Biochimica et Biophysica Acta, 468 (1977) 127—145
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BBA 77734

THE COMPUTATION OF SATURABLE AND LINEAR COMPONENTS OF INTESTINAL AND OTHER TRANSPORT KINETICS

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(Received November 30th, 1976)

Summary

1. Published data for absorption kinetics have been fitted by non-linear regression to (i) a single Michaelis-Menten function, (ii) a Michaelis-Menten function plus a linear term and (iii) a sum of two Michaelis-Menten functions. A series of criteria have been drawn up to establish the goodness of fit in each case.

2. In 17 out of 35 cases the Michaelis-Menten function was the "best fit". In nine cases the "best-fit" model also included a linear term, but never was the sum of two Michaelis-Menten functions accepted to be the "best-fit" model.

3. Linearity of a Lineweaver-Burk plot was of unreliable diagnostic value in assessing goodness of fit.

4. Since the fit of a Michaelis-Menten function was often poor, simulated data sets with error were used to study the influence of experimental design etc. on Michaelis-Menten parameter estimation.

5. Precision of estimation of K_m is increased by increasing the number of data points, reducing their variance, increasing the data range and by straddling K_m in the observations. For a given constant number of observations there is no advantage in using replicate observations at few concentrations or single values at relatively many concentrations, or in using single values rather than means.

6. The caution necessary in interpretation of kinetic models is emphasized.

Introduction

Ever since Fisher and Parsons [1] demonstrated that the kinetics of glucose absorption by isolated rat small intestine could be described by a function of the same form as the classical Michaelis-Menten equation for enzymic reactions

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innumerable authors have applied this same equation to intestinal absorption data. This equation is also frequently fitted to the kinetic data for carrier-mediated transport in many different tissues [2,3]. Sometimes the experimental data have been shown to be fitted by this type of function within limits imposed by the quality of the data; frequently however, values for K_m (half-saturation concentration) and V (maximum rate of transport) have been quoted without any evidence that the Michaelis-Menten model was appropriate in the particular circumstances.

Forster and Menzel [4] showed that the kinetics of glucose absorption by rat small intestine in vivo exhibited two components, one of which was phlorrhizin sensitive and saturable. A similar claim for absorption of glucose, galactose and α-methyl glucoside in vivo has been made subsequently by Debnam and Levin [5]. One component was phlorrhizin sensitive, saturable, and was associated with a transmural potential difference, while the second component was insensitive to phlorrhizin, apparently linear with respect to concentration, and non-electrogenic. Debnam and Levin [5] showed that subtraction of the rate of the phlorrhizin-insensitive component from the overall rate yielded a corrected rate for the saturable component by itself: these corrected rates gave a K_m very close to that obtained from measurements of electrical activity [5]. The contribution of the phlorrhizin-insensitive linear component to the total sugar absorption observed was clearly significant and both Forster and Menzel [4] and Debnam and Levin [5] pointed out that it was substantial at high sugar concentrations. This implies that the model:

$$v = \frac{V \cdot [S]}{K_m + [S]} + k \cdot [S]$$

(where V, K_m and k are constants) is more appropriate to describe the relationship between absorption rate, v, and luminal (mucosal) concentration, $[S]$, than the unmodified Michaelis-Menten equation under the conditions of these experiments. As both pairs of authors pointed out, correction of the data for the linear term made a substantial difference to the estimated values of K_m and V for the saturable function. We have therefore explored the possibility of fitting the above model directly to the data without the need for experimental manoeuvres such as the abolition of one component with phlorrhizin.

Another model used for intestinal absorption is a sum of two Michaelis-Menten terms. This has been claimed by Munck and Schultz [6,7] and by Honegger and Semenza [8] to describe their data for absorption of leucine and lysine and of glucose and galactose (but not 6-deoxyglucose nor 3-*O*-methylglucose), respectively.

Since such two component functions may be applicable to the data of other investigations we set out to explore whether the single Michaelis-Menten function really was appropriate in instances where it had been used, and also to see whether any unsuitability of this model would be immediately obvious from simple tests such as the Lineweaver-Burk plot. We used a versatile non-linear regression programme to fit the above three models to experimental data from a variety of published sources, and have attempted to assess which models are appropriate for each particular data set. Most of the data used are for the kinet-

ics of intestinal absorption of several sugars and amino acids as measured in a variety of preparations from different species.

It soon became apparent that many published data sets appeared to conform poorly or badly to any of the models tested including the single Michaelis-Menten equation. Therefore we also explored the effects of the introduction of "experimental error" at known levels on the accuracy of fitting the Michaelis-Menten equation to simulated data sets, the effects of using various numbers of data points, and also the effects of making experimental observations over limited ranges of substrate concentration. We attempted to assess whether the accuracy of curve-fitting may be influenced by the use of a relatively large number of data points at different concentrations or of replicate measurements at relatively few concentrations. It should therefore be possible to evolve an efficient strategy for experimental design including (i) collection of data, (ii) curve-fitting to observations, and (iii) the testing and the comparison of the goodness of fit of various models for the kinetics of absorption with the maximum precision.

Methods

Curve-fitting. The real data used in this survey were taken from experiments published by various authors. Some of the sets were taken directly from tables of results and the remainder were calculated from figures. References to the sources of the data are given in Table II.

The following three models were fitted to each data set by non-linear regression:

(i) Michaelis-Menten function

$$v = \frac{V \cdot [S]}{K_m + [S]} \qquad \text{(I)}$$

(ii) Michaelis-Menten function plus a linear term:

$$v = \frac{V \cdot [S]}{K_m + [S]} + k \cdot [S] \qquad \text{(II)}$$

(iii) Sum of two Michaelis-Menten functions:

$$v = \frac{V_1 \cdot [S]}{K_{m_1} + [S]} + \frac{V_2 \cdot [S]}{K_{m_2} + [S]} \qquad \text{(III)}$$

For six of the data sets a simple linear equation was also fitted:

$$v = k \cdot [S] + a \qquad \text{(IV)}$$

In the above equations V, V_1 and V_2 are apparent maximum rates of absorption, K_m, K_{m_1} and K_{m_2} are apparent substrate concentrations at half-maximum rates of absorption (Michaelis constants), k is a rate constant, and a is a constant.

The three non-linear functions were fitted to each of the data sets using unweighted non-linear regressions or a non-parametric technique. Eqn. I was fitted by digital computer versions of the methods of both Wilkinson [9] and

Eisenthal and Cornish-Bowden [10]. Wilkinson's method was used for all the real data and for the simulation study on the effect of replication of observations; it gives the same results as Atkins' [11] method applied to the Michaelis-Menten function and is simpler. Eisenthal and Cornish-Bowden's method was used for the subsequent investigation on the simulated data sets (except for the study on data replication, to which it was not directly applicable since intersections giving $K_m = [S]$ would be artifactually produced) in the light of the findings of Atkins and Nimmo [12]. Eqns. II and III were fitted by modifications of the general programme of Atkins [11]. The linear regressions for Eqn. IV were computed on an Olivetti P101 desktop calculator. The computer programmes calculated a S.D. for each parameter estimated on the assumption that the sum of squares function was symmetrical about the minimum. Hence a coefficient of variation (CV) was calculated for each parameter: CV = (S.D. × 100)/parameter.

The data were not weighted since this is uncommon in published studies on intestinal absorption and since the variance in the original data was not known.

Assessment of goodness of fit. Often an individual test for the goodness of fit of a model was inconclusive. Therefore we applied five tests to the results of each curve-fitting regression in order to decide which one of the equations tested (if any) was the best fit for each data set. The methods for, and difficulties in, assessment of goodness of fit have been discussed by Atkins [13].

Firstly, it was noted whether the incorporation of the additional term in Eqns. II and III made a significant contribution to the overall value of v. There were many instances where Eqns. II and III tended to become Eqn. I (or IV) owing to non-significance of the additional term. In these instances the model with the additional term (Eqns. II or III) was rejected forthwith, and without further testing (test 7 of Atkins [13]).

The second test was to note how quickly the non-linear regression converged to the minimum sum of squares and whether the computer programme proceeded during the fitting of the function without the output of any error or warning messages, (see Atkins [11]). Usually a model which is a good fit will allow a rapid and definite convergence (test 5 of Atkins [13]).

The third test was to note the coefficient of variation of each parameter. Usually the value will be less than 10% for a good fit, although this value is dependent on the variance of the original data. We also noted whether the coefficient of variation was similar for each of the parameters in the model under consideration, i.e. whether each parameter was approximately equally well determined (test 6 of Atkins [13]).

The fourth test was to calculate a value for the closeness of fit of the observed and predicted values of the dependent variable (test 1 of Atkins [13]):

$$X^2 = \sum \frac{(y_{obs} - y_{calc})^2}{y_{calc}}$$

where y_{obs} was the experimentally observed value for absorption rate, and y_{calc} was the predicted value calculated by the non-linear regression. The lower the value of X^2 the better is the goodness of fit, but this does not provide an absolute test for goodness of fit since it, unlike chi-squared, has dimensions. How-

ever, it is still valuable for comparing the goodness of fit of several models fitted to the same data set. (Atkins [13] was in error by integrating the chi-squared function to give a probability: this can only be done if X^2 is without dimensions [14].)

The fifth test was a sign test ("Runs test") on the distribution of the residuals about the fitted curve [15—17]. We counted the number of times that the residuals changed sign along their sequence and the number of positive and negative residuals, respectively. Hence the probability was calculated that the residuals were distributed randomly about the curve, i.e. that the model was a good fit to the data (test 3 of Atkins [13]).

In a few instances the Bliss [18] Rankit test was also applied (test 2 of Atkins [13]).

Each model was assessed according to each independent test as unacceptable, poor or acceptable. Each of these assessments were then considered together in arriving at a decision on the goodness of fit and in the choice (where possible) of the best-fitting model. In a few cases where a test gave equivocal results or where different tests gave conflicting categories the overall judgement was weighted subjectively in the light of the experience of one of the present authors (G.L.A.).

Computer. The computer programmes for the non-linear regressions, for Eisenthal and Cornish-Bowden's method and for the statistical tests were written in IMP (which is an advanced language evolved in Edinburgh from Algol and Atlas Autocode) and run on the I.C.L. system 4-75 at the Edinburgh Regional Computer Centre.

Simulation of data with "experimental" error. Sets of "perfect" (i.e. error free) data were formed using the Michaelis-Menten equation (Model I) and setting $V = K_m = 1.0$. (As pointed out by Endrenyi and Kwong [19] this assignation of specific parameter values does not invalidate the generality of the conclusions in the simple case of Model I, the single Michaelis-Menten function.) The values of $[S]$ were geometrically spaced within the range 0.25—3.0 so that the values of v were 0.2—0.75. Sets of simulated "experimental" data (i.e. containing error in v) were calculated from these perfect sets by using series of normally distributed pseudo-random numbers of known mean and standard deviation, generated by the Edinburgh Regional Computing Centre subroutine RANDOM. The "perfect" values of v were multiplied by random numbers of mean value 1.0 to give normally distributed errors of constant relative magnitude (cf. Atkins and Nimmo [12]). This type of error was used since the real data had been unweighted in accordance with current practice in intestinal transport studies. Anyway, it has been shown that correct weighting of observations, at least in enzyme kinetics, is a more difficult problem than has commonly been assumed [20,21]. However, it is recognised that the use of constant absolute error might have given somewhat different results (see ref. 19). In the common instance where different data points are contributed by observations on different animals the errors may not be normally distributed, but no allowance has been made for this complex situation. Fifty such data sets were used for each simulation.

For the first investigation into the effects of data error and of the number of data points on the accuracy and precision of the calculated parameters, either

3, 5, 7 or 9 values were used from the full range of $[S]$. The random numbers had S.D. = 0.005, 0.01, 0.05 or 0.1, i.e. corresponding to relative data error levels of 0.5, 1, 5 or 10%, respectively.

For investigating the effect of data range 19 points geometrically spaced in $[S]$ were calculated. Five sets of data were then selected as follows: A, lower 7 points; B, middle 7 points; C, upper 7 points; D, lower 7 odd-numbered points and E, upper 7 odd-numbered points. The S.D. of the random numbers was 0.02, i.e. corresponding to relative data error level of 2%.

For comparing the methods of Lineweaver and Burk [22] and Eisenthal and Cornish-Bowden [10] we used the data from the first simulation with S.D. = 0.05, i.e. corresponding to relative data error of 5%.

For the last investigation into the effect of replicating points the data were 12 single values, six duplicate values or four triplicate values. The S.D. values of the random numbers were 0.01, 0.02, 0.05 or 0.1.

Results

Direct computer fitting of saturable plus linear kinetics

When Atkins' non-linear regression programme [11] was used to fit the function $v = V \cdot [S]/(K_m + [S]) + k \cdot [S]$ to the overall data of Debnam and Levin [5] and of Akedo and Christensen [23] the parameters obtained were as shown in Table I. In each instance the computation was straightforward, and the fit of the model to the data was judged to be good. Fig. 1 shows the overall data of Debnam and Levin [5] for glucose absorption together with the saturable and the linear components as resolved from the computer curve-fitting separately plotted.

Since there is a major discrepancy between our computed estimates of V and the values experimentally derived by Debnam and Levin [5] for glucose and galactose absorption (Table I) we examined in greater detail their kinetics for the linear component in the presence of phlorrhizin (their Fig. 4). It appeared that these data were not strictly linear, but could lie on a curve. We therefore fitted a Michaelis-Menten function plus linear term (Model II) to the data for their "non-saturable" (i.e. phlorrhizin-insensitive) absorption. Fig. 2 shows the results and it is clear that their "non-saturable" absorption contains a propor-

TABLE I

COMPUTED PARAMETERS FOR SATURABLE PLUS LINEAR KINETICS (MODEL II)

Published data were fitted directly to the function $v = v \cdot [S]/(K_m + [S]) + k \cdot [S]$ by the non-linear regression programme of Atkins [11]. Values obtained experimentally by the original authors are shown in parentheses. Data sets 1—3 are for galactose, glucose and α-methyl glucoside absorption by rate rejunum in vivo (Debnam and Levin [5]); set 4 is for α-aminoisobutyric acid uptake into rat diaphragm in vitro (Akedo and Christensen [23]). (Units are those of original authors).

Data set	V	K_m	k
1	179.4 ± 21.0 (113 ± 11.1)	48.9 ± 6.13 (32.4 ± 2.3)	1.33 ± 0.0861
2	104.8 ± 11.7 (85.8 ± 4.0)	20.0 ± 3.61 (22.6 ± 1.3)	0.854 ± 0.0762
3	39.3 ± 1.35 (48.8 ± 3.9)	20.7 ± 0.808 (31.2 ± 1.8)	0.713 ± 0.0262
4	6.79 ± 0.553 (7.4)	1.40 ± 0.264 (1.55)	0.248 ± 0.0272 (0.22)

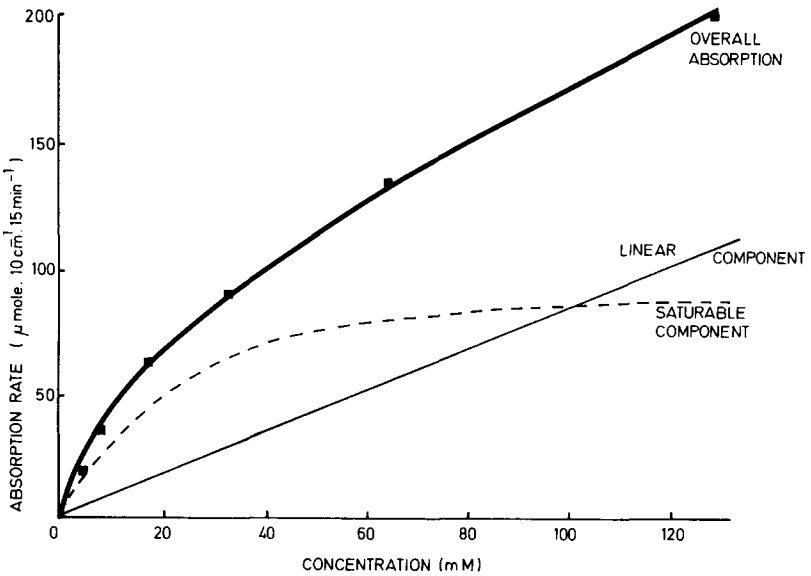


Fig. 1. Data of Debnam and Levin [5] for glucose absorption resolved into saturable and linear components directly by non-linear regression. ■———■, overall absorption; ———, linear component, $v = 0.854 \cdot [S]$; - - - - - -, saturable component, $v = 104.8 \cdot [S]/(20 + [S])$.

tion which obeys Michaelis-Menten kinetics. The K_m for this component is 20.0 ± 1.7 mM for glucose, and 45.8 ± 2.9 mM for galactose: these values are very close to those evaluated from our overall curve-fitting shown in Table I. A possible explanation is that phlorrhizin, under their conditions, has not completely inhibited the saturable absorption of glucose and galactose. The slopes of the linear components in Fig. 2 are not significantly different from the corresponding k values in Table I. Therefore the magnitude of the "true" linear component was probably the same in the presence and absence of phlorrhizin. The sum of the V values in Fig. 2 and those of the original authors agrees reasonably with our values in Table I.

Comparison of different kinetic models

Each of the three kinetic models was fitted where possible to each of a number of data sets taken from the literature. Table II summarizes the results and shows which model appears to be the best fit to each set of experimental data, which model(s) appear to be good fits to the data, and which model(s) clearly cannot fit acceptably to the data as judged from the criteria described in Methods above. The percentage effect on the estimated K_m of including the linear term in Model II (as compared to Model I) is also shown.

Diagnostic use of the Lineweaver-Burk plot in assessing goodness of fit

Many authors use the double-reciprocal plot of Lineweaver and Burk [22] to determine kinetic parameters although computerised methods of parameter estimation are now readily available and are acknowledged to be more efficient and precise. Therefore it was of interest to plot in this fashion data to which the simple Michaelis-Menten function (Model I) is known to be inappropriate

TABLE II

RESULTS OF MODEL FITTING TO PUBLISHED EXPERIMENTAL DATA FOR INTESTINAL ABSORPTION

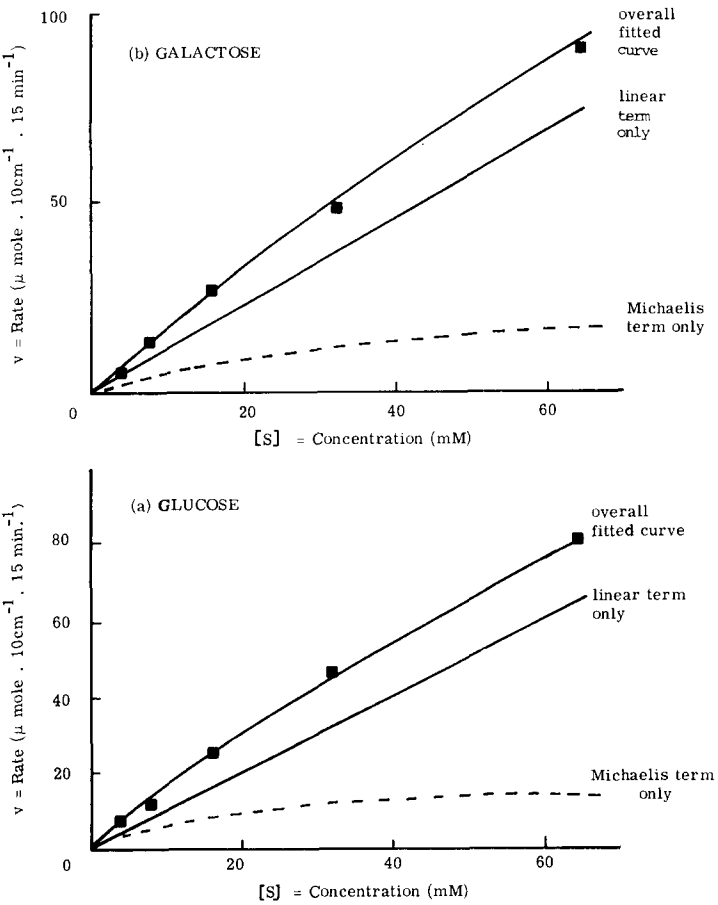
The three models considered were: I, Michaelis-Menten function; II, Michaelis-Menten equation plus a linear term; III, double Michaelis-Menten equation. The fit to each model was assessed as unacceptable (X), poor but probably acceptable (?) or good (√) according to the criteria described in Methods. The "best-fitting" model(s) are indicated by *. In the case of Model II the percentage effect of the inclusion of the linear term on the estimated K_m (compared to that estimated in Model I) is shown in parentheses in the instances where Model II was judged to be acceptable.

Author	Substrate	Species	Preparation †	Model I	Model II	Model III	See notes
1. Fisher and Gardner [24]	Glucose	Rat	b, c	√	√ (−21)	—	i
2. Fisher and Parsons [25]	Glucose	Rat	b, c	√	X	√	
3. Fullerton and Parsons [26]	Glucose	Rat	a, c	√ *	?	—	i
4; Rider et al. [27]	Glucose	Rat	a, c	X	X	X	ii. xi
5. Rider et al. [27]	Glucose	Rat	a, c	X	X	X	iii. xi
6. Rider at al. [27]	Glucose	Rat	a, c	?	? (−94)	X	iv
7. Rider et al. [27]	Glucose	Rat	a, c	?	? (−57)	X	v
8. Rider et al. [27]	Glucose	Rat	a, c	?	X	X	vi
9. Rider et al. [27]	Glucose	Rat	a, c	?	X	X	viii
10. Annegers [28]	Glucose	Dog	a, c	√ *	X	X	
11. Forster and Menzel [4]	Glucose	Rat	a, c	√ *	√ (−32)	?	xii
12. Forster and Menzel [4]	Glucose	Rat	a, c	√ *	X	X	xiii
13. Forster and Menzel [4]	Glucose	Rat	a, c	√ *	√ * (−21)	?	xiv
14. Debnam and Levin [5]	Glucose	Rat	a, c	?	√ * (−72)	X	
15. Debnam and Levin [5]	Galactose	Rat	a, c	?	√ * (−69)	X	
16. Annegers [28]	Galactose	Dog	a, c	√ *	X	X	
17. Barnett et al. [29]	Galactose	Hamster	b, e	√ *	? (−47)	X	
18. Honegger and Semenza [8]	Galactose	Hamster	b, g	?	? (−33)	?	
19. Honegger and Semenza [8]	3-Methyl glucoside	Hamster	b, g	? *	X	?	

20. Debnam and Levin [5]	α-Methyl glucoside	Rat	a, c	?	√ * (−73)	√	
21. Gracey et al. [30]	Fructose	Rat	b, (e)	√*	√ (−32)	?	
22. Adibi and Gray [31]	Methionine	Man	a, c	? *	? * (−20)	X	
23. Adibi and Gray [31]	Isoleucine	Man	a, c	? *	X	X	
24. Adibi and Gray [31]	Leucine	Man	a, c	? *	? (−28)	X	
25. Adibi and Gray [31]	Valine	Man	a, c	? *	X	X	
26. Adibi and Gray [31]	Phenylalanine	Man	a, c	√ *	X	X	
27. Adibi and Gray [31]	Tryptophan	Man	a, c	? *	X	X	
28. Adibi and Gray [31]	Threonine	Man	a, c	X	X	X	
29. Adibi and Gray [31]	Lysine	Man	a, c	? *	X	X	
30. Holdsworth [32]	Lysine	Man	a, c	√ *	X	?	
31. Munck and Schultz [6]	Lysine	Rabbit	b, f	?	√ * (−89)	√	viii
32. Munck and Schultz [6]	Lysine	Rabbit	b, f	?	√ * (−86)	?	ix
33. Munck and Schultz [7]	Leucine	Rabbit	b, f	?	√ * (−18)	√	
34. Larsen et al. [33]	Leucine	Rat	b,	√	√ (−86)	√	
35. Akedo and Christensen [23]	see note x			?	√ * (−67)	√	x

i, Too few data points for fitting Model III.
ii, Proximal intestine, very slow perfusion, radioactive measurement.
iii, Proximal intestine, very slow perfusion, chemical measurement.
iv, Proximal intestine, slow perfusion, radioactive measurement.
v, Distal intestine, very slow perfusion, radioactive measurement.
vi, Distal intestine, very slow perfusion, chemical measurement.
vii, Distal intestine, slow perfusion, radioactive measurement.
viii, Sodium present.
ix, Sodium absent.
x, Data for α-aminoisobutyric acid uptake into isolated diaphragm muscle.
xi, Model IV (straight line) was also fitted to data sets 4—9. For data sets 4 and 5 the fit, although poor, appeared to be better than that for Models, I, II or III.
xii, Their Fig. 4.
xiii, Their Fig. 5 (NaCl perfusate).
xiv, Their Fig. 7 ($MgSO_4$ perfusate).

† Types of preparation: a, in vivo; b, in vitro; c, perfusion; d, everted sacs; e, tissue slices; f, flux chamber; g, uni-directional fluxes.

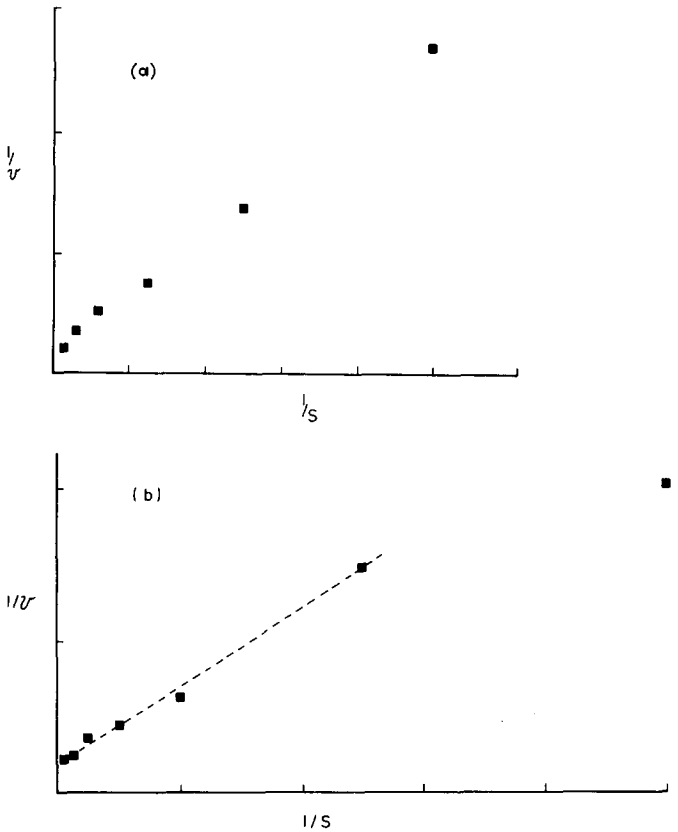


Figs. 2 (a and b). Data of Debnam and Levin's [5] "linear" component in their Fig. 4 for glucose (a) and galactose (b) absorption in presence of phlorrhizin resolved by non-linear regression into linear (———) and saturable (- - - - -) components. The equations for the overall fitted curves are: (a) glucose $v = 20.0 \cdot [S]/(20.0 + [S]) + 1.0 \cdot [S]$ and (b) galactose $v = 29.7 \cdot [S]/(45.8 + [S]) + 1.2 \cdot [S]$.

in order to see whether this would be obvious from the non-linearity of the plot.

In some instances (e.g. Munck and Schultz's data for lysine [6]; Debnam and Levin's data for galactose and α-methyl glucoside [5]) the plot was clearly curvilinear and would suffice to reject Model I. However, the data of Debnam and Levin [5] for glucose and of Munck and Schultz [7] for leucine (especially their lower 6 points) give fairly good straight lines (Fig. 3), and the use of this criterion would lead to the erroneous belief that Model I was appropriate.

In the light of the poorness of fit already experienced we turned our attention to features of data collection which may influence the precision and goodness of fit. These considerations are also important in enzyme kinetics and other applications of the Michaelis-Menten equation.



Figs. 3 (a and b). Lineweaver-Burk double reciprocal plots of data of (a) Debnam and Levin [5] for glucose absorption and (b) Munck and Schultz [7] for leucine absorption. The simple Michaelis-Menten function (Model I) was not a good fit to these data sets (see Table II).

Effect of data error and number of data points on fitting of the Michaelis-Menten equation

Data sets were computer simulated which had a mean relative "experimental error" of 0.5, 1.0, 2.0, 5.0 or 10.0%, and which had 3, 5, 7 or 9 data points geometrically spaced in $[S]$ over the concentration range from $0.25 \times K_m$ to $3.0 \times K_m$. Fifty data sets were simulated for each set of conditions, i.e. 1000 sets altogether (see Methods). A single Michaelis-Menten function (Model I) was then fitted to each using the computer version of Eisenthal and Cornish-Bowden's [10] method. The variability introduced into the estimated values of K_m, as expressed by the coefficient of variation of the estimated parameter, is shown in Fig. 4.

A further simulation study with 5% relative data error was made using an unweighted Lineweaver-Burk plot fitted by a first-order regression to estimate K_m since this simple method is commonly used in studies reported in the literature. Another simulation study was made at 5% relative error using Eisenthal and Cornish-Bowden's [10] method in order to test whether the 50 simulated data sets used were sufficient to give consistent results. The results in Table III show that the two replicate simulation studies using the Eisenthal and Cornish-

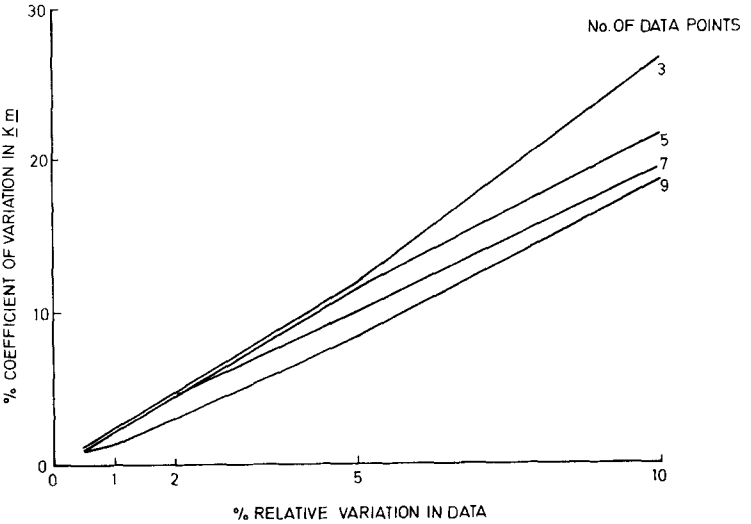


Fig. 4. Effect of data error and number of data points on precision of estimation of K_m in a simple Michaelis-Menten function (Model I) by Eisenthal and Cornish-Bowden's [10] method. Each point was constructed by the generation of 50 simulated data sets.

Bowden method gave very similar results, and that the relative error in K_m estimation was consistently substantially lower than when the unweighted Lineweaver-Burk plot was used.

Effect of range of experimental data points on fitting of the Michaelis-Menten equation

Since widely different ranges of experimental data points (relative to K_m) have been used by different investigators it was of interest to examine the effect of this choice on the fitting of a Michaelis-Menten function.

TABLE III

EFFECT OF NUMBER OF DATA POINTS AND COMPARISON OF THE EISENTHAL AND CORNISH-BOWDEN [10] AND UNWEIGHTED LINEWEAVER-BURK METHODS ON THE PRECISION OF ESTIMATION OF K_m FOR SIMULATED DATA SETS

In each case 50 data sets were simulated with 5% relative data error.

Number of data points	Coefficient of variation (%) of estimated K_m		
	Unweighted Lineweaver-Burk regression	Eisenthal and Cornish-Bowden [10] method	
		First simulation	Second simulation
3	16.2	11.7	11.3
5	15.0	11.5	11.6
7	13.7	9.9	9.0
9	11.7	8.4	8.2

TABLE IV

EFFECT OF RANGE OF EXPERIMENTAL DATA POINTS ON FITTING OF THE MICHAELIS-MENTEN EQUATION

Data sets were simulated with seven data points geometrically spaced over the range $[S] = 0.25 \times K_m$ to $[S] = 3.0 \times K_m$ or over part of this range, and with 2% relative data error. $K_m = 1.0$; $V = 1.0$. Fitting was by the Eisenthal and Cornish-Bowden method [10].

Range of data	Coefficient of variation (%) of estimated K_m
Whole range ($[S] = 0.25$—3.0)	4.6
First 2/3rds ($[S] = 0.25$—1.3103)	6.2
Second 2/3rds ($[S] = 0.5723$—3.0)	5.4
First 1/3rd ($[S] = 0.25$—0.5723)	15.6
Second 1/3rd ($[S] = 0.5723$—1.3103)	13.3
Third 1/3rd ($[S] = 1.3103$—3.0)	15.0

Data sets were simulated with seven data points and 2% relative data error. The data points were geometrically spaced in $[S]$ either in the range $[S]/K_m = 0.25$—3.0 or over part of this range, viz. either the first, second, or last third of the total range or over the first or last two-thirds of this range. The results using Eisenthal and Cornish-Bowden's [10] method are shown in Table IV. They show that the precision of estimation of K_m is substantially increased as the range over which observations are taken is increased, and that precision is improved if the experimental data straddle the value of K_m.

It was also noted that the mean value of the estimated K_m was never significantly different from the true value ($K_m = 1.0$) nor was it significantly different from the median value.

Comparison of curve-fitting to relatively few but replicated observations and to numerous unreplicated observations

A question which often occurs in the design of kinetic experiments is whether there is advantage in collecting observations at a large number of concentrations or in making replicate observations at relatively few concentrations. This was examined by a study in which data sets with 12 data points at relative data

TABLE V

EFFECT OF EXPERIMENTAL DESIGN ON PRECISION OF ESTIMATION OF K_m IN A SIMPLE MICHAELIS-MENTEN FUNCTION

Data sets containing 12 observations either as four, six or twelve points with 1, 2, 5 and 10% relative error were simulated. Fitting was by the Wilkinson method [9]. Coefficient of variation (%) in estimated K_m

Data type		Relative data error			
		1%	2%	5%	10%
A	12 individual points	2.19	3.95	11.52	19.40
B	6 points, each the mean of duplicates	1.80	3.91	10.61	21.01
C	4 points, each the mean of triplicates	1.91	3.39	9.19	20.14
D	12 points, 2 at each of 6 concentrations	2.00	4.96	9.76	18.62
E	12 points, 3 at each of 4 concentrations	1.58	3.90	7.75	18.83

error 1, 2, 5 and 10% were simulated. In one series of 50 simulated sets (A) the 12 observations were generated with $[S]$ geometrically spaced over the range $[S]/K_m$ = 0.25—3.0 (i.e. $[S]$ = 0.25—3.0, with K_m = 1.0). In the second series (B) six concentrations were used, again geometrically spaced over this range, and duplicate values were simulated at each concentration. The mean of two values of v was then used in the non-linear regression. In the third series (C) the mean of triplicate values at each of four concentrations was used. Series D was calculated using each duplicate value (not means) at six concentrations. Finally, series E was calculated using each triplicate value (not means) at four concentrations.

The results shown in Table V demonstrate that there is no significant advantage in any one of these forms of data collection.

In all cases the calculated mean K_m did not differ significantly from the true value.

Discussion

Direct fitting of non-linear kinetic functions

The fitting of each of the two-component models, the Michaelis-Menten plus linear term function, and the double Michaelis-Menten function proved to be straightforward using simple modifications of Atkins' [11] general programme for non-linear regression. Thus it is possible to fit directly a two-term function to data without the need to abolish one component experimentally as was done by Debnam and Levin [5]. Potentially this should increase the precision of the estimated parameters since it is dependent on fewer experimentally observed data points. On the other hand the use of the inhibitor gives additional information about the nature of the transport processes involved.

In several instances the estimated parameters differed appreciably from those originally reported (Table I). Thus Debnam and Levin [5], who used phlorrhizin to abolish the saturable component of absorption, obtained lower values for V than we obtained using direct computer fitting. However, analysis of their apparent linear components showed that they contained a saturable component, i.e. the inhibitor had apparently not completely abolished the saturable component (Fig. 2). This would account for the underestimate of V. The use of the direct fitting method with Model II is clearly better, in that it obviates the need for any assumption that one component has been annihilated experimentally.

Fitting of published absorption data to a Michaelis-Menten model

Table II shows that in many instances the simple Michaelis-Menten function was found to be the most appropriate of the three models tested, see also Table VI. However, in a large number of cases the fit was poor according to the criteria discussed in Methods even though the simple Michaelis-Menten function had been the model used by the original authors. Such poorness of fit could be due either to an inappropriate model or to inaccurate data. Our subsequent tests on simulated data suggested that the curve-fitting methods were generally tolerant of data with high relative error. Therefore the poorness of fit seen with many of the data sets may reflect an inappropriate choice of model.

TABLE VI

SUMMARY OF RESULTS FROM TABLE II

The number of data sets, out of a total of 35, which were accepted to be "a good fit" or were judged to be "the best fit" to each of the three models is shown.

	Model		
	I	II	III
Good fit [a]	13	12	6
The best fit [b]	17	9	0

[a] i.e. marked √ in Table II.
[b] i.e. marked * in Table II.

However, such incompatibility of data and model is only seen if one applies specific tests of goodness of fit as we have done. Fig. 3 shows that inspection of a Lineweaver-Burk double reciprocal plot for linearity is not a uniformly reliable criterion. Christensen (ref. 34, p. 140) also provides an example of a sum of two Michaelis equations (as our Model III) which gave an apparently linear Lineweaver-Burk plot with meaningless apparent K_m and V values. In some instances, however, this test did prove adequate for rejection of the simple Michaelis-Menten model. Walter [35] also showed that the Lineweaver-Burk plot was insensitive and suggested that the Hofstee [36] plot of v against $v/[S]$ was preferable. Obviously the choice of an inappropriate model leads to wildly inaccurate estimates of parameters. Thus we agree with Debnam and Levin [5] who showed how inclusion of the linear term in Model II could greatly influence the estimate of K_m for the saturable component. Table II summarizes the percentage effect of the additional term on the estimated K_m value. Therefore if one had chosen an inappropriate model, e.g. Michaelis-Menten only, the estimated K_m could be very inaccurate.

Fitting of published data to other models

Only in the cases of the data of Debnam and Levin [5], Munck and Schultz [6,7], Akedo and Christensen [23], and one set of Forster and Menzel [4] was Model II (Michaelis function plus linear term) found to be the "best fit" of the three models considered. Other data for sugar absorption do not lead to the same conclusions as Debnam and Levin's [5] results and so a phlorrhizin-insensitive "linear" component does not appear to be a general feature of sugar transport. Batt and Peters [37] observed 95 and 100% inhibition of jejunal and ileal absorption of galactose by the rat in vivo by phlorrhizin at 10^{-2} M and concluded that there was "no significant passive transfer component". In no instance did we accept the double Michaelis-Menten equation (Model III) as being the best-fit model, even though Munck and Schultz [6,7] and Honegger and Semenza [8] had concluded that such a model fitted their data.

The results of Table II are summarized in Table VI which shows that the single Michaelis-Menten function was the best-fit model in the greatest number of cases. However, only in 13 out of 35 cases was the fit judged to be good. The frequent occurrence of instances where more than one model was accepted

to be "a good fit" emphasizes the hazard in assuming that any particular model to which data seem to fit is necessarily the most appropriate one.

Influence of experimental design on fitting of the Michaelis-Menten equation

Since this equation (Model I) and many published data sets appeared to be incompatible, we used simulated data sets at various levels of relative "experimental" error and known values of K_m and V. As expected, the precision of K_m estimation is improved substantially as the variability in the data is decreased, on average the coefficient of variation of K_m was around twice the coefficient of variation in the original data points (Fig. 4). Also, precision was increased as the number of data points was increased (Fig. 4), although the magnitude of this effect was much less than expected. As this latter result was surprising the simulation study was repeated, and the results were closely similar to those already obtained and reported in Fig. 4. While this result may appear to support computations made with relatively few data points it must be stressed that non-conformity to a particular model may only be readily recognised when there are more numerous data points. Therefore we do not recommend the use of only, for example, 3 or 4 points for fitting a Michaelis-Menten equation. Precision of parameter estimation was also increased when the range of concentrations over which observations were made was increased, and especially when this range included the K_m, see Table IV. Endrenyi and Kwong [19] also concluded that as wide a range of concentrations as possible should be used where the relative data error was constant. On the other hand they showed that if the absolute error of observations is constant precision was gained by restricting observations to velocities greater than $0.2 \times V$. However, although the total number of observations made influences the precision of parameter estimation, there was no advantage either in taking replicate values at each of relatively few concentrations or in using single values at a relatively large number of individual concentrations (Table V). Where replicate observations are made either each individual value or their means can be used in the parameter estimation with equal precision (Table V). One advantage, however, in making replicate measurements at each concentration is that it allows the precision of the original experimental observations to be assessed. This is necessary if the observations are to be weighted on a rational basis, although it has been shown that this is only valid with large numbers of replicates [21]. The practice of weighting data for model-fitting is rare, especially in published studies of intestinal transport. Another advantage of replication of observations is that it allows error bars to be shown on kinetic plots.

Methods for curve-fitting

The availability of computers and their software makes the older graphical curve-fitting methods obsolescent. The results in Table III show the superiority of the method of Eisenthal and Cornish-Bowden [10] over the unweighted Lineweaver-Burk plot. They also confirm that 50 data sets were adequate to give valid results in each simulation study since the two separate series of simulated data gave closely similar results. Atkins and Nimmo [12] have already shown that Eisenthal and Cornish-Bowden's method [10] is more reliable than that of Lineweaver-Burk [22]; use of the latter ought nowadays to be restricted

to the evaluation of approximate initial estimates of parameters for use in computer methods. Endrenyi and Kwong [19] also concluded that use of the Lineweaver-Burk plot should be avoided. For further references see Fajszi and Endrenyi [38]. Non-linear regressions allow the direct fitting of data to non-linear functions such as the Michaelis-Menten equation without prior transformation (which itself would introduce biassed weighting into the raw data). Programmes such as that of Atkins [11] can be used with modifications in only a single statement to fit a wide variety of functions: they are therefore highly versatile. The success of using the Eisenthal and Cornish-Bowden [10] method for fitting the simulated data sets in this study suggests that the poorness of fit which was seen in many "real" published data sets was due to a genuine incompatibility of model and data rather than to inadequacies in our curve-fitting procedures or to too stringent model-testing.

Testing of goodness of fit

The results in Table II reinforce our view that tests of goodness of fit are necessary when experimental data are fitted to a model and estimates of parameters such as K_m and V are quoted. In assessing goodness of fit of the data reported in Table II we used a minimum of five independent tests described in Methods. Frequently, the use of only one or two of these tests would have led to a different conclusion. Therefore as many tests as possible should be applied before a particular model is claimed to be a "good fit" to experimental observations. At present few authors apply any such criteria, and our results suggest that one cannot confidently assume that intestinal absorption kinetics invariably obey the Michaelis-Menten equation. In some cases poorness of fit may be directly attributable to technical difficulties: for example, in perfusion experiments at very low flow rates radial diffusion within the fluid in the intestinal lumen may become rate limiting, and this might explain the almost linear kinetics observed by Rider et al. [27]. Thus unless this is overcome by a method such as the segmented-flow technique of Fisher and Gardner [24] the kinetics observed may be a property of the experimental system rather than of the absorbing cells (see Wingate [39]).

Interpretation of kinetic models

Great caution is required in the interpretation of best-fit models for the kinetics of absorption as was stressed by Fisher and Parsons [25] when they first showed that the Michaelis-Menten equation, or the Langmuir [40] adsorption equation, could describe the kinetics of glucose absorption. Transport across the intestinal mucosa is an extremely complex multi-stage process with many possible interactions: it is most unlikely that the kinetic characteristics of a rate-limiting step can be interpreted in terms of a model with physical significance. Additionally as pointed out above and by Wingate [39] the kinetics may be influenced by the experimental system. Therefore it is not legitimate to accept Model I as necessarily characteristic of a one-carrier process nor Model III as of a two-carrier process. Anyway, Fisher and Gilbert [41] demonstrated theoretically that "the appearance of fit to a single carrier hypothesis is no bar to the existence of a multiple carrier system". Similarly the linear term in Model II is not necessarily indicative of a passive diffusive process, although

Debnam and Levin [5] assumed that this was so. Indeed, Christensen and Liang [42] concluded that the non-saturable component of amino acid uptake into Ehrlich cells and rat jejunum was unlikely to be a diffusion component on the grounds that it showed (i) structure specificity, (ii) a high temperature coefficient, and (iii) a strong pH dependence.

Furthermore several studies have shown that the values of both K_m and V for several substrates, species and preparations differ in different regions of intestine (e.g. Batt and Peters [37] who give further references) although Fisher and Parsons [25] found variability only in V. There is no reason to suppose that these parameters change abruptly between jejunum and ileum. Indeed Fisher and Parsons [1] showed there to be a continuous linear relationship between glucose absorption rate and distance from the ileo-caecal valve: since these authors [25] found the K_m to be independent of position this could be interpreted in terms of a continuous gradient of V down the length of the intestine. Thus any attempt to express the overall absorption rate observed over a length of intestine in terms of constant K_m and V values is necessarily an oversimplification and the actual parameter values obtained will depend on the length of the segments used and the region from which they were taken as well as on the technique used. Also, even if the absorption rate of any single point along the length of the intestine observes Michaelis-Menten kinetics it does not follow that the overall absorption measured over a length of intestine will do so. This might contribute to the poorness of fit already discussed.

In spite of these reservations about interpretation, kinetic parameters remain valuable as operational definitions of observed transport phenomena.

Acknowledgments

We are grateful to Professor R.B. Fisher and Dr. I.A. Nimmo for helpful comments and to Miss Caroline Thompson for technical assistance. M.L.G.G. thanks the Medical Research Council for a project grant.

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