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COMPARTMENTAL ANALYSIS OF SULPHATE TRANSPORT IN *LEMNA MINOR* L., TAKING PLANT GROWTH AND SULPHATE METABOLIZATION INTO CONSIDERATION

A. THOIRON, B. THOIRON, M. DEMARTY and M. THELLIER

Faculté des Sciences et Techniques (Echanges cellulaires), L.A. C.N.R.S. 203, C.R.B.P.C., Université de Rouen, 76130 Mont-Saint-Aignan (France)

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The compartmental analysis of sulphate transport in cells of *Lemna* plants has been performed, taking into account the growth of the samples and the metabolization of sulphate into organic thiocompounds during the course of the experiment. The results obtained from efflux and influx experiments are fully consistent with one another. Both unidirectional fluxes between the external medium and the cell wall are very large (order of magnitude of 1 μmol/h per g fresh weight of plants). All the other unidirectional fluxes, including the flux of sulphate metabolization, are much smaller (from about 10 to 60 nmol/h per g). Over 70% of the total sulphur of the plant corresponds to that incorporated into organic thio compounds, and over 25% to free sulphate in the vacuola. The pool of free sulphate in the cytoplasm is only about 1% of the total sulphur, and the sulphate content of the cell wall (free spaces) is also about 1%. Two remarks of general relevance have been made concerning the influx curves. First, these curves exhibit a long (several hours), quasi-stationary phase after the first few minutes of absorption, though the slope of this straight line does not correspond to the unidirectional flux of sulphate entry through the plasmalemma (from cell wall to cytoplasm). Second, the *Lemna* plants seem to be sensitive to the effect of 'gas shock'.

Introduction

Compartmental analysis, which we have applied here to the study of sulphate transport by *Lemna minor* L., is a method allowing one to obtain cellular transport parameters from overall kinetic data [1]. This method is used conventionally at 'flux equilibrium', i.e., under conditions where influx (or efflux) is measured, by the use of convenient radioactive tracers, on plants put in an absorption (or desorption) medium, the chemical composition of which is identical to that of the medium in which the plants have been pre-equilibrated. Under such conditions it is customary to neglect sample growth. This simplification was, however, clearly untenable here, both because the growth of the *Lemna* plants is quite rapid under our usual experimental conditions, and because growth is accompanied by an almost irreversible metabolization of the absorbed SO_4^{2-} into organic thio compounds. It was thus necessary to build a theoretical model of compartmentation taking growth and metabolism into consideration.

Two different types of measurement, efflux and influx, were performed simultaneously. The cellular parameters of sulphate transport were determined from the kinetic data of efflux. Then a theoretical curve of influx of radioactivity was calculated from these parameters; and the internal consistency of our approach was tested by comparing this theoretical curve to the actual experimental points of the influx measurements.

Abbreviations: PPO, 2,5-diphenyloxazole; dimethyl POPOP, 1,4-bis[2-(4-methyl-5-phenyloxazolyl)]benzene.

Theoretical Section

Statement of the problem

As usual, the efflux experiments were performed on plants preloaded with a radioactive tracer of the studied substrate (here $^{35}SO_4^{2-}$), and transferred, at zero time of the experiment, to a medium of identical composition but unlabelled, in which they progressively released their radioactivity. Conversely, the influx experiments were performed with nonradioactive plants which were transferred to a radioactively labelled medium, in which they progressively absorbed radioactivity. The exit of radioactivity, in the first case, or its entry, in the second, easily led to the determination of kinetic parameters of the transport. However, the difficulty arose in calculating actual cellular parameters of sulphate transport from these kinetic parameters. This was overcome by assuming a compartmental model of the system, allowing the derivation of a set of theoretical equations relating the cellular to the kinetic parameters.

The compartmental model and significance of symbols

The *Lemna* plant is composed of a basal and an apical lamina of cells, interrelated by cellular trabeculae. All these cells look quite similar to one another [2], which makes it reasonable to use a serial model of compartmentation, as has already been proposed for reasonably homogeneous tissues [3]. By analogy with monocellular systems [4], three main compartments are generally considered: cell wall, cytoplasm and vacuola. Here, we have kept these three compartments for the inorganic sulfate, but we have added a fourth compartment corresponding to the metabolized sulphate. Fig. 1 gives the schematic representation of this compartmentation model.

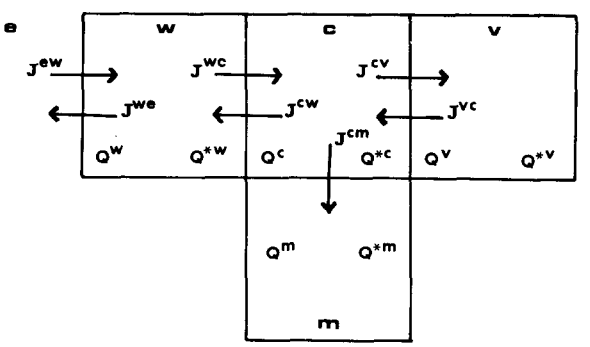

Fig. 1. Compartmental model for transport of sulphate by *Lemna minor*. J^{ij} represents densities of unidirectional fluxes of sulphate from any compartment i to any compartment j. Q^{*i} and Q^{i} represent the radioactivity and sulphur content, respectively, of any compartment i. Superscripts e, w, c and v denote inorganic sulphate in the exterior, the cell wall, the cytoplasm and the vacuola, respectively; superscript m denotes the metabolized (organic) forms of sulphur.

The meaning of the cellular parameters is as follows for the various compartments: Φ represents fluxes (nmol/h); J densities of fluxes (nmol/h per cm^2); Q contents in sulphur (nmol) and Q^* contents in radioactivity (cpm). The specific activities are designated S (cpm/nmol). The surface areas of the borders between the various neighbouring compartments are termed s, and it is assumed that the concept of area can be applied, at least formally, to the compartment of metabolized sulphate. Superscripts t, e, w, c, v and m stand for total, external, cell wall, cytoplasmic, vacuolar and metabolized, respectively. Subscripts 0, τ and ∞ correspond to zero time, to any time τ and to an interval of time much larger than the characteristic time of the system considered.

Derivation of the cellular parameters from the kinetic parameters obtained in efflux measurements

Basic assumptions. A certain number of simplifying assumptions can be made, some of which are justified in the Experimental Section, while others are fulfilled by the chosen experimental conditions.

a1. The specific radioactivity of the external medium is supposed to remain negligible throughout the course of an efflux experiment:

$$Q^{*e}/Q^{e} \doteq 0 \qquad (1)$$

a2. We assume that equilibration of the cell wall with the external medium is established after an interval of time, τ_1, sufficiently small that the radioactivity content of any other compartment is not significantly modified during this interval of time. Hence:

$$Q^{*w}_{\tau_1}/Q^{w}_{\tau_1} \doteq 0\,, \quad \tau \gtrsim \tau_1 \qquad (2)$$

a3. At zero time, the specific radioactivity of all

the cellular compartments is considered to be equal to the specific radioactivity, $\bar{S}$, of the pre-equilibration medium:

$$Q_0^{*w}/Q_0^w = Q_0^{*c}/Q_0^c = Q_0^{*v}/Q_0^v = Q_0^{*m}/Q_0^m = \bar{S} \quad (3)$$

a4. The *Lemna* are assumed to be under stationary conditions of growth, i.e., the total number of cells increases without the mean size of the cells significantly changing. Hence, all the extensive parameters, x, of the *Lemna* can be assumed to depend on time, τ, according to the same growth law which can be approximated [5,6] by:

$$x = x_0 e^{\gamma\tau} \quad (4)$$

where γ is the growth coefficient of the *Lemna*.

a5. The flux densities are supposed to be constant with respect to time:

$$J(\tau) \equiv J \quad (5)$$

a6. The metabolization of sulfate into organic thio compounds is taken as irreversible:

$$J^{mc} \equiv 0 \quad (6)$$

Equations of the system. According to assumption a2, the kinetics of compartment w can be described by the differential equation:

$$\frac{dQ^{*w}}{d\tau} = -\frac{J^{we}s^{we}}{Q^w} Q^{*w} \quad (7)$$

which holds for time $\tau < \tau_1$. Then, for $\tau \geqslant \tau_1$, compartments c, v and m are described by the differential equations:

$$\frac{dQ^{*c}}{d\tau} = -\frac{J^{cw}s^{cw} + J^{cv}s^{cv} + J^{cm}s^{cm}}{Q^c} Q^{*c} + \frac{J^{vc}s^{cv}}{Q^v} Q^{*v} \quad (8)$$

$$\frac{dQ^{*v}}{d\tau} = \frac{J^{cv}s^{cv}}{Q^c} Q^{*c} - \frac{J^{vc}s^{cv}}{Q^v} Q^{*v} \quad (9)$$

$$\frac{dQ^{*m}}{d\tau} = \frac{J^{cm}s^{cm}}{Q^c} Q^{*c} \quad (10)$$

According to assumption a4, any term s/Q is written:

$$s/Q = s_0 e^{\gamma\tau}/Q_0 e^{\gamma\tau} = s_0/Q_0 \quad (11)$$

and it is thus constant with respect to time. Under such conditions, it is well known that the solution of the above differential equations is given by combinations of exponential, and possibly of constant terms, which describe the kinetic behaviour of the system:

$$Q^{*1} = Q_0^{*1} e^{-\lambda_1\tau}, \quad \tau < \tau_1 \quad (12)$$

$$Q^{*t} - Q^{*1} = Q_0^{*2} e^{-\lambda_2\tau} + Q_0^{*3} e^{-\lambda_3\tau} + Q_0^{*4}, \quad \tau \geqslant \tau_1 \quad (13)$$

where Q_0^* and λ are the kinetic parameters and superscripts 1–4 refer to the various terms which can be obtained by separation of the overall experimental efflux curves into first-order or constant expressions.

Equations characterizing the limits of the system can be obtained by considering that there is a non-zero net influx of sulphate, which corresponds to growth. For instance, for compartment m, Eqn. 4 is written:

$$Q^m = Q_0^m e^{\gamma\tau} \quad \text{and} \quad s^m = s_0^m e^{\gamma\tau} \quad (14)$$

Hence:

$$J^{cm}s^{cm} = J^{cm}s_0^{cm} e^{\gamma\tau} = \frac{dQ^m}{d\tau} = \gamma Q_0^m e^{\gamma\tau} \quad (15)$$

or:

$$J^{cm}s_0^{cm} = \gamma Q_0^m \quad (16)$$

Similar equations can be obtained in the same manner for compartments w, c and v:

$$J^{ew}s_0^{ew} + J^{cw}s_0^{cw} - J^{wc}s_0^{cw} - J^{we}s_0^{ew} = \gamma Q_0^w \quad (17)$$

$$J^{wc}s_0^{wc} + J^{vc}s_0^{vc} - J^{cw}s_0^{wc} - J^{cv}s_0^{vc} - J^{cm}s_0^{cm} = \gamma Q_0^c \quad (18)$$

$$J^{cv}s_0^{cv} - J^{vc}s_0^{cv} = \gamma Q_0^v \quad (19)$$

Introducing Eqns. 16–19 in the course of the integration of Eqns. 7–10 giving Eqns. 12 and 13, eventually leads to the expression of the cellular

parameters as functions of the kinetic parameters:

$$Q_0^w = Q_0^{*1}/\bar{S} \tag{20}$$

$$\Phi_0^{we} = J^{we} s_0^{we} = \lambda_1 Q_0^w \tag{21}$$

$$\Phi_0^{cw} = (\lambda_2 Q_0^{*2} + \lambda_3 Q_0^{*3})/\bar{S} \tag{22}$$

$$\Phi_0^{cm} = J^{cm} s_0^{cm} = \gamma Q_0^m \tag{23}$$

where $\bar{S}$ is the specific activity of the growth medium and Q_0^m can be determined experimentally:

$$Q_0^c = \frac{\Phi_0^{cw} + \Phi_0^{cm} + \gamma(Q_0^t - Q_0^w - Q_0^m)}{\lambda_2 + \lambda_3 + \gamma - \dfrac{\lambda_2\lambda_3}{\Phi_0^{cw} + \Phi_0^{cm}}(Q_0^t - Q_0^w - Q_0^m)} \tag{24}$$

$$Q_0^v = Q_0^t - Q_0^w - Q_0^m - Q_0^c \tag{25}$$

$$\Phi_0^{vc} = J^{vc} s_0^{cv} = \frac{\lambda_2\lambda_3 Q_0^c Q_0^v}{\Phi_0^{cw} + \Phi_0^{cm}} \tag{26}$$

$$\Phi_0^{wc} = J^{wc} s_0^{wc} = \gamma(Q_0^c + Q_0^v + Q_0^m) + \Phi_0^{cw} \tag{27}$$

$$\Phi_0^{cv} = J^{cv} s_0^{cv} = \gamma Q_0^v + \Phi_0^{vc} \tag{28}$$

$$\Phi_0^{ew} = \Phi_0^{wc} + \Phi_0^{we} - \Phi_0^{cw} + \gamma Q_0^w \tag{29}$$

Moreover, from Eqns. 12 and 13, it is clear that Q_0^{*4} corresponds to the quantity of radioactivity remaining in the samples in the form of metabolized sulphur when time τ is large enough to make exponential terms $e^{-\lambda_1\tau}$, $e^{-\lambda_2\tau}$ and $e^{-\lambda_3\tau}$ not significantly different from zero. Hence:

$$Q_0^{*4} = Q_\infty^{*m} \tag{30}$$

Remarks about the effect of the growth coefficient γ. Numerical calculation has shown us that a modification of γ radically affected the value of Φ_0^{cm}, less significantly those of Φ_0^{wc}, Φ_0^{ew} and Φ_0^{cv}, and practically not at all, those of the other cellular parameters. Therefore, even when the growth coefficient γ cannot be determined very accurately, the estimation of the values of most of the cellular parameters remains approximately correct. Moreover, it is easy to see that, if the experiments were performed on plants which had ceased to grow, Eqns. 20–29 would then be simplified to a set of equations valid for any value of time τ:

$$Q^w = Q_0^{*1}/\bar{S} \tag{31}$$

$$\Phi^{we} = \Phi^{ew} = \lambda_1 Q^w \tag{32}$$

$$\Phi^{cw} = \Phi^{wc} = (\lambda_2 Q_0^{*2} + \lambda_3 Q_0^{*3})/\bar{S} \tag{33}$$

$$\Phi^{cm} = 0 \tag{34}$$

$$Q^c = \frac{\Phi^{cw}}{\lambda_2 + \lambda_3 - \dfrac{\lambda_2\lambda_3}{\Phi_0^{cw}}(Q^t - Q^w - Q^m)} \tag{35}$$

$$Q^v = Q^t - Q^w - Q^m - Q^c \tag{36}$$

$$\Phi^{cv} = \Phi^{vc} = \frac{\lambda_2\lambda_3 Q^c Q^v}{\Phi^{cw}} \tag{37}$$

which correspond to the conventional equations obtained in the cases when growth is negligible.

Elaboration of the theoretical equations for the influx measurements

Basic assumptions. We are interested now in the absorption of radioactively labelled sulphate by plants which have not been preloaded with radioactivity, but which are, in all other respects, identical to the plants considered for the efflux experiments. The general problem would be very cumbersome. We therefore limit ourselves to time values that are (i) larger than τ_1, which means that the specific activity of the cell wall is identical to that in the external medium (assumption a7):

$$S^w = S^e \tag{38}$$

but, (ii) still sufficiently small to allow the assumption that the specific activity of the vacuola has remained negligible (assumption a8):

$$S^v \doteq 0 \tag{39}$$

Derivation of the equations. The variation of the cytoplasmic radioactivity is written:

$$\frac{dQ^{*c}}{d\tau} = S^w\Phi^{wc} + S^v\Phi^{vc} - S^c(\Phi^{cw} + \Phi^{cv} + \Phi^{cm}) \tag{40}$$

Hence, according to Eqns. 38 and 39, when assumptions a7 and a8 are satisfied, one can write:

$$\frac{dQ^{*c}}{d\tau} = S^e\Phi^{wc} - S^c(\Phi^{cw} + \Phi^{cv} + \Phi^{cm}) \tag{41}$$

or:

$$\frac{dQ^{*c}}{d\tau} + \frac{\Phi^{cw} + \Phi^{cv} + \Phi^{cm}}{Q^c} Q^{*c} = S^e \Phi^{wc} \tag{42}$$

Using assumption a4 (Eq. 4), Eqn. 42 is written:

$$\frac{dQ^{*c}}{d\tau} + \frac{\Phi_0^{cw} + \Phi_0^{cv} + \Phi_0^{cm}}{Q_0^c} Q^{*c} = S^e \Phi_0^{wc} e^{\gamma\tau} \tag{43}$$

the integral of which is:

$$Q^{*c} = \frac{S^e Q_0^c \Phi_0^{wc}}{\gamma Q_0^c + \Phi_0^{cw} + \Phi_0^{cv} + \Phi_0^{cm}} \times \left(e^{\gamma\tau} - \exp - \frac{\Phi_0^{cw} + \Phi_0^{cv} + \Phi_0^{cm}}{Q_0^c} \tau \right) \tag{44}$$

The specific radioactivity of free sulphate in the cytoplasm is then expressed as:

$$S^c = \frac{Q^{*c}}{Q^c} = \frac{Q^{*c}}{Q_0^c e^{\gamma\tau}} = \frac{S^e \Phi_0^{wc}}{\gamma Q_0^c + \Phi_0^{cw} + \Phi_0^{cv} + \Phi_0^{cm}} \times \left\{ 1 - \exp - \left(\frac{\Phi_0^{cw} + \Phi_0^{cv} + \Phi_0^{cm}}{Q_0^c} + \gamma \right) \tau \right\} \tag{45}$$

Clearly, as Eqn. 45 has been derived under conditions satisfying assumption a7, this equation is meaningful only for those values of time, τ, greater than τ_1. The quantity of radioactivity gained by the cells, between time τ_1 and any further arbitrary time τ_2 satisfying assumption a8, is written:

$$Q^*_{\tau_1\tau_2} = \int_{\tau_1}^{\tau_2} (S^w \Phi^{wc} - S^c \Phi^{cw})\, d\tau \tag{46}$$

or:

$$Q^*_{\tau_1\tau_2} = \int_{\tau_1}^{\tau_2} \{(S^e \Phi_0^{wc} - S^e\beta)\, e^{\gamma\tau} + S^e\beta\, e^{-\alpha\tau}\}\, d\tau \tag{47}$$

with:

$$\beta = \frac{\Phi_0^{wc}\Phi_0^{cw}}{\gamma Q_0^c + \Phi_0^{cw} + \Phi_0^{cv} + \Phi_0^{cm}} \tag{48}$$

$$\alpha = \frac{\Phi_0^{cw} + \Phi_0^{cv} + \Phi_0^{cm}}{Q_0^c} \tag{49}$$

Eqn. 47 is integrated into:

$$Q^*_{\tau_1\tau_2} = \frac{S^e(\Phi_0^{wc} - \beta)}{\gamma} (e^{\gamma\tau_2} - e^{\gamma\tau_1}) - \frac{S^e\beta}{\alpha} (e^{-\alpha\tau_2} - e^{-\alpha\tau_1}) \tag{50}$$

If the quantity of radioactivity absorbed by the plants between time zero and time τ_1, $Q^*_{\tau 1}$, is determined experimentally, the total quantity of radioactivity, $Q^*_{\tau 2}$, absorbed by the plants from zero time to any time τ_2 satisfying assumption a8, is written:

$$Q^*_{\tau 2} = Q^*_{\tau 1} + Q^*_{\tau 1 \tau 2} \tag{51}$$

Therefore, the internal consistency of our approach can be tested by comparing the values of $Q^*_{\tau 2}$, calculated following Eqn. 51 with the aid of the parameters obtained from the efflux experiments, to the values of $Q^*_{\tau 2}$ experimentally determined in the influx experiments.

Remarks. According to Eqns. 48 and 49, Eqn. 45 can be written:

$$S^c = \frac{S^e\beta}{\Phi_0^{cw}} \{1 - e^{-(\alpha+\gamma)\tau}\} \tag{52}$$

Hence, S^c, the specific radioacity of the cytoplasm, will become quasi-constant when time, τ, is large enough for the condition:

$$\tau >> \frac{1}{\alpha + \gamma} \tag{53}$$

to be satisfied.

Moreover, Eqn. 50 can be written in simplified forms for the time values small enough to allow the exponential terms to be developed to the first order. Remembering that τ_1 is smaller than τ_2, one thus obtains for the three most interesting cases:

$$Q^*_{\tau_1\tau_2} \doteq S^e(\Phi_0^{wc} - \beta)(\tau_2 - \tau_1) - \frac{S^e\beta}{\alpha}(e^{-\alpha\tau_2} - e^{-\alpha\tau_1}), \quad \tau_2 << \frac{1}{\gamma} \tag{54}$$

$$Q^*_{\tau_1\tau_2} \doteq \frac{S^e(\Phi_0^{wc} - \beta)}{\gamma} (e^{\gamma\tau_2} - e^{\gamma\tau_1})$$

$$+S^e\beta(\tau_2-\tau_1)\,, \quad \tau_2 \ll \frac{1}{\alpha} \tag{55}$$

$$Q^*_{\tau_1\tau_2} \doteq S^e\Phi_0^{wc}(\tau_2-\tau_1)\,, \quad \tau_2 \ll \frac{1}{\alpha}, \frac{1}{\gamma} \tag{56}$$

Materials and Methods

Plants

The experiments were performed on *Lemna minor* L. grown under aseptic conditions [7], at constant temperature (22°C), under continuous light (4000 lx), in flasks containing 500 ml of solution. The composition of the nutrient solution is given in Table I. The *Lemna* plants were maintained in continuous, quasi-exponential [5,6] growth, by transferring every month two or three plants from their previous culture flask into a freshly prepared, sterilized new medium.

Experimental procedure

For each experiment, the efflux and influx experiments were run in parallel on *Lemna* batches grown simultaneously and under conditions as comparable as possible. During the month before the experiment, the plants were grown in the nutrient medium, ^{35}S-labelled in the case of the plants destined for the efflux measurements, and unlabelled in the case of those to be used for the influx measurements. The radioactive sulfate (SJS1) was furnished by the Radiochemical Center, Amersham. At the time of the experiment, the *Lemna* plants were separated from the growth medium by filtration on a fritted glass, then they were rapidly rinsed with distilled water and blotted on filter paper. They were transferred to the experimental medium, identical in composition to the growth medium, but ^{35}S-labelled in the case of the plants destined for the influx measurements, and unlabelled for those to be used for the efflux measurements. The size of the experimental medium (2 L) was large with respect to the mass of *Lemna* used in the experiment (approx. 1 g fresh weight). This permitted us to assume that external specific radioactivity remained constant for the influx measurements, and negligible (with respect to the internal radioactivity) for the efflux measurements, throughout the course of the experiment. Aliquots of the *Lemna* plants (for the influx measurements), and of the external medium (for the efflux measurements), were sampled at increasing intervals of time, and their radioactivity was measured by liquid scintillation spectrometry (Intertechnique SL 40).

The scintillation mixture was Triton X-100 (1 vol.), toluene (2 vol.), PPO (5 g/l), dimethyl POPOP (300 mg/l). The liquid samples were simply mixed into the scintillation solution. We were not able to solubilize the *Lemna* samples using the commercial solubilizing mixtures. We thus proceeded as follows. The *Lemna* samples (about 100 mg fresh weight each) were deposited in the scintillation flasks, dehydrated for 1 h at 100°C, and incubated for 1 h at 80°C in 1 ml of 32.5% HNO_3 (Merck Suprapur). After neutralization with 1.3 ml of 4 M NaOH, 10.5 ml of the scintillation mixture were added. The radioactivity measurements were performed, using the conventional methods of quenching correction.

The masses of the different forms of sulphur in the plants were determined from the measurement of the radioactivity of these different forms, at zero time,

TABLE I

COMPOSITION OF THE NUTRIENT AND EXPERIMENTAL MEDIUM

Macroelements	Concentration (M)	Oligoelements	Concentration (M)
KNO_3	$2.30 \cdot 10^{-3}$	Fe(EDTA, Fe^{3+})	$33 \cdot 10^{-6}$
$Ca(NO_3)_2 \cdot 4H_2O$	$0.77 \cdot 10^{-3}$	H_3BO_3	$9.30 \cdot 10^{-6}$
$MgSO_4 \cdot 7\,H_2O$	$0.54 \cdot 10^{-3}$	$MnSO_4 \cdot H_2O$	$3.28 \cdot 10^{-6}$
$NaH_2PO_4 \cdot 2\,H_2O$	$0.73 \cdot 10^{-3}$	$CuSO_4 \cdot 5\,H_2O$	$0.33 \cdot 10^{-6}$
		$ZnSO_4 \cdot 7\,H_2O$	$0.34 \cdot 10^{-9}$
		$(NH_4)_6Mo_7O_{24} \cdot 4\,H_2O$	$9.40 \cdot 10^{-12}$

in the plants grown in the ^{35}S-labelled medium. The method was derived from that of Vallée and Jeanjean [8]. The plants were dehydrated for 24 h at 110°C, and 200 mg (dry weight) were finely ground. They were then incubated for 3 h in 5 ml distilled water at boiling point. After cooling, measurement of the radioactivity in 1 ml of the homogenized suspension gave the radioactivity associated with the total sulphur. Consideration of the specific radioactivity of the growth medium, $\overline{S}$, gave the total quantity of sulphur in the plant, Q_0^t. The rest of the suspension was filtered onto a glass filter (Whatman GF/C), and the radioactivity retained on the filter gave that associated with the insoluble forms of sulphur; hence, with $\overline{S}$, it gave the quantity of insoluble sulphur in the plant, Q_0^{ins}. The filtrate was divided into two parts. The radioactivity of the first part gave that radioactivity associated with all of the soluble forms of sulphur, and hence the quantity of soluble sulphur in the plant, Q_0^{sol}. To the second part were added 5 μmol of unlabelled Na_2SO_4, and then treatment with 12% $BaCl_2$ was carried out. The precipitate thus obtained was separated by filtration on a Millipore membrane (0.2 μm), and washed in ethanol: its radioactivity corresponded to that associated with the free sulphate, and hence the quantity of free sulphate within the plant, $Q_0^{SO_4^{2-}}$. Finally, the quantity of metabolized sulphur in the plants, Q_0^m, was given by:

$$Q_0^m = Q_0^{ins} + Q_0^{sol} - Q_0^{SO_4^{2-}} \qquad (57)$$

Experimental Section

Preliminary measurements

Determination of the growth coefficient, γ. Under our usual experimental conditions, the period of time, T, necessary to double the number of *Lemna* plants was approx. 5 days (120 h). Hence:

$$\gamma = \frac{\ln 2}{T} \doteqdot 5.8 \cdot 10^{-3}\ h^{-1} \qquad (58)$$

This value of γ remained approximately constant provided the cultures were not older than about 1 month. With older cultures, growth progessively slackened, and γ thus diminished regularly. For cultures 41 days old, we (very approximately) estimated γ to be:

$$\gamma \doteqdot 2.9 \cdot 10^{-3}\ h^{-1} \qquad (59)$$

Chemical form of the effluent radioactive sulphur. In the efflux experiments, we never found any significant difference between the total radioactivity excreted into the external medium (total ^{35}S) and that which was liable to be precipitated by $BaCl_2$ ($^{35}SO_4^{2-}$). This means that the only form in which sulphur is re-excreted, is that of inorganic sulphate.

Determination of the cellular parameters of sulphate transport from the efflux measurements

Three experiments were performed. Expts. 1 and 2 were carried out with *Lemna* cultures 30 days old (coefficient γ given by Eqn. 58), while Expt. 3 was effected with a culture 41 days old, hence with a growth coefficient γ given by Eqn. 59. For each

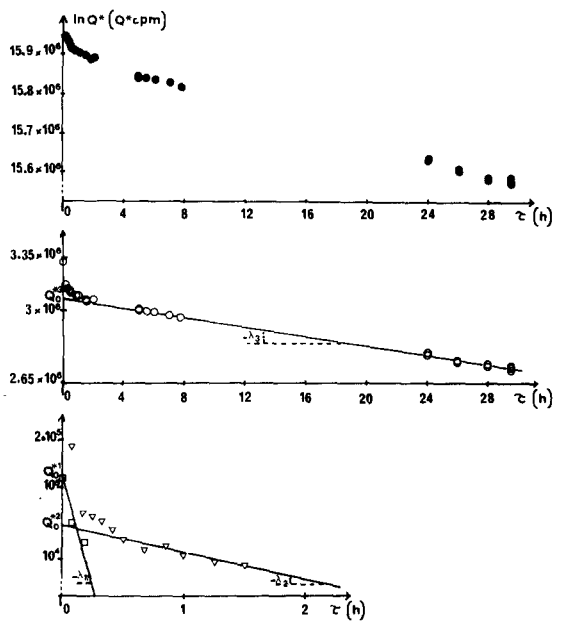

Fig. 2. Exit of radioactive sulphate from the *Lemna* plants, at flux equilibrium: separation of the overall experimental curve into a constant, Q_0^{*4}, and three first-order terms, Q^{*1}, Q^{*2} and Q^{*3}. Naperian logarithm of sulphate radioactivity is represented as a function of time. •, experimental points; ○, calculated values after subtracting the contribution of the constant term Q_0^{*4}; ▽, calculated values after subtracting the contribution of Q_0^{*4} and Q^{*3}; □, calculated values after subtracting the contribution of Q_0^{*4}, Q^{*3} and Q^{*2}. The figure corresponds to Expt. 3.

TABLE II

KINETIC PARAMETERS OF THE EFFLUX EXPERIMENTS

The Q_0 values are indicated with respect to fresh weight. The standard errors, σ, have been indicated (as $\pm\ \sigma$) for the parameters corresponding to the exponential terms which have been obtained by fitting the points with the least-square method.

Parameters	Expt. No.		
	1	2	3
$\gamma\ (h^{-1})$	$5.8 \cdot 10^{-3}$	$5.8 \cdot 10^{-3}$	$2.9 \cdot 10^{-3}$
$\lambda_1\ (h^{-1})$	12.6	23.2	12.9
$\lambda_2\ (h^{-1})$	1.1 ± 0.2	1.7 ± 0.2	$0.9,\pm 0.1$
$\lambda_3\ (h^{-1})$	$(8.0 \pm 1.9) \cdot 10^{-3}$	$(12.0 \pm 0.7) \cdot 10^{-3}$	$(4.0 \pm 0.1) \cdot 10^{-3}$
Q_0^{*1} (cpm/g)	$15.06 \cdot 10^4$	$5.44 \cdot 10^4$	$12.46 \cdot 10^4$
Q_0^{*2}((cpm/g)	$(9.26 \pm 1.69) \cdot 10^4$	$(4.02 \pm 0.49) \cdot 10^4$	$(3.20 \pm 0.42) \cdot 10^4$
Q_0^{*3} (cpm/g)	$(9.17 \pm 0.09) \cdot 10^5$	$(10.88 \pm 0.40) \cdot 10^5$	$(30.42 \pm 2.34) \cdot 10^5$
Q_0^{*4} (cpm/g)	$11.62 \cdot 10^6$	$13.60 \cdot 10^6$	$12.87 \cdot 10^6$
Q_0^t (nmol/g)	4491	5381	6141
$\bar{S}$ (cpm/nmol)	2846	2747	2616

experiment, Q_0^t and Q_0^m were obtained as indicated above. The specific radioactivity of the growth medium, $\bar{S}$, was easily determined by measuring the radioactivity of an aliquot volume of that medium. The kinetic parameters λ_1, λ_2, λ_3, Q_0^{*1}, Q_0^{*2}, Q_0^{*3} and Q_0^{*4} were determined by the method of separation of an overall curve into first-order functions (termed 1–3) and a constant term Q_0^{*4} [9]. Fig. 2 gives an example of such a separation. The numerical calculations were performed with a Hewlett-Packard 9815 A calculator and a plotter. Table II summarizes the experimental data thus

TABLE III

CELLULAR PARAMETERS OF SULPHATE TRANSPORT IN *LEMNA*

For the capacities, Q_0, the percentages with respect to Q_0^t are also indicated. All Φ values are expressed in nmol/h per g fresh weight. Except for Q_∞^{*m} (cpm/g fresh weight), all Q values are expressed in nmol/g fresh weight.

Parameters	Expt. No		
	1	2	3
Φ_0^{ew}	693	491	632
Φ_0^{we}	667	460	614
Φ_0^{wc}	64	61	33
Φ_0^{cw}	38	30	16
Φ_0^{cv}	20	37	14
Φ_0^{vc}	13	29	9
Φ_0^{cm}	18	23	13
Q_0^w	53(1.2%)	20(0.4%)	48(0.8%)
Q_0^c	70(1.6%)	52(1%)	48(0.8%)
Q_0^v	1 191(26.5%)	1 406(26.1%)	1 550(25.2%)
Q_0^m	3 177(70.5%)	3 903(72.5%)	4 495(73.2%)
Q_∞^{*m}	11 620 800	13 602 200	12 866 400

obtained for the three experiments. Eqns. 20–29 allowed the calculation of the cellular parameters of sulphate transport, as indicated in Table III. The determination of Φ_0^{ew} and Φ_0^{we} was not accurate, as those parameters were calculated from values of λ_1 obtained after subtracting three estimated constant or exponential terms from the experimental data. However, Φ_0^{ew} and Φ_0^{we} were clearly much larger than any other unidirectional flux of sulphate, which justifies assumption a2. For the plants in active growth (Expts. 1 and 2), the flux of free sulphate from the cell wall to the cytoplasm was about double that in the opposite direction, while the flux from the vacuola to the cytoplasm was not very much smaller than that from the cytoplasm to the vacuola. The flux of sulphate metabolization was of the same order of magnitude as the other intracellular fluxes. The contents of free sulphate in the cell wall and in the cytoplasm were very small (about 1% each), whereas more than 70% of the total sulphur was present in organic form, and more than 25% was free sulphate in the vacuola. When growth had significantly slackened (Expt. 3), the values of the capacities of the various compartments were not changed, nor were those of the fluxes between the external medium and the cell wall. The values of the fluxes involving transmembrane transport (Φ_0^{wc}, Φ_0^{cw}, Φ_0^{cv} and Φ_0^{vc}) or metabolism (Φ_0^{cm}) were hardly halved.

Comparison of the calculated values of influx to experimentally measured values

For each experiment (Expts. 1–3), the cellular parameters determined from the efflux experiments (Table III) can be used to calculate 'predicted values' of the radioactivity, $Q_{\tau 2}^*$, absorbed at any time, τ_2, in an influx experiment. This was effected by using

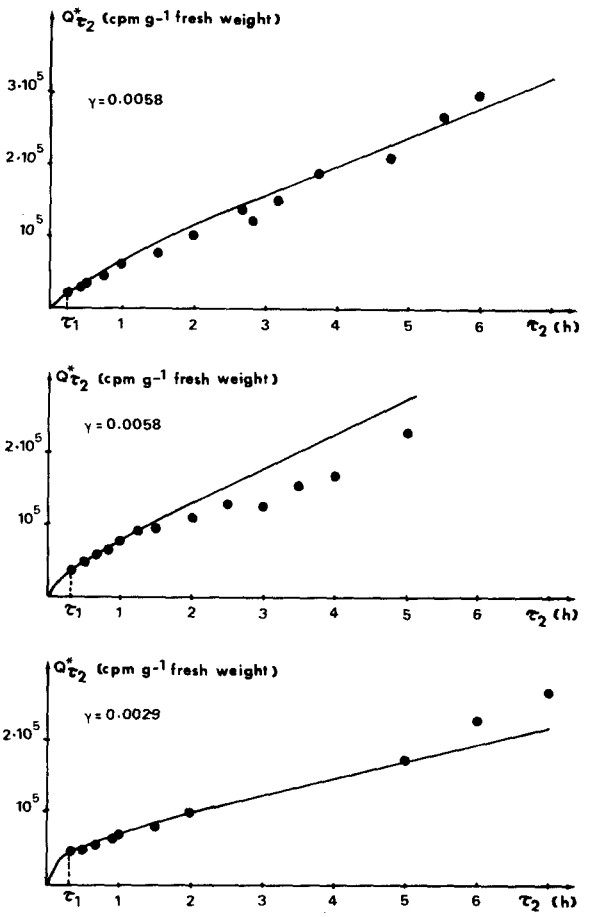

Fig. 3. Influx of radioactive sulphate in the *Lemna* plants. The full line represents the 'predicted' fixation of radioactivity, as calculated from the cellular parameters which were determined from the efflux experiments. •, actual values of influx experimentally measured, under experimental conditions comparable to those of the corresponding efflux experiments. τ_2 denotes time (a particular value of which is τ_1), $Q_{\tau 2}^*$ is the radioactivity accumulated during time τ_2, and γ is the growth coefficient. The upper curve corresponds to Expt. 1, the middle curve to Expt. 2 and the lower curve to Expt. 3.

TABLE IV

EXPERIMENTAL DETERMINATION OF THE RADIOACTIVITY, $Q_{\tau 1}^*$, ABSORBED IN AN INFLUX EXPERIMENT, AFTER A SHORT PERIOD OF TIME τ_1

Expt. No	τ_1 (min)	$Q_{\tau 1}^*$ (cpm/g)
1	15	21 800
2	20	39 500
3	20	45 688

Eqns. 48–51 and could be done by replacing Eqn. 50 with Eqn. 55 or 56 in the case when growth would be practically nil. The only parameter missing for using Eqn. 51 was $Q_{\tau 1}^*$, though it was easily determined experimentally (Table IV). The calculated values of $Q_{\tau 2}^*$ were then compared to those determined experimentally. The results, as indicated in Fig. 3, have been expressed after normalization of the

external specific activity S^e to the same value:

$$S^e = 1200 \text{ cpm/nmol} \quad (60)$$

for Expts. 1–3. This makes comparison of the results of the various experiments easier. Under the conditions when assumptions a7 and a8 were both satisfied, the ajdustment of the calculated values to the experimental data appears to be very good. This is a satisfactory test of the internal consistency of our approach. It is only when τ_2 became larger than 1.5 h for Expt. 1, or larger than 5–6 h for Expts. 2 and 3, that the theoretical curves and the experimental points diverged from one another.

Discussion and Conclusions

Interpretation of the data obtained in compartmental analysis always requires great care. The estimation of the unidirectional fluxes and of the compartment capacities especially would be meaningless if the compartment model (given here in Fig. 1) were not consistent with the actual cellular organization of the living sample studied. In the present case, we have seen that our estimations of unidirectional fluxes and compartment capacities were compatible with two sets of measurements, efflux and influx, performed independently. Clearly, this is not a sufficient condition for proving the unicity of the solution which we have arrived at (especially in the case of Expt. 3 where determination of γ was rather doubtful). However, this is at least necessary condition for unicity, and it supports the internal consistency of the approach followed. It is of interest, also, that three different experiments led to results fully consistent with one another, even when considering that the first two were performed on plants growing actively, while, in the third case, growth was much reduced. The results indicate also that almost three-quarters of the total sulphur of the *Lemna* plants is present in the form of thio organic compounds, while about one-quarter is made up of free sulphate stocked in the vacuola. Only about 1% of the total sulphur of the plants constitutes the pool of free sulphate in the cytoplasm. The sulphate content of the cell walls is also about 1% of the total sulphur content of the plants. Knowing [9,10] that the ratio dry weight : fresh weight is 0.05 for *Lemna*, that 1 g *Lemna* (fresh weight) corresponds to 0.026 g cell wall (fresh weight), and that 1 g cell wall contains 0.65 g swelling water, one can calculate the concentration of sulphate in the swelling water of the cell wall. From the values of Q_0^w given in Table III, one finds 3.1, 1.2 and 2.8 mM for Expts. 1, 2 and 3, respectively. This is significantly more than the sulphate concentration of the external medium (0.54 mM), whereas, given the Donnan effect, one would rather have expected it to be less. This might be due to the presence of proteins with an affinity for sulphate, in the cell wall or on the outer face of the plasmalemma. It might also correspond, at least in part, to the brief rinsing of the plants, when starting the desorption experiment, being insufficient to remove completely the diffusion layers along the surface of the plants. Apart from the unidirectional fluxes between the external medium and cell wall, which are always very large, the intracellular fluxes are all of the same order of magnitude, a few tens of nmol/h per g (fresh weight). In particular, the flux of sulphate metabolization is far from being negligible with respect to the other intracellular fluxes. This justifies a posteriori the necessity of us taking into account growth and metabolization in our compartmental model. It is likely that, even in the case of ions which are not incorporated into organic molecules (e.g., K^+, Na^+), it is not always possible to neglect growth when studying the cellular exchanges of rapidly growing samples. From this point of view, our approach might thus have a rather general relevance.

Let us consider the quantity of radioactivity which has been incorporated into organic sulphate during the course of an efflux experiment ($Q_\infty^{*m} - Q_0^m \bar{S}$), and the quantity of radioactivity which was associated with free sulfate at zero time of the efflux experiment, $(Q_0^t - Q_0^m)\bar{S}$. The percentage:

$$R = 100 \frac{Q_\infty^{*m} - Q_0^m \bar{S}}{(Q_0^t - Q_0^m)\bar{S}} \quad (61)$$

gives the fraction of the initial radioactivity of free sulphate which is finally incorporated into organic compounds. For Expts. 1 and 2, where the plants were actively growing, the values of R are 67 and 71%, respectively. This means that there is not

more than about 30% of the radioactivity, initially associated with free sulphate, which gets out of the plants during an efflux experiment, the rest being progressively immobilized in the plants as organic forms. This is probably the reason why previous authors [8] have indicated that absorption of radioactive sulphate was practically irreversible.

As regards the influx experiments, we have seen above (Eqns. 52 and 53) that the specific radioactivity of the cytoplasm, S^c, would become quasi-constant only for values of the time, τ, significantly greater than $1/(\alpha+\gamma)$. From the numerical values given in Table III, it is easy to calculate that $1/(\alpha+\gamma)$ equals about 55 min for Expt. 1, 35 min for Expt. 2 and 65 min for Expt. 3. Hence, S^c can be considered as quasi-constant, in an influx experiment, only after several hours of radioisotope absorption. Moreover, if we compare the values of $1/\alpha$ (about 0.92, 0.58 and 1.07 for Expts. 1, 2 and 3, respectively) with those of $1/\gamma$ (173, 173 and 346 for the same experiments), it is clear that:

$$\frac{1}{\alpha} << \frac{1}{\gamma} \tag{62}$$

Hence, in our experiments, when τ_2 is significantly smaller than $1/\alpha$ it is necessarily also much smaller than $1/\gamma$. In our case, the situation described by Eqn. 55 will never be encountered, and we shall use Eqn. 54 under conditions when:

$$\frac{1}{\alpha} \lesssim \tau_2 << \frac{1}{\gamma} \tag{63}$$

or Eqn. 56 under conditions when:

$$\tau_2 << \frac{1}{\alpha} << \frac{1}{\gamma} \tag{64}$$

It is notable in Fig. 3 that the influx of radioactive sulfate, $Q^*_{\tau 2}$, proceeds quasi-linearly with respect to time, τ_2, from about the 20th minute to at least the second hour (Expt. 1) and possibly until the fifth or sixth hour (Expts. 2 and 3) after the beginning of the influx experiment. This is an interval of time satisfying Eqn. 63, hence where Eqn. 54 must be used. Numerical calculation, using the values given in Table III, shows that the second term in the expression of $Q^*_{\tau 1 \tau 2}$ is then quasi-constant and significantly smaller than the first term. Hence, using Eqn. 51 with $Q^*_{\tau 1}$ being a constant term for Expts. 1–3, Eqn. 54 can be written:

$$Q^*_{\tau_2} \doteqdot S^e(\Phi_0^{wc} - \beta)(\tau_2 - \tau_1) + c^{te} \tag{65}$$

which is consistent with the experimental result, indicated above, of a quasi-linear relationship between $Q^*_{\tau 2}$ and τ_2. The theoretical slope of this linear relationship is $S^e\,(\Phi_0^{wc} - \beta)$; hence it is 39 600, 49 200 and 25 200 cpm/h for Expts. 1, 2 and 3, respectively. This is in relatively good agreement with the experimental slopes as indicated in Fig. 3 (42 300, 57 700 and 30 400 cpm/h for the same three experiments). This is again a test of the internal consistency of our approach.

Moreover, the above result indicates that the stationary phases of absorption which can be observed after the first few dozens of minutes, do not correspond to Φ_0^{wc}, but to a complex composition of the various transmembrane fluxes within the cells. For smaller values of the time, τ_2, obeying Eqn. 64, Eqn. 56 could be used in principle. The slope of a quasi-linear approximation obtained for $\tau_2 << \frac{1}{\alpha}$, if it exists, would then be $S^e\Phi_0^{wc}$, and would thus allow us to estimate Φ_0^{wc} very simply. However, given the values of $1/\alpha$ which we have indicated above, this would occur only for values of τ_2 of the order of magnitude of a few minutes, and hence equal to or even smaller than τ_1. This is precisely the range of time values where Eqns. 51 and 56 cannot be used any more, especially because the cell walls are not yet equilibrated with the external medium. Hence, we arrive at a conclusion of general relevance for those people studying the mechanisms of sulfate transport in cells: (i) quasi-stationary behaviour can be observed on the experimental influx curves, at any time after the first few minutes up to several hours, but (ii) this straight line has no simple biological significance, and, more particularly, it does not correspond to the unidirectional flux of sulfate, Φ_0^{wc}, from cell wall to cytoplasm; moreover (iii) the contribution of the cell wall equilibration cannot be neglected during intervals of time shorter than a few minutes. This might be valid for many types of living sample, other than the *Lemna* plant, and it is probably even more true under conditions

of non-equilibrium (which is most often the case for the conventional studies of sulphate influx as a function of the concentrations of sulphate in the bathing medium). If one wishes to obtain actual values of Φ_0^{wc}, from overall influx measurements, it thus seems advisable (i) to consider only the data obtained during the first few minutes of experiments, and (ii) to find a way of estimating and subtracting the contribution of the sulphate exchanges in the cell walls during the same interval of time.

As regards the influx curves (Fig. 3), it is not clearly understood why the theoretical curve, at the end of the experiments, went above the experimental points for Expt. 2, while it was beneath for Expts. 1 and 3. For time values, τ_2, large enough for assumption a8 to be no longer satisfied, the expected behaviour is that found in Expt. 2. A possible explanation of what occurred in Expts. 1 and 3, for the large time values, is that the *Lemna* plants might be sensitive to the phenomenon of gas shock (i.e., a transient change of their permeabilities for a few substrates, including sulfate, when they undergo a rapid change in their ambient atmosphere), in a way somewhat comparable to that observed with cell suspension cultures [11,12]. In the latter case, following gas shock, the rates of uptake of several substrates significantly decrease within a few minutes; then, after a few hours at the low value, the rates increase again and return to the initial value within 10–20 h. We have obtained other data (unpublished observations) which support the occurrence of gas shock effects with *Lemna*. Absorption curves with a positive curvature are also found sometimes in the literature [13], which would be consistent with the occurrence of gas shock on other types of living sample. Some authors [14] have disputed the role of a gas in this process, and prefer calling it manipulation shock. However that may be, people working on sulphate transport should thus pay attention to possible disturbances of their experiments by gas shock or manipulation shock effects, whether they use unicellular systems or samples originating from multicellular higher plants.

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