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STUDIES ON THE KINETICS AND MECHANISM OF THE
D-GLUCOSE-6-PHOSPHATE KETOL-ISOMERASE
CATALYZED REACTION

DISSERTATION
Presented in Partial Fulfillment of the Requirements for
the Degree Doctor of Philosophy in the Graduate
School of The Ohio State University

By
Joan Spinanger Davis, B.S., M.S.

The Ohio State University
1965

Approved by
Adviser
Department of Agricultural Biochemistry
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Those who have been most closely associated with my graduate work have given so very much that to attempt expressing appreciation in only a sentence or two seems quite inappropriate. May it be said that for those so briefly mentioned here, my gratitude is truly more than I am capable of expressing.

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For the years of sharing in the difficulties of graduate work and research, my husband deserves so very much more than can ever be repaid. To him goes my deepest appreciation.
VITA

February 17, 1937............Born. Staten Island, New York

1959.........................B.S., University of Cincinnati
Cincinnati, Ohio

1960.........................M.S., University of Cincinnati
Cincinnati, Ohio

FIELDS OF STUDY

Major Field: Biochemistry

Studies in Enzymes and Enzyme Kinetics.
Professors John E. Gander
Joseph E. Varner

Studies in Chemical Kinetics.
Professor Frank H. Verhoek

Studies in Thermodynamics.
Professor H.H. Jaffe
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ABBREVIATIONS

G-6-P.............Glucose-6-Phosphate
F-6-P.............Fructose-6-Phosphate
6-PG..............6-Phosphogluconate
G-1-P.............Glucose-1-Phosphate
M-6-P.............Mannose-6-Phosphate
2d-G-6-P..........2-deoxy-Glucose-6-Phosphate
G-1,6-diP.........Glucose-1,6-Diphosphate
F-1,6-diP........Fructose-1,6-Diphosphate
NAD+, NADH.......Nicotinamide Adenine Dinucleotide
and its Reduced Form
NADP+, NADPH.....Nicotinamide Adenine Dinucleotide
Phosphate and its Reduced Form
The factors which contribute to the structure, function, and metabolic control of an enzyme are now known to be numerous. Previously it was held that an enzyme was a rigid protein acting simply as an organic catalyst. This restricted concept has been altered by additions to the knowledge of the characteristics of these biologically active proteins. The ability of many enzymes to respond to the microenvironmental conditions of the cell is now recognized. This response to the needs of the cell and the translation of these responses into effective means of control may more aptly allow the enzyme to be considered as a type of biological "information transducer."

Within the old restricted concept, an enzyme was accepted as characterized when substrate and product, maximum velocity, and Michaelis constant were determined. It has been realized for some time, however, that the characteristics determined in vitro cannot be assumed to be representative of those in vivo. In reality, the methods of isolation were frequently severe and the resulting protein probably very little resemblance to the native state. Even under the more refined techniques and controlled conditions currently used, the characteristics of an enzyme, as
determined *in vitro*, cannot be strictly extrapolated to their *in vivo* significance.

The concept of the control of the rate of enzyme reactions, as currently understood, takes into consideration not only the effect that the rate of reaction has upon subsequent reactions, but also the microenvironmental conditions of the cell which influence its activity. It is therefore of interest and value to consider how an enzymatic reaction may be influenced in these respects.

There are various levels or types of control for an enzymatic reaction. A simplified classification would place them into two groups; those which influence the amount of enzyme present in a functional form, and those which affect the observed velocity of the existing enzyme. The levels of many enzymes have been found to be controlled by an inducible or repressible system. This usually implies that the synthesis of the enzyme depends upon the needs of the cell. Other enzymes have been found to be constitutive, that is, to have essentially the same level regardless of the changes in metabolism of the organism. In some cases the enzyme may also be partially controlled in its activity by alterations in its aggregate size or by its conversion from an inactive form to active form. Examples of this latter would be the conversion of trypsinogen to trypsin or the interconversion of the muscle phosphorylases.
The second group of this arbitrary classification of control of enzymatic activity is concerned with factors influencing the activity of existing enzymes. Most frequently placed in this category are: concentrations of reactants, products, and cofactors; product inhibition and inhibition by structurally similar compounds; inhibition by antimetabolites, or non-physiological compounds which are capable of permeating the cell; and (most recently added) that of feedback inhibition. This last type of inhibition allows for a wide range of control since it is not dependent upon structural analogues of the substrate or the immediate product of the reaction. In this case, the product of a reaction many steps removed may be an effective inhibitor, without bearing structural similarity to the substrate. The significance of this is that a product may aid in the conservation of the available energy and substrates of the cell by preventing further synthesis of a metabolite which is present in sufficient quantity.

Considering these types of control with particular reference to the initiation of carbohydrate metabolism via the actions upon G6P, the following observations should be made. Glucose-6-phosphate may be considered as a primary source of energy for the cell, and thus the enzymes which act directly upon it are in metabolic positions of unusual importance.
On the basis of data available on the relative activities of the enzymes in the paths of carbohydrate catabolism, the existence of rate limiting reactions is not clearly defined. As a result it cannot be determined if the fluctuations in the levels of those enzymes acting on F6P would affect the rates of carbohydrate metabolism.

Of the five enzymes acting on F6P, only G6Pase metabolizes it in such a way that the product produced is not of direct value to the cell, but produces glucose, which is transported from the cell for utilization at another locus. This reaction is thus controlled more by the requirement of other cells for the glucose than by the needs of the cell which contains the phosphatase activity. The action of phosphoglucomutase, on the other hand, responds to the availability of glucose or to the energy requirements of the cell. The path initiated by this enzyme leads to glycogen and is under rather stringent control. Its activity responds to fasting and refeeding conditions. In a normal cell, the glucose may be stored as glycogen when there is an excess of this hexose, e.i., the cell is taking in glucose at a rate greater than that at which it is metabolized. The oxidation of G6P to 6PG by the action of G6P dehydrogenase also changes with the needs of the cell. This conversion initiates the hexose monophosphate shunt, the function of which seems to lie
both in the production of reductive power in the form of NADPH and the production of pentose phosphates for DNA and RNA biosynthesis. However there is considerable question as to the relative contribution of the hexose monophosphate pathway to the overall metabolism of glucose in any given tissue.

The G6P may also be converted to glucosamine-6-phosphate, the further metabolism of which is mainly in the direction of mucopolysaccharides.

The remaining reaction of G6P is its isomerization to F6P by phosphoglucose isomerase. This initiates the Embden-Meyerhoff-Parnas pathway. Since the reaction is readily reversible, it allows for the redistribution through G6P of those metabolites which form F6P by the reverse of the glycolytic pathway. Although it is among those enzymes of primary importance for either aerobic or anaerobic metabolism, there has not been found for this isomerase, the types of controls which have been shown to exist for the other enzymes acting on G6P. In contrast to these others, the level of phosphoglucose isomerase does not change significantly in disease states. Under these abnormal conditions the levels of phosphoglucone mutase and G6P dehydrogenase changed several fold.

The only type of inhibition so far recognized for this enzyme is that produced by 6PG, and this would not be of significance under anaerobic conditions, when the
contribution of the hexose monophosphate shunt is minor.

In the studies reported herein, the source of the isomerase is *Saccharomyces cerevisiae*. This organism is capable of both an aerobic and anaerobic metabolism. Many of the early carbohydrate studies were initiated in yeast. This most likely occurred because of the practical importance of controlling conditions for the maximum synthesis of ethanol in the fermentation process. This anaerobic process of carbohydrate metabolism is similar in many aspects to glycolysis in muscle. Perhaps the most significant difference between the aerobic and anaerobic metabolic pathways is the amount of energy liberated from the metabolism of one mole of glucose: aerobic oxidation produces 473 kcal mole\(^{-1}\), while anaerobic gives only 22 kcal mole\(^{-1}\). Since yeast is capable of metabolizing carbohydrate under both conditions, many investigations have been performed to ascertain the metabolic differences between the two. Although the contributions of the various paths of glucose metabolism vary according to the type of metabolism, the relationship between the phosphoglucose isomerase activity obtained from cells grown in aerobic conditions has not been compared to that obtained from cells grown under anaerobic conditions.

Anaerobically, the degradation of glucose in *S. cerevisiae* proceeds along the Embden-Meyerhoff-Parnas scheme. Pyruvic acid undergoes the reactions characteristic
of yeast, resulting in the production of ethanol and carbon
dioxide. This is the main type of fermentation which is
observed in this organism.

When the yeast cells are metabolizing aerobically,
they have been shown to have the complete complement
of enzymes of the hexose monophosphate shunt. The
implementation of this pathway has been examined in many
aspects. The fact that yeast may catabolize G6P during
aerobic or anaerobic metabolism makes this organism a
particularly valuable one for examining the alterations and
control mechanisms involved in the transition from one
type of metabolism to the other.

In this respect some of the recent work of Chance\(^1\)
and Hess\(^2\) on *Saccharomyces carlsbergensis* and that of Pye
and Eddy\(^3\) on *Saccharomyces cerevisiae* has been of parti­
cular interest. These studies have involved the fluoro­
metric and spectrophotometric assays of NADH immediately
after the organism was transferred from an aerobic to an
anaerobic environment. Assays performed under these
conditions showed that changes in the levels of NADH and
ADP resemble a damped train of sinusoidal oscillations.
The oscillations appear to be started when the mitochon­
drial control of the ADP and Pi levels ceases because of
inhibition of respiration. Phosphoglycerokinase was
implicated as being the most influential enzyme in con­
trolling the oscillatory nature of glycolysis.
As previously mentioned, many enzymes which are responsive to metabolic control have more than one compound required for complete reaction. The concentration of reactants, products, and cofactors all influence the velocity of any given reaction and therefore may exert a regulatory influence upon the reaction. As far as has been determined, phosphoglucone isomerase has no known cofactor requirement, nor are its kinetic parameters influenced by fluctuations in Mg\(^+\) concentration. Therefore, apart from the possibility of contributions of control by allosteric effects,\(^4\) temperature, or pH changes, the rate of this isomerase reaction appears to be dependent upon substrate concentration, inhibitor (6PG) concentration, and the characteristic kinetic parameters of the enzyme. The kinetics would thus appear to be of unusual interest in view of the lack of other factors which are known to contribute to metabolic control.

A kinetic evaluation of the isomerase is to be examined herein. It should be mentioned that an enzymatically catalyzed reaction has no metabolic significance in itself, but only as it contributes to the overall function of the cell. For this reason, some aspects of metabolic control were included in the preceding discussion.

Kinetic investigations at best eliminate various postulated mechanisms, and more importantly are valid only in the system under investigation. It can be
dangerous to extrapolate to the complete metabolic pathway. However, it should be remembered that there are also examples where the mechanism *in vivo* appears to be similar to that observed in a highly purified and defined *in vitro* system. Therefore, the reader should realize that sections concerning metabolic control are given as guidelines for additional experimentation which might help correlate *in vivo* and *in vitro* data.
The existence of an enzymatically catalyzed isomerization between G6P and F6P was first reported by Lohmann in 1933. This enzyme, phosphoglucone isomerase (D-glucose-6-phosphate ketol-isomerase: E.C. 5.3.1.9) has been found to be essentially ubiquitous. The enzyme appears to show absolute specificity for its substrate and no cofactor or coenzyme requirement has been found for yeast phosphoglucone isomerase. If the phosphate group of G6P is replaced by sulfate or a methyl ether group, there is no isomerization to the corresponding 2-keto analogue (Tanko, 1952). These analogues do not inhibit the reaction. The substrate specificity of the isomerase was examined by Sols and Crane and reported in 1954. The enzyme was found not to act on free hexoses, M6P, G1P, G-1,6-diP, or F-1,6-diP.

The compounds which exert an action of competitive inhibition on phosphoglucone isomerase obtained from various sources are 6PG, sorbitol-6-P, glucosamine-6-P, 2-dG6P, and M6P. For the yeast isomerase only the 6PG inhibits at a concentration which might be in the physiological range. Kull (1962) observed that 6PG was a more effective inhibitor when competing with the substrate (F6P) which had the lower apparent Km. These results are not
compatible with the simple Michaelis-Menten kinetics. This difficulty in fitting these data into a model of a mono-molecular reaction obeying Michaelis-Menten kinetics led to the present investigations which further examine the kinetic properties of yeast phosphoglucone isomerase.

The pH optima of the isomerase from Phaseolus radiata were determined in both directions by Ramasarma and Giri (1956). Their report of pH 7.5 as the optimum in the direction of F6P production and pH 9.0 in the reverse direction is the only record found of different pH optima for the two directions of the reaction.

Since the enzyme was found to be so prevalent, and no differences had been found with respect to cofactor requirements for the reaction, the species specificity was finally determined by immunological techniques (Lippett, 1957). Antisera produced by rabbits immunized with purified dog liver isomerase showed no cross reaction with rat liver isomerase. However, the isomerase isolated from both the dog muscle and erythrocytes did precipitate the antisera to the dog liver isomerase.

Noltman and Bruns (1959) purified the yeast isomerase and carried out the initial studies on the kinetic parameters of this enzyme. The following year Klotzsch and Bergmeyer reported the crystallization of the enzyme.

The kinetic parameters of the mammary gland isomerase were investigated by Kahanna and a comparison
of the characteristics of mammary gland and yeast enzymes was made by Balch et al.\textsuperscript{17} The \textit{Km}'s for the animal source were found to be approximately one order of magnitude less than those of yeast.

Hines and Wolf\textsuperscript{18} examined the effect of \textit{pH} on the kinetic parameters of the mammary gland isomerase. From the shape of the curves produced by the data in this \textit{pH} study it is difficult to ascertain if there is only one optimum \textit{pH} as suggested by these investigators.

Differences in the kinetic parameters of yeast, red blood cell, and skeletal muscle isomerase have been reported by Rose and O'Connell.\textsuperscript{19} From these studies it was confirmed that animal phosphoglucose isomerase has \textit{Km} values approximately one order of magnitude less than those of yeast. In all cases so far reported, the \textit{Km} for G6P is greater than that for F6P. There are also other data suggesting the mechanism of the reaction depends upon the organism from which the isomerase is obtained.

For some time it was believed that the isomerization occurred without the intramolecular transfer of the hydrogen from C-1 of F6P to C-2 of G6P, as indicated in the scheme on the following page:
Apparently this conclusion was reached because of hydrogen exchange into the medium and because with prolonged reaction the effect of the intramolecular transfer was diluted out. The work of Rose and O'Connell showed the extent to which each type of reaction occurs in the three sources of isomerase, i.e. yeast, red blood cell, and skeletal muscle.

Equal in importance to the observation of intramolecular transfer is the difference in the relative contribution of this transfer catalyzed by red blood cell and skeletal muscle enzymes. Their method of reporting the data was to determine the ratio of exchange into the medium to the amount of intramolecular transfer of the tritium from C-1 of F6P to C-2 of 6PG (product of enzymatic oxidation of G6P). They expressed their results as percent of net reaction, which may also be considered as ratio of F6P/6PG. Their results showed that the two mammalian sources of isomerase behaved similarly and that the ratio of exchange/transfer was independent of the
ratio F6P/6PG. In the case of yeast isomerase, however, the ratio of exchange to transfer increased as the ratio F6P/6PG decreased. They interpreted this as an isotope effect. Another possibility is that this represents a change in the mechanism as the reaction progresses toward completion, or a change in relative contributions by each of more than one mechanism during the course of the reaction.

In addition to the difference in Km values and reaction mechanism, yeast and animal isomerases have been shown to differ in other characteristics. The molecular weight of the enzyme from animal is 48,000; that from yeast is 145,000. The turnover numbers differ by a similar order of magnitude; animal 15,300; yeast 36,000.

No study on this isomerase has demonstrated any cofactor or metal ion requirement. Metals have been shown to differ in their effects on the enzymes from various sources. The enzyme from *Phaseolus radiatus* was found to be unaffected by the following ions which were present at a concentration of 10^{-2}M: Mg^{2+}, Mn^{2+}, Co^{2+}, Ba^{2+}, AsO_{4}^{3-}, F^{-}. It was slightly inhibited by 2 \times 10^{-3}M Hg^{2+}, 10^{-3}M Zn^{2+}, and 2 \times 10^{-3}M CN^{-}. The enzyme from intestinal mucosa, on the other hand, is strongly inhibited by heavy metals.

The role of sulfhydryl groups in phosphoglucom isomerase has not been thoroughly examined. One can
note that the enzyme from *Phaseolus radiatus* is not affected by heavy metals, nor is it inhibited by $2 \times 10^{-3}$M iodoacetic acid. The enzyme from intestinal mucosa which is inhibited by metals has not been specifically examined with respect to the role of sulfhydryl groups.
METHODS

Chemical method

The chemical assay used for determination of fructose was derived from the procedure of Roe. This assay is based on the chromogenic substance produced in concentrated HCl by the reaction of resorcinol and the derivative of fructose, hydroxymethylfurfural. The mechanism of this reaction is not understood. A possible scheme is proposed as involving a condensation product formed as follows:

\[
\begin{align*}
\text{fructose} & \rightarrow \text{hydroxymethylfurfural} \\
& + \text{resorcinol} \\
& \rightarrow \text{derivative of hydroxymethylfurfural}
\end{align*}
\]

The reaction is not specific. A chromagen will be produced by any substance which is capable of being converted to hydroxymethylfurfural. Since glucose can also yield hydroxymethylfurfural, the conditions of the assay are critical for the substrates of the isomerase reaction. Thus the method was examined in an attempt to determine the best combination of maximum color development with minimum contribution by glucose to chromagen formation.

All of the parameters of the assay system were examined for their effect on chromagen formation from
glucose or fructose. Since the chromagen was known to have two broad absorbance maxima, absorbance was measured either with a spectrophotometer at 400μm and 490μm or with a Klett-Summerson colorimeter using filters 42 and 54.*

It had previously been shown by using solutions of equal concentration of glucose and fructose that chromagen formation by glucose was approximately 1-2 per cent that of the fructose. In order to produce absorbancies of similar magnitude, fructose solutions of 0.2μm/ml and glucose solutions of 10μm/ml were used.

The original method called for the use of 10.2N HCl, but as shown in Figure 1, concentrated acid increased the sensitivity about two fold. (3.0ml of 10.2N = 2.5ml 12.2N) Since one ml aliquots were used from the enzymatic assay, conditions for the chemical assay were sought which would yield maximum chromagen using this volume. Considering this, 3.0ml of acid was found to be the optimum amount. This quantity allows for maximum color development. Additional acid increases total color, but decreases the value one observes, i.e. absorbance/unit volume. The steepness of the acid vs. absorbance plot indicates one of the disadvantages of this method. Any error in amount

*These filters pass light over the range of 400μm to 450μm and 520μm to 580μm respectively and will be referred to hereafter as peaks at 420μm and 540μm respectively.
Figure 1

Effect of HCl on Chromagen Formation
o: 42 filter [glucose] 10 μM
u: 54 filter [glucose] 10 μM

x: 42 filter [fructose] 2 μM
δ: 54 filter [fructose] 2 μM

ml 2.2N HCl / 5ml assay mixture
of acid added to the reaction mixture produces a deviation in absorbance which is disproportionately large when compared to the resultant error in volume. In most assays one attempts to use an amount of reagent from a range where slight deviation in volume produces no more effect than that due to volume error. However, this ideal situation was not adhered to in order to achieve the increased sensitivity.

Since aqueous solutions decreased markedly the amount of color produced, absolute ethanol was used as the solvent for the resorcinol instead of 0.2M HAc. This further increased the sensitivity of the method by 70 per cent.

Absorption spectra shown in Figure 2 were obtained on reaction mixtures incorporating these two changes of acid and solvent. These showed that the peak at 400μm was greater than the previously used maximum at 490μm. The difference spectrum shows that this is also the range of maximum change. It was noted from the absorption spectrum (curve A) that the 40 filter might be expected to be more effective since it included the range 380-420μm. However, it gave only 70 per cent the absorbance of the 42 and the absorbance did not increase in proportion to the concentration of the ketose.

In view of these changes, the optimal time and temperature conditions were also re-examined. The time
Figure 2
Absorption and Difference Spectra of Chromagen
Absorption Spectra

Difference Spectrum

O.D.

A O.D.

wavelength
course of color development, as seen in Figure 3, shows that the relative absorbance at 400\(\mu\) or 490\(\mu\) is time dependent. Samples were heated at 75\(^\circ\) for 5, 10, 15, or 20 minutes. The figure shows that the broad 490\(\mu\) peak had a greater absorbance than the 400\(\mu\) peak during most of the 20 minute heating period.

The absorbance at 400\(\mu\) and 490\(\mu\) were plotted as a function of time. Figure 4 shows that there was almost a linear increase in absorbance at either 400\(\mu\) or 490\(\mu\) when glucose was being converted to chromagen. However during the first five minutes that the chromagen was derived from fructose, the rate of formation exceeded that from glucose. There appeared to be little increase in absorbance at 490\(\mu\) after 15 minutes, although the absorbance at 400\(\mu\) continued to increase. Prolonged heating shows a decrease in absorbance at 490\(\mu\) with a concomitant increase in absorbance at 400\(\mu\) (Figure 5).

The shapes of the curves in Figure 4 are significantly different. In view of the 50 fold factor in concentrations used, the difference in curves might be suspected to be due to the chromagen formation from fructose going from a zero order to a first order reaction, while the chromagen derived from glucose remained zero order over the entire time examined. However, the \(t_\frac{1}{2}\) for color development was shown to be independent of the carbohydrate concentration. Therefore, factors other than
Figure 3

Effect of Time on Absorption Spectrum of Chromagen
Figure 4

Effect of Time on Absorbance of Chromagen at 420μ and 540μ
Figure 5

Effect of Prolonged Heating on Chromagen Formation
concentration must be determining the rate of chromagen formation.

In order for glucose to produce the same chromagen as fructose it must first be converted to the ketose form. The isomerization is presumably the rate limiting step in the formation of chromagen by glucose. It is conceivable that the effect of each of the reaction parameters on this step may be qualitatively or quantitatively different from its effect on the actual chromagen formation. Conditions which selectively minimize the isomerization step, and have little influence upon formation of chromagen from fructose, should minimize chromagen formation from glucose. Temperature is likely to be the major factor and was thus first considered.

The rate of formation of chromagen from glucose and fructose was examined at 73° and 80°C. The results are represented in Figures 6 and 7. At 73° the chromagens derived from glucose contributed equally to each wavelength. Similar results were obtained with fructose; however fructose continued to yield about 50 fold more chromagen. At this temperature the rate of the conversion to the hydroxymethylfurfural may be reduced and thus play a more significant role in the observed rate of chromagen formation.

Although one might speculate about the influence of temperature upon the rate of formation of chromagen(s)
Figure 6

Time Course of Chromagen Formation at 73°C
Incubation Temperature 73°C

Source of Chromagen

- 620 fructose
- 540 "
- 430 glucose
- 540 "

KLETT UNITS

TIME (minutes)
Figure 7

Time Course of Chromagen Formation at 80°C
Incubation Temperature 80°C
absorbing at 420\textmu m and 540\textmu m, a lack of knowledge of the chemical reactions occurring and the structures of the end products makes speculation unjustified.

The attempt to determine optimum conditions, that is, maximum color with minimum contribution from glucose, by varying temperature was performed using the same 1:50 ratio of fructose:glucose concentrations. The reaction was heated for 10 minutes at the temperatures indicated in Figure 8. The ratio of color produced by fructose to that from glucose was plotted with the resulting biphasic curve. The temperature chosen for the routine assay was at the break in the curve. This gives the highest optical density which could reasonably be obtained, and still holds the contribution by glucose to less than 1 per cent when considered on an equal concentration basis.

An interesting observation was made with respect to maximum chromagen development for a given quantity of fructose. In a period of time ranging up to 20 minutes a maximum at 540\textmu m was reached which was not altered even by increasing the temperature above 75\degree C. When read at 420\textmu m, there was an almost linear increase in absorbance with increase in temperature up to 85\degree C, as shown in Figure 9. Chromagen formation from glucose increased with temperature over the entire range examined as shown in Figure 10.
Figure 8
Effect of Temperature on Relative Chromagen Formation from Glucose and Fructose
Figure 9

Effect of Temperature on Chromagen Formed from Fructose
Figure 10

Effect of Temperature on Chromagen Formed from Glucose
When reaction mixtures were heated at 33° and then transferred to 65° for a given period of time before being cooled in a 0° water bath, there was a decrease in the optical density when compared to those samples which were cooled immediately, as shown in Figure 11.

This type of decrease in absorbance at 540mu is different from that observed when the sample is heated for a prolonged period of time. In the latter case there is an increase in the absorbance at 420mu while in the former there is a comparable decrease in absorbance at 420mu. Therefore the final absorbance appears to be dependent only upon the temperature of the terminal heating period, and independent of the previous temperatures.

It is mentioned in Roe's article that the concentration of the resorcinol is unimportant as long as it is in sufficient excess. However, under the modified conditions described here, higher concentrations of resorcinol are shown to depress color development, particularly at 520 mu. (Figure 12)

In determining the optimal range of resorcinol concentration, a plot of log_{10} (resorcinol)/(fructose) showed a definite maximum (Figure 13). The ratio of resorcinol to fructose which allows for a relatively constant formation of chromagen is compatible with the concentrations of substrate used in the enzymatically catalyzed reaction. This optimal concentration of
Figure 11

Reversible Effect of Temperature on Absorbance of Chromagen
Figure 12

Effect of Resorcinol Concentration on Absorption Spectra of Chromagen
Figure 13

Effect of Relative Fructose and Resorcinol Concentration on Chromagen Formation
Resorcinol was found to be 0.05 per cent instead of 0.1 per cent.

Additional experiments showed that the optimal ratio of resorcinol/fructose changed over a ten fold range of fructose concentrations. The total effect was to give linearity over a range from 0.02umole to 0.25umole of F6P with a constant concentration of 4.5umoles of resorcinol.

The remaining factor to be considered in the modification of the method was time. Figure 14 shows that formation of chromagen derived from fructose is linear only for the first 4-6 minutes, depending upon the wavelength being recorded.

The conditions thus derived for the routine assay were as follows:

- Assay mixture: 3.0ml concentrated HCl
  1.0 0.05% resorcinol in absolute alcohol
  1.0ml from enzyme reaction mixture
- Color developed at 77° for 8 minutes and cooled immediately in ice-water bath

A standard curve run under these conditions was linear over the entire range used, as seen in Figure 15.

The results of the examination of the parameters of the Roe method shows that under the altered procedure the absorbance at 420mp exceeds that at 540mp. The relative absorbance at these two wavelengths is both time and temperature dependent. The absorbance is greater at
Figure 14

Time Course of Chromagen Formation by Glucose and Fructose Under Standard Conditions
Figure 15

Standard Curve of Fructose
the higher temperature, but there appears to be a reversible equilibrium established since the absorbance is decreased by slow cooling. Thus assay mixtures were immediately placed in an ice-water bath which had the effect of "trapping" the chromagen absorbing at 420mu.

Enzymatic assay

The extent of reaction in the phosphoglucose isomerase catalyzed reaction was determined by assaying for the amount of fructose. The initial reaction volume for all experiments unless otherwise stated was 9.0ml. This volume contained 600muoles of buffer, which was Tris-HCl unless specifically designated otherwise. The substrates and inhibitors were present in whatever concentrations were required for the particular experiment. A 0.9ml aliquot was withdrawn for the zero time assay, followed by the addition to the reaction mixture of 0.9ml of enzyme solution to initiate the reaction. One ml aliquots were taken every 10 seconds and pipetted into concentrated HCl to stop the reaction. Color was developed according to the modified Roe procedure, described in the previous section.

In order to estimate the initial velocities, a graph was made of product vs time, and a smooth line drawn. From this a secondary curve was made by plotting
$\Delta P/\Delta t$ vs time. Extrapolation to zero time provided the estimation of initial velocity.

For certain experiments the amount of product present when the velocity was one half the initial velocity was determined. Both primary and secondary plots were used for this. The time at which $v = \frac{1}{2} v_0$ was determined from the secondary plots, while the amount of product formed at that time was read from the primary plots.

Except for the specific studies involving the effect of altered temperature upon the enzyme stability or reaction rate, all experiments were performed at 25°C.

Reagents

Phosphoglucone isomerase was derived from the following sources:

1) Commercial yeast preparation from Calbiochem, prepared by Boehringer u. Sohne, Mannheim, Germany. This was obtained as a suspension in 2.4M ammonium sulfate. The average specific activity of these preparations was 400 E.U./mg protein. An enzyme unit is here defined as that amount of enzyme in 1.0ml of 25°C solution which irreversibly forms in 100 seconds 0.03 μmoles of NADPH in the presence of saturating quantities of F6P and an excess of glucose-6-phosphate dehydrogenase and NADP. For enzymatic studies an aliquot of the enzyme (in 2.4M ammonium sulfate) was diluted 20 fold with 0.02M Tris
pH 8.0 and dialyzed against 0.02M Tris pH 8.0, at 3°C for 24 hours. Additional dilutions were made from this according to the needs of the assay. Generally the enzyme as added to the final reaction mixture was approximately a 600 fold dilution of the original preparation.

2) Commercial muscle isomerase from Sigma Chemical Company, St. Louis, Mo. This was obtained as a crude powdered extract. Ten mg were dissolved in 5.0ml 0.02M Tris pH 8.0 and dialyzed against 1000 ml 0.02M Tris pH 8.0 for 24 hours at 3°C. This preparation was then diluted approximately ten-fold for use in enzymatic assays.

3) Isomerase prepared from the mold Penicillium charlesii G. Smith. Spores of this strain were originally received by our laboratory from the American Type Culture Collection (A.T.C.C. strain 1887) and were maintained by the weekly plating of spores on petri dishes of Czapek-Dox medium containing 2 per cent Bacto-Agar (Difco) and 5 per cent glucose. These were kept at 18°C in constant light. For the growth of the mycelia, two innoculating loops of spores were transferred to a 500 ml wide mouth erlenmeyer flask containing 200ml of autoclaved Raulin-Thom media. Flasks were plugged with sterile cotton. There was sufficient growth at the end of 5 days to use the mold as a source of enzyme. The pads were squeezed dry through cheese cloth, washed
two times with 0.1 M Tris pH 7.5, and ground in a mortar
with three weights of sand and three volumes of the Tris
to a consistency of a fine paste. The grindings were
then filtered through cheese cloth and the filtrate
centrifuged at 30,000xg for ten minutes. The supernatant
fluid was used directly for further study.

Glucose-6-phosphate, F6P, and 6PG were obtained
from Calbiochem, synthesized by Boehringer u. Sohne as
the barium salt. Each was prepared for use by dissolving
in 0.005N HCl. The barium was removed by passing the
solution through a Dowex-50 column. The pH of the solutions
of G6P and 6PG was adjusted to 6.5 with dilute KOH.
The concentration of G6P was determined spectrophotometri-
cally on a Beckman DB recording spectrophotometer. The
reaction mixture consisted of G6P, excess G6P-dehydrogenase,
0.6μm NADP, and 200μmoles Tris,pH 8.0, in a final volume
of 3.0ml. Phosphate was assayed according to the method
of Fiske and Subbarow. The concentrations of stock
solutions were adjusted to approximately 8-10μmoles G6P/ml.
6-phospho-gluconate was determined by the phosphate assay.

It was necessary to further purify the F6P.
After removal of the varlum according to the same method
described for G6P, the solution was concentrated on a
flash evaporator. The material was then streaked on
Whatman 3MM filter paper and the Bandurski-Axelrod acid
solvent or semi-stench solvent was allowed to ascend the paper. The phosphorylated compounds were detected by spraying with the phospho-molybdic acid spray reagent, and were developed under uv light. The location of the phosphorylated compounds was marked. The chromatograms, neutralized by holding over concentrated NH$_4$OH, were sprayed with a solution of concentrated HCl and 0.05 per cent resorcinol in absolute alcohol, and finally heated for five minutes in a moist chamber at 77°C. An orange color was obtained in areas of the chromatogram containing F6P or free fructose. An area was obtained that reacted with both resorcinol and the molybdic acid reagents. This was considered to be F6P. This area of the chromatogram was eluted with water and the concentration of F6P was estimated. The modified Roe method was used for determination of total fructose. Phosphate was determined by the Fiske-SubbaRow procedure. Fructose-6-phosphate was estimated by essentially the same procedure described for determination of G6P. The reaction medium contained 0.4 enzyme units of phosphoglucone isomerase in addition to the previously stated quantities of G6P-dehydrogenase, NADP, and Tris. The total carbohydrate content was estimated by the phenol-sulphuric acid method of DuBois et al. 26
Electrophoresis

The commercial yeast preparation of phosphoglucoisomerase was subjected to electrophoresis to determine if more than one major constituent was present. The migration was performed on polyacetate strips in barbital buffer, using a Shandon electrophoresis apparatus and VoKam power supply. The buffer was prepared according to Owens. Samples containing approximately 20μg of protein were run for 0.5, 1.0, or 2.0 hours and then stained with 0.025 per cent nigrosin in 3.0 per cent acetic acid. The results on strips removed at 0.5 hours suggested a separation of the protein into two bands. However, those developed longer produced only one diffuse zone. Assuming that the protein was separated into two bands, an attempt was made to ascertain whether both were enzymatically active. Therefore the strips were incubated in the dark for several hours in a mixture of F6P, G6P-dehydrogenase, NADP, Tris pH 8.0, phenazine methosulfate, and neotetrazoleum blue. However, minute quantities of phosphoglucoisomerase in crystalline G6P-dehydrogenase resulted in the conversion of F6P to 6PG and the reduced neotetrazoleum blue formed over the entire strip of cellulose acetate. This hindered the detection of any localized concentration of the isomerase activity.

Electrophoresis was also performed with polyacryl-
amide gel as the supporting medium. The apparatus for this, as well as the gels and buffers, were prepared according to the directions of Ornstein and Davis. For gel electrophoresis the VoKam power supply was again used. Approximately 12 μg of protein were applied to each of a maximum of 12 tubes. Of these, 2 were inverted to allow for the detection of possible components which might migrate toward the cathode. The migration was initiated at 1.5mA/tube at an initial emf of 150 volts. The voltage approached 190 volts at the end of the run. The protein was stained with 0.1 per cent amido schwartz in 7 per cent acetic acid. Destaining was accomplished electrophoretically at 5.0mA/tube.

Preparation of chromatographic columns

DEAE-Sephadex

DEAE-Sephadex A-50 Medium Grade was purchased from Pharmacia Fine Chemicals, Uppsala, Sweden. The characteristics of the lot used were: particle size 100-270 mesh, bed volume per gram, 10-15ml (in 0.2M phosphate at pH 7.0), and capacity of 5.5±0.5 milli-equivalents per gram. The gel was allowed to swell in an excess of water, and the fines removed by decantation.
The resin was converted to the salt form by:

1. washing with 5 volumes of 0.5N HCl
2. applying double distilled water until neutral to pH paper. (This required approximately ten volumes of water.)
3. treating with 5 volumes of 0.5N NaOH
4. washing with double distilled water until neutral reaction to pH paper (required approximately ten volumes of water)
5. allowing to equilibrate for 48 hours with the buffer to be used for chromatography

In order to maintain good flow rates it was found to be extremely important that equilibrium was truly attained.

For the initial elution of the protein, the gel was prepared at a pH of 6.6 in a 0.02M sodium phosphate buffer. A linear gradient of sodium chloride was applied which produced a final concentration of 0.4M.

The column, 1.1x30cm, was packed by adding the gel as a slurry from a reservoir where constant mixing was maintained. The flow rate was controlled at 3ml/hour by a screw clamp at the delivery tip. The packed column was washed with the buffer at this flow rate for 2 days prior to use. A sample of 200μg of protein was applied and 2 ml fractions were collected using a Gilson Medical Electronic Fractionator, volumetric model.

The conditions employed for the second elution were: pH 8.3; 0.02M Tris-glycine buffer; asymptotic gradient producing final concentration of 0.38M NaCl. Two hundred μg of protein were applied on a column
1x50 cm, and one ml fractions were collected. The location of the enzyme was determined by assaying 0.05ml of each fraction in a final volume of 1.0ml containing 60umoles Tris pH 8.0 and 0.23μmole G6P. This mixture was incubated 15 minutes at 25°0; the reaction was stopped by addition of concentrated HCl, and fructose concentration was estimated. When the peak of activity had been thus located, the activity of each fraction within the peak was measured by employing an incubation time of 10 seconds. Protein was determined by the Folin-Ciocalteu method.

Sephadex G-200

Sephadex G-200 for gel filtration was obtained from Pharmacia Fine Chemicals. The characteristics were: water retain 20±2g/g; particle size 40-120μ; bed volume/gram of dry gel, 30-40ml. The gel was allowed to swell for several days in the buffer used for chromatography (0.02M sodium phosphate pH 6.6, 0.2M NaCl). The column, 0.4x200cm, was packed under continuous flow and constant mixing. The packed column was washed 24 hours with the buffer. A sample of 50μg of protein was applied and 1.0 ml fractions collected.

The determination of enzyme activity and protein content were performed in the same way as for the DEAE-Sephadex fractions.
For the determination of the void volume, a crude preparation of globulins was used since a pure sample of gamma-globulin fraction II was not available. In order to prevent contamination by small proteins, the void volume was determined after the column had been used.
RESULTS

Previously in our laboratory (Kull, 1962) phosphoglucose isomerase had been shown to contain kinetic properties that did not fit the classical Michaelis-Menten concept of a catalyst. The study of this enzyme was continued by experiments of the following purposes: a) to determine by column chromatography, gel filtration, and electrophoresis if the enzyme as commercially isolated was a homogeneous preparation; b) to determine more fully the kinetic characterization of the enzyme; c) to study the effects of inhibitors, pH, and temperature upon the kinetics of the isomerase.

Electrophoresis

Electrophoretic migration of the isomerase on polyacetate strips gave only doubtful separation of the protein fraction during one half hour. Longer exposure to the voltage caused the bands to become diffuse and probably caused the denaturation of the protein since further migration was only very slight. Because of these difficulties, no additional separations were attempted using this supporting medium.

Electrophoresis on polyacrylamide gel showed two distinct bands which as judged visually appeared to
contain identical amounts of protein, as shown in Figure 16. There were also four other bands which were extremely faint and would appear not to contribute totally more than 2-3 per cent of the protein applied.

The two bands of protein were sufficiently close that if they were not fixed immediately the amount of diffusion prevented any detection of separation. Therefore, it was not possible to determine the location of the bands by staining half the gel and using the other half for further assay. The precluded the enzymatic assay of the separate fractions.

DEAE-Sephadex A-50

The initial elution on this media was performed at pH 6.6. Figure 17 shows that the peak produced by plotting the data from enzymatic assays is slightly skewed, while that for protein content is essentially symmetrical. When the fractions were assayed in the presence of 6PG the resulting peak of activity was also symmetrical. Thus the peak of activity is different for fractions assayed in the absence and presence of gluconate, but the difference is small, and is not taken to be significant.

Another DEAE-Sephadex A-50 column was prepared at pH 8.4 in Tris-glycine buffer, the same buffer system
Figure 16
Separation of Protein on Polyacrylamide Gel
Figure 17

Elution of Phosphoglucone Isomerase from DEAE-Sephadex A-50 at pH 6.6
used in the polyacrylamide gel system. The results of the column fractionation are seen in Figure 18. Enzymatic activity toward each substrate was determined for each fraction. A skewed curve was obtained when enzyme activity was plotted against fraction number. The significance of these data are not known other than it appears that at this pH the enzyme is not homogeneous. If this represents a separation of enzymatically active forms with different kinetic parameters, then several experiments can be done to examine this aspect.

Table 1 shows that the ratio of velocities measured at pH 8.3 and 6.6 was the same for both fractions. Dissimilar ratios would have been obtained if the two fractions contained unequal quantities of two enzymes with different pH optima. It should be noted, however, that the ratio of enzyme activities using a constant concentration of G6P and F6P does change with tube number (Figure 18).

**TABLE 1**

<table>
<thead>
<tr>
<th>EFFECT OF pH ON ACTIVITY OF DEAE FRACTIONS</th>
</tr>
</thead>
<tbody>
<tr>
<td>pH 6.6</td>
</tr>
<tr>
<td>--------</td>
</tr>
<tr>
<td>tube no.</td>
</tr>
<tr>
<td>157</td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td>169</td>
</tr>
<tr>
<td></td>
</tr>
</tbody>
</table>
Figure 18

Elution of Phosphoglucone Isomerase from DEAE-Sephadex A-50 at pH 8.3
G-200 Elution

Electrophoretic migration on polyacrylamide gel gives separation based on the effects of both emf and gel filtration. For this reason chromatographic columns were used containing gels which allowed for separation due to charge or molecular weight.

The results of migration on the G-200 column at pH 6.6 are seen in Figure 19. The shape of the peak as determined by enzymatic and chemical assay for protein content is symmetrical. There are no shoulders or other indication of a heterogeneous fraction. Gel filtration thus failed to separate the protein into fractions in a manner similar to that observed with polyacrylamide gel. There are several possible explanations. The two protein fractions may be so close in molecular weight as to elute simultaneously, or if the two bands were due to dissociation of one protein, then the relatively mild conditions used for the G-200 column may not have been sufficient to cause the dissociation.

Determination kinetic parameters

\( K_m \) and \( V_m \)

During the investigation of the kinetic parameters of the yeast isomerase a wider range of substrate concentrations was used than had been employed in the studies reported in the literature, or than had been previously
Figure 19

Elution of Phosphoglucone Isomerase from Sephadex G-200
used in this laboratory. This was feasible primarily as a result of the increased sensitivity and reproducibility obtained by the modifications of the Roe method. The double reciprocal plots utilizing this expanded range are presented in Figure 20. The biphasic character of the curve is evident in the plots of the reaction in either direction.

Reiner reports that when a biphasic Lineweaver-Burk plot is produced from a comparatively uncomplicated system involving two enzymes catalyzing the same reaction, the parameters of the separate enzymes can be estimated only at the extremes of the curve. Using slopes from this type of graph is an inaccurate method since the contribution of the "other" enzyme is significant over the entire range. Thus the values taken from these plots are at best only approximations.

In an attempt to obtain a better estimation of the Km and Vm, the data were also plotted as v vs v/(S) and (S) vs (S)/v as shown in Figure 21. These plots have a more pronounced biphasic nature. The calculations for the kinetic parameters from these graphs gave similar values as those resulting from the Lineweaver-Burk plots.

The Km and Vm for the muscle enzyme were determined under identical conditions to ascertain if the literature values could be reproduced. A linear relationship
Figure 20

Lineweaver-Burk Plots of Yeast Phosphoglucone Isomerase
Figure 21

Initial Velocity Data Graphed as $v$ vs $v/(S)$ and $(S)/v$ vs $(S)$
resulted when the data were plotted in the double reciprocal form. The experimental values for \( K_m \) and \( V_m \) closely approximated those of the literature.

Previous investigations in this laboratory (Kull, 1962) have shown that 6PG appears to inhibit the isomerase competitively when F6P is the substrate but has little inhibitory effect upon the reaction when G6P is the substrate. Based upon classical Michaelis-Menten kinetics, the \( K_i \) of a competitively inhibited mono-molecular reaction should be the same in both the forward and reverse directions.

The effect of this inhibitor was examined over the same wide substrate range used for the estimate of the kinetic parameters. Double reciprocal plots using the data obtained from the control and inhibited reactions are shown in Figure 20. There is to be observed a differential effect on the slopes of the two parts of the curve as examined in each direction, i.e. when G6P is the substrate, the effect on the slope is greatest in the low substrate range, while the converse is true when F6P is the substrate. The terminology of \((\text{intercept})^{-1}\) and \((\text{slope})(\text{intercept})^{-1}\) is used in Table 2. These are generally considered as representing the \( V_m \) and \( K_m \) respectively, but in a curvilinear plot the intercept and slope do not represent the same mathematical expressions.
### Table 2

**Estimated Kinetic Parameters of Yeast Phosphoglucone Isomerase**

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Range</th>
<th>(Intercept)⁻¹</th>
<th>(Slope)(Intercept)⁻¹</th>
<th>&quot;Kᵢ&quot; 6PG</th>
</tr>
</thead>
<tbody>
<tr>
<td>G6P</td>
<td>high</td>
<td>0.25</td>
<td>0.34</td>
<td>0.09</td>
</tr>
<tr>
<td>F6P</td>
<td>low</td>
<td>0.09</td>
<td>0.02</td>
<td>0.11</td>
</tr>
<tr>
<td>G6P</td>
<td>low</td>
<td>0.08</td>
<td>0.05</td>
<td>0.04</td>
</tr>
<tr>
<td>F6P</td>
<td>high</td>
<td>0.3</td>
<td>0.2</td>
<td>0.04</td>
</tr>
</tbody>
</table>

Values expressed as um/ml
of the kinetic parameters as in a linear Lineweaver-Burk plot. The values obtained here for high substrate concentrations compare favorably with those in the literature.

Similarly the biphasic nature of the curve makes impossible an accurate estimate of the Ki. (Because of the resulting inaccuracy, it is designated "Ki" on the Table). However, using the extremes of the curve where the slopes approach linearity, an estimate was made of the inhibitor constant to determine the influence of high and low substrate concentrations upon the degree of inhibition afforded by 6PG.

Table 2 contains the values as estimated from the graphs. It is interesting to note that the estimated "Ki" value obtained at high F6P concentrations was the same as that observed at low G6P concentrations. Similarly, the values obtained at high G6P and low F6P were the same, and were approximately three-fold greater than the other set.

The velocity of the isomerase catalyzed reaction was measured in the presence of either M6P or Gal6P. Neither compound altered the rate at concentrations as high as 1µmole/ml in a reaction mixture containing 0.3µm G6P/ml or 0.2µm F6P/ml.
Determination of product inhibition

In the routine determination of initial velocities it had been noted that the reaction rate as assayed in either direction decreased more than might be expected considering only the distance from equilibrium. Product inhibition, for the purpose of definition here, will be used only for the situation where the affinity of the product for the enzyme is greater than that of the substrate for the enzyme. Product inhibition, in which \( K_p \) is less than \( K_s \), should not exist in both directions of a reaction where there is only one set of kinetic parameters. In such a case the product and substrate will have their particular affinities for the enzyme and only in one direction can the product bind more tightly than the substrate.

Application of the Henri equation to data plotted \( p/t \) vs \( 1/t \) ln \( S_0/S_{0-p} \) and considering product as a competitive inhibitor of the reaction, should give a linear curve.\(^3\) The Henri equation is shown below:

\[
p/t = \frac{V_m}{K_m} (1 - K_i) - \frac{(1 + S_0 K_i)/(1 - K_i)}{1/t \ln S_0/S_{0-p}}
\]

Applewhite and Niemann\(^3\) have shown that the slopes of the lines obtained from data plotted in this manner are:

\[-K_s(K_p+S_0)/(K_p-K_s)\]

When \( K_p < K_s \) a positive slope is obtained, while when \( K_s < K_p \) a negative slope is obtained. Figure 22 shows that when
Figure 22

Henri Plot for Yeast Phosphoglucone Isomerase
either G6P or F6P served as substrate a positive slope was obtained, which should be indicative of product inhibition. Inspection of the Henri equation shows that a positive slope will also result as the reaction approaches equilibrium. Therefore a positive slope cannot be taken as strong evidence of product inhibition.

In order to further consider the aspect of product inhibition, the integrated rate equation was treated in such a manner as to make it applicable to the problem. The solution of the equation used here takes as the basis of comparison that point at which the velocity is one half the initial velocity, as well as the ratio of product:initial substrate concentration at that point. As derived by Gander, this solution takes into consideration the kinetic and thermodynamic parameters which contribute to the velocity of a reaction at any given time. This then allows for the estimation of product inhibition. The working form of this equation is:

\[ \frac{P}{S_o} \left( \frac{1}{K_f} + \frac{1}{K_b} + 2 \frac{K_f K_{eq}}{K_f + K_{eq}} \right) - \frac{1}{K_f} = \frac{P}{S_o} - \frac{2P}{3S_o^2} \left( 1 + \frac{1}{K_{eq}} \right) \]

In order to use this form, the only values required are substrate concentration, the equilibrium constant, and the amount of product present at the time when \( v \) is equal to one half \( v_0 \).

Calculations for a hypothetical reaction characterized by a single set of kinetic parameters gives
a straight line when $P/So$ is plotted against $P/So - 2P/So^2(1+1/Keq)$ as in Figure 23. When examined in the direction where there is no product inhibition, all ordinate values are negative. In the reverse direction all are positive. In both directions the value obtained for $P/So$ approaches a finite limit at low substrate concentrations. This limit is equal to $1/(2+2/Keq)$.

For the purpose of discussion and consideration of data, a vertical line through the limiting value and x-axis is used to divide the positive side of the abscissa into quadrants as shown in Figure 23. According to this equation values can fall only in the upper left quadrant (when $K_{mp} < K_{ms}$) or lower right quadrant (when $K_{mp} > K_{ms}$).

The isomerization catalyzed by the yeast enzyme was examined for product inhibition over a wide range of substrate concentrations. Data for these studies is shown in Table 3. Data from the reaction in either direction resulted in values which were in the upper left quadrant as indicated in Figures 24 and 25.

At low substrate concentrations the $P/So$ ratio approached the lower right quadrant. This would indicate that in both directions the product binds to the enzyme more than does the substrate, but that as the substrate concentration decreases, the product inhibition decreases. This invokes the requirement
Figure 23

Influence of $K_b/K_f$ Upon the Direction of Change in $P/So$ with Increasing Substrate Concentration
### TABLE 3

**INFLUENCE OF SUBSTRATE CONCENTRATION UPON P/So VALUES**

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Substrate</th>
<th>Concentration $x10^3$</th>
<th>P/So</th>
<th>$1/(2+2/Keq)$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Yeast</td>
<td>G6P</td>
<td>2.2</td>
<td>.038</td>
<td>.114</td>
</tr>
<tr>
<td></td>
<td></td>
<td>.45</td>
<td>.071</td>
<td>.114</td>
</tr>
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<td></td>
<td></td>
<td>.2</td>
<td>.074</td>
<td>.114</td>
</tr>
<tr>
<td></td>
<td>F6P</td>
<td>.2</td>
<td>.158</td>
<td>.385</td>
</tr>
<tr>
<td></td>
<td></td>
<td>.06</td>
<td>.271</td>
<td>.385</td>
</tr>
<tr>
<td>P.charlesii</td>
<td>G6P</td>
<td>.6</td>
<td>.055</td>
<td>.114</td>
</tr>
<tr>
<td></td>
<td>F6P</td>
<td>.2</td>
<td>.165</td>
<td>.385</td>
</tr>
<tr>
<td>muscle</td>
<td>G6P</td>
<td>.6</td>
<td>.059</td>
<td>.114</td>
</tr>
<tr>
<td></td>
<td>F6P</td>
<td>.2</td>
<td>.45</td>
<td>.385</td>
</tr>
</tbody>
</table>
Figure 24

Data from Yeast Phosphoglucone Isomerase (G6P Substrate)
Plotted According to Gander Equation
Figure 25

Data from Yeast Phosphoglucone Isomerase (F6P Substrate) Plotted According to Gander Equation
for more than one set of kinetic parameters. A graph was made for the hypothetical situation of a mono-molecular reaction catalyzed by two enzymes of different kinetic parameters. This situation produces a non-linear curve (Figure 26) similar to that obtained from the data of the yeast isomerase.

The kinetic properties of phosphoglucose isomerase obtained from rabbit muscle and *Penicillium charlesii* G. Smith were also examined by this procedure and are shown in Table 3.

Effect of pH

In view of the difficulty in interpreting double reciprocal plots for the estimation of kinetic parameters, the pH optima were determined by using $4 \times 10^{-4}$M G6P and $1.5 \times 10^{-4}$M F6P instead of employing a constant $S/K_m$ for each pH. The velocities obtained under these compromised conditions are not strictly comparable since the $K_m$'s are subject to change with pH. The pH optima are represented in Figure 27. When the enzyme reacts in the presence of $1 \times 10^{-4}$M 6PG when G6P is substrate or $2 \times 10^{-5}$M 6-phosphogluconate when F6P is substrate, the shape of the pH curve is altered (Figure 27, lower graph). In the absence of the gluconate the pH optima have essentially the same shape in each direction. The only significant difference is that the reaction with G6P as
Figure 26

Effect of Two Catalytically Active Sites on $P/So$ Values
Figure 27

pH Optima of Yeast Phosphoglucone Isomerase Catalyzed Reaction
substrate proceeds at a considerably lower pH than the reverse reaction. In the presence of gluconate the peak of the curve is shifted to a higher pH and is considerably sharpened. At a pH above 8.5, gluconate enhances the reaction velocity; at a pH lower than 6.2 in the forward direction, and 6.6 in the reverse direction it completely inhibits the reaction. These changes in the curve at these pH extremes were further investigated. The results of reactions run at three different substrate concentrations in the absence and presence of gluconate are given in Table 4.

Two important observations are recorded in this table; the activation effect of gluconate at pH 9.4 and its absolute inhibition effect at pH 6.7. These data strongly suggest that the catalytic properties of the enzyme are influenced by both pH and 6-PG.

Control experiments were carried out to determine whether the observed effects were in part due to inactivation at the pH extremes. Here, prior to the assay at pH 8.0, the enzyme was incubated for 5 minutes at the designated hydrogen ion concentrations. Table 5 shows that 6PG partially protects the enzyme from inactivation at pH 9.4. The apparent activation of the enzyme when incubated and assayed at pH 9.2 may be the result of the protection afforded during both the preincubation and assay period.
### TABLE 4

**EFFECT OF pH AND 6PG ON INITIAL VELOCITIES**

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Concentration</th>
<th>pH 6.7</th>
<th>6PG</th>
<th>pH 3.4</th>
<th>6PG</th>
<th>pH 9.4</th>
<th>6PG</th>
<th>6PG</th>
<th>+/-</th>
<th>+/-</th>
<th>+/-</th>
<th>+/-</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>-6PG</td>
<td>+6PG</td>
<td>+/-/</td>
<td>+/-/</td>
<td>-6PG</td>
<td>+6PG</td>
<td>+/-/</td>
<td>+/-/</td>
<td>+/-/</td>
<td>+/-/</td>
<td>+/-/</td>
</tr>
<tr>
<td>G6P</td>
<td>.068um/ml</td>
<td>1.9</td>
<td>.39</td>
<td>.21</td>
<td>2.3</td>
<td>2.3</td>
<td>.79</td>
<td>.79</td>
<td>.34</td>
<td>.34</td>
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<td>.34</td>
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<tr>
<td></td>
<td>.17</td>
<td>4.7</td>
<td>.64</td>
<td>.13</td>
<td>5.9</td>
<td>4.4</td>
<td>.75</td>
<td>.75</td>
<td>.16</td>
<td>.16</td>
<td>.16</td>
<td>.16</td>
</tr>
<tr>
<td></td>
<td>.34</td>
<td>4.9</td>
<td>1.1</td>
<td>.22</td>
<td>7.4</td>
<td>7.2</td>
<td>.97</td>
<td>.97</td>
<td>3.3</td>
<td>3.3</td>
<td>3.3</td>
<td>3.3</td>
</tr>
<tr>
<td>F6P</td>
<td>.05um/ml</td>
<td>2.4</td>
<td>0</td>
<td>5.8</td>
<td>3.0</td>
<td>.52</td>
<td>1.5</td>
<td>1.5</td>
<td>2.7</td>
<td>2.7</td>
<td>2.7</td>
<td>2.7</td>
</tr>
<tr>
<td></td>
<td>.11</td>
<td>3.1</td>
<td>0</td>
<td>6.2</td>
<td>5.9</td>
<td>.95</td>
<td>2.9</td>
<td>2.9</td>
<td>3.4</td>
<td>3.4</td>
<td>3.4</td>
<td>3.4</td>
</tr>
<tr>
<td></td>
<td>.21</td>
<td>8.7</td>
<td>0</td>
<td>11</td>
<td>6.3</td>
<td>.62</td>
<td>3.8</td>
<td>3.8</td>
<td>4.7</td>
<td>4.7</td>
<td>4.7</td>
<td>4.7</td>
</tr>
</tbody>
</table>

*Initial velocities as Klett units/ml/10 seconds*
TABLE 5

EFFECT OF 6PG UPON PHOSPHOGLUCOSE ISOMERASE INACTIVATION AT HIGH pH

<table>
<thead>
<tr>
<th>pH of Preincubation</th>
<th>pH of Assay</th>
<th>Velocity</th>
<th>% Inhibition by 6PG</th>
<th>% Increase by 6PG</th>
</tr>
</thead>
<tbody>
<tr>
<td>8.0</td>
<td>3.0</td>
<td>12.6 7.1</td>
<td>43</td>
<td>-</td>
</tr>
<tr>
<td>9.2</td>
<td>9.2</td>
<td>5.5 6.2</td>
<td>-</td>
<td>12.7</td>
</tr>
<tr>
<td>9.4</td>
<td>8.0</td>
<td>7.0 5.2</td>
<td>26</td>
<td>-</td>
</tr>
</tbody>
</table>

% inactivation of enzyme 40 27
The kinetic behavior of the enzyme preparation was investigated at pH 6.6 and was compared to that at pH 8.0 in the presence and absence of 6PG. The P/So values at pH 6.6 are recorded in Table 6 and show that product inhibition is observed only when F6P is the substrate. It should also be noted that product inhibition is observed when either F6P or G6P is the substrate in the presence and absence of 6PG at pH 8.0.

The effect of ionic concentration as produced by Tris or Tris+NaCl was also examined. There was found to be no alteration of initial velocity over the range of ionic concentration reported in Table 7. MgCl₂ was not found to affect the initial velocity (Table 7).

Effect of temperature

The effect of temperature on both the reaction rate and inactivation of the isomerase was examined. The influence of the presence of 6PG and preincubation temperature upon enzyme activity is shown in Figures 28 and 29. The enzyme was incubated for 5 minutes with 0.06M Tris HCl buffer at pH 8.0 at the temperatures indicated. The reaction mixture was cooled in an ice-water bath and then was returned to 25°C for determination of initial velocity.

Two tubes were incubated at each temperature, one with and one without gluconate. At the 25°C assay
TABLE 6

EFFECT OF pH ON P/So VALUES

<table>
<thead>
<tr>
<th>pH</th>
<th>Substrate</th>
<th>Concentration</th>
<th>P/So</th>
</tr>
</thead>
<tbody>
<tr>
<td>3.0</td>
<td>G6P</td>
<td>.45um/ml</td>
<td>.071</td>
</tr>
<tr>
<td></td>
<td>F6P</td>
<td>.20</td>
<td>.158</td>
</tr>
<tr>
<td>6.6</td>
<td>G6P</td>
<td>.42</td>
<td>.13</td>
</tr>
<tr>
<td></td>
<td>F6P</td>
<td>.06</td>
<td>.26</td>
</tr>
<tr>
<td>3.0 +6PG</td>
<td>G6P</td>
<td>.16</td>
<td>.07</td>
</tr>
<tr>
<td></td>
<td>F6P</td>
<td>.15</td>
<td>.2</td>
</tr>
</tbody>
</table>
### TABLE 7

**EFFECT OF IONIC CONCENTRATION ON INITIAL VELOCITY**

<table>
<thead>
<tr>
<th>Concentration of Tris·HCl</th>
<th>Initial Velocity</th>
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</thead>
<tbody>
<tr>
<td>0.01M</td>
<td>0.09μm/ml/min</td>
</tr>
<tr>
<td>0.02</td>
<td>0.09</td>
</tr>
<tr>
<td>0.04</td>
<td>0.088</td>
</tr>
<tr>
<td>0.06</td>
<td>0.91</td>
</tr>
<tr>
<td>0.08</td>
<td>0.09</td>
</tr>
<tr>
<td>0.10</td>
<td>0.092</td>
</tr>
<tr>
<td>0.12</td>
<td>0.091</td>
</tr>
<tr>
<td>0.14</td>
<td>0.09</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Concentration of NaCl</th>
<th>Initial Velocity</th>
</tr>
</thead>
<tbody>
<tr>
<td>(Tris=0.06M)</td>
<td>G6P Substrate</td>
</tr>
<tr>
<td>0.01M</td>
<td>0.09μm/ml/min</td>
</tr>
<tr>
<td>0.02</td>
<td>0.092</td>
</tr>
<tr>
<td>0.04</td>
<td>0.088</td>
</tr>
<tr>
<td>0.06</td>
<td>0.088</td>
</tr>
<tr>
<td>0.08</td>
<td>0.09</td>
</tr>
<tr>
<td>0.10</td>
<td>0.091</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Concentration MgCl₂</th>
<th>Initial Velocity</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>G6P Substrate</td>
</tr>
<tr>
<td>--</td>
<td>0.09μm/ml/min</td>
</tr>
<tr>
<td>0.05</td>
<td>0.088</td>
</tr>
<tr>
<td>--</td>
<td>F6P Substrate</td>
</tr>
<tr>
<td>0.05</td>
<td>0.125</td>
</tr>
<tr>
<td></td>
<td>0.127</td>
</tr>
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</table>
Figure 28

Effect of Heating on Inactivation of Enzyme as assayed with G6P as Substrate
Figure 29

Effect of Heating on Inactivation of Enzyme as Assayed with F6P as Substrate
temperature three reactions were run for each temperature of preincubation: 1) control - incubated without 6PG and assayed without 6PG; 2) incubated and assayed in presence of 6PG; 3) incubated in absence of gluconate, assayed in presence of gluconate. The last mixture was used to differentiate the effect of 6PG as a protective agent from its effect as an inhibitor. The data from these assays indicate that there is marked protection by the gluconate from 58° to 70°.

The actual progress of the reaction as carried out in the absence of 6PG is plotted as a function of time in Figures 30 and 31. These show that even when heated to a temperature of 80° there is some residual enzymatic activity. Controls containing no enzyme yielded no product over these extended experimental periods.
Figure 30

Time Course of Reaction Catalyzed by Heated Enzyme (G6P Substrate)
Figure 31

Time Course of Reaction Catalyzed by Heated Enzyme (F6P Substrate)
DISCUSSION

The one-substrate, one-product reaction catalyzed by phosphoglucone isomerase is not known to require any cofactors. Thus it should be one of the simplest types of enzymatically catalyzed reactions. However, several aspects of the data obtained in these studies have shown deviations from the type of results expected for a single substrate enzyme obeying Michaelis-Menten kinetics. As previously mentioned, the observation which initially led to these studies was that 6PG effected different Ki's for each direction. Also this inhibition was more effective when competing with the substrate having the lower apparent Km. As noted, this is in contradiction to the classic Michaelis-Menten kinetics.

In the experiments which stemmed from this observation, additional deviations from a simple kinetic system were noted. Lineweaver-Burk plots obtained from the data of the isomerase show a definite deviation from linearity. The graphs are essentially biphasic, approaching linearity only at the extremes of the plots. Though such a plot is not capable of giving much information about the more refined aspects of a mechanism, deviations from linearity can be taken as an indication that the reaction
is not characterized by a single set of kinetic parameters which is operative over the entire substrate range. Added to this is the influence of 6PG upon these double reciprocal plots. As noted, the effect was a differential one. The major change in the slope, and therefore the major inhibitory effect, occurred over the range of low substrate concentrations when G6P was the substrate, and high substrate when F6P was the substrate.

The influence of heat upon enzyme activity was investigated. In the absence of 6PG the catalytic ability of the enzyme was destroyed over the range 58°-70°C. The gluconate protected the enzyme from inactivation over this range.

Equations capable of detecting product inhibition were applied to the data. Foster and Niemanns state that a line with a positive slope will be obtained when data from a reaction in which $K_p<K_s$ is plotted according to the Henri equation; a negative slope will obtained with $K_p>K_s$. These authors imply that this method can be used for the complete course of a reaction. However, it should be noted that at equilibrium the $x$ and $y$ coordinates will be decreasing in a parallel manner and will intercept at the origin when $t$ is infinite. The result is a straight line of positive slope through the origin. Thus as a reaction is approaching equilibrium, it is not possible to separate
the contributions from product inhibition and approach to equilibrium toward the line of positive slope. The data from the isomerase, as examined in both directions, gave a positively sloped line, the extension of which passed through the origin. However, in view of the above interference, the existence of product inhibition of both directions could not be concluded solely from this evaluation.

The data were also treated with the Gander equation, which is a modified form of the integrated rate equation. As was shown in Figures 24 and 25, all of the experimental points plotted in this manner were in the upper left quadrant, indicative of product inhibition. However, when the reaction was carried out at pH 6.6 product inhibition was obtained only when F6P was the substrate. This indicates a change in the kinetic parameters with change in pH. It was also shown that product inhibition was observed with both substrates at pH 8.0 either in the presence or absence of 6PG. Therefore it appears that the pH effect is separate from the 6PG effect.

These preceding results are the experimental aspects which cannot be reconciled with Michaelis-Menten kinetics for a monomolecular reaction.

Before proposing a reaction model, it might be helpful to list the factors which may influence the kinetic
parameters of the catalytic process in the phosphoglucose isomerase catalyzed reaction. These are considered to be known variables which could exert an influence on the reaction in such a manner as to produce one or more of the experimental results observed.

1. Influence of ionic strength upon the ratio of various possible ionic species of substrate and product

2. Reversible association and dissociation of enzyme into various states of polymerization

3. Conformation of the substrate and product

4. Allosteric binding of substrate or metabolites

5. Factors influencing conformation of the enzyme (substrate, pH, metabolites, ionic strength)

6. Influence of conformation of the enzyme upon the catalytic process

7. Number of catalytic sites on the enzyme

8. Kinetic parameters of the catalytic sites or species of the enzyme

There are many reaction models which may result in non-linear Lineweaver-Burk plots. On the basis of currently accepted theories the following models may be considered in so far as they account for one or more parts of the data. There are certain aspects of the experimental results which make it necessary to consider each of these models. However, the data considered in toto eliminate most of them according to the interpretations given.
Possible reaction models:
Aspects of agreement or disagreement with experimental results

1. The change in ionic strength produced with increasing substrate concentration alters the kinetic parameters of the enzyme.

Changes in ionic strength can alter protein conformation, substrate conformation, the degree of ionization of a substrate, effective charge and/or change the observed affinity of the enzyme and substrate. Data obtained under such conditions could produce a non-linear Lineweaver Burk plot. However, several factors of experimental conditions and results eliminate this possibility as being the cause of non-linear plots observed here. The substrate range used was approximately $3 \times 10^{-5}$M to $4 \times 10^{-4}$M while the concentration of the buffer was approximately 0.1M. This is a difference of more than 1000-fold. Thus any increases in ionic strength due to substrate are insignificant. If the effect would be specifically due to the binding of additional molecules of substrate, this would not so much be classified as ionic strength effect but as an allosteric effect or non-specific binding. Furthermore, the velocity of the reaction was unaltered over a wide range of ionic strengths of Tris or Tris+NaCl.

The most convincing reason for eliminating ionic strength effects comes from the observation that 6PG
or hydrogen ion concentration differentially alters the kinetic parameters obtained for each substrate. If the effect were only ionic, the character of the plots should remain qualitatively the same in both directions. Therefore these data cannot be interpreted as resulting from changes in ionic concentration.

2. A reversible dissociation and association of subunits of the enzyme, under the influence of substrate or inhibitor concentration.

This type of situation could give non-linear double reciprocal plots. Additional support for such a mechanism could come from evidence of physical separation. Protein separation was effected by polyacrylamide gel electrophoresis, but it was not able to be ascertained if both the fractions were enzymatically active. The migration of the enzyme on DEAE-Sephadex A-50 at pH 8.3 produced an elution pattern which contained a shoulder, giving the appearance of a heterogeneous fraction. Other attempts at separation on DEAE-Sephadex A-50 at pH 6.6 or on Sephadex G-200 at pH 6.6 produced only a single symmetrical elution peak, which accounted for essentially all the protein applied.

In order to satisfy this model, it would have been necessary to show separation of subunits. With regard to this, no definite conclusions can be reached on the
basis of the experimental results obtained. Thus for this particular model, data was not obtained which could reasonably be considered to demonstrate subunit formation. The heterogeneity observed at pH 8.3 using gel electrophoresis and column chromatography may be due to a charge effect rather than subunit formation.

3. Two separate and unassociated enzymes, each containing a site with kinetic parameters distinct from the other.

As mentioned before, when two enzymes catalyze the same reaction, the data obtained from this result in a biphasic double reciprocal plot. However, in order to consider this model further, there should be evidence of physical separation of the two enzymes. The physical used and discussed with respect to the preceding model are applicable here. Again, the inconclusive evidence of separation rather prevents further consideration of this particular aspect. The factor of multiple catalytic sites apart from this particular case will be treated later.

4a. An enzyme capable of acting upon two forms of the substrate, with the conversion of the first form to the second being the rate limiting step.

Such a situation would be analogous to that of an enzyme catalyzing two reactions producing the same product,
or two enzymes catalyzing the same reaction. As has been shown by Reiner, data from this type of reaction results in a biphasic Lineweaver-Burk plot. Since the assay method here determines total fructose, i.e. sum of alpha and beta ring forms and chain form, there would appear only one product. Although the equilibrium amount of glucose in the straight chain has been determined to be approximately 0.0025 per cent of the total, the rate of the conversion between the two forms has not been found in the literature.

Figure 32 shows a hypothetical energy curve and the relative Km's and Vm's which would result. According to this the straight chain form would be the more reactive, and also bind more tightly. This would produce a biphasic Lineweaver-Burk plot in which high substrate concentrations would be expected to produce low Km's and high Vm's. Experimentally, high substrate concentrations gave high Vm's and High Km's. In this case the most convincing data in opposition to the model is the differential inhibition in the presence of 6PG. Since the catalysis of the isomerization of either the open or closed form would need to involve the catalytic center, the effect of 6PG upon Lineweaver-Burk plots should be qualitatively the same in both directions. Thus on this basis this model may be eliminated from further consideration.
Figure 32

Hypothetical Energy Diagram and Correlated Mechanism
4b. The ratio of the alpha/beta anomers of the substrates alters the kinetic parameters of the enzyme.

Since both substrates of the isomerase exist in solution as the alpha and beta anomers, there is the possibility of effect upon the reaction by the form which is not the substrate. In the event that the Keq between the forms is independent of the substrate concentration, then this would be analogous to a constant inhibitor/substrate ratio and a linear Lineweaver-Burk plot would result. However, if there is a concentration effect upon the equilibrium ratio between the forms, then there could be a non-linear plot produced. Nonetheless this model may be discarded on the basis of the gluconate, since it would not account for the differential effect.

5. An enzyme contains both a catalytically active site and a modifier (allosteric) site.

In this case the substrate or modifier may be bound in such a way as to alter the observed velocity of the reaction. Invoking this concept of two sites, the velocity of the reaction may be increased or decreased by the binding at the allosteric site. Figure 33 gives a representation of the two types of graphs which would result from the modifier effecting an increase or a decrease in the velocity of the reaction. In the lower
Figure 33

Hypothetical Allosteric Mechanism and Associated Plots
\[ V_{I2} = \frac{V_{P2}}{S} \left( \frac{K_{I1}}{K_{I2}} \right) \left( \frac{R_{S1}}{R_{S2}} \right) \]

\[ V_{I1} = 50 \quad V_{P1} = 100 \]
graph where the modifier increases the velocity, the doubly bound enzyme has both a higher $V_m$ and $K_m$. The double reciprocal plot resembles that which is produced from the experimental data of the isomerase. However, this type of allosteric effect, by having only one catalytically active site and one binding site would not be expected to show the differential effect that is observed with the gluconate and pH and therefore can be eliminated on this basis.

6. An enzyme containing two catalytic sites.

Double reciprocal plots of kinetic data obtained from a reaction catalyzed by two enzymes containing dissimilar kinetic parameters would be identical to data obtained from a reaction catalyzed by one enzyme containing two catalytic sites if the catalytic sites were independent. Furthermore it is not possible to ascertain from double reciprocal plots if the kinetic properties associated with the sites are independent or interdependent since qualitatively the data would appear quite similar.

The Lineweaver-Burk plots shown in Figures 20 are similar to those which would be obtained were the reaction catalyzed by either two separate species or one species of enzyme containing two catalytic sites.

Apart from nonlinearity of Lineweaver-Burk plots, the effect of gluconate on the reaction is also compatible
with a two site mechanism. The Lineweaver-Burk plots indicate that at high G6P concentrations the reaction is only slightly inhibited by the gluconate, while at low concentrations the reaction becomes disproportionately affected by the gluconate. This observation makes further modification of the model necessary. The postulate that gluconate is exerting strong inhibition at only one of the two catalytically active sites is surely one of the simpler and more attractive possibilities for this modification. The observation that gluconate appears to confer increased thermal stability upon the enzyme is in general agreement with this postulate although it does not directly bear upon the question of whether there are two catalytic sites or two separate species.

For this model to fit the data it is necessary to postulate that the free energy profiles for each catalytic site are such that there exists an inverse relationship between the Km's. That is, for one catalytic site \( K_{ms} > K_{mp} \) while at the other site \( K_{ms} < K_{mp} \). The Haldane equation then dictates the relationship that must exist between all the kinetic parameters for each catalytic site:

\[
\text{Keq} = \frac{V_{ms}K_{mp}}{V_{mp}K_{ms}}
\]
From this model it is apparent that experimental measurements of velocity would show product inhibition using either substrate. The gluconate, by inhibiting at only one site, the one which binds G6P most tightly (and therefore the site which most rapidly catalyzes the conversion of F6P to G6P) would show little inhibition of the conversion of G6P to F6P at high concentrations of G6P.

The differential effects of heat and pH are also compatible with the existence of two sites.

On the basis of this two site model, the existence of various species may be considered. These species represent the enzyme as it exists in the presence of those substances which modify its kinetics. From the data discussed these are 6PG and hydrogen ion concentration. The species as represented in Figure 34 indicate either 6PG or H⁺ as being bound to the site which experimental data have shown to be most affected by them. Their action upon these sites may actually be due to the binding, or they could produce conformational changes in the enzyme. Conformational changes, however, are not inherent in the proposal of this model, nor does experimental evidence necessarily support such an alteration.
Figure 34.

Enzyme Species Proposed as Part of Model
In the model as represented in Figures 34, 35, and 36 the "G6P site" is considered as that site to which G6P is bound more tightly. The "F6P site" binds F6P more tightly.
Figure 35

Product Formation under Various Experimental Conditions
Figure 36
Characteristics of Enzyme Species
The extrapolation of data obtained from in vitro experiments to an in vivo significance can be done only with great reservation. It has been indicated previously that there has been found no significant type of metabolic control over the phosphoglucone isomerase reaction, and for this reason the intrinsic character of the enzyme kinetics would appear to be of special importance. However it may also be speculated as to what significance such a controlled reaction would be to the cell. Many reactions which are under some degree of metabolic control are in one of the following categories: an irreversible reaction, which prevents the product from immediately re-entering the same metabolic scheme of the cell (frequently such a product only undergoes degradation); the reaction produces a product which is of special and limited metabolic purpose; the reaction involves the expenditure of a significant amount of energy. In this last case it may be of particular value to the cell not to invoke the reaction unless needed. None of these types of reactions is represented by the phosphoglucone isomerase catalyzed reaction. Since this reaction is readily reversible, and the equilibrium and ΔF of the order of magnitude of one, the cell does not lose much
energy when the reaction occurs to a greater extent than would appear to be needed. Another important consideration here is that other enzymes which act on G6P are under control, and the step immediately after the isomerase reaction, i.e. phosphofructokinase is also controlled. Therefore the reactions adjacent to the isomerization are all under some type of metabolic control.

Experimental data have resulted in the possibility of two types of control for the isomerase reaction. The inhibition by 6PG in the direction of F6P to G6P favors the further metabolism of F6P by the glycolytic scheme by slowing down the conversion of F6P to G6P. This has the value of a type of feedback inhibition by attempting to decrease the availability of G6P which can be converted to 6PG.

The evidence presented herein suggests that G6P will also act as a strong inhibitor of the conversion of F6P to G6P. The data indicate that the catalytic site which binds G6P more tightly than F6P is also the site which binds 6PG. Thus the effects of 6PG and G6P are additive in decreasing the rate of conversion of F6P to G6P.

The rate of the conversion of G6P to F6P appears to be influenced by two factors, pH and F6P. Both hydrogen ion and F6P appear to decrease the rate of F6P production
at the catalytic site binding F6P more tightly. Thus a lowered pH would result if there was an accumulation of acidic intermediate compounds during the metabolism of G6P.

The actual significance for \textit{in vivo} control by an enzyme of kinetic specifications described herein can only be proposed. \textit{In vitro} the amount of inhibition in both directions is significant at concentrations of G6P and F6P which would be expected to be in the physiological range. As indicated however, these studies were restricted to an isolated system and in order to extract more significant data for the purpose of deriving meaning for metabolic control, additional experimentation is necessary.
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