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ESTIMATION OF KINETIC PARAMETERS, AMOUNT OF ENDOGENOUS SUBSTRATE AND CONTAMINATING ENZYME ACTIVITY IN A TARGET ENZYME REACTION

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New analytical methods were devised to estimate the Michaelis constant (K_m), the maximum velocity (V), the concentration of endogenous substrate (x) and the activity of contaminating enzyme (u) in an impure enzyme reaction with single substrate. In the non-radiometric assay, the linear plot was developed on the basis of Eisenthal-Cornish-Bowden plot [1] by transforming the equation for reaction rate (v) consisting of K_m, V, x, u and the concentration of substrate (S'). To confirm the accuracy of the linear plot, the non-linear fitting method was simultaneously devised in terms of a modification of the method of Cleland [2]. In the radiometric assay, the linear and non-linear kinetic analyses were applied to the equation for the radiometric rate (v^*), expressed by K_m, V, x and S^* (concentration of radioactively labelled substrate) as in the non-radiometric assay. In both assays, the values of K_m, V and x (with u value in the non-radiometric assay) were obtainable at x_1 and x_2 with a known ratio of x_2/x_1. The validity of the above methods was proved by the model experiments with purified enzymes; and the radiometric model experiment offered a good example for a new enzymatic assay method of many substrates. These methods were successfully applied to the practical experiments.

Introduction

Crude enzyme samples (such as partially purified enzyme preparation, tissue homogenate, subcellular fraction etc.) are often used in an enzyme reaction to study the kinetic parameters (K_m and V) for a single substrate reaction obeying the Michaelis-Menten equation. The crude samples generally contain unknown amounts of endogenous substrate, reaction product and inhibitor; and the observed reaction rate is thought to deviate from the Michaelis-Menten equation, unless the concentrations of these endogenous components are made negligibly low by dilution in the reaction mixture. In addition, the crude samples sometimes contain the contaminating enzyme(s) which is different from the target enzyme, and the same product as that of target enzyme reaction is produced by the contaminating enzyme from the different substrate(s) present in the crude samples.

Under the condition that inhibition by endogenous product or inhibitor is negligible, the kinetic methods applicable to the impure reaction systems are described in this paper to estimate K_m, V, the unknown amount of endogenous substrate (x) and the contaminating enzyme activity (u) in the single substrate enzyme reactions, involving radioactively-labelled and non-radioactively-labelled substrates. The results of model experiments, using purified enzyme preparations with intentionally added substrates as substitutes for endogenous ones, are indicated to prove the validity of the methods; and

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then the examples are shown for application of the methods to analyze the enzyme reactions containing the crude samples, isolated cell nuclei and white blood cells.

Materials and Methods

Model experiments

Non-radiometric assay of galactose dehydrogenase (EC 1.1.1.48). Based on the determination method of Doudoroff [3], the activity of galactose dehydrogenase 487 (or 690) ng protein/ml was fluorimetrically determined in the reaction mixture (1.0 ml), containing 100 mM Tris-HCl (pH 8.5)/0.02% (w/v) bovine serum albumin/1 mM reduced glutathione (GSH)/2.55 mM NAD^+/varied concentration of galactose. Additional galactose (20–70 μM) was included as endogenous substrate. In this mixture, 5 mM sodium glutamate and 0.22 mM ADP were added with glutamate dehydrogenase (32.5 ng protein/ml, EC 1.4.1.2) as contaminating enzyme. The increase of fluorescence was read directly in a fluorimeter. The reaction rate of galactose dehydrogenase was linear for 20 min at 23°C under this condition, however the rate of glutamate dehydrogenase was not strictly linear but progressively decreased during this incubation period. In the reaction mixture containing both enzymes, the rate of NADH formation could be regarded as practically linear for the first 6 min and the initial velocity was determined during this period.

Radiometric assay of acetylcholinesterase (EC 3.1.1.7). [1-^{14}C]Acetic acid released from [1-^{14}C]-acetylcholine was measured according to McCaman et al. [4]. The reaction mixture (40 μl) consisted of 75 mM potassium phosphate buffer (pH 7.2)/0.0125% (w/v) bovine serum albumin/19.7 (or 39.4) ng protein of acetylcholinesterase/ml/varied concentrations of [^{14}C]acetylcholine chloride (specific radioactivity, 2.92 Ci/mol). Enzyme solution (10 μl) in 20 mM Tris-HCl (pH 8.0)/0.05% (w/v) bovine serum albumin was added at 0°C to the reaction mixture (30 μl). The mixture was incubated for 30 min at 37°C. The reaction was stopped by cooling in an ice bath and by adding 5 μl of 100 mM choline chloride in 1.5 M HCl and 20 μl of saturated ammonium reineckate, $(NH_4)[Cr(NH_3)_2(SCN)_4]H_2O$, solubilized in 0.5 M HCl. After centrifugation for 20 min at 1 500 × *g* at 4°C, 45 μl of the supernatant were collected and 5 μl choline chloride and 20 μl ammonium reineckate were added again as above. The radioactivity in a 50 μl aliquot of the supernatant was measured with 0.5 ml Soluen-350 (Packard, U.S.A.) and 10 ml toluene-scintillation fluid containing 0.4% (w/v) 2,5-diphenyloxazole and 0.025% (w/v) 1,4-bis(4-methyl-5-phenyloxazole-2-yl)benzene in a scintillation spectrometer (Packard Tri-Carb model 3 380, counting efficiency, 77.4%). The reaction rate was linear for 40 min in the range of enzyme concentration up to 100 ng protein/ml. Non-radioactively labelled acetylcholine chloride was added to the reaction mixture prior to assay for trial estimation of the amount of endogenous substrate.

Application experiments

Enzymatic assay of galactocerebrosidase (EC 3.2.1.46). Galactocerebrosidase in human white blood cells was fluorimetrically determined under the conditions reported previously [5]. White blood cells were collected from healthy adults (one female and four males) and the cell suspensions (3–5 mg protein/ml) were prepared in physiological saline according to the method of Snyder and Brady [6].

The suspension was sonicated for 1 min in an ice bath and directly added to the reaction mixture (46.8 μl) at the concentrations of 145–387 μg of protein/ml and incubated for 3 h to carry out the reaction by the enzyme contained in lysosomes of white blood cells. Saline was used as blank in the assay. The reaction was linear for at least 4 h. Galactose released was quantitatively converted to NADH by galactose dehydrogenase in the presence of excess NAD^+. The amount of NADH formed was so small that its fluorescence could not be measured directly because of the high background fluorescence from the other substances contained in white blood cells. Therefore NADH formed in a very small volume (1.4 μl) of galactose dehydrogenase reaction mixture was specifically amplified 6 000-fold to overcome the blank fluorescence by using NAD cycling reaction [7] and the amplified amount was fluorimetrically determined. The unknown amount of endogenous galactocerebroside in white blood cells was utilized as substrate by the endogenous galactocerebrosidase. The other enzyme activity (probably α- and β-galactosidases), which produced galactose from other undefined substrate(s) containing galactose residue

(ganglioside G_{M1}, ceramide-trihexoside, etc.) was present in white blood cell sample.

Radiometric assay of DNA ligase (EC 6.5.1.1). The DNA ligase activity in isolated cell nuclei was determined as described previously [8]. The glial cell nuclei isolated from cerebral cortex of guinea pig were incubated with [^{32}P]phosphoryl DNA as substrate, which had [5′-^{32}P]phosphomonoesters at the single-strand nicks of calf thymus DNA. After incubation, DNA was treated with alkaline phosphatase to remove unreacted and remaining 5′-phosphomonoesters. The amount of phosphatase-resistant 3′,5′-phosphodiesters was radiometrically determined and one unit of the enzyme activity was defined as that amount converting 1 fmol 5′-phosphomonoester into a phosphatase-resistant form per min under the reaction conditions. The endogenous nicks in chromatin DNA of isolated nuclei were utilized as substrate by the endogenous nuclear DNA ligase.

Other determinations. The concentration of acetylcholine in stock solution, used for acetylcholinesterase reaction, was determined photometrically by a modification of the enzyme coupling reactions reported by Browning [9]. The reaction mixture (1.0 ml) contained 100 mM Tris-HCl (pH 8.5)/10 mM $MgCl_2$/1.0 mM phospho*enol*-pyruvate/2.0 mM ATP/140 μM NADH/1.3 μg/ml lactate dehydrogenase (EC 1.1.1.27)/62 μg/ml pyruvate kinase (EC 2.7.1.40)/21 μg/ml choline kinase (EC 2.7.1.32)/47 ng/ml acetylcholinesterase/ about 100 μM acetylcholine chloride. Galactose solution, used for galactose dehydrogenase reaction, was standardized photometrically in the reaction mixture (1.0 ml) consisting of 100 mM Tris-HCl (pH 9.0)/1.0 mM GSH/0.26 mM NAD^+/25 μg/ml galactose dehydrogenase/about 100 μM galactose. The concentration of galactocerebroside (8.37 mM) solubilized in chloroform/methanol (2 : 1) was standardized by measuring galactose in a 53 μl aliquot, after the organic solvents were evaporated to dryness and galactocerebroside was hydrolyzed at 100°C, for 3 h, in 1.0 ml of 1 M HCl. For standardization of [^{32}P]phosphoryl DNA, see Inoue and Kato [8]. Protein was determined by the method of Lowry et al. [10] with bovine serum albumin as standard.

Materials. Acetylcholinesterase (acetylcholine hydrolase) from *Electrophorus electricus*, β-galactose dehydrogenase (D-galactose: NAD oxidoreductase) from *Pseudomonas fluorescens*, glutamate dehydrogenase (L-glutamate: NAD(P) oxidoreductase (deaminating)) from bovine liver, lactate dehydrogenase (D-lactate: NAD oxidoreductase) from porcine muscle, and pyruvate kinase (ATP: pyruvate phosphotransferase) from rabbit muscle were purchased from Boehringer-Mannheim, F.R.G. Choline kinase (ATP: choline phosphotransferase) from baker's yeast was obtained from Sigma, U.S.A. Galactocerebroside from bovine brain was supplied by Sedary Research Laboratory, Canada. [^{14}C]Acetylcholine chloride (specific radioactivity, 5.87 Ci/mol) was obtained from the Radiochemical Center, U.K. Other chemicals were of analytical grade.

Results

Model experiments

Fluorimetric assay of galactose dehydrogenase. Without addition of glutamate dehydrogenase as a contaminating enzyme ($u = 0$), the activities of galactose dehydrogenase (487 ng protein/ml) were determined at seven varied concentrations of substrate (0–1.44 mM) under the condition that two different concentration levels of galactose were added in advance as endogenous substrate ($x_1 = 42.0$ and $x_2 = 130$ μM). The plots of the reaction rates were hyperbolic curves with positive intercepts on the ordinate and the linear kinetic analysis was carried out in terms of Eqn. 4 (see Appendix). (Simultaneously, the same data were analyzed by the non-linear fitting method as described, following the linear method above). The median values of co-ordinates (A_1, B_1) and (A_2, B_2) for degenerated intersecting points (see Fig. 3) were (248.5, 4.89) and (341, 158), respectively. These median co-ordinates provided the values of V, K_m and x (with the results ± S.E. from the non-linear fitting method) as follows: $V_1 = 1.31$ [1.32 ± 0.038] and $V_2 = 1.34$ [1.36 ± 0.026] nmol/min per assay; $K_{m1} = 207$ [225 ± 22] and $K_{m2} = 209$ [213 ± 18] μM; $x_1 = 42.2$ [45.0 ± 6.0] and $x_2 = 132$ [132 ± 10] μM. The sizes and signs of the residuals (i.e., the differences between the data of v_i and the v_i values calculated from the fitted hyperbolic curves) were random and the σ (the average square of the residuals) was small enough (1.32% of V_1 and 0.74% of V_2). These results indicated that all the values of V, K_m and x obtained from the linear plotting

method coincided well with the results of the well-fitted non-linear curve, within a range of one standard error, and that there was no significant difference between the estimated values and the known values of x_1 and x_2.

On addition of 32.5 ng/ml glutamate dehydrogenase ($u = 0.529 \pm 0.024$ nmol/min per assay, average ± S.D. from three assays) as a contaminating enzyme with its substrate glutamate, part of NADH originated from this enzyme reaction as well as the formation by galactose dehydrogenase (690 ng/ml, and V is constant). Three hyperbolic curves, as a function of varied substrate concentration, with positive intercepts on the ordinate were expected at three different concentrations of galactose added in advance ($x_1 = 20.2$, $x_2 = 42.9$ and $x_3 = 70.4$ μM). These curves were subjected to the linear kinetic analysis in terms of Eqn. 4 (Appendix). (The same data were analyzed by the non-linear fitting method as described in Appendix). Three median co-ordinates were: ($A_1 = 232$, $B_1 = 2.64$), ($A_2 = 254$, $B_2 = 1.90$) and ($A_3 = 282$, $B_3 = 1.45$). Considering the known ratios (α_i) of any pair of x_i values ($i = 1$, 2 or 3), the corresponding pair of these three median values gave two values for V and u, but a single value for K_m and for each of paired x_i values. Thus, the remaining x_j value was calculated based on the known ratio α_j. Therefore, three couples taken from three median coordinates provided the numbers of results as given below in parentheses with the estimated values of V, K_m, x and u. For convenience to show the dispersions of the results, the averages of the estimated values are given with ± S.D. (and the results ± S.E. from the non-linear fitting method are simultaneously given in following brackets): $V = 1.74 \pm 0.12$ [1.93 ± 0.061] nmol/min per assay (6); $K_m = 212 \pm 1.9$ [213 ± 4.0] μM (3); $x_1 = 19.9 \pm 0.85$ [24.4 ± 1.8] μM (3), $x_2 = 42.3 \pm 1.7$ [49.2 ± 3.8] μM (3) and $x_3 = 69.3 \pm 2.9$ [81.6 ± 6.3] μM (3); $u = 0.561 \pm 0.021$ [0.420 ± 0.018] nmol/min per assay (6). In the non-linear fitting method, the tentative value, $K'_{mi} + x'_i$, corresponding to A_i, were calculated by fitting the data to Eqn. 1 with $u = 0$ (see Appendix). These ($K'_{mi} + x'_i$) values gave the S.E. ranged from 5.3 to 6.2% of their values. The S.E. of ($V + u$) ranged from 1.37 to 1.46% of their values and the residuals were randomly dispersed. The σ was small and 1.37–1.46% of V' values. These indicated that the hyperbolic curves fitted well to the data. The results from both methods were well in accord within a range of mostly two standard errors and there were no statistical significances in differences between the values of x_i in both methods and the known values of x_i added in advance. The u values in both methods were also statistically not different from the known values.

Radiometric assay of acetylcholinesterase. The activities of purified acetylcholinesterase at two different enzyme concentrations ($e_1 = 19.7$ and $e_2 = 39.4$ ng/ml, $e_2/e_1 = 2$) were determined at eight concentrations of radioactively labelled acetylcholine varied from 0 to 658 μM. The Eisenthal-Cornish-Bowden plot gave the following results (simultaneously the results ± S.E. from the non-linear fitting method are shown in brackets): $V_1 = 15.5$ [15.9 ± 0.22] and $V_2 = 31.7$ [31.6 ± 0.45] pmol/min per assay; $K_{m1} = 101$ [106 ± 4.1] and $K_{m2} = 105$ [104 ± 4.2] μM. An unknown amount of endogenous substrate, non-radioactively labelled acetylcholine was added further to the assay mixture at two different concentrations ($x_1 = 26.3$ and $x_2 = 52.7$ μM, $x_2/x_1 = 2$). The reaction rate curves were skewed from the normal hyperbolas as suggested by Eqn. 6 (see Appendix). The Eisenthal-Cornish-Bowden plot provided, as described in Appendix, the following median values (which are shown, for comparison, with the results ± S.E. from the non-linear fitting method in brackets): $K_m + x_1 = 129$ [128 ± 3.4] and $K_m + x_2 = 158$ [155 ± 6.2] μM; $V_1 = 15.9$ [14.4 ± 0.17] and $V_2 = 31.6$ [30.8 ± 0.54] pmol/min per assay. These results gave the following values based on the ratio of x_2/x_1: $K_m = 104$ [103] μM; $x_1 = 28.7$ [26.1] and $x_2 = 57.5$ [52.3] μM. In the non-linear fitting method, the residuals for all the hyperbolas above were random and the σ values were in the range from 0.83 to 1.06% of the V values above. These results indicated that the Eisenthal-Cornish-Bowden plot gave practically identical results as those for the well-fitted hyperbolic curves in the presence or absence of endogenous substrate. As a characteristic feature of the radiometric analysis, it can be pointed out that the dilution of radioactively labelled substrate with non-radioactively labelled endogenous substrate simplified the rate Eqn. 6 and made the kinetic analysis simpler compared with the non-radiometric rate Eqn. 1.

Application experiments

Enzymatic assay of galactocerebrosidase in white blood cells. White blood cells did not contain an appreciable amount of galactose (unpublished data) and the product-inhibition was regarded as negligible. In this practical case, the results of the present kinetic analysis were illustrated in detail (Fig. 1). The rate curves were fitted by eye to experimental activity plots and each observation value for enzyme activity (v_i) was read from these curves at each varied concentration of substrate (S_i') on the ordinate (Fig. 1a). The reason for this indirect reading is that if the actual activity values were used for analysis a very small fluctuation of the ordinate intercept of a straight line (which was given in a form of ratio between the difference of two observed values with

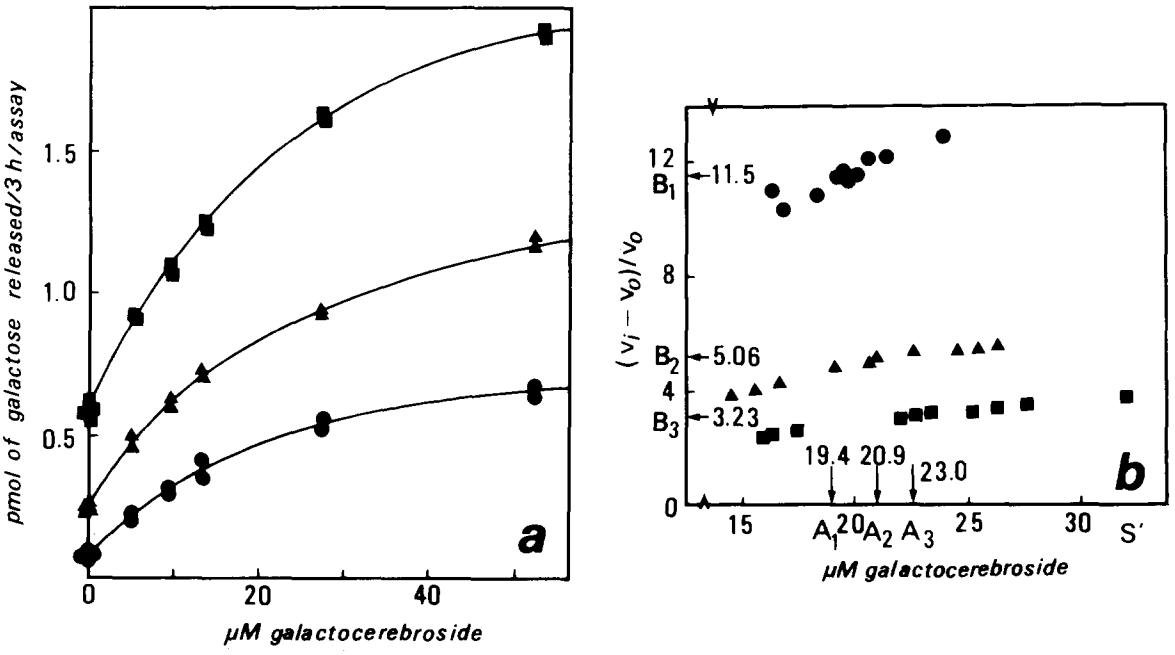

Fig. 1. Linear kinetic analysis of galactocerebrosidase activity in white blood cells. a. Sonicated suspension of white blood cells was assayed directly as enzyme sample at three concentration levels (●, 145; ▲, 242; and ■, 387 μg protein/ml). Three apparently hyperbolic curves, starting from the positive intercepts on the ordinate, were visually fitted to the plots of reaction rates as a function of varied concentration of galactocerebroside on the abscissa. Two plots at the same concentration represent the results of assay in duplicate, except for the four plots at the 0 concentration. Since v_0 was used as a basis for calculation of the ordinate intercepts (Y_i) in b, the quadruplicate assay was performed without addition of substrate and the average of its results was defined as v_0. b. Three groups of intersecting points of straight lines, produced from three curves in a were plotted as shown in Fig. 3 and the median co-ordinates of the points in each group (A_1, B_1), (A_2, B_2) and (A_3, B_3) were indicated in this figure (symbols are same as in a). (The hyperbolas were directly fitted to the plots in a and the values of $K_m + x$ and $V + u$ were calculated as described in the Appendix). These median co-ordinates provided the following averages ± S.D. with the numbers of results in parentheses (for the meaning of the numbers of results, see the 'Fluorimetric assay of galactose dehydrogenase'), after the values of V and x were, for comparison, normalized with respect to the amount of sample (the similarly expressed results ± S.E. from the direct non-linear fitting are given in brackets): V = 4.52 ± 0.05 [4.17 ± 0.70] μmol galactose released/kg protein per h (6); K_m = 17.4 ± 0.05 [17.1 ± 0.15] μM (3); x = 14.4 ± 2.9 [16.0 ± 0.58] μmol galactocerebroside/kg protein of white blood cell [6]; and $u/V\%$ = 3.88 ± 5.12 [5.28 ± 3.95] percent of V (6). (The $u/V\%$ was calculated to realize the degree of contamination with other enzyme activity). The non-linear fitting method provided the small S.E. for $K_m + x$ (5.74 to 7.71% of the values) and $V + u$ (1.67 to 2.35% of the values). The residuals were random and the σ values were small enough (0.61 to 0.75% of the values of $V + u$). When lipid substrate, galactocerebroside, is utilized by the impure enzyme sample such as white blood cells, the reaction rate curve was not precisely expressed by Eqn. 1, since x does not represent the true concentration of endogenous substrate, but is the summation of the concentration of endogenous substrate (10.0 ± 0.15 [11.8 ± 0.56]) and that of substrate-binding sites (4.38 ± 0.41 [4.16 ± 0.30]). The reason for this discrepancy is beyond the scope of this paper and will be dealt with in detail elsewhere (Kato and Suzuki, unpublished data).

similar magnitude ($v_i - v_0$) and v_0) very often produced an outlier in a group of straight lines. According to the method illustrated in Fig. 3 and as described in the Appendix, the intersecting points were plotted and three median co-ordinates of the intersecting points for three original curves were calculated by using a computer (Fig. 1b). As shown in the legend, the present linear kinetic method provided good results with small dispersions, which were supported by the results from the direct non-linear fitting. These small dispersions signified that the effect of inhibitors contained, if any, in white blood cells was too small to be appreciable (see Discussion). The K_m values in the legend were in the similar range of K_m values determined for the homogenates of mouse organs (cerebrum, 14.9 μM; liver, 22.9 μM, and kidney, 11.0 μM, unpublished data). The contaminating enzyme activity was so low that it could be neglected at the lower concentrations of white blood cell samples (u/V% less than 2% at 145 ng protein/ml).

Radiometric assay of DNA ligase in isolated glial cell nuclei. In this practical experiment, the results are also described in detail. The cell nuclei isolated by means of cell fractionation technique contained DNA ligase and its substrate nicks in chromatin DNA. When isolated nuclei were analyzed directly in the reaction mixture, the nuclear nicks worked as contaminating substrate and skewed the rate curves; then the nicks became an obstacle to estimation of kinetic parameters in the enzyme reaction. Under the assay condition, the reaction product (3',5'-phosphodiester bond) was far greater in amount than the substrate nicks (see the legend to Fig. 2); thus in addition to the effect of inhibitors, if present, that of the product must be taken into consideration. At two different concentrations of glial nuclei incubated, the double-reciprocal plots had two different intercepts on the abscissa and ordinate, respectively (Fig. 2). The ratio of V_2/V_1 was equal to that of sample concentrations ($C_2/C_1 = 2$). This was plausible since V is proportional to the sample concentration and signified that the appreciable effect on V of product and inhibitors was not detectable at the concentrations of crude samples. The calculated value of K_m was similar to the K_m values for the extract sample from glial nuclei (26.3 μg [^{32}P]phosphoryl DNA/ml; see Table II in Ref. 8), which was

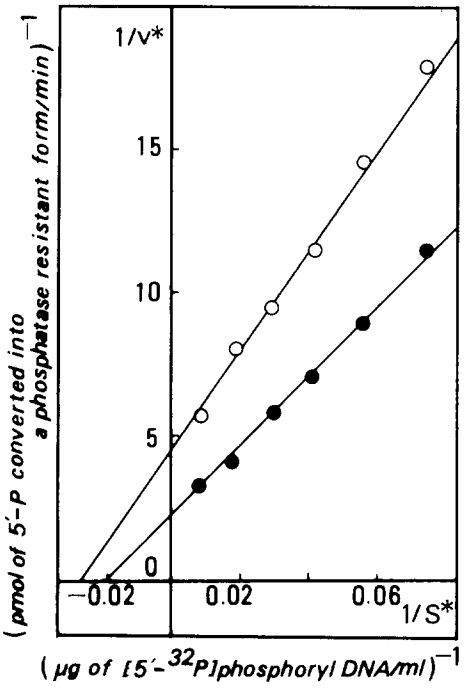

Fig. 2. Double-reciprocal plots for radiometric assay of nuclear DNA ligase. The double-reciprocal plot is given for the radiometric assay to show the validity of Eqn. 6 in the Appendix. At two concentration levels of glial nuclei (○, C_1 = 49.3 and ●, C_2 = 98.7 μg nuclear DNA/ml, $C_2/C_1 = 2$), DNA ligase activities of nuclear samples were measured. The short period of incubation (2 min) was adopted to prevent further breakage of chromatin DNA during incubation by endogenous DNAase. The Eisenthal-Cornish-Bowden plot gave the following median values of $K_m + x$ and V (the results ± S.E. from the non-linear fitting method were given simultaneously in the brackets, see the Appendix): $K_m + x_1 = 37.2$ [38.8 ± 3.7] and $K_m + x_2 = 48.7$ [49.3 ± 3.1] μg [^{32}P]phosphoryl DNA/ml; $V_1 = 222$ [223 ± 9.2] and $V_2 = 424$ [443 ± 31] units. The fitted hyperbolas had the random residuals and the small values of σ (1.9% of V_1 and 2.9% of V_2). These values provided the following values: $K_m = 25.6$ [28.4] μg [^{32}P]phosphoryl DNA/ml; $x_1 = 11.5$ [10.5] and $x_2 = 23.0$ [21.0] μg [^{32}P]phosphoryl DNA/ml. The average specific activity calculated from V_1 and V_2 was 38.6 units/μg nuclear DNA or 17.0 units/μg nuclear protein. The amount of nicks present in the radioactively labelled substrate DNA was determined (1.18 pmol/μg DNA; approx. 0.36% of the total 3',5'-phosphodiester bonds on the assumption that the average molecular weight of each deoxyribonucleotide is 330). Thus, the values of K_m, x_1 and x_2 could be expressed as 30.2, 13.6 and 27.2 pmol of nicks/ml of assay mixture, based on the results from the Eisenthal-Cornish-Bowden plot. Considering the amount of nuclear DNA in the reaction mixture, glial nuclei were estimated to contain 0.276 pmol of nicks/μg nuclear DNA (i.e., 0.084% of the total 3',5'-phosphodiester bonds in chromatin DNA).

obtained from the Lineweaver-Burk plot for the nuclear extract containing no endogenous substrate; this supported the validity of the present method and also indicated that any effect of product and inhibitor in nuclear sample, which is much cruder than extract sample, was apparently not present.

Discussion

Generally, the enzymes utilizing as substrates the substances with low molecular weights have relatively high contents (or specific activities, [11]) in tissue; and the sample concentrations can be made 100-fold, or more, lower with these enzymes than in the application experiments (Figs. 1 and 2), even though the tissue homogenate is directly added in the reaction mixture. In contrast, the contents of galactocerebrosidase and DNA ligase, catalyzing the reactions with high-molecular weight substrates, were so low that the sample concentrations could not be lowered enough by dilution in the reaction mixture to reduce the concentrations of endogenous substrates to negligible values. Therefore, the present kinetic methods are believed to be useful for analysis of an poorly active enzyme utilizing high-molecular weight substrate, when the highly sensitive determination methods (such as enzymatic cycling, radioenzymatic assay, mass spectrography, etc., [12,13]) are developed for the analysis.

Although it was assumed for developing the present methods that the inhibition by endogenous product and inhibitor is negligible (see Appendix), the effect of these components should be considered in the general case, in which the enzyme activity is so low that their concentrations cannot be diluted to be negligible. For instance, if the inhibition effect is of competitive type, the demominator of Eqn. 1 or 6 can be replaced by the expression, $K_m(1 + I/K_i) + x + S'$ (or S^*), where K_i represents the dissociation constant for I and I represents the concentration of inhibitor (or product), which is proportional to x; therefore the replaced denominator can be rewritten as $K_m + (\beta + 1)x + S'$ (or S^*; β is a constant coefficient and stands for $K_m I/K_i x$). In this case, the endogenous substrate, as determined in the present application experiments, would include the effect of a inhibiting factor. In the other cases (partially competitive, non-competitive, uncompetitive, etc.), the appropriate transformation of Eqn. 1 or 6 can be performed by the analogy of the established ways in the general enzyme kinetics [14,15]. The apparent K_m and V, including the effect of inhibiting factors, are estimated according to present kinetic methods; and these are replotted against the several levels of sample concentration (which is proportional to I), depending on the type of inhibition (detailed explanation on the replotting methods is omitted here since they are self-evident, considering the type of inhibition). The replot provides true K_m and V values, although the absolute values of I and K_i are not obtainable. Thus, the present methods are thought to be applicable to the general impure enzyme reactions.

In the galactocerebrosidase reaction (Fig. 1), the reaction product, galactose. was not sensibly contained in white blood cells and its effect on the reaction was neglected. If a competitive inhibitor was contained in the sample, the value of x was though to include its effect as discussed above. If the other type of inhibitor was included in the sample, K_m and V were though to be influenced to a greater extent with the increase in sample concentration, since the concentration of inhibitor was increased with the sample concentration. However the values of V, K_m and x were well in accord with small dispersions at three sample concentrations; then the effect of inhibitor could be neglected. In the DNA ligase reaction (Fig. 2), the substantial amount of product, 3′,5′-phosphodiester, was included in isolated nuclei and inevitably in the added substrate DNA. Thus, the effect of product should be considered as well as that of inhibitor in this case. However, this effect was detected as negligible as described in the Results and the present methods could be directly applied. In general, the application of the present methods is evidently straightforward and useful when the substrate of an enzyme is of high-molecular weight and the low-molecular weight substrate can be eliminated by dialysis with inhibitors.

As described in the model experiments, the linear plot for Eqns. 4 and 6 proved to be reliable by the support of the non-linear fitting method. The linear plotting method has the statistical basis on the generally unfamiliar non-parametric statistics [16] to obtain the median co-ordinates (A, B) in Fig. 3, but its application is easy and graphically demon-

strable; thus, it is intuitively understandable. The graphical plotting is practically useful to roughly estimate V, K_m and x values, although not precise because of the over-crowding intersecting points around the median point. In this practical sense, the double-reciprocal plot of Eqn. 6 is analogous to the Lineweaver-Burk plot and useful as a graphical method for non-statistical estimation of K_m and x (Fig. 2). The great mathematical difference between the linear and non-linear kinetic methods is that the average v_0 is employed as a fixed point in the linear plot, but a hyperbolic curve is fitted equally to v_0 and other v_i points in the non-linear fitting method.

As shown in Fig. 2, acetylcholine could be determined when [^{14}C]acetylcholine and purified acetylcholinesterase were available. Similarly, a large number of substrates can be enzymatically determined according to Eqn. 6, if the radioactively labelled substrates and enzymes catalyzing the reactions with the substrates are available. Where the radioactively labelled substrates are not available, the enzymatic method based on Eqn. 4 is useful for assays when the substrates have to be purified prior to determination by laborious procedures. For instance, galactocerebroside is usually determined by measuring galactose residue, after hydrolysis in acid following its purification by thin layer chromatography of lipid samples extracted with organic solvents. In addition, the substantial amount of original sample (e.g., about 10 mg protein of white blood cells) is necessary for these extraction and purification procedures. In contrast, only 300 μg protein of white blood cells were directly analyzed for their galactocerebroside content (Fig. 1).

In view of the basis for the present kinetic methods (namely enzyme kinetics), the determined values for endogenous substrates are thought to represent the available part for the target enzymes of the total substrates in the crude samples (Figs. 1 and 2). The available portion is thought to be biologically meaningful, because the impure enzyme reaction, including a sample of cells or their subfraction, mimics the enzyme reaction occurring in the cell and the estimated amount seems to be a better approximation in the in vivo situation (amount of endogenous nicks in chromatin DNA as shown in Fig. 2), compared with the amount of endogenous substrate determined chemically after complete disintegration of the cell structures [8].

Since the equation of Michaelis-Menten type can describe the receptor-binding of hormone, neurotransmitter and drug [17], the present methods are applicable to the crude receptor samples with low binding activities in order to determine the dissociation constant (K_d) and the maximum binding (B_{max}). The amount of ligand sample also becomes assyable in terms of the present methods if the purified ligands and the receptor preparations are available. Furthermore, the membrane transport can also be analyzed by applying the present methods to the crude samples, since the transport mechanism obeys the equation of Michaelis-Menten type [18]. However, u defined in Eqn. 1 is meaningless in the receptor-binding and membrane transport, since only the unique ligand and transportable component are involved in these cases and it is not necessary to consider the phenomenon provoked by other ligands and components (like the extra product formation in the impure enzyme reaction). In summary, the present kinetic methods are thought to be useful in the wide range of analysis of reaction systems.

Appendix

A crude enzyme sample is generally though to contain an unknown amount of endogenous substrate (concentration: x) and a contaminating enzyme(s) (activity: u), which produces the same product as that of the target enzyme reaction from contaminating substrate other than that for the target enzyme reaction. Under the condition that no product or inhibitor is included, the kinetic parameters, K_m and V, for the single substrate reaction can be determined by the linear kinetic method as follows, if the reaction obeys the Michaelis-Menten equation.

Non-radiometric determination. In this case, the Michaelis-Menten equation is rewritten as:

$$v_i = \frac{V(x + S_i')}{(K_m + x + S_i')} + u \tag{1}$$

where v_i is an observed reaction rate at a concentration of exogenously added substrate (S'). When the exogenous substrate is not added ($S_0' = 0$),

$$v_0 = \frac{Vx}{K_m + x} + u \tag{2}$$

Based on Eqns. 1 and 2, the following equation results:

$$\frac{v_i - v_0}{v_0(1 - u/v_0)} = \frac{K_m/x \cdot S_i'}{K_m + x + S_i'} \tag{3}$$

This can be rearranged in the form:

$$\frac{K_m/x \cdot (1 - u/v_0)}{(v_i - v_0)/v_0} - \frac{(K_m + x)}{S_i'} = 1 \; ; \quad i = 1 \text{ to } n \tag{4}$$

which is of the general form $y/b + x/a = 1$, representing a straight line in xy space with intercept a on the x axis and intercept b on the y axis. Thus, this equation represents a straight line when $-S_i'$ is plotted on the x axis as a and $(v_i - v_0)/v_0$ is plotted on the y axis as b (Fig. 3). For each observation (S_i', v_i), each straight line intersects at the same point whose co-ordinates are the only unique values of $(K_m + x)$ and $K_m/x \cdot (1 - u/v_0)$ that satisfy Eqn. 4. The figure of the lines for every observation is similar to the plot devised for the Michaelis-Menten equation by Eisenthal and Cornish-Bowden [1]. As indicated by these authors, the unique intersection point in Fig. 3 degenerates into $n(n-1)/2$ points in the actual experiment, if the observation is performed at n different concentrations of exogenously added substrate; the non-parametric statistics indicated that the best estimates of these $(K_m + x)$ and $K_m/x \cdot (1 - u/v_0)$ are the medians of the co-ordinates of these intersecting points [16]. The graphic method gives the rough estimates of the median co-ordinates

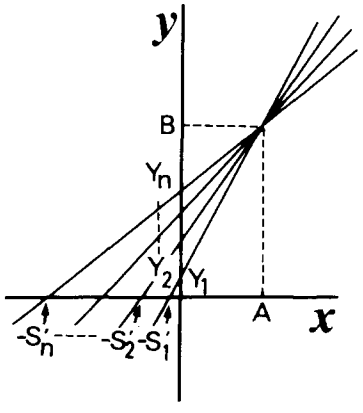

Fig. 3. Linear plot for non-radiometric assay.

but the intersecting points are too crowded around the point with median co-ordinates to exactly define the median values, which are indicated by the number-pair (A, B) in Fig. 3. In this paper, a computer was employed to calculate these values by solving any pair of Eqn. 4 as simultaneous equations representing two lines on two observations. In the case where no contaminating enzyme is present ($u = 0$), Eqn. 4 indicates that $A = (K_m + x)$ and $B = K_m/x$. Therefore, K_m and x are simply expressed as follows:

$$K_m = AB/(B + 1) \; ; \qquad x = A/(B + 1)$$

When other enzyme contaminates the target enzyme reaction ($u \neq 0$), Eqn. 4 indicates that $A = (K_m + x)$ and $B = K_m/x \cdot (1 - u/v_0)$. At two concentration levels of endogenous substrate (x_1 and x_2), two pairs of co-ordinates are obtained:

$$A_1 = K_m + x_1 \qquad A_2 = K_m + x_2$$

$$B_1 = K_m/x_1 \cdot (1 - u_1/v_{01}) \qquad B_2 = K_m/x_2 \cdot (1 - u_2/v_{02})$$

In the actual experiment where impure enzyme samples are analyzed, the ratio of x_2/x_1 or V_2/V_1 is equal to the ratio (α, known value) between sample concentrations at the two levels, because x and V are proportional to the sample concentration. Thus, all the values of K_m, V, x and u are calculated as follows:

$$\begin{cases} x_1 = (A_2 - A_1)/(\alpha - 1) \\ K_m = A_1 - x_1 \\ V_1 = A_1 B_1 v_{01}/K_m \\ u_1 = v_{01}(1 - B_1 x_1/K_m) \end{cases} \qquad \begin{cases} x_2 = \alpha x_1 \\ K_m = A_2 - x_2 \\ V_2 = A_2 B_2 v_{02}/K_m \\ u_2 = v_{02}(1 - B_2 x_2/K_m) \end{cases}$$

The concentration of other contaminating enzymes is also proportional to the sample concentration, but the activity is not necessarily proportional to the concentration as above, since the substrate for this enzyme originates from some contaminant in the impure sample and its concentration, generally far lower than its K_m value, is varied to influence the enzyme activity in such a way that the activity obeys the Michaelis-Menten equation.

To confirm statistically the reliability of the linear kinetic method as above, the non-linear fitting was performed, based on both the Gauss-Neuton and

the least-squares method as described by Cleland [2,19]. When $u = 0$, one may take partial derivatives of Eqn. 1 with respect to V, K_m and x in this order as follows:

$$Q_1 = (x + S'_i)/(K_m + x + S'_i)$$
$$Q_2 = (x + S'_i)/(K_m + x + S'_i)^2$$
$$Q_3 = K_m/(K_m + x + S_i)^2$$

where Q_1, Q_2 and Q_3, with $Q_4 = v_i$, give the matrix positions $S_{kj} = (\Sigma Q_k Q_j)$ for the solution of the set of linear Eqns. 7 used by Cleland [2]. By using these expressions of Q_i, the FORTRAN program can be written for fitting Eqn. 1 directly to the data. The provisional values for V, K_m and x are obtained in the exactly same way in the HYPRPLT program of Cleland [19], since the linear double-reciprocal equation is derived by rearranging Eqn. 1 as follows:

$$\frac{1}{v_i - v_0} = \frac{(K_m + x)}{VK_m}\left\{(K_m + x)\frac{1}{S'_i} + 1\right\} \qquad (5)$$

When $u \neq 0$, the non-linear fitting is impossible for Eqn. 1 by setting $Q_4 = 1$ and $Q_5 = v_i$ in addition to Q_1, Q_2 and Q_3 as above, since the estimated V, K_m, x and u values do not converge on certain values. In this case, a tentative equation with $u = 0$ [$v' = V'(x' + S'_i)/(K'_m + x' + S'_i)$] can be firstly fitted to the data, resulting in good convergence; and then the values of V, K_m, x and u are calculated at the two levels of x_1 and x_2, based on the following relations with a known ratio (α) of x_2/x_1 and V_2/V_1:

$$K'_m + x' = K_m + x\ ; \qquad V' = V + u\ ; \qquad V'K'_m = VK_m$$

These relations are evident since $V' = V + u$ when S'_i is infinity in Eqn. 1 and Eqn. 5 gives, irrespective of the value of u, the constant intercept on the abscissa (i.e., $K'_m + x' = K_m + x$) and accordingly the constant product, $V'K'_m = VK_m$.

Radiometric determination. In this case, u is negligible since the non-radioactively labelled product by contaminating enzymes is not measurable. The non-radioactively labelled endogenous substrate (x) dilutes the radioactively labelled substrate (its concentration: S^*) and the true reaction rate (v) is expressed in terms of the apparent radiometric reaction rate (v^*) as follows:

$$v = \frac{S^* + x}{S^*} \cdot v^*$$

According to this relation, the Michaelis-Menten equation is expressed in terms of v^* and S^* as:

$$v^* = \frac{VS^*}{K_m + x + S^*} \qquad (6)$$

The Eisenthal-Cornish-Bowden plot is useful by direct plotting $-S^*$ and v^* on the abscissa and ordinate, respectively, which gives $(K_m + x)$ and V as the co-ordinates for a median intersecting point (Fig. 3). However, a simple double-reciprocal plot is linear and provides $-1/(K_m + x)$ and $1/V$ as abscissa and ordinate intercepts, respectively. When the two abscissas $A_1 = K_m + x_1$ and $A_2 = K_m + x_2$ are graphically obtained or calculated by using a computer, the values of K_m, x_1 and x_2 are calculable at the two levels of x_1 and x_2 with $\alpha = x_2/x_1$ as follows:

$$K_m = \frac{\alpha A_1 - A_2}{\alpha - 1}, \qquad x_1 = \frac{A_2 - A_1}{\alpha - 1}, \qquad x_2 = \frac{\alpha(A_2 - A_1)}{\alpha - 1}$$

The parametric statistical analysis is performed by simply applying the non-linear fitting method with the aid of the HYPER program written by Cleland [19].

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