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# Fluxes of a Nonelectrolyte and Compartmentation in Cells of Carrot Root Tissue

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## ABSTRACT

**Permeation of thiourea into cells of carrot (*Daucus carota* L.) storage root discs was found to be governed by its concentration gradient only. The analysis of the course of thiourea efflux from preloaded discs shows two distinct exponential curves, apart from an initial very fast component. Reasons are given for relating the first exponential part of the curve to efflux from the cytoplasm and the second exponential part to efflux from the vacuole.**

**The rate constant for thiourea efflux through the tonoplast is markedly temperature-dependent (activation energy 13.2 kcal mole$^{-1}$) and is lowered by 2,4-dinitrophenol and by phenyl mercuric acetate treatments. The rate constant for its efflux through the plasmalemma, on the other hand, has a low activation energy (4.2 kcal mole$^{-1}$), which is equal to that of free diffusion of thiourea in aqueous solution, and is not affected by the metabolic inhibitors. Possible reasons for the difference in the properties of the two membranes are discussed.**

Using the technique of compartmental analysis (7) several workers (3, 9, 10) have succeeded in determining the fluxes of ions separately through the plasmalemma and the tonoplast in cells of higher plants. Because of the difficulties in determining the driving forces in such a system, the location of ion pumps and measurement of the permeabilities of the two membranes were not always conclusive. However, it is clear that the two membranes differ as far as transfer processes through them are concerned. By measuring the fluxes of neutral molecules, one overcomes some of the difficulties associated with ions, since it is generally accepted that the only driving force for the movements of the former molecules is the concentration gradient.

The present paper reports on measurements of thiourea fluxes through the plasmalemma and the tonoplast and demonstrates some differences in permeability characteristics between these two membranes.

## MATERIALS AND METHODS

Discs 1 mm thick and 14 mm in diameter, cut from the xylem tissue of the storage root of the carrot (*Daucus carota* L.), were used in these experiments. After being washed in running tap water for 1 hr, the discs were transferred to aerated distilled water containing 5 mM $CaSO_4$ plus 50 mg/l streptomycin and kept at 17 C for 1 or 2 days. To preload with thiourea, discs were incubated for 36 hr in 50 mM $^{35}$S-thiourea (plus $CaSO_4$ and streptomycin). After preloading, each disc was removed from the labeled solution, blotted, and weighed (weight range 145–160 mg). The $^{35}$S-thiourea efflux was followed by transferring each disc individually, sequentially through a series of 16 glass vials containing 5 ml of unlabeled solution. The vials were held in a temperature-controlled shaking bath. The time intervals between each transfer were selected, after pilot experiments, so that each washout volume contained 5 to 8% of the total radioactivity of the disc.

Samples of 1 ml were transferred from each vial to 15 ml of scintillation liquid (containing 4 g of PPO, 200 mg of POPOP, 60 g of naphthalene, 20 ml of ethylene glycol, 100 ml of methanol, made up to 1 liter with *n*-dioxane) and were counted in a Packard liquid scintillation spectrometer. The 16th washout lasted overnight, after which the disc itself was placed in scintillation liquid. The total radioactivity in the disc at the end of the loading period was calculated from the counts in the series of 17 vials.

The procedure used for estimating the amounts of isotope in each compartment and calculating the fluxes was basically that first used on higher plant tissues by Pitman (10) and since then in several other studies (3, 9). Using the efflux data, the logarithm of the amount of isotope remaining in the tissue was plotted against time (Fig. 2). The final straight part of the graph is believed to represent loss of isotope from the slowest exchanging compartment (10). The extrapolation of this straight line to zero time gives an intercept which represents the amount of isotope initially present in this compartment. Subtraction of the amount of radioactivity in this compartment from the activity in the whole tissue should give the amount of isotope in the remaining compartments. Replotting again the logarithm of the remaining activity against time (Fig. 3), gives a curve whose final straight section is believed to represent loss of isotope from the next compartment, and the extrapolated intercept indicates its initial isotope content.

From the slope of the line for each compartment the half-time for isotope exchange ($t_{0.5}$) and the efflux rate constant ($k = 0.693/t_{0.5}$) can be determined. When influx of $^{35}$S-thiourea or $^{14}$C-mannitol was measured, discs were immersed in the labeled solutions for selected periods of time. They were then taken out, blotted, weighed, and placed in scintillation liquid for counting.

## RESULTS

**Preliminary Observations.** The course of $^{35}$S-thiourea influx into the tissue is shown in Figure 1. Discs were immersed in labeled solution, and after each time interval, six of them were

blotted and placed in the scintillation fluid for counting. Quenching corrections were made when necessary.

It can be seen that in 36 hr the isotopic content of the tissue reached a constant value. This value was approximately 95% of the activity of the medium when 1 g tissue fresh weight was equated with 1 ml of medium. The course of influx was investigated from different thiourea concentrations in the bathing solution. Identical curves were obtained over the range between 1 and 100 mM when the ratio internal concentration/external concentration was plotted against time.

In order to test to what extent the radioactivity of the tissue represented its thiourea content, discs were transferred,

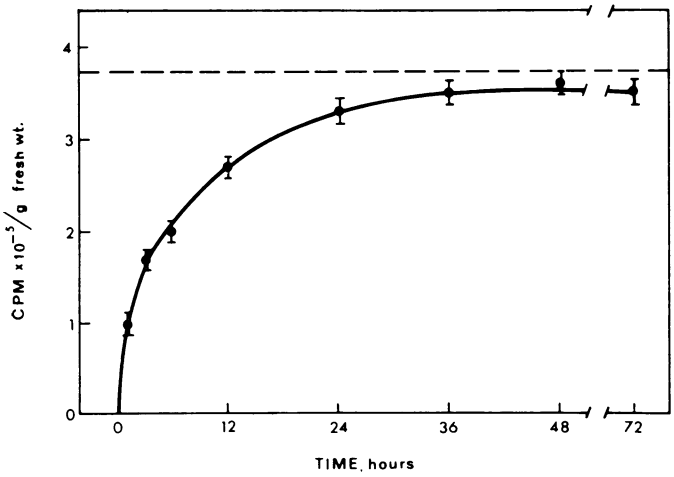


FIG. 1. Course of $^{35}$S-thiourea influx into discs of carrot tissue. Temperature was 17 C. Each point represents the mean of six determinations. Vertical bars indicate ± SE. The dashed line indicates the activity, cpm/ml, of the external solution.

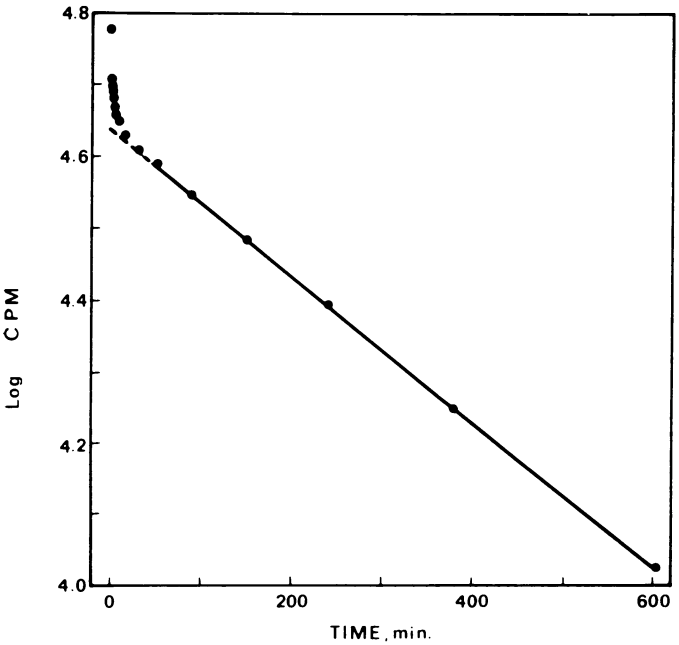


FIG. 2. Total activity of $^{35}$S-thiourea in a disc of tissue plotted on a logarithmic scale, as a function of time, during the course of efflux into non-radioactive thiourea solution. Each point represents the mean of 10 determinations. The broken line indicates extrapolation of the activity in the slowest exchanging compartment to zero time. Temperature was 17 C.

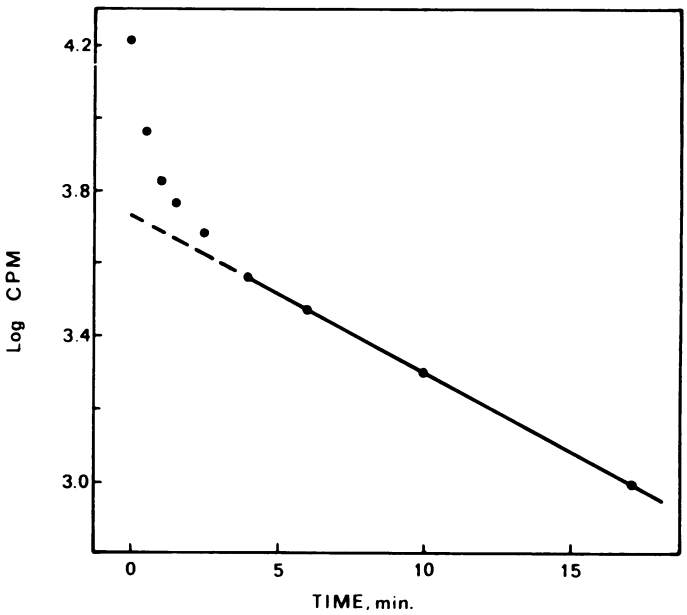


FIG. 3. Activity of $^{35}$S-thiourea in the tissue, after subtracting the activity in the slowest exchanging compartment, as a function of time, during the course of efflux into nonradioactive thiourea solution. The broken line indicates extrapolation of the activity in the next exchanging compartment to zero time. Temperature was 17 C.

Table I. *Efflux of $^{35}$S-Thiourea from Discs of Carrot Root Tissue*

Half-times and rate constants for each exchanging compartment together with the initial isotope content in each compartment are shown.

| Exchanging Compartment | Half-time | Rate Constant | Initial Content |
|---|---|---|---|
| | *min* | *min*$^{-1}$ × *10*$^{-3}$ | *cpm* × *10*$^{-3}$ |
| Slowest | 295 | 2.4 | 43.8 |
| Intermediate | 7 | 99 | 5.4 |
| Fastest | <1 | | 10.8 |

after 48 hr of influx, into a small volume of hot water. When most of the radioactivity had diffused into the liquid, the latter was analyzed by paper chromatography (running solvent *n*-butyl alcohol-acetic acid-water, 2:1:1 v/v). A fresh solution of $^{35}$S-thiourea was used as a reference. In both cases, the radioactivity was found in only one spot of identical $R_F$ (0.6).

**Compartmentation.** Figure 2 represents data from a typical efflux experiment. The compartmental analysis of the course of efflux (Figs. 2 and 3) distinguishes between an initial rapid loss and two distinct subsequent exponential loss phases. Table I summarizes the efflux characteristics calculated from these results.

Assuming that the thiourea is spread homogeneously through the whole volume of the tissue (an assumption which is justified in view of the fact that the steady state concentration of the isotope in the tissue equals that of the medium), the amount of radioactivity in each compartment indicates its relative volume, namely, 73, 9, and 18% for the slowest, intermediate, and fastest exchanging compartments, respectively. No value is presented for the rate constant of the fastest component because the loss was not exponential. It can only be said that the half-time for this exchange was less than 1 min. If the slowest and intermediate exchanging compartments are regarded as vacuole and cytoplasm respectively (10), the table

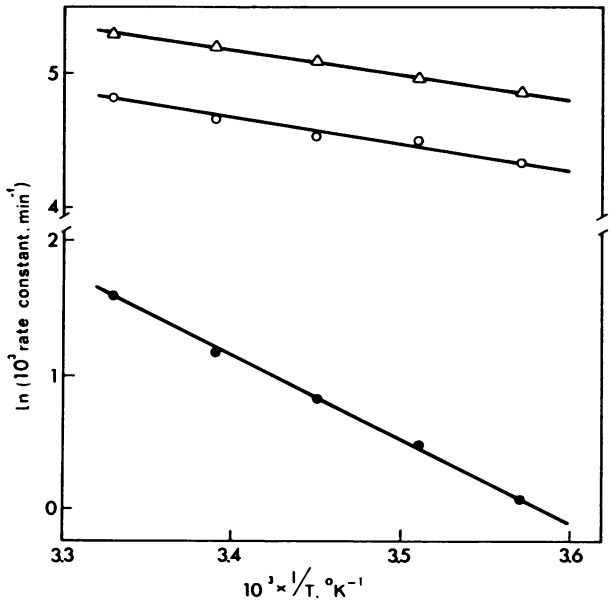


FIG. 4. Relationship between the natural logarithm of the efflux rate constant of $^{35}$S-thiourea from discs of carrot tissue and the reciprocal of the absolute temperature. Slowest compartment (●); intermediate compartment (○); killed discs (△). Each point represents the mean of 10 determinations.

shows that the rate constant for thiourea flux through the plasmalemma was about 40 times that through the tonoplast.

**Temperature Dependence.** Similar washout experiments were carried out at different temperatures in the range of 7 to 27 C and were analyzed in the manner described earlier. The discs were always loaded at 17 C, but during the final 2 hr of immersion in the radioactive solution, they were gradually brought to the desired temperature. The different temperatures did not alter the initial radioactive content of each compartment but did affect the efflux rate constants.

In Figure 4 these results are presented in a form that allows calculation of the activation energy of the flux process for each compartment from the slope of the line (according to the Arrhenius equation, ref. 5). There is a very pronounced difference between the slopes for the slowest and for the intermediate exchanging compartments. The activation energy for the $^{35}$S-thiourea fluxes from these two compartments were found to be 13.2 and 4.2 kcal $mole^{-1}$, respectively. Figure 4 also indicates the activation energy of $^{35}$S-thiourea efflux from 2 mm discs of dead carrot tissue. The discs were killed by exposure to chloroform vapor for 30 min, and then were immersed for 1 hr in the standard radioactive solution for equilibration. The washout procedure was carried out as usual, the time intervals for transfer of the discs being adjusted to the efflux rate. It can be seen that the activation energy of $^{35}$S-thiourea efflux from the dead tissue was comparable to that from the intermediate compartment of the live cells. The semilogarithmic plot of the course of efflux from the dead tissue showed an almost straight line for exit of the entire radioactive content.

**Effects of Metabolic Inhibitors.** The half-time for $^{35}$S-thiourea efflux from discs of tissue was increased by DNP[1] and by PMA (Table II). The only effect of these two substances was on the efflux from the slowest compartment. The flux from the intermediate compartment was not significantly affected. No difference in the relative volume of compartments under the two treatments was detected. Efflux measurements from killed tissue showed no effect either of DNP or of PMA.

**Free Space Determinations.** The low temperature dependence and lack of effect of DNP and PMA on the thiourea flux, apparently through the plasmalemma, emphasized the need to test the validity of the compartmental analysis interpretation, which relates the medium exchange compartment to the cytoplasm of the cells. It was important to determine whether the intermediate exchanging compartment is or is not a part of the free space of the tissue.

Figure 5 summarizes an experiment performed to measure the mannitol free space of the tissue. Discs were immersed in a solution of 50 mM mannitol, labeled with $^{14}$C held at 4 C to minimize metabolic mannitol absorption. At selected time intervals, individual discs were removed from the radioactive solution, blotted, weighed, and placed in scintillation fluid for counting. Quenching corrections were made when necessary. Figure 5 shows that the amount of $^{14}$C-mannitol in the disc very quickly reached a state of equilibrium. Calculation indicates that at this point 14% of the tissue's volume was in diffusional equilibrium with the external solution.

Parallel with the immersion of discs in mannitol, 10 discs were subjected to compartmental analysis for $^{35}$S-thiourea

Table II. *Effect of DNP and of PMA on the Half-time for $^{35}$S-Thiourea Efflux from the Exchanging Compartments of Carrot Root Tissue*

DNP or PMA were added to the preloading solution 3 hr prior to the start of efflux. Temperature was 27 C. The half-times are given as means ± SE of 10 determinations.

| Treatment | Half-time of Efflux | |
|---|---|---|
| | Slowest compartment | Intermediate compartment |
| | *min* | |
| DNP, 0.1 mM, pH 6.0 | 220 ± 12 | 6.2 ± 0.7 |
| None | 155 ± 15 | 5.6 ± 0.6 |
| PMA, 10 μM | 238 ± 16 | 5.9 ± 0.4 |
| None | 134 ± 10 | 6.1 ± 0.7 |

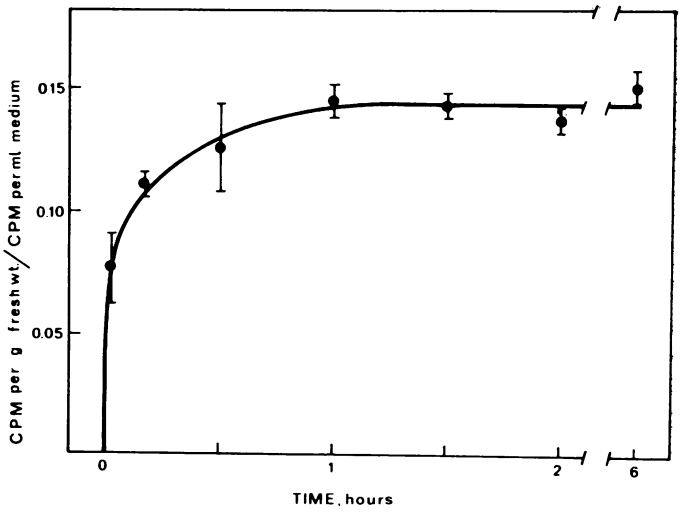


FIG. 5. Course of $^{14}$C-mannitol influx into discs of carrot root tissue. Temperature was 4 C. Each point represents the mean of five determinations. Vertical bars indicate ± SE.

[1] Abbreviations: DNP: 2,4-dinitrophenol; PMA: phenyl mercuric acetate.

efflux at the same temperature. The initial activities for the slowest, intermediate, and fastest compartments were found to be (percentage of total, mean ± SE) 75.1 ± 2.4, 8.3 ± 0.9 and 16.6 ± 2.0, respectively. It is obvious that the intermediate exchanging compartment was not included in the mannitol free space of the tissue.

## DISCUSSION

The use of labeled organic molecules for flux determinations may involve a source of error due to their metabolism, which has sometimes been overlooked. Up to 50% of the activity of cells immersed in $^{14}$C-urea for 30 min, may be found in compounds other than urea (12). It is especially important to take this point into consideration when treatments with radioactive compounds are long lasting. In our search for a labeled nonelectrolyte whose movement would be governed by concentration gradient only and whose metabolism would be as low as possible, $^{35}$S-thiourea was found to be extremely suitable.

The fact that identical courses of isotope influx were obtained from media of widely differing thiourea concentrations (1–100 mM), indicates that no saturating process was involved in uptake, and that no other driving forces except concentration gradients affect its fluxes. The $^{35}$S-thiourea molecule was found to be stable for at least 48 hr inside living carrot root cells. The fact that the activity in the tissue was equal to that in the external solution, indicates that no measurable amounts of thiourea were adsorbed by any part of the cells and it can reasonably be concluded that the thiourea was homogeneously spread through all the components of the tissue.

The analysis of the course of $^{35}$S-thiourea flux from the carrot discs reveals two distinct exponential curves apart from an initial very quick component which is not exponential. This picture is comparable to that observed for ion fluxes from tissue (3, 9). The accepted interpretation of such an analysis leads to the conclusion that the final exponential part of the curve indicates the flux from the vacuole, the intermediate exponential represents flux from the cytoplasm, while the initial part represents the flux from the free space of the tissue including the cell wall, intercellular spaces, and surface film of liquid. Accordingly, the rate constants for the two exponential parts of the curve relate to efflux through the tonoplast and the plasmalemma, respectively.

Our finding that the rate constant for flux of a nonelectrolyte from the cytoplasm far exceeds that for flux from the vacuole accords well with the observations for ion fluxes of higher plant cells (3, 9). In the alga *Nitella translucens*, the permeability of the plasmalemma to urea has been shown to be considerably higher than that of the tonoplast (4).

A striking finding presented in this work is that some characteristics of the plasmalemma differ from those of the tonoplast, as far as thiourea passage through them is concerned. The difference is expressed in the low activation energy for thiourea flux through the plasmalemma and the lack of effect of DNP and PMA treatments on plasmalemma fluxes.

The fact that the activation energy for thiourea flux through the plasmalemma is similar to that for its diffusion through dead tissue which in turn is comparable to its free diffusion in water; and further, that in contrast to the case of the tonoplast, no effect of an uncoupler and of a sulfhydryl inhibitor could be detected, raises the question whether the interpretation of the compartmental analysis is correct.

An alternative way of interpreting the compartmental analysis is to relate the final exponential part of the curve to the flux from the whole protoplast (=cytoplasm + vacuole), *i.e.* through the plasmalemma. If the permeability of the tonoplast is relatively high, no discrimination between the inner tonoplast and the other plasmalemma may be possible (11). The intermediate exponential part of the curve may then be looked upon as efflux from a part of the free space of the tissue, for example, the cell wall.

Strong evidence against the latter interpretation is the fact that the volume of the free space for mannitol is comparable to the initial quickly exchanging volume only. It is well estiblished that mannitol, as an osmotic agent, occupies all the extramembranal volume of the cells, and there is no reason to believe that its concentration in this volume, at equilibrium, should be lower than in the external solution. An additional indication that the supposed cytoplasm compartment is not a part of the free space, is its comparatively long half-time for thiourea exchange. The half-time for diffusion from a sheet can be expressed by the equation (8):

$$t_{0.5} = \frac{0.198\left(\frac{d}{2}\right)^2}{D}$$

where $D$ is the diffusion coefficient and $d$ is the thickness of the sheet. Substituting $9 \times 10^{-4}$ cm$^2$ sec$^{-1}$—the diffusion coefficient for thiourea in water (1)—for $D$ and 0.1 cm for $d$, $t_{0.5}$ becomes 50 sec.

The observed half-time of 420 sec is far too long to be accounted for by the possible additional barrier to diffusion provided by the microfibrils of the cell wall. Moreover, $d$ should practically, in fact, be equivalent to the thickness of the individual cell wall and then the calculated halftime would clearly fall even further short of the measured one.

In most of the work on permeability of plant cells (2), the entire protoplasm with its two boundary membranes has been treated as one complex membrane. It may be that the characteristic permeability properties attributed to the cytoplasmatic membranes as a whole are mainly properties of the tonoplast. In their study of nonelectrolyte permeability in different animal epithelia, Hingson and Diamond (6) showed that a high energy of activation indicates permeation through membrane lipids, while a low one suggests penetration through water-filled channels (= pores) in the membrane.

One way of interpreting the results presented in this paper would be to suggest that the passage of thiourea through the plasmalemma, in contrast to its passage through the tonoplast, takes place mainly through hydrophylic pores. It is well established that metabolic energy is required for the maintenance of membrane integrity and its permeability properties. It may be that the effect of the metabolic inhibitors lies in causing some lipids to peak more closely, and is virtually similar for the two membranes. In the case of the plasmalemma, however, permeability to thiourea is not altered because of the predominance of the pathway bypassing membrane lipids.

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