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A NOVEL METHOD FOR DETERMINING EQUILIBRIUM CONSTANTS CTP:PHOSPHORYLCHOLINE CYTIDYLTRANSFERASE

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Summary

A novel method for the determination of equilibrium constants for reversible reactions is described. The method is based on the measurement of initial velocities of isotope transfer for a given substrate-product pair of both the forward and reverse reactions as a function of the mass action of reactants. The reciprocal values of these initial velocities are plotted against the mass action ratios of reactants. The observed K_{eq} is the abscissa of the intersection point of these reciprocal plots, i.e. the mass action ratio at which the initial velocities of isotope transfer for both the forward and reverse reaction are identical, that is, when isotope exchange is occurring. In this manner, an observed K_{eq} of 0.2 was obtained from CTP:phosphorylcholine cytidyltransferase (CTP:cholinephosphate cytidyltransferase, EC 2.7.7.15) at 37°C and pH 7.5 under physiological conditions 1.0 mM free Mg^{2+} and 0.15 M salt concentration. A comparison of this value with the in vivo mass action of reactants calculated from published data indicates that this reaction is rate-limiting in the rat liver (Infante, J.P. (1977) Biochem. J. 167, 847—849).

The measurement of equilibrium constants (K_{eq}^{obs} *) of biochemical reactions under physiological conditions is fundamental for understanding the thermodynamics of biochemical pathways. Since only those reactions far

* The K_{eq}^{obs} is the observed equilibrium constant obtained under specified conditions such as ionic strength, pH, metal ion concentration and temperature, expressed as a function of total concentration of reactants rather than activities of individual species. In determining K_{eq}^{obs} for phosphoryl and nucleotidyl transfer reactions, it is particularly important that these conditions are clearly defined as they strongly influence the K_{eq}^{obs} [1—6].

Abbreviations used: CDP-choline, cytidine 5′-diphosphocholine; $[Mg^{2+}]_f$, free magnesium concentration; cytidyltransferase, CTP:cholinephosphate cytidyltransferase (EC 2.7.7.15); pyrophosphorylase, CDP-choline pyrophosphorylase (EC 3.6.1.-); $[R]_t$, total concentration of reactant R; E, enzyme; Γ, mass action ratio of reactants; v^*, initial velocity of isotope transfer; V_f and V_r, maximum forward and reverse velocities, respectively; F, net flux or velocity through a metabolic step; v_f and v_r, forward and reverse initial velocities of an enzymatic reaction.

displaced from equilibrium can be rate-limiting, the determination of K_{eq}^{obs} is essential to identify rate-limiting steps which, thus, have the potential to regulate the operation of metabolic pathways [7—10]. The rationale for this argument is that the flux ($F = v_f - v_b$) through an enzymatic step which is far from equilibrium is largely determined by its forward velocity, since $v_f \gg v_b$ under these conditions. Therefore, the increase in v_f produced by a higher enzyme activity will not produce any significant increase in v_b since the product concentration is too far from its equilibrium concentration. Hence, steps which are far from equilibrium will be highly responsive to changes in enzyme activity and consequently can be rate-limiting. On the other hand, the flux through near-equilibrium steps is lower than either v_f or v_b, since in these reactions v_f is only marginally higher than v_b (at equilibrium $v_f = v_b$). Therefore, an increase in enzyme activity will produce a significant compensatory increase in v_b because the product concentration is near equilibrium. Consequently, the flux through these steps is highly insensitive to changes in enzyme activity and thus are poor sites for metabolic regulation.

Studies on the regulation of the cytidine pathway for the synthesis of phosphatidylcholine in rat liver have determined that the MgATP:choline phosphoryltransferase enzyme catalyzes the most rate limiting reaction of the pathway and that this phosphorylation can be regulated by the intracellular level of free Mg^{2+} at physiological concentrations [11,16]. Herein, we report on the K_{eq}^{obs} of the MgCTP:phosphorylcholine cytidyltransferase reaction, at physiological conditions of temperature, ionic strength, pH and free Mg^{2+}, using a novel method based on the independent measurement of initial velocities of isotope transfer for the forward and reverse reactions as a function of the mass action ratio of reactants. A comparison of the obtained K_{eq}^{obs} with the intracellular value of Γ indicates that the cytidyltransferase reaction is also rate limiting in rat liver [11].

A preliminary report of this work has been published [34].

Materials and Methods

Materials

Adult albino male rats (4 months old, 400—450 g) were obtained from Holtzman Co. (Madison, Wisconsin) and fed a balanced rat diet ad libitum for 10 days. Sodium β,γ-methyleneadenosine 5′-triphosphate was purchased from P.L. Biochemicals, Inc., (Milwaukee, Wiconsin). Tris cytidine 5′-triphosphate, sodium cytidine 5′-diphosphocholine, phosphorylcholine, tetrasodium pyrophosphate and *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES) were obtained from Sigma Chemical Co. (St. Louis, Mo.). *O*-Phosphoryl-[*Me*-^{14}C]choline (specific activity 57 Ci/mol) and cytidine 5′-diphospho-[*Me*-^{14}C]choline (49 Ci/mol) were purchased from New England Nuclear (Boston, Massachusetts). $MgCl_2$ (1.0 M solution, Lot 766151) was obtained from Fisher Scientific, (Fair Lawn, New Jersey). The indicated concentration was confirmed by atomic absorption spectrophotometry, with a Fisher certified Mg standard (Lot 755639) as the reference solution.

Methods

Enzyme preparation. Rats were killed by decapitation. The high-speed super-

natant was obtained by differential centrifugation of the homogenized liver [16]. This material was lyophilized and stored at −25°C. All subsequent operations were carried out in ice or at the temperatures indicated. Appropriate amounts of the freeze-dried material were dissolved in glass double distilled and deionized water using a Teflon-pestle micro homogenizer. This solution was subsequently centrifuged at 3000 × *g* for 10 min, at 4°C to remove the undissolved material. The clear supernatant was adjusted to pH 5.2 and the enzyme was fractionated as previously described [12,13].

Measurement of enzyme activity. Forward reactions of isotope transfer were measured from the incorporation of *O*-phosphoryl-[*Me*-^{14}C]choline into CDP-choline. Reverse reactions (including isotope transfer rates) were measured from the conversion of cytidine 5′-diphospho-[*Me*-^{14}C]choline to labeled phosphorylcholine. Pyrophosphorylase activity on CDP-choline was assayed in the same manner as the cytidyltransferase back reaction except that PP_i was omitted. Therefore its hydrolytic activity was measured by the PP_i-independent formation of phosphorylcholine from CDP-choline. Initial velocities (about 5% isotope transfer of substrates to products) for the above enzymatic activities were measured with incubation times between 15 and 40 min. 1 nmol formed labeled CDP-choline and phosphorylcholine represents 22 000 cpm and 97 000 cpm, respectively.

Unless otherwise specified, enzyme assays were carried out at 37°C in the presence of 1 mM $[Mg^{2+}]_f$, 0.25 mM Mg · methyleneATP^{-2} and 80 mM HEPES/Tris (pH 7.5) in a total volume of 375 μl. Ionic strength was adjusted to 0.15 M with KCl. Reactions were initiated by the addition of enzyme (0.2—0.3 mg of protein), stopped with 50 μl 1 M trichloroacetic acid and the mixture subsequently centrifuged at 5000 × *g* for 5 min at 4°C. Aliquots of the protein free supernatant were subsequently analyzed for substrates and products.

Analytical. Phosphorylcholine and CDP-choline were resolved by paper chromatography (10 × 10 inch, Whatman 3MM) using 95% ethanol/1 M ammonium acetate (pH 9.0) saturated with tetrasodium borate (60 : 40, v/v) as the developing solvent. Phosphorylcholine was visualized with the phosphorus spray of Dawson [14] and CDP-choline by ultraviolet absorption. R_F values of 0.22 and 0.48 were obtained for authentic standards of CDP-choline and phosphorylcholine, respectively. The paper strips containing the appropriate spots were cut and their radioactivity was determined by a Packard (model 3385) scintillation spectrometer in a toluene-based scintillation solution containing 5 g/l 2,5-diphenyloxazole and 0.3 g/l 1,4-bis[2/4-methyl-5-phenyloxazolyl)]. Isotope transfer was determined from the ratio of cpm found in product over the sum total of cpm in product plus unreacted substrate. Recovery of radioactivity was determined for each assay and was better than 99%. Radiochemical purity of substrates which was routinely checked was 99%. Protein was determined with Folin phenol reagent [15] with crystalline bovine serum albumin as standard.

Other methods. The equations to determine the total concentrations of metal and ligands required to obtain a given level of free metal and metal-ligand concentration have been described [16]. The apparent Mg-binding constants, i.e. K_{assoc}, used in these calculations are presented in Table I.

Initial velocities of isotope transfer as a function of mass action ratio of

TABLE I

APPARENT Mg^{2+}-BINDING CONSTANTS OF THE CYTIDINE TRANSFERASE LIGANDS AT pH 7.5

Reaction	K_{assoc}^{app} (M^{-1})	Ref.
$CTP^{4-} + Mg \rightleftarrows MgCTP^{2-}$	10 200	17
$HPP_i^{3-} + Mg \rightleftarrows MgHPP_i^{1-}$	1 150	18
CDP-choline$^-$ + $Mg \rightleftarrows$ MgCDP-choline$^+$	20	19
Phosphorylcholine$^-$ + $Mg \rightleftarrows$ MgP-choline$^+$	28	20
MethyleneATP^{4-} + $Mg \rightleftarrows$ Mg · methyleneATP^{2-}	10 250 *	

* This value was assumed to be identical to the K_{assoc} obtained for $MgATP^{2-}$ at 37°C and 0.15 M [21] since the anhydride oxygens have little influence in the metal coordination properties of nucleotides [22,23].

reactants were analyzed by the least squares method assuming a proportional variance. A Hewlett Packard computer (Model 9862A) with the appropriate accessories was used to calculate and plot the best fit functions. The method to derive isotope transfer rate equations has been described [24].

Theory

The equilibrium of a chemical reaction is reached when there is no net conversion of reactants, that is, when the rates of the forward and reverse reactions, measured in the presence of all reactants, are identical. In multireactant systems, another property of the equilibrium condition is that the rates of isotope transfer (the term isotope transfer rather than isotope exchange will be used throughout, since the latter in its stoichiometrical meaning only occurs at equilibrium) for the forward and reverse reactions of any given substrate product pair, will be identical [25].

In a reaction of the type A + B ⇋ P + Q, the value of $\Gamma = [P][Q]/[A][B]$ can be varied by changing the initial concentration of one reactant, i.e. A, while the concentration of all other reactants is kept constant. If the reciprocal velocities of isotope transfer from labeled A to Q ($A^* \rightarrow Q$) are plotted against varying values of Γ or $1/[A^*]$, a linear function will be obtained for compulsory mechanisms as well as for random rapid equilibrium sequences [25,26]. However, for steady-state alternate sequences, non-linear functions will be generated. The isotope transfer and exchange rate equations for some of these mechanisms have been reviewed [25,26]. When the reciprocal velocities of isotope transfer for the reverse reaction ($Q^* \rightarrow A$) are plotted against the same varying labels of Γ or $1/[A]$, a concave function may be observed, i.e. the reaction velocity will decrease with increasing [A], since this reactant is a product of the reverse reaction and competes with Q for the same enzyme form. This can be predicted from Eqn. 1, which was derived for a bireactant ordered mechanism (for the meaning of the kinetic constants in terms of rate constants, see ref. 26). However a function of different form will be obtained for other kinetic mechanisms, in double reciprocal plots of initial velocities versus the concentration of other products, in the presence of all other reactants. For instance it can easily be shown that when the reciprocal value of initial velocities of isotope transfer from P^* to A are plotted aganst $1/[A]$ (all other reactants

present at a constant concentration), a linear pattern will be obtained for a compulsory mechanisms where A adds first and Q is released last. This can be verified by inspecting Eqn. 2, derived for such a mechanism. Therefore, these plots can also be used as diagnostic tools for the elucidation of kinetic mechanisms.

If the reciprocal values of initial velocities of isotope transfer from $A^* \rightarrow Q$ and from $Q^* \rightarrow A$ are plotted in the ordinate against varying values of Γ in the abscissa, K_{eq}^{obs} can be obtained from the abscissa coordinate of the intersection point of these two velocity functions. In other words, K_{eq}^{obs} will be equal to the value of Γ at which the initial velocities of isotope transfer for the forward and reverse reactions (for the chosen substrate-product pair) are identical. Under these conditions isotope exchange in occurring. Of course, the K_{eq}^{obs} can be obtained in this manner, regardless of the mechanism, the shape of the plots or the substrate-product pair chosen, since it is based on the thermodynamic properties of the system.

$$\frac{[E]_t}{v^*_{Q^* \rightarrow A}} = \frac{\frac{1}{[A]}\left[K_p([Q]+K_{iq})+2K_q[P]+\left(\frac{K_q}{K_{iq}}+1\right)[P][Q]+[B]\left(\frac{K_{iq}K_p}{K_{ia}K_b}\left(2K_a+\frac{K_a}{K_{ia}}+1\right.\right.\right.}{\frac{[P][Q^*]}{[A]}\left(1+\frac{K_q}{K_{iq}}[P]+\frac{K_a}{K_{ia}K_b}\right)V_r}$$

$$\left.\left.\left.+\frac{2K_a}{K_{ia}K_b}(K_p[Q]+K_q[P])+\frac{[P][Q]K_a}{K_{ia}K_b}\left(\frac{K_q}{K_{iq}}+1\right)\right.\right.\right.$$

$$\left.\left.\left.+\frac{[P][Q]}{K_{ib}}+\frac{K_q}{K_{iq}K_pK_{ib}}[P]^2[Q]\right)+[B]^2\left(\frac{K_a^2K_p}{K_{ia}^2K_b^2}(K_{iq}+[Q])\right.\right.\right.$$

$$\left.\left.\left.+\frac{K_a}{K_{ia}K_{ib}K_b}[P][Q]\right)\right]+\frac{[B]}{K_{ia}K_b}\left(K_pK_{iq}\left(1+\frac{K_a}{K_{ia}}+\frac{[P]}{K_{ip}}\right)\right.\right.$$

$$\left.\left.+K_q[P]\left(1+\frac{K_a}{K_{ia}}+\frac{[P]}{K_{ip}}\right)\right)+\frac{K_aK_pK_{iq}}{K_{ia}^2K_b^2}[B]^2\left(1+\frac{[P]}{K_{ip}}\right)+\frac{1}{K_{ia}}(K_pK_{iq}+2K_p[P])\right.$$

$$\frac{[E]_t}{v^*_{P^* \rightarrow A}} = \frac{\frac{1}{[A]}\left[\frac{K_{ip}K_q}{[P][Q]^2}\left(1+\frac{[B]}{K_{ib}}\right)+\frac{2K_p}{[P][Q]}\left(1+\frac{[B]}{K_{ib}}\right)+\frac{K_q}{[Q]^2}+\frac{1}{[Q]}\left(1+\frac{K_p}{K_{ip}}\right)\right.}{\frac{V_r}{[A]}\left(\frac{1}{[Q]}+\frac{K_p}{K_{ip}K_q}\right)+\frac{K_bV_r}{K_aK_{ib}[Q]}} \quad (2$$

$$\left.+\frac{K_p^2}{K_{ip}K_{iq}[P]}\left(1+\frac{[B]}{K_{ib}}\right)+\frac{K_p}{K_{ip}K_q}\right]+\frac{K_p}{K_{ip}K_qK_{ia}}+\frac{K_{ip}K_q}{K_aK_{ib}[P][Q]^2}(K_b+[B])$$

$$+\frac{1}{K_aK_{ib}[P][Q]}(K_pK_b+K_{ip}K_q[B]+K_p[B])+\frac{K_p[B]}{K_{iq}K_aK_{ib}[P]}$$

$$+\frac{1}{[Q]}\left(\frac{1}{K_{ia}}+\frac{K_qK_b}{K_{ip}K_qK_{ib}}\right)+\frac{K_qK_b}{K_aK_{ib}[Q]^2}$$

Results and Discussion

Preliminary experiments

Initial experiments indicated the presence of a pyrophosphorylase activity on CDP-choline. MethyleneATP was found to be a specific inhibitor of this enzyme (K_i^{app} = 38 μM) while the cytidyltransferase remained fully active in its presence (Fig. 1A). Therefore, all assays were carried out in the presence of 0.25 mM Mg · methyleneATP^{-2} which produced a 90% inhibition of the pyrophosphorylase. Its residual activity accounted for less than 1.0% conversion of the initial CDP-choline concentration and it was subtracted from the cytidyl transferase activity (reverse reaction) to obtain the net rate of PP_i dependent phosphorylcholine synthesis. Initial velocities for both the forward and reverse reactions were linear functions of protein concentration up to at least 0.5 mg. There was no need to take into account the pyrophosphorylase activity in the forward cytidyltransferase reaction since the activity on CTP and phosphorylcholine was negligible and the change in CDP-choline concentration was also very small, thus the reaction rates were not significantly changed by the inhibitor (Fig. 1B).

Determination of the K_{eq}^{obs}

Initial velocities of isotope transfer for both the forward and reverse reactions at varying Γ (determined by varying phosphorylcholine alone) were measured at 37°C, pH 7.5, 1.0 mM free magnesium concentration and 0.15 M salt concentration (Fig. 2). The abscissa of the intersection point of the two lines in this figure indicates a Γ value of 0.20 which is the K_{eq}^{obs} of the reaction. Therefore, at this Γ value, the initial rates of label transfer from CDP-choline to phosphorylcholine are identical to the initial rates of label transfer from phosphorylcholine to CDP-choline.

The concave function produced for the initial velocities of isotope transfer from CDP-choline to phosphorylcholine is consistent with the pattern predicted by Eqn. 1, i.e. the isotope transfer from Q* to A. On this basis it may be suggested that the cytidyltransferase may follow a compulsory sequence where phosphorylcholine adds first and CDP-choline is released last. However, other independent evidence will be needed to confirm this suggestion, since the possibility of homeomorphic mechanisms cannot be discarded.

The most common technique for the determination of the K_{eq}^{obs} of enzymatic reactions involves the incubation of the enzyme only in the presence of the reactants needed for the reaction to proceed in one direction. This reaction mixture is incubated for the period of time required for the reaction to reach equilibrium (see refs. 27—29 for examples of this technique). However, this method has the following shortcomings: First, it often required long incubation times (up to several hours) which may introduce errors due to the instability of enzymes and reactants. Second, in the case of metal-dependent reactions, the large changes in reactant concentration produced to reach equilibrium will cause significant changes in the free metal concentration when limiting, if the metal binding constants of substrates and products are widely different. As this is often the case for phosphoryl and nucleotidyl transferases, the free metal concentration cannot be set as an independent parameter by the

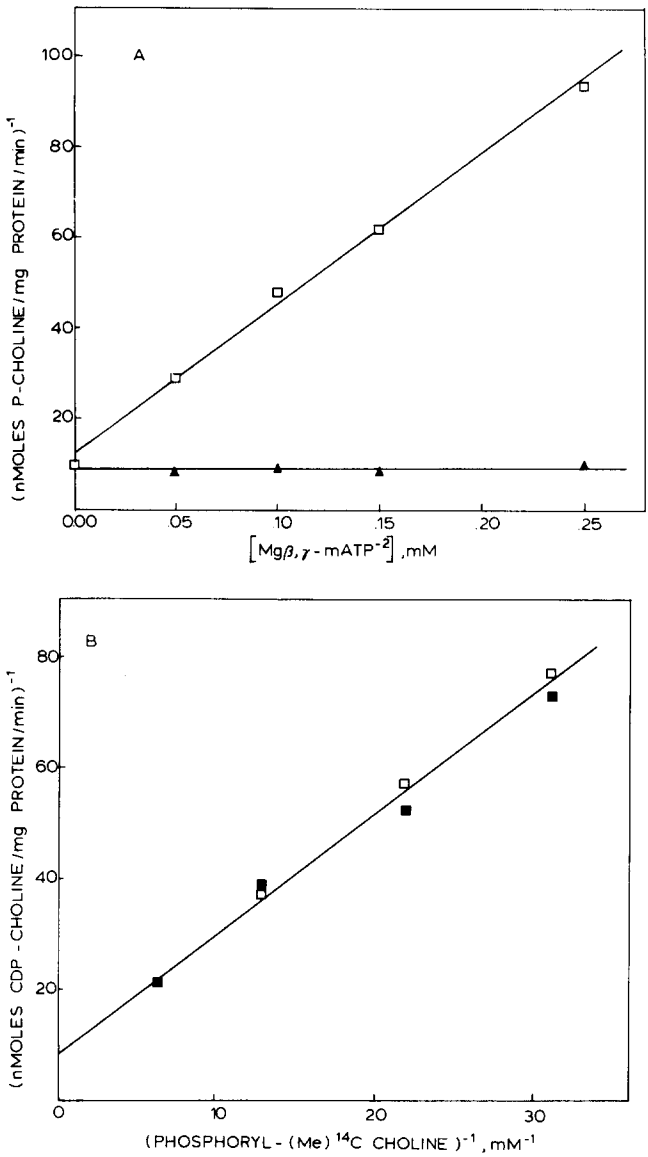

Fig. 1. (A) Reciprocal plots of initial velocities of the cytidyl transferase reverse reaction (▲———▲) and pyrophosphorylase (□———□) activities vs. Mg · methyleneATP^{-2} concentration in the presence of 11 μM cytidine 5′-diphospho-[*Me*-^{14}C]choline and 1 mM free Mg^{2+}. Cytidyl transferase assays were performed in the presence of 1.1 mM total PP_i while the pyrophosphorylase assays were carried out in its absence. (B) Double reciprocal plots of initial velocities of isotope transfer the cytidyl transferase forward reaction vs. *O*-phosphoryl-[*Me*-^{14}C]choline as the variable substrate, in the presence (■———■) and absence (□———□) of 0.25 mM Mg · methyleneATP^{-2}. Concentrations of other reactants were: 0.67 mM total CTP, 1.1 mM PP_i, 11 μm total CDP-choline and 1 mM free Mg^{2+}.

experimenter (unless an effective metal buffer is used). In this case, its concentration can only be calculated as a response variable from the equilibrium concentrations of substrates and products and their appropriate metal binding constants. Third, the equilibrium method also requires the quantitative analysis

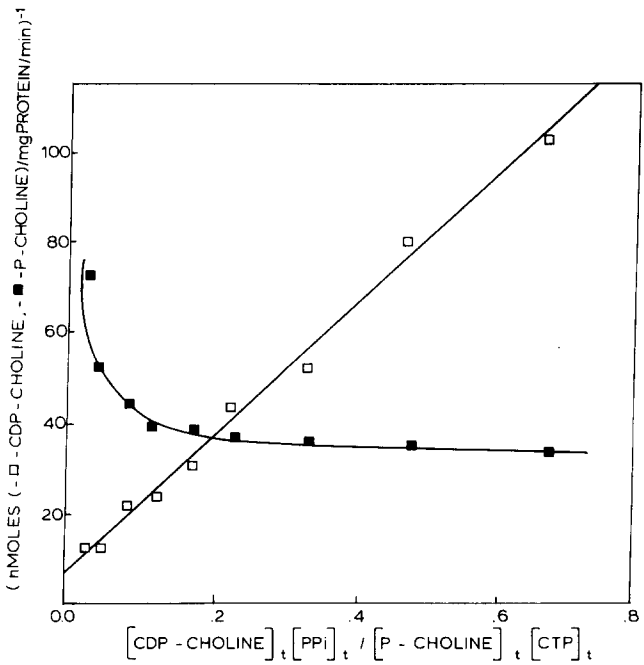

Fig. 2. Reciprocal plots of initial velocities of isotope transfer for the cytidyl transferase forward (□———□) and reverse (■———■) reaction vs. mass action ratios: [CDP-choline]$_t$ [PP$_i$]$_t$/[phosphorylcholine]$_t$ [CTP]$_t$, generated by a variable phosphorylcholine concentration in the presence of constant levels of 0.67 mM total CTP, 1.1 mM total PP$_i$, 11 μM total CDP-choline and 1 mM free Mg^{2+}.

of at least one reactant to determine the final concentration of the others.

The method described by Plowman [26] eliminates the first two disadvantages of the equilibrium method. It is based on measurements of small net changes in reactant concentrations produced by short incubations of assay mixtures with varying Γ values. However, it still requires the quantitative analysis of at least one reactant [26,30]. In this method the K_{eq}^{obs} is identified as the Γ value which does not generate a net change in reactant concentrations. As smaller concentration changes are produced when equilibrium is approached, a sensitive experimental design and analytical technique are required by this approach.

The method reported here introduces a sensitive technique for the determination of K_{eq}^{obs} of enzymatic reactions under simulated physiological conditions and eliminates all the disadvantages of the methods discussed above. Since only initial velocities are measured, short incubation times are required, thus minimizing the problems associated with the instability of enzyme and reactants. Another consequence of the initial velocity condition is that the known initial concentrations of reactants, i.e. Γ, can be assumed to remain unchanged and thus their quantitative analysis is not required to determine Γ or K_{eq}^{obs}. This is particularly advantageous for reactions where the K_{eq}^{obs} is dependent on metal cations, as the free metal concentration at which the K_{eq}^{obs} wants to be determined can be set as an independent parameter by the experimenter. As this method is not based on the measurements of net velocities but on initial velocities of isotope transfer for the forward and reverse reactions, its

sensitivity is not impaired near equilibrium conditions.

The method described here has one limitation. Since initial velocities for the forward and reverse reactions are required, more sensitive velocity measurements will be required for reactions which are not readily reversible.

The K_{eq}^{obs} found here for the CTP:phosphorylcholine cytidyltransferase, forward direction, is in the range of values reported for other transferases catalyzing similar reactions; 0.46 for the CTP:ethanolamine cytidyltransferase [29] and 0.25 for the GTP:hexose-1-*P* guanosyltransferase [31]. However, comparisons must be made with caution since these equilibrium constants were not obtained in the presence of known constant concentrations of this cation. Turner and Turner [32], for instance, obtained K_{eq}^{obs} values for the UTP:Glu-1-*P*:uridyltransferase which varied from 0.12 to 0.26 according to the concentration of Mg^{2+}.

A disequilibrium ratio value of 0.013 has been obtained from the K_{eq}^{obs} obtained here, under physiological conditions, and the intracellular mass action ratio of the reactants involved in the cytidyltransferase reaction [11]. On the basis of this parameter and other supporting data, it has been concluded that this reaction is rate-limiting in rat liver [11]. Since the CTP:phosphorylethanolamine cytidyltransferase (EC 2.7.7.14) reaction is also far from equilibrium in rat liver [11], the argument that this enzyme [29] and the phosphorylcholine cytidyltransferase, in their reverse reactions, may contribute to the net synthesis of CTP is untenable.

As the intracellular concentration of total PP_i in rat liver (6.2 ± 0.3 nmol/g wet weight) is the lowest of all the reactants involved in the cytidyltransferase reaction [6,33], it is conceivable that its extremely low concentration may be the principal determining factor causing the mass action rates of the cytidyl transferase reactants to be far from equilibrium. On this basis it is tempting to suggest that a very active pyrophosphorylase may be responsible for these low levels of PP_i, and thus may play a critical role in maintaining the cytidyl transferase and other PP_i-producing reactions as rate-limiting steps.

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