Inactivation of Phosphorylase by Cyanate*

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ABSTRACT: Phosphorylases *a* and *b* have been found to be inactivated by potassium cyanate. The course of the inactivation follows pseudo-first-order kinetics. The rate of inactivation varies directly with the concentration of cyanate, but the variation of rate is less than first order with respect to protein concentration. The rates of incorporation of [^{14}C]cyanate into phosphorylases *a* and *b* parallel the rates of inactivation of the two enzymes, but those for *b* are somewhat greater. Identical pseudo-first-order constants for both incorporation and inactivation are obtained for each of the two proteins if it is assumed that 52 residues per molecule of *a* and 23 per molecule of *b* must be carbamylated to yield 100% inactivation. The reaction of cyanate with phosphorylase has been shown to involve the carbamylation of the ϵ-amino groups of lysine to form homocitrulline residues. The sulfhydryl groups of phosphorylase do not react, nor is pyridoxal 5′-phosphate displaced from the protein. The K_m of glucose-1-P for phosphorylase *a* did not change significantly through the greater part of the inactivation process while the K_m values of glucose-1-P and adenosine monophosphate (AMP) for phosphorylase *b* did increase. Although glucose-1-P and AMP decrease the rate of inactivation by cyanate while glycogen does not, there is as yet no evidence that the ϵ-amino groups of lysine play a direct role in the catalytic process of this enzyme. Ultracentrifugal analyses of carbamylated phosphorylase *a* suggest that the completely inactivated enzyme exists in a dimeric form rather than the usual tetrameric form, while the partially inactivated enzyme shows a partial conversion to the dimeric form.

Stark *et al.* (1960) found that ribonuclease not only lost enzymatic activity but also showed an altered amino acid composition when it was incubated with 8 M urea at 40° and then dialyzed to remove the urea. They found that some of the lysine residues had been carbamylated, to form homocitrulline, by the cyanate present in the urea solution. Further investigation showed that potassium cyanate would react rapidly with the amino groups of proteins under mild conditions of pH and temperature. It reacts even more rapidly with sulfhydryl groups, so that this must be borne in mind when using the reagent to alter the amino groups of proteins containing free sulfhydryl groups. Subsequent work by Stark (1964, 1965a,b; Stark and Smyth, 1963) has been concerned with a detailed analysis of the chemistry of the reactions between cyanate and various functional groups of proteins or model compounds.

This study was undertaken to investigate the effects of cyanate on various enzymatic and physical characteristics of phosphorylases *a* and *b* in an effort to determine the role which lysine might play in this enzyme. Information on the role of various specific amino acid residues in the normal functioning of phosphorylase is sparse, but it is known that intact sulfhydryl groups are required for activity, probably because of their role in maintaining the quaternary structure of the protein (Madsen and Cori, 1956; Madsen and Gurd, 1956). Phosphorylase *a*, with a molecular weight of 495,000 and an $s_{20,w}$ of 13.2 S (Keller and Cori, 1953), was found to be a tetramer which could be dissociated to a monomer of molecular weight 135,000 and $s_{20,w}$ of 5.6 S by reaction with *p*-mercuribenzoate (PMB)[1] or other sulfhydryl reagents. Similarly, phosphorylase *b*, with a molecular weight of 242,000 and an $s_{20,w}$ of 8.2 S (Keller and Cori, 1953), was shown to be a dimer. In addition, it is known that phosphorylase *a* contains four serine phosphate residues while phosphorylase *b* contains none (Krebs and Fischer, 1956). Each form of the enzyme contains 1 mole of pyridoxal phosphate bound to one specific lysine for each 125,000 molecular weight (Baranowski *et al.*, 1957; Fischer *et al.*, 1958).

Materials and Methods

Crystalline phosphorylase *b* was prepared from rabbit muscle as described by Fischer and Krebs (1962) and crystalline phosphorylase *a* was prepared from rabbit muscle by the method of Green and Cori (1943) or from phosphorylase *b* by the method of Krebs *et al.* (1958). These enzymes were recrystallized four or five times and passed through a Sephadex G-25 gel filtration column, pre-equilibrated with 0.02 M sodium glycerophosphate–0.0015 M EDTA buffer (pH 6.8)

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[1] The following abbreviations are used: PMB, *p*-mercuribenzoate; AMP, adenosine monophosphate; glucose-1-P, glucose 1-phosphate; TCA, trichloroacetic acid.

in order to remove adenosine monophosphate (AMP), Mg^{2+}, and other impurities.

Glucose-1-P and rabbit liver glycogen were purchased from Mann Research Laboratories, Inc. The latter was purified by passing it through a Dowex 1-Cl column. AMP and PMB were obtained from the Sigma Chemical Co. Sephadex G-25 was a product of Pharmacia, Uppsala, Sweden. KCNO was bought from the Fischer Scientific Co. and used without further purification. Sufficient KCNO to make a 1.0 M solution was weighed into a 10-ml volumetric flask and dissolved in 0.1 M sodium glycerophosphate–0.0015 M EDTA buffer, pH 6.8, just before use. [^{14}C]KCNO (7 mcuries/mmole) was obtained from the Radiochemical Centre, Amersham, England.

It was found that buffers of 0.02 M glycerophosphate–0.0015 M EDTA were inadequate to maintain a constant pH when 0.1 M KCNO reacted with phosphorylase. Typically, the pH increased from 6.8 to 8.5 during the reaction. Increasing the sodium glycerophosphate concentration to 0.1 M sufficed to maintain the pH at 6.8. To determine the inhibition of phosphorylase by KCNO, the enzyme and 1 M KCNO in 0.1 M sodium glycerophosphate–0.0015 M EDTA were preincubated for 10 min at 30°. The carbamylation was started by the addition of KCNO to the enzyme solution to make the reaction mixture 0.1 M in cyanate. The enzymatic activity of aliquots diluted 100 times in 0.02 M sodium glycerophosphate–0.0015 M EDTA, pH 6.8, was determined at zero time and at various intervals. The enzymatic activity of a control reaction, minus KCNO, remained fairly constant for 24 hr.

Phosphorylase activity in the direction of glycogen synthesis was determined by the method of Cori *et al.* (1943), in the absence of cysteine, however Absorbance spectra were determined with a Zeiss PMQ II spectrophotometer. Protein concentrations were determined from the absorbance at 280 mμ, using a value of 11.8 for the $E_{cm}^{1\%}$ (Velick and Wicks, 1951).

The [^{14}C]CNO content of phosphorylase was determined by precipitating the reaction mixture with cold 5% TCA and washing the protein with 5% TCA on a glass fiber type E filter from Gelman Co. The denatured protein on the filter was then transferred to a scintillation vial with 10 ml of scintillation fluid (Bray, 1960), and the radioactivity was counted on a liquid scintillation counter (Model 8260, Nuclear Chicago). The total incorporation of [^{14}C]CNO into phosphorylase *a* or *b* was calculated from the ^{14}C count in the precipitated protein as compared to the specific radioactivity of the chemical as determined on controls.

Amino acid analyses of control or carbamylated phosphorylases were carried out according to the procedures which Stark *et al.* (1960) employed in order to obtain an adequate resolution of homocitrulline from valine. The values obtained for homocitrulline were corrected for the 24% reversion to lysine which the above authors found to occur during the 22-hr hydrolysis at 110° in 6 N HCl.

Ultracentrifugal studies of sedimentation coefficients were made with a Spinco Model E analytical ultracentrifuge equipped with an RTIC unit for temperature regulation, employing a 12-mm single sector Kel F cell at a rotor speed of 59,780 rpm. The temperature of the rotor during most runs was maintained at 20°. The buffer system was 0.033 M sodium glycerophosphate, 0.0015 M EDTA, and 0.13 M KCl, pH 6.8. Part of the KCl was occasionally replaced by an equivalent amount of KCNO. Sedimentation constants were corrected to the standard sedimentation constant, $s_{20,w}$, by standard procedures. The percentage of components with different sedimentation coefficients was determined by estimation of areas of empirically resolved components. No corrections for possible effects of multiple components on the sedimentation coefficients or areas were made, nor have such corrections been made in previous studies on the phosphorylase system (Keller and Cori, 1953; Madsen and Gurd, 1956). The protein concentration was in the range 0.4–0.7%. No attempt was made to determine the effect of protein concentration on the sedimentation analyses, but it may be noted that previous studies have shown that the sedimentation constants of phosphorylases *a*, *b*, and the monomer resulting from reaction with PMB show little dependence on protein concentration (Keller, 1953; Madsen and Cori, 1956).

Pyridoxal 5-phosphate was liberated from phosphorylase by extraction with 0.3 N perchloric acid (Krebs *et al.*, 1958). Treatment of phosphorylase *a* or *b* with sodium borohydride at pH 4.5 in order to reduce the linkage of pyridoxal 5-phosphate to lysine was carried out according to the procedure of Fischer *et al.*

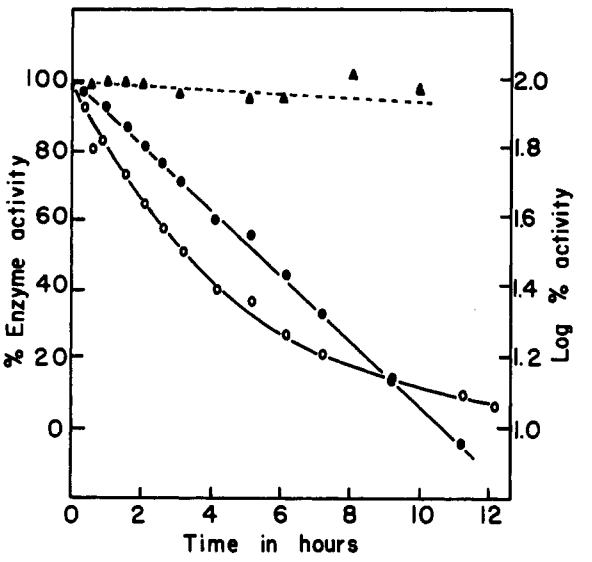

FIGURE 1: Inactivation of phosphorylase *a* activity by 0.1 M KCNO in 0.1 M sodium glycerophosphate–0.0015 M EDTA, pH 6.8, 30° (6.4 mg/ml of protein). ○, Enzymic activity as per cent of zero time activity; ●, logarithm of per cent enzymic activity; ▲, control enzymic activity in 0.1 M KCl.

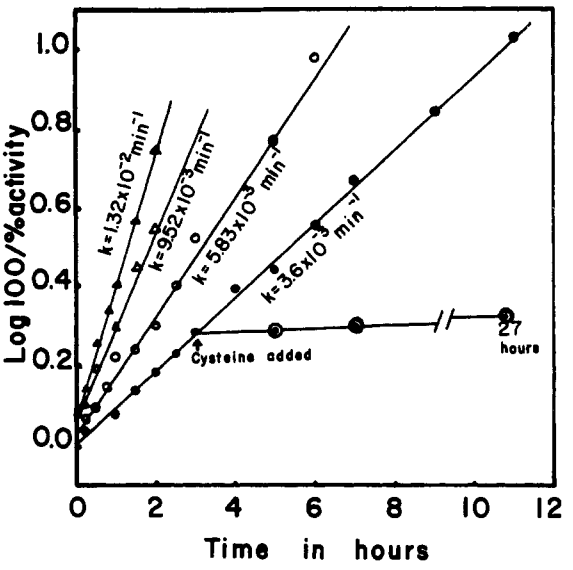

FIGURE 2: Comparison of phosphorylase *a*, *b*, and their reduced forms with respect to inactivation by 0.1 M KCNO. ●, Phosphorylase *a*, 6.4 mg/ml; ○, phosphorylase *b*, 7.0 mg/ml; △, reduced phosphorylase *a*, 1.9 mg/ml; ▲, reduced phosphorylase *b*, 2.1 mg/ml; ⊙, freshly neutralized cysteine added to a concentration of 0.1 M to a portion of the reaction mixture containing phosphorylase *a*.

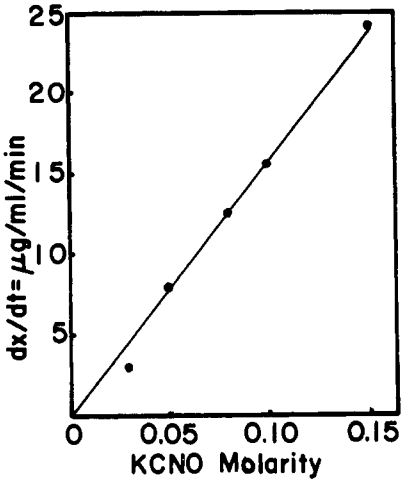

FIGURE 3: The effect of cyanate concentration on the rate of inactivation of phosphorylase *a* (4.8 mg/ml), pH 6.8, at 30°.

(1958). Such borohydride-treated enzyme will be referred to as reduced phosphorylase.

Disk electrophoresis on polyacrylamide gels was carried out according to the procedures of Davis and Ornstein (1961). A 5% gel in 5×10^{-3} M Tris–4×10^{-2} M glycine, pH 8.2, in columns 5 mm × 7 cm, was found most suitable. The entire apparatus was refrigerated at 4° and the voltage was kept at 115 v between gel ends for 1.5 hr.

Results

Kinetic Studies

The inactivation of phosphorylase *a* by 0.1 M KCNO is shown in Figure 1, compared to a control reaction mixture in which KCl replaced the cyanate. As shown in Figure 1, the inactivation of phosphorylase activity by 0.1 M KCNO follows kinetics typical of a first-order reaction. A comparison of rabbit muscle phosphorylase *a*, *b*, and reduced phosphorylase *a* and *b* has been made and is shown in Figure 2. The kinetics of inactivation of these four enzymes appear to be similar except that the inactivation of phosphorylase *b* is faster than that of phosphorylase *a*. The larger pseudo-first-order constants obtained with the reduced enzymes are due primarily to their being present at lower concentrations in the reaction mixture.

Figure 2 also shows that if neutralized cysteine sufficient to exceed the KCNO is added at any point during the reaction the enzymatic activity remains at a constant level. This convenient and rapid means of arresting the inactivation has been used whenever partially inactivated enzymes were required. It would appear that the cysteine reacts rapidly with the free KCNO and thus prevents further carbamylation and inactivation. Furthermore, the inactivation of phosphorylase by cyanate is not reversible by cysteine.

The pseudo-first-order constants (K') for the inactivation of phosphorylase *a* were determined for individual experiments at varying concentrations of KCNO at a constant enzyme concentration of 4.8 mg/ml. The initial rates in terms of micrograms of protein inactivated per minute per ml were calculated from the formula $dx/dt = K'$ (μg of protein/ml) and are shown plotted against the concentration of KCNO in Figure 3. The linear relationship between the initial rate of reaction and the concentration of KCNO is the expected effect of changing the concentration of the components of a bimolecular reaction, i.e.,

$$dx/dt = K(\text{KCNO})(\text{enzyme})$$

Here K is the second-order constant which is equal to $K'/(\text{KCNO})$. The second-order constant was found to be 2.0, 3.2, 3.25, 3.25, and 3.30×10^{-2} M^{-1} min^{-1} for 0.03, 0.05, 0.08, 0.1, and 0.15 M KCNO, respectively. This uniformity of the second-order constant at various concentrations of KCNO indicates that the rate of inactivation is directly proportional to the concentration of KCNO. It may also indicate an inverse proportionality of amount of activity to the amount of KCNO which has reacted.

The experiment was repeated using various concentrations of phosphorylase *a* at a constant concentration of KCNO of 0.1 M. The results were analyzed as discussed above and are shown in Figure 4. The peculiar effect of protein concentration on the rate of inactivation may mean that the reaction is less than first order with respect to enzyme. This may be due to increasing

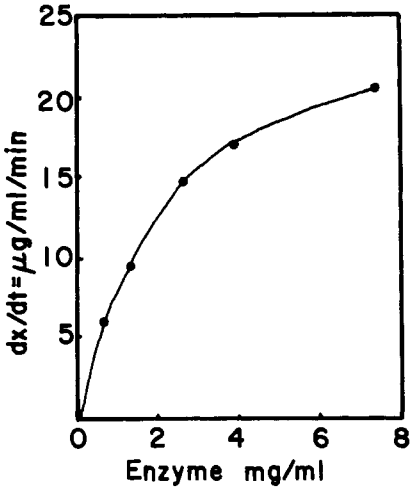

FIGURE 4: The effect of phosphorylase *a* concentration on the rate of its inactivation by 0.1 M KCNO.

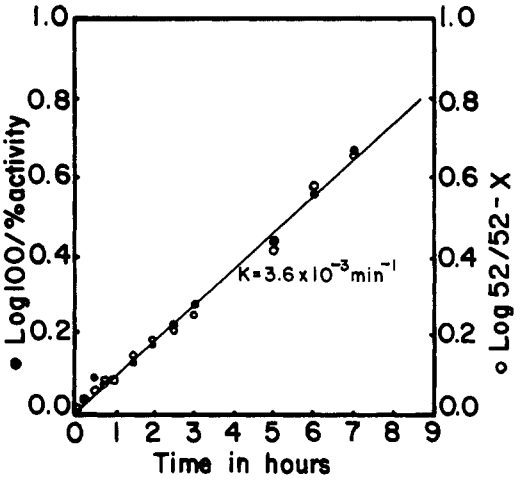

FIGURE 5: The first-order rate constant calculated from either the loss of enzymic activity or the equivalents of cyanate incorporated per molecule of phosphorylase *a* (6.5 mg/ml of protein and 0.1 M KCNO containing 4 μcuries of [^{14}C]CNO per milliliter).

enzyme concentration resulting in decreasing the accessibility of the sites reacting with cyanate. One might speculate that the reagent reacts with a dissociated form of the enzyme which is always present in a very small proportion in equilibrium with associated oligomer. As the concentration of protein is increased, the proportion in the dissociated form would decrease, even though its absolute concentration would increase.

The relationship between the extent of the reaction between cyanate and phosphorylase and the loss of enzymatic activity was investigated by following the incorporation of [^{14}C]CNO into protein at the same time that activity losses were determined. The results for phosphorylase *a* are shown in Figure 5. The loss of activity follows the usual first-order kinetics. The incorporation of [^{14}C]CNO also follows first-order kinetics with the same rate constant if it is assumed that the incorporation of 52 moles of cyanate/mole of protein results in complete inactivation. A similar experiment with phosphorylase *b* is shown in Figure 6, except that the data suggest that 23 moles of cyanate/mole of enzyme must be incorporated to achieve inactivation. Evidence presented below indicates that it is unlikely that cyanate reacts with any amino acid residue other than lysine. The data in Figures 5 and 6 therefore suggest that there are 13 lysine residues/monomer unit of phosphorylase *a* which are equivalent with respect to their effect on enzymatic activity, while the corresponding value for phosphorylase *b* is 12. The data for the two forms of phosphorylase are thus consistent in spite of the differences in molecular weights and rates of reaction with cyanate.

Sulfhydryl Groups

Fraenkel-Conrat (1944) and Stark *et al.* (1960) have shown that the sulfhydryl groups of cysteine and glutathione react rapidly with cyanate. Because, as discussed in the introduction, the reaction of the sulfhydryl groups of phosphorylase with various reagents results in the loss of enzymatic activity and structural integrity, it was necessary to determine if the inactivation of phosphorylase by cyanate was due to the carbamylation of sulfhydryl groups. As was demonstrated in Figure 2, the inactivation is not reversible with cysteine, whereas the reaction of sulfhydryl groups with cyanate is readily reversible (Stark, 1964). Carbamylated phosphorylase *a* was titrated with PMB by the method of Boyer (1954), and a value of 19.4 sulfhydryl groups/molecule of protein was obtained, in agreement with previous determinations (Madsen and Cori, 1956). Furthermore, the rate of reaction of PMB with the sulfhydryl groups of carbamylated phosphorylase *a* was similar to that found for the native enzyme. Finally, the rates of incorporation of [^{14}C]CNO into phosphorylases *a* and *b* which had been pretreated with sufficient PMB to cause complete inactivation were compared to the rates of incorporation into the native proteins. As can be seen in Figure 7, the protection of the sulfhydryl groups did not affect the rate of carbamylation of the protein.

These experiments all point to the same conclusion, that the immediate, primary cause of the inactivation of phosphorylase by cyanate is not a reaction of the reagent with the sulfhydryl groups of the enzyme. This conclusion is reached in spite of the fact that cyanate reacts very rapidly with the free sulfhydryl groups of simple organic molecules. However, there is considerable evidence that the sulfhydryl groups of phosphorylase are not freely available to react rapidly with reagents which would normally do so (Madsen and Cori, 1956).

Pyridoxal Phosphate

If the cyanate had displaced the pyridoxal phosphate

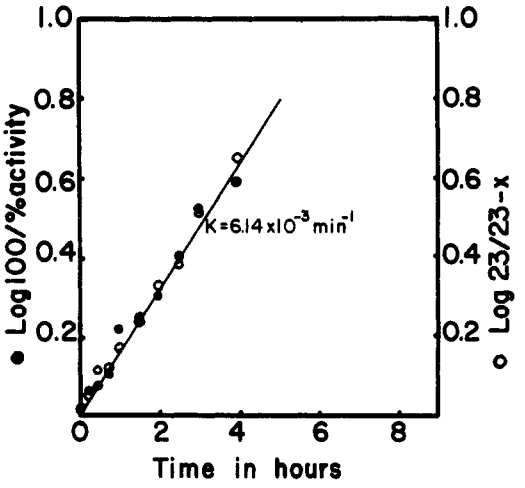

FIGURE 6: The first-order rate constant calculated from either the loss of enzymic activity or the equivalents of cyanate incorporated per molecule of phosphorylase *b* (7.0 mg/ml of protein and 0.1 M KCNO containing 4 μcuries of [^{14}C]CNO per milliliter).

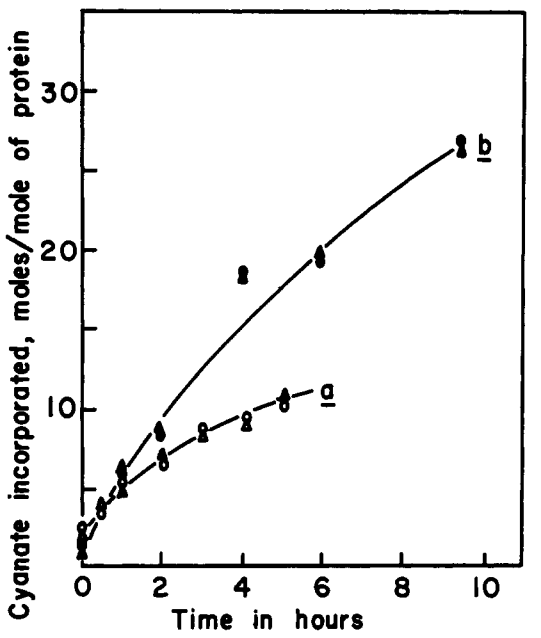

FIGURE 7: Incorporation of [^{14}C]CNO into phosphorylases preincubated with PMB, as compared to the native enzymes. Preincubation for 90 min resulted in complete inactivation. Phosphorylase *a* samples were added directly to 5% TCA for radioactivity measurements, while phosphorylase *b* samples were first added to cysteine buffer and dialyzed overnight. △, Phosphorylase *a*, 5.3 mg/ml; ○, phosphorylase *a*, 5.3 mg/ml plus 2.4 × 10^{-4} M PMB; ▲, phosphorylase *b*, 5.8 mg/ml; ●, phosphorylase *b*, 5.8 mg/ml plus 3.3 × 10^{-4} M PMB.

moieties from the lysine residues to which they are bound, then an inactivation would occur. This possibility was explored by spectrophotometric means, based on the fact that pyridoxal phosphate at neutral pH has an absorption maximum at 333 mμ when bound to phosphorylase while the free coenzyme has an absorption maximum at 388 mμ (Kent *et al.*, 1958). Furthermore, the dissociation of the coenzyme proceeds through an intermediate Schiff base form with an absorption maximum above 400 mμ. The absorption spectrum of phosphorylase *b* at a concentration of 9.23 mg/ml was determined and was then repeated at 2, 4, and 5.5 hr after the addition of KCNO to a concentration of 0.1 M at 30°. The enzymatic activity at these times was 47, 19, and 14% of the original activity. The spectra showed two peaks, at 278 and 333 mμ, and did not show any alteration during the period in which the enzyme was being inactivated by cyanate. The native enzyme had an absorbance of 0.621 at 333 mμ for a 1% solution of protein while the carbamylated enzyme had an absorbance of 0.625. These values agree well with those reported by Kent (1959), which are in the range of 0.52 to 0.63.

Borohydride-treated phosphorylases *a* and *b*, in which the linkage of pyridoxal phosphate to lysine has been reduced to a more stable amine form, still retain from 60 to 80% of their original activity (Fischer *et al.*, 1958). As was shown in Figure 2, such "reduced" enzymes are inactivated by cyanate in the same manner as the original enzymes.

These experiments indicate that the inactivation of phosphorylase by cyanate does not involve the pyridoxal phosphate components of the enzymes. Nevertheless, it was found that phosphorylase *b* which had been fully inactivated by cyanate lost as much as 45% of its content of pyridoxal phosphate when dialyzed for 24 hr in 0.02 M glycerophosphate–0.0015 M EDTA, pH 6.8, at 0°. Since reagents such as 8 M urea, sodium dodecyl sulfate, or 2.5 M NaCl also cause the loss of pyridoxal phosphate, it may be suggested that the carbamylation of lysine residues has induced conformational changes.

Amino Acid Analyses

When samples of phosphorylase which had been inactivated with cyanate were subjected to amino acid analyses it was found that a new amino acid peak appeared at exactly the same location (375 ml of effluent from the Amberlite IR-120 column) as did the homocitrulline from carbamylated ribonuclease (Stark *et al.*, 1960). Furthermore, the appearance of this new peak was accompanied by a proportionate decrease in the amount of lysine found in the protein. No significant changes were found in the quantities of the other amino acids in phosphorylase (no analyses were performed for cysteine and tryptophan). Experiments to rule out the possibility of reaction with the sulfhydryl groups of cysteine were reported above, and there is a possibility that phosphorylase does not contain any free α-amino groups (Appleman *et al.*, 1963). It was concluded that the primary, if not the sole, action of cyanate on phos-

TABLE I: Homocitrulline Contents of Carbamylated Phosphorylases.

Experiment	Conditions[a]	Residues of Homocitrulline/Molecule of Protein	Lysines carbamylated (%)
1	Phosphorylase *a*, 2.5 hr in 0.1 M KCNO, 42% inactivated	26	10.6
2	Phosphorylase *a*, 23 hr in 0.1 M KCNO, 100% inactivated	102	41.5
3	Phosphorylase *a*, 24 hr in 0.1 M KCNO and 8 M urea	115	46.2
4	Phosphorylase *b*, 1.5 hr in 0.1 M KCNO, 60% inactivated	12	9.1
5	Phosphorylase *b*, 24 hr in 0.1 M KCNO, 100% inactivated	35	27.5
6	Phosphorylase *b*, 24 hr in 0.1 M KCNO and 8 M urea	50	40

[a] All reactions at pH 6.8. 30°.

phorylase is to carbamylate the ε-amino groups of lysine residues to form homocitrulline residues.

Table I shows the quantities of homocitrulline which were found in samples of phosphorylase *a* or *b* which had been inactivated partially or completely by cyanate. The partially inactivated enzymes have homocitrulline contents which are in reasonable agreement with those predictable from the experiments on the incorporation of [^{14}C]CNO. It is obvious that carbamylation of lysine residues continues after those which are necessary for activity have reacted, but the conditions used here, even including 8 M urea, did not result in more than 50% of the total number of lysine residues becoming carbamylated.

Relationship of Substrates

Possible protection against inactivation was tested by preincubating phosphorylase *a* with the following at 30°: (1) 1.1% glycogen; (2) 0.018 M glucose-1-P; (3) 0.0011 M AMP; (4) 0.0011 M AMP plus 0.018 M glucose-1-P. After 10 min of preincubation, 0.1 volume of 1.0 M KCNO was added. In the control experiments 0.1 M KCl replaced the KCNO. The results are shown in Figure 8. It may be seen that 1% glycogen did not provide any protection against inactivation by cyanate, while glucose-1-P and AMP provided significant protection of a similar degree. Even more protection occurred when both glucose-1-P and AMP were present together. The increased protection afforded by the combination of these two compounds might indicate that they have separated binding sites and that these sites have some relationship to lysine residues. This is presumably not true for the glycogen binding site(s).

It should be noted, however, that the protection afforded by glucose-1-P and AMP against inactivation by cyanate is not an absolute effect but is manifested merely by a decrease in the rate of inactivation by no more than 60% at most. Thus, one is not justified in invoking a direct relationship with lysine residues because other possibilities may be visualized for an enzyme composed of four subunits in which interactions between substrates and activators have been observed (Helmreich and Cori, 1964; Lowry *et al.*, 1964), as well as effects of the latter upon protein conformation (Wang and Graves, 1963). The phenomenon noted here, there-

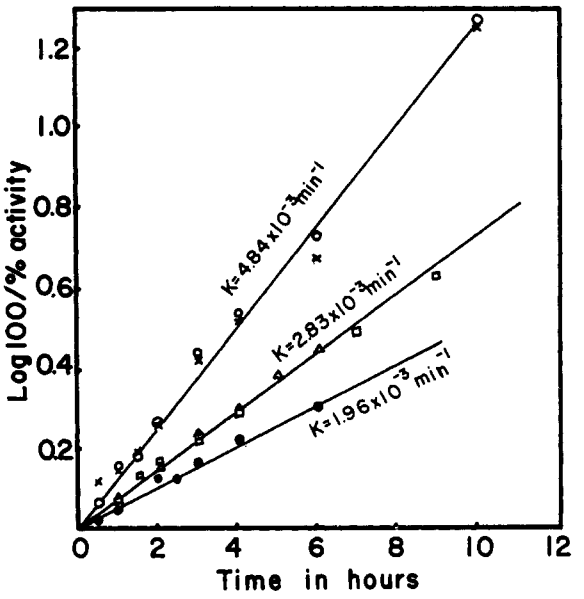

FIGURE 8: The effect of substrates on the rate of inactivation of phosphorylase *a* (4 mg/ml) by 0.1 M KCNO. ○, Control; ×, plus 1% glycogen; △, plus 0.016 M glucose-1-P; □, plus 0.001 M AMP; ●, plus 0.016 M glucose-1-P and 0.001 M AMP.

TABLE II: K_m and V_{max} Values of Glucose-1-P and AMP for Phosphorylases *a* and *b* at Various Carbamylation Times.

Exposure to 0.1 M KCNO (hr)	Phosphorylase *b*				Phosphorylase *a*	
	V_{max} of AMP[a]	K_m of AMP ($M \times 10^{-5}$)	V_{max} of Glucose-1-P[a]	K_m of Glucose-1-P ($M \times 10^{-3}$	V_{max} of Glucose-1-P[a]	K_m of Glucose-1-P ($M \times 10^{-3}$)
0	9.7	3.2	14.7	3.3	5.88	2.2
1	6.4	3.9	9.25	6.4	5.5	2.3
3	2.8	6.3	5.5	13.0	3.42	2.4
5	1.55	6.78	2.82	13.1	2.02	3.3
7	0.79	6.25			1.65	8.1
0 (control)			13.3	3.6	6.3	2.3
5 (control)	11.3	2.1	14.7	4.5		

[a] Micrograms of inorganic phosphate per minute. AMP concentrations in reaction mixtures were 0.4, 0.6, 1.0, 2.0, and 10.0×10^{-4} M at 1.6×10^{-2} M glucose-1-P. Glucose-1-P concentrations in reaction mixtures were 0.4, 0.8, 1.6, and 2.4×10^{-2} M at 10×10^{-4} M AMP.

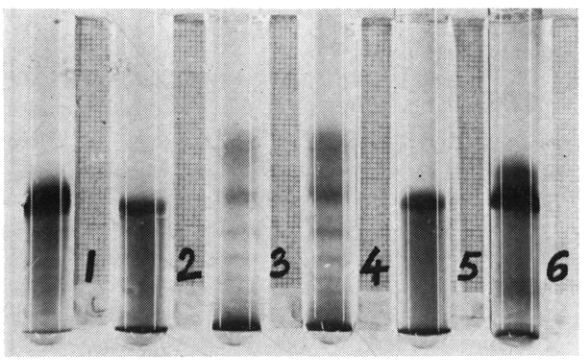

FIGURE 9: Disk electrophoresis patterns of various phosphorylases. (1) Phosphorylase *a*; (2) liver phosphorylase; (3) carbamylated phosphorylase *b*; (4) carbamylated phosphorylase *a*; (5) PMB-treated phosphorylase *b*; (6) phosphorylase *b*. The origins may be seen at the bottoms of the tubes and migration was toward the anode.

fore, may be due to the effects of the compounds on protein conformation, similar to the nonspecific type of protection of an enzyme by its substrate such as is commonly observed in connection with such denaturing agents as heat. In this connection, it is interesting to note that AMP but not glucose-1-P decreases the rate of inactivation of phosphorylase *a* by PMB (M. Battel *et al.*, 1965, unpublished data). Here, of course, sulfhydryl groups rather than the ε-amino groups of lysine are involved, but gross changes in protein structure are observed to occur in both cases.

If the inactivation of phosphorylase were due to a specific blocking of the active sites or a change in their conformation, then this might be manifested by a change in the Michaelis–Menten kinetics during the course of the inactivation. Phosphorylase *a* or *b* was inactivated with 0.1 M KCNO as described in Materials and Methods. Aliquots were withdrawn at intervals and mixed with excess cysteine to stop the reaction. Enzymatic activities were then determined at various concentrations of AMP or glucose-1-P for phosphorylase *b* or glucose-1-P only for phosphorylase *a*. The data were analyzed by the reciprocal plot method of Lineweaver and Burk (1934) and the results are shown in Table II.

To consider phosphorylase *a* first, the decrease in the V_{max} for glucose-1-P with increasing time of carbamylation follows a first-order type of kinetics. The K_m for glucose-1-P does not show any significant change over the greater part of the inactivation. Not until some 75% of the activity has been lost does the K_m show a marked change.

The decrease in the V_{max} values for phosphorylase *b* also follows first-order kinetics, but the changes in the K_m values are more difficult to interpret. Certainly a change in the K_m of glucose-1-P from 3.3 to 13 mM cannot be dismissed as insignificant. The change in the K_m for AMP is less dramatic and could be the result of the change in the binding of the glucose-1-P, since these two compounds have reciprocal effects on each other's K_m values (Madsen, 1964). There is the possibility, then, that during the inactivation of phosphorylase *b* by cyanate various intermediate species of protein appear which have reduced ability to bind substrate or activator but which still retain some catalytic properties. The disk electrophoresis experiments, reported below, indicate that there are indeed intermediates of which some may retain some enzymatic activity. This interpretation is at variance with the results obtained from the kinetics of inactivation and cyanate incorporation.

There would also appear to be a distinct difference between the behavior of the two phosphorylases in this respect.

When the AMP is bound to phosphorylase *a* there is a pronounced alteration in its spectrum, with a decrease in the molar extinction coefficient from 16,000 to 10,000 and a shift in the maximal absorbancy from 259 to 264 mμ (Madsen and Cori, 1957). The alteration in the spectrum of AMP which is produced by carbamylated phosphorylase *a* was found to be less than 20% of the alteration caused by the native enzyme, and it was concluded that the cyanate-inactivated enzyme has lost most of the AMP-binding characteristics of the native enzyme. Although a change in the nature of the binding could produce this result, it is considered more likely that the dissociation constant for AMP and protein has been greatly increased. It has similarly been found that phosphorylase *a* which has been inactivated by PMB no longer affects the spectrum of AMP (M. Battel, 1965, unpublished data).

Disk Electrophoresis

Disk electrophoresis in acrylamide gel indicated that phosphorylases *a* and *b* from muscle are homogeneous by this criterion, as shown in Figure 9. A sample of purified liver phosphorylase also showed only one band. The monomer resulting from the treatment of phosphorylase *a* or *b* with PMB also proved to be homogeneous upon disk electrophoresis and corroborated earlier evidence which suggested that the four monomer units from phosphorylase *a* are identical (Madsen and Cori, 1956; Nolan *et al.*, 1964).

In contrast to these results, it was found that the inactive proteins resulting from the reaction of phosphorylase *a* or *b* with cyanate showed nine bands when subjected to disk electrophoresis (Figure 9, tubes 4 and 3). Thus the carbamylated enzymes are quite heterogeneous by this criterion, in spite of their relative homogeneity in the ultracentrifuge. The proteins which have had a large proportion of their lysine residues carbamylated would be expected to have a greater negative charge and should therefore have migrated a greater distance toward the anode. It is therefore puzzling that seven of the nine bands showed less mobility than the original native enzymes.

Ultracentrifugal Sedimentation Studies

Ultracentrifugal sedimentation analyses of phosphorylase *a* which had been inactivated with cyanate revealed that the protein had been converted into a form with an $s_{20,w}$ of 8.4 S (Figure 10, B and C), as compared with the original $s_{20,w}$ of 13.2 S (Figure 10A). In the light of considerable earlier experimental analyses of the phosphorylase system (Keller and Cori, 1953; Wang and Graves, 1963), one would conclude from these results that the tetrameric form of the native phosphorylase *a* has been converted into a dimeric form upon having some of its lysine residues carbamylated. As is shown in Figure 10C, 90% of the completely inactivated phosphorylase *a* has an $s_{20,w}$ of 8.4 S and only 10% appears as a peak with an $s_{20,w}$ of 5.9 S. If the sulfhydryl groups had been modified, then all of the protein should have been in the form of a monomer with an $s_{20,w}$ of approximately 5.6 S (Madsen and Cori, 1956).

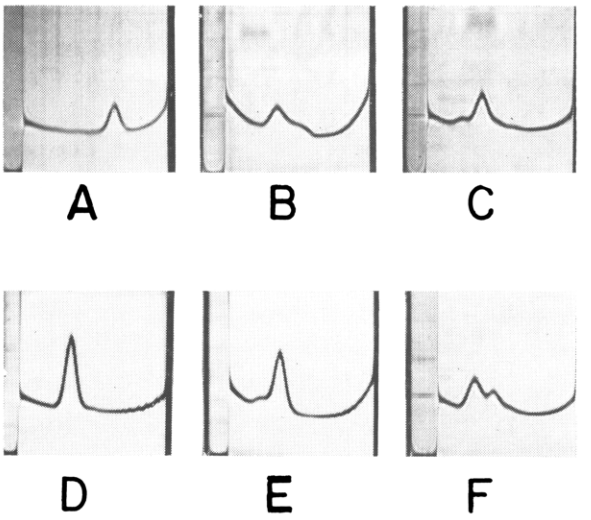

FIGURE 10: Ultracentrifugal patterns of phosphorylase which has been reacted with cyanate. Pictures were taken 32 min after the ultracentrifuge reached a speed of 59,780 rpm. Sedimentation direction is from left to right. Experimental conditions and analyses of the patterns are given in Table III.

Figure 10B shows that a partially inactivated phosphorylase *a* preparation shows a partial conversion to the dimeric form. It may be seen, however, that the two boundaries are not well defined at 32 min, whereas Keller and Cori (1953) have shown that mixtures of phosphorylase *a* and *b* exhibit well-defined boundaries at this time. On the other hand, Wang and Graves (1963, 1964) have reported patterns similar to Figure 10B with phosphorylase *a* under such conditions as high ionic strength or higher temperatures (34°), and they concluded that interacting components were present, rather than two noninterconvertible molecular forms with $s_{20,w}$ values of 8 and 13 S.

Figure 10 also indicates the effect of treating phosphorylase *b* with cyanate. In this case the tendency of the protein to dissociate into monomers is greater than was the case with phosphorylase *a*. However, this dissociation into monomers does not appear to be related to the loss in enzymatic activity since a 60% inactivated preparation showed only an 11% conversion to monomer (Figure 10E), and the completely inactivated enzyme still retained some dimer (Figure 10F). The cause of this dissociation into monomer is not clear, but the situation is quite different from the effects seen as a result of the inactivation by PMB.

Discussion

The evidence presented in this paper indicates that the various effects of cyanate upon phosphorylase are

TABLE III: Experimental Conditions and Analyses of Ultracentrifugal Patterns of Phosphorylase.[a]

	Type of Enzyme	Hours in 0.1 M KCNO	Enzyme Activity	Distribution of Protein and $s_{20,w}$ of Components (%)		
				Tetramer	Dimer	Monomer
A	*a*	0	100	100 (13.7 S = 90) (17.7 S = 10)	0	0
B	*a*	2.5	44	31 (12.7 S)	69 (8.4 S)	0
C	*a*	10.5	0	0	90 (8.4 S)	10 (5.9 S)
D	*b*	0	100	0	100 (8.6 S)	0
E	*b*	2.0	40	0	89 (8.4 S)	11 (5.4 S)
F	*b*	10.5	0	0	25 (8.7 S)	75 (5.6 S)

[a] As shown in Figure 10.

due primarily to the carbamylation of the ϵ-amino groups of lysine. The kinetics of both the inactivation and the carbamylation are in reasonable accord with what one might expect from the investigations of Stark *et al.* (1960; Stark, 1965b). Only the effect of protein concentration on the rate of inactivation is not in agreement with a conventional interpretation.

Approximately 12 lysine groups/125,000 molecular weight appear to be involved in maintaining both the structure and activity of phosphorylase *a*. These groups are more involved in keeping dimer units associated as tetramers rather than monomer units associated. Nolan *et al.* (1964) have suggested that the esterification of phosphate to two serine residues of phosphorylase *b* results in the neutralization of positive charges on nearby lysine and arginine residues, thereby facilitating the association of the dimer units. Wang and Graves (1963) also presented evidence for an electrostatic interaction between the two dimers composing phosphorylase *a* by showing that the latter dissociates into a dimeric form at high ionic strength. Further work by these authors (1964, 1965) indicated that a dimeric form of phosphorylase *a* is produced by dilution or by low concentrations of glucose and that this dimer is more active catalytically than the tetramer.

The work presented here suggests strongly that lysine residues are involved in the electrostatic interaction between the two dimer units composing phosphorylase *a*. The various treatments discussed above all result in the dissociation of phosphorylase *a* into two subunits, whereas the blocking of some 18 sulfhydryl groups with PMB or other reagents causes a dissociation into four subunits of 125,000 molecular weight. It is apparent, then, that the forces holding each pair of monomers together in a dimer differ from those holding two dimers together in a tetramer, and that the first forces are stronger than the second. Sulfhydryl groups would appear to be involved in some as yet unknown manner in maintaining the monomer units associated into dimers, but they are probably not involved in the further association of dimers to tetramers. Phosphorylase fits very precisely the description of an oligomeric protein given by Monod *et al.* (1965) in which there is an "isologous" association between two protomers to form a dimer and a subsequent association between two dimers to form a tetramer. The latter association utilizes different binding sites from those involved in the first association to the dimer stage. This model requires that all the protomers be identical. This requirement is probably fulfilled for phosphorylase since the subunit formed as a result of the reaction with PMB appears to be homogeneous when examined by various physical criteria, and the analyses of the four peptides containing serine phosphate, as well as the four containing the lysine pyridoxal phosphate, show that the four peptides within each set are identical.

The actual mechanism by which the carbamylation of some 52 lysine residues in phosphorylase *a* causes a loss of catalytic activity is not immediately apparent from the experiments reported here. The dissociation of the protein to a dimeric form does not provide an answer because phosphorylase *b*, which is fully active in the presence of AMP, is a dimer. Furthermore, as discussed above, the literature provides examples of the dissociation of phosphorylase *a* into dimers without loss of activity. While it might seem reasonable to suggest that the positively charged ϵ-amino groups of lysine could be involved in binding the negatively charged substrates or activator of this enzyme, no differentiation of the small number of residues expected to be involved in such binding could be seen in this work. The change in the binding characteristics of AMP could be due to conformational changes rather than the loss of a residue involved in the binding.

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