A Kinetic Study of Thymine 7-Hydroxylase from *Neurospora crassa*†

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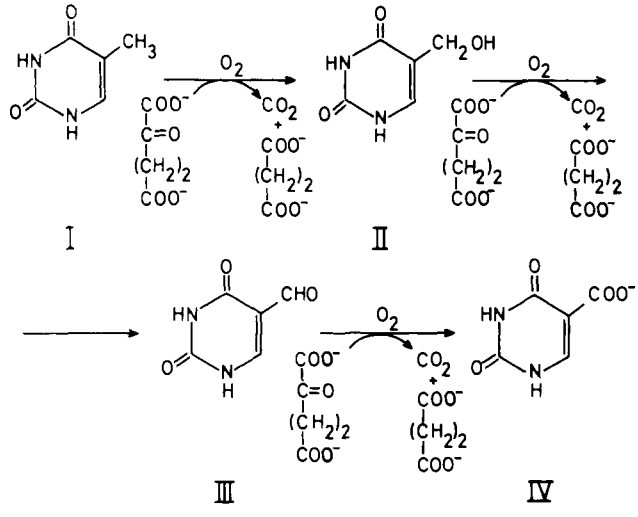
ABSTRACT: The steady-state kinetics of thymine 7-hydroxylase (thymine, 2-oxoglutarate dioxygenase, EC 1.14.11.6) has been investigated. Initial velocity plots were all found to be linear and intersecting. Variation in concentration of two of the substrates, when the third substrate was at a constant high or low concentration, gave initial velocity plots that conform to an ordered sequential mechanism, where thymine is the second substrate to add. With 5-carboxyuracil, which is the end product in the sequential oxygenation of thymine, a competitive inhibition pattern was observed when 2-ketoglutarate was the variable substrate. When either thymine or oxygen was the variable substrate a noncompetitive inhibition pattern was obtained. When either 2-ketoglutarate or thymine was the variable substrate the inhibition patterns observed with bicarbonate were noncompetitive. With succinate noncompetitive inhibition patterns with hyperbolic intercept replots were obtained. These results are consistent with an ordered sequential kinetic mechanism, where 2-ketoglutarate is added first, followed by thymine and oxygen, and the products are released in the order: bicarbonate, succinate, and 5-hydroxymethyluracil. The order of the two last mentioned products, however, is changed in the presence of succinate.

A number of 2-ketoglutarate dependent hydroxylases have been shown to catalyze oxidative decarboxylation of the keto acid concomitantly with hydroxylation of the other substrate (Lindstedt et al., 1968; Rhoads and Udenfriend, 1968; Holme et al., 1970; Bankel et al., 1972b). Two reaction mechanisms for this type of oxygenases have been proposed. In one of these the first step is a peroxide formation of the substrate to be hydroxylated (Holme et al., 1968), and in the other the first step is the formation of a peracid from the keto acid (Hamilton, 1971). A kinetic study might give a clue to which is the most likely mechanism, but very little is known about the kinetics of these reactions. It has been proposed that a substrate-induced change of iron-containing mono- and dioxygenases is necessary before oxygen can combine with these enzymes (Peisach et al., 1973). The 2-ketoglutarate dependent oxygenases so far studied have all required ferrous ion. In view of the above mentioned reports it is of particular interest to establish if oxygen or one of the organic substrates binds first to the enzyme.

The present paper reports kinetic studies on the hydroxylation of thymine to 5-hydroxymethyluracil catalyzed by an enzyme from *Neurospora crassa* (thymine, 2-oxyglutarate dioxygenase, EC 1.14.11.6). Purified preparations of thymine 7-hydroxylase catalyze the sequential oxygenation of thymine (I) to 5-hydroxymethyluracil (II), 5-formyluracil (III), and 5-carboxyuracil (IV) coupled to oxidative decarboxylation of 2-ketoglutarate (Scheme I). Studies with heavy oxygen have revealed that one atom of molecular oxygen is incorporated into the products viz. hydroxylated pyrimidine and succinate (Holme et al., 1971). A stoichiometric relation of 1:1 for thymine hydroxylation and 2-ketoglutarate decarboxylation has been demonstrated (Holme et al., 1970). Purification of the enzyme about 3000 times by a series of chromatographic procedures has failed to separate the three enzymatic activities catalyzing the three steps in the oxygenation of thymine to 5-carboxyuracil. Together with previously reported studies with mutant strains of *Neurospora crassa* these findings indicate that one single enzyme is catalyzing the sequential oxygenation of thymine to 5-carboxyuracil (Bankel et al., 1972a).

Scheme I



Experimental Procedure

Materials. Compounds were obtained from the following sources: thymine and 5-hydroxymethyluracil from Calbiochem AG, Luzern, Switzerland; sodium ascorbate and 5-carboxyuracil from Dr. Theodor Schuchardt GmbH, Munich, West Germany; 2-ketoglutaric acid and catalase from Boehringer Mannheim GmbH, Mannheim, West Germany; 2-keto[1-^{14}C]glutaric acid from New England Nuclear Corp., Frankfurt/Main, West Germany; [2-^{14}C]thymine from the Radiochemical Centre, Amersham, Buchs, England; gas mixtures from AGA AB, Vällingby, Sweden; hydroxylapatite from Bio-Rad Laboratories, Inc., Richmond, Calif.; DEAE-Sephadex A-50 and Sepharose 4B from

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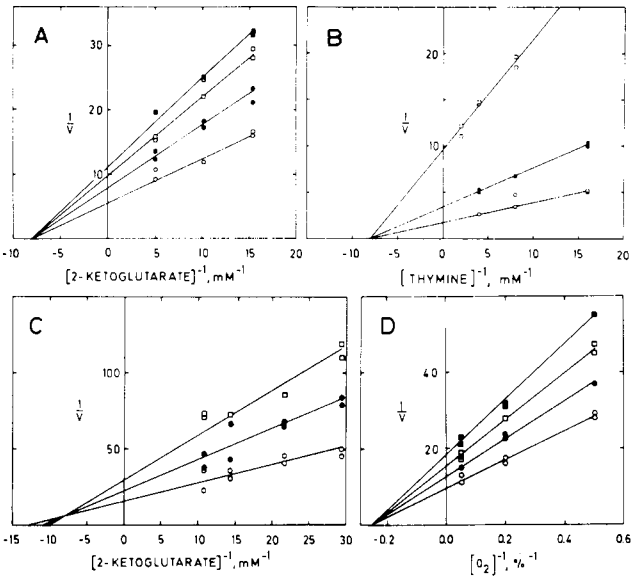


FIGURE 1: Initial velocity plots. (A) 2-Ketoglutarate is the variable substrate. Concentrations of thymine are: (■) 0.05 m*M*; (□) 0.07 m*M*; (●) 0.10 m*M*; and (○) 0.20 m*M*. (B) Thymine is the variable substrate. Concentrations of 2-ketoglutarate are: (□) 0.02 m*M*; (●) 0.05 m*M*; and (○) 0.15 m*M*. (C) 2-Ketoglutarate is the variable substrate at a constant oxygen concentration of 2.1% in the gas phase. Concentrations of thymine are: (□) 0.07 m*M*; (●) 0.10 m*M*; and (○) 0.20 m*M*. (D) Oxygen is the variable substrate at a constant and low thymine concentration of 0.02 m*M*. Concentrations of 2-ketoglutarate are: (■) 0.04 m*M*; (□) 0.05 m*M*; (●) 0.07 m*M*; and (○) 0.10 m*M*. The $^{14}CO_2$ assay has been used. Velocity is expressed as nanomoles of CO_2 formed per minute.

Pharmacia Fine Chemicals AB, Uppsala, Sweden; 5-aminouracil from Cyclo Chemical Division, Travenol Laboratories, Inc., Los Angeles, Calif.

Enzyme. Neurospora crassa strain STA 4 (FGSC 262 A, Fungal Genetic Stock Center, Humboldt State College, Arcata, Calif.) was cultured, harvested, and homogenized as described previously (Holme et al., 1971). Purification of the enzyme was achieved through fractionation on a hydroxylapatite column followed by fractionation on DEAE-Sephadex as reported previously (Bankel et al., 1972a). The enzyme was further purified on a column of Sepharose 4B to which had been coupled 5-aminouracil via 6-aminocaproic acid by the method of Cuatrecasas (Cuatrecasas and Anfinsen, 1971). The specific activity of the final preparations varied from 30 to 60 U per g.

Incubations. The basic incubation mixture contained varying concentrations of thymine and 2-ketoglutarate, in 100 m*M* potassium phosphate buffer at pH 7.5. In all experiments the buffer contained: ferrous sulfate (1 m*M*), ascorbate (5 m*M*), and catalase (2 mg/ml). The incubations were carried out in air at 37°C for 10 to 15 min in a final volume of 0.2 ml except when otherwise stated. The assay of enzymic activity was based either on $^{14}CO_2$ formation from 2-keto[1-^{14}C]glutaric acid or on the formation of labeled 5-hydroxymethyluracil from [2-^{14}C]thymine. The $^{14}CO_2$ formed was trapped on a piece of filter paper attached to a wire in the rubber stopper of the test tube used for incubation. Twenty microliters of a 1 *M* solution of Hyamin in methanol had been pipetted onto the filter paper. When bicarbonate had been added to the incubation 50 μl of Hyamin was used. The enzymic reaction was stopped by the addition of 0.2 ml of a 10% aqueous trichloroacetic acid solution. Diffusion of carbon dioxide was allowed to proceed for 60 min at 37°C, and the filter paper was then transferred to a scintillation vial and counted. When the formation of 5-hydroxymethyluracil was to be measured the reaction was stopped by the addition of 4 vol of ethanol. 5-Hydroxymethyluracil and thymine were separated by descending paper chromatography with the upper phase from a mixture of water and *sec*-butyl alcohol (Cline et al., 1959) as moving phase. Unlabeled thymine and 5-hydroxymethyluracil were added as markers. The spots were made visible by their ultraviolet (uv) absorption and then cut out and transferred to a scintillation vial for counting.

The same amount of enzyme was added in each single series of incubations. It was selected to give significant reaction rates and yet to be small enough so that all the velocities measured were within the linear part of the product-time progress curve. Not more than about 20% of any of the substrates were consumed in any of the incubations. Under these conditions there is a stoichiometric relation of 1:1 for the production of CO_2 and 5-hydroxymethyluracil, provided no extra 5-hydroxymethyluracil has been added to the incubation mixture (Holme et al., 1970). All reactions, except those where the oxygen concentrations were varied, were initiated by addition of the enzyme. Each experimental point was determined in duplicate or triplicate. Each experiment where there was any doubt about the pattern in the reciprocal plot was repeated two to several times to establish a consistent pattern.

Variation in Oxygen Concentration. The tubes with the complete incubation mixture including the enzyme were placed on ice. The tubes were then evacuated with a water pump, refilled with the appropriate gas mixture, and flushed with gas for 20 to 30 min before the incubation was started by putting the tubes in a water bath at 37°C. The concentrations of oxygen given are those in the gas phase.

Data Processing. The nomenclature used in this paper is that of Cleland (1963a, 1970). Reciprocal velocities were plotted graphically against the reciprocals of substrate concentrations to determine the pattern. Slopes and intercepts were replotted against the reciprocal of the nonvaried substrate—for initial velocity experiments—and the inhibitor concentration—for inhibitor experiments. For initial velocity experiments these replots were used to determine the numerical value of constants. The replots were linear except in those cases where otherwise specially indicated. In the inhibition experiments, where linear inhibitions were indicated by the replots of intercepts and slopes, the data were fitted to the equations for linear competitive inhibition and linear noncompetitive inhibition by the least-squares method using the Fortran programs of Cleland (1963b). The rate equation giving the best fit was chosen as that with the least variance of the fitted constants. In experiments where nonlinearity was suspected the data were also fitted to the equation for S-linear I-hyperbolic noncompetitive inhibition. In figures showing the inhibition patterns the lines have been calculated from the fits of the data to the corresponding rate equation. The computer generated constants are given in the figure legends of these plots.

Results

Initial Velocity Patterns. Initial velocity plots with 2-ketoglutarate as the variable substrate and with different concentrations of thymine as the fixed substrate in air give an intersecting pattern (Figure 1A). When thymine is plotted as the variable substrate with 2-ketoglutarate as the chang-

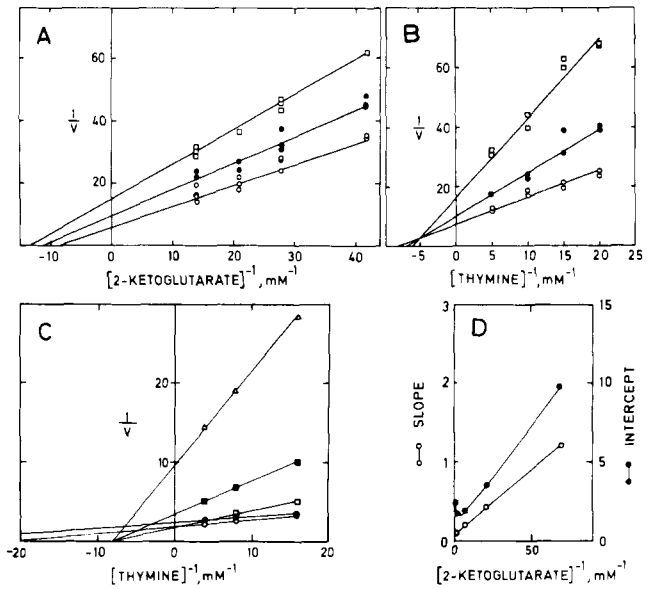


FIGURE 2: (A) Initial velocity plot with 2-ketoglutarate as the variable substrate at a constant thymine concentration of 0.20 mM. The concentrations of oxygen are: (□) 2.1%; (●) 5.2%; and (○) 20%. (B) Initial velocity plot with thymine as the variable substrate at a constant concentration of 2-ketoglutarate of 0.20 mM. The concentrations of oxygen are the same as in A. (C) Substrate inhibition by 2-ketoglutarate with thymine as the variable substrate. The concentrations of 2-ketoglutarate are: (△) 0.02 mM; (■) 0.05 mM; (□) 0.15 mM; (○) 0.74 mM; and (●) 1.48 mM. For clarity only the mean values have been indicated. (D) Replot from C of the slopes and intercepts. The $^{14}CO_2$ assay has been used. Velocity is expressed as nanomoles of CO_2 formed per minute: slope unit, min mM nmol^{-1}; intercept unit, min nmol^{-1}.

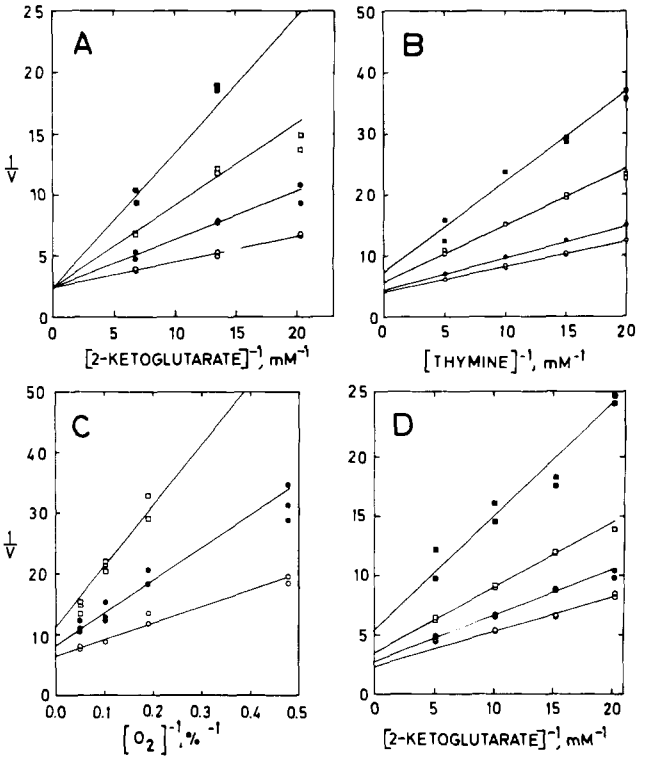


FIGURE 3: (A) Inhibition by 5-carboxyuracil with 2-ketoglutarate as the variable substrate at a constant concentration of thymine of 0.12 mM. The concentrations of 5-carboxyuracil are: (■) 10.0 mM; (□) 5.0 mM; (●) 2.0 mM; and (○) 0.0 mM. Kinetic constants: K = 0.09 mM, V = 0.42 nmol min^{-1}, and K_{IS} = 2.3 mM. (B) Inhibition by 5-carboxyuracil with thymine as the variable substrate at a constant concentration of 2-ketoglutarate of 0.05 mM. The concentrations of 5-carboxyuracil are: (■) 10.0 mM; (□) 5.0 mM; (●) 1.0 mM; and (○) 0.0 mM. Kinetic constants: K = 0.10 mM, V = 0.25 nmol min^{-1}, K_{IS} = 4.0 mM, and K_{II} = 1.2 mM. (C) Inhibition by 5-carboxyuracil with oxygen as the variable substrate at a constant concentration of 2-ketoglutarate of 0.05 mM and of thymine of 0.20 mM. The concentrations of 5-carboxyuracil are: (□) 5.0 mM; (●) 2.0 mM; and (○) 0.0 mM. Kinetic constants: K = 4.2%, V = 0.16 nmol min^{-1}, K_{IS} = 2.0 mM, and K_{II} = 7.6 mM. (D) Product inhibition by bicarbonate with 2-ketoglutarate as the variable substrate at a constant concentration of thymine of 0.10 mM. The concentrations of bicarbonate are: (■) 100 mM; (□) 50 mM; (●) 20 mM; and (○) 0 mM. Kinetic constants: K = 0.08 mM, V = 0.34 nmol min^{-1}, K_{IS} = 42 mM, and K_{II} = 140 mM. The $^{14}CO_2$ assay has been used. Velocity is expressed as nanomoles of CO_2 formed per minute.

ing fixed substrate the same type of pattern is obtained (Figure 2). The data of Figure 1A and Figure 1B can be represented by eq 1:

$$v = \frac{VAB}{K_aB + K_bA + K_{ia}K_b + AB} \quad (1)$$

In this equation K_a and K_b are the Michaelis constants for 2-ketoglutarate and thymine, respectively, and A and B the concentrations of 2-ketoglutarate and thymine, respectively. K_a and K_b are both found to be 0.12 mM. K_{ia} is the inhibition constant for 2-ketoglutarate; it was equal to K_a. When initial velocity experiments were carried out at reduced oxygen concentrations (Figure 1C), the data could be represented by eq 2.

$$v = \frac{VAB/(1 + K_c/C)}{K_aB/(1 + K_c/C) + K_bA + K_aK_b + AB} \quad (2)$$

In this equation K_c is the Michaelis constant for oxygen and C is the concentration of oxygen. K_c was approximately 0.06 mM (4% oxygen in the gas phase) (Figure 1D). An intersecting pattern was obtained with the crossover point on the abscissa when oxygen was the variable substrate and 2-ketoglutarate was the changing fixed substrate at low concentration of thymine. The data fit an equation of the same form as eq 1, the difference being that V is divided by the factor $(1 + K_b/B)$. An increase in thymine concentration did not affect the Michaelis constants of 2-ketoglutarate and oxygen (Figure 2A), but the crossover point occurred beneath the abscissa. The data can be represented by

$$v = \frac{VAC/(1 + K_b/B)}{K_aC + K_cA + K_aK_c/(1 + B/K_b)} \quad (3)$$

When thymine was the variable substrate and oxygen was the changing fixed substrate at a fixed concentration of 2-ketoglutarate (Figure 2B) the data could be represented by an equation corresponding to eq 2. Thus, the Michaelis constant found for thymine is independent of the 2-ketoglutarate concentration, whereas the Michaelis constant of oxygen is divided by the factor $(1 + K_a/A)$.

Inhibition Patterns. An increase in the concentration of 2-ketoglutarate resulted in substrate inhibition of the uncompetitive type (Figures 2C and 2D). 5-Carboxyuracil, which is the end product in the total reaction sequence, is an inhibitor of thymine 7-hydroxylase. When 2-ketoglutarate was the variable substrate an inhibition pattern of the competitive type was obtained (Figure 3A). With thymine as the variable substrate a noncompetitive inhibition was found (Figure 3B), as was also the case when oxygen was the variable substrate (Figure 3C). Bicarbonate was a noncompetitive inhibitor when the concentration of either 2-ketoglutarate (Figure 3D) or thymine (Figure 4A) was varied. Inhibition experiments with oxygen as the variable substrate have not been performed for technical reasons. 5-

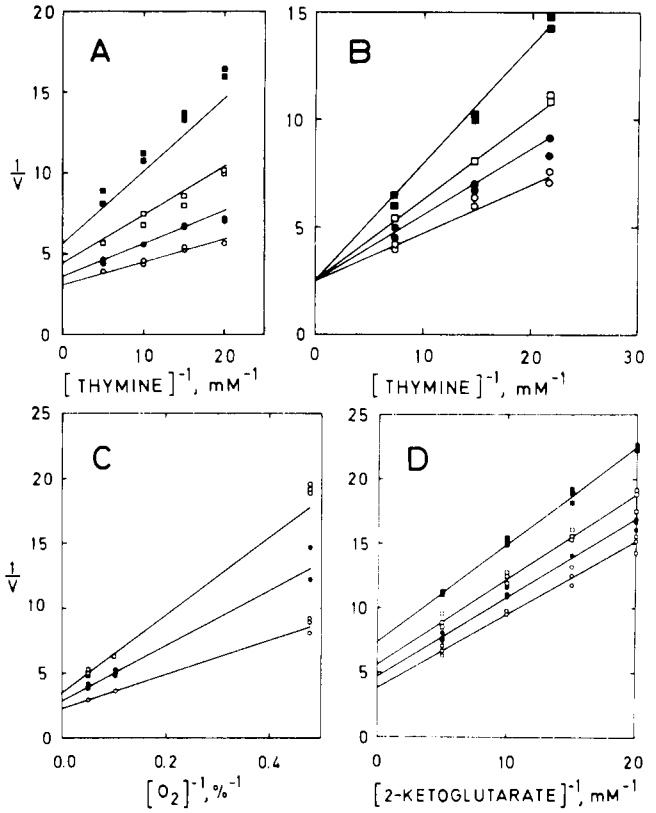


FIGURE 4: (A) Product inhibition by bicarbonate with thymine as the variable substrate at a constant concentration of 2-ketoglutarate of 0.15 mM. The concentrations of bicarbonate are: (■) 100 mM; (□) 50 mM; (●) 20 mM; and (○) 0 mM. The $^{14}CO_2$ assay has been used. Velocity is expressed as nanomoles of CO_2 formed per minute. Kinetic constants: K = 0.05 mM, V = 0.32 nmol min^{-1}, K_{IS} = 49 mM, and K_{II} = 124 mM. (B) Inhibition by 5-hydroxymethyl with thymine as the variable substrate at a constant concentration of 2-ketoglutarate of 0.10 mM. The concentrations of 5-hydroxymethyluracil are: (■) 0.40 mM; (□) 0.20 mM; (●) 0.10 mM; and (○) 0.00 mM. Kinetic constants: K = 0.09 mM, V = 0.39 nmol min^{-1}, and K_{IS} = 0.29 mM. (C) Inhibition by 5-hydroxymethyluracil with oxygen as the variable substrate at a constant concentration of 2-ketoglutarate of 0.20 mM and of thymine of 0.05 mM. The concentrations of 5-hydroxymethyluracil are: (□) 0.20 mM; (●) 0.10 mM; and (○) 0.00 mM. Kinetic constants: K = 5.7%, V = 0.44 nmol min^{-1}, K_{IS} = 0.17 mM, and K_{II} = 0.38 mM. (D) Inhibition by 5-hydroxymethyluracil with 2-ketoglutarate as the variable substrate at a constant concentration of thymine of 0.05 mM. The concentrations of 5-hydroxymethyluracil are: (■) 0.20 mM; (□) 0.10 mM; (●) 0.05 mM; and (○) 0.00 mM. Kinetic constants: K = 0.15 mM, V = 0.26 nmol min^{-1}, K_{IS} = 0.62 mM, and K_{II} = 0.21 mM. The velocity unit is nanomoles of 5-hydroxymethyluracil formed per minute (B–D).

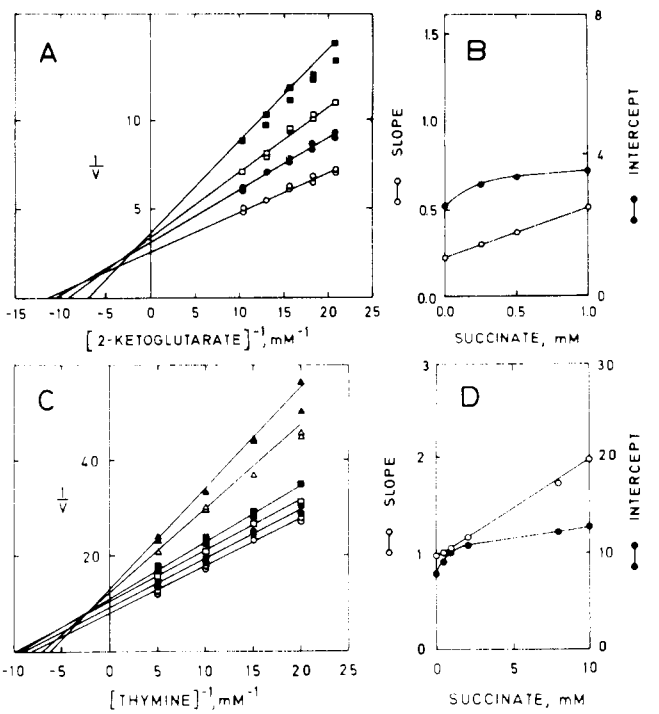


FIGURE 5: (A) Product inhibition by succinate with 2-ketoglutarate as the variable substrate at a constant concentration of thymine of 0.10 mM. The concentrations of succinate are: (■) 1.00 mM; (□) 0.50 mM; (●) 0.25 mM; and (○) 0.00 mM. Kinetic constants: K = 0.09 mM, V = 0.39 nmol min^{-1}, K_{IS} = 0.78 mM, $K_{I,num}$ = 0.17 mM, and $K_{I,denom}$ = 0.25 mM. (B) The slopes and intercepts replots from A vs. succinate concentration; slope unit, min mM nmol^{-1}; intercept unit, min nmol^{-1}. (C) Product inhibition by succinate with thymine as the variable substrate at a constant concentration of 2-ketoglutarate of 0.05 mM. The concentrations of succinate are: (▲) 10.0 mM; (△) 8.0 mM; (■) 2.0 mM; (□) 1.0 mM; (●) 0.5 mM; and (○) 0.0 mM. (D) The slopes and intercepts replots from C vs. succinate concentration; slope unit, min mM nmol^{-1}; intercept unit min nmol^{-1}. The $^{14}CO_2$ assay has been used. Velocity is expressed as nanomoles of CO_2 formed per minute.

Hydroxymethyluracil, which is both a substrate and a product in the reaction catalyzed by thymine 7-hydroxylase, was a competitive inhibitor against thymine (Figure 4B) and a noncompetitive inhibitor against oxygen (Figure 4C). A noncompetitive inhibition with 5-hydroxymethyluracil against 2-ketoglutarate (Figure 4D) was also found, but the slope effect was very slight. The patterns obtained with succinate as inhibitor and either 2-ketoglutarate or thymine as the variable substrate are shown in Figures 5A and 5C. Hyperbolic intercept effects are seen in both cases (Figures 5B and 5D), but the intercept replot when thymine is the variable substrate seems to be a 2:1 function (Cleland, 1963c).

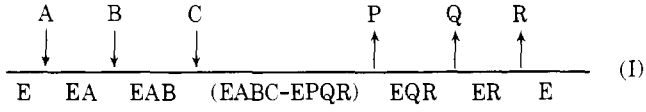
Discussion

The initial velocity patterns have all been linear and intersecting which is characteristic for a sequential reaction mechanism in which all substrates have to be bound to the enzyme before any product is released. Linear intersecting initial velocity patterns are seen in both random equilibrium and ordered sequential terreactant mechanisms. The cumulative results are best represented by the following rate equation (eq 4):

$$v = VABC/(K_aBC + K_bAC + K_cAB + K_aK_bC + K_bK_cA + K_aK_bK_c + ABC) \quad (4)$$

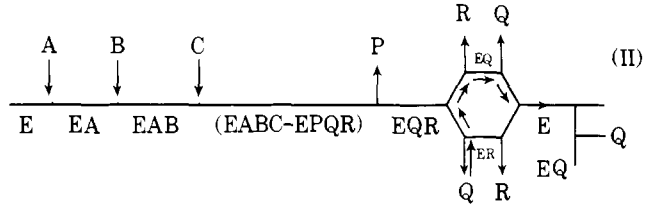
This is the equation for an ordered sequential terreactant mechanism and it is in fact only a rearranged form of eq 2 and 3. Substrate B could be identified since initial velocity plots with the crossover point on the abscissa are obtained at high concentrations of A or C when the concentration of one of the other two substrates is varied, whereas the same type of pattern is obtained at low concentrations of B and varying concentrations of either A or C. On the other hand, at high concentrations of B when the concentration of A or C is varied a set of parallel lines in the initial velocity plot is predicted (Frieden, 1959). Thus, thymine can be identified as substrate B, since at a low concentration of thymine, when the concentration of 2-ketoglutarate or oxygen was varied, an initial velocity plot with the crossover point on the abscissa was obtained. When the thymine concentration was raised the lines in the initial velocity plot approached a parallel pattern. It would have been desirable to use a wider range of substrate concentrations in the initial velocity stud-

ies, but the appearance of substrate inhibition made this impossible. However, the conclusions drawn from the initial velocity studies are supported by the inhibition experiments which are discussed below. A sequential ordered ter-ter mechanism is most simply represented by the following scheme in which the symbolism of Cleland (1963a) is used (mechanism I).



(I)

5-Carboxyuracil, which is the product when 5-formyluracil is hydroxylated, has been used as an inhibitor instead of 5-hydroxymethyluracil, since it is not further metabolized by the enzyme. It should be noticed, however, that under the assay condition used 5-hydroxymethyluracil formed during incubations is not further metabolized to any significant degree (Holme et al., 1970). The data obtained with this inhibitor conform to the pattern predicted for an ordered mechanism with A corresponding to 2-ketoglutarate and R to the hydroxylated pyrimidine substrate. Thus, 2-ketoglutarate can be identified as substrate A, thymine as substrate B, and oxygen as substrate C. The inhibition patterns obtained with bicarbonate as the inhibitor indicate that bicarbonate corresponds to P in the scheme. As the inhibition patterns with oxygen as the variable substrate are not known, a Theorell–Chance mechanism cannot be excluded. As indicated by the substrate inhibition seen with 2-ketoglutarate, a dead-end complex could be formed between 2-ketoglutarate and the enzyme, probably with the same enzyme form that binds succinate (ER). The uncompetitive inhibition seen is thus in agreement with a scheme with succinate corresponding to Q. The interpretation of the inhibition obtained with 5-hydroxymethyluracil is complicated by the fact that 5-hydroxymethyluracil is a substrate as well as a product. The competitive inhibition seen when thymine is the variable substrate indicates that there is no appreciable classical product inhibition of 5-hydroxymethyluracil in the concentration range used in the experiments. A competitive inhibitor against thymine (B) should give a linear noncompetitive inhibition against substrate C if no EAIC complex is formed. Since 5-hydroxymethyluracil is a substrate for the enzyme it is obvious that such a complex could be formed, which would result in a nonlinear inhibition pattern (Fromm, 1967). However, the nonlinearity could not be detected under the experimental condition used. An uncompetitive inhibition is predicted against substrate A, but the inhibition pattern obtained when 2-ketoglutarate is the variable substrate shows a noncompetitive pattern although the slope effect is very slight. The slope effect indicates that there is a slight product inhibition, which is too small, however, to give a significant intercept effect when thymine is the variable substrate. The results obtained with succinate as product inhibitor could be represented in the following scheme (mechanism II). Succinate (Q) could



(II)

form a dead-end complex with the free enzyme (E) and in the presence of succinate the order of product release would then be partially altered so that the hydroxylated pyrimidine substrate is released before succinate.

The findings are in agreement with the proposal that the iron-containing oxygenases have to be modified by substrate binding before oxygen can combine with the enzymes. The results, however, do not permit any conclusions to be drawn about the nature of the reaction intermediates, which would have been the case if the reaction mechanism had been found to be of the ping-pong type. It would further be of interest to find out if the other 2-ketoglutarate dependent oxygenases have the same kinetic mechanism. In fact, preliminary results in our laboratory with γ-butyrobetaine hydroxylase and with thymidine-2′-hydroxylase indicate that these enzymes also have sequential initial velocity patterns, which indicates that this is a general feature of the 2-ketoglutarate-dependent oxygenases.

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