Letters to the Editors

Amino acid partitioning and milk protein synthesis

Study of the regulation of milk protein synthesis has focused primarily on the transcriptional control of gene expression. The possibilities for translational or post-translational regulation are still largely overlooked despite increasing evidence that mammary protein secretion is not constitutive (Turner *et al.* 1992) and that synthesis depends on an intact secretory mechanism (Rennison *et al.* 1993). Therefore, it was encouraging to read the paper by Bequette *et al.* (1994) which explored the possibility that regulation of milk protein output is exerted through precursor supply for protein translation. The paper reported results which were interpreted to indicate either a contribution of systemic dipeptides to mammary protein synthesis or a recycling of amino acid from degraded intracellular protein through an intermediate precursor pool. The authors' conclusions were based wholly on the observed time course of casein-amino acid isotopic enrichment in milk after continuous infusion of radiolabelled leucine, phenylalanine or methionine: enrichment reached a plateau only after about 12 h. However, we must offer a more prosaic explanation for the kinetics of casein–amino acid labelling they observed.

Given the regimens employed to obtain milk in the two series of experiments, a more likely explanation for the findings is that the long time-course of labelling was simply related to incomplete removal of unlabelled milk either before the infusions were begun (residual milk) or each hour. In other words, the protracted increase in isotope enrichment may reflect the sampling of milk in which isotopically labelled casein progressively dilutes unlabelled casein in residual milk. The presence of residual milk would have been even more important in the second experiment described since no exogenous oxytocin was used before the infusion.

It was in order to tackle the sort of questions that Bequette *et al.* (1994) attempted to answer that Linzell (1967) developed the hourly-milking technique in goats since he realised the importance of complete emptying of the mammary gland both before and during such experiments. These studies were extended by Linzell & Peaker (1971) who also showed, together with Maltz *et al.* (1984), the effects of hourly milking without administering a physiological dose of oxytocin each hour. The technique had also been used to show the relatively rapid and contemporaneous appearance of those milk constituents secreted by the golgi route, including casein from leucine (Neville & Peaker, 1979).

It is, therefore, incumbent on the authors to demonstrate that completeness of milk removal was not a factor contributing to the casein–amino acid enrichment profile observed in their study.

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Danger in the interpretation of polyester-bag method parameters fitted by computer software

Computer software is a useful tool nowadays. Several programs are readily available when non-linear curve fitting is needed, such as for describing disappearance in the polyester-bag method. Data obtained from rumen-incubated polyester bags are generally described using a first-order kinetics model as described by Ørskov & McDonald (1979). The model is represented as follows:

$$y_t = A + B(1 - e^{-ct}), \quad (1)$$

where y_t is disappearance from incubated bag at time t (h), A denotes the soluble fraction, B denotes the degradable but insoluble fraction and c denotes the fractional degradation rate of B. Typically, A, B and c are constants fitted according to iterative procedures where the goal is to minimize the residual sum of squares (RSS). Therefore, A and B really denote computer-fitted parameters for the non-linear equation best describing the data set. The asymptotic ruminal degradation y_∞ is estimated as:

$$y_\infty = A + B. \quad (2)$$

A and B being computer-fitted parameters, may not be equal to the real measurement of the soluble fraction (a) or insoluble but potentially degradable fraction (b). Software curve fitting occasionally may yield estimated parameters where $A < a$. As a consequence it can result in values for B larger than b due to the relationship:

$$B = y_\infty - A. \quad (3)$$

To solve this problem we have to estimate b according to our measurement of the soluble fraction as:

$$b = y_\infty - a. \quad (4)$$

This procedure does not affect y_∞ as:

$$y_\infty = a + b = A + B. \quad (5)$$

In additional to this, McDonald (1981) and Dhanoa (1988) introduced a lag phase into the equation to account for this fact. The model now becomes:

$$y_t = A + B(1 - e^{(-c(t-T))}), \quad t \geqslant T \quad (6)$$

where $T \geqslant 0$ becomes lag time (h) before the start of degradation of b.

No importance has been given before to the probability of $A > a$, even though it can be a common case. As a consequence of $A > a$, computer software will yield negative T values. This fact had been seen as apparently not having an impact on the estimation of the other parameters. However, if we are trying to describe particular kinetics, it is obviously wrong to assume the possibility that there is a negative lag phase which means that the disappearance from polyester bags starts before incubation. According to equations 3, 4 and 5, whatever the estimates of a, b, A and B, no change occurs in y_∞. In spite of this fact there is no ground to assume that it is the same case for c. If the fraction a has been experimentally measured and b can be estimated with certain degree of accuracy from

Table 1. *Parameters for ruminal disappearance in the polyester bag method, fitted according to the eqn* $y_t = A + B\,(1 - e^{-c(t-T)})$ *by different procedures*

Parameters...	A (%)	B (%)	c (%/h)	T (h)	y_∞ (%)
Without restriction	17·94	27·51	1·72	−6·8	45·46
With restriction*	14·49	29·16	2·31	0·0	43·65
'Manual procedure†	14·50	30·96	2·46	0·0	45·46

* Restriction on iterative procedure where $A > a$ is not accepted, $a = 14{\cdot}50$.

† c estimated according to equation 7; with y_t value for $t = 12$, and $y_\infty = 45{\cdot}46$: $c = -\{\ln[(45{\cdot}46 - 22{\cdot}43)/30{\cdot}96]\}/12$.

equation 4, certainly only the parameter c is needed. The first and older approach for solving equation 1 was a manual procedure where:

$$c = -\{\ln[(a + b - y_t)/b]\}/t. \tag{7}$$

In the manual procedure the observed values of disappearance were plotted against time and then a point selected 'by eye' in time t where the values of y_t still were changing rapidly. This point, so-called 'the most sensitive area of the curve', should yield a good estimate of c (Ørskov *et al.* 1980). This procedure has been superseded by the use of more 'accurate' computer software data handling. Let us assume that in addition to the 'best' least squares equation, it is desired that the estimate of A cannot be larger than a, as the latter is an experimentally measured quantity. Therefore, let us use an interactive procedure such as a computer spreadsheet and the Steepest Descent method, with the restriction on the iterative procedure that $A > a$ is not permissible as it will yield negative T values. An example is given in Table 1, where data from *Pennisetum purpureum* is used and $t = 12$, 24, 48, 72, 96 and 120 h; $y_t = 22{\cdot}43$, 28·73, 32·02, 38·32, 39·91 and 42·08; $a = 14{\cdot}50$.

The restriction in the iterative procedure certainly will result in larger RSS, little or no change in y_∞, but a change in c when compared with an unrestricted iterative procedure. The implication of this result is a change in the rumen degradation kinetics of the feed, with a 35% increase in c in this example; in some cases the difference may be too insignificant to be of any consequence; unfortunately in other cases the increase in c, when the iterative procedure is restricted, can be many times the value estimated without any restriction. The interpretation of the parameters can change priorities, from looking first to increase c as a way to improve the rumen utilization and degradation of feed, and then as a consequence an increase in its outflow, to looking to increase the rumen outflow of the feed as first priority or as equal priority with c.

With the widespread use of nutritional models where the nutrient supply from a feed is based on data generated from polyester bag methodology, care should be taken in correct data manipulation and interpretation. Moreover, we should not forget that computer software is a tool and not the definitive answer, not only for this methodology but for anyone using a non-linear analysis where the result $A > a$ is unacceptable.

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Consequences of model choice when analysing polyester bag data

Physiologically unacceptable results can be indicative of an inappropriate model or that the data are not compatible with the requirements of the selected model. In biology very few models are applicable universally. The defining conditions and constraints of a model need to be recognized if erroneous conclusions are to be avoided. Such considerations are necessary when selecting a model to analyse polyester bag data. The simple exponential model (t = incubation time, y_t = cumulative loss, A and B are constants and c (/h) is the fractional rate of degradation):

$$y_t = A + B(1 - e^{-ct}), \tag{1}$$

as advocated by Ørskov & McDonald (1979) and later extended to include a discrete lag (McDonald, 1981; Dhanoa, 1988), is not appropriate for all substrates because the degradation kinetics involved are more complex. To this end other models (Robinson *et al.* 1986; Dhanoa *et al.* 1995) have been developed which allow for underlying kinetics that may vary from diminishing returns to sigmoidal type.

The problem (fitted intercept greater than the measured soluble fraction y_w) highlighted by Sandoval-Castro (1996) is not too common when evaluating animal feeds; however, it is predictable. It is possible that some fractions of the substrate are more rapidly degradable compared with the remaining fractions and as a consequence modification or enhancement of polyester bag profiles in the earlier stages of fermentation leads to departures from first-order kinetics. Thus there may be accelerated or enhanced fermentation due to the presence of rapidly degrading fractions. On the other hand, retarded or inhibited degradation leads to discrete lag type or sigmoidal profiles. In the latter case, if the simple exponential model (equation 1) is fitted then the intercept A will generally be less than the value y_w (Dhanoa, 1988). This is because the mathematical properties of the simple exponential allow only the diminishing returns type shape not the sigmoidal. Significantly lower or higher values of the intercept compared with the wash value point to incompatibility between the data and the selected model. If the data are deemed to be adequate then one should look at variants of the simple exponential model or switch to other suitable models. Unfortunately this process is open to subjectivity and great care is needed in the decision-making. To avoid this subjective input, Dhanoa *et al.* (1995) used an extra time-dependent parameter to modify the fractional rate so that most shapes are catered for. Procedure for modelling zero-order kinetics was proposed by France *et al.* (1990). Such profiles were reported by Mosimanyana & Mowat (1992) for xylose-treated soyabean meal (beware of typographical error in the formula quoted).

Examination of the data given by Sandoval-Castro (1996) suggests inadequacy of the experimental design. Presumably these data were collected on the assumption that the simple exponential model was applicable. This is unlikely to be true given the problems encountered. Departures from first-order kinetics usually manifest themselves in the earlier stages of degradation and to identify the shape, adequate data are required over this period.

Unfortunately in this example no such data were recorded and the opportunity to test an alternative model is slight. In this example and generally, at least two more samples are required between zero and 12 h. Mertens (1973) considered this aspect and suggested 0, 3, 6, 12, 18, 24, 36, 48 and 72 h as a suitable design that permits use of many models. For low-quality feeds, 96 and 120 h values may also be necessary. To minimize and place sampling times (e.g. by using optimal design criterion; Box & Lucas, 1959) one needs to assume a model that is to be fitted. As no single model is applicable or acceptable universally, enough data should be collected to test the suitability of other models which may have more parameters to describe a variety of shapes in the earlier stages of fermentation.

Least squares fit of the simple exponential model (equation 1) to the data on *Pennisetum purpureum* given by Sandoval-Castro (1996) generates estimates of the parameters A, B and c as 17·94 (SE 2·438), 27·50 (SE 2·648) and 0·0173 (SE 0·00629) respectively. Statistical estimates must be interpreted with reference to their uncertainty range, such as 0·95 confidence intervals. Fig. 1 shows the fitted curve with ranges corresponding to twice and three times the calculated standard errors. Now, as a second step, it is necessary to use inverse interpolation to calculate the lag time using the wash value y_w (normally excluded when fitting the selected model). Rearranging equation 1 for this purpose, a formula for lag time (T) is:

$$T = -\frac{1}{c}\log_e\left[\frac{B-(y_w-A)}{B}\right]. \qquad (2)$$

Because $B \gg A$ or y_w, there are only three outcomes depending on the value of the expression inside the square brackets:

(1) If $A = y_w$, the expression equals 1 giving $T = 0$.
(2) If $A < y_w$, the expression will be < 1 giving a positive lag time.
(3) If $A > y_w$, the expression will be > 1 giving a negative value of T.

When the estimated intercept is larger than the wash value, the lag time must be taken as zero because negative lag times are inadmissible. However, given that such analyses are largely done using computer programs which fit (say) equation 1 and print out predictions such as T using equation 2, the user must decide if the predictions are sensible or not. In the case of the present data, only three error degrees of freedom are available and the 0·95 confidence interval for the intercept is 10·63 to 25·26, which just includes the wash value indicating no significant difference, therefore suggesting zero lag time. Nevertheless, there is doubt about the suitability of the simple exponential model in this case. Unfortunately lack of data in the interval 0–12 h makes it difficult to apply other models. The question of lag can be looked at in another way using a t test to test the significance of the estimate of lag time. From the example data analysed above, $T = -6{\cdot}8$ (SE 6·40) which is obviously not significantly different from zero. It should be remembered that extrapolations carry large errors and they can be misleading.

To illustrate the above points and to comment on the question of constrained least squares, simulation was used to generate degradation profiles enhanced in the earlier stages of fermentation by the use of the double exponential model:

$$y_t = A + B_1\{1-\exp(-k_1 t)\} + B_2\{1-\exp(-k_2 t)\}, \quad k_2 \geqslant k_1, \qquad (3)$$

with $A = 10$, $B_1 = 50$, $B_2 = 20$, $k_1 = 0{\cdot}01$(step = 0·01)0·15 and $k_2 = k_1$(step = 0·01)0·30 for values of $t = 12$, 24, 48, 72, 96 and 120. Here the effect of the fast degrading fraction B_2 is to enhance 'observed' degradation in the earlier stages. If these data are wrongly analysed using the simple exponential model, it will result in distortions of various quantities. For example the rate constant c (/h) has a value that lies between k_1 and k_2 and its size depends on the relative sizes of the two rates and the degradable fractions B_1 and B_2 (Table 1). Furthermore, the fitted intercept is also larger than the assumed value of 10 (Table 2). Note

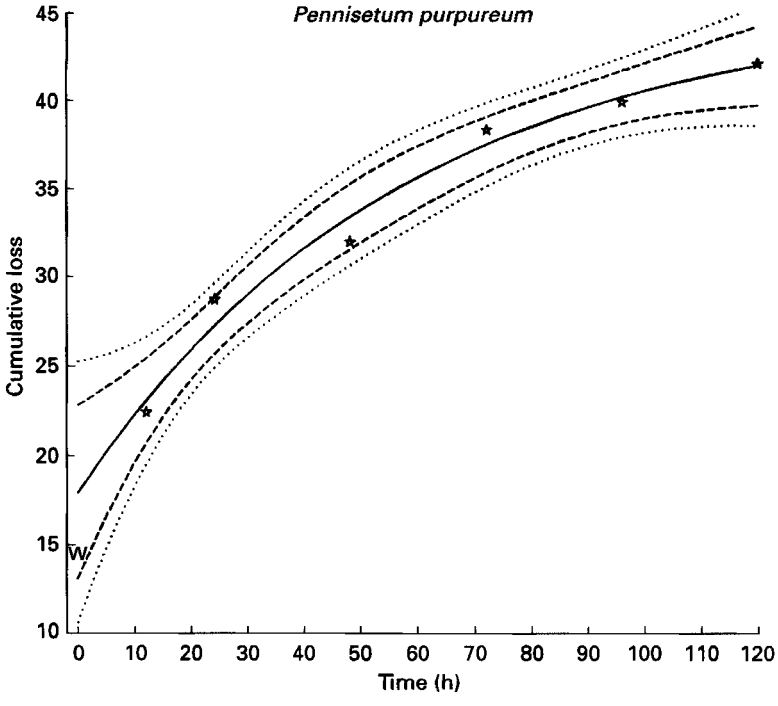

Fig. 1. Least squares fitted curve (continuous line) according to the model $y_t = A + B(1 - e^{-ct})$, excluding the wash value, to the cumulative loss data from polyester-bag study of *Pennisetum pupureum* (Sandoval-Castro, 1996). Also shown are the ranges corresponding to two (– – – –) and three (· · · ·) standard deviations. The wash value is marked by W. The outer range (· · · ·) is approximately the 0·95 confidence interval.

that the model proposed by Dhanoa *et al.* (1995) contains an extra parameter d so that enhanced (positive values of d) or attenuated (negative values of d) degradation in the earlier stages can be modelled.

Another question raised by Sandoval-Castro (1996) was whether the fitted curve should be constrained to pass through a point lower than or equal to (0,14·5). As the unconstrained fitted intercept is greater than 14·5, the indications are that the data come from a process of enhanced rather than simple first-order degradation. In such a situation the estimate of the rate constant c (/h) is probably already an overestimate (see Table 1). It appears that constrained fitting is unwarranted in this case as it will necessarily lead to an even more inflated estimate of the rate constant.

It is not always possible to develop models where all parameters have direct biological meaning. The next best thing to aim for is derived functions of model parameters that are biologically relevant. In polyester bag studies, calculation of the extent of ruminal degradation (E) combines information from the parameters together with the relevant rate of passage (k, /h) for the feed according to the formula:

$$E = y_w + e^{-kT} bc/(c+k), \tag{4}$$

where b is the difference between estimate of the asymptote ($A+B$) and the wash value, rather than the fitted intercept. Generally $b \neq B$, except when $T = 0$. Direct use of B in equation 4 can lead to erroneous results. For a small lag time the scaling factor e^{-kT} may

Table 1. *Estimated values of rate constant* c *(/h) when applying the model* $y_t = A + B(1 - e^{-ct})$ *to the data generated from* $y_t = A + B_1\{1\text{-}exp(-k_1 t)\} + B_2\{1\text{-}exp(-k_2 t)\}$ *with* $A = 10$, $B_1 = 50$ *and* $B_2 = 20$

k2 k1→ ↓	0·01	0·02	0·03	0·04	0·05	0·06	0·07	0·08	0·09	0·10	0·11	0·12	0·13	0·14	0·15
0·01	0·010														
0·02	0·014	0·020													
0·03	0·018	0·023	0·030												
0·04	0·022	0·026	0·033	0·040											
0·05	0·025	0·029	0·036	0·043	0·050										
0·06	0·028	0·031	0·038	0·045	0·053	0·060									
0·07	0·030	0·033	0·040	0·047	0·055	0·063	0·070								
0·08	0·032	0·035	0·041	0·049	0·057	0·065	0·073	0·080							
0·09	0·034	0·036	0·043	0·051	0·059	0·067	0·075	0·083	0·090						
0·10	0·035	0·037	0·044	0·052	0·061	0·069	0·077	0·085	0·093	0·100					
0·11	0·036	0·038	0·045	0·053	0·062	0·070	0·079	0·087	0·095	0·103	0·110				
0·12	0·037	0·038	0·045	0·054	0·063	0·072	0·080	0·089	0·097	0·105	0·113	0·120			
0·13	0·037	0·038	0·046	0·054	0·064	0·073	0·082	0·091	0·099	0·107	0·115	0·123	0·130		
0·14	0·037	0·039	0·046	0·055	0·064	0·074	0·083	0·092	0·101	0·109	0·117	0·125	0·133	0·140	
0·15	0·037	0·039	0·046	0·055	0·065	0·074	0·084	0·093	0·102	0·111	0·119	0·127	0·135	0·143	0·150
0·16	0·037	0·039	0·046	0·056	0·065	0·075	0·085	0·094	0·103	0·112	0·121	0·129	0·137	0·145	0·153
0·17	0·037	0·039	0·046	0·056	0·066	0·075	0·085	0·095	0·105	0·114	0·123	0·131	0·139	0·147	0·155
0·18	0·037	0·038	0·046	0·056	0·066	0·076	0·086	0·096	0·105	0·115	0·124	0·133	0·141	0·150	0·157
0·19	0·036	0·038	0·046	0·056	0·066	0·076	0·086	0·096	0·106	0·116	0·125	0·134	0·143	0·151	0·160
0·20	0·036	0·038	0·046	0·056	0·066	0·076	0·087	0·0·97	0·107	0·117	0·126	0·135	0·144	0·153	0·162
⋮															
0·30	0·031	0·035	0·044	0·054	0·065	0·076	0·087	0·098	0·110	0·121	0·132	0·142	0·153	0·163	0·173

Table 2. *Estimated values of intercept* A *when applying the model* $y_t = A + B(1 - e^{-ct})$ *to the data generated from* $y_t = A + B_1\{1\text{-}exp(-k_1t)\} + B_2\{1\text{-}exp(-k_2t)\}$ *with* $A = 10$, $B_1 = 50$ *and* $B_2 = 20$

k2 k1→ ↓	0·01	0·02	0·03	0·04	0·05	0·06	0·07	0·08	0·09	0·10	0·11	0·12	0·13	0·14	0·15
0·01	10·0														
0·02	10·0	10·0													
0·03	10·2	10·1	10·0												
0·04	10·5	10·2	10·1	10·0											
0·05	10·8	10·5	10·2	10·1	10·0										
0·06	11·2	10·9	10·5	10·2	10·1	10·0									
0·07	11·7	11·3	10·9	10·5	10·2	10·0	10·0								
0·08	12·2	11·8	11·3	10·8	10·4	10·2	10·0	10·0							
0·09	12·7	12·3	11·7	11·2	10·7	10·4	10·2	10·0	10·0						
0·10	13·3	12·9	12·2	11·6	11·1	10·6	10·3	10·1	10·0	10·0					
0·11	13·8	13·4	12·7	12·0	11·4	10·9	10·6	10·3	10·1	10·0	10·0				
0·12	14·3	14·0	13·2	12·5	11·8	11·3	10·8	10·5	10·3	10·1	10·0	10·0			
0·13	14·9	14·5	13·7	12·9	12·2	11·6	11·1	10·7	10·5	10·2	10·1	10·0	10·0		
0·14	15·4	15·0	14·2	13·4	12·7	12·0	11·4	11·0	10·7	10·4	10·2	10·1	10·0	10·0	
0·15	15·9	15·5	14·7	13·9	13·1	12·4	11·8	11·3	10·9	10·6	10·4	10·2	10·1	10·0	10·0
0·16	16·4	16·0	15·2	14·3	13·5	12·8	12·1	11·6	11·2	10·8	10·5	10·3	10·2	10·1	10·0
0·17	16·9	16·5	15·7	14·8	13·9	13·2	12·5	11·9	11·4	11·0	10·7	10·5	10·3	10·2	10·1
0·18	17·4	17·0	16·1	15·2	14·3	13·5	12·8	12·2	11·7	11·3	10·9	10·7	10·4	10·3	10·2
0·19	17·9	17·5	16·6	15·7	14·8	13·9	13·2	12·6	12·0	11·5	11·2	10·8	10·6	10·4	10·2
0·20	18·3	17·9	17·0	16·1	15·2	14·3	13·6	12·9	12·3	11·8	11·4	11·1	10·8	10·5	10·4
⋮															
0·30	21·8	21·3	20·5	19·6	18·7	17·8	16·9	16·1	15·3	14·7	14·1	13·5	13·0	12·6	12·2

be omitted. It is preferable if authors of polyester bag studies quote a figure for E even for an assumed rate of passage because individual parameters can vary in repeated runs whilst collectively they may give similar estimates of E. From Sandoval-Castro (1996, table 1), the calculated estimates of E for (say) $k = 0{\cdot}01$/h for the three solutions, i.e. without restriction, with restriction and manual, were found to be 34·1, 34·8 and 36·5% respectively. For the higher rate of passage $k = 0{\cdot}02$/h the corresponding values of E become 28·8, 30·1 and 31·6 respectively. However, note that in the above calculations of E the wash value of 14·5 contributes some 40–50% of its value. Therefore, more attention should be paid to the determination of the wash value as described by Cockburn *et al.* (1993) and Lopez *et al.* (1994).

The point of statistical regression methodology is to provide an objective basis from which inferences are made; fitting by eye or manual methods only serve to undermine that process. As far as the graphical methods are concerned, readers should consult Mertens (1973, 1993), Nocek (1988) and Miller (1982). These authors suggest logarithmic transformation of residue remaining and fitting a straight line to data in the earlier stages, e.g. Miller (1982) recommends 0–12 h whereas Mertens (1973) used residue remaining at 48 or 72 or 96 h as an estimate of undegradable fraction. These methods generally assume that the simple exponential model is adequate which, however, is not always true. By the use of a curve peeling technique it is possible to identify if one or more rates apply (Hartley & Dhanoa, 1981).

In conclusion it must be stressed that appropriate experimental design of polyester bag studies is fundamental to obtaining good-quality information and it is prudent to make allowance for the use of alternative models. Uncritical use of any model is inadvisable.

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