Effect of Polyamines on Glycogen Phosphorylase. Differential Electrostatic Interactions and Enzymic Properties*

Jerry Hsueh-ching Wang,† Paul M. Humniski, and William J. Black‡

ABSTRACT: The activation of glycogen phosphorylase *b* by adenosine monophosphate (AMP) is stimulated by the polyamines: spermine, spermidine, and putrescine. This stimulation is more pronounced at low AMP concentrations. Kinetic studies indicate that while the affinity of the enzyme for AMP is enhanced, neither the Hill coefficient for AMP nor the K_m for glucose 1-phosphate is significantly influenced. Although the polyamines have little effect on the sedimentation pattern of phosphorylase *b* in the absence of AMP, the partial association caused by AMP is greatly enhanced in the presence of the polyamines. In contrast to phosphorylase *b*, phosphorylases *a* and *b'* are not significantly affected by spermine. Fischer *et al.* (Fischer, E. H., Graves, D. J., Crittenden, E. S., and Krebs, E. G. (1959), *J. Biol. Chem. 231*, 65) have pointed out that phosphorylases *a*, *b*, and *b'* differ in a hexapeptide fragment and that removal of this particular hexapeptide from phosphorylase *a* by trypsin results in the production of phosphorylase *b'*. Kinetic data, ultracentrifugal analyses, and polyacrylamide gel patterns all indicate that trypsin-treated phosphorylase *b* behaves like phosphorylase *b'*. Amino acid analysis of the major tryptic peptide liberated by incubation of phosphorylase *b* with trypsin also strongly suggests that trypsin may split the same hexapeptide from phosphorylase *b* as it does from *a*. Only the *b* to *b'* conversion is significantly enhanced by spermine. In the light of these observations, a model relating the chemical structure of phosphorylase to electrostatic interactions is discussed.

The conversion of glycogen phosphorylase *b* into phosphorylase *a* results in the incorporation of 2 moles of phosphate/mole of phosphorylase *b* (Krebs *et al.*, 1958), with the concomitant appearance of catalytic activity (Green and Cori, 1943). The mechanism of activation through phosphate incorporation however is not clear. The phosphate groups probably do not participate directly in enzyme catalysis, since there is no exchange of these phosphate groups with inorganic phosphate supplied as substrate (Krebs *et al.*, 1958). The fact that the two forms of the enzyme differ in their stability (Graves *et al.*, 1965; Shaltiel *et al.*, 1966) as well as their properties of association (Keller and Cori, 1953), suggests they exist in different conformational states. This may account for their differences in catalytic activity. The observation that AMP,[1] a cofactor for phosphorylase *b*, minimizes the differences in physical properties of these two forms of phosphorylase (Graves *et al.*, 1965; Shaltiel *et al.*, 1966) supports this hypothesis.

Although attempts have been made to understand the conformational differences between phosphorylases *a* and *b* (Hedrick, 1966; Helmreich and Cori, 1964), the nature of these differences has not yet been established. From studies on the proteolytic hydrolysate of phosphorylase *a*, it has been shown that a specific serine residue is phosphorylated during the *b* into *a* conversion. Many of the amino acid residues in the vicinity of this serine group are positively charged (Fischer *et al.*, 1959; Nolan *et al.*, 1964). Fischer *et al.* (1959) have postulated that the negatively charged phosphate groups may neutralize some of these positive charges. Thus the *b* and *a* forms of phosphorylase may differ in their electrostatic interactions in this particular region of the enzyme molecule. The findings that protamine (Krebs, 1954) and salt solutions (Wang and Graves, 1963, 1964; Sealock and Graves, 1967) have a profound effect on the structure and kinetic property of the enzyme suggest that such differences in electrostatic interactions may be directly related to catalytic properties of this enzyme.

In the present study, the effect of some polyamines on the structure and activity of glycogen phosphorylase has been investigated in order to obtain a greater understanding of the relationship between the electrostatic interactions and the enzyme catalysis. Tryptic digestion of phosphorylase has also been studied in some detail to examine the involvement of a trypsin-released hexapeptide in these electrostatic interactions.

Materials and Methods

Materials. Crystalline phosphorylase *b* was isolated from rabbit muscle by the procedure of Fischer and Krebs (1958). Phosphorylase *a* was prepared from phosphorylase *b* using phosphorylase *b* kinase (Fischer and Krebs, 1962). The three- or four-times-crystallized phosphorylase was treated with Norit A to remove firmly bound AMP. For ultracentrifugation and tryptic digestion experiments the enzyme solution was dialyzed

* From the Department of Biochemistry, Faculty of Medicine, University of Manitoba, Winnipeg, Manitoba, Canada. *Received July 31, 1967.* Supported by Grant MA 2381 from the Medical Research Council of Canada.

† Medical Research Council scholar.

‡ Predoctoral fellow of the Muscular Dystrophy Association of Canada.

[1] Abbreviations are as listed in *Biochemistry 5*, 1445 (1966).

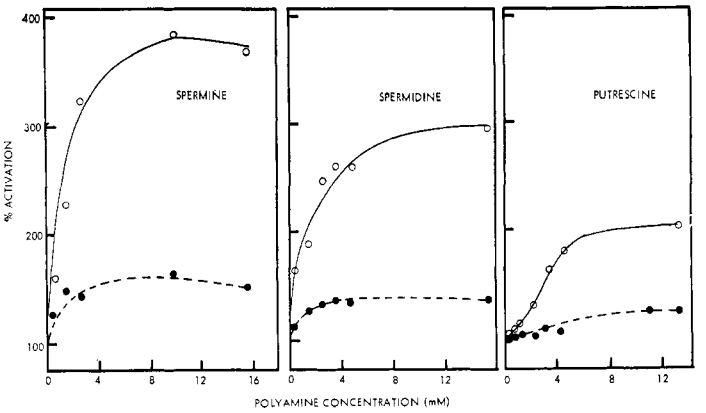

FIGURE 1: Effect of polyamines on the activation of phosphorylase *b*. The enzyme was assayed at 30° in 0.015 M cysteine–0.02 M glycerophosphate buffer (pH 7.0) with 0.016 M G-1-P, 1% glycogen, and containing either 2.5 mM (●) or 5 × 10^{-5} M (○) AMP. The enzyme activities without polyamines were taken as 100%.

against 0.04 M sodium glycerophosphate–0.03 M cysteine buffer (pH 7.0) in order to remove Mg^{2+} ions used in the crystallization of phosphorylase *b*. Phosphorylase *b'* was prepared by controlled tryptic digestion of phosphorylase *a* (Cori and Cori, 1945). The digestion was stopped by addition of trypsin inhibitor and the enzyme was used without prior removal of trypsin (bovine pancreas) and trypsin inhibitor (egg white).

Cysteine hydrochloride, sodium glycerophosphate, glucose 1-phosphate (G-1-P), adenosine monophosphate (AMP), inosine monophosphate (IMP), and shellfish glycogen were obtained from Sigma Chemical Co. The glycogen was purified according to Sutherland and Wosilait (1956) to free it from AMP contamination. Spermine tetrahydrochloride and putrescine dihydrochloride were obtained from Mann Research Laboratories. Spermidine trihydrochloride was obtained from Nutritional Biochemicals Corp. Trypsin and trypsin inhibitor were obtained from Calbiochem Co. Polyacrylamide gel reagents were obtained from Canal Industrial Corp.

Methods. Enzyme concentrations were determined spectrophotometrically as described by Velick and Wicks (1951). Phosphorylase activities were measured by a modification of the procedure of Illingworth and Cori (1953). The enzyme stopping reagent contained 1.0 M potassium chloride in addition to the molybdate and sulfuric acid. The potassium chloride prevented precipitation of the enzyme in the presence of the polyamines and had a negligible effect on the colorimetric assays.

Sedimentation velocity experiments were carried out with a Spinco Model E analytical ultracentrifuge using a 12-mm single-sector cell at a rotor speed of 60,000 rpm and a rotor temperature of 20°. For sedimentation constants, boundary movements were obtained from direct microcomparator measurements of the schlieren diagram.

Polyacrylamide gel electrophoresis was carried out at pH 8.3 essentially according to the method of Ornstein (1964). Columns 0.5 cm in diameter were used. Phosphorylase samples (125 μg/60 μl) were added to the tops of the gels. The current was run at 3 mA/tube for 70 min. Protein was stained with Amido Black.

For the preparation of large amounts of peptides, phosphorylase *b* (1.1 g/25 ml) in 0.03 M β-mercaptoethanol and 0.04 M NH_4HCO_3 at pH 7.6 was treated with 4.4 mg of trypsin for 30 min at 30°. The reaction was then stopped by addition of 13.2 mg of trypsin inhibitor. Small peptides were separated from core protein by gel filtration. Aliquots of the reaction mixture (5 ml) were applied on a Sephadex G-25 column (1.5 × 80 cm) and eluted with the β-mercaptoethanol and NH_4HCO_3 buffer. Fractions containing peptides were detected by the ninhydrin method (Moore and Stein, 1954) after alkaline hydrolysis (Hirs *et al.*, 1956). These fractions were pooled and then dried on a flash evaporator with several washings with double-distilled water.

High-voltage paper electrophoresis was carried out according to the procedure of Ryle *et al.* (1955) in pyridine acetate buffer pH 4.7 for 35 min at 3000 V. Sheets of Whatman No. 3MM paper were used, on which up to 40 μl of peptide mixture (2 mg/ml) was spotted. Papers were stained with ninhydrin–collidine spray (Block *et al.*, 1955). Essentially the same conditions were used for the preparative high-voltage electrophoresis.

Samples for amino acid analysis, were hydrolyzed in 6 N HCl under nitrogen for 30 hr at 110°. The hydrolysate was then dried in an evacuated desiccator over solid NaOH at room temperature. Quantitative amino acid analyses were performed on a Technicon Autoanalyzer.

Results

Effect of Polyamines on the Activity of Phosphorylase b. It has been shown that binding of protamine or polylysine to glycogen phosphorylase *b* results in an enhanced affinity of the enzyme toward AMP (Krebs, 1954; Madsen and Cori, 1954). In order to further understand the mechanism of such interaction between phosphorylase *b* and these macromolecules, the effect of relatively small polycationic molecules on the catalytic and structural properties of the enzyme has been examined. Figure 1 shows that all polyamines tested activate phosphorylase *b* at either saturating (2.5 mM) or suboptimal (5 × 10^{-2} mM) concentrations of AMP. The effectiveness of the polyamines in activating the enzyme

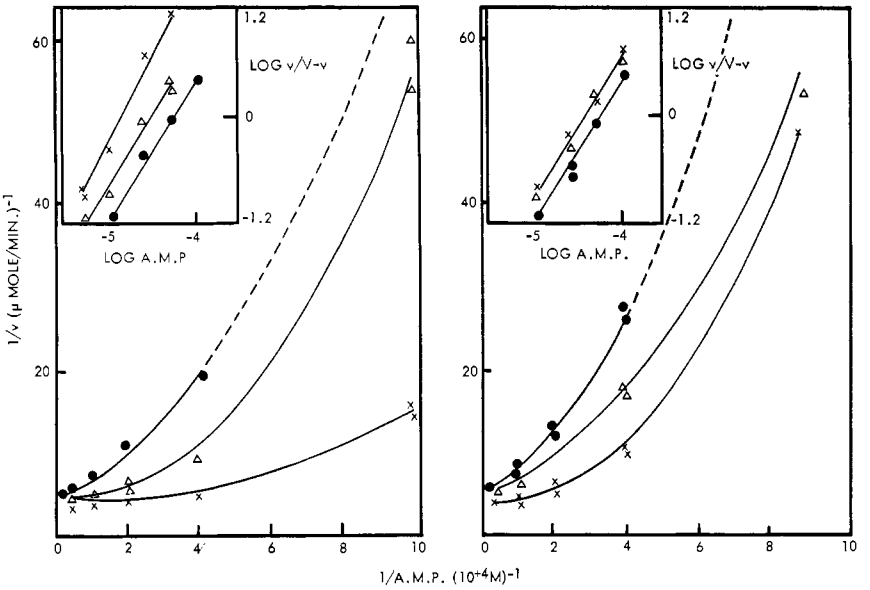

FIGURE 2: Effect of polyamines on the reciprocal plots for AMP activation. The assay mixture contained, in addition to AMP 0.016 M G-1-P, 1% glycogen, 0.015 M cysteine with either (A) 0 (●), 0.5 (△), or 2.5 mM (x) spermine; or (B) 0 (●), 3.5 (△), or 4.5 mM (x) spermidine. Reactions were carried out at 30° and buffered at pH 7.0 with 0.02 M glycerophosphate.

is related to their positive charges and/or their molecular size. Spermine is the most effective in the activation of the enzyme while putrescine is the least effective. The enzyme using the suboptimal concentration of AMP is activated to a much higher extent than that using the saturating AMP concentration. This suboptimal concentration of AMP activates phosphorylase *b* to half its maximal activity (Krebs, 1954). In the presence of 5 mM or higher concentration of spermine the final enzyme activities were essentially the same, regardless of which AMP concentration was employed in the assays. These results suggest that the polyamines enhance the affinity of the enzyme for AMP. The fact that slight activation of phosphorylase *b* may be observed even at the saturating concentration of AMP indicates that the maximum velocity of the enzyme is also increased.

Since the AMP concentration *vs.* initial velocity plot for phosphorylase *b* is sigmoidal (Helmreich and Cori, 1964; Sealock and Graves, 1967), it may be suggested that there is cooperative interaction between AMP binding sites on the enzyme (Monod *et al.*, 1965). The possibility that the apparent enhancement of AMP affinity of the enzyme is a result of partial or total elimination of the interactions between the AMP binding sites has been examined. Figure 2 shows that the Lineweaver–Burk plots for AMP are nonlinear regardless of the presence of polyamines. The Hill coefficients for this nucleotide phosphorylase interaction, ranging from 1.6 to 1.8, are also independent of the presence of polyamines. Sealock and Graves (1967) in a study on the effect of salt solutions on glycogen phosphorylase have found that phosphorylase *b* may also be activated by NaF at low concentrations of AMP. This activation is associated with a change in kinetic order for the nucleotide.

In contrast to its effect on the affinity of the enzyme for AMP, spermine proved to have a negligible effect on the K_m of G-1-P. At 2.5 mM AMP, the K_m for G-1-P in the presence or absence of 2.5 mM spermine has been found to be 2.5 or 2.3 mM, respectively. These results suggest that these polyamines have a specific effect on the binding of AMP to the enzyme. Neither the binding of G-1-P nor the interaction between AMP binding sites is significantly altered by these polyamines.

Effect of Polyamines on the Structure of Phosphorylase b. In order to see the relationship between the change in catalytic property and the enzyme structure, the effect of polyamines on the association of phosphorylase *b* has been examined ultracentrifugally. Figure 3 shows the schlieren patterns of six phosphorylase *b* samples. While the free enzyme sediments as a homogeneous material with a sedimentation constant of 8.2 S (Figure 3A), addition of 2 mM AMP results in the appearance of a new sedimenting component with a $s_{20,w}$ of 13.7 S (Figure 3B) which consists of approximately 50% of the total enzyme. Although 2 mM spermine does not cause significant alteration of the ultracentrifugal pattern of phosphorylase *b* (Figure 3C *vs.* 3A), the addition of both spermine and AMP results in almost complete conversion of the enzyme into the 13.7S material (Figure 3D). Similar effects are also observed with spermidine and AMP (Figure 3E). Putrescine, the least effective polyamine, shows little or no effect on the ultracentrifugal pattern of the enzyme. Addition of 2 mM putrescine to an enzyme sample containing AMP does not increase the amount of fast-sedimenting components (Figure 3F). However the association of phosphorylase *b* by AMP may be enhanced by higher concentrations of putrescine. The amount of the faster sedimenting component at 3 and 5 mM putrescine (Figure 3G,H) has been estimated as 76 and 89% respectively. Further increase in putrescine concentrations from 5 to 10 mM does not result in additional alteration of the sedimentation pattern. Thus the increase in the amount of the 13.7S material of phosphorylase *b* shows a dependence on putrescine concen-

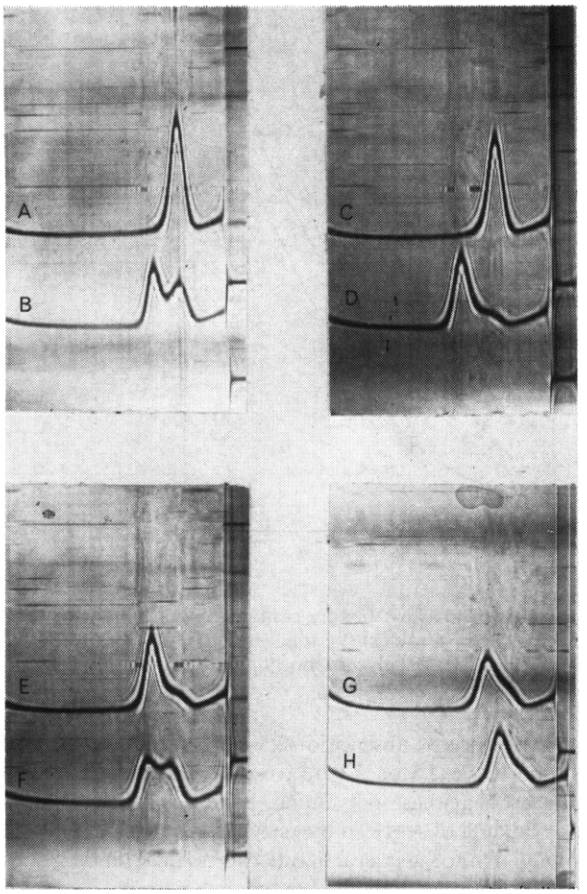

FIGURE 3: Effect of polyamines on the sedimentation patterns of phosphorylase *b*. (A) Phosphorylase *b* (5 mg/ml) was centrifuged at 60,000 rpm at 20° in 0.04 M glycerophosphate–0.03 M cysteine (pH 7.0); (B) same as A with 2 mM AMP; (C) as A with 2 mM spermine; (D) as B with 2 mM spermine; (E) as B with 2 mM spermidine; (F) as B with 2 mM putrescine; (G) as B with 3 mM putrescine; (H) as B with 5 mM putrescine. Direction of the sedimentation was from right to left.

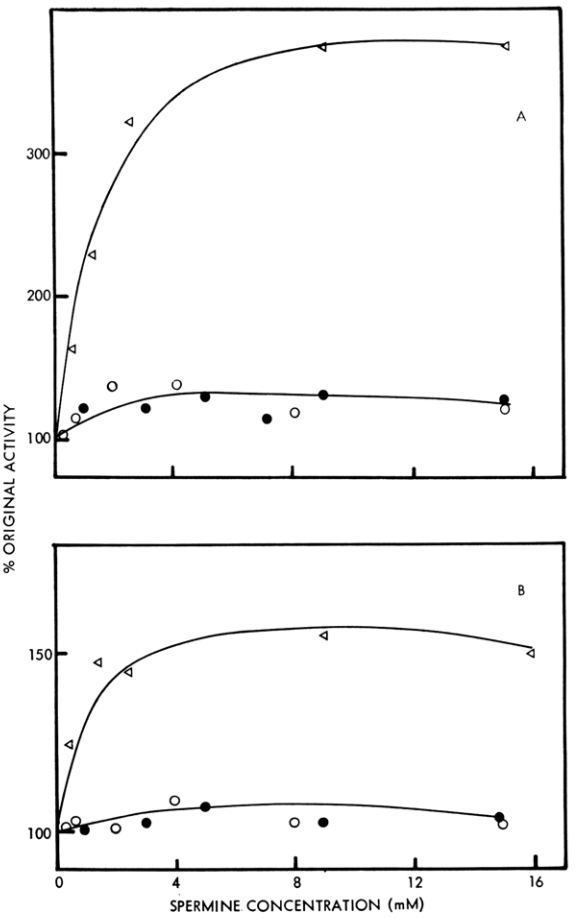

FIGURE 4: Differential effect of spermine in the activation of phosphorylase *a*, *b*, and *b'*. Phosphorylase *a* (●), *b* (△), and *b'* (○) were assayed with 5×10^{-5} M (A) or 2.5 mM (B) AMP in the presence of various concentrations of spermine. Other conditions as in Figure 1.

tration similar to that of enzyme activity (Figure 1c). Since the sedimentation constants of 13.7 and 8.2 S correspond to those of dimeric and tetrameric species of the enzyme, respectively (Keller and Cori, 1953; Madsen and Cori, 1956), these results suggest that polyamines enhance the formation of a tetrameric species of phosphorylase *b* in the presence of AMP.

Differential Response of Phosphorylases a, b, and b' to Spermine. In contrast to phosphorylase *b*, phosphorylase *b'* has been shown to be insensitive to the addition of protamine, whereas phosphorylase *a* is inhibited rather than activated by protamine (Krebs, 1954). A differential response toward polyamines among the three forms of the enzyme has also been observed in this study. Figure 4 shows that neither phosphorylase *a* nor phosphorylase *b'* is significantly activated by spermine at either high or low concentrations of AMP. Nor is there significant activation of phosphorylase *a* by spermine in the absence of AMP. At saturating concentrations of spermine, a 10% activation of phosphorylase *a* is achieved when assayed without AMP. In addition to the lack of activation of phosphorylase *a* and phosphorylase *b'* by spermine, the ultracentrifugal patterns of these two forms of the enzyme are also unaltered by this polyamine. Nor are they altered by the combination of spermine and AMP (Figure 5). Although AMP may cause partial aggregation of phosphorylase *b* into a tetrameric species (Figure 3B), no association may be induced by AMP with phosphorylase *b'*. There is no formation of tetrameric species in the presence of spermine alone (Figure 5C) or with both AMP and spermine (Figure 5D).

In addition to AMP, IMP has been shown to slightly activate phosphorylase *b* (Cori *et al.*, 1938). The extent of this activation may be greatly increased with protamine (Krebs, 1954). Figure 6 shows that similar stimulation of IMP activation of phosphorylase *b* is brought about by spermine. At a saturating concentration of spermine phosphorylase *b* activity measured with 5 mM IMP is increased to over 200%. In contrast, the activation of phosphorylase *b'* by IMP is only slightly affected by spermine. The maximum increase in IMP activation of phosphorylase *b'* brought about by spermine is less than 10%. Thus although both phosphorylases *b* and *b'* show similar catalytic properties they may be distinguished by their differential response toward spermine.

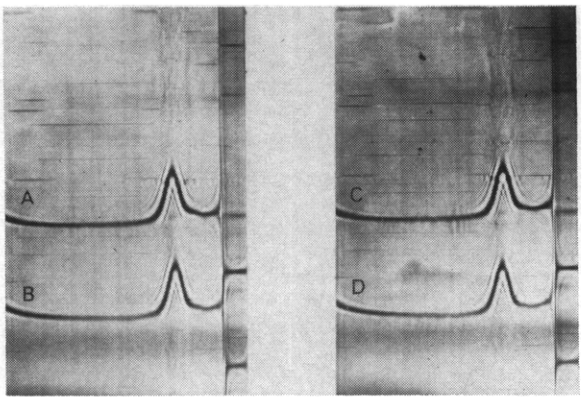

FIGURE 5: Effect of spermine on the ultracentrifugal patterns of phosphorylase *b'*. Conditions for ultracentrifugation are similar to those in Figure 3 except the enzyme concentration was 4 mg/ml. (A) Phosphorylase *b'* alone; (B) as A plus 2 mM AMP; (C) as A plus 2 mM spermine; (D) as B plus 2 mM spermine.

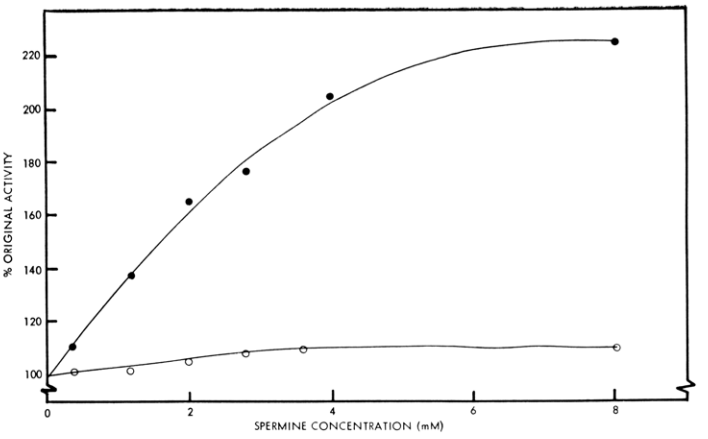

FIGURE 6: Differential effect of spermine on the activation of phosphorylase *b* and *b'* by IMP. Phosphorylase *b* (●) and *b'* (○) were assayed with 2.5 mM IMP, 25.6 mM G-1-P, and various concentrations of spermine. Other conditions as in Figure 1.

Effect of Spermine on Tryptic Digestion of Phosphorylase. Although it has been found that removal of a hexapeptide containing a specific phosphoseryl residue from phosphorylase *a* results in the formation of phosphorylase *b'* (Cori and Cori, 1945; Fischer *et al.*, 1959), the possibility of producing phosphorylase *b'* from *b* has not previously been investigated. While AMP is an absolute requirement for phosphorylase *b'* activity, phosphorylase *a* may be active in its absence. The conversion of phosphorylase *a* into *b'*, therefore may be followed by the change in the ratio of enzyme activity assayed in the presence and absence of AMP. Since both phosphorylases *b* and *b'* are active only in the presence of AMP, this assay method cannot be used to follow the conversion of phosphorylase *b* into *b'*. As an alternative, the differential response of phosphorylases *b* and *b'* toward spermine may be employed. In the presence of 4 mM spermine, the ratio of phosphorylase *b* activity assayed with 5 mM IMP to that assayed with 2 mM AMP is approximately 90% (Table I). The same activity ratio for phosphorylase *b'* is 30% (also Table I). Therefore if phosphorylase *b'* can be formed from phosphorylase *b*, a decrease in this activity ratio would be observed.

Figure 7a shows the time dependence of the trypsin effect on phosphorylase *b*. At various times after addition of trypsin (20 μg) to the enzyme (5 mg), aliquots of the mixture were placed in buffer solution containing trypsin inhibitor (60 μg). Assays were then performed in the presence of 4 mM spermine plus 5 mM IMP or 1 mM AMP. As can be seen, the activity of the enzyme, as measured with either AMP or IMP, decreases rapidly. This decrease in activity is accompanied by a decrease in the ratio of activities assayed with IMP to that assayed with AMP. After 10-min incubation, the enzyme activity starts to level off and the activity ratio has dropped from the original 89 to 27%. These results suggest that phosphorylase *b'* may be produced from tryptic digestion of phosphorylase *b*. If the trypsin phosphorylase *b* incubation medium contains 4 mM spermine, a more rapid initial loss in activity results, although final activity levels reached are the same. Figure 7b shows the effect of trypsin digestion of phosphorylase *a* in the presence and absence of 4 mM spermine. The conversion of phosphorylase *a* into *b'*, in contrast to the *b* into *b'* conversion, is only slightly facilitated by spermine. Thus the increased rate of phosphorylase *b* into *b'* conversion in the presence of spermine is not due to a direct interaction of spermine with trypsin. These results further support the notion that catalytic property changes of phosphorylase *b* are associated with a structural change of this form of the enzyme.

TABLE I: Differential Nucleotide Activation of Phosphorylases *b* and *b'*.[a]

Forms of Phosphorylase	% IMP Activation[b]	
	No Spermine	4 mM Spermine
b	33	88
b'	23	30

[a] Activity measurements were carried out with 24 mM G-1-P and either 5 mM IMP or 1 mM AMP. Other conditions as in Figure 1. [b] AMP activation was taken as 100%.

Further Evidence of the Conversion of Phosphorylase b into Phosphorylase b'. Since phosphorylase *b* but not phosphorylase *b'* can be converted into phosphorylase *a* by phosphorylase *b* kinase (Brown and Cori, 1961), the action of the kinase on phosphorylase *b* both before and after tryptic digestion has been examined. Under the standard conditions employed by Fischer and Krebs (1962) phosphorylase *b* is quantitatively converted into phosphorylase *a*. The trypsin-treated phosphorylase *b* shows no change in its catalytic properties.

Although AMP and polyamines cause partial association of phosphorylase *b* into a tetrameric species (Figure 3), these compounds do not affect phosphorylase *b'* (Figure 5) in this respect. After treatment of phosphoryl-

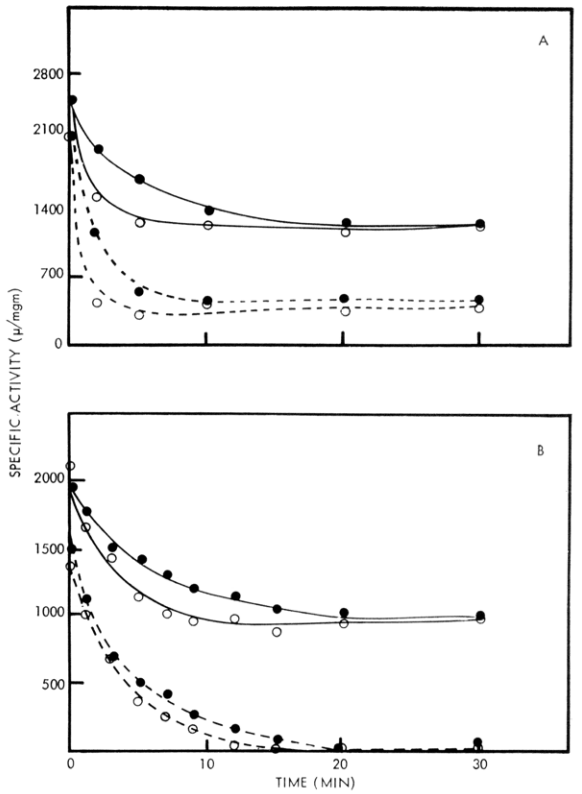

FIGURE 7: Tryptic digestion of phosphorylase. (A) Phosphorylase *b* (5 mg/ml) in 0.04 M glycerophosphate–0.03 M cysteine (pH 7.0) with 4 mM (●) or without spermine (○) was incubated with trypsin (20 μg/ml) at 30°. At various time intervals aliquots were then diluted in the same buffer containing (60 μg/ml) trypsin inhibitor. These samples were then assayed with 25.6 mM G-1-P, 1% glycogen, 4 mM spermine, and either 1 mM AMP (——) or 5 mM IMP (- - -). (B) Phosphorylase *a* (4 mg/ml) alone (●) or with 4 mM spermine (○) was incubated with 10 μg/ml of trypsin. Aliquots of the reaction mixture were withdrawn at the time intervals and diluted as in A. Activity measurements were carried out with 0.016 M G-1-P, 1% glycogen, and with 1 mM AMP (——) or without AMP (- - -). Other conditions as in A.

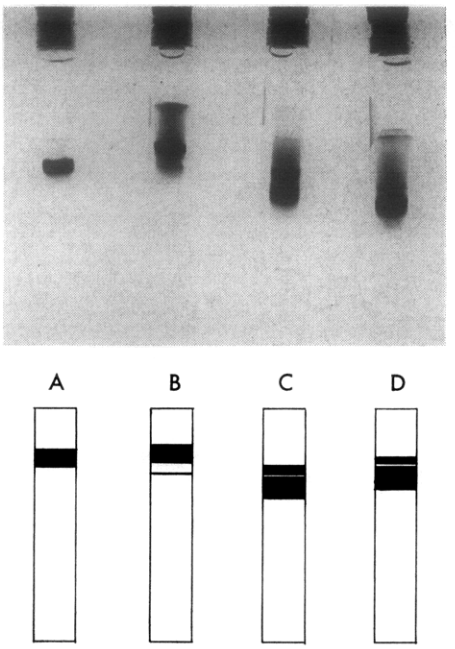

FIGURE 8: Effect of trypsin on the gel electrophoresis patterns of phosphorylase. (A) Phosphorylase *a* or (B) phosphorylase *b* was added to the tops of the gels in 60 μl (125 μg); (C) as A after trypsin treatment; (D) as B after trypsin treatment. Phosphorylase (5 mg/ml) was treated with trypsin (20 μg/ml) at 30°. After 10 min the reaction was stopped with trypsin inhibitor (60 μg), then diluted in the sample gel. Resolution was obtained at pH 8.3, at 3 mA/tube for 70 min. Below the photograph of the gels a diagrammatic representation is presented of each gel perfectly aligned.

ase *b* (4 mg/ml) with 20 μg of trypsin for 10 min, the ultracentrifugal pattern of the enzyme shows a symmetrical peak with a sedimentation constant of 8.2 S, which is identical with that of the untreated enzyme. Addition of 1 mM AMP or 1 mM AMP plus 2 mM spermine has no effect on this ultracentrifugal pattern.

In addition phosphorylase has been characterized by polyacrylamide gel electrophoresis. Figure 8A,B shows that a slight difference in mobility between native phosphorylase *a* and *b* can be detected. This difference is reproducible. After trypsin digestion, however, the gel patterns of these two forms of the enzyme become more similar in respect to their mobilities (Figure 8C,D). Two distinct protein bands also appear in both cases. The relative distribution of the two bands are similar in that the predominant ones possess greater mobility. It is known that the mobility of proteins on polyacrylamide electrophoretic gels depends on their molecular charge as well as size (Sober *et al.*, 1965). The sedimentation velocity experiments, however, suggest that trypsin causes little change in the molecular size of phosphorylase *b*. The observation that the enzyme moves farther to the anode after tryptic digestion, therefore, suggests that positively charged peptides have been removed from the enzyme.

Chemical Characterization of One of the Peptides Liberated by Tryptic Digestion of Phosphorylase b. Figure 9 shows that the peptides released by tryptic digestion of phosphorylase *b* can separate into six ninhydrin-positive components by high-voltage electrophoresis. That all components migrate to the cathode suggests that they consist of positively charged amino acids or peptides at pH 4.7. The peptides corresponding to the major component (spot 5) have been isolated by preparative electrophoresis and its amino acid composition was determined. Nine amino acids have been identified and their ratios to the six major ones are: $Ala_{0.20}$, $Arg_{0.97\ (1)}$, $Asp_{0.23}$, $Glu_{1.07\ (1)}$, $Gly_{0.23}$, $Ile_{0.97\ (1)}$, $Lys_{0.98\ (1)}$, $Ser_{1.07\ (1)}$, $Val_{0.92\ (1)}$. The six major amino acids correspond to those of the hexapeptide released by tryptic digestion of phosphorylase *a*. These results, along with the other evidence, offer strong support for the postulate that phosphorylase *b'* may be produced by tryptic digestion of phosphorylase *b*.

Discussion

The effect of polyamines on the activity of glycogen phosphorylase is similar in most respects to the protamine or polylysine effect observed by Krebs (1954).

Both protamine and polyamines enhance the affinity of phosphorylase *b* for AMP. Since these effectors have one common characteristic in that they are polycationic to a high degree, the results strongly support the argument that the interactions of protamine and polylysine as well as polyamines with glycogen phosphorylase are electrostatic in nature. Presumably then there exist regions on the enzyme surface which are negatively charged, and can bind these polycationic molecules. The use of the smaller molecules such as polyamines, however, may facilitate the investigation of this molecular interaction. For instance the precipitation of phosphorylase *a* by protamine (Madsen and Cori, 1954), which inhibits phosphorylase *a* (Krebs, 1954), was not observed in our experiments using polyamines instead of protamine. If, as suggested by Madsen and Cori (1954), precipitation by protamine is due to an interaction analogous to an antigen–antibody reaction, then our results indicate that polyamines have only a monovalent binding site for the enzyme. Furthermore ultracentrifugal determination of structural changes in the enzyme which may be caused by these electrostatic interactions are facilitated by the use of the polyamines. While polyamines alone do not affect the ultracentrifugal pattern of phosphorylase *b*, the combination of AMP and a polyamine enhances the partial aggregation caused by AMP (Figure 3). Since phosphorylase *a* shows a higher affinity for AMP (Green and Cori, 1943), as well as a higher tendency to associate into a tetrameric species (Keller and Cori, 1953), it may be postulated that binding of polyamines to phosphorylase *b* results in an enzyme form which is intermediate between phosphorylase *a* and phosphorylase *b*.

In contrast to the results observed with phosphorylase *b*, the structure and activity of phosphorylases *a* and *b'* are not significantly altered by polyamines. It is known that the main chemical difference among these forms lies in a hexapeptide fragment containing a specific serine residue. Our results, therefore, indicate that the effect of polyamines on phosphorylase is closely related to this hexapeptide. Fischer and Krebs and their coworkers (Fischer *et al.*, 1959; Nolan *et al.*, 1964) have shown by amino acid sequence studies that there are a large number of positively charged amino acids in the vicinity of this serine residue. In this particular region of the enzyme, phosphorylase *a*, because of phosphorylation of the serine residue, and phosphorylase *b'* by removal of a positively charged hexapeptide are less positively charged than phosphorylase *b*. The activation of phosphorylase *b* by polycationic molecules, then, cannot be explained by their direct binding to this region. It may be expected, however, that differences in positive charges in this portion of the enzyme cause differences in the environment of the three enzyme forms. While in phosphorylase *b* the hexapeptide fragment may electrostatically interact with a highly negatively charged region of the enzyme, such interaction would be partially or totally eliminated in the other two forms. When polyamines bind to this polyanionic site, a displacement of the particular polycationic region of phosphorylase *b* could result. Such displacement could cause phosphorylase *b* to assume some of the characteristics of phosphorylase *a*. Although this model is not the only means of interpreting our data, it may be used as a working hypothesis for further experimental tests.

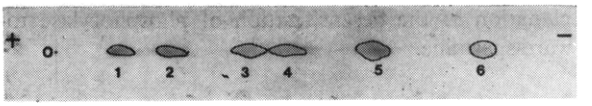

FIGURE 9: High-voltage electrophoresis of the tryptic peptide from phosphorylase *b*. Resolution was obtained in pyridine acetate buffer (pH 4.7) at 3000 V for 35 min. Peptide mixture (2 mg/ml) was spotted (40 μl) at point 0. The paper was stained with ninhydrin collidine.

The effect of polyamines on the tryptic digestion of phosphorylase has been used to test this model. Conversion of phosphorylase *a* into *b'* by trypsin results in the removal of a hexapeptide fragment (Fischer *et al.*, 1959). In the present study changes in catalytic properties as well as other data suggest that phosphorylase *b'* can also result from the tryptic digestion of phosphorylase *b*. The fact that only the *b* into *b'* conversion is significantly facilitated by spermine can be explained on the basis that spermine in this case exposes the hexapeptide in question.

Both phosphorylases *a* and *b* may exist in equilibrium between a less active tetrameric and a more active dimeric form (Kent *et al.*, 1958; Wang and Graves, 1963, 1964; Wang *et al.*, 1965a,b). In contrast, phosphorylase *b'* is shown to exist as a dimer even under conditions known to induce the formation of a tetramer using phosphorylase *b*. These results suggest that the hexapeptide is essential for the association of the dimeric into a tetrameric form.

Recently Sealock and Graves (1967) have also placed emphasis on the phosphoseryl residue, in explaining the effect of salt solutions on phosphorylase. In their model the activation of phosphorylase *b* by NaF is attributed to the "salting out" of a specific region of the enzyme surrounding the serine residue. Since the mechanism of salting out of proteins is not well understood (Dixon and Webb, 1961), it is difficult to compare their model with that presented in the present study.

Since spermine enhances the affinity of phosphorylase *b* toward AMP presumably through its effect on protein conformation, it may be considered an allosteric effector (Monod *et al.*, 1963). The interaction between the polyamines and the nucleotide is not compatible with the model recently proposed by Monod *et al.*, (1965), in that the Hill coefficient for AMP is not affected by spermine. Our data are more compatible with a postulation of a conformational change of the enzyme induced by the binding of an effector (Atkinson *et al.*, 1965). The allosteric interaction of glucose and AMP with phosphorylase *a* has also been suggested to agree with this postulation (Black and Wang, 1967).

Although it is generally accepted that "desensitization" of allosteric enzymes is associated with an alteration of enzyme structure, the molecular mechanism is not clear. The sensitivity of phosphorylase to spermine has been shown to depend on the chemical structure of a small fragment of the enzyme, the hexapeptide. Furthermore our molecular model seems to provide an ex-

planation of the desensitization of phosphorylase toward spermine.

Acknowledgments

The authors are grateful to Dr. F. C. Stevens for his advice on the chemical study of the peptides, to Dr. F. S. LaBella for the use of the autoanalyzer, and to Mr. Garry Queen for performing the amino acid analysis. The authors also wish to acknowledge Mr. D. Zaleski and Mr. C. Anema for performing the ultracentrifugal experiments.

References

Atkinson, D. E., Hathaway, J. A., and Smith, E. C. (1965), *J. Biol. Chem. 240*, 2682.

Black, W. J., and Wang, J. H. (1967), *Proc. Can. Federation Biol. Soc. 10*, 20.

Block, R. J., Durrum, E. L., and Zweig, G. (1955), A Manual of Paper Chromatography and Paper Electrophoresis, New York, N. Y., Academic.

Brown, D. H., and Cori, C. F. (1961), *Enzymes 3*, 1.

Cori, G. T., Colowick, S. P., and Cori, C. F. (1938), *J. Biol. Chem. 123*, 381.

Cori, G. T., and Cori, C. F. (1945), *J. Biol. Chem. 158*, 321.

Dixon, M., and Webb, E. C. (1961), *Advan. Enzymol. 16*, 197.

Fischer, E. H., Graves, D. J., Crittenden, E. S., and Krebs, E. G. (1959), *J. Biol. Chem. 231*, 65.

Fischer, E. H., and Krebs, E. G. (1958), *J. Biol. Chem. 231*, 65.

Fischer, E. H., and Krebs, E. G. (1962), *Methods Enzymol. 5*, 369.

Graves, D. G., Sealock, R. W., and Wang, J. H. (1965), *Biochemistry 4*, 290.

Green, A. A., and Cori, G. T. (1943), *J. Biol. Chem. 151*, 21.

Hedrick, J. L. (1966), *Arch. Biochem. Biophys. 114*, 21.

Helmreich, E., and Cori, C. F. (1964), *Proc. Natl. Acad. Sci. U. S. 51*, 131.

Hirs, C. H. W., Moore, S., and Stein, W. H. (1956), *J. Biol. Chem. 219*, 623.

Illingworth, B., and Cori, G. T. (1953), *Biochem. Prepn. 3*, 1.

Keller, P. J., and Cori, G. T. (1953), *Biochim. Biophys. Acta 12*, 235.

Kent, A. B., Krebs, E. G., and Fischer, E. H. (1958), *J. Biol. Chem. 232*, 549.

Krebs, E. G. (1954), *Biochim. Biophys. Acta 15*, 508.

Krebs, E. G., Kent, A. B., and Fischer, E. H. (1958), *J. Biol. Chem. 231*, 73.

Madsen, N. B., and Cori, C. F. (1954), *Biochim. Biophys. Acta 15*, 516.

Madsen, N. B., and Cori, C. F. (1956), *J. Biol. Chem. 223*, 1055.

Monod, J., Changeux, J.-P., and Jacob, F. (1963), *J. Mol Biol. 6*, 306.

Monod, J., Wyman, J., and Changeux, J.-P. (1965), *J. Mol. Biol. 12*, 88.

Moore, S., and Stein, W. H. (1954), *J. Biol. Chem. 211*, 907.

Nolan, C., Novoa, W. B., Krebs, E. G., and Fischer, E. H. (1964), *Biochemistry 3*, 542.

Ornstein, L. (1964), *Ann. N. Y. Acad. Sci. 121*, 321.

Ryle, A. P., Sanger, F., Smith, L. F., and Kitae, R. (1955), *Biochem. J. 60*, 541.

Sealock, R. W., and Graves, D. J. (1967), *Biochemistry 6*, 201.

Shaltiel, S., Hedrick, J. L., and Fischer, E. H. (1966), *Biochemistry 5*, 2108.

Sober, H., Hartley, R., Carroll, W., and Peterson, E. (1965), *Proteins 3*, 1.

Sutherland, E. W., and Wosilait, W. D. (1956), *J. Biol. Chem. 218*, 459.

Velick, S. F., and Wicks, L. F. (1951), *J. Biol. Chem. 190*, 741.

Wang, J. H., and Graves, D. J. (1963), *J. Biol. Chem. 238*, 2386.

Wang, J. H., and Graves, D. J. (1964), *Biochemistry 3*, 1437.

Wang, J. H., Shonka, M. L., and Graves, D. J. (1965a), *Biochem. Biophys. Res. Commun. 18*, 131.

Wang, J. H., Shonka, M. L., and Graves, D. J. (1965b), *Biochemistry 4*, 2296.