THE DETERMINATION OF THE SALT RELATIONS OF THE CYTOPLASMIC PHASE IN CELLS OF BEETROOT TISSUE

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[*Manuscript received December* 17, 1962]

Summary

An analysis of the rate at which isotope diffuses out of disks of beetroot tissue shows that there are at least two components of the non-free space. As these components are not due to differences in cell type within the tissue, it is suggested they are due to a cytoplasmic phase in the parenchymatous cells, and to the vacuoles.

Evidence is given to support the suggestion that, as in the characean cell, these phases are in a series with the free space, so that ions pass to the vacuole in salt uptake through the cytoplasmic phase.

Using this serial model for the cell it has been estimated from the amount of isotope diffusing out of labelled tissue that the cytoplasmic phase contains about 3 m-equiv/kg K^+ or Na^+, and 0·1–1·0 m-equiv/kg Br^-, when the tissue is brought to equilibrium with potassium or sodium bromide solution whose concentration is 10–50 m-equiv/l. The time for 50% exchange of K^+ in this phase is about 2 hr at 2°C, and for Br^- about 40 min. At 25°C the exchange of both K^+ and Br^- is some three times faster.

The fluxes into and out of the cell have been estimated for K^+ and Br^- when either the concentration in the solution or the content of the vacuoles was varied. It was shown that "salt saturation" of the tissue was mainly due to an increase in efflux as concentration in the vacuole increased. The permeability of the boundaries of the cytoplasmic phase to K^+, estimated from the fluxes, was about 10^{-8} cm/sec, and it is suggested these boundaries are due to the plasmalemma and the tonoplast membranes respectively.

I. INTRODUCTION

It has been suggested by MacRobbie and Dainty (1958) that as far as the movement of ions between the solution and the vacuole is concerned, the cell of *Nitellopsis obtusa* behaves as a system of three phases in series. These phases are the free space and two components of the non-free space, namely, a cytoplasmic phase with a relatively rapid turnover, and the vacuole, containing the major part of the salt in the cell and equilibrating only slowly with the solution. The contents of these phases were estimated from an analysis of the isotope diffusing into a series of non-radioactive solutions from tissue that previously had been in a radioactive solution. In this method the cytoplasmic phase was detected as a component with a rate of equilibration intermediate between that of the free space and the vacuole,

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and sensitive to changes in temperature. The serial model has subsequently been supported by Diamond and Solomon (1959) working with *Nitella axillaris*, and the cell wall has been shown to be part of the free space in *Chara australis* by Dainty and Hope (1959). The location of the cytoplasmic phase is not well defined but by a process of elimination it must be part of the cytoplasm, for the bulk of the non-free space with a slow turnover is in the vacuole, and the cell walls are part of the free space.

This paper gives the results of similar experiments carried out with beetroot disks (i.e. analysis of the rate at which isotope diffuses out of labelled tissue). In general, these results are similar to those found for the Characeae, that is, three phases can usually be detected; a free space component, a cytoplasmic phase whose tempo of equilibration is much more rapid at 25°C than at 2°C, and a slowly exchanging component from the vacuoles. These phases are not due to any difference in cell type within the beet disks (cf. p. 660) and they are considered to be characteristic of the salt relations of the parenchymatous cells that make up the major part of the tissue.

It has been shown elsewhere (Briggs, Hope, and Pitman 1958*a*) that the free space in beet disks 1 mm thick has the exchange properties associated with a Donnan phase occupying about 2% of the tissues, and containing 10 m-equiv/kg of non-exchangeable anion, together with a "water free space" in which both anions and cations have the same concentration as the solution, and occupying about 20% of the tissue. This Donnan space has since been shown to be located in the cell walls, while the water free space is mainly due to cut cells (15%) and to intercellular spaces (5%) (Pitman, unpublished data). Thus, as in the characean cell, the only available region in which to locate the component with the intermediate tempo of exchange is the cytoplasm.

There is, however, no *a priori* reason for equating the cytoplasmic phase with the whole, or any particular region, of the cytoplasm, and two extreme configurations can be devised. In one the phases form the series free space–cytoplasmic phase–vacuole but in the other the cytoplasmic phase is connected to the vacuole only via the free space, i.e. is in "parallel" with the vacuole. The first arrangement would occur if the cytoplasmic phase were the whole of the cytoplasm, so that salt taken in to the vacuole had to pass through the cytoplasmic phase. The second arrangement would apply if the cytoplasm as a whole were free space, and the "cytoplasmic phase" some particulate component (e.g. mitochondria, plastids) within the cytoplasm. To emphasize the relation of the cytoplasmic phase to the vacuole the two models are called "serial" and "parallel" in what follows.

If the isotope diffusing out of the tissue is to be used to estimate the content and fluxes in the cytoplasmic phase, it is necessary to know which of these models applies to beet tissue. For example, the proportion of isotope in the cytoplasmic phase that diffuses to the solution depends on the ratio of the fluxes to solution and vacuole, and may be much smaller if the phases form a series than if they are in parallel. Evidence is given below to show that the phases behave as if in series, but, for convenience, is presented after the description of experimental procedure and the methods of estimating the K^+ or Br^- content of the cytoplasmic phase. The relationship between the cytoplasm and the cytoplasmic phase is then considered.

II. Experimental Details and Procedure

(a) *Material*

Disks 1 mm thick and about 15 mm in diameter were cut with a hand-microtome from cylinders of beet tissue. The cylinders were cut with a cork-borer in the general direction of the vascular bundles, and were taken as far as possible from the parenchyma between the rings of vascular bundles. The disks were rinsed in distilled water and kept in continuously aerated distilled water for a period of about 7–10 days; during this time the ability to take up salt by the tissue had developed to a maximum.

(b) *Isotopes*

The isotopes used were ^{42}K, ^{22}Na, ^{24}Na, and ^{82}Br. The ^{42}K and ^{24}Na were obtained from the Atomic Energy Research Establishment, Harwell, as irradiated carbonates which were converted to chlorides by titration with hydrochloric acid, and boiling the solution to remove the carbon dioxide. The ^{82}Br was also obtained from Harwell but as irradiated NH_4Br. This was used as a solution of NH_4Br and diluted with KBr solutions to the required activity. In this way the concentration of the NH_4^+ ions in the labelling solutions was not appreciable.

These three isotopes were irradiated for only 24–48 hr to reduce radioactive impurities to a minimum compatible with adequate isotope production. Irradiation for 48 hr produces 93% maximal activity of ^{42}K and 89% ^{24}Na.

^{22}Na was obtained from the Radiochemical Centre at Amersham as a solution of NaCl, and was used in this form, with suitable dilution with carrier NaCl.

(c) *Methods of Estimation*

Radioactive concentrations were determined by counting the pulses from about 10 ml of solution with a Mullard MX 124 Geiger–Müller tube. Where possible the solutions were counted for a period sufficiently long such that the error due to random variation in the rate of isotope disintegration was less than 1%: for some solutions of low activity it was not practicable to count more than 1000 disintegrations.

Concentrations of sodium and potassium were determined with an "EEL" flame-photometer. The accuracy of the determinations was better than $\pm 0{\cdot}02$ m-equiv/l when the scale was set to 1 m-equiv/l KCl or NaCl, and $\pm 0{\cdot}01$ m-equiv/l when set to $0{\cdot}2$ m-equiv/l potassium in 5 m-equiv/l NaCl. There was no detectable interference by sodium with potassium determinations.

Bromide and chloride were determined by potentiometric titration with silver nitrate solutions. A silver–silver halide electrode was used. The accuracy of these determinations was between 1 and 2%.

(d) *Experimental Procedure*

The experimental procedure can be divided into three parts: the pretreatment of the tissues, the uptake of isotope, and the subsequent elution and measurement of the loss of isotope. The purpose of the pretreatment was first to remove the divalent cations from the free space and to equilibrate the free space with the solutions to be

used in later stages, and then to bring the fluxes in the system to steady values under the conditions of the experiment. To this end the tissue was put into a 50 m-equiv/l KCl solution at 2°C for three periods of about 30 min, followed by three similar treatments with solution of the same content as the labelling solutions. The tissue was then left in aerated solutions of this concentration for about 7 hr with occasional changes.

The tissue and unlabelled solutions were then brought to the appropriate temperature for uptake of isotope. When disks are accumulating labelled salt but losing salt at a lower specific activity from the vacuoles, the specific activity of the free space towards the centre of the disks will be lower than that towards the surface, because of the greater length of the diffusion path to the solution from cells in the centre. This difference in specific activity, and in isotope uptake, will increase with the flux into the cell, but will be much lower at 2°C than at 25°C, so the temperature of isotope uptake was 2°C wherever possible.

The ratio of solution to tissue was relatively large during isotope uptake to reduce the changes in specific activity to a minimum compatible with determination of the net salt uptake. At the end of about 15–18 hr the tissue was removed from the solution, lightly blotted, and put into a series of non-radioactive solutions for elution of isotope. Samples of about 2 g of tissue (10 disks) were used in a volume of solution varying from 100 ml at the start of the elution, when there was a lot of isotope in the free space, to 10 or 12 ml during the later stages of the elution, when the rate of loss from the tissue was small. For similar reasons, the length of each period of elution was varied from about 20 min at the start of the elution to 1 hr or more towards the end. Throughout the experiment, the solutions were stirred and aerated by a stream of moist air. Samples were taken at the end of the periods of elution by removing as much as possible of the solution, and replacing it with an equal amount of unlabelled solution at the same temperature. All experiments were at the same temperature throughout, with the exception of the determination of potassium content at 25°C. In these experiments there was a series of elutions at 2°C lasting 2 hr and then the temperature was changed to 25°C for the non-free-space elution. In this way, during the first 2 hr, the isotope in the free space was reduced to about 0·1% but only 10–40% had diffused out of the cytoplasmic phase; at 25°C a similar elution would have removed the same amount of isotope from the free space, but 60–90% from the cytoplasmic phase.

At the end of the experiment the disks were removed from the solutions, blotted, weighed, and then treated to extract the isotope and salt in the tissue by first boiling with water to remove as much bromide as possible, and then boiling with 5 ml 0·5N nitric acid to remove the rest of the sodium and potassium. These two extracts were kept separate. The concentrations of isotope, and of sodium, potassium, and halide were determined by the methods described above.

(e) *Experimental Errors*

Primary determinations were measured to within 1–5%, depending on the concentrations of salt or isotope involved. As duplicate samples were used, a more useful value was the mean difference between the duplicates. For the apparent con-

tent of the cytoplasmic phase (A_c^*), and for the net uptake rate (ϕ), when ϕ was relatively large (1–2 m-equiv/kg/hr), this difference was between 5–10%. For the content of the cytoplasmic phase (Q_c), for lower values of ϕ, and for the fluxes into and out of the cytoplasmic phase, it was about 10–15%.

III. Theoretical Basis of Estimation of the Properties of the Cytoplasmic Phase

(a) *Symbols Used*

A_c^*, "amount" of isotope apparently in the cytoplasmic phase (counts/min/20 ml/g);

C, concentration (m-equiv/l): C_o in solution, C_c in the cytoplasmic phase, C_v in the vacuole;

E, potential (mV);

F, Faraday's constant;

k_c, rate constant for exchange of the cytoplasmic phase (hr^{-1});

Q, amount of an ion (m-equiv/kg): Q_c in the cytoplasmic phase, Q_s in the free space, Q_v in the vacuole;

Q^*, "amount" of isotope (counts/min/20 ml/g), subscripts as in preceding definition;

R, universal gas constant;

s, specific activity [(counts/min/20 ml)/(μ-equiv/20 ml)]: s_c in the cytoplasmic phase, s_v in the vacuole, s_i inside, s_o outside, or in the solution;

T, absolute temperature (°K);

t, period in solution (hr);

$t_{\frac{1}{2}}$, time for half-exchange of the cytoplasmic phase (hours or minutes);

ϕ, flux (m-equiv/kg/hr): ϕ_{sc}, from free space to cytoplasmic phase; ϕ_{cs}, from cytoplasmic phase to free space; ϕ_{cv}, from cytoplasmic phase to vacuole; ϕ_{vc}, from vacuole to cytoplasmic phase; ϕ (no subscript), net flux or net uptake rate.

(b) *Estimation of Contents of Cytoplasmic Phase*

The amount of isotope in the tissue throughout the elution can be estimated from the series of measurements of isotope diffusing out of the tissue, and from its content at the end of the experiment. In general three stages can be distinguished:

(1) A period in which the isotope diffusing out of the tissue is mainly from the free space, but also from the cytoplasmic phase and the vacuole, and in which the rate of exchange is limited by the diffusion in the free space.

(2) Eventually isotope lost from the tissue is limited by the exchange between the cytoplasmic phase and the free space, and so has the tempo of exchange of the cytoplasmic phase. In this stage the isotope comes both from the vacuole and the cytoplasmic phase.

(3) Finally when the specific activity in both free space and the cytoplasmic phase has fallen to a value intermediate between that of the vacuole and the solution, isotope diffusing out of the tissue is limited by the flux out of the vacuole, and all the isotope appearing in the solution is from this region.

When these phases can be separated, the analysis of the isotope diffusing out of the tissue can conveniently be made by the method described by MacRobbie and Dainty (1958). Briefly, the logarithm of the amount of isotope in the tissue is plotted against the time from the start of elution. The vacuolar contribution is then the final straight part of the graph and its extrapolation to $t = 0$. Subtraction of these values from the total amount of isotope in the tissue gives estimates at different times of the amount of isotope in the free space and cytoplasmic phase together. These

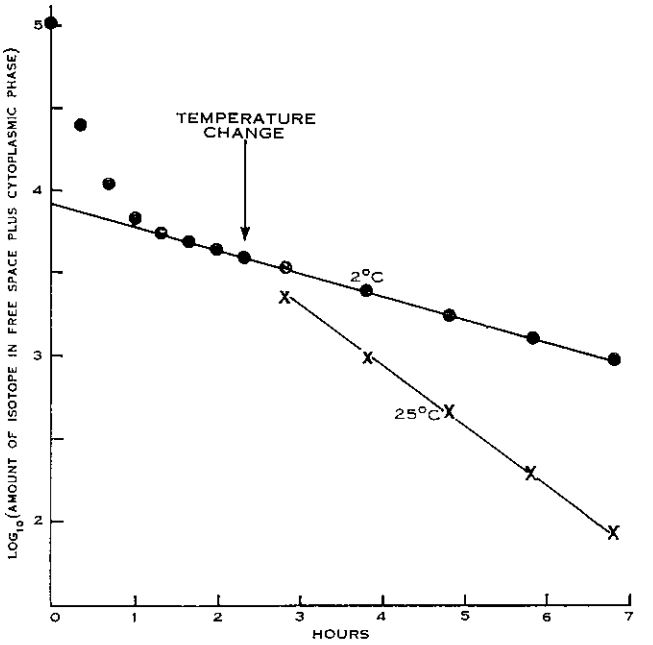

Fig. 1.—Amount of isotope estimated to be in the free space and cytoplasmic phase together plotted semi-logarithmically (see text). ● Disks at 2°C throughout. × Disks changed from 2°C to 25°C at $t = 2{\cdot}33$ hr.

two phases can also be separated by a semilogarithmic plot, but now the final straight part is characteristic of the exchange of the cytoplasmic phase (Fig. 1). During the first part of the elution the tissue was at 2°C, but the temperature of one set of samples was changed at $t = 2{\cdot}33$ hr to 25°C: the two straight lines from this stage onwards represent isotope lost from the cytoplasmic phase at the two temperatures.

From these analyses of the experimental results, the following quantities can be estimated, as well as the content of the free space and the rate constant for its exchange with the solution:

(i) *The rate constant for exchange between the cytoplasmic phase and the solution*, estimated from the slope of the straight part of the graph (cf. Fig. 1, when $k_c = 0{\cdot}33$ at 2°C, and $0{\cdot}85$ at 25°C).

(ii) *The amount of isotope estimated to have been in the cytoplasmic phase at the start of the elution* (A_c^*) from the extrapolate to $t = 0$ (in Fig. 1, 8500±500 counts/min/20 ml at 2°C).

(iii) *The amount of isotope in the tissue at the end of the elution, and its average specific activity.*

(iv) *The rate of loss of isotope from the tissue at the end of the experiment, when loss is predominantly from the vacuole.*

(v) Other quantities that can be determined are *the specific activity of the labelling solution*, and, from the change in concentration, *the net uptake rate* of salt.

These quantities can be related to the fluxes into and out of the phases and their contents, but the details of the relationship depend on the spatial arrangement. In what follows only the serial model is considered in detail, and Figure 2 shows the

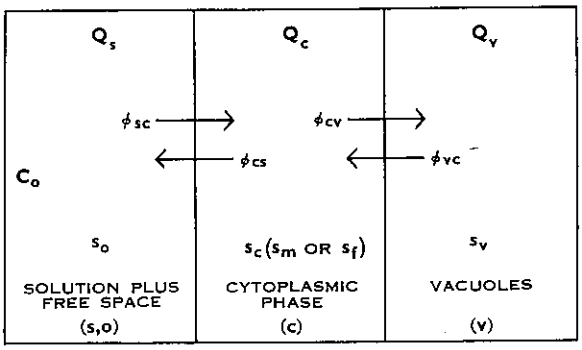

Fig. 2.—Schematic representation of the serial model of free space–cytoplasmic phase–vacuoles.

quantities involved. The conditions of the experiments were arranged so that several simplifying assumptions can be made. Firstly, the net uptake was reduced to small proportions by using tissue that was at 2°C or salt-saturated; as a result, the content of the vacuole increases by not more than 10% over the course of the experiment, and the fluxes into and out of the cytoplasmic phase and its content are assumed to be steady. Furthermore there will be little change in the concentration in the free space due to the diffusion path at different distances from the surface.

Secondly, it is assumed that the specific activity of the free space in all parts of the tissue is equal to that of the solution s_o, so that the equation for the rate of change of isotope in the cytoplasmic phase is

$$dQ_c^*/dt = \phi_{sc} \cdot s_o + \phi_{vc} \cdot s_v - s_c(\phi_{cs} + \phi_{cv}). \quad (1)$$

During the period following transition from one solution to another of different specific activity, the specific activity in the free space will not be equal to that in the solution and the equation will not be true. As the time for half-exchange of isotope in the free space is much less (*c.* 10 min) than that in the cytoplasmic phase (60–100 min), this error has been ignored.

More serious errors could arise when dQ_c^*/dt is small. On the assumptions of equation (1), the net flux of isotope across the outer cytoplasmic phase boundary will be $(\phi_{sc} \cdot s_o - \phi_{cs} \cdot s_c)$ inwards. If the cell is losing salt from a region of different specific activity while taking it up from the solution, the specific activity adjacent to the cytoplasmic phase will be some value intermediate between that of the solution and the phase, and determined by the diffusion path to the solution, the fluxes into and out of the phase, and the content of the free space adjacent to the phase. The difference between s_o and the relevant specific activity in the free space will be larger for anions than for cations, as there is less anion in the free space. For example, when $C_o = 5$ m-equiv/l KBr, the free space will contain about 0·5–1·0 m-equiv/kg of Br^- and 12–13 m-equiv/kg of K^+; if the region adjacent to the cytoplasmic phase were the cell wall containing the Donnan free space, then the difference would be greater, about 0·1 m-equiv/kg Br^- to 12 m-equiv/kg K^+. This source of error is not considered to be important for K^+ at 2°C and possibly not at 25°C, an assumption that is supported by the good agreement between the observed and calculated values of the final efflux from the tissue (cf. p. 655 and Table 2). For Br^- there is some evidence that the specific activity in the free space during elution is appreciably larger than s_o; one method of correcting for this is to put $a \cdot s_c$ as the relevant specific activity, where a is a constant for a particular set of fluxes and concentrations. Equation (1) then becomes

$$dQ_c^*/dt = \phi_{vc} \cdot s_v - (\phi_{cs} + \phi_{cv} - a \cdot \phi_{sc}) s_c. \quad (2)$$

In this case $\phi_{cs} \cdot s_c$ can no longer be taken as an approximation for the net isotope flux out of the cytoplasmic phase but $(\phi_{cs} - \phi_{sc} \cdot a)\, s_c$ may be used instead (cf. p. 661). Using the symbols given in Figure 2, and assuming that equation (1) is true, it can be shown that the quantities determined in the experiments have the following relationships:

(i) *The rate constant for exchange of the cytoplasmic phase*, k_c, estimated from the slope of the graph in Figure, 1 is

$$k_c = (\phi_{cs} + \phi_{cv})/Q_c. \quad (3)$$

The time for half-exchange of the cytoplasmic phase, $t_{\frac{1}{2}}$, equals $0{\cdot}694/k_c$.

(ii) At the start of the elution *the amount of isotope in the cytoplasmic phase* is Q_c^* and equals $Q_c \cdot s_c$. If the time in the labelling solution, t_1, is more than about five times $t_{\frac{1}{2}}$, then the value of s_c at t_1 is more or less steady at

$$s_m = (\phi_{sc} s_o + \phi_{vc} s_v)/(\phi_{cs} + \phi_{cv}). \quad (4)$$

At the end of the elution Q_c^* will have fallen to $Q_c \cdot s_f$ where, as s_o is very nearly zero, under the conditions of the experiment

$$s_f = \phi_{vc} s_v/(\phi_{cs} + \phi_{cv}). \quad (5)$$

The apparent content of the cytoplasmic phase, A_c^*, is the extrapolate to $t = 0$ of the straight part of Figure 1, and is related to Q_c by

$$A_c^* = Q_c(s_m - s_f)\phi_{cs}/(\phi_{cs} + \phi_{cv}). \quad (6)$$

(iii) *The specific activity of the tissue as a whole at the end of the experiment* when nearly all the isotope is in the vacuole is $Q_v^*/(Q_s+Q_c+Q_v)$. As Q_s is estimated separately in the experiment, and as Q_c is small compared with Q_v, an approximate value of Q_c can be used to estimate $s_v = Q_v^*/Q_v$. The amount of isotope in the vacuoles, Q_v^*, is related to the fluxes and specific activity in the cytoplasmic phase by

$$Q_v^* = \phi_{cv}\left(\int_0^{t_2} s_c \cdot \mathrm{d}t\right) - \phi_{vc}\left(\int_0^{t_2} s_v \cdot \mathrm{d}t\right), \tag{7}$$

where t_2 is the time at the end of the experiment. If $\phi_{vc} \cdot s_v$ is small, and if the period of labelling (t_1) is about the same as the period of elution (t_2-t_1), then equation (7) is very nearly

$$Q_v^* = \phi_{cv}(s_m-s_f)t_1. \tag{8}$$

(iv) *The rate of loss of isotope from the tissue* at the end of the experiment, or the "apparent efflux" is

$$\phi_{cs} \cdot s_f = \phi_{vc} \cdot s_v \therefore \phi_{cs}/(\phi_{cs}+\phi_{cv}). \tag{9}$$

(v) *The net uptake rate*, estimated from the change in concentration of the solution, is

$$\phi = (\phi_{sc}-\phi_{cs}) = (\phi_{cv}-\phi_{vc}). \tag{10}$$

From equations (8), (6), and (3), $(\phi_{cs}+\phi_{cv})(s_m-s_f)$ can be determined, and as

$$(\phi_{cs}+\phi_{cv})(s_m-s_f) = \phi_{sc} \cdot s_o \tag{4,5}$$

$$= (\phi+\phi_{cs})s_o, \tag{10}$$

ϕ_{cs} can be calculated if ϕ is known. By substitution in other equations, values of the other fluxes and the content of the cytoplasmic phase can be estimated. From these quantities a value for the apparent efflux (eqn. (9)) can be calculated and compared with the value found experimentally (from the rate of loss of isotope at the end of an elution experiment when the specific activity of the cytoplasmic phase is at a steady value intermediate between that of the free space and the vacuoles, s_f). This comparison has been made for potassium in Table 2.

IV. Experimental Results

(a) *Potassium and Sodium in the Cytoplasmic Phase*

To ensure that s_o has reached the value s_m given above, the tissue must be in the isotope solution for at least five times $t_{\frac{1}{2}}$. Figure 3 gives values for the apparent isotopic content of the cytoplasmic phase, A_c^*/s_o, when tissue is eluted after varied periods in the labelling solution, showing that it reaches a more or less steady value. The line is calculated from the final determination ($t = 20\frac{1}{2}$ hr) and is in reasonable agreement with the other determinations. Similar values for the apparent uptake, Q_v^*/s_o are also given. These results are not taken to differentiate between the two models but only to show that the serial model is capable of explaining the observations.

Most of the determinations described below have been made with potassium instead of sodium for two reasons. Firstly, tissue that has been in sodium chloride solution for periods as long as are used in the experiments sometimes loses some of the red pigment from the vacuoles, and can no longer be assumed to be in the same state throughout the experiment; secondly, at 25°C the turnover of sodium in the cytoplasmic phase is too fast to make separation of this phase from the free space practicable. For example, the time for 50% turnover of sodium at 2°C is about

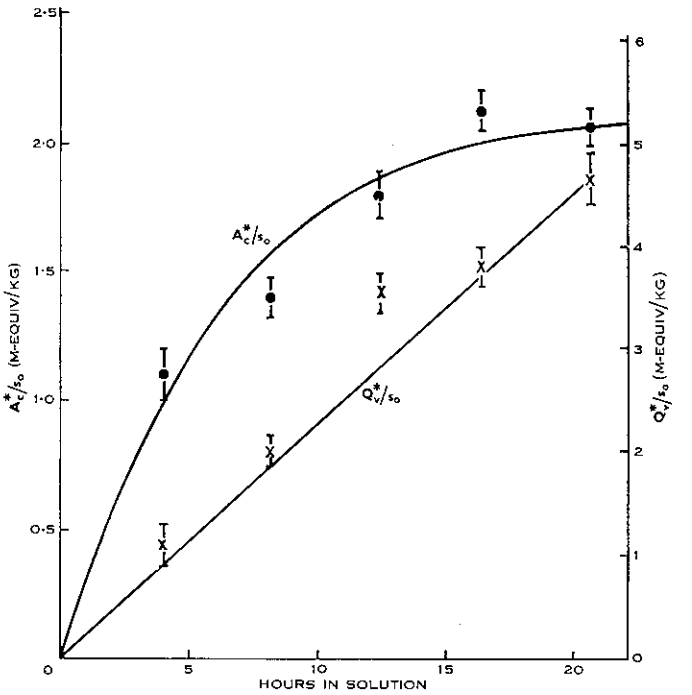

Fig. 3.—Values found for the apparent content of the cytoplasmic phase, A_c^*/s_o (●), and for the apparent uptake, Q_{vl}^*/s_o (×), after varied periods in the labelling solution. The line gives values calculated from the uptake at 20½ hr.

1–1·25 hr but only 20–30 min at 25°C; the time for 50% exchange of the free space is about 10 min for 1-mm thick disks at both 2 and 25°C, but as the content of the free space is some 10 times the apparent content of the cytoplasmic phase, the two phases are not separable at 25°C.

The effect of temperature on the fluxes of potassium in the system is shown in Table 1, together with some results for fluxes of sodium at 2°C. There are two sets of results for potassium fluxes: one when the tissue was salt-saturated and net uptake was low, the other when the tissue contained less salt and showed appreciable potassium uptake to the vacuole. In both cases the turnover of potassium was faster at 25°C than at 2°C, due to the increase in the fluxes ϕ_{cs} and ϕ_{cv}. The temperature coefficient for these fluxes is about 1·5–1·9 and is about the same for each

flux. This is smaller than the temperature coefficient of net uptake (2·5–3·0), but ϕ is the difference between the fluxes into and out of the vacuole, and so these may well have different temperature coefficients.

TABLE 1

COMPARISON OF THE CONTENT AND FLUXES IN THE CYTOPLASMIC PHASE FOR SODIUM AT 2°C AND FOR POTASSIUM AT BOTH 2°C AND 25°C, AT HIGH AND LOW VACUOLE CONCENTRATION

C_o = 5 m-equiv/l; duplicate samples

Solution	Temp. (°C)	A_c^*/s_o (m-equiv/kg)	Q_c (m-equiv/kg)	$t_{\frac{1}{2}}$ (hr)	ϕ (m-equiv/kg/hr)	ϕ_{cs} (m-equiv/kg/hr)	ϕ_{cv} (m-equiv/kg/hr)	Q_v (m-equiv/kg)
NaCl	2	0·33	2·2	1·25	0·2	0·33	0·90	35
KCl	2	0·72	2·9	2·25	0·1	0·40	0·50	70
KCl	25	0·70	2·9	0·75	1·2	1·1	1·65	85
KCl	2	1·9	4·0	2·0	0	0·92	0·45	205
KCl	25	2·3	4·3	0·7	0	2·95	1·40	205

The net rate of salt uptake by beet tissue increases with external salt concentration until it reaches a maximum at room temperature of about 4 m-equiv/kg/hr when the external concentration is about 30–50 m-equiv/l (KCl) (cf. for example Briggs,

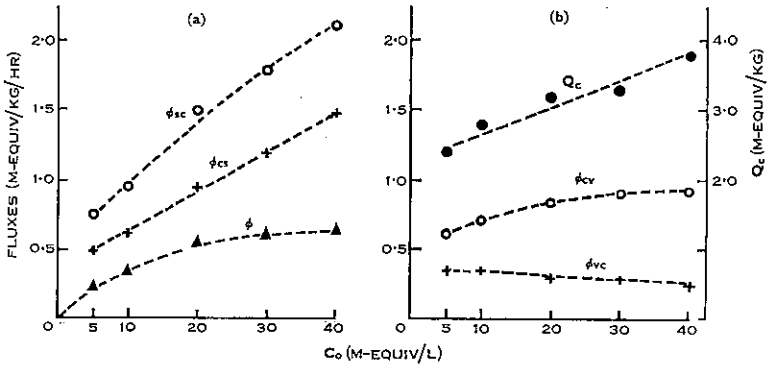

Fig. 4.—Effect of potassium chloride concentration (C_o) on potassium fluxes into and out of the cytoplasmic phase, and on Q_c. Temperature 2°C. (*a*) Net uptake rate (▲) and the fluxes across the outer boundary, ϕ_{sc} (○) and ϕ_{cs} (+). (*b*) Content of the cytoplasmic phase (●), and the fluxes at the inner boundary, ϕ_{cv} (○) and ϕ_{vc} (+).

Hope, and Robertson 1961, p. 134). At lower temperatures the net uptake rate also rises to a maximum but to a lower value. Figure 4 gives the results of an experiment at 2°C to measure the fluxes into and out of the cytoplasmic phase and the potassium content in the cytoplasmic phase, when the external potassium chloride concentration

was varied from 5 to 40 m-equiv/l. The concentration of potassium in the vacuoles* was 60, 63, 67, 68, and 69 m-equiv/kg, so in effect the only factor varied was the concentration in the solution. Over this range the amount of potassium in the cytoplasmic phase increased from 2·4 to 3·8 m-equiv/kg, and, as may be expected, the fluxes across the outer boundary increased as well (Fig. 4). The net uptake to the vacuole also increased with concentration, but at the inner boundary this was

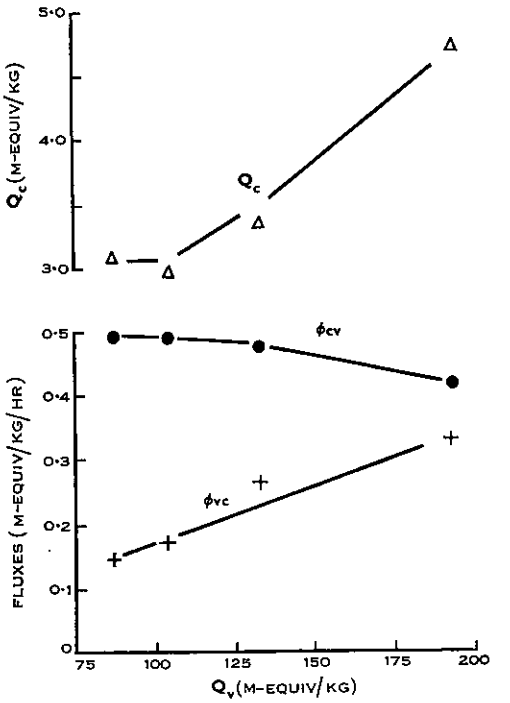

Fig. 5.—Values of Q_c (△), ϕ_{cv}(●), and ϕ_{vc} (+) for potassium at different levels of potassium in the vacuoles (Q_v). Temperature 2°C.

mainly due to an increase in the flux ϕ_{cv}. The efflux from the vacuole, ϕ_{vc}, showed a small decrease from 0·35 to 0·25 m-equiv/kg/hr, although the concentration in the vacuole increased from 60 to 69 m-equiv/kg.

In spite of this decrease in efflux, measurements made with tissue that had been allowed to take up varied amounts of potassium chloride from a solution containing 5 m-equiv/l showed an increase in efflux (ϕ_{vc}) with increasing vacuole content (Fig. 5). At the highest content the net uptake has been reduced to a small proportion of that when $Q_v = 88$ m-equiv/kg and this decrease in ϕ was due mainly to an increase in ϕ_{vc}, but also to a decrease in ϕ_{cv}. Q_c also increased with internal (vacuolar) concentration.

* The vacuoles occupy about 0·70 ml/g fresh weight of disks, so 1 m-equiv/kg is approximately equal to 1·4 m-equiv/l.

The apparent efflux, $\phi_{vc} \cdot s_v \cdot \phi_{cs}/(\phi_{cs}+\phi_{cv})$ (cf. eqn. (9)) can be measured directly in these experiments and compared with the value calculated from the other fluxes (cf. p. 655). Table 2 gives values of the calculated and observed apparent efflux from the results of Figure 4, showing that there is a reasonable agreement.

TABLE 2

OBSERVED AND CALCULATED VALUES OF THE APPARENT EFFLUX OF POTASSIUM OVER A RANGE OF EXTERNAL POTASSIUM CHLORIDE CONCENTRATIONS

Duplicate samples

Potassium Chloride Concn. (m-equiv/l)	Apparent Potassium Efflux (m-equiv/kg/hr)	
	Observed	Calculated
5	0·18	0·16
10	0·14	0·16
20	0·13	0·16
30	0·13	0·17
40	0·13	0·15

Disks cut transversely from a beetroot contain sections through vascular bundles and so are not strictly homogeneous in cell type, i.e. the cytoplasmic phase could be due to this cellular difference rather than a difference in subcellular organization. Table 3 gives results of an experiment to show that the cytoplasmic phase

TABLE 3

AMOUNT OF POTASSIUM ELUTED BY SODIUM CHLORIDE SOLUTION FROM BEETROOT DISKS, FROM VASCULAR REGION OF DISKS, AND FROM THE PARENCHYMA ONLY

Tissue Eluted	Sample Weight (g)	Free Space Potassium (m-equiv/kg)	Q_c (m-equiv/kg)	Potassium Efflux from Vacuoles (m-equiv/kg/hr)
Beetroot disks:				
Sample 1	2·25	11·2	2·9	0·32
Sample 2	2·15	11·1	3·1	0·31
Vascular region	1·77	11·7	3·5	0·43
Parenchyma	2·69	10·2	2·5	0·23

is a property of the parenchymatous cells, by comparing the behaviour of:

(1) whole disks of tissue (two samples);
(2) tissue cut from two samples of disks so as to contain mainly vascular tissue;
(3) the remainder of the tissue from the two samples, which was entirely parenchyma.

The samples had first taken up ^{42}K and were then eluted with a sodium chloride solution. Each sample showed the same pattern of salt relations, but there was a small difference in absolute amounts. For example, each elution gave free space, cytoplasmic phase, and vacuolar components, but the cytoplasmic phase and efflux from the vacuole for sample (2) are larger than for sample (3). This difference is probably due to the smaller size of the parenchymatous cells near the vascular bundles, which have a larger surface area per volume of tissue than the larger cells in the regions between vascular bundles.

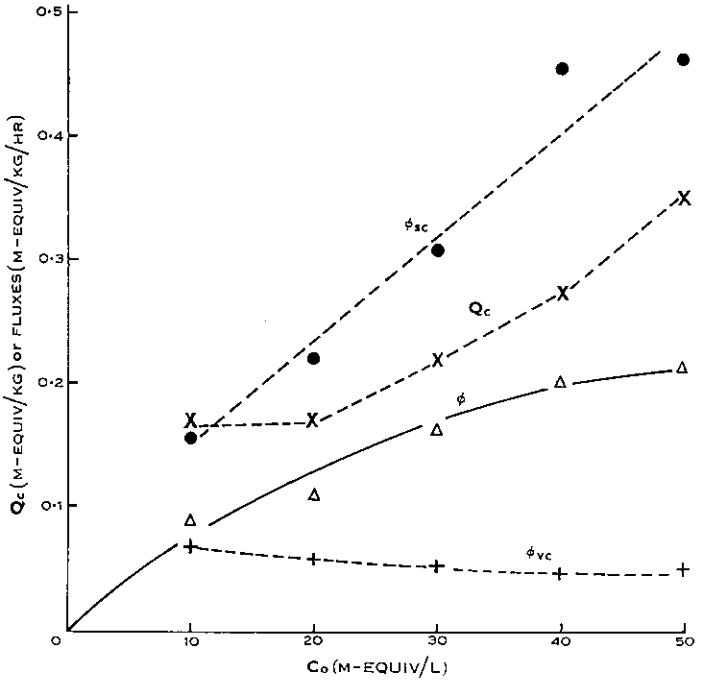

Fig. 6.—Effect of variation in potassium bromide concentration (C_o) on bromide fluxes and content of the cytoplasmic phase at 2°C. Q_c (×); ϕ_{sc} (●); ϕ_{vc} (+); and ϕ (△).

(*b*) *Bromide in the Cytoplasmic Phase*

Although good agreement is found between observed and calculated values for the apparent efflux of potassium (Table 2), this is not always so for anion exchange when the observed efflux may be 30–50% of the calculated value. These observations apply to chloride, iodide, and bromide, but bromide has been used in these experiments as its isotope has the most suitable half life and energy of radiation. As discussed above, under some conditions the specific activity adjacent to the cytoplasmic phase could be much larger than that in the solution, and the isotope diffusing out of the tissue would then not be $\phi_{cs}s_c$, but $(\phi_{cs}-\phi_{sc} \cdot a)s_c$. This effect leads to underestimation of ϕ_{cs} and overestimation of Q_c, and may be expected to be greater at 25°C (when the fluxes are larger) than at 2°C.

Allowance can be made for the underestimation of ϕ_{cs} by assuming that the observed efflux from the tissue is in fact

$$(\phi_{cs}-\phi_{sc} \cdot a)s_c = \phi'_{cs} \cdot s_c$$

and using this relationship together with the other measurements given above to estimate ϕ'_{cs}. As ϕ_{sc} equals $(1/s_o)(s_m-s_f)(\phi'_{cs}+\phi_{cv})$ [cf. eqn. (2), (4), and (5)] and ϕ_{cs} equals $\phi_{sc}-\phi$, ϕ_{cs} can be estimated and compared with ϕ'_{cs}. For the tissue used at 2°C (Fig. 6) there was little difference in observed and calculated efflux, presumably as the influx was small, but for other tissue (Tables 4 and 5) when C_o was only 5 m-equiv/l, and ϕ_{sc} was larger, in some cases $\phi_{sc} \cdot a$ was about 50% of ϕ_{cs}.

TABLE 4

EFFECT OF TEMPERATURE ON THE BROMIDE CONTENT AND FLUXES IN THE CYTOPLASMIC PHASE OF BEETROOT TISSUE

Duplicate samples of tissue placed in potassium bromide solution of concentration 5 m-equiv/l

Temp. (°C)	Q_v (m-equiv/kg)	ϕ‡	Q_c (m-equiv/kg)	ϕ_{sc}‡	ϕ_{cs}‡	ϕ_{cv}‡	ϕ_{vc}‡	ϕ'_{cs}‡
2	83	0·06	0·35	0·12	0·06	0·19	0·13	0·06
25	92	0·40	1·00	0·60	0·20	1·35	0·95	0·11
Temperature coefficient†		2·3		2·0	1·7	2·3	2·3	1·3

† For a temperature rise of 10 degC.
‡ Units: m-equiv/kg/hr.

Table 4 gives some values for Q_c and for the fluxes in the system at 2°C and at 25°C, when C_o was 5 m-equiv/l, estimated by making allowance for the difference between observed and calculated efflux. As for potassium, the exchange of bromide in the cytoplasmic phase was more rapid at 25°C than at 2°C, and the temperature coefficient for net uptake was about 2·5. The temperature coefficient for ϕ'_{cs}, was only about 1·3, but if allowance is made for the diffusion effect, i.e. by estimating ϕ_{cs}, the temperature coefficient for all the fluxes was about 2, as shown in Table 4. The change in the fluxes with increased temperature was accompanied by a threefold increase in Q_c of about 0·65 m-equiv/kg. If the same increase in potassium content had taken place in the cytoplasmic phase, it would have been detected with difficulty, as it is only a little larger than the experimental error in Q_c for potassium.

The bromide content and the fluxes into and out of the cytoplasmic phase when the concentration of the potassium bromide solution was varied are shown in Figure 6. The net uptake of bromide is generally much smaller than the uptake of potassium (Fig. 4) although the vacuolar content of each was about the same in both experiments. In these results the vacuolar content of bromide increased from 13 to

17 m-equiv/kg, and of potassium from 66 to 77 m-equiv/kg as C_o was increased from 10 to 50 m-equiv/l KBr. The pattern of results is generally similar to that in Figure 4, but Q_c was much smaller for bromide than for potassium, and increased more with increasing C_o. Again the fluxes across the outer boundary, and ϕ_{cv}, increased with increasing C_o, but ϕ_{vc} showed a small decrease with rising net uptake.

In comparison with Figure 5, Table 5 gives some results of the effect of varying the bromide content of the vacuole. As with potassium, increase in Q_v decreased the net uptake, mainly due to an increase in the flux from the vacuole, ϕ_{vc}. The increase in Q_v was accompanied by an increase in Q_c from 0·75 to 1·25 m-equiv/kg. Values of Q_c for potassium are also given in the table.

TABLE 5

EFFECT OF BROMIDE CONTENT OF VACUOLE ON THE BROMIDE CONTENT AND FLUXES IN THE CYTOPLASMIC PHASE OF BEETROOT TISSUE

Tissue placed in potassium bromide solution of concentration 5 m-equiv/l. Temperature 2°C

Q_v† (Br^-)	ϕ‡	Q_c† (Br^-)	ϕ_{sc}‡	ϕ_{cs}‡	ϕ_{cv}‡	ϕ_{vc}‡	ϕ'_{cs}‡	Q_v† (K^+)	Q_c† (K^+)
32	0·40	0·75	0·50	0·10	0·63	0·23	0·05	86	2·8
95	0·15	1·25	0·25	0·10	0·72	0·57	0·04	150	3·6

† Units: m-equiv/kg. ‡ Units: m-equiv/kg/hr.

V. Spatial Relationship of the Free Space, Cytoplasmic Phase, and the Vacuole

(a) *Experiments with Radioactive Sodium*

Tissue was allowed to take up salt from a sodium chloride solution of concentration 5 m-equiv/l, and which was labelled with ^{22}Na, until a relatively high concentration was reached in the vacuole. It was then put into solutions of inactive sodium chloride of the same concentration to reduce the specific activity of the free space and the cytoplasmic phase to as low a value as possible. After a suitable period it was transferred to a sodium chloride solution labelled with ^{24}Na and then, after a period long enough for the cytoplasmic phase to have reached a relatively steady specific activity, the radioactive sodium was eluted from the tissue by inactive sodium chloride solutions as described above. ^{22}Na and ^{24}Na concentrations were estimated by counting the solutions at two different times, and the ratio of $[^{22}Na]/[^{24}Na]$ determined in the components of the free space, cytoplasmic phase, and the vacuole. In the free space this ratio was about 1/6000; in the tissue at the end of the experiment (and in the efflux from the tissue at the end of the experiment) the ratio was only 1/4, but in the cytoplasmic phase it was between 1/30–1/50. This low ratio could have been maintained only if there was a large flux between the cytoplasmic phase and the vacuole, i.e. as if the phases were in series.

(*b*) *Experiments with Radioactive Potassium*

Similar experiments with radioactive potassium are not easy to carry out as the potassium isotopes other than ^{42}K that are available at low specific activity. An alternative approach is to examine the effect of different treatments on the apparent content (A^*_c) of the cytoplasmic phase, i.e. on the amount of isotope diffusing out of the tissue as if from the cytoplasmic phase. For example, tissue was allowed to take up ^{42}K from a labelled potassium chloride solution of concentration 5 m-equiv/l, and then eluted with either potassium chloride or sodium chloride at 2 or 25°C. Table 6 gives the amounts of potassium and its isotope that diffused out of the non-free space during a 9-hr period of elutions, following elution of the

TABLE 6

AMOUNTS OF POTASSIUM AND OF POTASSIUM ISOTOPE DIFFUSING FROM THE NON-FREE SPACE OF BEETROOT DISKS IN 9 HR INTO SOLUTIONS OF POTASSIUM CHLORIDE OR SODIUM CHLORIDE AT 2 OR 25°C

Tissue allowed to take up $^{42}K^+$ from a labelled potassium chloride solution of concentration 5 m-equiv/l prior to elution with sodium chloride or potassium chloride solution

	Elution with Sodium Chloride		Elution with Potassium Chloride	
	2°C	25°C	2°C	25°C
Amount of $^{42}K^+$ diffusing from tissue (arbitrary units)	5·8	10·1	1·9	2·6
Amount of potassium diffusing from tissue (m-equiv/kg)	3·55	10·0	—	—
Specific activity (arbitrary units) of:				
Labelling solution	5·0	5·0	5·0	5·0
Potassium in vacuoles at end of elution period	0·45	0·45	0·37	0·38
Minimal estimate of Q^*_c	4·6	6·2		

free space at 2°C for 2 hr (cf. p. 650). The specific activity of the labelling solutions and of the potassium in the vacuole were also measured. It is evident that the sodium is having some effect other than simply replacing potassium in the non-free space, and furthermore there is a large contribution from some other region than free space and the vacuoles, as the average specific activity of potassium diffusing into the sodium chloride solution is higher than that in the vacuoles. A minimal estimate of the contribution of the cytoplasmic phase can be made by assuming that the specific activity in this phase is as high as that in the labelling solution (an extreme value). Even this conservative estimate is some 2·5 times the amount of isotope diffusing out of the tissue to the potassium chloride solutions from both parts of the non-free space together.

This difference can be explained simply on the serial model as sodium inhibits the uptake of potassium to the vacuoles, making the ratio $\phi_{cs}/(\phi_{cs}+\phi_{cv})$ more nearly equal to unity. As a result the amount of isotope diffusing to the solution, A^*_c, becomes

more nearly equal to Q_c^*. As an example of this inhibition, uptake of potassium from a mixture of sodium and potassium chloride solutions (concn. of each 5 m-equiv/l) is negligible until the sodium concentration has fallen (as a result of sodium uptake) to about 40% of the original concentration (Briggs, Hope, and Pitman 1958*b*).

The inhibition of potassium influx also has an effect on the apparent efflux from the tissue (eqn. (9)). For example, Table 7 gives values of the apparent efflux measured by loss of potassium or sodium isotopes from tissue in a range of mixtures of potassium chloride and sodium chloride, of total chloride concentration 5 m-equiv/l. As the sodium concentration increased, the apparent efflux of potassium became larger.

TABLE 7

APPARENT EFFLUX OF POTASSIUM AND SODIUM FROM TISSUE ACCUMULATING SALT FROM SOLUTIONS CONTAINING VARIED RATIOS OF POTASSIUM TO SODIUM BUT WITH TOTAL CHLORIDE CONCENTRATION OF 5 M-EQUIV/L

Solution Concn. (m-equiv/l)		Content of Vacuoles (m-equiv/kg)		Apparent Efflux (μ-equiv/kg/hr)	
Sodium	Potassium	Sodium	Potassium	Sodium	Potassium
5	0	29	90	25	620
4	1	26	92	24	400
2·5	2·5	22	95	22	310
1	4	21	98	24	270
0	5	16·5	98	18	170

The establishment of the higher efflux when tissue was transferred from potassium chloride to sodium chloride solutions of the same concentration was rate-limited by some process much slower than the equilibration of the free space, and with a temperature coefficient for k_c of between 2 and 3. For example, Figure 7 shows the pattern of increase in ^{42}K diffusing out of the tissue when the solution was changed from potassium chloride to sodium chloride of the same concentration at either 25 or 16°C. The tissue, which had taken up the isotope from a labelled potassium chloride solution of concentration 5 m-equiv/l, was put into a series of unlabelled potassium chloride solutions until the efflux of isotope fell to a steady value, which is shown in the figure. The apparent efflux is plotted as a percentage of the final steady value in sodium chloride, which at 25°C was 1·3 m-equiv/kg/hr, and at 16°C was 0·9 m-equiv/kg/hr. The equilibration of the free space is shown on the same scale, and can be taken as the same at each temperature.

The inhibition of potassium uptake could increase the isotope diffusing out of the tissue if the diffusion path to the solution was such that a large proportion of the isotope diffusing out of cells at the centre of the disk was accumulated by cells nearer the surface. This effect can be estimated only roughly as both the diffusion path and the diffusion coefficient of potassium in the free space are somewhat

indeterminate, but taking a low value of the diffusion coefficient (10^{-7}cm^{2}sec^{-1}), and an influx of 1 m-equiv/kg/hr, the underestimation would be only about 15% when the tissue was in potassium chloride solutions of the concentrations used. It is considered that the results given above support the suggestion that the tissue behaves as if the three phases are in series, rather than in parallel.

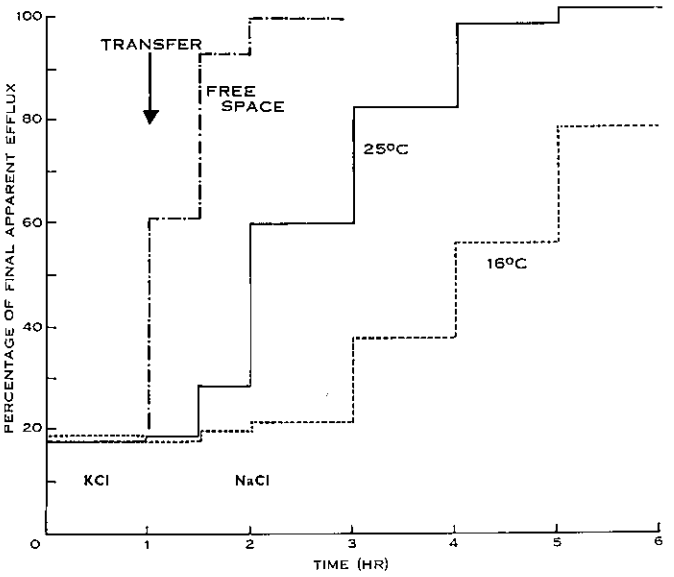

Fig. 7.—Apparent potassium efflux from tissue at 25 and 16°C when transferred from a potassium chloride to a sodium chloride solution of the same concentration, 5 m-equiv/l. ——— at 25°C; — — — at 16°C; —·—·— equilibration of the free space. Values are plotted as a percentage of the final steady values reached at each temperature.

VI. Discussion

The first object of this paper has been the demonstration that beet tissue contains two non-free space components—the cytoplasmic phase and the vacuoles—and that these phases are in series. It is suggested that the phases detected in salt-uptake studies are due to differences in the salt relations of parts of the cells and not to a plurality of cell type within the tissue. As used in these studies, beet disks contain both vascular tissue and parenchyma. The main argument in favour of relating these phases to parts of the cell is that the three-phase system can be demonstrated in beet disks cut so as to contain only parenchymatous cells (Table 4).

On these assumptions the isotope diffusing out of labelled tissue can be analysed to calculate the fluxes into and out of the cytoplasmic phase if some assumptions are also made about the uniformity of labelling in the tissue. It is realized that these fluxes are "average" values for all the cells in the tissue, but the comparison of the fluxes when conditions are varied can give some qualitative information on the relation of the cytoplasmic phase to salt uptake to the vacuoles.

The cytoplasmic phase must be located in the cytoplasm but may be the whole of it, bounded by the plasmalemma and the tonoplast membrane, or a small part cut off by, say, the endoplasmic reticulum and the tonoplast membrane. However, a reasonable working hypothesis is that the cytoplasmic phase is the whole of the cytoplasm. This assumption has the advantage that there are obvious boundaries, which have a suitable organization to account for the high resistance to ion diffusion found for the cytoplasmic phase boundaries.

The cytoplasm in beet cells comprises about 5% of the tissue, showing in electron micrographs as a layer of about the same thickness as the cell walls (Chambers, unpublished data) and, as far as can be estimated, contains about 5–10% of mitochondria. Beet disks 1 mm thick have about 15–20% of cut or damaged cells, and 5% of intercellular space all of which acts as the water free space. The cell walls occupy about 5% of the tissue and contain the Donnan free space in about half this volume, the rest being solid material or water free space. There is therefore about 70% available for the vacuoles.

The cytoplasm is certainly not a homogeneous phase, and whatever the location of the cytoplasmic phase, the concentrations of potassium and bromide are not likely to be simply $Q_c/5\%$. Regions within the cytoplasmic phase but with a turnover more rapid than that of the phase as a whole would contribute to Q_c but would not be detectable from the rate of exchange. For example, uptake of bromide to the mitochondria could account for a large part of Q_c; a reasonable concentration in mitochondria might be about 100 m-equiv/l, which if there are 10% mitochondria in the cytoplasm, would be equivalent to 0·5 m-equiv/kg. This is not an extreme estimate, and shows that care must be taken in attempting to locate active transport at either boundary from arguments based on Q_c and an estimated volume of the phase.

This criticism does not apply to the flux determinations which are independent of the distribution of Q_c, and so may be used to investigate the properties of the boundaries of the cytoplasmic phase. The temperature coefficients of the fluxes across both boundaries are between 1·7 and 2·3, i.e. much higher than the temperature coefficients for diffusion in solution. The high value is not taken to show that these fluxes are active, but only that the two boundaries may have the same "activation energy" for penetration. The similarity between the membranes is also shown by estimation of the permeability, assuming that the potassium fluxes ϕ_{sc} and ϕ_{vc} are not active. The diffusion flux across the outer boundary, assuming uniform potential gradient, is

$$\phi_{sc} = -P \cdot \frac{zFE/RT}{\{1 - \exp(zF\bar{E}/RT)\}} \cdot C_o, \tag{11}$$

where

$$P = uRT/Fl,$$

u being the mobility of the ion, and l the thickness of the boundary. The relevant value of $\bar{E}$ is not known, but it is likely to be about −58 to −116 mV, when P would be 2×10^{-8} cm/sec or $1 \cdot 1 \times 10^{-8}$ cm/sec, respectively, if ϕ_{sc} is taken as 0·75 m-equiv/kg/hr* when C_o is 5 m-equiv/l (Fig. 4). Taking the same values of E, and again using

* 1 m-equiv/kg/hr = 0·3 p-equiv/cm²/sec for beet tissue.

the data of Figure 4, where $Q_v = 60$ m-equiv/l, estimates can be made from ϕ_{vc} giving $P = 0{\cdot}49 \times 10^{-8}$ or $2{\cdot}7 \times 10^{-8}$ cm/sec. If the membrane were about 100 Å thick the value of the diffusion coefficient would be 10^{-14} cm^2/sec, and so about that found for lipoprotein membranes.

The similarity in resistance of the two boundaries can also be seen from the dependence of Q_c on both C_o and on C_v. If one boundary had been very much more permeable than the other, Q_c would have been related more closely to the concentration in the solution separated from the cytoplasmic phase by the lower resistance.

If the influx, ϕ_{sc}, is assumed to be passive, the relationship between ϕ_{sc}/C_o and C_o gives an estimate of the concentration of non-exchangeable anions of about 100 m-equiv/l in the membrane, or, if the negative charge were on a surface, about 10^{-6} coulombs/cm^2. This charge density is about that found for many organelles, such as chloroplasts, mitochondria, or blood cells. A negatively charged membrane (or surface) would thus not be unlikely and would be convenient to explain the low permeability of the cytoplasm to divalent anions, and the stimulation of uptake of chloride ions by divalent cations (Pitman, unpublished data).

As there are so many different ways of distributing potassium and bromide ions within the cytoplasmic phase, there does not seem to be much point in using these results to attempt to locate active uptake of bromide or potassium at either boundary, as they can be made to support many models by suitable choice of a volume for the cytoplasmic phase and distribution within the cytoplasmic phase.

The demonstration that the tissue behaves as a three-phase system means that in some cases the estimation of fluxes suggested by Briggs (1957) and used by Briggs, Hope, and Pitman (1958*b*) and by Van Stevenick (1962) will not be valid. This derivation was based on a two-phase system and

$$\phi_i = K_i(\mathrm{d}s_i/\mathrm{d}t)/(s_o - s_i),$$

where ϕ_i is the influx, s_i, s_o are the specific activities inside and outside, and K_i is the concentration in the vacuoles. In fact, s_o should be replaced by s_c, if ϕ_i is made ϕ_{cv}; as s_i is about 5% of s_o in most cases, and s_c may be about 50–70% of s_o (particularly when there is a net loss of salt from the vacuoles), this difference may lead to underestimation of the influx to the vacuole by 45/95 to 65/95, depending on the value of s_c.

VII. Acknowledgments

I am grateful to Professor G. E. Briggs and to other colleagues in the Botany Department, University of Cambridge, for their helpful comments.

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