Use of Added Labeled Substrate Conversion Ratio to Measure Endogenous Substrate Concentration and Enzyme Activity[1] (34478)

DAVID T. SCHWARTZ, JOHN S. BRITTEN, RICHARD C. MASON
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Departments of Physiology and Urology, Columbia University College of Physicians and Surgeons, New York, New York 10032

A method for measuring the concentration of an enzyme's substrate was developed from the Michaelis-Menten hypothesis, and its validity demonstrated with the hydrolysis of *p*-nitrophenylacetate by α-chymotrypsin.

To arrive at a simple method for measuring angiotensin in the blood, we have made use of the competition of endogenous angiotensin and added ^{14}C labeled angiotensin for plasma angiotensinase (1). Because plasma angiotensinase was not isolated, we first tested the validity of the method with a model system. The method requires that both substrate and enzyme concentrations be rate-limiting, and the enzymatic reaction be free from significant product inhibition. Under these conditions, an increase in the amount of substrate will result in a decrease in the conversion ratio (the amount of product formed per unit of substrate). If a known amount of exogenous substrate (S^*), identical to endogenous substrate except that it is radioactively labeled in a moiety which gives a labeled product, is added to the otherwise unchanged reaction mixture, then the ratio of radioactive product (P^*) to radioactive substrate is the same as the conversion ratio for all substrate. From this conversion ratio, the amount of (labeled) substrate added, and from the reaction rate measured in the presence of excess substrate, one can calculate the amount of unlabeled (endogenous) substrate present, by the following equation:[2]

$$X = S^*/P^*\,(P_{max}) - S^* - K_m \qquad (1)$$

P_{max} can be measured by running the reaction with a large excess of labeled substrate, and measuring the labeled product. In addition, both K_m and P_{max} can be determined by measuring P when the reaction is run in absence of endogenous substrate at different labeled substrate concentrations, using the method of Lineweaver and Burk.

P_{max} gives a measure of enzyme concentration, and X (P^*/S^*) gives the amount of unlabeled substrate converted to product by the enzyme during the test reaction.

Substrate and enzyme concentrations might be of interest under conditions where P_{max} and K_m could not be measured directly. Under such conditions, P_{max} can be determined by

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[2] This equation was derived by assuming the Michaelis-Menten hypothesis (2) holds

(a) $S/V = K_m/V_{max} + S/V_{max}$

(where S = substrate concentration in moles/liter, V_{max}= velocity of the reaction when a large excess of substrate is present relative to the amount of enzyme, and K_m = Michaelis-Menten constant, in the absence of product inhibition.)

If the reaction is run under standard conditions over a fixed short time interval, t, then product, P, may be substituted for the velocity, V, by dividing both sides of equation (a) by t, since $P = V \cdot t$, and $P_{max} = V_{max} \cdot t$

(b) $S/P = K_m/P_{max} + S/P_{max}$

The conversion ratio (S/P) may be calculated from Formula b.

If the reaction is run in the presence of an added amount of substrate (s^*) which has been radioactively labeled in a moiety which leads to a labeled product, P^*, then the conversion ratio will now be given by S^*. The total amount of substrate in the reaction mixture is equal to the added labeled substrate, S^*, plus the unlabeled endogenous substrate, X. Equation 2 then becomes,

(c) $S^*/P^* = K_m/P_{max} + (S^* + X)/P_{max}$

Solving this equation for x gives formula 1.

running the reaction at two different added labeled substrate concentrations.[3]

$$P_{max} = (S_1 - S_2)/(S_1/P_1 - S_2/P_2) \quad (2)$$

When K_m is unknown, X cannot be measured. However, the quantity $(K_m + X)$ can be determined, and when K_m is constant, the absolute variations in $(K_m + X)$ are equal to variations in X. $(K_m + X)$ can be determined from labeled substrate and product concentrations when the reaction is run at two different labeled substrate concentrations.

$$K_m + X = (P_2 - P_1)/(P_1/S_1 - P_2/S_2)^4 \quad (3)$$

In order to test these formulas, the kinetics of the hydrolysis of *p*-nitrophenylacetate to *p*-nitrophenol and acetic acid by α-chymotrypsin were investigated.

p-Nitrophenylacetate-1-^{14}C was obtained from New England Nuclear Corp., Boston, Massachusetts. *p*-Nitrophenol and *p*-nitrophenylacetate were obtained from Nutritional Biochemical Corp., Cleveland, Ohio. α-Chymotrypsin was obtained from Schwarz Bioresearch, Inc., New York.

A standard reaction mixture contained 20 μg (9.1×10^{-4} mmoles) of α-chymotrypsin, 0.082–3.62 mg (0.45 to 20 μmoles) of *p*-nitrophenylacetate-1-^{14}C, and 0.362–3.62 (2–20 μmoles) of unlabeled *p*-nitrophenylacetate, and was brought to 5 ml in 0.1 *M* sodium phosphate buffer pH 7.4. In order to demonstrate that the same kinetics applied to the reaction when it was run in the presence of plasma, reactions were run with phosphate buffer omitted, and 0.5 ml of fresh heparinized dog plasma added to the mixture instead, and the volume brought to 5 ml with water.

The reaction mixture was incubated at 25 cr 37° for 26-60 min, after which 1 ml of 40% trichloroacetic acid was added to stop the reaction and to precipitate the plasma proteins. The mixture was shaken, centrifuged twice, and the supernatant fraction was sampled for radioactivity. This gave a measure of S^*, since the original labeled substrate concentration equals the labeled product plus the remaining labeled substrate. The supernatant fraction was then shaken twice with ethyl ether, leaving only the labeled acetate in the aqueous phase.[5] The radioactivity in this phase (P^*) was measured.

Adherence to Michaelis-Menten kinetics was demonstrated by showing that when the reaction was conducted in phosphate buffer for 20 min at various substrate concentrations, a plot of $1/V$ vs $1/S$ gave a straight line (Fig. 1).

When the reaction was run with plasma for 60 min at 37° with 10 mg/ml of chymotrypsin, the Lineweaver-Burk formulation also held (Fig. 2). The K_m for the reaction in plasma, 1.1×10^{-3} was identical to that calculated from Fig. 1, the Lineweaver-Burk plot for the reaction run in phosphate buffer.

In order to test the accuracy of formula 1, the reaction was run in plasma with a 30-fold variation in concentration of labeled *p*-nitrophenylacetate and a 10-fold variation in concentration of concurrently added unlabeled

[3] Since x is the same in both instances,

$$X = S_1/P_1(P_{max}) - S_1 - K_m$$
$$= S_2/P_2(P_{max}) - S_2 - K_m$$

Solving for P_{max} gives formula 2.

[4] Formula (3) was derived from formula c,

(d) $$S^* = [(K_m + S^* + X)/P_{max}]P^*$$

(e) $$P_{max} = P^*K_m/S^* + P^* + P^*X/S^*$$
$$= P^*/S^*(K_m + X) + P^*$$

When the reaction is run at two different concentrations of S^* and P^*, $K_m + X$ remains the same. So the right-hand member of the equation can be set equal for two test reactions with different added substrate concentrations.

(f) $$P_1/S_1(K_m + X) + P_1 = P_2/S_2(K_m + X) + P_2$$

(g) $$(K_m + X)(P_1/S_1 - P_2/S_2) = P_2 - P_1$$

Solving for $(K_m + X)$ gives formula 3.

[5] Under these conditions, labeled acetate was found to remain in the aqueous phase. Labeled *p*-nitrophenylacetate was distributed 98.4% into the ether phase and 1.6% into the aqueous phase. For this reason, control tubes without enzyme were always compared to tubes with enzyme and the difference in radioactivity between the two in aqueous phase taken as the radioactivity of the product.

TABLE I. Hydrolysis of *p*-Nitrophenylacetate by α-Chymotrypsin in Plasma, Using Varying Amounts of Labeled and Unlabeled Substrate.[a]

Experiment	S^* *p*-Nitrophenylacetate-1-^{14}C (mg/5 ml reaction mixture)	P^*/S^* (conversion ratio)	x Unlabeled *p*-nitrophenylacetate (calcd mg)	Unlabeled *p*-nitrophenylacetate actually added (mg)
A	0.00362	.1603	.413	.362
	0.00362	.1177	.841	.905
	0.00362	.0780	1.50	1.81
B	0.0905	.0407	3.00	2.715
	0.0905	.0296	4.16	3.62
C	0.492	0.087	1.03	0.905
	0.492	0.082	1.07	0.905
	0.492	0.063	1.74	1.81
	0.492	0.062	1.82	1.81

[a] Reactions were run for 20 min at 37°, in 5 ml of reaction mixture containing 20 mg of α-chymotrypsin.

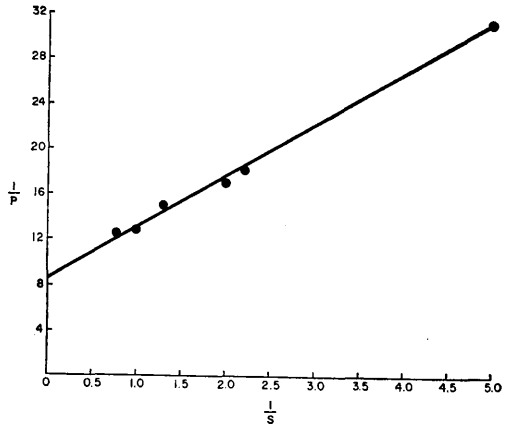

FIG. 1. Lineweaver-Burk plot of reciprocals of substrate and product concentrations during hydrolysis of *p*-nitrophenylacetate by α-chymotrypsin in phosphate buffer. Data are expressed in milliliters of *p*-nitrophenylacetate at 1.64 mg/cc.

p-nitrophenylacetate. The mixture was incubated at 37°, for 20 min, and contained 20 mg of chymotrypsin. P_{max} was determined separately by using a large excess of labeled substrate in the presence of unlabeled substrate. The results are presented in Table I. A regression analysis of the calculated amount of unlabeled *p*-nitrophenylacetate vs the amount actually added showed a slope of + 1.12, with a standard error of 0.07, while the y-intercept was calculated as —0.13, with standard error of 0.14.

In order to see whether Eq. 1 was still valid in calculating the amount of endogenous unlabeled substrate at conversion ratios as low as 1:2000, the reaction was run with 0.2 mg of chymotrypsin (instead of 20 mg), for 20 min at room temperature (instead of 37°) in plasma. The data in Table II indicate that even under these conditions formula 1 was accurate to at least 8% in calculating the amount of unlabeled substrate added.

From Tables I and II it may be seen that in four test reactions, 20 μmoles of unlabeled paranitrophenylacetate were added, and a calculated value for the unlabeled material

TABLE II. Hydrolysis of *p*-Nitrophenylacetate-1-^{14}C by α-Chymotrypsin with and without Additional Unlabeled *p*-Nitrophenylacetate.

Expt.	Substrate (mg)[a]	Product (mg)[a]	Unlabeled added *p*-nitrophenylacetate (mg)
A	.156	.0005687	0.0
	.84994	.001540	0.0
	K_m (calculated) = 0.5418 mg		
B	.51473	.000227	1.81
	3.28	.001528	1.81
	P_m = .00153 (measured)		

[a] From formula 4, x = 1.95 mg (calculated unlabeled *p*-nitrophenylacetate). Error = (1.95—1.81)/1.81 = 7.7%.

TABLE III. Hydrolysis of *p*-Nitrophenylacetate with and without Added Unlabeled Substrate.[a]

Tubes	$[S]$ (μg/5 ml)	$[P]$	P_m	$X + K_m$	Actual X	Calculated X
1	3.75	0.124				
2	15.0	0.375	1.15	31.37	0	
3	11.23	0.2246			15.	
4	7.5	0.16			15.	
5	1.5	0.036			15.	
3 + 4			1.19	48.57	15.	17.20
4 + 5			1.15	46.44	15.	15.07
5 + 3			1.17	47.12	15.	15.75

[a] Mean calculated $X = 16.0$; accuracy $= 6.7\%$. Standard error for $X = 0.996 = 6.6\%$. Standard error for $K_m = 3.0\%$ ($n = 4$). Standard error for $P_m = 1.0\%$ ($n = 4$).

obtained. The average deviation of the calculated value from the actual amount added in the four test reactions was 7%.

In order to test the accuracy of formulas 2 and 3, the reaction was run at 37° for 1 hr at three different labeled substrate concentrations in the presence of a constant amount of unlabeled substrate. For this experiment, K_m was measured as $K_m + X$ calculated from formulas 2 and 3, and S^* and P^* when the reaction was run at two different labeled substrate concentrations without added unlabeled substrate. The results are presented in Table III. The accuracy and standard deviation of the determination of unlabeled substrate were both about 7%.

TABLE IV. Hydrolysis of *p*-Nitrophenylacetate with and without Added Unlabeled Substrates, in Phosphate Buffer.

Tube	S^* (mg $\times 10^4$)	P^* (mg $\times 10^4$)	P_m (calcd)	$X + K_m$ (calcd)	Actual X	X (calcd)
1 + 2	18.75	0.1656	1.78	36.12	10.0	11.24
1	3.75	0.138				
2	15.	0.450				
3	18.75	0.500				
1 + 2			1.78	44.5	10	
1 + 3			1.50	36.12	10	
2 + 3			1.01	16.7	10	
Mean			1.43	32.44	10.	11.24
			±.39	±13.7		
4	3.75	0.1656				
5	7.5	0.292				
6	11.25	0.394				
7	18.75	0.543				
4 + 5			1.27	25.3	5	
4 + 6			1.28	25.4	5	
4 + 7			1.28	25.2	5	
5 + 6			1.26	25.5	5	
5 + 7			1.28	25.1	5	
6 + 7			1.29	24.8	5	
Mean			1.28	25.2	5.0	
			±1.3	±0.25		
8	3.75	0.1725	1.15	21.2	0	
9	7.5	0.300				

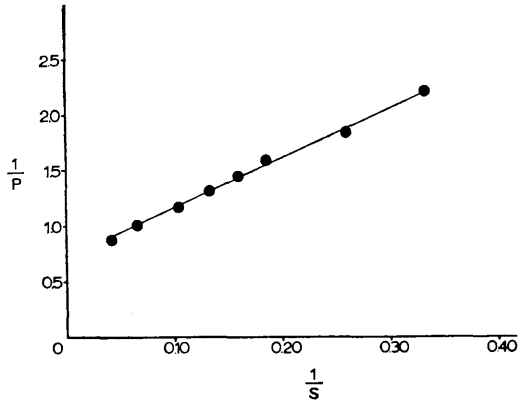

FIG. 2. Lineweaver-Burk plot of reciprocals of substrate and product concentrations during hydrolysis of *p*-nitrophenylacetate by α-chymotrypsin in plasma.

When this experiment was performed with two smaller concentrations of unlabeled substrates (as unknowns), the absolute accuracy remained the same (10^{-4} mg/5 cc) (Table IV) but represented errors of 12.4% and 20% in the two determinations. We attribute most of the error to that involved in the separation of the *p*-nitrophenylacetate (*S*) from acetic acid (*P*).

The assay method proposed here rests on the detailed knowledge of the nonlinear dependence of a typical enzyme reaction on substrate concentration given in the Michaelis-Menten equation. This relation is expected to hold only under steady-state conditions in the absence of significant product inhibition. Our data confirm that the enzymatic hydrolysis of *p*-nitrophenylacetate conforms to these requirements and so provides an adequate test model in evaluating the theory.

While isotope dilution methods and enzyme kinetics have been used to measure concentrations of substances or enzymes, we could find reference to no system to measure both substrate concentration and enzyme activity without chemical isolation of either substrate or a product of enzyme action. The assay method described here allows measurement of substrate concentration and enzyme activity in equivalent units, and requires separation—not isolation—of substrate and one product. The use of this method to measure plasma angiotensin is described elsewhere (1).

1. Schwartz, D. T., Britten, J. S., and Mason, R. C., Proc. Soc. Exp. Biol. Med. **132**, 855 (1969).

2. Michaelis, L. and Menten, M. L., Biochem. Z. **49**, 333 (1913).

3. Lineweaver, H. and Burk, D., J. Am. Chem. Soc. **56**, 658 (1934).

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