74-108

SCHWAB, Alfred H., 1936-

OXIDATION AND SULFONATION OF THE RAPIDLY REACTING SULFHYDRYL GROUPS OF MUSCLE PHOSPHORYLASE B.

The City University of New York, Ph.D., 1973
Biochemistry

OXIDATION AND SULFONATION
OF THE RAPIDLY REACTING SULFHYDRYL GROUPS
OF MUSCLE PHOSPHORYLASE B

by

ALFRED SCHWAB

A dissertation submitted to the Graduate Faculty in Biochemistry in partial fulfillment of the requirements for the degree of Doctor of Philosophy, The City University of New York.

1973

This manuscript has been read and accepted for the Graduate Faculty in Biochemistry in satisfaction of the dissertation requirement for the degree of Doctor of Philosophy.

5 23 73
date

Aaron Lukton
Prof. Aaron Lukton
Chairman of Examining Committee

5 23 73
date

Aaron Lukton
Prof. Aaron Lukton, Executive Officer

George H. Fried
Prof. George H. Fried

Morton D. Glantz
Prof. Morton D. Glantz

James F. Hogg
Prof. James F. Hogg

Norman Indictor
Prof. Norman Indictor

Supervisory Committee

The City University of New York

Acknowledgements

To Professor Aaron Lukton for stimulating my interest in enzymology, and for helpful support and advice during the course of this work.

To my wife, Eleanore, whose patience and understanding were essential for the completion of this work.

To Dr. Allan Reiss and Mr. Stephen Plotch for helpful discussions.

Table of Contents

Page

List of Tables..... vi

List of Figures..... vii

List of Abbreviations..... viii

Abstract..... ix

Introduction..... 1

Materials and Methods..... 14

- Removal of AMP from Phosphorylase b..... 15
- Determination of Protein Concentration..... 15
- Phosphorylase b Assays..... 16
- Preparation of S-Sulfonated Phosphorylase b..... 17
- Determination of Radioactivity..... 18
- Ultracentrifugation..... 19
- DTNB Titration of Sulfhydryl Groups..... 20

Results..... 21

- Inactivation of Phosphorylase b by Mn^{2+} and Sulfite..... 21
- Effect of AMP and Substrates..... 21
- Nature of the Reaction of Phosphorylase b with Mn^{2+} and Sulfite..... 21
- Effect of Thiols on Mn^{2+}-Sulfite-Inactivated Phosphorylase b..... 22
- Quaternary Structure of Mn^{2+}-Sulfite-Inactivated Phosphorylase b..... 23
- Inactivation of Phosphorylase b by Cu^{2+}..... 23
- Characteristics of Cu^{2+}-Inactivated Phosphorylase b..... 24
- Inactivation of Phosphorylase b by o-Iodosobenzoate..... 24
- Reactivation of o-Iodosobenzoate-Inactivated Phosphorylase b by Reducing Agents..... 25
- Quaternary Structure of o-Iodosobenzoate-Inactivated Phosphorylase b..... 25
- Effect of Dithiothreitol on the Quaternary Structure of o-Iodosobenzoate-Inactivated Phosphorylase b..... 26
- Effect of Sulfite on the Quaternary Structure of o-Iodosobenzoate-Inactivated Phosphorylase b..... 27

Table of Contents

Page

Incorporation of $^{35}SO_3^=$ into o-Iodoso-benzoate-Inactivated Phosphorylase b..... 27
Titration of the SH Groups of Native and S-Sulfonated Phosphorylase b with DTNB..... 28
Sedimentation Behavior of S-Sulfonated Phosphorylase b..... 28
Effect of o-Iodosobenzoate on Phosphorylase b Treated with 1-^{14}C-Iodoacetamide..... 30
Incorporation of 1-^{14}C-Iodoacetamide into S-Sulfonated Phosphorylase b..... 30
Effect of AMP and Substrates on the Inactivation of Phosphorylase b by o-Iodosobenzoate..... 31
Effect of Dithiothreitol on the $^{35}SO_3^=$ Content and Activity of S-Sulfonated Phosphorylase b..... 31
Kinetics of S-Sulfonated and Native Phosphorylase b..... 32
Effect of Dithiothreitol on the Kinetic Response of S-Sulfonated Phosphorylase b to AMP..... 33

Discussion..... 35

Inactivation of Phosphorylase b by Mn^{2+} and Sulfite..... 35
Inactivation of Phosphorylase b by Cu^{2+}..... 36
Inactivation of Phosphorylase b by o-Iodosobenzoate..... 37

Summary and Conclusions..... 49

Bibliography..... 70

List of Tables

Page

I The effect of various compounds on the inactivation of phosphorylase b by Mn^{2+} and sulfite.....52

II The effect of reducing agents on native and o-iodosobenzoate-inactivated phosphorylase b.....53

III The effect of o-iodosobenzoate on 1-^{14}C-iodoacetamide-treated phosphorylase b.....54

IV Incorporation of 1-^{14}C-iodoacetamide into native and S-sulfonated phosphorylase b.....55

V The effect of substrates and AMP on the inactivation of phosphorylase b by o-iodosobenzoate.....56

VI The effect of dithiothreitol on the activity and $^{35}SO_3^=$ content of S-sulfonated phosphorylase b.....57

List of Figures

Page

1. Inactivation of phosphorylase b in the presence of Mn^{2+} and sulfite..... 58

2. The effect of AMP and substrates on the inactivation of phosphorylase by Mn^{2+} and sulfite..... 59

3. Sedimentation velocity pattern of Mn^{2+}-sulfite-inactivated phosphorylase b..... 60

4. Inactivation of phosphorylase b by Cu^{2+}..... 61

5. The stoichiometry of o-iodosobenzoate inactivation of phosphorylase b..... 62

6. Sedimentation velocity patterns of native and o-iodosobenzoate-inactivated phosphorylase b..... 63

7. The effect of sulfite on the sedimentation velocity pattern of o-iodosobenzoate-inactivated phosphorylase b..... 64

8. Titration of native and S-sulfonated phosphorylase b with DTNB..... 65

9. Sedimentation velocity patterns of native and S-sulfonated phosphorylase b, with, and without, dithiothreitol..... 66

10. Double-reciprocal plots of the activation of native and S-sulfonated phosphorylase b by AMP..... 67

11. Hill plots for the activation of native and S-sulfonated phosphorylase b by AMP..... 68

12. Hill plots for the activation of dithiothreitol-treated, native and dithiothreitol-treated, S-sulfonated phosphorylase b by AMP..... 69

List of Abbreviations

P_i	orthophosphate
AMP	adenosine-5'-monophosphate
ATP	adenosine triphosphate
PLP	pyridoxal-5'-phosphate
PMB	p-chloromercuribenzoate
DTNB	5,5'-dithiobis(2-nitrobenzoic acid)
IAM	iodoacetamide
BSA	bovine serum albumin

Abstract

The reaction of rabbit skeletal muscle phosphorylase b with a three-fold molar excess of o-iodosobenzoate leads to aggregation of the enzyme, and a 95% loss of enzyme activity. Substrates and AMP have little or no effect on the reaction of phosphorylase b with o-iodosobenzoate. Dithiothreitol restores the activity of o-iodosobenzoate-inactivated phosphorylase b to 92% of the control activity, and causes re-formation of the dimeric structure which is characteristic of the native enzyme. These results are consistent with the idea that phosphorylase b activity may be regulated *in vivo* by the oxidation of enzyme -SH groups to disulfides.

Treatment of oxidized phosphorylase b with $^{35}SO_3^=$ results in S-sulfonation of an average of 1.4 -SH groups per molecule of phosphorylase b dimer. The S-sulfonated enzyme has an average of 1.6 fewer -SH groups available for rapid reaction with iodoacetamide than native phosphorylase b.

Phosphorylase b that has 3.0 to 3.2 rapidly reacting -SH groups alkylated, by reaction with iodoacetamide, retains 73-85% of its activity after reaction with o-iodosobenzoate.

It is concluded that those -SH groups of phosphorylase b that react rapidly with iodoacetamide can be oxidized by o-iodosobenzoate, and subsequently sulfonated by reaction

with sulfite.

S-Sulfonated phosphorylase b has 59-68% of the control activity, and sediments primarily as a dimer in the ultracentrifuge. The S-sulfonated enzyme shows a hyperbolic activity response to AMP, and a decreased affinity for AMP; these effects are completely reversed by dithiothreitol.

Since modification of the same -SH groups of phosphorylase b by different reagents causes diverse effects on enzyme activity, it is suggested that these effects are due to changes in enzyme conformation.

Introduction

Glycogen phosphorylase (E.C.2.4.1.1) catalyses the following reaction:

$$\text{Glycogen}_{(n \text{ glucose units})} + P_i = \text{Glycogen}_{(n-1 \text{ glucose units})} + \text{Glucose-1-phosphate} \quad (1)$$

Although the enzyme has been isolated from numerous plant and animal tissues, much of the recent work has been carried out on rabbit skeletal muscle phosphorylase (1).

It is known from the work of Cori et. al. (2) that rabbit skeletal muscle phosphorylase exists in two forms: phosphorylase a, a tetramer, which is active in the absence of AMP, and phosphorylase b, a dimer, which is active only in the presence of AMP. Recent investigations have indicated that phosphorylases b and a have subunit sizes of 100,000, and molecular weights of 200,000, and 402,000 respectively (3).

Krebs and Fischer (4) have shown that phosphorylase b can be converted to phosphorylase a in a reaction that is catalyzed by phosphorylase kinase, and requires Mg-ATP. The conversion of phosphorylase b to a involves the phosphorylation of one seryl residue per subunit. Dephosphorylation of the seryl residues of phosphorylase a, through the action of phosphorylase phosphatase, results in the formation of phosphorylase b (5).

Glycogen phosphorylase is a complex regulatory enzyme

containing numerous sites that are involved either in determining or controlling the activity of the enzyme (6).

All known phosphorylases contain one mole of PLP per mole of enzyme subunit (6). It is known that in phosphorylase b, PLP is linked, by means of its carbonyl group, to an ϵ-amino group of a lysyl residue (7). Spectral studies of Schiff base derivatives of PLP in nonaqueous solvents have led to the proposal that, at neutral pH, PLP in phosphorylase b is in a hydrophobic environment, and is bound to the enzyme through a hydrogen-bonded Schiff base structure (8,9). More recently, in agreement with earlier proposals (7), Honikel and Madsen (10) have concluded that PLP is covalently bound to two groups on phosphorylase b, in the form of a carbinolamine-like structure.

Regardless of the nature of PLP bonding in native phosphorylase b, it is known that under appropriate conditions the coenzyme can be bound to the enzyme as a Schiff base (11). Reduction of this Schiff base with sodium borohydride leads to an enzyme which has about 60% of the activity of the native enzyme. These studies indicated that the carbonyl function of the cofactor could not serve as a catalytic group (12).

Thus, the function of PLP in phosphorylase is different from its function in other PLP-containing enzymes (6). Nevertheless, PLP is indispensable for the catalytic activity

of phosphorylase b, and its removal from the enzyme, by denaturing or deforming agents, causes dissociation of the enzyme into inactive monomers (6).

In addition to the PLP site, each subunit of phosphorylase b contains binding sites for the substrates, glucose-1-phosphate, P_i and glycogen, as well as a binding site for the modifier, AMP (1).

Kinetic studies have indicated that AMP activates phosphorylase b by increasing the affinity of the enzyme for its substrates, glycogen, glucose-1-phosphate, and P_i (13). Furthermore, the binding of each substrate causes an increased affinity of the enzyme for the activator, AMP. Thus, there is heterotropic cooperativity between each substrate binding site and the AMP site. In addition, there is heterotropic cooperativity between the unlike binding sites for different substrates (14). Whereas heterotropic cooperactivity is readily observed, homotropic cooperativity (the ability of a substrate to cause an increase in affinity for itself) is more difficult to demonstrate (14).

Extensive studies have been carried out on glycogen phosphorylase in an effort to understand how the enzyme mediates the cooperative effects described above, and in order to determine which sites on the enzyme are involved. Chemical modification of amino acid side chains of phosphorylase have indicated that lysyl and sulfhydryl residues play a role in determining the structure and

function of the enzyme (14).

Wang and Tu (15) modified phosphorylase b with glutaraldehyde and obtained enzyme derivatives containing 7 to 11 modified amino groups. Kinetic studies of these derivatives indicated that the homotropic cooperativity of AMP sites was abolished, and there was a decrease in the maximum velocity of the enzyme. Further studies indicated that homotropic cooperativity of glucose-1-phosphate sites was also abolished in the glutaraldehyde-modified enzyme, but no effect on heterotropic cooperativity was observed (16).

Fasold et al. (17) completely inactivated phosphorylase b with dinitrofluorobenzene at pH 6.5, and found that one lysyl residue per monomer was dinitrophenylated. Binding studies on the modified enzyme indicated that the dissociation constant, K_{dis}, for AMP was 0.01M as compared with 5×10^{-5} M for native phosphorylase b. The affinity of the dinitrophenylated enzyme for glucose-1-phosphate and glycogen was the same as that of the native enzyme.

It has been shown recently that native phosphorylase b, which contains one mole of PLP per subunit, can incorporate one additional mole of PLP per mole of subunit (18). Reduction of PLP-phosphorylase b with sodium borohydride yields a stable derivative which exhibits fluorescence spectra characteristic of a pyridoxamine derivative. Kinetic studies of this modified enzyme indicate that homotropic interactions between AMP sites are abolished, and the homotropic cooperactivity

of substrate sites is altered (19).

The -SH groups of phosphorylase were first studied by Madsen and Cori (20-22). These investigators showed that the reaction of 13-14 -SH groups of phosphorylase a with the mercurial, PMB, led to complete inactivation of phosphorylase a, and dissociation of the phosphorylase a tetramer into monomers. Comparable results were obtained with phosphorylase b.

Battell et al. (23) studied the reactivity of the -SH groups of phosphorylase b with a variety of -SH reagents, both in the presence and absence of denaturing agents. This study indicated that the number of titratable -SH groups varied with different -SH reagents. Moreover, -SH titrations with the same reagent gave different results in the presence of different denaturing agents. Cysteic acid determinations on performic acid-oxidized phosphorylase b showed that each phosphorylase b monomer contains 9 -SH groups.

Analysis of the peptide mixture obtained after reaction of phosphorylase b with radioactive iodoacetamide and pepsin digestion, indicated that only 9 unique cysteine-containing peptide sequences are present per mole of phosphorylase b monomer (24). Since there are 9 -SH groups per subunit of phosphorylase b, these results support the view that the subunits are identical.

The reaction of phosphorylase b with 0.01 M iodoacetamide leads to almost complete inactivation of the enzyme (23).

Extensive studies of this reaction have indicated that 2 to 3 -SH groups per dimer of phosphorylase b are rapidly alkylated with no loss in enzyme activity, while the slower alkylation of an additional 4 -SH groups per dimer leads to almost complete loss of activity and monomerization (25-27).

It has been shown that the slowly reacting -SH groups belong to two unique amino acid sequences of phosphorylase b, which have been designated as peptide N and peptide A (25). Furthermore, the rate of alkylation of peptide N could be correlated with the loss of enzyme activity and dissociation of phosphorylase b dimer into monomers (26). These results suggest that the alkylation of only one -SH group per monomer of phosphorylase b is responsible for the loss of activity, and dissociation of the enzyme into monomers (26).

Further studies on the 2 to 3 -SH groups which are rapidly alkylated by iodoacetamide have indicated that these -SH groups belong to two unique amino acid sequences of phosphorylase b, designated as peptides B_1 and B_2 (27). Alkylation of these -SH groups with iodoacetamide does not lead to loss of enzyme activity (27), and apparently does not change the allosteric response of phosphorylase b to AMP, ATP, or glucose -1- phosphate (25).

Gold (28) has shown that chlorodinitrobenzene or fluorodinitrobenzene react rapidly with 4 -SH groups of phosphorylase b dimer. Kinetic studies have indicated that the major effect of dinitrophenylation on phosphorylase b is to decrease the affinity of the enzyme for AMP and

glucose-1-phosphate. Isolation of the radioactive dinitrophenyl peptides, after enzymatic digestion of dinitrophenylated phosphorylase b, has led Gold (28) to conclude that those -SH groups of phosphorylase b which react with chlorodinitrobenzene are the same as those which react rapidly with iodoacetamide (29). The exact number of rapidly reacting -SH groups that react with iodoacetamide or chlorodinitrobenzene varies with different enzyme preparations, and with the age of a particular enzyme preparation (27, 29).

Kastenschmidt et al. (30) have reacted phosphorylase b with DTNB, a reagent that forms mixed disulfides with -SH groups. These investigators isolated a phosphorylase b dimer which contained up to 3.2 modified -SH groups. Kinetic studies on this modified enzyme indicated that the maximum velocity of the enzyme was unchanged, but the homotropic cooperativity of AMP sites was abolished. It is not clear whether DTNB reacts with the same rapidly reacting -SH groups of phosphorylase b that react with iodoacetamide (14). Alkylation of the rapidly reacting -SH groups with iodoacetamide does not lead to the changes in kinetic properties that occur when the enzyme is reacted with DTNB or chlorodinitrobenzene (25). It has been suggested that these kinetic differences may be due to the different nature of the substituting groups (29).

Although the relationship between the rapidly reacting

-SH groups of phosphorylase b and allosteric control of enzyme activity is not yet known, their reactivity with various reagents indicates that they are fully exposed on the enzyme surface (23). It has been suggested that these -SH groups may form intramolecular or intermolecular disulfide bonds in vivo, and may have an affect on the regulation of enzyme activity (23, 25). In support of this hypothesis is the finding that aggregates of phosphorylase b, which dissociate upon addition of excess mercaptoethanol, are sometimes seen in the ultracentrifuge (20).

In summary, of the 18 -SH groups present per molecule of phosphorylase b dimer, 3 to 4 react rapidly with various reagents and may have a regulatory function, while 4 additional -SH groups react more slowly with iodoacetamide. As indicated previously, the rate of alkylation of the slowly-reacting -SH groups of peptide N has been correlated with the rate of enzyme inactivation and the rate at which the enzyme dissociates into monomers (26). The role of the other two slowly reacting -SH groups of peptide A in the structure and function of phosphorylase b is not known (26). In addition, there are approximately 10 other -SH groups per molecule of phosphorylase b dimer which do not readily react with various -SH reagents unless the enzyme is denatured (23). The function of these -SH groups of phosphorylase b is also unknown. A need for clarification of the role of -SH groups in the structure and function of phosphorylase b is, therefore,

apparent.

The general purpose of the following study was to selectively modify particular -SH groups of phosphorylase b and to correlate the modification of these -SH groups with possible changes in the structure and/or activity of the enzyme.

The best kind of modifying agent appeared to be a mild oxidizing agent which could oxidize -SH groups of phosphorylase b to disulfide groups. Reasons for such a choice included the following: 1) The formation of disulfide bonds in proteins requires proper spatial orientation of the reacting -SH groups (31), so that a limited number of -SH groups would be expected to react in native phosphorylase b. 2) It has been suggested that the rapidly reacting -SH groups of phosphorylase b may have a regulatory role dependent on the formation of intermolecular or intramolecular disulfide bonds (23, 25).

The oxidation of thiols to disulfides proceeds according to the following general equation:

$$2\ RSH + X = RSSR + XH_2 \qquad (2)$$

where, X, is a hydrogen or electron acceptor (32). Oxidants employed in this reaction have included metal ions, in the presence, or absence of oxygen, iodine, tetrathionate and o-iodosobenzoic acid (32).

The air oxidation of thiols to disulfides proceeds rapidly in the presence of basic catalysts (33). The

following course of base-catalyzed thiol oxidation can be visualized (33):

$$RSH + R'O^- = RS^- + R'OH \quad (R' = H \text{ or alkyl}) \qquad (3)$$

The thiolate ion reacts with O_2 via an electron transfer mechanism to give thiyl radical and peroxide ion.

$$RS^- + \cdot O_2 \cdot \longrightarrow RS\cdot + \cdot O_2^- \qquad (4)$$

Dimerization of the thiyl radicals leads to the disulfide.

$$2\ RS\cdot \longrightarrow RSSR \qquad (5)$$

The peroxide ion formed in equation 4 can also oxidize the thiol as indicated below.

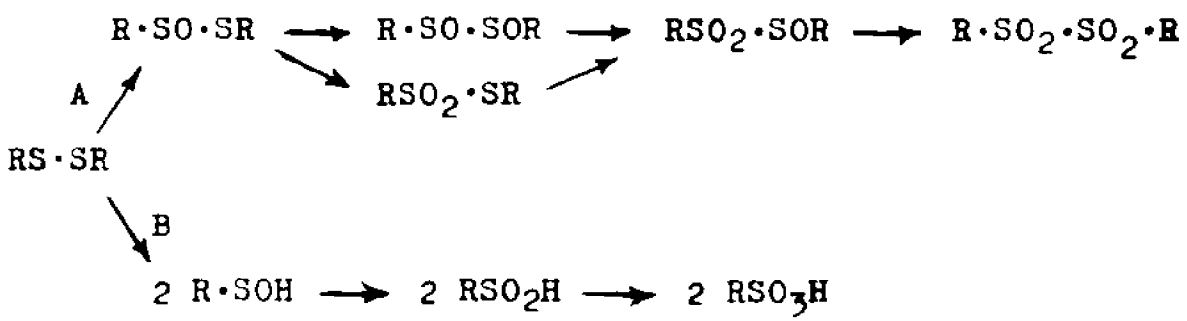
$$RSH + \cdot O_2^- \longrightarrow RS\cdot + HO_2^- \qquad (6)$$

$$RSH + HO_2^- \longrightarrow RS\cdot + HO^- + \cdot OH \qquad (7)$$

Wallace et al. (34) investigated the base-catalyzed oxidation of a large number of thiols of different structure and acidity. These studies indicated that aliphatic thiols are oxidized to disulfides more readily than aromatic thiols.

The oxidation of cysteinyl residues in proteins sometimes leads to oxidation products other than disulfide; such oxidation products are not usually identified (35). Studies on the oxidation of model compounds provide information regarding the kind of products that might result when cysteinyl residues in proteins are oxidized.

Two pathways for the oxidation of disulfides by S-S fission are possible in hydroxylic solvents (36):



In pathway A, complete oxidation precedes hydrolytic fission of the disulfide bond. In pathway B, disulfide fission precedes oxidation.

Cystine S-monoxide (Cy·SO·S·Cy) can be obtained by oxidising cystine (Cy·S·S·Cy) with performic acid in dilute sulfuric acid at 0°C. Aqueous solutions of cystine S-monoxide are generally unstable; the eventual products of hydrolysis at ordinary temperatures are cystine and alanine-3-sulfinic acid (36).

$$3\ Cy{\cdot}SO{\cdot}S{\cdot}Cy + H_2O \longrightarrow 2\ Cy{\cdot}SO_2{\cdot}H + 2\ Cy{\cdot}S{\cdot}S{\cdot}Cy$$

Cystine S-monoxide reacts with organic mercurials at pH 3-7 giving the mercaptide and alanine-3-sulfinic acid (37). Thiols react readily with cystine S-monoxide at pH 3-6 to yield cystine (36). Cystine S-monoxide is known to react with sulfite over a wide range of pH (2-9) to give the S-sulfonate (37).

$$Cy{\cdot}SO{\cdot}S{\cdot}Cy + 2\ HSO_3^- \longrightarrow 2\ Cy{\cdot}SSO_3^- + H_2O$$

At pH 0-1 cystine S-monoxide disproportionates to form cystine dioxide (Cy·SO$_2$·S·Cy) and cystine (38).

$$2\ Cy{\cdot}SO{\cdot}S{\cdot}Cy \longrightarrow Cy{\cdot}SO_2{\cdot}S{\cdot}Cy + Cy{\cdot}S{\cdot}S{\cdot}Cy$$

Cystine dioxide can also be split by sulfite to yield cysteine S-Sulfonate and alanine-3-sulfinic acid (36).

$$Cy{\cdot}SO_2{\cdot}S{\cdot}Cy + SO_3^{=} \longrightarrow Cy{\cdot}S{\cdot}SO_3^{-} + Cy{\cdot}SO_2^{-}$$

While oxidation of disulfides can lead to a variety of products, the reduction of a disulfide yields the corresponding thiol. This reaction appears to involve a two-step ionic displacement (39).

$$R_1\text{-}SH + R_2\text{-}S\text{-}S\text{-}R_2 = R_1\text{-}S\text{-}S\text{-}R_2 + R_2\text{-}SH \qquad (3)$$

$$R_1\text{-}S\text{-}S\text{-}R_2 + R_1\text{-}SH = R_1\text{-}S\text{-}S\text{-}R_1 + R_2SH \qquad (4)$$

In addition, disulfides can be split by nucleophiles such as CN^- or SO_3^{2-} (40). The reaction of a disulfide with sulfite is reversible and proceeds as follows (40):

$$R\text{-}S\text{-}S\text{-}R + SO_3^{=} = R\text{-}S^- + R\text{-}S\text{-}SO_3^- \qquad (5)$$

Although in this reaction only half of the original disulfide is converted to the S-sulfonate, $R\text{-}S\text{-}SO_3^-$, the addition of an appropriate oxidizing agent causes oxidation of the product thiol in equation 4, so that under these conditions all the disulfide can be converted to the S-sulfonate, according to the following equations:

$$R\text{-}S\text{-}S\text{-}R + 2\ SO_3^{=} = 2\ R\text{-}S\text{-}SO_3^- + 2e \qquad (6)$$

$$2e + \text{oxidant} = \text{reductant} \qquad (7)$$

This two-step reaction, employing sulfite and an oxidant, has been termed "oxidative sulfitolysis" by Leach and Swan (41).

These investigators have studied the oxidative sulfitolysis of cystine, insulin, and keratins (41, 42). Oxidative

sulfitolysis of cystine has been shown to proceed readily with Cu^{2+}, or Mn^{2+} in the presence of atmospheric oxygen (41). The oxidative sulfitolysis of insulin is effectively catalyzed by Cu^{2+} in the presence or absence of atmospheric oxygen (42).

Sulfitolysis has been recently used for the complete S-sulfonation of cysteine residues in rabbit muscle aldolase (43). The S-sulfonated aldolase is inactive, but can be completely reactivated with mercaptoethanol.

In the following study, the first group of experiments is concerned with the inactivation of phosphorylase b in the presence of Mn^{2+}, sulfite, and atmospheric oxygen. The second set of experiments deals with the inactivation of phosphorylase b by Cu^{2+}.

The third group of experiments is concerned with the reaction of phosphorylase b with o-iodosobenzoate. Evidence is presented which suggests that this reagent inactivates phosphorylase b by oxidizing those -SH groups that react rapidly with iodoacetamide.

Materials and Methods

The sodium-β-glycerophosphate (Calbiochem) used for the preparation of buffer solutions which did not contain EDTA was freed of trace metal ions. For this purpose, sodium- -glycerophosphate (200g.) was dissolved in a final volume of 300ml. of water, and stirred with a slurry (10-15 ml.) of Dowex 50W-X8, in the sodium form, for 10 minutes. After filtration, the sodium-β-glycerophosphate was crystallized from 33% ethanol-water.

Oyster glycogen (Calbiochem) was purified according to the method of Somogyi (44).

o-Iodosobenzoic acid (Mann) was purified as described by Chinard and Hellerman (45). Stock solutions of o-iodosobenzoate (0.1 M) were prepared in 0.1 M NaOH.

^{35}S-Labeled sodium sulfite (lot 659-152), which had an initial specific activity of 27 mC/mmole, and 1-^{14}C-iodoacetamide (lot 252-152), with a specific activity of 3.1 mC/mmole, were purchased from New England Nuclear Corp. 1-^{14}C-Labeled iodoacetamide was dissolved in hot water, and crystallized with an excess of non-radioactive iodoacetamide (Calbiochem), which had previously been recrystallized from water.

Imidazole (Baker Chemical Co.) was recrystallized from ethyl acetate. Glucose-1-phosphate, dipotassium salt, AMP, DTNB, and bovine serum albumin were A grade, and were obtained from Calbiochem. All other chemicals were of the highest grade commercially available.

Phosphorylase b was prepared from frozen rabbit skeletal muscle (Pel-Freez Biologicals, Inc.) by the method of Fischer and Krebs (46) as modified by DeLange et al. (47), except that 0.005 M dithiothreitol replaced mercaptoethanol. The enzyme was recrystallized three times after preparation, and weekly thereafter as described by Gold (29).

Removal of AMP from Phosphorylase b.

In order to remove AMP, Mg^{2+}, and dithiothreitol, enzyme crystals were centrifuged out of suspension, dissolved in the appropriate buffer, and passed through Sephadex G-25 (.9 x 23 cm.) equilibrated with the same buffer. For experiments in which phosphorylase was to be inactivated with Mn^{2+} and sulfite, the buffer was 0.05 M sodium-β-glycerophosphate (pH 6.7), equilibrated with nitrogen (Matheson, prepurified). A buffer consisting of 0.03 M imidazole-HCL-1 x 10^{-5} M EDTA (pH 7.2) was used for studies dealing with Cu^{2+} inactivation of phosphorylase b. When phosphorylase b was oxidized with o-iodosobenzoate, the buffer was 0.05 M sodium barbital-0.001 M EDTA (pH 8.0).

Determination of Protein Concentration.

The protein concentration of samples of phosphorylase b or S-sulfonated phosphorylase b was determined, after dilution with water, from the absorbance at 280nm. Protein concentrations were calculated using the extinction coefficient,

$E^{1\%}$ = 13.1 at 280nm (3).

Phosphorylase b Assays.

Routine assays of enzyme activity were carried out according to the zero order method of Hedrick and Fischer (48). The buffer used for dilution of enzyme samples consisted of 0.1 M maleate-0.002 M EDTA- 1mg./ml. BSA (pH 6.5). The substrate solution consisted of 0.15M glucose-1-phosphate, 2% glycogen, 0.002 M AMP, and 0.1 M maleate (pH 6.5). The enzyme assay was initiated by pipetting 0.2 ml. of substrate solution, at 30°C., into a large test tube containing 0.2 ml. of enzyme solution at 30°C. The enzyme reaction was stopped after 5 minutes by addition of 8.0 ml. of .064 N sulfuric acid. Inorganic phosphate was determined by the method of Fiske and SubbaRow (49) except that 0.9 ml. of 5 N H_2SO_4 containing 2.78% ammonium molybdate was added to the acidified enzyme solution, instead of 1.0 ml. 5 N H_2SO_4 and 1.0 ml. of ammonium molybdate solution. Enzyme activity was expressed as μ moles of phosphate formed per minute per mg. of enzyme.

Enzyme activities were also measured according to the method of Cori et al. (2), for determination of the kinetic constants of phosphorylase b and 3-sulfonated phosphorylase b. The reaction mixture consisted of 0.008 M sodium-β-glycerophosphate, 0.003 M EDTA, 0.048 M glucose-1-phosphate, 0.6% glycogen, various amounts of AMP, and 25μg. of enzyme

in a final volume of 1.0 ml. The temperature was 23°C. When the kinetic constants of dithiothreitol-treated enzymes were determined, the reaction mixture also contained 0.001 M dithiothreitol. At various times, aliquots of the reaction mixture were removed and pipetted into 8.0 ml. of 0.064 N sulfuric acid. Inorganic phosphate was determined as described above for the routine assay of phosphorylase b activity. First order rate constants were calculated using the equation given by Cori et al. (2). Knowing the value of k, the percent glucose-1-phosphate converted to P_i after 1 min. was calculated. Initial velocities were then expressed as μmoles of phosphate formed per minute per mg. of enzyme.

Preparation of S-Sulfonated Phosphorylase b.

Phosphorylase b, 4 mg. per ml., was oxidized with o-iodosobenzoate (6×10^{-5} M) in 0.05 M sodium barbital-0.001 M EDTA-1.0 M NaCl (pH 8.0) for 10 minutes at 25°C. Immediately after oxidation, an aliquot was removed and placed in a 15 ml. Corex centrifuge tube (No. 8441) for reaction with ^{35}S-labeled sodium sulfite. The reaction mixture for S-sulfonation contained oxidized phosphorylase b, 3.2 mg. per ml., and 0.01 M sodium sulfite. The buffer was 0.05 M sodium barbital-0.001 M EDTA-0.8 M NaCl (pH 8.0). After incubation for 30 minutes at 25°C., the enzyme was precipitated by addition of an equal volume of 0.05 M sodium-β-glycerophosphate-0.001 M EDTA saturated with ammonium

sulfate at pH 6.8 Following centrifugation at 0°C., the pellet was dissolved in 1 ml. of 0.05 M sodium-β-glycerophosphate-0.001 M EDTA (pH 6.8) and re-precipitated by addition of an equal volume of 0.05 M sodium-β-glycerophosphate-0.001 M EDTA saturated with ammonium sulfate at pH 6.8 The pellet obtained after centrifugation, was dissolved in glycerophosphate buffer, and insoluble barbital was removed by centrifugation at 0°C. The enzyme was passed through Sephadex G-25, which had been previously equilibrated with 0.05 M sodium-β-glycerophosphate-0.001 M EDTA (pH 6.8).

Parallel samples of unoxidized phosphorylase b, which were carried through the same procedure, usually contained 0.04 moles of ^{35}S per mole of enzyme.

Determination of Radioactivity.

Incorporation of radioactive reagents into phosphorylase b, or modified phosphorylase b, was determined by pipetting aliquots of enzyme solutions into 5 ml. of cold 5% trichloracetic acid. After filtration through a Millipore HAWP 02400 filter (pore size .45), the precipitated protein was washed four times with 5 ml. portions of cold 5% trichloroacetic acid. In two experiments with ^{35}S-labeled phosphorylase b, aliquots of enzyme which had been freed of unreacted $^{35}SO_3^=$, were applied directly to Millipore filters. The filters were placed in scintillation vials and dried with a stream of warm air.

After addition of 10 ml. of scintillation fluid, samples were counted to an error of 1% in a Picker Nuclear Liquimat 220 liquid-scintillation counter. When ^{35}S was the isotope being counted, the scintillation fluid was a toluene solution containing 4 g. of 2,5-diphenzloxazole and 50 mg. of p-bis 2-(5-phenyloxazolyl) -benzene per liter. Bray's solution (50), without ethylene glycol, was the scintillation fluid used when samples of ^{14}C were counted. The counts per minute were corrected to 100% efficiency, using quench correction curves obtained by the channels-ratio method (51). The number of moles of radioactive reagent incorporated per mole of phosphorylase b dimer were calculated by dividing the specific radioactivity of the enzyme by the specific radioactivity of the reaction mixture.

Ultracentrifugation,

Sedimentation velocity experiments were carried out at 20°C. in a Spinco Model E analytical ultracentrifuge equipped with Schlieren optics and a Rotor Temperature Indicating and Control System. Generally, two single sector cells, one of which contained a +1° wedge window, were used in an An-D rotor.

Measurements of radial distances on photographic plates were done with a Nikon Microcomparator. Sedimentation coefficients were calculated using equations given by Chervenka (52), and corrected for the viscosity and density of buffer relative to water. Viscosities were determined

with an Ostwald viscometer, and densities were measured with a pycnometer.

In the calculations of $S_{20,w}$, the partial specific volume, $\bar{v}$, of phosphorylase b was taken as 0.746 when the enzyme was dissolved in buffer which did not contain sodium chloride (3). When the enzyme was sedimented in solutions containing high concentrations of salt, a value of 0.765 was assumed for $\bar{v}$ (3).

DTNB Titration of Sulfhydryl Groups

Spectrophotometric titrations of the -SH groups of native phosphorylase b and S-sulfonated phosphorylase b were carried out according to Ellman (53). The concentrations of protein and DTNB were 5.0×10^{-5} M and 1.0×10^{-4} M respectively. The buffer was 0.05 M sodium-β-glycerophosphate-0.001 M EDTA (pH 6.8) at 24°. Corrections were made for the absorbance of DTNB and unreacted protein at 412 nm; the absorbance of native phosphorylase b was 0.007, while the absorbance of S-sulfonated phosphorylase b was 0.010. An extinction coefficient of 13,600 was used to calculate the number of moles of -SH groups which had reacted (53).

Results

Inactivation of Phosphorylase b by Mn^{2+} and Sulfite.

As shown in Fig. 1, phosphorylase b is almost completely inactivated in the presence of Mn^{2+} and sulfite, but retains its activity in the presence of Mn^{2+} alone.

Effect of AMP and Substrates.

Since the extensive loss of enzyme activity indicated in Fig. 1 could be due to modification of amino acid residues at or near the active site of the enzyme, it was of interest to determine the effect of AMP and substrates on the rate of enzyme inactivation. Fig. 2 shows that AMP, or glucose-1-phosphate, has little or no effect on the rate of inactivation of the enzyme. Glycogen appears to decrease the rate of inactivation to some extent. The strongest protection against enzyme inactivation occurs when both glucose-1-phosphate and AMP are present in the reaction mixture.

Nature of the Reaction of Phosphorylase b with Mn^{2+} and Sulfite.

It can be seen in Table I that inactivation of phosphorylase b does not occur in the presence of sulfite alone, nor in the presence of Mg^{2+} and sulfite. This suggests that a specific effect of Mn^{2+}, in the presence of sulfite, is responsible for enzyme inactivation.

It is known that the aerobic oxidation of sulfite to

sulfate occurs by way of a free-radical chain mechanism, and can be initiated by metal ions (54,55). Yang (56) has recently shown that methionine is oxidized to methionine sulfoxide during the Mn^{2+}-catalyzed, aerobic oxidation of sulfite ion. Substances which induced sulfoxide formation were found to induce sulfite oxidation, while agents which inhibited sulfoxide formation also inhibited sulfite oxidation.

The inactivation of phosphorylase b by Mn^{2+} and sulfite is enhanced by oxygen, and is inhibited by nitrogen (Table I). This suggests that atmospheric oxygen is required for enzyme inactivation. Mannitol, a known inhibitor of the aerobic oxidation of sulfite ion (55), was found to effectively retard the inactivation of phosphorylase b. On the basis of the data shown in Table I, it appears likely that phosphorylase b inactivation is dependent on the aerobic oxidation of sulfite.

Effect of Thiols on Mn^{2+}-Sulfite-Inactivated Phosphorylase b.

In order to determine whether phosphorylase b inactivation by Mn^{2+} and sulfite was due to oxidation of enzyme thiol groups to disulfides, several attempts were made to reactivate the enzyme with reducing agents. Incubation of inactivated phosphorylase b with mercaptoethanol or dithiothreitol under various conditions did not result in any significant restoration of enzyme activity. Dilution of inactivated phosphorylase b

with solutions containing PLP and mercaptoethanol was also ineffective.

Quaternary Structure of Mn^{2+}-Sulfite-Inactivated Phosphorylase b,

Battell et al. (25) have shown that 0.01 M iodoacetamide inactivates phosphorylase b and causes the enzyme to dissociate into monomers. These investigators found that monomerization of the enzyme proceeded at the same rate as enzyme inactivation.

In view of these observations, it was of interest to determine whether inactivation of phosphorylase b by Mn^{2+} and sulfite causes dissociation of the enzyme into monomers. Sedimentation of a partially inactivated sample of phosphorylase b (20% of initial activity) in the analytical ultracentrifuge at 60,000 rpm and 20°C. suggested that the enzyme sedimented as a mixture of monomers and dimers (see Fig. 3).

Inactivation of Phosphorylase b by Cu^{2+},

The effect of Cu^{2+} on the activity of phosphorylase b is illustrated in Fig. 4. It can be seen that the enzyme loses more than 90% of its activity when the molar ratio of free Cu^{2+} to enzyme is 1:2.

It is reasonable to assume that enzyme inactivation is due to free Cu^{2+}, since no loss of enzyme activity was observed in reaction mixtures which contained equimolar amounts of Cu^{2+} and EDTA (not shown).

Characteristics of Cu^{2+}-Inactivated Phosphorylase b.

Dilution of Cu^{2+}-inactivated phosphorylase b in solutions containing 0.001 M EDTA did not result in any regain of enzyme activity. However, a five-fold dilution of inactivated enzyme in a solution containing 0.05 M sodium-β-glycerophosphate-0.001 M EDTA and 0.02M dithiothreitol at pH 7.0, resulted in restoration of at least 90% of enzyme activity after 15 minutes. These results suggest that inactivation of phosphorylase b by Cu^{2+} is due to disulfide bond formation.

In view of this possibility, it was of interest to determine whether changes in the molecular size of the enzyme occurred as a result of intermolecular disulfide bond formation. For this purpose, a sample of phosphorylase b, which had been inactivated with Cu^{2+}, was sedimented in the analytical ultracentrifuge at 60,000 rpm and 20°C. The inactivated enzyme sedimented under these conditions as a broad boundary which spread out rapidly with time; the sedimentation velocity pattern was similar to the pattern shown in the lower portion of Fig. 6B. This suggests that the inactive enzyme is heterogeneous with respect to molecular size, and supports the hypothesis that the reaction of Cu^{2+} with phosphorylase b leads to the formation of intermolecular disulfide bonds.

Inactivation of Phosphorylase b by o-Iodosobenzoate.

The inactivation of phosphorylase b by o-iodosobenzoate

is complete in 10 minutes at 25°C.; no further loss of activity was observed after 30 minutes. Fig. 5 shows that the loss of enzyme activity is linear with o-iodosobenzoate concentration until the concentration of this reagent is 1.5 times the concentration of the enzyme. Further inactivation requires more than a stoichiometric amount of o-iodosobenzoate. Incubation of phosphorylase b with a three-fold molar excess of o-iodosobenzoate results in the loss of about 94% of enzyme activity. An increase in o-iodosobenzoate concentration to 4 times the molar concentration of phosphorylase b does not lead to further inactivation.

Reactivation of o-Iodosobenzoate-Inactivated Phosphorylase b by Reducing Agents.

As shown in Table II, the inactivation of phosphorylase b by o-iodosobenzoate is almost completely reversed by dithiothreitol or glutathione. Treatment of oxidized phosphorylase b with sodium sulfite also resulted in considerable restoration of enzyme activity, while sodium cyanide was almost ineffective in restoring enzyme activity. The reactivation of o-iodosobenzoate-treated phosphorylase b by reducing agents suggests that enzyme inactivation is associated with disulfide bond formation.

Quaternary Structure of o-Iodosobenzoate-Inactivated Phosphorylase b.

In order to determine whether inactivation of phos-

phorylase b by o-iodosobenzoate leads to changes in quaternary structure, the sedimentation behavior of native and o-iodosobenzoate-treated phosphorylase b was determined, after gel filtration as described in Fig. 6A. The activity of the o-iodosobenzoate-treated enzyme did not change after gel filtration. As shown in Fig. 6A, the sedimentation velocity pattern of o-iodosobenzoate-inactivated phosphorylase b is considerably altered in comparison with that of the untreated enzyme. The polydisperse nature of the inactivated enzyme is apparent from the broad sedimentation pattern. The sedimentation coefficient, $S_{20,w}$, was 8.1 S for the control enzyme, and 12.2 S for the oxidized enzyme.

Effect of Dithiothreitol on the Quaternary Structure of o-Iodosobenzoate-Inactivated Phosphorylase b.

Since dithiothreitol had been shown to reactivate o-iodosobenzoate-inactivated phosphorylase b, it was of interest to determine the effect of this reagent on the quaternary structure of the oxidized enzyme. Fig. 6B (top) shows that, after incubation of o-iodosobenzoate-inactivated phosphorylase b with dithiothreitol, the sedimentation velocity pattern is comparable to that of the native enzyme. The sedimentation coefficient, $S_{20,w}$, was 11.6 S for the oxidized enzyme and 8.5 S after incubation with dithiothreitol. The specific activity of oxidized phosphorylase b in this experiment was 4% of the control activity. After treatment of the oxidized enzyme and native phosphorylase b with dithiothreitol, the

oxidized enzyme had 90% of the activity of native phosphorylase b

Effect of Sulfite on the Quaternary Structure of o-Iodosobenzoate-Inactivated Phosphorylase b.

Incubation of o-iodosobenzoate-inactivated phosphorylase b with 0.01 M sodium sulfite changes the sedimentation velocity pattern of the oxidized enzyme (Fig. 7), and restores its activity to 68-78% of the control activity. While the sulfite-reactivated enzyme appears to sediment as a single boundary in the presence of sulfite, the $S_{20,w}$ value, under the conditions described in the legend to Fig. 6, is 11.2 S. Native phosphorylase b was found to have an $S_{20,w}$ of 8.2 S under these conditions. This suggests that sulfite-reactivated phosphorylase b associates under the conditions described in the legend to Fig. 7.

Incorporation of $^{35}SO_3^=$ into o-Iodosobenzoate-Inactivated Phosphorylase b.

Since sulfite is known to cleave disulfide bonds (40), the reactivation of o-iodosobenzoate-inactivated phosphorylase b by this reagent supports the view that inactivation of phosphorylase b by o-iodosobenzoate involves disulfide bond formation. In order to gain information on the number of disulfide bonds formed, the oxidized enzyme was incubated with ^{35}S-labeled sodium sulfite as described under Materials and Methods. After separation from the reaction mixture, the ^{35}S-labeled enzyme contained an average

1.4 moles of $^{35}SO_3^=$ per 200,000 g. of protein (5 experiments). This value is corrected for nonspecific binding of $^{35}SO_3^=$ to unoxidized phosphorylase b (see Materials and Methods). The enzyme activity of ^{35}S-labeled phosphorylase b varied from 59 to 68% of the control activity. In view of the stoichiometry of the reaction of sulfite with a disulfide (40), the data suggest that at least 1.4 disulfide bonds are formed as a result of the reaction of phosphorylase b with o-iodosobenzoate.

Titration of the SH Groups of Native and S-Sulfonated Phosphorylase b with DTNB.

The titration curves of native and S-sulfonated phosphorylase b with DTNB are shown in Fig. 8. Based on a molar extinction coefficient of 13,600 (53), 4.1 -SH groups of native phosphorylase b were found to react with DTNB. In contrast, only 1.9 -SH groups per 200,000 g. of S-sulfonated phosphorylase b were found to react with DTNB. The ^{35}S-labeled enzyme used in this experiment contained 1.6 moles of ^{35}S per 200,000 g. of protein. These data suggest that approximately 2 -SH groups per molecule of phosphorylase b are blocked in S-sulfonated phosphorylase b.

Sedimentation Behavior of S-Sulfonated Phosphorylase b.

The sedimentation velocity pattern shown in Fig. 9A (top) indicates that S-sulfonated phosphorylase b is a mixture of two components. The major, slowly-moving, component has an $S_{20,w}$ of 9.3 S while the more rapidly sedimenting species

has an $S_{20,w}$ of 13.0 S. These sedimentation coefficients were obtained using ^{35}S-labeled phosphorylase b and a control sample which had been stored for two days at 4° prior to ultracentrifugation. The ^{35}S content of the S-sulfonated enzyme decreased during this period from an initial value of 1.3 moles of ^{35}S per 200,000 g. of protein to 1.0 moles of ^{35}S per 200,000 g. of protein on the day of centrifugation. This decrease in the ^{35}S content could be due to the attack of enzyme -SH groups on the $-S-^{35}SO_3^=$ group (42). Enzyme activity of ^{35}S-labeled phosphorylase b was 62% of the control activity at the time of centrifugation.

Fig. 9A (bottom) shows the sedimentation velocity pattern of native phosphorylase b which, except for treatment with o-iodosobenzoate, was treated exactly like the ^{35}S-labeled enzyme. It can be seen that in addition to the major peak ($S_{20,w}$ = 8.7 S), a small amount of rapidly sedimenting material ($S_{20,w}$ = 11.6 S) is also present.

In order to determine whether the rapidly-sedimenting components shown in Fig. 9A were the result of intermolecular disulfide bond formation between phosphorylase b dimers, S-sulfonated phosphorylase b and the control enzyme used for the experiments shown in Fig. 9A, were treated with dithiothreitol. It is apparent from Fig. 9B that, after treatment with dithiothreitol, both ^{35}S-labeled phosphorylase b and the control enzyme sediment as dimers having $S_{20,w}$ values of 8.7 S, and 8.8 S respectively. This suggests that the rapidly sedimenting species shown in Fig. 9A are the

result of intermolecular disulfide formation.

Effect of o-Iodosobenzoate on Phosphorylase b Treated with 1-^{14}C-Iodoacetamide.

Zarkadas et al. (27) have shown that freshly prepared phosphorylase b can incorporate 3.1 moles of 1-^{14}C-iodoacetamide per mole of dimer, with no loss in enzyme activity.

As shown in Table III, the reaction of o-iodosobenzoate with IAM-phosphorylase b results in only a small loss of enzyme activity. This suggests that o-iodosobenzoate reacts with those -SH groups of phosphorylase b that react rapidly with iodoacetamide.

Incorporation of 1-^{14}C-Iodoacetamide into S-Sulfonated Phosphorylase b.

Reaction of S-sulfonated phosphorylase b with 1-^{14}C-iodoacetamide leads to the incorporation of 1.2 to 1.4 moles of reagent per 200,000 g. of enzyme (Table IV), with no loss of enzyme activity. On the other hand, alkylation of control samples of native phosphorylase b with 1-^{14}C-iodoacetamide results in the incorporation of 2.7 to 3.2 moles of ^{14}C per 200,000 g. of protein, without loss of enzyme activity.

Previous results have indicated that reactivation of oxidized phosphorylase b with $^{35}SO_3^=$ leads to incorporation of an average of 1.4 moles of ^{35}S per 200,000 g. of protein. Since S-sulfonated phosphorylase b has an average of 1.6

fewer -SH groups available for rapid reaction with 1-^{14}C-iodoacetamide than the control enzyme, it is reasonable to conclude that the reaction of oxidized phosphorylase b with $^{35}SO_3^=$ leads to modification of the same -SH groups that are rapidly alkylated by iodoacetamide.

Effect of AMP and Substrates on the Inactivation of Phosphorylase b by o-Iodosobenzoate.

The data presented in Table V indicate that there is no significant protective effect of glucose-1-phosphate, AMP, or glycogen on the inactivation of phosphorylase b by o-iodosobenzoate. Only a very small protective effect was observed in the presence of both AMP and glucose-1-phosphate. These results suggest that the -SH groups of phosphorylase b that are oxidized by o-iodosobenzoate are not involved in the binding of substrates or AMP to the enzyme.

Effect of Dithiothreitol on the $^{35}SO_3^=$ Content and Activity of S-Sulfonated Phosphorylase b.

Kastenschmidt et al. (30) observed that the reaction of phosphorylase b with DTNB leads to a mixture of monomer, dimer, and aggregates which contains an average of 4 modified -SH groups per mole of phosphorylase b dimer. While thionitrobenzoate groups could be removed with dithiothreitol in several minutes, the full restoration of enzyme activity required several hours.

The results shown in Table VI indicate that treatment of S-sulfonated phosphorylase b with dithiothreitol restores enzyme activity to 79% of the control activity after 1 hour. At this time, the S-sulfonated enzyme still contains about 0.4 moles of $^{35}SO_3^=$ per mole of phosphorylase b. Further incubation with dithiothreitol leads to a progressive decrease in the $^{35}SO_3^=$ content, but no further increase in enzyme activity occurs. These results suggest that the increase in activity of S-sulfonated phosphorylase b, in the presence of dithiothreitol, is primarily due to reduction of disulfide bonds (see Fig. 9B).

Kinetics of S-Sulfonated and Native Phosphorylase b.

Gold (28, 29) observed that dinitrophenylation of those -SH groups of phosphorylase b that react rapidly with iodoacetamide, causes a decrease in the affinity of the enzyme for AMP and glucose-1-phosphate.

Kastenschmidt et al. (30) treated phosphorylase b with DTNB and isolated a phosphorylase b dimer containing up to 1.6 modified -SH groups per subunit. Kinetic studies on the modified enzyme indicated that homotropic cooperativity between AMP binding sites was abolished, and the affinity of the modified enzyme for AMP was decreased. In view of these observations, it was of interest to determine the activity response of S-sulfonated phosphorylase b to AMP.

It can be seen in Fig. 10 that the double reciprocal plot for unmodified phosphorylase b shows upward curvature,

expressing homotropic cooperativity between AMP binding sites. This homotropic cooperativity appears to be eliminated in the case of S-sulfonated phosphorylase b. Hill plots (58, 59) of these data, shown in Fig. 11, indicate that the Hill coefficient, n, is 1.3 for unmodified phosphorylase b in the range of AMP concentrations from 2×10^{-6} M to 1×10^{-5} M, while the K_m for AMP is 1.9×10^{-5} M. In contrast, the K_m for AMP of S-sulfonated phosphorylase b is 6.1×10^{-5} M, and the Hill coefficient, n, is 1.1. There appears to be only a small difference in V_{max} between native and S-sulfonated phosphorylase b (see Fig. 10).

These results suggest that the homotropic cooperativity of AMP sites is almost completely abolished in S-sulfonated phosphorylase b, and that the affinity of this enzyme for AMP is decreased relative to the native enzyme.

Effect of Dithiothreitol on the Kinetic Response of S-Sulfonated Phosphorylase b to AMP.

Kastenschmidt et al. (30) observed that dithiothreitol restored the homotropic cooperativity of AMP sites in DTNB-treated phosphorylase b, and decreased the K_m for AMP of this enzyme.

Therefore, S-sulfonated phosphorylase b and a control sample of phosphorylase b were treated with dithiothreitol as indicated in the legend to Fig. 12. The K_m values for AMP of native and S-sulfonated phosphorylase b, after treatment

with dithiothreitol, were 1.9×10^{-5} M and 1.5×10^{-5} M respectively. The Hill coefficient, n, of S-sulfonated phosphorylase b increases from 1.1 to 1.4 after treatment with dithiothreitol, while there is no change in n for the native enzyme. The V_{max} values for native and S-sulfonated phosphorylase b, after treatment with dithiothreitol, were 44 μmoles per min. per mg. and 34 μmoles per min. per mg. respectively, as determined by assay in the presence of saturating concentrations of AMP.

Discussion

Inactivation of Phosphorylase b by Mn^{2+} and Sulfite.

The data obtained in this study suggest that inactivation of phosphorylase b, in the presence of Mn^{2+} and sulfite, is a result of the aerobic oxidation of sulfite. This hypothesis is supported by the finding that substances that promote sulfite oxidation (56) also promote enzyme inactivation, and by the observation that inhibitors of sulfite oxidation (55, 56) also retard enzyme inactivation.

The aerobic oxidation of sulfite proceeds by way of a free-radical chain mechanism which is similar to the chain reaction involved in the dye-sensitized photooxidation of amino acids (60). The inactivation of enzymes as a result of the dye-sensitized photooxidation of certain amino acid residues is well known (61, 62). In view of the similarity in the mechanisms involved in the aerobic oxidation of sulfite and in the dye-sensitized photooxidation of amino acids, it is likely that phosphorylase b inactivation is caused by atmospheric oxygen or by free radicals that are formed during the aerobic oxidation of sulfite.

No evidence has been obtained regarding the amino acid residues of phosphorylase b that are modified as a result of aerobic oxidation of sulfite. It is possible that aromatic amino acid residues as well as sulfur-containing amino acid residues are oxidized. It is unlikely that

inactivation of phosphorylase b, in the presence of Mn^{2+} and sulfite, is due to oxidation of -SH groups to disulfides, because treatment of modified phosphorylase b with mercaptoethanol does not lead to reactivation of the enzyme.

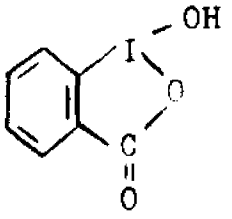
The sedimentation velocity pattern of the inactivated phosphorylase b indicates that the enzyme is partially dissociated into monomers, suggesting that inactivation may be due in part to monomerization of the enzyme. Dissociation of phosphorylase b into monomers, during the aerobic oxidation of sulfite, may be due to oxidation of the cysteinyl residue in the N-peptide to oxidation states higher than disulfide. This interpretation is consistent with the finding of Battell et al. (25), that alkylation of the cysteinyl residue in the N-peptide of phosphorylase b with iodoacetamide can be correlated with inactivation and monomerization of the enzyme.

Inactivation of Phosphorylase b by Cu^{2+}.

The evidence presented in this study suggests that inactivation of phosphorylase b by Cu^{2+} occurs as a result of disulfide bond formation. Restoration of more than 90% of enzyme activity after treatment with dithiothreitol, supports this conclusion. Since the sedimentation velocity pattern of Cu^{2+}-inactivated phosphorylase b indicates that enzyme aggregation has occurred, it is likely that this aggregation is the result of intermolecular disulfide bond formation.

Inactivation of Phosphorylase b by o-Iodosobenzoate.

o-Iodosobenzoate was first prepared by Meyer and Wachter (63). Absence of the characteristic "iodoso" smell and its stability to further oxidation led to the formulation of this compound as (63, 64):



Infra-red spectra of o-iodosobenzoate show a low value for the carbonyl stretching band (1620 cm^{-1}), presumably because of an I-O-CO system in the structure, and do not contain the hydrogen-bonded hydroxyl bands characteristic of carboxylic acids (65). These studies support the view that o-iodosobenzoate has the cyclic structure shown above.

Hellerman et al. (66) showed that o-iodosobenzoate oxidizes cysteine or glutathione to the corresponding disulfides at pH 7. It does not noticeably react with methionine, cystine, tyrosine, serine, tryptophan, proline, or hydroxyproline at pH 7; however at pH 5 it oxidizes methionine (45).

o-Iodosobenzoate has been frequently used as an inhibitor of enzymes containing -SH groups (67). The ability of myosin to associate with actin and to split ATP depends on -SH groups, and is inhibited by o-iodosobenzoate (68).

The reaction of o-iodosobenzoate with yeast ferrocytochrome *c* at pH 8.5 and 20°C. has recently been studied (69). This enzyme contains only 1 free -SH group per molecule. Little and O'Brien (69) have shown that the reaction of ferrocytochrome *c* with 0.36 mM o-iodosobenzoate at pH 8.5 results in the conversion of 82% of the enzyme to a dimer. However, oxidation of ferrocytochrome *c* with 1.2 mM o-iodosobenzoate caused a decreased level of dimerization, suggesting that under these conditions oxidation beyond the disulfide stage occurred.

The evidence presented here suggests that o-iodosobenzoate oxidizes a limited number of -SH groups of phosphorylase *b* to the corresponding disulfides, and causes the enzyme to aggregate. It has been shown that dithiothreitol restores the activity of oxidized phosphorylase *b* to at least 90% of the control activity. This result is consistent with the idea that inactivation of the enzyme occurs primarily as a result of disulfide bond formation.

The broad sedimentation pattern of oxidized phosphorylase *b* (Fig. 6) indicates its polydisperse nature, and suggests that the disulfide bonds formed during the inactivation of the enzyme by o-iodosobenzoate, are intermolecular. Further support for this conclusion is derived from the fact that dithiothreitol not only restores the activity of oxidized phosphorylase *b*, but also causes reformation of the original dimeric structure of phosphorylase *b*

(Fig. 6B). It has been shown that o-iodosobenzoate-treated phosphorylase b incorporates an average of 1.4 moles of $^{35}SO_3^=$ per mole of phosphorylase b dimer. In view of the stoichiometry of the reaction of sulfite with a disulfide (40), the results presented here suggest that at least 1.4 disulfide bonds are formed per molecule of phosphorylase b dimer, during the inactivation of phosphorylase b by o-iodosobenzoate.

The observation that sulfite can restore the activity of oxidized phosphorylase b to 68-78% of the control value is noteworthy. Since sulfite is presumably incorporated into the same -SH groups that were oxidized by o-iodosobenzoate, substitution of these -SH groups cannot be the primary cause of enzyme inactivation. Rather, it is more likely that the change in enzyme conformation, due to intermolecular disulfide bond formation, is primarily responsible for the enzyme inactivation.

It has been shown that, in the presence of sulfite, the broad sedimentation pattern of oxidized phosphorylase b (Fig. 6) is replaced by a single peak (Fig. 7). This finding is consistent with the conclusion that sulfite reacts with intermolecular disulfide bonds in the oxidized enzyme, and causes the formation of S-sulfonated phosphorylase b. At pH 8.0, and in the presence of a high salt concentration, the S-sulfonated enzyme has an $S_{20,w}$ value of 11.2 S in the presence of sulfite, while the $S_{20,w}$ of native phosphorylase

b under identical conditions is 8.2 S.

The tendency of phosphorylase b dimer to associate into tetramer in the presence of AMP is well known (70). Cohen et al. (3) recently obtained an $S_{20,w}$ of 12.4 S for native phosphorylase b in the presence of AMP. In the absence of this nucleotide, native phosphorylase b tetramerizes only at high enzyme concentrations and at low temperatures (30).

The fact that S-sulfonated phosphorylase b has an $S_{20,w}$ of 11.2 S suggests that, under the conditions described in the legend to Fig. 7, the S-sulfonated enzyme has a tendency to associate into a tetramer even in the absence of AMP. The difference between the $S_{20,w}$ of native phosphorylase b in the presence of AMP and the $S_{20,w}$ of the S-sulfonated enzyme may be due to a salt effect. Cohen et al. (3) observed that the $S_{20,w}$ of native phosphorylase b decreases with increasing salt concentration. A similar affect of sodium chloride on S-sulfonated phosphorylase b may occur.

It is unlikely that association of S-sulfonated phosphorylase b, at pH 8.0 and in the presence of sulfite, involves intermolecular disulfide bond formation. After separation of the S-sulfonated enzyme from unreacted sulfite by ammonium sulfate precipitation and gel filtration at pH 6.8, the enzyme sediments primarily as a dimer with an

$S_{20,w}$ of 9.3 S.

The difference between the sedimentation coefficients of S-sulfonated phosphorylase b and native phosphorylase b at pH 8.0 indicates that S-sulfonation of 1 to 2 SH groups of phosphorylase b causes changes in the dimer-dimer interactions of the enzyme, which facilitate association.

The sedimentation velocity pattern of S-sulfonated phosphorylase b in the absence of sulfite (Fig. 9A) suggests that some intermolecular disulfide bond formation occurs during separation of the enzyme from unreacted sulfite. This conclusion is supported by the observation that, in the presence of dithiothreitol, the S-sulfonated enzyme sediments as a single boundary with an $S_{20,w}$ of 8.7 S (Fig. 9B). Since the reaction of sulfite with a disulfide is reversible, it is probable that during separation of S-sulfonated phosphorylase b from unreacted sulfite, free -SH groups of phosphorylase b react with S-sulfonate groups according to the following equation (40):

$$RS^- + R'\text{-}S\text{-}SO_3^= = R'\text{-}S\text{-}S\text{-}R + SO_3^=$$

This conclusion is supported by the finding that the ^{35}S content of S-sulfonated phosphorylase b decreases from 1.3 to 1.0 moles per mole of phosphorylase b dimer after 48 hours. However, since the native enzyme also has a tendency to form high molecular weight species in the absence of reducing agent (Fig. 9A), some intermolecular disulfide bond formation may also occur as a result of air oxidation of -SH groups during the separation of S-sulfonated phos-

phorylase b from unreacted sulfite.

Battell et al. (25) found that 1.6 -SH groups per molecule of phosphorylase b dimer react rapidly with iodoacetamide, with no loss of enzyme activity. More recently, Zarkadas et al. (27) reported that, with freshly prepared enzyme, up to 3.1 -SH groups per molecule of phosphorylase b could be reacted with iodoacetamide with no change in enzyme activity. The observations of Zarkadas et al. are confirmed by the data presented in this study (Table III).

It has been demonstrated that the reaction of o-iodosobenzoate with iodoacetamide-treated phosphorylase b results in only a small loss of enzyme activity. This suggests that o-iodosobenzoate oxidizes the same -SH groups of phosphorylase b which react rapidly with iodoacetamide. The results of the alkylation of S-sulfonated phosphorylase b with 1-^{14}C-iodoacetamide support this conclusion. These data indicate that a molecule of S-sulfonated phosphorylase b has 1.6 to 2.0 fewer -SH groups available for rapid reaction with iodoacetamide than a molecule of native enzyme. It appears very likely, therefore, that the -SH groups of phosphorylase b that are oxidized by o-iodosobenzoate and sulfonated by sulfite are the same as those which react rapidly with iodoacetamide.

Gold and Blackman (29) have presented evidence which indicates that chlorodinitrobenzene reacts with the same -SH groups of phosphorylase b that react rapidly with iodo-

acetamide. Gold (28) found that dinitrophenylation of these -SH groups results in a decrease in the affinity of the enzyme for AMP and glucose-1-phosphate.

Kastenschmidt et al. (30) treated phosphorylase b with DTNB, and isolated a dimeric species that contained up to 3.2 modified -SH groups. Kinetic studies of this modified enzyme showed that homotropic cooperativity for AMP was abolished, and the K_m for AMP was increased, with no change in maximum velocity. Treatment of the modified enzyme with dithiothreitol caused a rapid release of thionitrobenzoate, but several hours were required for the full return of activity and the restoration of homotropic cooperativity of AMP sites.

The data presented here suggest a similarity between S-sulfonated phosphorylase b and the DTNB-treated enzyme studied by Kastenschmidt et al. (30). S-Sulfonation of phosphorylase b results in a three-fold increase in the K_m for AMP and a hyperbolic response to AMP, with little change in the maximum velocity (Fig. 10).

After treatment of S-sulfonated phosphorylase b with dithiothreitol, the kinetic response of the enzyme to AMP becomes similar to that of the native enzyme. The K_m for AMP and the Hill coefficient for dithiothreitol-treated, S-sulfonated phosphorylase b are comparable to the corresponding values obtained for dithiothreitol-treated native phosphorylase b. However, dithiothreitol-treated, S-sul-

fonated phosphorylase b has a V_{max} equal to about 80% of the value obtained for dithiothreitol-treated native phosphorylase b.

Since dithiothreitol-reactivated, S-sulfonated phosphorylase b has been shown to sediment as a dimer in the ultracentrifuge, it is unlikely that the decrease in maximum velocity is due to intermolecular disulfide bond formation. It is possible, however, that some intramolecular disulfide bond formation occurs when S-sulfonated phosphorylase b is reactivated with dithiothreitol.

Eldjarn and Pihl (71) and Kolthoff (72) have shown that mixed disulfides are intermediates in the reduction of disulfides by thiols, and that the concentration of such mixed disulfides can be high even in the presence of a large excess of thiol. Under appropriate conditions, unsymmetrical disulfides can also be formed by the reaction of thiols with alkyl thiosulfates (73).

When intermolecular disulfide bonds are formed by oxidation of the rapidly-reacting -SH groups of phosphorylase b, the enzyme is almost completely inactivated. If intermolecular disulfide bond formation can markedly lower the maximum velocity of phosphorylase b, it is possible that intramolecular disulfide bond formation might account for the small reduction in V_{max} observed for dithiothreitol-treated, S-sulfonated phosphorylase b.

On the other hand, the concentration of o-iodosobenzoate

used to inactivate phosphorylase b was in excess of the amount required to oxidize 2.8 -SH groups per molecule to the corresponding disulfides. It is possible, therefore, that some -SH groups are oxidized to higher oxidation states than disulfide, (e. g. RSO_2^-, R·SO·SR, or R·SO_2·SR), and that such modified groups are responsible for the lower V_{max} observed in dithiothreitol-treated, S-sulfonated phosphorylase b. Another possibility is that some amino acid residue at the active site of the enzyme is partially oxidized and this results in a small decrease in the maximum velocity of the S-sulfonated enzyme.

On the basis of the kinetic data presented here, it appears likely that the -SH groups of phosphorylase b, that are oxidized by o-iodosobenzoate and subsequently sulfonated are the same as those that react with DTNB. The effects of DTNB treatment and S-sulfonation on the K_m for AMP and on the homotropic cooperativity of AMP sites in phosphorylase b are almost identical. These effects can be completely reversed by dithiothreitol in both DTNB-treated and S-sulfonated phosphorylase b.

Gold (29) presented evidence which indicates that chlorodinitrobenzene reacts with the same -SH groups of phosphorylase b which react rapidly with iodoacetamide. The data presented here suggest that these rapidly-reacting -SH groups can be oxidized to the corresponding disulfides and subsequently sulfonated with sulfite. Furthermore, S-sulfonated

phosphorylase b shows a kinetic response to AMP which is almost identical with that shown by the DTNB-modified enzyme studied by Kastenschmidt et al. (30). It appears likely, therefore, that all of these reagents modify the same rapidly-reacting -SH groups of phosphorylase b.

However, modification of these -SH groups with different reagents produces different effects on enzyme activity. Reaction of phosphorylase b with 0.001 M iodoacetamide does not cause any significant change in enzyme activity (25). Reaction with DTNB (30), chlorodinitrobenzene (28, 29), and sulfite (after oxidation) causes a decrease in the affinity of the enzyme for AMP and alters the homotropic cooperativity of AMP sites. Dinitrophenylation with chlorodinitrobenzene, and S-sulfonation, also lowers the V_{max} of phosphorylase b slightly.

On the other hand, oxidation of an average of 2.8 -SH groups per molecule of phosphorylase b to the corresponding disulfides causes almost complete inactivation of the enzyme. Since glycogen, glucose-1-phosphate, and AMP do not provide any significant protection against o-iodosobenzoate-inactivation of the enzyme, it is unlikely that amino acid residues at the active site of phosphorylase b are modified.

It is apparent that modification of the rapidly-reacting -SH groups of phosphorylase b with different reagents causes different effects on enzyme activity. It is likely

that these effects are allosteric in nature and result from changes in enzyme conformation. The ability of S-sulfonated phosphorylase b to associate under conditions in which the native enzyme is a dimer, supports this conclusion.

Battell et al. (25) have suggested that the -SH groups of phosphorylase b that react rapidly with iodoacetamide may be involved in regulating the activity of phosphorylase b in vivo. The results reported here support this hypothesis.

It is known that in resting muscle, phosphorylase is present mainly in the b form (74), and the enzyme has very little activity, even though sufficient AMP is present for activity (75). The results presented in this study suggest that this lack of activity may be due to the formation of intermolecular disulfide bonds in phosphorylase b.

The activation of phosphorylase by AMP, and by the conversion of phosphorylase b to phosphorylase a is well known (1). Phosphorylase is rapidly activated at the onset of muscle contraction, and activation is associated with the conversion of phosphorylase b to phosphorylase a (76). However, the increase in glycogenolysis which occurs in anoxic muscle appears to result mainly from activation of phosphorylase b (77, 78). Morgan and Parmeggiani (78) have attributed the activation of phosphorylase b under these conditions to increased levels of AMP, and decreased levels of ATP and glucose-6-phosphate. However, Helmreich and Cori (75) have shown, from measurements in vivo, that a 60-

fold increase in phosphorylase activity occurs during muscle contraction under anoxic conditions, while only a 2-fold decrease in the ATP/AMP ratio is observed. This decrease in the ATP/AMP ratio cannot account for the increase in phosphorylase activity which occurs during muscle contraction.

I-strain mice provide a useful tool for studying the regulation of phosphorylase activity, since skeletal muscle from these mice does not show any phosphorylase a activity (79). This lack of phosphorylase a activity is presumably due to a deficiency in phosphorylase b kinase (80).

Danforth and Lyon (78) found that tetanic stimulation of skeletal muscle from I-strain mice results in activation of glycogenolysis, but the extent of activation is less than that observed in control muscle. Helmreich and Cori (74) have discussed these data, and suggest that, while the conversion of phosphorylase b to phosphorylase a plays a role in the activation of glycogenolysis during tetanic stimulation of mouse skeletal muscle, a mechanism that allows phosphorylase b to be activated during muscle contraction must exist.

The data presented here suggest that such a mechanism could involve the reduction of intermolecular disulfide bonds in phosphorylase b.

Summary and Conclusions

Phosphorylase b loses 95% of its activity after oxidation with a three-fold molar excess of o-iodosobenzoate. Inactivation is accompanied by aggregation of the enzyme. Dithiothreitol restores the activity of o-iodosobenzoate-inactivated phosphorylase b to 92% of the control activity, and causes re-formation of the dimeric structure which is characteristic of the native enzyme. These results suggest that inactivation of phosphorylase b by o-iodosobenzoate is associated with the formation of intermolecular disulfide bonds.

Reaction of the oxidized enzyme with $^{35}SO_3^=$ leads to S-sulfonation of an average of 1.4 -SH groups per molecule of phosphorylase b. After separation from the reaction mixture, S-sulfonated phosphorylase b has 59 to 68% of the control activity and sediments primarily as a dimer in the ultracentrifuge. These results support the conclusion that o-iodosobenzoate causes the formation of intermolecular disulfide bonds in phosphorylase b.

The sulfhydryl groups of phosphorylase b that are oxidized by o-iodosobenzoate, and subsequently sulfonated by sulfite, are identical with those that react rapidly with iodoacetamide. This conclusion is supported by the following: 1) Iodoacetamide-treated phosphorylase b retains 73 to 85 percent of the control activity after reaction with o-iodosobenzoate. 2) S-sulfonated phosphorylase b

has 1.3 to 2.0 fewer -SH groups available for rapid reaction with iodoacetamide than native phosphorylase b.

The activity response of S-sulfonated phosphorylase b to AMP suggests that S-sulfonation of the rapidly-reacting -SH groups of phosphorylase b causes a decrease in the affinity of the enzyme for AMP, and nearly abolishes the homotropic cooperativity of AMP sites. Oxidation of the rapidly-reacting -SH groups of phosphorylase b by o-iodosobenzoate leads to a 95% loss in enzyme activity, while alkylation of these -SH groups with iodoacetamide has little or no effect on enzyme activity. Apparently, modification of the same -SH groups with different reagents leads to different effects on enzyme activity.

Since substitution of the rapidly-reacting -SH groups of phosphorylase b never leads to a complete loss of enzyme activity, these -SH groups cannot be essential for enzyme activity. The inability of AMP or substrates to protect these -SH groups against oxidation by o-iodosobenzoate suggests that they are not at, or near, the AMP or substrate binding sites.

Therefore, it is likely that modification of the rapidly-reacting -SH groups of phosphorylase b indirectly affects the enzyme active site. Accordingly, each modifying agent causes a characteristic change in enzyme conformation which in turn causes the observed effects on enzyme activity.

This hypothesis is supported by the following: a) It has been shown that oxidation of the rapidly-reacting -SH groups of phosphorylase b results in enzyme aggregation. b) S-sulfonated phosphorylase b associates under conditions in which the native enzyme does not.

The data presented in this study are consistent with the hypothesis that the rapidly-reacting -SH groups of phosphorylase b play a role in regulating the activity of this enzyme *in vivo*.

Table I

The effect of various compounds on the inactivation of phosphorylase b by Mn^{2+} and sulfite

Reaction mixtures contained phosphorylase b, 5.0×10^{-6} M, 0.05 M sodium-β-glycerophosphate and 0.005 M Na_2SO_3 at pH 6.7. Reactions were carried out in a 25 ml. Erlenmeyer flask at 0°C. The final volume was 2.0 ml.

Additions to the reaction mixture	% Activity remaining after 20 min.[a]
None	39
$MnSO_4$ omitted	100
$MgSO_4$ replaced $MnSO_4$	100
O_2 atmosphere[b]	9
N_2 atmosphere[b]	81
Mannitol, 0.02 M	89

[a]Aliquots of the reaction mixtures were diluted 100-fold in a buffer consisting of 0.1 M maleate, 0.002 M EDTA, and 1 mg./ml. BSA (pH 6.5) for assay of phosphorylase b activity. Activities are expressed as a percentage of the zero time activity in each experiment.

[b]After removing zero time aliquots, the reaction vessels were flushed with oxygen or nitrogen and stoppered.

Table II

The effect of reducing agents on native and o-iodosobenzoate-inactivated phosphorylase b

Phosphorylase b, 4.0 mg. per ml., was incubated with, and without o-iodosobenzoate, 6.0 x 10^{-5} M, in a buffer consisting of 0.05 M sodium barbital, 0.001 M EDTA, and 1.0 M NaCl (pH 8.0) for 10 minutes at 25°C. At this time, aliquots containing 200 µg. of phosphorylase b were diluted to 1.0 ml. with 0.05 M sodium barbital 0.001 M EDTA (pH 8.0), containing the indicated concentration of reducing agent. After 30 minutes at 25°C. aliquots were diluted 10-fold in 0.1 M maleate-0.002 M EDTA-1 mg./ml. BSA at pH 6.5 for assay of phosphorylase b activity.

Reducing agent	% Activity	
	Native phosphorylase b	o-iodosobenzoate treated phosphorylase b
None	100	6
Dithiothreitol, 0.002 M	105	97
Glutathione, 0.02 M	99	90
NaCN, 0.02 M	104	10
Na_2SO_3, 0.02 M	103	76

Table III

The effect of o-iodosobenzoate on 1-^{14}C-iodoacetamide-treated phosphorylase b.

Phosphorylase b, which had been recrystallized from 0.015 M dithiothreitol, was freed of AMP by gel filtration on a Sephadex G-25 column, which had been previously equilibrated with 0.02 M sodium-β-glycerophosphate-0.0015 M EDTA (pH 6.8). Phosphorylase b, 10 mg. per ml., was reacted with 0.001 M 1-^{14}C-iodoacetamide for 60 minutes at 30°C. in 0.02 M sodium-β-glycerophosphate-0.0015 M EDTA at pH 6.8 (57). The reaction was terminated by addition of mercaptoethanol to a final concentration of 0.05 M. The ^{14}C-labeled enzyme was passed through a Sephadex G-25 column which had been previously equilibrated with 0.05 M sodium barbital-0.001 M EDTA (pH 8.0). Phosphorylase b, or iodoacetamide-treated phosphorylase b, 1.0 x 10^{-5} M, was reacted with o-iodosobenzoate, 3.0 x 10^{-5} M, for 10 minutes, in 0.05 M sodium barbital-0.001 M EDTA-1.0 M NaCl at 25°C.

Moles of ^{14}C-iodoacetamide incorporated / Mole of phosphorylase b	% Activity after o-iodosobenzoate treatment
None	7
3.2	85[a]
3.0	73[a,b]

[a]Activities are expressed as a percentage of the activity of iodoacetamide-treated phosphorylase b, which had not been oxidized with o-iodosobenzoate.

[b]After reaction with o-iodosobenzoate for 1 hour, this enzyme retained 61% of the control activity.

Table IV

Incorporation of 1-^{14}C-iodoacetamide into native and S-sulfonated phosphorylase b

Phosphorylase b was S-sulfonated with non-radioactive sodium sulfite as described under Materials and Methods. After the second ammonium sulfate precipitation, S-sulfonated phosphorylase b was dissolved in 1.0 ml. of 0.02 M sodium-β-glycerophosphate-0.0015 M EDTA (pH 6.8), and passed through a Sephadex G-25 column (0.9 x 23 cm.) which had been previously equilibrated with the same buffer. Native phosphorylase b, which had not been oxidized with o-iodosobenzoate, was carried through the same procedure. The reactions of native and S-sulfonated phosphorylase b with 0.001 M 1-^{14}C-iodoacetamide were carried out for 60 minutes in 0.02 M sodium-β-glycerophosphate-0.0015 M EDTA at pH 6.8 (57); enzyme concentrations ranged from 2.7 mg. per ml. to 3.2 mg. per ml.

	Moles of ^{14}C-iodoacetamide incorporated / Mole of phosphorylase b	
Experiment number	Native enzyme	S-sulfonated enzyme
1	2.7	1.4
2	3.2	1.2
3	2.7	1.3

Table V

The effect of substrates and AMP on the inactivation of phosphorylase b by o-iodosobenzoate

Phosphorylase b, 2.0 mg. per ml., was inactivated with o-iodosobenzoate, 3.0×10^{-5} M, in 0.05 M sodium barbital-0.001 M EDTA-1.0 M NaCl (pH 8.0) at 25°C. After 10 minutes aliquots were diluted 100-fold with 0.1 M maleate-0.002 M EDTA-1mg. per ml. BSA (pH 6.5) for assay of phosphorylase b activity.

Additions to the reaction mixture	% Inactivation[a]
None	95
AMP, 0.001 M	93
Glucose-1-phosphate, 0.02 M	96
Glycogen, 1%	93
Glucose-1-phosphate, 0.02 M + AMP, 0.001 M	88

[a]Inactivation of phosphorylase b is expressed as a percentage of the control activity in each experiment.

Table VI

The effect of dithiothreitol on the activity and $^{35}SO_3^=$ content of S-sulfonated phosphorylase b.

S-sulfonated phosphorylase b, 1.0 mg. per ml., was incubated with 0.01 M dithiothreitol in 0.05 M sodium-β-glycerophosphate-0.001 M EDTA (pH 6.8) at 25°C. The S-sulfonated enzyme was prepared 2 days prior to reaction with dithiothreitol and stored at 4°C. At the time of preparation, the enzyme contained 1.3 moles of ^{35}S per mole of phosphorylase b and its activity was 67% of the control activity. At the indicated times, aliquots were diluted 100-fold in a buffer consisting of 0.1 M maleate, 0.002 M EDTA, 0.002 M dithiothreitol, and bovine serum albumin, 1 mg. per ml. (pH 6.5), for enzyme assay. Enzyme activities were measured in the presence of 0.001 M dithiothreitol.

Incubation time (hours)	% Activity[a]	Moles $^{35}SO_3^=$ / Mole of enzyme
0	59	1.0
1	79	0.42
2	80	0.35
4	84	0.21
5.5	80	0.15

[a]Enzyme activity is expressed as a percentage of the activity of native enzyme which had also been treated with dithiothreitol.

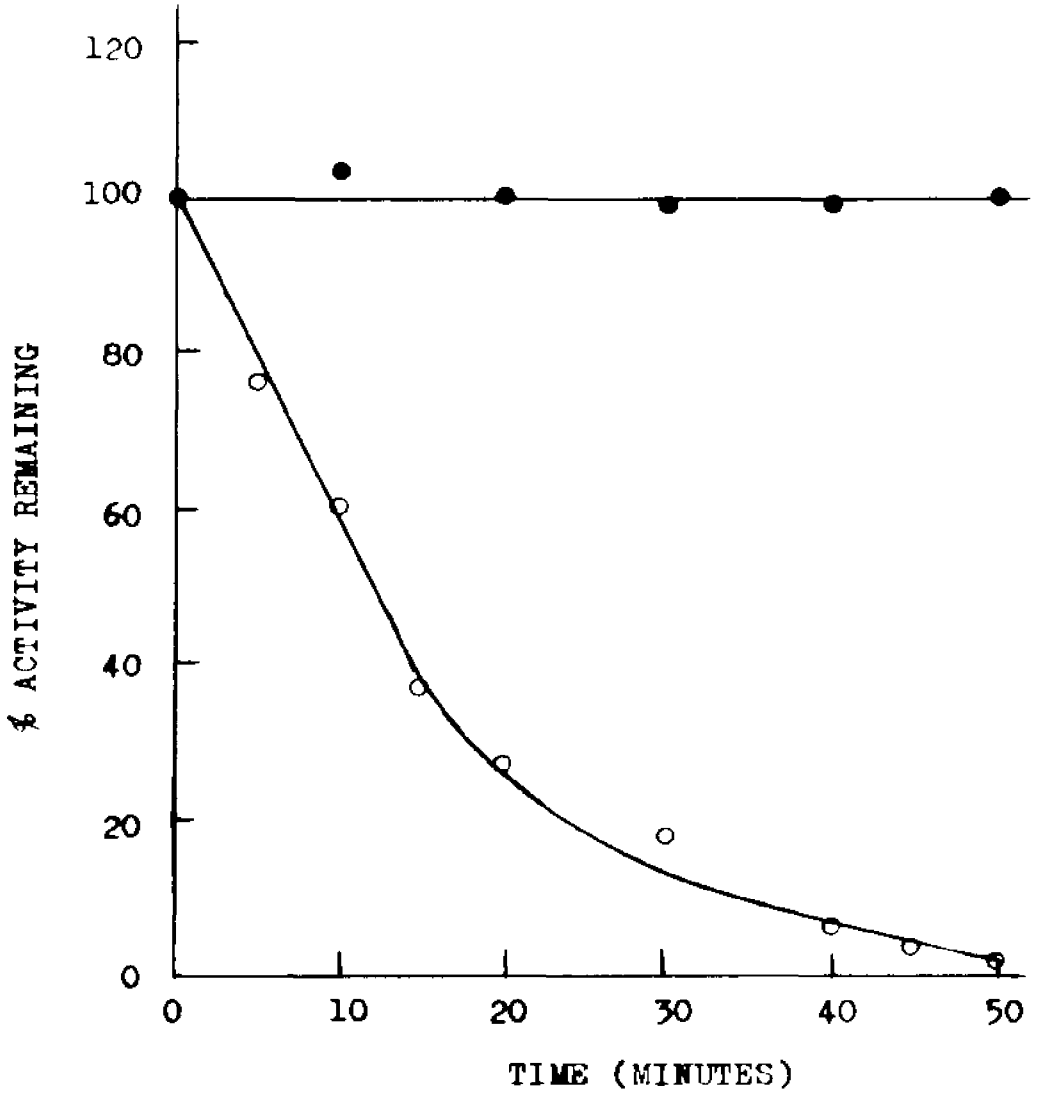


FIG. 1. Inactivation of phosphorylase b in the presence of Mn^{2+} and sulfite. Reaction mixtures contained phosphorylase b, 5.0×10^{-6} M, 0.05 M sodium-β-glycerophosphate, and 5.0×10^{-5} M $MnSO_4$, at pH 6.7 and 0°C., with 0.005 M Na_2SO_4 (●) or 0.005 M Na_2SO_3 (o). The final volume was 2.0 ml., and the reaction was carried out in a 25 ml. Erlenmeyer flask. At the indicated times, aliquots were diluted 100-fold in a buffer consisting of 0.1 M maleate, 0.002 M EDTA, and 1 mg./ml. BSA (pH 6.5) for assay of phosphorylase activity.

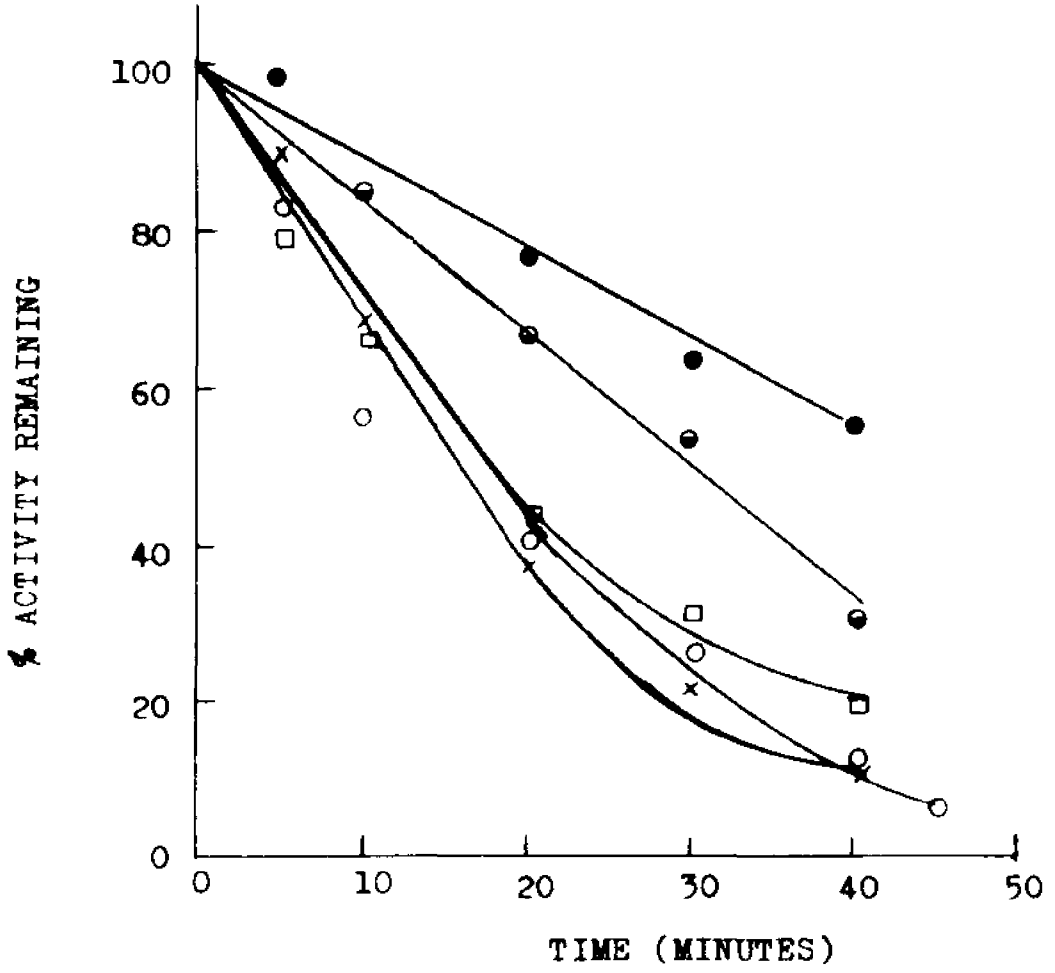


FIG. 2. The effect of AMP and substrates on the inactivation of phosphorylase by Mn^{2+} and sulfite. Reaction mixtures contained phosphorylase b, 5.0×10^{-6} M, 0.05 M sodium-β-glycerophosphate, 5.0×10^{-5}M $MnSO_4$, and 0.005 M Na_2SO_3 (○) no additions, (✕) also contained 0.025 M glucose-1-phosphate, (□) also contained 0.001 M AMP, (●) also contained 0.025M glucose-1-phosphate and 0.001 M AMP, (◒) also contained 1% glycogen. Reactions were carried out at 0°C. in a 25 ml. Erlenmeyer flask; the final volume was 2.0 ml. At the indicated times, aliquots were diluted in a buffer consisting of 0.1 M maleate-0.002 M EDTA-1mg./ml. BSA (pH 6.5) for assay of phosphorylase activity.

FIG. 3 Sedimentation velocity pattern of Mn^{2+} sulfite inactivated phosphorylase b. Phosphorylase b, 3.0×10^{-5} M, was inactivated by incubation in the presence of 5.0×10^{-4} M $MnSO_4$, and 0.005 M Na_2SO_3 for 70 min. at 0°C. The buffer was 0.05 M sodium-β-glycerophosphate-0.001 M EDTA at pH 6.7. The final volume was 2.0 ml., and the reaction was carried out in a 25ml. Erlenmeyer flask. Ultracentrifugation was carried out in the same buffer at 20°C. The direction of sedimentation is to the left. The picture was taken 16 min. after the rotor reached a speed of 60,000 rpm.

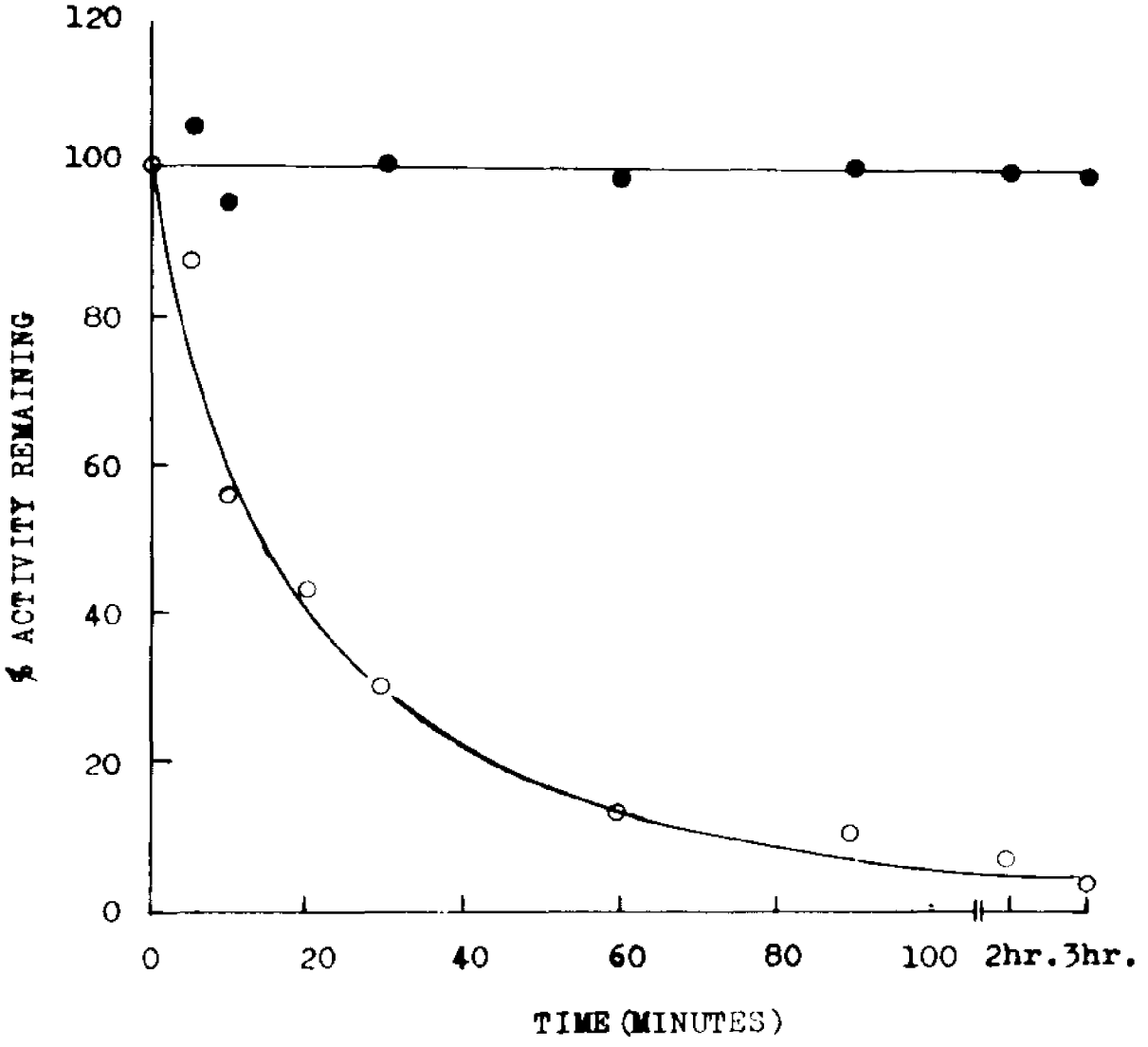


FIG. 4. Inactivation of phosphorylase b by Cu^{2+}. Reaction mixtures contain phosphorylase b, 1.0×10^{-5} M, .03 M imidazole-HCl, 1.0×10^{-5} M EDTA, at pH 7.2 and 23°C. with 1.5×10^{-5} M $CuSO_4$ (○), and 1.5×10^{-5} M Na_2SO_4 (●). Aliquots were diluted 100-fold in a buffer consisting of 0.1 M maleate, 0.002 M EDTA, and 1 mg./ml. BSA (pH 6.5) for assay of phosphorylase activity.

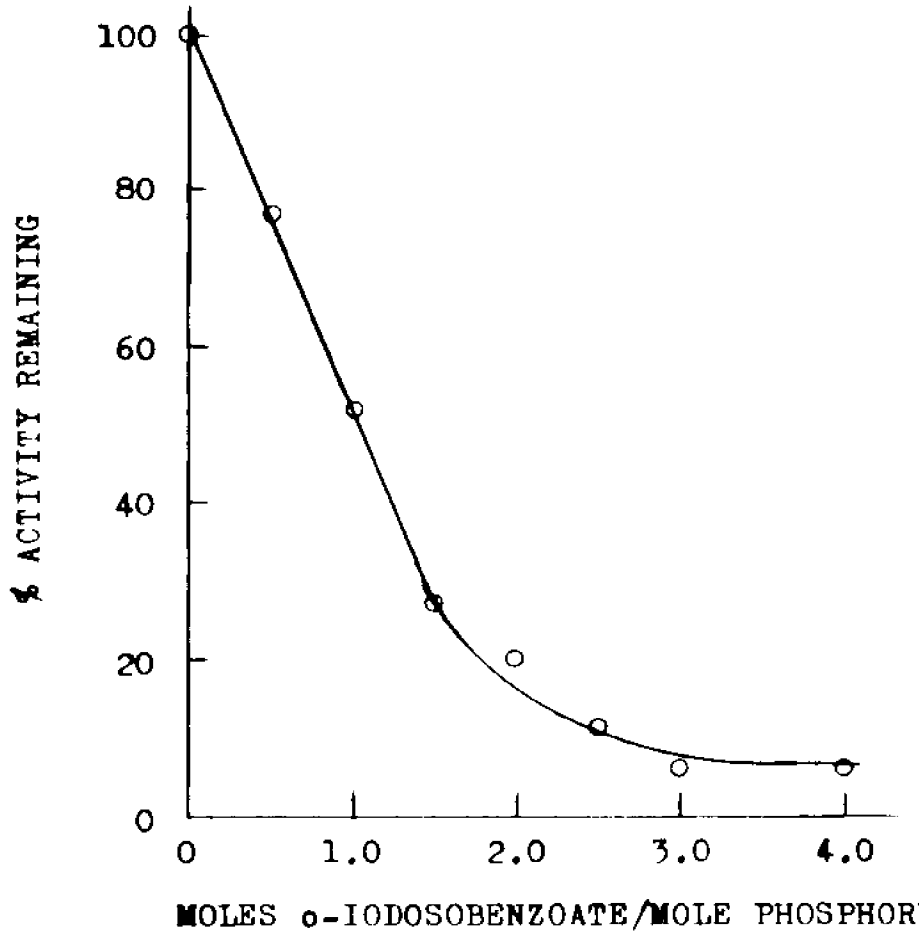


FIG. 5. The stoichiometry of o-iodosobenzoate inactivation of phosphorylase b. Reaction mixtures contained phosphorylase b, 1.0×10^{-5} M, 0.05 M sodium barbital, 0.001 M EDTA, 1.0 M NaCl, and o-iodosobenzoate at pH 8.0. After incubation for 10 min. at 25°C., aliquots were diluted 100-fold in a buffer consisting of 0.1 M maleate, 0.002 M EDTA, and 1 mg./ml. BSA (pH 6.5) for assay of phosphorylase activity.

A B

FIG. 6. Sedimentation velocity patterns of native and o-iodosobenzoate-inactivated phosphorylase b. Ultracentrifugation was carried out at 48,000 rpm and 20°C. Sedimentation proceeds to the left. Phosphorylase b, 4.0 mg. per ml., was oxidized with o-iodosobenzoate, 6.0 x 10^{-5} M, for 10 min. in 0.05 M sodium barbital-0.001 M EDTA-1.0 M NaCl (pH 8.0) at 25°C., and treated as described below immediately after inactivation. (A) Native and o-iodosobenzoate-treated phosphorylase b were subjected to gel filtration on a Sephadex G-25 column (0.9 x 23 cm.), which had been previously equilibrated with 0.05 M sodium barbital-0.001 M EDTA-1.0 M NaCl (pH 8.0); ultracentrifugation was carried out in the same buffer. Upper pattern: phosphorylase b, 3.3 mg. per ml. Lower pattern: o-iodosobenzoate-inactivated phosphorylase b, 3.5 mg. per ml. The picture was taken 16 min. after the rotor reached speed. (B) Aliquots of o-iodosobenzoate-treated phosphorylase b were incubated with and without dithiothreitol at 25°C. for 30 min., before ultracentrifugation. The buffer was 0.05 M sodium barbital-0.001 M EDTA-0.9 M NaCl (pH 8.0). Upper pattern: o-iodosobenzoate-inactivated phosphorylase b, 3.6 mg. per ml., with 0.002 M dithiothreitol. Lower pattern: o-iodosobenzoate-inactivated phosphorylase b, 3.6 mg. per ml. The picture was taken 39 min. after the rotor reached speed.

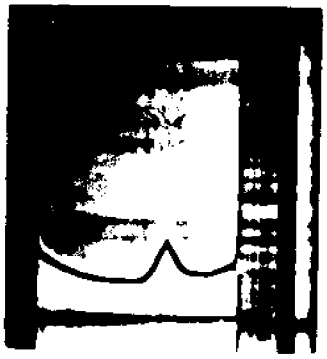


FIG. 7. The effect of sulfite on the sedimentation velocity pattern of o-iodosobenzoate-inactivated phosphorylase b. Phosphorylase b, 4.0 mg. per ml., was reacted with o-iodosobenzoate, 6.0×10^{-5} M, for 10 min. in 0.05 M sodium barbital-0.001 M EDTA-1.0 M NaCl (pH 8.0) at 25°C. Immediately after oxidation, the inactivated enzyme, 3.2 mg. per ml., was incubated with 0.01 M sodium sulfite for 30 min. at 25°C. The buffer was 0.05 M sodium barbital-0.001 M EDTA-0.8 M NaCl at pH 8.0. Ultracentrifugation was carried out in this buffer at 56,000 rpm and 20°C. The direction of sedimentation is to the left. The picture was taken 34 min. after the rotor reached speed.

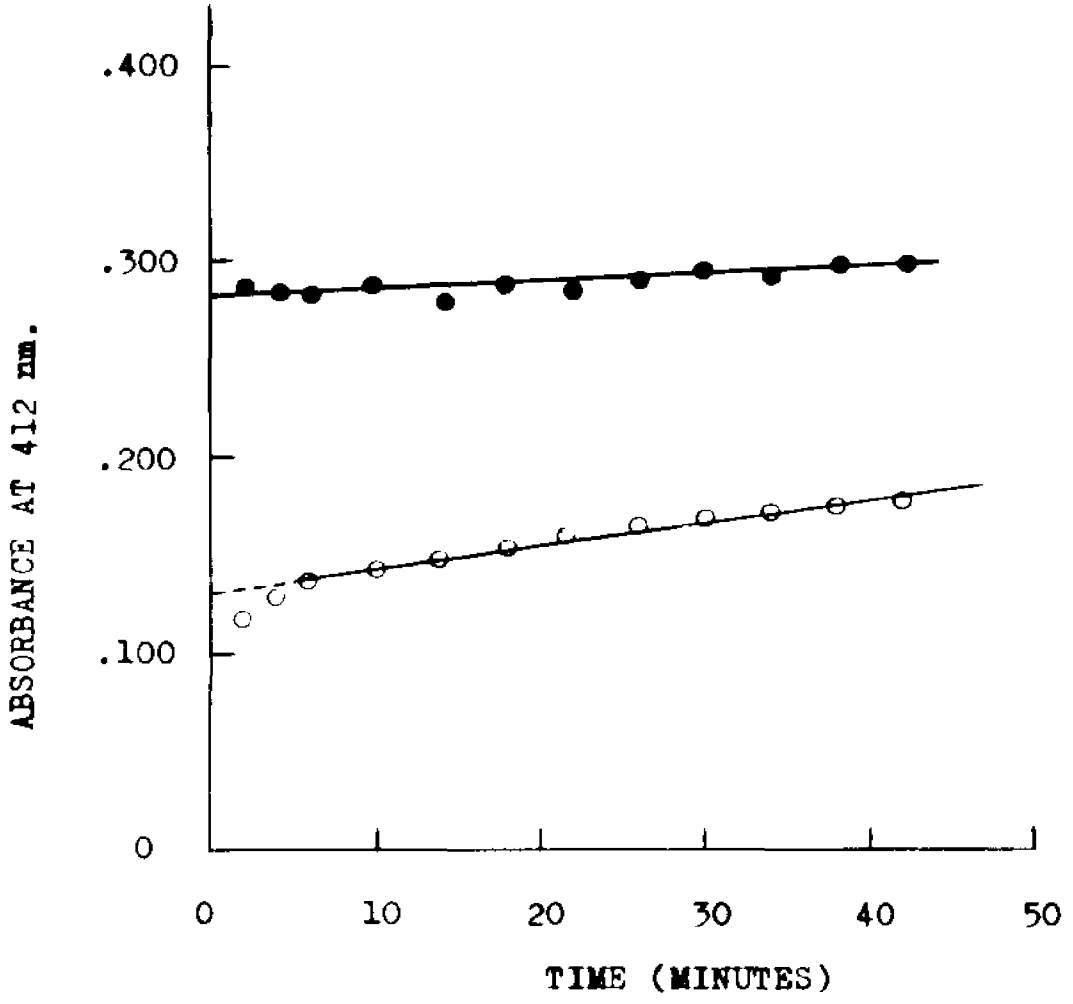


FIG. 8. Titration of native (●) and S-sulfonated (○) phosphorylase b with DTNB. The reaction conditions for DTNB titrations, and the method of preparation of S-sulfonated phosphorylase b, are described under Materials and Methods.

A B

FIG. 9. Sedimentation velocity patterns of native and S-sulfonated phosphorylase b, with and without, dithiothreitol. Phosphorylase b, 4.0 mg. per ml., was oxidized with o-iodosobenzoate, 6.0×10^{-5} M, for 10 min. in 0.05 M sodium barbital-0.001 M EDTA-1.0 M NaCl (pH 8.0) at 25° C.; the volume was 3.0 ml. S-Sulfonation of phosphorylase b was carried out as described under Materials and Methods. Phosphorylase b which had not been oxidized with o-iodosobenzoate was taken through the same procedure. Ultracentrifugation was carried out in 0.05 M sodium-β-glycerophosphate-0.001 M EDTA (pH 6.8). (A) Samples of native, and S-sulfonated phosphorylase b were stored at 4°C. for 48 hours before ultracentrifugation. Upper pattern: S-sulfonated phosphorylase b, 4.0 mg. per ml. Lower pattern: native phosphorylase b, 4.0 mg. per ml. The picture was taken 33 min. after the rotor reached speed. (B) Aliquots of the enzyme solutions used for Fig. 9A were treated with 0.005 M dithiothreitol for 1 hour at 25°C., before ultracentrifugation. The buffer was 0.05 M sodium-β-glycerophosphate-0.001 M EDTA (pH 6.8). Upper pattern: S-sulfonated phosphorylase b, 2.7 mg. per ml. Lower pattern: native phosphorylase b, 2.7 mg. per ml. The picture was taken 37 min. after the rotor reached speed. Ultracentrifugation in (A) and (B) was carried out at 56,000 rpm and 20°C.; the direction of sedimentation is to the left.

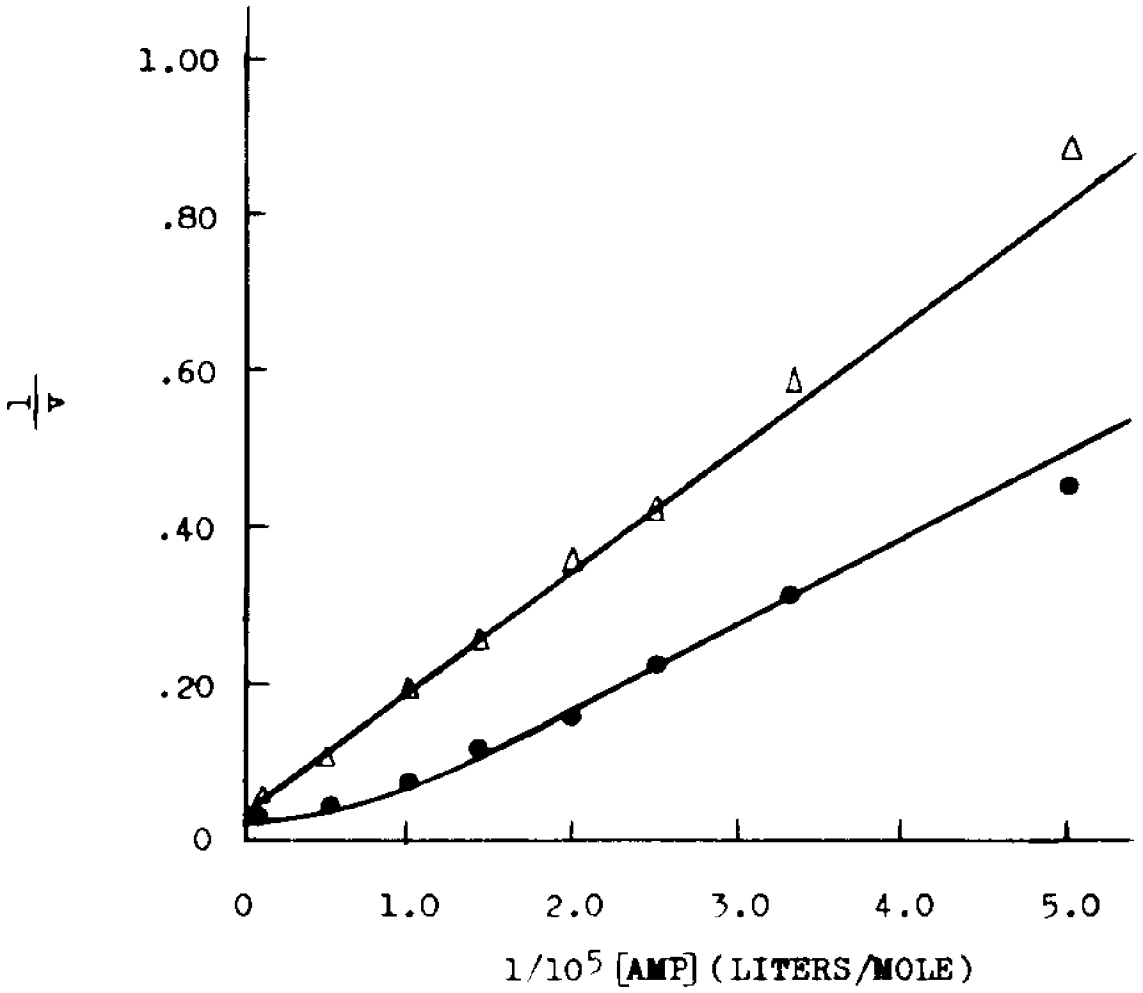


FIG. 10. Double-reciprocal plots of the activation of native (●) and S-sulfonated (△) phosphorylase b by AMP. Activity measurements were carried out in 0.008 M sodium-β-glycerophosphate-0.003 M EDTA buffer (pH 6.8), containing 0.6% glycogen, 0.048 M glucose-1-phosphate, and various amounts of AMP at 23°C. The enzyme concentration was 25 μg. per ml. S-Sulfonated phosphorylase b was prepared as described under Materials and Methods.

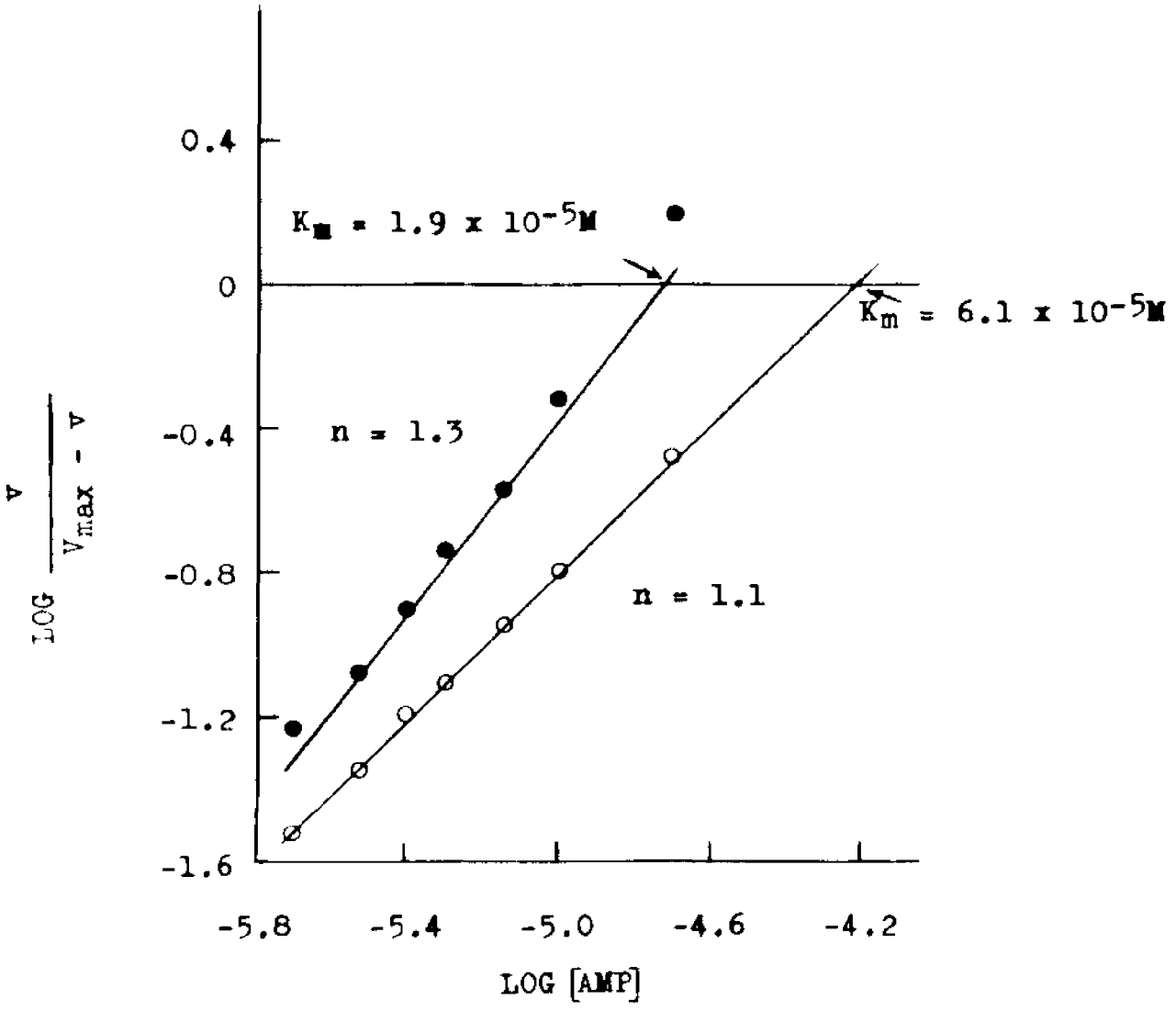


FIG. 11. Hill plots for the activation of native (●) and 6-sulfonated (○) phosphorylase b by AMP. The data were taken from Fig. 10.

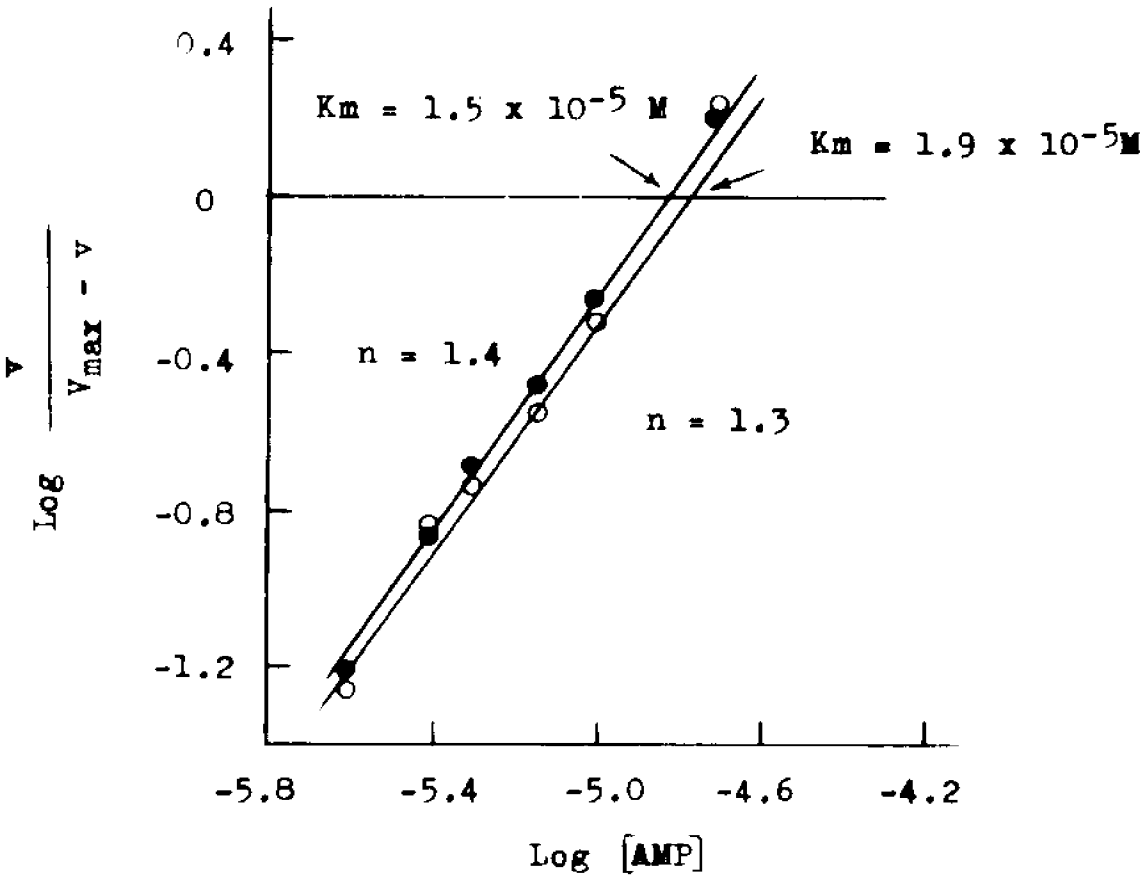


FIG. 12. Hill plots for the activation of dithiothreitol-treated, native (○) and dithiothreitol-treated, S-sulfonated (●) phosphorylase b by AMP. Native phosphorylase b, 1.5 mg. per ml., and S-sulfonated phosphorylase b, 1.5 mg. per ml., were incubated with 0.01 M dithiothreitol for 17 hours in 0.05 M sodium-β-glycerophosphate-0.001 M EDTA (pH 6.8) at 23°C. At this time, aliquots were diluted 1:12 in 0.008 M sodium- -glycerophosphate, 0.003 M EDTA, 0.002 M dithiothreitol, for assay of phosphorylase activity. Activity measurements were carried out in 0.008 M sodium-β-glycerophosphate-0.003 M EDTA-0.001 M dithiothreitol buffer (pH 6.8), containing 0.6% glycogen, 0.048 M glucose-1-phosphate, and various amounts of AMP at 23°C. The enzyme concentration was 25 μg. per ml.

Bibliography

1. Ryman, B. E., and Whelan, W. J. (1971) Adv. Enzymol. 34, 285.
2. Cori, C. F., Cori, G. T., and Green, A. A. (1943) J. Biol. Chem. 151, 39.
3. Cogen, P., Duewer, T., and Fischer, E. H. (1971) Biochemistry 10, 2683.
4. Krebs, E. G., and Fischer, E. H. (1956) Biochim. Biophys. Acta 20, 150.
5. Cori, G. T., and Cori, C. F. (1945) J. Biol. Chem. 158, 321.
6. Fischer, E. H., Pocker, A., and Saari, J. C. (1970) in Essays in Biochemistry (Campbell, P. N., and Dickens, F., eds.) Vol. 6, p. 23, Academic Press, New York.
7. Kent, A. B., Krebs, E. G., and Fischer, E. H. (1958) J. Biol. Chem. 232, 549.
8. Shaltiel, S., and Cortijo, M. (1970) Biochem. Biophys. Res. Commun. 41, 594.
9. Jones, D. C., and Cowgill, R. W. (1971) Biochemistry 10, 4276.
10. Honikel, K. O., and Madsen, N. B. (1972) J. Biol. Chem. 247, 1057.
11. Fischer, E. H., Kent, A. B., Sneider, E. R., and Krebs, E. G. (1958) J. Am. Chem. Soc. 80, 2906.
12. Fischer, E. H., Forrey, A. W., Hedrick, J. L., Hughes,

R. C., Kent, A. B., and Krebs, E. G. (1963) in Chemical and Biological Aspects of Pyridoxal Catalysis (Snell, E. E., Fasella, P. M., Braunstein, A., and Rossi-Fanelli, A., eds.) p. 543, Pergamon Press, Oxford.

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

14. Helmreich, E. (1969) Federation of European Biochemical Societies Symposium 19, 131.

15. Wang, J. H., and Tu, J. I. (1969) Biochemistry 8, 4403.

16. Wang, H. H., and Tu, J. I. (1970) J. Biol. Chem. 245, 176.

17. Fasold, H., Keller, F., and Halbach, M. (1969) Abstract 458 6th Meeting of Fed. of Europ. Biochem. Soc., 7-11th April, Madrid, Spain.

18. Avramovic-Zikic, O., and Madsen, N. B. (1972) J. Biol. Chem. 247, 6999.

19. Avramovic-Zikic, O., and Madsen, N. B. (1972) J. Biol. Chem. 247, 7005.

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

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

21. Madsen, N. B. (1956) J. Biol. Chem. 223, 1067.

22. Madsen, N. B., and Gurd, F. R. N. (1956) J. Biol. Chem. 223, 1075.

23. Battell, M. L., Smillie, L. B., and Madsen, N. B. (1968) Can. J. Biochem. 46, 609.

24. Zarkadas, C. G., Smillie, L. B., and Madsen, N. B. (1968) J. Mol. Biol. 38, 245.

25. Battell, M. L., Zarkadas, C. G., Smillie, L. B. and Madsen, N. B. (1968) J. Biol. Chem. 243,

26. Avramovic-Zikic, O., Smillie, L. B., and Madsen, N. B. (1970) J. Biol. Chem. 245, 1558.

27. Zarkadas, C. G., Smillie, L. B., and Madsen, N. B., (1970) Can. J. Biochem, 48, 763.

28. Gold, A. M. (1968) Biochemistry 7, 2106.

29. Gold, A. M., and Blackman, D. (1970) Biochemistry 9, 4480.

30. Kastenschmidt, L. L., Kastenschmidt, J., and Helmreich, E., (1968) Biochemistry 7, 3590.

31. Webb, J. L. (1966) Enzyme and Metabolic Inhibitors, Vol. 2, p. 655, Academic Press, New York.

32. Webb, J. L. (1966) Enzyme and Metabolic Inhibitors, Vol. 2, p. 642, Academic Press, New York.

33. Oswald, A. A. and Wallace, T. J., (1966) in The Chemistry of Organic Sulfur Compounds, (Kharasch, N. and Meyers, C. Y., eds.) Vol. 2, p. 205, Pergamon Press, New York.

34. Wallace, T. J., Schriesheim, A., and Bartok, W., (1962) J. Org. Chem. 27, 1514.

35. Lane, R. S., and Dekker, E. E. (1972) Biochemistry 11, 3295.

36. Savige, W. E., and Maclaren, J. A. (1966) in The Chemistry of Organic Sulfur Compounds, (Kharasch, N. and Meyers, C. Y., eds.) Vol. 2, p. 367, Pergamon Press, New York.

37. Maclaren, J. A., Savige, W. E., and Sweetman, B. J., (1965) Austral. J. Chem. 18, 1655.

38. Savige, W. E., Eager, J., Maclaren, J. A., and Roxburgh. C. M., (1964) Tetrahedron Lett. 3289.

39. Webb, J. L. (1966) Enzyme and Metabolic Inhibitors, Vol. 2, p. 639, Academic Press, New York.

40. Cecil, R., and McPhee, J. P. (1959) Advan. Protein Chem. 14, 255.

41. Leach, S. J., and Swan, J. M. (1962) Austral. J. Chem. 15, 365.

42. Swan, J. M. (1961) Austral. J. Chem. 14, 69.

43. Chan, W. (1968) Biochemistry 7, 4247.

44. Somogyi, M. (1957) Methods Enzymol. 3, 3.

45. Chinard, F. P., and Hellerman, L. (1954) in Methods of Biochemical Analysis (Glick, D. ed.) Vol. 1, p. 10, Interscience, New York.

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

47. DeLange, R. J., Kemp, R. G., Riley, W. D., Cooper, R. A., and Krebs, E. G. (1968) J. Biol. Chem. 243, 2200.

48. Hedrick, J. L., and Fischer, E. H. (1965) Biochemistry 4, 1337.

49. Friske, C. H., and Subbarow, Y. (1925) J. Biol. Chem. 81, 629.

50. Bray, G. A. (1960) Anal. Biochem. 1, 279.

51. Bush, E. T. (1963) Anal. Chem. 35, 1024.

52. Chervenka, C. H. (1969) A Manual of Methods for the Analytical Ultracentrifuge, p. 27, Spinco Division of Beckman Instruments, Palo Alto.

53. Ellman, G. L. (1959) Arch. Biochem. Biophys. 82, 70.

54. Abel, E. (1951) Monatsh. Chem. 82, 815.

55. Fuller, E. C., and Crist, R. H. (1941) J. Am. Chem. Soc. 63, 1644.

56. Yang, S. F. (1970) Biochemistry 9, 5009.

57. Hasinoff, B. B., Madsen, N. B., and Avramovic-Zikic, O. (1971) Can. J. Biochem. 49, 742.

58. Hill, A. H (1913) Biochem. J. 7, 471.

59. Atkinson, D. E. (1966) Ann. Rev. Biochem. 35, 89.

60. Fridovich, I., and Handler, P. (1960) J. Biol. Chem. 235, 1835.

61. Ray, W. J., Jr., and Koshland, D. E., Jr. (1960) Brook-Haven Symp. Quant. Biol. 13, 135

62. Weil, L., James, S., and Buchert, A. R. (1953) Arch. Biochem. Biophys. 46, 266.

63. Meyer, V., and Wachter, W. (1892) Ber. 25, 2632.

64. Askenasy, P., and Meyer, V. (1893) Ber. 26, 1354.

65. Bell, R., and Morgan, K. J. (1960) J. Chem. Soc. 1209.

66. Hellerman, L., Chinard, F. P., and Ramsdell, P. A. (1941) J. Am. Chem. Soc. 63, 2551.

67. Webb, J. L. (1966) Enzyme and Metabolic Inhibitors, Vol. 2, p. 704, Academic Press, New York.

68. Bailey, K., and Perry, S. V. (1947) Biochim. et. Biophys. Acta 1, 506.

69. Little, C., and O'Brien, P. J. (1967) Arch. Biochem. Biophys. 122, 406.

70. Assaf, S. A., and Yunis, A. A. (1971) Biochem. Biophys. Res. Commun. 42, 865.

71. Eldjarn, L., and Pihl, A. (1957) J. Biol. Chem. 225, 499.

72. Kolthoff, I. M., Stricks, W., and Kapoor, R. C. (1955) J. Am. Chem. Soc. 77, 4733.

73. Swan, J. M. (1957) Nature 180, 643.

74. Krebs, E. G., and Fischer, E. H. (1955) J. Biol. Chem. 216, 113.

75. Helmreich, E. and Cori, C. F. (1965) Advan. Enzyme Regulat. 6, 245.

76. Danforth, W. H., Helmreich, E., and Cori, C. F. (1962) Proc. Nat. Acad. Sci., U. S. A., 48, 1191.

77. Morgan, H. E., and Parmeggiani, A. (1964) J. Biol. Chem. 239, 2435.

78. Morgan, H. E., and Parmeggiani, A. (1964) J. Biol. Chem. 239, 2440.

79. Danforth, W. H., and Lyon, J. B., Jr. (1964) J. Biol. Chem. 239, 4047.

80. Lyon, J. B., Jr. (1970) Biochem. Gen. 4, 169.