Rate-Limiting Steps in the Cytidine Pathway for the Synthesis of Phosphatidylcholine and Phosphatidylethanolamine

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An analysis of the available data on the cytidine pathway for the synthesis of phosphatidylcholine and phosphatidylethanolamine, by the logic derived from the theoretical principles of metabolic regulation, shows that the first two reactions catalysed by choline (ethanolamine) kinase and phosphocholine (phosphoethanolamine) cytidylyltransferase are rate-limiting, whereas the phosphocholine (phosphoethanolamine) transferase step is near equilibrium in rat liver.

Phosphatidylcholine and phosphatidylethanolamine are the major phospholipid constituents of plasma and organelle membranes in mammalian cells (McMurray, 1973; White, 1973) and play an essential role in regulating the biochemical and physico-chemical properties of such membranes, as well as the activity of many enzymes and enzyme systems (Coleman, 1973a; Fourcans & Jain, 1974). In addition, these phospholipids are essential components of the lipoproteins involved in lipid transport and secretion (Dawson, 1966; Eisenberg & Levy, 1975; Fourcans & Jain, 1974). Although the reactions of the cytidine pathway for the synthesis of phosphatidylcholine and phosphatidylethanolamine were described more than two decades ago (Kennedy & Weiss, 1956; Whitenberg & Kornberg, 1953), very little on the regulation *in vivo* of this biosynthetic pathway in mammalian cells has been learned [see Coleman (1973b), Hill & Lands (1970), Lennarz (1970), McMurray & Magee (1972), Thompson (1973) and Van den Bosh (1974) for general reviews].

The present paper shows that an analysis of the available data by the logic derived from the theoretical principles of metabolic regulation leads to the conclusion that the first two enzymes of the cytidine pathway, choline kinase (MgATP²⁻-choline phosphotransferase, EC 2.7.1.32) and cytidylyltransferase (MgCTP²⁻-phosphocholine cytidylyltransferase, EC 2.7.7.15), catalyse rate-limiting steps. The phosphocholinetransferase (CDP-choline-1,2-diacyl-*sn*-glycerol phosphocholinetransferase, EC 2.7.8.2) catalyses a near-equilibrium reaction, and thus is not rate-limiting. The same conclusions are reached for the analogous reactions leading to the synthesis of phosphatidylethanolamine.

The identification of far-from-equilibrium and near-equilibrium steps is essential to the elucidation of the regulatory mechanisms of metabolic pathways. Only reactions far from equilibrium are rate-limiting, as the flux through these steps is highly sensitive to

changes in enzyme activity; therefore these are the sites with the highest potential to control the flux through a metabolic sequence (Ferdinand, 1976; Newsholme & Crabtree, 1973; Rolleston, 1972). Near-equilibrium reactions, on the other hand, are extremely resistant to even large changes in enzyme activity and thus are poor sites for flux regulation (Ferdinand, 1976; Newsholme & Crabtree, 1973). A corollary of this is that flux changes through a pathway are not necessarily expected to be accompanied by changes in the activity of enzymes catalysing near-equilibrium reactions. Consequently the monitoring of changes in enzyme activities catalysing these near-equilibrium steps is of little diagnostic value, since even fairly large changes will not be able to affect the flux of the pathway in question to any significant extent.

The extent to which a reaction is rate-limiting can be quantitatively determined by the value of its disequilibrium ratio (Hess & Brand, 1965): $\rho = (\text{mass-action ratio})/K_{\text{eq}}^{\text{eq}}$, where ρ is the disequilibrium ratio and $K_{\text{eq}}^{\text{eq}}$ is the observed equilibrium constant for that reaction, measured under physiological conditions. Rolleston (1972) has suggested that values of $\rho \leq 0.05$ indicate rate-limiting steps, since the rate of the back reaction (v_b) can be regarded as negligible when it is less than 5% of the forward velocity (v_f). Therefore the flux $F = v_f - v_b$ through that step is almost totally dependent on the forward rate in a rate-limiting reaction. The mass-action ratio for choline kinase in normal rat liver can be obtained from published data on the pool sizes of the reactants involved. The average tissue concentrations ($\mu\text{mol/g wet wt.}$) of total ATP and choline are 1.95 ± 0.06 (Veech *et al.*, 1970) and 0.23 ± 0.1 (Sundler *et al.*, 1972) respectively; and the values for phosphocholine and total ADP are 1.4 ± 0.2 (Sundler *et al.*, 1972) and 1.1 ± 0.03 (Veech *et al.*, 1970) respectively. In the same tissue, the free Mg²⁺ concentration has been calculated to be between 0.5 and 0.9 mM (Veloso

et al., 1973). From these data a mass-action ratio of 3.4 can be calculated in the forward direction, which compared with the K_{eq}^{obs} of 1.24×10^4 (Guynn, 1976), calculated for 1.0 mM-free Mg^{2+} , gives a disequilibrium ratio $\rho = 2.77 \times 10^{-4}$. This clearly indicates that the choline kinase step is far displaced from equilibrium in rat liver, and consequently is rate-limiting. It is unlikely that such a small value of ρ could be accounted for by cellular compartmentalization. In fact the ρ value will be even smaller if compartmentalization is considered, since Sundler *et al.* (1972) have calculated that the actual pool size of choline available for phosphorylation is 29–39 nmol/g wet wt. of rat liver. It is noteworthy that these values are in the range of the apparent and true K_m values reported for choline kinase from the same tissue (Infante & Kinsella, 1976a), suggesting that choline is limiting for this enzyme. From the relationship $\rho = v_b/v_f$ (Hess & Brand, 1965), it can be calculated that the choline kinase forward reaction is 3.7×10^3 -fold the velocity for the back reaction; therefore the flux is almost totally dependent (>99.9%) on the forward reaction, as is expected for a physiologically irreversible step.

In the case of the cytidylyltransferase, the tissue concentrations (nmol/g wet wt.) of the reactants involved have also been measured in normal rat liver, and they are: 83 ± 5.0 and 51 ± 3.0 for total CTP and CDP-choline respectively (Domschke *et al.*, 1971); 1404 ± 308 for phosphocholine (Sundler *et al.*, 1972); 6.2 ± 0.3 for total pyrophosphate (Flodgaard & Fleron, 1974). From these data, the mass-action ratio of 2.71×10^{-3} in the forward direction can be calculated. When this is compared with its K_{eq}^{obs} of 0.20, obtained at 37°C, in the presence of 1.0 mM-free Mg^{2+} and 0.15 M ionic strength (J. P. Infante & J. E. Kinsella, unpublished work), a value of $\rho = 1.36 \times 10^{-2}$ is obtained. This indicates that this reaction is also far displaced from equilibrium and consequently is effectively irreversible, i.e. the forward reaction is 77-fold faster than the back reaction (the flux through this step depends 98.6% on the forward reaction). As it is unlikely that such a small value of ρ can be accounted for by cellular compartmentalization, it may be concluded that the cytidylyltransferase step is also rate-limiting. However, the choline kinase step is 49-fold more rate-limiting than the cytidylyltransferase reaction when the ρ values are compared. This is expected since the choline kinase is the first unique step of the cytidine pathway for the synthesis of phosphatidylcholine.

There is other evidence consistent with the conclusion that the choline kinase and cytidylyltransferase reactions are rate-limiting in rat liver. Flux changes in rate-limiting steps generate similar changes in the pool sizes of the substrates involved, whereas near-equilibrium reactions can absorb great changes in flux with little modification of metabolite concen-

trations (Ferdinand, 1976; Newsholme & Crabtree, 1973). In the choline-deficient state the decreased rate of phosphatidylcholine synthesis is accompanied by a 5-fold decrease in the free choline pool size (Noffsinger, 1975) and a similar decrease (4–5-fold) in the concentrations of phosphocholine (Noffsinger, 1975; Thompson *et al.*, 1969). On the other hand, the concentrations of CDP-choline in choline-deficient rats have been reported to remain unchanged (Wilgram *et al.*, 1960) or to change very little (Noffsinger, 1975). On the basis of the above rationale, these data strongly suggest that the phosphocholine-transferase step is near equilibrium. This is also consistent with the data showing that this reaction is freely reversible *in vivo* (Bjørnstad & Bremer, 1966; Kanoh & Ohno, 1973a,b; Treble *et al.*, 1970; Weiss *et al.*, 1958), since this is only a property of near-equilibrium steps (Ferdinand, 1976; Newsholme & Crabtree, 1973). The maintenance of a near-equilibrium condition in the phosphocholinetransferase step will make the monitoring of the activity of this enzyme a poor choice for detecting flux changes in the cytidine pathway as near-equilibrium steps can absorb large flux changes with little change in enzyme activity (Ferdinand, 1976; Newsholme & Crabtree, 1973). A better approach, in this case, would be to determine the pool sizes of the reactants involved as response variables of experimental treatments since the flux through near-equilibrium steps is very sensitive to changes in reactant concentration (Ferdinand, 1976). However, a sensitive experimental design and methods of analysis are required since small differences are expected. The regulatory behaviour of the cytidine pathway for the synthesis of phosphatidylethanolamine seems to be analogous to the phosphatidylcholine case. Rat hepatocytes subjected to increasing concentrations of ethanolamine have a nearly constant CDP-ethanolamine pool size, whereas the intracellular phosphoethanolamine concentration increases drastically (Sundler & Åkesson, 1975). These data, on the basis of the rationale indicated before, suggest that $MgATP^{2-}$ -ethanolamine phosphotransferase and $MgCT^{2-}$ -phosphoethanolamine cytidylyltransferase catalyse rate-limiting steps, whereas the CDP-ethanolamine-1,2-diacyl-*sn*-glycerol phosphoethanolaminetransferase step is a near-equilibrium reaction.

In conclusion, the kinase and cytidylyltransferase reactions leading to the synthesis of phosphatidylcholine and phosphatidylethanolamine are rate-limiting steps, and therefore are the logical sites for the regulation of the respective pathways. Consequently the monitoring of these reactions in metabolic studies, as well as the detailed kinetic study of these enzymes, warrant much more attention than given in the past (Infante & Kinsella, 1973, 1976a,b; Sundler *et al.*, 1972; Sundler, 1975; Weinhold & Rethy, 1974), since the flux through these pathways is

almost totally dependent on the activity of these enzymes. The kinetic mechanism for choline kinase, the most rate-limiting enzyme in phosphatidylcholine synthesis in rat liver, has been elucidated (Infante & Kinsella, 1976a). An analysis of the proposed rate equation in relation to the intracellular pool of reactants indicates that all ligands are present in limiting concentrations and therefore have the potential to control the activity of the enzyme. Mg^{2+} is required as an enzyme activator as well as an ATP ligand; therefore this cation controls the concentration both of active enzyme and of active substrate. The calculated kinetic parameters indicate that the velocity is extremely sensitive to changes in free Mg^{2+} concentration in the physiological range. All these kinetic properties strongly suggest a regulatory role for choline kinase in the synthesis of phosphatidylcholine.

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