APPENDIX

A Computer-Assisted Spectrophotometric Enzyme Assay

A METHOD FOR THE CALCULATION OF K_m AND V FROM A SINGLE REACTION PROGRESS CURVE BY THE INTEGRATED FORM OF THE LINEWEAVER-BURK PLOT WITH PARTICULAR REFERENCE TO β -LACTAMASE

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The utility of u.v. spectrophotometry for penicillin assay was re-confirmed by Waley (1974) and Samuni (1975), who obtained the kinetic parameters of β -lactamase comparable with those reported in the literature. Yun & Suelter (1977) described a manual method for the calculation of K_m and V from a single reaction progress curve by the integrated Michaelis-Menten equation. In the present Appendix a new computer-assisted enzyme assay method is described in which a reaction progress curve expressed in time-absorbance is converted into time-(molar substrate) concentration data and systematically combined for the determination of statistically reasonable values of K_m and V by the integrated form of the Lineweaver-Burk plot. This new assay method has the following advantages: (1) K_m and V are precise and accurate; (2) it takes less time and less material to get a set of the kinetic constants; (3) no calculation error is probable; (4) In the final-absorbance method for conversion of absorbance readings into molar substrate concentrations, some causes of assay errors (e.g. increase in u.v. absorbance by addition of enzyme and additives, disturbance of u.v. absorption by improper washing or setting of cuvettes, and pipetting error of substrate solution) are automatically cancelled; (5) additional enzymological information (e.g. fitting to the Michaelis-Menten equation, substrate inhibition, product inhibition, type of inhibition by inhibitor, kinetic parameters for equilibrium reactions and two-substrate reactions and accuracy of the assay method) is obtainable.

Theory (Yun & Suelter, 1977)

The irreversible two-step enzyme reaction:

should obey the integrated Michaelis-Menten eqn. (1):

$$Vt = ([S]_0 - [S]_t) + K_m \cdot \ln([S]_0/[S]_t) \quad (1)$$

where V is the maximum reaction velocity, t is the time of reaction, $[S]_0$ is the substrate concentration

at zero time, $[S]_t$ is the substrate concentration at time t and K_m is the Michaelis constant.

For two pairs of time-(molar substrate) concentration data, $(t_i, [S]_i)$ and $(t_j, [S]_j)$, along a single reaction progress curve, eqn. (2) without $[S]_0$ can be obtained from eqn. (1):

$$V(t_j - t_i) = ([S]_i - [S]_j) + K_m \cdot \ln([S]_i/[S]_j) \quad (2)$$

Division of both sides by $V([S]_i - [S]_j)$ gives the integrated form of the Lineweaver-Burk plot (eqn. 3):

$$\frac{t_j - t_i}{[S]_i - [S]_j} = \frac{1}{V} + \frac{K_m \cdot \ln([S]_i/[S]_j)}{V([S]_i - [S]_j)} \quad (3)$$

Conversion of Absorbance into Molar Substrate Concentration*Final-absorbance method*

The absorbance reading of product at the end of reaction (A_∞) is used as zero concentration of substrate as follows:

$$[S]_i = (A_i - A_\infty)/\Delta\epsilon$$

where $[S]_i$ is the molar substrate concentration at time t_i , A_i is the absorbance reading at time t_i , A_∞ is the final absorbance reading and $\Delta\epsilon$ is the molar differential absorption coefficient.

Initial-absorbance method

When the product is spectrophotometrically unstable, the final-absorbance method cannot be applied successfully. As an alternative basis for conversion, the initial absorbance reading (A_0), independent of reaction time, after being appropriately corrected for the absorbance change due to the added enzyme, is employable as follows:

$$[S]_i = (A_0/\epsilon) - [(A_0 - A_i)/\Delta\epsilon]$$

where $[S]_i$ is the molar substrate concentration at time t_i , A_0 is the initial absorbance reading (corrected for the absorbance change due to the added enzyme, if necessary), A_i is the absorbance reading at time t_i , ϵ is the molar absorption coefficient and $\Delta\epsilon$ is the molar differential absorption coefficient.

Time-A-molar substrate concn. table

TIME	ABS	MOL SUB CONC
6.00	1026.000	7.45E-01
6.50	966.000	6.51E-01
7.00	908.000	5.60E-01
7.50	851.000	4.70E-01
8.00	796.000	3.84E-01
8.50	743.000	3.00E-01
9.00	694.000	2.23E-01
9.50	651.000	1.56E-01
10.00	614.000	9.75E-02
10.50	586.000	5.35E-02

Sweep mode of data combination

CONSTANT-DIFFERENCE METHOD		METHOD	
DIF	COR COEFF	V MAX	KM
1	.9996	2.16E-01	1.07E-01
2	.9997	2.17E-01	1.08E-01
3	.9998	2.18E-01	1.10E-01
4	.9999	2.18E-01	1.09E-01
5	.9999	2.17E-01	1.08E-01
6	.9998	2.17E-01	1.08E-01
7	.9999	2.16E-01	1.06E-01

CONSTANT-POINT METHOD		METHOD	
POINT	COR COEFF	V MAX	KM
1	.9993	2.17E-01	1.08E-01
2	.9988	2.16E-01	1.07E-01
3	.9992	2.16E-01	1.06E-01
4	.9995	2.17E-01	1.07E-01
5	.9991	2.17E-01	1.07E-01
6	.9994	2.19E-01	1.12E-01
7	.9991	2.20E-01	1.13E-01
8	.9994	2.15E-01	1.06E-01
9	.9999	2.16E-01	1.06E-01
10	.9999	2.16E-01	1.07E-01

ALL-COMBINATION METHOD		METHOD	
COR COEFF		V MAX	KM
.9996		2.17E-01	1.08E-01

Lineweaver-Burk plot by the all-combination method

R= .9996 V MAX=2.17E-01 KM=1.08E-01
1/V MAX=4.61E+00 1/KM=9.27E+00

Y UNIT = 4 X UNIT = 4

Combination of Time-(Molar Substrate) Concentration Data for the Calculation of K_m and V by the Integrated Form of the Lineweaver-Burk Plot

Although any combination of assay data is valid in the integrated Michaelis-Menten equation, three systematic methods of data combination are employed for the effective analysis of assay errors.

Constant-difference method (Yun & Suelter, 1977)

The t -[S] data are combined at a given constant difference between data numbers. Thus the larger the constant difference is the smaller the number of combinations that are obtained.

Constant-point method (T. Ishikura, unpublished work)

The kinetic constants are calculated by combination of a selected data number with all the rest. In this method, the number of combinations remains the same for all the constant points.

All-combination method (T. Ishikura, unpublished work)

All the possible combinations of data pairs [from n data pairs, $(n-1) \times n/2$ combinations] are used to give one set of K_m and V values.

Computer Program

For the IBM CALL/370 ON-LINE TIME SHARING SERVICE SYSTEM (System 370, model 155), a program named YF6TAIM was written as an improved form of TI6BLASE (Y. Fukagawa & T. Ishikura, unpublished work) in the CALL:BASIC language. A copy of this program is obtainable from the author on request or as Supplementary Publication SUP 50100 (12 pages) from the British Library Lending Division, Boston Spa, Wetherby, West Yorkshire LS23 7BQ, U.K., on the terms indicated in *Biochem. J.* (1978) **169**, 5.

Fig. 1. Computer analysis of the hydrolysis curve of 1.96 mM-benzylpenicillin (excerpt from the print-out) After the non-Michaelian data pairs were trimmed in the Scatchard space [Fig. 3 of the main paper (Fukagawa *et al.*, 1980)], the time course from 6.0 to 10.5 min of hydrolysis of 1.96 mM-benzylpenicillin by *Bacillus licheniformis* 749/C β -lactamase was converted into the t -[S] data by the final-absorbance method, and then combined by the sweep mode of data combination for the computation of K_m and V values. The attached plot was drawn by the all-combination method of data combination.

Example of Data Processing

As an example of data processing by the computer program YF6TAIM, the time course of hydrolysis of 1.96 mM-benzylpenicillin by *Bacillus licheniformis* 749/C β -lactamase in Table 1 of the main paper (Fukagawa *et al.*, 1980) was chosen. After the non-Michaelian data pairs were deleted in the Scatchard space, the time course of reaction from 6.0 to 10.5 min was converted into time-(molar substrate) concentration data by the final-absorbance method, and then combined by the sweep mode of data combination (all the possible combination steps

of the constant-difference, constant-point and all-combination methods) (A_{∞} 0.552 at 240 nm; $\Delta\epsilon$ 636 M⁻¹·cm⁻¹; $n = 10$). Essential parts of the computer print-out are shown in Fig. 1.

References

- Fukagawa, Y., Takei, T. & Ishikura, T. (1980) *Biochem. J.* **185**, 177–185
Samuni, A. (1975) *Anal. Biochem.* **63**, 17–26
Waley, S. G. (1974) *Biochem. J.* **139**, 789–790
Yun, S.-L. & Suelter, C. H. (1977) *Biochim. Biophys. Acta* **480**, 1–13