THE STEADY-STATE RELAXATION TIME AND THE PRODUCT AT EQUILIBRIUM

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SUMMARY

1. According to BÜCHER and his co-workers, the steady-state relaxation time (*i.e.* the reciprocal of the first-order process by which an enzyme reaction reaches equilibrium) determines, in part, the apparent metabolic flow rate through a given enzymatic step *in vivo*.

2. A new method for the determination of the kinetic constants required for the calculation of the apparent metabolic flow rates according to BÜCHER, is developed. The method is based on the measured concentrations of substrate or product in the course of incubation, and has been adapted for use by a digital computer.

3. The relevance of the data thus obtained to the calculated metabolic flow rates are briefly discussed.

INTRODUCTION

It has been suggested by BÜCHER AND RÜSSMAN[1] that the apparent metabolic flow rate through a two-partner reaction of the form $A \rightleftharpoons P$ near equilibrium can be calculated from the following formula:

$$V_{st} = \frac{(V_1)_{tissue}}{(\tau_{ss} V_1)} \frac{\bar{P} (K_{app} - \Gamma)}{K_{app} (1 + \Gamma)} \qquad (1)$$

where V_{st} is the metabolic flow rate (in terms of unit mass per weight per unit time), $(V_1)_{tissue}$ is the maximal activity of the enzyme measured in the tissue, $\bar{P}$ is the concentration of the product at equilibrium[2], K_{app} the equilibrium constant, and Γ an expression based on the deviation of the substrates from equilibrium. τ_{ss} (see refs. 3, 4) is the steady-state relaxation time (*i.e.* the time taken for the deviation from equilibrium to decrease by one e-th part of its original value), and V_1 the maximal activity of the enzyme used for the determination of the steady-state relaxation time

in vitro. Determinations of the apparent metabolic flow rates are made possible by the demonstration[1] that $(\tau_{ss} V_1)$ is related to $\bar{P}$ by a linear equation of the form:

$$\tau_{ss}V_1 = \alpha \bar{P} + \beta \tag{2}$$

where α and β are the slope and intercept respectively of the linear regression of $(\tau_{ss} V_1)$ on $\bar{P}$.

The development of Eqn. 1 is based on the assumptions[1] that (1) Equilibrium is approached by a first-order process. (2) Michaelis–Haldane kinetics apply *in vivo* as well as *in vitro* regardless of the much greater concentration of the enzyme *in vivo* compared with that used *in vitro*, and on the additional assumption. (3) That the deviation of the substrate from equilibrium (ΔA) is small. The latter is used by BÜCHER AND RÜSSMAN[1] in the development of their Eqn. 8 which postulates that

$$\Delta A \left(\frac{1}{K_a} - \frac{1}{K_p} \right) \approx 0 \tag{3}$$

where K_a and K_p are the Michaelis constants of substrate and product, respectively. Furthermore, in the experimental determination of the constants for Eqn. 2 certain difficulties may arise:

(1) If P_j is the concentration of the product at any time t_j, the calculation of the steady-state relaxation time depends on $\ln(\bar{P}-P_j)$. If $\bar{P}$ is only slightly larger than P_j, minimal errors in reading the optional density result in significant changes in the calculated relaxation times.

(2) Two lines, each representing approximately first-order kinetics may be obtained from the same set of data. These lines are characterized by slightly different slopes and therefore different relaxation times.

As first-order kinetics may be obeyed even for quite large deviations from equilibrium calculations of the metabolic flow rate would be feasible for a wider range of metabolite concentration if the conditions of Eqn. 3 were not obligatory (*i.e.* if no stipulations were made as to the magnitude of ΔA and if $K_a \neq K_p$).

In this paper a method for the calculation of Eqn. 2 is developed which is independent of Assumption 3, and which attempts to overcome some of the experimental difficulties outlined above. This method will be exemplified by the phospho-pyruvate hydratase (2-phospho-D-glycerate hydrolase, EC 4.2.1.11) reaction.

METHODS

Experimental

Materials: substrates, co-factors and enzymes were purchased from Boehringer Mannheim Inc.

Phosphopyruvate hydratase was prepared as follows: approx. 50 g of human liver obtained at autopsy were homogenized in 0.25 M sucrose using a Potter Elvehjem homogenizer with a teflon pestle. For the preparation of the sucrose medium and throughout the preparative procedure, double-distilled water made 1 mM in EDTA (disodium salt) was used. The final volume was made to 250 ml and the homogenate centrifuged at 10 000 × g. The pH of the supernatant was then adjusted to 5.0 with a few drops of 1 M HCl and the concentration of protein[5] adjusted to 2.2 mg/ml. The material was then warmed to 37° and the 0.65% to 0.75% (NH₄)₂SO₄ precipitate

collected. This precipitate was resuspended in 0.1 M $(NH_4)_2SO_4$. Any trace of pigment remaining was readily removed by extraction with a small amount of $CHCl_3$ and without loss of enzyme activity. Except where otherwise stated, all procedures were carried out at 0–4°. The resultant preparation contained phosphopyruvate hydratase and no other enzymes capable of acting on either 2-phosphoglycerate or phosphoenolpyruvate in the absence of added co-factors. When kept in the cold, the preparation lost about 5% of its activity per day.

For the kinetic studies a suitably dilute sample of the enzyme preparation was incubated with a known concentration of 2-phosphoglycerate in 0.2 M Tris (pH 7.2) at 37° in a Beckman DU spectrophotometer. The reaction was initiated by the addition of $MgCl_2$ in 3.3 mM final concentration, and the formation of phosphoenolpyruvate recorded at 1-min intervals at 240 mμ until no further change in absorbance occurred (i.e. until equilibrium had been reached).

The maximal activity of the phosphopyruvate hydratase preparation in the forward direction was measured on the basis of the method described by CZOK AND ECKERT[6] using lactate dehydrogenase (L-lactate NAD oxidoreductase, EC 1.1.1.27) as the indicator for the forward reaction and glyceraldehyde phosphate dehydrogenase (D-glyceraldehyde-3-phosphate NAD oxidoreductase (phosphorylating) EC 1.2.1.12) for the reverse reaction.

The apparent equilibrium constant was determined at pH 7.2 and 37°, and in the Tris buffer using commercial phosphopyruvate hydratase. Following the addition of $MgCl_2$ and either 2-phosphoglycerate or phosphoenolpyruvate, incubation was continued for 30 min. Samples were then taken at 5-min intervals for up to 45 min, inactivated in about 5 vol. of chilled 6 M $HClO_4$, neutralized and centrifuged. The ratio of phosphoenolpyruvate to 2-phosphoglycerate in the neutralized extract was then determined[6].

Mathematical

The calculations depend on the following principles:

(a) The data obtained in the course of incubation of enzyme with substrate are fitted to the integrated form of the Michaelis–Menten equation for a two-partner reaction expressing the concentration of the product formed as a function of time.

(b) A series of such equations were obtained in which the initial substrate concentrations and the enzyme activities were varied independently. Each of these was used for the calculation of τ_{ss} and the product of τ_{ss} and V_1 was then plotted against P thus obtaining the regression line given by Eqn. 2.

If E is the concentration of the enzyme, A the concentration of the substrate and P the concentration of the product then, near equilibrium, when both substrate and product are present, the reaction may be formulated as follows:

$$E + A \rightleftharpoons EA \rightleftharpoons EP \rightleftharpoons E + P$$
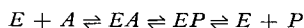
The steady-state velocity is given by[7]

$$-\frac{dA}{dt} = \frac{dP}{dt} = \frac{V_a A/K_a - V_p P/K_p}{1 + \dfrac{A}{K_a} + \dfrac{P}{K_p}}$$

where V_a and V_p are the maximal velocities in the direction A to P and P to A,

respectively, and K_a and K_p are their respective substrate constants. Integration of this equation[7] yields:

$$t\left[\frac{V_a}{K_a} + \frac{V_p}{K_p}\right] = \left[1 + \frac{A_0}{K_p} + \frac{\left(\frac{1}{K_a} - \frac{1}{K_p}\right)\frac{V_p}{K_p}A_0}{\frac{V_a}{K_a} + \frac{V_p}{K_p}}\right] \ln \frac{A_0 - \bar{A}}{A - \bar{A}} + \left[\frac{1}{K_a} - \frac{1}{K_p}\right](A_0 - A) \quad (4)$$

where A_0 is the initial substrate concentration (*i.e.* the substrate concentration when $P = 0$). The concentrations of the metabolites A and P at various times t are obtained from the experimental data. $\bar{A}$ then is the concentration of the substrate at equilibrium. A mathematical expression which permits the experimental data to be fitted to Eqn. 4 was developed as follows:

In the absence of interfering reactions, the sum of substrate and product is constant

$$\bar{A} = A_0 - \bar{P}$$
$$A = A_0 - P$$

substituting these equations into Eqn. 4 and simplifying:

$$t = \left[\frac{K_a K_p}{V_a K_p - K_a V_p}\right]\left[1 + \frac{A_0(V_a + V_p)}{V_a K_p + K_a V_p}\right]\ln\frac{\bar{P}}{\bar{P} - P} + \left[\frac{K_p - K_a}{V_a K_p - K_a V_p}\right]P \quad (5)$$

Using the well-known HALDANE–ALBERTY relationship[8]

$$K_{app} = \frac{V_a K_p}{V_p K_a} = \frac{\bar{P}}{\bar{A}} = \frac{\bar{P}}{A_0 - \bar{P}} \quad (6)$$

or

$$K_p = \frac{\bar{P} V_p}{(A_0 - \bar{P})V_a}K_a \quad (7)$$

and substituting into Eqn. 5 and simplifying:

$$t = \left[\frac{\bar{P}}{A_0 V_a}\right]\left[K_a + \frac{(V_a + V_p)(A_0 - \bar{P})}{V_p}\right]\ln\frac{\bar{P}}{\bar{P} - P} + \left[\frac{P(V_a + V_p) - A_0 V_a}{A_0 V_a V_p}\right]P \quad (8)$$

The only unknown parameter in Eqn. 8 is K_a because both V_a and V_p have been measured experimentally. To obtain the maximum likelihood estimate of this value, the least-squares regression criterion was employed as follows:

A function $G(K_a)$ was defined such that

$$G(K_a) = \sum_{j=1}^{n} (t_{0_j} - t_{p_j})^2 \quad (9)$$

where j is an index on the data points, t_0 identifies the observed data, and t_p the predicted points. The value of K_a for which $G(K_a)$ is a minimum is given by $\dfrac{dG(K_a)}{dt} = 0$

Substituting the expression on the right of Eqn. 8 for t_{p_j} into Eqn. 9 differentiating, simplifying and solving for K_a we obtain:

$$K_a = \frac{A_0 V_a V_p \sum_j t_j \ln \dfrac{\bar{P}}{\bar{P} - P_j} - \bar{P}(V_a + V_p)(A_0 - \bar{P})\sum_j \ln^2 \dfrac{\bar{P}}{\bar{P} - P_j} - [\bar{P}(V_a + V_p) - A_0 V_a]\sum_j P_j \ln \dfrac{\bar{P}}{\bar{P} - P_j}}{V_p \bar{P} \sum \ln^2 \dfrac{\bar{P}}{\bar{P} - P_j}} \quad (10)$$

It is then possible to calculate K_p from Eqn. 10 and Eqn. 7. These values are then substituted into Eqn. 4.

Eqn. 4 is then used for the calculation of the steady-state relaxation time, τ_{ss}, as follows:

By definition[1] τ_{ss} is that interval of time (t_2-t_1) for which the corresponding value (P_2-P_1) represents an increase of P_1 by one e-th of its value, *i.e.*

$$P_2 = \left(P_1 + \frac{P_1}{e}\right) \tag{11}$$

First-order kinetics on which these calculations are based are best met near equilibrium (*i.e.* when $P_2 = \bar{P}$). However, as $P \to P$, $t \to \infty$, and for this reason a constant c is selected such that $P_2 = cP$ where c is near unity (*e.g.* 0.99). If $P_2 = cP$ then from Eqn. 11

$$c\bar{P} = \left(1 + \frac{1}{e}\right) P_1$$

and

$$P_1 = \left(\frac{e}{e+1}\right) c\bar{P}$$

Therefore

$$\tau_{ss} \text{ (in minutes)} = \left[t(c\bar{P}) - t\left(c\,\frac{e}{e+1}\,\bar{P}\right)\right]$$

Substituting into Eqn. 4, multiplying by V_1, factoring and collecting terms:

$$\tau_{ss}V_1 = V_1\left\{\left[\frac{K_aK_p}{V_aK_p + K_aV_p}\right]\left[1 + \frac{A_0(V_a+K_p)}{V_aK_p+K_aV_p}\right]\ln\left[\frac{e(1-c)+1}{(e+1)(1-c)}\right] + \frac{K_p-K_a}{V_aK_p-K_aV_p}\,c\,\frac{1}{e+1}\,\bar{P}\right\} \tag{12}$$

The relationship between $(\tau_{ss} V_1)$ and $\bar{P}$ should be linear and of the form given by Eqn. 2. From the maximum likelihood estimates of the coefficient and intercept of linear regression equations, α and β are given be Eqns. 13 and 14.

$$\alpha = \frac{m\sum_{K=1}^{m}[(\tau_{ss}V_1)_K\,\bar{P}_K] - \left[\sum_{K=1}^{m}(\tau_{ss}V_1)_K\right]\sum_{K=1}^{m}\bar{P}_K}{m\sum_{K=1}^{m}\bar{P}_K^2 - \left(\sum_{K=1}^{m}\bar{P}_K\right)^2} \tag{13}$$

$$\beta = \frac{1}{m}\left\{\sum_{K=1}^{m}(\tau_{ss}V_1)_K - \alpha\sum_{K=1}^{m}\bar{P}_K\right\} \tag{14}$$

where k is an index on the data points for m number of experiments of the type described earlier.

Eqns. 10, 12–14 are readily translated into suitable computer language. (A program written in the FORTRAN II compiler language and suitable for an IBM 7090 digital computer may be obtained from the authors upon request.)

RESULTS AND DISCUSSION

In a mean of five experiments K_{app} was found to be 5.13, fairly close to the value of 4.8 which can be calculated from the data of BÜCHER AND RÜSSMAN[1] (*op. cit.* Fig. 4). The ratio of V_a/V_p was 3.72, somewhat higher than that given for ascites tumor cells[9].

The experimental data fitted the curve given by Eqn. 4 with a standard error of the mean of 10.7% based on 22 experiments using enzyme prepared from one specimen of human liver. The linear relationship between $\tau_{ss}\,V_1$ and $\bar{P}$ was computed from these experiments and is illustrated in Fig. 1. It is evident that the data fit a linear regression line which would be anticipated if Eqn. 2 were valid, and with sufficient precision to be used as a basis for the computation of metabolic flow rates.

As will be seen from inspection of Eqns. 1 and 2 the apparent metabolic flow rate is inversely related to α, the slope of Eqn. 2. The slope of the regression line is
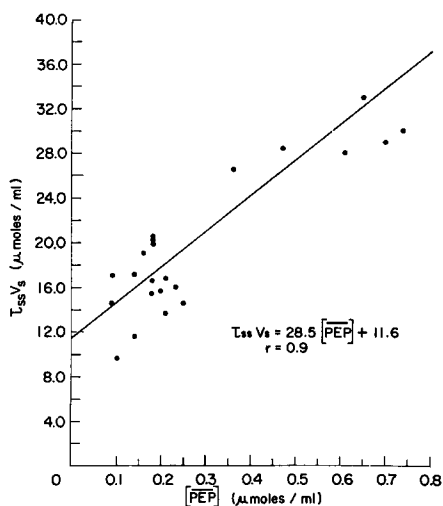

Fig. 1. Plot of $(\tau_{ss}V_1)$ against $\bar{P}$. Enolase from normal human liver. PEP stands for phospho-enolpyruvate.

higher and the calculated metabolic flow rate therefore lower than in the experiments of BÜCHER AND RÜSSMAN[1] which were based on enolase derived from muscle. Whether this difference is due to the more sluggish rate of glycolysis which characterizes liver compared with muscle, due to the differences in the mathematical treatment of the data, or both remains to be determined.

The procedure outlined in this paper clearly could be extended to other two-partner reactions such as the phosphoglucose isomerase (D-glucose-6-phosphate ketol isomerase, EC 5.3.1.9) reaction and studies to explore this possibility are in progress.

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