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Responses of Microorganisms to Physical and Chemical Gradients [and Discussion]

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Responses of microorganisms to physical and chemical gradients

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Most microbial ecosystems are spatially organized heterogeneous structures where microbes proliferate in gradients of biologically active solute molecules as well as in physical gradients of temperature, pressure, light, ionic strength, redox potential, pH and so on. Some of these ecosystems are discussed in this paper; however, the importance of investigating them in the laboratory is stressed. My group has developed a number of model systems. Seven of these are discussed and include four experimental, two numerical and one conceptual models. These are briefly described.

(1) The gradostat consists of a number of bidirectionally linked fermenter vessels fed with solutes from each end of the array. Steady-state solute counter-gradients are established. A number of results in which different microbes are grown in different gradient systems are described. (2) The gel-stabilized system: organisms are grown in a solute gradient diffusing from a source agar layer beneath a semisolid layer containing agar and cells. (3) A constant dimension thin film fermenter. (4) The bacterial colony. (5, 6) The two numerical models, devised to simulate growth in the gradostat and in gel-stabilized systems respectively. (7) A conceptual model in which cells are regarded as compartments surrounded by activity domains; the importance of vectorial solute transfer in natural ecosystems is stressed.

Introduction

The science of microbial ecology seems now to be in conflict with attitudes and approac dominated on the one hand by classical field biologists and on the other by microbial physiologists schooled in the use of pure cultures of bacteria cultivated in what are, at any rate macroscopically, homogeneous culture conditions. Examination of most microbial ecosystems indicates that they are far from pure cultures and generally inhomogeneous, showing elements of spatial and temporal order that it is not possible to simulate in well agitated laboratory microcosms. The heterogeneity of the natural ecosystem in contrast to the laboratory culture system means that organisms proliferate in gradients of biologically active solute molecules, and often in physical gradients of temperature, light, pH, redox potential (*Eh*), ionic strength, pressure and so on.

Natural ecosystems that are spatially organized are reviewed in some detail by Wimpenny (1981) and will therefore only be mentioned briefly in this article. A family of habitats exist that are essentially flat or horizontally stratified. These include many water bodies such as lakes, which when rich in organic matter have an anaerobic hypolimnion lying below a warmer aerobic epilimnion. A few lakes, such as Gek Gel' in Russia (Sorokin 1968), Knaack Lake, Wisconsin, U.S.A. (Winfrey & Zeikus 1979), and in addition the Black Sea, are stably stratified or meromictic and here the establishment of physical and chemical gradients is at its most elaborate and the consequent distribution of microorganisms at its most beautiful. These systems represent the most spatially extensive gradient systems, however, there are many others that are shallower but quantitatively much more important to the wellbeing of the biosphere. The addition of particulate matter to water or to air–water mixtures generates on the one hand

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sediments and muds, and on the other soil. The former may be entirely anaerobic if the sediment is at the base of an eutrophic lake but is often differentiated, having aerobic surface and anaerobic subsurface zones. Muds and sediments exhibit large changes in redox potential and biologically conditions range from highly aerobic at the surface through layers where nitrate and nitrite, sulphate and finally carbon dioxide are successively employed as electron acceptors. The presence of particulate matter in an aqueous environment adds an extra degree of complexity since it provides surfaces often with strong electrical charges, which allow the attachment and growth of microorganisms. Soil is a degree or so more complex than this even, since it consists of a range of mineral particles with qualitatively different identities and a wide range of sizes. The presence of varying ratios of water and air can lead to huge changes in oxygen transfer rates since diffusion is about five orders of magnitude slower through water than through air. Vertical stratification from aerobic to anaerobic is augmented by the spherical heterogeneity of soil crumbs, which may themselves have aerobic external layers and anaerobic centres. Another class of linearly stratified gradient systems is microbial films. These are found in a huge range of habitats. In the ocean, films are associated with the air–water interface, with the surfaces of minerals and plants and are an economic nuisance as fouling on the hulls of ships. Microbial film is common on plant and animal surfaces. It is important to the dental profession as dental plaque, it appears on many other internal and external surfaces of animals; it is also found around the roots of plants in the rhizosphere, on their leaves (the phylloplane) and on their germinating seeds (the spermosphere). Microbial film has considerable commercial importance. An early application was in the 'quick vinegar' process in which *Acetobacter* was cultivated on the surface of beech chips percolated with an ethanolic solution. Modern sewage treatment plants incorporate filter beds where layers of organisms are responsible for removing organic matter from polluted water.

There are a number of spatially heterogeneous ecosystems that are spherical or irregularly spherical that also deserve attention. These include the soil crumbs already mentioned, mycelial pellets often found in the controlled conditions of an industrial fungal fermentation and in microbial floc, which can often form naturally but which may be generated deliberately to remove microbes from an aqueous suspension. All these may under some conditions demonstrate steep concentration gradients for oxygen tension, nutrients, products and so on. Another interesting microbial ecosystem whose composition and shape are dominated by diffusion gradients are microbial colonies. Here we start to recognize that diffusion phenomena may have an important part to play in the morphogenesis of multicellular arrays, though in colonies this is a haphazard and unsophisticated process. Most of the examples cited so far can be resolved to what ecologists call 'stratification'. Other physical and chemical gradients may lead to horizontal changes in ecology. Classical examples of 'zonation' include the alteration in populations of microorganisms in a river or stream away from a rich source of organic substrates, say from a sewage outfall, or the change in microflora down a thermal gradient near the edge of a hot sulphur spring. Gradients in radiant energy include the penetration of light into water bodies. Such gradients may be large at the surface of an estuarine sediment or small in a clear oligotrophic lake.

An understanding of the organization of structured ecosystems of the type described above requires a conceptual shift away from the currently accepted paradigm of homogeneity. A knowledge of the cellular units of microbial ecology so strikingly and effectively pursued by using stirred batch and continuous culture systems of single and mixed microbial species can

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have only limited success in affording an understanding of how these same organisms occupy the three-dimensional space of a microbial ecosystem. It is not that the rules governing the behaviour of a single cell are wrong or even irrelevant, it is simply that an additional family of rules must be formulated to define the behaviour of multicellular arrays of organisms. It must be clear that this is an anti-reductionist approach which is not necessarily obvious to all practising microbiologists.

The investigation of structured microbial ecosystems

Structured or heterogeneous ecosystems are, as has been emphasized, ubiquitous in Nature and their practical investigation poses severe problems to microbiologists. There are two main lines of approach to these problems, which, though complementary in nature, ought to converge somewhere near an exact understanding of the biology of particular microbial ecosystems. The first investigates the system *in situ*, using whatever tools are available. The latter include on the one hand sampling and the laboratory examination of natural material, and on the other the practical investigation of the system in the field while disturbing it as little as possible. The second main approach is the use of laboratory analogues that incorporate at least some of the characteristics of natural material as closely as possible. It is not claimed here that either route can ever be completely successful on its own. It is clear, however, that laboratory model systems that incorporate the elements of heterogeneity – solute diffusion gradients – are very scarce. It is for this reason that I and my group have concentrated almost exclusively on the development of such a methodology.

A methodology for investigating spatially organized microbial ecosystems

Linked homogeneous laboratory microcosms

The stirred laboratory fermenter is parent to the continuous culture system first described by Monod and by Novick & Szilard in 1950. The continuous flow system in its commonest form (the chemostat) consists of a single vessel; however, various workers have included two or more stages so that medium flows from a reservoir through a number of vessels, in which environmental conditions can vary widely to a receiver at the end of the array. Margalef (1967) has investigated estuarine conditions in such a multistage continuous culture apparatus. The incorporation of bidirectional flow in a multistage chemostat allows us to investigate growth in opposing solute gradients. Such a linked system was developed as an ecological tool by Cooper & Copeland (1973). Their system consisted of five 9 gallon (*ca.* 41 l) plastic containers linked together by glass tubing couplers that allowed transfer of material between vessels in two directions by a combination of mixing and diffusion. Cooper & Copeland studied estuarine ecology in salinity gradients by using this apparatus.

Lovitt & Wimpenny (1979, 1980, 1981*a*) approached the same problem as an attempt to incorporate opposing solute gradients in a multistage continuous culture apparatus. We described such a bidirectional compound chemostat as the 'gradostat', unaware at that time of the work of Cooper & Copeland.

Flow in the gradostat, in contrast to the Cooper–Copeland model, is by tubing pumps in one direction and over weirs in the other. The gradostat is sketched in figure 1 and shown in

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figure 2. An analysis of gradostat kinetics indicates that when all the vessels have the same volume, all the flow rates are the same and steady-state conditions have been reached, the concentration of a solute whose source is the first reservoir falls linearly across the array away from the reservoir. Naturally the concentration of a second solute whose source is the second reservoir also falls linearly along the series of vessels but in the opposite direction. It is easy to see that such an opposing gradient system can be a powerful tool in investigating the responses of pure or mixed cultures of bacteria to the types of gradient seen in many natural microbial habitats.

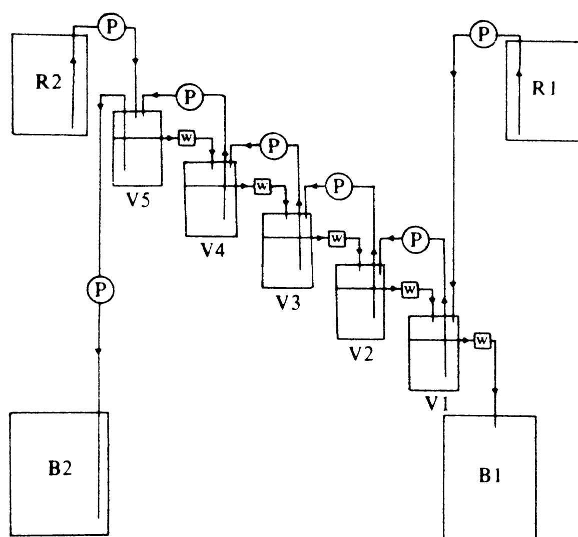

FIGURE 1. Schematic representation of the gradostat. The five culture vessels (V1 to V5) are fed with medium from reservoir R1 in an upward direction, with transfer between the vessels by a series of pumps (P). Medium from reservoir R2 is fed down the sequence of vessels over a series of weirs (w). B1 and B2 are the effluent culture vessels.

When two essential nutrients are fed into the gradostat from opposite directions, growth occurs in the centre of the array where the solutes meet. This was confirmed by using succinate and nitrate in cultures of *Paracoccus denitrificans*. When *Escherichia coli* was grown in opposing gradients of glucose and oxygen plus nitrate, the organism responded by inducing and repressing different oxidative activities in different vessels.

When more than one organism is present in the gradostat, each vessel represents a separate niche where growth can take place if the conditions are suitable. An obvious experiment is to grow a mixture of aerobic and anaerobic species in opposing gradients of glucose and oxygen. The organisms segregate in the expected directions.

The possibility of using the gradostat to investigate interactions in natural enrichment cultures was also examined by using opposing gradients of lactate and sulphate plus nitrate. All vessels were incubated anaerobically. The ecosystem became stably organized after about 2 weeks and demonstrated sulphate reduction at the reducing end of the array, and sulphide oxidation at the oxidizing end. A change in nutrient levels, decreasing oxidant and increasing reductant, led to interesting transients in the levels of cells and metabolites, especially in sulphide concentration.

The gradostat, as the few experiments described here show, is clearly a powerful tool in

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investigating the organization of spatially structured microbial ecosystems. Its main advantages are that (1) interactions can be investigated under true steady-state conditions, and (2) each vessel contains sufficient material to allow an accurate determination of solute concentrations and a reliable assessment of the physiological properties of cells in it. Its main disadvantage is that cells as well as solutes are transferred between vessels and this is not a natural property of

FIGURE 2. The gradostat.

most microbial ecosystems. This problem may be overcome if the cells are separated from the solutes by a semipermeable membrane. The chamber containing cells may itself be open or closed to flow. If each cell chamber were open the device as a whole would operate under true steady-state conditions. Other forms of gradostat are possible and include multidimensional arrays. Even the simplest two-dimensional gradostat needs four vessels, however, and construction and operating problems may make these variations impracticable. Applications of the gradostat concept are legion and centre around its ability to provide separate habitats each

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connected to its neighbours so that homogeneously distributed material, including cells, are transferred reciprocally between them. Early indications suggest that even with a pure culture of one bacterial strain the provision of different environmental stresses leads to the selection of genotypes adapted to each niche, which may become established as the dominant population in each vessel. (R. Lovitt & J. W. T. Wimpenny, unpublished.)

Gel-stabilized laboratory microcosms

A second major experimental approach is to construct a model system in which diffusion is the only significant solute transport mechanism. Such a system duplicates conditions in many ecosystems, including most aquatic sediments, although it is a 'pure' solution and cannot include disruptive elements such as the burrowing activities of annelid worms! The use of gelling agents in microbiology has a long history beginning with developments in Koch's laboratory. Beijerinck (1889) acknowledged the importance of diffusion in agar when he developed 'auxanographie'. Williams (1938*a*, *b*, 1939*a*, *b*), in work that had sadly sunk into oblivion until now, demonstrated the formation of multiple growth bands in agar-stabilized test tube cultures of various bacterial species under different atmospheric conditions. Later Tschapek & Giambiagi (1954) reported a similar phenomenon in nitrogen-fixing shake cultures of *Azotobacter vinelandii*. These bands appear to be a biological form of the well known Liesegang ring phenomenon (Liesegang 1898; Hedges 1932; Crowle *et al.* 1963). Such culture systems have been used to investigate the responses of organisms to oxygen gradients, mostly to determine the sensitivity of anaerobic or microaerophilic species to the toxic effects of the oxidant (Whittenbury 1963; de Vries & Stouthamer 1969).

We believed that such a laboratory microcosm could be successfully employed to investigate growth of, and interactions between, microorganisms cultivated in opposing solute gradients. As we currently employ them, our gels are usually established in 250 cm^3 glass beakers, in the bottom of which is poured a gel layer containing basal nutrients, a diffusing solute source (often glucose), and agar at 10 g l^{-1}. The upper layer contains the same basal nutrient concentration, no diffusing solute, a cell inoculum initially homogeneously dispersed throughout the layer and agar at 4 g l^{-1}. When incubated aerobically, cells in the upper layer proliferate in opposing gradients of glucose and oxygen.

While investigating growth of various bacterial species in such a system we observed periodic growth bands similar to those described above. Banded growth can be quite easily determined photographically or by simple measurement, or by scanning the gel with a gel scanner (figure 3*a*). It is also possible to take core samples from the gel, to section these and to measure growth in each gel slice turbidimetrically. When a number of different species of bacteria were examined in this way, patterns were produced that appeared to be species-specific in those species in which bands were actually seen. In general, band formation was characteristic of facultative anerobes and microaerophilic species and was comparatively rare among the obligate aerobes. One microaerophilic organism, *Lactobacillus confusus*, produced no surface growth but generated a family of subsurface bands, including a major one at the interface between the bottom and the semisolid layers. All others showed surface growth with or without band formation. We have investigated banded growth in considerable detail by using in particular cultures of *Bacillus cereus* because initially this species generated more bands than were seen with any other species (Wimpenny & Whittaker 1979; Coombs & Wimpenny 1980; Wimpenny *et al.* 1981). Since choosing *B. cereus* one of my colleagues (H. Abdollahi) noted bands of *E. coli* grown on a gel

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gradient containing yeast extract as diffusing solute. A culture of *Escherichia coli* generated up to 20 bands in this gel after about 10 weeks of incubation (figure 3*b*) (J. P. Coombs, unpublished data). Investigations into the *B. cereus* system revealed that band formation is dependent on the establishment of opposing gradients of glucose and oxygen and that such bands were formed sequentially (figure 3*c*) rather than together. Increasing the source glucose concentration compressed the bands nearer the surface, whereas reducing the basal nutrient concentration did the same thing. Changes in agar concentration in the semisolid layer between 4 and 15 g l^{-1} had virtually no effect on band formation. This fact, together with the observation that non-motile species including *Streptococcus faecalis* also produced bands, led us to conclude that motility is unimportant in band formation since it is inconceivable that the most motile organisms can move through 15 g l^{-1} agar preparations.

Physicochemical changes in gel models have been investigated by using needle pO_2, pH and *Eh* electrodes and by assaying solutes and cell density in sliced core samples removed from the gel. It was clear from these experiments that all the observed chemical gradients vary smoothly in contrast to the 'chopping' seen in cell distribution. Use of different types and concentrations of agar did not lead to significant changes in band position, and similarly different strains of *B. cereus* were examined and although some variations were seen they were not judged to be significant.

Band formation is almost certainly one form of a periodic phenomenon seen when certain reacting solutes diffuse through gel-stabilized aqueous media. This was first reported in 1896 by Liesegang and has been reviewed in detail by Hedges (1932). Liesegang rings are produced for example when silver nitrate diffuses into a gel containing small amounts of chloride ions and consists of a periodic precipitation of silver chloride. Many examples of Liesegang ring formation have been cited in the early literature and it has been suggested that such phenomena may be involved in pattern formation in biological morphogenetic systems. So far no satisfactory explanation of the process has been forthcoming, though a number of theories have been proposed. Numerical models of growth in solute diffusion gradients throw some light on band formation and are discussed later.

We have investigated other deep gel-stabilized systems including the establishment of natural enrichment cultures. One of the most interesting was an attempt to duplicate conditions in the water phase found at the base of an oil storage tank. The microbial flora of this habitat includes anaerobic sulphate-reducing bacteria responsible for the corrosion of the steel base of the tank. In addition there are aerobic species capable of utilizing hydrocarbons as nutrients in the presence of oxygen. Many other species, both aerobic and anaerobic, can be isolated from such an environment. It is clear that such an ecosystem has a reducing 'end' and an oxidizing 'end', and therefore that gradients in oxygen tension, in *Eh* and in speciation must exist. This system is easily established in the gel-stabilized model. A steel plate is placed at the base of 250 cm^3 beakers and a layer of semisolid agar containing a basal nutrient solution together with a natural cell inoculum is poured over it. After this has set, gas oil is poured carefully over the agar layer. The system is incubated and then examined by using needle electrodes, gel slicing and the assay of cell numbers and metabolite concentrations. The diffusion of ferrous ions makes an ideal internal marker of redox conditions because the ions in the reduced form react with oxygen and precipitate at the aerobic–anaerobic interface as ferric salts. This shows clearly as a band or family of red bands separating the aerobic upper layer from the anaerobic lower layer. Sulphide and iron are measured near the steel plate and sulphate reducers are also

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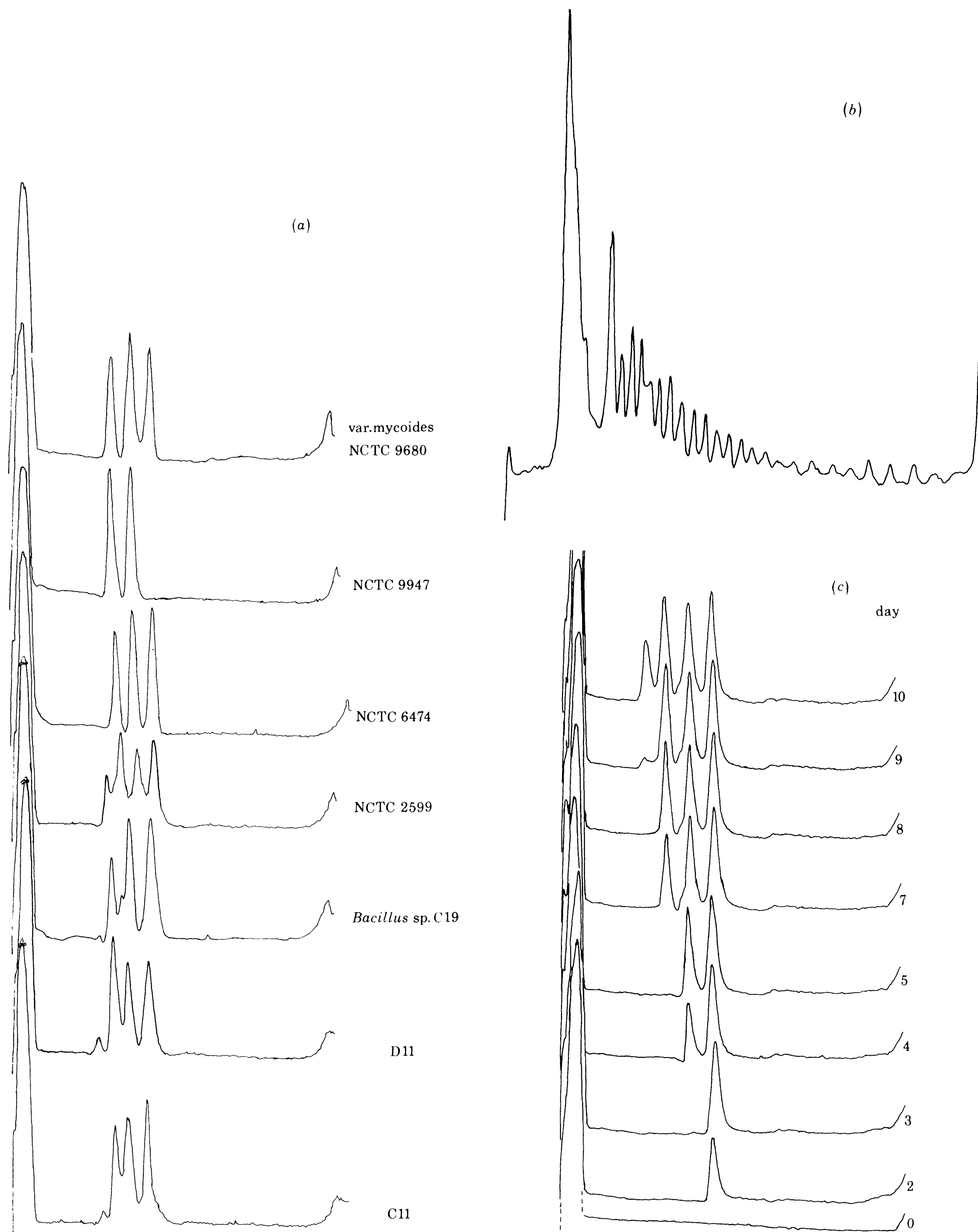

FIGURE 3. Scans of banded growth in a gel-stabilized laboratory model ecosystem. (*a*) Strains of *Bacillus cereus* incubated in casamino acids – yeast extract – salts (c.y.s.) medium in opposing gradients of oxygen and glucose, for 5 days at 37 °C. (*b*) *Escherichia coli* K12 grown in a salts medium in opposing gradients of oxygen and yeast extract and incubated at 30 °C for 10 weeks. (*c*) Time course for band formation in cultures of *B. cereus* grown in c.y.s. medium in opposing gradients of oxygen and glucose. All gel scans were performed with a Unicam SP500 spectrophotometer with a Hilger–Gilford scanning attachment.

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found in this region. Hexadecane oxidizers are found only in the upper layer, while a family of aerobic and anaerobic heterotrophs are present at different concentrations throughout the whole gel. Further details of these experiments are presented by Wimpenny *et al.* (1981).

Other gel-stabilized systems have been devised that are capable of generating diffusion gradients in more than one direction. Caldwell & Hirsch (1973) and Caldwell *et al.* (1973) developed a sophisticated steady-state two-dimensional gradient plate, which they showed could segregate related species of bacteria and had considerable potential in defining the niche of microorganisms. This work has so far not been followed up.

We have examined a simpler form of two-dimensional gradient plate in which steady-state gradients are not obtained. Particular solutes are incorporated in troughs in the agar on adjacent edges of 10 cm diameter petri dishes. In some experiments one of the gradients was established by using the well known wedge plate technique. Diffusion proceeds for long enough to establish a two-dimensional gradient system. The surface of the agar is then inoculated with organisms, and a stainless steel template is inserted into the gel. The latter divides the gel into a number of separate compartments. In our experiments a 7×7 template provides 49 separate units, each containing distinct levels of each of the two solutes. Using this system we have investigated microbial growth in two-dimensional gradients of sodium chloride and pH and in a variety of combinations of biocide, EDTA and pH to investigate possible synergism between these agents. It seems possible that diffusion methodology along the lines of the Caldwell plate or the stopped time-dependent gradient plate just described, if sophisticated and developed in terms of reliability and ease of manipulation, could play an important part in investigating the habitat profiles of different microbial species.

Thin-film fermenters

The ubiquity of microbial films in natural ecosystems suggests that they could profitably be studied under the more controlled conditions of the laboratory if suitable laboratory model systems existed. Atkinson and his colleagues (see, for example, Atkinson & Fowler 1974) have extensively reviewed microbial film formation, and have themselves designed a number of alternative forms of film fermenter. In one version the film grew on a surface in recesses in a template that was placed over the plate. The film depth was kept constant by using a wiping system. These film fermenters have been studied in detail in terms of their chemical dynamics, and applications include organic effluent treatment. Another major area where models are important is in the study of dental plaque. A number of dental film models have been described, some for use *in vitro* and some for *in vivo* examination. The validity of some of these models is questionable since the conditions of the laboratory experiment are for one reason or other quite unrelated to conditions in the oral cavity.

We have designed a laboratory model film fermenter. The film surface is swept so that films of constant thickness are generated and it is probable that given long enough these films will enter some sort of steady-state where growth on the one hand is balanced by removal of cells plus death and recycling of a fraction of the population on the other.

The film fermenter (figure 4) is enclosed in a glass fermentation vessel to provide a sterile environment. Film is grown in a number of shallow pans made at present out of PTFE plastic. The depth of each pan depends on the thickness of film required. Our system was set up to examine the properties of dental plaque and the pan depth was 300 μm. The pans, six in all,

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are located in a larger PTFE disc so that each pan is absolutely flush with the surface of disc. Nutrient solution percolates over the surface of the disc from small holes drilled in a hollow PTFE wiper blade, which slowly rotates over the surface of the disc. Medium is pumped from a sterile holding reservoir by a peristaltic tubing pump into the chamber and from there into the axle of the rotating wiper. Another peristaltic pump operating at a faster rate removes excess

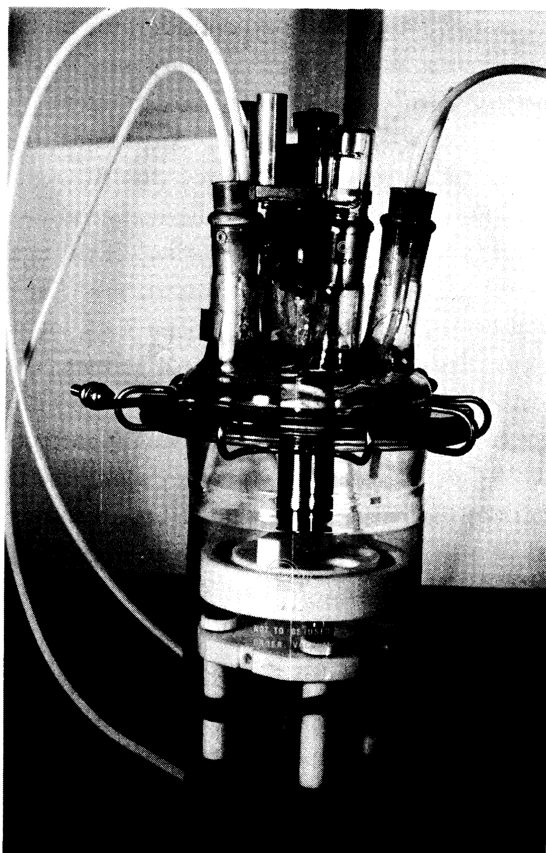

FIGURE 4. The thin-film fermenter vessel. Nutrient is fed into a hollow drive shaft and distributed over the disc containing six film pans by a hollow PTFE wiper blade. The medium is then collected in a ditch around the periphery of the film disc and removed by a fast-running peristaltic pump. (R. A. Coombe, A. Tatevossian & J. W. T. Wimpenny, unpublished.)

medium from an overflow ditch near the edge of the fermenter disc. The waste material passes into a receiver containing disinfectant. The system is thus entirely closed. Sterile air is bled into the fermenter chamber; of course this may be replaced by other gas mixtures, e.g. nitrogen to investigate the effect of anaerobiosis on film activity.

Our first experiments have been confined to examining the appearance of thin sections of films produced by pure cultures of plaque organisms. Electron micrographs reveal some changes in packing density at the base of films and there are suggestions that the frequency of cell division decreases with depth in the film. We hope to use mixed-culture films and to continue the cytological investigation. We also plan to measure changes in oxygen tension, pH and redox potential between the surface and the base of the film. This can be done by using microelectrodes. We also intend to section frozen film horizontally and vertically, in the former case

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to determine the proportion of cell types present at different depths, to measure solute concentrations at different points in the film and to determine the activity of several different enzyme systems, and in the latter with enzyme histochemical techniques to confirm the distribution of catalytic activities as a function of film depth.

The microbial colony

Colony form depends on several factors, some intrinsic (such as cell morphology, motility, and production of extracellular material) and some extrinsic, including a family of solute diffusion gradients into and out of the structure. As a paradigm of other biological phenomena

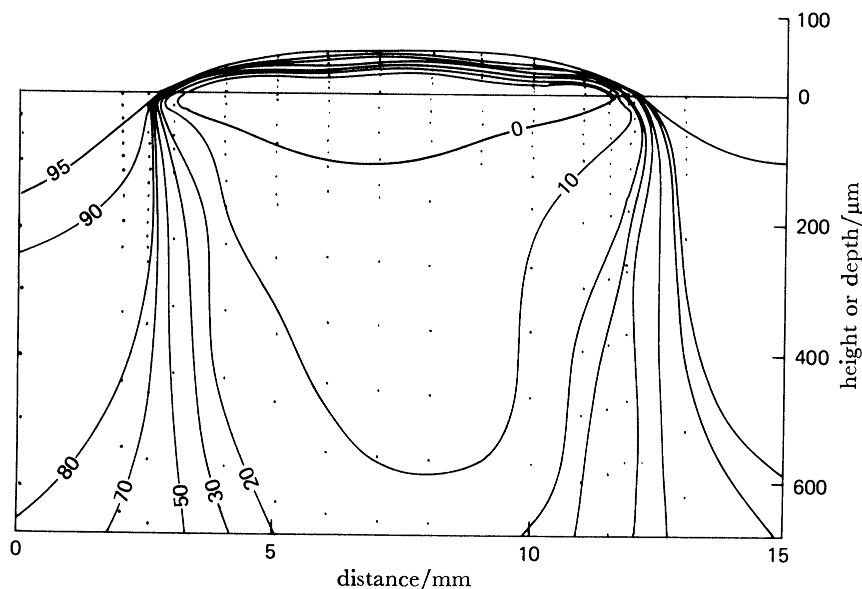

FIGURE 5. The distribution of oxygen in and around a colony of *Bacillus cereus* growing at 37 °C in a tryptone–soya agar. Isopleths are shown as a percentage of air-saturated value. Data were obtained with a Transidyne oxygen sensor and microelectrode (Transidyne General Corporation, Ann Arbor, Michigan).

we could perhaps consider them as containing elements of film structure and organization and at the same time having the attributes of a rather limited but sometimes quite beautiful morphogenesis.

Colonies have been used throughout the history of microbiology for diagnostic, enumerative and genetic ends, but rather rarely as objects of scientific scrutiny for their own sake. Legroux & Magrou (1920) examined sections of old colonies of *Vibrio* sp. and reported on cytological differentiation between cells adjacent to the atmosphere and those in more remote regions. Colonial growth kinetics have been studied in detail by Pirt (1967), Palumbo *et al.* (1971), Cooper *et al.* (1968) and Wimpenny (1979). The following generalizations seem valid. The growth rate is near its maximum at the leading edge of the growing colony. In profile this often generates a steep or upwardly curving leading edge region. Edge growth leads to a linear increase in colony diameter. The internal regions of the colony also appear to grow, but at a rate that is probably limited by solute diffusion. Cells have nowhere else to go in this region but up, and this leads to elevation of the central zone. The colony profile now rises in a gentle sweep to a flattened or dome-shaped peak. J. P. Coombs and I have examined oxygen and pH gradients in growing colonies of various species. Figure 5 shows a typical result of using *Bacillus*

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cereus colonies growing on a rich medium, and clearly demonstrates the steep oxygen gradients in and around the colony. Using a freeze sectioning technique we have analysed oxidative enzyme activity across large colonies of *Enterobacter cloacae* and have demonstrated clear biochemical differentiation across the structure. The greatest activities of the enzymes tested were near the upper surface of the colony (Wimpenny & Parr 1979).

The use of numerical models

Each of the experimental laboratory model systems described above incorporates, it is hoped, some of the fundamental characteristics of natural microbial ecosystems. It is also possible to construct mathematical models that can apply directly to the natural ecosystem, or they can apply to the laboratory system which is itself a model! The gradostat seems to us to be a powerful research tool when applied to heterogeneous ecosystems; however, it is complicated to assemble and sterilize and takes days (depending on the experiment) to settle down to steady-state conditions. An experiment must be clearly defined to enter into the commitment to run it. On the other hand, with a computer simulation numerous experiments can be run in minutes. From these simulations it is then possible to decide on the most appropriate real-life experiment. Significant differences between results from the simulation and results from actual experiments can then be further investigated.

Mathematical models have been used by us in conjunction with normal computer software including graph-plotting routines and are discussed below. GRADSIM was developed by S. Jaffe as a completely versatile program that could easily be used by comparatively inexperienced operators. It was designed to follow changes in up to 10 substrates, products or cells in a gradostat consisting of as many as ten vessels. The versatility of the program is illustrated by what follows: while Monod growth kinetics is normally used in the standard program there is a built-in option to use any equations describing growth that the operator can devise. The flow between vessels is usually the same but may be varied, as may the volume of each vessel. In fact zero flow may be specified, in which case the system behaves as one or more separate batch cultures. If flow is specified in a single vessel in one direction only, the system becomes a chemostat, or if more than one vessel is used a multistage continuous culture system is the result. A few examples illustrate the use of GRADSIM.

We were interested in the effects of altering the ratio of the flow rates in each direction in the gradostat because it appeared likely that under these conditions the steady-state distribution of solutes would become nonlinear. The simulation was run by using a variety of ratios, and the results showed that distribution was strongly dependent on the ratio of flow rates in each direction (figure 6). We are also interested in the outcome of competition experiments where two organisms with crossing Monod growth curves grew together in the gradostat. In the chemostat one or other organism is eliminated from the system, depending on dilution rate, except at the crossover point. The outcome of this simulation is also dependent on dilution rate in the gradostat; however, the crossover point in the gradostat is not simply related to the crossover point in a single-stage chemostat.

Another simulation that will be investigated experimentally in the gradostat is the growth of two organisms under conditions where both must occupy different habitats, yet where both organisms depend on one another for survival. For example, an anaerobe fermenting glucose coming from one end of a gradostat array generates lactate, which diffuses to an aerobe using

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oxygen, which enters the system from the other end. The aerobe removes oxygen, which itself inhibits growth of the anaerobe. Such a system can easily be simulated in the GRADSIM program. Two points must be noted. The program allows product formation to be simulated in the normal Monod version of the program simply by incorporating a negative value for growth yield. This means that instead of a substrate disappearing as cells grow, a product is formed.

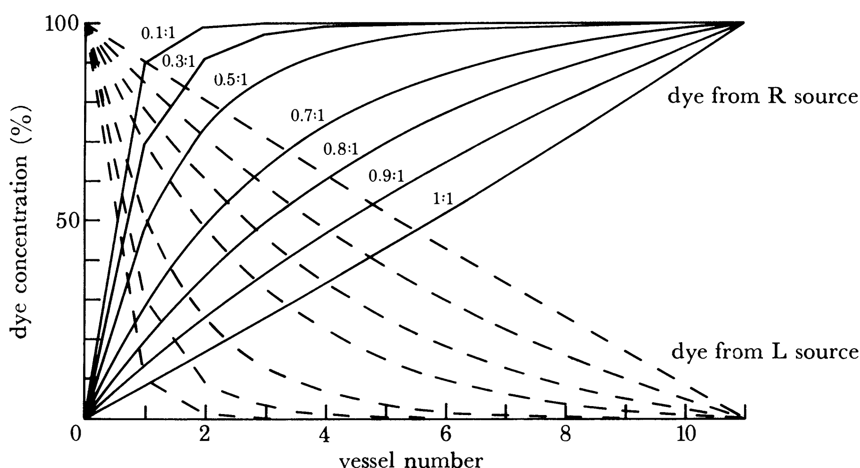

FIGURE 6. The distribution of two dyes in a gradostat in which the ratio of opposing flow rates was varied from 0.1:1 to 1:1. The results from the use of ten vessels and two sources and sinks (vessels '0' and '11') were calculated by using a computer simulation program (GRADSIM).

The only restriction is that product formation is directly related to cell growth. Inhibition can be specified by incorporating a specific constant in a growth equation that describes inhibition kinetics. The simulation demonstrates segregation of the two organisms, as expected. A control simulation in which the aerobe is omitted demonstrates elimination of the anaerobe as oxygen diffuses to the anaerobic end of the array.

S. Jaffe has developed a second and similarly flexible program to simulate microbial growth in stable diffusion gradients such as are found in the gel-stabilized laboratory microcosms. As in GRADSIM, GELSIM allows the choice between *table d'hôte* Monod growth and an *à la carte* menu of equations to one's own choice. It is fully interactive and allows output to either the plotter or the terminal. The main assumptions of the model are that solutes diffuse through the agar according to Fick's laws. GELSIM or earlier versions of this program have been used by S. Jaffe to investigate the banded growth phenomenon described earlier. Under conditions of a typical experiment, one band of growth is seen where the two diffusing solutes meet (figure 7*a*). Thereafter the growth zone moves smoothly down through the agar and no distinct periodicities are seen. Altering the conditions of the simulations to a rather narrowly defined set of values does generate periodic behaviour, but it remains to be seen whether these are related directly to the observed bands (figure 7*b*). We considered that the involvement of a third limiting substrate might be necessary for band formation, and alternatively the production of an inhibitor by cells in each band might generate periodic structures. These possibilities were tested in the computer simulation, again with negative results. One explanation for Liesegang ring formation is the possibility that supersaturation occurs when reacting solutes meet. Once precipitation is initiated it is completed rapidly and depletes the zone adjacent to the band of

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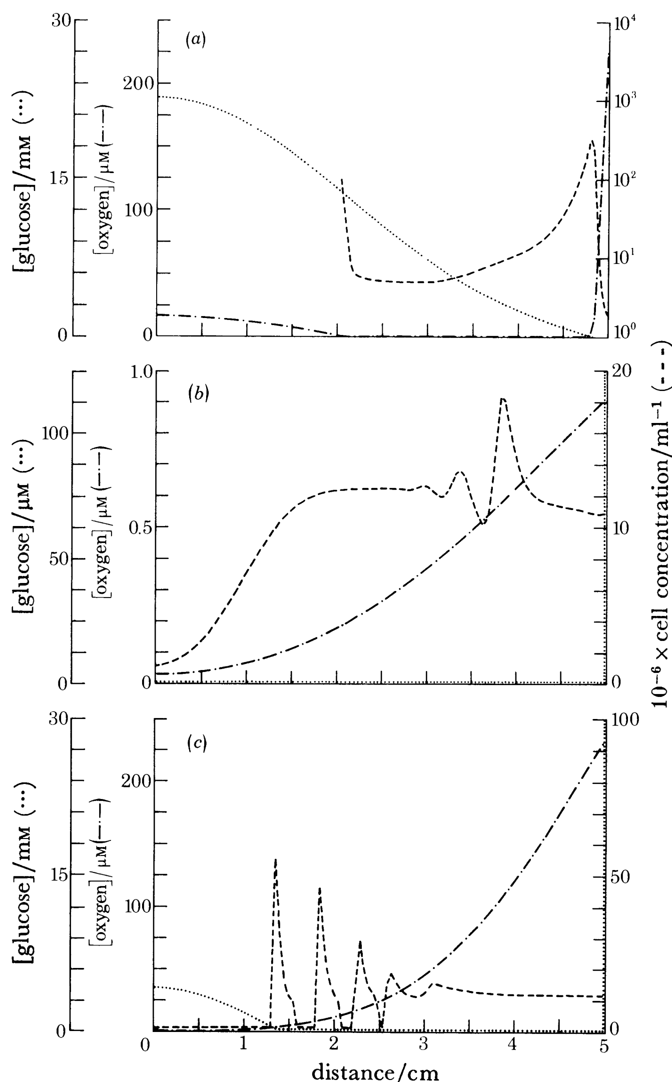

FIGURE 7. Numerical simulations of microbial growth in gel systems. Growth kinetic constants and yield and diffusion coefficients are close to values cited by various authors in the literature. Incubation time 50 h. (*a*) Two-layer gel as used experimentally; (*b*) single-layer gel with low initial glucose concentration; (*c*) single gel with normal glucose concentration and asymmetric activation threshold. (S. Jaffe & J. W. T. Wimpenny, unpublished.)

precipitate of a limiting nutrient. The second nutrient must now diffuse across this zone where no precipitation occurs until it reaches another region where supersaturation and precipitation can once again take place. This is formally equivalent to the inclusion of a threshold in substrate concentration below which growth cannot take place in the microbial system. The incorporation of asymmetric thresholds in the computer model did indeed lead to the development of banded growth; however, rather large thresholds were needed that seemed unrealistic physiologically (figure 7*c*). We must conclude that further work is needed to understand the banded growth phenomenon.

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Towards reality

We are conscious that the laboratory models described here may appear to be quite atypical and unrelated to natural microbial ecosystems. I shall therefore discuss some of these systems and ask how relevant these are to the real world. I have chosen to start with the gel model of the ecosystem in the water at the base of an oil tank because it illustrates some of the good and some of the bad points about laboratory models. First of all, the gel-stabilized model undoubtedly includes many of the elements of the natural ecosystem. Thus there are oxidizing and reducing regions, the proliferation of sulphate-reducing bacteria and the growth of hexadecane-oxidizing species. The major problems are that there is no vertical mixing between layers in the gel model. This leads to a 'pure' solution to a particularly 'impure' ecological problem. It also generates beautiful structures in space that are really totally obscured or perhaps even absent under real conditions. This is because of mixing on the one hand and because organisms at the oil–water interface form clumps or large aggregates that then become detached and fall to the base of the oil tank by gravity. They enter an anaerobic zone, so the cells die and are recycled to support the growth of the anaerobic species. The addition of any kind of mixing alters the quantitative dynamics of the system, which are probably accelerated since the system is no longer restricted by rather slow diffusion rates. On the one hand, therefore, we can develop a reliable idea of the qualitative and quantitative nature of the ecosystem, one that may be modified predictably and understood completely; on the other hand, we have an ill-defined chaotic system that nevertheless shares many of the attributes of the model but which cannot be defined quantitatively except at the highest level, that is as a black box embracing the activity of the oil tank as a whole.

Another example is the banded growth phenomenon, common in the laboratory gel-stabilized models and probably a manifestation of Liesegang ring formation. Are these bands just a pretty trick, a parlour game for microbes to perform in the confines of the laboratory, or do they really say something about events in the real world? Fortunately the answer is almost certainly the latter. One of rather few published examples of band formation is Perf'ilev's description of bands of *Gallionella*, which he observed with the capillary peloscope inserted into the structured environment of a lake sediment (Perf'ilev & Gabe 1969). It seems that banded growth needs reacting solutes (growth substrates), one of which is probably present in small amounts, diffusing towards one another. Such circumstances must be common in most sedimentary ecosystems and I predict that band formation could be very common in these and in similar habitats.

In this example the laboratory model displays a clear revelatory characteristic, which may be used to predict the behaviour of the natural ecosystem. In practical terms only, the model is transparent and no sophisticated techniques are needed, beyond the naked eye, to reveal its behaviour. The real-life system contains, among other things, mineral particles and microbial growth is obscured. The next step is to develop the model a stage further to include mineral particles. We may need to use other techniques, such as gel coring and slicing, to estimate microbial growth but at least we know what to look for. The system will now be closer to the real-life system since it contains surfaces upon which microbial growth can occur. The final step is to return to the natural ecosystem and to search for banded growth there.

The gradostat is a laboratory model with somewhat unusual characteristics. Of these the most remote from reality is the transport of cells from vessel to vessel at the same rate as that

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of all the solutes. This is unlikely ever to occur except perhaps in well mixed water bodies, where steep solute gradients are not likely. One might say that gradostat conditions could never occur naturally. On the other hand there are powerful reasons for investigating the behaviour of organisms in habitats that, though separate, have inputs and outputs between them, and which, like the chemostat, can achieve steady-state conditions. Experiments vindicate the gradostat. Thus it seems clear that natural enrichment cultures such as the lactate:sulphate plus nitrate experiment already discussed organize the space of a gradostat in a manner consistent with what we know of the behaviour of the same system in Nature. Other experiments have shown that a pure culture adapts to changes in environmental condition by changes in level of key enzymes. This was shown by using cultures of *Escherichia coli* growing in opposing gradients of glucose and oxygen plus nitrate. There are indications in some experiments with pure cultures that different stresses in different vessels can select for different genotypes. This aspect of the gradostat is potentially exciting because the exchange of material along an array of vessels allows cells exposed to quite stressful environments to be exchanged backwards and forwards from high to low concentrations of the effector. Such effects may be amplified by altering the ratio of flow rates, which could lead to changes in the degree of stress in a particular vessel.

Space and time: the new dimensions in microbiology

The subject of this paper, perhaps alone among contributions to this meeting, is literally concerned with the dimensions of time and space and in the context of microbiology today these dimensions might be described as 'new'. The ability of cells to absorb nutrients, to increase in size and to divide given an appropriate physicochemical environment means that in a particular region in space, seeded with a selection of mixed microbial genotypes, the system will inexorably develop in an orderly sequential manner to solve the specific problems posed by that particular habitat. When diffusion is the major or only solute transport system in operation, such an orderly unfolding can lead to complex and beautiful patterns in space. Such patterns include the development of the bacterial colony, the production of multiple growth bands, and the microbial colonization of a deep meromictic lake.

An awareness of the importance of space emphasizes that the properties of living organisms are predicated upon not only their genetic make-up, but on a region of space immediately surrounding them whose physicochemical composition determines their phenotypic behaviour. Such a cell can be regarded as a 'compartment' surrounded by a region over which it has some influence and which in turn influences it. This region is literally a 'domain' for that organism. It is possible to speak of the 'activity domain' of a given organism, where the latter is a source or a sink for particular solutes. We may also speak in a passive sense of the 'habitat domain' of a cell that defines a region in space whose physicochemical composition permits its growth. Such a terminology may be needed to allow a quantitative analysis of microbial interactions in terms that include dimensions of domains as well as solute transfer between compartments, not only in scalar but in vectorial terms. This terminology may also help to tidy up rather imprecise terms such as 'habitat', and especially 'niche'. This subject has been discussed a little more fully by Wimpenny (1981).

The use of the term 'domain' is useful in investigating microbial interactions. Thus two organisms may interact if their activity domains overlap, irrespective of any overlap in habitat domain. However, if habitat domains do overlap, organisms may occupy the same physico-

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chemical space, which means that they may be physically very close, and the coupling between each compartment can be very tight. This is true, for example, of syntrophic associations like *Methanobacillus omelianskii* (Bryant *et al.* 1967), *Chloropseudomonas ethylica* (Gray *et al.* 1973) or *Pelochromatium roseoviride* (Kuznetsov 1975). Where habitat domains do not overlap, for example where obligate anaerobes are interacting with obligate aerobes, it is still possible for solutes to diffuse between them and their activity domains may be said to overlap. The activities of anaerobic sulphate-reducing bacteria may be coupled with those of aerobic sulphide-oxidizing species to generate a functional sulphur cycle, for example. This is common in stratified ecosystems such as those found in sediments. Thus each species is represented by a layer or zone with a no man's land between them, through which sulphur compounds diffuse. The complete array represents a physiological transducer driven by oxygen from one direction and by organic substrates from the other.

The terminology outlined briefly above needs further precision and ought to form the basis for a complete quantitative analysis of spatially organized microbial ecosystems. It is hoped that the experimental and numerical model systems described in this paper will be a step towards this end.

I recognize with pleasure and gratitude all the valuable contributions to the work described here made by my colleagues R. W. Lovitt, J. P. Coombs, H. Abdollahi, A. Tatevossian, M. W. A. Lewis, S. Jaffe and R. A. Coombe. I also acknowledge financial help from Esso Research Ltd., N.E.R.C. and the S.R.C.

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Discussion

J. Wiegel (*University of Gottingen Institute of Microbiology, F.R.G.*). Has Dr Wimpenny applied his first two methods mentioned on the isolation and/or cultivation of organisms using H_2S and O_2 simultaneously (e.g. *Thiothrix*)? If not, please can he make some comments on which of these methods would have some advantages for such purposes?

J. W. T. Wimpenny. We have never isolated or cultivated H_2S-oxidizing bacteria such as *Thiothrix*. Both the gradostat and gel-stabilized models could be used here. The gradostat, fed with oxygen and H_2S from opposite ends of the array, allows continuous enrichment but is relatively complex to assemble and operate. Gels, on the other hand, are very simple to establish but they are closed systems.

J. G. Kuenen (*Laboratory of Microbiology, Delft University of Technology, The Netherlands*). I wish to comment on the gradostats described by Dr Wimpenny. It must be stressed that many interesting questions concerning microbial life in *gradients* of nutrients are directed at growth

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under nutrient *limitation*. The gradostat cannot maintain gradients of the growth-limiting nutrients. For example, if we have a five-stage gradostat with a medium lacking two essential ingredients for growth, one of the two is fed into the system at one end, the other at the other end. Somewhere the two ingredients will meet and organisms will start to grow. One of the two will become growth-limiting and as a consequence only minute quantities will pass to the next stage. As a result we will have *growth* of organisms in only one of the centre vessels, and starvation in all the others. The one vessel in which growth occurs is essentially a chemostat. Of course one might then start to add additional or alternative nutrients to the other vessels, but then, of course, we have a very interesting chain of chemostats where organisms having different histories will meet. Studies with these kinds of system will certainly provide interesting information. Gradients *can* be maintained with those nutrients that are conservative, such as sodium chloride.

J. W. T. Wimpenny. Professor Kuenen has made a valid and interesting point concerning the operation of the gradostat. To restate his case in more general terms: in any spatially organized ecosystem an essential nutrient if it limits growth will not be transferred in measurable amounts beyond the region of cell growth. This is clearly true not only for the gradostat but for any other heterogeneous ecosystem whether they are laboratory models or are found in natural habitats. The growth region must act as a sink for all essential nutrients and there are therefore gradients of nutrients from sources in the environment to cellular sinks. The gradostat reproduces this characteristic of natural systems exactly. Where two solutes, both needed for growth, are transferred in the gradostat towards one another, growth will only take place at the point where they meet. There will be gradients of both solutes towards this point. Organisms in these other vessels may still be affected by these solute gradients.

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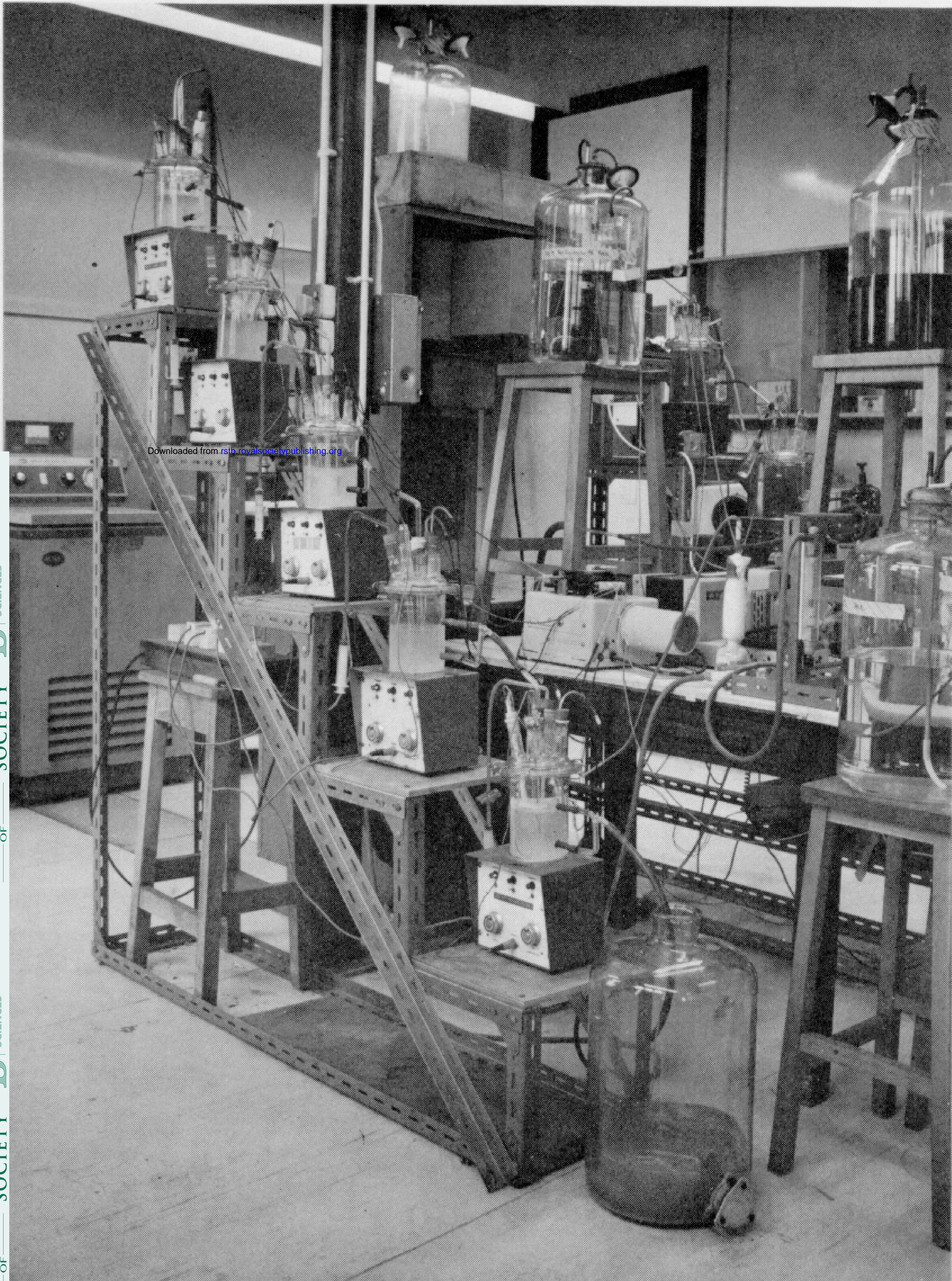

FIGURE 2. The gradostat.

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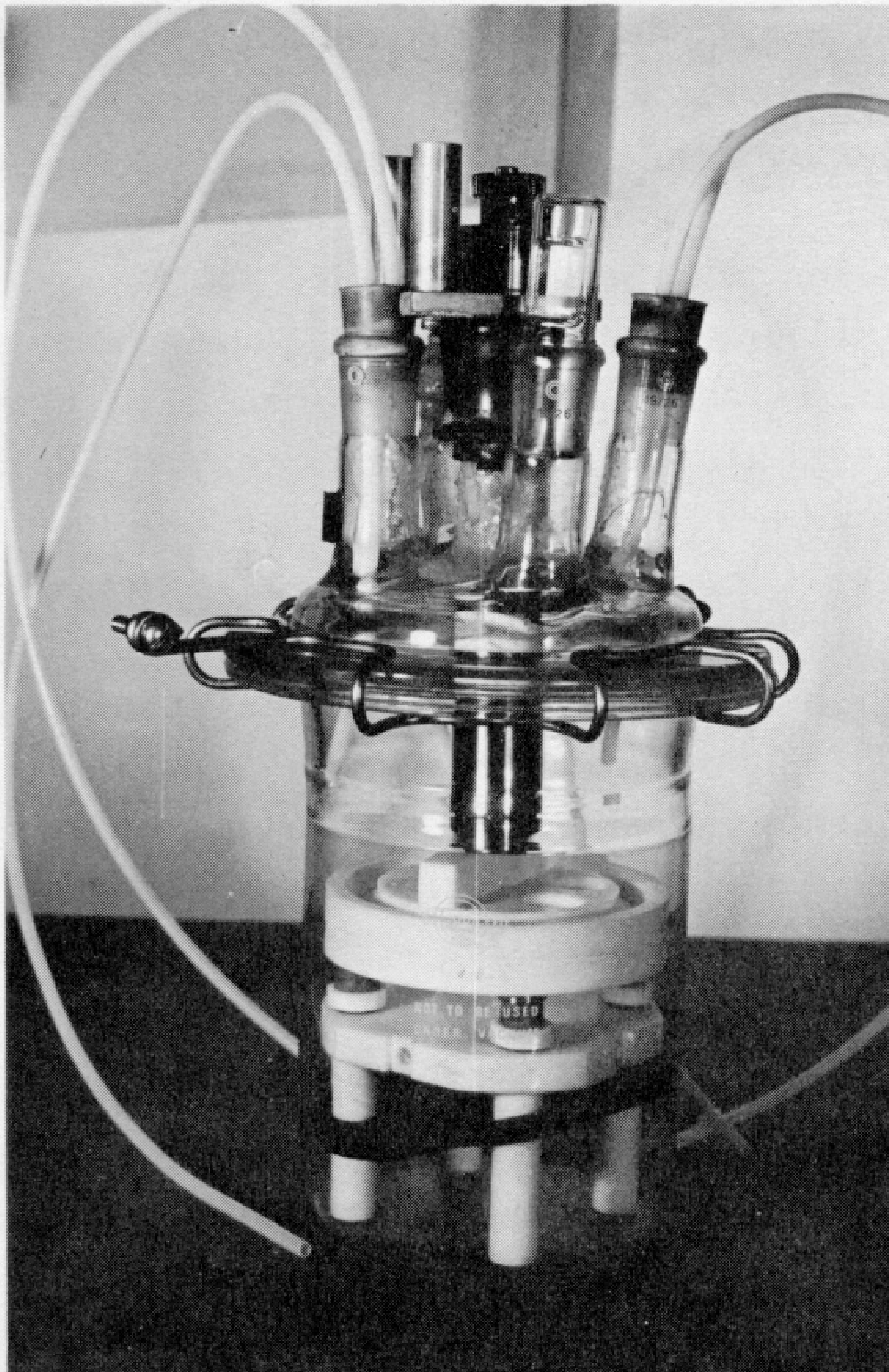

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FIGURE 4. The thin-film fermenter vessel. Nutrient is fed into a hollow drive shaft and distributed over the disc containing six film pans by a hollow PTFE wiper blade. The medium is then collected in a ditch around the periphery of the film disc and removed by a fast-running peristaltic pump. (R. A. Coombe, A. Tatevossian & J. W. T. Wimpenny, unpublished.)