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NEW METHOD FOR THE MEASUREMENT OF MEMBRANE PERMEABILITY FOR ^3H_2O IN PLANT TISSUES

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Key Word Index—Membrane permeability; tritiated water; flow-through cell; half-time.

Abstract—The efflux of ^3H_2O from segments of etiolated mung bean seedlings was measured using a flow-through cell designed to collect a large number of fractions. A computer program was developed that split the diffusion curve into its three components: one curve representing a very fast diffusion out of the free space ($t_{\frac{1}{2}} = 0.21$), one of an intermediate diffusion rate ($t_{\frac{1}{2}} = 2.09$) and one of a slower diffusion rate ($t_{\frac{1}{2}} = 11.41$).

INTRODUCTION

In the past, qualitative as well as quantitative studies on plant membrane permeability have been undertaken. The permeation of applied dyes into the vacuole, and the study of plasmolysis and deplasmolysis are processes easily observed by light microscopy. However, the chemical nature of the dye and the rather high concentrations of plasmolyticum used may disturb the normal function of the cell and its membranes [1].

By using liposomes with known composition, the influence of some membrane components on transport processes can be studied and it is assumed that their function is similar *in vivo*. The use of deuterium and tritium isotopes offers many advantages over the above cited techniques. It may be assumed that labelled compounds behave as unlabelled ones and that they do not influence membrane characteristics. We developed a method for measuring passive membrane transport of tissues saturated with tritiated water. We do not pretend that water-transport itself is a limiting factor in tissues, but it certainly gives an indication of the general permeability of the membranes of a tissue.

RESULTS

Plant material and incubation medium

Five mm segments of etiolated mung bean hypocotyls were used in our study. The seedlings were grown in moist vermiculite in a dark room at 25°. After cutting, batches of 10 segments were rinsed for three hr by incubating them at 25° (dark) in petri-dishes (5 cm diam.) containing a filter paper soaked with 0.5 ml salt solution ($1.72\ 10^{-2}$ M NaCl, $8.64\ 10^{-4}$ M Na_2SO_4, $9.84\ 10^{-2}$ M $NaNO_3$). This solution approximates the salt concentration of the cells [2]. After this treatment, the segments were saturated with tritiated water in 12.5 ml salt solution containing 37.5 μCi ^3H_2O. In preliminary experiments, it was found that after a 90 min loading sufficient ^3H_2O was taken up by the tissues. The influx and efflux of water mainly occurs through the cut surfaces, as the cuticula prohibits water diffusion.

After the incubation period, the segments were immersed for five sec in salt solution to remove most of the adhering ^3H_2O. Then the segments were placed in a flow-through cell.

Flow-through cell

An inverted 2 ml disposable syringe was used as a flow-through cell (A). To obtain a hollow plunger for the solvent to flow through, a 1 ml syringe was used. Its needle was punched through the original rubber septum of the 2 ml plunger, and cut off at the rubber surface. Then the syringe was fixed at the foot of the septum by an appropriate gum. The needle of the flow-through cell was bent downwards (Fig. 1). In preliminary experiments, it was found necessary to use the flow cell upside down to ensure rapid removal of air and an efficient exchange and discharge of ^3H_2O. The end of the hollow plunger was connected to a reservoir (B) placed above the flow cell and containing non-radioactive salt solution. This flowed by

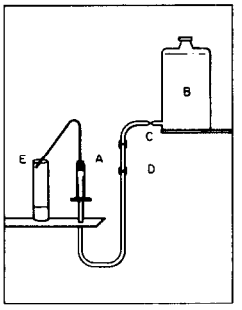

Fig. 1. Flow-through cell for measuring the efflux of tritiated water from plant tissue as a function of time. Non-radioactive medium flows from reservoir B through the cell A containing the segments. The tritiated water in the segments is exchanged with the non-radioactive water. The flow is regulated by clamp C on the rubber tubing. Clamp D is opened when the experiment starts. Over a period of 30 min, samples of 1 ml are collected in E and the tritium is counted in a liquid scintillation counter.

gravity through the cell at 2 ml/min. The flow was regulated by a clamp (C) on the rubber tubing. Ten plant segments were placed in the cell after stopping the flow by a second clamp (D) on the tubing. The plunger was then pushed into the syringe leaving a cell volume of 1 ml, thus allowing for optimal contact of the segments with the non-radioactive water. After opening clamp D the ^3H_2O of the segments was exchanged with the water of the medium. Over a 30 min period, samples of 1 ml were collected every 30 sec. This was done in a modified fraction-collector allowing four simultaneous experiments. An experienced person can start up an experiment in about five sec after the segments have been placed in the cells.

In our experiments, rigorous control of the 30 sec interval was critical, but not the exact volume of 1 ml water collected. This was only important for obtaining a good emulsion with 3.4 ml of the scintillation fluid (6.4 g PPO:800 ml Triton X-100:1600 ml Xylol). The radioactivity was measured in a liquid scintillation counter.

Effect of gravity

Because the non-radioactive water flowed from the reservoir through the cell by gravity, the experimental set-up might be criticised on the grounds that the pressure on the segments affects the membrane permeability of the tissue. The pressure on the segments is given by eqn (1).

$$p = p_o + \rho g h \tag{1}$$

where p_o is the atmospheric pressure, ρ the density of water, g, the gravity constant and h the difference in height between the segments and the water-surface of the reservoir ($h = 0.2$ m). This gives us:

$$\begin{aligned} P &= 1 \text{ atm} + 1000 \text{ kg/m}^3 \times 9.8 \text{ m/sec}^2 \times 0.2 \text{ m} \\ &\approx 1 \text{ atm} + 2000 \text{ kg/m sec}^2 \\ &\approx 1 \text{ atm} + 2000 \text{ N/m}^2 \qquad \text{as } 1 \text{ N} = 1 \text{ kgm/sec}^2 \\ &\approx 1 \text{ atm} + 2000 \text{ Pa} \qquad \text{as } 1 \text{ Pa} = 1 \text{ N/m}^2 \\ &\approx 1 \text{ atm} + 0.02 \text{ atm} \qquad \text{as } 1000 \text{ Pa} \approx 0.01 \text{ atm} \\ &\approx 1.02 \text{ atm} \end{aligned}$$

The additional pressure of $\pm$ 0.02 atm is negligible because it is in the range of the natural fluctuations of the atmospheric pressure. We also carried out experiments in which the reservoir was placed at different heights above the plant segments (5, 10, 20, 30 cm). The flow was maintained at 2 ml/min by adjusting clamp C. None of these heights had any influence on membrane permeability (results not shown).

Calculations

When the radioactivity content of the fractions was plotted against time an exponential curve was obtained, a result to be expected from a passive diffusion of water (Fig. 2a). It is possible to calculate the best fitting exponential curve (dotted line in Fig. 2a) by use of eqn (2):

$$y = a\, e^{bx} \quad (a > 0 \text{ and } b < 0) \tag{2}$$

where y is the radioactivity in the fractions (dpm), x, the time (min) a, the intercept with the ordinate and b, the slope of the curve.

$$a = \exp\{(\sum \ln y_i)/n - b(\sum x_i)/n\} \tag{3}$$

$$b = \frac{\{\sum(x_i \ln y_i) - [(\sum x_i)(\sum \ln y_i)/n]\}}{\{\sum x_i^2 - \sum^2 x_i/n\}} \tag{4}$$

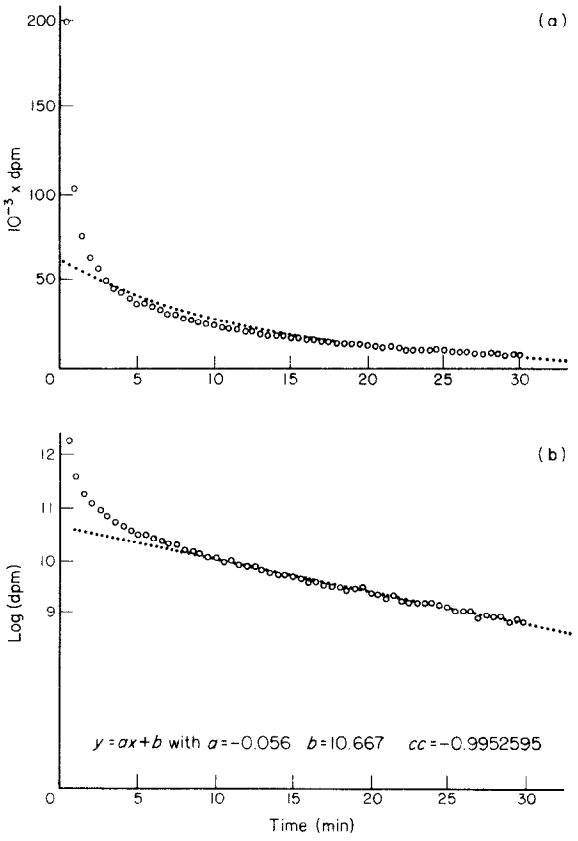

Fig. 2. (a) Radioactivity content of the fractions obtained every 30 sec plotted against time. The best fitting exponential curve is drawn by a dotted line. (b) The logarithms of the radioactivity plotted against time. The best fitting straight line (points 17 to 60) is drawn by a dotted line. The equation of the best fitting straight line is given.

The correlation between the curve obtained from the measured values and the best fitting exponential curve is given by the following coefficient of correlation [eqn (5)]:

$$r^2 = \frac{\{(\sum x_i \ln y_i) - [(\sum x_i)(\sum \ln y_i)]/n\}}{\{[\sum x_i^2 - (\sum^2 x_i/n)][\sum \ln^2 y_i - (\sum^2 \ln y_i/n)]\}} \tag{5}$$

This coefficient of correlation gives values between 0 and 1. The closer to 1, the better the curve with the experimental data relates to a pure exponential curve.

The best fitting exponential curve obtained permitted the calculation of the half-time, a measure of the permeability. This is the time needed to halve the efflux of the tritiated water from the tissue. If the half-time is low, the efflux of the tritiated water occurs very quickly i.e. the tissue has a high permeability for water. A high value for the half-time shows a low permeability for water.

The equation for the exponential curve $y = ae^{bx}$ was used to calculate the half-time. After 0 minutes the radioactivity in the fractions is $y = a$ dpm; after $t_{1/2}$ minutes the radioactivity is given by $y = a/2$ dpm. It follows that:

$$\begin{aligned} a/2 &= a\, e^{bt_{1/2}} \\ \text{or } t_{1/2} &= -\ln 2/b \quad (b < 0) \end{aligned} \tag{6}$$

When the above calculations were performed on our experimental data, it was found that the experimental

curve did not fit very well with a pure exponential curve. The calculated coefficient of correlation was rather low (± 0.93). By elimination of the first points, the best correlation coefficient was found after the elimination of the first 16 points, i.e. the points from the fractions collected during the first eight min.

Plotting the logarithms of an exponential curve against time should give a straight line. Figure 2b shows that, again, the first 16 points do not fit with the straight line that is obtained with the points 17 to 60. Careful examination of Fig. 2b shows that the first 16 points lay on one or two straight line(s) with a much steeper slope than the next points.

For the exponential curves, the best fitting straight line as well as the correlation coefficient between the experimental and best fitting line can be calculated. The best fitting straight line (dotted line in Fig. 2b) is calculated by eqn (7):

$$y - Y = k(x - X) \tag{7}$$

where

$$k = \frac{[\sum x_i y_i - (\sum x_i y_i/n)]}{[\sum x_i^2 - (\sum^2 x_i/n)]} \tag{8}$$

and

$$X = \sum x_i/n \tag{9}$$

and

$$Y = \sum y_i/n \tag{10}$$

The coefficient of correlation is obtained by eqn (11).

$$r = \frac{\sum\{[\ln y_i - (\sum \ln y_i/n)][x_i - (\sum x_i/n)]\}}{\{\sum[x_i - (\sum x_i/n)]^2 \sum[\ln y_i - (\sum \ln y_i/n)]^2\}^{1/2}} \tag{11}$$

To perform all the above calculations and associated plots, a computer program has been developed that is available on request.

The first 16 measurements most probably demonstrate the diffusion of the water in the apparent free space. This water is exchanged very rapidly with the water in the medium. This explains the low half-time. After the efflux from the apparent free space, the diffusion through the membranes is measured. The half-time calculated from these points is a measure of the permeability of the membranes to water. To obtain the best measure of this permeability, we have to discard the data for the diffusion of the apparent free space.

In our computer program, the first points are eliminated one by one, and each time the coefficient of correlation is calculated between the remaining points and the best fitting curve. When the highest correlation is found, the half-time is calculated and this is taken as the best estimation of the water permeability through the membranes. The coefficient of correlation reaches very high values sometimes up to 0.999. This means that by calculating the half-time of such good fitting curves, a very good estimation of the water permeability through the membranes can be made. This is also reflected by the very low s.e. of half-times calculated in different experiments (10.23 $\pm$ 0.30, mean and s.e. of 10 measurements). Because of the high coefficient of correlation resulting from the high number of collected fractions, it was possible to discard every two collected fractions without significantly influencing the half-time. This could reduce excessive use of the liquid scintillation counter. However, to keep the same precision in the curves, it is not advisable to collect 2 ml fractions every minute instead of 1 ml fractions every 30 sec, because this leads to a loss of resolution.

Detailed analysis of the diffusion curves

In experiments on the diffusion of radioactive potassium chloride, Lüttge [3] considers three straight lines when the log curves are plotted. The first one shows a half-time of a few minutes, the second one of a few hours and the third one of up to a few days. The second straight line was distinguished from the first one by measuring the diffusion of potassium chloride at 4°. Lüttge interprets the three distinct curves as the diffusion from the apparent free space (fastest $t_{\frac{1}{2}}$), through the plasmalemma ($t_{\frac{1}{2}}$ of a few hours) and the tonoplast ($t_{\frac{1}{2}}$ from a few hours to a few days).

As Fig. 2b suggested three straight lines, a few additional experiments were done in which we collected the ^3H_2O every 15 sec, giving 120 data points for each experiment (Fig. 3a). The computer program was modified to allow for extented calculations.

The analysis was started in the usual way. After the elimination of the first 48 points (i.e. from the fractions collected during the first 12 min), the $t_{\frac{1}{2}}$ was calculated for the last points (49–120) (line 3 in Fig. 3b, $t_{\frac{1}{2}} = 11.41$ min, $cc = -0.994$). In a control calculation, the $t_{\frac{1}{2}}$ of line 3 was also calculated with the last 50 data only. This gave a very similar $t_{\frac{1}{2}}$ ($t_{\frac{1}{2}} = 11.56$, $cc = -0.973$). This proves that there is no significant influence of the fast diffusion occuring

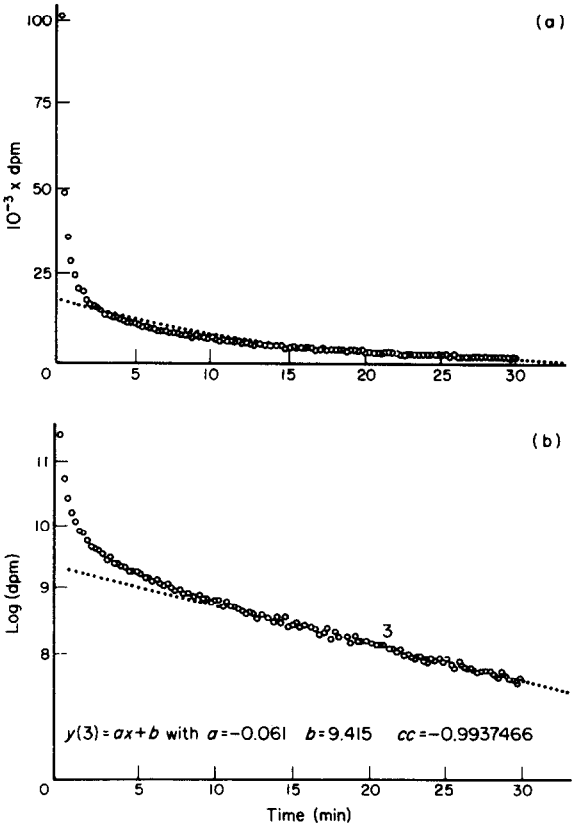

Fig. 3. (a) Radioactivity content of the fractions obtained every 15 sec plotted against time. The best fitting exponential curve is drawn by a dotted line. (b) The logarithms of the radioactivity plotted against time. The best fitting straight line, line 3 (points 49 to 120), is drawn by a dotted line. The equation of the best fitting straight line is given.

during the first 12 min on the diffusion occuring after the first 12 min. The diffusion after 12 min (represented by line 3) on the contrary, has a pronounced influence on the data of the first 12 min and provokes an understimation of the $t_{\frac{1}{2}}$ of this fast difusion. From the equation of line 3 ($y3 = -0.061x + 9.415$), the contribution of line 3 to the data of the first 12 min can be calculated by extrapolation. After substraction of this calculated contribution of line 3 from the former 48 data points, a new plot of the former 48 (corrected) points is obtained (fig. 4a). The log of these data plotted against time fails to show one straight line, indicating that the plot is still a mixture of two curves (Fig. 4b). By elimination of the former data (12, i.e. the first 3 min), the best fitting exponential curve is obtained for the remaining data (13 to 48) and the $t_{\frac{1}{2}}$ is calculated for the second exponential diffusion curve ($t_{\frac{1}{2}} = 2.09$, $cc = -0.946$). The log of this curve gives a straight line which allows us to substract the contribution of the second line from the first line. Then the $t_{\frac{1}{2}}$ of this first diffusion curve can be calculated ($t_{\frac{1}{2}} = 0.21$, $cc = -0.986$) (Fig. 5a and b).

All these calculations and plots were done by the computer program. Besides the $t_{\frac{1}{2}}$, the computer was programmed to print out the 'end' of the three different diffusion curves. As the practical end of the diffusion, the intersection of the log-curve with the abscis is taken, corresponding to a negligible diffusion of 1 dpm (3.65, 28.48 and 154.98 min for resp. the first, second and third diffusion curve). This end of the different diffusion curves

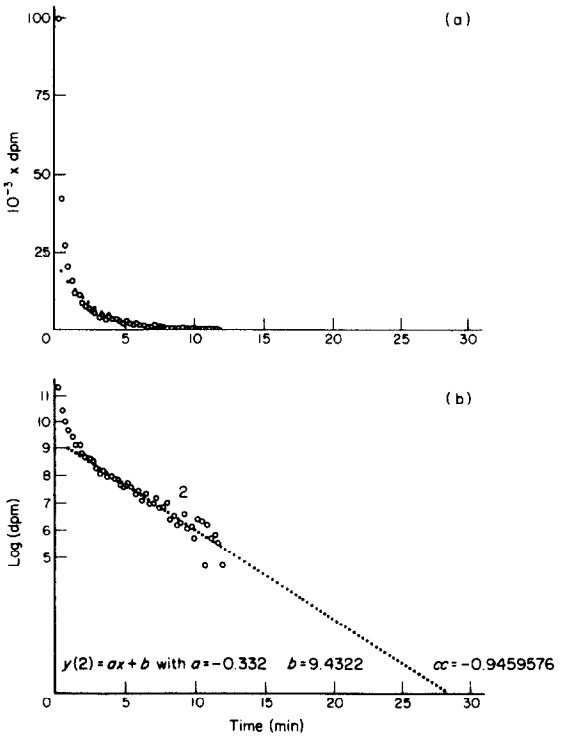

Fig. 4. (a) Radioactivity content of each of the first 48 fractions after subtraction of the contribution of line 3 from Fig. 3b. The best fitting exponential curve is drawn by a dotted line. (b) The logarithms of the radioactivity content of the first 48 fractions plotted against time. The best fitting straight line, line 2 (points 13 to 48), is drawn by a dotted line. The equation of the best fitting line is given.

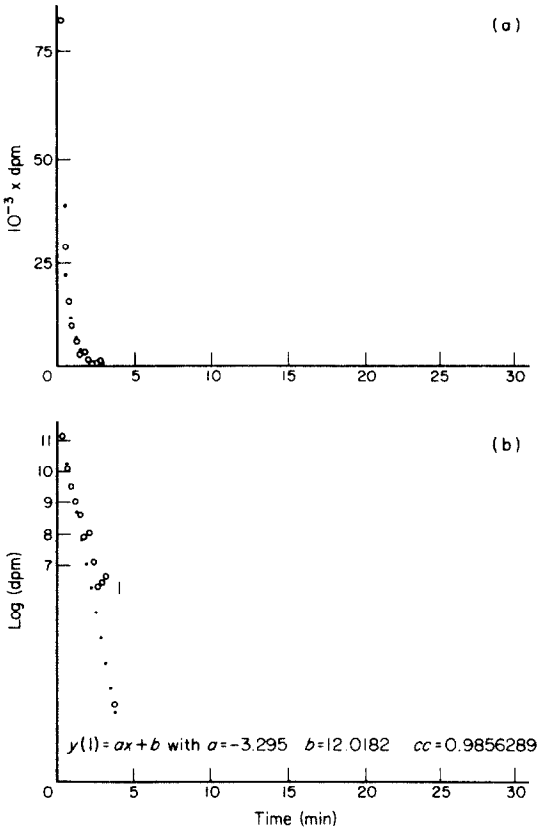

Fig. 5. (a) Radioactivity content of each of the first 12 fractions after subtraction of the contribution of line 2 from Fig. 4b. The best fitting exponential curve is drawn by a dotted line. (b) The logarithms of the radioactivity content of the first 12 fractions plotted against time. The best fitting straight line, line 1 (points 1 to 3), is drawn by a dotted line. The equation of the best fitting straight line is given.

allowed us to verify the correctness of the calculated $t_{\frac{1}{2}}$, and to make an estimate of a possible error in the calculation of $t_{\frac{1}{2}}$. The $t_{\frac{1}{2}}$ of curve 3 was calculated with the data 49 to 120 (12.25 min to 30 min). As the second diffusion curve 'ends' at 28.48 min only, its influence on curve 3 can be estimated. From equation $y(2) = -0.332x + 9.432$ of curve 2, its contribution to the amount of radioactivity measured from 12.25 min on can be calculated. It was calculated that its contribution to the third curve was only 3.5% at 12.25 min and then gradually decreased as a function of time. This proves that the calculated $t_{\frac{1}{2}}$ of the third curve is a very good measure of the diffusion from 12.25 min on.

In the same way, the influence of the first diffusion curve on the second curve can be estimated. The $t_{\frac{1}{2}}$ of the second curve was calculated with the data 13 to 48 (3.25 to 12 min). As the first diffusion curve 'ends' at 3.65 min, its influence on the data from 3.25 min on was calculated. At 3.25 min the contribution of the first diffusion curve on the radioactivity measured was only 0.08%, and it then diminished as a function of time. It may be concluded that the calculated $t_{\frac{1}{2}}$ of the second diffusion curve is quite correct.

As the $t_{\frac{1}{2}}$ of the third and second diffusion curves are accurate, we may assume that the data obtained after the

two substractions of the influence of the third and second curve on the first curve, gives a good representation of the fast diffusion curve (first curve with equation $y(1) = -3.295x + 12.018$). Therefore, its calculated $t_{\frac{1}{2}}$ is a good measure of this fast diffusion.

From these experiments and extended calculations, we may conclude that the initial exponential curve plot (Fig. 3) is in fact the reflection of the sum of three different diffusion processes. The first exponential curve indicates a very fast diffusion ($t_{\frac{1}{2}} = 0.21$), and might be ascribed to the diffusion of free water from the apparent free space. This first curve most probably corresponds to the first curve as described by Lüttge [3] and Greenway [1]. So far, we do not know if the second and the third diffusion curves are reflections of the diffusion of water through the plasmalemma (second curve) or the tonoplast (and plasmalemma) (third curve) as suggested by Lüttge [3] and Greenway [1]. Additional experiments have to be invented to discriminate between diffusion through the plasmalemma and the tonoplast.

DISCUSSION

In this study, we have used segments from higher plants to study the diffusion of water through plant cell membranes. The computer program enabled us to separate the diffusion curve into its three components. It is however, still difficult to correlate definite diffusion curves with definite compartments. By using a suspension culture of *Phycomyces* spores, we were able to discount objections concerning the diffusion from the apparent free space and through the different membranes. In this study, we had to adapt the flow-through cell to take 3 μm frits made of stainless steel. We also used a HPLC-pump to maintain the flow through the frits. A small magnetic stirrer was used to prevent the spores from sticking to the frits. The *Phycomyces* spores were taken at an early stage of germination before the visual appearance of vacuoles. Under these conditions only one diffusion curve was observed (no vacuoles and no AFS) (see Fig. 6).

Some criticism might arise about the difference in diffusion of 'free' and 'bound' water (e.g. in cell compartments containing large amounts of protein). The incubation period with ^3H_2O is kept relatively short (90 min).

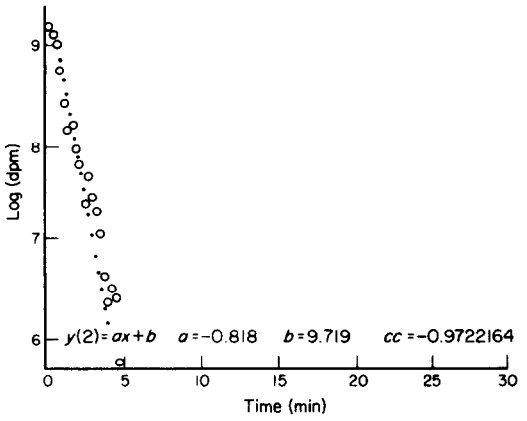

Fig. 6. The logarithms of the radioactivity content of the fractions from the efflux of ^3H_2O out of *Phycomyces* spores plotted against time. The best fitting straight line is drawn by a dotted line. The equation of the best fitting straight line is given.

Therefore, it may be expected that the exchange with 'bound' water is neglible. We suspect that 'bound' water is important in diffusion studies with longer incubation periods of several hours or days.

All the experiments are done at 20°. This temperature is held constant because lower or higher temperatures do influence the water permeability of segments of mung bean hypocotyls. Recently, Guye [4] described a loss of membrane integrity of mung bean seedlings at chilling temperatures (0 to 15°). Therefore, we incubated mung bean sections at 1° for 2.5 hr. After loading the segments with ^3H_2O, the diffusion was measured at 1°. In these experiments it was shown that the permeability for ^3H_2O increased by 35%. This effect may be explained by the loss of membrane integrity at low temperature [4].

Our method using a flow-through cell to measure ^3H_2O-efflux has a number of advantages over published methods [2,5,6]. These are: (i) unlike other authors who have constructed diffusion curves with a very limited number of data (6–10), often taken at the beginning of the experiments, when the fast diffusion curve of the apparent free space still strongly influences the data obtained, we are able to monitor the efflux continously over periods of minutes up to hours or days. It is possible to select sampling times of a few seconds up to several minutes, thus providing us with the large number of data necessary for accurate calculations of half-times and intercept points with ordinate and abscisa for the three different diffusion processes.

(ii) By the use of fast sampling times, it is possible to split up the original diffusion curve obtained with 'raw' data into its three constituent diffusion curves each with its own half-time and intercept points. Very high correlation coefficients are obtained. In the future it might be possible to demonstrate that these three curves describe the diffusion of the apparent free space (first curve), of the plasmalemma (second curve) and of the tonoplast (third curve).

(iii) By the use of a relatively high flow rate, good aeration of the tissues is provided, thus excluding possible problems with respiration and hence with cell metabolism.

(iv) This technique can also be used for studying ion transport, or the diffusion of glycerol and other 'permeants'.

(v) By careful temperature control at lower temperatures, it will certainly be possible to obtain a better separation of the three diffusion curves and even to determine the membrane transition temperatures of certain membranes.

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REFERENCES

1. Greenway, H. (1974) *Aust. J. Plant Physiol.* **1**, 247.
2. Lecock, F. and Buffel, K. (1986). *Biochem. Physiol. Pflanzen* **181**, 459.
3. Lüttge, U. (1973) *Stoffentransport der Pflanzen.* Springer, Berlin.
4. Guye, M. (1987) *News Bull. BPGRG* **9**, 10.
5. Glinka, Z. and Reinhold, L. (1972). *Plant Physiol.* **49**, 602.
6. Kang, B. G. and Burg, S. P. (1971). *Proc. Natl Acad. Sci. U.S.A.* **68**, 1730.