The Kinetic Properties of Human Erythrocyte Glucose 6-Phosphate Dehydrogenase*

Steven J. Soldin and Doris Balinsky

ABSTRACT: Erythrocyte glucose 6-phosphate dehydrogenase was purified 17,830-fold. The dissociation and Michaelis constants of the enzyme for nicotinamide–adenine dinucleotide phosphate (NADP) and glucose 6-phosphate (G-6-P) were determined at pH values between 5.7 and 10.0. Product inhibition studies using reduced nicotinamide–adenine dinucleotide phosphate (NADPH) as inhibitor were carried out at pH 8.0. The data obtained were consistent with the postulate that the mechanism of this enzyme is sequential. The mechanism would appear to be either a compulsory order mechanism or a rapid equilibrium, random order mechanism with a dead-end enzyme–glucose 6-phosphate–NADPH complex. Logarithmic plots of V_f, V_f/K_a', V_f/K_b', and K_a *vs.* pH (where V_f is the maximum velocity of the reaction, K_a' and K_b' are the Michaelis constants for NADP and G-6-P respectively, and K_a is the dissociation constant of the enzyme–NADP complex) indicate the possible participation in the reaction mechanism of unprotonated imidazolyl and un-ionized sulfhydryl groups.

Human erythrocyte glucose 6-phosphate[1] dehydrogenase (D-glucose 6-phosphate : NADP oxidoreductase, EC 1.1.1.49) is of interest because a genetically determined deficiency of this enzyme leads to various haemolytic disorders (Beutler, 1959). Purification factors of 8300 (Balinsky and Bernstein, 1963), 10,000 (Kirkman, 1962), 43,500 (Chung and Langdon, 1963), and 63,500 (Yoshida, 1966) have been reported.

G-6-PD catalyzes reaction 1. The occurrence of a

$$\text{NADP} + \text{G-6-P} \rightleftharpoons \text{NADPH} + \text{6-PGL} \quad (1)$$

complex of free enzyme with NADP has been shown fluorimetrically by Marks *et al.* (1961) and Kirkman (1962). In the equation for the initial velocity of the reaction

$$v = \frac{V_f}{1 + \frac{K_a'}{[A]} + \frac{K_b'}{[B]} + \frac{K_a K_b'}{[A][B]}} \quad (2)$$

v is the initial velocity, V_f is the maximum velocity in the forward direction, and [A] and [B] can be taken to refer to the concentrations of NADP and G-6-P, respectively. K_a' is the limiting Michaelis constant for NADP when the concentration of G-6-P is sufficiently high that further increasing it will not affect the rate. Similarly K_b' is the limiting Michaelis constant for G-6-P. K_a is the dissociation constant of the enzyme–NADP complex. Equation 2 holds if linear Lineweaver–Burk plots are obtained (Alberty, 1953).

The present paper describes kinetic studies on the purified enzyme. The nature of the reaction has been investigated from product inhibition studies and from dissociation and Michaelis constants measured for the forward direction. The effects of pH on log V_f, log V_f/K_a', log V/K_b', and $-\log K_a$ were determined with a view to obtaining evidence as to the nature of the groups at the active site of the enzyme.

Methods and Materials

NADP monosodium salt, glucose 6-phosphate disodium salt, and 6-phosphogluconate sodium salt were purchased from the Sigma Chemical Co. DEAE-cellulose (floc) and CM-cellulose (floc) were obtained from the Whatman Co. Sephadex G-200 and DEAE-Sephadex A-50 were purchased from Pharmacia, Uppsala. Bio-Gel P-300 was obtained from the Bio-Rad Laboratories. Bovine plasma albumin was a product of the Armour Pharmaceutical Co. and 2-amino-2-methylpropane-1,3-diol (Ammediol) was obtained from the British Drug House and recrystallized from ethanol.

The ion-exchange celluloses were purified as described by Peterson and Sober (1956). Calcium phosphate gel was prepared according to the method of Keilin and Hartree (1938).

Bantu blood, group O, was obtained from the Human Serum Laboratories of the South African Institute for Medical Research. The blood had been stored at 4° for periods of 0–2 weeks in acid–citrate–dextrose to prevent clotting.

Throughout the purification procedure G-6-PD was assayed spectrophotometrically using a Beckman Model DB spectrophotometer adapted for recording and modified to expand the absorbance reading by factors of 2, 5, or 10. The enzyme was assayed by following the

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[1] Abbreviations used: G-6-P, glucose 6-phosphate; G-6-PD, glucose 6-phosphate dehydrogenase; 6-PGL, 6-phosphogluconolactone; Ammediol, 2-amino-2-methylpropane-1,3-diol; NADP, nicotinamide–adenine dinucleotide phosphate; NADPH, reduced NADP.

TABLE I: Purification of Glucose 6-Phosphate Dehydrogenase.

Fraction	Vol. (ml)	Total Act. (units)	Protein (g)	Sp Act. (units/mg of protein)	Yield (%)	Purifcn Factor
Haemolysate	22,740	14,720	7,400	0.00199	100.0	1.0
DEAE-cellulose eluate	18,550	6,760	78.5	0.086	45.9	43.2
$(NH_4)_2SO_4$ fractionation and dialysis	626	4,200	28.0	0.150	28.5	75.4
CM-cellulose eluate	5,810	2,940	2.97	0.990	20.0	497
$(NH_4)_2SO_4$ fractionation and dialysis	46	1,600	0.78	2.05	10.9	1,030
Calcium phosphate gel eluate	40	1,060	0.132	8.02	7.2	4,030
Sephadex G-200 eluate	41	773	0.049	15.7	5.3	7,890
Bio-Gel P-300 eluate	43	648	0.037	17.6	4.4	8,840
DEAE Sephadex A-50 eluate	8.4	288	0.0082	35.5	2.0	17,830

NADPH production at 340 mμ and 25°. Quartz cuvets of 1-cm light path contained, in 3 ml, 0.09 M Tris-HCl buffer (pH 8.2), 1.40 mM G-6-P, 0.16 mM NADP, and enzyme. One enzyme unit is defined as that quantity of enzyme which catalyses the reduction of 1 μmole of NADP/min under the above assay conditions. Specific activity is the activity per milligram of protein; protein concentration was measured at 280 mμ.

The G-6-PD activity in the haemolysate was measured by a modification of the method of Glock and McLean (1953). Two assays were carried out, one in the presence of 1.26 mM 6-phosphogluconate in addition to the other components of the usual assay mixture, the other containing the 6-phosphogluconate instead of G-6-P. G-6-PD activity was calculated as the difference between the two assays. During the first step in the purification procedure, 6-phosphogluconate dehydrogenase was eliminated so that the assay with G-6-P only could subsequently be used.

The dissociation and Michaelis constants were obtained using a Beckman DU spectrophotometer with fluorescence attachment and automatic recorder. A Schott UG 11 filter was used to select the exciting wave bands, and the NADPH production was assayed at 450 mμ and 25°. The recorder zero was set with 0.1 N H_2SO_4 and the 100% setting with 0.2-ppm quinine sulfate in 0.1 N H_2SO_4. The relationship between concentration of NADPH (dissolved in 0.01 M Tris-HCl, pH 7.6) and fluorescence emission over the above range was found to be linear. The product inhibition was studied at 30° using this instrument.

Purification Procedures

Unless otherwise stated all operations were performed at 4°. All water used was glass distilled and then deionized. Throughout the purification all buffers and enzyme solutions contained 10^{-5} M NADP and 10^{-4} M EDTA.

For each purification procedure 60 pt of Bantu blood (group O) was obtained. The blood was first screened for G-6-PD activity using the method of Bernstein (1962). Approximately 5% of the blood obtained was deficient in G-6-PD activity and was discarded. The red cells were collected, the buffy layer was removed, and the cells were washed as described by Kirkman (1962). The washed erythrocytes were lysed in an equal volume of 0.01 M potassium phosphate buffer (pH 7.0) by freezing and thawing (Chung and Langdon, 1963).

DEAE-cellulose and First Ammonium Sulfate Fractionation. The method of Chung and Langdon (1963) was followed except that the enzyme was eluted from the cellulose with 0.4 M potassium phosphate buffer (pH 6.3) and the ammonium sulfate precipitate was redissolved in 0.05 M potassium phosphate buffer (pH 6.0). This solution was then dialyzed for 8 hr against 0.005 M potassium phosphate buffer (pH 6.0).

CM-cellulose Fractionation and Second Ammonium Sulfate Precipitation. The dialysate was added to CM-cellulose (500 g dry wt) which had been equilibrated in 0.005 M potassium phosphate buffer (pH 6.0). After being well stirred at regular intervals for 1 hr, the cellulose was filtered on a Buchner funnel and washed with 6 l. of 0.005 M potassium phosphate buffer (pH 6.0). The enzyme was eluted from the cellulose using 0.2 M potassium phosphate buffer (pH 6.0) and precipitated with 420 g of solid $(NH_4)_2SO_4$/l. of enzyme solution. The precipitate, collected by centrifugation, was redissolved in a minimum volume of 0.05 M potassium phosphate buffer (pH 7.0) and dialyzed for 8 hr against 0.005 M potassium phosphate buffer (pH 7.0).

Calcium Phosphate Gel Fractionation. The method used was similar to those outlined by Kirkman (1962) and Chung and Langdon (1963). Successive quantities of calcium phosphate gel were added to the enzyme preparation until the enzyme was almost completely adsorbed. The enzyme was then eluted from the gel with 0.2 M potassium phosphate buffer (pH 5.8). At this stage the enzyme was extremely stable and could be stored in the deep freeze for months without loss of activity. The

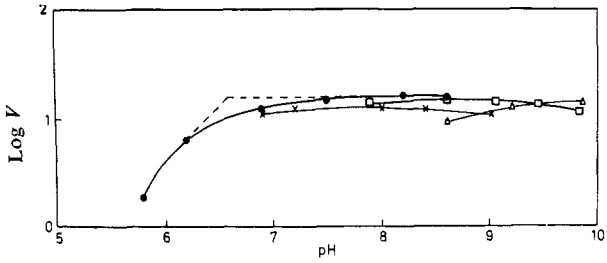


FIGURE 1: The effect of pH on log V. (●—●) Tris-maleate buffer; (□—□) Ammediol-HCl buffer; (△—△) glycine-NaOH buffer; (×—×) Tris-HCl buffer. Assays were carried out spectrophotometrically by following the rate of NADPH production at 340 mμ and 25°. Quartz cuvets of 1-cm light path contained 0.046 M buffer, 0.24 mM NADP, 1.20 mM G-6-P, and a constant amount of enzyme (specific activity 8.02).

eluate was concentrated by the addition of 455 g of solid $(NH_4)_2SO_4$/l. of solution and the precipitate, collected by centrifugation, was dissolved in a minimum volume of 0.02 M potassium phosphate buffer (pH 7.0).

Sephadex G-200 and Bio-Gel P-300 Columns. The concentrated enzyme was then placed onto a Sephadex G-200 column of dimensions 32 × 3 cm, and the enzyme was eluted in 0.02 M potassium phosphate buffer (pH 7.0). The active fractions were combined and the protein was precipitated with solid $(NH_4)_2SO_4$ (490 g/l. of enzyme solution). After centrifugation the precipitate was dissolved in 0.02 M potassium phosphate buffer (pH 7.0). The procedure was repeated using a Bio-Gel P-300 column of dimensions 30 × 3 cm. The resulting $(NH_4)_2SO_4$ precipitate obtained was dissolved in and dialyzed against 0.02 M potassium phosphate buffer (pH 7.0) for 5 hr.

DEAE Sephadex A-50 Column. The enzyme solution was then placed on a DEAE Sephadex column of dimensions 10 × 1 cm and buffer (0.02 M potassium phosphate, pH 7.0) passed through the column until no further protein was eluted. This process was repeated using the same buffer containing (a) 0.05 M KCl, (b) 0.1 M KCl, and (c) 0.2 M KCl. The enzyme was eluted in c.

Results

Table I shows the results of a typical purification procedure. A final purification factor of 17,830 was obtained. The enzyme lacked stability at this purification and 0.5% bovine plasma albumin was added to the preparation to stabilize the enzyme sufficiently for kinetic experiments. The albumin added had no G-6-PD or 6-phosphogluconate dehydrogenase activities. For recent work the enzyme was only purified 4030 times (specific activity 8.02), *i.e.*, to the end of the calcium phosphate gel step. The stability at this stage was high without the addition of an extraneous protein. 6-Phosphogluconate dehydrogenase and glucose 6-phosphatase activities were absent in the purified enzyme preparations. The enzyme was found to be unstable below pH 5.2 in 0.05 M citrate and 0.05 M Tris-maleate and above pH 10.0 in 0.05 M glycine-NaOH and 0.05 M Ammediol-HCl buffers. All kinetic experiments had therefore

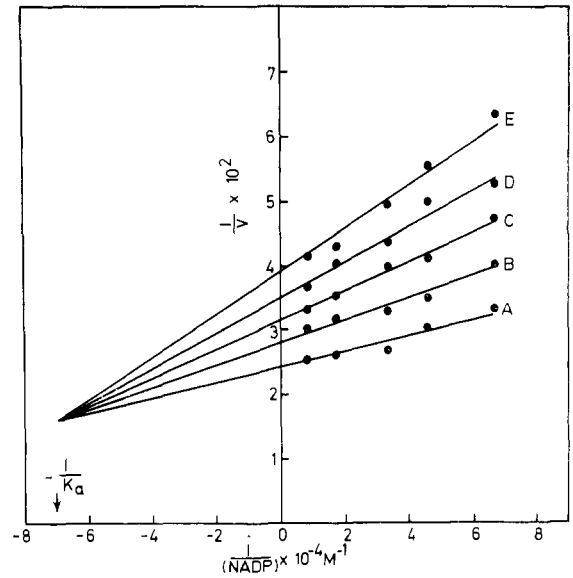


FIGURE 2: Double-reciprocal plots of initial velocity *vs.* NADP concentration at various constant levels of G-6-P. The concentrations of G-6-P used were: (A) 3.00 × 10^{-4} M, (B) 1.00 × 10^{-4} M, (C) 6.00 × 10^{-5} M, (D) 4.29 × 10^{-5} M, and (E) 3.33 × 10^{-5} M. The assays were followed spectrofluorimetrically at 450 mμ and 25° in 0.048 M Tris-maleate buffer (pH 7.05). Enzyme of specific activity 8.02 was used.

to be carried out between pH 5.2 and 10.0. Owing to the instability of NADPH at low pH values, assays below pH 5.7 were not made.

The Effect of pH on Maximum Velocity (V). The effect of pH on maximum velocity was measured using both the enzyme preparation purified 17,830-fold, stabilized with albumin, and that purified 4030-fold. Identical results were obtained with both enzyme preparations. Assay mixtures contained an excess of both substrates and the study was carried out over the pH range of 5.7–10.0.

The effect of pH on log V can be seen in Figure 1. Lines of integral slope were drawn to the curve and intersected at a point giving a pK value of 6.6.

Determination of the Kinetic Parameters K_a, K_a', and K_b'. The dissociation and Michaelis constants were determined graphically by the method of Florini and

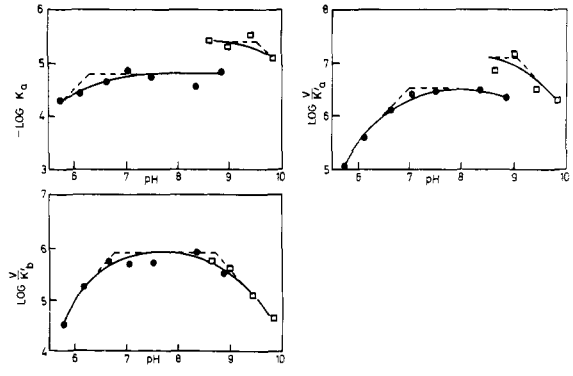


FIGURE 3: The effect of pH on $-\log K_a$, $\log V/K_a'$, and $\log V/K_b'$ in Tris-maleate (●—●) and Ammediol-HCl (□—□) buffers.

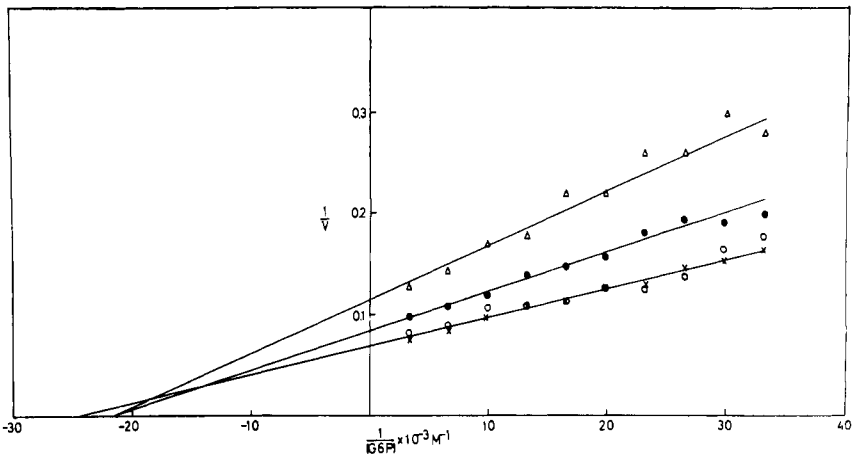


FIGURE 4: Product inhibition effects of NADPH. Double-reciprocal plots of initial velocity *vs.* G-6-P concentration. The concentrations of NADP and NADPH used were: (×—×) 0.637 mM NADP, no NADPH; (○—○) 0.637 mM NADP, 10 μM NADPH; (●—●) 20 μM NADP, no NADPH; (△—△) 20 μM NADP, 10 μM NADPH. The reaction was assayed spectrofluorimetrically at 450 mμ in 0.048 M Tris-maleate buffer (pH 8.0) at 30°. Enzyme of specific activity 8.02 was used.

Vestling (1957). A series of double-reciprocal plots was obtained for NADP at various constant concentrations of G-6-P, as shown in Figure 2. The family of lines obtained intersected at a point giving $-1/K_a$. A secondary plot of the intercepts on the ordinate *vs.* reciprocal G-6-P concentration gave $-1/K_b'$ as the intercept on the abscissa. The experimental values of Figure 2 were replotted to give the apparent maximum velocities at fixed NADP concentrations. K_a' was obtained by a secondary plot of these values. The experiment was repeated at various pH values between 5.7 and 10.0. The values obtained for the constants can be seen in Table II.

Within the limits of experimental error the constants obtained were independent of the order of addition of the substrates and enzyme to Tris-maleate buffer at pH 7.5. It was found, however, that if the enzyme was added to the Ammediol buffer prior to the addition of NADP it was inactivated, rapidly and irreversibly. The presence of NADP even in small quantities prevented this inactivation. Therefore for experiments carried out in Ammediol buffer the NADP was added to the buffer prior to the enzyme.

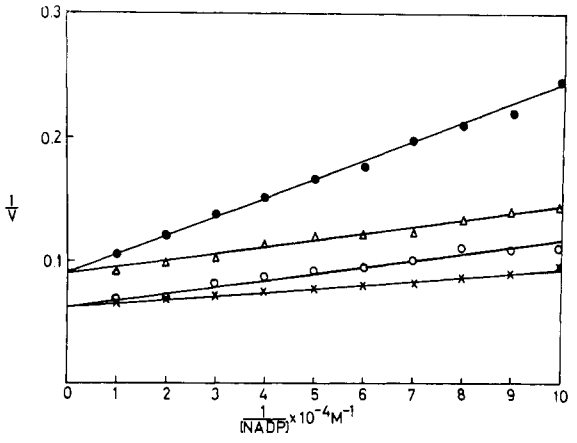


FIGURE 5: Product inhibition effects of NADPH. Double-reciprocal plots of initial velocity *vs.* NADP concentration. The concentrations of G-6-P and NADPH used were: (×—×) 3.43 mM G-6-P, no NADPH; (○—○) 3.43 mM G-6-P, 10 μM NADPH; (△—△) 0.1 mM G-6-P, no NADPH; (●—●) 0.1 mM G-6-P, 10 μM NADPH. Other assay conditions were as described in Figure 4.

The effects of pH on $-\log K_a$, $\log V/K_a'$ and $\log V/K_b'$ can be seen in Figure 3. Lines of integral slope were drawn to these curves (Dixon and Webb, 1964). These lines intersected to give pK values of 6.3 and 9.5, 7.0 and 9.0, and 6.8 and 8.7, respectively.

Product Inhibition Studies. A study of inhibition by NADPH was carried out. Figure 4 shows the effect of 10 μM NADPH on the velocity of the reaction when the concentration of G-6-P was varied at both saturating (0.637 mM) and unsaturating (20 μM) NADP levels. No inhibition occurred with NADP saturating while noncompetitive inhibition was observed at the unsaturating NADP level. Figure 5 shows the effect of 10 μM NADPH on the rate of the reaction on varying the NADP concentration at both saturating (3.43 mM) and unsaturating (0.10 mM) G-6-P levels. Competitive inhibition was found to occur in both cases.

TABLE II: Dissociation and Michaelis Constants at Various pH Values.

Buffer	pH	$K_a \times 10^5$ M	$K_a' \times 10^5$ M	$K_b' \times 10^5$ M
Tris-maleate	5.75	5.3	1.6	5.5
	6.15	3.4	1.4	3.3
	6.65	2.2	0.8	1.8
	7.05	1.4	0.5	2.5
	7.50	1.9	0.5	2.7
	8.35	2.8	0.5	1.8
	8.85	1.4	0.7	4.9
Ammediol-HCl	8.63	0.4	0.2	2.4
	9.00	0.5	0.1	3.5
	9.45	0.3	0.4	11.0
	9.85	0.8	0.6	27.0

Discussion

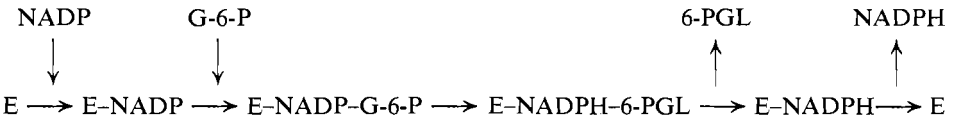
The dissociation and Michaelis constants for the forward reaction were determined but the constants for the reverse reaction could not be measured due to the instability of 6-phosphoglucono-δ-lactone, which has a half-life of about 1.5 min at pH 7.4 (Horecker and Smyrniotis, 1953). The plots in Figure 2 meet in a point and there is a decrease in the apparent Michaelis constants with increase in the level of nonvaried substrate. These results indicate that the mechanism of the reaction is sequential. Results of product inhibition are consistent with a compulsory order mechanism of the "ordered Bi Bi" type (3). A rapid equilibrium, random order mechanism is ruled out except in the case of the formation of a dead-end enzyme–G-6-P–NADPH complex (Cleland, 1963).

In the case of a compulsory order mechanism, Frieden (1957) has shown that results similar to those in Figure 2 yield an enzyme–substrate dissociation constant (K_a) for at least one of the substrates (NADP). If the rapid equilibrium, random order mechanism with the dead-end complex holds, then the dissociation constant for the second substrate (G-6-P), *viz.*, K_b, can also be obtained from the data in Figure 2. K_b is related to the other three parameters by the equation $K_aK_b' = K_a'K_b$ (Alberty, 1953). By thermal inactivation experiments, Marks *et al.* (1961) obtained a K_s value for NADP of 6.5 μM, which is very similar to the value for K_a obtained in these experiments at a corresponding pH. However, the K_s value for G-6-P determined by these workers was 1.5 mM, which is approximately 17 times higher than the K_b value of 9.0×10^{-5} M obtained in our experiments at the same pH, indicating that any random mechanism can probably be ruled out. The broad pH optimum for the reaction is similar to the results of Marks *et al.* (1961) and Balinsky and Bernstein (1963).

The variation of V with pH shows the effect of ionization of groups on those enzymes forms present at infinite substrate concentrations. Figure 1 shows an apparent drop in V in the low pH region (6–7) but no clear-cut drop at high pH values. Lines of integral slope drawn to the pH *vs.* log V curve intersect to give a pK value of 6.6 which may represent an unprotonated imidazole group in the ternary complex.

Plots of log V/K_a', log V/K_b', and $-\log K_a$ *vs.* pH show pK values at 7.0 and 9.0, 6.8 and 8.7, and at 6.3 and 9.5, respectively. The variations of these parameters with pH indicate the effects of ionizations in the free substrates and the enzyme forms reacting with them. The pK values in the low region could represent the ionizations of phosphate groups in the substrates. G-6-P has a pK value of 6.1 (Kumler and Eiler, 1943) while the pK values of NADP are not known. Alternatively they could indicate the participation of unprotonated imidazole groups in the reaction mechanism. The sulfhydryl group exhibits a pK value of 9.20 in glutathione and 9.09 in cysteine ethyl ester (Benesch and Benesch, 1955). Therefore pK values around 9 visible on $-\log K_a$, log V/K_a', and log V/K_b' *vs.* pH plots suggest that unionized sulfhydryl groups participate in the binding of both substrates to the various enzyme forms.

Evidence for the presence of imidazole and sulfhydryl groups at the active center has previously been advanced by Soldin and Balinsky (1966) who obtained pK values from pK_m ($-\log$ apparent Michaelis constant) *vs.* pH plots of 6.2 and 9.0, and of 6.7 and 9.1 for G-6-P and NADP, respectively. Additional evidence of a sulfhydryl group near the active site is the inactivation of the enzyme by *p*-mercuribenzoate (Balinsky and Bernstein, 1963). NADP protects the enzyme from *p*-mercuribenzoate inhibition (Chung and Langdon, 1963) indicating further that sulfhydryl groups may be involved in NADP binding.



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Acknowledgments

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Subunit Interaction in Native and Modified Muscle Phosphorylases*

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ABSTRACT: It is shown by sedimentation and gel electrophoresis that both muscle phosphorylases *a* and *b* exist under normal circumstances in equilibrium between dimeric and tetrameric forms. The equilibrium is displaced in favor of the tetramer by the addition of 5′-adenylic acid. The nature of the sedimenting boundaries indicates that the association equilibrium is not in general a rapid process, and may thus involve a conformational change in the protein. Reaction with *p*-mercuribenzoic acid (PMB) is known to lead to monomers from either phosphorylase; these monomers are still found to interact with adenylic acid, the phosphorylase *b* giving predominantly dimer and some polydisperse aggregates, phosphorylase *a* only large aggregates. On lowering the ionic strength, the PMB monomers of phosphorylase *b* give place to dimer, whereas phosphorylase *a* produces a remarkable series of polymers, which are shown to include odd species, notably the trimer. Nine bands are resolved by gel electrophoresis, and the first four are resolved in the analytical ultracentrifuge. In the electron microscope native dimeric phosphorylase *b* is shown to consist of two indistinguishable, roughly ellipsoidal subunits, and the phosphorylase *a* and tetrameric form of phosphorylase *b*, of four such subunits, arranged with their centers at the corners of a rhombus. Each subunit corresponds to a monomer of 92,500 mol wt. Examination of phosphorylase *a* from crystals immediately after dissolving in a medium of low cysteine content shows polymers consisting apparently of dimers assembled with their long axes parallel. It is concluded that certain lattice contacts can become association sites in the dispersed state. The PMB aggregates of phosphorylase *a* take the form of clusters of monomers, which indicates that cyclic rather than linear aggregation is favored. By all available criteria the PMB monomers are intact, and not unfolded, protein; arguments are given for the presence of at least three different binding sites in each phosphorylase monomer, even in the native state, two of which are weakened when PMB is bound. Application of the hydrodynamic theory of Kirkwood for assemblages of monomeric beads leads to calculated sedimentation coefficients and frictional ratios which agree remarkably with experimental data, and are more compatible with cyclic than with linear structures.

The native muscle phosphorylases exist in two aggregation states, a dimer (Keller and Cori, 1953; Keller, 1955) which has now been found (Seery *et al.*, 1967) to have a molecular weight of 185,000, and a tetramer of mol wt 370,000. Phosphorylase *a* differs from *b* in having one seryl residue in each subunit in the form of a phosphate ester. Phosphorylase *a* is normally tetrameric, but is capable of dissociating at high ionic strength into a still active dimer (Wang and Graves, 1963). Phosphorylase *b*, in the absence of 5′-adenylic acid (AMP)[1], is a dimer, which on activation with AMP is converted into the active tetrameric form (Kent *et al.*, 1958). As with other such systems, it is therefore evident that the function of the enzyme is intimately connected with its aggregation state. When phosphorylase *a* is treated with organomercurials, such as *p*-mercuribenzoic acid (PMB), the enzyme is reversibly inactivated and the monomer (which is not necessarily however a single polypeptide chain) is generated (Madsen and Cori, 1956). The same authors also reported that these monomers slowly associate to form very large aggregates. Slow aggregation has likewise been observed in the native protein when no protective sulfhydryl compounds are present (Krebs and Fischer, 1962). We have observed that under well-defined conditions of ionic strength the PMB-treated monomer gives rise to a series of discrete polymers. We report here a study of the nature of the polymerization process, and the relation

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[1] Abbreviations used: PMB, *p*-mercuribenzoic acid; AMP, 5′-adenylic acid.