The Evaluation of Initial Rates in the Presence of Background Noise

Fritz JAUKER

Institut fuer Tierphysiologie, Wartweg 95, 6300 Giessen, Federal Republic of Germany

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A three-parameter model was used to test a curvilinear regression for its y -intercept. It was applied in the kinetic analysis of a model reaction and of real experiments. The model reaction consisted of a dialysis experiment with increasing amounts of background added at t_0 . In the presence of this background, initial rates of solute exchange were determined precisely enough to be in agreement with Fick's law of diffusion. The biological experiments were concerned with the saturation kinetics of the acid-soluble radioactivity of cells exposed to ^3H -labelled amino acids. Two models of regression were applied, facilitated and physical diffusion, both consistent with the assumption of a substantial amount of background. This background was assumed to be caused by adsorption of ^3H -labelled amino acids to cell-surface proteins. Without further consideration it caused a systematic overestimation of the initial rates.

In studies of transport kinetics one has to deal with rates of uptake. These rates, the dependent variables, are measured as functions of external concentrations of substrates or competitors, of temperature, state of growth etc. Usually the rates of transport are estimated from the increase in the intracellular concentration of a radioactively labelled solute with time. This is a curvilinear saturation process, and the times of observation have to be in the range of a few seconds in order to record linearity (Plagemann *et al.*, 1978). This apparent linearity suffices for most practical purposes, but mathematically it is still curvilinear according to the biophysical models of tracer fluxes between two compartments. Therefore the most common measure of the rate of transport is the slope of the initial tangent (Kotyk & Janáček, 1975).

The purpose of the present paper is to demonstrate that there may be reasons for a misinterpretation of the initial slope and how to obtain a more likely estimation of its true value. The general concept is that the observed solute concentration, y , as a function of time, t , has the general structure

$$y = f(t) \pm N \quad (1)$$

The term N here is not considered a random experimental or instrumental variable with a zero mean ('white noise'), but to deviate significantly from zero. If it is positive, biologists most commonly refer to it as 'background'. In tracer experiments such background may be caused by radioactive contamination, cosmic radiation, or incom-

plete removal of the radioactive supernatant. This source of error is usually eliminated by referring to external standards or by performing appropriate controls. More serious, however, is adsorption of the isotope to the surface of the specimen. Amino acids, for example, tend to become adsorbed to proteins. This is a well known effect in radioautography and has already caused misinterpretations of the amount of amino acid incorporation (Peters & Ashley, 1967). If in transport studies radioactive amino acids are not completely removed by the washing procedure, they eventually become released during the final treatment and may cause N to become intolerably large.

It is evident from eqn. (1) that a given amount of background does not change the increments of y upon t . There are two ways, then, to filter out the disturbing background:

(1) The relevant parameters are estimated from the partial derivatives of the functions involved.

(2) The co-ordinates are transformed and the first data point, t_1/y_1 , is made the 0/0 origin. Depending on convenience and on experimental needs the further analysis may be based on any of the functions illustrated in Fig. 1.

Both procedures were applied in the present investigation and worked satisfactorily compared with the accuracy obtained in an iterative fitting procedure.

As outlined below, three parameters lead to a significantly closer fit than the minimum of two required to describe the regression of a time-dependent saturation process. As is to be expected, a

Fig. 1. Set of functions employed in regression analysis in the presence of background

If the original function cuts the ordinate away from the origin the transformed function will pass this point at $(x'_0 - x_1)$. The corresponding $|y'|$ will not equal y_1 . The difference, $(y - y')$, is termed N and is the mathematical correlate of the background. The observed regression of y on x , then, is expressed by $y = f(x) + N$. According to convenience and needs, any one of the functions illustrated may be used in the data evaluation.

two-parameter estimation in the presence of background caused a heavy overestimation of the initial rates.

Materials and Methods

The model reaction

This reaction served to produce data of a time-dependent saturation process allowing tight control of experimental conditions. The actual subject of the kinetic analysis was the increase in external radioactivity released from a dialysis chamber.

The chamber was filled with 0.8% NaCl containing 50 μ l of ^3H -labelled amino acids (disc chamber model B; 500 μ l; E. Schuett, Göttingen, Germany). Membranes were punched from Visking tubing, and had an effective surface area of 127.23 mm². The ^3H -labelled-amino-acid mixture (TRK 440; Amersham Buchler, Braunschweig, Germany) contained the 15 amino acids specified in the manufacturer's catalogue. The specific radioactivity as calculated from the batch analysis was usually about 0.1 Ci/mmol of the total amino acids. At t_0 the chamber was placed into 150 ml of 0.8% NaCl and rotated at 25°C. At each time indicated, two 50 μ l samples were removed from the external

fluid and their radioactivity determined in a liquid-scintillation counter.

A 'background' was simulated by adding a certain amount of the ^3H -labelled amino acids at t_0 to the outer compartment.

Transport studies

Stock cultures of the ciliate *Tetrahymena pyriformis* HSM were grown in the defined synthetic medium (Elliott *et al.*, 1954) containing the 11 essential amino acids at 0.5 mmol/litre each (arginine, histidine, isoleucine, leucine, lysine, methionine, phenylalanine, serine, threonine, tryptophan and valine). All experimental cultures were at the transition stage between exponential and stationary growth. This phase lasts about 12 h and is characterized by a balanced duplication of cell number and cellular matter with duplication times of about 7 h at 29°C.

The labelling experiments were performed without external depletion of substrates or isotopes. Small portions of each individual ^3H -labelled amino acid were mixed after the individual specific radioactivity had been adjusted to 1 Ci/mmol (all ^3H -labelled L-amino acids from Amersham Buchler, including L-[2(n)- ^3H]methionine, L-[^3H]Threonine was purchased from New England Nuclear Corp., Boston, MA, U.S.A.). A portion (100 μ Ci) of this mixture was applied to 55 ml of cell suspension. At the times specified in the Figures or Tables, 10 ml samples were drawn off and analysed for the amount of soluble and insoluble radioactivity.

Quantitative procedures

Radioactive labelling was terminated by pipetting the sample into conical tubes kept in a mixture of 1 vol. of crystalline CaCl_2 and 3 vol. of crushed ice at -25°C. The cells were immediately pelleted for 1 min at 800g in a Christ swinging-bucket laboratory centrifuge. The soft pellet was washed twice by suspending it in 10 ml of ice-cold phosphate-buffered saline (see below) and centrifuged as described above. The best yield was obtained by using the following solution: 9.9 mM-NaCl, 0.33 mM-KCl, 4.9 mM-MgCl₂·6H₂O, 0.9 mM-CaCl₂·2H₂O, 2.4 mM-KH₂PO₄, 9.2 mM-Na₂HPO₄, 1.7 mM-spermidine and 36.5 mM-sucrose. The sucrose serves to make the fluid iso-osmotic with respect to the cells (120 mosm). The final precipitate was treated with hot HClO₄ (0.6 M, 1 ml/10⁶ cells) for 20 min at 75°C. The supernatant was recovered after centrifuging the samples at 20 000g for 15 min at 2°C in a Beckman J 21A refrigerated centrifuge. The pellet was suspended in 1 M-NaOH (1 ml/5 × 10⁵ cells) at 30°C for 18 h and subjected to protein determination by Coakley & James' (1978) modification of the method of Lowry *et al.*

(1951). The ninhydrin method as described by Rosen (1957) was used to measure the molarity of the summated amino acids and to obtain the correct basis for converting extracellular radioactivities (c.p.m.) into mol of amino acids. The total yield was calibrated against a reference consisting of the 11 essential amino acids. Linearity of the absorbance readings (on a Beckman SP. 25 spectrophotometer set to single beam) was obtained between a total of 10 and 500 μM . The radioactivity was determined by placing 50 μl portions of the acidic extracts and the protein hydrolysates into 10 ml pre-mixed scintillator (Unisolve I; Zinsser, Frankfurt/Main, Germany). The alkaline extracts had to be acidified by adding an equal volume of 1.2 M-HClO₄. Under the special condition of isotope depletion (no unlabelled amino acids present) the following recoveries were obtained: 50 μCi (1.11×10^8 d.p.m.) added at t_0 produced 4.400×10^7 c.p.m. (39.6% yield) in the external medium. After 15 min they were decreased to 3.164×10^7 c.p.m., which is a loss of 28.1% of the initial radioactivity. At the same time 6.192×10^6 c.p.m. were recovered from the acid-soluble and 7.096×10^6 from the high-molecular-weight material. These radioactivities together amount to 30.2% of the initial radioactivities. These values are not in themselves relevant for the actual experiments where the isotope concentration remained constant within the limits of accuracy, but they prove a sufficiently precise additivity of the radioactivity counts in the three relevant phases: medium, soluble fraction, and insoluble fraction.

The non-linear regression analysis was based on the general procedure outlined by Atkins (1971). The data were weighted with their errors and subjected to the iterative approximation with the provisional estimates as initial values of the parameters. All data sets led to converging minima. The program was stated in FORTRAN and is available from myself on request.

Results

The model reaction

The results of 16 independent experiments are summarized in the y column of Table 1. Each value of the external concentration, y , is the arithmetic mean of an apparently normally distributed sample with a common s_y of 0.093. If physical diffusion prevails, its rate depends on the concentration difference and approaches zero at equilibrium, E . In the dialysis experiment, therefore, the external concentration, y , changes as a function of time, t , according to:

$$y = E(1 - e^{qt}); \quad q < 0 \quad (2)$$

where q is a dimensionless rate constant. This notation implies that the concentration difference decreases by a constant factor per time interval. The rate of this process at any time is obtained from the first derivation of eqn. (2):

$$\frac{dy}{dt} = y' = -E q e^{qt} \quad (3)$$

Table 1. *Tracer net fluxes as a function of time*

t , Time (min); y , external concentration ($10^{-3} \times \text{c.p.m.}/50 \mu\text{l}$) and 95% confidence limits; y_a, y_b, y_c , as y except that an amount of radioactivity, n , was added at t_0 into the outer compartment; E and q , coefficients of regression (eqn. 2); N , mean difference ($y - Y$) and the third parameter; VR, variances of residuals; k_{+1} , initial rate for three-parameter regression, 95% confidence limits = $\pm 4 \times 10^{-3}$; k_{+2} , initial rate for two-parameter regression (the 95% confidence limits increase with k_{+2} and amount to 3×10^{-2} in the last column). The rates are listed in the units of y/min .

	t	y	n	y_a	y_b	y_c
		—	...	0.55	1.20	1.61
	60	1.69 ± 0.08		2.06	2.41	2.69
	120	2.71 ± 0.10		2.93	3.13	3.31
	180	3.25 ± 0.10		3.41	3.54	3.64
	240	3.60 ± 0.10		3.73	3.79	3.85
	300	3.86 ± 0.09		3.89	3.94	3.97
	360	3.97 ± 0.08		4.01	4.03	4.04
Three-parameter regression	E	4.101		3.538	2.944	2.485
	q	-8.45×10^{-3}		-8.76×10^{-3}	-8.76×10^{-3}	-9.13×10^{-3}
	N	0.065		0.620	1.209	1.645
	VR	8.313×10^{-4}		1.944×10^{-4}	2.750×10^{-5}	7.757×10^{-5}
Two-parameter regression	E	4.164		4.029	3.956	3.931
	q	-8.68×10^{-3}		-1.11×10^{-2}	-1.42×10^{-2}	-1.75×10^{-2}
	VR	7.130×10^{-4}		5.033×10^{-3}	1.388×10^{-2}	1.755×10^{-3}
	k_{+1}	3.47×10^{-2}		3.099×10^{-2}	2.579×10^{-2}	2.269×10^{-2}
	k_{+2}	3.60×10^{-2}		4.470×10^{-2}	5.620×10^{-2}	6.880×10^{-2}

The value of q was estimated from the ratios of the equidistant differences where E cancels out and q equals the slope of the linear plot $\ln \Delta y/t$. The first difference, $\Delta y_1 = y_1$, was omitted and its expected value, Y_1 , was obtained from the antilogarithm of the y -intercept. E was calculated by using eqn. (2): $E = Y_1/(1 - e^{q\Delta t})$. The background, N , was equated with the arithmetic mean of the differences $y - Y$. The final fitted values of these parameters are listed in Table 1. They served to calculate the initial rate, k_{+1} , by solving eqn. (3) for $t = 0$. Its 95% confidence limits were derived with respect to the covariance matrix of E , q and N .

Table 1 includes three sets of experiments, y_a , y_b , and y_c , where a background, n , was added at t_0 . It consisted of a small volume of ^3H -labelled amino acids placed into the outer compartment and producing the radioactivities indicated. Each value of n and y is the arithmetic mean from four independent experiments. The terms E , q , and N were estimated as above. All four sets of data were subjected to an alternative evaluation by approximating only two parameters, E and q . As judged from the one-sided F-test of the variances of the residuals and with three and four degrees of freedom respectively, the three-parameter fit is far superior in the presence of background. As a consequence the initial rates, k_{+1} and k_{+2} , increasingly diverge with increasing background.

Because the total radioactivity does not change, the internal concentration can be inferred from the external radioactivity and the rate of exchange stated as a function of the concentration difference at any time interval. The linearity of this function was overwhelmingly significant ($P < 0.00001$), which, of course, is trivial in Fick's diffusion. The alternative calculation, k_{+2} , was not consistent with Fick's law. It should be clear from the experimental set-up that N , here, does not have the meaning of a real background. Actually it causes the equilibrium to occur earlier in proportion to the amounts of amino acids added into the outer compartment. But regardless of the true nature of N , it has a strong effect on the initial rate, as demonstrated in Table 1.

Biological experiments

For *Tetrahymena*, as for other organisms, facilitated diffusion and active transport of amino acids are well established in the literature (Rasmussen, 1976). In ideal experiments it is possible to discriminate whether pool saturation occurs by facilitated or by physical diffusion (LeFevre & McGinniss, 1960). In reality, however, variation is very likely to interfere with the making of an easy decision. This is especially true if essential terms are still unknown, which would be needed in a data analysis of the model of a simple asymmetrical carrier (Nimmo, 1979). In the present case, there-

fore, facilitated diffusion was dealt with in a phenomenological fashion. Both diffusion and facilitated diffusion have in common that the rates of solute exchange tend to an equilibrium. But with a given equilibrium in facilitated diffusion the regression of the internal concentration on time initially has a stronger curvature (LeFevre & McGinniss, 1960). This causes the ratios of equidistant differences, $\Delta y_i/\Delta y_{i+1}$, to increase with time and to tend to unity. One empirical regression equation that takes this into account has the notation:

$$y = E \frac{t}{t + A} \quad (4)$$

(y = concentration at the time t , E = maximal value of y , A = the value of t at $\frac{1}{2}E$ = time of half-saturation).

In fact, eqn. (4) has the structure of the Michaelis-Menten equation. Therefore its coefficients, E and A , are easily derived from the direct linear plot as introduced by Cornish-Bowden & Eisenthal (1974). The slope of the initial tangent, then, is obtained from the first derivative of eqn. (4):

$$\frac{dy}{dt} = y' = E \frac{A}{(t + A)^2} \quad (5)$$

Data from 16 experiments are summarized in Table 2. Each value of y is the arithmetic mean of an apparently normally distributed sample. The regression was analysed as described in the introductory comments by applying both models, physical diffusion (eqn. 2) and facilitated diffusion (eqn. 4). The fitted values of the relevant parameters are listed in Table 2.

The least-square criterion revealed two facts. Again the three-parameter approximation is the closer estimate, as judged from the one-sided F-test of the variances of the residuals with two and three degrees of freedom respectively, and physical diffusion does better agree with the data than does facilitated diffusion. This latter statement, however, refers to a difference in the fit that is just nominal and not significant. Actually such agreement is to be expected, as both models deal with asymptotic approaches. Additional information would be needed if both models were to be distinguished. The same relations, consequently, are reflected by the initial rates. The 95% confidence limits were calculated from the respective covariance matrix. Although there is no significant difference within the three- and two-parameter approximations, the two-parameter estimate systematically leads to higher rates.

It is therefore concluded that, regardless of the model, a two-parameter regression analysis is ineffective and introduces a systematic overesti-

Table 2. *Amino acid-pool saturation as a function of time*

t, Time (min); *y*, summated amino acids ($\text{mol} \times 10^4$)/g of protein, with 95% confidence limits; *E*, maximal values of *y* at equilibrium; *q* and *A*, regression coefficients for the models of diffusion and facilitated diffusion respectively; *N*, 'background' and the third parameter; *VR*, variances of the residuals; *k*, initial rates of pool saturation with 95% confidence limits as derived from the covariance matrix. Rates are listed in the units of *y*/min.

<i>t</i>	5	15	25	35	45			
<i>y</i>	2.466 ± 0.19	3.263 ± 0.22	3.656 ± 0.19	3.824 ± 0.17	3.957 ± 0.21			
	Physical diffusion (eqn. 2)				Facilitated diffusion (eqn 4)			
No. of parameters	<i>E</i>	<i>q</i>	<i>N</i>	<i>VR</i>	<i>E</i>	<i>A</i>	<i>N</i>	<i>VR</i>
3	2.225	−0.070	1.808	4.156 × 10 ^{−4}	3.048	11.48	1.542	5.105 × 10 ^{−4}
2	3.759	−0.195	—	5.063 × 10 ^{−2}	4.220	3.76	—	6.627 × 10 ^{−3}
	Initial rates (<i>k</i>)							
No. of parameters	Physical diffusion				Facilitated diffusion			
3	0.156 ^{+0.071} _{−0.062}				0.262 ^{+0.097} _{−0.056}			
2	0.733 ^{+0.173} _{−0.132}				1.123 ^{+0.269} _{−0.182}

Fig. 2. Rate of uptake as a function of temperature (a) The rate is defined by the slope of the initial tangent and was calculated with respect to background as outlined in the text. (b) The rate is defined by the initial tangent as calculated (without any assumptions *a priori*) by means of Newton's interpolation. Each point represents the mean value for three independent experiments and each experiment contributed five evenly spaced data points.

mation of *k* if a background, *N*, becomes significantly larger than zero.

Neglecting the existence of *N* may strongly impair experimental evaluation. This is demonstrated by the experiments summarized in Figs. 2(a) and 2(b). At 1 h before the application of the radioactive amino acids, the total culture was transferred into the indicated temperatures and the initial rates were obtained from the means from three independent replicate experiments. In Fig. 2(a) the rates were obtained as described in the introductory comments but without further fitting. These data obey a linear plot ($\ln k$

versus $T/10$), where a Q_{10} of 1.66 is obtained from the antilogarithm of the slope. In Fig. 2(b) the rates are equal to the initial slope derived from Newton's polynomial interpolation (for details refer to textbooks of applied mathematics) and using the 0/0 origin as point of support. Regardless of the different scale of the ordinate, no meaningful Q_{10} value can be calculated from the scattergram. In this respect the rates in Fig. 2(b) are not compatible with van't Hoff's rule. This is very remarkable, because the contours of the individual polynomials look rather smooth.

Discussion

A method has been described that enables one to obtain the slope of the initial tangent in tracer experiments in the presence of background. Actually, the assumption of a non-zero recording at zero time appears to be rather unexpected. It is easy to demonstrate, however, that ^3H -labelled amino acids indeed are retained by albumin in a dialysis bag. By far the largest proportion of this radioactivity was washed free from the precipitated protein with cold HClO_4 . But even repeated washings in the cold did not remove a residual amount of radioactivity, which exhibited concentration-dependent saturation behaviour and was competitively decreased by addition of unlabelled amino acids.

This effect could not be quantitatively repeated with $[^3\text{H}]$ uridine or $[^3\text{H}]$ UMP. Though these labelled compounds also caused a background, its existence was more of academic interest, and it only changed the initial rates within the experimental error.

Therefore background due to adsorption so far seems to be specific for amino acids.

In many papers reporting rates of uptake under several conditions, the term 'rate' is not always explicitly defined, nor are the smooth curves connecting three or four data points with the origin. Some authors chose short labelling times in order 'to be in linear range' of time-dependent pool saturation. The linear range, however, is scarcely observed but assumed to occur between t_0 and the shortest pulse possible (Freemann & Moner, 1976; Wolfe, 1975; Dall'asta *et al.*, 1978). In some instances a background due to incomplete removal of the label is considered and subtracted from the data, with reference to inulin experiments (Reiser & Christiansen, 1972; Winter & Christensen, 1964). The best method available so far, and that certainly meets most biometric requirements, is not easily applied in studies of growth-specific rates of uptake because of rapid external depletion of the solute (Hoffmann & Rasmussen, 1972). After all, if one agrees to the existence of a background, the problem is not solved by pulses in the order of a few seconds.

It should be mentioned that more attention was given to the slope of the initial tangent than to the determination of the background itself. Most biological data are not likely to be as precise as the model reaction and therefore the amount of background will depend on the type of regression assumed (Table 2). Therefore, if the background is of real importance, more information would be required if the estimates are not to remain rather provisional. But as the initial rates are based on the first derivative of the functions involved, they are less inclined to change with the fitting. Therefore if the initial rate is of major importance, a procedure such as that outlined in the introduction is likely to suffice. Even a provisional estimate would still be closer than a two-parameter fit, whereas a one-point pulse experiment should be out of the question.

Though the prevailing theme of the present paper is mainly methodological, a few comments should be made regarding the biological background. As suggested by its Q_{10} of 1.66, the uptake of amino acids is very close to a value to be expected if diffusion is rate-limiting, whereas it should be above 2 if chemical catalysis is rate-limiting. This certainly opposes the findings of Hoffmann & Rasmussen (1972), who reported uptake of L-phenylalanine against a concentration gradient in *Tetrahymena*. In this organism, nutrient uptake in general has been found to be ATP-dependent (Rasmussen *et al.*, 1978), and uptake of amino acids occurs by facilitated diffusion (Orias & Rasmussen, 1979). However, the uptake of nutrients in *Tetrahymena* does not seem to occur via directly energized carriers, but rather to be driven by intracellular ATP-

dependent covalent modifications of the solutes and by pool consumption (results not shown). The obviously small Q_{10} of 1.66, therefore, appears to be related to a low rate of protein synthesis, owing to the growth conditions applied. At maximal growth rates the Q_{10} of amino acid uptake was 2.45 when measured at four evenly spaced temperatures within a 10 to 30°C interval. Preliminary work has shown that even under these conditions, some of the criteria attributed to facilitated diffusion are the result of dynamic interactions between uptake, pool size, and rate of utilization.

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