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# SELECTIVE COMPARTMENTATION OF THE HYDRATION PRODUCTS OF CARBON DIOXIDE IN LIPOSOMES, AND ITS ROLE IN REGULATING WATER MOVEMENT

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## Summary

Using liposomes, some possible consequences of auxin-stimulated proton secretion in plant tissues are modelled. Liposomes, made specifically permeable to protons and potassium ions, but containing at least one impermeant, swell at a rate which is proportional to the availability and hydration rate of carbon dioxide; the gas functions as a source of proton and dehydrated anion and this enables a net transfer of $KHCO_3$ to take place.

While it is recognised that one benefit to living systems derived from respiratory carbon dioxide is its provision of an almost inexhaustible supply of buffering capacity [1], it is here suggested that the unique chemistry of $CO_2$ offers a further advantageous feature to living systems. As a gas, $CO_2$ will exert an osmotic pressure in solution, but like water, it presumably freely permeates biological membranes. By contrast, hydration of $CO_2$ to form proton and bicarbonate ions produces two species with considerably reduced permeabilities to most biological membranes [2,3]. In itself, such a fundamental property is of little relevance to osmotic water movements, because of the rapid equilibration of $CO_2$ across membranes and the capacity for the reverse dehydration reaction to occur.

$$CO_2 + H_2O \rightleftharpoons H^+ + HCO_3^-$$

But if the dehydration process is discouraged by vectorial separation of the two hydration products, e.g. by proton translocation, there is scope for inducing bulk water movement, energy necessarily being expended in the selective compartmentation process.

To demonstrate experimentally the feasibility of such a selective com-

Abbreviation: FCCP, carbonylcyanide *p*-trifluoromethoxyphenylhydrazone.

partmentation of the hydration products of $CO_2$ and its role in regulating osmotically driven water movement, a series of experiments were devised exploiting the cell membrane-like properties of liposomes [4].

Since liposomes represent a non-energised system, and therefore cannot be expected to do the work required for ion translocation, their prior manipulation into a non-equilibrium state is essential. To this end, two basic types of experiments have been performed: one, in which the energy is initially put into the system by a potassium bicarbonate-induced shrinkage of liposomes. In the second, liposomes are prepared with $NaHCO_3$ inside but $KHCO_3$ outside; the driving force for subsequent events then arises by permitting the unilateral diffusion of the potassium ion inwards down its concentration gradient while impermeability to sodium ions is maintained.

In both forms of experiments, net salt movement ($KHCO_3$) into the sequestered compartment is achieved by addition of valinomycin and carbonylcyanide *p*-trifluoromethoxyphenylhydrazone (FCCP), making the liposomes specifically permeable to potassium ions and protons respectively [5]; $CO_2$ functions, in effect, as a permeant source of dehydrated anion. The swelling (or reswelling) behaviour of liposomes is then regulated by the abundance of $CO_2$ in solution (Table I) and its rate of hydration within the inner compartment (Table II; Fig. 1) and can be assayed spectrophotometrically by changes in absorbance at 450 nm [6]. All solutions were prepared from $CO_2$-free distilled water. Fig. 2 summarises the movements of molecular and ionic species common to all the experiments described.

The relative impermeability of liposomal membranes to undissociated carbonic acid was established by challenging a liposome preparation osmotically with $NH_4HCO_3$. The prolonged shrinkage and slow recovery observed is in sharp contrast to the behaviour of liposomes upon addition of ammonium acetate. In the latter case salt equilibration across the membranes (as ammonia gas and acetic acid) is so rapid that osmotic clamping of liposomes is scarcely observable [6].

While it could be contended that the results presented in Tables I and II,

TABLE I

DEPENDENCE OF RESWELLING ON $CO_2$ ABUNDANCE

Mean values are given, followed by standard error of mean and number of values. 30 μmol lipid (5 mol% phosphatidic acid from Lipid Products, South Nuffield, Surrey, and 95 mol% egg yolk lecithin, prepared according to ref. 4) were taken to dryness from stock chloroform solution, and resuspended by gentle agitation in 3 ml $CO_2$-free distilled water. After dilution to an appropriate volume the liposome preparation was divided into two equal portions; their relative absorbance was taken as the zero point. The "control" batch was diluted with a known volume of water, while the other was shrunk with an equivalent volume of $KHCO_3$ solution to give a final concentration of 16.7 mM $KHCO_3$. Liposomes approached maximal shrinkage after 2 h, but were left overnight to equilibrate. Each assay is based on a 3-ml aliquot containing 1 μmol of lipid. Valinomycin and FCCP were added as ethanolic solutions to both control and sample cuvettes (0.30 and 3.9 μM final, respectively); final ethanol concentration did not exceed 0.67%. Gas, where used, was bubbled into the top of both cuvettes at 50 ml·min$^{-1}$ each. Liposomal volume changes at 27.5 ± 0.5°C were followed at 450 nm using a Perkin-Elmer dual beam spectrophotometer model 402; a change of 0.21 absorbance units accompanied the 50% recovery from shrinkage, which did not occur in the absence of ionophores.

| $CO_2$ availability | Time for 50% recovery from shrinkage (min) |
|---|---|
| Uncatalyzed dehydration of $HCO_3^-$ | 191.9 ± 5.5 (4) |
| 5% $CO_2$ (+95% $O_2$) | 33.2 ± 0.85 (4) |
| 100% $CO_2$ | 9.6 ± 0.5 (6) |

TABLE II

DEPENDENCE OF RESWELLING ON RATE OF HYDRATION OF $CO_2$, AS INFLUENCED BY SEQUESTERED CARBONIC ANHYDRASE ACTIVITY

Mean values are given, followed by standard error of mean and number of values. Lipids as described for Table I, but resuspended in 3 ml 10 mM potassium phosphate buffer, pH 7.2 (KP buffer) containing 30 μg bovine carbonic anhydrase (EC 4.2.1.1., Sigma). Free and sequestered enzyme were separated by gel chromatography on a Sephadex (Pharmacia) G-100 column, total volume 90 ml, with KP buffer as eluant. Liposome fractions were combined, diluted with KP buffer to an appropriate volume and divided into two equal portions, the relative absorbance being taken as zero. The "control" batch was diluted with a known volume of KP buffer, while the other was shrunk with an equivalent volume of $KHCO_3$ solution in KP buffer to give 16.7 mM $KHCO_3$ final. Assays were based on 2.5 ml aliquots containing 0.22 μmol lipid. Valinomycin and FCCP were added as ethanolic solutions to both control and sample cuvettes (0.36 μM and 4.6 μM final, respectively). Final ethanol concentration did not exceed 0.8%.

| Final Diamox* concentration (μM) | Time for 50% recovery from shrinkage (min) |
|---|---|
| 0 | 1.18 ± 0.29 (4) |
| 1 | 3.65 ± 1.15 (4) |
| 10 | 7.9 ± 0.87 (4) |

*The specific carbonic anhydrase inhibitor, Diamox (Lederle) [1] was added as a KP buffer solution to sample cuvettes only, and permitted an equilibration and interaction time of 6 min before reswelling was initiated by addition of valinomycin and FCCP. The 5-fold amplification mode of a Perkin-Elmer Spectrophotometer was used to detect liposomal volume changes by light scattering at 450 nm, 27.5 ± 0.5°C (see Fig. 1). Liposomes lacking sequestered carbonic anhydrase but swollen in KP buffer alone, and treated in an otherwise similar fashion required an average of 28.9 ± 2.3 min ($n$ = 6) for 50% recovery from shrinkage.

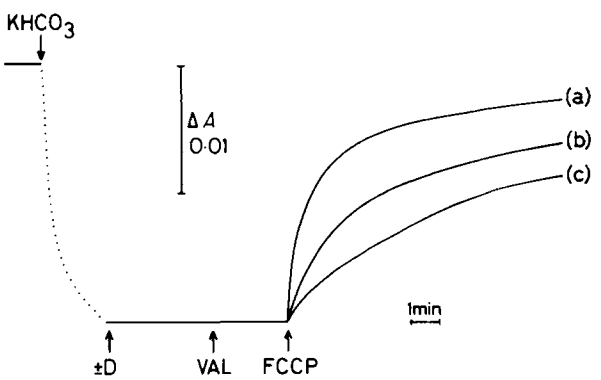


Fig. 1. Dependence of Reswelling on Rate of Hydration of $CO_2$, as influenced by sequestered Carbonic Anhydrase activity. Recovery from $KHCO_3$-induced shrinkage upon addition of valinomycin (VAL) and FCCP in the presence of (a) Zero Diamox, (b) 1 μM Diamox, (c) 10 μM Diamox. Sample preparation and experimentation as described in legend for Table II.

and Fig. 1 merely represent a specialized contrivance for the redistribution of $KHCO_3$ according to chemical and osmotic gradients, the same criticism is less justified for experiments permitting unilateral equilibration of potassium ions, with consequent over-swelling from an initial osmotic equilibrium. Liposomes with sequestered 10 mM sodium bicarbonate but external 10 mM $KHCO_3$ (prepared with the chromatographic method described for Table II) will swell beyond their equilibrium volume upon treatment with valinomycin and FCCP and in the presence of $CO_2$, as the external salt moves down its concentration gradient. The cation selectivity of valinomycin for potassium [5], coupled with the bilayer impermeability to sodium ensure that reciprocal movements cannot take place. For the conditions described, such liposomes

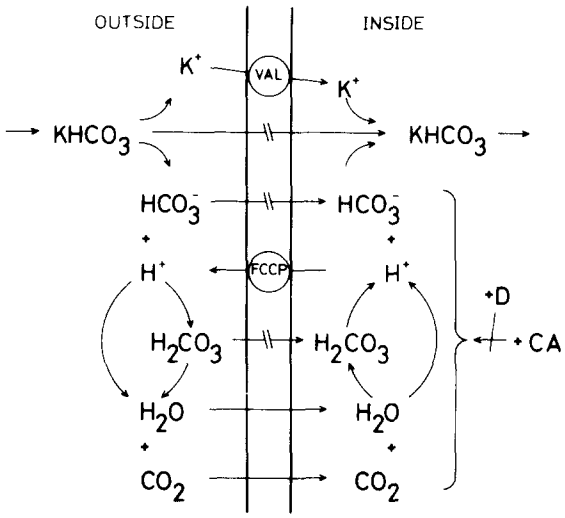


Fig. 2. Flow diagram describing the movement of $KHCO_3$ across a bilayer in the presence of valinomycin (VAL) and carbonylcyanide *p*-trifluoromethoxyphenylhydrazone (FCCP) CA, carbonic anhydrase; D, Diamox.

(approx. 1 μmol lipid per assay) showed a constant rate of swelling for almost 20 min ($\bar{x}$ = 0.0019 absorbance units · $min^{-1}$; $n$ = 3) at 27.5°C ± 0.5.

These experiments were undertaken to investigate possible consequences of auxin-stimulated proton secretion in *Avena* coleoptile tissue [7]. It has been proposed, though not without criticism [8—10], that the primary site for auxin action in plants is a proton pump located at the plasma membrane [11], and that protons secreted in response to auxin action serve as "second messengers" [11,12], diffusing to the cell wall region where the acidification promotes chemical or enzymatic cleavage of specific bonds in the wall, thereby permitting enhanced growth.

Within the framework of this proton secretion theory and the results presented here, demonstrating that proton translocation and counter-cation movement in the presence of $CO_2$ will initiate swelling responses in liposomes, two situations of physiological relevance may ensue. Where auxin-stimulated proton secretion and growth are concurrent processes, tissue bicarbonate production may be an accompanying homeostatic mechanism for maintenance of cellular osmotic pressure during increases in tissue volume. At the other extreme, if auxin-induced proton secretion occurs in the presence of deliberate restrictions on tissue volume changes (for example, by an absence of the hypothetical "second messenger" receptor system in the wall or its inhibition by other mechanisms), then the increased cellular osmotic pressure could be utilized for enhanced water uptake into the plant, or movement between tissues or compartments; it would be dependent on respiration, both for the production of carbon dioxide and the synthesis of adenosine triphosphate, if this in fact drives the proposed proton pump. The scheme would also contribute to resolving much of the long-standing controversy over respiration-linked water uptake by plant tissue and its regulation by auxins [13,14]. Similarly some of the discrepancies over acid- and carbon dioxide-induced growth of

tissue [8] appear more reasonable when it is kept in mind that applied $CO_2$ can freely permeate tissue and generate its hydration products within cellular compartments, whereas the same possibility is not open to applied protons.

Applications of this model for regulating water movements are not restricted to plant physiology, and Maren [1], in reviewing the physiological roles of carbonic anhydrase, has emphasised several situations in which similar principles might apply.

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## References

1 Maren, T.H. (1967) Physiol. Rev. 47, 595–781
2 Mitchell, P. (1976) Biochem. Soc. Trans. 4, 399–430
3 Carlisky, N.J. and Lew, V.L. (1970) J. Physiol. 206, 529–541
4 Bangham, A.D., Hill, M.W. and Miller, N.G.A. (1974) in Methods in Membrane Biology (Korn, E.D. ed.) Vol. 1, pp. 1–68, Plenum, New York
5 Henderson, P.F.J., McGiven, J.D. and Chappell, J.B. (1969) Biochem. J. 111, 521–535
6 Bangham, A.D., De Gier, J. and Greville, G.D. (1967) Chem. Phys. Lipids 1, 225–246
7 Rayle, D.L. (1973) Planta 114, 63–73
8 Evans, M.L. (1974) Annu. Rev. Plant Physiol. 25, 195–223
9 Penny, P., Dunlop, J., Perley, J.E. and Penny, D. (1975) Plant Sci. Lett. 4, 35–40
10 Perley, J.E., Penny, D. and Penny, P. (1975) Plant Sci. Lett. 4, 133–136
11 Hager, A., Menzel, H. and Krauss, A. (1971) Planta 100, 47–75
12 Cleland, R.E. (1973) Proc. Natl. Acad. Sci. U.S. 70, 3092–3093
13 Kramer, P.J. (1955) Annu. Rev. Plant Physiol. 6, 253–272
14 Galston, A.W. and Purves, W.K. (1960) Annu. Rev. Plant Physiol. 11, 239–276