3. Production curve. Example of graphical solution by using method of Bischoff (1966).

The reaction rate of product formation is—

$$r_P(x, s, \ldots) = kx = dP/dt \tag{17}$$

By plotting the reciprocal value of dP/dt against P, the production curve (Fig. 3) can be constructed. Areas of rectangles with bases and heights read off on the abscissa and ordinate give the nominal holding times in pertinent reactors.

It is necessary that the values of x and P should be plotted in the same units (mg/ml or g/litre).

4. *Application of single-stream system*

(a) *Simultaneous study of two physiological states.* The distribution of two enzymatic systems in two-stage cultures of *Escherichia coli* grown on rich medium without inducing saccharides (Fig. 2), could serve as an example. The cells taken from the first stage at D_1x_1 and from the second stage at D_2x_2 are transferred into medium containing the inducer. Cells taken from the first stage have the shortest induction time and the maximum specific activity for β-galactosidase; but for amylomaltase the cells taken from the second stage have the shortest induction period and the highest specific activity.

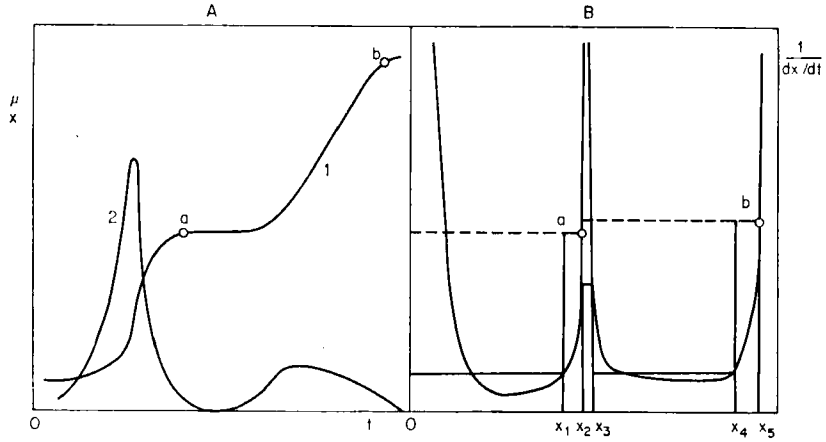

FIG. 4. Diauxic growth on a mixture of two saccharides. 1, growth curve; 2, specific growth rate, μ; a, one saccharide preferentially exhausted; b, the other saccharide exhausted.

In a similar way it is possible to distribute, into different stages, different enzymatic systems formed in different growth phases and by controlling the temperature or pH, stimulate or repress the formation of any one.

(b) *Utilization of a mixture of substrates.* Fig. 4 shows a diauxic growth curve of a culture growing on a mixture of two saccharides, one of which is utilized preferentially and inhibits the utilization of the other. One saccharide is utilized in the first stage, point *a* (dashed line in Fig. 4B), and the other in the second stage (point *b*) of a two-stage system. The rectangular areas representing the holding times have the bases x_2–0 and x_5–x_2, respectively. As another alternative, a five-stage system consisting of reactors of unequal volumes is given (Fig. 4B), where the sum of the holding times is much lower than with a two-stage system. If the lag phase between the two growth phases is important for adaptation on the second substrate, then the holding time between x_2 and x_3 should equal the time interval of the lag.

(c) *Product formation.* According to the kinetics of product formation in batch culture it may be considered to what extent the formation of product is associated with the individual growth phases (Humphrey, 1965, 1966) or whether the product is a result of energetic or secondary metabolism (Gaden, 1959; Humphrey, 1965). Both viewpoints are important and must be taken into consideration when employing the graphic solution of design of multi-stage systems. Failures in continuous production of industrially significant products are often due to insufficient knowledge of metabolic pathways controlling product formation. When the culture is divided between several stages it is desirable that the key reactions should take place in a particular stage (reactor) with maximum rate, so that the individual holding times should correlate with this demand. In individual stages, particular metabolic reactions can be stimulated or repressed by controlling the temperature, pH, concentration of dissolved O_2 and CO_2, precursors, etc. In continuous processes producing secondary metabolites, medium of the same composition as that used in the corresponding production batch process is often used, but it is often forgotten that the inoculum for the batch culture was a spore suspension seeded on media of different composition. Therefore some modification of the medium composition may be necessary.

For sufficient product formation, the first requisite is a sufficient supply of active cells. When using graphic representation it is advantageous to correlate product formation with both the growth rate and the concentration of organisms and divide the processes into two types—

(i) The maximum concentrations of organisms and of the product are achieved at the same time (Fig. 5). For example: sorbose, organic acids, lipids, polysaccharides, vitamins, enzymes, etc.

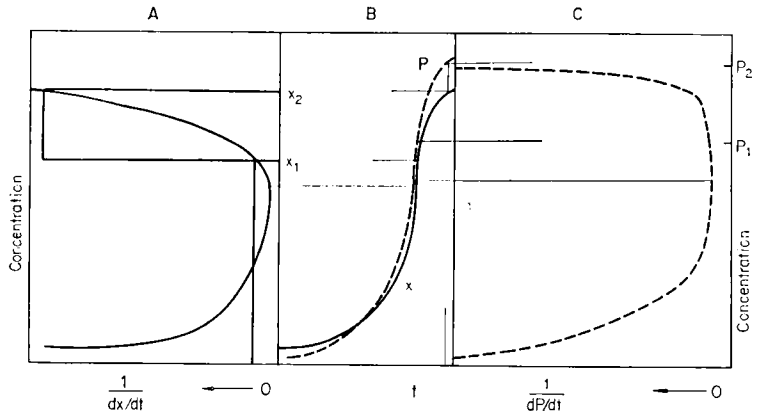

FIG. 5. Maximum concentrations of both product and cells are achieved at the same time (graphical interpretation similar to Maxon, 1960).

(ii) The maximum concentration of product is achieved in the stationary phase or in the phase of negative acceleration (Fig. 6). For example: polysaccharides, enzymes, secondary products (antibiotics), vitamins, amino-acids, etc.

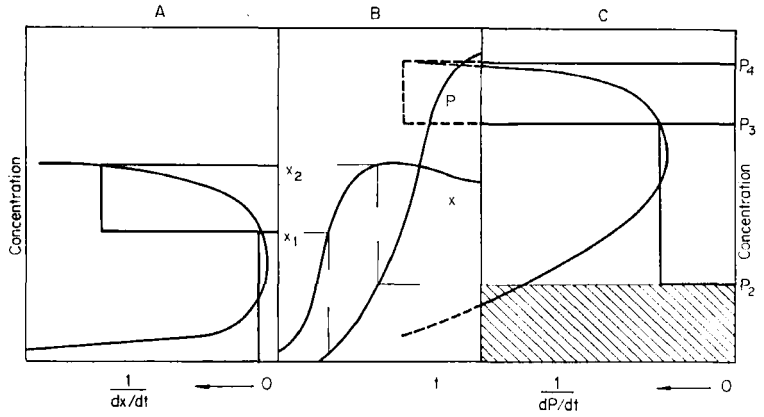

FIG. 6. Maximum product concentration is achieved in stationary or negative acceleration phases.

Type (*i*). With this type, the growth and the production curve are similar (Fig. 5). If the points of inflexion are identical or close to each other, then in the first reactor the maximum output of both cells and product may be obtained (rectangle with base of x_1–0, Fig. 5A). The final product concentration is then achieved in the second reactor (rectangle with base x–x_1, Fig. 5). If the distance between the inflexion points is significant then a three-stage system can be employed. The maximum output, Dx, occurs in the first stage, the maximum output, DP, (short mean holding time) in the second stage and the completion of accumulation of the product in the third stage (in Fig. 6 this alternative is not included).

Type (*ii*). Biosynthesis with this type being complex, the process must be divided into several phases. Fig. 6 shows a four-stage system, but according to the character of the process other alternatives may be employed, as well. First, let us follow the growth curve (Fig. 6A). In the first stage (rectangle with base x_1–0), for the sake of safe operation the dilution rate is kept behind the point of inflexion, but quite near to it. In this phase rapid cell mass formation, intensive metabolism (nucleic acid synthesis, respiration and rapid decrease of sugar and α-amino-nitrogen, etc.) occur, but there is no formation of the secondary product. In the second stage (rectangle with base x_2–x_1), both nucleic acid synthesis and respiration decrease, sugar is depleted, pH decreases, the content of ammonia nitrogen

considerably diminishes, the synthesis of secondary product is just starting and growth approaches the stationary phase. Now, consider the production curve (Fig. 6C). In the third stage (rectangle with base P_3–P_2) growth has stopped, respiration decreases considerably, pH begins to rise and product formation rate is high. The rectangle area begins at P_2 because product synthesis began in the growth reactors (hatched area, Fig. 6C). In the fourth stage (rectangle with base P_4–P_3) the metabolic level is very low, but product synthesis is proceeding. It follows from the graphical illustration that, when using a stirred reactor, the maximum concentration is theoretically reached at an infinitely large holding time, i.e., an infinite volume (the curve ends at infinity). Owing to the dilution effect, in a reactor of defined volume the yield is lower than that calculated theoretically.

With some actinomycete antibiotic-producers, the growth period is divided into three phases: (i) growth of the primary mycelium, (ii) mycelium fragmentation and (iii) growth of the secondary mycelium. Accordingly, the growth period must be divided into three or more stages.

(d) *Production of spores.* In cultures of unicellular micro-organisms, spores are formed in the stationary phase and in the phase of negative acceleration (Fig. 7); it is thus possible to employ the graphical solution shown in Fig. 6.

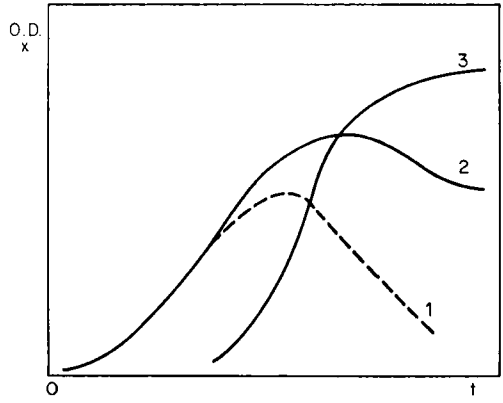

FIG. 7. Spore formation. 1, vegetative cells; 2, absorbance; 3, spores.

In the first stage, intensive growth is maintained (near to maximum Dx) and no spore formation occurs. In the second stage, growth continues at a lower rate (optical density is still increasing), but the number of vegetative cells decreases and sporulation is just starting. In the third stage, the optical density reaches its maximum, growth stops, the number of vegetative cells decreases rapidly and spores are formed at maximum rate. In the fourth stage, the accumulation of spores is completed. It is obvious that in a

continuous system production of spores must be performed in several stages. However, the optimum holding time in individual stages is influenced by the past history of the vegetative cells, by the composition of the medium and sometimes also by formation of a lytic factor produced by some *Bacillus* species.

If we simplify this complex process, a mass balance equation of vegetative cells, x_n, for the nth stage in a series may read—

$$\text{increase} = 0 = \text{input} + \text{growth} - \text{decrease} - \text{output}$$

$$\frac{dx_n}{dt} = 0 = D_n x_{n-1} + \left(\frac{dx_n}{dt}\right)_{\text{growth}} - \left(\frac{dx_n}{dt}\right)_{\text{decrease}} - D_n x_n \qquad (18)$$

In the period of maximum sporulation the growth rate of vegetative cells, $dx_n/dt = \mu_n x_n$, equals zero. The rate of decrease of vegetative cells is given by the sum of sporulation and lysis—

$$-\frac{dx_n}{dt} = -\sigma_n x_n = -x_n(k_n + \lambda_n) \qquad (19)$$

where σ_n, k_n and λ_n are the specific rates of decrease of vegetative cells, sporulation and lysis which equal zero in the first stage. A similar equation may be written for the formation of spores, P—

$$\text{input} + \text{formation} = \text{output}$$

$$D_n P_{n-1} + k_n x_n = D_n P_n \qquad (20)$$

The specific rate of spore formation, k_n, can also be used for the specific rate coefficient of maturation of vegetative cells.

The areas of rectangles, i.e., the individual holding times, should be chosen so that they exceed the growth curve and the sporulation curve as little as possible (Fig. 6A, C). The larger the portion of the rectangle area above the curve, the larger are the discrepancies between the predicted values and those achieved in continuous culture.

Producing spores in a continuous system is not easy, since both the mechanism and the kinetics of spore formation are in contradiction to the laws of mixed reactors. The tubular plug flow reactor is more likely to be compatible with sporulation. The necessary experimental data are still lacking.

(e) *Production of viruses* (*bacteriophages*). The host cells serving as a substrate for virus multiplication are produced in the first stage, the performance of which is the same as a single-stage chemostat. When employing the dilution rate at which the maximum output, $D_1 x_1$, occurs, we obtain cells having the optimum properties required for infection, since the synthetic rate of nucleic acids is maximal. The second stage is inoculated with virus. As not all cells are lysed (similar to equation 18), both lysis and increase of virus titre continue in the following stages (Jacobson and Jacobson, 1966).

If in the second stage the dilution rate is lower than the specific rate of lysis (i.e., bursting of cells and releasing of virus), reinoculation and establishment of a steady level of virus titre take place. If the specific rate of virus formation for a batch culture is known, it is possible to calculate the dilution rate for the second stage at which the maximum output of virus occurs. For promoting virus production, the second stage may be continuously inoculated with a batch virus culture.

B. Branched systems (multi-stream)

If to a simple single-stream chain further inflows are added, different types of multi-stream systems can be produced, some of which are illustrated in Fig. 8. Fresh medium is supplied not only into the first, F_1, but also into the second, F_{02}, and eventually into some further stage. The second stage is continuously inoculated from the preceding stage, Fx_1. Type b employing feed-back is an exception, as the first stage is inoculated by cells taken from the following nth or from the last stage.

The amount of organisms x_1, flowing into the second stage (with the type b into the first stage, Fig. 8), is diluted by the sum of all inflows to the concentration of x_{02}—

$$x_{02} = \frac{F_1 x_1}{F_1 + F_{02}} \tag{21}$$

For the steady state we may write—

$$\text{input} + \text{growth} = \text{output}$$

$$D_2 x_{02} + \mu_2 x_2 = D_2 x_2 \tag{22}$$

where $D_2 = (F_1 + F_{02})/V_2$.

If the product, P_1, is present in the inflowing medium then in the second stage of types a, c and d and in the first stage of type b (Fig. 8) under steady state we have—

$$P_{02} D_2 + k_2 x_2 = D_2 P_2 \tag{23}$$

where $P_{02} = F_1 P_1 / F_1 + F_{02}$ and k_2 is the specific rate of product formation.

The substrate $s_{02} = (F_1 s_1 + F_{02} s)/(F_1 + F_{02})$ is utilized by growing organisms according to the equation—

$$D_2 s_{02} - \frac{\mu_2 x_2}{Y_2} = D_2 s_2 \tag{24}$$

where s_1 is the residual substrate unutilized in the preceding stage (with type b, $s_1 = 0$), and s the fresh substrate fed into the second stage (with type b into the first one).

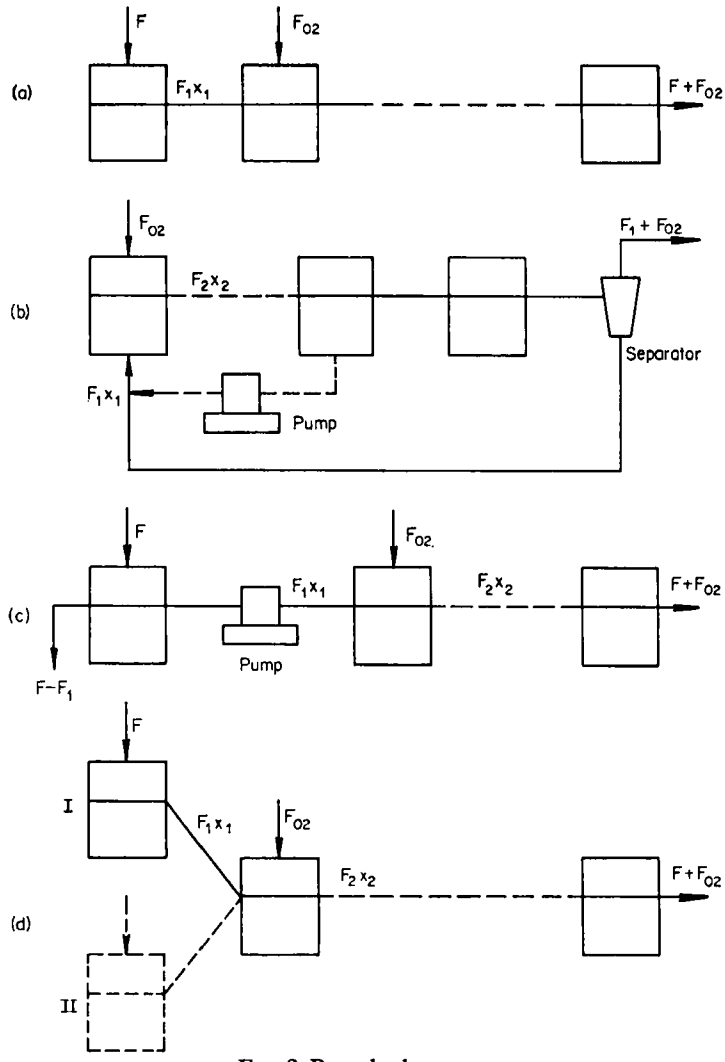

FIG. 8. Branched systems.

1. *Application of multi-stream systems*

(a) *Growth in excess of substrate.* For these experiments, two stage systems, types a and c, are employed (Fig. 8). Type c has an advantage over type a, since in the first stage the physiological state of the culture may be changed at will by means of different dilution rates, without influencing the dilution rate in the second stage. F_1x_1 is kept constant by a dosing device. There is also a possibility of supplying substrates of different composition to the first and second stages.

(i) *Increase of culture activity.* If throughout the period of maximum growth a product or an enzymatic system are formed at a maximum rate, then by increasing the inflow of fresh medium, F_{02}, into the second stage conditions can be attained under which the specific rates of formation and growth reach maximum values in a given medium, e.g., dehydrogenation of sorbitol to sorbose with *Acetobacter suboxydans*, β-galactosidase activity with *E. coli*, glucose-oxidase activity with *Aspergillus niger* (Řičica, 1964; Řičica *et al.*, 1967; Fencl *et al.*, 1967).

(ii) *Transient states.* System c (Fig. 8) enables us to increase the inflow of fresh substrate into the second stage, from $F_{02} = 0$ up to high values at which the organisms are grown in excess of the substrate. In theory it can be shown that complete washing-out of cells does not occur. In this way, cells of a certain physiological state originating from the first stage, are shifted down or up into different conditions in the second stage. If the holding time of cells is very short, then, due to the steady state conditions, the transient state between limited and unlimited growth (eventually the transient state of changing metabolism evoked by transfer from one substrate to another) is fixed.

(b) *Growth curve imitation.* In cultivation system b (Fig. 8) the growth curve can be imitated. From the nth stage, an aliquot of the culture is taken continuously and transported as inoculum into the first stage. At the same time fresh medium is added. In order to maintain the ratio $F_1 : F_{02}$ as low as possible, the inoculum can be concentrated in a continuous centrifuge. If the system consists of several stages with a short holding time, the culture passes through different development phases down the chain. Sometimes an inoculum possessing specific properties is desired and in this case type c (Fig. 8), with different media added into the first and second stage, may be employed.

(i) *Product formation.* In system b (Fig. 8) the development of the culture is similar to that in batch process. Cells leaving the last stage may be concentrated and recycled into the first stage as a significant part of the population. The concentration of active cells is high at the very beginning and substrate utilization is therefore more effective (Powell and Lowe, 1964). Since it follows that either the flow rate or the substrate concentration may be increased resulting in an increase in the productivity of this system, the system is attractive for accumulation of certain products, for the efficient conversion of solutes which are available as by-products, and for the elimination by microbial action of toxic substances from waste waters.

(ii) In system d (Fig. 8), loss of the ability to form a product (e.g., antibiotics) can be eliminated.

There are two first reactors, in one of them (I) a batch culture is grown. In a certain development phase a fresh substrate inflow is applied and batch cultivation is changed to the continuous one. The culture passes through the successive stages. Before the culture loses its production capacity, a new batch culture is prepared in another reactor (II) and supply of fresh medium is started. The inflow of medium into reactor I is stopped, product formation is completed under batch conditions and the product is then isolated. Interchanging of reactors I and II in the role of the first stage is repeated several times (Reusser, 1961; Ping Shu, 1966).

C. Heterogeneous continuous systems

In an ideal tubular reactor with plug flow, the microbial culture is variable in space along the tube, but is constant in time. Several mathematical analyses of heterogeneous continuous cultivators have been elaborated and reasons given for a plug flow reactor with recycle of organisms and for a stirred reactor with a plug flow in combination (Fig. 9) being very attractive for the cultivation of micro-organisms (Herbert, 1960; Grieves *et al.*, 1964; Powell and Lowe, 1964; Bischoff, 1966). However, a comparison of experimental and theoretical results is still lacking. The main disadvantage of plug flow reactors is the difficulty of providing an adequate gas supply throughout the system. Therefore the tubular reactor could be of advantage only for anaerobic cultivation. But even for anaerobic cultivation in the medium of Newtonian character no tubular reactor has been devised in which settling of cells and longitudinal and radial irregular mixing caused

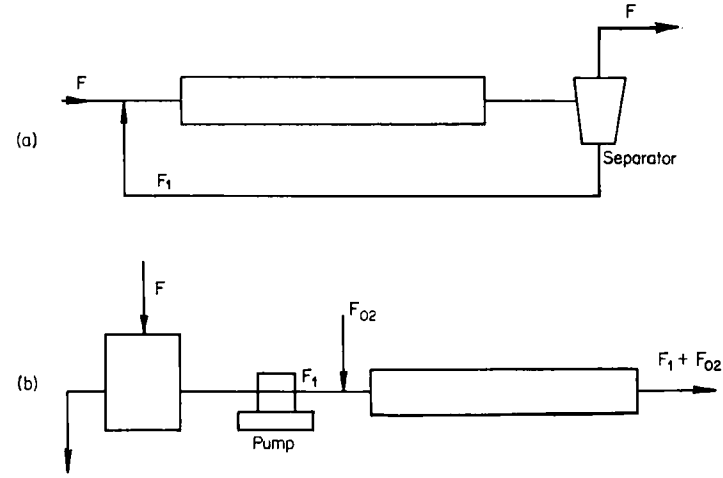

FIG. 9. Heterogeneous continuous systems. (a) Tubular plug flow reactor with feed-back; (b) stirred and plug flow reactors in combination.

by gas bubbles and by flow instabilities, can be prevented. In practice therefore an ideal plug flow reactor becomes an imperfectly mixed one, variable both in space and time.

It can be said that the time, θ_t, for a given element of the culture to flow a distance L along the tube or the time necessary for a desired conversion is given by—

$$\theta = AL/F = V/F = 1/D \tag{25}$$

where A is the cross-sectional area, and F is the volumetric flow rate.

It should be noted that, in spite of all above mentioned imperfections, an effective continuous beer fermentation process in pilot-scale tower fermenters, similar to a tubular reactor, has been developed (The A.P.V. Company, Ltd., Crawley, Sussex, England).

II. CONSTRUCTION OF MULTI-STAGE SYSTEMS

The individual stages, reactors, of a multi-stage system are perfectly mixed chemostats. A detailed description of their design, of dosing, of level control, of pH and of temperature control devices, as well as description of operational difficulties, can be found elsewhere in this Volume (pp. 259 and 277), and by J. Řičica in the monograph "Theoretical and methodological basis of continuous culture of micro-organisms" (1966). A multi-stage system consisting of agitated, horizontally positioned compartments was described by Means *et al.* (1962).

A. Level control and connecting tubes

In Fig. 10, several ways of interconnecting individual reactors in series are given (for simplicity only two are drawn). The culture level is maintained constant by means of internal and external overflow mechanisms (Fig. 11), some details of which are to be found in the Chapter by Evans, Herbert and Tempest (this Volume, page 277).

If the level is controlled by an internal overflow tube (Fig. 11b) tubes of different internal diameters should be employed. At a certain difference in hydrostatic pressure both culture liquids and air are sucked into the tube and the organisms transported into the next stage do not lack oxygen. In cultures of filamentous organisms tubes of internal diameter exceeding 6 mm are used. Air leaving the culture should flow through a separate way (Fig. 11c). When intercoupling reactors by methods II and III (Fig. 10), there is no aeration of the culture in the tubing and therefore the holding time of the culture in the tube should be as short as possible.

Control of the level of a medium consisting of several mutually immiscible components (e.g., gas–water solution–hydrophobic hydrocarbon) is very

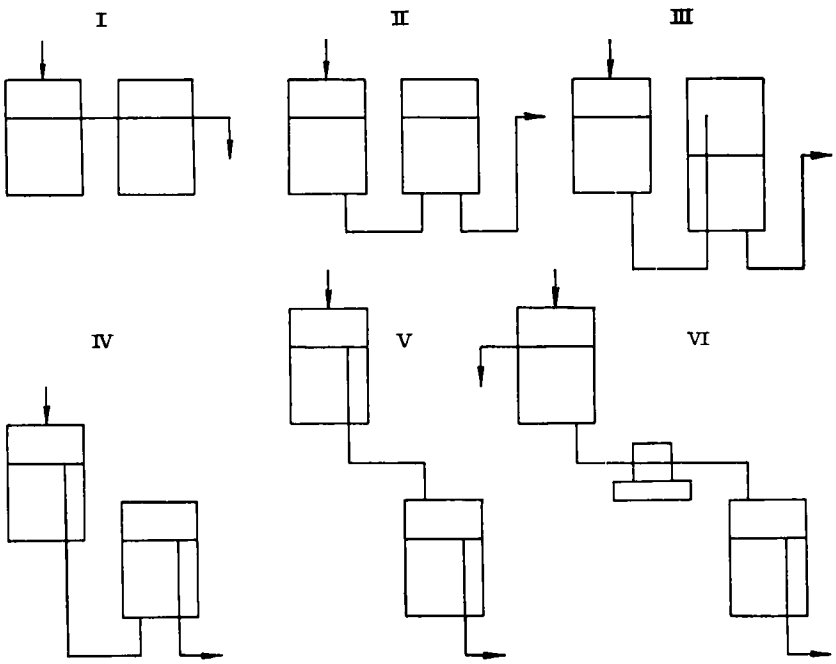

FIG. 10. Connecting tubes and level control in multi-stage systems.

difficult in systems (a), (b) and (c); only system (d) can be recommended (Fig. 11). A solenoid valve (2) controlling the outflow is monitored by an electrode (1). As soon as the culture level reaches the electrode, the electric circuit is closed and the valve opens. The culture liquid flows out as long as the electrode is in contact with the liquid.

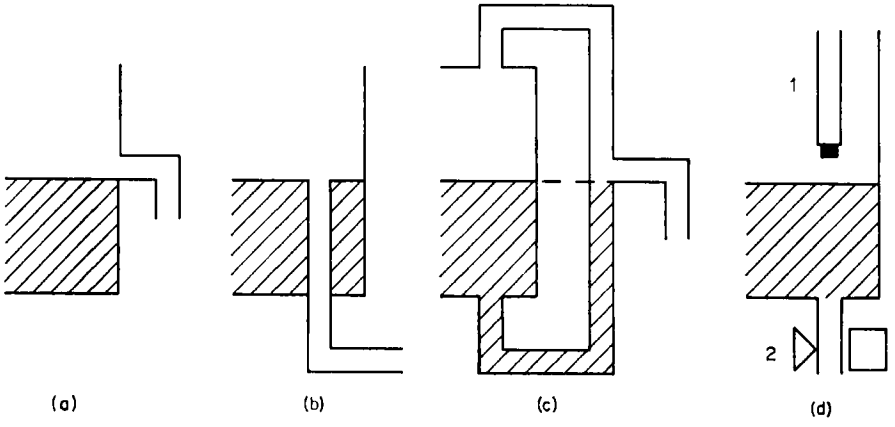

FIG. 11. Some types of overflow mechanisms. 1, electrode; 2, solenoid valve.

B. Active transport of the culture

The rheological properties of cultures of filamentous organisms change throughout the growth period. The culture liquid becomes viscous and therefore active transport of the culture is necessary. Active transport of the culture is also preferred for accurate dosing into another culture (Fig. 8 b, c; Fig. 9a, b). The best device for this purpose is a hose metering pump. If the culture is to be transported for a long distance and the cells might suffer from the lack of oxygen, it is possible to employ the device shown in Fig. 12. By means of a hose metering pump (2) the culture is pumped through the sampling tube (1) and transported into the next reactor.

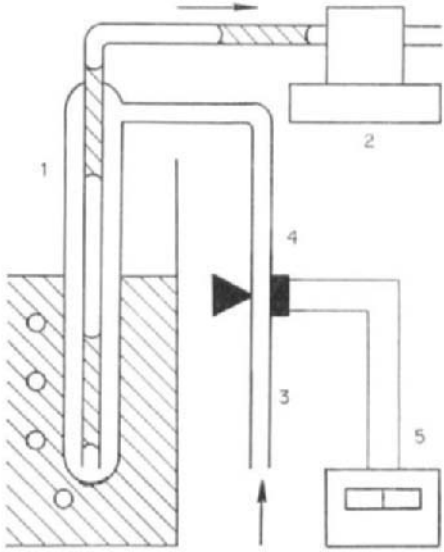

FIG. 12. Active transport of the culture. 1, sampling tube (open at bottom with air escaping); 2, hose metering pump; 3, air inlet; 4, solenoid valve; 5, timer.

The sampling tube (1) consists of two tubes, an inner and an outer. If air passes through tube (3) there is no connection of the inner tube with the culture, and air is pumped into the inner tube. When interrupting the air stream by the solenoid valve (4), the fluid level rises and the culture is pumped through the inner tube. By means of a timer (5) the intervals of alternative pumping of culture and of air are adjusted. The volume ratio of air to culture is dependent on the volumetric pumping rate and on the time period during which valve (4) is open.

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CHAPTER XV

Turbidostats

R. J. MUNSON

Medical Research Council Radiobiological Research Unit, Harwell, Didcot, Berks., England

I. INTRODUCTION

An experimental bacteriologist requiring a culture of micro-organisms growing under constant physiological conditions would generally find that a chemostat would meet his need. If he were interested in an effect dependent on the average generation time, he could readily adjust this parameter within a wide range of values by alteration of the dilution rate. He would find however that each increase in dilution rate would lead to a lower stable population density, until finally, at a certain dilution rate, the population density could no longer reach a stable value, but would decrease steadily. This critical population density would correspond to the upper end of the so-called

logarithmic growth phase of a batch culture. When the growth rate is no longer dependent upon population density, a constant density can only be maintained by a system that monitors some property dependent on population density and which adjusts the dilution rate accordingly. One such device is the turbidostat (Bryson, 1952; Anderson, 1953).

II. PRINCIPLE OF OPERATION

A turbidostat is a continuous-culture apparatus in which a photoelectric monitor detects deviations from some desired culture turbidity and passes a signal calling for a compensatory increase or decrease in dilution rate to a pump or valve controlling the flow of growth medium. Apart from the photoelectric control system and some simple optical requirements with respect to the culture tube, all the components of a turbidostat have similar functions to those of a chemostat. Other parts of this Volume should therefore be consulted for information on suitable designs for such common components, and also on such matters as sterilization procedures, the storage of growth medium and sample collection.

A. Light scattering by a bacterial suspension

The turbidity of a suspension of bacteria or other micro-organisms is a manifestation of the combined effect of light scattering by individual organisms. The fraction of the incident light scattered by a bacterium depends upon several factors. It is approximately proportional to the difference in the refractive indices of the suspending fluid and the intracellular cytoplasm. The relative intensity of light scattered in different directions is dependent upon its wavelength, the size and shape of the bacteria, and, in the case of a non-spherical bacterium, its orientation with respect to the directions of the incident and scattered light.

The influence of wavelength and bacterial size on the scattering of light can be readily observed with very simple apparatus set up in a dark room as illustrated in Fig. 1. A projection lamp or other suitable intense source of white light illuminates part of a bacterial suspension, which occupies a spherical glass flask. The aperture, A, restricts the illumination to a small diametral region of the sphere. On looking at the illuminated suspension one would see that the scattered light is not white but perceptibly coloured. The hue would change with the angle of scattering, θ, the colours having the same sequence as in the rainbow. For example with *Escherichia coli* growing logarithmically in minimal medium, the first fairly pure colour observed as θ increases from zero would be blue at an angle of approximately 40°. Green would follow at 45° and red at approximately 58°. The intensity of scattered light would fall rapidly as θ increases, but one would usually observe a

second colour sequence, commencing with blue green at $\theta = 76°$ and finishing with red at $\theta = 105°$. With *Bacillus megaterium*, which has a diameter nearly twice that of *E. coli*, the corresponding angles would be about half as large.

Light transmitted by a suspension is almost as white as the source unless a very thick suspension is used. Rayleigh scattering by ultramicroscopic particles, such as enzymes and polysomes, is much stronger for blue than for red light, so the transmitted light through a thick suspension is noticeably deficient in blue, i.e., it is reddish.

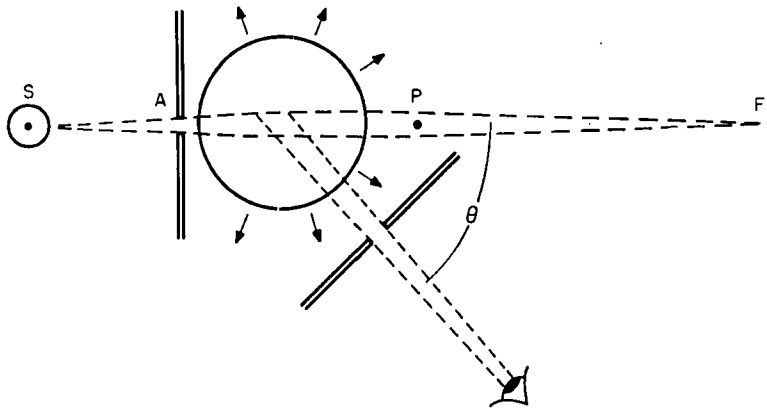

FIG. 1. Scattering of white light by a bacterial suspension. A narrow pencil of light from source S passes through aperture A into the suspension contained in a spherical flask. The fraction of light scattered in the direction θ depends upon its wavelength and upon the shape and size of the bacteria, but the fraction diminishes as θ increases. When the suspension is not too dense, the colours of the light scattered at any particular angle θ are fairly pure. F is the real image of S formed by the flask and its contents.

The other important fact that may be observed is that the intensity of scattered light diminishes rather rapidly as θ increases, particularly when θ is small.

The intensity of illumination at a point just outside the flask and within the transmitted beam (e.g., P in Fig. 1) diminishes rather slowly as the density of the suspension is increased since the reduction in transmitted light is largely compensated by a comparable increase in scattered light. By contrast, at a fairly remote point, such as F, the scattered component is relatively small and the total illumination is much more sensitive to changes in turbidity of the suspension.

Experimental measurements of the turbidity of a suspension are only meaningful when the intensity of scattered light at the receiving photocell is small compared with the intensity of unscattered (transmitted) light. Ideally

the scattered intensity should be negligible, in which case the fraction of light transmitted by a suspension could be expressed as $e^{-\alpha t}$, where α is the extinction coefficient associated with scattering, i.e., the *turbidity*, and t is the optical path length in the sample. The turbidity of a bacterial suspension defined in this way is strictly proportional to the population density provided other factors are not altered such as average cell size and refractive index of the suspending fluid and also provided the orientation of non-spherical organisms is not biased by movement of the suspension (Powell and Stoward, 1962).

B. Turbidity: transmitted light versus scattered light

A turbidostat can be designed to operate when the controlling photocell receives (i) light transmitted by the culture or (ii) light scattered by the culture.

(i) The intensity of light transmitted by a culture can be quite high when turbidity of the suspension is not too large, and a relatively insensitive photocell may then be employed, e.g., a barrier layer photovoltaic cell or a cadmium sulphide photoresistive cell. The transmitted light can also be focused on the sensitive surface at a point sufficiently remote from the culture to ensure that light scattered by it is only a small fraction of the total light reaching the photocell. The response of the photocell to a given change in turbidity is then close to the optimum.

(ii) When the scattering angle, θ, is greater than, say, 30°, the intensity of scattered light is usually so small that a sensitive vacuum photocell or even a photomultiplier may be required for a satisfactory signal. The main advantage of detecting scattered light lies in the fact that the photocurrent is proportional to the population density of the culture when this is fairly low, whereas with transmitted light the photocurrent slowly approaches a fixed value as the turbidity diminishes. In principle, scattered light should provide the more sensitive means of controlling cultures of low population density. In practice, there may be difficulties from light scattering by particulate foreign material suspended in the growth medium. Electronic amplification of the very small photocurrents and the avoidance of electrical leakage currents under humid conditions also add to the problems of this type of turbidimeter.

With both (i) and (ii) it is customary to employ a second, or reference, photocell, which receives light from the same source as the controlling photocell, but by some other path that does not include the culture. The illumination of the reference cell can be adjusted by a suitable stop or filter so that both cells constitute an optical balance or differential photometer.

This balance is almost unaffected by large changes in the luminosity of the lamp, but small alterations in turbidity of the culture produce electrical signals proportional to the alterations.

III. TURBIDOSTAT DESIGN

A. Early forms

The first recorded turbidostat was that of Myers and Clark (1944) who described "an apparatus for the continuous culture of *Chlorella*". The differential photometer consisted of two barrier layer photovoltaic cells, T_1 and T_2, connected in opposition to a mirror galvanometer as in a conventional colorimeter (see Fig. 2). Cell T_2 received light from a flourescent lamp via the culture, and T_1 was illuminated by the same lamp through suitable adjustable screens. After inoculation of the culture tube, the population density was allowed to increase to the desired value and the photometer was then balanced by adjusting the illumination of T_1. A further increase in turbidity

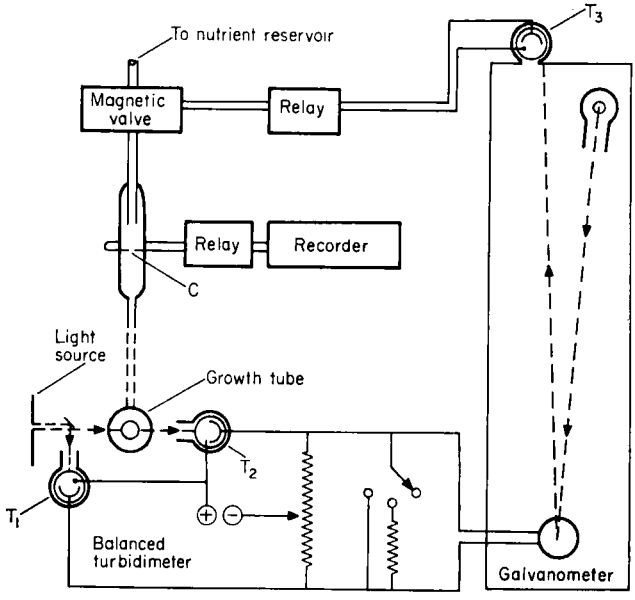

FIG. 2. Schematic diagram of a turbidostat (from Anderson, 1953). The photocells T_1 and T_2 each form an arm of a bridge circuit which constitutes the balanced turbidimeter. When the balance is sufficiently disturbed by growth of the culture the galvanometer spot falls on photocell T_3. The relay operates the magnetic valve and sterile medium is added to the culture drop by drop until the balance of the turbidimeter is restored. Drops are counted as they fall across Pt contacts C.

caused the light spot of the galvanometer to fall on the cathode of a vacuum photoemissive cell (T_3). The greatly increased current through this cell opened a solenoid (magnetic) valve, so allowing growth medium to flow through a tube from the elevated reservoir to the culture. The dilution of the culture continued until the photometer was again balanced. After further growth the cycle of operations repeated itself automatically as long as the supply of medium allowed. During periods when the valve was closed the turbidity rose and when it was open the turbidity fell, but the rise and fall in any one cycle only corresponded to a 1% change in population density. The performance of the system can be judged from the fact that the measured volumes occupied by cells per ml of suspension showed a standard deviation of less than 2% during one run that lasted 3 weeks.

In the apparatus of Myers and Clark (1944), the culture initially occupied only a small part of the whole culture vessel, the culture volume being allowed to increase steadily between the times when relatively large samples were withdrawn. By contrast, the "turbidostatic selector" of Bryson (1952) maintained a culture of constant volume by the provision of a self-levelling overflow. This feature has been adopted in the culture tubes of all later turbidostats.

B. Later forms

The more important considerations and modifications in the later designs of turbidostats can be conveniently classified under three headings: 1, culture vessel; 2, turbidimetry and control system; and 3, measurement of dilution rate.

1. *Culture vessel*

There are several requirements that must be met in the design of a satisfactory culture vessel.

(a) *Mixing and aeration.* Firstly, there should be efficient mixing of the incoming nutrient with the culture so that the equivalent amount of suspension passing down the overflow is a representative sample of the culture. With vigorous stirring, the turbulent movements of the suspension give good mixing, and it is only necessary to ensure that incoming medium does not immediately pass down the overflow tube.

Secondly, aeration should be adequate, and if it involves bubbling through the suspension it should be so arranged that it does not interfere with the chosen optical path for turbidimetry. When the culture volume is small and the population density less than a few times 10^8/ml, vigorous stirring of the suspension will ensure adequate aeration from above without recourse to bubbling. Even air dissolved in the medium entering the culture at, say,

23°C should be sufficient for a continuous culture of a strict aerobe with a population density of about 10^8/ml (Johnson, 1959).

(b) *Optical considerations.* Some attention to the optical characteristics of a culture vessel can produce a considerable improvement in the overall performance of the turbidimetric system.

The photocell T_2 (Fig. 2), positioned to receive unscattered light transmitted by the culture, will also receive unwanted scattered light. As we have seen earlier, this can be minimized by placing the photocell as far as possible from the culture. If in the process the unscattered beam comes to a focus on the photocell, the relative contribution by scattered light can be still further reduced.

A simple and optically satisfactory shape of culture vessel is suggested by Fig. 1. Most standard spherical flasks give adequate focusing when the diameter of the light beam is restricted to half the radius of the sphere. A spherical culture vessel of 500 ml capacity has been used in a turbidostat designed for short-term experiments on mutants induced with low frequencies (P. Gray and R. J. Munson, unpublished results).

The commonly used cylindrical culture tube (see Figs. 3, 4 and 5) has an optically undesirable shape, since it has no focusing power in a direction parallel to its axis. It can only provide reasonably good focusing when it is combined with another cylindrical lens of suitable radius placed with its axis at right angles to that of the culture tube. This arrangement has been used successfully with a cylindrical culture tube of 1·2 cm radius when the photocell T_2 was necessarily at least 20 cm from it (Munson and Jeffery, 1964).

The main problem with turbidostats that operate by scattered rather than by transmitted light is to minimize the amount of unwanted transmitted light reflected or scattered from external surfaces after passing through the culture. Turbidostats of this type have been described by Cooper *et al.* (1959) and by Bjorklünd *et al.* (1961). In both systems a photocell received light scattered through approximately 90° by a mammalian cell culture. Intense illumination of a small part of the culture near the controlling photocell and the large culture volume (1–2 litres) reduced troubles from reflected light and also provided a sufficient intensity of scattered light to permit the use of cadmium sulphide photocells.

A completely different plan was necessary in a system used to control a bacterial culture on the central axis of a lead cylinder of 60 cm diameter while it was growing under γ-radiation (R. J. Munson and R. J. C. Hudson, unpublished results). An external lamp and collimator were used to project a narrow parallel pencil of light along a radius of the lead cylinder and thence across a diameter of a cylindrical culture tube of the type shown in Fig. 5. Light scattered through 90° by bacteria in a volume of 0·5 ml at the centre of

the culture was focused by a lens onto the cathode of an externally mounted photomultiplier. The photomultiplier was necessary because the useful intensity of scattered light was probably three or four orders of magnitude smaller than in the turbidostats of Cooper *et al.* (1959) and of Bjorklünd *et al.* (1961).

(c) *Wall growths.* One of the most persistent problems to which there appears to be no general solution is the elimination of those films of bacteria that adhere to practically all solid surfaces in contact with the suspension. If such bacterial films consist of only one layer of cells, the rate of growth of the bacteria composing them should be virtually the same as that of bacteria in suspension. If the numbers of bacteria attached to walls and in suspension are n and N, respectively, then the dilution rate, D, should exceed the growth rate, μ, by the factor $(n+N)/N$ (see Tempest, this Volume, p. 259).

Adherent films of bacteria, such as *E. coli*, appear to be of two classes. One class produces large bush-like filamentous growths of a form suggesting that the bacteria stick to each other. These easily visible growths often include far more bacteria than the suspension, i.e., $n/N \gg 1$. The apparent growth rate of the culture is then several times greater than μ.

The other class of surface films has such a low surface density that it is normally invisible. Films of this type have been studied by Munson and Bridges (1964). They found that with a "non-sticky" strain of *E. coli* at a population density of 10^8/ml, the net rate at which bacteria adhere to a clean Pyrex glass culture tube is about 10^5 bacteria/cm^2/min until the surface density is around 10^6 bacteria/cm^2. The value of n/N would then usually be of the order of 10^{-2}, and the associated change in apparent growth rate would scarcely be detectable.

Observations by Larsen and Dimmick (1964) using a chemostat indicated that *Serratia marcescens*, when grown in broth, sticks to glass to form similar films with a larger surface density than *E. coli*. Their results showed that the surface density was approximately 10^8 bacteria/cm^2 when the population in suspension had a density between 5×10^7/ml and 10^9/ml.

Various devices designed to deal with these surface films have been described. Anderson (1953), whose culture tube rotated slowly about its axis, fitted stationary wipers that were held against the inner cylindrical surface of his culture tube. This remedy met with only partial success as bacteria also adhered to other surfaces. In a later design Anderson (1956) provided scrapers for the flat base of the culture tube as well (Fig. 3) and increased the rotation rate to 1·5 rpm. Wall growth on the culture outflow tube was also reduced by arranging that the outgoing air passed along the same tube. Continuous runs for periods up to 120 h showed no increase in growth rate after these precautions had been taken.

Fox and Szilard (1955) employed a fixed culture tube and a rotating magnetic stirrer enclosed in glass. All glass surfaces in contact with the culture were coated with a silicone "drifilm" (General Electric Drifilm No. 9987) and a detergent (0·05% Tween 80) was added to the culture medium. The presence of detergent led to excessive foaming with aeration, and in order to limit the height of the foam a platinum filament was maintained at red heat above the culture surface.

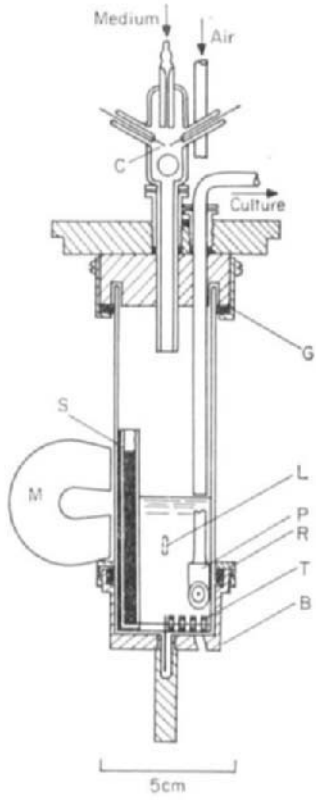

FIG. 3. Rotating culture tube (from Anderson, 1956). The tube is clamped in base B by rubber O rings R and is rotated at 1·5 rpm. Teflon- or rubber-enclosed iron rod, S, is rotated by frictional gearing to wall, dislodges cells and is self-cleaning. Teflon retained by Pt wire T scour the floor; P, rubber policeman on air tube; L, light beam; G, silicone-rubber gasket; C, Pt drop counting contacts; M, Alnico magnet.

A very simple mechanical wiper was used by Northrop (1954) consisting of a small number of split rungs of rubber on a simple frame that fitted fairly closely inside the cylindrical culture vessel. The wiper could be operated continuously at 150–200 strokes/min. In a later version (Northrop 1960),

a greater speed was achieved, and for short periods it was possible to reach 2000–3000 strokes/min (Fig. 4).

Massive sticky growths occur only rarely with some strains of bacteria, and a simple agitation system is then feasible. In the writer's experience this is true of *E. coli* when grown in minimal medium. Maclean and Munson (1961)

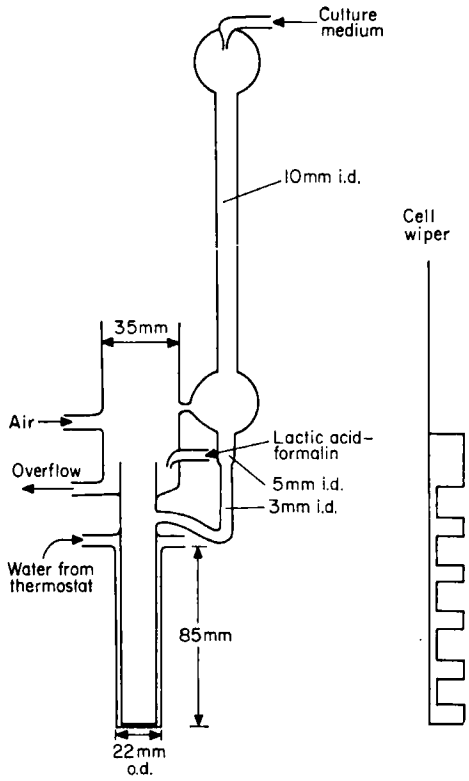

FIG. 4. Improved cell for steady-state apparatus with fixed culture tube, with mechanically operated wipers, designed to fit a standard colorimeter (from Northrop, 1960). The wiper, cut from a single piece of Lusteroid tube closely fits the inside of the culture tube and is operated at 300–500 strokes/min. It prevents the accumulation of wall growths and also aerates the culture. Lactic acid-formalin eliminates contamination from the over-flow tube.

successfully used small cylindrical magnetic stirrers encased in polythene for runs of 15–20 h duration. After use, these were disinfected by storage in ethanol for a few days. In cultures operated over longer periods, thick local growths sometimes appeared on the surface of the polythene, and a modified form of case made entirely of stainless steel was preferred. This had a free-running stainless-steel "tyre" at one end and a fixed one at the other, so that

as the permanent magnet was forced to revolve at 300 rpm, it was also subject to a rolling frictional couple which caused it to rotate at about 1000 rpm (Fig. 5). This rolling motion almost eliminated troubles from attachment of bacteria to the surface of the stirrer, although occasionally it still occurred.

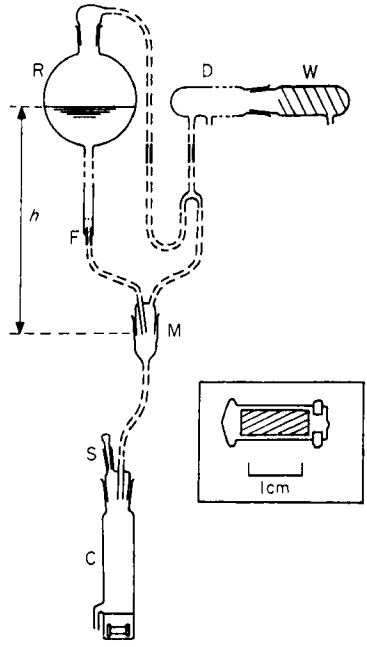

FIG. 5. Magnetically stirred culture tube and connections (from Maclean and Munson, 1961). Silicone-rubber tubes are shown by dashed lines, narrow glass tubes by heavy black lines. C, culture vessel; S, sampling port; M, mixer through which falling drops are counted optically; D, distributor of sterile humid air; F, filter; R, reservoir of medium; W, cotton-wool plug. Light beam for turbidimetric control passes between the surface of the culture and the upper edge of the magnetic stirrer (inset).

2. *Turbidimetry and control system*

Northrop (1954) slightly modified a standard commercial photoelectric colorimeter and used this in place of a purpose-built turbidimeter in a system otherwise very similar to that of Anderson (1953) (Fig. 2). The population densities of *B. megaterium* usually employed were 10^7–10^8/ml and the variations allowed by the control system were $\pm 5\%$. Later, Northrop (1960) modified his original turbidity-control system.

One of the disadvantages of the simple control circuit of Fig. 2 is that near the balance point the valve relay and the valve itself tend to chatter, partly as

a result of minor fluctuations in light transmission by the culture. Anderson (1956) eliminated this chatter by incorporating an electronic device in his control system, which allowed it to approach the balance point in small steps from one direction only.

Fox and Szilard (1955) adopted a control system that automatically examined the turbidity of the culture at regular intervals of 4 min and, if required, diluted the culture by adding a fixed amount of fresh medium. The 4-min cycle of operations was dictated by microswitches that were actuated by cams mounted on a shaft driven by a synchronous motor at $\frac{1}{4}$ rpm. The stirring motor was first switched off and the culture illumination then switched on for 15 sec. During this time, the differential photometer passed the appropriate signal to the galvanometer, which triggered or did not trigger the solenoid valve. After the lamp was switched off, the valve remained in its previous state for the remaining $3\frac{1}{2}$ min of the cycle. The stirrer was switched on for the last $\frac{1}{2}$ min only of each cycle. Although aeration was intermittent it was apparently adequate at the population densities of *E. coli* used, viz., 10^8 to 2×10^8/ml.

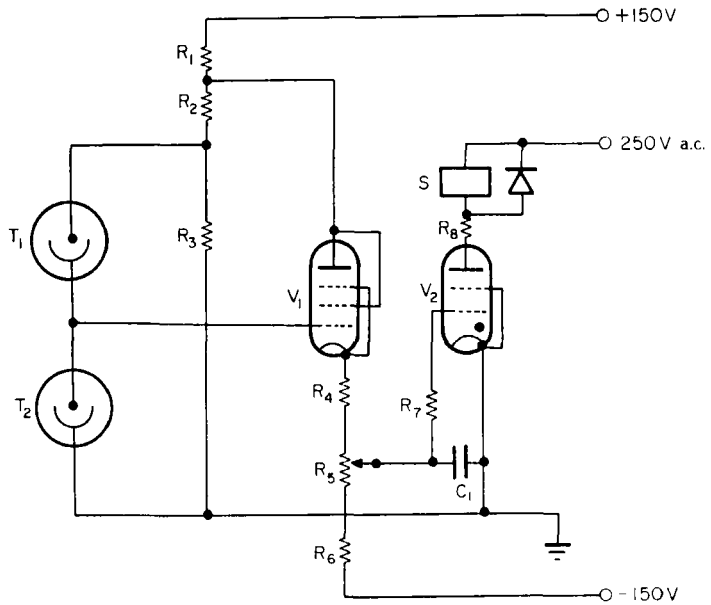

FIG. 6. Circuit diagram of differential photometer and amplifier used by Munson and Jeffery (1964). Photocells T_1 (reference) and T_2 (culture) are of vacuum photoemissive type (90CV); V_1, electrometer pentode (ME 1400); V_2, thyratron (20A3); S, relay (2·5 kΩ), operating pinch cock in medium supply line; C_1, 0·25 μF; R_1, 15kΩ; R_2, 3·9kΩ; R_3, 10kΩ; R_4, 270kΩ; R_5, 250kΩ; R_6, 1·5MΩ; R_7, 500kΩ; R_8, 10kΩ.

Bryson (1959) has given some details of later refinements in his turbidostat. Whenever the continuously monitoring turbidimeter gave the out-of-balance signal demanding more medium, a relay was tripped and a 1 min cycle of operations was initiated. A pump delivered a pre-set amount of medium during a short time, adjustable between 0 and 60 sec, and a commercial time stamp clock recorded the operation on a paper tape. Northrop (1960) has incorporated a similar operation cycle in his turbidostat and has pointed out that this modification obviates the chattering of the control valve, which occurred in most early turbidostats and tended to blur the onset and termination of each period of flow.

The control system of Munson and Jeffery (1964) differed from those described above in two respects. The photocells of the differential photometer were connected in series across a fixed voltage (Fig. 6) and not in opposition, and the out-of-balance detector was an electronic amplifier instead of a sensitive galvanometer. In addition to the portability and general convenience of an electronic amplifier, the system has considerably greater sensitivity (P. Gray and R. J. Munson, unpublished results). Recently P. Gray and R. J. Munson (unpublished results) have introduced an entirely different control system, whereby the dilution rate is regulated directly by the out-of-balance signal from the differential photometer. An essential component of this system is a peristaltic pump (Watson-Marlow Air Pump Co., Marlow, Bucks) whose speed is determined by the magnitude of the signal from the photometer.

3. *Measurement of dilution rate*

Dilution rate, D, is defined as the volume of medium flowing into the culture per unit time, divided by the culture volume. When the number (or total mass) of bacteria per unit volume remains constant, the dilution rate is equal to the average number growth rate (or mass growth rate) of the bacteria (Powell, 1956). The average mass and number growth rates become equal when the average size of a bacterium is constant. One may then refer simply to average growth rate, μ, in a turbidostat.

The mass growth rate of a turbidostatic culture usually increases during the first few generation times and then tends to a constant value, μ_m, which is characteristic of the organism and environment. The bacterial viability is then not measurably different from 100%, and the mean generation time for the population, τ, is $\log_e 2/\mu_m$. The value of μ_m is independent, or almost independent, of population density and of the concentration of the constituents of the growth medium, provided the latter are present in considerable excess. The quantity μ_m can be determined accurately by measuring the volume of the culture tube and the volume of effluent collected over a measured period of a few generation times. On the other hand if the culture

is subject to an environmental disturbance, the mass growth rate may change rapidly, and an automatic device for recording flow rates is essential.

In some turbidostats, medium flows from the reservoir to the culture under the influence of a pressure difference between the air in the reservoir and air in the culture vessel (Fox and Szilard, 1955), or simply by gravity feed from an elevated reservoir (Northrop, 1954; Maclean and Munson, 1961). In either case, when the control valve is open the flow rate should remain practically constant during a long experiment. If the out-of-balance signal from the turbidimeter that opens the control valve is also fed into a recording voltmeter, the trace will show the times at which medium flows. The average growth rate during any time interval is then proportional to the fraction of that interval for which medium flows (Northrop, 1954, 1960).

Another method applicable to cultures of small volume is to count individual drops of medium at a convenient point in the supply line. The accuracy of this technique depends upon the constancy of the average drop size. This may be readily checked, and in practice variations during one run are usually less than 1%.

The action of a counter described by Anderson (1953) depended upon the electrical conductivity of the growth medium. Each drop fell across a pair of platinum electrodes (Fig. 3) connected to a low voltage supply and an automatic current-pulse register that recorded the total count in each 30-min interval of an experiment. The same counter could be used to estimate the volume of the culture tube, and in this way one could determine the dilution rate without knowing the average volume of a drop.

Munson and Jeffery (1964) also measured flow rates by drop counting using specially made counters. Each drop, after falling 1 cm, interrupted a light beam between a lamp and an ORP 60 (Mullard) cadmium sulphide photocell (Fig. 5). After amplification, the current pulse actuated a ratchet motor coupled mechanically with a pen that was lifted 0·5 mm by each signal. This produced a short vertical stroke on a recording chart which was driven horizontally by a synchronous motor at a rate of 1 in./h. The flow rate was then indicated by the average gradient of the trace (Fig. 7). When the pen reached the top of the chart, a microswitch closed, the ratchet motor drive was uncoupled and the pen fell rapidly to the bottom of the chart in readiness for the next drop.

The advent of commercial, precision-made, peristaltic pumps has rendered other methods of estimating and of adjusting flow-rate somewhat obsolete. Such pumps are now available (Watson-Marlow Air Pump Co., Marlow, Bucks) that provide flow rates adjustable by a factor of up to 100. The flow rate with a given speed setting and given rubber tube remains constant to 1%, but the setting can be automatically controlled by a turbidimeter, if required, to give a continuously regulated flow rate (P. Gray and

R. J. Munson, unpublished results). The time record of operation of a pump set for a constant rate can be made by using the signal from a turbidimeter as described above. With a continuously regulated system, a record of the number of pump revolutions against time is necessary for following changes in growth rate.

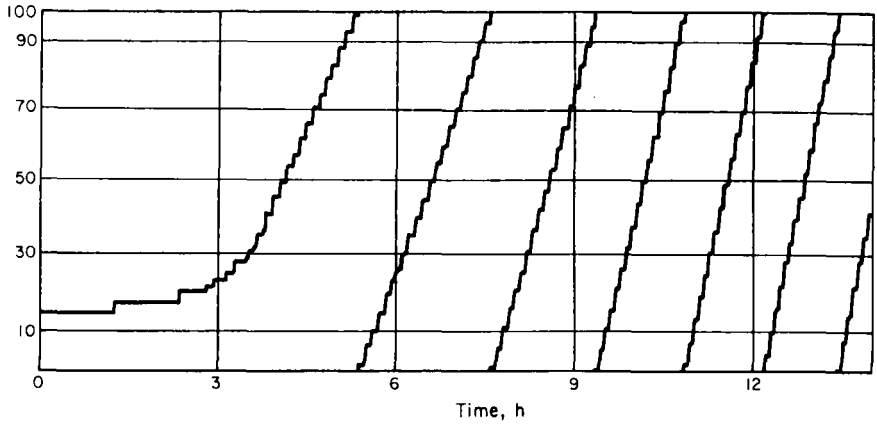

FIG. 7. Typical drop counter record (R. J. Munson and A. Jeffery, unpublished results). Overnight culture of *E. coli* is used as inoculum for turbidostatic culture at time zero. One vertical traverse corresponds to 240 drops (= 11·7 ml). The height of each step in the record indicates the number of drops supplied at each demand. This average number is around 11 in this record. It can be reduced by shortening the tube connecting the mixer and culture (see Fig. 5), so that fewer drops are in transit. The dilution rate has not reached its final value corresponding to μ_m during the first 13½ h.

IV. APPLICATIONS OF TURBIDOSTATS

Many of the problems for which turbidostats have been used have involved the accurate estimation of growth rates. Since relative values of dilution rate can be measured to better than 1% without difficulty, quite small changes in growth rate can be detected and correlated with the concentrations of inhibitors or antibiotics or with the action of bacteriocidal agents.

A. Effect of γ-radiation on growth rate of turbidostatic cultures of bacteria

There is now considerable evidence that the bacteriocidal effect of γ-radiation is very largely due to genetic damage (Munson and Bridges, 1966; Bridges and Munson, 1968). If lethal damage to a genome led to an appreci-

able reduction in the rate of cytoplasmic growth during the first subsequent generation time, this could be observed as a reduction in the growth rate of a turbidostatic culture.

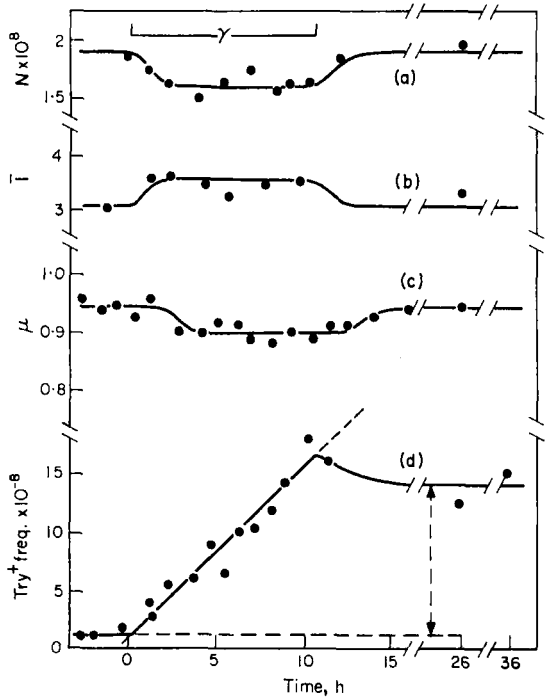

FIG. 8. (a). Changes in population density; (b), average length (in μm); (c), dilution rate; (d), revertant frequency [try$^+$/(try$^-$ + try$^+$)], of *E. coli* WP2 during an exposure to γ-radiation at 425 rad/h. The culture was grown at 37°C in M medium + 6 μg of DL-tryptophan/ml + amino acids "pool". The fall and subsequent rise in population density occurred at the beginning and end of the irradiation and coincided with the rise and fall in average length as expected for a constant opacity of the culture. Changes in growth rate showed a delay of one to two generation times. Mutation rates were estimated from the slope of the dashed line and from the overall change of frequency. (From Munson and Jeffery, 1965.)

The results of an experiment in which a turbidostatic culture of *E. coli* WP2 try$^-$ was grown in a minimal medium with added tryptophan and amino-acids "pool" (Kada *et al.*, 1961) for many hours at 37°C are shown in Fig. 8. After a few generation times, γ-irradiation was commenced at 425 rad/h and continued for 10·6 h. During the first generation time under radiation, there was a scarcely perceptible fall in mass growth rate, although there was a marked rise in average bacterial length and a concomitant fall in

population density. The existence of this lag in the response of the mass growth rate suggests that there are reserves of metabolites that can maintain the initial rate of growth for roughly one generation time after radiation damage to DNA has interfered with their synthesis. The occurrence of a similar lag in recovery of the initial growth rate after the end of irradiation would also be consistent with a rather slow restoration of a metabolic pool before the maximum growth rate could be resumed.

The extent of the depression in the growth rate which is finally reached has been measured at different dose rates and at temperatures of 37°, 22° and 16°C. The results of an experiment at 16°C, shown in Fig. 9 are qualitatively

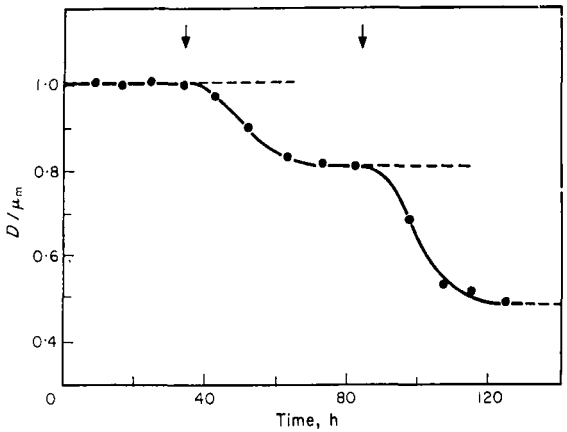

FIG. 9. Dilution rate of a turbidostatic culture of *E. coli* WP2 following alterations of γ-ray dose rate. Growth medium was M + 6 μg/ml of DL-tryptophan, population density 4×10^7/ml and temperature 16°C. After 35 h growth without radiation the dose rate became 700 rad/h (first arrow) and at 84 h was increased to 1290 rad/h. Dilution rate expressed as fraction of that without radiation (D/μ_m).

similar to those in Fig. 8, but the reductions in growth rate due to radiation are relatively much greater. These reductions in growth rate can be explained in terms of radiation damage to bacterial genomes, which are the most radiation-sensitive sites in *E. coli* (Munson and Bridges, 1966).

B. Estimation of concentration of required supplement for unrestricted growth of an auxotroph

For most purposes it would appear that the chemostat provides a simple means of estimating the amount of any supplement required by an auxotrophic bacterium. It would be necessary only to add a limiting amount of the particular supplement to the minimal growth medium and after a few

generation times determine the total population of the culture. One could then calculate the mass of supplement required for the production of one bacterium growing at an arbitrarily imposed rate less than optimal and at the growth-restricting concentration of the supplement. A turbidostat could not provide this information, but, as we have already noted in the introduction, it can maintain the culture at its optimal growth rate indefinitely, so that the earliest stage of starvation of the particular supplement (which might follow an increase in population) becomes evident from a slight fall in growth rate. This was the problem that interested us (Munson and Jeffery, 1964).

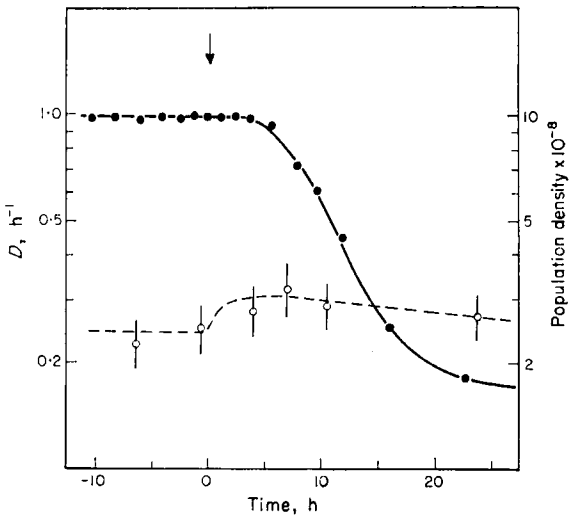

FIG. 10. Slow change in dilution rate (●) of a turbidostatically controlled culture of *E. coli* WP2 try⁻ following an increase in turbidity setting when tryptophan was near the point of exhaustion. Culture medium, M + 2μg/ml of DL-tryptophan; temperature 37°C. Population density (○), $2{\cdot}4 \times 10^8$/ml until time zero when setting increased to correspond to a density of approximately $2{\cdot}9 \times 10^8$/ml.

In one experiment the tryptophan auxotroph *E. coli* WP2 was grown at 37°C in M medium (Haas and Doudney, 1957) supplemented with 2μg/ml of DL-tryptophan. The population density was increased in stages to $2{\cdot}4 \times 10^8$/ml without appreciable alteration of growth rate. The absorbance in the light path to the reference photocell was increased again, and the population density rose in 2–3 h to approximately $2{\cdot}9 \times 10^8$/ml (Fig. 10). At the same time, the dilution rate began to fall and continued to decrease for about 24 h and then levelled off at 19% of its initial value. The fact that the culture required about 15 generation times to make the change from one steady state to another suggests a slow adaptation to tryptophan starvation.

There was evidence of a slight inhibition in cell division in the final steady state, the average bacterial length being slightly increased.

Other experiments were made with tryptophan concentrations of 6μg/ml and 20μg/ml (Fig. 11). These showed a gradual fall in growth rate at densities

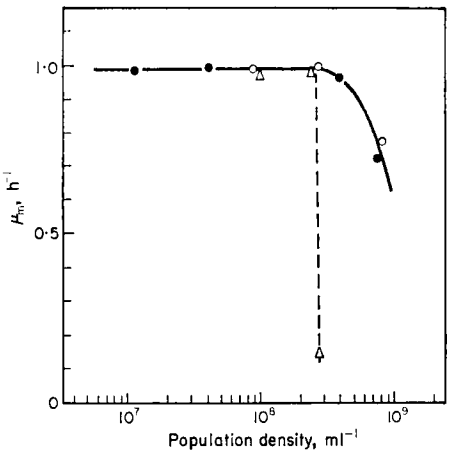

FIG. 11. Final growth rate of turbidostatic culture of *E. coli* WP2 as a function of population density in M medium with different tryptophan supplements (△, 2μg/ml; ○, 6μg/ml; ●, 20μg/ml).

greater than 3×10^8/ml, very similar to that previously found for *E. coli* B/r in minimal medium (Maclean and Munson, 1961) and presumably due to similar factors other than tryptophan limitation.

C. Mutations during turbidostatic growth

Most gene mutations that arise during active growth in the absence of a known mutagen, i.e., "spontaneous" mutations, are almost certainly the result of a random error in DNA replication (Novick and Szilard, 1951). Such errors may occur more frequently under the influence of chemical mutagens, ultraviolet light or ionizing radiation.

Mutation rate can be defined as the probability of a mutant arising per cell division. In bacteria there are often two or more copies of the same gene per bacterium, and it would therefore be preferable to define mutation rate as the probability of a mutant arising per replication of a genome. This distinction is of practical importance when the average number of genomes per bacterium changes with cultural conditions.

Mutation rates observed in the presence of a mutagenic agent can be regarded as consisting of two components; the spontaneous mutation rate

and the induced mutation rate. Both of these components usually have small values, in which case the induced mutation rate is equal to the arithmetical difference between the observed and spontaneous rates. With chemical mutagens, the induced mutation rate increases with concentration of the mutagen at low concentrations. Induced mutation rates for radiation also increase with the intensity or with the radiation dose, the rate for X- or γ-radiation being proportional to dose (Munson and Jeffery, 1964) and for ultraviolet light proportional to the square of the dose in some cases (Bridges *et al.*, 1967).

The frequency of spontaneous mutants in a culture composed initially of wild type bacteria increases linearly with time when there is no selection for or against the mutants. If the growth rate of the mutants exceeds that of the wild types, the rise of mutant frequency becomes accelerated as time progresses, until the culture contains a vanishingly small number of wild types. On the other hand, if the mutants have the smaller growth rate, the rate of increase of mutant frequency slows down, until eventually the frequency reaches a plateau. This "equilibrium" mutant frequency is proportional to the spontaneous mutation rate and inversely proportional to the difference in the two growth rates. This relation can be expressed as

$$(M/W)_{\text{eq}} = \lambda\mu_W/(\mu_W - \mu_M) \qquad (1)$$

where M is the number (assumed large) of mutants in culture, W is the number (assumed very large) of wild-type bacteria in culture, λ is the mutation rate, μ_W is the growth rate of wild-type bacteria and μ_M is the growth rate of mutant-type bacteria.

1. *Mutations induced by γ rays*

An example of a linear rise in mutant frequency is shown in Fig. 8. The mutants are prototrophic revertants of the auxotroph *E. coli* WP try$^-$ induced by γ-radiation over a 10 h period (Munson and Jeffery, 1964). The growth rates of the auxotrophs and prototrophs were subsequently compared and found to differ by less than the experimental error of $\pm 1\%$. This is a particularly straightforward example of mutagenesis without selection and with negligible interference from spontaneous mutants.

The induced mutation rate, indicated by the slope of the graph, was proportional to dose rate. At a given dose rate it was found to decrease with decreasing temperature of the culture. Subsequently it was shown that this temperature dependence could be largely explained in terms of a "loss" of mutants, particularly at low culture temperatures (5°–16°C). This loss appeared to be due to the operation of a repair process (low-temperature mutation loss) between the time when the radiation damage occurred and the time of plating at 37°C.

The mutation rate deduced from the linear increase in mutant frequency was slightly smaller when nutrient broth was added to minimal medium + tryptophan. This effect was probably associated with a slightly greater repair of pre-mutational radiation lesions.

2. *Spontaneous mutation to phage resistance*

Fox (1955) grew turbidostatic cultures of *E. coli* B/1/t, a tryptophan auxotroph resistant to phage T1, in a minimal medium containing 20μg/ml of tryptophan. The spontaneous mutation rate to resistance to phage T5, given by the rate of linear rise in mutant frequency, was $1{\cdot}3 \times 10^{-8}h^{-1}$ or $1{\cdot}2 \times 10^{-8}$ per generation (τ = 67 min). Chemostat experiments with much larger values of τ showed that the mutation rate per generation was proportional to the generation time. When nutrient broth was used as growth medium, the mutation rate was $4{\cdot}0 \times 10^{-8}h^{-1}$ or, since τ = 29 min, the mutation rate per generation was $1{\cdot}9 \times 10^{-8}$.

Mutations to T6 resistance were also observed. In minimal medium the mutation rate was $0{\cdot}6 \times 10^{-8}$ per generation and in broth $0{\cdot}87 \times 10^{-8}$.

Mutagens, such as theophyline, and antimutagens, such as adenosine, did not affect either mutation rate in complex media, although in chemostatic cultures (Novick, 1956) they did.

These results, although inexplicable at the time, may be due to the fact that two classes of mutation are involved. (Kubitschek and Bendigkeit, 1964a,b). Mutations may occur during synthesis of the gene concerned ("error" hypothesis) or it may occur after synthesis has been completed. The mutation rate per generation should therefore be independent of generation time for the first class and proportional to generation time for the second.

D. Mutations to antibiotic resistance: selection in the presence of the antibiotic

1. *Neomycin*

Bryson (1952) combined his turbidostat with a device that added to the culture medium an exponentially increasing concentration of any desired antibiotic or toxic agent. In order to overcome the difficulty of bacteriostasis consequent upon a too rapid rise in concentration of the antibiotic, nutrient alone was supplied at a steady, rather low, rate through another tube in parallel with the main supply tube. This parallel steady feed rescued the culture if its growth rate became too low and diluted the antibiotic until turbidostatic control was resumed.

With this "turbidostatic selector", Bryson (1952) was able to select a culture, *E. coli* B, that was able to grow at a concentration of antibiotic 16 times greater than its parent. This was achieved in one 10 day run.

2. *Terramycin*

Spontaneous mutation to terramycin resistance in turbidostatic cultures of *B. megaterium* 899a has been studied by Northrop (1957). Resistant mutants were scored on plates containing 4μg/ml of terramycin. With no terramycin in the growth medium (yeast extract peptone) the mutant frequency increased from a low value, rapidly at first, but then more slowly, until a constant frequency was reached after more than 100 generation times.

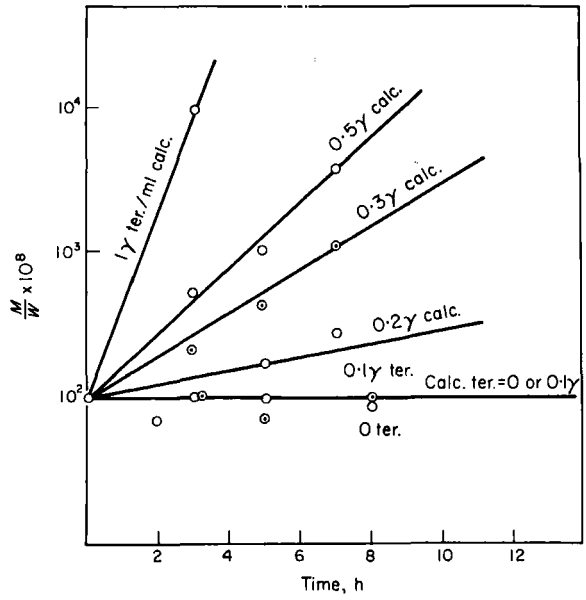

FIG. 12. Frequency of terramycin-resistant mutants of *B. megaterium* (M/W) in batch cultures growing at 35°C in yeast extract peptone with different concentrations of added terramycin. Lines through the experimental points were calculated assuming (i) $\lambda = 2 \times 10^{-8}$/generation and (ii) values of $(\mu_W - \mu_M)$ in Table I. The close fit of these lines provides good evidence that selection is due almost entirely to a reduction in growth rate of sensitive wild types in the presence of terramycin. (From Northrop, 1957.)

The slow approach to an equilibrium mutant frequency suggested that there was slight selection against the mutants, probably owing to their growth rate being marginally smaller than the sensitive wild types. On the other hand, when terramycin at concentrations between 0·2 and 1·0μg/ml was added to the growth medium the resistant mutants eventually became more numerous than the sensitive wild types, possibly because the selection pressure was then reversed.

Supplementary experiments with batch cultures established that the growth rate of the terramycin resistant mutants decreased only slightly as the terramycin concentration was increased, whereas the growth rate of the sensitive wild types decreased rapidly and became virtually zero at 1·0μg/ml. Figure 12 shows the observed mutant frequency versus time over the first

TABLE I

Growth rates in yeast extract peptone and terramycin of *B. megaterium* 899a (μ_W) and its terramycin resistant mutants (μ_M).

	Terramycin, μg/ml					
	0	0·1	0·2	0·3	0·5	1·0
μ_W, h^{-1}	1·7	1·7	1·5	1·1	0·9	0
μ_M, h^{-1}	1·7	1·7	1·6	1·6	1·5	1·5
$\mu_W - \mu_M$	0	0	−0·1	−0·5	−0·6	−1·5
$(\mu_W - \mu_M)$†	0·05	0·05	−0·08	−0·34	−0·53	−1·5

† These were the values of $(\mu_W - \mu_M)$ used in drawing the graphs of Fig. 12. From Northrop (1957).

few generations in different concentrations of terramycin and Table I lists the corresponding growth rates of sensitive and resistant bacteria. It is apparent that the rate of increase in mutant frequency is roughly proportional to the difference between the growth rates of mutant and wild types. The lines through the experimental points were drawn assuming values of $(\mu_W - \mu_M)$ close to the approximate experimental ones (Table I) and taking 2×10^{-8} as the "best" value of λ. The main factor in the observed selection of terramycin-resistant mutants is therefore the inhibitory effect of terramycin on growth of the sensitive wild types.

There is evidence that terramycin interferes with the process of detachment at the ribosome of the amino-acid moiety of amino-acyl sRNA, so presumably this interference is much more severe in sensitive than in resistant bacteria.

E. Selection of bacteria by attachment to surfaces of culture tube

During experiments on the induction of prototrophic reversions of *E. coli* WP2 by γ-rays (Munson and Bridges, 1964) it was very occasionally found that the initial rise in mutant frequency was followed after a variable period by a second and much larger increase. Examples of this phenomenon are represented in Figs. 13 and 14. In the first experiment (Fig. 13), two cultures were simultaneously irradiated at the same dose rate over a period of 4 h, during which the mutant frequency in each rose from 10^{-8} to 2×10^{-7}. After

a short pause, the mutant frequency in one culture began to rise again and continued steadily until it reached a constant value of about 50%. The mutant frequency in the second culture rose at the same rate after a delay of 12 h. The growth rates of both cultures during these periods of "take-over" were constant to within 1%.

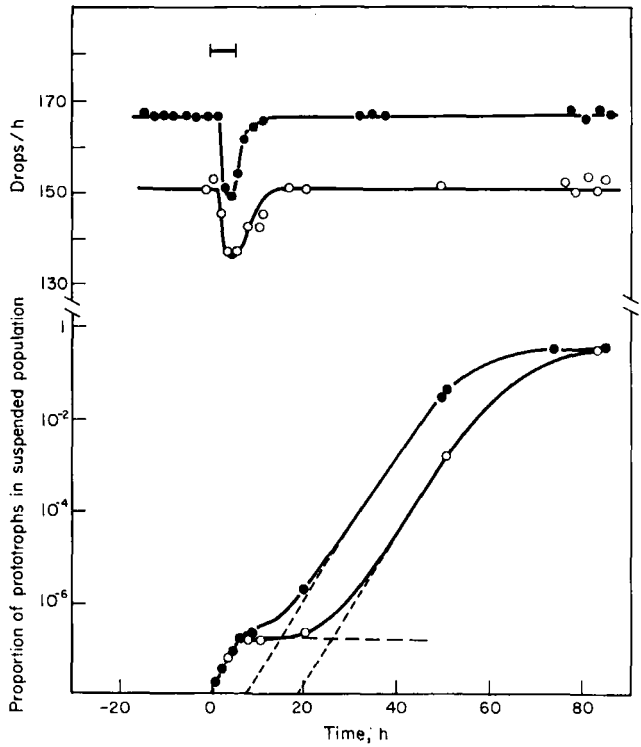

FIG. 13. Increases in prototroph frequency ("take-over") in two continuous cultures of *E. coli* WP2 try$^-$. The cultures were simultaneously exposed to 4500 rads of γ-radiation during the period 0–4 h. The broken line indicates the contribution from normal prototrophs and the dotted lines the contributions from the hypothetical variant "sticky" prototrophs, their slopes being 0·36 h^{-1}. The growth rates (drops/h) shown in the upper graphs show no changes except during the irradiations. (From Munson and Bridges, 1964.)

In another experiment two small doses were given to one culture (Fig. 14). There was no evidence of a change in mutant frequency in three days following the first dose, but after the second dose a rapid rise began and a final prototroph frequency of 10^{-2} was reached.

In the course of further tests designed to check possible hypotheses relating

to "take-over", two facts came to light. When a culture in an early stage of "take-over" was transferred to a fresh tube, the rise in prototroph frequency was delayed by several hours. This suggested that the suspension contained only a small fraction of something essential for "take-over". Also, bacteria

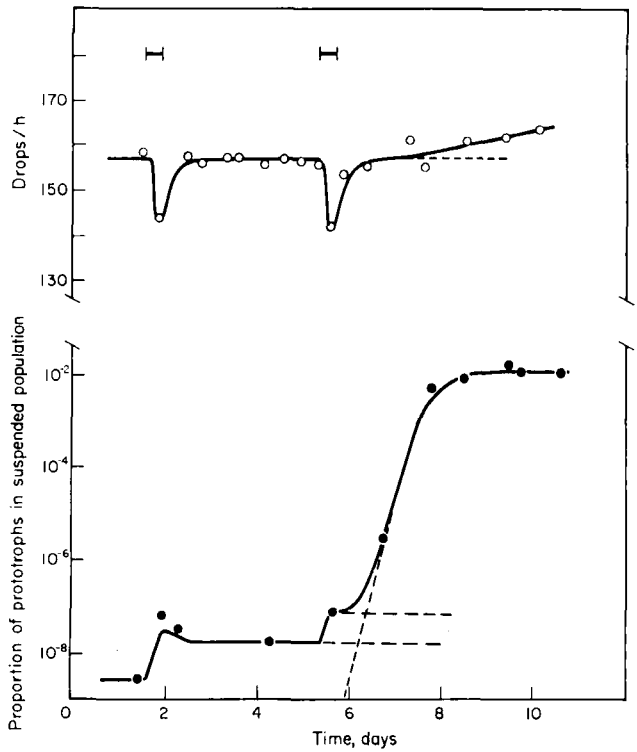

FIG. 14. "Take-over" following two irradiations of a culture of *E. coli* WP2 try$^-$. Two doses each of 600 rad were given during the periods indicated by the horizontal lines. The dotted line representing the contribution from the hypothetical variant prototrophs has a slope of 0·35 h^{-1}. After "take-over" the growth rate (drops/h) increased slowly by approximately 3%, suggesting that there was an increase in the number of bacteria on the walls equivlaent to 3 × of the whole population. (From Munson and Bridges, 1965.)

removed from the walls of the old culture tube and from its stirrer contained prototrophs at a frequency 10 or more times greater than the transferred suspension.

Munson and Bridges (1964) were able to explain their findings by supposing that both try$^-$ and normal try$^+$ bacteria may each become attached to the walls of a culture tube with a certain probability α per unit time and that

having become attached, there is a probability β per unit time that they become detached again. After a sufficiently long time, the number of bacteria attached to the walls would become constant and the frequency of prototrophs in the suspension would then be equal, on average, to the frequency in the surface film. Since the total number of bacteria in the surface film was of the order of 10^7 and the frequency of prototrophs at the onset of "take-over" was about 10^{-7}, it would follow that at this time the number of prototrophs attached to the wall could be no more than a very few. Munson and Bridges (1964) then suggested that one of these prototrophs might be a rather rare variant having the same attachment probability as the rest of the population ($\alpha' = \alpha$), but a smaller detachment probability ($\beta' < \beta$). If so there was a possibility that "take-over" would commence. After a few generation times, an appreciable number of these "sticky" variants would be attached to the walls, and their frequency would increase logarithmically at a rate $(\mu - \beta')$ until other factors intervened. Analysis of the curves of Fig. 13 and 14 led to the conclusion that $(\mu - \beta)$ was negative for the normal population whereas $(\mu - \beta')$ was positive and equal to 0·35 h^{-1}.

Hence "take-over" appeared to be a result of the variant revertants having a detachment rate smaller than their rate of multiplication, whereas for the rest of the population the detachment rate was greater than the rate of multiplication.

F. Selection of mutants resistant to bacteriophage

Cocito and Bryson (1958) grew a proline-requiring auxotroph of *E. coli* B (E12–100) as a turbidostatic culture in nutrient broth and observed the frequency of mutants resistant to phage T_3. These B/3 mutants outgrew their phage-sensitive parents at an exponential rate of 0·19 h^{-1}, until they reached a final constant frequency of 0·06. The graph of the logarithm of mutant frequency versus time was similar in form to those of Figs. 13 and 14 for "take-over" prototrophs of *E. coli* WP2. One possible hypothesis considered by Cocito and Bryson (1958) for this phenomenon was the liberation by the parental strain of a colicine selectively inhibitory to itself.

In support of this hypothesis, they then sought evidence for a colicine that had the required selective characteristic. Colicine synthesis was induced by ultraviolet irradiation of *E. coli* sub-cultured from the turbidostat and grown in a synthetic medium. After further growth, the culture was centrifuged, concentrated by lyophilization, dialysed at 3°C and reconcentrated by lyophilization of the non-dialysable fraction. Using *E. coli* B as the indicator, protein obtained by fractionation with ammonium sulphate and sodium sulphate gave a zone of inhibition by the paper-disc method at dilutions of 10^{-7}. The inhibitory substance was heat labile, inactivated by papain at pH 7 and was relatively more resistant to ultraviolet inactivation than phage T3.

In a mixed logarithmic culture of strains B and B/3, B was inhibited. The hypothesis was thus verified.

V. SOME FACTORS RELEVANT TO THE CHOICE OF TURBIDOSTAT OR CHEMOSTAT FOR PARTICULAR PURPOSES

Continuous cultures have been used broadly for two purposes. Firstly, to generate populations of cells with characteristics that are constant over the period of an experiment for which they are needed. Secondly, to investigate effects of toxic or mutagenic agents and to study the utilization of substrates in steady-state populations.

Several factors should be considered before deciding whether a chemostat, a turbidostat or a combination of both would be most suitable for a particular purpose. The relative simplicity of a chemostat would make this the instrument of choice, unless there were good reasons to the contrary. One such reason could be the requirement that the culture should grow at a rate that does not depend upon the concentration of the chosen constituents of the growth medium. This condition could be satisfied by a turbidostat or, if constancy of turbidity is not essential, by a chemostat with manual adjustment of the dilution rate to match the growth rate of the culture (Maclean and Munson, 1961). Another reason, relevant to the continuous culture of mammalian cells (Cooper *et al.*, 1959), is that the range of population densities over which the growth rate is near optimum may be quite narrow and rather unpredictable. Maladjustment of the dilution rate of a simple chemostat may then lead either to a stationary culture or to wash-out. Cooper *et al.* (1959) overcame this difficulty by deliberately making the chemostatic dilution rate greater than the growth rate and providing a turbidimetric control to stop dilution when the turbidity became too low (chemo-turbidostat).

An early example of a hybrid culture system was the "turbidostatic selector" of Bryson (1952) mentioned above, which provided two independent dilution systems, one (turbidimetrically controlled), which supplied growth medium and antibiotic, the other (chemostatic), which supplied medium alone at a fixed low rate.

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CHAPTER XVI

Harvesting and Clarification of Cultures—Storage of Harvest

R. O. THOMSON AND W. H. FOSTER

The Wellcome Research Laboratories, Langley Court, Beckenham, Kent, England

I. INTRODUCTION

The methods used for harvesting bacterial cultures fall into two main categories, filtration and centrifugation. Each can be used for laboratory- or production-scale work, and the choice may be regulated as much by the equipment available to the operator as by the nature of the culture and the product to be harvested. It is the purpose of this Chapter to describe these methods and others that, in certain circumstances, may be of value. In so doing, experience gained at these laboratories in the harvesting of cultures of bacteria for the preparation of human and veterinary vaccines will be called upon.

The purpose of the harvesting stage in the course of isolating the required product of growth is to obtain the product in a state of relative stability

and in a volume usually much smaller than that of the culture, so that it may if necessary be conveniently stored for some time without fear of losing its required properties. Harvesting, therefore, not only involves the physical separation of two phases by the methods already mentioned, but includes further treatment of the bacteria. If the desired product is present in the culture filtrate, concentration by some method or other may be necessary in order to remove the bulk of unwanted fluid or to minimize the destructive effect of enzymes present in the filtrate. Although the emphasis will be on separation of bacteria from culture fluid, it is proposed to give some consideration to further treatment of bacteria and soluble products of growth.

An important part of microbial culture and one associated with the harvesting stage is the sterilization of the product to be retained and of the fractions to be discarded. Unwanted material must be sterilized, preferably by autoclaving, before disposal. The method used for sterilizing bacteria will depend upon the purpose for which they were grown; such techniques as heating or treatment with formalin may be deleterious to the desired property. Culture filtrates or supernatants or fractions therefrom should be sterilized by filtration if they are to be kept, provided of course that in so doing the desired properties of the material are not lost. A preservative may be added to prevent bacterial contamination and here it must be remembered that many of these have properties that may prove to be injurious. For example organic mercurials such as thiomersalate, which rely for their action on combination with sulphydryl groups may, because of this property, inhibit several enzymes, including some bacterial proteinases. Methods for sterilization have been fully dealt with by Sykes (this Series, Vol. 1) and by Mulvany (this Series, Vol. 1).

When working with bacterial cultures, certain safety precautions are necessary to ensure the adequate containment of organisms and the safe disposal of unwanted noxious products of growth. These precautions are necessary, not only for the protection of the operator, but also to prevent cross-contamination. Although general laboratory safety is discussed by Darlow (this Series, Vol. 1), some consideration will be given to the subject in this Chapter, since it is during harvesting more than at any other stage that safety precautions must be rigidly applied.

Finally, in an article of this kind, discussions of processes necessarily include descriptions of commercially available equipment. It would clearly be a monumental task to cover the whole range of such equipment and for the most part procedures and apparatus at present being used at these laboratories will be described in greatest detail. Although such equipment has proved to be adequate for the purposes described, it should not be implied that approval of it in any way detracts from the merits of other similar equipment not mentioned.

II. FILTRATION

Filtration in one form or another is one of the most widely used procedures in the laboratory and in the process plant. Its use for harvesting bacterial cultures has found wide application, particularly when the filtrate is required rather than the bacteria. Although its main advantage is the relatively low capital and running cost involved, the speed and efficiency with which cultures can be harvested often make it the method of choice. In some cases it may be the only method that can be used, for example, in the clarification of liquids of high specific gravity, since in these cases sedimentation by centrifugation can be achieved only with great difficulty if at all.

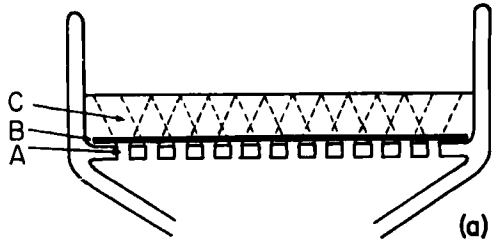
The use of gravity-flow filtration is totally inadequate for harvesting large quantities of culture. In order to achieve a satisfactory rate of flow it is necessary to force fluid through the filter bed by means of either vacuum or pressure. If the liquid is drawn through the filter bed by means of a vacuum-pump, the maximum pressure differential over the pressure gradient set up cannot exceed about 78 cm Hg (atmospheric pressure), whereas if the liquid is pushed through the filter by positive pressure the differential is limited only by the size of the pressure pump and the safety limits of the equipment. Much higher rates of flow can therefore be obtained with the latter procedure.

Filtration is self-limiting. The volume of culture that can be clarified depends on the density of the bacterial suspension, since the organisms form a layer on the surface of the filter that very quickly becomes impervious to the free flow of fluid. At this stage the application of increased pressure is without effect. For practical purposes the quantity of bacteria retained on the filter bed is the limiting factor to further filtration and one would expect each filter to have a capacity, in terms of the volume of organisms necessary to cause cessation of flow. This volume is surprisingly small and filtration would be quite impracticable were it not for the availability of certain materials, described as filter media or filter aids, that help to increase the through-put of a filter to a practical level.

A. Filtration media

Any material that can be freely dispersed in aqueous fluid and that can form a pad of a porosity small enough to retain bacteria may be used as an aid to filtration. Although the most common filter aids are those based on cellulose or on the diatomaceous earth, kieselguhr, other materials, such as asbestos, kaolin, talc and sintered or fibre glass are of value in certain cases. In practice filter aids are added to the culture before harvesting, so that during filtration they will form with the bacteria a porous layer on the surface of the filter bed. Because of this the filtration rate will fall off much more

gradually than if no filter aid were used. Filter aids can in addition be used to prepare filtration beds. Whatever the medium, whether it be a layer of asbestos or kieselguhr, or simply filter paper, it must be supported by a rigid structure adequate to withstand the pressure differential applied during filtration. The support in the case of the Buchner funnel is a perforated porcelain disc, whose filtration area is the sum of the areas of the perforations. By forming a layer of filter aid above this disc, the filtration area is increased to that of the surface of the bed. This is illustrated in Fig. 1a. Since the filtration medium used is freely dispersible, an important



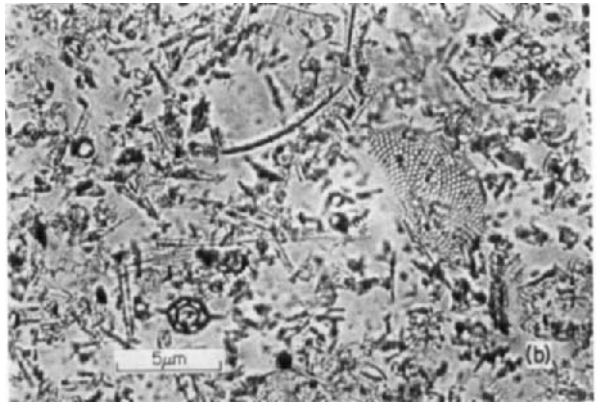


FIG. 1. (a) Buchner funnel: A, primary support, perforated porcelain; B, secondary support, paper, lint, etc.; C, filter bed, kieselguhr. Broken lines indicate the extent of surface area available for filtration. (b) Photomicrograph of kieselguhr.

property of these beds is that the surface can be removed by scraping without necessarily damaging the filtering properties of the bed. This is of practical importance when the rate of filtration is decreased by the formation of a layer of retained bacteria on the surface of the bed. Such layers can be contin-

uously removed by scraping with a palette knife or similar tool. The limit to the amount of scraping will depend on the thickness of the bed.

By using these aids as described above, considerable volumes of culture may be harvested fairly rapidly either through Buchner funnels or industrial presses and metafilters as described below. In the absence of filter aids, the quantity of bacteria that can be removed from cultures by pressure filtration is small, amounting to about 5 mg (wet wt)/cm^2 of filter bed for *Clostridium perfringens*. When kieselguhr is mixed in with the culture before filtering, the quantity of bacteria separated depends on the ratio of organisms to filter aid. About ten times the above weight may be harvested by using a ratio of 0·1 on a dry weight basis of *Cl. perfringens* to Hyflo Supercel (see below). The figures obtained, of course, vary with the species of organism being harvested, as well as with the age of the culture, degree of lysis, etc. For the filtration of almost all types of culture, the diatomaceous earth kieselguhr is the medium *par excellence*. As can be seen in Fig. 1b it is composed of various rigid and porous particles which when packed together form a porous conglomerate ideal for filtering bacterial cultures. It is not attacked by weak acids or alkalies and does not generally adsorb material during filtration. At these laboratories the product Hyflo Supercel (Johns–Manville, Lompoc, California) has been used for many years with complete satisfaction.

B. Filtration equipment

1. *Buchner filter*

This is the most commonly used apparatus for vacuum filtration on a laboratory scale, although with a battery of several large Buchner funnels, large volumes of culture (up to 500 litres) may be harvested in a working day. The main disadvantage of this apparatus is that as it is normally used aseptic filtration is not possible. For the best results and most rapid filtration a bed of filter aid, such as that illustrated in Fig. 1a, should be prepared on the funnel. A secondary support to this bed is necessary, and for this purpose a layer of filter paper (Whatman No. 54; H. Reeve Angel Co. Ltd, New Bridge Road, London, E.C.4.) or absorbent lint is placed on the perforated bottom of the funnel. The bed is then prepared by filtering a slurry of Hyflo Supercel through this support. The Hyflo forms a porous bed that can be used without further treatment. It has generally been found necessary, however, to compact the bed before use by applying manual pressure, since otherwise there is a tendency for it to contract and possibly crack during the course of filtration. When cultures or other protein-containing fluids are being filtered, a layer of liquid must always be maintained over the Hyflo bed to prevent air being taken up. This creates air pockets that not only slow the filtration rate, but also result in softening of the bed.

Although, as has already been stated, kieselguhr does not appear to adsorb substances from culture filtrates, this is not necessarily true of the filter paper or other materials used as the secondary support to the bed. In addition to the retention of particles that occurs as a result of differences in the porosity of the paper and the Hyflo bed, some adsorption may also take place and the paper soon becomes blocked. When this happens the filter bed softens, since the pressure gradient is no longer being applied to it and in such cases pads must be discarded and remade. The danger of this happening can be minimized by using a secondary support of fairly large porosity such as lint, but even when this has been used blocking up has sometimes occurred.

In large-scale filtration, funnels of 10 in. dia. on flasks of 10 litre capacity are linked to 10 litre bottles acting as reservoirs for the filtrate. These bottles can be replaced when they are full without interrupting the filtration. Since the apparatus is mainly glass and is under fairly high vacuum (50–70 cm Hg) a safety hazard exists. Buchner flasks are made sufficiently robust to withstand this vacuum, but a constant check is necessary to ensure that damage in the form of star cracks, for example, has not occurred since they were last used. Bottles, especially large ones, on the other hand, are not designed to be used under vacuum. Walls are of variable thickness, in some cases they have been found to be less than $\frac{1}{32}$ in. in parts. The danger of implosion is therefore very real and several such incidents have occurred, fortunately without injury to personnel. The hazard is particularly dangerous when pathogenic bacteria or toxic materials are being filtered. In the circumstances, since the chance of implosion cannot be completely eliminated, steps must be taken to minimize the effects. This can be done by keeping flasks and reservoir bottles under vacuum in strong containers, such as wooden boxes or prefabricated jackets made from heavy-duty webbing. These have been found to be completely satisfactory and can be stored more easily than boxes.

2. *Filter candles*

Earthenware candles of the Doulton (Doulton Industrial Porcelains Ltd, Tamworth, Staffs.) or Berkefeld (Berkefeld Filter GmbH, Celle, W. Germany) type are of limited use in the harvesting of cultures, since they are very easily clogged by the bacteria they are filtering. However, they have some application not only in the filtration of low-density cultures, but in the clarification of supernatants after centrifugation. To some extent what has been said about Buchner filtration can be applied to candles. Filtration can be aided by adding kieselguhr to the culture and by scraping the candle with a sharp tool, such as a 3×1 in. microscope slide. This, of course, results in irreparable damage to the sterilizing efficiency of the candle, and for this

reason only old discarded candles are used. A simpler method of removing impervious layers of bacteria, and one that does no damage, is to release the vacuum and allow the filtrate contained in the candle to flow out and wash off the bacteria.

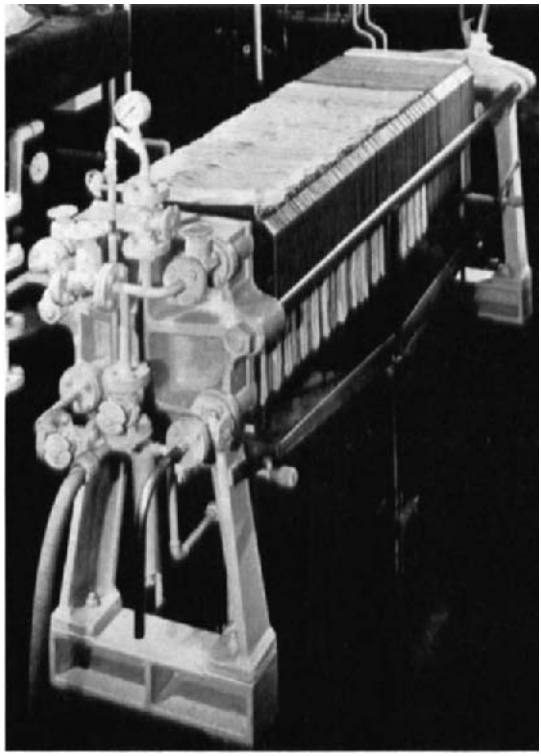


FIG. 2. Filter press. (Reproduced by permission of S. J. Johnson & Co. Ltd.)

3. *Large scale equipment*

One of the determining factors in the capacity and flow rate of filtration equipment is the surface area of the filter bed and in the design of such equipment for large-scale work, i.e., 50 litres and upwards, some means of substantially increasing this surface area must be incorporated. In so doing, manufacturers must cope with the problem of keeping the apparatus within manageable proportions. This has usually been done at the expense of the filter cake capacity.

Two pieces of equipment that exemplify the two main types that have been developed to handle large-scale filtration will be described here. In

both types the principle is the same; they provide a series of beds through which filtration can proceed rapidly.

(a) *Filter presses* (S. J. Johnson & Co. Ltd, Carpenters Road, Stratford, London E.15). These consist of several prefabricated filter plates made from such materials as heavy-duty cloth or asbestos–paper mixtures. The plates are stacked together, but kept apart from each other by separators, the whole being built up on a horizontal framework and held together by a screw clamp under sufficient pressure to permit only the slightest leakage of fluid. Figure 2 illustrates such a press. As can be seen the capacity can be increased by increasing the number of plates in the press.

As with all filtration techniques, filter aids are used to good advantage in these presses either as a coating on the filter screens or incorporated into the culture to be harvested or both.

(b) *Metafilters* (Stella-Meta Filters Ltd, Laverstoke, Hants). Whereas the filters in the equipment just described were prefabricated, those in the Metafilters are prepared before filtration by coating a manifold of porous columns with filtration medium such as kieselguhr. The columns (Fig. 3), manufactured from stainless steel, consist of a central fluted rod supporting a vertical stack of rings, each one separated from its neighbour by several

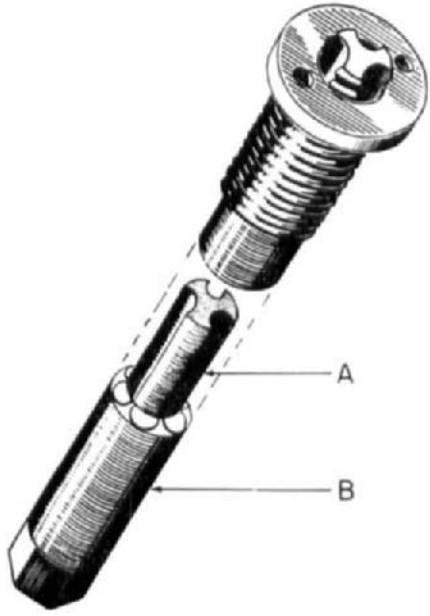


FIG. 3. Metafilter Ring Pack: A, fluted rod; B, metafilter rings. (Reproduced by permission of Stella-Metafilters Ltd.)

scallops raised above the surface of the ring. In this way liquid can pass between the rings and down the fluted core to be discharged at the base of the apparatus. A variable number of these columns are housed in a hermetically sealed and sterilizable cylinder. The columns are coated by filtering through them under pressure a slurry of kieselguhr in water, the filtrate being re-circulated until it has been clarified. The material to be filtered must now be introduced into the machine without releasing the pressure, or the prepared filter beds may be damaged.

In contrast to the filter press, the Metafilter has a limited capacity for filter cake that cannot be increased by the addition of further filters. The equipment, however, has the advantage of being sterilizable, and the filter cake can easily be obtained by back-flushing with filtrate or other fluid. The manufacturers claim that, used properly, these filters need be dismantled only rarely.

The rate of flow and efficiency of both types of filter depend on the surface area of the filter bed and the capacity of the equipment for filter-aid coating and retained material. Thus, a 16 column Metafilter with a filtering area of 6 ft^2 (5600 cm^2) after coating with a slurry containing about 1500 g of Hyflo Supercel is capable of clarifying a maximum of 300 litres of *Clostridium tetani* culture—ca. Brown's Tube 6—in 3 h using a pressure of 15 psi (1100 g/cm^2). During the filtration, the flow rate drops considerably because of the filters becoming blocked with retained organisms. On the other hand if a comparatively clear liquid, e.g., culture medium, is being filtered before sterilizing filtration, the flow rate is about 100 litres/h at a pressure of 7 psi (510 g/cm^2). It has been found that similar flow rates can be obtained with filter presses. Other factors, such as temperature and pH, particularly outside normal working limits, have some bearing on the rate of filtration because of their effect on the viscosity of the culture.

The filtrates obtained from both types of equipment described will be clarified but not sterile. Whether they should now be filtered through a sterilizing press will depend on the further treatment they are to receive.

III. CENTRIFUGATION

In this Section centrifugation will be treated from a practical viewpoint. For the mathematical theory of centrifugal sedimentation readers are referred to papers by Ambler (1959) and Hayter (1962).

Although filtration is a satisfactory method for producing clarified culture filtrates on a laboratory or a production scale, it suffers from the disadvantage that the bacteria retained by the filter bed are admixed with considerable quantities of filter aid from which they cannot readily be separated. Since, as has been shown earlier, filter aids are a practical necessity, filtration cannot

therefore be recommended for collecting bacteria from cultures, unless of course admixture of the bacteria with filter aid can be tolerated. Centrifugation on the other hand offers a method for obtaining both the culture fluid and the organisms unadulterated by the presence of extraneous material. Generally speaking the separation obtained by centrifugation is not so complete as that obtained by filtration and supernatants invariably contain a certain proportion of organisms, albeit negligible when compared to the numbers originally present in the culture, but often sufficient to require secondary clarification by filtration. During harvesting, centrifuges are normally run at their maximum permitted speed and any attempt to increase the efficiency of separation must involve increasing the time of centrifugation. For practical as well as economic reasons, this is not always possible, particularly when large production batches are to be harvested.

Centrifuges fall into two categories: those which fractionate a continuous flow of material into sediment, collected in the bowl, and supernatant that is discharged and those which operate non-continuously, being reloaded between runs with the culture to be harvested. Comprehensive ranges of centrifuges are available with capacities of from less than 10 ml to 6 litres for the non-continuous type, see for example the lists of Measuring & Scientific Equipment Ltd, Crawley, Sussex (MSE), and from about 200 ml to many litres for the continuous type—these capacities being the volume of sediment capable of being collected per run. For some time now there has been a tendency on the part of manufacturers to provide multipurpose machines, usually refrigerated, which are capable of using a range of angle and swing-out heads, and in some cases able to take continuously operating rotors. Although it will be some considerable time before relative centrifugal forces (RCF) similar to those obtained with analytical centrifuges can be achieved in preparative machines *of large capacity* it is commendable that a centrifuge with a maximum capacity of 3,300 ml can now be obtained which is capable of developing 33,000 ***g*** (Lourdes Instrument Corp., 656 Montauk Ave, Brooklyn, New York 11208).

A. Non-continuous centrifuges

Non-continuous centrifuges can handle volumes of up to 6 litres per run and are therefore not suited for large-scale harvesting. They are, however, particularly useful when it is necessary to centrifuge cultures aseptically since sealed containers, such as polypropylene bottles, that are autoclavable can be used to hold the culture during the run.

Many types of non-continuous centrifuges are available, and there is no reason to believe that one model is any better or worse than another. Apart from the multipurpose machines already mentioned, refrigeration is usually available for other large capacity instruments.

1. *Angle rotors*

Two kinds of rotor can be used in non-continuous centrifuges, viz. angle and swing out. In angle centrifuges tubes or bottles containing the material to be centrifuged are held in a fixed position at an angle to the vertical, the base of the container being nearer the circumference than the head. This type of centrifuge is often preferred because of its greater sedimenting efficiency. The rotors offer little resistance to the surrounding air, since they are streamlined and high rotational speeds can be obtained. However, they suffer from the disadvantage that during deceleration turbulence occurring in the supernatant is sufficient to cause disturbance to some types of sediment, even when these have been well compacted. Tubes and bottles must be matched and balanced before centrifuging and it is worth remembering that the surface of the liquid, horizontal when at rest becomes vertical at fairly low centrifugal speeds. Since the angle of the container is fixed the meniscus rises towards the lip of the vessel on the circumferencial side during acceleration. It is essential therefore to make sure that the liquid does not overflow into the bowl by (*a*) using the containers at about 2/3 their capacity and (*b*) sealing them with a liquid-tight screwcap.

2. *Swing-out rotors*

This type of rotor, because of its shape, causes considerable turbulence in the surrounding air and the greater frictional resistance results in slower speeds than those obtained with angle rotors. This is balanced to some extent by the larger diameter of these heads when they are in motion. In this type of rotor, pots that hold the liquid containers pivot on their horizontal axis so that when the machine is in motion the longitudinal axis of the pot assumes a horizontal position. The surface of the fluid thus remains in the same relative position to the vessels and these can be completely filled without fear of them losing their contents. The sediment is formed as a layer over the bottom of the container and suffers no disturbance when the centrifuge is decelerating.

3. *Abuses and precautions*

When one considers the kinetic energy of a centrifuge when running fully loaded it is surprising but true that these machines are abused more than any other piece of laboratory equipment. It is a tribute to manufacturers that their equipment stands up to misuse so well even to the extent of surviving runs with unbalanced tubes which cause the machine to "walk" from one side of the room to the other. Whatever type of rotor is used it must always be balanced about the centre of rotation. Thus a tube or bottle containing the fluid to be centrifuged must have opposite it on the rotor a similar container with the same weight of fluid of similar specific gravity.

This procedure is regarded by most laboratory workers as tedious and many rough and ready methods have been used to deal with it. Perhaps the most widely practised habit is to achieve balance in pairs of pots containing the culture bottles by adding water or any other liquid at hand into the lighter of the two pots until they balance. This technique is to be deplored since it creates dirty working conditions and creates serious microbiological contamination hazards. It usually results in the eventual corrosion of the pots, since it is unusual for the liquid to be removed and the pots dried after a run. An even more deplorable habit, although one not practised so frequently, is to balance bottles against others containing such dense materials as lead shot. Persistent use of these methods is likely to shorten the life of the instrument because although the rotor is balanced when at rest, the fact that the balanced pots have different centres of gravity means that they are unbalanced when spinning. In this respect it has been found on occasion that pairs of balanced tubes, one of which contains water, become unbalanced during a run because of the shift in the centre of gravity of the other as the organisms sediment.

Another practice that has been observed is that of keeping the whole complement of lining tubes or pots in the rotor head—probably because this is the most convenient place for them—when using the centrifuge to spin one pair of bottles. There is a tendency to do this particularly on angle rotors. When these tubes are kept as matched pairs there is no danger, but in laboratories where the techniques described above are used for balancing, the centrifuge may be under considerable chronic strain.

In addition to these abuses other more obvious ones are practised, such as running the machine too fast. Manufacturers are well aware of these, and manuals supplied with centrifuges fully describe their limitations. Although these usually allow for a wide margin of error, it is advisable to stay within the limits imposed, since not to do so can be dangerous and expensive.

B. Continuous centrifuges

Figure 4 illustrates the general principle used in all models of continuous centrifuges. Fluid entering the vessel is clarified by the centrifugal force created by the motion of the rotor, the sediment being forced to the outer wall and held there while the supernatant is expelled by displacement with more suspension entering the chamber. Some method of preventing the mixing of clarified supernatant with unseparated suspension is necessary, and in Fig. 4 this is done by means of a rudimentary partition. In practice more elaborate designs of rotor are found to be essential not only to prevent internal mixing during runs, but also to increase the efficiency of separation,

without necessarily increasing the speed of rotation. It is clear from what has been stated that the ability of these centrifuges to cope with the separation of large quantities of suspension is determined by the space available in the rotor for holding the sediment. Again, the efficiency of separation will decrease somewhat during a run since the effective diameter of the rotor is decreased by the deposition of sediment on the wall.

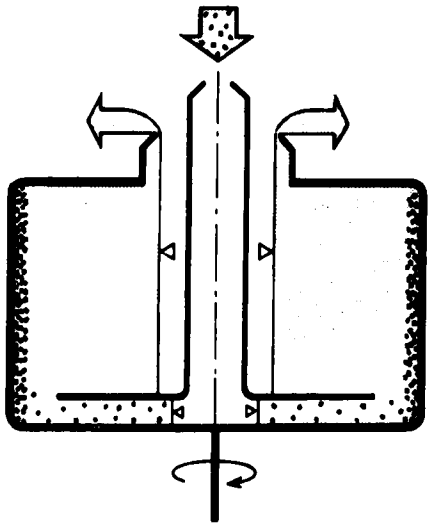


FIG. 4. Diagrammatic representation of simple continuous rotor, showing the direction of liquid flow. (Reproduced by permission of Alfa-Laval Co. Ltd.)

1. *Laboratory models*

Laboratory-scale continuous-flow rotors can be used in the large high-speed machines at present available. In general they consist of a stainless-steel bowl containing a well-fitting polyethylene or polypropylene liner in which the sediment collects. The design of these liners is of importance as far as the efficiency of the rotor is concerned, since it not only determines how effectively clarified supernatant can be prevented from mixing with incoming suspension but it also has some bearing on the rate of sedimentation, since this can be increased by any procedure that shortens the path length over which particles must travel before settling. Unfortunately, however, it is usually the case that in producing a high-efficiency rotor some capacity must be sacrificed. Because of this, some manufacturers have provided liners of different design, having the properties of low efficiency/high capacity and the reverse. The former are perfectly adequate for the rapid separation of many types of suspension and have the advantage of

holding as much as 50% more precipitate than their more efficient counterpart. Rotors of the type described have capacities from 100 to 1800 ml and can be used in refrigerated machines capable of producing RCFs of up to 28,000*g*.

Continuous-flow centrifuges may suffer from two major disadvantages that could preclude their use for certain purposes. Firstly, with some models, supernatants are expelled partly as a foam owing to the large amounts of air taken into the rotor during the run. Not only does this create a problem in the disposal of this foam, but it may be deleterious to certain products, particularly proteins, in the supernatant. Secondly, aerosol may be produced during separation, and this could be extremely hazardous when bacterial cultures are being centrifuged, since it could lead to widespread cross-contamination, not to mention the danger to personnel if the cultures are pathogenic. On the whole this hazard can be minimized or eliminated by the use of safety seals in the rotor and with most of the modern rotors properly assembled and used it need not be a problem.

Because of the high speed necessary for running these rotors efficiently, large amounts of heat are evolved. Refrigeration built into the machine is adequate to cope with this, but where centrifuges are not refrigerated it is advisable to operate in a cold room. It may be noted here that refrigeration, while it prevents overheating, does little to lower the temperature of the rotor below that of the suspension being centrifuged and the temperature of expelled supernatant is usually similar to that of the material entering.

Certain models of continuous flow rotors are capable of being sterilized by autoclaving, and sterility can be maintained during runs. Although it may not be a great advantage to be able to obtain supernatants and sediments aseptically at this stage it allows the rotor to be quickly and effectively sterilized after use and is more convenient than the chemical sterilization necessary for the disinfection of non-autoclavable rotors.

In Table I several models of laboratory-scale continuous centrifuges are compared. Throughputs have not been listed, since these depend to a great extent on the material being centrifuged. In general their use is limited by the capacity of the receptacle, and for separating large quantities of culture it may be necessary to stop the centrifuge several times in order to empty the bowl. This is of course obviated if there is some means of continuously removing separated fractions during a run, as in the Westfalia model. Recently Judson *et al.* (1968) described a closed centrifuge with which it is possible to separate a continuous flow of suspension at a constant centrifugal force and at the same time collect all the separated components. Although the rotor was developed for the separation of blood components, the authors felt that it had many applications, including the continuous collection of bacteria and supernatant from culture fluids.

TABLE I

Comparison of several types of laboratory-scale continuous centrifuges

	Description	Bowl capacity, ml	Speed, rev/min.	RCF × *g*	Refrigeration	Rotor
De Laval	1225	200	$9{\cdot}1 \times 10^3$	—	No	Multiple plate
Lourdes	Betafuge	100–1800	13×10^3–18×10^3	20×10^3–$27{\cdot}5 \times 10^3$	Yes	Single compartment plastic bag sludge holder
MSE	Superspeed 8	200–300	18×10^3	21×10^3	Yes	Single compartment polypropylene liner
Sorvall†	KSB : R	250–350	20×10^3	48×10^3	Yes	8 Tube type
Sharples	1A Lab. Super Centrifuge	. .	23×10^3	13×10^3	No	Spindle motor driven
Westfalia‡	LWA 205	Continuous desludging	12×10^3	$7{\cdot}5 \times 10^3$	No	Multiple plate

† Ivan Sorvall Inc., Norwalk, Conn.

‡ Westfalia Separator (Great Britain) Ltd, Salisbury House, London Wall, London E.C.2.

2. *Large-scale continuous-flow centrifuges*

In order to obtain the high throughputs of supernatant that are necessary when volumes of 500 litres and above are to be separated, two types of centrifuge have been designed. The first, typified by the Sharples (Pennsalt Ltd, Camberley, Surrey), relies on sheer speed of rotation of a long narrow spindlelike rotor to achieve this. The ratio of bowl length to bowl diameter ranges from 4 to 8 and speeds vary from 15,000 to 50,000 rpm, corresponding to centrifugal forces of 12,000 to 65,000 ***g***. In these machines, the sediment is deposited on the wall of the rotor. Several methods may by used for ensuring its quantitative recovery. For example, a slip of fixed X-ray film may be inserted into the rotor before use so that it covers the the entire internal surface. After the run it is a simple matter to pull out the film and unroll it with the harvest. The rotor is left completely clean and the harvest easily accessible. Another method has been described by Vallet (1960) in which sediments are suspended or dissolved without first removing them from the bowl. More recent models of Sharples have been developed from which the heavy bacterial slurry is continuously expelled during the run. In addition they are hermetically sealed in order to prevent the uptake air which would result in the formation of foams and aerosol.

The second type of centrifuge and one that has been used extensively for clarifying cultures relies for its efficiency on the introduction of plates or baffles into the rotor. This technique, which has been exploited particularly by AB Separator of Sweden, is designed to reduce the distance through which particles have to travel before settling, thus allowing good clarification at relatively low speeds. An example of this kind of instrument is the De Laval centrifuge (Alfa-Laval Co. Ltd, Great West Road, Middlesex) (Fig. 5). The rotor of the centrifuge consists mainly of a stack of numerous stainless-steel conical discs ; the liquid being separated flows outward from the central hollow spindle, then upward and inward at an angle of 45° to the axis of rotation. The sediment moves outward in the gravitational field until it impinges on the underside of the conical discs. It continues its outward motion down the discs and eventually accumulates on the wall of the rotor.

Machines of the type illustrated have been used extensively for clarifying cultures of the pathogenic clostridia. At a maximum speed of 7500 rpm a throughput of about 5 litres/min can be obtained, the supernatant being passed from the centrifuge through a bacteriological filter. The rotor is hermetically sealed during operation and this has the advantage of permitting centrifugation of potentially dangerous cultures without fear of aerosol or foam formation.

For harvesting cultures of pathogenic bacteria for vaccine production, the cultures are fed into the centrifuge from holding vessels, either by air

pressure or by pump. The emerging supernatant fluid passes via a sight glass flowmeter through a filter press (e.g., British Filters Ltd, Maidenhead, Berks, England) containing clarifying and sterilizing asbestos–cellulose filter mats (e.g., Carlson-Ford, Ashton-under-Lyme, Staffs, England) into

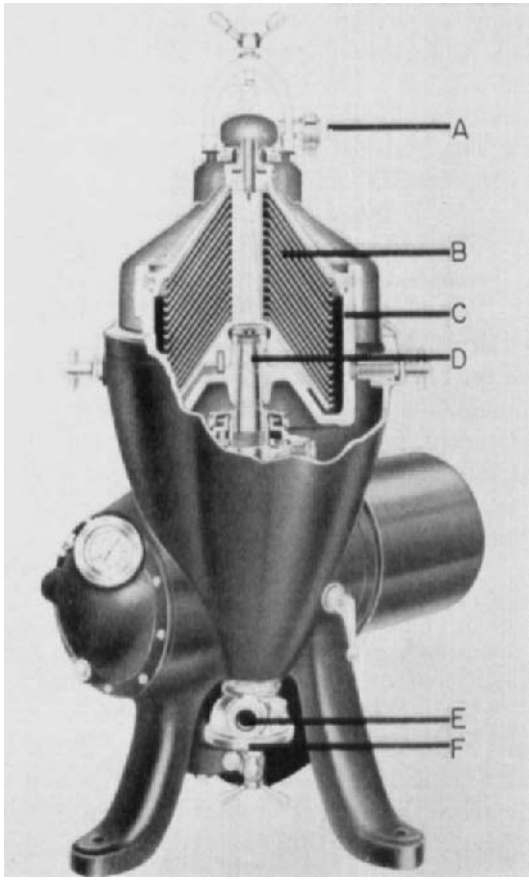


FIG. 5. Sectional elevation of the BRH 409×30H De Laval Separator: A, liquid discharge; B, intermediate discs; C, bowl; D, hollow spindle through which the liquid is fed into the bowl; E, liquid inlet, F, built-in feed pump.

sterile containers. The limiting factor in the process regarding throughput is the pressure that the filter mats can sustain. The manufacturers recommend about 5 psi (350 g/cm^2). At this level it has been found possible to harvest tetanus toxoid at the rate of 4 litres/min or *Cl. perfringens* Type D cultures at 2 litres/min. These throughputs will be modified as the filter

pads become blocked, and for this reason care is taken to ensure that the material leaving the centrifuge is as clear as is practically possible. This is done by re-circulating the supernatant fluid through the centrifuge until it can be seen to be clear in the sight-glass flowmeter. The vessels, pump and centrifuge are assembled 2 or more days before use and completely filled with 2% (v/v) formalin (40% formaldehyde solution B.P.), air pockets being eliminated by starting the centrifuge and re-circulating the formalin for a short period. Before the equipment is to be used the formalin is flushed out and replaced with air entering through a sterilizing filter. If necessary the apparatus can be washed through with sterile water. The filter press is steam-sterilized. The bacterial culture is pumped into the reservoir and thence into the centrifuge. The supernatant is re-circulated through the centrifuge until it is seen to be sufficiently clear by examining it as it passes through the flowmeter. It is then fed from the centrifuge through the bacteriological filter and collected in suitable containers.

After use the whole of the equipment is again sterilized chemically with formalin, then completely dismantled and cleaned.

The design of the centrifuge makes it suitable for collecting supernatant rather than sediment. The bowl capacity for bacterial sludge is about 5 litres and this limits the centrifuge to separating volumes of culture resulting in this quantity of sediment. A more recent model has been developed that allows for the periodic removal of sludge while the machine is running, so that continuous operation is possible over long periods.

IV. OTHER HARVESTING PROCEDURES

Apart from filtration and centrifugation, several techniques have been developed that may be found to have useful application in the harvesting of particular types of culture. In general these methods have been used for concentrating and recovering organisms rather than culture filtrates. They have the advantage over other methods in that large-scale harvesting can be undertaken without the need for expensive equipment.

A. Co-precipitation

This is a fairly old and well-established procedure, having its origins probably in the use of silica gel or egg albumin for settling coffee grounds. Many compounds may be used for this purpose, the precipitation of the bacteria being due to their coagulation in the presence of oppositely charged micellar particles and their subsequent rapid deposition. In recent years a considerable amount of developmental research has gone into the use of co-precipitation agents for clarifying sewage and industrial waste and purifying water, and some of the materials used have been applied successfully to the harvesting of bacteria.

Nakamura (1961) described the use of various inorganic flocculating agents that he found to be effective in the precipitation of yeasts, bacteria, algae, etc. These agents were the sulphates, chlorides or insoluble hydroxides of several polyvalent cations, and their mode of action appeared to be due either to neutralization of the anionic charge on the surface of the organism or to adsorption of the organism on the surface of insoluble hydroxides. Concentrations as low as 0·0005 M were effective, the efficacy presumably being dependent on the valency of the metals; Ti^{4+} was effective as this level, whereas with Ca^{2+} 0·2 M was necessary.

Extensive studies on the use of polyelectrolytes for flocculating clays and other materials in water by workers at the Massachusetts Institute of Technology have led to some understanding of the reaction mechanisms involved. From this work, Michaels (1954) and Michaels and Morelos (1955) were able to elucidate the type of chemical structure likely to be necessary for promoting optimum flocculation. In general, while some polycationic polymers (Johnson, 1956), polyanionic and non-ionic water-soluble polymers and gums (Quastel, 1952) were effective flocculants, others, such as sodium polymethacrylate or sodium polystyrenesulphonate were not. Ruehrwein and Ward (1952) stated that polyionic polymers must also carry nonionic polar groups, for example, hydroxyl or amide. According to these authors, flocculation of polymer with anionic particles occurs because of anion interchange so that carboxyl ions on the polymer replace adsorbed anions (presumably OH^-) on the micellar surface. Two other possible mechanisms were proposed by Michaels (1955): (a) that adsorption occurs by hydrogen bonding between the suspended solid and hydroxyl, amide or un-ionized carboxyl groups on the polymer, the function of the ionic groups being to extend the polymer chain; and (b) that polyvalent cations (e.g., Ca^{2+}) present on the solid act as electrostatic bridges between the solid surface and polyanions. It is likely that all three possibilities are factors in co-precipitation. On the other hand, some electrostatic repulsion and, therefore inhibition of co-precipitation must occur between electronegative micellar surfaces and ionized carboxyl groups, and one would expect flocculation to be favoured by lowering of pH. Although polyanions or polycations are in general poor flocculants, so too are the water-soluble non-ionic polymers, and from what has been said it would appear that both non-ionic and ionic groups are essential. Thus most linear polyhydroxy or polyamide polymers, for example, polyvinyl alcohol or polyvinyl cyclohexanetriol can be converted into good flocculants by the introduction of, or copolymerization with, controlled concentrations of ionic constituents, for example, carboxyl, sulphate, sulphonate, phosphate, xanthate, primary, secondary, tertiary or arylamino-groups and quaternary ammonium salts (Michaels, 1954). Such compounds are effective as coagulants in con-

centrations as low at 0·01% (w/w) of the solid in suspension being flocculated.

Although the work described above was with clay suspensions it is obviously relevant to problems associated with co-precipitation of micro-organisms.

In our experience the technique of co-precipitation, using inorganic or polymeric organic compounds has been found to be reasonably efficient for separating organisms from cultures. The rate of deposition is fairly dependent on the type of material used, but even so the treated culture must be allowed to stand for several hours before a clear supernatant can be decanted. This time factor may be unacceptable if the organisms are unstable.

B. Foam separation

This method has been used by several investigators for collecting organisms from culture, e.g., Boyles and Lincoln (1959), Gaudin *et al.* (1962a) and Bretz *et al.* (1966). As in the technique of co-precipitation described above, the organisms are agglutinated by a molecule of the opposite charge, such as a hydrophobic colloid (Grieves and Bhattacharyya, 1965). Instead of the suspension then sedimenting, however, it is brought to the surface by air sparged into the culture, and is entrapped in foam, which is continuously being formed. After collection, the foam can be collapsed and the organisms recovered as a concentrated suspension. By the use of the cationic surface-active agent ethylhexadecyldimethylammonium bromide, Grieves and Wang (1967) studied the foam separation of six species of bacteria, viz, *Escherichia coli*, *Proteus vulgaris*, *Serratia marcescens*, *Pseudomonas fluorescens*, *Bacillus cereus* and *Bacillus subtilis* var. *niger*. In contrast to the agglutinating effect of hydrophobic colloids, these authors were unable to show any evidence of this with the surfactant. In addition, foam volumes were much smaller when the surfactant was used, and, depending on the species, concentrations of 30–3000 fold were obtained with yields for organisms in the collapsed foam of between 75 and 100%. The apparatus used for this technique was simple and inexpensive (Grieves and Wang, 1966), and consisted of a Pyrex cylinder 9·6 cm dia. × 82 cm high. Filtered nitrogen gas was sparged into the cylinder through twin sintered-glass aerators of 50 μm porosity. The foam formed was removed continuously from a port located about 20 cm above the initial bacterial suspension level, i.e., about 35 cm above the base of the cylinder. Refinements in the technique were made by Rubin *et al.* (1966) by the use of a "frother", ethanol, to refine the size of gas bubble produced by the sparger and a "flocculant", such as alum, to coagulate the bacteria before collection. Gaudin *et al.* (1962b) investigated the effect of NaCl and other inorganic salts on the flotation of *E. coli*, and found these to improve the technique considerably.

V. FURTHER TREATMENT OF HARVESTED PRODUCTS

It is usually necessary, after harvesting, to submit the required portion of the culture to further treatment in order to be able to store it for prolonged periods in a stable condition. With respect to the bacteria themselves, suitable preservation can be achieved by several methods, all of which cause considerable, if not complete loss of viability of vegetative forms. Soluble products of growth, on the other hand have still to be concentrated in order to remove the bulk of unwanted fluid. The concentrates can then be stored either as sterile or preserved solutions, or dried. Since these procedures are an essential part of harvesting some consideration will now be given to them.

A. Preservation of organisms

The preservation of viability in dried cultures is adequately discussed by Lapage *et al.* (this Series, Vol. 3A). Here we are concerned with stabilizing the harvested mass of organisms. Unless the organisms in question are spore forms, it must be accepted that, whatever the treatment, some loss of viability is unavoidable, even when the organisms are stored simply as a suspension in culture fluid or isotonic buffer.

As well as suffering loss of viability, organisms in suspension tend to disintegrate because of the presence of autolytic enzymes within them. Steps must therefore be taken to prevent this if they are required intact and the methods used will depend upon the purpose for which the organisms are being stored. Heating or formalin treatment are the agents most commonly used for killing bacteria and these normally prevent subsequent lysis. As a general rule, one would advocate using the minimum quantities that are found to be adequate for the purpose, since other properties and constituents are progressively destroyed by such treatments.

The maintenance of organisms in the dry state is perhaps the most satisfactory way of achieving long-term stability. Several methods of drying may be used, the simplest being to dehydrate the harvested mass by storage in a desiccator under vacuum in the presence of P_2O_5 or other drying agent. By this method about 200 g of dry product may be obtained in 1 week by desiccating with 3 changes of 100 g of P_2O_5. Other techniques include the replacement of water from suspensions by several washes with acetone, the latter being finally removed by drying at room temperature. Deep freezing of bacterial pastes at temperatures less than $-20°C$ is successful in preserving enzymes, antigens and even viability. Repeated thawing and freezing can be deleterious, and it may be worthwhile when storing organisms in this state to do so in small aliquots, so that after thawing any unused portions may be discarded.

Freeze-drying is another technique that has been used with considerable

success and is perhaps the mildest of all procedures used for preserving bacteria. The organisms after suitable pretreatment, such as washing, are suspended in buffer and quickly frozen with a mixture of ethanol or acetone and solid CO_2. The application of a high vacuum—200 microns Hg or less—to the frozen mass results in the removal of water by sublimation, leaving the bacteria and buffer salts in the dry state. Many monographs have been written on the subject of freeze drying, and of these the reader is referred in particular to those by Flosdorf (1949), Harris (1954), Meryman (1966) and Fry (1966).

B. Concentration and preservation of soluble products of growth

Products, such as antigens, or pharmacologically active agents, such as toxins, are normally present in culture filtrates in relatively low concentration. It is necessary to concentrate them, therefore, not only to remove large quantities of unwanted fluid but to have material of sufficient potency for subsequent processing. Two methods that have found widespread use in the concentration of macromolecular products, such as proteins, are salt precipitation (salting out) and ultrafiltration.

1. *Salt precipitation*

The theoretical aspects of salting out of proteins are discussed by Cohn and Ferry (1943) and practical considerations by Taylor (1953). In simple terms, salting out consists of the removal from protein molecules of their surrounding shells of water resulting in the aggregation of these molecules to form micelles of increasing size, which eventually precipitate. The ability of salts to effect salting out of proteins depends on their solubility and on the ionic strength of their concentrated solutions. Salts of di- and trivalent anions, such as sulphate or phosphate, are more efficient than those of monovalent anions, such as chlorides. It is not surprising, therefore, that the salts that have been used most commonly are those that combine high solubility with multivalency, i.e., the phosphates of sodium and potassium and the sulphates, particularly those of ammonia and sodium. Of these, the phosphates, being comparatively expensive, are normally restricted to small-scale concentration and fractionation, whereas sodium sulphate, because of its relatively low solubility can generally only be used effectively at temperatures of 20°C and above. The remaining salt, ammonium sulphate, is cheap and very soluble and has found widespread use in the concentration of culture filtrates. Unlike the phosphates, however, it is not a buffer, and on prolonged storage solutions of the salt may become acid because of loss of ammonia. This can be corrected by the addition of a small amount of sodium bicarbonate to the solution.

Most laboratories engaged in this type of work hold stocks of saturated

ammonium sulphate that can be used when required, and details of precipitation procedures often cite concentrations in terms of percentage saturation. While the absolute salt concentration of saturated ammonium sulphate varies considerably with the ambient temperature, it may be regarded as being in the region of 55% w/v or approximately 4M. This method of using the salt is convenient, but it suffers from the disadvantage of increasing the volume to be filtered; for example, precipitation at 50% saturation doubles the volume. When precipitating large bulks of material it is usual to use solid ammonium sulphate.

Precipitation of protein by ammonium sulphate proceeds fairly rapidly and is complete in less than 1 h. After this time, the precipitate can be collected, but it may be advantageous to allow the mixture to stand longer, so that the precipitate can coacervate, thus assisting its collection. This is best done at room temperature or higher. Storage in the cold should be avoided, not only because coacervation proceeds much more slowly but also because many proteins are more soluble in the cold than at room temperature. In certain cases, however, it may be necessary to work in the cold in order to minimize the destruction of unstable products. As a general rule, large variations in temperature during the precipitation and harvesting stages should be avoided, since this could lead to a variable product both quantitatively and qualitatively.

Collection of precipitates can be done either by filtration or by centrifugation. Filtration through a Buchner funnel with a filter aid is satisfactory, since the latter can be removed very easily when the precipitate has been collected and re-dissolved. Centrifugation is often used when the volumes being handled are fairly small and when the presence of filter aid in the precipitate is inconvenient. Coacervation of the precipitate is often an essential step in the procedure if the protein is to be collected by centrifugation, since otherwise the difference in density between the precipitate and the salt solution may not be large enough to allow satisfactory sedimentation.

During precipitation with salt, in particular with ammonium sulphate, most of the dissolved oxygen present in the solution is released. Bubbles that form are entrained in the precipitate, and raise it to the surface where it can be skimmed off. This is made use of in the collection of precipitates from large volumes of precipitated culture filtrate, and the completeness of separation can be further facilitated by sparging the mixture with air or by incorporating air during the precipitation by the formation of a vortex. The precipitate that rises to the surface coagulates together to form a fairly solid crust that can be easily skimmed off. In this way as much as 80% of the material can be collected without having recourse to filtration or other laborious techniques.

Concentrates obtained by salting out can be stored as sterile or preserved solutions or can be freeze-dried. Whatever method is adopted, it is necessary to remove the considerable quantities of precipitating salt that are present in the preparations. In the past, one of the procedures that was adopted for the further treatment of concentrate, particularly that obtained by collecting precipitate crusts, as described above, was to dry it in a desiccator over P_2O_5, pulverize the solid in a mortar and float the powder in a beaker of chloroform. Salts that were present in the powder sank to the bottom of the chloroform, leaving the protein on the surface. This method has been largely superseded by dialysis to remove salt, the concentrated solution then being freeze dried. With regard to concentrates obtained by precipitation with ammonium sulphate, a word of caution is necessary. If these contain residual quantities of ammonium sulphate, and have not been buffered, freeze drying will result in the removal of ammonia and the dried material will be acidic. It is always necessary when such concentrates are to be dried either to dialyse against a neutral pH buffer or to add a buffer to the solution before drying.

2. *Ultrafiltration*

Macromolecules, such as proteins and carbohydrates, can be concentrated by the forced filtration of fluid and small-molecular-weight compounds through semi-permeable membranes. The technique has many applications where the material to be concentrated is particularly labile, and although it is usually regarded as a fairly slow procedure, by the use of sufficient surface area very large volumes indeed can be concentrated rapidly. Any semi-permeable membrane can in theory be used, provided it has sufficient strength to withstand the pressure gradient applied during the filtration or provided it can be supported on a sufficiently strong porous structure. The membranes most commonly used are those fabricated from cellulose derivatives, particularly the acetate and nitrate. Such membranes can be used with or without support. Cellophane tubing, for example, can act as an ultrafilter under fairly high vacuum (50–70 cm Hg) provided that air pockets are carefully excluded from the bag. Cellulose nitrate thimbles (e.g., Membranfiltergesellschaft GmbH, Göttingen, W. Germany), which are more strongly constructed can be obtained for small-scale ultrafiltration. These can be used repeatedly. Less rigid membranes that have larger average pore size, but still exclude macromolecules ultrafilter more rapidly. These do not have the strength to withstand pressure differences and must be supported either on filter paper or on porcelain candles.

At the Wellcome Research Laboratories considerable use has been made of cellulose nitrate membranes formed over filter candles. The candles used, either Berkefeld or Doulton, or other similar manufacture, are

sterilizing candles which have been rejected because they are no longer effective.

The cellulose nitrate (collodion) is used as a 7% (w/v) solution in glacial acetic acid.

Preparation of ultrafilters. Candles used for the preparation of ultrafilters are stored in an incubator, so that when they are required they will be perfectly dry. A tight fitting rubber band, about 1 in. wide, is used to cover the cemented joint between the candle and its metal cap. The prepared candle is now immersed completely, except for its threaded end, in a bath of collodion, removed and the excess of collodion solution is drained off. Care must be taken to ensure that too much draining does not occur, or not only will the final membrane be exceedingly thin, but it may have dried in parts. The candle with its coating is now submerged in running tap water for several minutes to harden the membrane, after which time it may be removed and examined for flaws. The membrane should be uniformly thick and should cover the whole of the filtering area of the candle. The presence of small air bubbles in the membrane is not regarded as troublesome, since, if they break down under pressure or vacuum the amount of material they allow to pass through is generally negligible; and in any case they soon become blocked during the course of ultrafiltration.

After the membrane has been examined, the candles are returned to the cold-water bath and washed with running water to remove the acetic acid in which the cellulose nitrate was dissolved. For the same reason the membrane and candle are flushed through with water by the application of vacuum. The washing procedure generally takes about 30 min, and after this time the candle is ready for use. The collodion membrane is fragile and particular care must be taken with it. It may be grasped carefully but firmly so that a piece of rubber tubing can be pushed over the end of the candle. The ultrafilter is now immersed in the material to be concentrated and filtration is initiated by the application of vacuum (50–70 cm Hg).

By the use of different concentrations of cellulose nitrate in acetic acid, membranes of different average porosities can be prepared. These will of course determine the minimum size of retained macromolecules and in addition will determine the rate of filtration. A concentration of 7% (w/v) has been found to be a practical minimum, since below this value the membranes obtained tend to be fragile and difficult to prepare. On the other hand membranes prepared from higher concentrations are thicker and the filtration rate correspondingly slower.

During the ultrafiltration of culture filtrates or other solutions, the flow rate will decrease considerably as the fluid becomes more concentrated and as a build up of retained material over the surface of the membrane

takes place. This can be minimized by the use of some means of continuous agitation of the fluid in order to attempt to keep the membrane surface clean. Material that has coated the surface of the ultrafilter can be effectively removed by releasing the vacuum for a short period of time, the reverse flow of filtrate that occurs being sufficient to wash off the skin which has built up.

Ultrafiltration has been used for large-scale concentration of tetanus toxoid, and for this purpose a battery of thirty six 10×2 in. candles is used. These ultrafilters are immersed in a tank of 50 litre capacity stirred at three points by stirrers connected by pulley to a common motor. The tank is filled from a 300 litre holding vessel by a pump controlled by a relay activated by constant-level electrodes. The filtrate is collected in another tank of the same dimensions kept under vacuum. With this equipment 1000 litres of tetanus toxoid can be concentrated per week.

This may be regarded as a slow process compared with other methods of concentration. It is, however, quite innocuous to the constituents being concentrated and once it is in operation it requires only minimal attention. Filtration can continue for weeks and is usually terminated because, despite the constant stirring, the candles eventually become irreversibly blocked. Nevertheless during this time, about two weeks, 2000 litres will have been concentrated to 30 litres. With a replacement set of candles the equipment can be reassembled in less than a day.

After ultrafiltration the concentrates may be stored sterile or they may be freeze dried.

3. *Concentration by dialysis*

Culture fluids may be concentrated by dialysing against a solution of a higher osmotic pressure. This can be considered as a type of ultrafiltration in which the force applied is osmotic pressure. Although concentrated salts or other small molecules may be used for this purpose, these diffuse through the dialysis membrane into the material being concentrated. Not only is this inconvenient but it leads to a fairly rapid equilibration of the system.

In recent years several high-molecular-weight hydrophilic polymers have been used successfully for concentrating by dialysis. The advantage of such materials as carboxymethylcellulose (Palmstierna, 1960) dextran (Schneider and Wallenius, 1951; Rossi and Schneider, 1953), polyvinyl pyrolidine and polyethylene glycol (Kohn, 1961) is that although they are high-molecular-weight products they exert considerable osmotic pressure because of their numerous hydrophilic groups. Water is therefore rapidly removed from inside the dialysis sac in order to balance osmotic pressures, and the protein solution is thereby concentrated. This is so effective that the material may become concentrated to dryness within the dialysis sac.

This technique is capable of being scaled up for concentrating large volumes, and for this purpose polyethylene glycol—Carbowax 20M (Union Carbide Inc., 270 Park Ave., New York, N.Y., U.S.A.)—is generally used since it is comparatively inexpensive. The material to be concentrated is filled into dialysis tubing and this is then immersed in a bath either of the solid polymer or of an aqueous slurry. During concentration the bag is kept filled from a reservoir. After the material has been concentrated, the polyethylene glycol will contain the water that has diffused from the bag. This can be removed by drying the wax in an incubator in order to regenerate the polymer.

These polymers can be obtained in several molecular-weight ranges, and it is usual to choose a size that will not diffuse through the membrane. Nevertheless, it is not unlikely that whatever the average molecular weight is, there will be some fraction of sufficiently small size to pass through the membrane and contaminate the product. Since the concentration is due to the osmotic-pressure differential, these low molecular-weight fractions will be actively transported through the membrane by the pressure gradient. Similarly the normal diffusion of small molecules other than water from inside the membrane will be minimized by the pressure gradient, with the result that they will become concentrated with the macromolecules within the sac. With buffered solutions this may lead to saturation and crystallization of salts.

Another technique that may be useful for concentrating fluids on a small scale is to absorb water and low-molecular-weight materials by the addition of dry molecular-sieve gels of low exclusion limits, for example Sephadex G-10 (Pharmacia, Uppsala, Sweden) or Biogel P2 (Bio-Rad Laboratories, Richmond, Calif.). Although large losses in concentrated material may occur because of the difficulty of separating it quantitatively from the gel, this can be minimized by drawing it off under vacuum on a small Buchner.

VI. CONCLUSIONS

In selecting a particular technique from the comprehensive range that has been described in the previous Sections, several factors must be considered. Of prime importance is the question of whether the organisms, the culture fluid or both are required since the best technique used for harvesting of one of these does so to the detriment of the other. In addition, other factors must be borne in mind, and these are dependent on the purpose to which the primary products are to be put.

Filtration is a major technique in harvesting, but is almost impracticable without the use of a filter aid. Before ruling it out, however, one must look to the primary product and its subsequent treatment to determine whether

the presence of filter aid admixed with the organisms can be tolerated. Included in the purpose to which organisms can conceivably be put are the provision of soluble antigens, enzymes and other cell constituents by lysis, disintegration or extraction, the provision of whole cells for serological studies, etc., and the provision of sub-cellular particles, e. g., flagella, cell walls or mesosomes. Where the bacteria themselves or subcellular particles are required, the presence of filter aid is undesirable, whereas it may help in the provision of soluble products in aiding the removal of insoluble debris. The problem is simplified by the use of centrifugation, but unless some means of continuous centrifugation is available, large volumes of culture might best be handled by co-precipitation or foam separation. Again one must be careful in the selection of the procedures to adopt. If either of the two latter techniques is to be employed the choice of surface-active agent must be considered in the light of possible modification or destruction of the desired property of the organism. Like filter aids, these surfactants may be difficult to remove.

Where the culture fluid or a constituent thereof is the desired product the choice is between filtration and centrifugation, bearing in mind that after the primary separation clarification and sterilization may be necessary, particularly after centrifugation. Because of the use of surfactants, or hydrophobic and hydrophilic colloids, the use of co-precipitation or foam separation cannot be recommended, but here again there will obviously be some cases in which the presence of these is not harmful. Like centrifugation, filtration of the culture fluids from these procedures will be necessary to remove residual organisms.

Further treatment of the primary separation products is not without its hazards, the most obvious being the loss of viability of organisms attendant upon considerable lysis in some cases. Less obvious but of equal importance is the complete or partial loss of certain components in the culture fluid that may occur with the various methods of concentration. Macromolecular constituents may be lost during filtration by adsorption on filter aids, during precipitation by destruction or irreversible denaturation because of the presence of high salt concentrations and during dialysis and freeze drying. There is no means of telling beforehand how various fractions will survive the methods used. One must be prepared for such losses and attempt to circumvent them by the use of other techniques that may prove to be innocuous for the particular constituent under investigation.

This has been a Chapter in which prime consideration has been given to the practical aspects of harvesting. Most of the techniques set out may be described as "know-how", and as such it is to be hoped that sufficient detail has been given to enable newcomers to the field to apply them successfully.

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