Cellular membranes

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Numerous reports have been published on the study of cellular membranes using microelectrode techniques[1]. As this present review must of necessity be brief, it would perhaps be of value to explore the more recent interests in the electrical characteristics of accessable surfaces, as a means of understanding membrane and cellular behaviour under normal and chemically or physically stressed conditions. The rationale for this approach is that generally the 'metabolic' function within the cell is reflected by the electrical characteristics of the surface membranes. This has been adequately demonstrated with microbial systems[2] and with red blood cells[3].

An excellent review on the central role of the electric field in the functional membrane of photosynthesis, using electric pulse methods has recently been published[4]. Methodology for the examination of the plant photosystems, using longer periods of irradiation and electrochemical detection techniques, has also been reviewed[5].

It was shown, for example, that by imposing potentiostasis (voltage clamp) on irradiated thylakoid membrane suspensions an increasing current with time was produced. This electric current was obtained as a result of the oxidation of water by the irradiated thylakoids with resultant production of oxygen and reducing equivalents (electrons).

The results of studies using chemical substances known to inhibit charge transfer at specific sites within the plant photosystems, suggested that the charges being transferred to the electrode under normal conditions came from the reducing side of photosystem I. Of interest too were the findings, using catalase as a radical scavenger, which suggested the formation of · OH (ads) as a result of the oxidation of water by the irradiated thylakoids. This was verified by irradiating a thylakoid suspension containing allyl alcohol. At the end of the experiment the presence of glycerol in the medium was detected[6].

One of the interesting observations made as a result of attempts to establish a reproducible base-line for analytical studies using thylakoid membranes, was the fact that although the absolute coulombic outputs, obtained by integration of the current vs time curves for the 1st 1-min light period, varied from day to day, the 2nd, 3rd, etc. light period gave essentially a constant fractional value of the 1st period. Thus it was possible to use the coulombic output fractional value for the 2nd light period, as a control for experiments in which thylakoid behaviour was affected by a phytotoxic agent added immediately after the 1st light period.

The procedure followed in a blind test of the validity of the method involved exposure of a buffered thylakoid suspension (15 cm^3 0.1 M KH_2 PO_4 – K_2H PO_4, pH 6.8 + 0.2 cm^3 thylakoid concentrate equivalent to 200 µg chlorophyll) to 2 successive light pulses of 1 min each separated by a 15-min dark period. In instances where a chemical compound was to be evaluated, 10 mm^3 of a 3×10^{-3} M (0.03 µmoles) solution was added immediately after the 1st light period.

The comparative results obtained for a number of chemical compounds of unknown activity vs the inhibitor 2-chloro-4 cyano-isopropyl-amino-6-ethylamino-1,3,5-triazine (Bladex, Shell) and methyl viologen (Paraquat, ICI) a potentiator of electron flow in the plant photosystems are shown in tables 1 and 2.

The results of the bioelectrochemical assays were qualitatively similar to those obtained in the greenhouse testing on whole plants.

Null potential voltammetry

The earlier results were achieved with a relatively inefficient electrochemical cell system requiring comparatively large volumes of solutions, and non-ideal for the uniform irradiation of the thylakoid mem-

Table 1. Agents inhibiting electron flow

	Coulombic output % period 1	Corrected for % decrease of control	Activity fraction Bladex
Control	92.9	–	–
Bladex	27.6	29.7	1.00
BS1	77.6	83.5	0.35
BS2	67.1	72.2	0.41
BS3	74.1	79.7	0.37
BS4	23.4	25.1	1.18
BS5	16.6	17.8	1.66

Table 2. Agents potentiating electron flow

	Coulombic output % period 1	Corrected for % decrease of control	Activity fraction Paraquat
Control	90.6	–	–
Paraquat	554.8	612.3	1.0
PS1	452.6	499.5	0.81
PS2	391.6	432.2	0.70
PS3	360.9	398.3	0.65
PS4	342.9	378.0	0.61

brane suspension. Therefore for the studies using null potential voltammetry a new thin-film cell of greater efficiency was designed (figure 1). In addition to the coulombic behaviour of the system, as manifested by the current vs time recording, it was also possible to determine the power output of the system from an xy recording of the current vs the applied voltage across the cell's electrodes. Other parameters such as resistance change of membrane material as a result of irradiation, as well as changes in the potentials of charge transfer to the detecting electrode under normal conditions, and in the presence of chemical agents affecting charge transfer processes could also be obtained[7].

The electrochemical cell used in these investigations was mounted in a horizontal position so that the light beam, reflected from a mirror, completely covered the 1 cm^2 exposed electrode surface. Prior to use, the platinum working electrode (f in figure 1) was treated for 3 min with 50% aqueous aqua regia (50 °C), washed with distilled water, dried and then, together with the counter electrode, immersed in concentrated nitric acid (50 °C). After exhaustive washing in distilled water and drying, the working electrode was mounted in the cell between the silicone gasket and the cooling block. The 0.05-cm^2 counter electrode (e in figure 1) was mounted in a corner of the 1-cm^2 window between the perspex cover and the silicone gasket. The salt-bridge tip, an extension of the reservoir containing a saturated calomel electrode (SCE), consisted of an appropriate length of 1 mm bore PVC tubing packed at the open end with asbestos fibres. The bridge was filled with 0.1 M phosphate buffer, pH 6.8.

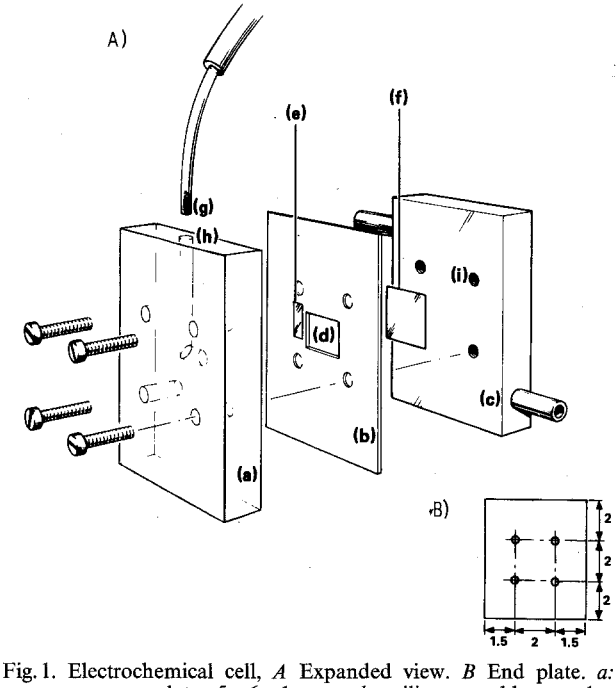


Fig. 1. Electrochemical cell, *A* Expanded view. *B* End plate. *a:* perspex cover plate 5×6×1 cm; *b:* silicone rubber gasket 5×6×0.2 cm; *c:* stainless steel cooling block 5×6×1 cm contains two 3 mm ID tubular openings for water circulation; *d:* window in silicone rubber gasket 1×1 cm; *e:* Pt counter electrode; *f:* Pt working electrode; *g:* salt bridge inserted into opening h; *h:* channel (3 mm ID) leading to irradiation compartment; *i:* tapped to accept 3 mm nylon screw.

A 24-V/250-W quartz-halogen lamp incorporating a Wratten No. 29 filter transmitting light of a wavelength predominantly greater than 630 nm, the most effective region for activation of the photosystems, served as the light source. The duration of illumination was determined by a solenoid-operated shutter controlled by an electronic time activated relay.

The instruments used in the system (figure 2) consisted of a potentiostat, a potential meter and XY and XT recorders. The recorder's drive and pen units were synchronized with the shutter solenoid via the electronic timer-relay system.

After assembly of the electrochemical cell, 200 mm^3 of the thylakoid suspension (equivalent to 50 µg chlorophyll) was introduced into the irradiation compartment. The cell was positioned for illumination and equilibrated to temperature for 10 min. At the end of this period the electrode is apparently plated with a layer of thylakoid material. The 'dark' potential of the working electrode vs SCE (U_R) was determined and this potential then imposed on the working electrode with the potentiostat (U_C). The response of a thylakoid suspension (Th) to 3 successive light periods of 1 min intercepted by a 15-min dark period is shown in figure 3.

The coulombic output for light periods 1, 2 and 3 in figure 3 were 314, 377 and 429 µC respectively. By treating these data in the same fashion as described earlier for the comparative assay of agents inhibiting or potentiating photosystem activity, it was possible to use this more refined system for the

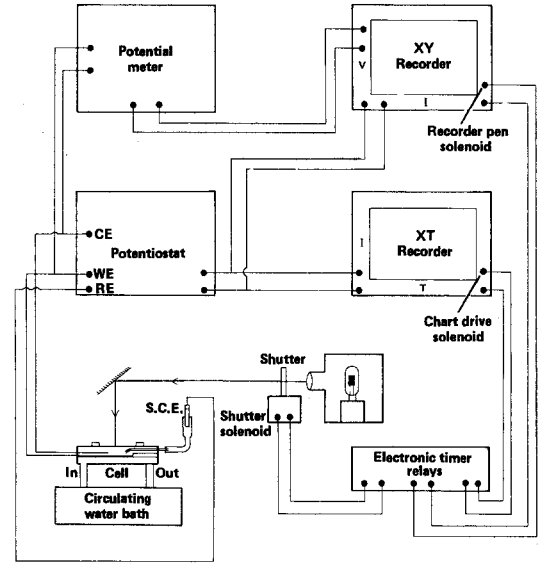


Fig. 2. Schema of electrochemical instrumentation.

quantitative evaluation of the dose-response behaviour of the thylakoids to these chemical substances.

Of further interest is the voltammetric responses of the irradiated thylakoid suspension (figure 4). The biphasic I vs curves are similar in character to the I vs t curves obtained after irradiation of a thylakoid suspension in the presence of peroxidase[6]. The explanation given previously, based on fluorescence emission (680 to 710 nm) biphasic curves obtained as a function of time during irradiation with blue light, was that the initial portion of the curve represented electron transport along the redox components of photosystem II and the 2nd portion of the curve, photosystem I.

If the intermediate plateau in the biphasic curves represents a distinction between the 2 segments of the photosystems, then the midpotentials (U_m) obtained from this region of the null potential voltammetric curves, may represent the electrochemical events occurring during charge transfer from the reducing side of photosystem II to the oxidizing side of photosystem I. As the potential of the working electrode is 'clamped', it is then the potential of the counter-electrode which is changing. Therefore these counter electrode potentials are related to the reduction of biochemical species formed as a result of the photo-oxidative processes, and the U_m values then relate to the composite redox potential of the bridge between the two photosystems. Reference to the accepted 'Z' scheme of electron flow would indicate the potential range from the reducing side of photosystem II to the oxidizing side of photosystem I to be approximately $U_0'=0$ to $U_0'=+385$ mV at pH 7.0[8]. On this basis then, for example, the U_0 value for L-1 period of -212 mV (sign of the counter electrode) when adjusted to the normal hydrogen electrode (NHE) (assuming the working electrode to be the reference electrode for the counter-electrode) gives a $U_0'=+386$ mV which essentially falls within the range of the potentials quoted.

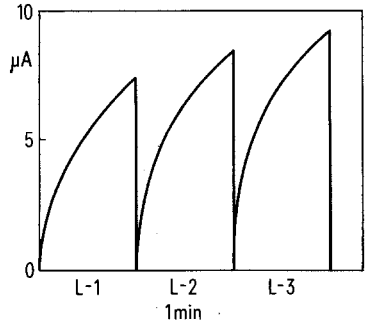


Fig. 3. Current vs time responses of irradiated thylakoid suspension. Reaction conditions 200 mm³ (μl) Th suspension in pH 6.8 buffer; $U_R - U_C = 351$ mV vs SCE; L/D-1/15 min, 20 °C.

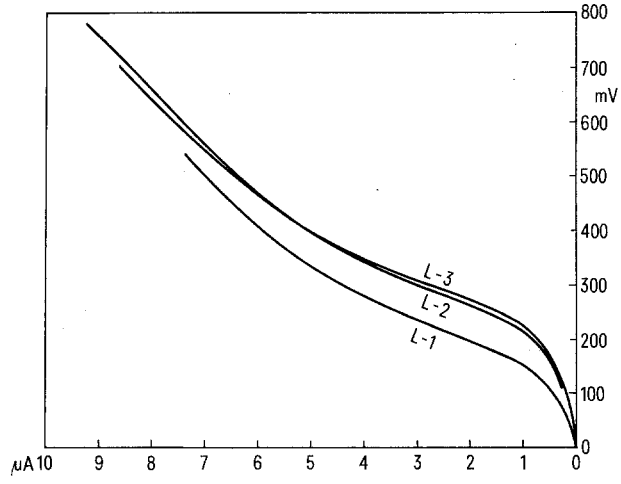


Fig. 4. Null potential voltammetric responses of irradiated thylakoid suspension. Reaction conditions as in figure 3.

The triazine mentioned earlier is known to inhibit electron flow beyond the reducing side of photosystem II. Thus the absence of a biphasic response, in the voltammetric curve obtained from an irradiated thylakoid system containing 5 nmoles of the triaze, adds strength to the possibility that the plateau separating the curves normally obtained, represents the bridge between the 2 photosystems.

It has also been found possible by using null potential voltammetry, to elucidate the site of action of various herbicidal substances on the plant photosystems.

Transport of biocides through leaf membranes

A neo-biological system which approximated the total leaf behaviour was developed for the electrochemical investigations of the passage of photosystem toxicants in suitable dispersants, through leaf cuticle and epidermis, to the sites of action within the leaf, namely the thylakoid membranes[9].

Earlier it was shown that the irradiated thylakoids' electrophysiological responses are affected by the presence of agents which inhibit or potentiate charge transfer processes. For that reason it was appropriate to incorporate this transducer into a total system consisting of:

SCE/Pt(WE) Thylakoid membrane suspension Pt(CE)//Diluted biocide formulation

Leaf epidermis-cuticle preparation

The electrochemical cell and schema of the instrumentation used are shown in figures 5 and 6.

Prior to the initiation of an experiment, a segment of the desired leaf is cut away and its abaxial surface is gently rubbed with a moistened cotton bud applicator until essentially all the green material has been removed. After being rinsed and inserted between blotters to remove excess moisture, the preparation

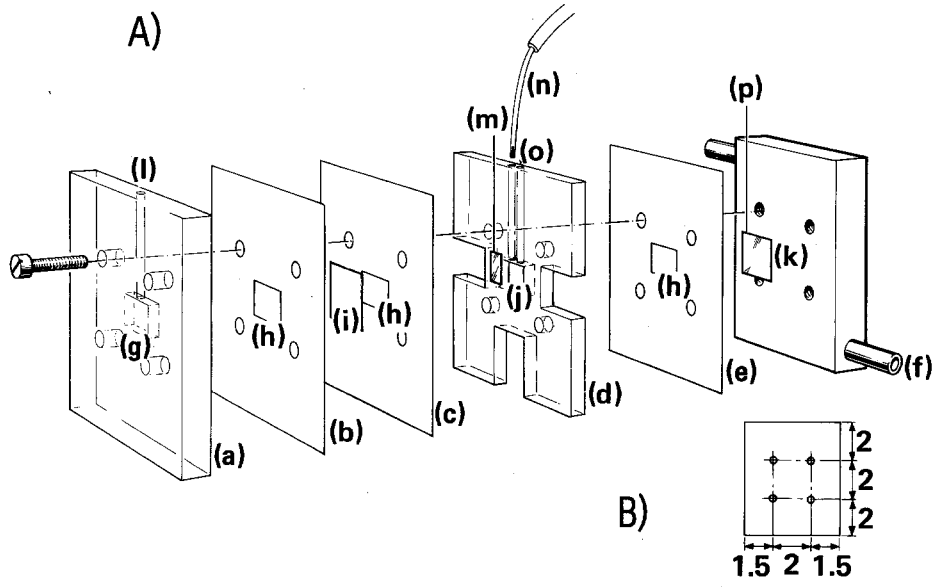


Fig. 5. Electrochemical cell. *A* expanded view. *a:* perspex cover plate 5×6×0.6 cm with inner surface compartment, g, 1×1×0.3 cm and channel, l (2 mm ID); *b:* silicone rubber gasket 5×6×0.15 cm; *c* and *e:* silicone rubber gaskets 5×6×0.1 cm; *d:* electrode compartment perspex block 5×6×0.45 cm containing 2 side slots, 1.6×1.3 cm and bottom slot, 2.5×1.3 cm for insertion of fibre optic light guides with centrally located window; *j:* 1×1 cm optically polished on side and bottom sections with 2 channels, *o*, (3 mm inner diameter); *h:* windows in silicone rubber gaskets 1×1 cm; *k:* stainless steel cooling block 5×6×1 cm containing 2 3 mm inner diameter tubular openings, *f*, for water circulation; *i:* membrane preparation; *m:* Pt counter-electrode; *n:* salt-bridge inserted into opening; *p:* Pt working electrode. *B* End plate, screw centres tapped to accept 3 mm nylon screws, dimensions in cm.

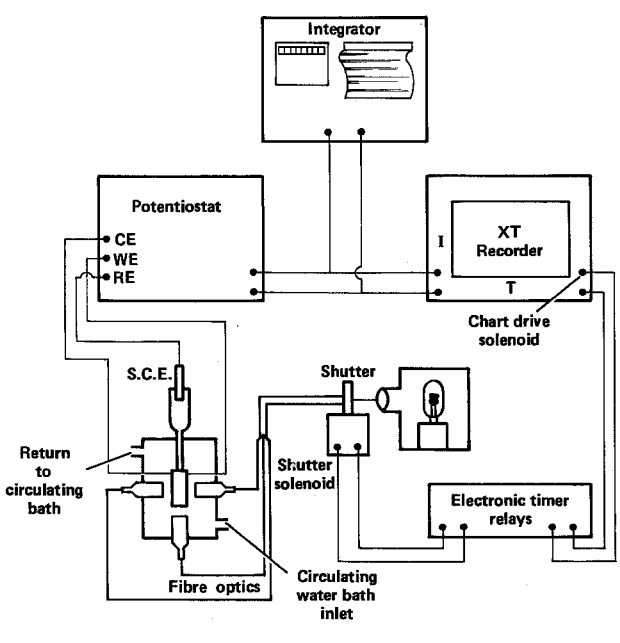


Fig. 6. Schema of electrochemical instrumentation.

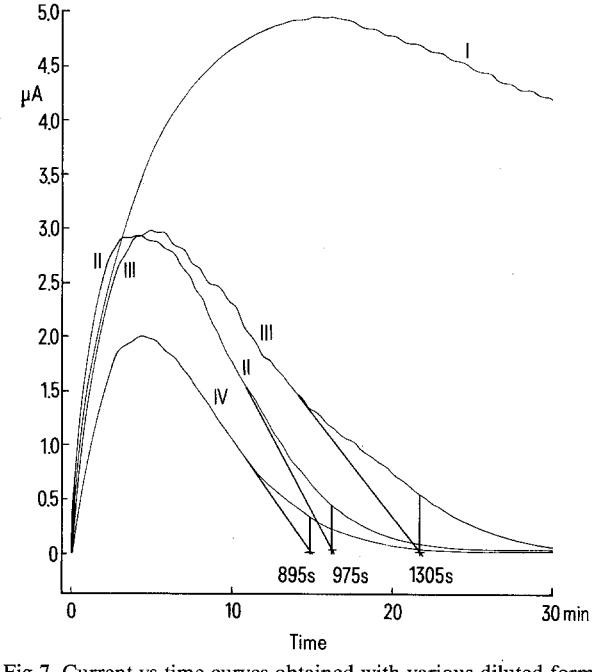


Fig. 7. Current vs time curves obtained with various diluted formulations containing 1 mg triazine biocide/400 mm³. I control - no formulation in contact with cuticle-epidermis preparation; II, formulation A; III, formulation B; IV, reference formulation EC. The electrode compartment contained 600 mm³ thylakoid suspension (equivalent to 100 μg chlorophyll) in pH 6.8 0.1 M phosphate buffer.

is clamped into the electrochemical cell in such a fashion that the epidermal portion of the leaf is exposed to the electrode compartment (j in figure 5), and the cuticle to the compartment into which will subsequently be added the diluted biocide formulation (g in figure 5).

An example of the current vs time curves obtained using soybean leaf (Amsoy strain) cuticle epidermis preparations with various formulations vs a standard formulation are shown in figure 7. These formulations consisted of the biocide, oil, emulsifiers and solvent which were prepared for use by dilution with standard

hard water (5.56 g $MgCl_2$, 12.5 g $CaCl_2$ in 1 l distilled H_2O) to give a 1.25% V/V emulsion containing 1 mg biocide/400 mm^3.

Integration of the current vs time recordings to the intersect gave the coulombic outputs from which were obtained the relative activities of the unknown formulations vs the reference formulation EC (table 3).

The results obtained with the example in table 3 and other biocide formulations were correlated with similar studies in the greenhouse with soybean plants. In all these instances, the results obtained using the electrochemical technique were qualitatively in agreement with those from the total plant responses. Similar correlations were also obtained using membranes from the french bean, broad bean, cotton, maize, etc.

The bio-electrochemical system developed is such that it is analogous to the living system, both physiologically and thermodynamically. It is likely that it would be quite possible to utilize similar approaches with other physiologically active materials providing that the biological receptors can also serve as electrical transducers.

Effects of surfactants and biocides on membrane systems

The studies related to the passage of various biocide formulations through leaf cuticle-epidermis tissue, led to speculation about the actual effects of the various components of these formulations on this membrane system.

In order to undertake such a study, an electrochemical system was developed in which the thylakoid transducer used in the previous penetration studies, because of its susceptibility to phytotoxic agents, was replaced by a voltage scan generator which simulated a thylakoid-like electrical behaviour pattern. Also designed into the system was a scheme of control-produced data which could be related to the change in the various electrical parameters due to the introduction of a membrane into the electrical field of the electrochemical cell[10].

The electrochemical cell used in these studies was similar to that described in figure 5 but also included a 2nd counter electrode (CE2) inserted between the perspex cover plate (a in figure 5) and the silicone gasket (b in figure 5).

The instrumentation system used in conjunction with the various plant cuticle epidermis preparations (figure 8) gave current vs voltage curves similar to those shown in figure 4.

An important item in studies in which the absolute values of the electrical parameters will vary from one membrane species to another within a particular leaf species, is the design of adequate controls. In an earlier section of this review it was shown that by relating the 2nd and subsequent scan coulombic outputs to the 1st scan, essentially a constant fractional value was obtained. A similar behaviour was observed using this system to obtain integrated currents. The integrated voltage change between the working electrode and the appropriate counter electrode followed a similar pattern. The method used to determine the transfer equivalents attributable to the presence of the membrane in the electrochemical cell involved 2 separate experiments.

A control experiment consisted of an initial scan (L-1) on the electrochemical cell clamped at its null potential using counter electrode, CE 1.

600 mm^3 pH 6.8
SCE/Pt - phosphate buffer (0.1 M) - PT (CE 1) // After L-1 added
500 mm^3
solution to be tested
Membrane

The integrated current and voltage data obtained serve as the baseline for the subsequent scans. For each successive scan the integrated current and voltage is related to L-1 data as a fraction of that current and voltage value. In addition, from the data

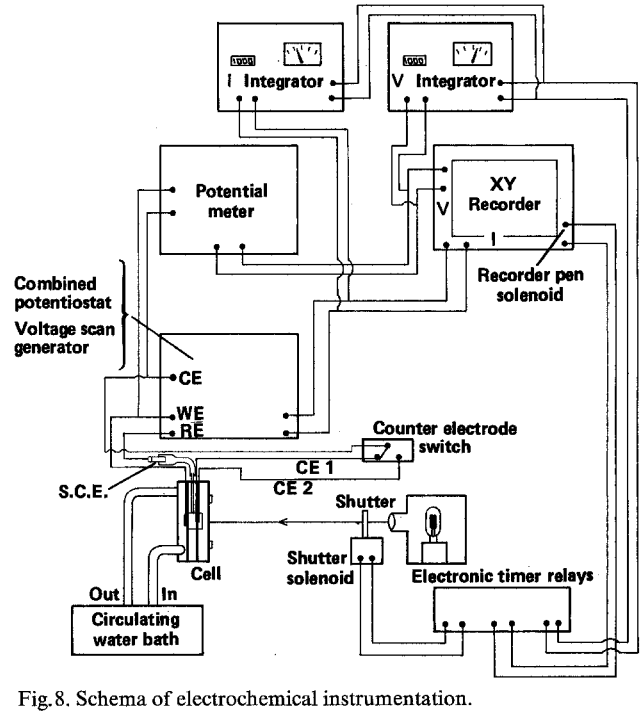


Fig. 8. Schema of electrochemical instrumentation.

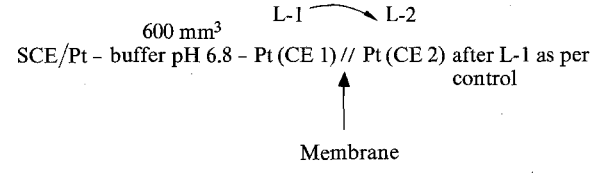
Table 3. Coulombic outputs obtained with various formulations

Formulation	Coulombic output (μC)	Relative activity
EC	1098	1
A	1898	0.58
B	2370	0.46

is calculated the capacitance of the system using the equation

$$C = \frac{\int A(ts)}{\int V} = \text{Farads}$$

The 2nd experiment differs from the control in that immediately after addition of the test solution, CE 1 is switched to CE 2.



This now introduces the membrane between the working and counter electrodes. The following scans reflect the differences due to the presence of the membrane. In this instance the 1st value obtained (L-1) is corrected by use of the fraction values for each of the scans of the control experiment. This then yields values as if the membrane were not in the system, and the difference between the adjusted values and those actually obtained from L-2 onward in the 2nd experiment reflects the effect of the membrane on the electrical measurement.

The following is by way of illustration.

Control experiment (I)
L-1 a mV/min, a′ μF
L-2 b (fr $^{b}/_{a}$), b′ (fr $^{b'}/_{a'}$)

2nd experiment (II)
L-1 A mV/min, A′ μF
L-2 B mV/min, B′ μF

Calculation
L-1 II correction factor
L-2 A x (fr $^{b}/_{a}$) = corrected value (mV)
L-3 A′ x (fr $^{b'}/_{a'}$) = corrected value (μF)

The ΔmV and ΔμF can be obtained from the difference between the corrected control and the experimental voltage and capacitance values in the 2nd experiment. The equivalents transferred due to the presence of the membrane can be obtained from $(\Delta mV \times 10^{-3}) \times (\Delta \mu F \times 10^{-6})/96{,}500$.

Some of the results obtained with soybean leaf (SBLEC) and broad bean leaf (BBLEC) cuticle-epidermis preparations in the presence of the surfactant, Emulsogen (HOECHST) and the phytotoxic triazine, PT[9] in pH 6.8 buffer, are given in table 4.

Table 4. Equivalent transferred across various leaf-membrane systems under artificially induced active transport

System	p-equivalents
SBLEC/pH 6.8	34.9
SBLEC/Emulsogen	42.8
SBLEC/PT	6.4
BBLEC/pH 6.8	169.0
BBLEC/Emulsogen	269.5
BBLEC/PT	26.2
BBLEC/PT + Emulsogen	62.6

In all instances in which Emulsogen and other non-phytotoxic surfactants were introduced on the cuticle side of the membrane preparation, an increase in the number of transport equivalents was obtained. The magnitude of the increase was dependant on the type of surfactant used and undoubtedly due to some degree to the partial or complete removal of lipid material from the leaf surface.

A more interesting observation is related to the effect of phytotoxic biocides, such as PT. As there is essentially no photosystem activity associated with the membrane systems used, the pronounced inhibition noted with PT and other phytotoxic agents can only be considered as a separate and distinct effect on the normal behaviour of the epidermal membrane systems. This is restricted to the epidermal membrane as onion epidermis, a membrane lacking cuticle material, demonstrates this same behaviour. A possible explanation for this observation is that these agents may affect the gateing mechanisms of the membrane pores, inducing a partial or complete stasis of these gates, which results in a restricted flow through the epidermal membrane. The limited reversal of inhibition, when a mixture of PT and Emulsogen is used, cannot be construed as an indication that the surfactant is inhibiting the metabolic poisoning effect of the biocide. In this instance the surfactant is countering the biochemical effects of the phytotoxic agent by some physico-chemical means.

It is appropriate now to suggest boldly that 'the end of the beginning', a comment made some time ago in relation to the application of electrochemical techniques to the study of biological phenomena[11], has long since passed. Bioelectrochemistry is now an established discipline which undoubtedly will continue to flourish and expand its activities from purely biochemical areas to the study of the more challenging problems of total biosystems taking into account the changing environment to which these systems are exposed.

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