BIOCHEMICAL LETTERS

Kinetic characteristics of nitric oxide synthase from rat brain

The recent article by Knowles *et al.* [1] describes an assay for NO synthase activity that is based on an indirect assay of the NO by guanylate cyclase (GC). They use an apparent Michaelis–Menten relationship between GC stimulation and NO concentration, so that a measurement of the former can be used to estimate the latter. Although the overall rationale is valid, the authors do not adequately test some of their assumptions, and also make critical mathematical errors. A careful examination of Fig. 1(b) shows that it is inappropriate for several reasons.

First, the authors' assumption is wrong that when a Michaelis–Menten relationship exists between the GC stimulation and the NO concentration (which, if NO has a second-order disappearance rate, would be proportional to the square root of the NO production rate), then this relationship would also be apparent in a plot of the square of the GC stimulation versus the NO production rate (as represented by the nitroprusside concentration). It is mathematically false to expect a hyperbolic curve when plotting the square of the velocity versus the square of the NO concentration. Only a graph of the GC stimulation versus the square root of the nitroprusside concentration would accurately show such a Michaelis–Menten relationship.

Second, the plot should be in a double-reciprocal form. Not only would this help to validate the assumption of a Michaelis–Menten relationship (which neither the current plotting method nor any other evidence yet supports), it would provide a more straightforward display of the data and the important constants. Also, it would allow a visual estimate of the range of experimental error in the measurement of low GC activities (which are critical K_1 estimates derived from Fig. 3).

Third, the authors propose that the loss of NO in solution is via the termolecular reaction $2\text{NO} + \text{O}_2 \rightarrow \text{N}_2\text{O}_4$, so that at constant O_2 concentration, the rate of NO loss is second order. Several facts argue that NO has a first-order disappearance in physiological buffers and not second order, making its concentration linearly related to its rate of generation. Evidence includes:

(a) Feelisch & Noack [2] compared NO production to GC stimulation in very similar experiments. The data in Fig. 5 of that paper shows a direct relationship between NO production rate and GC stimulation, which was only compatible with a first-order loss of NO.

(b) A first-order assumption leads to a good fit of NO/EDRF loss measured by other authors (Fig. 3 in [3]; Fig. 2B in [4]) and even when reported by the authors themselves previously (Fig. 1 in [5]).

(c) Although the authors used the assumption of a second-order loss in a previous paper to obtain a tolerable fit to the data (Fig. 1 in [6]) the data were incorrectly analysed (i.e. a straight line was fitted to the graph when a hyperbolic curve was expected). An appropriate analysis shows that the data are more consistent with a first-order loss.

(d) The deviation of the data points from the curve in Fig. 1(b)

show just the sort of slight sigmoidal deviation expected if NO loss is first-order, and the graph thereby plotted inappropriately.

(e) The reference used as evidence of a second-order loss [7] dealt with the reaction of pure, dry NO with O_2 cooled to about 85 K in a near vacuum. In reviewing this and other industrial reactions, Beattie [8] noted that even small amounts of water, trace elements or glass surfaces may alter the homogeneous termolecular reaction between NO and O_2 .

The next question is to what extent the conclusions of Knowles *et al.* [1] are affected if the data have been plotted incorrectly in Fig. 1(b). First, the estimate of the NO-production rate (i.e. that from 1.45 μM -nitroprusside) which causes half-maximal GC stimulation would be nearly correct. However, the NO-production rates which give other velocities are poorly estimated by the equation obtained from the figure and used by the authors. For example, if the NO loss is first-order, then a graph of the unsquared velocity versus nitroprusside concentration would have yielded a line fitting the equation:

$$(v =)y = (100 \times x)/(1.45 + x)$$

rather than the equation that was used:

$$(v^2 =)y = (10000 \times x)/(4.35 + x)$$

Therefore, at 5% maximal velocity, the current equation would underestimate the true rate of NO production by 7-fold, and at 2% maximal velocity (common in Fig. 3), the underestimate would be 17-fold. Such a progression of errors would partly explain why the data in Fig. 2 deviate from the expected straight line. The estimate for the K_m for arginine might thereby be too high, and the later estimates of K_1 values accordingly affected.

On the other hand, if the loss of NO is indeed second-order, and the authors have only plotted the data wrongly, then the proper graph of the data would have yielded a line fitting the equation:

$$(v =)y = (100 \times \sqrt{x})/(\sqrt{1.45} + \sqrt{x})$$

At 5% and 2% of maximal velocity, then, the current equation would have overestimated the actual NO production rate by 2.71- and 2.88-fold, respectively. However, the deviation of the actual data points from the present curves (Fig. 1b and Fig. 2) is the opposite of that expected from this error.

As a final point, the overall rationale of the method requires that the rate of NO loss remains unchanged under different conditions. However, tissue homogenates contain varying amounts of compounds which can react with NO, such as thiol groups (in addition to the dithiothreitol added to all incubations), haem iron and sources of superoxide. The rate of NO loss might vary among tissues or treatments (e.g. with the addition of reducing equivalents such as NADPH). This basic limitation of the method must be kept in mind, since it is critical to all further calculations.

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Kinetics of nitric oxide synthase: a reply

We believe that the points raised by Murphy [1] are irrelevant to our article [2] which was a study using a standard curve of the guanylate cyclase stimulation caused by different rates of NO formation in order to calculate rates of NO formation by NO synthase. It is clear that the equation used fits the data in Fig. 1(b) very well, permitting this calculation of NO synthase rates. Our experiments were not intended to test whether NO breakdown is first- or second-order under our experimental conditions.

Any deviations from our standard curve at very low rates of cyclic GMP formation are not likely to be important since these values are, in any case, subject to relatively large errors because of the limitations of the assay for cyclic GMP at very low cyclic GMP concentrations. It is well known that the transformation of

velocity (v) to $1/v$ in reciprocal plots distorts the effect of experimental errors at low v so that such plots are an inappropriate way to determine kinetic constants (discussed in [3–5]). In our study, although we presented data as reciprocal plots for easy interpretation by the reader, we determined the kinetic constants by non-linear least-squares fitting to untransformed velocity data in order to avoid this problem of undue emphasis on the least accurate data. In any case, the data in Figs. 2 and 3 of our article show no *consistent* pattern of error at low rates, suggesting that the standard curve used does not give rise to a systematic error.

The question whether NO breakdown follows first- or second-order kinetics in physiological buffers remains unresolved. None of the papers referred to by Murphy distinguish between these possibilities by comparing first- and second-order fits to the data, and none claim to have proven the kinetic order. We chose to use an assumption of second-order kinetics since the reaction of NO with O_2 is thought to be the major route of NO breakdown in the absence of significant concentrations of haemoglobin or O_2^- , neither of which was present in our experiments.

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