The Kinetics of Bicarbonate and Malate Exchange in Carrot and Barley Root Cells

W. J. CRAM
School of Biological Sciences, The University of Sydney, Sydney, N.S.W. 2006, Australia

and

G. G. LATIES
Department of Biology and Molecular Biology Institute, University of California, Los Angeles, U.S.A.

Received 1 May 1973
Accepted for publication 8 August 1973

ABSTRACT
The time course of loss of $^{14}$C from $^{14}$CO$_3^-$-labelled carrot tissue has been measured. The graph of log ($^{14}$C remaining in the cells) versus time can be fitted by two exponential components. The graph of log (rate of $^{14}$C loss over successive periods) versus time can also be fitted by two exponential components using the same fitting procedure. However, the half times of the components fitting the two types of graph are not the same, and therefore the apparently good fit is not valid. Three exponential components can be fitted to the two types of plot such that their rate constants are equal and their intercepts in the correct theoretical relationship. Their rate constants are about 7 h$^{-1}$, 1.5 h$^{-1}$, and less than 0.1 h$^{-1}$, and they probably correspond to the total HCO$_3^-$-CO$_2$ in the cell, the malate in the cytoplasm, and the malate in the vacuole, respectively. From these data it is shown that one can calculate the influx of HCO$_3^-$-CO$_2$ across the plasmalemma, and the influx of malate across the tonoplast during accumulation of endogenously produced malate.

The time course of uptake of $^{14}$C-malate is estimated as the sum of $^{14}$C accumulated in the tissue and $^{14}$C evolved as CO$_2$. At 1 mM external malate total uptake is linear with time, suggesting that uptake is limited by and equal to the influx across the plasmalemma. At higher external malate concentrations the evolution of $^{14}$CO$_2$ saturates but accumulation in the tissue continues to rise. Under these conditions it is concluded that the tonoplast influx of malate can be calculated and that this influx does not saturate at high external malate concentrations.

The net efflux of malate is very small but measurable. The efflux of $^{14}$C-labelled malate is not stimulated by external malate, Cl$^-$, NO$_3^-$ or SO$_4^2$-. There are therefore no plasmalemma systems exchanging internal malate for these anions.

INTRODUCTION
Three methods have been used in investigating compartmentation and rates of transport of organic acids in plant cells.
12  Cram and Laities—The Kinetics of Bicarbonate and Malate Exchange

(1) Kinetic methods. Kinetically definable components of CO$_2$ or malate evolution from plant tissues were observed by Ting and Duggar (1965, 1966, 1968) and Osmond and Laities (1969).

Osmond and Laities (loc. cit.) further examined the sizes of the intercepts of the wash-out components at $t = 0$. Analysis of such wash-out curves has uses and limitations which will be discussed in the present paper. For instance, while Osmond and Laities’ conclusion that external salts altered the fraction of fixed CO$_2$ which was transferred to the vacuole is valid (provided the majority of the fixed product remained in the cell), it would not be valid to take the size of an intercept as equal, or even proportional, to the content of a compartment. It is also easy to fit false exponential components to a curve, as shown in the present paper.

Essentially the same method was used by Lips and Beevers (1966a, 1966b), with the analytical difference that the internal malate rather than the malate or CO$_2$ flowing to the external solution was measured, and much less accurate values of the kinetics were obtained. A rapidly and a slowly falling component of the labelled malate were distinguished in maize roots, which were differentially labelled with acetate-[$^{14}$C] and $^{14}$CO$_2$ and which had a flux between them stimulated by low pH and malonate. Quantitative interpretation is even more difficult than with the accurate wash-out curves, and Jacoby and Laities (1971) have shown that the effect of malonate is in fact more complex than appears from the earlier work.

(2) The ideal situation is one in which each compartment may be analysed separately, when the contents of all phases and the fluxes between them may be calculated. MacLennan, Beevers and Harley (1963) used the specific activity of CO$_2$ evolved from the cell as a measure of the specific activity of the organic acid pools from which it came. At a steady state the specific activity of CO$_2$ was greater than that of total malate or other organic acids in the cells, and after various corrections the results give an estimate of the fraction of the total cell organic acid outside the respiratory pool, assuming that the extra-respiratory pool has a specific activity of zero. The observation that the fraction outside the respiratory pool in maize roots increased as vacuolation increased is most persuasive that the method gives at least qualitatively valid results. However, this measurement does not tell how many pools there are outside the respiratory pool, and does not give values of fluxes between them or of their total content, since the specific activity of the external pool(s) is not known.

(3) Comparison of fully vacuolate and less vacuolate cells (Torii and Laities, 1966). Such results are suggestive, but quantitative conclusions cannot be obtained.

In this paper the kinetics of HCO$_3^-$-malate exchange in carrot root cells are examined, and it is concluded that they are more complex than are the kinetics of inorganic ion exchange, but can be fairly rigorously described and used to obtain reliable values of the influx across the plasmalemma. The frequently used measure of the rate of CO$_2$ fixation into malate—the steady accumulation (presumably in the vacuole) of $^{14}$C from external HCO$_3^-$—is considered in relation to these results. The influx of malate to barley and carrot root tissues is also examined, and malate–malate and malate–anion exchange systems have been looked for.
MATERIALS AND METHODS

Carrot (Daucus carota L.) root xylem tissue was cut into 1 mm thick slices and washed in aerated distilled water at room temperature for 4–6 d before use. Barley (Hordeum vulgare L.) seedlings were grown in aerated 0.5 mM CaSO\(_4\) solutions and excised after 6 d, as previously described (Cram and Laties, 1971).

Cl\(^-\) influx was measured as previously described (Cram and Laties, 1971). H\(^{14}\)CO\(_3\)\(^-\) and L-malate-\(^{14}\)C influxes were measured similarly except that the tissue was maintained in a sealed conical flask during the uptake and washing periods to prevent loss of \(^{14}\)CO\(_2\), and the solution was stirred by shaking rather than by a stream of air. In one experiment, Cl\(^-\) influx from a solution mixed by shaking was 108 ± 14 (s.d.) per cent of the Cl\(^-\) influx from a solution mixed by passing an air stream through it. The two treatments are therefore not significantly different. During malate-[\(^{14}\)C] influx, \(^{14}\)CO\(_2\) was collected on KOH impregnated silica paper, as during efflux.

\(^{14}\)C efflux was measured as the counts appearing in the external solution and in CO\(_2\). \(^{14}\)CO\(_2\) was collected on 10 per cent KOH impregnated silica paper suspended above the tissue and medium in the sealed conical flasks. The solution and silica paper were replaced at intervals. Radioactivity in the silica paper, in aliquots of the washing solution, and in barley roots dried down under slightly alkaline conditions, was estimated by scintillation counting in toluene or dioxane-naphthalene plus PPO and POPOP as described by Osmond and Laties (1969).

The net efflux of malate was estimated from the malate found in the solution in which tissue had been immersed for several hours. The malate in a 10 mM KHCO\(_3\) washing solution was separated from the large excess of KHCO\(_3\) by precipitating the K\(^+\) ions as K\(^+\) perchlorate at 0 °C as follows. The 250 ml of washing solution was evaporated down to 10 ml and perchloric acid, at 0 °C, was added to the evaporated washing solution, also at 0 °C, until the pH of the solution was 7. The K perchlorate crystals were filtered off, and the titrations repeated until the pH of the solution was 7.3 after filtering off the K perchlorate crystals. The solution was then further evaporated to 2 ml, centrifuged to remove the debris and assayed for malic acid. When the malate content was to be estimated, the tissue was extracted in boiling distilled water. Malic acid was assayed enzymatically (Hohorst, 1963).

To examine the distribution of \(^{14}\)C in the components of the tissue after fixation of \(^{14}\)CO\(_3\), 4 g of carrot tissue were extracted in three 50 ml aliquots of boiling 80 per cent ethanol, each extraction lasting 30 min. The total extract was evaporated to a small volume and the particulate matter spun down. The pellet was resuspended in 1 ml H\(_2\)O, and the radio-activity in it was counted. The supernatant was made up to 50 ml and an aliquot counted. The amino acids and organic acids were separated on ion exchange columns, eluted and counted, as described by Torii and Laties (1966). The organic acid fraction was again evaporated down and the individual organic acids separated on a paper chromatogram with butanol: H\(_2\)O:formic acid as solvent (Torii and Laties, 1966). The organic acids were revealed by bromophenol blue staining, but only malate was positively identified by running a parallel malate standard on the paper. The various acids, and substances at the origin, were washed off the paper chromatogram and the activity in them was counted. The malate spot was also assayed for malate.

RESULTS

Malate contents and distribution of \(^{14}\)C after H\(^{14}\)CO\(_3\)\(^-\) labelling

Three-day water-washed carrot tissue was shaken in 1 mM KH\(^{14}\)CO\(_3\) for 15 h. The tissue was then washed in water to remove extracellular contents and the cellular contents extracted. The activity of \(^{14}\)C in the various fractions of the tissue extract are shown in Table 1. Total malate concentration in the tissue was 36 µmol g\(^{-1}\) (fresh wt). The initial malate content would have been about 10 µmol g\(^{-1}\) (Cram, 1973a).

Most of the count in the tissue is found in malic acid so that, at least over long periods, accumulation and loss of \(^{14}\)C corresponds to accumulation and loss of malate.
The kinetics of $^{14}$C loss after labelling carrot tissue with $^{14}$CO$_3^-$

An analysis of one series of results will be presented.

In this experiment carrot tissue was labelled with $^{14}$CO$_3^-$ for about 3 h, and then the time-course of loss of $^{14}$C (to gas phase and solution) was measured. Fig. 1 shows the $^{14}$C activity in the tissue plotted against time, and Fig. 2 shows the corresponding graph of $^{14}$C efflux plotted against time. Both curves are fitted reasonably well by the sum of three exponentials. The initial rapid component must include loss from extracellular spaces, and will not be considered further.

As shown in the Theoretical Section, in a system exchanging tracer at a near-steady-state the content versus time and the efflux versus time graphs must be fitted by exponential terms having the same rate constants, and with intercepts in proportion to the rate constants. The exponential terms fitting the log content versus time graph in the present case have half-times of 22-3 h and 25 min whereas the terms fitting the log efflux versus time plots have half times of 114 min and 6 min. This means that neither curve is an adequate representation of the data, and the apparently good fit of exponential terms in Figs 1 and 2 is deceptive.

The discrepancy might be removed if there were also a large, slowly exchanging fraction of the $^{14}$C in the tissue. If its rate of loss were small enough it would be negligible compared with the rates shown in Fig. 2, which would then in fact be a correct fit to the data. A constant amount subtracted from the data of Fig. 1 would leave two phases of the log content versus time plot with shorter half times, and if subtracting a large slow phase is valid one should be able to obtain two exponential phases having the same half times as those of Fig. 2. It has proved possible to do this with all the experimental curves obtained.

The fit of exponentials to these 'adjusted content' versus time graphs appears as good as the fit in Figs. 1 and 2. Table 2 shows that, in one experiment, the slopes of the fitted exponentials were the same in the log efflux versus time and log 'adjusted content' versus time graphs, and the intercepts to a first approximation in the correct theoretical relationship. The uncertainty in the values of the intercepts is greater than in the values of the rate constants, and so the greater variability in the ratio of intercept ratios is not unexpected.

A three-compartment system is too complex to be analysed without access to individual compartments (see e.g. Rescigno and Segre, 1965). However, a validly fitted curve allows one to estimate the efflux at the beginning of the wash-out by

### Table 1. $^{14}$C activity in various fractions of carrot cells after $^{14}$CO$_3$ labelling

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Percentage of activity in original extract</th>
</tr>
</thead>
<tbody>
<tr>
<td>Particulate</td>
<td>0.8</td>
</tr>
<tr>
<td>Amino acids</td>
<td>6.5</td>
</tr>
<tr>
<td>Malic acid</td>
<td>88</td>
</tr>
<tr>
<td>Other organic acids</td>
<td>4.6</td>
</tr>
<tr>
<td></td>
<td>99.9</td>
</tr>
</tbody>
</table>
Fig. 1. The time course of loss of $^{14}$C from $^{14}$CO$_3^-$-labelled carrot root tissue. Tissue was loaded for 124 min in 10 mM KHCO$_3$ + 0.5 mM CaSO$_4$, and then washed in aliquots of inactive solution of the same composition. The activity lost in successive washing periods was added to the final tissue activity to give the total activity in the tissue at the beginning of each washing period. (A) Log (total activity in the tissue) versus time. The slowest component is extrapolated to $t = 0$ (---) and its content subtracted from the total to give the content of the faster exchanging components. (B) Content of the faster exchanging components plotted on a log scale versus time.

extrapolation, from which the plasmalemma influx can be calculated (see Theoretical Section).

As an example, parameters of the $^{14}$C wash-out curves and the fluxes calculated from them in a single experiment are shown in Table 3. These values will be referred to in the Discussion.
FIG. 2. The same primary data as in Fig. 1, but rates of loss over successive washing periods are plotted against time. (A) Total rate plotted on a log scale versus time. The slowest component is extrapolated (— — —) and subtracted from the total. (B) The faster components of 14C loss plotted on a log scale versus time.

**Net malate efflux**

The rate of loss of malate from carrot tissue, estimated as malate by the enzymatic assay, was measured in two experiments. Malate efflux was about 5 nmol g⁻¹ l⁻¹. The rate constants were nearly the same (2–3 × 10⁻⁴ h⁻¹) in the two experiments.
Table 2. Comparison of the parameters of exponential components fitted to 'rate' and 'adjusted content' wash-out curves

<table>
<thead>
<tr>
<th>Component</th>
<th>$k_{\text{content}}$</th>
<th>$I/I_i$</th>
<th>$k_{\text{rate}}$</th>
<th>$I_i/I_i'$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Slow</td>
<td>1.11</td>
<td>0.92</td>
<td>0.97</td>
<td>0.83</td>
</tr>
<tr>
<td>Fast</td>
<td>1.01</td>
<td>0.98</td>
<td>0.93</td>
<td>0.83</td>
</tr>
<tr>
<td>Mean</td>
<td>0.96</td>
<td>1.18</td>
<td>1.03±0.06</td>
<td>1.02±0.11</td>
</tr>
</tbody>
</table>

Table 3. Parameters of $^{14}$C efflux from 1-d water-washed carrot tissue

<table>
<thead>
<tr>
<th>Parameter*</th>
<th>1 mM KHCO$_3$†</th>
<th>10 mM KHCO$_3$†</th>
</tr>
</thead>
<tbody>
<tr>
<td>$t_i$ (min.)</td>
<td>134</td>
<td>114</td>
</tr>
<tr>
<td>$t_f$ (min.)</td>
<td>3.5</td>
<td>6.0</td>
</tr>
<tr>
<td>$I_i$ (µmol g$^{-1}$ h$^{-1}$)</td>
<td>0.0056</td>
<td>0.015</td>
</tr>
<tr>
<td>$I_f$ (µmol g$^{-1}$ h$^{-1}$)</td>
<td>0.24</td>
<td>0.57</td>
</tr>
<tr>
<td>$M_{\text{ve}}$ (µmol g$^{-1}$ h$^{-1}$)</td>
<td>0.042</td>
<td>0.20</td>
</tr>
</tbody>
</table>

* Tissue was loaded for 123 min in $^{14}$C-labelled solution, then washed from $t = 0$ in unlabelled solution of the same composition. The data were analysed as described in the text. The plot of log (efflux) versus time was fitted by a fast ($f$) and a slow ($s$) exponential term. $t_i =$ half time; $I_i =$ intercept at $t = 0$.
† All solutions also contained 0.5 mM CaSO$_4$.
‡ Rate of $^{14}$C-labelled entry to the slowly exchanging phase (final tissue content divided by loading time). This is thought to represent vacuolar filling and is therefore designated $M_{ve}$.

The external solution varied, but since this has no influence on efflux of $^{14}$C to the solution (next section) it probably does not influence net malate loss.

The rate of CO$_2$ loss is about 20 times greater than malate loss under several conditions (e.g. Table 4 and Cram, unpublished), so the rate constant for malate loss from the vacuole over the relatively long period of these measurements would be 10$^{-2}$ h$^{-1}$ or less. This is about the rate expected of the large, very slowly exchanging component postulated to reconcile the log content versus time and log efflux versus time graphs.

The specific activity of the malate lost to the external solution was 96±3 per cent (mean of four replicates,± standard deviation) of the specific activity of the internal malate under conditions of no net malate synthesis. This assumes that the distribution of $^{14}$C is the same in the malate in the vacuole and in the external solution.

Exchange processes

The effect of external malate on the efflux of malate from carrot tissue was examined. If a major malate–malate exchange process were present at the plasmalemma, one would expect to find a transient burst of malate efflux followed by a

...
continued large stimulation of malate efflux after malate was added to the external solution (similar to the external Cl− stimulation of Cl− efflux found in carrot—Cram, 1968). As shown in Fig. 3, there was only a slight stimulation of [14C]-malate efflux, and no transient burst of malate efflux, after adding external malate. It therefore appears that there is no specific malate-malate exchange system in carrot root cells.

There is little difference between malate efflux in KCl and in K2SO4 (Table 4). In another experiment no difference was found between malate efflux in 1 mM KCl+0·5 mM CaSO4 and in 1 mM KNO3+0·5 CaSO4, there being only a gradual

---

**Fig. 3.** The effect of 2 mM L-malate on 14C efflux from KH4CO3 labelled carrot tissue. After the efflux of CO₂ and malate was steady in 10 mM KCl, the washing solution was changed to 10 mM KCl+2 mM L-malate. pH 5·0, 24 °C. (A) Efflux of malate. (B) Efflux of CO₂.

**Table 4**

<table>
<thead>
<tr>
<th>A. Stimulation of malate efflux by external malate*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Temperature (°C)</td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td>-----------------</td>
</tr>
<tr>
<td>24</td>
</tr>
<tr>
<td>15</td>
</tr>
<tr>
<td>5</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>B. Stimulation of CO₂ efflux by external malate*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Temperature (°C)</td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td>-----------------</td>
</tr>
<tr>
<td>24</td>
</tr>
<tr>
<td>15</td>
</tr>
<tr>
<td>5</td>
</tr>
</tbody>
</table>

* Measured simultaneously on the same tissue.
† Steady efflux after 2 mM malate addition divided by steady efflux before malate addition.
increase by 2–3-fold after 4 h. No transient stimulation of malate efflux was found after transfer of the tissue from water to KCl, K₂SO₄, or KNO₃. Hence there appear to be no non-specific malate–anion exchange systems in carrot root cells.

The influx of malate to barley and carrot root tissue

The time course of uptake of ¹⁴C-labelled malate to barley roots is shown in Fig. 4. After the first 12 min the rate of uptake by the tissue (¹⁴C accumulation in the root, plus that which is taken up and then lost as CO₂) is constant until at least 80 min.

The intercept of this steady uptake on the content axis is 0.17 μmol g⁻¹, as though 17 per cent of the volume of the root rapidly equilibrates with the external solution, i.e. equivalent to an extracellular space of 17 per cent. This agrees with estimates with other anions (Pitman, 1965).

The fact that after 5–10 min the total malate taken up increases linearly with time in the absence of any washing in inactive solution before counting, shows that the influx across the plasmalemma is the rate-limiting step in influx to the cells at 1 mM. At lower concentrations, the plasmalemma influx will be smaller, and must still be the rate-limiting step. At higher external malate concentrations the plasmalemma is probably not the rate-limiting step, as shown below.

Fig. 5 shows the rate of malate accumulation and the rate of evolution of CO₂ from malate over a range of external malate concentrations in barley roots. The scale is expanded below 1 mM only to allow the points to be distinguished. There is no discontinuity in the line at 1 mM. Fig. 6 shows more restricted but similar results in excised carrot tissue.

It will be assumed that malate flows to the vacuole and to CO₂ from the same cytoplasmic pool, with specific activity s₂. At higher external malate concentrations the rate of ¹⁴C evolution in CO₂ remains constant, so s₂ must remain constant.
Since the influx of $^{14}C$ to the vacuole increases at the same time, the flux from cytoplasm to vacuole must increase, presumably due to an increase in the malate concentration in the cytoplasm ($C_c$). An increase in $C_c$ while $s_s$ remains constant would result from a plasmalemma influx increasing with external concentration only if $s_s = s_o$, the specific activity of the external solution. Hence Figs. 5 and 6 suggest that the $CO_2$-evolving process in barley and carrot saturates with respect to cytoplasmic malate, and that above 1 mM external malate the influx to the vacuole ($M_{ov}$) is equal to the tonoplast influx ($M_{eq}$) (see Theoretical section).

The lag in $^{14}CO_2$ evolution (Fig. 4) must be related to the time taken to fill the pool from which $CO_2$ is evolved. If this is an exponentially filling pool, with rate constant $k$, then the intercept on the time axis equals $1/k$ (see Theoretical section). In 1 mM malate at 20 °C the half time ($= 0.7/k$) for exchange of the phase from which $^{14}CO_2$ is evolved is $11 \pm 3$ min (mean of seven expt. standard deviation). This is equivalent to a rate constant of 3.8 h$^{-1}$. 

---

**Fig. 5.** The isotherm for malate influx to barley roots. Details as for Fig. 4, but only one sample taken at each concentration, between 70 min (70 mM) and 116 min (0.1 mM) after loading had begun. The root samples were then washed in inactive solution for 10 min to remove malate from extracellular spaces. Pretreatment: 2.5 h in inactive solution of the same composition. $K^+$ salt. pH 5.0. Note expanded scale below 1 mM $K$ malate.

**Fig. 6.** The isotherm for malate influx to excised carrot root tissue. Tissue washed in water for 5 d after excision. Details as in Fig. 5. Final samples taken after 105-30 min of loading with $K$ malate-$^{14}C$. Each point is the mean of three samples. Standard errors of the mean are less than 7 per cent.
The contents of this pool can be calculated from the relationship

\[
\text{Rate constant } (k, \text{ h}^{-1}) = \frac{\text{Efflux } (M_s - \text{d/min g}^{-1} \text{ h}^{-1})}{\text{Content } (Q_c - \text{d/min g}^{-1})}.
\]

Assuming that the efflux from the pool equals the steady rate of CO\textsubscript{2} evolution plus the steady rate of accumulation in the vacuole (which can both be obtained from Fig. 4), and that the cytoplasmic specific activity \(s_c\) equals that in the external solution \(s_o\), the cytoplasmic malate pool is calculated to contain \((0.22 + 0.15)/3.5 = 0.11 \mu\text{mol g}^{-1}\). If \(s_c\) is less than \(s_o\) due to endogenous CO\textsubscript{2} fixation into malate, then \(Q_c\) will be greater than this. If the pool occupies 2 per cent of the tissue, the malate concentration in it will be 5.5 mM.

**THEORETICAL SECTION**

**Symbols used**

Subscripts \(o, c\) and \(v\) refer to external solution, cytoplasm, and vacuole, respectively,

- \(M\)—chemical flux (\(\mu\text{mol g}^{-1} \text{ h}^{-1}\))
- \(s\)—specific activity (d/min \(\mu\text{mol}^{-1}\))
- \(Q\)—content (\(\mu\text{mol g}^{-1}\))
- \(k\)—rate constant (h\(^{-1}\)) = \(\left(\frac{\sum M_i}{Q_i}\right)\)
- \(M^*\)—label flux (d/min \(\mu\text{mol}^{-1} \text{ h}^{-1}\)) = \(M_{ij} \cdot s_i\).

**Relationship between ‘content’ and ‘rate’ graphs of wash-out**

In a compartmented system at a steady state the exchange of a substance, measured with the use of some tracer such as a radio-actively labelled molecule, will obey first order kinetics. After loading in a labelled solution and transferring to an unlabelled one the content of the system at time \(t\) will be expressible as \(Q_t = \sum I_i \exp(-k_i t)\), and the efflux will be \(\frac{dQ_i}{dt} = -\sum k_i I_i \exp(-k_i t)\). In such a system, therefore, the relation of content \(v.\) time and of efflux \(v.\) time must be fitted by exponential terms having the same rate constants and having intercepts in a ratio proportional to the ratio of rate constants. If the intercepts on the adjusted content plot are \(I_1^*\) and \(I_2^*\) for components having rate constants \(k_1\) and \(k_2\), then the intercepts on the rate plot (\(I_1^* / I_2^*\)) will equal \(k_1/k_2\) times the ratio of the intercepts on the ‘adjusted content’ plot (\(I_1 / I_2\)).

The estimation of the plasmalemma influx, \(M_{oc}\)

At a quasi-steady state (when the fluxes and compartment contents change negligibly during an experiment), the net influx of tracer, \(M_{oc} \cdot s_o\) d/min g\(^{-1}\) h\(^{-1}\)), will equal the plasmalemma influx minus the plasmalemma efflux:

\[
M_{oc} \cdot s_o = M_{oc} \cdot s_o - M_{co} \cdot s_c.
\]

The first term, the quasi-steady influx of tracer, and the external solution specific activity, \(s_o\), can be measured. The third term, the plasmalemma efflux during quasi-
steady influx, can be obtained as the initial efflux of tracer immediately after transferring to zero external specific activity. This initial efflux is swamped by the rapid loss from extracellular spaces, and so has to be obtained by extrapolation of the efflux curve to \( t = 0 \). From these three values \( M_{oc} \) can be calculated.

This estimate of \( M_{oc} \) depends on the existence of a quasi-steady state with regard to net and tracer influx, and on the validity of the extrapolation to obtain the initial efflux value, \( M_{oc} \). It does not depend on assumptions about the arrangement of compartments within the cell.

The quasi-steady influx to the vacuole

At a quasi-steady state the influx of tracer to the vacuole is \( M_{oc} \cdot s_o \) (d/min g\(^{-1}\) h\(^{-1}\)). If there is no 'straight through' component of the influx to the vacuole, then this can be equated with the influx from the cytoplasm.

\[
M_{oc} \cdot s_o = M_{co} \cdot s_c
\]

\( M_{oc} \cdot s_o \) and \( s_o \) can be measured. If \( s_o \) is known, \( M_{co} \) can then be calculated.

The evolution of CO\(_2\) from an exponentially filling cytoplasmic pool

If \(^{14}\)CO\(_2\) is evolved from an exponentially filling cytoplasmic pool, then the flux of \(^{14}\)CO\(_2\) out of the tissue (the rate of \(^{14}\)CO\(_2\) evolution) at any instant, \( M_{CO_2} \cdot s \) (d/min g\(^{-1}\) h\(^{-1}\)), can be written as

\[
M_{CO_2} \cdot s_{CO_2} = M_{CO_2} \cdot s_{CO_1}[1 - \exp(-kt)]
\]

where \( M_{CO_2} \) is the rate of evolution of total CO\(_2\) (\( \mu \)mol g\(^{-1}\) h\(^{-1}\)), \( s_{CO_2} \) is the specific activity of the pool from which CO\(_2\) comes (d/min \( \mu \)mol\(^{-1}\)), \( s_{CO_1} \) is the specific activity of this pool at a time long compared with \( 1/k \), and \( k \) (h\(^{-1}\)) is the rate constant for exchange in the pool. The total \(^{14}\)CO\(_2\) evolved up to time \( t \) (d/min g\(^{-1}\)) is then given by

\[
Q_{CO_2}^* = \int_0^t M_{CO_2} \cdot s_{CO_2} \, dt
\]

\[
= M_{CO_2} \cdot s_{CO_1} \left[ t - \frac{1}{k} (1 - \exp(-kt)) \right].
\]

At \( t \) large compared with \( 1/k \), the total CO\(_2\) evolved is

\[
Q_{CO_2}^* = M_{CO_2} \cdot s_{CO_1} \left[ t - \frac{1}{k} \right]
\]

so that the intercept of the linear part of the CO\(_2\) evolution curve on the time axis is at \( 1/k \), and hence \( k \) and \( t_4 \) of the phase can be obtained. (In Fig. 4, \( Q^* \) (d/min g\(^{-1}\)) is divided by \( s_o \) to give \(^{14}\)CO\(_2\) evolved in terms of the equivalent amount of malate taken up from the external solution.)

DISCUSSION

Compartmentization of carrot tissue

The conclusion that there are three distinguishable kinetic compartments in carrot root tissue is based on fitting three exponential components to the wash-out curve. This is justified by the comparison of the content and efflux plots of the wash-out data. It cannot be justified by an increased closeness of fit of three exponential
components to the data. A curve with six independent parameters (three intercepts and three rate constants) is almost bound to fit closely any monotonically falling and naturally variable set of points. The results of these experiments re-emphasize that curve fitting is not a sensitive test of whether a biological system properly obeys first order kinetics. A seemingly good fit to exponentials may be misleading, but the comparison of content and efflux plots provides some check on this (cf. Cram, 1973b).

The compartments may be physical pools, such as cytoplasm, vacuole or organelles, or they may be chemical pools, such as malate or CO$_2$-HCO$_3^-$. The CO$_2$–HCO$_3^-$ exchange is fast enough, even in the absence of carbonic anhydrase, not to complicate the kinetics (Kern, 1960). Since malate and CO$_2$ are interconverted in the mitochondria (Klingenberg, 1970) and in the ground cytoplasm (Ting, 1971), $^{14}$C label probably occurs in both molecules in both compartments. Malate at 35 $\mu$mol g$^{-1}$ must be partly in the vacuole since it would be at an impossibly high concentration (700 mM) if exclusively in the cytoplasm. CO$_2$–HCO$_3^-$ also probably occurs in the vacuole. $^{14}$C may also occur in compounds other than malate, particularly amino acids, but most of the label, at least after several hours of loading, is in malate. The minimum compartmentation of $^{14}$C from H$^{14}$CO$_3$ taken up by plant cells can therefore be represented as in Fig. 7.

Since in carrot the exchange of $^{14}$C can be adequately described by three exponential terms, some of the pools must either be very nearly in isotopic equilibrium with each other, or be negligible in size. There is a rapid exchange of malate between mitochondria and external medium in vitro (Hoek, Lofrumento, Meyer and Tager, 1971; Palmieri, Prezioso, Quagliariello, and Klingenberg, 1971; Coleman and Palmer, 1972). The probable rapid exchange and/or the small size of a mitochondrial compartment makes it probable that it does not contribute to the kinetics. It seems likely that CO$_2$ would exchange rapidly across mitochondrial and cytoplasmic membranes (though values of P$_{CO_2}$ do not appear to have been published). It therefore seems likely that $^{14}$C equilibrates rapidly with CO$_2$–HCO$_3^-$ throughout the cell. Thus the model that is favoured to account for the three components of
\(^{14}\)C exchange in carrot root cells is as shown in Fig. 8, the rate of turnover of CO\(_2\)-HCO\(_3^-\) being faster than cytoplasmic malate, which in turn is faster than vacuolar malate.

The presence of two malate pools in plant cells has been proposed several times and based on various types of evidence (see Introduction). The conclusion of the present paper agrees with that of Jacoby and Laties (1971), based on a comparison of root tip and highly vacuolated root cells. Lips and Beevers (1966b), have proposed that there are two compartments of malate within the cytoplasm. However, Fig. 2 of Jacoby and Laties (1971) shows that there is no need to postulate more than a single cytoplasmic and a vacuolar compartment to account for the results in Fig. 3 of Lips and Beevers (1966b).

Other results of Lips and Beevers (1966a) suggest a modification to Fig. 8. They showed that, in maize root segments, malate taken up from outside and malate synthesised from CO\(_2\) appear to be in the same pool, and that malate synthesised from external acetate is in a qualitatively distinct pool. The acetate-labelled malate is in the more rapidly exchanging pool, the cytoplasm in Fig. 8. The CO\(_2\)-labelled malate is mostly in the more slowly exchanging pool, the vacuole in Fig. 8. However, CO\(_2\) is assumed to be fixed to malate in the cytoplasm, and therefore it appears that a larger fraction of CO\(_2\)-labelled malate than of acetate-labelled malate moves to the vacuole during the labelling period. This suggests the possibility of a pathway for malate uptake similar to that for Cl\(^-\) uptake in *Nitella* and *Chara* (MacRobbie, 1969; Findlay, Hope and Walker, 1971), in which CO\(_2\) fixation enzymes are also located. Such a small cytoplasmic pool would not be apparent in the wash-out curve, and would be shown in Fig. 8 as an arrow from CO\(_2\)-HCO\(_3^-\) to vacuolar malate. This, and several other modifications, will not be discussed further, since they are almost indistinguishable kinetically. It should be emphasized, however, that rapid randomization of molecules between phases does not mean that the concentrations in them are necessarily equal or even proportional.

*The estimation of fluxes*

One can summarize the HCO\(_3^-\) and malate fluxes that can be measured as follows:

1. The plasmalemma influx of CO\(_2\)-HCO\(_3^-\)—from exchange kinetics at a quasi-steady-state.
2. The plasmalemma influx of malate from 1 mM malate solutions—from the quasi-steady influx (M\(_{\text{in}}\)). The linear time course of uptake suggests that M\(_{\text{in}}\) is rate-limited by the plasmalemma influx at and below 1 mM malate.
3. The plasmalemma efflux of malate—from chemical determinations of the malate appearing in the external solution (only possible in solutions initially containing no malate).
4. The tonoplast influx of malate accumulated from external malate solutions—
from the quasi-steady influx. The constancy of $^{14}\text{CO}_2$ evolution above 10 mM strongly suggests that the cytoplasmic specific activity then equals the external solution specific activity, so that the quasi-steady influx ($M_{m0}/s_0$) equals the tonoplast influx ($M_{m0}$).

5. The tonoplast influx of malate in $\text{HCO}_3^-$ solutions—from the influx of tracer to the vacuole ($M_{m0}$) plus an estimate of the specific activity of the malate produced by $\text{CO}_2$ fixation in the cytoplasm, $s_c$.

6. The net influx or accumulation of malate—by chemical analysis at a steady or quasi-steady state. If influxes are known under the same conditions, corresponding effluxes could be calculated.

The only problem which needs further discussion is that of estimating the specific activity of malate produced by $\text{CO}_2$ fixation in the cytoplasm, which is used in calculating the tonoplast influx of malate in $\text{HCO}_3^-$ solutions (flux 5 above). This can be done by measuring the specific activity either of malate or of $\text{CO}_2$ flowing to the external solution. The specific activity of lost malate can be measured directly, as in the results section. It then remains to show that this is the same as in the fixed $\text{CO}_2$ pool from which malate flows to the vacuole. This approach will not be considered further here. Alternatively, the specific activity of $\text{CO}_2$ flowing out of the tissue can be estimated from the rate of $\text{CO}_2$ evolution (as $\text{CO}_2$) and the rate of $^{14}\text{CO}_2$ evolution, which is obtained as the initial efflux of $^{14}\text{C}$ after transfer from a labelled to an unlabelled $\text{HCO}_3^-$ solution, when most of the $^{14}\text{C}$ is lost as $\text{CO}_2$ (e.g. Table 4 and Cram, unpublished). The initial efflux equals $I_f+I_s$, as in calculating $M_{m0}$ above. The $C$-4 of malate produced by $\text{CO}_2$ fixation in the cytoplasm will be at this specific activity. If there is no randomization of $^{14}\text{C}$ in the malate molecule, $x$ moles of $^{14}\text{CO}_2$ fixed would equal $x$ moles of malate produced. The distribution of $^{14}\text{C}$ in malate produced by $\text{CO}_2$ fixation in carrot tissue is mainly in C-4 (Splittstoesser, 1967), and may be almost exclusively in C-4 (Sutton and Osmond, 1972).

$^{14}\text{C}$ will be assumed to be exclusively in C-4 of malate for the purpose of calculation in this and a subsequent paper (Cram, 1974). If $^{14}\text{C}$ is not exclusively in C-4 of malate, $M_{m0}$ would be overestimated by these calculations. The problem of calculating $M_{m0}$ would then be more complex, involving also the specific activity of the 3-carbon compound to which $\text{CO}_2$ is fixed, and exchange with mitochondrial malate.

**Physiological conclusions**

Calculated values of the $\text{HCO}_3^-$-$\text{CO}_2$ influx across the plasmalemma and of the malate influx to the vacuole in carrot tissue are shown in Table 5. The influx of $\text{HCO}_3^-$-$\text{CO}_2$ across the plasmalemma is less than the influx of malate to the vacuole and hence cannot be a controlling step in the rate of malate production. There is a more than sufficient supply of $\text{CO}_2$ from within the tissue. On the other hand, the malate influx across the tonoplast increases with the increase in external $\text{KHCO}_3$ concentration. The increased rate of malate production at higher $\text{KHCO}_3$ concentrations is therefore probably related to the increased external K concentration, either via a K limitation on K malate transport or via a change in cytoplasmic pH accompanying K uptake (cf. Jacoby and Laties, 1971; Cram, 1974).

The influx of malate to the vacuole of barley root cells is limited by the influx of
malate across the plasmalemma at low external concentrations. At higher external concentrations the results do not show whether the tonoplast becomes the rate limiting step or not. One can conclude that the influx of malate to the vacuole does not saturate at high external malate concentrations. This is not sufficient to characterize the transport of malate, although it does distinguish it from the transport of Cl" (Cram and Laties, 1971; Cram, 1973b).

**Table 5. Calculation of the influx of malate across the tonoplast in carrot tissue water washed for 1 d**

<table>
<thead>
<tr>
<th></th>
<th>1 mM KHCO₃</th>
<th>10 mM KHCO₃</th>
</tr>
</thead>
<tbody>
<tr>
<td>$M_{in}$ (μmol g⁻¹ h⁻¹)*</td>
<td>0.042</td>
<td>0.20</td>
</tr>
<tr>
<td>qCO₂ (μmol g⁻¹ h⁻¹)†</td>
<td>5.6</td>
<td>6.4</td>
</tr>
<tr>
<td>$M_{in}$ (μmol g⁻¹ h⁻¹)*</td>
<td>0.25</td>
<td>0.59</td>
</tr>
<tr>
<td>$s_c$</td>
<td>0.25</td>
<td>0.59</td>
</tr>
<tr>
<td>$M_{in}$ ($M_{in} + M_{in}'$−μmol g⁻¹ h⁻¹)</td>
<td>0.29</td>
<td>0.79</td>
</tr>
<tr>
<td>$M_{in}$ ($M_{in}'$−μmol g⁻¹ h⁻¹)</td>
<td>1.0</td>
<td>2.4</td>
</tr>
</tbody>
</table>

* From Table 3.
† From Adams and Rowan (1972).

It was of interest to see if there were any anion exchanges involved in the movement of malate molecules similar to the Cl"-Cl" exchange found at the plasmalemma in carrot, barley and maize root tissues (Cram, 1968; Cram and Laties, 1971; Weigl, 1968), or similar to the malate-anion exchanges found in mitochondria (Klingenberg, 1970). The absence of transient or significant long-term stimulations of malate efflux by external malate, Cl", SO₄²⁻, or NO₃⁻ differs markedly from the Cl" stimulation of Cl" efflux in carrot tissue. It therefore appears that there is no specific malate-malate exchange and no non-specific malate-anion exchanges at the plasmalemma in carrot root tissue. These results show that the slight stimulation of ¹⁴C loss from roots of whole plants by NO₃⁻ (Ben Zioni, Vaadia and Lips, 1971) is probably not due to a coupled NO₃⁻-malate exchange.

**ACKNOWLEDGEMENTS**

We are grateful for a grant from the United States Atomic Energy Commission to G. G. L., and from the Australian Research Grants Commission to W. J. C, and also for a Fulbright travel grant from the United States–United Kingdom Educational Commission to W. J. C.

**LITERATURE CITED**

Cram and Laties—The Kinetics of Bicarbonate and Malate Exchange