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A Comparison of Methods for Determining Compartmental Analysis Parameters[1]

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ABSTRACT

The traditional method for determining compartmental analysis parameters relies on a visual selection of data points to be used for regression of data from each cellular compartment. This method is appropriate when the compartments are kinetically discrete and are easily discernible. However, where treatment effects on compartment parameters are being evaluated, a more objective method for determining initial parameters is desirable.

Three methods were examined for determining initial isotopic contents and half-times of ^{86}Rb elution from cellular compartments using theoretical data with known parameters. Experimental data from roots of Douglas fir (*Pseudotsuga menziesii* [Mirb.] Franco) and barley (*Hordeum vulgare* L.) intact seedlings were also used. The three methods were a visually assisted, linear regression on data of semilog plot of isotope elution *versus* time, a microcomputer-assisted, linear regression on semilog plot where maximization of the square of the correlation coefficient (r^2) was the criterion to determine data points needed for each regression and a mainframe computer-assisted, direct nonlinear regression on elution data using a model of the sum of three exponential decay functions. The visual method resulted in the least accurate estimates of compartmental analysis parameters. The microcomputer-assisted and nonlinear regression methods calculated the parameters equally well.

The technique of compartmental analysis of radioisotope elution from plant cells and tissues has been used extensively to estimate the number of cellular compartments participating in solute exchange and to estimate the solute content of each compartment. Under appropriate circumstances (12), solute fluxes to and from the various compartments may also be estimated. The first reported use of compartmental analysis was 1822 by Fourier (see 13) who described heat flow. Since then, compartmental analysis has arisen in various subdivisions of physiological sciences. In the botanical literature, compartmental analysis was applied to algal cells and subsequently to excised and intact higher plant organs and more recently to tissue culture cells (9).

Typically, the tissue is loaded with labeled solute for at least five cytoplasmic half-times. Next, the tissue is transferred to nonlabeled solution wherein the pattern of isotope efflux is monitored for several hours. A semilog plot of the radioisotope content of the tissue as a function of time commonly reveals three components in the elution profile (12), corresponding to fast, medium, and slow exchange compartments. These three compartments may have a rough correspondence to three cellular compartments—the CW+FS[4], cytoplasm, and vacuole. The customary methodology is to do 'curve-peeling' by performing a linear regression or visual fit upon the straight line portion of the semilog plots of the elution data (Fig. 1). The extrapolation of this line to the y axis provides an estimate of the apparent isotopic content at the beginning of the washout period (A_v) of this slowly exchanging compartment as well as the rate constant (k_v) for isotope exchange from this compartment. After subtraction of this slowly exchanging (possibly vacuolar) component from the total isotope content of the tissue at each interval, the remaining isotopic contents are replotted to give estimates of A_c (apparent isotopic content of the intermediate, possibly cytoplasmic compartment) and the rate constant k_c. After subtraction of the cytoplasmic component, A_w and k_w for CW+FS are obtained. This method generally relies upon a subjective selection of the number of data points to be included in regressions of each phase. If too many or too few data points for each phase are included, slope and intercept may be altered and error is increased. Nevertheless, where $t_{1/2}$ values for solute exchange for the various compartments are very different, the method is quite adequate, particularly when separate and independent estimates of various fluxes are obtained as checks of the method. However, where the method is employed to evaluate treatment effects e.g. effects of hormones, mycorrhizal infection (10), or even genotypic differences (3), a less subjective method for determining 'cut-off' points for regression is essential. For large data sets, use of computers is advantageous.

In this paper, we compare a nonlinear regression method with the more traditional linear regression method. In addition, we describe a microcomputer method in which maximization of r^2 for linear regression serves as the criterion for determining data points to be included in the regression line for each component. These methods were tested using a theoretical data set. In addition, these methods were used to compare ^{86}Rb radioisotopic elution data from roots of intact barley and Douglas fir seedlings.

MATERIALS AND METHODS

Methods of Data Analysis. The efflux data were analyzed using three procedures: (A) that of Poole (8) and Cram (1); (B)

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[3] Copies of the r^2 maximization microcomputer program may be obtained by writing directly to A.D.M. Glass.

[4] Abbreviations: CW+FS, cell wall + free space; $t_{1/2}$, half-time; r^2, square of the sample correlation coefficient.

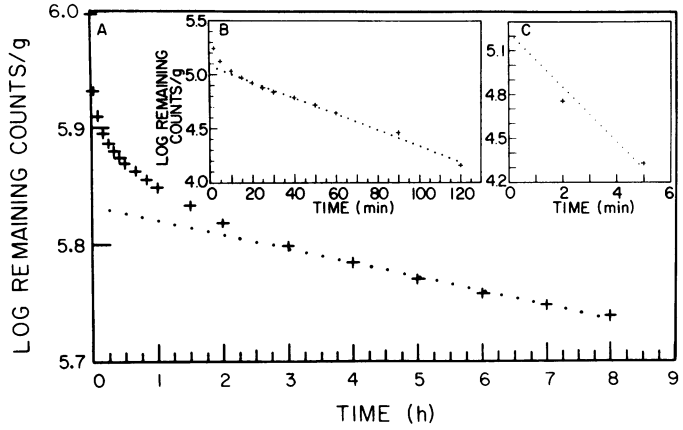

FIG. 1. Linear regression on semilog plot of ^{86}Rb elution data for barley roots: (A) linear regression on final straight line portion of semilog data to determine slow compartment parameters; (B) linear regression on log cpm remaining in tissue after subtraction of slow phase, (C) linear regression on data from fast compartment. These regressions were done using the r^2 maximization program described in the text.

that of Poole (8) and Cram (1) assisted by a microcomputer; and (C) a nonlinear regression analysis.

The first method (A) was the traditional, visual estimation of slopes and intercepts using linear regression on a semilog transformation of the data (6 and many others). The second method (B) utilized a microcomputer program (identified as EFFANP) to determine automatically data cut off points for linear regression. The program was written in BASIC for a Hewlett Packard HP87 microcomputer with 32K memory. Plotting of the resulting regressions was done with a Hewlett Packard 7470A plotter (Fig. 1). This program requires that the operator enter all elution data, which is then stored on file. Then, the program determines the total isotopic content of tissue at $t = 0$ and subsequent times. Log transformations are made and regression begins from the last three (vacuolar) data points. Additional data points are added one at a time until three successive data points cause a reduction in the regression r^2. The program back-tracks to the last point before r^2 begins to decline and calculates the required parameters (slope, intercept, r^2) and provides a plot of the regression which can be sent to plotter or printer. Next, the slow regression is subtracted from remaining tissue contents to obtain data for medium-fast regression. Finally, the program subtracts the medium-fast regression from the remaining tissue contents to obtain the fast or cell wall regression. There is an option to specify particular points for regression so that obviously anomalous data points can be dropped from inclusion in regression.

The third method (C, a nonlinear regression) is a direct fit of untransformed data to the sum of three exponential decay functions. Nobel (7), who described solute removal from cell wall and other cellular compartments where the external solute concentrations was zero, used Fick's first law of diffusion to describe these processes. The solution of the diffusion equations for the CW+FS is a series of exponentials which, after a short time, become a single exponential; little error is involved in representing the CW+FS by an exponential term. If one considers the tissue as a series of three compartments, the model of the sum of three exponential decay functions can be employed.

$$\text{cpm} = A_w e^{-k_w t} + A_c e^{-k_c t} + A_v e^{-k_v t} \quad (1)$$

where cpm = cpm of activity remaining in the tissue at time t; A_w, A_c, A_v: estimated initial isotopic content of fast (A_w), medium-fast (A_c), and slow (A_v) compartments at the beginning of the washout period; k_w, k_c, k_v: rate constants for isotopic exchange of each compartment (subscripts as above).

An added convenience of the three exponential decay equation is the use of a data reduction software program. We used the Biomedical Data Processing (BMDP) software programs of the Health Sciences School, University of California, Los Angeles[5] (2) to determine the initial parameters for subsequent compartmental analysis. The BMDP program varies the rate constants (k) and initial isotopic contents (A_o) according to a prescribed algorithm until the residual sums of squares are minimized. By using this software program, the mathematical solution for the initial parameters for all three compartments is determined simultaneously and objectively, unlike methods A and B, where calculations are made for each compartment individually. If an error is made during an earlier regression using methods A and B, compounded errors may result for the subsequent compartments.

Both methods A and B, described previously, transform the efflux data to a semilog plot, after which three linear regressions (one for each compartment) are performed. Method C does not transform the efflux data but uses it directly in a nonlinear regression model which is the sum of three exponential decay functions.

Plant Material. Seedlings of Douglas fir (*Pseudotsuga menziesii* [Mirb.] Franco) were grown for 6 months in a peat/vermiculite/perlite mixture (1:1:1) contained in plastic tubes (2.5 × 16.5 cm) in a greenhouse with supplemental high pressure sodium lighting. Before experimentation, roots were gently washed free of the planting medium.

Barley (*Hordeum vulgare* L.) seedlings were germinated on plastic gauze stretched across 10-cm Plexiglas discs in moistened sand as described previously (3). After 3 d, discs with seedlings were transferred to hydroponic facilities. The hydroponic tanks, containing 36 L of 0.5 mM $CaSO_4$ plus 0.1 mM KCl, were placed in temperature-controlled growth rooms maintained at 26° ± 2°C on a 16-h day/8-h night. Light (spectrally equivalent to sunlight) was supplied by banks of fluorescent lamps which provided an irradiance of 5 mw·cm^{-2} at plant level.

Pretreatment and Isotope Uptake. Douglas fir seedlings were pretreated in aerated dilute nutrient solution (0.5 mM $CaSO_4$ plus 0.1 mM KCl) for 24 h under continuous light in a water bath at 18°C. Roots of intact plants were transferred to 500 ml of aerated isotope labeling solution (0.5 mM $CaSO_4$, 0.1 mM KCl, and 0.10 μCi $^{86}Rb \cdot \mu mol\ K^{-1}$) and maintained in this solution at 18°C under continuous light for 18 h.

When barley plants were 6 d old, groups of approximately 20 plants (~2 g fresh weight of root) were gently transferred from discs and roots were immersed in fresh aerated dilute nutrient medium. After 2 h, plants were transferred to 500 ml of aerated isotope labeling solution (0.5 mM $CaSO_4$ plus 0.1 mM KCl with 1.6 μCi $^{86}Rb \cdot \mu mol\ K^{-1}$, 24° ± 2°C).

Elution and Counting of ^{86}Rb. Roots of intact Douglas fir seedlings were removed from the loading solution and washed for 5 s in 1 L of dilute nutrient solution to remove surface water containing radioisotope. Each seedling was transferred to a 30-ml syringe fitted with a value for drainage of efflux solutions. Successive 18-ml volumes of efflux solution (dilute nutrient medium, 18°C) were added to the syringe and incubated with the root material with aeration for gradually increasing periods during the 10-h efflux period. Initial elution periods were 2 min and were gradually increased to 1 h.

Barley plants were transferred to 60 ml glass columns (i.d. 2.5 cm) fitted with drainage valves for elution. Successive 18-ml aliquots of the dilute nutrient medium were delivered to these columns by means of syringes. Roots were completely immersed

[5] These programs were developed at the Health Sciences Computing Facility, UCLA, and were supported by NIH Special Research Resources Grant RR-3. We used subprogram BMDP3R, revised November 1978.

by this aerated solution which was maintained at 26°C. At the intervals shown in Figure 1, the eluate was drained from the columns into glass scintillation vials.

At the end of the elution period, roots were blotted gently, weighed, dry-ashed at 500°C, and the resulting ashes resuspended in 1 N HCl (Douglas fir) or distilled H_2O (barley) for Cerenkov counting of ^{86}Rb. Samples were counted to less than 1.5% counting error in a Packard Tri-Carb liquid scintillation counter (Douglas fir) or a Searle Isocap 300 counter (barley). Quenching losses were estimated using the channels ratio technique. Barley and Douglas fir data were expressed on root fresh weight basis.

This elution technique, like other elution methods, does allow for some small amount of carry-over of solution adhering to the root surfaces and to the walls of the elution chamber. This carry-over of radioisotope does not affect the methodological comparisons. In addition, during the efflux period, some isotope flux through the stele to the shoot will occur, although stelar flux is apparently small. Other workers have used excised roots to eliminate this transfer, but separation of root and shoot can affect root metabolism and there can be appreciable differences between fluxes in intact and excised roots (4, 5). This loss to the stele will not interfere with the comparison of the three methods.

RESULTS AND DISCUSSION

Comparison of the Three Methods. The two linear methods A and B and the nonlinear method C were compared using data which was calculated from prescribed parameters created by one author (A.D.M.G.) using the following values: half-times of 1.1 min, 39 min, and 13 h and A values of 316,200, 100,000, and 1,000,000 cpm·g^{-1} for compartments which roughly correspond to CW+FS, cytoplasm, and vacuole, respectively (Table I). The calculated data were then used to test the three methods by determining the parameters. Estimates of parameters for the three compartments were within 2.2% (mean error) of the actual prescribed values (Table I). Generally, all three methods estimated A values more accurately than half-times.

Table I. *Half-Times and A Values: Comparisons of Three Regression Methods for Determining Compartmental Analysis Parameters for Isotopic Exchange in Three Compartments—Fast, Medium, and Slow*

Methods employed were a visual estimation of slopes (A), a microcomputer-assisted maximization of r^2 values for linear regression (B), and direct computer fit of a nonlinear, three-component exponential decay equation (C).

Data Set	Method	Parameters		
		Fast	Medium	Slow
		min		*h*
Half-time				
Prescribed values		1.1	39.0	13.0
Calculated values				
Unaltered	A	1.0	35.0	12.9
	B	0.96	36.6	13.0
	C	1.1	39.0	13.0
Altered (±5% error)	A	1.0	33.5	13.1
	B	1.0	34.9	13.1
	C	1.1	37.9	13.2
		cpm g^{-1} dry wt		
A Values				
Prescribed values		316,200	100,000	1,000,000
Calculated values				
Unaltered	A	319,900	98,900	1,002,300
	B	325,100	105,000	1,000,000
	C	316,200	99,800	1,000,000
Altered (±5% error)	A	319,900	98,900	1,009,300
	B	318,400	98,900	1,009,300
	C	312,600	98,400	1,004,600

The nonlinear method C was the most accurate since the parameter estimates were within 0.1% of the prescribed values; methods A and B were also quite accurate, within 6 to 7% of the prescribed values. Thus, all three methods estimated the parameters closely; however, methods A and B could not estimate the parameters as closely as method C, even when exact data were used. When the raw data were altered by introducing 5% random error (see Table I, 'altered' data set), the estimates of parameters were less accurate than when unmodified data were used. Method C again provided the most accurate estimates of half-times, while all three methods provided similar estimates of A values.

To assign data points to a particular phase by the subjective, visual method may introduce errors in the slope and/or intercept. Moreover, because these parameters are used in calculating data for subsequent regressions, any errors introduced in the initial regression will be compounded in analyses of subsequent phases. Thus, there may be greater doubt associated with estimates of the faster compartments (cytoplasmic and cell wall) parameters than with estimates for the slow compartment. This is particularly unfortunate since cytoplasmic characteristics are frequently those of greatest interest.

There are also two other potential sources of error. As with any analytical technique, errors are present in the counting estimates of radioactivity; low counts result in underestimates of the true mean (Poisson distribution) while high total counts more accurately estimate the mean (Gaussian). Last, the best fit for the logarithm of a function is not quite the best fit for the original function. When data scatter badly, the errors arising from these sources tend to steepen the slope of the regression line (see discussion in Simon (11).

As stated earlier, provided that the experimenter is evaluating compartments which differ substantially in their $t_{1/2}$ values, errors of the above types may not be significant. When the purpose is to compare parameters for the same compartment under various treatment conditions, more objective methods are preferred and methods B and C are recommended. The use of a microcomputer (method B) or a large mainframe computer (method C) can greatly facilitate data manipulations and provide more objective estimates of the parameters.

Sources of Error. Method A, using visual fit of the data, introduced substantial error in estimating half-times and was the least reliable method. This error was less apparent with unaltered data than with data altered by introduction of 5% random error (Table I). Estimates provided by methods B and C were extremely close; however, the r^2 maximization method (B) sometimes required that the experimenter make subjective decisions regarding the data.

For example, with the altered data set, completely automatic regression gave a $t_{1/2}$ value of 28 min for cytoplasmic exchange (theoretical value = 39 min). As Figure 2 demonstrates, the error associated with one point (at t = 180 min) resulted in a steeper slope of the regression line (Fig. 2A). As a consequence, the 60-min data point and subsequent points were automatically rejected by the microcomputer program and excluded from the regression. The fact that these subsequent points lay below the regression line was clear indication of the inappropriateness of this regression which was based on only three data points. By elimination of the 180-min data point, $t_{1/2}$ was increased to 35 min and a good fit was obtained for 10 sequential points before the r^2 began to decrease (t = 10 min; Fig. 2b).

In our first attempt at developing this program, we selected an automatic regression procedure in which data points were added to the regression until r^2 decreased by some value specified by

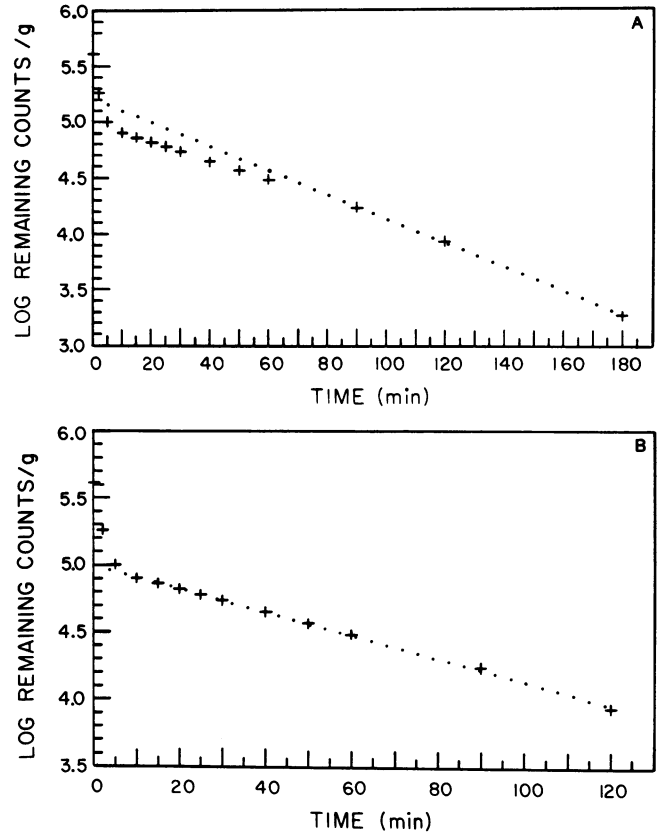

FIG. 2. Linear regression on second compartment of the theoretical model data using the r^2 maximization method where (A) includes the 180-min datum point and (B) does not.

Table II. *Half-Times and A Values for Three Compartments*

Parameters were calculated using ^{86}Rb efflux data from roots of intact seedlings of Douglas fir and barley. All conventions as in Table I.

Experimental Data Set	Method	Parameters		
		Fast	Medium	Slow
		min		*h*
Half-time				
Douglas fir	A	9.2	60.0	8.7
	B	1.8	22.8	8.4
	C	1.5	17.8	8.2
Barley	A	3.7	64.0	31.0
	B	1.6	40.4	25.0
	C	1.2	39.0	25.3
		cpm g fresh wt $\cdot 10^{-3}$		
A Values				
Douglas fir	A	69.8	13.1	128
	B	126	37.9	131
	C	132	42.9	132
Barley	A	135	12.9	653
	B	168	122	676
	C	187	129	678

the operator. However, it soon became apparent that under some conditions experimental error might cause sufficient deviation from the regression in a single point to terminate regression of that phase. This could occur even though several subsequent points might lie on the regression line. To avoid premature termination of the regression by such a source of error, the programs were modified so that termination does not occur until three consecutive points reduce the r^2. When this criterion is satisfied, the program goes back to the last point before the r^2 value begins to decline and determines the regression parameters.

By contrast, the nonlinear method required no such decision making on the part of the experimenter. The estimates obtained by this method using both altered and unaltered data, judged on the basis of deviation from the prescribed parameters, appeared to be slightly more accurate than those given by the r^2 maximization method, particularly at early time periods. The nonlinear regression method C provided better estimates of the CW+FS and cytoplasmic compartment parameters. Parameter estimates for the vacuolar compartment were similar between the two methods.

Barley and Douglas Fir. Methods A, B, and C were used to estimate half-times and apparent isotopic contents for ^{86}Rb efflux from roots of intact barley and Douglas fir seedlings (Table II). Unlike the theoretical data analyzed previously, we do not know what the actual parameter values are, since only estimated values may be calculated from the experimental data. These results show that methods B and C produced similar values, while those of method A were different. The half-times for method A were longer than those calculated by methods B and C. Since the 'real' values are not known, we cannot determine which method is the most accurate.

The half-times for Douglas fir were generally faster than those of barley, while the apparent isotopic contents were less. These data show that complex tissue such as roots of barley and Douglas fir provide ^{86}Rb elution data which can be separated into three phases and that methods B and C give rather similar estimates of parameters for compartmental analysis.

SUMMARY AND CONCLUSIONS

The r^2 maximization, linear regression method B, and the nonlinear regression method C are preferred over the traditional, visual method A for estimating subcellular compartment parameters for subsequent compartmental analysis. The nonlinear method C offers the advantage of complete objectivity on the part of the experimenter for considering anomalous data, but this method does require use of a complex program and a large computer which may not be easily accessible. The r^2 maximization, linear regression method B was designed for the increasingly more available microcomputers and permits intelligent interaction between experimenter and analytical method.

When compartmental analysis experiments are designed to evaluate differences between vastly different subcellular compartments within one tissue, the visual method A is adequate. However, if differences between treatments are required, the r^2 maximization and nonlinear methods (B and C) offer more objective determinations of compartmental analysis parameters.

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