PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES

Measurement of PH and Ionic Composition of Pericellular Sites [and Discussion]

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Measurement of pH and ionic composition of pericellular sites

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The development of ion selective microelectrodes has made it possible to measure the normal steady state in the pericellular environment together with ion fluxes in response to physiological or pathological disturbances. Combined intracellular and extracellular measurements indicate that there is a considerable range of ability between various types of cells in the efficiency with which they can tolerate changes in pericellular conditions. Macrophages are extremely tolerant while cells of the cerebral cortex require a very finely controlled local environment. Combination of ion selective probes with microelectrodes which measure substrate and oxygen availability extend the information which can be obtained about ionic composition of cellular environment and the factors which are important in its homostasis.

Introduction

The concept of the 'milieu interieur' has been widely accepted since it was propounded by Claude Bernard, but only recently has it become possible to make direct continuous measurement of the pericellular environment. Developments of ion specific and other types of microelectrodes, together with a variety of optical 'probes' have made it feasible to measure highly localised conditions in the microenvironment of cells, with considerable precision. In addition to the measurement of ion activities (Hinke 1959; Thomas 1970; Walker 1971; Khuri 1967; Zeuthen, Hiam & Silver 1974), oxygen tension (Davies & Brink 1942; Cater & Silver 1961; Silver 1965, 1972*a*; Kunze 1966; Lübbers 1969) carbon dioxide tension (Silver 1972*a*) and local blood flow by hydrogen clearance methods (Lübbers & Baumgärtl 1967) it has also become possible, with the aid of enzyme microelectrodes (Silver 1975*a*) to determine the local tissue concentration of certain metabolites, particularly glucose, for which specific oxidases have been isolated.

The combination of optical and electrode techniques now available permits the measurement of the local variations in the physical environment of cells with a great deal more accuracy than has been possible hitherto not only of steady state conditions but also of relatively rapid changes associated with altered local metabolism or systemic effects. Perhaps the most important feature of the pericellular environment that has been demonstrated by electrode methods is the existence of steep gradients of many substances between the microvasculature and the cytoplasm, and the necessary corollary of this situation, that all the cells in a group may not, and often do not, live under precisely the same conditions. Furthermore, the microenvironment does not remain constant and in some tissues may vary over wide ranges depending on cellular activity and vascular control. It is therefore of particular interest to establish what are the normal limits of these environmental fluctuations, to determine at what stage deviations from the normal lead to reversible pathological changes in the cells, and in which pericellular conditions permanent or lethal damage may be inflicted on the local cell population.

While it is obvious that the strict control of pericellular conditions is an essential part of general bodily homeostasis, it is also clear that unusual changes in local pericellular conditions

may have the effect of stimulating cellular or systemic responses which are of a protective or reparative nature. It is not known for instance what is the stimulus for repair in damaged tissue but it can be shown clearly that marked changes occur in the pericellular environment in a damaged area (Hunt & Zederfeldt 1969; Niinikoski 1969; Silver 1969, 1972*a*, *b*) and it may well be that accumulations of metabolites such as lactic acid, which cause large changes of extra and intracellular pH, trigger proliferative and synthetic activities in local fibroblasts in contra-distinction to the rather more passive role which these cells appear to play under normal environmental conditions. In a more dramatic context the results of conditions such as surgical shock or acute hypoxia of the brain may lead to rapid changes in pericellular environment which are potentially lethal to the cell but whose effects can trigger c.n.s. mediated systemic reactions which not only serve to improve conditions locally in the brain, but also throughout the whole body. These large systemic effects can be related to changes of intra and pericellular ionic activities in the c.n.s.

Finally the performance of enzymes at the surface of cells and indeed the state of many enzyme substrates may be affected by the hydrogen and other ionic activities in the pericellular environment. Thus a knowledge of pH and ionic concentrations in pericellular sites is of primary importance in understanding how metabolism may be controlled and the way in which cells may respond to normal and abnormal situations.

Methods

The methods available for continuous measurement of pericellular environment are of two types; (1) microelectrode systems which are based on ion selective membranes or liquid ion exchangers and (2) optical probes in the form of dyes whose fluorescent or absorbative properties are altered by the conditions in which they are situated. Optical probes have the advantage over microelectrodes in that they are non-destructive but on the other hand they are limited in application to the surface of tissues or to very thin layers of tissue. Surface electrodes have similar advantages and disadvantages and do not have the excellent resolution of optical probes.

(*a*) *Microelectrodes*

(i) *All glass electrodes*

(1) Standard micropipettes with tip diameters ranging from 0.1 to 1.0 μm were pulled with a vertical electrode puller from pyrex glass o.d. 1.8 mm, i.d. 1.2 mm. These were filled either with 2 M KCl or 2 M NaCl and used as reference electrodes, or were filled with Procion yellow (Stretton & Kravitz 1968) for marking recording sites by iontophoresis (100 V for 0.5 s).

(2) Micro pH electrodes were constructed by the methods of Hinke (1959) figure 1 or of Thomas (1970) figure 2. The detector membrane was made of Corning 0150 glass and the outer pipette was Corning 0120. The two glasses were fused together in a microforge and a positive air pressure was maintained inside the pipette during the heating process to prevent collapse of the pH glass cone.

(3) Micro sodium glass electrodes were constructed in the same manner as for pH electrodes except that NAS-11 glass was used as the detector cone. No internal pressure was required since the sodium sensitive glass had a higher melting point than the pyrex of the outer capillary.

(4) CO_2 sensitive electrodes were constructed as for the 'Thomas' type pH microelectrode and the cavity at the tip of the probe was filled with 100 mM $NaHCO_3$ in 1% agar. The tip of the electrode was covered with a silicone membrane (Silver 1972*a*).

(ii) *Liquid ion-exchanger electrodes*

Double barelled electrodes were pulled from pyrex glass by the method of Zeuthen *et al.* (1974) (figure 3). One barrel was coated on the inside with dichlorodimethylsilane to render it hydrophobic. The tip of this barrel was filled with an appropriate ion exchanger (Corning 477317 for potassium; 477315 for chloride). The shaft of the barrel was filled with 1 M KCl and the reference barrel was filled with 2 M KCl.

In all these electrodes mentioned above, and silver/silver chloride internal reference was used.

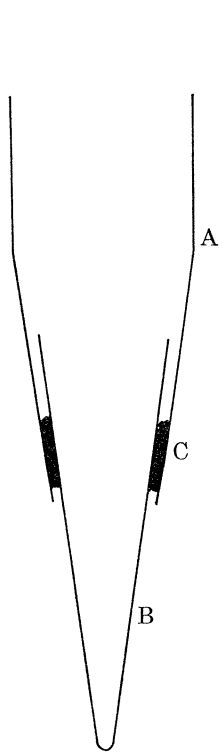

FIGURE 1

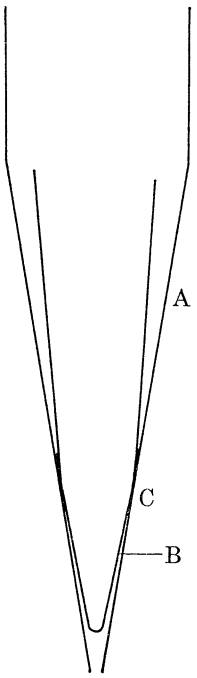

FIGURE 2

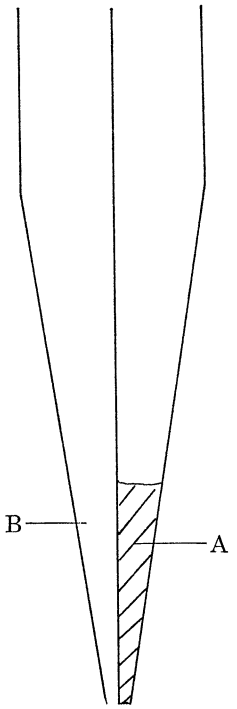

FIGURE 3

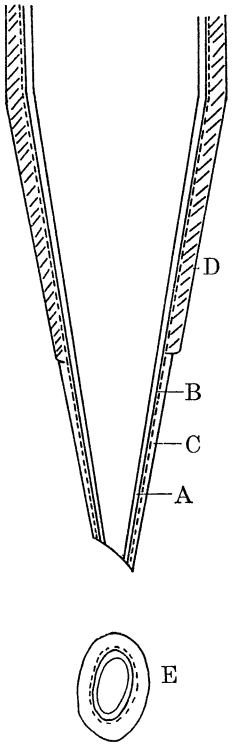

FIGURE 4

FIGURE 1. Hinke-type electrode for pH. A, Pyrex capillary; B, pH glass cone; C, Glass to glass seal. Tip diameter 3 μm.

FIGURE 2. Thomas-type pH electrode. A, Pyrex capillary; B, pH glass cone; C, Glass to glass seal. Tip diameter 0.3 μm.

FIGURE 3. Double barrelled liquid ion exchanger electrode. A, Liquid ion exchanger in siliconized barrel; B, reference barrel. Tip diameter 0.6 μm.

FIGURE 4. Microenzyme electrode for glucose detection. A, inner glass capillary; B, platinum layer; C, outer glass insulation; D, resin insulation; E, end-on view of electrode tip. Tip diameter 2 μm.

(iii) *Glass and metal electrodes*

(1) *Oxygen electrodes* were constructed either by the method of Silver (1965) or that of Whalen, Riley & Nair (1967). Both types of probe have an effective area of about 0.2–1.0 μm and were polarized at −0.7 V.

(2) *Glucose microelectrodes* were miniaturized from the design of Clark (1973) and were in two forms. The first, which could be used in well oxygenated tissue was a platinum-iridium needle, insulated with glass and similar to an oxygen microelectrode. It was coated with catalase-

free glucose oxidase (Sigma Chemical Corp.) by dipping in a 1% solution of glucose oxidase in water, and drying in air. The second type, which was used in anaerobic situations was a glass micropipette, vacuum coated with platinum. The tip of the pipette was insulated with a glass cap which was fused to the platinum surface without collapsing the inner pipette. The tip of the pipette was cut on a diamond wheel to expose the platinum ring (figure 4) to give a tip diameter of 1–2 μm. This was coated with glucose oxidase as already described. When used in hypoxic tissue the electrode was filled with oxygen and a pressure of approximately 20 cm water applied to it through a flexible tube. This supplied O_2 to the glucose oxidase by diffusion (Silver 1975*a*). Both types of glucose electrodes were polarized as anodes at 0.6 V (Chance 1949; Clark 1973). The glucose electrode functions by the oxidation of glucose at the electrode surface by the glucose oxidase and the consequent formation of H_2O_2. This latter is destroyed at the polarized surface of the platinum to give a current proportional to the H_2O_2 concentration. The enzyme reaction is oxygen dependent, hence the need for a supply of oxygen to the functional surface of the electrode in anaerobic tissues.

(iv) *Multiparameter electrodes*

Because of the considerable variation in microenvironment over short distances and times, it is inadequate to make either serial measurements in one place or multiple simultaneous measurements of different parameters in different places. Multi-purpose probes with the capacity to detect several different ionic activities, or oxygen tension together with ion activity, within a total probe of 0.5–1.5 μm have considerable advantages. Kessler, Hajek & Simon (1975) and Silver (1975*b*) have described multi-purpose electrodes. These are either multi-barrelled liquid ion exchanger probes, or they contain one metal-filled barrel, two ion exchanger barrels and a reference barrel.

Before and after use *in vivo* and in tissue cultures, electrodes were tested for specificity and linearity of response. Micro reference electrodes with tip potentials which altered more than 2–3 mV when changed from one test solution to another (see Zeuthen & Monge 1975) were discarded.

Electrode output was recorded through appropriate high input impedance d.c. amplifiers.

The sites of electrode recording were examined by histological reconstruction at the end of each experiment. Some sites were marked with Procion yellow.

New and more specific ion exchangers are constantly being synthesized. Simon and his colleagues (Ammann, Pretsch & Simon 1973; Kessler *et al.* 1975) have produced new sodium sensitive and calcium sensitive lipophilic neutral ion exchangers of high specificity. A proton exchanger (W. Simon 1974, personal communication) has been synthesized and will probably displace the all glass pH microelectrode for many purposes. Its specificity for hydrogen ions in the presence of interfering ions has not yet been fully evaluated.

Electrode response times. Hinke type sodium or pH electrodes have a response time of 1–5 s for 95% response. The Thomas type of electrode design shows a much longer response time, which is dependant on the length of the recess at the tip of the electrode. On average our electrodes had a 95% response in about 30–50 s. A similar response was recorded with CO_2 electrodes based on the Thomas pH electrode design. Liquid ion exchanger electrodes have a short response time of the order of milliseconds but owing to their high impedance it is possible to take advantage of this rapid response only with negative compacitance amplification systems. The response time of our recording system including the electrode was of the order of 0.5 s.

PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES

Micro-oxygen electrodes showed 97 % response in approximately 0.15 s, while glucose microelectrodes showed a similar response to a 50 % step change in glucose concentration in a simple stirred solution in between 2 and 8 s. The response time of the glucose microelectrode depended on whether or not the surface was covered with a diffusion membrane.

'Poisoning' of electrodes. Most microelectrodes showed progressive changes in sensitivity after prolonged insertion in tissue. This was usually associated with the build-up of protein layers at the electrode tip and, in the case of liquid ion exchanger electrodes was sometimes associated with 'creeping' of the ion exchanger from the hydrophobic electrode cavity. In the case of oxygen microelectrodes poisoning is not only associated with protein deposition but also with the interaction of sulphydryl groups on the active surface of the platinum cathode. Similar problems were occasionally encountered with the glucose electrodes, although these appeared to be very much more stable. The performance of commercially available liquid ion exchangers such as the Corning series used in these investigations have been discussed by Walker (1975) and by Khuri, Agulian & Wise (1970). These investigators and our own findings suggest that the potassium ion exchanger shows a rejection of sodium of approximately 50–1 in favour of potassium.

All-glass electrodes which are contaminated by protein films may be rejuvenated by cleaning. The problems associated with these electrodes have been discussed by Thomas (1975).

(*b*) *Optical probes*

Among dyes which change their fluorescence or absorbative properties quantitatively under different conditions, two groups have shown promising results in the investigation of tissue micro-environment. (1) The merocyanin series of dyes may be synthesized in suitable configurations either to dissolve in biological membranes or to pass through them or to remain outside them. These dyes when excited by ultraviolet light give a fluorescence which, according to the structure of the dye, may characterize the potential across the membrane in which they are dissolved or may indicate the ionic concentration in which they find themselves. When these dyes have been adequately developed it will be possible to use them for measuring kinetic changes in ionic concentration in the same way as they may now be used for the detection of rapid changes in membrane potential. (2) The pyrene butyric acid series has the characteristic that the quenching of the u.v. induced fluorescence is proportional to the oxygen tension in which the dye is situated over a range which is commonly found in biological tissues. This characteristic has been exploited by Longmuir & Knopp (1973) and is extremely useful in detecting oxygen gradients in the pericellular and intracellular situation. The great advantage of the pyrene system is that an instantaneous picture of gradients may be obtained so that the internal oxygen tension may be referred to the external tension at any given time. The chief limitation of optical systems is that they are only suitable for the investigation of phenomena at the surfaces of tissues or in thin layer cultures.

Results

The observations to be reported have all been obtained with single or multiple electrode systems and a few of these have been checked against optical probes. Most of the measurements have been made either in brain or in soft or hard connective tissues growing in thin layers in rabbit ear chambers (Wood, Lewis, Mulholland & Knaack 1966). Other observations relating

PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES

intracellular to extracellular conditions in the gut have been reported by my colleague Dr Zeuthen (Zeuthen & Monge 1975).

(*a*) *Observations in the brain*

When microelectrodes are inserted into brain tissue they inevitably cause a certain amount of damage, even when the tip of the electrode is not larger than 0.1 μm. Insertion of these electrodes into the brain produces an artificial extracellular space, probably left after the explosion of a cell penetrated by an electrode. It has been our experience that conditions in these artificial spaces equilibrate very rapidly with the conditions found in the natural gross extracellular space provided by the ventricles of the brain. Most of the observations to be reported here have been obtained in rats or cats under anaesthesia and held in stereo tactic instruments, and are related to changes found in the cerebral cortex and elsewhere during periods of acute hypoxia and haemorrhagic or endotoxic shock.

(i) *Normal pericellular conditions*

A striking feature of pericellular environment in the brain is its constancy. Readings from multiple insertions of ion selective electrodes in different parts of the cortex, thalamus and hypothalamus all indicated that the extracellular potassium concentration was *ca.* 3.0 mM and that the sodium activity was *ca.* 145 mM (Dora & Zeuthen 1975). The pH, whether measured with a high resolution Thomas-type electrode, or with a rather coarser Hinke-type probe was approximately 7.40 and was closely similar to that of the cerebrospinal fluid in the lateral ventricles of the brain. Similarly, carbon dioxide tensions showed little variation from place to place, or in time, and averaged, under our conditions of anaesthesia, 5.6 kPa (42 mmHg), which was almost the same as that in jugular blood and c.s.f. Little or no evidence of gradients in ionic concentrations pH or CO_2 tension could be demonstrated. By contrast, there were considerable variations of oxygen tension, not only between different anatomical areas, but also over very short distances, within well defined microanatomical sites. For instance in the cingulate cortex of the rat, arteriolar capillaries showed a range of P_{O_2} of between 10.65 and 16 kPa (80 and 120 mmHg), yet tissue P_{O_2} levels of *ca.* 0.16 kPa (1–2 mmHg) occurred frequently within 30 μm of such vessels. Furthermore, the P_{O_2} gradient did not remain constant at one site and varied markedly with changes in local blood flow and cellular activity. However, there appeared to be some kind of 'set point' about which the fluctuations took place, and homoeostatic mechanisms, presumably in the form of local vascular flow adjustments ensured that deviations from the 'set point' were rapidly reversed. The following experimental results may be quoted to indicate the fine nature of the control of pericellular conditions: A neuron was identified by recording its spontaneous electrical activity through the reference barrel of a multibarrelled electrode while local P_{O_2}, and $[K^+]$ and $[H^+]$ activity were recorded simultaneously. The cell activity was then stimulated by applying a sensory input to the area via a peripheral stimulus (cold to the tail). The increase in cell firing rate was rapidly followed by a decrease in extracellular P_{O2} during a period of 1.5 s. This decrease was followed by a return to normal values and then by an 'overshoot' of about 10% and a second return to normal. When the stimulus to cell activity was removed the sequence of P_{O_2} changes was repeated in reverse and the original 'resting' value reappeared about 3 s after the cessation of the stimulus. During the increase and decrease of the firing rate a slight change from resting $[K^+]$ activity

PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES

was detected (increase and decrease of 5 %) but no change of CO_2 or pH was seen. No change was noted in glucose electrode readings close to stimulated cells.

(ii) *Changes in pericellular conditions during acute hypoxia and shock*

(1) In acute severe arterial hypoxia, the P_{O_2} of the brain dropped rapidly to zero. During the initial phase of the anoxia there was a transient rise of cell membrane potential (hyperpolarization) which was concommitant with a fall in pericellular [K^+] activity. This stage lasted for about 20 s and was followed by a progressive, slow rise of extracellular [K^+] for a period of 2.5–4 min. At this time there was a slight increase of hydrogen ion concentration and an equally small fall of sodium activity. After about 3 min of hypoxia there was a sudden drastic increase of extracellular [K^+] and [H^+] and a decrease of [Na^+] activity. Unless this situation was rapidly reversed by administration of O_2, permanent cell damage and death of the animal occurred (see Dora & Zeuthen 1975). During this late phase of acute hypoxia a 'spreading depression' (Leão 1947) sometimes appeared, which was characterized by large changes of extracellular [K^+] and corresponding changes of intracellular redox state (Mayevsky, Zeuthen & Chance 1974; Crowe, Mayevsky, Mela & Silver 1975). Concomitant with these ionic changes, cell activity in many parts of the brain was first stimulated and later depressed. In some regions, such as the lateral hypothalamic area, stimulation without subsequent depression was marked until the hypoxia became lethal (Cross & Silver 1963) and this activity was associated with sympathetic arousal and attempts at homoeostasis. These included increased cerebral blood flow, hyperventilation and hyperglycaemia. These responses were reflected in changes in the pericellular environment as follows: there was a fall in P_{CO_2} from 5.6 kPa (42 mmHg) to about 4.67 kPa (35 mmHg) and a rise of about 80 % in pericellular glucose concentration. This rise was sustained until either the animal died or oxygen supply was restored. Preliminary observations suggest that although the general level of glucose increased there may be a greater gradient in the glucose concentration between blood vessels and cells. This was not so obvious in brain as the situation seen in connective tissue during underperfusion.

(2) *Hypoperfusion of the brain.* This occurs in haemorrhagic shock, usually develops slowly, and, in distinction to acute hypoxia, is irregular in its effects. There was a slow fall in P_{O_2} which was dramatic in some areas and negligible in others. Similarly, [K^+] increased obviously in the hypoxic areas but not at all in the non-hypoxic zones. The hypoxic areas were those in which blockage of the circulation occurred due to microthrombi. In late shock there was a general rise in pericellular [K^+] which was reflected in raised blood potassium levels. Hydrogen ion concentration increased and it appeared that a change of 30 mV in pH was the maximum change in extracellular environment that could be tolerated in the short term, without the occurrence of obvious, morphologically detectable cell damage.

(*b*) *Observations in connective tissue*

The following observations have been made using a variety of electrodes in thin layers of connective tissue growing in rabbit ear chambers. The technique used was in general that reported by Silver (1969) for measurements of oxygen gradients. Measurements were made in growing granulation tissue, in 'wound fluid', in established fibrous connective tissue and in fragments of articular cartilage transplanted into an ear chamber after the cavity had been fully vascularized.

PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES

(i) *Normal pericellular values*

In established connective tissue which was adequately perfused gradients of pH, [K^+] activity, glucose, and CO_2 could not be detected. There were shallow gradients of oxygen tension, the high points of which were found over small blood vessels and the low points in relation to cell aggregates. Unlike the situation in the central nervous system, there were wide differences of absolute values of O_2 and pH which depended on anatomical site and the degree of perfusion of local blood vessels. These values showed large long term fluctuations as compared to the much smaller short term changes seen in brain. Although pH values were variable (6.95–7.4), extracellular [K^+] and [Na^+] activity was constant and similar to that in brain: glucose concentration ranged from 4 to 8 mM.

Measurement in small but growing fragments of cartilage showed that while [Na^+] and [K^+] activity was the same as for soft tissue, the hydrogen ion concentration was on average at the higher end of the soft tissue range, while glucose concentration was slightly lower than the soft tissue average. Oxygen gradients in cartilage were relatively steep compared with those in fibrous connective tissue and absolute values were of the order of *ca.* 0.85 kPa (5–8 mmHg) in the centre of 0.5 mm cartilage blocks, as compared with an average of approximately 15–20 mmHg P_{O_2} in the central intercapillary space of soft tissue.

(ii) *Values in healing tissue*

Measurements in growing granulation tissue showed marked variation in pericellular environment according to the state of tissue and its age. In the wound cavity, which was occupied by tissue fluid, polymorphonuclear cells and macrophages, the P_{O_2} was near zero, the P_{CO_2} was about 7.35–8.0 kPa (55–60 mmHg) and the pH varied from 6.9 to 5.2. Glucose concentrations were less than 1 mM and [K^+] activity was high (approx. 15 mM). In the growing edge of the new tissue the P_{O_2} gradients were very steep and ranged from 10.65–12.0 kPa (80–90 mmHg) over vessels to 0 at the tissue edge; pH was 6.9–7.3 while [K^+] was variable but usually less than 5 mM. Glucose concentrations varied according to the local P_{O_2}, being highest when the P_{O_2} was above 1.33 kPa (10 mmHg). Growing tissue is very susceptible to changes in microcirculation which occur constantly. This results in large changes in P_{O_2} gradients and sometimes, if flow ceases altogether for more than a few seconds, in alterations in local pH and glucose concentration. As granulation tissue matures, pericellular environment becomes more stable, gradients of metabolites become flatter; pH approximates to 7.4 and P_{CO_2} to 5.33 kPa (40 mmHg).

(iii) *Effects of inflammation, hypoxia and shock*

(1) *Inflammation.* During the development of the inflammatory reaction in response to direct sterile minor trauma in established vascular connective tissue, a characteristic sequence of events occurs in the local microenvironment. This may be summarized as follows: At the site of damage there is an immediate rise in [K^+] concentration due to release of cell contents and this is immediately followed by a rise in local P_{O_2} due to vasodilatation, and a return of [K^+] to normal. After a variable interval depending on the severity of the damage, there is a fall in P_{O_2} which is accompanied by an increase in hydrogen ion concentration and a secondary increase in [K^+] activity, together with a fall in extracellular glucose levels. In cases of minor damage resolution and removal of cellular debris takes place over 12–24 h and the normal

PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES

resting situation is restored. Where gross damage or infection occurs, further much more severe changes can be observed in cellular microenvironment.

(2) *Hypoxia*. In contrast to the neural cells in the central nervous system, connective tissue cells appear to be very resistant to short term hypoxia, and consequently the changes in pericellular environment during oxygen lack differ in connective tissue and brain. In arterial hypoxia, connective tissue in rabbit ear chambers suffers rapid falls in P_{O_2}, but little other obvious change. If the hypoxia persists, there is an increase of hydrogen ion concentration if the circulation is reduced, but a decrease if the perfusion rate remains high. Changes of P_{CO_2} could not be measured even when vasoconstriction was prolonged and widespread. Minor changes in glucose levels could sometimes be detected, but these, like the pH changes, were variable and appeared to be dependant on changes in blood flow. The most obvious difference in the response to hypoxia between connective tissue and brain was that no change in extracellular $[K^+]$ activity could be detected even when P_{O_2} was reduced to zero for several minutes.

(3) *Shock*. Haemorrhagic and endotoxic shock both result in severe underperfusion of connective tissues and this causes progressive changes in tissue microenvironment. The first effect of underperfusion is a fall in tissue P_{O_2} and this is followed by a slow increase in hydrogen ion concentration. In severe, prolonged hypoperfusion P_{CO_2} rises and may reach 7.34–8.0 kPa (55–60 mmHg). In healing wounds, hypoperfusion may lead to a P_{CO_2} of as much as 10.65 kPa (80 mmHg) and a change of pH to 5.0 or less in the wound cavity. When tissue pH falls below 6.7 there is a gradual rise of pericellular $[K^+]$ concentration which accompanies a failure of cells to maintain their membrane potential. These pericellular conditions lead to irreversible cell damage, at least in actively growing fibroblasts, within 3–4 h. On the other hand, macrophages have been observed to survive in wound cavities with a pH of less than 5.3 for many hours without damage. They do however appear to have a reduced ability to kill microorganisms in hypoxic situations although their phagocytic activity is unimpaired.

Discussion

Most if not all cellular activity is closely related to the pericellular environment. Such activity may be electrical, synthetic, secretory or motile and cell types with different activities are more or less affected by changes in their environment. It is clear that the neurons of the cerebral cortex require a very closely controlled microenvironment in which to operate efficiently while connective tissue cells can at least survive, if not flourish, under a far greater range of conditions.

At the present time, systems for investigating various aspects of tissue microenvironment range from the identification of specific antigens by immunofluorescence techniques to measurement of simple ions by microelectrodes. The physical methods outlined in this paper have considerable limitations when applied to complex situations such as occur in tissue. It is not yet clear to what extent the performance of some types of microelectrode differs in tissue from that in the test situations and there are still technical problems in identifying the exact position of the electrode tip in relation to a cell when a particular measurement has been made. There are also difficulties in the interpretation of data from optical probes, because of the effects of different concentrations of dye in different cell and pericellular compartments. Nevertheless, in spite of these limitations, we now have tools for investigation of tissue environment at the cellular level and we can expect constant improvements in the methods available as they

PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES

become more widely used and development of increasingly specific detector systems for all the common tissue constituents (Kessler *et al.* 1975).

The results of investigation of pericellular environment reported here must be viewed with caution, since very large numbers of measurements must be made before we can be sure of the normal range of conditions in tissue. To date the only systematic surveys that have been carried out have concerned frequency histograms of P_{O_2} levels in tissues (Lübbers 1973). Similarly detailed and systematic measurements are now required for other parameters in normal and abnormal tissues. There is a degree of unanimity on resting extracellular conditions in regard to [Na^+] and [K^+] activity in brain among several workers (Bito & Meyers 1972; Vyskocil, Kriz & Bures 1972) but there have been few attempts to investigate pericellular conditions outside the central nervous system (Khuri *et al.* 1970; Silver 1968, 1972*a*, *b*, 1973; Heuser & Betz 1975).

What does emerge from this study is that in the connective tissues investigated, microenvironment varies within wide limits both in time and space. This variation is most obviously linked to vascular perfusion as might be expected but must also depend on cellular activity. The mechanism of the rapid feedback between cell activity and perfusion was not detected by the electrode studies, but seems likely to be associated with changes of pH and P_{CO_2} locally and also with [K^+] and [Ca^{2+}] activity (Betz 1975). In damaged, growing and healing connective tissues the steep gradients which appear in the pericellular environment may well be features which attract or repel cell migration and which stimulate or depress synthetic activity, or which determine the direction of differentiation of a pluripotential cell. Similarly, the smoothing of these gradients by the establishment of adequate vascularisation may be associated with the change of behaviour of cells from a proliferative to a synthetic activity, or this may be a consequence not of reduction of gradients but of a relatively small change of ionic activity. pH changes for instance are a feature of wounds and clearly occur because of faulty vascular perfusion in the damaged zone combined with the glycolytic activity of inflammatory cells. Such a fortuitous combination may well provide the stimulus to the initiation of repair. The advance of fibroblasts into an acid area seems limited by their intolerance of hydrogen ion concentrations of greater than pH 6.7 (Silver 1972*a*), but removal of debris by macrophages from these zones is not so limited. We do not yet understand what is the mechanism which allows the macrophage its relative immunity to acid environments, but the differing tolerance of these two cells to local conditions ensures that wound healing proceeds in an orderly sequence with removal of debris preceding reconstruction of tissue.

Finally, since synthesis of pericellular ground substance, fibres, and mineralisation depends on an adequate supply of glucose and amino acid, together with the correct pH and other ionic activity for enzyme function, a knowledge of pericellular environment will help in the understanding of normal and abnormal connective tissue functions.

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Discussion

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With reference to pH variations, we would like to draw attention to the effect of fixed charged groups on pH. It can be shown from considerations of Donnan equilibrium (W. D. S. C. Freeman & A. Maroudas, in preparation) that for a tissue containing fixed charged groups the internal pH is different from that of the external solution, this difference being given by the formula

$$\mathrm{pH} = \lg K_{\mathrm{Na}} + \lg \{\bar{\gamma}_{\mathrm{Na}}/\gamma_{\mathrm{Na}}\}, \qquad (1)$$

where Δ pH is the external pH minus the internal pH, K_{Na}, the molal distribution coefficient for sodium ion, $\bar{\gamma}_{\mathrm{Na}}$, the activity coefficient of Na^+ inside the tissue water, γ_{Na}, the activity coefficient of Na^+ in external medium.

The r.h.s. of equation (1) depends on fixed charge density and we have been able to show experimentally that this variation is linear, at any rate within the range of fixed charge densities, $\bar{C}_x$, such that $-0.3\ \mathrm{mmol/g} < \bar{C}_x \angle 0$. For a fixed charge density of 0.25 mmol/g (a typical value for the middle zone of human articular cartilage from the femoral head) Δ pH = 0.4 and hence for a pH of 7.4 in synovial fluid, the $\overline{\mathrm{pH}}$ in the middle zone of cartilage would be only 7.0. The pH around the cell lacunae, where the glycosaminoglycan content is probably even higher (Stockwell & Scott 1965; Maroudas 1972), would be lower still.

Apart from the simple Donnan equilibrium effect, further differences in pH could be expected, for instance, from lactic acid gradients, but we have no quantitative data as yet.

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