General Kinetic Mechanism of Microsomal Carbamyl Phosphate:Glucose Phosphotransferase, Glucose 6-Phosphatase, and Other Associated Activities†

James D. Lueck,‡ John L. Herrman, and Robert C. Nordlie*

ABSTRACT: The kinetic mechanism of carbamyl phosphate:glucose phosphotransferase and associated hydrolytic and synthetic activities of microsomal D-glucose-6-phosphate phosphohydrolase (EC 3.1.3.9) has been investigated in detail at pH 7 and μ = 0.1. Classical "two-substrate" kinetic studies were carried out with the phosphotransferase activity. In addition, inhibitions by carbamyl phosphate of glucose-6-phosphate phosphohydrolase activity and by glucose of both carbamyl phosphatase and glucose-6-phosphate phosphohydrolase activities of the multifunctional enzyme were studied. The modifying effects of detergent treatment on inhibition by glucose of the latter activity at pH 6 also were investigated. Although kinetic patterns from the present studies differ in certain respects (described in detail in the text) from those of earlier studies of various activities of this enzyme, they are shown to be compatible with a generally applicable kinetic mechanism involving, successively, the following steps: (a) formation of binary enzyme–phosphoryl substrate complexes, (b) dissociations of such enzyme-bound compounds to produce a common phosphoryl–enzyme intermediate, and (c) transfer of phosphoryl group from phosphoryl–enzyme alternatively to glucose (phosphotransferase) or water (phosphohydrolase) to complete the process. Certain apparent differences in patterns of results obtained in these and earlier kinetic studies of various activities of this biological catalyst by us and others are rationalized mechanistically. Such variations appear to arise due to modifications by detergents and/or alterations in assay pH on the relative magnitude (ratio) of the individual rate constants corresponding with the terminal step (see C, above) in, respectively, the phosphohydrolase and phosphotransferase reactions.

Over the past several years the multifunctional nature of microsomal D-glucose-6-phosphate phosphohydrolase (EC 3.1.3.9; reaction 1) has been elucidated in detail (see Nordlie, 1971). The enzyme displays significant phosphotransferase as well as phosphohydrolase action. The most potent of these phosphotransferase activities, carbamyl phosphate:glucose phosphotransferase (reaction 2), has been the subject of intensive study in this laboratory for the past 2 years (Lueck and Nordlie, 1970; Nordlie *et al.*, 1971; Herrman *et al.*, 1971).

$$\text{glucose-6-P} + H_2O \longrightarrow \text{glucose} + P_i \quad (1)$$

$$\text{carbamyl-P} + \text{glucose} \longrightarrow \text{glucose-6-P} + \text{carbamate} \quad (2)$$

This activity is of particular interest, we believe, because (a) it appears to be widely distributed in higher organisms,[1] (b) it involves physiologically established metabolites as substrates, (c) it is highly active even at and above pH 7, in contrast with such activity with PP_i or ATP as phosphoryl donor (Nordlie *et al.*, 1971), (d) it is the most potent activity yet discovered for the enzyme, maximally exceeding quite significantly the enzyme's capacity even for glucose-6-P hydrolysis (see below), (e) apparent K_m,Glc,trf values approaching physiological blood sugar levels have been observed with certain species,[2] and (f) its kinetics appear on first examination to differ in certain important details from those of other activities previously studied (Arion and Nordlie, 1964; Hass and Bryne, 1960; Segal, 1959).

With regard to this last point, apparent K_m values for carbamyl-P and glucose with the enzyme from a variety of sources have been observed to vary directly with concentration of second substrate (Herrman *et al.*, 1971; also see text below), while such parameters were found independent of second substrate concentration in studies of PP_i–glucose phosphotransferase activity of the enzyme previously carried out at pH 6 (Arion and Nordlie, 1964).

In an attempt to resolve in a mechanistic fashion these latter apparent variations, as well as to rationalize certain additional subtle differences in the kinetics of inhibition by glucose of various phosphohydrolase activities of this enzyme (see Hass and Byrne, 1960; Segal, 1959; Arion and Nordlie, 1964), the rather extensive kinetic studies described in this paper were carried out.

On the basis of these studies and a consideration of additional information in the literature, a general kinetic treatment, which appears to resolve these apparent differences and which is consistent with all kinetic observations made to date

† From the Guy and Bertha Ireland Research Laboratory, Department of Biochemistry, University of North Dakota Medical School, Grand Forks, North Dakota 58201. *Received February 18, 1972.* This work was supported in part by the Hill Family Foundation, United States Public Health Service Research Grant AM-07141, National Science Foundation Research Grant GB-14752, and the American Diabetes Association.

‡ American Cancer Society Postdoctoral Fellow.

[1] Highly significant levels of carbamyl-P:glucose phosphotransferase activity have been observed in liver preparations of all of 18 species of mammals, birds, amphibians, fish, reptiles, and arachnoids (man, beef, cat, mouse, rat, guinea pig, deer, rabbit, chicken, duck, pigeon bullfrog, grass frog, bullhead, mudpuppy, turtle, garter snake, and crayfish) which have thus far been investigated (J. L. Herrman and R. C. Nordlie, unpublished observations).

[2] For example, K_m',Glc,trf values as low as 23 mM have been noted (J. L. Herrman and R. C. Nordlie, unpublished observation) with preparations from livers of chicken, in which blood sugar levels between 250 and 300 mg % (14–17 mM) routinely are observed (Sturkie, 1965)).

with the various activities of this multifunctional catalyst, is proposed.

Materials and Methods

All substrates used in these studies were obtained from Sigma Chemical Co. *N*-2-Hydroxyethylpiperazine-*N'*-ethanesulfonic acid[3] (Hepes) was purchased from Calbiochem. Young, male, adult albino rats weighing between 150 and 250 g were obtained from Sprague-Dawley, Inc., Madison, Wis. The substrates glucose-6-P (Nordlie and Arion, 1966), glucose (Nordlie and Soodsma, 1966; and Nordlie and Arion, 1966), and carbamyl-P (Herries, 1967) were assayed as previously described. Solutions of the latter were freshly prepared just prior to assay and were kept in ice until used.

Preparations of rat liver microsomal glucose-6-phosphatase were obtained as described earlier (Nordlie and Arion, 1964) and were utilized, except in the studies depected in Figures 5A and B. The preparation described had a specific activity of 0.25 unit[4] per mg of protein. A wide variety of studies over the past 8 years all support the catalysis of a rather imposing number of hydrolytic and synthetic reactions by this classical membrane-bound microsomal enzyme (see Nordlie, 1971). Reaction mixtures containing an aliquot of the enzyme preparation (0.048 unit) were incubated for 10 min (5 min in the case of the carbamyl-P phosphohydrolase reaction) at 30 ± 0.1°, with shaking. All reaction mixtures, at pH 7.0, contained 40 mM Hepes and sufficient NaCl such that the ionic strength equaled 0.1; other reaction mixture components are indicated in the legends to the figures.

Phosphotransferase activity was assessed by measuring glucose-6-P produced (Nordlie and Arion, 1966), while glucose-6-P phosphohydrolase was monitored by measuring either P_i (Nordlie and Arion, 1966) or glucose liberated (Nordlie and Soodsma, 1966). Further details are as in earlier papers (Nordlie and Arion, 1966; Nordlie and Soodsma, 1966).

Carbamyl-P phosphohydrolase activity was measured as follows. Reaction mixtures were kept in ice until assayed. After a 3-min preincubation of assay mixtures at 30 ± 0.1°, enzyme was added; boiled enzyme was added to a corresponding set of control assay mixtures to compensate for nonenzymic hydrolysis of carbamyl-P and for endogenous P_i. Inorganic phosphate liberated during a 5-min incubation was determined in aliquots of assay mixtures by the method of Herries (1967); addition of the aliquot to the acid molybdate–isobutyl alcohol reagent as the preliminary step in P_i determination terminated the reaction.

All activities were determined under conditions of linearity with respect to enzyme concentration and time. Initial reaction velocity, v, is in all cases expressed in terms of 10 × μmoles of glucose-6-P formed (phosphotransferase) or phosphate substrate hydrolyzed (phosphohydrolase) per 1.5 ml of reaction mixture per min.

The kinetic data (Figures 1 and 3–5) are presented as conventional double-reciprocal plots (Lineweaver and Burk, 1934). Experimental lines were constructed freehand by inspection of experimental points as in our earlier studies (Arion and Nordlie, 1964; Nordlie and Arion, 1964; Nordlie and Soodsma, 1966; Herrman *et al.*, 1971). Data presented here were obtained with enzyme preparations from rat liver microsomes; all studies were repeated at least three times, and identical kinetic patterns were obtained. In addition, entirely comparable patterns of kinetics were obtained in supplementary studies with rat kidney microsomal preparations and in repeated studies with liver microsomal preparations from beef, cat, mouse, guinea pig, rabbit, chick, and duck (J. L. Herrman and R. C. Nordlie, unpublished observations, 1972). Abbreviations for the various kinetic expressions employed are defined, for convenience, in Table I.

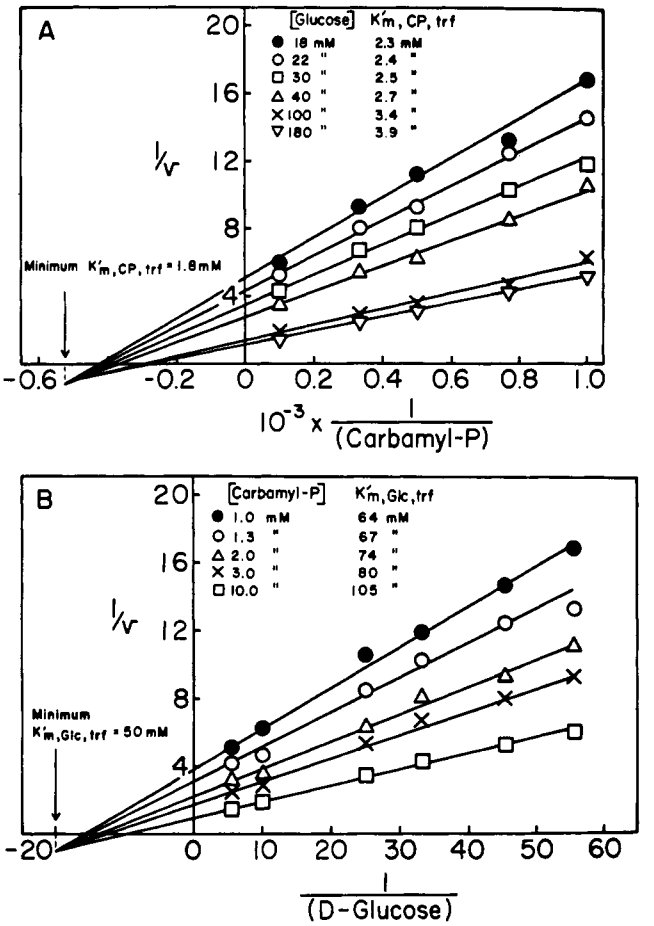

FIGURE 1: Primary plots of reciprocals of initial reaction velocities (v) against reciprocals of molar concentrations of carbamyl-P (A) or glucose (B). In A, D-glucose concentrations were held constant at 1.80×10^{-2} M (●), 2.20×10^{-2} M (○), 3.00×10^{-2} M (□), 4.00×10^{-2} M (△), 1.00×10^{-1} M (×), or 1.80×10^{-1} M (▽), and v determined as a function of carbamyl-P concentration which was varied from 1.00×10^{-3} M to 1.00×10^{-2} M. In B, carbamyl-P concentrations were held constant at 1.00×10^{-3} M (●), 1.30×10^{-3} M (○), 2.00×10^{-3} M (△), 3.00×10^{-3} M (×), or 1.00×10^{-2} M (□), and v was determined as a function of D-glucose concentration which varied from 1.80×10^{-2} M to 1.80×10^{-1} M. Velocity, v, is expressed as 10 × μmoles of glucose-6-P produced/1.5 ml of reaction mixture per min. Apparent Michaelis constant values for carbamyl-P (K_m',CP,trf) and glucose (K_m',Glc,trf), calculated as $-1/x$ axis intercepts of extrapolations of experimental plots, are indicated on the figures along with concentrations of second substrate with which they correspond. Additional details are given in the text.

Results and Discussion

Carbamyl-P:Glucose Phosphotransferase Kinetics. The results of kinetic studies of the carbamyl-P:glucose phosphotransferase reaction (eq 2) are presented in Figures 1A and B.

[3] Abbreviations used are: Hepes, *N*-2-hydroxyethylpiperazine-*N'*-ethanesulfonic acid; glucose-6-P, glucose 6-phosphate.

[4] One unit of enzymic activity is that amount catalyzing the hydrolysis of 1 μmole of glucose-6-P per 1.5 ml of reaction mixture per min under conditions previously defined (Nordlie and Arion, 1964).

TABLE I: Definitions of Kinetic Symbols.

Term	Definition
v	Initial reaction velocity
V_{max},Glc-6-Pase	Maximal reaction velocity for the D-glucose-6-P phosphohydrolase reaction
V_{max},CPase	Maximal reaction velocity for the carbamyl-P phosphohydrolase reaction
V_{max},trf	Maximal reaction velocity of the carbamyl-P: glucose phosphotransferase reaction; concentrations of carbamyl-P and glucose infinite
V_{max}',trf	Apparent maximal reaction velocity for the carbamyl-P: glucose phosphotransferase reaction; applicable for infinite concentration of carbamyl-P and specified finite concentration of glucose
V_{max}'',trf	Apparent maximal reaction velocity for the carbamyl-P: glucose phosphotransferase reaction; applicable for infinite concentration of glucose and specified finite concentration of carbamyl-P
K_m,CP,trf	Michaelis constant for carbamyl-P in the carbamyl-P: glucose phosphotransferase reaction; that concentration of carbamyl-P for which $v = {}^1/_2V_{max}$,trf (concentration of glucose infinite)
K_m',CP,trf	Apparent Michaelis constant for carbamyl-P in the carbamyl-P: glucose phosphotransferase reaction; that concentration of carbamyl-P for which $v = {}^1/_2V_{max}'$,trf (concentration of glucose at a stated, finite level)
K_m,CP,CPase	Michaelis constant for carbamyl-P in the carbamyl-P phosphohydrolase reaction; that concentration of carbamyl-P for which $v = {}^1/_2V_{max}$,CPase, for the carbamyl-P phosphohydrolase reaction
K_i,CP,Glc-6-Pase	The inhibitor constant for carbamyl-P acting as a competitive inhibitor of glucose-6-P phosphohydrolase
K_m,Glc-6-P	Michaelis constant for glucose-6-P in the glucose-6-P phosphohydrolase reaction; that concentration of glucose-6-P for which $v = {}^1/_2V_{max}$,Glc-6-Pase
K_m,Glc,trf	The Michaelis constant for D-glucose in the carbamyl-P: glucose phosphotransferase reaction; that concentration of glucose for which $v = {}^1/_2V_{max}$,trf (concentration of carbamyl-P infinite)
K_m',Glc,trf	Apparent Michaelis constant for glucose in the carbamyl-P: glucose phosphotransferase reaction; that concentration of glucose for which $v = {}^1/_2V_{max}''$,trf (concentration of carbamyl-P at a stated, finite level)
K_i,Glc,Glc-6-Pase	Inhibitor constant for glucose acting as an inhibitor of glucose-6-P phosphohydrolase
K_i,Glc,CPase	Inhibitor constant for glucose acting as an inhibitor of carbamyl-P phosphohydrolase activity
$K_{CP\cdot Glc,trf}$	The "complex constant" (Florini and Vestling, 1957)
CP	Carbamyl phosphate
Glc	D-Glucose
Glc-6-P	D-Glucose 6-phosphate
trf	Carbamyl-P: glucose phosphotransferase
( ) or [ ]	Molar concentration
PP_i[a]	Inorganic pyrophosphate
PP_iase[a]	Inorganic pyrophosphate phosphohydrolase
RP[a]	Generalized abbrevation for carbamyl-P, PP_i, or other phosphoryl substrate other than glucose-6-P

[a] Kinetic expressions in the text involving these abbreviations are readily identifiable by analogy with their counterparts specifically involving carbamyl-P as defined above.

Initial reaction velocities were determined in a series of reaction mixtures at several constant glucose concentrations with carbamyl-P concentration varied in the indicated range (Figure 1A), and in another series of reaction mixtures at several constant carbamyl-P concentrations with glucose concentration varied in the indicated range (Figure 1B). Note that, in contrast with PP_i: glucose and mannose-6-P: glucose phosphotransferase activities which previously have been studied (Arion and Nordlie, 1964), extrapolations of the primary double-reciprocal plots converge at common points *below* the x axis (*i.e.*, in the third quadrant).

K_m'CP,trf values for carbamyl-P (those concentrations of carbamyl-P giving 50% of apparent maximal velocity with the corresponding concentrations of glucose) may be calculated as negative reciprocals of x-axis intercepts of extrapolations of experimental plots in Figure 1A. Similarly, K_m',Glc,trf values, applicable in the presence of the indicated concentrations of carbamyl-P, may be calculated from data in Figure 1B. These values are recorded on Figures 1A and B, along with concentrations of second substrate to which they relate.

Minimum K_m',CP,trf and K_m',Glc,trf values—those obtained with glucose or carbamyl-P concentration, respectively, approaching zero—calculated as $-1/x$ coordinates of common points of convergence, also are given on Figures 1A and B.

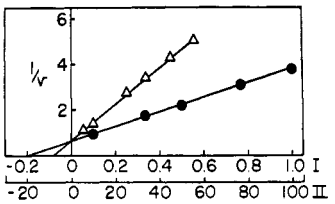

FIGURE 2: Secondary plots of y-axis intercepts ($1/V_{max}$,trf) from Figures 1B and A against reciprocals of concentrations of carbamyl-P (●; abscissa I) or D-glucose (△; abscissa II), respectively. Axis of abscissas I indicates 10^{-3} × reciprocals of molar concentrations of carbamyl-P, while axis of abscissas II refers to reciprocals of molar concentrations of D-glucose. V_{max},trf = $1/y$ axis intercept = 0.154 μmole of glucose-6-P formed/1.5 ml of reaction mixture per min; K_m,CP, trf and K_m,Glc,trf, evaluated as $-1/x$ axis intercepts, = 5.0 mM and 125 mM, respectively.

Secondary plots (Florini and Vestling, 1957) of y-axis intercepts ($1/V_{max}'$,trf) from Figures 1A *vs.* reciprocals of the several constant concentrations of glucose employed and of y-axis intercepts from Figure 1B *vs.* reciprocals of carbamyl-P concentrations are given in Figure 2. The V_{max},trf value (velocity noted when infinite concentrations of both glucose and carbamyl-P are present) is given by the reciprocal of the y-axis intercept of this secondary plot (Florini and Vestling, 1957) and is calculated as 0.154 μmole of glucose-6-P produced per 1.5 ml of reaction mixture per min. The K_m,CP,-trf, that concentration of carbamyl-P giving a velocity one-half of V_{max},trf with glucose concentration infinite, may be calculated as $-1/x$-axis intercept of an extrapolation of the line indicated by closed circles in Figure 2. K_m,CP,trf = 5.0 mM. K_m,Glc,trf, similarly calculated from data indicated by open triangles in Figure 2, = 125 mM.

The "complex constant," $K_{CP\cdot Glc,trf}$, was calculated on the basis of the x-coordinate values of the points of convergence of experimental plots as described in detail by Florini and Vestling (1957). $K_{CP\cdot Glc,trf}$ = 250 mM[2].

Patterns of kinetic results similar in form to those above previously have been obtained with human liver preparations (Herrman *et al.*, 1971). As a further check on the generally applicable nature of the observations as described in Figures 1A and B and 2, above, identical kinetic studies were carried out with detergent-activated liver microsomal preparations from a variety of sources—beef, cat, mouse, guinea pig, rabbit, chicken, and duck. In all instances, results obtained were basically as in Figures 1A and B and 2 (J. L. Herrman and R. C. Nordlie, unpublished observations, 1972). Because such preparations have been employed most extensively in our earlier studies of this multifunctional enzyme, rat liver microsomal enzyme was utilized in the additional kinetic studies described below.

Inhibition Kinetics. Kinetic studies of the inhibition by glucose of the hydrolysis of glucose-6-P and carbamyl-P are described in Figures 3A and B, respectively. Data are again presented as conventional double-reciprocal plots. P_i liberated enzymically from phosphate substrates in the absence and presence of various indicated concentrations of glucose was in each instance measured.

In contrast with earlier observations of Arion and Nordlie (1964), Segal (1959), and Hass and Byrne (1960), at pH 6 or 6.4, extrapolations of the sets of experimental plots obtained in the glucose-6-P phosphohydrolase study (Figure 3A) converged in a point *above* the x axis (*i.e.*, in the second quadrant) rather than *on* (Segal, 1959; Arion and Nordlie, 1964) or

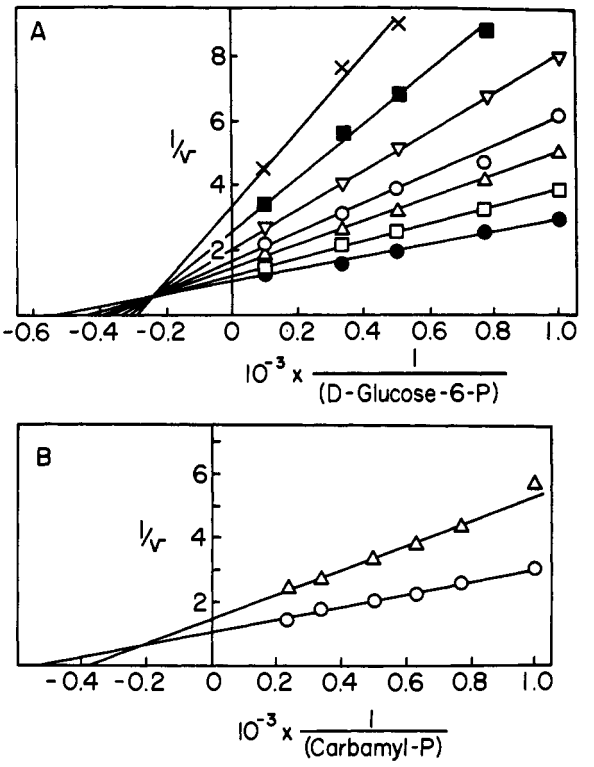

FIGURE 3: Kinetics of inhibition by D-glucose of glucose-6-P phosphohydrolase (A) and carbamyl-P phosphohydrolase (B). In A, v for glucose-6-P phosphohydrolase was measured in the absence (●) and presence of 3.00 × 10^{-2} M (□) 6.00 × 10^{-2} M (△), 9.00 × 10^{-2} M (○), 12.0 × 10^{-2} M (▽), 18.0 × 10^{-2} M (■), and 27.0 × 10^{-2} M (×) D-glucose. D-glucose-6-P concentration was varied from 1.00 × 10^{-3} M to 1.00 × 10^{-2} M. K_m,Glc-6-P,Glc-6-Pase = 1.8 mM and V_{max},-Glc-6-Pase = 0.100 μmole/1.5 ml per min. K_i,Glc,Glc-6-Pase values, corresponding to the indicated finite concentrations of glucose and calculated as described in the text, were 120 mM, 110 mM, 113 mM, 115 mM, 117 mM, and 114 mM, respectively. In B, v values for carbamyl-P phosphohydrolase were measured in the absence (○) and presence of 4.00 × 10^{-2} M D-glucose (△). Carbamyl-P concentrations were varied from 1.00 × 10^{-3} M to 1.00 × 10^{-2} M. K_i,Glc,-CPase = 2.0 mM, K_m,CP,CPase = 2.0 mM, and V_{max}, CPase = 0.100 μmole/1.5 ml per min. In both A and B, v is expressed as 10 × μmoles of P_i produced per 1.5 ml of reaction mixture per min.

below (Hass and Byrne, 1960) the x axis. A similar observation also was made in the study of the effects of glucose on carbamyl-P phosphohydrolase activity (see Figure 3B). The significance of these observations is considered in detail below. K_m,Glc-6-P,Glc-6-Pase ($-1/x$ intercept of plot of data obtained in the absence of glucose), V_{max},Glc-6-Pase ($1/y$ intercept of plot of data obtained in the absence of glucose), K_i,Glc,Glc-6-Pase values (calculated from the relationship given in eq 3 (Dixon and Webb, 1964)) are presented in the legend to Figure 3A for the glucose-6-P phosphohydrolase study. The K_i value for glucose functioning as an inhibitor of carbamyl-P phosphohydrolase, calculated in an analogous manner, is given in the legend to Figure 3B.

$$y\text{-axis intercept} = 1/V_{max},\text{Glc-6-Pase} \times (1 + (\text{Glc})/K_i,\text{Glc,Glc-6-Pase}) \quad (3)$$

The competitive nature of the inhibition by carbamyl-P of glucose-6-P phosphohydrolase activity is apparent from the convergence on the y axis of the various experimental lines in

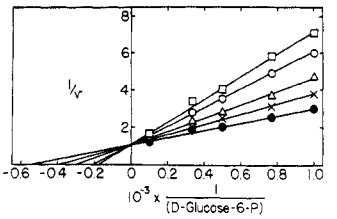

FIGURE 4: Kinetics of inhibition by carbamyl-P of glucose-6-P phosphohydrolase. Initial reaction velocity, v, was measured in the absence (●) and presence of 1.00×10^{-3} M (×), 2.00×10^{-3} M (△), 4.00×10^{-3} M (○), or 6.00×10^{-3} M (□) carbamyl-P. D-Glucose-6-P concentration was varied from 1.00×10^{-3} M to 1.00×10^{-2} M. Data are presented as conventional double-reciprocal plots (Lineweaver and Burk, 1934). Velocity, v, is expressed as $10 \times$ μmoles of glucose liberated/1.5 ml of reaction mixture per min. K_i, CP,Glc-6-Pase values, calculated for a competitive inhibitor by the method described by Dixon and Webb (1964), were, respective to the concentrations of carbamyl-P indicated above, 2.2 mM, 2.3 mM, 2.2 mM, and 2.6 mM.

Figure 4. In this study, glucose-6-P phosphohydrolase activity, measured on the basis of glucose liberation (Nordlie and Soodsma, 1966), was studied in the absence and presence of various concentrations of carbamyl-P. K_i,CP,Glc-6-Pase values were calculated from the relationship given in eq 4 (Dixon and Webb, 1964). Such values, which averaged 2.3 mM, are given in the legend to Figure 4. A summary of kinetic param-

$$x\text{-axis intercept} = -1/K_m,\text{Glc-6-P,Glc-6-Pase} \times (1 + (\text{CP})/K_i,\text{CP,Glc-6-Pase}) \quad (4)$$

eters is presented in Table II.

Mechanistic Considerations. To be applicable to this complex system, any proposed reaction mechanism must reconcile the following experimental observations as reported above: (a) K_m,CP,trf > K_m,CP,CPase = K_i,CP,Glc-6-Pase; (b) V_{max},trf > V_{max},CPase = V_{max},Glc-6-Pase; (c) K_m,CP,trf × K_m,Glc,trf > $K_{CP \cdot Glc,trf}$; and (d) K_m,Glc,trf = K_i,Glc,-CPase = K_i,Glc,Glc-6-Pase (see Table II). (e) Apparent K_m values for glucose are directly dependent upon concentration of the second substrate carbamyl-P (see Figure 1B). (f) Apparent K_m values for carbamyl-P are directly dependent upon concentration of glucose (see Figure 1A). (g) Carbamyl-P inhibits competitively glucose-6-P phosphohydrolase activity (see Figure 4). (h) The y coordinates of the common points of intersection of extrapolations of primary double-reciprocal plots for carbamyl-P:glucose phosphotransferase are negative and equal (see Figures 1A and B). (i) Inhibition by glucose of glucose-6-P phosphohydrolase and carbamyl-P phosphohydrolase activities is not of a conventional classical type (see Figures 3A and B). Rather, extrapolations of double-reciprocal plots of data obtained in the absence and presence of various concentrations of glucose converge in a common point, in each case, in the second quadrant. The y coordinate of this point is positive. In addition, the y coordinate of this point obtained in the experiment in which glucose inhibition of carbamyl-P phosphohydrolase was studied is equal, but opposite in sign, to the y coordinate of the common point of convergence of primary double-reciprocal plots obtained with the carbamyl-P:glucose phosphotransferase activity (see Figures 1A and B and 3B).

These observations differ in several rather significant ways from kinetic observations with various activities of the enzyme made under somewhat different conditions in this laboratory (Arion and Nordlie, 1964), and elsewhere (Hass and Byrne, 1960; Segal, 1959). Arion and Nordlie (1964) in earlier studies at pH 6 noted that K_m values for PP_i and glucose in the PP_i-glucose phosphotransferase were independent of second substrate concentrations; that V_{max},trf, V_{max},Glc-6-Pase, and V_{max},PP_iase were equal; that K_m,PP_i,trf = K_m,PP_i,PP_iase; and that K_m,PP_i,trf × K_m,Glc,trf = $K_{PP_i \cdot Glc,trf}$. These workers, as well as Segal (1959), noted inhibition by glucose of glucose-6-P phosphohydrolase to be of the classical noncompetitive type, while Hass and Byrne (1960) observed that experimental double-reciprocal plots of data obtained with glucose-6-P phosphohydrolase in the absence and presence of glucose were not indicative of any classical type of inhibition.

All of these experimental observations, present and past, even though significantly different in some respects, can be rationalized in terms of a common kinetic mechanism, as will be pointed out in detail below. This mechanism, which previously has been proposed to describe certain activities of this multifunctional enzyme, is described briefly in eq 5a–7b for convenience of reference.

$$\text{enzyme} + \text{glucose-6-P} \underset{k_2}{\overset{k_1}{\rightleftharpoons}} \text{glucose-6-P–enzyme} \quad (5a)$$

$$\text{enzyme} + \text{R-P} \underset{k_7}{\overset{k_6}{\rightleftharpoons}} \text{R-P-enzyme} \quad (5b)$$

$$\text{glucose-6-P–enzyme} \underset{k_4}{\overset{k_3}{\rightleftharpoons}} \text{P-enzyme} + \text{glucose} \quad (6a)$$

$$\text{R-P-enzyme} \underset{k_9}{\overset{k_8}{\rightleftharpoons}} \text{P-enzyme} + \text{R} \quad (6b)$$

$$\text{P-enzyme} + \text{H}_2\text{O} \underset{k_{10}}{\overset{k_5}{\rightleftharpoons}} \text{enzyme} + \text{P}_i \quad (7a)$$

$$\text{P-enzyme} + \text{glucose} \underset{k_3}{\overset{k_4}{\rightleftharpoons}} \text{glucose-6-P–enzyme} \quad (7b)$$

$$\text{glucose-6-P–enzyme} \underset{k_1}{\overset{k_2}{\rightleftharpoons}} \text{enzyme} + \text{glucose-6-P} \quad (5a)$$

In accordance with this mechanistic scheme (eq 5a–7b), R-P (carbamyl-P or other phosphoryl substrate) and glucose-6-P compete for the active site of the free enzyme producing kinetically significant binary enzyme–substrate complexes (eq 5a and 5b) and thus exhibit mutually competitive inhibitions as seen in Figure 4, for example, and as described in rate eq 11, below. The enzyme–phosphoryl substrate complex then dissociates to produce a phosphoryl–enzyme intermediate (eq 6a and 6b). Glucose and H_2O compete for the enzyme–phosphoryl intermediate (eq 7a and 7b) resulting in apparent inhibitions by glucose of the glucose-6-P phosphohydrolase and carbamyl-P phosphohydrolase reactions (see Figures 3A and B).

Steady-state consideration of this kinetic mechanism leads to the following rate equations (R-P = carbamyl-P in this case) for carbamyl-P:glucose phosphotransferase (eq 8), carbamyl-P phosphohydrolase in the presence or absence of glucose (eq 9), glucose-6-P phosphohydrolase in the presence or absence of glucose (eq 10), and glucose-6-P phosphohydrolase in the presence of carbamyl-P (eq 11). These equations are presented in linear, "double-reciprocal" fashion for convenience of reference in the discussion to follow. Definitions

TABLE II: Kinetic Parameters; Definitions in Terms of Individual Rate Constants and Experimental Values.

Kinetic Parameter	Definition in Terms of Individual Rate Constants and (E_0)[a]	Experimentally Determined Values
K_m,CP,trf	$\frac{k_2(k_7 + k_8)}{k_6(k_2 + k_8)}$	5.0 mM (Figure 2)
K_m,CP,CPase	$\frac{(k_7 + k_8)k_5(H_2O)}{[k_5(H_2O) + k_8]k_6}$	2.0 mM (Figure 3B)
K_i,CP,Glc-6-Pase	$\frac{(k_7 + k_8)k_5(H_2O)}{[k_5(H_2O) + k_8]k_6}$	2.3 mM (Figure 4)
K_m,Glc,trf	$\frac{(k_2 + k_3)[k_5(H_2O) + k_8]}{k_4(k_2 + k_8)}$	125 mM (Figure 2)
K_i,Glc,CPase	$\frac{(k_2 + k_3)[k_5(H_2O) + k_8]}{k_4(k_2 + k_8)}$	120 mM (Figure 3B)
K_i,Glc,Glc-6-Pase	$\frac{k_3 + k_5(H_2O)}{k_4}$	115 mM (Figure 3A)
K_m,Glc-6-P,Glc-6-Pase	$\frac{k_5(H_2O)(k_2 + k_3)}{k_1[k_5H_2O) + k_3]}$	1.8 mM (Figures 3A and 4)
K_i,Glc-6-P,CPase	$\frac{k_5(H_2O)(k_2 + k_3)}{k_1[k_5(H_2O) + k_3]}$	Not determined
$K_{CP\cdot Glc,trf}$	$\frac{k_5(H_2O)(k_2 + k_3)(k_7 + k_8)}{k_4k_6(k_2 + k_8)}$	250 mM (Figure 1A and 5)
K_m,CP,trf × K_m,Glc,trf	$\frac{k_2(k_7 + k_8)(k_2 + k_3)[k_5(H_2O) + k_8]}{k_6(k_2 + k_8)k_4(k_2 + k_8)}$	625 mM (Figure 2)
V_{max},trf	$\frac{k_2k_8(E_0)}{k_2 + k_8}$	0.154 (Figure 2)
V_{max},CPase	$\frac{k_8k_5(H_2O)(E_0)}{k_5(H_2O) + k_8}$	0.100 (Figure 3B)
V_{max},Glc-6-Pase	$\frac{k_3k_5(H_2O)(E_0)}{k_3 + k_5(H_2O)}$	0.100 (Figure 3A and 4)

[a] (E_0) = Total enzyme concentration.

of all kinetic parameters in terms of individual rate constants are presented in Table II, along with experimentally determined values for the various K_m, K_i, and V_{max}.

$$\frac{1}{v} = \frac{1}{V_{max},trf}\left[1 + \frac{K_m,CP,trf}{(CP)} + \frac{K_m,Glc,trf}{(Glc)} + \frac{K_{CP\cdot Glc,trf}}{(CP)(Glc)}\right] \quad (8)$$

$$\frac{1}{v} = \frac{1}{(CP)}\left[\frac{K_m,CP,CPase}{V_{max},CPase} + \frac{1}{V_{max},CPase} \times \frac{(Glc)}{K_i,Glc,CPase} \times K_m,CP,trf\right] + \frac{1}{V_{max},CPase}\left[1 + \frac{(Glc)}{K_i,Glc,CPase}\right] \quad (9)$$

$$\frac{1}{v} = \frac{1}{V_{max},Glc\text{-}6\text{-}Pase}\left[1 + \frac{(Glc)}{K_i,Glc,Glc\text{-}6\text{-}Pase}\right] + \left[\frac{k_2}{k_1} \times \frac{(Glc)}{K_i,Glc,Glc\text{-}6\text{-}Pase} \times \frac{1}{V_{max},Glc\text{-}6\text{-}Pase} + \frac{K_m,Glc\text{-}6\text{-}P,Glc\text{-}6\text{-}Pase}{V_{max},Glc\text{-}6\text{-}Pase}\right]\frac{1}{(Glc\text{-}6\text{-}P)} \quad (10)$$

$$\frac{1}{v,Glc\text{-}6\text{-}Pase} = \frac{1}{V_{max},Glc\text{-}6\text{-}Pase} + \frac{1}{(Glc\text{-}6\text{-}P)} \times \frac{K_m,Glc\text{-}6\text{-}P,Glc\text{-}6\text{-}Pase}{V_{max},Glc\text{-}6\text{-}Pase} \times \left[1 + \frac{(CP)}{K_i,CP,Glc\text{-}6\text{-}Pase}\right] \quad (11)$$

It is immediately apparent from the observed experimental relationship, V_{max},Glc-6-Pase = V_{max},CPase (see Table II), that $k_3 = k_8$, as previously observed in studies with glucose-6-P phosphohydrolase and inorganic pyrophosphatase (Arion and Nordlie, 1964).

Expressions for the x and y coordinates of the common points of convergence of extrapolations of experimental primary double-reciprocal plots, obtained with the carbamyl-P:glucose phosphotransferase system (see Figures 1A and B), may be determined by the method outlined in detail by Florini and Vestling (1957). For example, eq 8 may be written for two different concentrations of glucose and the resulting equations solved in turn for $1/v$ (*i.e.*, the common y coordinate) and for 1/(Glc) (*i.e.*, the common x coordinate). The y coordinate of this common point of intersection thus can be shown equal to: $[K_{CP\cdot Glc,trf} - (K_m,CP,trf \times K_m,Glc,trf)]/(K_{CP\cdot Glc,trf} \times V_{max},trf)$, while the x coordinate of this common point = $-K_m,Glc,trf/K_{CP\cdot Glc,trf}$. Similarly, the y and x coordinates, respectively, of the point of intersection of experimental plots in studies such as depicted in Figure 1B, are

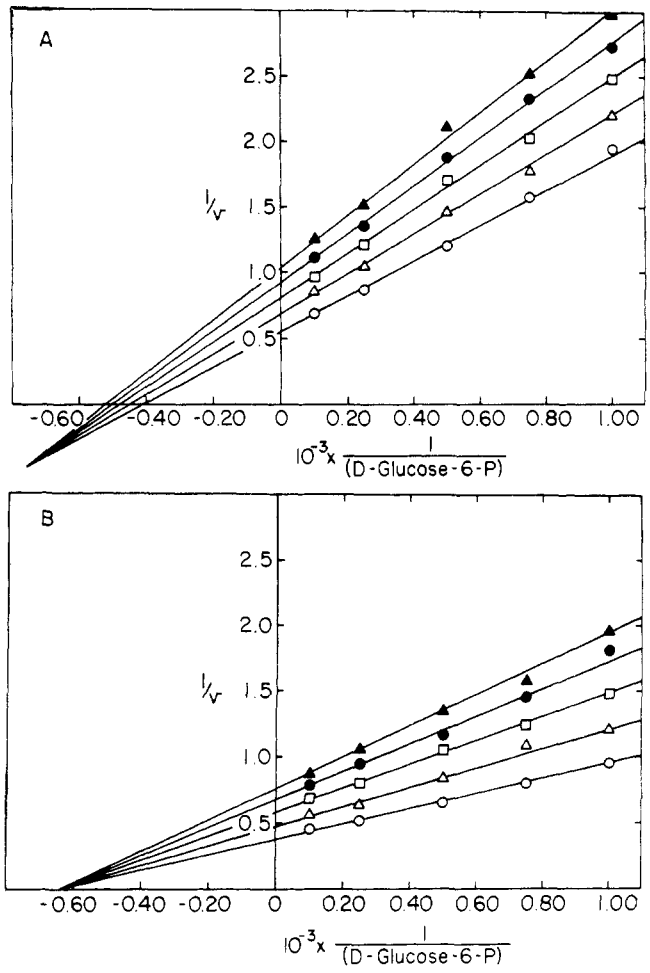

FIGURE 5: Effects of detergent on kinetics of inhibition by glucose of glucose-6-P phosphohydrolase activity. In both A and B, assay mixtures, pH 6.0, contained 1.5 ml of 40 mM sodium cacodylate buffer and 0.8 mg of rat liver microsomal protein. D-Glucose-6-P concentration was varied between 1.00×10^{-3} M and 1.00×10^{-2} M, as indicated. Activity, v, was measured in the absence (○), and presence of 3.00×10^{-2} M(△), 6.00×10^{-2} M(□), 9.00×10^{-2} M(●), or 12.0×10^{-2} M(▲) D-glucose. In A freshly prepared, unsupplemented microsomal suspensions were employed as enzyme source while in B, such suspensions which had been supplemented to 0.2%, w/v, with sodium deoxycholate were employed.

$[K_{CP\cdot Glc,trf} - (K_m,CP,trf \times K_m,Glc,trf)]/(K_{CP\cdot Glc,trf} \times V_{max},trf)$ and $-K_m,CP,trf/K_{CP\cdot Glc,trf}$.

Substituting the equivalent rate constant expressions (see Table II) for the various kinetic parameters, it may be shown that the y coordinate of this common point of intersection equals $[k_5(H_2O) - k_2]/[k_2k_5(H_2O)(E_0)]$. From this relationship it follows that, when $k_2 = k_5(H_2O)$, this y coordinate of the common point of intersection of experimental primary plots is zero [*i.e.*, the plots converge on the x axis, as previously observed by Arion and Nordlie (1964) with the PP_i–glucose phosphotransferase system at pH 6], and Michaelis constants for both phosphoryl donor and phosphoryl acceptor are independent of concentration of alternate substrate. Under such circumstances, $K_{RP\cdot Glc,trf}$ also $= K_m,RP,trf \times K_m,Glc,trf$.

In contrast, when $k_2 > k_5(H_2O)$, this common y coordinate of the point of intersection will be *negative*, as seen in the present studies (see Figures 1A and B). Under these circumstances, $K_{CP\cdot Glc,trf} < K_m,CP,trf \times K_m,Glc,trf$. This same inequality, *i.e.*, $k_2 > k_5(H_2O)$, also may be deduced by algebraic consideration of the following inequalities: $V_{max},trf > V_{max},CPase$, *i.e.*, $k_2k_8(E_0)/(k_2 + k_8) > k_5(H_2O)k_8(E_0)/[k_5(H_2O) + k_8]$; or $K_m,CP,trf > K_m,CP,CPase$; *i.e.*, $k_2(k_7 + k_8)/k_6(k_2 + k_8) > k_5(H_2O)(k_7 + k_8)/k_6[k_5(H_2O) + k_8]$ (see Table II).

An analogous consideration of rate eq 9 relating to carbamyl-P hydrolysis in the presence or absence of glucose reveals that the y and x coordinates of the common point of convergence of experimental double-reciprocal plots obtained without and with various concentrations of glucose are, respectively, equal to $(K_m,CP,trf - K_m,CP,CPase)/(K_m,CP,trf \times V_{max},CPase)$ and $-1/K_m,CP,trf$. The former of these two expressions, that for the common y coordinate, may be shown equal to $[k_2 - k_5(H_2O)]/[k_2k_5(H_2O)(E_0)]$ by substitution of the various applicable combinations of rate constants (see Table II) for the corresponding K_m or V_{max} which they define. This common y coordinate is thus equal in magnitude, but opposite in sign, to the common y coordinate for the point of intersection noted in carbamyl-P:glucose phosphotransferase primary double-reciprocal plots (see above). When $k_2 > k_5(H_2O)$, this common y coordinate for the phosphohydrolase activity will be positive. Experimentally, these relationships are obvious from an examination of Figures 1A and B and Figure 3B.

In a completely analogous fashion, the y and x coordinates of the common point of intersection of experimental double-reciprocal plots of data describing glucose-6-P phosphohydrolase in the absence and presence of various concentrations of glucose may be shown to be, respectively, $(1/V_{max},Glc\text{-}6\text{-}Pase)[1 - (k_1/k_2)K_m, Glc\text{-}6\text{-}P,Glc\text{-}6\text{-}Pase]$ and $-k_1/k_2$.

When $k_2 = k_5(H_2O)$, the expression for $K_m,Glc\text{-}6\text{-}P,Glc\text{-}6\text{-}Pase$, *i.e.*, $k_5(H_2O)(k_2 + k_3)/\{k_1[k_3 + k_5(H_2O)]\}$, becomes equal to k_2/k_1, and the common y coordinate consequently becomes zero [*i.e.*, experimental double-reciprocal plots converge on the x axis, in a manner analogous with classical noncompetitive inhibition, as experimentally observed by Arion and Nordlie (1964) and also earlier by Segal (1959)].

Further, it follows similarly that when $k_2 > k_5(H_2O)$, then $K_m,Glc\text{-}6\text{-}P,Glc\text{-}6\text{-}Pase < k_2/k_1$, and this y coordinate of the common point of intersection becomes equal to $1/V_{max},Glc\text{-}6\text{-}Pase$ (1 – a positive number less than 1); *i.e.*, this y coordinate is positive, as experimentally seen in the present study carried out at pH 7 (see Figure 3A). The equilibrium constant for the dissociation of the enzyme–glucose-6-P complex (*i.e.*, k_2/k_1), calculated as the negative reciprocal of the x coordinate of the common point of intersection in Figure 3A, is 4.2×10^{-3} M, a value more than double the $K_m,Glc\text{-}6\text{-}P,Glc\text{-}6\text{-}Pase$ (1.8×10^{-3} M) calculated from these same data.

A third situation, relating to Hass and Byrne's (1960) observations on the effects of glucose on glucose-6-P phosphohydrolase activity (freshly prepared, unsupplemented microsomal suspension, assayed at pH 6.0), also may be reconciled on the basis of the above mechanistic considerations. These workers noted an inhibition by the hexose which they concluded did not fit any classical type of inhibition; they also calculated from their data that k_2/k_1 (as defined here) $< K_m,Glc\text{-}6\text{-}P,Glc\text{-}6\text{-}Pase$. Extrapolation of their experimental double-reciprocal plots (Figure 3 in Hass and Byrne, 1960) by the present authors indicates a convergence at a common point in the third quadrant (*i.e.*, the y coordinate of this point of convergence is negative). Both of these observations are consistent with the mechanistic treatment presented above, when $k_2 < k_5(H_2O)$.

Effects of Detergent on Glucose-6-P Phosphohydrolase Inhibition Kinetics. The experiments described in Figures 5A and B were conducted in an effort to resolve experimentally the basically different patterns of inhibition by glucose of glucose-6-P phosphohydrolase activity noted by (a) Hass and Byrne

(1960), (b) Segal (1959), (c) Arion and Nordlie (1964), and (d) by us in the present paper (see Figure 3A). Of particular interest in this regard was the fact that enzyme preparations used by the latter three groups of workers were activated by detergent treatment (deoxycholate, or, in Segal's (1959) studies, digitonin), while microsomal preparations employed by Hass and Byrne (1960) were not. Accordingly, inhibition studies were carried out at pH 6 [as employed previously by Hass and Byrne (1960)] with freshly isolated microsomal suspensions (see Figure 5A) and with such preparations which had been supplemented with sodium deoxycholate to 0.2%, w/v, prior to assay of enzymic activity (Figure 5B). In both instances, glucose-6-P phosphohydrolase activity was measured in the absence and presence of various concentrations of added glucose, and data obtained were plotted in double-reciprocal fashion.

When freshly prepared, unsupplemented microsomal preparations were employed as enzyme source (Figure 5A), a family of double-reciprocal plots was obtained which intersected at a common point *below* the x axis (*i.e.*, in the third quadrant) as previously seen with similar unsupplemented preparations at this same pH by Hass and Byrne (1960).

In contrast, when detergent-treated preparations were assayed, a set of such double-reciprocal plots intersecting in a point *on* the x axis was obtained (see Figure 5B), as in the earlier studies of Segal (1959) and Arion and Nordlie (1964). In supplementary studies identical with those described in Figure 6B, except that pH was 7.0 instead of 6.0, intersection *above* the x axis was noted, as in Figure 3A.

Detergent treatment thus would appear to affect significantly the ratio $k_2:k_5(H_2O)$, increasing it from an original value of <1 (Figure 5A, this study; Hass and Byrne, 1960) to a value of 1 (Segal, 1959; Arion and Nordlie, 1964) or >1 (see above and Figure 3A, this study). This effect on this rate-constant ratio also is mechanistically consistent with the earlier observations of Lueck and Nordlie (1970) that carbamyl-P:glucose phosphotransferase activity of the enzyme (substrate concentrations held high) is considerably more responsive to detergent activation than is glucose-6-P phosphohydrolase activity of the catalyst. Such differential patterns of activation are consistent with a detergent-induced increase in the ratio of $k_2:k_5(H_2O)$.

Some Final Conclusions. The relative size of k_2 compared with $k_5(H_2O)$ thus appears to be a deciding factor dictating the type of kinetics exhibited by the enzyme under any set of experimental conditions. The relative size of these rate constants, in turn, may be affected by such variables as assay pH and the physical state of the enzyme as influenced by such modifying factors as detergents. It is further apparent that if water concentration in the vicinity of the hydrophobic endoplasmic reticulum of which this catalyst is a part (Ernster *et al.*, 1962) may vary, a further influence on the catalytic behavior of the enzyme also may be manifest.

The mechanistic concepts and relationships outlined in eq 5a–7b, rate eq 8–11, and Tables I and II thus appear to reconcile a rather large and diverse number of kinetic observations (see this paper; Herrman *et al.*, 1971; Arion and Nordlie, 1964; Hass and Byrne, 1960; and Segal, 1959); and, *in toto*, would appear to the authors to constitute the most complete, unified kinetic treatment yet presented to describe this unique, complex, multifunctional enzyme.

References

Arion, W. J., and Nordlie, R. C. (1964), *J. Biol. Chem. 239*, 2752.

Dixon, M., and Webb, E. C. (1964), Enzymes, New York, N. Y., Academic Press, p 327.

Ernster, L., Siekevitz, P., and Palade, G. E. (1962), *J. Cell Biol. 15*, 541.

Florini, J. R., and Vestling, C. S. (1957), *Biochim. Biophys. Acta 25*, 575.

Hass, L. F., and Bryne, W. L. (1960), *J. Amer. Chem. Soc. 82*, 947.

Herries, D. G. (1967), *Biochim. Biophys. Acta 136*, 95.

Herrman, J. L., Nordlie, P. E., and Nordlie, R. C. (1971), *FEBS (Fed. Eur. Biochem. Soc.) Lett. 18*, 241.

Lineweaver, H., and Burk, D. (1934), *J. Amer. Chem. Soc. 56*, 658.

Lueck, J. D., and Nordlie, R. C. (1970), *Biochem. Biophys. Res. Commun. 39*, 190.

Nordlie, R. C. (1971), *Enzymes 4*, 543.

Nordlie, R. C., and Arion, W. J. (1964), *J. Biol. Chem. 239*, 1680.

Nordlie, R. C., and Arion, W. J. (1966), *Methods Enzymol. 9*, 619.

Nordlie, R. C., Lueck, J. D., Hanson, T. L., and Johns, P. T. (1971), *J. Biol. Chem. 246*, 4807.

Nordlie, R. C., and Soodsma, J. F. (1966), *J. Biol. Chem. 241*, 1719.

Segal, H. L. (1959), *J. Amer. Chem. Soc. 81*, 4047.

Sturkie, P. D. (1965), Avian Physiology, Ithaca, N. Y., Cornell University Press, p 43.