Ion-Selective Electrode Rev. 1982, Vol. 4, pp. 75-131
Printed in Great Britain.

0191-5371/82/010075-57$28.50/0

THE ROLE OF ION-SELECTIVE ELECTRODES IN MICROBIAL PROCESS CONTROL

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KEYWORDS: Ion-selective electrodes for fermentation control; microbial process control; computerisation of ISEs in microbial process control; enzyme sensors in microbial process control; gas sensors in microbial process control; pH in microbial processes.

1. GENERAL INTRODUCTION

Although ion-selective electrode (ISE) and related technologies are perhaps the most significant recent advances in the exploitation of transducers by life scientists, their use is far from widespread. The crossing of the inter-disciplinary barriers between their origins in mainstream electrochemistry to their many biological applications has proved difficult, as is acutely demonstrated in fermentation science by the present lack of appropriate on-line transducers. Some knowledge of the relevant aspects of electrochemistry, instrumentation, microbiology and bioengineering is therefore essential if one is to promote successfully the applications of ISEs to fermentation processes, and we apologise in advance for periodically boring the specialist reader. However, our major aim is to provide a practical discussion of the special problems of ISE application in this field, which we believe to be the major obstacle to urgently needed progress.

2. INTRODUCING THE PROBLEM

In the interests of bringing the skills of the microbiologist, bioengineer and electrochemist to a common focus, it is worthwhile summarising the conceptual and practical problems currently faced by fermentation technologists. Whilst increasing use is being made of microorganisms in a wide variety of industrial processes, operation of these has hitherto required surprisingly little understanding of the process dynamics. Instead, success has been based on careful attention to the empirical needs of the organism. The complexities of microbial processes are subject to a high degree of intrinsic control, which is often sufficient for acceptable productivity in the exploitation of a near-natural process. However, progressive interest in the exploitation of minority or unnatural processes, as well as commercial considerations, have demanded more manipulative skills of the industry. Understandably, the extensive accumulation of knowledge concerning the nature of the metabolic pathways involved has given rise to the ability to select, and more recently to engineer, the genotype for productivity in existing and new processes. On the other hand, lack of understanding of the dynamics of a microbial process largely precludes its effective control by physiological or genetic means and, once proven, the process is for the most part left to its own devices.

Technology has developed through the ability to build defined units into a complex process, amenable to reductive analysis. On the other hand, Biotechnology must seek to provide operational simplification of an existing complex, often ill-understood process. Once the microbiologist has provided the appropriate organism, technological considerations largely dictate the operation of the fermenter plant. Biochemical assessment is then largely based on empirical experience, tempered with some off-line analysis. Meagre reliance on on-line monitors allows only rudimentary dynamic process assessment and control.

Adoption of chemical engineering principles by the bioengineer in the application of mathematical models for the design, optimisation and control of reactors has become widely accepted (1-3). However, one of the major problems always faced in such kinetic modelling is the complexity of reality. Technological understanding of the process has, therefore, required simplification of models so that they provide testable representations of reality. The main simplification strategy used is based on the concept of relaxation times, which delete from the model all mechanisms not contributing to the dynamics of the model system in the relevant time scale. The "unstructured models" are, therefore, the most widely used both in academic and industrial circles, simplifying the properties of the biomass to a single entity viz. the amount of biomass itself, and logical progression of such macroscopic theory employs mass balance equations as the main analytical tool (1,2,4,5). Although the microbiologist has an extremely "structured" understanding of the biomass black box, its largely static nature does not contribute to the bioengineer's model of reality. However, since even exclusion from such models should only be based on prior assessment of the dynamic relevance of parameters, it must be realised that models are only simplifications of those aspects of reality which are testable. Further, it must even be questioned whether validation of extant models can be reliably made. Incomplete on-line measurement is a major problem even in the attainment of the mass balances of "unstructured" models (e.g. 3,4) and recourse to indirect or long time-constant discontinuous measurement of samples (which themselves may not be representative) is generally unsatisfactory.

The biomass is clearly geared in nature to its own productive ends, and the pragmatic microbiological test is whether these are also the needs of the technologist. The difficulties, for example, of optimisation and upscaling, as well as of the day-to-day irreproducibility of many established fermentations, is beginning to undermine the industry's near total reliance on the technological sense of the microorganism (e.g. 3,4). Indeed, as many more minor or 'alien metabolisms' become 'forced' on the microorganism the situation must worsen. In concert with the recent increased awareness of the importance of on-line measurement (9,10), it is necessary for transducer technologists to devote considerable effort to overcoming the difficult measurement problems of the operating fermenter. ISE and derivative technology would appear to provide a relatively cheap, yet powerful, method of biochemical measurement (11). Yet, although the clinical application of ISEs are found to be of considerable commercial and practical interest, little development effort is apparently being directed towards their use in fermentations. The extremely demanding problems of aseptic on-line operation and of the analysis of complex solutions in transducer-fouling and sensor-poisoning broths have greatly retarded progress. It is our intention to examine the role and application of ISE measurement technology in these practical terms. Although no methodology alone is likely to provide the complete answer and could never straightforwardly match the performance criteria of the more customary temperature and pressure transducers (largely because of the complexity of the determinands being analysed), this should not preclude application of the methodologies that are already available.

A microbial fermentation may be simply regarded in terms of the medium and biomass bulk compartments. This of course makes a number of assumptions. In

the majority of large-scale fermenters mixing of the contents is imperfect, and the consequent stratifications give rise to a number of dynamically-changing sub-compartments ("distributed" models (3)). Similarly, the biomass itself cannot be assumed to be uniform. Many of the metabolic reactions occur on membranes and the membrane-bounded cytosol itself is far from free of microenvironments (12). Further, increasing use is being made of mixed culture fermentations (13-15), wherein a number of biomass compartments must be envisaged.

Currently, the determination of a mass balance (substrate input, product output and conversion) is the prime aim of measurement, yet it is not difficult to perceive that more thorough measurement, assessment, control and even manipulation of the organism's environment are necessary, and would no doubt be applied if reliable on-line transducers were available. Although the measurement of cytosol-located parameters is clearly more difficult to accomplish and interpret, the problems are not insurmountable. It is our belief that it is only with this type of perspective that microbial process technology can improve, and that despite the great promise of genetic manipulation, the microbial physiologist should not consider his role diminished, but in its infancy. Although physiological manipulation forms the basis of some fermentations and will undoubtedly prove the key to the exploitation of many more, it is also clear that knowledge of the dynamic physiological and biochemical properties of the system is a major feature currently lacking in microbial strain design, in small-to large-scale optimisations, in routine maintenance of productivity and in quality control. This without doubt will necessitate direct on-line monitoring of all important parameters.

3. PRESENT USE OF ISEs AND ALTERNATIVES

The pH, dissolved oxygen and platinum redox probes have long been commercially available and have been used at all levels of fermentation. As Kell (11) has pointed out, "..less prevalent...even to the point of non-existence..." is use made of other, even commercially available ISEs. Although general awareness of these and other measurement methods has increased (e.g. 9), practical usage has not kept pace with this increased knowledge. We thus extend our discussion to the scope and practical application of these transducers.

The pH, dissolved oxygen and, to a lesser extent, the redox electrodes have been long established as input elements of fermentation feedback control loops. Although there has rarely been any well established molecular basis for the success of these control practices (e.g. 11,16), the resulting improvements in product yield can only be considered dramatic. Changes in biomass and product yield, as well as shifts in metabolism, may be effected by quite subtle alteration of the set points of these control loops (e.g. pH (17-20), oxygen tension (16, 21-30) and redox potential (31)). The reasons for choosing these parameters would appear to be as much related to transducer availability and commercial instrumentation supply (10) as they are to any conceived fermentation process theory; in this regard it is worth noting the claimed superiority of certain redox glass electrodes that are not commercially available (332,333). The fact that transducers measuring temperature (32) and pressure (33) have also been available, and that these are the only other parameters which have received any attention, would tend to suggest that the development onus has been placed with commercial fermentation system suppliers. Fermentation transducer technology is perhaps too far removed from the already-stretched multidisciplinary front of Biotechnology for the industry itself to be able to perceive the benefits of more rational development in this field.

It is quite clear that a considerable number of other "environmental parameters" greatly affect microbial metabolism; just as clear is the corollary that similar control of these parameters may be expected to improve dramatically

the optimisation of fermentations. In a number of published reports off-line manipulations of the microbial culture medium have been demonstrated to engender key metabolic changes which in some cases have a discernible biochemical basis (e.g. 8 and see section 8). Before proceeding to ISEs, it is perhaps germane to place other measurement methodologies in perspective.

Certain automatable analytical procedures which have been designed for routine, off-line discrete sample analysis, and which have therefore been used in off-line fermentation analysis, have also been applied on-line. By far the most significant of these procedures is mass spectrometry linked to some form of biochemical separation method, historically gas-chromatography (GCMS). The development of cheaper and less bulky GCMS systems has encouraged their use in the larger organisations (34). Semi-continuous sampling of the fermentation gas phase through special gas permeable septa has enabled excellent off-gas (or volatile) analysis (35-37). The technique is clearly more versatile (and more expensive) than infra-red gas analysis (10,38). The development of further rapid separation methods and extensive analytical programs will no doubt extend the range of this tool. Its high sophistication and cost, commissioning time and inability to measure all important parameters simultaneously will no doubt confine its more general use to those specialised cases where chemically-similar species must be analysed (e.g. organoleptic components (39)). GC has similar considerable benefits with less complex detection methods (40). High performance liquid chromatography (HPLC) must also be considered a similarly powerful analytical procedure employing biochemical separation prior to detection, and there is every reason to believe that more versatile and diagnostic HPLC detection methods will shortly become available (41,42).

Calorimetry is a similar high-cost, indirect but potentially versatile single-unit measurement system. Dynamic fermentation heat release as a diagnostic tool (43-45) has thermodynamically interesting possibilities (46) although the interpretation of data and the practical implementation are somewhat difficult. The "enzyme thermistor" transduces specific biochemical reaction enthalpy (47). When the measurement is performed in a stirred or flow stream a steady-state reading, proportional to concentration, rather than a discrete sample reaction rate, is measured (48). Measurement against a similar non-enzyme thermistor as reference may provide a better on-line substrate (or enzyme) assay principle than spectrophotometric methods, which always suffer from poor sensitivity and drastic interference from opaque broths, bubbles and fouling of optical surfaces.

The wide range of colorimetric and enzyme-linked spectrophotometric discrete-sample assay procedures can be applied using multichannel autoanalyser equipment. The assays may be made semicontinuous with acceptable periodicity for monitoring (9,10). The methods, although versatile, have grave disadvantages which, as well as the above optical problems, include high capital and running costs requiring many reagents/enzymes, difficult on-line calibration, semi-continuous remote and indirect measurement with a time lag and reaction poisoning. These rather inelegant systems are being steadily replaced by direct ISE procedures in clinical and routine analytical laboratories (49). On-line use is, however, still difficult. Atomic absorption/emission spectrometry may also be applied on-line for individual inorganic ion analysis and can often replace ISE analysis where total concentration, rather than free ionic activity, is required (49,50).

4. SCOPE OF DIRECT ISE MEASUREMENTS

It is convenient to classify the various types of ISEs as summarised in Table 1.

TABLE 1. Direct measurement of biologically relevant determinands by ISE and related techniques

GLASS MEMBRANE ISEs (e.g. 51, 52)

H^+, Na^+, K^+, NH_4^+

PRECIPITATE- AND CRYSTAL-BASED MEMBRANE ISEs (e.g. 51-55)

Ca^{2+}, Mg^{2+}, K^+, Na^+, NH_4^+, Co^{2+}, Ni^{2+}, Cu^{2+}, Mn^{2+}, Zn^{2+}, Pb^{2+}

Cl^-, F^-, I^-, Br^-, PO_4^{3-}, NO_3^-, SCN^-, S^{2-}

ION EXCHANGE AND NEUTRAL CARRIER MEMBRANE ISEs (e.g. 52, 56-62, 307)

Liquid membrane, plastic matrix-enclosed liquid membrane and coated-wire constructions for:-

H^+, K^+, Na^+, NH_4^+, Mg^{2+}, Ca^{2+}

Cl^-, NO_3^-, Br^-, I^-, SCN^-, PO_4^{3-}

Organic acids (e.g. acetate, oxalate, phthalate and butyrate);

Amino acids (e.g. phenylalanine and leucine);

Vitamins (e.g. nicotinate and vitamin B_6) and

Drugs (e.g. chloramine T and aspirins)

ISFET DEVICES (e.g. 63-64, 145-152)

H^+, K^+, Na^+ and other membrane and sensitised methods (see text)

ENZYME-SENSITISED ISEs (e.g. 65-69)

Many different types linked to other ISE types (see Table 2); classically, urea, penicillin and glucose electrodes

GAS SENSING ISEs (e.g. 70-72, 94)

Conventional or air gap constructions for CO_2, NH_3, SO_2, nitrogen oxides and volatiles

POLAROGRAPHIC ELECTRODES (e.g. 65, 73-76, 154-156)

O_2, H_2, H_2O_2 and various

enzyme-sensitised (e.g. sugars, alcohols and vitamins)

OTHER DEVICES

Antibody/antigen electrodes (e.g. 78)

Substrate electrodes (e.g. 79)

Piezoelectric transducers (e.g. 80)

Chemically-modified electrodes (e.g. 81-87)

Since it is our purpose only to refer to the means of operation of each ISE type, the more correct classifications recommended by IUPAC (e.g. 52,85) will only briefly be alluded to below and the reader will be referred to other excellent

expositions where appropriate. The present interdisciplinary problem is well illustrated by the fact that the well established ISEs generally respond to inorganic ions, whilst the biologist often requires to assay the activities of organic ions. However, many of the inorganic ion-selective electrodes have applications in fermentation technology since some determinands (e.g. potassium, sodium, magnesium, calcium, phosphate, nitrate, ammonium, ammonia, carbon dioxide and hydrogen) are of great importance as environmental fermentation parameters; certain trace elements in culture media can also be assayed by ISEs.

More recent biomedical and other interest has stimulated examination of organic molecule sensors, some of which are of wider biochemical and fermentation significance. These direct measurement transducers can also be linked to single or multiple enzyme reaction sequences where the initial substrate or terminal product is the specific determinand of the transducer. Of course, true potentiometric ISEs do not consume determinand (or reagents) and can operate under unstirred conditions. However, these sensitised ISEs, and the amperometric types, consume determinand (and occasionally reagent) and therefore require effective stirring so that electrode surface layers do not become depleted.

4.1 Permselectivity and general mechanism

An ISE is an electrochemical half-cell separated from the bulk solution by a membrane exhibiting permselectivity to a restricted number of species. Where two phases with electrically charged species come into contact a Nernst potential is developed across the interface (e.g. 89). In the case of the ISE it may be envisaged as developing across the permselective membrane which has at its outer surface the determinand-containing solution and at its internal surface a standard activity of determinand (the internal reference). This potential may be measured at a metallic electrode (usually a silver wire) dipped into the internal reference solution (usually a chloride salt). The half-cell potential is then the sum of the potentials at the silver wire/(silver) chloride solution (ideally constant) and the variable determinand activity-dependent Nernst potential (plus certain other small and essentially constant junction potentials).

The potential difference between the working ISE half-cell and another, generally reference electrode, half-cell is measured by an arrangement as shown in Fig. 1.

Since the reference side of the measurement cell is also designed to be stable, the only factor that should cause a change in potential difference between the pair is the Nernst potential of the working electrode (89). The species in the permselective membrane of the transducer electrode that governs its selective response is referred to as the sensor by electrochemists; this is not to be confused with the fermentation technologist's use of "sensor" to mean transducer.

Various constructions of ISEs are schematically represented in Fig. 2.

4.2 Primary fixed-site ISEs

There are a number of ways of preparing solid-state electrodes, but the majority are of the primary or fixed-site electrode type (52). In the rigid matrix, glass electrode membrane, the ion-exchange and steric properties of the glass constitute the sensor (Fig. 2a), and various well-recognised cation-selective glasses have been developed (52). Glass electrodes sensitive to oxidation potential have also been described(333). The precipitate- (or crystal-) based electrodes can have membranes constructed of homogeneous sensor (homogeneous crystalline (52,55)) or may be heterogeneous (52,55), where the sensor is entrapped in another matrix material (e.g. polyvinyl chloride (PVC), wax, silicone rubber, etc.). The sensor may also be non-crystalline in the pressed sensor pellet varieties (53). The

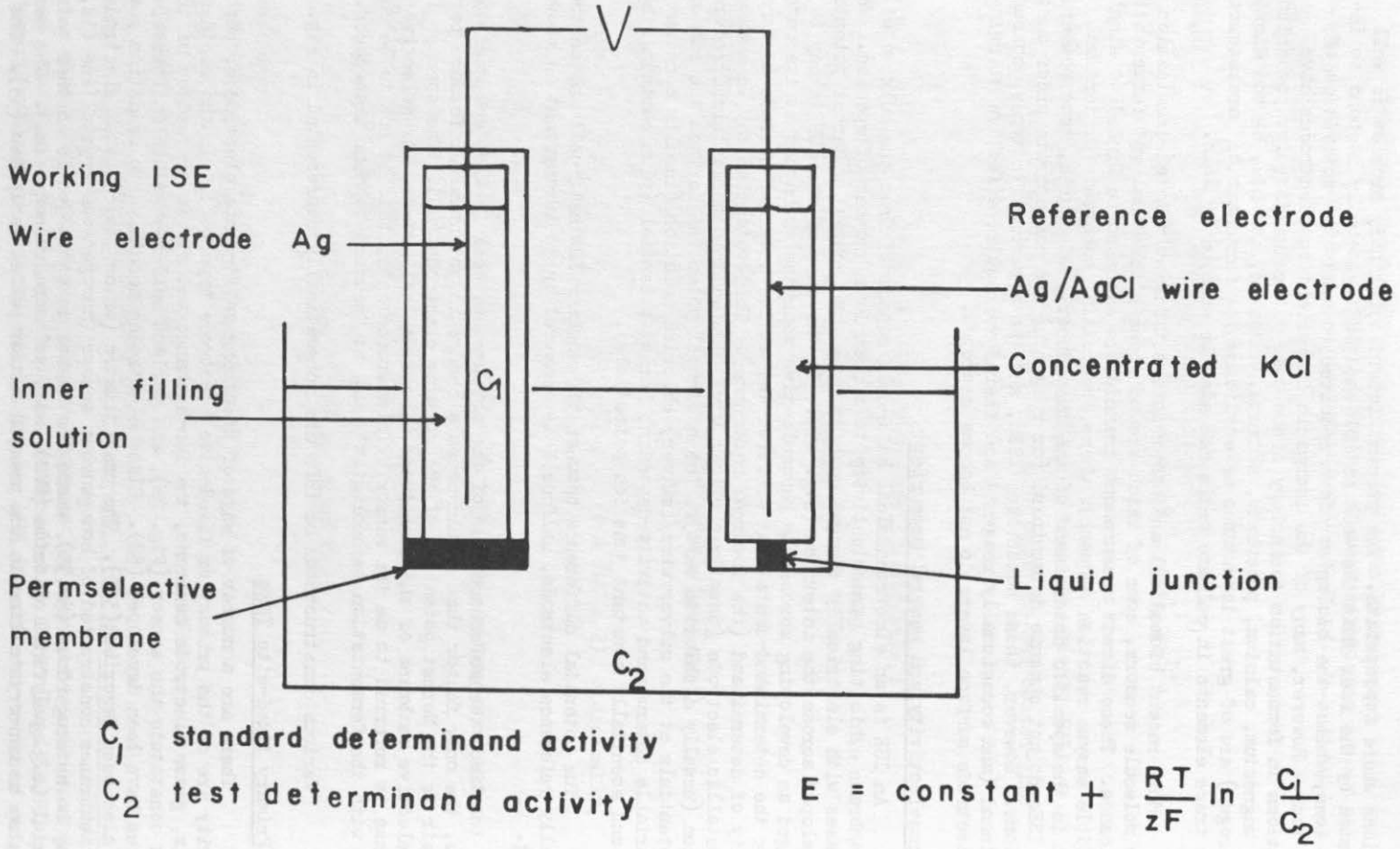


Fig. 1. Differential measurement of the potential of an ion-selective electrode versus that of a reference electrode

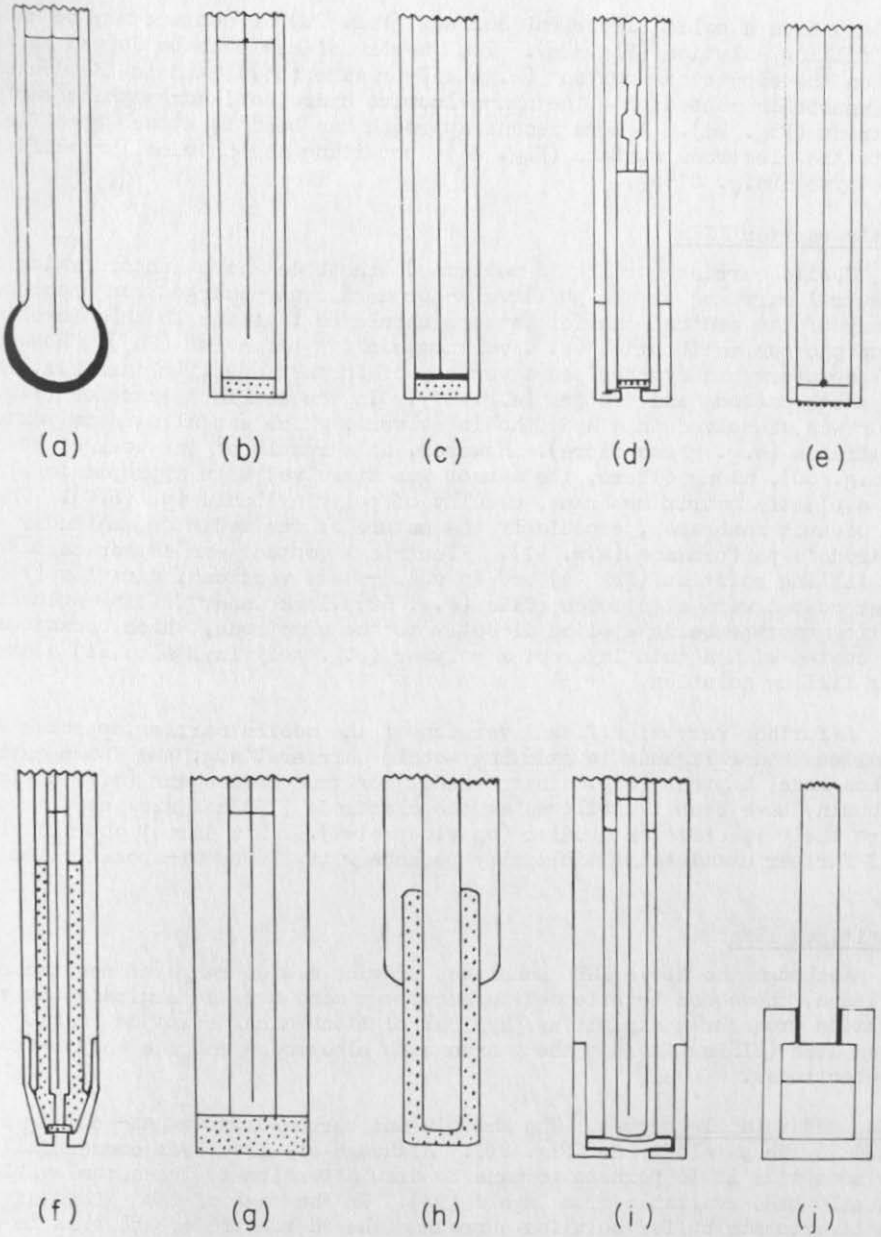


Fig. 2. The construction of various ISEs:- (a) glass membrane sensor, homogeneous or heterogeneous polymer-supported precipitate/crystal sensor membrane with liquid (b) or solid (c) contact; (d) 'selectrode' with sensor on end of hydrophobised graphite rod; (e) chemical modification of electrode surface with sensor; (f) liquid membrane with sensor dissolved in water-immiscible solvent, sensor dissolved in plasticiser of a plastic membrane with liquid contact (g) and solid contact in coated wire electrode (h); (i) gas-(e.g. CO_2-)sensing electrode with inner ISE (e.g. pH) sensitised with a solution (e.g. HCO_3^-) held behind a gas-permeable membrane; (j) ISE separated from solution by an air gap.

membrane may have a solid electrical contact (Fig. 2b) or contact may be made via an inner filling solution (Fig. 2c). The 'membrane' may even be formed as a precipitate on the electrical contact (e.g. silver wire (55)), and in the 'Selectrode' (90) the sensor is rubbed onto the non-selective hydrophobised-graphite surface of the electrode (Fig. 2d). A more recent approach has been to attach specific chemical ligands to the electrode surface (Fig. 2e), providing the chemically-modified electrode types (e.g. 81-87).

4.3 Mobile carrier ISEs

Mobile carrier (or liquid membrane) electrodes have sensors which are either neutral carriers or are positively- or negatively-charged ion-exchangers (52). Many of the neutral carrier sensors should be familiar to the microbiologist as the ionophorous antibiotics (e.g. valinomycin for potassium (20)). However, Simon's laboratory has synthesised a variety of further specific carriers for the alkaline earth cations and protons (e.g. 61). In the earliest examples (Fig. 2f) the sensor was dissolved in a hydrophobic solvent, which was allowed to permeate inert membranes (e.g. glass fibre). However, as a result of the work of Moody and Thomas (e.g. 60), among others, the sensor was dissolved with appropriate plasticisers in a plastic matrix membrane, usually of polyvinyl chloride (PVC). The composition of such membranes, especially the nature of the mediator, markedly affects the electrode's performance (e.g. 91). Electrical contact can either be made via an inner filling solution (Fig 2g) or, in solid-state versions, directly (Fig 2h). The latter coated wire electrodes (CWEs (e.g. 62)) lack inner filling solutions, the plastic membrane being applied directly to the electrode, which occasionally is first coated with a thin layer of a polymer (e.g. polyvinyl alcohol) soaked in the inner filling solution.

A further very significant version of the mobile carrier approach is to attach various other ligands to existing mobile carriers, e.g. the Crown carriers (92). Thus model haptens (e.g. dinitrophenol) or true immunogens (e.g. bovine serum albumin) have been immobilised at the electrode (78) and are able directly to measure their specific antibodies (or vice versa). This direct approach is worthy of further examination, initially perhaps with the sugar-specific lectins (93).

4.4 Sensitised ISEs

Although the above ISE techniques provide a wide range of measurement possibilities, these can be extended to encompass many more determinands by various sensitisation procedures exploiting physical or biochemical reaction links. The sensitised ISEs (SISEs) include the gas-sensing electrodes and the enzyme-(substrate-) sensing electrodes.

4.4.1 Gas and volatile probes. The ammonia and carbon dioxide gas-sensing electrodes are linked to the pH electrode (Fig. 2i). Although all have been commercially available for some time it is perhaps germane to draw attention to the autoclavable variety recently made available from Ingold (94). In the case of the carbon dioxide sensor a bicarbonate buffer solution surrounds the pH electrode, and this is separated from the determinand solution by a gas-permeable membrane. Dissolved carbon dioxide is in reasonably rapid equilibrium with bicarbonate ions; entry of dissolved gas into the entrapped bicarbonate buffer will disturb the equilibrium, producing a pH change. At steady state this will represent a particular dissolved gas concentration and the Ingold electrode has further elegant modifications to improve performance and to ease calibration (94).

A further modification of this type of approach is the air-gap electrode (Fig. 2j), which separates the determinand solution from the sensitised or ordinary ISE by an air space (70), but which is unlikely to be of great utility in analysing

the gas/soluble phase equilibria in fermentations. Significantly, PVC-membrane electrodes have been used to monitor the gaseous oxides of nitrogen (72) (and cf. (322) for a polarographic method), which suggests that it will be possible to monitor directly some of the more volatile organic compounds in the fermenter gas phase using similarly-constructed electrodes.

4.4.2 Enzyme-sensitised ISEs. There are potentially many enzyme-linked (or sensitised) ISE probes (i.e. ESISEs). The ISEs of most importance are perhaps pH (including ammonia and carbon dioxide), ammonium, phosphate, iodide, redox (and oxygen) and many of the organically-selective ISEs. Since these ISEs provide the means to measure the substrate or product of a very large number of enzymes, there appears little point in cataloguing the possibilities, since the microbiologist will be aware of the available enzymes, reactions and co-factors etc. and the electrochemist can easily discover them (e.g. 95, 138). Table 2 summarises the basic types of mechanisms involved and draws attention to examples of the relative few that have been constructed. It is perhaps more appropriate to discuss some of the key features of such methods.

TABLE 2. Bioselective probes

MICROORGANISM- AND ORGANELLE-SENSITISED ELECTRODES

The transduction of the effect of determinand on the microbial production/release of the determinand(s) of a variety of primary electrochemical transducers, including fuel cell and ISE types e.g.:-

acetate (313)
phenols (315)
nitrate (312)
succinate (288)
alcohols (314)
antibiotics (316)
vitamins, amino acids and cofactors (158, 159)
biomass (160)

ENZYME-SENSITISED POTENTIOMETRIC AND AMPEROMETRIC ELECTRODES

The substrate, product or co-factor of a (multi-) enzyme reaction as the determinand of the primary electrode, which includes potentiometric redox electrodes, and amperometric cathodic (e.g. polarographic/voltammetric and self-polarising galvanic oxygen-type electrodes) or anodic (e.g. peroxide anode) electrodes e.g.:-

glucose (106-107, 124-127, 130, 140, 141, 278-279, 288-289, 295-297, 301-303, 313, 316-318)
galactose (285)
lactate (105, 286, 304)
ascorbic acid (114)
phenols (98, 298)
xanthine (287)
NAD(P)H linked enzymes (e.g. 288)
gluconate (305)
alcohols (288)
amino acids (277, 288)
cholesterol (128, 287)
pyruvate (287)
oil in water (291)

Regenerating co-substrate/co-factor systems (e.g. 140,141,300) and co-product removal systems (e.g. 302) have been developed for this class of bio-selective probes.

ENZYME-SENSITISED ISEs

The substrate or product of a (multi-) enzyme reaction as the determinand of the primary ISE including:-

TABLE 2 contd...

TABLE 2 contd.

NH_3 and CO_2 gas sensing pH electrodes e.g.:-

creatine (99)
amino acids (113,288)
urea (102,104-105,281,284)

pH electrode e.g.:-

penicillin (101-102)
glucose (102)
lipids (129)

NH_4^+ electrode (nonactin and glass types) e.g.:-

urea (103,282-283)
amino acids (282)

CN^- electrode e.g.:-

amygdalin

ENZYME-SENSITISED ISFETS

Directly ion-sensing ISFETS or ion-selective membrane modified ISFETS are being converted to "enzyme transistors" by analogous methods e.g.:-

"penicillin transistor" (148)

ENZYME-SENSITISED CONDUCTOMETRIC PROBES

Many enzyme reactions change the measured conductivity of the substrate-product solution e.g.:-

antibiotics (163)

ENZYME-SENSITISED THERMISTORS

Enzymic reaction enthalpy can be used as the specific transduction mechanism (e.g. 47) for many potential bio-selective probes.

ENZYME-SENSITISED PIEZOELECTRIC TRANSDUCERS

Coated piezoelectric ctystals (80) could be used to transduce the substrate-product of enzyme reactions.

ENZYME GRAFT OR MODIFIED ELECTRODES

Attachment or grafting of enzymes to electrodes can provide a more directly transducing enzyme electrode (e.g. 292) for many potential bioselective probes (viz. 308).

ENZYME/SUBSTRATE-SENSITISED ELECTRODES

Enzyme substrates can be attached to electrode surfaces to transduce the activity of enzymes (viz. 308) e.g.:-

hydrolases (99)

ENZYME-SENSITISED OPTICALLY-TRANSPARENT ELECTRODES

Optically-transparent electrodes (e.g. 320,321), chemically modified with redox or pH, etc. dyes could have some advantages when used as the primary transducer of bio-selective probes.

LIGAND-SENSITISED ELECTRODES

Simple binding of determinand to ligand- (e.g. enzyme-, antibody-, antigen-, enzyme inhibitor-, hapten-) modified electrodes avoids the biochemical reactions associated with other sensitisation methods (e.g. 79, 308).

Many of the reported ESISEs have required the cofactor and non-determinand substrate (even the enzyme) to be added to the determinand solution (e.g. 96-100), which, in truth, is an ISE-linked off-line assay procedure and is clearly of diminished benefit to on-line fermentation analysis. The majority of true ESISEs have accordingly used enzymes with single substrates (the determinand) and no cofactor or further substrate requirement (the only other requirement being an appropriate pH and metal ions etc.). Classically the penicillin (101) and urea (102) ESISEs are of this type and a variety of other similar enzymes could be used (e.g. 65-68).

The simplest construction involves macro-entrapment of the enzyme solution in a dialysis membrane sac surrounding the ISE (101,104) and in this respect many commercial gas-sensing electrodes may be converted for ESISE use (102,104). Ideally the enzyme compartment should be made as thin as possible. This ensures more rapid attainment of the steady-state concentration of the measured product, (rapid response), which itself will leak from the compartment. The sensitivity of the overall electrode is clearly related to the specific activity and concentration of the enzyme.

Various more sophisticated enzyme entrapment and immobilisation methods are available. A common entrapment procedure is to add the enzyme solution to a gelling acrylamide, gelatin or agar solution (e.g. 105,106). This results in rather thick enzyme membranes which can all too rapidly lose their enzyme by diffusion (e.g. 68). A more elegant approach involves the entrapment of the enzyme in a synthesised ultrafilter membrane of variable porosity and relatively good mechanical properties (107).

Other excellent methods of entrapment are available but have not as yet apparently been used (e.g. 108-112). Immobilisation by entrapment clearly offers the least chance of enzyme denaturation. However, bifunctional chemical modifying reagents are finding more widespread use. Glutaraldehyde/enzyme/inert protein membrane preparation methods have been, at this early stage of ESISEs, very popular and may be made relatively thin, although friable and partially denatured single and mixed enzyme membranes have been prepared (e.g. 113). Similar methods enable enzymes to be attached to inert (e.g. polyamine) filters (114). Glutaraldehyde is a rather crude immobilisation reagent, but alternative methods exist for chemical attachment to a wide variety of surfaces, including most plastics, natural polymers (e.g. cellulose and collagen), metals and semiconductors (117-123); these often gentler procedures will find increasing use (e.g. 124-130). Techniques for preparing enzyme multi-layers (131) and asymmetrical layers (127) for multi-enzyme sensitised ISEs have been developed. It is also highly significant that enzyme immobilisation methods (and intra-chain cross-linking (e.g. 132-135)) tend to slow the quite high denaturation rates of mesophilic enzymes (136-138), such that enzyme membranes may be expected to achieve sufficient longevity (under optimal conditions 1 to 14 months (e.g. 67)) for use in process control. More naturally stable enzymes (e.g. some enzymes from thermophiles (e.g. 139)) are already of interest both to the microbial technologist and the bioelectrochemist.

It should be noted that some of the above methods do not protect against unfavourable environmental conditions. ESISE destruction by microbial proteases is particularly to be expected, and those methods in which the probe is protected by an ultrafiltration membrane or other steric occlusion methods should be used. It is also worth noting that considerable retention of cofactors is expected by use of ultrafiltration approaches, making it possible to design a wider range of ESISEs. Particularly significant is the real possibility of regenerating redox couples in otherwise reagent-requiring EISESs (140,141). Avoidance of sub-optimal assay conditions andinhibitors is also important (see flow methods). Reappraisal of the enzyme-sensitisation approach in favour of more direct primary coupling methods is of clear benefit and should be receiving more attention.

As far as the fermentation technologist is concerned, the optimal construction of ESISEs should be based on either cheap disposable complete transducers or on more expensive ISEs with cheap replacable enzyme membranes with a long shelf-life. The latter clearly has commercial and practical benefits and should be borne in mind when developing semiconductor micro-transducers. In any event, in our view commercial suppliers will have to reassess their ISE prices to encourage a healthier, larger and wider market.

4.5 Semiconductor field effect devices

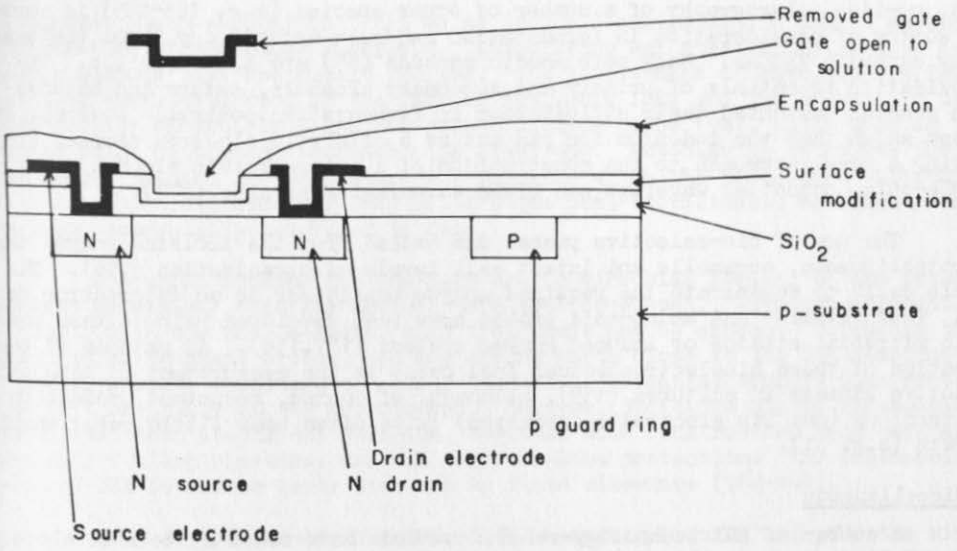
(Bio)chemically-sensitive semiconductor devices (CSSDs) are extremely important prospective devices; multitransducer chips with inbuilt integrated circuitry are already conceived. The basic mechanism of these devices may be classified as primary, mobile carrier or sensitised (see above). Ion-selective field-effect transistors (ISFETs) are a modified version of the metal oxide field-effect transistor (MOSFET) devices and are the present major starting point in CSSD application to ISE technology (323). However, the extent of chemical modifications that are presently being undertaken extend much further than any prior electronic classification, and it is not too difficult to perceive that the information capacity of 'biomolecular semiconductors' will be of considerable importance in the future development of microelectronics.

The surface field of conventional ISFETs is produced by polar or ionic material on the surface of the FET and, for example, simple exposure of its gate will render the device sensitive to H^+, K^+ and Na^+ (63,64). Clearly basic double layer theory (e.g. 143) applies to CSSD electrolyte-semiconductor interfaces (144, 145), as it does to similar ISE interfaces and as long as ionic currents through the semiconductor gate can be considered to be negligible, a similar treatment may be expected to apply (e.g. 64, 145). Accordingly the field generated in the insulating layer (Si_3N_4 or SiO_2 etc.) affects the flow of current between the source and drain (Fig. 3)

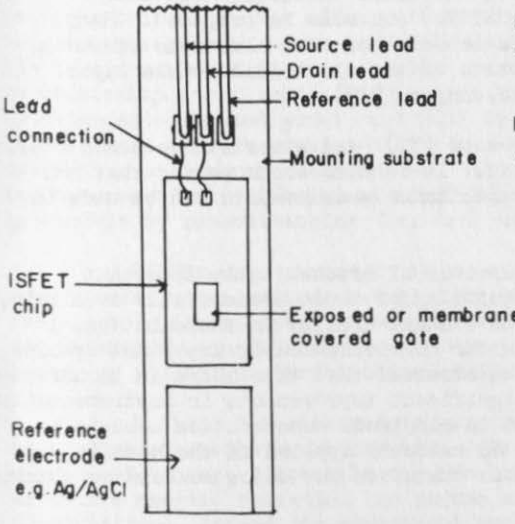
Compensation for electrochemically-driven changes in drain current by change in applied voltage is the best means of measurement, although the analogous, constant voltage, current measurement is also possible (64). There is presently much deliberation concerning the need of a reference CSSD for the various types of 'working' CSSDs and especially for the electroneutral modifications (146,147). As well as pH- and pK-sensitive ISFETs (64), Pd-MOSFETs sensitive to hydrogen, and ammonia (148), oxygen (149), sulphate (150), alcohols (151), carbon dioxide (325), carbon monoxide (326) and various volatile hydrocarbons (326,327) MOSFETs are under development as primary CSSDs. Many mobile carrier and sensitised varieties are also possible (see 64), making use of more conventional ISE technology, including the "enzyme transistor" (152)

4.6 Amperometric methods

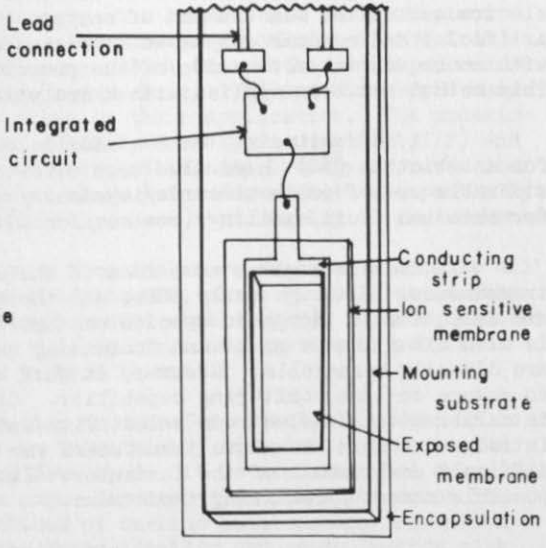
A variety of non-ISE, amperometric methods are included here because in fermentation terms they provide electrochemical probes of considerable merit. Fermentation technologists tend to employ the self-polarising galvanic oxygen probe, in preference to the Clark-type polarographic system. Since both methods pose considerable problems, especially when applied in fermentation, it is worthwhile noting some more recent improvements to the polarographic types. It is often overlooked that the measurement of hydrogen with an 'oxygen' electrode in microbial cultures is possible by altering the polarising potential (73), and it is significant that drift and noise can be minimised by employing electrochemical conditioning cycles (74). Pulsed-polarographic regimes are predicted to improve the sensitivity drift and surface mixing properties of these electrodes, and headway has been made in their practical implementation (75). Improved measurement of dissolved oxygen tension, even in thixotropic or poorly-mixed broths is therefore a possibility.



Surface modified gate ISFET



Sensing tip of an ISFET probe



Thick film hybrid ion sensing electrode

Fig. 3. Exploitation of field effect transistors as and in ISEs.

Indeed, on-line polarography of a number of other species (e.g. 153-155) is certainly worthy of consideration in fermentation and such methodology forms the basis of many cathodic ESISEs. Many more anodic methods (65) are also feasible. The high oxidation potentials of primary and secondary alcohols, esters and ketones has in general prevented their exploitation in fermentation control. However, it has been shown that the iodonium ion can act as a catalytic electron carrier (76), providing a novel approach to the construction of a polarographic alcohol probe. A triple-pulse potential waveform has given encouraging results (272).

The use of bio-selective probes can extend from the isolated enzyme through the proteoliposome, organelle and intact cell levels of organisation (156). The use of whole cells to regenerate the required enzyme sensitiser is an interesting approach. Thus vitamin- and amino-acid probes have been developed using either auxotrophic microbial strains or induced enzyme systems (157,158). An example of the application of these bioelectrochemical fuel cells is the measurement of metabolically-active biomass in cultures (159), although, of course, reductant production by microorganisms (and its electrodic oxidation) quite often bear little relationship to growth (e.g. 31).

4.7 Miscellaneous

A number of ISE technology-related methods have recently been developed. The use of piezoelectric (quartz) crystals is common in environmental transducers (e.g. pressure). The characteristics of crystal oscillation are significantly changed after surface adsorption of a variety of molecules (80) to provide a powerful transducer mechanism. The coating of such quartz crystals for selective measurement of a number of gases (e.g. ammonia, hydrogen chloride and hydrogen sulphide) and volatiles (80) is likely to contribute significantly to bio-transducer technology.

Enzymic activity can be monitored by ISEs although this will require intricate arrangements if it is to be achieved on-line. However, substrate electrodes for the measurement of enzymes (SSISEs) can also be prepared. Many artificial and natural enzyme substrates can be adsorbed onto electrode surfaces, with consequential alteration of the properties of the electrical double layer. This method has been applied with hydrolytic enzymes (79).

A conductimetric carbon dioxide sensor (52) and a resistance-based sensor for antibiotics (52) have also been reported. It is also worth noting that considerable use of conductimetric/impedance/capacitance measurements can be made in fermentation fluid handling (see section 8).

The above gives some idea of the armoury of biochemically important transducers. Although early ISEs, and the majority of those commercially available, are selective to inorganic species and are of evident utility in fermentation, it is also clear that a great and increasing number of biochemically important species are directly measurable. However, it must be stressed that this alone is insufficient to ensure on-line monitoring capability. Significant improvements in instrumentation, in calibration, in electrode selectivity and in electrode construction need to be introduced before the above transducers can be reliably applied in the hostile and difficult environment of the fermenter. It is therefore our major concern to examine possible means of realising these aims.

5. PRACTICAL CONSTRUCTION OF ISEs FOR USE IN FERMENTATION

5.1 Batch probes

Microbiologists are understandably preoccupied with sterilising all devices

that contact the fermentation fluid so as to avoid contamination and to ensure containment of biohazardous materials. Autoclaving and steam sterilisation are the only methods that are widely trusted. The development of autoclavable ISEs is a major task, evidenced by the lack of available ISEs and the efforts to provide a commercially acceptable dissolved carbon dioxide probe (94), which used as its starting point an already-acceptable pH electrode. Autoclaving of course involves considerable changes in temperature and pressure which the probe must withstand, and this perhaps explains why only solid-state heat-sterilisable membrane probes have been commercially developed to date.

A special problem in long-term use is microbial fouling of the probe surfaces; mechanical scraping devices have been developed. Although apparently acceptable with the pH electrode, such treatments may be expected to change the properties of membrane probes, and any moving parts close to the ISE membrane will of course generate streaming and other electrical potential changes (see section 7), which will seriously affect measurement. However, judicious use of ultrasonics or even intermittent electrical currents, together with construction from more appropriate anti-fouling plastics, may well provide good protection. The engineering design of ISE fermenter ports etc. can be found elsewhere (164-168).

Many of the non-rigid membrane ISEs contain liquids and pose the kind of problem which has been overcome in the oxygen, pH and reference electrodes. These should not then require reinvention. Those direct electrical contact types do not pose this problem. However, the construction and composition of the permselective membranes requires considerable attention. Reinforcement with inert plastic mesh in the interstices of which is the permselective membrane is an apparently little tried but valid approach (169); the problem is also overcome by the use of coated wire-type electrodes.

Autoclaving of PVC has undesirable effects on its structure, and other materials which are more resistant to wet heat must be introduced. The most critical problem is the effect of autoclaving on the sensors and mediators, and this is particularly acute in mobile carrier ISEs; evaporation of mediator from the liquid membrane is particularly troublesome. Such transducers could of course be chemically sterilised. Many sterilising agents (e.g. 170) are chaotropic and/or detergent-like and great care must be taken in their application. The undesirable effects of such treatment are therefore likely to be poisoning (171,172) and extraction of membrane components. Determinand conditioning broadly improves the performance of all ISEs and although some of these poisoning effects are partially reversible by reconditioning they will obviously cause rapid ageing of the membranes.

EISESs have purposely been omitted from the above discussion because all such sterilisation methods are clearly less applicable, although it is noteworthy that certain immobilised enzymes are remarkably heat-stable (288). Gentle chemical sterilisation methods such as exposure to wide spectrum antibiotics or ethylene oxide, or radiation sterilisation, could perhaps be used.

Methods for electrode sterilisation do therefore exist. However, it is to be doubted whether such practice will be industrially acceptable, unless pre-sterilised, cheap, disposable sensors are developed. It is apparent that ensuring effective aseptic operation has become a major impediment to ISE application in fermentation. Indeed the additional problems of on-line calibration, prevention of sensor poisoning and probe fouling, the impracticality and contamination risk of a large number of fermenter probe ports and the difficulty of probe replacement/servicing might seem to preclude any forseeable widespread and reliable ISE application. However, such a prognosis is based on the misconception that probes can only be introduced through numerous fermenter ports, directly into the fermentation mixture. If this historical conception is discarded a number of appropriate alternatives may be seen to exist.

5.2 Flow methods

"Only by constant flow does a mountain stream remain clear"

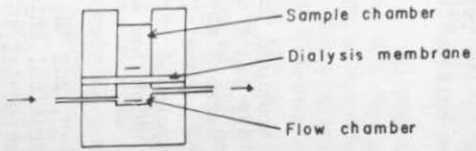
Japanese Proverb

Continuous flow-line (163-168) sampling of the fermentation through a one-way valve into a non-sterilised array of minature flow probes overcomes most of the above problems. On-line calibration is now possible, and as long as the analyser 'dead volume' is small and the sample is not returned, the major problem remaining is probe fouling and poisoning. In research-scale fermentation, loss of even a relatively large proportion of culture for analysis purposes may not be considered important, and in larger fermentations the amounts involved are certainly insignificant. Removal of samples from cultures has been shown to effect significant and progressive physiological changes (e.g. as a consequence of changes in aeration and temperature). Whilst feedback control concerns the engineer in terms of the time-lag that such external loops produce, the relative sloth of most microbial systems and the necessary rapid sampling should surely preclude major problems.

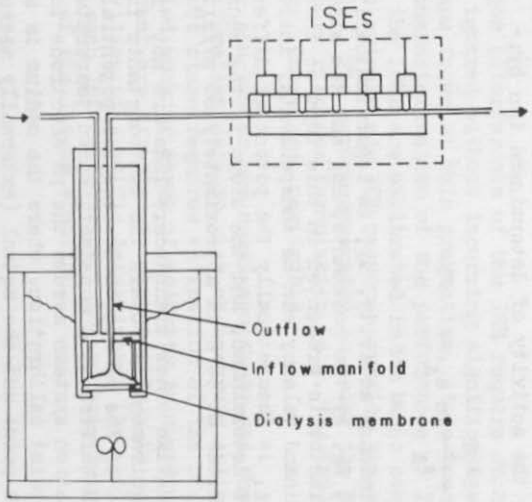
Flow dialysis (173) provides a versatile continuous sampling method which has found use in microbiological research (e.g. 174-176) and sporadic use in on-line reagent-requiring analysers (177,273). The conventional two-compartment cell consisting of a reaction compartment separated from the flow compartment by some form of ultrafiltration membrane (Fig 4a) can be converted for fermenter use by removal of the reaction compartment, presenting the membrane-separated flow compartment directly to the fermentation fluid (Fig. 4b). Judicious design of the dialysis half-cell probe can eliminate the need for mechanical stirring, the flow properties of the cell inflow and outflow providing sufficient turbulent mixing for uniform continuous sampling. The dialysing surface area:volume ratio of the half cell, the permeability properties of the membrane and the fluid flow rate all contribute to the dilution of the sample in the cell effluent (173). Since the major purpose of the membrane is to exclude all microorganisms, permeability to small molecules can be high and therefore overdilution becomes irrelevant. The steady-state response time of the cell itself is linearly dependent on flow rate, and if the analysing transducers are placed downstream a further proportional delay is added. Cells should be designed such that typical dilutions of 50- to 1000-fold can be achieved by changes in flow rate.

The logarithmic response of ISEs enables measurement over at least 3 to 4 decades of determinand activity with routine limit of detection close to 1 μM. This range is ideally suited to fermentation processes, since most environmental biochemicals are present within the millimolar to molar range and generally only vitamins and bases are provided in the micromolar range. The above dilutions are not only possible but necessary in certain cases; indeed there may be a positive advantage in cases where the effects of poisons are also minimised by dilution. The cell influent medium can also be chosen (e.g. for pH and ionic strength) for optimal performance of the electrode array (see section 6). This approach successfully overcomes all of the above problems, providing aseptic operation without electrode sterilisation, on-line calibration, variable sample dilution, minimisation of electrode poisoning, avoidance of transducer fouling, easy probe removal and servicing, avoidance of electrical interference and optimal assay conditions. Still further improvement of this approach in the minimisation of response time at all flow rates can be achieved by accommodating the transducer battery in the flow cell compartment (Fig. 4c). Flow dialysis is therefore capable of providing an effective ISE analysis system suitable for real-time control of microbial processes.

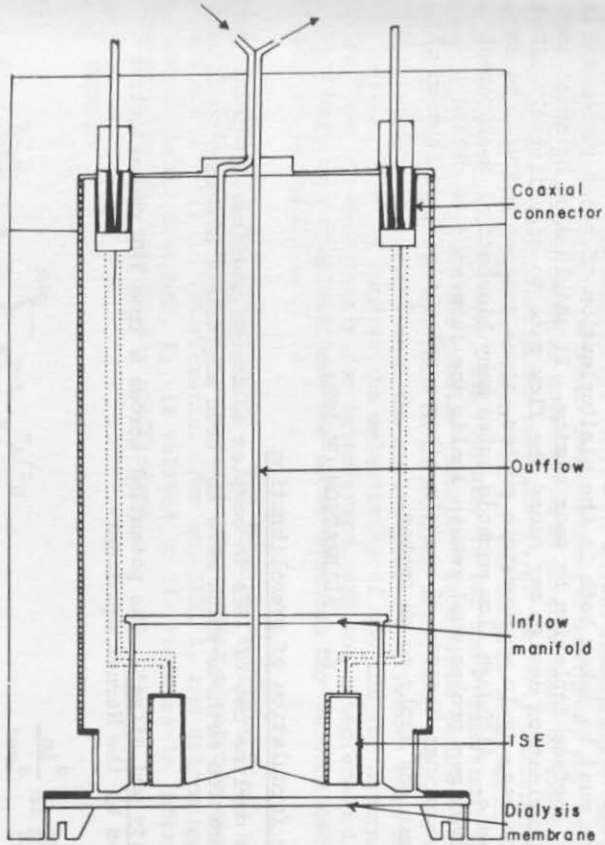
All of the flow methods ideally require considerable reductions in transducer size. True ISE sensing surfaces can be reduced to around 1 to 2 mm diameter (in many cases less) without significantly affecting measurement properties (e.g.



(a) Flow dialysis cell



(b) Flow dialysis half-cell with downstream ISEs



(c) Flow dialysis half-cell with integral ISEs

Fig. 4. (a) Continuous sampling using flow dialysis cell. (b) Principles, with ISEs in the outflow stream, and (c) within the flow compartment of the dialysis cell.

178,179). Reduction of ISE size increases their output impedance and care must be exercised in the choice of instrumentation (see section 7). However, the sensitivity of amperometric methods is directly dependent on electrode surface area and considerable care must be taken both in the miniturisation of these probes and in the avoidance of surface depletion by poor mixing. It should also be noted that sub-optimal miniaturisation design may cause the flow rate to significantly affect sensitised-ISE probes, as in amperometric probes, since determinand will be consumed in both cases. Although flow methods solve many biochemical measurement problems, dissolved gas probes must remain inside the fermenter to obtain representative sampling; because of certain other complexities (see section 6) similar considerations apply to pH probes.

6. CALIBRATION OF ISEs

6.1 Algorithmic formulation of precalibration

Direct on-line use of ISEs in complex solutions requires the development of special calibration methods which have not been adequately attended to in most ISE methodologies

6.1.1 Sensitivity and offset. The potential across a true ISE permselective membrane is given by the Nernst equation:-

$$E = \text{constant} + \frac{RT}{zF} \ln \frac{a_{in}}{a_{out}} \qquad (1)$$

Where R, T and F have their usual meanings, z is the unitary charge of the determinand ion and the activities of determinand in the internal reference and external solutions are a_{in} and a_{out} respectively. If a_{in} and the potential of the reference electrode remain constant the potential difference between the two half-cells is related to the activity of determinand ion by:-

$$E_A = Eo_A + \frac{S'_A T}{z_A} \ln a_A \qquad (2)$$

where determinand activity, a_A, is logarithmically related to the measured potential difference, E_A, by two constants, Eo_A and the slope, S where $S' = R/F$ and $S = S'T/z_A$. The slope factor is presented in this form so that temperature (in degrees absolute) and determinand valency can be independently introduced. Thus, the linear offset factor, Eo_A, is theoretically the potential difference at an extrapolated zero activity of determinand, and the slope is the sensitivity factor, which for an ideal electrode at 30°C is approximately 60 mV/z_A.

Unlike other transducers ISEs are not "factory calibrated", and the minimum requirement to obtain the two constants is a 2-point calibration, which in the case of the pH electrode requires potential difference measurements in two standard pH buffers at a constant, known temperature. However, many fermenter instrumentation systems assume the pH electrode to have ideal sensitivity and only allow a 1-point calibration, where the medium is adjusted close to the desired control set point and the actual (externally measured) pH is entered to obtain the offset factor. Periodic sampling and external pH measurement then allow the instrumentation to be reset against drift.

The measured potential difference of ISEs versus a reference electrode is also dependent on medium ionic strength and interfering ions. The electrochemist tends to characterise ISEs in constant ionic strength media of sufficient

strength to minimise noise and other extraneous effects, and containing only the determinand and perhaps a few interfering ions. These calibration methods have been well covered elsewhere (e.g. 180). In direct measurement the microbiologist cannot use manufacturer's constant ionic strength mixtures, nor can anything but a dynamically changing complex solution with interference be expected. Where ISEs have been used in microbiological research (e.g. 50, 181) calibration requires that the known pI changes are performed in the complex interfering solution. In the presence of interferents, ISE response is far from Nernstian; the slope and offset factors obtained are greatly different from those obtained under "ideal" conditions (Fig 5a) and can only be considered valid over a narrow working range (usually around 1 pX unit) when the activity of interferents must remain constant (Fig. 5b-d). Thus as long as the selectivity of the ISE for determinand is much greater than its selectivity for interferent, and the interferent is not present in large varying concentrations, the above approach may be considered valid, if not completely reliable.

6.1.2 Selectivity and interference. The Nicolski equation (183) is a semi-empirical expansion of the Nernst equation describing electrode response to determinand as well as interferents. For any ISE, A, and determinand activity, a_A, the electrode response, E_A, is related to its response to interferents, B of activity a_B by the selectivity coefficient

$k^{pot}_{A,B}$ where:-

$$E_A = Eo_A + \frac{S'_A T}{z_A} \ln a_A + \sum_{B=1}^{B=m} k^{pot}_{A,B} \; a_B^{z_A/z_B} \qquad (3)$$

The purpose of any calibration of an array (A=1 to A=n) of ISEs is to estimate the constants Eo_A, S'_A and $k^{pot}_{A,B}$ for interferents.

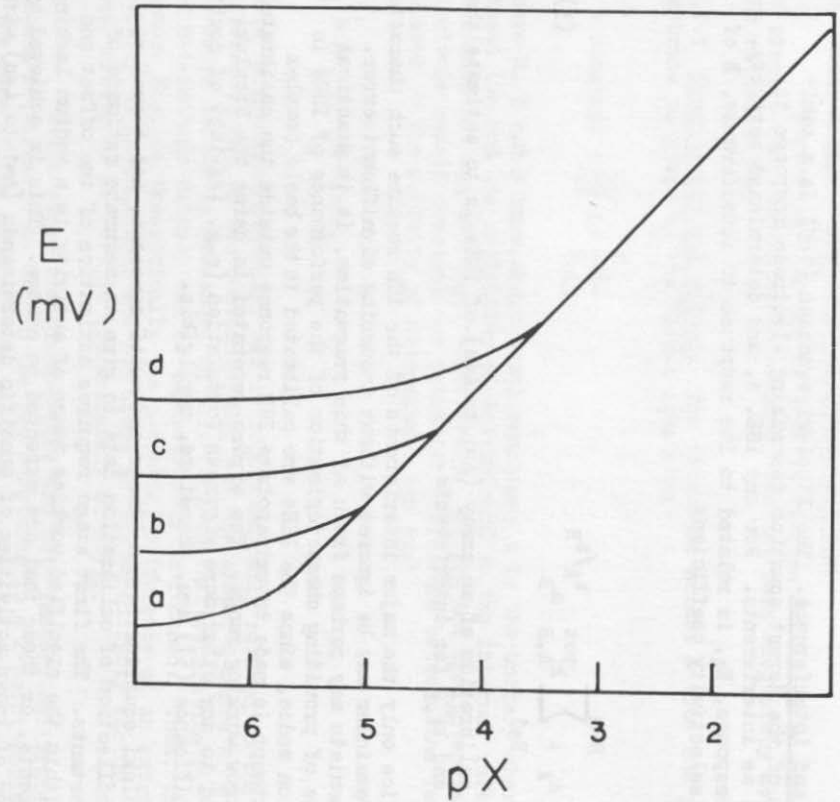
In practice only the major interferents of the ISE require such characterisation, and the remainder may be ignored without incurring significant error. Although electrochemists may perhaps frown at this pragmatism, it is practical within the confines of providing characterisation of the performance of ISEs in complex fermentation media, since the ISEs are calibrated in the basic complex solution and no attempt is made to extrapolate ISE response outside the calibrated and relatively narrow working range. The errors generated in using the Nicolski equation as opposed to any other more rigorous formulation (e.g. 184-185) of doubtful practical significance (51) are, therefore, negligible.

The Nicolski equation is non-linear and multi-constant, and therefore requires careful collection of calibration data to give an accurate estimate of the underlying constants. The first stage requires estimation of the offset and slope constants within the specified working range of activity in a medium lacking the gross interferents, or those that are expected to change. This is achieved by sequential addition of known activities of specific determinands (A=1 to A=n) with fresh medium being introduced for each ISE calibration. Logarithmic transformation then allows least squares fitting of the linear equation.

Let $a'_A = \ln a_A$

Therefore $E_A = Eo_A + S_A \cdot a'_A$

For V observations (K)-



$$E = E_o + \frac{RT}{zF} \ln \left[a_A + \sum_{B=1}^{B=m} k^{pot}_{A,B} \, a^{Z_A/Z_B} \right]$$

Fig. 5. The response of a typical ISE to its determinand in the presence of increasing concentrations (traces a to d) of an interfering ion.

$$S_A = \frac{\sum_{K=1}^{K=V} E_{A,k} \cdot a'_{A.k} - V_A \cdot \bar{E}_A \cdot \bar{a}'_A}{\sum_{K=1}^{K=V} a'^{2}_{A,K} - V_A \cdot \bar{a}'^{2}_{A}} \quad \text{and}$$

$$Eo_A = \bar{E}_A - S_A \cdot \bar{a}'_A \qquad (4)$$

The medium is then supplemented with low activities of determinand and the activity of the B'th ion is sequentially increased whilst the A'th ISE is monitored for interference. Nicolski equation for any interferent can now be scaled into a linear, single-constant, least-squares fit:-

$$E'_{A,B} = \exp\left(\frac{E_{A,B} - Eo_A}{S_A}\right)$$

$$E'_{A,B} = k^{pot}_{A,B} \cdot a_B + \text{unknown}$$

and the least squares fit for V observations is:-

$$k^{pot}_{A,B} = \frac{\sum_{K=1}^{K=V} E'_{A,B,K} - V_{A,B} \bar{E}'_{A,B} \bar{a}_{A,B}}{\sum_{K=1}^{K=V} a^{2}_{A,B,K} - V_{A,B} \bar{a}^{-2}_{A,B}} \qquad (5)$$

A matrix of selectivity coefficients may therefore be calculated for the ISE array, including interferences:-

1	$k_{1,2}$	$k_{1,3}$	$k_{1,4}$	....	$k_{1,m}$
$k_{2,1}$	1	$k_{2,3}$	$k_{2,4}$	....	$k_{2,m}$
$k_{3,1}$	$k_{3,2}$	1	$k_{3,4}$	....	$k_{3,m}$
$k_{4,1}$	$k_{4,2}$	$k_{4,3}$	1	....	$k_{4,m}$
.	.	.	.		.
.	.	.	.		.
.	.	.	.		.
.	.	.	.		.
$k_{n,1}$	$k_{n,2}$	$k_{n,3}$	$k_{n,4}$	....	1

6.1.3 Microbial relevance of solution complexation and buffering. ISEs respond to activities and not to concentrations. Further, any chelated (or buffered) fraction is not measured. The 'sensors' possessed by microorganisms are, not

surprisingly, analogous and the appropriate ionic fraction would therefore appear to be measured. However, certain situations can arise during calibration, calibration data interpretation or during monitoring, when the free activity and bound concentration should be independently known. Ionic activities can be related to (free) concentrations by various relationships having their roots in the extended Debye-Hückel formulation (e.g. 186,187). The total ionic strength (I) of the solution enables estimation of the activity coefficient (γ), which in turn relates activities a_A to concentrations c_A where a straightforward interpretation is:-

$$I = \tfrac{1}{2}\sum_{A=1}^{A=n} c_A \cdot z_A^2 \quad \text{and} \tag{7.1}$$

$$\log \gamma = -z^2 \frac{Y\sqrt{I}}{1 + 1.5\sqrt{I}} - 0.2I \tag{7.2}$$

(N.B. if the total ionic strength is greater than 100 mM then other formulations must be used for greatest accuracy)

where Y=0.507 at 20°C and where

$$a_A = \gamma c_A \tag{7.3}$$

Total ionic strength (I) can be either predicted from the 'calibrating' additions (using the first expression in equation (7.1)) or be estimated, with reasonable error in assumptions, by additional conductivity measurements or mass balancing.

Buffered ionic species (e.g. calcium, magnesium, protons) are often present in fermentation media, and certain measurable ionic species may be inherently buffers (e.g. carboxylic and amino acids). Expression of stock calibration solutions in total concentration terms will therefore require conversion to free activities, in some cases. Otherwise in a typical case of error, calibrating data may be obtained that characterised ISE interference when in fact solution complexation was responsible. It is fortunate that in this situation, the cation-selective ISEs will usually only experience cationic interference, whereas complexation is largely to be expected between cations and anions (and the reverse is likely to be true for anion-selective electrodes). Chelation is also important microbiologically, since, if the ISE is being used to determine either substance utilisation or production by the microorganism, it is clearly important to convert free activities into total concentrations. Characterisation of the solution properties of the medium is therefore just as important as calibrating the ISEs. Although theoretically this could easily become very complex (188-190), practically a number of working situations can usefully be identified:

(a) Calibration of ISEs with known pI buffers
(b) The natural presence or production/removal during fermentation of buffers/chelators, where these are not independently measured
(c) The measurement of both (or all) chelating species.

Commonly both microbiological media and ISE calibration solutions employ buffers to maintain steady activities of determinand species. The preparation of such buffers for pH and other cations has been described elsewhere (191). Various pI buffers can be adequately provided over typical microbial and ISE working ranges by mixing various concentrations of ligand and ion of interest according to, for example, the Henderson-Hasselbalch expression:

$$pI = pK_a + \log \frac{L_f}{IL} \tag{8}$$

where pK_a is the logarithimic association constant and IL and L_f are the

concentrations of bound and free ligand (respectively), which may be expressed in terms of the conjugated ion by use of the law of conservation of mass. Treatments of multiple ionisations of, for example, pH buffers are well covered in biochemical texts. However, analysis of multiple conjugations and dynamic assessment of their metabolic alteration has received little attention.

If the case of two ligands of concentration L_1 and L_2 with association constants, Ka_1 and Ka_2 and one conjugation ion of concentration I, with conjugates of concentrations $I'L_1$ and $I'L_2$ are taken, the following formulae relating their free (f), bound (b) and total (t) concentrations may be written:-

$$Ka_1 = \frac{I'L_1}{I_f \, Lf_1} \quad \text{and} \quad Ka_2 = \frac{I'L_2}{I_f \, L_{f_2}} \tag{9.1}$$

$$\text{that is} \quad Lf_1 = \frac{I'L_1}{I_f \, K_{a_1}} \quad \text{and} \quad Lf_2 = \frac{I'L_2}{I_f \, K_{a_2}} \tag{9.2}$$

Law of conservation of mass:-

$$L_{t_1} = L_{f_1} + I'L_1 \quad \text{and} \quad L_{t_2} = L_{f_2} + I'L_2 \tag{9.3}$$

$$\text{That is} \quad I'L_1 = L_{t_1} - L_{f_1} \quad \text{and} \quad I'L_2 = L_{t_2} - L_{f_2} \tag{9.4}$$

Combining 9.2 and 9.4 and rearranging gives:-

$$I'L_1 = \frac{L_{t_1} \, I_f \, K_{a_1}}{1 + I_f \, K_{a_1}} \quad \text{and} \quad I'L_2 = \frac{L_{t_2} \, I_f \, K_{a_2}}{1 + I_f \, K_{a_2}} \tag{9.5}$$

For both ligands in solution together the law of conservation of mass gives:-

$$I_f = I_t - I'L_1 - I'L_2 \tag{9.6}$$

Substituting from 9.5 into 9.6 and rearrangement gives:-

$$\frac{I_t}{I_f} = 1 + \frac{L_{t_1} \, K_{a_1}}{1 + I_f \, K_{a_1}} + \frac{L_{t_2} \, K_{a_2}}{1 + I_f \, K_{a_2}} \tag{9.7}$$

Such a description was developed to calibrate a calcium electrode in the presence of of succinate and phosphate (192). Equation 9.7 may be expanded to the more general case:-

$$\frac{I_t}{I_f} = 1 + \sum_{i=1}^{i=n} \frac{L_{t_i} \, K_{a_i}}{1 + I_f \, K_{a_i}} \tag{9.8}$$

When a strong buffer is present, I_f will be relatively small and a constant, large fraction, of ion will be bound to the ligand. The denominator of equation 9.8 will then be close to unity and can therefore be ignored (192).

The solution used for the initial ISE calibrations (equations 9.1 to 9.6) can be designed such that no solution chelation occurs. However, the various calibrating solutions must, in the general case, be expected to present ligands to other added ions. If the association constants of these are not known under the operating conditions, they must be estimated from 'calibration'. The total concentration of ion (I_t) and ligand (L_t) will be known and the free concentration will be estimated from ISE activity measurements. When I_{t_i}/I_{f_i} is greater than one, chelation may be expected (see 9.8). Therefore, during similar calibrations to those allowing estimation of the selectivity coefficient matrix (equation 6), a matrix of association constants ($K_{a_{A,B}}$) may be obtained for each ion, A for the purported ligands, B, where $K_{a_{m,n}} = K_{a_{n,m}}$. Thus, rather than analysing for interference between cation-sensing ISEs and added anionic interferent, solution complexation is estimated. The situation is slightly more involved, in that calibrating solutions will consist of salt solutions, not just the determinand ion, and therefore the effects of the partner ion(s) may also need to be taken into account.

Frequently, supplementation with complex media may serve to supply amino acids, vitamins and other factors at relatively low concentrations; measurement of endogenous levels of measured ions is necessary and additional estimation of the bulk chelation properties of the added complex medium for these ions will suffice. This is especially true where higher concentrations of either non-metabolisable chelators (equation 9) or measured ligands are added, as is the case with most semi-defined media:

$$\frac{I_t}{I_f} = 1 + \sum_{i=1}^{i=n} \frac{L_{t_i} K_{a_i}}{1 + I_f K_{a_i}} + K_a' \qquad (9.9)$$

where K_a' represents the degree of complex medium chelation, which has been abbreviated from the factor LK_a (for the medium) in the shortened version of equation 9.8.

However, in extreme cases it may be necessary to use the calibrated ISEs in the titration of the complex chelating polyelectrolyte to determine the most significant association constants. Various sophisticated algorithms have been developed for this purpose (188-190,193).

The above procedures enable the most significant features of the characterisation of both the ISE array and the solution to be estimated. Although this provides a quantitative assessment of the efficacy of the application of particular ISE arrays in particular media, it is now worthwhile discussing how these various constants may be used dynamically in on-line measurements.

6.2 Inversion procedures for real time operation

6.2.1 ISE characterisation. Estimated values of the offset E_{o_A}, and the slope factor, S_A ($= S'_A T/z_A$), constants have been obtained for each of the ISEs (A=1 to A=n). The measured cell e.m.f.'s for any timed data point, $E_{A,t}$, can be appropriately scaled for these constants to $E'_{A,t}$:-

$$E'_{A,t} = \exp\left(\frac{E_{A,t} - Eo_A}{S_A}\right) \qquad (10)$$

Introduction of the selectivity coefficients then provides the following matrix formulation for each data point:-

$$\begin{bmatrix} E'_{1,t} \\ E'_{2,t} \\ E'_{3,t} \\ E'_{4,t} \\ \cdot \\ \cdot \\ \cdot \\ E'_{n,t} \end{bmatrix} = \begin{bmatrix} 1 & k_{1,2} & k_{1,3} & k_{1,4} & \cdots & k_{1,m} \\ k_{2,1} & 1 & k_{2,3} & k_{2,4} & \cdots & k_{2,m} \\ k_{3,1} & k_{3,2} & 1 & k_{3,4} & \cdots & k_{3,m} \\ k_{4,1} & k_{4,2} & k_{4,3} & 1 & \cdots & k_{4,m} \\ \cdot & \cdot & \cdot & \cdot & & \cdot \\ \cdot & \cdot & \cdot & \cdot & & \cdot \\ \cdot & \cdot & \cdot & \cdot & & \cdot \\ k_{n,1} & k_{n,2} & k_{n,3} & k_{n,4} & \cdots & 1 \end{bmatrix} x \begin{bmatrix} a_{1,t} \\ a_{2,t} \\ a_{3,t} \\ a_{4,t} \\ \cdot \\ \cdot \\ \cdot \\ a_{n,t} \end{bmatrix}$$

Or in matrix abbreviation, when m=n:-

$$\underset{\sim}{E'}_{A,t} = \underset{\sim}{k}\,\underset{\sim}{a}_{A,t} \qquad (11)$$

Prior inversion of the selectivity coefficient matrix, $\underset{\sim}{k}$, will therefore enable the measured activities to be corrected for interferences:-

$$a_{A,t} = \underset{\sim}{k}^{-1} E'_{A,t} \qquad (12)$$

Correction for dynamically changing interferents can obviously only be performed if the interferents themselves are being independently measured and in the simplest case the matrix vectors m and n are therefore equal. Where the interferent is not being measured only fixed, non-adaptive correction is possible. This inversion procedure will clearly fail when the selectivity of any ISE for its specified ion is not greater than selectivity for interferents (which fortunately defines an ISE) and when extrapolated outside the 'calibrated limits'.

6.2.2 Solution characterisation. The corrected activity data may be expressed in (free) concentration terms by inversion of equation 7.3 following estimation of the activity coefficient (equation 7.2) from simultaneous conductivity measurement, allowing estimation of total ionic strength.

The main purpose of solution characterisation, other than for peculiarities of precalibration, is to estimate total concentration, I_{t_A}, changes from free activity measurements. I_{t_A} may therefore be estimated from the appropriate version of equation 9.9. The concentration of non-metabolised medium ligands and buffers (e.g. ethylene-diamine tetra-acetic acid (EDTA), nitriloacetic acid (NTA) or some pH buffers) will not change in an unknown manner and since temperature etc. are usually kept constant, there need be no measurement of the ligand concentration or correction of association constants. However, the concentration of the ligands in the undefined and unmeasured complex medium component must be expected to change, since nutrients will be removed and metabolites produced. The effect of these changes will usually be kept small by the presence of known chelators/buffers (above). However, the dynamic relevance of the factor $K'a_A$ may be re-estimated from the immediate effect of small additions of ion (equation 9.9). However,

where both ion and major ligand are expected to change, both concentrations must be measured. For instance, a measured increase in the free concentration of any ion could represent an increase in the ion or a decrease in the ligand concentration. To simplify description the case of two complexing ions of concentrations I_1 and I_2 is taken:-

From the law of conservation of mass:-

$$I_{t_2} = I_{f_2} + I_{b_2} \quad \text{and} \quad I_{t_1} = I_{f_1} + I_{b_1} \tag{13.1}$$

In this special circumstance $I_{b_1} = q.I_{b_2}$ where q is an integer defining the stochiometry of complexation and will be equal to 1 in the most common case. Combining equations 13.1 gives:-

$$I_{t_2} = I_{t_1} - I_{f_1} + I_{f_2} \quad \text{and} \quad I_{t_1} = I_{t_2} - I_{f_2} + I_{f_1} \tag{13.2}$$

From equation 9.9:-

$$I_{t_1} = I_{f_1} + \frac{I_{f_1}.I_{t_2}.K_{a_{1,2}}}{1 + I_{f_1}.K_{a_{1,2}}} \quad \text{and} \quad I_{t_2} = I_{f_2} + \frac{I_{f_2}.I_{t_1}.K_{a_{2,1}}}{1 + I_{f_2}.K_{a_{2,1}}} \tag{13.3}$$

Substituting equations 13.2 into equations 13.3, rearranging and presenting for each timed (t) data point gives:-

$$I_{t_{1,t}} = I_{f_{1,t}}.(1 + I_{f_{2,t}}.K_{a_{1,2}}) \quad \text{and} \quad I_{t_{2,1}} = I_{f_{2,t}}(1 + I_{f_{1,t}}.K_{a_{2,1}}) \tag{13.4}$$

The total concentration of either ion in the complex can therefore be calculated from the measured free concentrations of both ions at any data point. This relationship can be expanded to the more general relationship represented by equation 9.9.

6.3 On-line calibration and performance checking

All ISE and related transducers cannot be expected to maintain their calibrations for lengthy periods and large linear drifts as well as changes in selectivity can be expected. It is well known, although often routinely ignored, that even the pH electrode displays significant drift and the offset factor is commonly corrected by external measurement of pH in a single point calibration (equations 1 and 2). However, this procedure is far from ideal when a number of ISEs are being used on-line.

Flow line arrays of ISEs are especially suited to automatic and frequent on-line calibrations and performance checks. In the case ofthe flow dialysis, continuous sampling arrangement, calibrating solutions can be presented to the ISEs without disturbing the fermentation at all. If the ISEs are slightly remote then switching to the calibrating line is simply achieved, whereas, if the ISEs are mounted in the flow dialysis cell (to minimise lag) the compartment influent must be briefly supplemented with calibrating solution. Dialysed determinand levels must then be subtracted from the calibrating step changes. One and 2-point calibrations will correct offset and sensitivity (respectively) when offset recalibration is normally expected to be the most frequent. Presentation of a single complex mixture of all ions of known composition will enable the performanc of the selectivity matrix to be checked. These procedures are simple in concept and need no further description. However, certain ISEs must be introduced directl

into the fermentation broth itself and require in situ, on-line precalibrations. It is necessary to know the pH in the broth as well as in the flow dialysate so that, for example, the degree of carboxylic acid ionisation may be corrected in the dialysed measurement.

Certain other procedures for the correction of pH and dissolved gas electrodes have been described. Although a number of variations may be envisaged, their basis lies in the following relationship (11):-

$$N = k_1 (C - C') \qquad (14)$$

Where N is the rate of microbial usage/production of the determinand, C' is the control set point of determinand concentration and C is the concentration achieved after appropriate actuation (upscale or downscale against the microbial trend). N is obtained from the rate of determinand addition to maintain the set point and k_1 is therefore obtained from equation 14. If the reciprocal time taken for the calibrating addition to fall to the set point is compared to another similar operation the slope factor is obtained, as long as N and Eo in equation 2, remain constant (11). These procedures are dependent on the precision of actuators and on the absence of stochastic events.

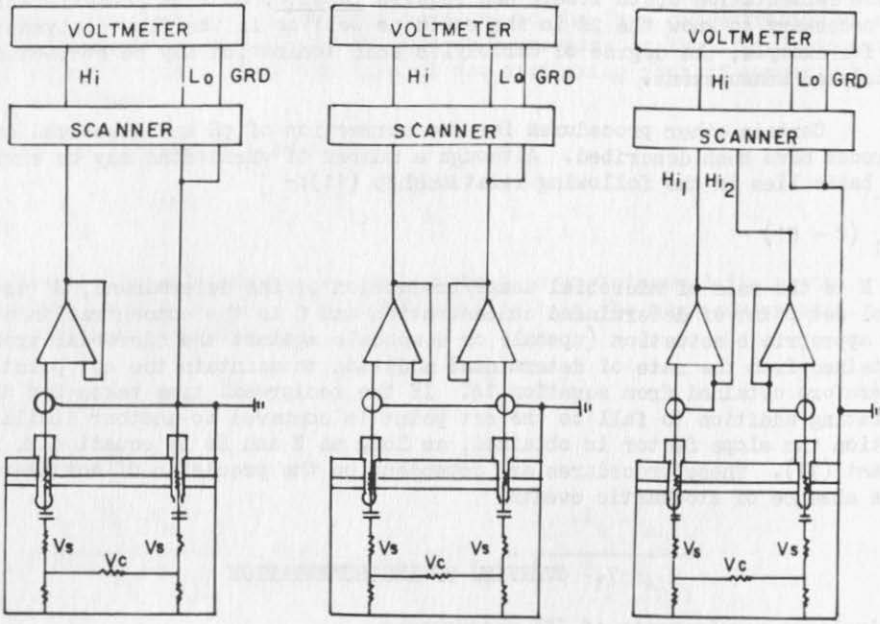
7. OVERVIEW OF INSTRUMENTATION

7.1 Analogue requirements of ISE measurement

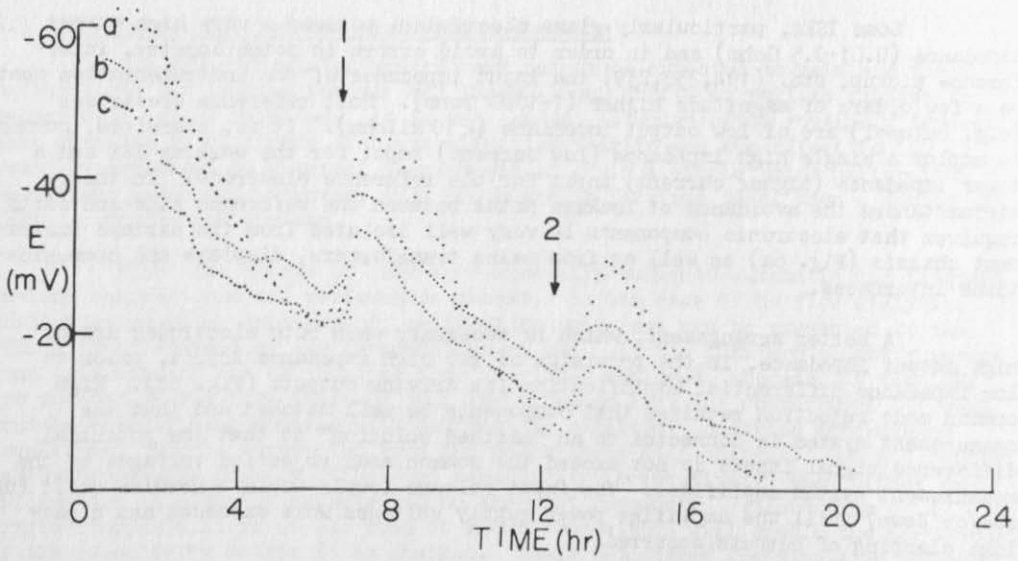
The electronic requirements of analogue ISE measurement instrumentation have been excellently described elsewhere (e.g. 194,239), and the basic configurations are only summarised in this review (Fig. 6). These were designed for stand-alone, relatively (electrical) interference-free, discrete sample, laboratory use. On-line fermentation applications are characterised by much higher levels of series and common mode electrical interference and a requirement for longer term stability and reliability of the measurement system. Their performance requirements should therefore be higher, although, commercially, the same types of instrumentation as in the former case are generally provided.

Some ISEs, particularly glass electrodes, possess a very high output impedance (0.01-0.5 Gohm) and in order to avoid errors in potentiometry, interference pickup, etc. (194,238,239) the input impedance of the instrumentation must be a few orders of magnitude higher (1-1000 Tohm). Most reference electrodes (e.g. calomel) are of low output impedance (<10 kilohm). It is, therefore, common to employ a single high impedance (low current) input for the working ISE and a lower impedance (higher current) input for the reference electrode. In these circumstances the avoidance of leakage paths between the reference side and earth requires that electronic components be very well isolated from the earthed instrument chassis (Fig. 6a) as well as from mains transformers, displays and communications interfaces.

A better arrangement, which is necessary when both electrodes are of high output impedance, is the provision of two high impedance inputs, prior to low impedance differential amplification for driving outputs (Fig. 6b). High common mode rejection requires that components be well matched and that the measurement system is connected to an "earthed solution" so that the potential difference signal inputs do not exceed the common mode rejection voltages of the measurement system amplifiers. The input voltage levels would otherwise drift (up and/or down) until the amplifier power supply voltages were exceeded and diode-like clamping of outputs occurred.



	NOISE	DRIFT/DAY	NOISE	DRIFT/DAY	NOISE	DRIFT/DAY
mV	2-5	20-30	0.05-0.2	1.5-4	0.04-0.08	1-2
pH	0.034 0.086	0.34-0.52	0.0009 0.003	0.026-0.069	0.0007 0.0014	0.017 0.03



In both the above configurations the input stages are fully floating with good isolation from earth and high input impedance, so that any variable electrical interference generated in the solution is more or less equally picked up by the working and reference electrode sides of the measurement, being therefore common mode. Any difference in interference pickup in any side of the measurement will result in series mode interference, caused by either differences in interference input between electrodes and leads or by poor instrument isolation/earthing (see Fig. 6.). Individual coaxial leads may have guarded or driven screens, so that the potential difference between the screen and the core signal is held steady and small to minimise lead leakage and lead noise pickup.

All fermenters, even small-scale versions, have high-power, coil-operated switchgear, or motors with direct chassis connection to the fermentation broth, and transient (interfering) voltages greater than 200 V (hopefully common mode) are likely. Similarly significant and variable potential differences exist across the complex impedance of the solution and more significantly across the solution/metal interface, one side of which (i.e. the fermenter) will be earthed. These interferences will vary with the chemical and gaseous (e.g. oxygen) composition of the broth and with the rate of stirring. Rejection of electrical interferences is in principle improved by the use of combined, rather than pairs of single, electrodes because the distance, and, therefore, any differences in interference pickup between the reference and working electrode half-cells, is minimised.

Placement of ISE probes close to surfaces over which fluid is passing will cause localised and variable streaming potentials (195-198) to be introduced into the measurement and these are extremely likely to be series mode. This mode of interference is a significant proportion of 'stirrer noise' even in well-positioned electrodes and may be reduced by increasing the conductivity of the broth, as predicted from streaming potential theory (195). Furthermore, any leakage paths will allow electrochemical cell currents to flow, and this may either involve the ISEs themselves and/or other metal-solution interfaces in the broth.

The significance of some of the more demonstrable effects is illustrated in Fig. 6. The fermentation broth is clearly challenged with large, unstable and heterogenous interferences, even in small-scale systems. Meter displays or outputs to chart recorders can appear to avoid much of the noise in the signal by

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Fig. 6. (Opposite). The reliability of monitoring two combined pH electrodes (Pye, U.K.) was estimated in 0.1M KH_2PO_4 buffer, pH 6.8, held in a typical small-scale fermenter (2 l model 2000 type vessel, L.H. Engineering, U.K.). A digital voltmeter (Model 1051, Datron, U.K.) with a well-isolated analogue scanner (model 1200, Datron, U.K.) was used in conjunction with battery-powered, high input impedance voltage followers to estimate average noise (in 40 msec, filtered samples taken over 1 sec) and long term drift (centre) in 3 measurement configurations (top):- differential measurement with low impedance reference electrode input (left); differential measurement with high impedance reference electrode input (centre); measurement of both pH and reference electrodes against solution earth, difference by software (right). The latter configuration (right) allowed measurement of the source of electrical interferences (bottom):- (a) reference cell of probe 1 with low impedance input; (b) pH cell of probe 1 with high impedance input; (c) reference cell of probe 2 with high impedance input. Agitation was changed to 1500 rpm at time 1 and both probes were moved close (3-5mm) to the glass fermenter wall at time 2. Measurements were averaged over 1 minute prior to presentation.

either mechanical or long time-constant electronic damping. Although mains frequency noise is a component of ISE signal noise, much of that caused by agitators and the other above effects is of very low frequency and, therefore, more sophisticated signal filter networks become difficult to implement because of the necessary long settling times. Digital instrumentation sampling times are necessarily rapid and any signal noise will be faithfully reproduced. The use of analogue instrumentation, therefore, can falsely condition the user into believing that ISE signal quality is high, allowing less than optimal measurement configurations to be implemented. Thus, it is very common in analogue fermenter instrumentations to employ a single relatively high impedance input for the pH ISE and a low impedance reference electrode input and, further, to use the screen of the coaxial cable for the reference electrode signal. This encourages interference pickup, especially when leads pass close to mains-powered actuators. Electrical leakage pathways are also encouraged, particularly in the latter configuration and by the use of poorly insulated coaxial connectors in a wet environment; thus great care must be taken in keeping dry coaxial connectors local to ISEs.

Although this common measurement configuration can be considered to be sub-optimal, it appears sufficient for low-performance pH measurement. However, although noise is not a problem because of damping, it is likely that interference will cause measurement drift. Measurement drift is widely believed to emanate from the electrodes, especially the reference electrode (e.g. 199). Although, electrode performance can be improved by exercising great care (169,199) or by using better reference electrodes (e.g. 200) a substantial component of the drift would appear to result from sub-optimal measurement configuration (Fig. 6).

If the potential difference of each half-cell of two identical, combined pH electrodes is measured against the solution earth (rather than differentially) substantial drifts can be demonstrated. When all electrodes are connected to high input impedance signal-conditioning amplifiers, the individual long-term drifts in well-buffered and -stirred media track well and the calculated differential readings barely drift at all. However, if the low impedance reference electrodes are diverted directly into the measurement system, even with 1 Gohm input impedance, the drifts measured against earth did not track and calculated differential long-term pH drift and very low frequency 'noise' was noted. Similar, simultaneous measurements made directly (i.e. differentially) display similar drifts, even in the combined electrode configuration. In these latter two cases the interference pickup is clearly not balanced, resulting in series mode drift and low frequency noise which is faithfully reproduced in the differential measurement, even when using a high performance measurement system (50; see Fig. 6). Although the required pH measurement resolution is not particularly high in most fermentations, the longer term reliability of measurement is a significant problem, requiring the frequent recalibration of the measurement system. It would appear (Fig. 6) that reliability and calibration longevity is significantly improved by the employment of better measurement configurations. The use of ISEs to measure non-logarithmic activities, rather than pI, together with the lower measurement resolution of some ISEs, inherently requires both higher resolution and more reliable measurement configurations than are commonly employed for the measurement of pH in present fermenters.

Although flow electrodes can be well guarded electrically from extraneous interferences, streaming potentials can be a problem. Where the size of ISEs is reduced, the output impedance is increased and higher performance measurement is essential. The sensing membranes of all such ISEs are very close to the flow cell surfaces. The flow of fluid across these surfaces will cause variable potentials to be developed close to the electrode surfaces. Careful attention to the electrode and flow cell geometries and materials is necessary to minimise these

potential differences (and to make them common mode).

7.2 From analogue to digital instrumentation

The interfacing of fermentation measurement (and control) systems to computers or the use of microprocessor control is of undoubted importance to all scales of fermentation. The introduction of microprocessors holding commercially-defined machine code software must be frowned upon at this early stage of instrumentation development as premature. System requirements and algorithmic formulations of measurement and control are not fully developed and the use of hard-wired microprocessors will, therefore, inhibit further necessary developments because users will have little access to the software and commercial suppliers will find it too expensive to provide the necessary microprocessor development backup (182,201).

Instrumentation exploiting microcomputers has undoubted benefits in cost, flexibility and development terms, and many manufacturers have therefore adopted an intermediate stage. Existing analogue measurement and control instrumentation has been retained and interfaced to microcomputers; data acquisition is here achieved by analogue to digital converter (ADC) multiplexing of the scaled meter outputs. 'Supervisory control' of the existing analogue control loops is then achieved by multichannel digital to analogue converters (DACs) which provide a signal proportional to the control set point and override the manual potentiometer controls.

The 12-bit resolution of most ADC multiplexes allows approximately 1 mV resolution over the working range (e.g. -2.0 V to +2.0 V) of ISEs or pX resolution of approximately 0.02. This is barely acceptable particularly as practical commercial precision specifications usually round up the last resolved digit, and the above 4-digit resolution is practically $3\frac{1}{2}$-digit. As far as most fermentation measurements, especially ISEs, are concerned the ADC multiplex approach requires a complete meter configuration as the signal conditioning unit. The implementation can, therefore, be expensive despite the relatively low cost of microcomputers and interfaces.

The cost of improving analogue performance, let alone digital resolution, is even greater at the present time. ADC multiplexes are presently ideal for rapid data acquisition applications where signal quality is high; neither condition is apparent in fermentation applications.

Other industries have solved the requirement for high performance multichannel measurement by analogue scanning digital multimeters. The exploitation of a single, high performance, multifunction digital meter seems particularly appropriate in fermentation technology, since the majority of the above signal conditioning requirements are now redundant. Transducer outputs (AC or DC voltage, resistance or current) can be measured directly with better performance and resolution (e.g. $5\frac{1}{2}$-digit). Sampling times of these instruments are necessarily short and as well as employing selectable filter networks which will increase sampling times, the better instruments inherently reject noise, such that data acquisition rates of 20 independent transducer measurements per second are routinely achieved. Although the high (e.g. 1 μV) resolution of these devices requires that isolation etc. be very good, as noted above, an appropriate measurement configuration can improve this further by making the ISE half-cell measurements individually against 'solution earth' and acquiring the conventional (working-minus-reference) differential measurement by software subtraction. The performance of this configuration in a running fermenter is high with an average noise of 40 μV - 80 μV between consecutive, non-filtered 40 msec readings and long term drift cycles consisting of approximately 1 mV per day excursions, even with uncombined electrodes. Since the cost of such equipment for many channels, including a computer interface, is

equivalent to two high quality, non-interfaced pI meters of comparable quality, it is an obviously cost-effective, flexible solution to fermentation instrumentation.

7.3 Instrumentation for fermentation systems

A system implementing the above philosophy is diagrammed in Fig. 7. This differs from conventional supervisory control systems in that both measurement and control can be effected by direct inputs and outputs. Such a system allows for direct high-level software control of both switched and proportional feedback elements. The use of hard-wired microprocessor control for input measurement and output control algorithms can lead to inflexibility, and diminished ability to keep pace with relatively rapid changes in fermentation system requirements. However, when the machine level software is written such that the essential variables can be passed from a microcomputer to a battery-backed read only memory (ROM), more flexibility and resistance to power failure is achieved.

The flexibility, cost-effectiveness and power of the former types of system (Fig. 7) lies in the use of high level software at the expense of hardware. In such systems all device input (remote programming of the device) and output (measured data) is controlled by the system microcomputer by standard interface bus links of which the IEEE 488 (1978 version (202)) is becoming standard. In these systems essential data may be maintained during power failure either by disc storage with an autostart mechanism or by a battery-powered ROM. Whilst it may be argued that computer failure in these systems results in complete loss of control, a networked supervisory and backup microcomputer completely overcomes this as well as providing useful background job computational facilities. The available flexibility is illustrated by, for example, the simple provision of a sophisticated digital polarograph largely using existing instrumentation (Fig. 8). Polarising voltages may be provided by a precision fully floating DAC unit which most systems will possess. The driving of various polarising voltage waveforms and ramps and the appropriate triggering of measurement is handled at the software level, and enables the improved measurement procedures mentioned earlier (see e.g. 274).

8. THE PROSPECT OF BIOCHEMICAL CONTROL

8.1 Widespread importance of biochemical assessment

It would be a lengthy and complex task even to begin outlining the broad wealth of biochemical and physiological knowledge of microbial growth processes that evidence the importance of on-line biochemical assessment, let alone its application in real-time control. However, in truth this vast literature provides the microbiologist with little more than stochastic peeps into the dynamics of microbial physiology.

Although well provided with the means of studying the nature and properties of individual events, microbial biochemists are largely unable to study simultaneously, continuously and in real time the interrelated events of growth. Direct multiparameter monitoring is a facility lacking both in research and in technological applications. That the majority of the benefits of this approach remain to be discovered should be sufficient of itself to encourage its more widespread application. However, the purpose of this discussion is to justity in general terms the application of multiparameter monitoring to the ends of understanding, maintaining and improving fermentation yields.

The profound effects of all environmental parameters on microbial metabolism must be considered a major justification for the introduction of multicomponent biochemical monitoring. The following discussion is intended to

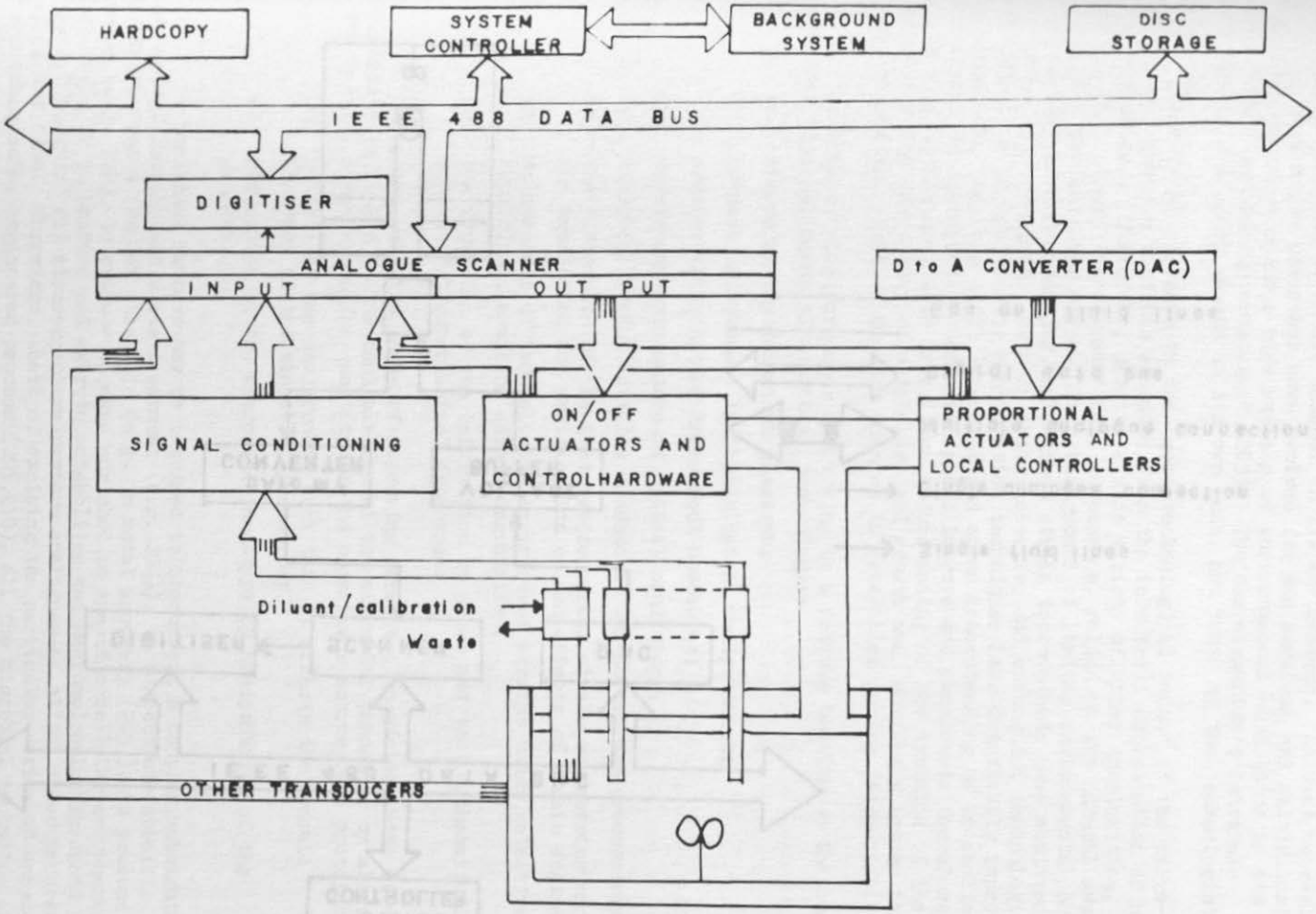

Fig. 7. Microcomputer-controlled fermentation system using high performance digital measuring techniques as the input elements of feedback control loops closed at the software level.

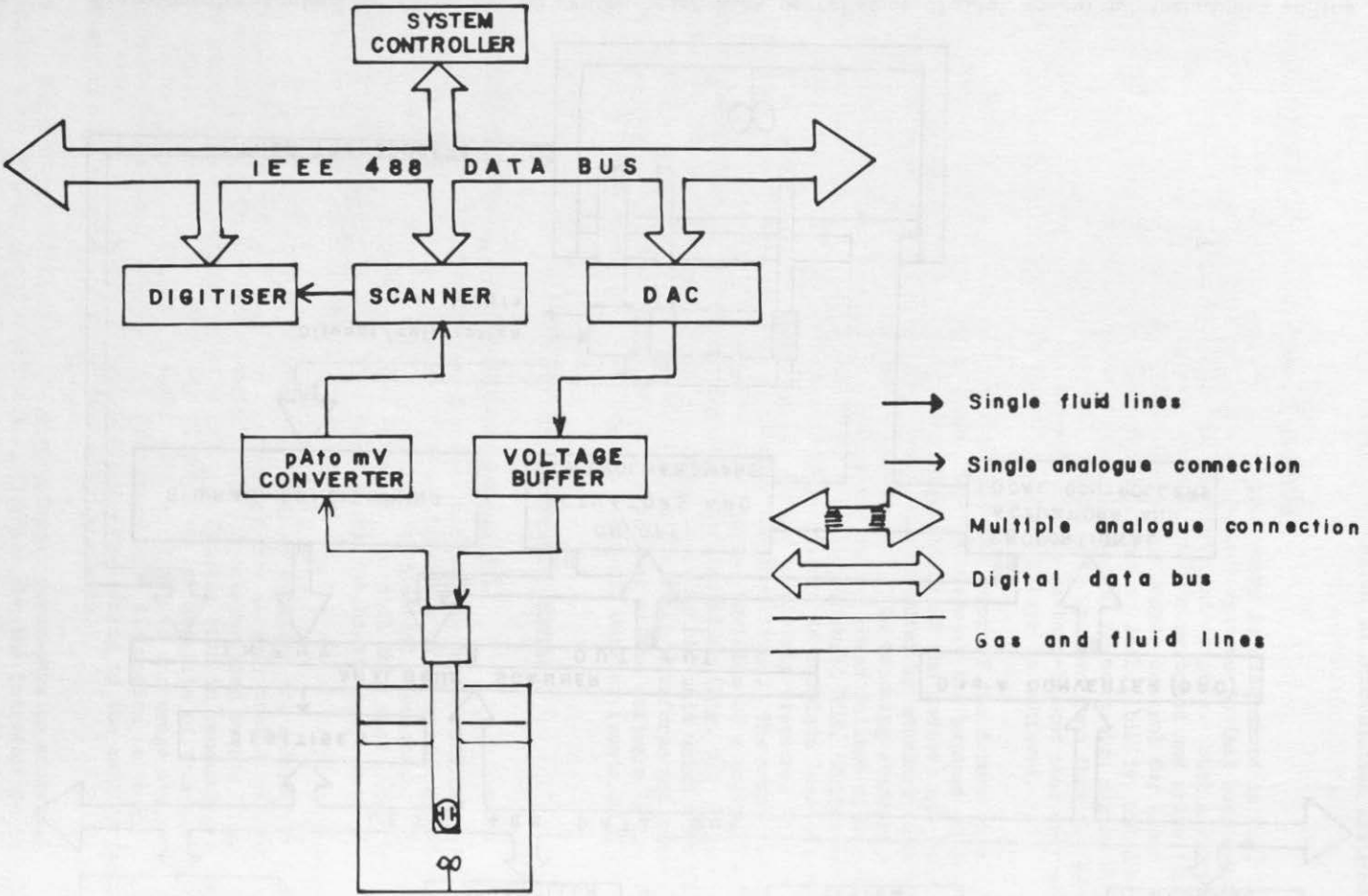

Fig. 8. Software-directed polarograph as a subsystem of the fermentation control system represented in Fig. 7.

underline the fact that the microorganism will continuously seek to alter its metabolism in response to subtle changes in its environment. It must be realised that these regulatory phenomena have evolved for the survival and multiplication of the microorganism under a wide variety of environmental conditions in its natural "feast and famine existence" (203). The microorganism's intrinsic regulation is selfishly geared to its own ends, not those of the technologist.

Near total reliance on the 'good technological sense' of the microorganism is a concept that has emanated from the industrial exploitation of near-natural processes. Therefore, despite a wide variety of other complexities, provision of a strain that 'works' has, in essence, relied on the natural availability of a suitable phenotype. In a background of limited environmental control and physiological understanding, subsequent strain improvements have admitted a high degree of serendipity, both in their selection and subsequent technological performance. Although genetic manipulation techniques have drastically improved this situation, they are still largely based on an understanding of metabolism which itself is incomplete. Genotypic yield improvement procedures therefore lack the means of obtaining a more definitive understanding of the dynamics of the biochemistry and physiology of metabolism and growth and obviously ignore the effects on product yield of dynamic changes in metabolism during "fermentation".

In effect, multiparameter monitoring is a feature bearing on the success of the following key areas of microbial technology:-

(a) microbial physiological research;

(b) process improvement by physiological manipulation;

(c) process optimisation throughout upscaling;

(d) day-to-day maintenance of optimal conditions;

(e) microbial product quality assessment and downstream processing;

(f) identification of the features requiring strain engineering and the prediction and verification of the effects of strain engineering;

(g) maintenance of technologically improved strains under selective conditions, avoiding strain degradation;

(h) the provision of the information to erect and test mathematical models of "fermentation" processes.

There are only two means of improving fermentation yields, both of which are inescapably linked to the manipulation of the microbial phenotype by alteration of the nature, activity and/or quantity of the organism's enzymes and proteins:-

(a) control of the environment such that it affects the dynamic function of the microbial phenotype;

(b) alteration of the microbial phenotype by manipulation of the genome.

A microbial process may be required to bioconvert a complex substrate (e.g. detoxification and waste treatment (e.g. 204)), to concentrate specific components from a complex substrate (e.g. in metal recovery (205)), to produce biomass (e.g. 206), to produce biomass enriched in specific cellular components (e.g. 207), or to produce and excrete metabolites and macromolecular products (e.g. 8,208,209). All microorganisms perform analogues of all of these processes most of the time. Therefore, short of excising the particular series of enzymes in a biochemically engineered process (209,210), all the microbial technologist can hope to achieve is to manipulate the phenotype to maximise predictably any one process at the expense of all the others. The genetic engineering of strains:-

(a) to introduce previously non-synthesised products (e.g. 211,212)

(b) to provide higher productivity by introduction of multiple gene copies (e.g. 213,214); or

(c) to alter the regulation of metabolic pathways (e.g. 215,216)

are powerful tools whose success depends on the ill-understood response of the microorganism to its environment and to many other resultant influences on its metabolism. Even when the genotype is expressed as the desired phenotype, mutation, plasmid loss and unfavourable regulation (i.e. strain degradation) can confound productivity during "fermentation" (e.g. 217-219) in the absence of appropriate 'environmental selective pressure', since "from the organism's point of view, strain degradation is often strain improvement" (8). Further, it is important to realise that the more alien or minor the process being optimised, the more likely it is to be overriden by the primary metabolism of the microorganism.

It is unfortunate that the physiologist/bioengineer has not been availed of the tools to advance understanding of microbial processes at a level to complement the manipulative powers of genetic engineering. It is similarly unfortunate that the "New Biotechnology" has an all-too-common tendency to lose perspective in the present onslaught of "cloned market insights...and...sticky-ended stockmarket projections" (220); statements such as "fortunately today's experienced industrial geneticist clearly understands this demanding interdependence... and it falls on him to demand, lead, or coerce others to provide the means for translating esoteric research into practical products" (221) draw attention to the problems of interdisciplinary work of this type.

8.2 A general scheme of biochemical assessment revisited

The practice of providing general schemes is particularly dangerous in view of the complexity of the processes which are summarised. However, in the interests of illustration certain features common to all microbial processes (summarised in Fig. 9) can be identified and employed to gauge the prospect of improved biochemical assessment and control.

Some of the most important properties of microbial systems of interest to the improvement of fermentation yields are:-

(a) nutrient uptake from the environmental compartment and waste product removal from the microbial compartment (membrane transport);

(b) the provision of energy by catabolism and its consumption in transport, assimilation and growth;

(c) provision of molecular building blocks for assimilatory pathways;

(d) the intrinsic regulation of transport, catabolism and assimilation.

8.2.1 Transport. The cell membrane is a selectively permeable ultrathin lipoprotein membrane, which is impermeable to most of the intracellular metabolites; however, many other metabolites or biochemicals can be treated as nutrients or waste products by different organisms and tend to be maintained at optimal concentrations within the cellular compartment. The majority of the latter require active transport to drive nutrient import or waste product export against an established concentration gradient (19,20,222,223). These environmental parameters are intuitively the link to biochemical control as they provide the major means of 'communicating' with the organism's interior; indeed they are believed to provide the organism with the means of sensing its environment (224). Transport must therefore be considered a crucial aspect of physiological assessment, evidenced by the estimation that microorganisms themselves can devote as much as 50% of their energy to maintaining transport processes (225), even in a nutrient-rich

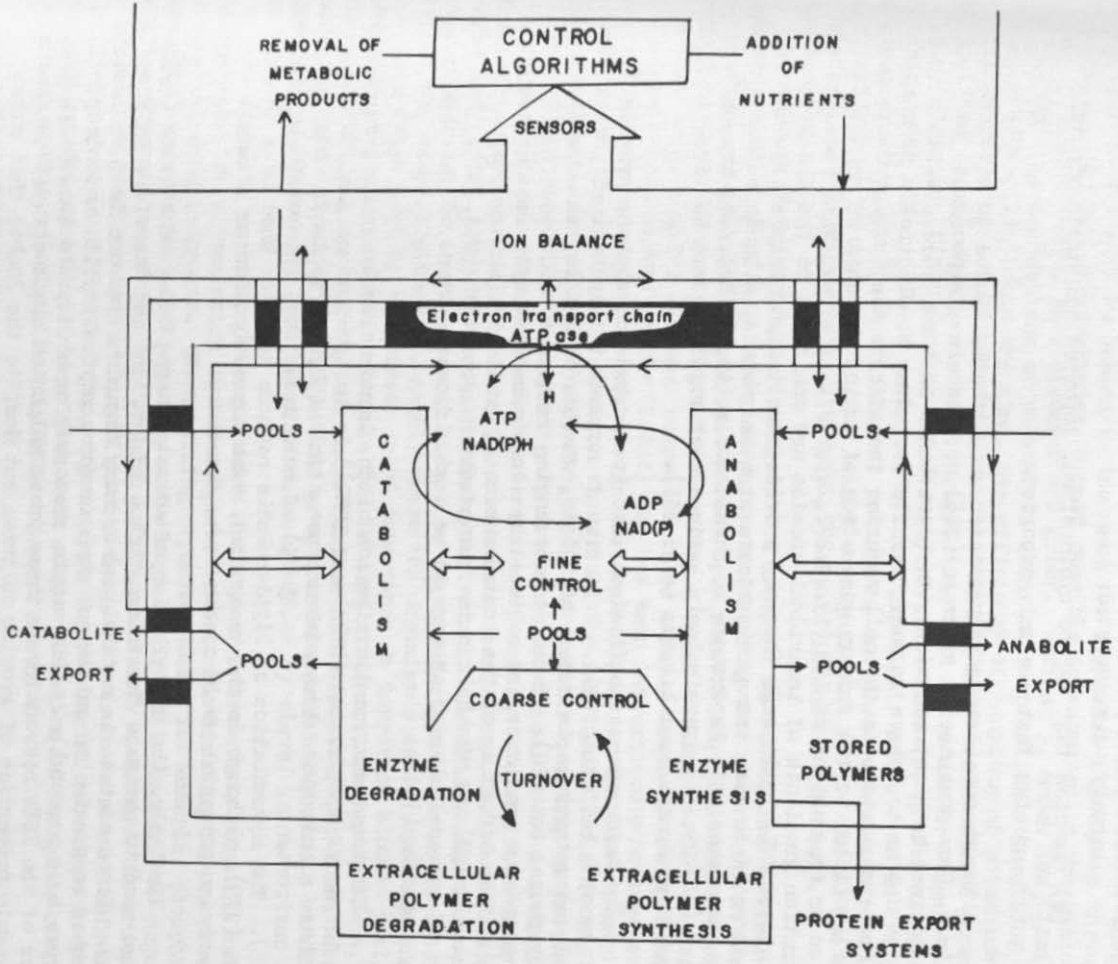

Fig. 9. Schematic representation of some of the interrelated parameters of the biomass compartment which are or could be accessible to biochemical control.

environment. They are therefore worthy of more detailed consideration.

The transported species may be divided into:-

(a) species not modified by the organism at all and involving import and export;

(b) species that are otherwise not synthesised by metabolism and are largely only imported;

(c) species that may be synthesised, imported and exported; and

(d) species that are only exported.

Although some transport processes are directly linked to biochemical energy (e.g. from phosphoenol pyruvate (226) or adenosine triphosphate (ATP) (227)), most are drived by electrochemical ion (especially proton (228)) gradients (e.g. 20), which appear to behave in many respects according to electrodic principles (12). Protein carriers in the cell membrane specific to the transport species are secondarily linked to the primary generation of proton currents by metabolite oxidation or consumption of ATP (19,20,222,223,228). The resulting steady-state concentration gradients of individual species are analogous to the electrochemical potentials of ISEs. Although this may provide some insight into the amount of energy diverted to each transport process at steady-state, allusions to its thermodynamic and mechanistic importance in chemiosmosis (19,222,223,228) are perhaps premature (12,229-231), particularly because microorganisms are believed to operate far from thermodynamic equilibrium (e.g. 232).

The major non-metabolised cations (potassium and sodium) provide the bulk of osmotic buffering (20). Potassium is accumulated by bacteria, at least, to total concentrations in excess of 200 mM, whereas sodium is, in general, actively pumped out (20). Under non-scavenging transport conditions (233), the sodium-potassium gradients are believed by some to be the major factors responsible for "the buffering of the transmembrane pH gradient" (maintaining a constant intracellular pH) and the bulk phase transmembrane potential (234,334). The maintenance of intracellular pH appears to be a significant feature of the physiology of many bacteria (334).

The transmembrane distribution of the lipid-soluble cations, butyl and methyl triphenyl phosphonium ($BTPP^+$ and $TPMP^+$) is believed to respond to the "bulk phase steady-state transmembrane potential" (50,234-236,246). ISEs selective to low environmental levels (5 to 25 μM) of both probe ions are available (50,235, 236,246). The accumulation of lipid-soluble cations has been used as a measure of biomass (235), although in the case cited, some correction for any changes in transmembrane potential would appear to be necessary.

Similarly, the distribution of acetate (among other weak acids (20)), has been used to estimate the transmembrane pH gradient and therefore intracellular pH (19). Since acetate is metabolised by many organisms its use is questionable and probes responsive to substances such as for acetylsalicyclic acid (aspirin) may prove more acceptable. ISEs may be prepared for both probe ions (e.g. 62). Neither of the ISEs responsive to these probe molecules has been used to assess the dynamic properties of growing cultures but despite the doubts that exist concerning the mechanistic and quantitative thermodynamic significance of the measured gradients, highly useful on-line information of the physiological state of the organisms would be forthcoming were such measurements undertaken.

In the anaerobes (e.g. Clostridia) operating a true fermentative metabolism (e.g. glycolysing glucose to acetate and butyrate and lacking any respiration (19)), the protonmotive coupling of energy (from ATP) to potassium

accumulation would appear to be linked to the environmental potassium activity. Potassium leakage pathways constitute a considerable dissipation of energy primarily derived from ATP via the membrane adenosine triphosphatase (ATPase (237)), especially under potassium-replete conditions, and this was further characterised by a high rate of glycolysis and insignificant bulk transmembrane potential. Since in the same organism specific inhibition of the ATPase results in inhibition of glycolysis (presumably resultant from ATP feedback inhibition), futile, uncoupled ATP hydrolysis may well be linked to the need for a greater glycolytic throughput to provide other key intermediates, a need possibly accentuated in the glucose minimal medium in which this organism was grown. Similarly, under low (limiting) environmental concentrations of potassium, when the intracellular levels of potassium remain very much the same, the efficiency of coupling of potassium transport to ATP hydrolysis was high, as was the apparent transmembrane potential, and the rate of glycolysis was lower, suggesting that there was no longer the ability to dispel excess ATP and enhance glycolysis. However, growth on richer media supports higher growth rates, with lower glycolytic rates even in the presence of low environmental concentrations of potassium, and glycolytic inhibition as a result of ATPase inhibition was not nearly so significant (50). The ATPase itself is also allosterically activated by key glycolytic intermediates (241).

It is extremely interesting to the prospect of biochemical control that the manipulation of an environmental parameter, such as the potassium ion concentration, should have such a profound effect upon metabolism. Protonmotive energy spillage to heat production (itself presenting a major cooling problem to the microbial technologist) has been suggested to be a major means of energy dissipation under excess carbon supply (8,242) and has been inferred to encourage overflow metabolism (secondary metabolite production) (8), as in the above example. As well as the possibility of variable "slip" in the functional cycle of the primary protonmotive pumps (243,275-276,324), the variable coupling of protonmotive energy to specific transport systems would also appear to be a mechanism of energy spillage. The maintenance of relatively constant "adenylate energy charge" in many organisms under many environmental conditions (328) can be exploited to assess the microorganism's physiological state. In this regard the overflow metabolisms of poorly coupled ATPase or transport mutant strains is of interest (245). The reverse situation, namely where the organism's energy spillage and overflow metabolism must be minimised, is of importance in improving biomass yields. More extensive study of the interrelation between transport, energy spillage, inherent "ATP pool control" by the above Pasteur-like effects (8,247) and their relationship to overflow metabolism and growth is of central importance to all microbial processes and their biochemical control.

In many anabolic pathways which consume environmentally supplied nutrients, the transport or accumulation of the nutrient is the rate-limiting factor in that pathway and even in growth. In respiring organisms when, for example, phosphate, magnesium or potassium limits growth, and energy supply is in excess, the organism's specific oxygen consumption rate is high. This presumably maintains the protonmotivated transport systems with sufficient energy to overcome the more unfavourable ionic gradients. Maintaining oxygen supply to industrial processes is a difficult problem and it is clear that even simple variation of inorganic composition of the medium can significantly effect oxygen demand.

Much evidence has suggested that there is a more subtle coupling between protonmotive energy and transport (e.g. 12), and local protoneural networks have been suggested as the means of coupling protonmotive sources and sinks (229,329). These mechanisms allow close kinetic and thermodynamic control to be exerted on transport, and explain why the accumulation of various nutrients bear only a qualitative or no relationship to the apparent bulk phase protonmotive force (e.g. 50,233). More significantly, such a model provides a means of understanding

how the organism exerts variable energy coupling to individual transport systems and how energy spillage may actually occur (275,276).

Similar transport processes are also involved in metabolite export. Although fewer studies have been made, the basic mechanisms are believed to be the same (20). Of particular importance are the organic acids whose environmental accumulation has long been known to inhibit growth and metabolism (248) as do many other "staling factors". Species such as non-ionised acetic acid are soluble in the cell membrane and can be excreted to significantly high concentrations and act to dissipate the transmembrane pH gradient (20). As well as decreasing growth rate and increasing oxygen consumption rate in response to the apparent uncoupling effect (8), the excretion of some metabolites (e.g. lactate) has even been shown to drive the uptake of other nutrients (249). The above are merely a few instances of the profound importance of transport in microbial processes; many other examples can be found.

8.2.2 Fine and coarse metabolic control. The microbial cell operates two interrelated metabolic regulation mechanisms. Fine control of the activity of preformed enzyme molecules involves either allosteric activation or inactivation of specific enzymes by certain key metabolites or their reversible covalent modification. This can involve alteration of the affinity of the enzyme for substrate and/or the maximum reaction velocity at saturating substrate concentrations, and competitive, non-competitive, mixed and cooperative effects have been established (e.g. 250,251). Most of these regulatory mechanisms have been quantified by *in vitro* assay of purified enzymes, and the information then extrapolated to the whole cell. However, certain features can be qualitatively demonstrated in whole cells, some of which are alluded to in this discussion.

The coarse control mechanisms not only involve induction or repression of enzyme synthesis itself but also specific degradation of enzyme molecules which are also effected by key metabolites. Such regulatory mechanisms often involve whole metabolic pathways (e.g. 252,254). These mechanisms were first demonstrated in whole cells, phenomena of which diauxie is the classical example (255), and many more have been identified. More recently, it has proved possible to engineer certain operons and so manipulate regulation mediated at the level of DNA transcription (e.g. 252-256).

Further discussion of these regulatory mechanisms is beyond the scope of this presentation; however, such mechanisms are largely responsible for effecting any proposed environmentally applied biochemical control. Although some of the gross effects of these control mechanisms have been demonstrated there is little understanding of how they interact in the growing cell. It is the role of the physiologist and biochemist to provide more insight into the factors affecting these regulatory mechanisms. At present one of the major tools in studying the metabolism of growing cells is the chemostat (8,46). Although this has provided a great deal of understanding, it is not a culture method traditionally favoured by the technologist. However, certain examples are worthy of mention to underline further the effect of the microorganism's environment upon its metabolism.

Transport systems themselves, as well as undergoing specific regulation (above), are prone to dramatic non-specific regulation under environmental stress. Many nutrient limitations induce synthesis of high affinity transport systems (256) which may even alter subsequent metabolic routes (257). Many organisms synthesise chelating agents (siderophores) in response to inorganic cation insufficiency (especially of iron or manganese) (258). However, unless transport mechanisms are chronically limiting growth, specific regulatory mechanisms usually predominate.

Some of the effects of carbon source and oxygen have been mentioned with

respect to transport. However, one rather obvious but important example is the difference between anaerobic fermentative and aerobic respiratory modes of growth. Under aerobic conditions the major end product of sugar metabolism is biomass and carbon dioxide, but under anaerobic conditions fermentation pathways yield a variety of other partially oxidised metabolites (e.g. ethanol, succinate, lactate, acetate). One of the major metabolic bottlenecks in such growth is the production of excess reducing equivalents, both in overflow metabolites and in the excess of NAD(P)H (e.g. 8). Anaerobes therefore 'waste' reducing power by production of hydrogen, a process which is therefore of importance in the control of anaerobic processes (259). Significant variations in fermentation end product yield may be expected upon alteration of environmental conditions (8). The classical example is the production of more basic metabolites in acidic media and the production of more acidic metabolites in alkaline media (260).

The routes of assimilation of many nutrients can suggest the reason for switches in metabolism to accommodate the environmental changes. Many organisms employ glutamine synthetase and glutamate synthase to assimilate ammonia. Under ammonia limitation the substrates for driving assimilation (ATP, NADPH and 2-oxoglutarate) must be present in excess. This often results in overproduction of 2-oxoglutarate and in storage polymer synthesis in response to high ATP levels (e.g. 8). Limitation of sulphate as well as of certain vitamins would similarly be expected to limit seriously the operation of those enzymes requiring these substances as precursors of cofactors, and either metabolic rerouting or metabolite excretion then occurs (3).

Sufficient evidence has been accumulated even with the currently inadequate procedures to underline the profound effects of most of the usually unmeasured environmental parameters on metabolism. Many of these must be expected to change dynamically during "fermentation" as a result of microbial growth. A significant example is provided by the fact that many "fermentations" even rely on the microorganisms to change its environment before significant product yield is achieved (e.g. 261,262). The heuristic role of multiparameter biochemical assessment and control would therefore appear to be unquestionable.

8.3 Practical considerations

8.3.1 Measurement. Nearly all of the non-sensitised ISEs have a possible role in fermentation analysis, and the prospect of enzyme-sensitised ISEs must surely result in biochemical assessment systems being largely based upon ISEs for all the reasons summarised in Table 3.

In addition to the need for monitoring and controlling the constitution of the fermenter fluid, there is also a requirement to achieve some estimate of certain metabolites within the microbial compartment. This is more difficult, but it is possible, selectively or totally, to permeabilise the cell membrane, so as to achieve measurement of internal components or to effect entry of environmentally provided substrates without transport (263-265). Many of these procedures are gentle, easily performed and provoke rapid equilibration of the intracellular and extracellular compartments.

The microbial volume fraction of many fermentations varies from as little as 0.01% to over 35%. Since many key metabolites (e.g. ATP) are present at intracellular concentrations in the millimolar range (e.g. 266), their dilution after equilibration of aliquots containing the microbial volume fraction with the total volume still allows direct ISE measurement. Although many detergents are interferents of most ISEs, and in the long term may extract mobile carriers (see section 5), very low levels of the stronger (e.g. sodium dodecyl sulphate) and low levels of the weaker non-ionic detergents are sufficient to effect rapid

TABLE 3. Some advantages of ISE-based transducer technology in microbial process assessment and control

(a)	Continuous, real-time assay suitable for feedback control
(b)	Potential selectivity for most important biological determinands
(c)	Electrode sterilisation methods available, although they can be avoided completely
(d)	Probe fouling can be minimised or avoided
(e)	Good sensitivity (ca 1%) over a wide range ($<10^{-6}$M to >1M) and automatic on-line dilution methods possible
(f)	Responsive to thermodynamically- and microbially-important free activities rather than total concentration
(g)	No requirement for added reagents, except with a few indirect enzyme electrodes
(h)	Assay is non-destructive or determinand consumption is negligible
(i)	Electrodes are, or can be made, biologically inert
(j)	Selectivity good to poor, where interference can be corrected or minimised
(k)	Acceptable response time, even with enzyme electrodes
(l)	Good shelf-life and acceptable in-use life time
(m)	Relatively low cost and disposable designs possible
(n)	Can be used in turbid and viscous broths as well as in the gas phase
(o)	Can be effectively 'home made' where small market size does not encourage commercial interest
(p)	Calibration possible on-line
(q)	Can be miniaturised and incorporated into multi-ISE probes to avoid multiple fermenter entry ports
(r)	Largely steady-state methodologies, even pseudo-equilibrium methods (e.g. enzyme electrodes) can be made acceptably unresponsive to flow or stirring rates.

equilibration (e.g. 267). Furthermore, under such conditions the major proportion of detergents enters the membrane phase and relatively little is available in free solution (267). It is therefore quite conceivable, that, in many cases, premixing of small samples of cell suspensions with detergent in a flow line, followed by direct analysis, would provide effective, reliable and near real-time analysis of the intracellular compartment. A very valuable development would be the ability to measure biomass on-line and, although direct methods are available, all are possessed of chronic problems (268,330). Although there are many 'units' of

biomass, intracellular volume fraction would appear to be the most suitable, so that intracellular concentrations of metabolites may be estimated in real time.

8.3.2 Actuation. Addition pumps are widely used to effect environmental change. However, in the case of most nutrients these may only effect 'upscale actuation'. Although addition of chelators or metabolism itself may effect 'downscale control', it is not widely applicable. In continuous culture basal medium continually dilutes the culture and limited 'downscale control' may be effected by this mechanism. However, it should be noted that peristaltic pumps are notoriously inaccurate and the ability to monitor the volume of the fermentation and/or the volumetric addition of titrants would be essential, especially when the likelihood is that set point feedback control is replaced with more sophisticated control algorithms. Here, the downscale aspect of control is therefore a great problem; nonetheless, dialysis culture, at least at the small scale, would appear to provide a means of external removal of unwanted metabolites. Indeed, where such culture methods have been used, cell yields and densities in particular have been greatly increased (e.g. 269). Various methods of performing controlled dialysis culture are available (270).

8.3.3 Control loops. Set-point, single-loop feedback control has proved extremely useful in fermentation for parameters such as pH and temperature, but its application has been guided by the availability of electronic controllers. Although these may possess sophistications such as proportional, differential and integral control (271,331) it is often difficult for the fermentation technologist to engineer full use of such intricacies, especially in the case of biochemical parameters. The sophistication that is inherent in these control loops is that most should be multi-input and involve multiple actuations, and in truth this holds even for those presently used in fermentation (e.g. oxygen).

This area is clearly the province of computer-directed digital control and is another reason to reject the prevalence of analogue controllers in computer-supervised systems. The use of true computer control allows the control loops to be algorithmically formulated in software terms, and bestows flexibility, sophistication and greater cost-effectiveness on the system. Since all input signals are converted to physically meaningful units, and since the magnitudes or rates of nearly all actuations may be calculated or measured rapidly enough to represent real time to the microbiologist, appropriate and elegant fermentation control may readily be achieved. As well as making actuation adaptive to changing conditions, control loops can easily be made mixed, or responsive to derived parameters as well as to individual input signals. This type of approach is essential to biochemical control and places control in the hands of the microbiologist and not the instrument designer. Many of the arguments traditionally raised against such control mechanisms are based on systems with much more rapid response times than those generally present in microbial processes.

9. FUTURE PROSPECTS

Multiparameter monitoring would appear to be inextricably linked to improvements in many areas of microbial research and industrial processes, for which purpose ISE technology has been awaiting exploitation for some time (11). It is hoped that the practical considerations presented in this review will at least create an awareness of the problems of the application of ISEs in fermentation, and underline the fact that the first few steps can be taken now. Digitally instrumented computer techniques would appear to be a prerequisite for effective ISE use, and there are demonstrable benefits from using the inherent flexibility of software for real-time operation as well as for off-line analysis.

The future prospects of ISE technology appear to depend on the further development of electronic transduction systems and the provision of a wider variety of more selective sensor elements. The latter have been, from the electrochemical point of view, relatively difficult to find or to synthesise. Biology is in this sense fortunate in the possession of a great number of highly specific ligands, and it would be beneficial both to ISE technology and to biotechnology if this feature could be exploited more extensively. Proteinaceous ligands as sensor elements in ISEs require considerable study if they are to find widespread and reliable use, and the explotiation of thermophilic or chemically-stabilised enzymes is a rather obvious first step. It is also of the greatest interest to note the functional similarity between microbial transport proteins and ISE sensors, and a considerable number of reagentless bio-sensors could perhaps be developed from these ligands. The possibility of developing regenerating reagent/co-factor ISEs has already been demonstrated. The potential of modern molecular biological methodologies should not be overlooked in solving some of these problems. As well as improving protein/ligand preparation methods, it is also clear that DNA- and protein-sequencing may shortly provide an understanding of the molecular structure of the ligand sites of proteins and allow rather unstable proteins to be discarded in favour of shorter, even 'designed' polypeptides, which may then be further modified to comprise sensor elements.

In the bioelectrochemist's preoccupation with providing transducers, the direct bioconversion of chemical, electromagnetic and electrical energies should not be overlooked when considering future appropriate technologies. There is already very little difference between bio-transducers and bio-fuel cells other than that of scale and purpose; improved biomolecular design and engineering will surely lead to improved signal-transducing systems.

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11. ACKNOWLEDGEMENTS

We are grateful to the Science and Engineering Research Council, U.K. for financial support. We thank Mr Anthony Pugh for photographic assistance, and Joan Crawford for typing the manuscript

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