The Activation of Phosphoglucomutase by Denaturing Agents, Urea, Guanidine Hydrochloride, and Heat*

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ABSTRACT: Phosphoglucomutase (PGM) in crystalline preparations or in fresh muscle extract was activated severalfold by prior exposure to urea, guanidine hydrochloride, or heat. The resulting increase in catalytic activity was greater in samples having lower specific activity than in those with higher specific activity. The maximum activation attainable in each case approached similar levels which approximate the values obtained by activation with magnesium–imidazole. The rate of activation observed in urea was faster at higher than at lower concentrations of enzyme. This was not true with guanidine hydrochloride. No difference in the energy of activation of the enzyme was obtained between the activated and nonactivated state.

It has been repeatedly demonstrated that in the absence of substrate the enzyme phosphoglucomutase (PGM)[1] can be activated to a considerable extent, up to sixfold, by very short preincubation with magnesium and a metal-complexing agent such as histidine, imidazole, and cysteine (Robinson and Najjar, 1960, 1961; Najjar, 1962).

A detailed study of the mechanism of activation with magnesium and imidazole revealed that the activation resulted from the interaction of PGM with the complex composed of one atom of magnesium per imidazole. The rate and extent of activation depended on the concentration of this complex. Neither alone effected any significant measure of activation (Robinson *et al.*, 1965; Harshman *et al.*, 1965). It has also been demonstrated that EDTA can activate the enzyme (Milstein, 1960). While the latter may well result from the removal of inhibitory metals, the activation by magnesium–imidazole complex was shown not to be affected by prior dialysis with EDTA (Harshman *et al.*, 1965). Based on these findings, it was suggested that the activation by the magnesium complexes was the result of a subtle change in conformation of the enzyme at the active site. Consequently, various means known to effect a change in conformation of the protein were studied. PGM was subjected to high hydrogen ion and hydroxyl ion concentrations (Bocchini *et al.*, 1964; Harshman *et al.*, 1964), and in this communication urea and guanidinium treatment prior to the addition of substrate, all of which produced considerable increase in the catalytic activity of the enzyme. All this supports the conclusion that the process of activation involves a change in conformation of the molecule that results in the increase of the turnover rate of the enzyme. Because activation was obtained in extracts of fresh or frozen muscle at magnitudes similar to those observed with the crystalline enzyme, the suggestion was made that the process is not an artifact of isolation but may represent a physiological regulatory mechanism (Harshman *et al.*, 1965). Of interest in this connection is the recent observation by Hashimoto *et al.* (1967), showing that activation *in vivo* can be brought about rapidly with the injection of insulin to rats. The enzyme assayed under these conditions displays the same level of activity obtained by the *in vitro* activation of the enzyme in the nontreated controls. This *in vivo* activated enzyme cannot be further activated *in vitro* by magnesium–histidine.

This paper presents a detailed study of the activation of PGM by urea and guanidine hydrochloride and demonstrates the variable extent of activation in numerous preparations. This remarkable and consistent difference may well result from the varying physiological state of the animals at the time of isolation (Harshman *et al.*, 1965; Hashimoto *et al.*, 1967).

Experimental Procedure

Crystalline PGM was prepared from frozen rabbit muscle (Pel Freez, Rogers, Ark.) as described previously (Najjar, 1948, 1962). Each of the preparations used in this study was shown to be homogenous by ultracentrifugation and paper electrophoresis in Veronal buffer at pH 8.4. The protein concentration was estimated by optical density measurement at 278 mμ using an extinction of 0.77/mg (Najjar, 1948). All the chemicals were of reagent grade. Glucose 1-phosphate

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[1] Abbreviations used: PGM, phosphoglucomutase; G-1-P, glucose 1-phosphate; G-1,6-P, glucose 1,6-diphosphate; G-6-P, glucose 6-phosphate.

(G-1-P) (Nutritional Biochemical Corp., Cleveland, Ohio) contained sufficient amounts of glucose 1,6-diphosphate (G-1,6-P) to saturate the enzyme during the conditions of assay. Urea (Mallinckrodt Chemical Works Co., St. Louis, Mo.) and imidazole (Aldrich Chemical Co., Milwaukee, Wis.) were both recrystallized either from 70% ethanol or demineralized water. Solutions (10 M) of recrystallized urea contained less than 2×10^{-3} M cyanate as assayed according to Werner (1923). Guanidine hydrochloride (Eastman, Rochester, N. Y.) and potassium cyanate (Fisher Scientific Co., St. Louis, Mo.) were used without further purification. Aqueous solutions of both urea and guanidine hydrochloride were prepared just before use. Enzyme activity was assayed by the usual standard method (Najjar, 1948) in a reaction mixture containing 5×10^{-3} M G-1-P, 4×10^{-2} M imidazole, 1×10^{-3} M $MgCl_2$, and 2×10^{-3} M Mg^{2+}–EDTA (pH 7.4) and at 30° unless otherwise indicated. Enzymatic activity is expressed as micromoles of glucose 6-phosphate (G-6-P) formed per minute per milligram of enzyme at 30°. Optical rotation measurements were made with the Rudolph recording spectropolarimeter Model 260/65802/810.609 equipped with a 450-w xenon lamp. In all measurements the cell housing was cooled by circulating ice-cold water and the temperature was kept at 5 ± 1°. A cell with a light path of 1 cm was used in all the experiments.

Results

The Activation of Crystalline Phosphoglucomutase by Urea and Guanidine Hydrochloride. All experiments were carried out at 0° at enzyme concentrations of 3.76–3.92 mg/ml. An aliquot was removed at various intervals after exposure to the denaturing reagent and immediately added to the reaction mixture for assay of catalytic activity. In this process the reagent was diluted 400–2000-fold, far too low to affect the activity of the enzyme.

Urea at 1–2 M concentrations showed little or no activation of PGM. However, at 3 M a slow activation occurred reaching a maximum of 1.7 times the activity of the control. Figure 1 shows the rate of activation at 4–7 M concentrations. It is apparent that with increased urea concentration, the rate of activation was correspondingly increased. The maximum level of activation was approximately the same in each case 2.1–2.5-fold. This was attained at 0.25 min with 7 M and 8 min with 4 M urea. The rate of decay of enzymatic activity also increased with increasing concentration of urea. After exposure for 15 min, the activity of the enzyme declined to levels below the control value except at 4 M urea where activation was maintained well above the control sample, even after 20 min. The decay of activity cannot be reversed either by dilution or dialysis of the urea at 0 or 23°.

Guanidine hydrochloride exhibited essentially the same general type of activation except that the maximum level attained was considerably higher; four to five times the control values. The highest value, over

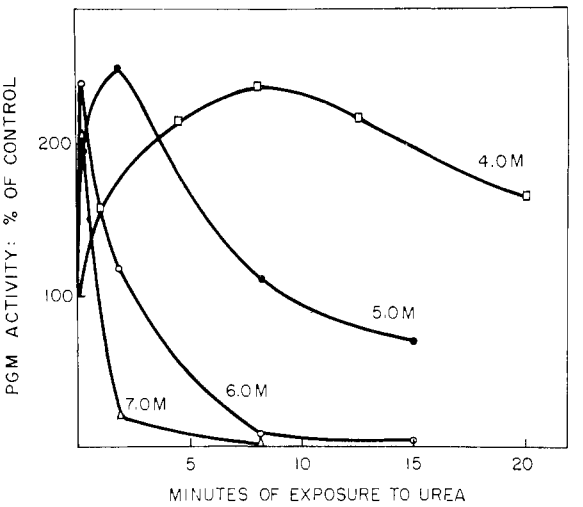

FIGURE 1: The activating effect of urea on crystalline PGM. The crystalline enzyme was obtained from 60% ammonium sulfate suspension by centrifugation and dissolved in a minimal volume of demineralized distilled water. It was then added to aqueous solutions of urea at 0° at a final concentration of 3.76 mg/ml in the desired molarity of urea. At the indicated time intervals, aliquots were assayed for catalytic activity. In this process, the enzyme and urea were diluted 400-fold. The resulting concentration of urea does not influence the enzymatic activity, inasmuch as 1 M urea fails to show an effect. Control aliquots of the enzyme were prepared in water at 3.76 mg/ml and 0°. These were assayed in the same reaction mixture containing the same amounts of urea and guanidine hydrochloride as in the experimental samples. The untreated native enzyme had a specific activity of 63.

fivefold activation, was attained at 1 and 2 M concentration. At 0.5 M a twofold activation resulted. Again the rate of decay of catalytic activity was pronounced only at high concentrations. However, unlike urea, the effect of guanidine hydrochloride was more salutory. It produced a higher level of activation as a consequence of the slower rate of decay. These results are shown in Figure 2. Only 3 M concentrations showed a rapid rate of decay and at 4 M the enzyme was completely inactivated after 15 sec. Exposure of the enzyme for 15 sec to guanidine effects maximum activation at 2 M at which concentration little or no activation with urea is observed.

The Possible Relationship of Activation to Tertiary Structure. A similar comparative study of the effect of urea and guanidine hydrochloride on the optical rotation of the enzyme was made at a fixed wavelength of 300 mμ and fixed reaction times of 1.4 and 45 min at variable concentrations of the denaturing agents 1–6 M. Figure 3 is illustrative of the data obtained. The increase in the negative rotation with concentration observed with each agent parallels the increase in activation of the enzyme. Thus the enzyme

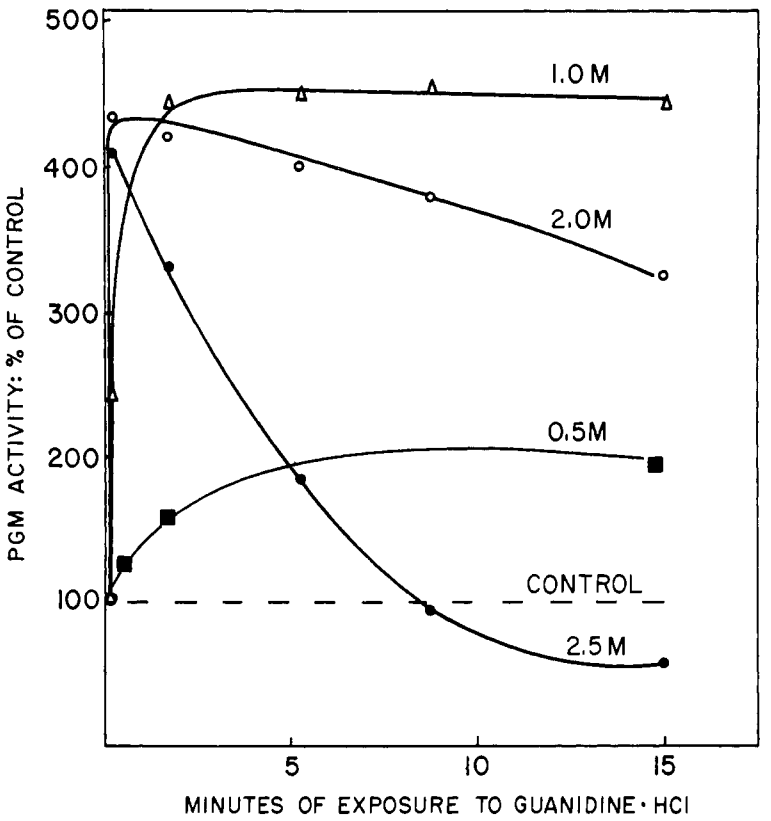

FIGURE 2: The activating effect of guanidine hydrochloride on crystalline PGM. The same enzyme preparation and procedure as in Figure 1 were used. The final concentration of PGM was 3.92 mg/ml at the molar concentrations of the guanidinium salt indicated in the figure. The final dilution of the enzyme and guanidine hydrochloride for activity assay was 2000-fold.

which is readily activated at low concentrations of the guanidinium compound also displays a change in conformation at these concentrations. On the other hand, urea activates PGM only at higher concentrations and affects its conformation only at these high concentrations.

A comparison of the activation patterns in Figures 1 and 2 with those of the rotation in Figure 3, reveals that in the case of guanidine, the process of activation is a good deal more sensitive to the effect of this agent than the change in optical rotation. At 1 M no rotational change was detectable even after 45 min, while activation was pronounced after 15 sec. Further, at 2 M activation reached maximal values after 15 sec while the increase in rotation was well below half maximal after 85 sec. In view of these results it became desirable to perform simultaneous kinetic studies of both parameters concurrently.

Figure 4 shows the effect of 1 and 2 M guanidine hydrochloride on the change in the optical rotation with simultaneous measurements of the activation of the enzyme. Values are given as per cent of the control enzyme. After 1 min in 2 M there was a definite and rapid increase of levorotation measured at 578 mμ with a simultaneous rapid increase in the activation of the enzyme. This was followed by a slow increase in the rotation to a value approaching 1.7 times the control in about 40 min. During this time, there was a rapid decay in the activated state of the enzyme. At 1 M guanidine hydrochloride, the change in optical rotation was hardly measurable before the lapse of 10 min and even at 40 min did not approach the level attained after 1 min in 2 M. Here again, the rapid increase in the state of activation was followed by a definite decay paralleling the slow increase in levorotation. It is reasonable to assume that activation of PGM is the result of a change in conformation of the enzyme. That a limited region or a limited extent of conformational change is needed for activation, is suggested by the two distinct slopes in the levorotation curve denoting two types of conformational change; a rapid and slow change. The rapid rate of levorotation occurs simultaneously with the rapid increase in activation. No such biphasic rate is observed at 1 M guanidine hydrochloride. In view of the limited magnitude of the rotation at this concentration any change in rate would not be detected. It is noteworthy that at this concentration activation is slower and its maximal value is lower than is observed at 2 M. There is little doubt however that the decay in the level of activation is related to the increased unfolding of the enzyme molecule.

The Activation of PGM in Fresh Muscle Extract by

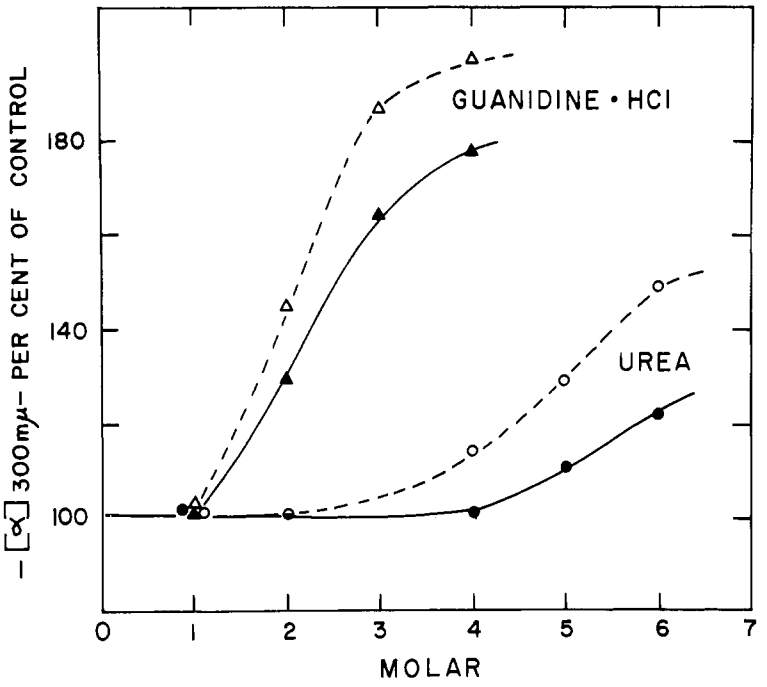

FIGURE 3: The effect of various concentrations of urea and guanidine hydrochloride on the optical rotation of crystalline PGM at 300 mμ. The enzyme concentrations used were 4.10 mg/ml in the urea solutions and 5.73 mg/ml in the guanidine hydrochloride solutions at the indicated molarities. Measurements were recorded 1.4 (———) and 45 min (– – – –) after the addition of the enzyme. Temperature at 5 ± 1°.

Urea and Guanidine Hydrochloride. The activation of crystalline PGM by Mg complexes, extreme pH levels, urea, and guanidine hydrochloride raises the obvious question as to whether the isolation procedure alters the enzyme in such a way as to confer upon it the property of activation. This question was answered earlier with respect to the activation by magnesium–histidine. It was shown at the time that fresh muscle extracts obtained within a few minutes after the animal is sacrificed, indeed show considerable activation to the maximal values obtained with the crystalline enzyme (Najjar, 1962). It was important therefore to study this aspect of the activation phenomenon with urea and guanidine hydrochloride. Fresh muscle was extracted with cold demineralized distilled water at 4–6° and adjusted to pH 5.0 (Najjar, 1948). Incubation at 0° in 1 M guanidine hydrochloride or 5 M urea was performed as soon as possible and sampled for activity assay at various time intervals. Quantitatively as well as qualitatively the resulting activation was indistinguishable from that observed with the isolated crystalline enzyme.

The Effect of Cyanate on the Activation of PGM. Cyanate is known to be a contaminant in urea solutions, formed as an intermediate in the hydrolysis of urea to ammonium carbonate (Warner, 1942). A freshly prepared 8 M solution contains less than 1×10^{-3} M cyanate. This increases to about 2×10^{-2} M after heating for 1 hr at 100° (Stark *et al.*, 1960). For that reason and in order to avoid this possible complication in our urea studies, we have used freshly recrystallized urea throughout, such that 10 M solutions contained less than 2×10^{-3} M cyanate. Nevertheless we studied the possible effect of cyanate on the activation of the enzyme because of its reactivity with functional groups of proteins (Stark, 1964, 1965a,b). Table I shows representative data in this regard. The effect of cyanate was studied in the presence and absence of urea. Again the enzyme was added to prepared solutions of one or both reagents at 0° and samples were obtained at intervals at which the activation process was incomplete and near complete, 15 and 75 sec, respectively.

It is evident that cyanate at 1 and 3×10^{-2} M shows no activating effect. However, at 3×10^{-2} M in conjunction with 5 M urea a definite increase in activation beyond what is exerted by urea alone is obtained. Whether cyanate increases the rate of unfolding of the molecule by reaction with unmasked functional groups or stabilizes a particular conformation, cannot be ascertained.

The Heat Activation of PGM. During the original isolation of the enzyme, it was noted that the activity tended to increase upon heating of enzyme solutions at pH 5.0 (Najjar, 1948). At the time it was assumed that a heat-sensitive inhibitor was inactivated. However, no dissociable inhibitor was detectable. In view of the effect of denaturing agents discussed above, the heat effect was deemed worthy of further investigation. Fresh muscle extract was prepared and adjusted

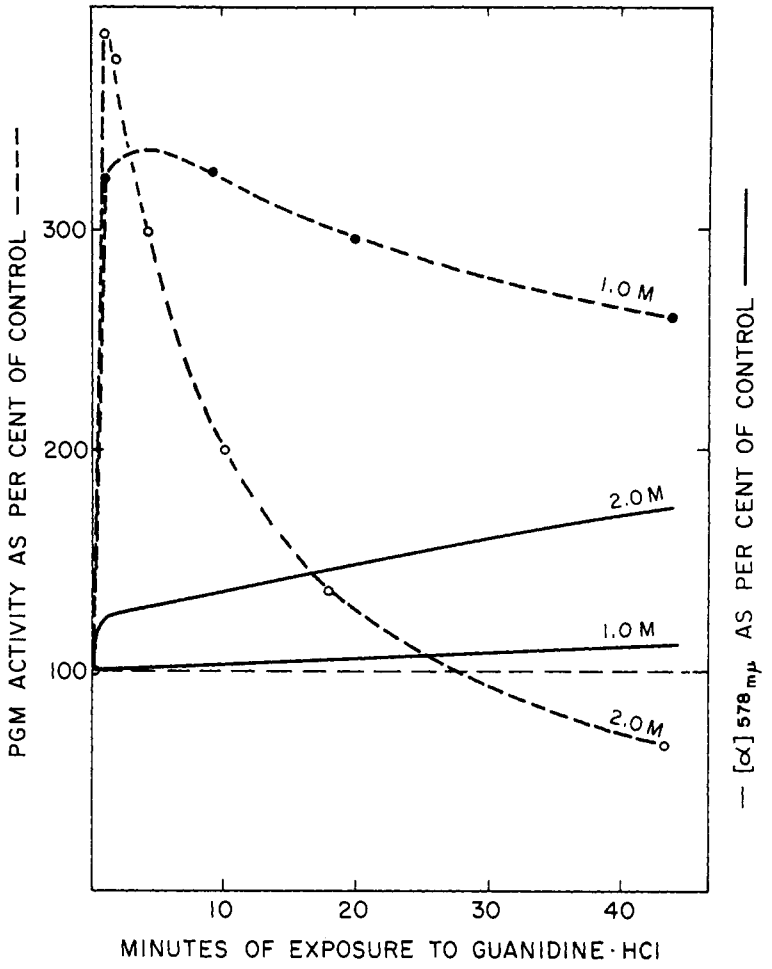

FIGURE 4: The extent of activation and optical rotatory change of crystalline PGM during exposure to guanidine hydrochloride. Optical rotation was recorded 1 min after the addition of enzyme which achieved a final concentration of 5.84 mg/ml in 1 or 2 M guanidine hydrochloride. Aliquots were removed directly from the open cell for enzymatic assay. Recorder sensitivity was set at 50 cm/deg of rotation and a speed of 1 cm/min. Temperature 5 ± 1°.

to pH 5.0 with acetic acid. The solution was placed in a water bath at 60° and identical aliquots were removed for direct assay at various intervals. The activities of the enzyme obtained are shown graphically in Figure 5. There is no doubt that heat causes an increase in the activity of the enzyme and like other denaturing agents, eventually exhibits an adverse effect on catalysis.

The Influence of Enzyme Concentration on the Response to Guanidine and Urea Activation. It is a common observation that in general proteins tend to maintain their native state in solutions at high concentration better than at low concentration. It would be expected therefore that this phenomenon would be operable at conditions where a denaturing agent is included in the solution. Because of the activation effect of these agents, we have studied the rate of activation and decay with guanidine hydrochloride and urea at two widely different concentrations of the enzyme.

Solutions of PGM (0.096 and 5.1 mg per ml) were prepared in 1 M guanidine hydrochloride and incubated at 0° for 15 min. Activity assays were made at various time intervals. Figure 6 shows that activation reaches completion in the dilute enzyme solution within 1 min, after which decay ensues. On the other hand, at the high concentration, PGM continues to increase in activity after the first minute of exposure, though slowly, to reach a higher activation level. No sign of decay appears even after 15 min of exposure. These observations are quite in accord with the expected behavior of proteins. However, such was not the case with urea.

Similar experiments with PGM concentrations of 0.053 mg and 6.7 mg per ml in 5 M urea showed quite unexpectedly the reverse effect. At the higher concentration, activation was very rapid reaching a maximum of 2.5-fold in 3 min, after which decay followed. At the low enzyme concentration, activation was very slow. It attained a 2.3-fold activation maximum after 15 min of exposure to urea, at which time the activation state of the concentrated enzyme had decayed to half the maximum level. This was repeatedly obtained

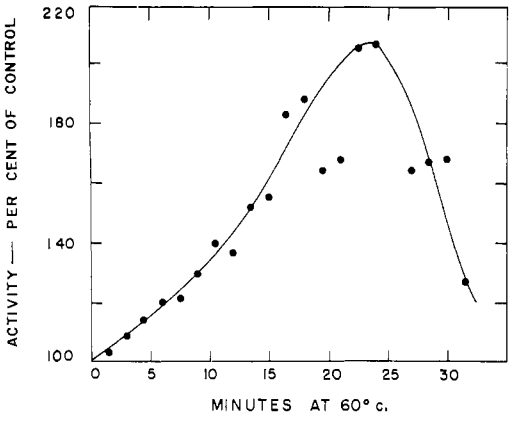

FIGURE 5: The activating effect of heat on PGM in fresh muscle extract. An extract of fresh rabbit muscle was prepared and adjusted to pH 5. It was diluted fivefold in demineralized distilled water and placed in a water bath at 60°. Aliquots were then taken for enzymatic assay at intervals of 90 sec.

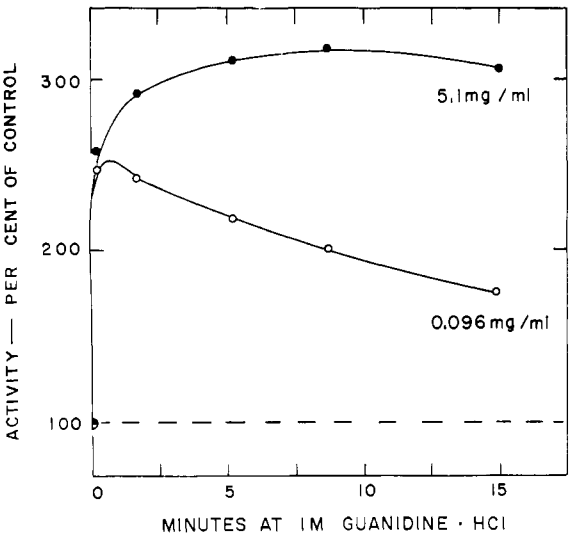

FIGURE 6: The effect of the concentration of crystalline PGM on the rate of activation by guanidine hydrochloride. Samples were assayed as usual with the appropriate dilutions in the reaction mixtures. For details of procedure, see Figure 1 and the text.

and intermediate rates were obtained with intermediate concentrations of 0.22 and 1.49 mg per ml. It is certain therefore that the rate of urea activation varies directly with the concentrations of the enzyme. These results are shown in Figure 7.

TABLE I: The Effect of Potassium Cyanate on the Activation of PGM in the Presence and Absence of Urea.[a]

Expt	Urea (M)	KCNO (M×10²)	Sp Act. of PGM: Time of Exposure (sec) 15	75
1			92	95
	5[b]		134	158
	5[b]	1	117	159
	5[b]	3	150	234
2			91	94
		3	94	96
	5		157	178
	5	3	220	271
3			97	95
		3	100	97
	5		135	170
	5	3	120	202

[a] The enzyme concentration ranged from 2.74 to 3.42 mg/ml. Following the transfer into the reaction mixture an 800-fold dilution was achieved. See Figure 1 for details of experimental conditions. Urea was recrystallized from 60% ethanol or water. [b] Recrystallized from demineralized water.

The correspondence between urea activation and denaturation of PGM suggests that denaturation follows a similar pattern relative to the enzyme concentration. Similar concentration effects were observed during studies of the denaturation of ovalbumin by urea (Simpson and Kauzman, 1953). With decreasing protein concentration, the half-time for maximal change in optical rotation increased. This was presumed to be due to the dependence on protein concentration of the activity coefficients of the protein in the native and activated state.

The Inverse Relationship between Specific Activity and Extent of Activation. It has been repeatedly observed in this laboratory that different preparations vary in the extent of activation with magnesium–imidazole (Robinson and Najjar, 1960, 1961). Two- to sixfold activation has been observed with different preparations. It was suggested that this may relate to the physiological state of the particular animal (Harshman *et al.*, 1965). During the course of this study with 5 M urea activation, several observations were noted indicating that activation appears to relate to the specific activity of the individual enzyme preparations on hand. A total of 12 crystalline enzyme preparations were analyzed. Catalytic activity of each sample was measured before and after exposure to urea for 2 min, at 0° and 3.7 mg/ml. The enzyme in these preparations was crystallized only once under identical conditions and stored in 0.60 saturation of ammonium sulfate at 4° for variable periods of time, 4–36 months. All samples were treated and assayed under identical conditions using the same stock solutions and buffers. Figure 8 depicts a definite inverse relationship between the per cent activation and the

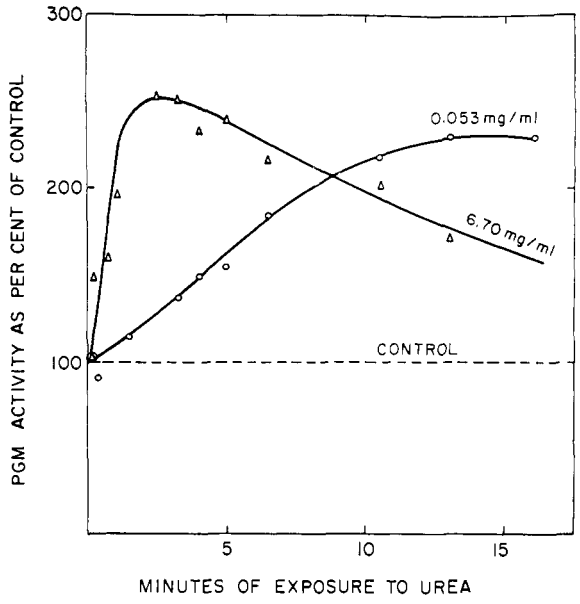

FIGURE 7: The effect of the concentration of crystalline PGM on the rate of activation by urea. Details as in Figures 1 and 6.

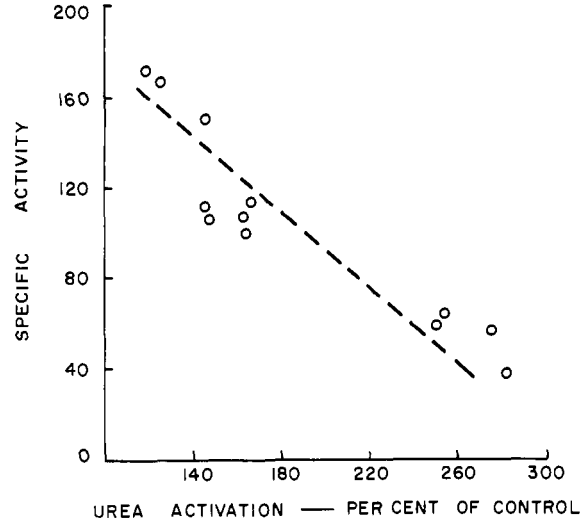

FIGURE 8: The existence of an inverse relationship between the specific activity and the extent of activation. Several crystalline preparations possessing different specific activities and stored in ammonium sulfate for various periods of time were assayed before and after exposure to 5 M urea for 105 sec in a concentration of 3.7 mg/ml at 0°. Samples were then removed for the assay of catalytic activity. In this figure the activity induced by urea, expressed as the per cent of the untreated control enzyme, is plotted against the specific activity of the untreated enzyme control. See text for details.

specific activity of the sample. Neither the specific activity nor the extent of activation showed any correlation with the age of the sample. It appears certain that the lower the specific activity of the enzyme preparation, the higher the extent of activation. This relationship is consistent with the possibility that the physiological state of the donor animal may be involved (Harshman *et al.*, 1965) and in line with the recent reported hormonal effects on such activation (Hashimoto *et al.*, 1967). It is noteworthy that the specific activity of the various preparations before urea treatment showed a considerable spread (37–171), around a 5:1 ratio between the maximum and minimum values. The specific activity after urea activation became more uniform (147–223) with a ratio of approximately 1.5. The possibility that different preparations are activated at different rates was ruled out by kinetic studies on 4 of the 12 preparations; two samples representing highly activatable preparations possessing low specific activity and two other samples representing high specific activities with low activation. These were exposed to 5 M urea and the time course of activation was followed for 15 min as usual. All showed substantially similar kinetics of activation as depicted in Figures 1 and 7.

The Energy of Activation of PGM before and after Exposure to Urea. The catalytic activity of the enzyme was measured before and 2 min after exposure to 5 M urea at 0, 10, 20, and 30°. Samples treated with urea were twice as active as the untreated controls at all temperatures. The energy of activation derived from an Arrhenius plot of the data gave a value of 15.7 kcal for the native enzyme and 15.1 kcal after urea treatment. The value for the native enzyme is in reasonable agreement with that reported earlier of 19.3 (Harshman *et al.*, 1965). It thus appears that activation by urea does not affect the energy of activation of the enzyme.

Discussion

The general effect of urea, guanidine hydrochloride, and heat on protein is to alter the tertiary structure through the rupture of the stabilizing bonds and unfolding of the molecule. All these agents are capable of denaturing phosphoglucomutase at extreme conditions as evidenced by increased levorotation and the destruction of its catalytic activity. However, in the early stages of this process or when these agents are applied at controlled or mild conditions, the enzyme becomes more catalytically active to a maximal level, over fivefold the rate obtained in the native untreated state. This activation was observed with several crystalline preparations as well as with fresh muscle extracts. It is quite unlikely, therefore, that the enzyme acquires this characteristic as a result of the purification procedure.

The activation observed with these three denaturing agents, in addition to other denaturing procedures reported earlier, such as controlled incubation at pH 2.5–3.5 and 9.5–10.5 (Harshman *et al.*, 1964), support

the conclusion that activation results from a change in its conformation which in some manner involves the active site to augment its catalytic efficiency to a considerable degree.

The activation by magnesium along with a complexing agent such as cysteine, histidine, and imidazole, displays many of the characteristics of this type of activation and reaches the same magnitude. We have found the K_m of glucose diphosphate for the magnesium–imidazole-activated enzyme to be 1.3×10^{-7} M as compared to 4.7×10^{-8} M for the same but nonactivated sample. This threefold increase in the concentration of the G-1,6-P that is required to attain half-maximal activity also denotes a conformational change at the active site which affects the dissociation of the G-1,6-P or its accessibility. It appears that such a change of conformation at the catalytic site does not and need not affect the energy of activation.

The possibility that denaturing agents such as urea and guanidine hydrochloride may well alter the state of aggregation of the enzyme and thereby influence the process of activation was investigated. Several sedimentation velocity measurements in the analytical ultracentrifuge were performed at activation conditions of temperature and concentration. Urea (4–6 M) and 1 M guanidine hydrochloride in the presence of varying concentrations of NaCl (1×10^{-3}–1×10^{-1} M) at 0° were used. Other than minimal quantities of denatured material, only one symmetrical peak was observed in all experiments. This had approximately the same sedimentation characteristics obtained for the native enzyme. The range of salt concentration that was used did not affect activation by either agent. These experiments are being verified and extended and will be the subject of a separate communication.

The wide variations in the level of activation of different preparations of this enzyme (Robinson and Najjar, 1960, 1961) prompted our earlier suggestion that this variation might relate to the physiological state of the donor animal (Harshman *et al.*, 1965). This has now been made more significant by the present demonstration that preparations with low specific activity undergo severalfold activation, while those with high specific activity show proportionally much less activation. However, in either case following activation, the enzyme approaches approximately the same maximal level of activity. That the physiological state of the donor animal influences the state of activation of phosphoglucomutase has since been demonstrated by the injection of insulin which results in near-maximal level of activation (Hashimoto *et al.*, 1967).

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