STUDIES ON PHOSPHOGLUCOMUTASE
AND PHOSPHOFRUCTOKINASE FROM BRAIN

by

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We accept this thesis as conforming to the
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ABSTRACT

It has recently been established that the activity of crystalline muscle phosphoglucomutase can be greatly stimulated by preincubation of the enzyme with a Mg++-imidazole complex. This observation has aroused interest in the physiological significance of such a system in the possible cellular control of phosphoglucomutase activity. The present study constitutes, in part, an investigation of the properties of phosphoglucomutase from brain tissue. A procedure for the purification of phosphoglucomutase from beef brain is described. The brain enzyme appears to be similar to that from skeletal muscle. Evidence is also presented which indicates that the "activation" produced by Mg++-imidazole is probably of no physiological importance in brain. This observation is consistent with the more recent reports that the phosphoglucomutase reaction is likely not involved in cellular regulatory mechanisms.

It is well established that phosphofructokinase is intimately involved in the cellular regulation of glycolysis and the citric acid cycle. Control mechanisms of the phosphofructokinase reaction in mammalian tissues have been postulated on the basis of the complex kinetics of the enzyme. In yeast, however, two enzymatically inter-convertible forms of the enzyme have been reported. Preliminary experiments in this study failed to demonstrate a phosphofructokinase system in brain similar to that found in yeast.
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ABBREVIATIONS

The following abbreviations have been used: F6P, fructose 6-phosphate; FDP or fructose diphosphate, fructose 1,6-diphosphate; glucose diphosphate, α-glucose 1,6-diphosphate; ATP, adenosine triphosphate; ADP, adenosine diphosphate; AMP, adenosine monophosphate; cyclic 3',5'-AMP, adenosine 3',5'-monophosphate; Pi, orthophosphate; Tris, tris-(hydroxymethyl)-aminomethane; DPNH, the reduced form of diphosphopyridine nucleotide.
INTRODUCTION

Recent years have seen a great deal of attention devoted to the cellular regulation of enzyme activity. In the light of present knowledge concerning metabolic pathways, properties of enzymes and the chemistry of the cell, the ultimate aim of many biochemists is no longer to focus attention on isolated cell components or biochemical reactions alone, but rather to examine the mechanisms by which the intricate cellular machinery is controlled. It has been well established that the methods available to the cell for regulation of metabolism are both numerous and diverse. Studies on the flux of substrates, co-factors and end-products during metabolic activity, interconversions of multiple enzyme forms, conformational changes during enzyme activity and hormonal effects have provided an abundance of information on the nature of metabolic control mechanisms. It has become apparent from recent reports that disturbance of the delicate metabolic balance which exists in the cell under normal conditions may result in the "turning on" of some enzymes and the "turning off" of others. In microorganisms this phenomenon has been investigated mainly from the point of view of controlled enzyme synthesis. Enzyme regulation in mammalian tissues is unique in many respects and a fruitful approach has been the study of factors controlling enzyme activity directly. This area of endeavor is rapidly gathering momentum and the importance of these problems is emphasized by the number of recent international symposia (1,2,3) on the subject.

The control of carbohydrate and energy metabolism has been of particular interest in a number of laboratories. Of the mammalian tissues
studied in this regard, however, brain has not attracted wide attention, primarily because glucose has long been considered the primary energy source of this tissue. In addition, technical problems involved in isolating enzymes from this material have also discouraged metabolic studies on brain. Recent evidence obtained in this and other laboratories (4,5), however, indicates that mammalian brain is richly endowed with glycogen phosphorylase which exists in an active and an inactive form and that the enzymes for interconversion of these forms are also present. Moreover, it is clear from the work of Breckenridge and Crawford (6) and of Villar-Palasi and Lerner (7) that the enzymes of the glycogen cycle in brain are present with sufficient activity to account for maximal rates of glycogen accumulation and disappearance in intact brain.

Information concerning the enzyme phosphoglucomutase in these studies was limited to the observation that the activity of this enzyme exceeds the activities of the glycogen cycle enzymes. As the link between the glycogen cycle and the Embden-Meyerhof pathway, phosphoglucomutase could well be regarded as a logical site for physiological control. This enzyme has been extensively studied in skeletal muscle (8) and a method has been described for its activation in vitro (9). The lack of knowledge regarding phosphoglucomutase in brain and the possibility of its importance in physiological regulation of glycolysis suggested the need for a study of this enzyme in brain tissue. This thesis describes studies to determine the possible significance of an activating system for the control of phosphoglucomutase activity in brain.

In the past two years, the glycolytic enzyme phosphofructokinase has received wide attention, primarily as a result of the observation that this
enzyme is rate-limiting and that its peculiar and complex kinetics suggest the possibility of an exceedingly fine mechanism for the cellular regulation of glycolysis. It has been demonstrated by various workers (10-15) that control of phosphofructokinase activity is provided by a delicate balance of substrate, inhibitor and end-product levels. In addition, there appear to be two enzymatically interconvertible forms of the enzyme in yeast (16), thus introducing another parameter for regulation of glycolysis. A further objective of the work described in this thesis was to make preliminary observations on the possible existence of multiple forms of phosphofructokinase in brain.

PHOSPHOGLUCOMUTASE

Phosphoglucomutase, the enzyme that catalyzes the conversion of glucose 1-phosphate to glucose 6-phosphate, has been found in the extracts of various mammalian tissues as well as in microorganisms, plants and fish (8). The enzyme was first described by Cori, Colowick and Cori (17), crystallized from rabbit muscle by Najjar (18) and has since been studied extensively.

Metabolically, the phosphoglucomutase step is directly in the path of glycogen breakdown and synthesis (Figure 1). Phosphoglucomutase activity has been found in excess of the activities of the glycogen cycle enzymes in all tissues studied (19). This finding agrees with its postulated role of supplying the glycogen-synthesizing system with enough glucose 1-phosphate against an unfavorable equilibrium and constituting a rapid step in the utilization of the glucose 1-phosphate derived from glycogen.

Phosphoglucomutase requires both Mg$^{++}$ and a metal-binding agent, in addition to $\alpha$-glucose 1, 6-diphosphate for full activity. Sutherland (20)
Figure 1. Diagram showing the relationship of the phosphoglucomutase reaction to the glycogen cycle. Abbreviations used are: glucose 6-phosphate, glucose 6-P; glucose 1-phosphate, glucose 1-P; uridine triphosphate, UTP; uridine diphosphate, UDP; ortho phosphate, $P_i$; pyrophosphate, PP.
demonstrated that a large number of metal-binding agents were effective as co-factors and it was suggested that they functioned by removing trace amounts of inhibitory metals bound by the enzyme protein. More recently, Najjar (9) has reported that the role of the metal-complexing substance is not to remove inhibitory metals but rather to form an activator complex with Mg++. The mechanism of the catalytic reaction has been studied in some detail by various groups of workers since 1949 and considerable controversy has arisen regarding the sequence of events in the phosphoglucomutase pathway (21-24). Najjar and Pullman (22) suggested a 2-step reaction through the alternate formation of phospho- and dephosphoenzyme:

\[
\begin{align*}
\text{Glucose 1-phosphate} + \text{phosphoenzyme} & \quad \text{Step 1} \\
\text{Glucose 1,6-diphosphate} + \text{dephosphoenzyme} & \\
\text{Glucose 6-phosphate} + \text{phosphoenzyme}
\end{align*}
\]

Cleland (24), however, has proposed a sequential pathway as an alternative to the "ping-pong" type of pathway (so designated because one or more products are released before all substrates have added to the enzyme) of Najjar and Pullman. This proposal has now been rejected by Ray and Roscelli (25) who recently performed elaborate kinetic studies on phosphoglucomutase and concluded that a ping-pong-like pathway exists in which dephosphoenzyme-glucose 1,6-diphosphate complexes are obligatory intermediates. Their experiments indicate that free glucose 1,6-diphosphate is not a necessary reaction intermediate and is infrequently formed during the reaction (about once in 20 turnovers). Hence "added" glucose
diphosphate serves only to ensure that the phosphoenzyme does not become depleted in phosphate via the production of "free" glucose diphosphate. In view of these findings, glucose 1,6-diphosphate may no longer be referred to as a coenzyme for the phosphoglucomutase reaction.

Robinson and Najjar (9) described the time dependent activation of phosphoglucomutase by Mg$$^{++}$$ and imidazole. They observed that preincubation of the crystalline enzyme with Mg$$^{++}$$ and imidazole or histidine at pH 7.5 resulted in a two to five-fold activation of the enzyme. Activation reached a maximum in 5 to 10 minutes at 0° but it occurred much more rapidly at 30°. Mg$$^{++}$$ or imidazole alone did not produce significant activation. The data indicated that activation was effected by a Mg$$^{++}$$-imidazole complex and the possibility of inhibitory metal chelation was discounted. Marked differences were observed in the Mg$$^{++}$$ concentrations required by the 2 forms of the enzyme for maximal catalytic activity. Furthermore, the optimal concentration required by the non-activated enzyme was inhibitory to the activated form. An explanation for this phenomenon was tendered on the basis of kinetic data, but some question still remained regarding the possible significance in physiological regulation of this enzyme.

The first part of this thesis constitutes an attempt to purify phosphoglucomutase from brain and to compare the nature of the activation process with that described for the enzyme from muscle in the hope of clarifying its importance in the control of the phosphoglucomutase reaction.

During the course of this investigation several reports appeared in the literature indicating that phosphoglucomutase is likely not under
hormonal control (26) and that the reaction it catalyzes is probably maintained near equilibrium under all circumstances (27).

**PHOSPHOFRUCTOKINASE**

Phosphofructokinase is the unique enzyme of the Embden-Meyerhof glycolytic pathway in that it is not involved in any phase of carbohydrate metabolism other than the conversion of fructose 6-phosphate to fructose 1, 6-diphosphate. Thus phosphofructokinase plays a key role in the metabolism of rapidly glycolyzing cells.

Although the enzyme has been studied by numerous investigators since its discovery thirty years ago, many of the problems concerning its purification, properties and mechanism of action have remained largely unsolved (28). Several early reports in the literature indicated that phosphofructokinase plays an important role in the regulation of glycolysis in the cell (29,30,31). The significance of these observations, however, has only recently been realized. Re-examination of this enzyme and its function in metabolic control has attracted wide attention since Mansour (32) first reported the regulation of glycolysis in the liver fluke by phosphofructokinase and Passonneau and Lowry (11) confirmed earlier observations implicating this enzyme with the Pasteur effect.

In the early studies of Mansour (33) it was found that serotonin stimulated glucose uptake and lactic acid production in the liver fluke *Fasciola hepatica*. In his subsequent communication (32) it was reported that serotonin stimulates glycolysis in homogenates of this organism. The results suggested that this was accomplished through stimulation of phosphofructokinase, a rate-limiting enzyme of glycolysis in the liver fluke. This effect could be duplicated by adenosine 3',5'-monophosphate.
In further experiments with phosphofructokinase from the liver fluke Mansour and Mansour (10) found that activation of the enzyme by serotonin or Ca\textsuperscript{++} was contingent on the presence of a heavy particulate fraction, whereas activation by cyclic 3',5'-AMP could be demonstrated in a high-speed supernatant fraction prepared after incubation of the homogenate with ATP and Mg\textsuperscript{++}. This activating effect of cyclic 3',5'-AMP was characterized by a decrease in the concentration of fructose 6-phosphate required for activity in the presence of high ATP levels. ATP in concentrations greater than the optimal caused inhibition of the phosphofructokinase activity. In the presence of cyclic 3',5'-AMP, inhibition by ATP occurred at relatively higher concentrations. In an earlier report, Lardy and Parks (34) described the inhibition by ATP of the enzyme from rabbit muscle. They suggested that the concentrations of ATP in living cells may have a direct regulatory influence on this enzyme.

The Pasteur effect has been of interest to numerous investigators since it was first described. In more recent studies evidence points to the phosphofructokinase reaction as being the enzyme reaction primarily responsible for this phenomenon (31,35,36,37). Passonneau and Lowry (11) have examined the kinetics of a partially purified phosphofructokinase preparation from muscle and have concluded that they may explain the Pasteur effect. It was noted that in the presence of inhibitory concentrations of ATP, activity was increased many-fold by cyclic 3',5'-AMP, AMP, or P\textsubscript{i}, but not ADP. In the presence of P\textsubscript{i}, however, ADP was also effective. The phosphofructokinase stimulators appeared to act chiefly by lowering, as much as 25-fold, the concentration of fructose 6-phosphate required for activity, whereas increasing the ATP concentration had the
opposite effect. Phosphofructokinase in mouse brain preparations behaved similarly, although no data were reported. The product of the reaction, fructose 1,6-diphosphate, was the best stimulator studied. It was effective at minute concentrations when ATP was present in inhibitory concentrations. With ATP concentrations in excess of the inhibitory level, however, fructose diphosphate was without effect, even at relatively high concentrations. From the kinetic data obtained it was postulated that there are two ATP sites on the enzyme surface, a primary active and an inhibitory site, and that the stimulators can compete with ATP for the second site. This would indicate that glycolysis in the cell could be turned on if either ATP falls or P_i, ADP or AMP levels rise. It was suggested that such a mechanism would provide multiple protection in emergency situations. These findings were recently corroborated by Wu (38) in various tumor preparations.

Mansour (12) has described the properties of a 143-fold purified preparation of phosphofructokinase from guinea pig heart. Inhibition of the enzyme could only be observed below pH 7.5 and at low fructose 6-phosphate concentrations. Activation by cyclic 3',5'-AMP required low substrate concentrations, and inhibitory levels of ATP, and could not be observed at alkaline pH. Mansour concluded that activation by cyclic 3',5'-AMP and related compounds is dependent on kinetic characteristics of the enzyme, rather than participation of another activating system. This hypothesis was supported by the finding that these effects were not reduced with purification of the enzyme.

In a study of muscle from diabetic rabbits Parmeggiani and Bowman (13) demonstrated that it was not possible to account for phosphofructo-
kinase inhibition on the basis of altered nucleotide ratios or $P_i$ content. Their data indicated that the inhibition of glycolysis in diabetes may be mediated, in part, by an elevation of tissue citrate which acts as a direct inhibitor of phosphofructokinase.

In a continuation of studies on phosphofructokinase, Passonneau and Lowry (14) obtained evidence to support the view that the enzyme may control not only the rate of glycolysis but also the levels of substrates in the citric acid cycle. Physiological concentrations of citrate were shown to markedly inhibit purified phosphofructokinase from sheep brain; the degree of inhibition could be increased by ATP and decreased by fructose 6-phosphate as determined by Parmeggiani for the muscle enzyme. In the case of brain enzyme, other members of the citric acid cycle may also be inhibitory, but to a lesser extent. AMP and $P_i$ were capable of relieving citrate inhibition and fructose diphosphate was an exceedingly potent antagonist. Ammonium ion, as shown by Muntz (39), increases absolute enzyme velocity but is not antagonistic to citrate. Although phosphofructokinase activity is very low in liver, it possesses properties very similar to the brain enzyme.

The importance of phosphofructokinase in the regulation of cell metabolism is emphasized in the following statements of Passonneau and Lowry (14):

"Phosphofructokinase emerges as a key enzyme subject to a multiplicity of coarse and fine adjustments. Under normal conditions its activity is kept low by ATP. When high activity is demanded (by anoxia, for example) this can be brought about by a decrease in ATP and increase in $P_i$, AMP, possibly $NH_4^+$, and secondarily fructose diphosphate. When fine control of the low normal rate is required, this can be provided by a delicate balance between citrate and fructose 6-phosphate levels without necessary changes in ATP, $P_i$ or AMP. Phosphofructokinase is thus responsive, on the one hand, to the balance between $\sim P$ expenditure and $\sim P$ formation, and on the other, to the metabolic mixture offered to the cell."
It has recently been demonstrated that the phosphofructokinase system in yeast, although similar in several respects to that of mammalian tissues, possesses a number of strikingly different properties. Vinuela, Salas and Sols (40) have observed inhibition of yeast phosphofructokinase by ATP in a manner which is competitive with fructose 6-phosphate. They suggested that end-product inhibition by ATP can act as a feed-back control in glycolysis. Curiously, no appreciable effect on the enzyme could be observed with P_i, cyclic 3',5'-AMP, AMP, ADP or fructose diphosphate even at inhibitory levels of ATP.

In a further communication, these authors (16) reported the identification of two forms of yeast phosphofructokinase which are enzymatically interconvertible. The two forms, insensitive and sensitive to inhibition by ATP, were designated as a and b respectively. The a form was also insensitive to inhibition by citrate. Incubation of a crude yeast extract in the presence of fluoride led to the disappearance of the sensitivity of phosphofructokinase to inhibition by ATP. This effect could be reversed by dialysis or prevented by EDTA. Incubation of a dialyzed extract with fluoride plus ATP-Mg^{++} and cyclic 3',5'-AMP also resulted in the appearance of an ATP-insensitive form. A separation of phosphofructokinase from an "activating enzyme" was also achieved.

In view of these findings the exciting possibility arose that a similar system exists in mammalian tissues, and that some of the peculiar kinetics observed could perhaps be the result of enzymatic interconversions of multiple enzyme forms of phosphofructokinase. A further objective of this thesis was to initiate a study of phosphofructokinase in brain and to make preliminary observations concerning the possibility of a multi-enzyme system in this
EXPERIMENTAL PROCEDURE

MATERIALS

α-D-Glucose 1-phosphate (Sigma Chemical Company) was purified by recrystallization according to the procedure of Sutherland and Wosilait (41). It contained glucose 1,6-diphosphate in sufficient quantity to saturate the enzyme and thereby obtain full activity under the conditions of the assay. α-Glucose 1,6-diphosphate as the barium salt, was a gift of Dr. H.L.A. Tarr, Fisheries Technological Station. It was converted to the potassium salt by treatment with Dowex-50 (K-form) prior to use. Imidazole was obtained from the California Corporation for Biochemical Research (Calbiochem). Glycylglycine, egg albumin, and ATP (dipotassium salt) were purchased from Nutritional Biochemicals. Adenosine 3',5'-monophosphate was the product of Sigma Chemical Company. Fructose 6-phosphate and the enzymes aldolase, triosphosphate isomerase and α-glycerophosphate dehydrogenase were purchased from Boehringer and Sons through Calbiochem.

PHOSPHOGLUCOMUTASE ASSAY SYSTEM AND DEFINITION OF UNIT

The activity of enzyme preparations was measured by the conversion of the acid-labile phosphate of the 1-ester to the acid-stable phosphate of the 6-ester. The method was essentially that of Najjar (18). For standard assay, an appropriate aliquot of the enzyme was added to a reaction mixture consisting of 4 mM glucose 1-phosphate containing sufficient glucose 1,6-diphosphate, 6 to 10 mM magnesium acetate, 40 mM imidazole, pH 7.5, 50 mM Tris-HCl buffer, pH 7.5, in a total volume of 1.0 ml and at 30°. After a 10 minute incubation at 30° the reaction was stopped by the addition of 1.0 ml of 5N H2SO4. Water was then added to bring the volume to 5.0 ml. The reaction tubes were immersed in a boiling water bath for 3 minutes and then
rapidly cooled. The inorganic phosphate liberated during acid hydrolysis (equivalent to unchanged 1-ester) was estimated by the method of Fiske and Subbarow (42). Color intensity was read in the Klett-Sommerson colorimeter, using the 660 μ (red) filter. The acid-stable phosphate, representing the amount of glucose 6-phosphate formed, was calculated from the difference in readily hydrolyzable phosphate before and after incubation.

Protein was estimated by the spectrophotometric method of Warburg and Christian (43) or by the modified biuret reaction of Gornall et al (44).

The unit of activity is defined as that amount of enzyme which catalyzes the formation of 1 μmole of glucose 6-phosphate in 10 minutes when assayed under conditions whereby the course of the reaction is linear throughout the reaction period.

The specific activity was defined as the number of units per mg of protein.

**ACTIVATION OF PHOSPHOGLUCOMUTASE**

Maximally activated enzyme was prepared by the method of Najjar (8). Enzyme was preincubated at 0° with 1 mM Mg++ and 40 mM imidazole, pH 7.5, for 15 minutes prior to the start of the reaction. A suitable aliquot was then added at zero time to the reaction mixture described.

**PURIFICATION OF PHOSPHOGLUCOMUTASE FROM BEEF BRAIN**

Beef brain was obtained immediately after slaughter and transported in ice to the laboratory. The cerebral lobes were removed and the remainder discarded. The tissue was stripped of its outer membranes and surface blood vessels. Tissue which was not to be used immediately was then frozen in powdered dry ice and stored in the deepfreeze.
Preparation of extract. 140 g of fresh or fresh-frozen brain were cut into small pieces and placed in the 400 ml cup of the Servall Omni mixer with 280 ml of 0.02 M Tris-HCl, pH 7.0. A creamy homogenate was obtained by homogenizing at full velocity for 2 one-minute periods, allowing an interval of 1 minute chilling in the ice-water bath. The homogenate was centrifuged for 45 minutes at 65,000 x g, 0°, in the Spinco Ultracentrifuge, Model L, using the #30 rotor. The clear, red supernatant fluid was removed and the precipitate discarded.

Heat treatment. The crude supernatant solution was adjusted to pH 5.0 by the cautious addition of 12 N acetic acid. The suspension was allowed to stand for 45 minutes at 0°. The precipitate was removed by centrifugation for 20 minutes at 20,000 x g, 0°, and discarded. The supernatant fluid was transferred to a stainless steel beaker, brought to 60° by heating in an 85° water bath and then chilled to 4° in an ice-water bath. Constant vigorous stirring was maintained throughout the heating and cooling procedures. The resulting suspension was centrifuged for 20 minutes at 20,000 x g, 0°, and the precipitate discarded.

First Ammonium sulfate fractionation. The heat-treated supernatant solution was brought to 40% saturation at 0° by the gradual addition of powdered ammonium sulfate (28.2 g per 100 ml) with efficient mechanical stirring. The suspension was allowed to equilibrate for 20 to 30 minutes at 0° with gentle stirring. It was centrifuged for 20 minutes at 20,000 x g, 0°, and the precipitate was discarded. The addition of ammonium sulfate (14.2 g per 100 ml of original heat-treated supernatant) was continued until the solution reached a saturation of 60% at 0°. Equilibration and centrifugation were carried out as before and the precipitate was dissolved in a
minimal volume of 0.05 M acetate, pH 5.0 (8-10 ml, final volume). The reddish and faintly turbid fraction was dialyzed 3 to 4 hours against 2 changes of 1 liter each of the same buffer. Following dialysis, the flocculent precipitate was removed by centrifugation for 10 minutes at full velocity, 0°, in the Clay-Adams table centrifuge, and discarded.

**Treatment with calcium phosphate gel.** The dialyzed solution was diluted with 0.05 M acetate, pH 5.0, to obtain a protein concentration of 8 mg per ml. Sufficient calcium phosphate gel (dry weight, 20 mg per ml) was added to provide a protein/gel ratio of 10/15. Stirring was continued for 10 minutes at 0° and the suspension centrifuged at 20,000 x g, 0°, for 20 minutes. The precipitate was discarded.

**Second ammonium sulfate fractionation.** The clear, faintly straw-coloured supernatant solution was brought to 45% saturation by dropwise addition of a saturated (at 0°), unbuffered solution of ammonium sulfate with efficient stirring. After an equilibration period of 45 minutes, the precipitate, if any, was removed by centrifugation. The supernatant fluid was dialyzed against 2 liters of a 50% saturated solution of ammonium sulfate at 0° for 4 hours. The suspension was centrifuged at 20,000 x g, 0°, for 20 minutes. The precipitate was dissolved in a small volume of 0.05 M acetate, pH 5.0 (approximately 3 ml) and dialyzed for 4 hours against 2 changes of 1 liter each of the same buffer to give a clear, almost colourless solution of the enzyme.

**PHOSPHOFRUCTOKINASE ASSAY SYSTEM AND DEFINITION OF UNIT**

Phosphofructokinase activity was measured by determining the amount of fructose diphosphate formed by the enzyme. This was carried out at 25°
to $25^\circ$ in a reaction volume of 1.5 ml in a Beckman DU spectrophotometer through a light path of 0.5 cm. The enzyme was coupled with aldolase, triosphosphate isomerase and glycerophosphate dehydrogenase systems. The rate of disappearance at DPNH was followed at a wavelength of 340 mp for 10 minutes.

The reaction conditions used for determining maximal enzyme activity were essentially those of Mansour (12). The reaction mixture had a final concentration of glycylglycine buffer, 50 mM, pH 8.2; egg albumin, 0.013%; cysteine, 15 mM, pH 8.2; MgCl$_2$, 1 mM; fructose 6-phosphate 1 mM; ATP, 1 mM; DPNH, 0.5 mM; aldolase, 100 µg; triosphosphate isomerase and glycerophosphate dehydrogenase mixture, 10 µg. The "blank" contained DPNH, 0.25 mM; and all components except substrates and coupling enzymes.

The unit of phosphofructokinase activity is defined as that amount of enzyme which catalyzes the formation of 1 µmole of fructose diphosphate per minute.

**PREPARATION OF PHOSPHOFRACTOKINASE FROM RABBIT BRAIN**

A rabbit was deeply anesthetized with 300 mg of pentobarbital and immediately bled by severing the blood vessels of the neck. The whole brain was quickly removed and chilled in ice for a few minutes. A 4 to 5 g portion was homogenized in 4 volumes of cold, unbuffered 0.25 M sucrose solution at full velocity in the Omnimixer for 1 minute. The homogenate was centrifuged either at 20,000 x g for 20 minutes or at 65,000 x g for 45 minutes, $0^\circ$. The supernate was stored at $2^\circ$. The remainder of the brain tissue was rapidly frozen in powdered dry ice
and stored in the deepfreeze.

RESULTS

PURIFICATION OF PHOSPHOGLUCOMUTASE FROM BRAIN

Our first approach to a study of phosphoglucomutase was an attempt to achieve a high degree of purification of the enzyme from beef brain. Repeated attempts to adapt the method of Najjar (8) for the muscle enzyme to fractionation of brain tissue were unsuccessful. The enzyme lost considerable activity during the procedure and appeared to be less stable to heat than muscle phosphoglucomutase. The use of organic solvents (ethanol and acetone) resulted in a considerable loss of activity. Adsorption procedures employing alumina C y gel were also unsuccessful.

Satisfactory purification was achieved by the use of heat-treatment, salt fractionation and calcium phosphate gel adsorption (see under "Methods"). This procedure is summarized in the "flow-sheet" (Figure 2) and the data on the purification are shown in Table I. All attempts to crystallize the enzyme met with failure.

Table I

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Protein mg</th>
<th>Specific Activity Units/mg</th>
<th>Total Activity Units</th>
<th>Yield %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crude supernatant</td>
<td>4,000</td>
<td>4.8</td>
<td>17,500</td>
<td>&quot;100&quot;</td>
</tr>
<tr>
<td>Heat-treated supernatant</td>
<td>1,500</td>
<td>10</td>
<td>14,900</td>
<td>85</td>
</tr>
<tr>
<td>First (NH₄)₂SO₄, 40-60% sat.</td>
<td>215</td>
<td>51</td>
<td>10,900</td>
<td>62</td>
</tr>
<tr>
<td>Calcium phosphate gel supernatant</td>
<td>105</td>
<td>93</td>
<td>9,200</td>
<td>53</td>
</tr>
<tr>
<td>Second (NH₄)₂SO₄, 45-50% sat.</td>
<td>16</td>
<td>230</td>
<td>3,650</td>
<td>21</td>
</tr>
</tbody>
</table>
Extract

pH 5.0
To 60° and chill

P          S
(Discard)  (NH₄)₂ SO₄
40-60% saturation

P          S
Dissolved in  (Discard)
ph 5.0 buffer and
dialyzed

P          S
(Discard)
Ca₃(PO₄)₂ gel
protein/gel = 10/15

P          S
(Discard)
(NH₄)₂ SO₄
45-50% saturation

P          S
Dissolved in  (Discard)
ph 5.0 buffer and
dialyzed.

Final Fraction

Figure 2. Flow-sheet showing a summary of the phosphoglucomutase purification procedure. P represents precipitate and S is the supernatant solution.
Several attempts were made to determine the homogeneity of the purified enzyme. Electrophoresis on acrylamide gel gave some indication of a single protein component, but the results were inconclusive. Sedimentation in the Spinco, Model E, analytical ultracentrifuge was attempted. A sharp peak was not obtained, however, possibly as a result of protein aggregation in the presence of the relatively high (0.5 M) salt concentration. This was not repeated with a dialyzed preparation due to lack of time and partly due to the unavailability of the centrifuge when required.

Stability of Purified Phosphoglycomutase. The purified enzyme was stable for 2 weeks at 2° in 0.05 M acetate, pH 5.0, when the protein concentration was 3 to 5 mg per ml. The enzyme then gradually lost activity. In the presence of 0.5 M ammonium sulfate the activity declined much more rapidly, presumably as a result of protein aggregation. Increasing the protein concentration did not increase stability. The enzyme lost 80% of its activity after a single freezing and thawing. It was equally unstable at -15° in the presence of 50% glycerol.

It is pertinent to note that the crude extract was stable for only a few hours and could not tolerate freezing and thawing. None of the intermediary fractions retained full activity after a few days at 2°.

PROPERTIES OF PURIFIED PHOSPHOGLUCOMUTASE FROM BRAIN

Phosphoglucomutase from muscle and yeast has been extensively studied (8) (45) but little information is available on this enzyme from other sources. The kinetics of the mutase reaction and the strange activation properties of the enzyme have been studied almost exclusively in crystalline preparations. While it has been accepted by some workers
that activation of phosphoglucomutase by pre-incubation with Mg\textsuperscript{++} and a metal-binding agent involves the orientation of the "active site" of the enzyme, this phenomenon has aroused some interest regarding its physiological importance. Since these properties have not previously been examined in brain, experiments were designed to compare the purified enzyme from this tissue with that of muscle. It was felt that such a study would indicate whether or not there is a close resemblance between phosphoglucomutase of these two distinct tissues and thereby further our scant knowledge of the biochemistry of the brain.

**Enzyme Concentration.** Conditions for the reaction were chosen so that the reaction rate was directly proportional to the enzyme concentration in order to maintain zero order kinetics (Figure 3).

**Time Course.** In all experiments enzyme concentrations were selected to ensure that the course of the reaction was linear with respect to time throughout the 10 minute incubation period. The reaction always proceeded in a linear fashion up to the point where 55% of the 1-ester had been converted to 6-ester (Figure 4).

**Equilibrium of the Reaction.** The equilibrium of the phosphoglucomutase reaction was determined with the purified preparation. When equilibrium was reached at 30° the relative amounts of glucose 1-phosphate and glucose 6-phosphate were 6 and 94 per cent respectively. This agrees closely with the finding of Najjar (18) for the muscle enzyme.

**Effect of pH.** The effect of the hydrogen ion concentration on the activity of the enzyme is shown in Figure 5. The activity was maximal at pH 7.5. Above or below this optimal value there is a precipitous decline in
Figure 3. Effect of enzyme concentration on the rate of glucose 6-phosphate formation. Standard assay conditions were employed. The purified phosphoglucomutase preparation was used.
Figure 4. Time course for the phosphoglucomutase reaction. Standard assay conditions were employed. The purified phosphoglucomutase preparation was used.
Figure 5. Effect of pH on the activity of purified phosphoglucomutase under otherwise standard conditions.
activity. This decline is not due to denaturation of the enzyme during catalysis since it is stable for several hours at these pH values. The pH curve corresponds closely to that obtained for the enzyme from other sources.

**Mg**\(^{++}\) and Metal-binding Agent Requirements.** The purified phosphoglucomutase exhibited slight activity in the absence of Mg\(^{++}\) or a metal-binding agent even after 18 hour dialysis against several changes of buffer. This confirms Najjar's finding (18) that the crystalline muscle enzyme is slightly active without added Mg\(^{++}\), probably as a result of traces of the ion in the assay components or bound to the protein. The optimum range of Mg\(^{++}\) concentration between 6 and 12 mM produced a 3-fold increase in activity (**Figure 6**).

Imidazole, histidine and cysteine have been the most widely used metal-binding agents for the phosphoglucomutase reaction, although numerous other agents have been used successfully (8) (20). Imidazole has been used in most recent studies and was used throughout this investigation.

When the dialyzed, purified enzyme was assayed in the absence of added Mg\(^{++}\) the addition of imidazole resulted in a further decrease in activity (**Figure 7**). In the presence of optimal Mg\(^{++}\) levels imidazole produced an increase in activity as high as 40-fold (**Figure 7**). This is consistent with the observations of Najjar (9).

**ACTIVATION OF BRAIN PHOSPHOGLUCOMUTASE BY MAGNESIUM AND IMIDAZOLE**

It has been demonstrated by Robinson and Najjar (9) that crystalline muscle phosphoglucomutase can be activated 2 to 5-fold by pre-incubation
Figure 6. Effect of Mg$^{++}$ on the activity of the exhaustively dialyzed, purified phosphoglucomutase in the absence of a metal-binding agent under otherwise standard conditions.
Figure 7. Effect of a metal-binding agent, imidazole, on the activity of exhaustively dialyzed, purified phosphoglucomutase in the absence of added Mg$^{++}$ (•—•) and in the presence of 1 mM Mg$^{++}$ (x—x), under otherwise standard conditions.
with Mg\(^{++}\) in the presence of imidazole or histidine in a manner dependent on time. They observed that no measurable activation occurred during catalytic activity. A Mg\(^{++}\)-coordination complex appeared to be the activating factor; neither Mg\(^{++}\) nor imidazole alone produced this activation. Differences in the Mg\(^{++}\) requirement for catalytic activity of the activated and non-activated enzymes were also demonstrated. Although this phenomenon could apparently be explained on the basis of the kinetics of the reaction mechanism, a possible role in the physiological regulation of the enzyme was also indicated.

In our experiments the purified brain phosphoglucomutase could be activated 1.5 to 2.5-fold by the procedure of Najjar (8). The activated enzyme had a pH curve (Figure 8) similar to that of the non-activated enzyme (cf. Figure 5) and exhibited maximal activity at pH 7.5.

The effects of pH on the degree of activation of the enzyme by Mg\(^{++}\) and imidazole have never been reported in the literature. Figure 9 demonstrates the effect on the catalytic activity of enzyme which has been activated by Mg\(^{++}\) and imidazole at various pH values. Activation appeared to be maximal at pH 7.5 to 9.

An important and perhaps physiologically significant feature of the activated phosphoglucomutase reported by Najjar (9) was the difference in its Mg\(^{++}\) requirement for catalytic activity as compared to that of the non-activated enzyme. We have observed some differences, in this respect, between the two forms of the brain phosphoglucomutase system (Figure 10). The activated enzyme has a fairly sharp optimum with a maximum at 6 mM Mg\(^{++}\), whereas the non-activated enzyme exhibits a
Figure 8. Effect of pH on the activity of purified phosphoglucomutase which had been "activated" by pre-incubating the enzyme at 0° for 15 minutes with 1 mM Mg++ and 40 mM imidazole, pH 7.5. Standard assay conditions were employed except that 6 mM instead of 1 mM Mg++ was used.
Figure 9. Effect of pH on the "activation" of phosphoglucomutase from brain by Mg$^{++}$ and imidazole. The purified enzyme was preincubated at 0° for 15 minutes and 1 mM Mg$^{++}$ and 40 mM imidazole at various hydrogen ion concentrations, and assayed under otherwise standard conditions.
Figure 10. Effect of various concentrations of Mg$^{++}$ on the catalytic activity of "activated" (●—●) and "non-activated" (x—x) phosphoglucomutase when assayed in the presence of 40 mM imidazole under otherwise standard conditions. The activation procedure was that described under "Methods".
broader optimum for Mg$^{++}$ in the range of 10 to 20 mM. Robinson and Najjar (9) reported a sharp peak with a maximum at 60 mM for the non-activated form as compared to a very broad peak with a maximum near 4 mM for the activated enzyme. Further, at the level of maximal activity of the activated brain enzyme, the non-activated form shows suboptimal catalytic activity, although this effect is slight as compared to that described by Najjar. Some inhibition of the activated enzyme at levels of Mg$^{++}$ which are optimal for the non-activated form was also observed, although this effect again was not profound.

No activation was observed during enzyme catalysis, as is evident from the time course (cf. Figure 4) in which no autocatalytic response could be demonstrated.

**OBSERVATIONS ON PHOSPHOGLUCOMUTASE IN CRUDE EXTRACTS OF BRAIN**

It seemed worthwhile to compare the activation of a crude enzyme preparation with that observed in the purified system, since this approach could feasibly point to the physiological significance of the activation phenomenon.

Pre-incubation of the crude extract with Mg$^{++}$ and imidazole under conditions identical to those applied to the purified enzyme produced a mere 15% activation, and would appear to be of little importance.

The enzyme is only slightly active in the absence of Mg$^{++}$ or imidazole and is stimulated 3-fold by the addition of Mg$^{++}$ alone. The presence of optimal concentrations of both the cation and the metal-binding agent, similar to those required by purified mutase, results in a 25-fold increase in activity (Figure 11). The crude brain phosphoglucomutase preparation therefore appears to be sufficiently active
Figure 11. Effect of Mg$^{++}$ on phosphoglucomutase activity in a crude brain extract, in the presence (----) and absence (x-x) of imidazole. The crude extract was prepared as described under "Methods". The conditions of the reaction were those of the standard assay.
without the special procedures required to produce the activation phenomenon.

PHOSPHOFRUCTOKINASE FROM RABBIT BRAIN: PRELIMINARY EXPERIMENTS

Phosphofructokinase is currently receiving wide attention as a result of recent observations that the complex kinetic properties of this enzyme provide a reasonable explanation for the control of glycolysis beyond fructose 6-phosphate as well as the levels of substrates in the citric acid cycle. Mansour (12) has reported on studies on heart phosphofructokinase in which he showed that the enzyme can be strongly inhibited by ATP in concentrations which may well exist in the cell, and that this inhibition may be counteracted by the other substrate and both products, as well as by 5'-AMP, cyclic 3',5'-AMP, ADP and P_i. Increases in activity of 10 or 20-fold are produced by small increases in these compounds. These results have been confirmed for skeletal muscle phosphofructokinase (11) and purified sheep brain enzyme (14). It has also been reported that citrate at physiological levels inhibits the enzyme from muscle (13), heart (46), brain and liver (14), and that the degree of inhibition is increased by ATP and decreased by fructose 6-phosphate. No less sensational has been the recent discovery of Vinuela et al (16) that yeast phosphofructokinase, which possesses many of these kinetic properties, exists as two enzymatically interconvertible forms with different sensitivities to end-product inhibition.

In view of the latter observation, a preliminary investigation was undertaken to examine phosphofructokinase in rabbit brain in an effort to determine whether or not the complex kinetics reported for mammalian systems could be explained on the basis of a multi-enzyme system.
Enzyme Concentration and Time Course. The crude extract described under "Methods" was used in all these experiments. The conditions of the reaction were chosen so that the reaction rate was directly proportional to the enzyme concentration. The enzyme showed a variable degree of self-activation in the first few minutes of the reaction (Figure 12). This has also been observed by Parmeggiani and Bowman (13). The reaction rate was linear with respect to time in the range of 3 to 10 minutes.

DPNH Oxidase Activity. The oxidation of DPNH in the absence of substrate was low. The small amount of oxidase activity could be compensated for by the presence of DPNH in the "blank".

Stability of Brain Extract. The crude brain extract retained full phosphofructokinase activity for 3 days at 2° and pH 6.8 to 7. A single freezing and thawing of the extract resulted in a 50% loss of activity.

Effect of pH on Phosphofructokinase Activity. The activity of the enzyme was tested at different hydrogen ion concentrations in the presence of substrate levels described as optimal by Mansour (12). Under these conditions enzyme activity was maximal at pH 8.2 to 9.0 (Figure 13), in accordance with the observations of Mansour.

Effect of ATP. The effect of varying the ATP concentration under otherwise optimal conditions is shown in Figure 14. At pH 8.2 and with the substrate concentrations described the optimal ATP concentration was in the range of 1 to 2 mM, while 8 to 10 mM ATP resulted in a 2-fold inhibition of the enzyme. Similar results were obtained by Parmeggiani and Bowman (13).

The inhibition of purified phosphofructokinase by ATP has been shown to be critically dependent on the concentration of F6P (11) (12) and more
Figure 12. Time course for the phosphofructokinase reaction. The reaction was carried out at pH 8.2 under the conditions of the standard assay.
Figure 13. Effect of pH on the activity of brain phosphofructokinase. Enzyme activity was measured spectrophotometrically as described under "Methods", in the presence of F6P (1 mM), ATP (1 mM), Mg++ (1 mM) and a suitable buffer (50 mM).
Figure 14. Effect of ATP on phosphofructokinase activity in crude brain extract. The standard conditions described under "Methods" were used in the assay.
readily detectable at pH values close to neutrality. In view of this observation the effects of ATP were determined in the presence of a more physiological F6P concentration (0.2 mM) at pH 7.0. Maximal activity was obtained under these conditions when the ATP concentration was 1 to 2 mM. A 2.5-fold inhibition was observed in the presence of 10 mM ATP (cf. Figure 14). This effect is much less profound than that reported by Mansour, who observed a 20-fold inhibition by 2 mM ATP under similar conditions using a purified heart enzyme. These experiments were repeated with F6P levels as low as 0.06 mM. In all instances the nature of the ATP curve remained unchanged.

Similar experiments were performed with an extract that had been dialyzed 15 hours against 0.01 M Tris buffer, pH 7.5, containing 0.1 mM EDTA, as described by Vinuela et al (16). The results were virtually the same. Similarly, the preparation of an extract by homogenization of the tissue in the presence of 50 mM EDTA did not alter the response of the enzyme to ATP.

It is pertinent to mention at this point that Passonneau and Lowry (11) found the most active stimulator of phosphofructokinase to be the product fructose diphosphate (FDP) itself. As little as 0.001 mM FDP tripled activity at an inhibiting level of ATP (1 mM) while at high ATP levels (4 mM) FDP was ineffective even at high concentrations. This fact emphasizes the need for very low reaction rates to maintain a low steady-state level of the product, a condition which may not be met in our experiments.

Effect of Cyclic 3',5'-AMP. Mansour (12) has reported that cyclic 3',5'-AMP is a potent stimulator of phosphofructokinase activity in crude as
well as purified heart preparations under carefully defined conditions. These conditions required the presence of inhibitory concentrations of ATP, a pH of 6.9 and low F6P levels. Similar observations were described earlier by Passonneau and Lowry, using purified muscle phosphofructokinase (11). In a subsequent communication these authors reported that cyclic 3',5'-AMP acts chiefly by enhancing phosphofructokinase activity at all ATP levels (14).

In our preliminary investigations of the enzyme in rabbit brain we were unable to show appreciable stimulation by this nucleotide (0.1 mM) under conditions resembling those described by Passonneau, namely, in the presence of 0.2 mM F6P, 2 mM ATP, pH 7.0. Caffeine (0.5 mM) was also included in our experiments to prevent any hydrolysis of cyclic 3',5'-AMP by the brain diesterase. Similar results were obtained when these experiments were repeated with lower F6P levels (0.04 mM).

**Effect of Fluoride.** Vinuela et al (16) have recently reported the identification of an ATP-sensitive and an ATP-insensitive form of yeast phosphofructokinase. When a crude yeast extract was incubated in the presence of fluoride, the sensitivity of the enzyme to inhibition by ATP disappeared. This "activation" could be reversed by EDTA or dialysis. The authors suggested an enzymatic conversion of the ATP-sensitive to the less sensitive form of phosphofructokinase in a manner reminiscent of the phosphorylase phosphatase reaction. Further, cyclic 3',5'-AMP appeared to stimulate enzymatic reversal of this conversion.

A single attempt was made to determine the effect of fluoride on the crude rabbit brain extract (Table II) using conditions similar to those described by Vinuela et al. Under these conditions fluoride was
completely ineffective in altering the control values.

Table II

Effect of Fluoride on Brain Phosphofructokinase

A fresh extract was prepared as described under "Methods". 1 ml of the extract was incubated at room temperature for 20 minutes with and without sodium fluoride. Phosphofructokinase activity was assayed under standard conditions for optimal activity. Activity is expressed as μmoles of fructose diphosphate formed per minute.

<table>
<thead>
<tr>
<th>Preincubation Additions</th>
<th>Phosphofructokinase Activity</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1 mM ATP</td>
</tr>
<tr>
<td>None</td>
<td>22</td>
</tr>
<tr>
<td>20 mM NaF</td>
<td>19.8</td>
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</tbody>
</table>

DISCUSSION

PHOSPHOGLUCOMUTASE

The experiments reported in this thesis indicate that the general properties of phosphoglucomutase from brain are quite similar to those possessed by the muscle enzyme. Considerable effort was expended in the direction of purification of the brain enzyme and a successful procedure was developed. However, all attempts to crystallize the enzyme were unsuccessful.

The approach used to study phosphoglucomutase was designed primarily to determine the effect of the activation phenomenon described by Robinson and Najjar (9) on the brain enzyme, rather than to study its
reaction kinetics and mechanism. Our interest in this aspect arose as a result of the possibility that the time-dependent stimulating effect of the Mg$^{++}$-metal-binding agent complex on phosphoglucomutase in the absence of substrate could have significance in the cellular control of hexose phosphate levels. The suggestion by Ellis (47) of hexose phosphate involvement in neural function appeared to emphasize the need for such a study in brain tissue.

The purified brain phosphoglucomutase can be activated approximately 2-fold by pre-incubation with Mg$^{++}$ and imidazole, but this effect is not as startling as the 5-fold activation achieved by Robinson and Najjar. The fact that a mere 15% activation of the enzyme in crude brain extract was demonstrated in our experiments would appear to minimize the importance of this response in physiological systems.

More interesting perhaps, in terms of mutase regulation, was the demonstration by Robinson of different Mg$^{++}$ requirements for catalytic activity of the activated and non-activated muscle enzyme. The importance of a system in which cations perform a regulatory role by activating or inhibiting an enzyme is evident in the discussion by Wyatt (48) of allosteric inhibitions involving cations. Our experiments showed, however, that differences in Mg$^{++}$ requirements of the activated and non-activated brain phosphoglucomutase were slight and likely of little importance.

While these studies were in progress, two reports appeared in the literature which reduced the possibility of metabolic control at the phosphoglucomutase step. Lowry and Passonneau (27) demonstrated that several steps of the glycolytic pathway in mouse brain, including the
phosphoglucomutase reaction, were always maintained near equilibrium. This, of course, would obviate the need for a control mechanism at these points. Very recently Craig and Larner (26) determined that phosphoglucomutase activity in diaphragm muscle was not influenced by epinephrine or insulin, the two key hormones involved in the control of glycogen metabolism.

PHOSPHOFRUCTOKINASE

Our preliminary experiments on phosphofructokinase indicate that this enzyme is quite active in rabbit brain extracts and that its properties under optimal conditions resemble those of the enzyme from heart (12) muscle (28) and yeast (40).

In order to observe the complex kinetics of phosphofructokinase in these systems it has been found necessary to approximate conditions as they might occur within the cell. In heart the control of phosphofructokinase activity by ATP, cyclic 3',5'-AMP and various other nucleotides was demonstrated to be critically dependent on suboptimal pH levels and low fructose 6-phosphate concentrations. The enzyme from muscle and sheep brain similarly required conditions which could exist in the cell, for these controlling factors to be observed. In addition, these reports emphasized the importance of low reaction rates, in order to maintain low steady-state levels of fructose diphosphate, a potent antagonist of various inhibitors.

Our preliminary attempts to simulate physiological conditions were unsuccessful, partly as a result of the technical problems involved in measuring low reaction rates spectrophotometrically and partly because
of the limited time available. An attempt to demonstrate interconvertible forms of phosphofructokinase, as described by Vinuela et al (16), failed to show that these do exist and it cannot be concluded that the control of phosphofructokinase activity in brain is solely dependent on the kinetic properties of the enzyme. A careful study of the factors which may be involved in such a regulatory mechanism is indicated.
BIBLIOGRAPHY


3. Advances in Enzyme Regulation, Volumes 1 and 2, 1963-64.


