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(Signed) C. G. Zarkadas

PERMANENT ADDRESS:
Department of Biochemistry
ALBERT EINSTEIN MEDICAL CENTER
YORK AND TABOR ROADS
PHILADELPHIA, PA. U.S.A.

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STUDIES ON THE THIOL SEQUENCES AND SUBUNIT STRUCTURE OF MUSCLE PHOSPHORYLASE

by

CONSTANTINOS GEORGE ZARKADAS

A THESIS

SUBMITTED TO THE FACULTY OF GRADUATE STUDIES

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FACULTY OF GRADUATE STUDIES

The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies for acceptance, a thesis entitled "Studies on the Thiol Sequences and Subunit Structure of Muscle Phosphorylase" submitted by Constantinos George Zarkadas in partial fulfilment of the requirements for the degree of Doctor of Philosophy.

L. B. Smillie . . .
Supervisor

C. M. Kay . . .

Neil Madsen

G. S. Marks . . . .

. . . .

D R Whitaker
External Examiner

Date July 4, 1969 . . .

ABSTRACT

Skeletal muscle glycogen phosphorylase *a* and *b* have previously been shown to consist of 4 and 2 subunits respectively, each having a molecular weight of 92,500. Evidence that the subunits of phosphorylase may be chemically identical has come from several lines of investigation, including studies on the physical-chemical properties and molecular weight determination of the protein, and from the isolation from phosphorylase of the unique peptides involved in the binding of phosphate, PLP, and AMP. However, the possibility of non-identity of subunits has been suggested by molecular weight determinations of phosphorylase in 7.2 M guanidine hydrochloride and in the presence of 8 M urea and SDS and by electroscopic observations of its rhombic shape.

To shed further light on this question the amino acid sequences of all thiol groups in phosphorylase have been studied in order to establish the total number of unique half-cystine groups. Peptic digests of phosphorylase have been incubated with cystine-3-^{14}C or diethanol disulfide at pH 8.0 and 37°, to promote disulfide interchange. The digests were separated into several fractions on columns of Sephadex G-25. Each fraction was subjected to the diagonal electrophoretic procedure and the cysteic acid peptides were isolated and characterized.

The results indicated that there is a minimum of 8 and a maximum of 9 unique half-cystine sequences in phosphorylase, which provides additional evidence that the subunits are identical or

alternatively are identical subunits composed of non-identical polypeptide chains of molecular weight of less than 92,500. However, there is no reliable evidence for such a possibility.

From the known sequences of the nine sulfhydryl groups it was also possible to isolate and characterize the reactive sulfhydryl peptides in phosphorylase. Careful column chromatographic purifications of alkylated peptides showed that there are two cysteine residues per monomer of phosphorylase *b* whose rate of reaction with iodoacetamide approaches that of model compounds and whose alkylation does not affect significantly the enzymic properties of the protein. These two cysteines have been identified in sequences corresponding to numbers 2 and 5 previously elucidated. These sequences have now been extended in the present work and may be written as:

No. 2 Asn-Gln-Lys-Ile-CMC-Gly-Gly-Try-Gln-Ser

No. 5 Gly-CMC-Arg-Asp-(Val,Pro)-Arg-Thr-(Asn,Phe).

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LIST OF ABBREVIATIONS

ADP	Adenosine 5'-diphosphate
AMP	Adenosine 5'-monophosphate
Asn	Asparagine
ATP	Adenosine 5'-triphosphate
CMC	S-carboxymethylcysteine
Cya	Cysteic acid
$D^{\circ}_{20,w}$	Intrinsic diffusion coefficient in water at 20° (Fick, $cm^2 sec^{-1}$)
DEAE-cellulose	Diethylaminoethyl cellulose
DNFB	2,4-Dinitrofluorobenzene
DNP-AA	2,4-Dinitrophenyl-amino acid
DNS-AA	1-dimethylaminonaphthalene-5-sulphonyl amino acids
DNS-Cl	1-dimethylaminonaphthalene-5-sulphonyl chloride
DTNB	5,5'-dithiobis (2-nitrobenzoic acid)
$E^{1\%}_{1cm.}$	Extinction coefficient of protein
EDTA	Ethylene diaminetetra-acetate
Et-S-S-Et	Diethanol disulfide
f/fo	frictional ratio
Gln	Glutamine
glucose-1-P	α-D-glucose-1-phosphate
K_m	Michaelis constant
L	"allosteric constant"
M_n	The number-average molecular weight (g./mole)
M_w	The weight-average molecular weight (g./mole)
M_z	The z-average molecular weight (g./mole)
NEM	*N*-ethylmaleimide

LIST OF ABBREVIATIONS (continued)

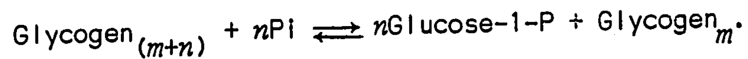
Pi	Orthophosphate
PCMB	*p*-chloromercuribenzoate
$S^{\circ}_{20,w}$	Intrinsic sedimentation coefficient in water at 20° (in Svedberg units, S)
SDS	Sodium dodecyl sulphate
SerP	Serine phosphate
TPCK	1-tosylamido-2-phenylethyl chloromethyl ketone
Try or Trp	Tryptophan
$\bar{v}_{20}$	partial specific volume of anhydrous particle ($ml.g^{-1}$.)
Vmax	Maximum velocity when the enzyme is saturated with substrate

All temperatures are given in Centigrade degrees.

CHAPTER I

INTRODUCTION

Phosphorylase (α-1, 4-glucan:orthophosphate glycosyltransferase, EC.2.4.1.1) is an allosteric enzyme system in animal cells, which catalyzes the first step in the reversible reaction (Cori *et al.*, 1939) for the synthesis and degradation of glycogen:



$$\text{Glycogen}_{(m+n)} + n\text{Pi} \rightleftharpoons n\text{Glucose-1-P} + \text{Glycogen}_m.$$

This reaction, initially reported by Parnas and Baranowski (1935), was mainly elucidated by Cori and Cori (1936; 1937). The enzyme was so named because glucose-1-phosphate was found (Cori and Cori, 1937) to be the primary end product of the phosphorolysis reaction (Parnas, 1937) of glycogen, and required adenylic acid (AMP) for activity (Cori *et al.*, 1938). In most of the early studies dialyzed rabbit extracts from skeletal muscle were used.

Although the equilibrium of this reaction favors glycogen synthesis, phosphorylase operates *in vivo* towards the degradation of glycogen because of the high intracellular inorganic phosphate in the cell relative to the low concentration of glucose-1-phosphate (Larner *et al.*, 1960). Morgan and Parmeggiani (1964) indicated that the intracellular concentration of inorganic phosphate imposes a significant restraint on the rate of phosphorolysis of glycogen. The Pi concentration was estimated to be about 3mM, but studies of the binding of inorganic phosphate to muscle protein would suggest

that the level of free Pi in the cell may be much lower (Hasselbach, 1957, Gergely and Maruyama, 1960).

In addition, the regulation of glycogen breakdown in muscle, as well as in other tissues, involves several other factors, including (a) levels of substrates, allosteric effectors and inhibitors of phosphorylase; (b) the concentration of the product of the reaction, hexose monophosphate; and (c) enzymes, ultimately leading to enzymatically active or inactive phosphorylase molecules. Phosphorylase is now known to be regulated hormonally as well as non-hormonally (Krebs and Fischer, 1956; Sutherland and Rall, 1960; Krebs and Fischer, 1962; Danforth, 1965; and Harwood and Drummond, 1969).

The principal subject of this thesis is the subunit structure of muscle phosphorylase. The most pertinent evidence related to the chemical structure and function of this native protein and of the component subunits will be discussed in this and later chapters where they will be integrated with the present work. Studies of purely historical significance will not be listed or described.

Our present concepts of the enzymatic forms of muscle phosphorylase and of the component subunits started about thirty years ago, and were developed by the pioneering studies of Green, Sutherland, Fischer, Keller, Krebs, Madsen, and most prominently the Cori's. The Cori's were convinced that the activation and deactivation of phosphorylase was a reversible associating process, and they set about to develop methods for the isolation of these enzymatic forms that are involved in this reaction. Since then the

molecular weights of the proteins have been redetermined and the methodology of handling the protein has been enormously enriched. However, the Cori's fundamental ideas and experiments on the molecular forms of phosphorylase are still valid.

The intensive investigation of the protein chemistry of phosphorylase began with Green *et al.* (1942) and Green and Cori (1943). These studies led to the purification and crystallization of a new form of the enzyme, phosphorylase *a*. Oncley (1943) assigned a molecular weight of 340,000 to 400,000 to phosphorylase *a*. This molecular form of the enzyme possessed considerable activity in the absence of adenosine-5′-phosphate (AMP). It was thus shown that skeletal rabbit muscle enzyme exists in two molecular forms: phosphorylase *a*, which exhibits 60 to 70 per cent of maximal activity without the addition of AMP (Green and Cori, 1943); and phosphorylase *b*, which has an absolute requirement for the nucleotide (Cori and Green, 1943). This was probably the first allosteric enzymic mechanism to have been discovered.

In subsequent experiments phosphorylase *b* was crystallized (Cori and Cori, 1943), and Green (1945) reported that phosphorylase *a* and *b* had different electrophoretic migration velocities in potassium phosphate buffer.

The discovery by the Cori's and their associates of methods for the isolation and crystallization of the two forms of the enzyme yielded many significant results, and opened a new era in the study of structure-function relationships of this allosteric protein.

TABLE I

COMPARISON OF PHYSICOCHEMICAL PROPERTIES OF PHOSPHORYLASES

(Keller, 1953)

Constant	Protein			
	Phosphorylase *a*	Phosphorylase *b*	Phosphorylase *b'*	Subunits
$S^{\circ}_{20,w}$ ($\times 10^{13}$ Svedberg units)	13.2	8.2	8.2	5.6[a]
$D^{\circ}_{20,w}$ ($\times 10^{7}$ cm.^{2}/sec.$^{-1}$)	2.6[b]	3.3[b]	3.1	4.0
$\bar{v}_{20}$ (ml.g.$^{-1}$)	0.751	0.751		0.751
f/fo	1.55	1.55	1.65	
Mol. Wt. (10^{3})	495	242	257	135[c]

[a]Madsen and Cori (1956)

[b]Determined by Green (1944)

[c]Madsen and Gurd (1956)

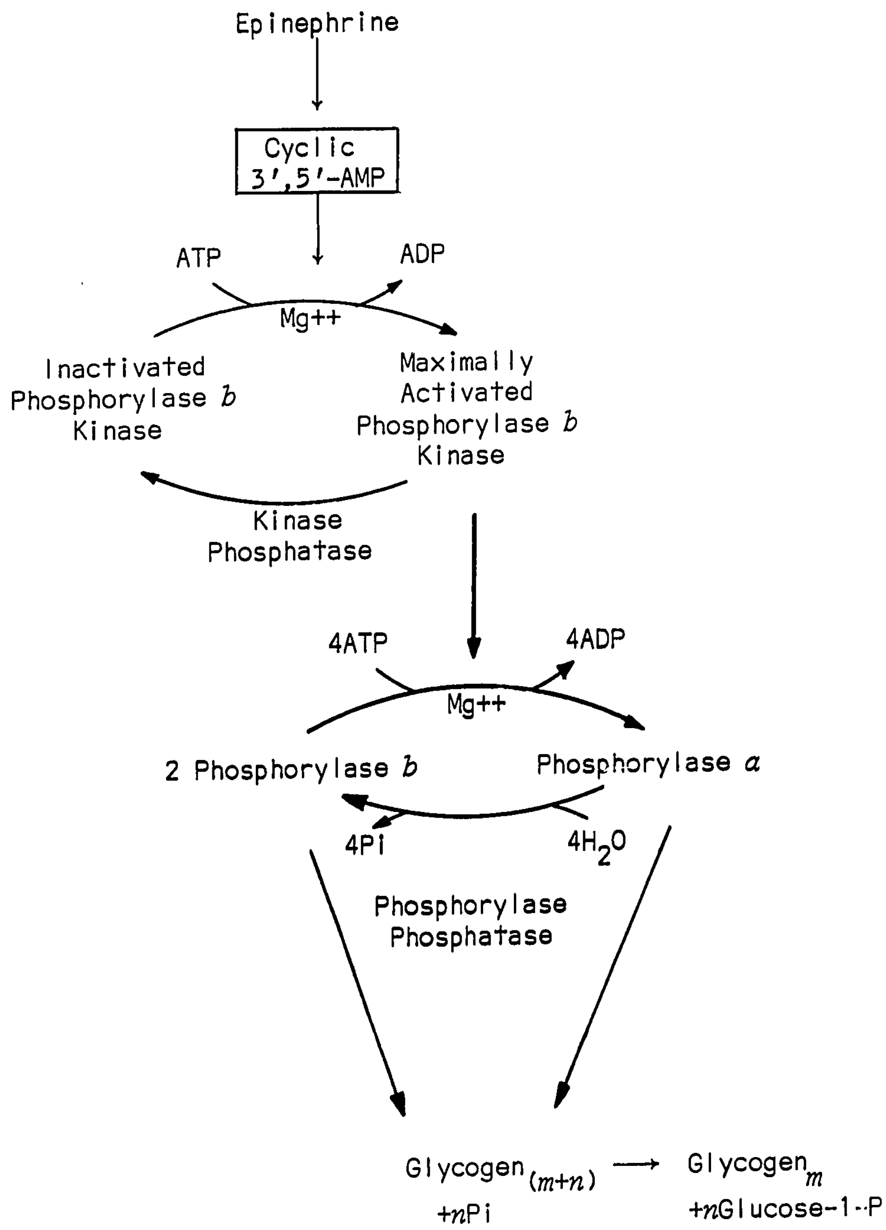


Figure 1. The Mechanism of Hormonal Action on Glycogenolysis in Rabbit Muscle Accordking to Krebs *et al.* (1966).

First, came the outstanding contribution of Keller and Cori (1953) who, using purified PR (phosphorylase rupturing) enzyme preparations, investigated the time course of the conversion of phosphorylase *a* to *b* in the ultracentrifuge. They observed the occurrence of concomitant changes in the molecular properties and in the enzymic behaviour, and demonstrated that the two molecular forms are readily distinguishable in the ultracentrifuge. Their sedimentation velocities and diffusion data, summarized in Table I, showed that phosphorylase *a* has twice the molecular weight of phosphorylase *b*. A third molecular form, referred to as phosphorylase *b'*, was observed by Keller (1953; 1955), when trypsin reacts with phosphorylase *a*, which is ultracentrifugally indistinguishable from phosphorylase *b* and requires AMP for activity.

The two important reactions concerning the mechanism of the interconversion reaction of the two forms of skeletal muscle phosphorylase deserve mention and these relationships are shown in Figure 1. The enzyme catalyzing the phosphorylase *a* to *b* reaction, which had originally been designated as PR enzyme (Cori and Cori, 1945), was called phosphorylase phosphatase (EC.3.1.3.17) (Graves *et al.*, 1960) and the enzyme catalyzing the *b* to *a* reaction was referred to as phosphorylase *b* kinase (EC.2.7.1.38) (Krebs *et al.*, 1958). It is seen (Figure 1) that the chemical nature of the interconversion reactions of phosphorylase involves phosphorylation and dephosphorylation of the enzyme (Krebs and Fischer, 1956), and the relative rates of these reactions (Figure 1) determine which form of phosphorylase predominates in tissue.

Thus, as dephosphorylation proceeds during the conversion of phosphorylase *a* to *b* by phosphorylase phosphatase, dissociation of the tetrameric *a* species is favoured to the extent that only phosphosylase *b* dimers are found. Phosphorylase *b* dimer is converted to phosphorylase *a* through phosphorylation of a particular serine of the protein in the presence of magnesium (Mg^{++}), ATP, and phosphorylase *b* kinase (Krebs *et al.*, 1958) and requires adenylic acid. The work of Krebs *et al.* (1959, 1964), Delange *et al.* (1968), and Riley *et al.* (1968) indicated that the phosphorylase *b* kinase is probably the more dynamic of the converting enzymes and that the principal site of action of epinephrine in its glycolytic role and of cyclic 3′,5′-AMP appeared not to be on phosphorylase itself but rather on phosphorylase *b* kinase, which activates phosphorylase.

Trypsin, which mimics the action of phosphorylase phosphatase during the phosphorylase *a* to *b* conversion, liberates a phosphoserine hexapeptide and in some way "freezes" the molecule in the active conformation (Fischer *et al.*, 1959; Nolan *et al.*, 1964). This form of the enzyme is referred to as phosphorylase *b′* dimer, requires 5′-AMP for activity, and can no longer be reconverted to phosphorylase *a* by phosphorylase *b* kinase.

The work of Madsen and Cori (1956) and of Madsen and Gurd (1956) demonstrated that phosphorylase *a* dissociates into four subunits and phosphorylase *b* into two subunits of a molecular weight of approximately 125,000, when treated with *p*-chloromercuribenzoate (PCMB), and their results are summarized in Table I. That phosphorylase *a* must be regarded as a tetramer and *b* as a dimer is

further indicated by the work of Krebs *et al.* (1958) who showed that in the conversion of two moles of phosphorylase *b* to one mole of phosphorylase *a* not two but four phosphates were introduced into the protein during the kinase reaction. These investigators also found that phosphorylation of the protein by ATP produced four moles of ADP which enabled them to write stoichiometric equations for the interconversion reactions. Furthermore, phosphorylase *a* and phosphorylase *b* bind four and two moles of AMP respectively (Madsen and Cori, 1957), and the number of moles of the prosthetic group, pyridoxal-5′-phosphate (PLP), bound to the enzyme is shown by Baranowski *et al.*(1957) to be two for the *b* and four for the *a* form of the enzyme.

All of the above studies clearly show that the activities of either phosphorylase *a* or *b* are profoundly dependent upon the binding of small molecules at specific sites which are distinct from the substrate catalytic binding sites. For example, the work of Parmeggiani and Morgan (1962), Madsen (1964), and Helmreich and Cori (1964a) has shown that muscle phosphorylase *b* depends upon AMP for activity, and that phosphorylase *a* displays an augmentation of activity in the presence of AMP. On the other hand, the earlier studies of Cori and Illingworth (1957) and Baranowski *et al.* (1957) and more recently Shaltiel *et al.* (1966) and Hedrick *et al.* (1966) have shown that pyridoxal 5′-phosphate (PLP) is indispensable for enzymatic activity although it is not known whether it is involved either in direct catalysis or control. This remains to be elucidated.

Direct experimental evidence, in support of the concept that phosphorylase *a* and *b* consist of four and two subunits, respectively, came from sequence investigations concerning the sites of peptide chains that are involved in some way in determining or controlling enzymic activity.

<u>The phosphoserine site</u>. Fischer *et al.* (1959) found that tryptic attack on ^{32}P-labelled phosphorylase *a* released a ^{32}P-labelled phosphohexapeptide fragment, which upon purification on Dowex 50 columns followed by paper chromatography and high voltage electrophoresis have been shown to have the following sequence: Lys-Gln-Ile-Ser(P)-Val-Arg. Although this phosphohexapeptide was shown to be dephosphorylated, though very slowly, the dephosphopeptide could not be rephosphorylated by phosphorylase *b* kinase and ATP (Graves *et al.*, 1960). From this evidence it was concluded that a unique seryl residue, presumably present in each of four very similar if not identical subunits, was phosphorylated in the conversion reaction (Fischer *et al.*, 1959).

In an attempt to extend the sequence of the site phosphorylated by the conversion of phosphorylase *b* to *a*, and in order to establish whether the longer dephosphorylated fragments could be rephosphorylated by phosphorylase kinase, Nolan *et al.* (1964) isolated larger phosphoserine-containing peptide fragments from chymotryptic digests of ^{32}P-labelled phosphorylase *a*. From the closely related phosphopeptides, isolated by column chromatography and purified by paper electrophoresis, they extended the amino acid

sequence to fourteen residues. These results suggest strongly that this unique phosphorylated site is in a particularly exposed position or less organized region of the subunit surface, and that the primary sequence of this site, shown below, was required for recognition, as mentioned before, by phosphorylase *b* kinase:

```
                        T   T   T         O-P       T
                        ↓   ↓   ↓          |        ↓
Ser-Asp-Gln-Glu-Lys-Arg⋮Lys-Gln-Ile-Ser-Val-Arg⋮Gly-Leu
                         ⋮                       ⋮
```

The unique nature of the phosphoserine hexapeptide is also suggested by kinetic experiments. Partial tryptic digestion of phosphorylase *a*, indicated by the letter T over an arrow, cleaves several of the basic amino acid residues surrounding the phosphoserine site. The desensitized form of the enzyme, phosphorylase *b'*, is missing the region of the primary structure containing the seryl residue, as indicated by the dotted lines, and does not possess either the kinetic or self-association properties of phosphorylase *b* (Graves *et al.*, 1968).

<u>Pyridoxal 5'-phosphate site</u>. The most notable characteristic of phosphorylase is that it contains 1 mole of pyridoxal 5'-phosphate (PLP) per mole of enzyme monomer (Baranowski *et al.*, 1957; Cori and Illingworth, 1957; Kent, 1959). PLP is attached to the ε-amino group of a lysyl residue adjacent to a phenylalanyl residue as a disubstituted aldamine derivative (Fischer *et al.*, 1958; Fischer *et al.*, 1963; Nolan *et al*, 1964) and is indispensable to the activity of the enzyme.

The isolation of the pyridoxal-phosphate peptide from a chymotryptic digest of "reduced" phosphorylase *b* was achieved by ion exchange chromatography on Dowex 50-X2. The single large peak was observed in the 330 mμ profile, and was then purified by high voltage electrophoresis at pH 6.5, followed by chromatography in the butanol-acetic acid-water (4:1:5) system (Kent, 1959). Fischer and Krebs (1959) reported the sequence of a unique substituted dipeptide ε-N-pyridoxylphosphatelysylphenylalanine, and Nolan *et al.* (1964) have confirmed the structure of the dipeptide as originally reported by Fischer and Krebs (1959). Their results showed only one such unique dipeptide, which might suggest that there is only one site for PLP binding per monomer of phosphorylase.

Addition of acid, base, or deforming agent causes conformational changes of the protein, the formation of the C=N azomethine bond and the production of the Schiff's base form. The Schiff's base is highly polarized and tends to hydrolyze rapidly, giving a mixture of apoenzyme and free PLP. On the other hand, reduction of the protein (Fischer *et al.*, 1958) *in situ* with sodium borohydride, fixes the cofactor to the protein, irreversibly modifies the aldehyde group of PLP, and leads to a molecule with reduced activity (60 per cent active) and allosteric responses.

The removal of PLP required two successive operations (Hedrick *et al.*, 1966; Shaltiel *et al.*, 1966). In the presence of 0.4 M imidazolium citrate and L-cysteine at pH 6.0, phosphorylase *b* was converted to its apoenzyme, known as apophosphorylase *b*, and dissociated freely into subunits. The energy of activation is 11 kcal.,

and there was no evidence of the formation of a Schiff's base during the conversion. The apoenzyme was crystallized in the presence of AMP and magnesium.

Apophosphorylase *b* behaves as a typical associating-dissociating system. This suggests that the protein retained all the structural properties of the parent holoenzyme, except catalytic activity. The properties of the PLP site have been summarized elsewhere (Shaltiel *et al.*, 1966; Hedrick *et al*, 1966; Fischer and Krebs, 1966).

The AMP site. The work of Madsen and Cori (1957), Parmeggiani and Morgan (1962), Madsen (1964), and Helmreich and Cori (1964a), indicated that the binding of AMP to the enzyme involves a specific site in each polypeptide chain, near the active site. This site also binds ATP. Okazaki *et al.* (1968) in an attempt to elucidate the chemical nature of the interaction between the functional atoms of AMP involved in the binding to protein, concluded that the amino group at position 6 of AMP and the nitrogen atom at position 1 were effective in the binding. The hydroxyl group at position 5 of the ribose moiety contributed to the binding, while the phosphate was absolutely required for activation. It seems that there are numerous points of close contact, involving both polar and nonpolar interactions.

Recently, Fasold *et al.* (1969) have shown that one mole of DNFB reacted with a specific site in the polypeptide chain per mole of monomer of phosphorylase *b*, presumably the AMP binding site, and caused complete inactivation of the enzyme. These authors have

succeeded in isolating a unique peptic ^{14}C-DNFB peptide from phosphorylase *b* and its sequence is being determined.

Allosteric properties. The kinetic properties of phosphorylase have been a subject of intensive study for several years. Much of the rapid progress on the relationships between structure and function of phosphorylase has been due to investigations of the binding of ligands to the enzyme, and the determinations of the equilibrium of these reactions. From the data obtained, it can be concluded that phosphorylase has many of the fundamental characteristics predicted for allosteric proteins (Monod *et al.*, 1963; 1965). In addition to the subunit structure, the activities of the two forms of the enzyme are profoundly altered by small molecules (effectors), which are totally unrelated structurally either to substrates or products, and are bound at sites that are distinct from the catalytically active sites (*supra*, p. 10).

The work of Parmeggiani and Morgan (1962), Madsen (1964), and Helmreich and Cori (1964a) have shown that the dependence of muscle phosphorylase on AMP for activity, absolute in the case of phosphorylase *b* and relative in the case of phosphorylase *a*, is explained in either case by an increased affinity of the enzyme for the substrates and *vice versa*. These effects are observed kinetically in large changes in K_m under conditions where there is little change in V_{max} (Helmreich and Cori, 1964a; Madsen, 1964; Lowry *et al.*, 1964), or by an increased affinity for bromthymol blue in the presence of AMP (Ullmann *et al.*, 1964) and by an alteration of the ultraviolet spectrum of its bound pyridoxal-5-P (Bresler *et al.*, 1966). The activation of

phosphorylase *b* by AMP is partly attributable to a decrease in the apparent K_m of the enzyme for its substrates; i.e., inorganic phosphate and glycogen (Helmreich and Cori, 1946b) and conversely, the binding of either substrate to the enzyme decreases the apparent K_m for AMP.

Homotropic interactions of this type and between the binding sites of substrates and effectors or inhibitors were first observed for phosphorylase *b* by Madsen (1964). This investigator showed that at fixed ATP concentrations (9.0×10^{-3}M), a competitive inhibitor of AMP, the saturation curve of phosphorylase *b* for glucose-1-P had a sigmoidal shape. In the absence of the inhibitor the usual hyperbolic Michaelis-Menten formulation was described. Possibly related to these observations is the kinetics described by Cori *et al.* (1943) for the inhibition of phosphorylase *a* by glucose and the AMP counteraction of this inhibition. Helmreich *et al.* (1967) presented evidence in which the negative effector, glucose, caused homotropic cooperativities in phosphorylase *a*.

The strength of the cooperative interaction was estimated thermodynamically. Madsen and Cori (1957) and Helmreich and Cori (1964b) indicated that AMP lowered both the energy of activation from 25.0 to 20.0 *K*cal., and the positive entropy of activation from 37 to 6 e.u. However, the binding sites of glucose-6-P on phosphorylase *a*, and of ATP on phosphorylase *b* have not yet been revealed. From Parmeggiani and Morgan (1962), Helmreich *et al.* (1967), Madsen (1964), and Madsen and Shechosky (1967) it could be assumed that the allosteric inhibitors bind at the activator binding site. This

remains to be elucidated (Fasold *et al.*, 1969). Hedrick (1966) and Johnson and Graves (1966), on the basis of optical rotatory dispersion studies and circular dichroism measurements, concluded that the binding of substrates, allosteric effectors, and inhibitors caused no significant changes in the secondary structure of the protein.

From limited digestion of phosphorylase *a* with trypsin, which cleaves the phosphoserine hexapeptide and possibly certain of the basic amino acids from the clusters adjoining the serine residue, Graves *et al.* (1968) showed that the desensitized active phosphorylase *b'* (Figure 2, squares) does not possess the homotropic properties of phosphorylase *b*. These authors suggested that the seryl-phosphate site is implicated with the strong homotropic interactions observed in phosphorylase *b*. In support of this view, Graves *et al.* (1967) showed that the sigmoidal saturation curve previously described for phosphorylase *b* (Madsen, 1964; Sealock and Graves, 1967) could be converted to a hyperbolic form in the presence of NaF. This evidence suggests that the surrounding basic residues of the seryl site mediated the allosteric properties in phosphorylase *b* dimers, presumably as a result of electrostatic interactions involving these basic amino acid residues of one monomer to complimentary negatively charged residues of an adjacent monomer. If, on the other hand, these positive charges are neutralized to some extent by the presence of the phosphate, phosphorylase *a* can exhibit heterotropic cooperativities only, or the weak homotropic interactions described by Helmreich *et al.* (1967).

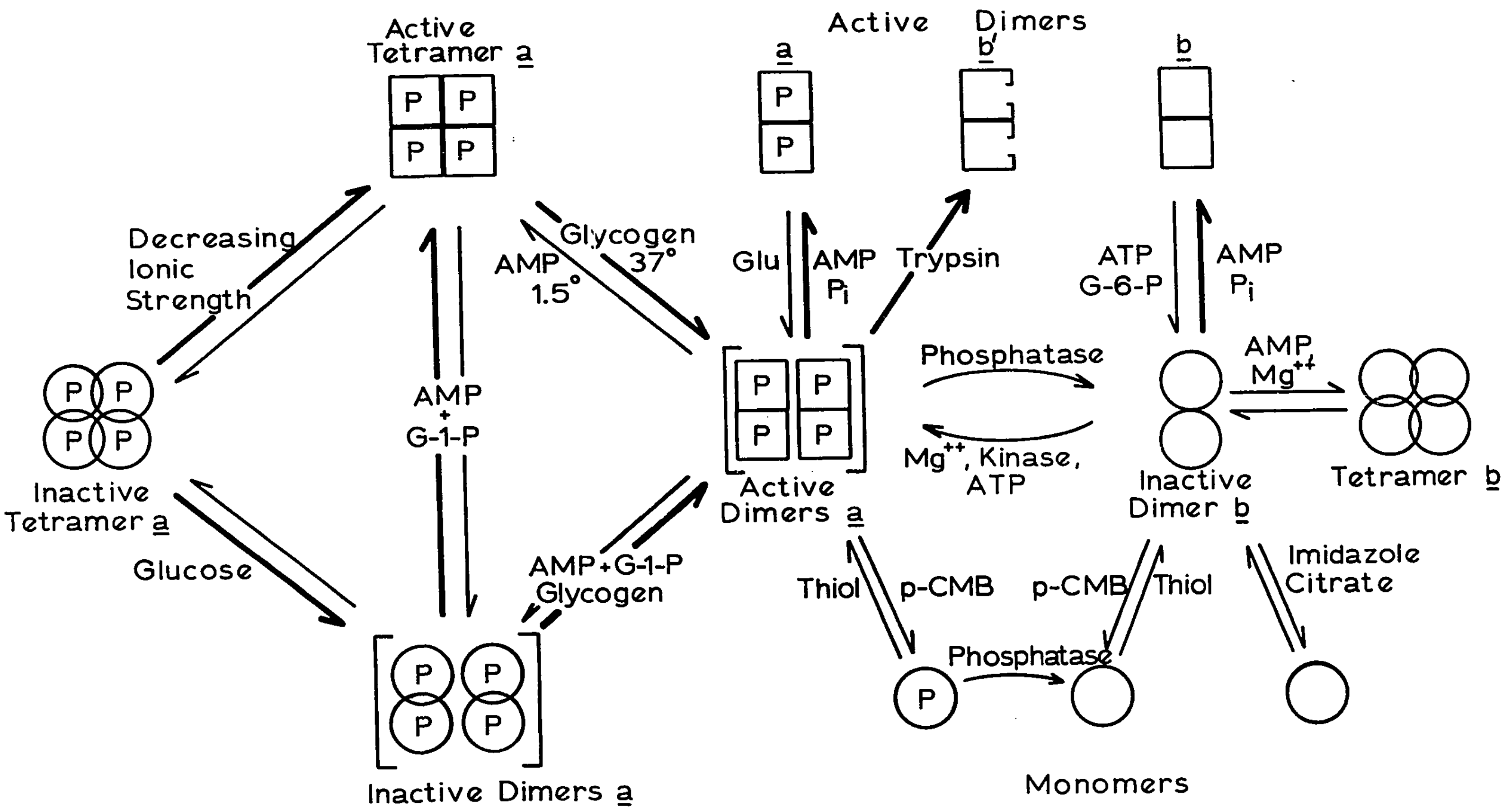


Figure 2. Molecular Forms and Interconversions of Phosphorylase *a* and *b*.

Tertiary and quaternary structure. It may be noticed from the above discussion that a wide variety of treatments have been observed to produce similar changes in the allosteric and catalytic properties of phosphorylase. In addition, the state of aggregation of phosphorylase may be influenced by protein concentration, ionic strength, pH, temperature, and the presence or absence of specific ligands. Wang and Graves (1963) and Helmreich *et al.* (1967) characterized the profound dependence of the state of aggregation upon the ionic composition of the medium, and concluded that the observed effects may be a result of a distortion of the enzyme's quaternary and possibly tertiary structure in response to the ionic strength.

According to the scheme presented in Figure 2, high salt concentrations (Wang and Graves, 1963), low protein concentration at 30° (Wang and Graves, 1964), and the presence of glycogen as substrate (Metzger *et al.*, 1967) causes the tetrameric active (squares) form of phosphorylase *a* to dissociate into active (squares) dimeric forms of phosphorylase *a*. Conversely, low temperature and low ionic strength (Helmreich *et al.*, 1967) favour the association of phosphorylase *a* into active tetrameric forms (squares). Wang *et al.* (1965a; 1965b) reported that under certain conditions, 0.05 M glucose promotes the dissociation of phosphorylase *a* tetramers (circles) into inactive dimeric forms of phosphorylase *a* (circles). However, Helmreich *et al.* (1967) have shown that phosphorylase *a*, in the absence of reactive ligands, exists to a considerable extent in the active form (squares) (L varied from 3 to 13) which explains why phosphorylase *a* can be catalytically more active. These authors and

the studies of Metzger *et al.* (1967) suggested that the phenomenon might be related to a mechanism of *in vivo* regulation of phosphorylase activity; whether or not this apparent physical dissociation-association and sigmoidal relationship is a function of the quaternary structure of the protein remains to be elucidated.

In contrast, the non-phosphorylated form of the enzyme, phosphorylase *b* dimers in the absence of specific ligands, i.e. AMP, exists mainly in the inactive form (circles). Madsen and Shechosky (1967); Helmreich *et al.* (1967), and Kastenschmidt *et al.* (1968) have shown that for phosphorylase *b* the allosteric constant is high ($L > 500$). Thus, the dependence of catalytic activity of phosphorylase *b* upon homotropic cooperativity of the substrate, the activator, and inhibitor may be a function of the quaternary or tertiary structure of the protein.

Similar, although more specific, physical dissociation-association relationships exist in the non-phosphorylated enzyme. Phosphorylase *b*, in the presence of 0.01 M solutions of magnesium acetate, can be induced by their allosteric effector, i.e. AMP, Mg^{++} to associate into tetrameric forms of phosphorylase *b* (circles) (Kent *et al.*, 1958; Appleman, 1962). Both of these forms have been recently crystallized and will be discussed in a later section (Matthews, 1967; Valentine and Chignell, 1968).

As seen in Figure 2 both phosphorylase *a* and *b* dissociate into monomer units (Madsen and Cori, 1956) and this dissociation can be reversed by the addition of excess cysteine which restores enzymic activity (Figure 2). Similar effects were described by Baranowski

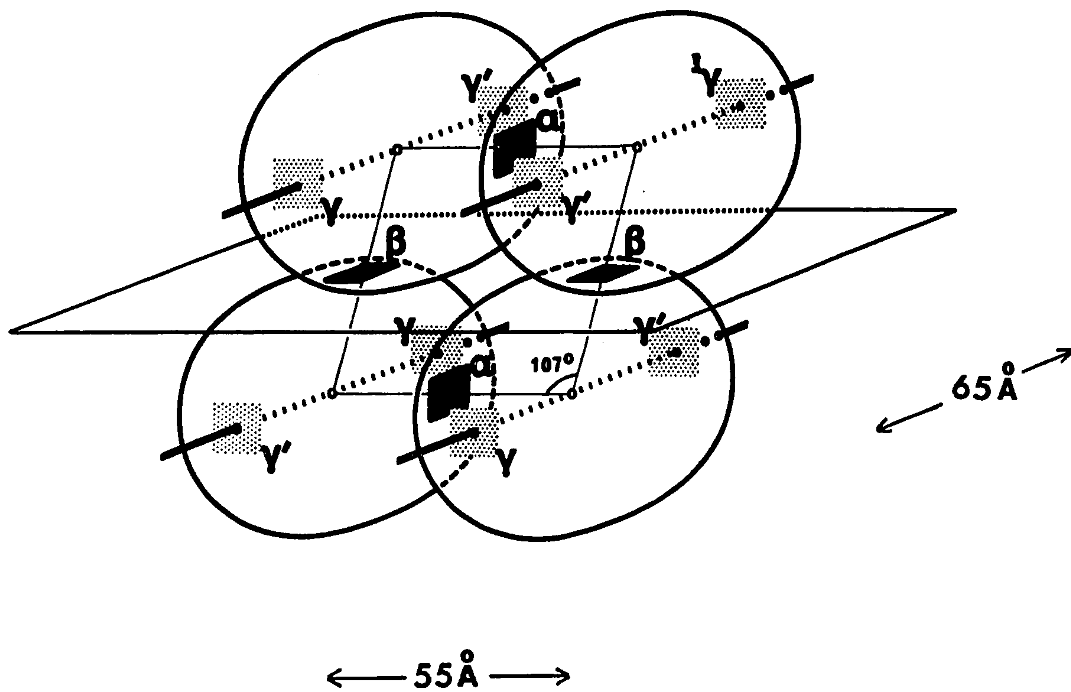


Figure 3. Schematic Representation of Subunits and Binding Sites of Muscle Phosphorylases According to Chignell *et al.* (1968).

et al. (1957) and Cori and Illingworth (1957), who found imidozole-citrate causes monomerization of phosphorylase *b*, and as was shown by Shaltiel *et al.* (1966) in the presence of L-cysteine (pH 6.0), phosphorylase *b* was converted into apophosphorylase *b* that dissociated into subunits (Figure 2).

Thus, as shown in Figure 3, the modes of isologous association of the protomer and the domain of bonding is defined by Chignell *et al.* (1968) as the $\alpha\alpha'$ primary binding sites in a dimer, the $\beta\beta'$ binding sites between the dimers in a tetramer, and a third set of $\gamma\gamma'$ binding sites which are probably involved in the polymerization of phosphorylase during the formation of higher aggregates.

Recent molecular weight determinations of enzyme and subunits. The discovery by Madsen and Cori (1956) that phosphorylase *a* and *b* dissociate into subunits which exhibit identical behaviour and molecular weight both by ultracentrifugation and light scattering techniques (Madsen, 1956) suggests that this may represent the protomeric form of the enzyme, and that the actual structural changes in the subunits may not be extensive. This was the first substantial evidence to show that the sulfhydryl groups are involved in maintaining the quaternary structure of the protein, and that blocking of these thiol groups produced an unstable molecule leading to dissociation into protomers.

For the purposes of this thesis, the term "subunits of phosphorylase" is considered to be the physically or chemically identifiable intact sub-molecular polypeptide chains within the protein

as was first proposed in Monod's nomenclature (Monod *et al.*, 1965). In their proposed model, identical subunits are designated as protomers, and the term "monomer" is used to describe the fully dissociated protomer, or the non-identical subunits.

The findings mentioned above stimulated extensive investigations in the quaternary structure of the molecule, and the molecular weight of rabbit muscle phosphorylase *a* and *b*, long accepted to be 495,000 and 247,000 respectively (Table I, p. 4), has now been reassessed by a number of modern physicochemical methods.

The dimer, as it is envisioned by electronmicroscopy (Chignell *et al.*, 1968) is a rod-like globular protein with indications of a cleft across the middle, suggesting contacts between the two appreciably compact ellipsoidal subunits. The rod-shaped dimers are 110 Å x 55 Å to 65 Å depending on their orientation, whereas phosphorylase *b* tetramers consist of two dimers side by side and they are about 55 Å thick, and measured 110 Å on each side. Valentine and Chignell (1968) suggested that the center of each of the subunits lies at the corners of a rhombus inclined with a 107° angle. Chignell *et al.* (1968) found no evidence of any further subunit structure within each monomer in the electron microscope.

Preliminary X-ray crystallographic reports (Matthews, 1967) indicated that phosphorylase *b* dimer crystals, formed in strong salt solutions, gave space group symmetry $P2_1$ with cell dimensions a = 120 Å, b = 190 Å, c = 89 Å, and β = 107°. In the electron micrographs, the arrangement of phosphorylase *b* tetrameric needle-shaped crystals, formed in the presence of AMP at 4° in 1×10^{-2} M

solution of magnesium acetate (Kent *et al.*, 1958) is such as to give space group symmetry $P2_1$ and the following periodicities were observed: a = c = 120 Å, b = 95 Å, and β = 107°, and in a perpendicular plane, a = 120 Å, b = 95 Å, and γ = 90° (Valentine and Chignell, 1968).

The problem of developing a consistent picture of the architecture and function of the protein is essentially dependent upon accurate determinations of the molecular weights of the enzyme and its constituent subunits. This has been difficult for a number of reasons, including the sensitivity of the thiol groups to oxidation, the reversible association-dissociation characteristics of the system, and the high molecular weights.

However, from the work of Seery *et al.* (1967), De Vincenzi and Hedrick (1967), Buc and Buc (1967), Ullmann *et al.* (1968), Metzger *et al.* (1968), and Chignell *et al.* (1968), a consistent picture of the molecular weights of the several forms of phosphorylase has emerged. In Fischer's laboratory (Seery *et al.*, 1967) the molecular weight of phosphorylase was determined by sedimentation equilibrium and sedimentation diffusion under a number of conditions in which temperature, pH, buffer, substrate, and effector were varied. The resulting data indicated that the molecular weight of the tetrameric molecule of phosphorylase *a* is 370,000 and the dimeric phosphorylase *b* in the order of 185,000. From measurements in 3.6 and 5.4 M guanidine hydrochloride, it was concluded that each of the subunits has a molecular weight of approximately 92,500.

De Vincenzi and Hedrick (1967) using gel-filtration techniques, reported a molecular weight for phosphorylase *a* and *b* in the order of

TABLE II

MOLECULAR WEIGHTS OF PHOSPHORYLASES DETERMINED BY CENTRIFUGATION EQUILIBRIUM AND BY SEDIMENTATION-DIFFUSION

(Seery *et al.*, 1967)

Protein	$S^{\circ}_{20,w}$ ($\times 10^{13}$ Svedberg Units)	$D^{\circ}_{20,w}$ ($\times 10^{7}$ cm.^{2}/sec^{-1})	$\bar{v}_{20}$ (ml.g.$^{-1}$)	*f/fo*	Molecular Weight (10^3 g. mole^{-1})		
					M_n	M_w	M_z
Phosphorylase *a*	13.1 S	3.3	0.737[a]	1.35	354	365	373
Phosphorylase *b*	8.4 S	4.2	0.737[a]	1.35	187	188	196
Phosphorylase *b* monomer							
3.6 M G-HCl					96.6	92	84
5.4 M G-HCl					90	83	94
7.2 M G-HCl					78.5	73	64.9

[a]Determined from amino acid analysis (Table III, page 21) **Appleman** *et al.* **(1963) and Metzger** *et al.* (1968).

($\bar{v}_{20}$ = 0.739 ± 0.004 measured by Cahn electrobalance)

367,000 and 177,000, respectively. These results are almost identical with the values obtained by Seery *et al.* (1967). Buc and Buc (1967) assigned to phosphorylase *b* dimer a weight average molecular weight ($\overline{M}_w$) of 188,000 by the Archibald approach to equilibrium, by sedimentation diffusion, and by the light scattering methods. Ullmann *et al.* (1968) believed that rabbit skeletal phosphorylase *b* dissociates in 6.0 M guanidine hydrochloride to yield subunits with a weight average molecular weight ($\overline{M}_w$) of 94,000 with no detectable trace of heterogeneity. The molecular weight of skeletal muscle phosphorylase *b* from frog, *Rana pipiens*, a poikilothermic animal, was measured by sedimentation equilibrium. Metzger *et al.* (1968) reported a molecular weight of 188,000, a value in close agreement with the mammalian enzyme. From crystal density determinations and a partial specific volume ($\bar{v}_{20}$) of 0.74, Chignell *et al.* (1968) calculated a molecular weight of 365,000 for the tetrameric form, which agrees closely with the hydrodynamic values obtained by Seery *et al.* (1967).

Thus, in the opinion of most workers, molecular weight estimates of 370,000 and 185,000 for phosphorylase *a* and *b*, respectively, are more realistic values, and each of the subunits, shown to be hydrodynamically identical, has a molecular weight of 92,500. Therefore, the first diffusion measurements (Table I, page 4) on phosphorylase *a* and *b* made in the absence of thiol compounds by Green (1944) differed significantly from the diffusion coefficients determined by Oncley (1943) and Seery *et al.* (1967)(Table II). These

differences were attributed to aggregation phenomena encountered during the determinations in the Tiselius apparatus.

Recent evidence for non-identical subunits. Somewhat at variance with these findings, Seery *et al.* (1967) found that phosphorylase *b* in 7.2 M guanidine hydrochloride and in the presence of 0.1 M 2-mercaptoethanol dissociated into subunits, yielding molecular weight estimates in the range of 69.8 x 10^3g. per mole, for the smallest major component. In order to resolve inherent difficulties associated with molecular weight determinations of reversible associating systems in the presence of denaturants, Seery *et al.* (1967) treated their data by the presently available methods (Casassa and Eisenberg, 1964; Yphantis, 1964) for eliminating such problems as preferential solvation, thermodynamic non-ideality and macromolecular heterogeneity (Schachman and Edelstein, 1966; Hade and Tanford, 1967; Kielley and Harrington, 1960; and Tanford *et al.*, 1967). However, when the combined data of $2Mn - Mw$ for four samples was plotted against guanidine concentrations, and was extrapolated to zero concentration, the observed downward curvature of the line at 7.2 M guanidine hydrochloride indicated heterogeneity of subunits. This was interpreted as due to heterogeneity of either $\bar{v}_{20}$ or molecular weight. They pointed out that in order to resolve these difficulties, further studies were required.

Further evidence suggesting non-identity of subunits has come from experiments done with sodium dodecyl sulphate (SDS). Livanova *et al.* (1966a) indicated that in the presence of SDS at pH below 3

TABLE III

AMINO ACID COMPOSITION OF MUSCLE PHOSPHORYLASES

(Appleman *et al.*, 1963; Metzger *et al.*, 1968)

Amino Acid	Residues per monomer of phosphorylase *b* (mol. wt. 92,500)		
	Rabbit[a]	Human[a]	Frog[b]
Lysine	48.2	47.0	40.3
Histidine	22.2	23.3	18.6
Ammonia	(71.1)	(68.4)	(62.6)
Arginine	62.7	63.4	49.7
Aspartic Acid	89.8	94.0	86.9
Threonine	31.3	31.7	32.3
Serine	23.3	24.5	33.6
Glutamic Acid	88.7	87.9	79.8
Proline	33.3	32.9	40.9
Glycine	44.3	45.9	44.4
Alanine	58.5	61.5	52.1
Half-cystine[c]	8.4	8.0	10.1
Valine	56.9	56.2	54.7
Methionine	19.9	21.4	18.8
Isoleucine	45.9	46.2	40.6
Leucine	74.9	74.9	66.0
Tyrosine	33.6	32.5	32.5
Phenylalanine	36.7	39.4	33.3
Tryptophan[d]	11.5	11.1	11.7
Total	790	801	746.3

[a]From data of Appleman *et al.* (1963).
[b]Determined by Metzger *et al.* (1968).
[c]Determined as cysteic acid.
[d]Spectrophotometric measurements.

and above 10, phosphorylase *a* and *b* may be further dissociated into smaller fragments of molecular weight of approximately 60,000, and $\bar{v}_{20}$ 0.816. They concluded that phosphorylase *a* and *b* consist of 8 and 4 subunits respectively (Livanova *et al.*, 1966b). However, inherent difficulties in determining molecular weights of proteins in detergents, such as SDS, and the size of phosphorylase, make conclusions very tentative (Seery *et al.*, 1967).

The amino acid composition. The amino acid composition of rabbit and human skeletal muscle phosphorylase *b* determined by Appleman *et al.* (1963) is given in Table III, and is calculated on the basis of a molecular weight of 92,500. For comparison, data are also given in Table III on the amino acid composition of frog skeletal muscle phosphorylase *a* reported by Metzger *et al.* (1968). Although from amino acid composition and assumed pK values of ionizable groups, phosphorylase should be isoelectric at pH 10.0 (Velick and Wicks, 1951), Keller (1953) found that the isoelectric point of phosphorylase *b* is approximately pH 5.6 in sodium β-glycerophosphate buffer. This value is in close agreement with pH 5.7 reported by Madsen and Cori (1954).

As seen in Table III, the number of half-cystine residues, measured as cysteic acid, has been estimated by Appleman *et al.* (1963) to be 8.3 residues per molecular weight of 92,500. Recently, Battell *et al.* (1968a) redetermined the half-cystine of phosphorylase *b* and showed that this figure is slightly increased to 9. These investigators also indicated the absence of disulfide linkages in the protein. Their studies on the importance of the sulfhydryl groups

in the activity and solubility of phosphorylase are discussed in the following chapters where they will be integrated with the present work.

Although considerable work has been done on skeletal muscle glycogen phosphorylase, much concerning the chemistry and substructure of this protein is unknown. The physicochemical evidence previously described indicated that phosphorylase *a* and *b* consist of four and two subunits, respectively, each having a molecular weight of approximately 92,500 (Seery *et al.*, 1967; De Vincenzi and Hedrick, 1967; Ullmann *et al.*, 1968; Buc and Buc, 1967; and Chignell *et al.*, 1968). Evidence that the subunits may be chemically identical has come from the isolation from phosphorylase of a unique dipeptide (ε-*N*-pyridoxylphosphatelysylphenylalanine), the elucidation of the structure of a ^{32}P-labelled phosphoserine tetradecapeptide from phosphorylase *a* (Nolan *et al.*, 1964) and more recently from the isolation of a unique peptic ^{14}C-DFNB peptide concerned with the binding of AMP (Fasold *et al.*, 1969).

However, the possibility of non-identity of subunits is suggested by the observation that in 7.2 M guanidine hydrochloride some heterogeneity of molecular weight is observed (Seery *et al.*, 1967); by the electron microscope observations of Valentine and Chignell (1968), who have interpreted the rhombic shape of the phosphorylase *b* tetramer in terms of two kinds of subunits; and by the reports that phosphorylase dissociated into smaller fragments (molecular weight of approximately 60,000) in the presence of urea and SDS (Livanova *et al.*, 1966a, 1966b).

To shed further light on this question, the aim of the present thesis was the identification and characterization of all of the thiol sequences in the protein, in order to establish the total number of unique thiol groups, for the purpose of deriving information on the basic structural subunit of the enzyme, and the pertinent data is summarized in Chapter II. The rationale was that, in the event that a total of 9 unique half-cystine sequences is found, it would be concluded that the molecule consists of identical subunits of molecular weight of 92,500. In the event that the subunits are non-identical, the value would be expected to exceed 9. Thus, the number actually found should provide an experimental chemical basis for preferring one of these two hypotheses.

In addition to giving information concerning the subunit structure, such sequence analyses would be a significant contribution for the identification of those thiol residues which are reactive and those thiols associated with the maintenance of the quaternary structure and enzymic activity of phosphorylase. Such studies were concurrently in progress in Professor Madsen's laboratory and these results have been reported (Battell *et al.*, 1968a; 1968b).

Finally, knowledge concerning the half-cystine sequences of phosphorylase should also be a valuable contribution to the ultimate elucidation of the primary and tertiary structure of this protein. Specific labelling or isomorphous replacement of these thiols with heavy atoms or organomercurials can yield valuable information in mapping the relative positions of these thiol groups in the three-dimensional structure of the molecule.

CHAPTER II

THE NUMBER AND AMINO ACID SEQUENCES OF HALF-CYSTINE PEPTIDES FROM PHOSPHORYLASE *b*

1. INTRODUCTION

The sulfhydryl groups have frequently been considered *inter alia* as contributing appreciably to the maintenance of protein structure and the evidence available on the subject has been recently appraised by Webb (1964), and previously published in comprehensive reviews (Boyer, 1959; Benesch and Benesch, 1962; Cecil, 1963; Johnstone, 1963; and Madsen, 1963). The essential structural role of certain sulfhydryl groups in phosphorylase was first suggested by Madsen and Cori (1956), Madsen and Gurd (1956), and Madsen (1956), who found that mercaptide formation was accompanied by structural changes in the protein, and these changes were correlated with loss of phosphorylase activity.

Further evidence that certain sulfhydryl groups *per se* do contribute to the stability of the protein, emerged from chemical modifications of phosphorylase, which have been carried out by Jokay *et al.* (1965) and Damjanovich and Kleppe (1966). These investigators studied the kinetics of the reactions of phosphorylase with PCMB, NEM, and DTNB. Their data showed that maximum protection by the substrates and by AMP against chemical modification of the sulfhydryl groups of the enzyme was afforded. The same

evidence has been reported for phosphorylase *a* by Kudo and Shukuya (1964), and Ballin and Lukton (1965) using PCMB, NEM, and iodoacetate.

All of the above data clearly showed that in studies involving the effects of specific modifications of the sulfhydryl groups on activity, it is the reaction of the sluggish sulfhydryl groups which leads to structural change, and that the structural changes of phosphorylase lag considerably behind the chemical modifications (Madsen, 1956). Also pertinent is the observation that in the presence of the substrates and AMP, maximum protection against chemical modifications is afforded.

This general approach to the delineation of the functional sulfhydryl groups in the molecule, by studying the effects of specific modifications on activity, by no means proves that the residues in question form part of the active center. Inactivation may be a consequence of secondary changes in the structure of the protein that result from the modification, and the active site may in fact reside at a point quite distant from the residue modified, as is noted by Madsen (1956). It follows from this line of reasoning, that not only must one take into consideration the particular residues acted upon, since phosphorylase *b* contains nine half-cystine residues per monomer (Table III, page 21), but also the primary sequence surrounding the thus strategically located half-cystine residues.

With this work as a background, an attempt to isolate the half-cystine peptides from phosphorylase *b* was begun. These studies were carried out to ascertain the number of unique half-cystine

peptides in phosphorylase *b* and to relate this information to the subunit structure of the molecule. As a consequence of this work, it was also hoped that it would eventually be possible to identify the individual thiol residues that collectively affect activity.

Thus, one of the objectives of this investigation was to develop methods for the selective purification of the half-cystine peptides in order to ultimately elucidate their amino acid sequence. The major experimental procedure employed in this thesis for the isolation of half-cystine peptides was based on the diagonal electrophoretic procedure developed by Brown and Hartley (1963; 1966). This method relies on the change of electrophoretic mobility when cystine peptides are oxidized by performic acid to pairs of cysteic acid peptides.

The first attempts to develop a suitable method for their isolation and characterization explored the possibility whereby all thiol groups could be converted into disulfides. The rationale was not only to protect the half-cystine peptides from oxidation during the isolation procedures, but also to introduce a radioactive label which would facilitate the identification of these peptides either on column chromatograms or on paper electrophoerograms and would allow use of the diagonal electrophoretic method of Brown and Hartley (1963; 1966).

While this work was in progress a method described by Weeds and Hartley (1967; 1968) was developed for the disulfide interchange of rabbit skeletal muscle myosin, a very large protein of molecular weight about 500,000 (Tonomura *et al.*, 1966) with S^{35}-cystine and

S^{35}-cystamine. These authors incubated myosin for 92 hours with 100-fold excess of either radioactive cystine or cystamine, in which 75 per cent incorporation was reported. When in the last phase of the interchange reaction, the reaction mixture was made 5.0 M with respect to guanidine hydrochloride, the half-cystine incorporation was increased to well over 90 per cent. Their data clearly indicated that certain sulfhydryl groups are well protected in the interior of the protein and that they are not made available to disulfide reagents even in the presence of denaturants. Similar effects have been reported for phosphorylase *a* and *b* by Madsen and Cori (1956) and by Battell *et al.* (1968a).

Towards this end, phosphorylase was subjected to proteolytic digestion. Pepsin was chosen (Smyth, 1967) because the digestion could be performed under acidic conditions which reduces the incidence of disulfide interchange and oxidation of sulfhydryl residues (Spackman *et al.*, 1960). Thus, the protein was degraded to a soluble peptide mixture, and it was then exposed to an excess of cystine-3-^{14}C (25-fold) at pH 8.0, to promote disulfide interchange reactions. As a result, each of the available sulfhydryl residues was converted to a disulfide in which one half-cystine of the pair was labelled with C^{14}. The peptide mixture was then subjected to a preliminary sub-fractionation on a Sephadex G-25 column, at pH 2.0, and the peptides were located by examination of the effluent by absorbancy measurements at 280 and 260 mμ and radioactivity. Each fraction was then submitted to the diagonal electrophoretic procedure of Brown and Hartley (1963; 1966). On such a diagonal peptide "map",

each half-cystine of the original protein was found off the diagonal as a cysteic acid peptide and associated with a radioactive cysteic acid residue vertically in line with it. Each cysteic acid peptide, thus located, was isolated and sequenced by the Dansyl-Edman procedures described by Gray and Hartley (1963a; 1963b). However, during the course of the work it became apparent that cystine-3-^{14}C could not be removed effectively from the reaction mixture after adjustment of the pH to 1.8. Hence, during the gel-filtration, cystine-3-^{14}C emerged together with Fraction II (Figure 4, page 49), from the column and interfered with subsequent electrophoretic separation of cysteic acid peptides from that fraction. This difficulty has been circumvented subsequently by using diethanol disulfide instead of cystine. This material did not interfere with subsequent purification steps by paper electrophoresis.

The results of this study have been reported briefly elsewhere (Zarkadas *et al.*, 1968) and the experimental evidence for these findings is presented in this chapter.

2. MATERIALS AND METHODS

A. Materials

(1) Solvents and General Chemicals

Cystine-3-^{14}C, 17 mc. per mmole, a product of Service Molecules Marquees, Fabrique per CEA-France, and L-cystine were purchased from Calbiochem, Los Angeles, California, U. S. A. Diethanol disulfide (2-hydroxyethyl disulfide, 2, 2′-dithiodiethanol), was

purchased from Aldrich Chemical Company, Inc., Milwaukee, Wisconsin, U. S. A. α-D-Glucose-1-phosphate, AMP, sodium β-glycerophosphate, 2-mercaptoethanol, and EDTA were products of Sigma Chemical Company. Rabbit liver glycogen, a product of Sigma Chemical Company, was routinely purified through a Dowex 1-X2 chloride column. It was assayed against a glucose standard by the method of Dische as described by Ashwell (1957), and its concentration is expressed as a molar equivalent of its glucose residues. Sephadex G-10 and G-25 (fine bead type) and Blue Dextran 2000 were obtained from Pharmacia, Fine Chemicals, Uppsala, Sweden. AG1-X2 (Dowex 1-X2) (200 - 400 mesh, actual wet mesh size 80-200) was obtained from Bio-Rad Laboratories, Richmond, California, U. S. A. *N*-Ethylmorpholine (practical) was purchased from Eastman Organic Chemicals, Rochester, New York, U. S. A. and was redistilled (b.p. 134.0°) prior to use. Pyridine, spectro grade, and naphthalene were also products of Eastman Organic Chemicals. The scintillators, 2,5-diphenyloxazole and *p*-Bis[2-(5-phenyloxazolyl)]-benzene, were products of the New England Nuclear Corporation, Boston, Massachusetts, U. S. A. DNP-amino acids were prepared according to Sanger (1945). Dansyl amino acids were purchased from Calbiochem, Los Angeles, California, U. S. A., or were prepared by the method described by Gray (1964). All other chemicals in this study were reagent grade and were used without further purification.

(2) Enzymes

Pepsin (twice crystallized from ethanol), trypsin (twice crystallized; 50 per cent $MgSO_4$), α-chymotrypsin (three times crystallized) and carboxypeptidase A suspension (di-isopropyl phosphorofluoridate treated) were purchased from Worthington Biochemical Company, Freehold, New Jersey. TPCK-trypsin (devoid of chymotryptic activity) was prepared according to the method of Schoellmann and Shaw (1963). An α-lytic protease preparation from *Sorangium sp.* was kindly donated by Dr. D. R. Whitaker.

B. Methods

(1) Preparation of Muscle Phosphorylase *b*

Crystalline phosphorylase *b* was prepared from back and hind leg muscles of adult female rabbits of the New Zealand White strain by the method of Fischer and Krebs (1962) except that 2-mercaptoethanol replaced cysteine in the procedure. The enzyme was recrystallized three times, and the final crystallization was carried out in 0.02 M sodium β-glycerophosphate, 0.02 M 2-mercaptoethanol, 0.001 M AMP, 0.01 M magnesium acetate and 0.0015 M EDTA buffer at pH 6.8 (2 to 4°). The crystal suspension was stored at 2 to 4° under toluene vapor. Prior to use, the crystals were centrifuged out of suspension, the supernatant fluid was removed and the crystals were then dissolved in 0.02 M sodium β-glycerophosphate, 0.0015 M 2-mercaptoethanol and 0.0015 M EDTA buffer at pH 6.8 (35°).

The concentrated protein solution was passed through a Sephadex G-25 column (40 cm. x 2.5 cm.) pre-equilibrated with the same buffer, by the purification method recommended by Huang and Madsen (1966), and AMP, magnesium, excess 2-mercaptoethanol and other impurities were removed. It was found that such treatment did not cause loss of protein or enzymatic activity. To rule out any possible effects of aging of the samples, freshly prepared and chromatographically purified phosphorylase *b* was used for each set of experiments.

a. Protein Concentration

Following the Sephadex G-25 treatment of phosphorylase *b*. the protein concentration in solution was determined spectrophotometrically at 280 mμ, using a Zeiss PMQII spectrophotometer. A value for $E_{1cm.}^{1\%}$ of 13.2 calculated by Buc and Buc (1967) from the data of Appleman *et al.* (1963) for the amino acid composition of rabbit muscle phosphorylase *b*, was used. Samples of the enzyme (0.1 ml.) were suitably diluted with 0.03 M 2-mercaptoethanol (0.9 ml.) and aliquots (0.2 ml.) were further diluted (1.8 ml.) in the cuvette tube. Absorbancy ratios $A_{280m\mu}:A_{260m\mu}$ were also calculated to ensure that the preparation was free of nucleic acid.

b. Phosphorylase *b* Activity

Activity assays of phosphorylase *b* were routinely determined in the direction of glycogen synthesis, following the appearance of inorganic phosphate during the conversion of glucose-1-phosphate to glycogen, by the method of Cori *et al.* (1943) as described by

Illingworth and Cori (1953). For routine assay, 0.1 ml. of the enzyme (50 μg. per ml.) suitably diluted in 0.02 M sodium β-glycerophosphate, 0.02 M 2-mercaptoethanol, and 0.015 M EDTA buffer at pH 6.81 was placed in a Klett tube. After preincubation at 30° for 20 minutes, 0.1 ml. of the substrate was added. The usual composition of the reaction mixture was 1 per cent glycogen (which corresponds to 5 x 10^{-3} M glycosyl groups at the nonreducing end of the chains), 0.016 M glucose-1-phosphate, 0.001 M AMP and 5 μg. of the enzyme per assay. The reaction was stopped after 5 minutes by the addition of 0.07 M H_2SO_4 (7.0 ml.). The inorganic phosphate released in the reaction was determined in the Klett tube by the method of Fiske and Subbarow (1925).

The percentage of glucose-1-phosphate incorporated into glycogen was calculated and the velocity constant k for a first order reversible reaction was evaluated from the equation:

$$k = \frac{1}{t}\log_{10}\frac{x_e}{x_e - x}$$

where x_e is the percentage of the glucose-1-phosphate converted to inorganic phosphate and polysaccharide at equilibrium, and x is the percentage converted at time t (5 minutes). Arbitrarily, k multiplied by 1,000 has been defined as the number of enzyme "units" of activity per milliliter of original solution. At pH 6.8, x_e has the value of 78 per cent, and at pH 6.0 x_e = 86. x_e varies with pH; hence, the pH of the reaction mixture must be known and kept constant.

In a typical preparation, 2 to 3 million units of phosphorylase *b* were obtained in the crude muscle extract, and the yield of re-crystallized enzyme was 1 to 1.5 million units. The specific activity of the crystalline phosphorylase *b* used throughout the work reported in this thesis was 1700 to 2000 units per milligram of protein. All phosphorylase *b* preparations showed a single symmetrical peak on acrylamide gel electrophoresis and were also homogeneous in the ultracentrifuge.

(2) Digestion of Protein with Pepsin

This was always carried out in 5 per cent (v/v) formic acid. In a typical experiment, following the Sephadex G-25 treatment, freshly prepared phosphorylase *b* (0.5 to 1.5 g.) from rabbit skeletal muscle (Fischer and Krebs, 1962) in 0.0015 2-mercaptoethanol and 0.0015 M EDTA buffer, pH 6.8, was sufficiently diluted with water and enough 90 per cent (v/v) formic acid to give a final concentration of 5 mg. per ml. protein and 5 per cent (v/v) with respect to formic acid (pH 1.8). Freshly dissolved pepsin, in 5 per cent (v/v) formic acid (10 mg. per ml.), was added (ratio of pepsin to protein, 1.10) and the protein was digested at 37° for 24 hours. Control experiments with pepsin alone gave no indication of the liberation of peptides from its autolysis.

(3) Disulfide Interchange Reaction

a. Cystine-3-^{14}C

The disulfide interchange reaction of the peptic digest of phosphorylase *b* was carried out under conditions similar to those described by Battell *et al.* (1968a) for the protein.

The cystine-3-^{14}C, 0.2 mc. (17 mc. per mmole), was dissolved in 1.0 N HCl, diluted to 2.5 ml. with water, and mixed with a calculated weight of unlabelled L-cystine in 1.0 N HCl to give a radioactivity of approximately 1.6 μc. per mmole.

The peptic digest of phosphorylase *b* in 5 per cent formic acid, at a concentration of 5 to 10 mg. per ml. was adjusted to pH 8.0 with 10 N NaOH and exposed to an excess (25-fold over protein sulfhydryl groups) of cystine-3-^{14}C at 30° for 17 hours to promote disulfide interchange. The reaction mixture was adjusted to pH 2.0 with 6.0 N HCl, and the precipitate of excess cystine and denatured pepsin was removed by centrifugation.

b. Diethanol Disulfide

The disulfide interchange reaction of the peptic digests of phosphorylase *b* was carried out under conditions similar to those described for cystine-3-^{14}C except that the diethanol disulfide was unlabelled. The peptic digest of phosphorylase *b* in 5 per cent formic acid was added to a flask containing a calculated weight of diethanol disulfide (25-fold over protein sulfhydryl groups). The reaction mixture was adjusted to pH 8.0 with concentrated NH_4OH, incubated at 30° for 17 to 20 hours and then adjusted to pH 2.0 with

99 per cent (v/v) formic acid. A flocculent precipitate appearing after acidification and attributed to pepsin denaturation, as judged from control experiments, was removed by centrifugation. This method had the advantage that the solvents used and the diethanol disulfide did not interfere with subsequent fractionation of peptides.

(4) Peptide Purification Procedures

a. Group Separation of Peptides on Sephadex G-25

i. Preparation of the column:-

A Pyrex glass tube (195 cm. x 4.3 cm.) was fitted with a sintered glass plate at one end. Smaller columns (150 cm. x 1.1 cm.) of similar design were also constructed. A smaller (40 cm. x 2.5 cm.) column of similar design (Sephadex precision borosilicate glass) was used for peptide fractionation or protein purification. Sephadex G-25 (200 mesh "fine" beads) was allowed to swell in 0.05 M acetic acid solution, pH 2.0, the very fine particles were removed and the gel slurry and solutions were de-aerated. Before packing, the column was filled with the same de-aerated solution, a conical funnel with a rubber stopper attached to its stem was positioned at the top of the column, and the gel slurry, sufficiently dilute, was poured and allowed to pack under flow conditions induced by gravity. An electric stirrer, lowered into the funnel, kept the suspension agitated throughout the packing operation while the liquid was permitted to run freely through the sintered glass disc at the bottom. The packed column was then equilibrated with several column volumes of the same solution by the procedure recommended by Flodin (1961).

ii. Operation of the large column (195 cm. x 4.3 cm.):-

The column was mounted in a vertical position over an automatic fraction collector (Beckman Model 130; Beckman Instruments, Inc., Palo Alto, California, U. S. A.) (250 tube capacity). The fraction collector was housed in a chamber constructed of polyethylene film. This prevented excessive evaporation of the eluate and also prevented dust from entering.

The pepsin digest which was to be fractionated was added (25 ml.; 250 mg.) to the top of the column without disturbing the surface of the gel, and was then eluted with 0.05 M acetic acid solution. The flow was maintained at 178 to 180 ml. per hour and the effluent was collected (14.6 ml. fractions) in 20 ml. tubes. The total volume of eluate was approximately 5 to 6 liters.

iii. Operation of the small columns (150 cm. x 1.1 cm.; 40 cm. x 2.5 cm.):-

The preliminary fractionation experiments were conducted by means of small columns (150 cm. x 1.1 cm.; 40 cm. x 2.5 cm.) which were similar to the 195 cm. column in design and operation. The column was eluted with the same solution or as specified in the text. The flow was maintained at approximately 13 to 15 ml. per hour and the effluent was collected (2.2 to 3.0 ml. fractions) in 20 ml. tubes. The total volume of eluate was approximately 300 ml.

iv. Location of peptides:-

The fractionation was monitored by absorbancy measurements of the effluent fractions at 280 mμ and 260 mμ and by radioactivity

determinations. Aliquots (200 µl.) of the effluent fractions were transferred to a scintillation vial. Ten milliliters of Bray's (1960) scintillation fluid without ethylene glycol was added to the vials, and the samples were counted in a Nuclear-Chicago Mark I liquid-scintillation counter. Since measurements of effluents were carried out over a period of several months and under differing conditions of pH, corrections were made by counting standard samples in parallel with the other samples. The counts per minute were then corrected to disintegrations per minute using a quench correction curve determined for the system being used. Corrections for the decay of the nuclide were not necessary.

v. Concentration of column fractions:-

The radioactive fractions from the Sephadex columns were concentrated by freeze-drying. In some cases the final product was an oily liquid, which was diluted with water and further freeze-dried. This procedure was repeated until most of the oil was removed. Although some of the salts and/or diethanol disulfide remained, they did not interfere in the subsequent paper ionophoresis at pH 6.5. Drying by rotary evaporation was not a successful method for concentration and removal of salts because of excessive frothing and loss of peptides.

b. High Voltage Paper Electrophoresis

The vertical strip high voltage electrophoresis apparatus similar to that described by Mihl (1951) and by Ryle *et al.* (1955) was used with the pH 6.5 buffer system. The electrophoresis of

peptides at pH 3.5 and 1.8 was carried out in a Gilson High Voltage Electrophorator Model D (Gilson Medical Electronics, Middleton, Wisconsin, U. S. A.) equipped with a large bifurcated Fiberglass tank, as described by Dreyer and Bynum (1967). The buffer systems and coolants at pH 6.5, 3.5, and 1.8 were as described by Ambler (1963) except that the toluene was 8 per cent (v/v) with respect to pyridine. The radioactive peptides were purified by paper electrophoresis on Whatman No. 1 and 3MM filter paper. Peptides were applied as wide bands to filter paper with internal fluorescent markers at a loading of about 2 mg. per cm. for Whatman 3MM paper or 0.03 μmole of peptide per cm. of Whatman No. 1 paper, and dried in a stream of warm air. The papers were wetted with the appropriate buffers, the bands sharpened and ionophoresis was carried out at 80 v. per cm. for the appropriate time. The peptides were located by the cadmium-ninhydrin reagent of Heilmann *et al.* (1957), the starch-iodine reagent of Rydon and Smith (1952), the Pauly reagent for histidine of Dent (1947), the Ehrlich's reagent for tryptophan of Harley-Mason and Archer (1958), the 1-nitroso-2-naphthol reagent for tyrosine, and the Sakaguchi reaction for arginine of Jepson and Smith (1953). Electrophoresis strips were analyzed for radioactivity with a Nuclear-Chicago Actigraph III strip scanner with 4π geometry.

c. The Diagonal Purification Procedure

The major experimental procedure employed in these studies for the purification of disulfide interchanged half-cystine peptides from peptic digests of phosphorylase *b* was the diagonal electrophoretic procedure described by Brown and Hartley (1963; 1966).

i. Preparative separation of peptide mixture by electrophoresis:-

After a preliminary sub-fractionation of the peptic digest on a Sephadex G-25 column, samples from each fraction were applied as a band to the middle of Whatman No. 3MM filter paper at a loading of approximately 2 mg. per cm. and submitted to electrophoresis at pH 6.5 (see above). Guide strips were stained with cadmium-ninhydrin reagent (Heilmann *et al.*, 1957) to reveal the position of the peptides.

ii. Performic acid oxidation of the paper strips:-

A 3 cm. band from a preparative sheet was cut parallel to the direction of electrophoresis at pH 6.5. The strip was subjected (for two hours) to performic acid vapours generated in a dessicator by an admixture of 19.0 ml. of 98 per cent (v/v) formic acid and 1.0 ml. 30 per cent (v/v) hydrogen peroxide. After oxidation the Petri dish containing the performic acid was replaced by NaOH pellets, and the strip was allowed (under vacuo) to dry for an additional two hours.

iii. Diagonal electrophoretic peptide "maps":-

Diagonal electrophoretic peptide "maps" of cysteic acid peptides were prepared as follows: The oxidized strip, when dry, was stitched to a larger sheet and was submitted to electrophoresis at pH 6.5 at right angles to the original direction. The sheets were stained with cadmium-ninhydrin reagent (Heilmann *et al.*, 1957), to reveal the position of the cysteic acid peptides. On such a diagonal peptide "map", each half-cystine peptide of the original

protein was found off the diagonal as a cysteic acid peptide. Where the original disulfide interchange had been carried out with cystine-3-^{14}C, each cysteic acid peptide was associated with a radioactive cysteic acid vertically in line with it.

iv. Isolation of cysteic acid peptides:-

After location of the cysteic acid peptide on such a diagonal peptide "map" the corresponding bands from the original electropherogram were cut out and oxidized, and the strip stitched to a new sheet of paper for electrophoresis at pH 6.5. Further purification of the bands of cysteic acid peptides was frequently necessary, because of diagonal contaminants or because of contaminating peptides with similar mobilities. This was achieved by electrophoresis at pH 1.8, 3.5, and 6.5, in which case very pure peptides were obtained. The peptides were then eluted from the paper with water.

d. Further Digestion of Peptides with Proteolytic Enzymes

Tryptic, chymotryptic, and α-lytic protease digests of peptides were carried out in 0.05 M *N*-Ethylmorpholine-acetic acid buffer, pH 8.0, with a 1:50, or 1:100 molar ratio at 37° for 5 hours depending on the extent of digestion desired. In certain cases the ratio of enzyme to peptide was increased, and also prolonged periods of incubation were used when peptides were found to be digested slowly by the protease. The final concentration of peptide was about 0.20 to 0.50 μmole per ml.. Fresh aqueous enzyme solutions (1 mg. per ml.) were prepared in the same buffer for each digestion.

Carboxypeptidase A suspension (treated with diisopropyl fluorophosphate) were dissolved essentially by the method of Fraenkel-Conrat *et al.* (1955) as follows: 1.25 mg. of suspension in 100 µl. of 1 per cent sodium bicarbonate (w/v) were cooled in ice. Solution was achieved by addition of 0.1 M NaOH dropwise, and immediately an equal number of drops of 0.1 M HCl were added to restore the pH of the solution. The digestions were carried out in 0.05 M *N*-Ethylmorpholine formate at pH 8.0 for 5 hours at 37°, with approximately 0.01 mg. of enzyme per 0.02 to 0.05 µmole of peptide.

After digestion with these enzymes the digested peptides were submitted to electrophoresis at pH 6.5, and isolated as described above.

e. Amino Acid Analysis of Peptides

Quantitative determination of the amino acid composition of peptides was made on Beckman Spinco Model 120C automatic amino acid analyzer equipped with the accelerated system. About 0.01 to 0.05 µmole of peptide was hydrolyzed in 0.2 to 0.3 ml. constant boiling HCl for 16 to 24 hours at 110° in sealed evacuated tubes (10 mm. x 75 mm.). The hydrochloric acid was then removed under vacuo (5 to 50 µ) in a vacuum dessicator at room temperature (21°), and analyzed in the amino acid analyzer.

f. Determination of N-Terminal Groups of Peptides and Sequence

The N-terminal groups were determined by their reaction with DNS chloride (Gray and Hartley, 1963a; 1963b). This method, coupled with the Edman procedure (designated below as the Dansyl-Edman

procedure) as described by Gray and Hartley (1963a; 1963b) and by Smillie and Hartley (1966), was extremely valuable for determining the sequence of the peptides isolated in this work. Approximately 0.2 to 0.5 μmole of peptide in water or volatile buffer was evaporated to dryness in ground glass test tubes, and the peptide was then dissolved in 0.2 ml. of pyridine water solvent (ratio of pyridine to water, 1:2). A 0.02 ml. sample was transferred to a 30 mm. x 6 mm. Durham fermentation tube (A. Gallenkamp and Company, Ltd., London, EC2) and the remainder frozen and stored at -10°. To the Durham tube was added 0.02 ml. of 0.1 M $NaHCO_3$, the contents were thoroughly mixed, and the solution evaporated to dryness in a dessicator under vacuum. Deionized water (0.02 ml.) and 0.02 ml. of DNS chloride (2.5 mg. per ml. in acetone) were added, the solutions were mixed, and the tubes sealed with Parafilm and incubated at 37° for 3 hours. After drying under vacuum (water aspirator), constant boiling HCl (0.05 ml.) was added, and the tube was sealed under vacuum and incubated at 110° for 16 to 20 hours. The tube was opened, the contents were evaporated to dryness, and, after solution in 1 N NH_4OH, about half was used for identification of the DNS-amino acid by electrophoresis at pH 4.38.

The phenyl isocyanate degradation was essentially as described by Gray and Hartley (1963b) except that the reaction with the reagent was at 37° for 2 to 3 hours. Anhydrous trifluoroacetic acid instead of acetic acid-anhydrous HCl was employed for one hour at room temperature. After removal of the trifluoroacetic acid under vacuum, the degraded peptide was again dissolved in water and pyridine and a

further sample taken for reaction with DNS chloride. In none of the N-terminal degradations described in this work was it necessary to repurify the peptide at each step. Unequivocal identification of the N-terminal amino acid was obtained after as many as seven or eight degradations. When more than one DNS derivative was observed after electrophoresis at pH 4.38 or when the fluorescent spots became faint, the series was discontinued.

3. RESULTS

A. Peptide Nomenclature

The sequence of purification steps for the isolation and characterization of the disulfide interchanged unique half-cystine peptic peptides were the following:

(1) The preliminary group sub-fractionation by gel-filtration on Sephadex G-25. Five fractions were assigned Roman numerals, I to V.

(2) Each fraction was then submitted to the diagonal fingerprint procedure. Capital letters A to J are added to these designations in alphabetical order to indicate their relative mobility on electrophoresis at pH 6.5 in the first dimension. The cysteic acid peptides from Bands I-A and I-B are basic, those from band I-C are neutral, and the acidic peptides are designated I-D to I-J.

(3) Final purification by electrophoresis. Each cysteic acid in a given band is assigned an Arabic number in consecutive order, according to its relative mobility at pH 6.5 or 1.8 after performic acid oxidation, the most basic peptide having the lowest number. The

products of peptides subsequently digested with trypsin have been designated by the letter T, or those degraded by α-lytic protease (of *Sorangium sp.*) have been designated by the letter α-LP. An Arabic numeral is assigned to each of the products to indicate the relative mobility of the peptide on electrophoresis at pH 6.5, the most basic peptide of the digest having the lowest number. Mobilities were measured relative to that of lysine, indicated by a positive sign (m = + 1.0) and relative to that of aspartic acid, indicated by a negative sign (m = - 1.0). The mobility of peptides at pH 1.8 was measured relative to that of serine, indicated by a positive sign (m′ = 1.0) (Ambler, 1963; Weeds and Hartley, 1968).

An example illustrates the system: peptide I-B-3αLP5 was obtained from Fraction I of the Sephadex gel-filtration step, was the second basic band in the diagonal "map", and was eluted as the third cysteic acid peptide from the pH 6.5 electropherogram. After α-lytic protease digestion of this fraction, it was recovered as the fifth most basic component upon electrophoresis at pH 6.5.

B. Choice of Proteolytic Enzyme

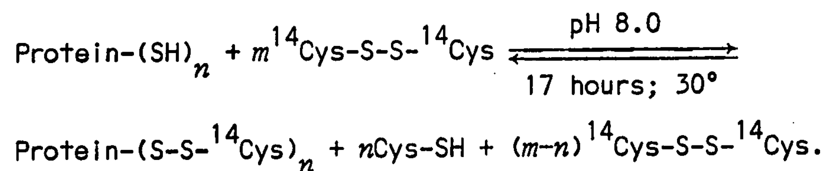
Proteolytic enzymes were first systematically employed to probe the accessibility and general exposure of the peptide bonds of phosphorylase *b* to proteolysis. Exploratory small scale experiments were carried out with endopeptidases of wide specificity, such as pepsin and chymotrypsin, as well as TPCK-treated trypsin. Since trypsin serves better for the production of peptides of high molecular weight, owing to its narrower specificity, fewer derivatives

are formed. However, it was found that its use complicated the analysis because of the production of large insoluble peptides. Similar results were obtained with chymotrypsin. However, pepsin in its action on phosphorylase *b* produced peptic peptides that were completely soluble, suggesting that no part of the molecule had escaped its action. Another advantage for its use on phosphorylase *b*, was that the digestion was performed in 5 per cent formic acid (pH 1.8) and therefore it afforded maximum protection of the thiol groups during the 24 hour proteolysis. Thus pepsin has been successfully used throughout this work.

C. Disulfide Interchange Reactions

(1) Cystine-3-^{14}C Interchange of Phosphorylase *b*

Disulfide interchange experiments were therefore attempted with cystine-3-^{14}C on peptic digests of phosphorylase *b* as described in the Methods (page 34). A simplified example to illustrate the method follows: The protein, after pepsin digestion, was adjusted to pH 8.0 and was exposed to an excess of cystine-3-^{14}C (25-fold) over protein sulfhydryl groups to promote disulfide interchange. The reaction of the thiols with cystine-3-^{14}C proceeds according to the equation:



$$\text{Protein-(SH)}_n + m\,^{14}\text{Cys-S-S-}^{14}\text{Cys} \xrightleftharpoons[\text{17 hours; 30°}]{\text{pH 8.0}}$$

$$\text{Protein-(S-S-}^{14}\text{Cys)}_n + n\text{Cys-SH} + (m-n)\,^{14}\text{Cys-S-S-}^{14}\text{Cys.}$$

As a result, each of the available sulfhydryl residues was converted to a mixed disulfide, in which one half-cystine of the pair was labelled with C^{14}. The reaction was terminated by adjusting the reaction mixture to pH 2.0 and the precipitate of excess cystine-3-^{14}C and denatured pepsin was removed by centrifugation. Therefore, the standard conditions described in the Methods (page 34) were adopted for the exchange reaction. The peptide mixture was then subjected to a preliminary sub-fractionation on a Sephadex G-25 column, and each fraction thus obtained was then submitted to the diagonal fingerprint procedure outlined (*vide infra*). On such a diagonal peptide map, each half-cystine of the original protein was found off the diagonal as a cysteic acid peptide and associated with a radioactive cysteic acid residue vertically in line with it. Each cysteic acid peptide, thus located, could be isolated by high voltage electrophoresis.

The large scale disulfide interchange experiments of peptic digests of phosphorylase *b* with cystine-3-^{14}C represent results from two different phosphorylase *b* preparations and the reaction conditions were identical to those described in the Methods (page 34). Phosphorylase *b* (0.79 g.; 4.29 μmole), prepared from rabbit skeletal muscle as described in the Methods (page 31) was digested with pepsin (ratio of pepsin to protein, 1:10) at a concentration of 10 mg. per ml. in 5 per cent formic acid. A sample of cystine-3-^{14}C (472.0 mg. 1.96 mmole) was dissolved in 26.3 ml. of 1 N HCl and the digest was then adjusted to pH 8.0 as described in the Methods (page 34). The reaction mixture was sufficiently diluted to ensure that cystine-3-

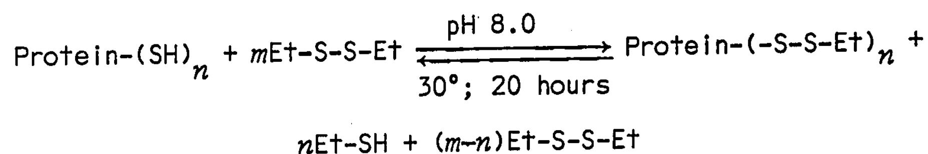
^{14}C remained in solution during incubation and for this the solution was agitated continuously throughout. The reaction was carried out under standard conditions (page 34), the final concentration being:

Phosphorylase *b*	3.61 mg./ml. 0.35 mM phosphorylase *b* sulfhydryl groups
Cystine-3-^{14}C	2.146 mg./ml. = 8.91 mM
2-mercaptoethanol	0.05 mM
EDTA	0.05 mM

The pH of the reaction was 8.0.

(2) Diethanol Disulfide Interchange of Phosphorylase *b*

The large scale disulfide interchange experiments of phosphorylase *b* represent average results from three different phosphorylase *b* preparations and were carried out as described in the Methods (page 34). The reaction proceeds according to the equation:



$$\text{Protein-(SH)}_n + m\text{Et-S-S-Et} \xrightleftharpoons[30°;\ 20\ \text{hours}]{\text{pH } 8.0} \text{Protein-(-S-S-Et)}_n + n\text{Et-SH} + (m-n)\text{Et-S-S-Et}$$

where $n{:}m = 1{:}25$.

In this experiment, 1.29 g. (6.97 μmole) of phosphorylase *b* was digested with pepsin (ratio of pepsin to protein, 1:10) at a concentration of 5 mg. per ml. in 5 per cent formic acid, 0.14 mM 2-mercaptoethanol and 0.2 mM EDTA at 37° for 20 hours. After pepsin digestion, the digest was transferred to a flask containing 0.48 g. (3.14 mmole) of diethanol disulfide to promote disulfide interchange

reactions. The final concentration of the reaction mixture (411.3 ml.) was:

Phosphorylase *b*	3.156 mg. per ml. = 0.307 mM phosphorylase *b* sulfhydryl groups
Diethanol disulfide	1.178 mg. per ml. = 7.640 mM
2-mercaptoethanol	= 0.139 mM
EDTA	= 0.209 mM.

The reaction was terminated by adjusting the pH to 2.0.

D. Gel-Filtration of the Peptic Digest of Disulfide Interchange Phosphorylase *b*

Because of the large number of peptides produced by proteolysis of phosphorylase *b*, it was necessary to perform preliminary group fractionation of the radioactive peptides by gel-filtration at acid pH as suggested by Porath (1960). However, in order to establish the optimal operational conditions of the gels, small scale preliminary filtrations for the purification of cysteic acid peptides were performed through small columns (150 cm. x 1.1 cm.; 40 cm. x 2.5 cm.) packed with either Sephadex G-10 or G-25 gels. From these results it was evident that maximum efficiency of peptide molecular sieving was obtained with long columns packed with Sephadex G-25 gels. Thus, it was decided to adopt the gel-filtration method in conjunction with the diagonal electrophoretic procedure for the selective purification of cysteic acid peptides.

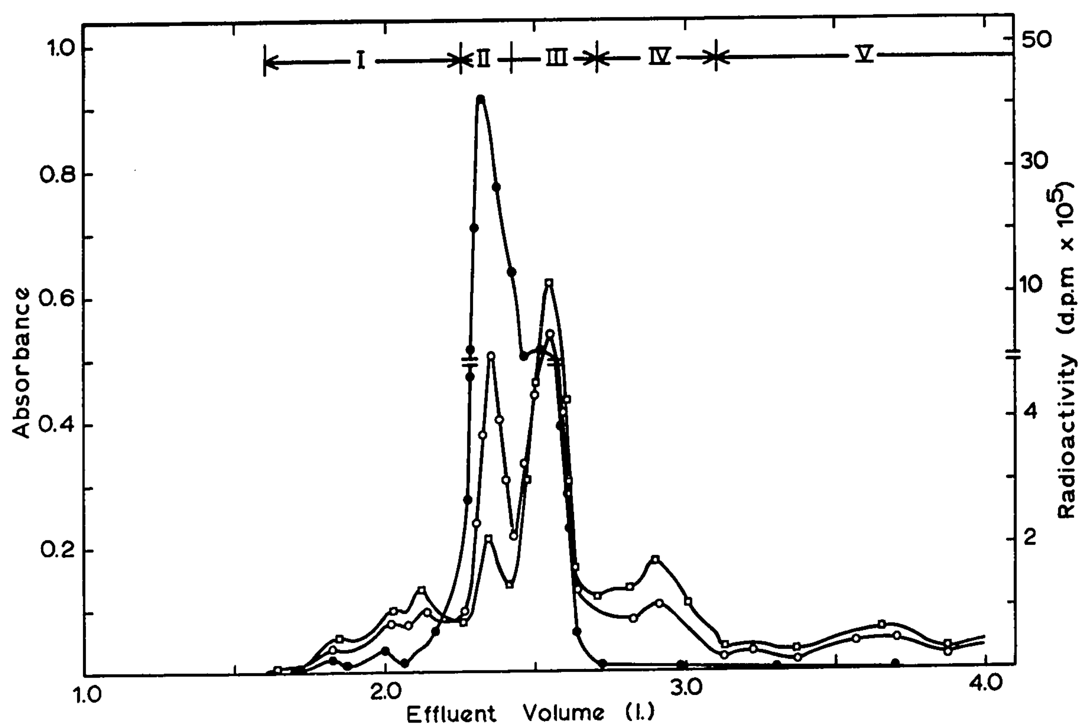


Figure 4. Preparative Fractionation of Cystine-3-^{14}C Interchanged Peptic Digest (0.25 gm.) of Phosphorylase *b* by Gel-filtration on Sephadex G-25.
The effluent was monitored for radioactivity (●——●) and absorbancy measurements at 280 mμ (o——o) and at 260 mμ (□——□). Fractions I to V were taken as indicated by the arrows.

(1) Preparative Gel-Filtration of the Cystine-3-^{14}C Interchanged Phosphorylase *b*

After adjustment of the reaction mixture to pH 2.0, the excess cystine and denatured pepsin were removed by centrifugation (12,000 x g) and the peptic digest was then subjected to a preliminary sub-fractionation by gel-filtration as described in the Methods (page 36). The material was eluted with 0.05 M acetic acid, and these operations were conducted at room temperature (21°). The results of the large scale experiments, and the elution profile of one such column fractionation is shown in Figure 4. The products of digestion and disulfide interchange were easy to fractionate as all were soluble, and no "core" was produced to complicate the sequence investigations. The recoveries of the radioactive peptides were quantitative as judged by radioactivity measurements. On the basis of the elution pattern shown in Figure 4, five fractions designated I to V were separated and suitable cuts were then pooled and lyophilized.

(2) Preparative Gel-Filtration of the Peptic Digest of Diethanol Disulfide Phosphorylase *b*

The disulfide-diethanol-exchanged peptic digest was then subjected to a preliminary sub-fractionation on a Sephadex G-25 column (195 cm. x 4.3 cm.) as described earlier (page 36) to give the elution pattern shown in Figure 5. On the basis of the optical density at 280 mμ and 260 mμ the effluent was divided into five

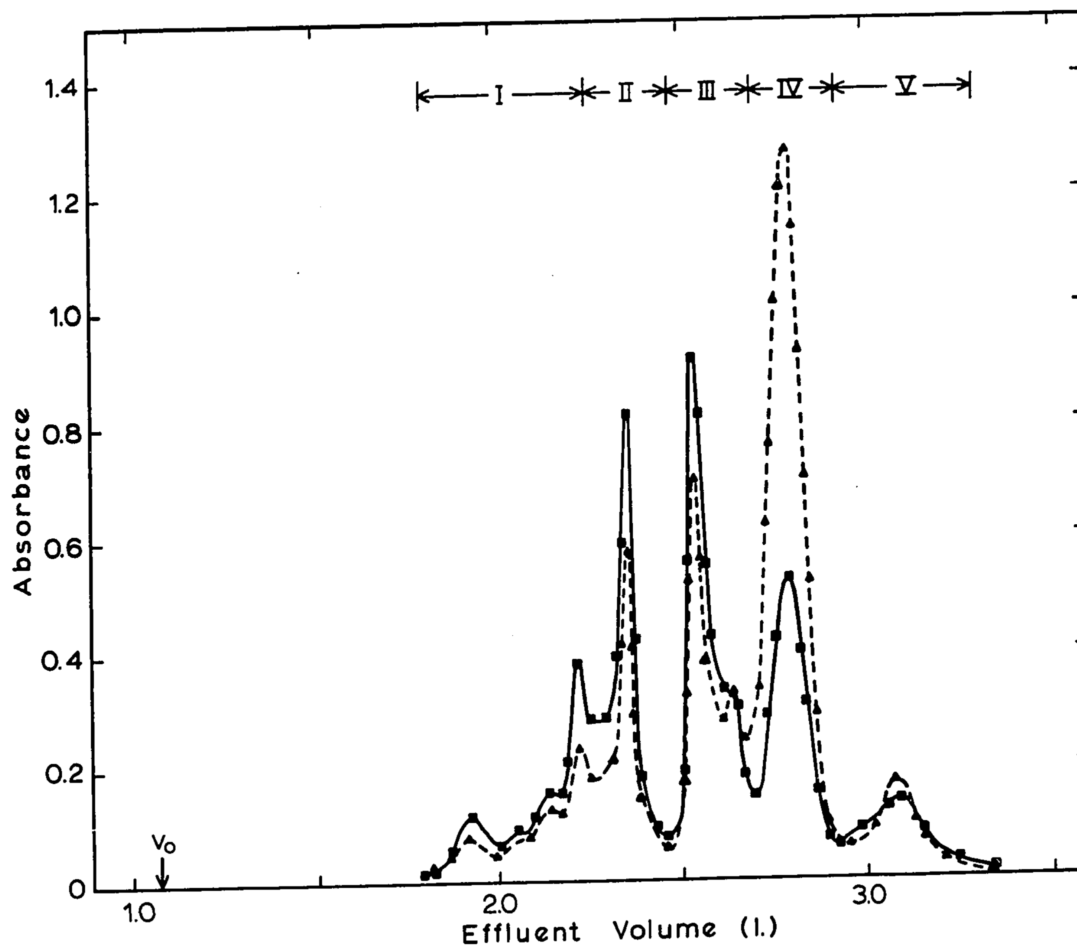


Figure 5. Preparative Fractionation of Diethanol Disulfide Interchanged Peptic Digest (0.25 mg.) of Phosphorylase *b* by Gel-filtration on Sephadex G-25.
The effluent was monitored for absorbancy at 280 mμ (■——■) and at 260 mμ (▲----▲). Fractions I to V were taken as indicated by the arrows.

fractions, I to V, shown in Figure 5. Each fraction was pooled, lyophilized, and was then submitted to the diagonal fingerprint procedure, and was further purified by high voltage electrophoresis as described in the Methods (page 37).

E. Purification, Isolation, and Characterization of the Unique Cysteic Acid Peptides

Most of the sequence results on the unique cysteic acid peptides reported in this chapter were obtained from characterization of those peptides recovered from Fraction I of the peptic digest of phosphorylase *b* disulfide exchanged with cystine-3-^{14}C. As previously described and as is evident from Figure 4, Fractions II and III of this digest were contaminated with excess cystine-3-^{14}C which interfered with subsequent purification attempts by paper electrophoresis. For this reason, new peptic digests of phosphorylase *b* were disulfide interchanged with diethanol disulfide and fractionated on Sephadex G-25 in the usual way. Fraction I in this case yielded many of the same cysteic peptides as with the cystine-3-^{14}C exchanged preparation and the results presented below for the purification and characterization of these peptides have been combined for simplicity of presentation. Although diethanol disulfide contaminated Fraction IV, as is evident from the data of Figure 5, it was not an impediment to further purification of the peptides of this fraction by paper electrophoresis, presumably because of its solubility in the toluene-pyridine mixture used as coolant in this procedure. The results

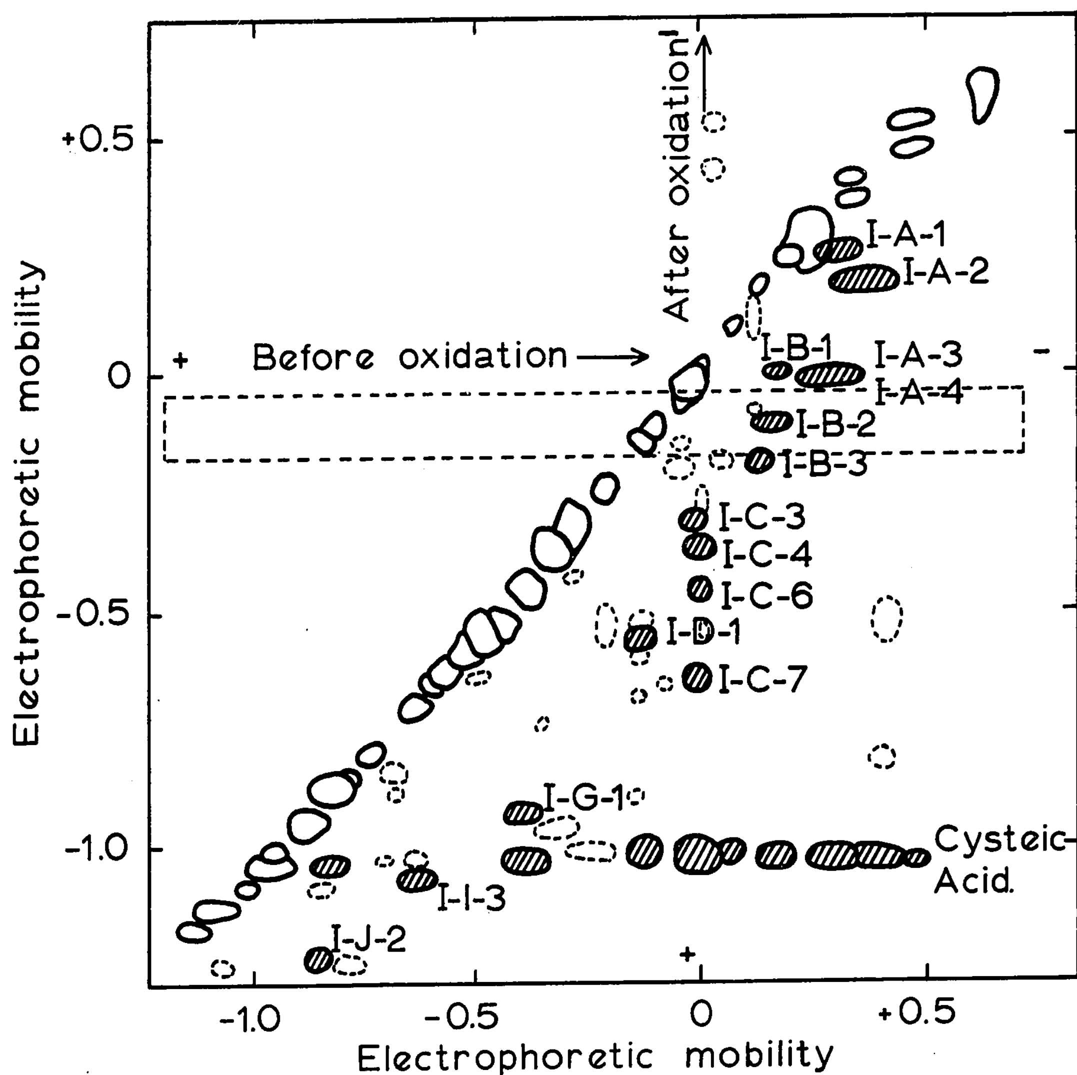


Figure 6. Diagonal Peptide "Map" of Fraction I Cystine-3-^{14}C Interchanged Peptic Digest of Phosphorylase *b*. Electrophoresis was at pH 6.5 in both dimensions. The major cysteic acid peptides are hatched. The conditions and nomenclature are as described in the text, and in Tables IV and X, pages 51 and 60.

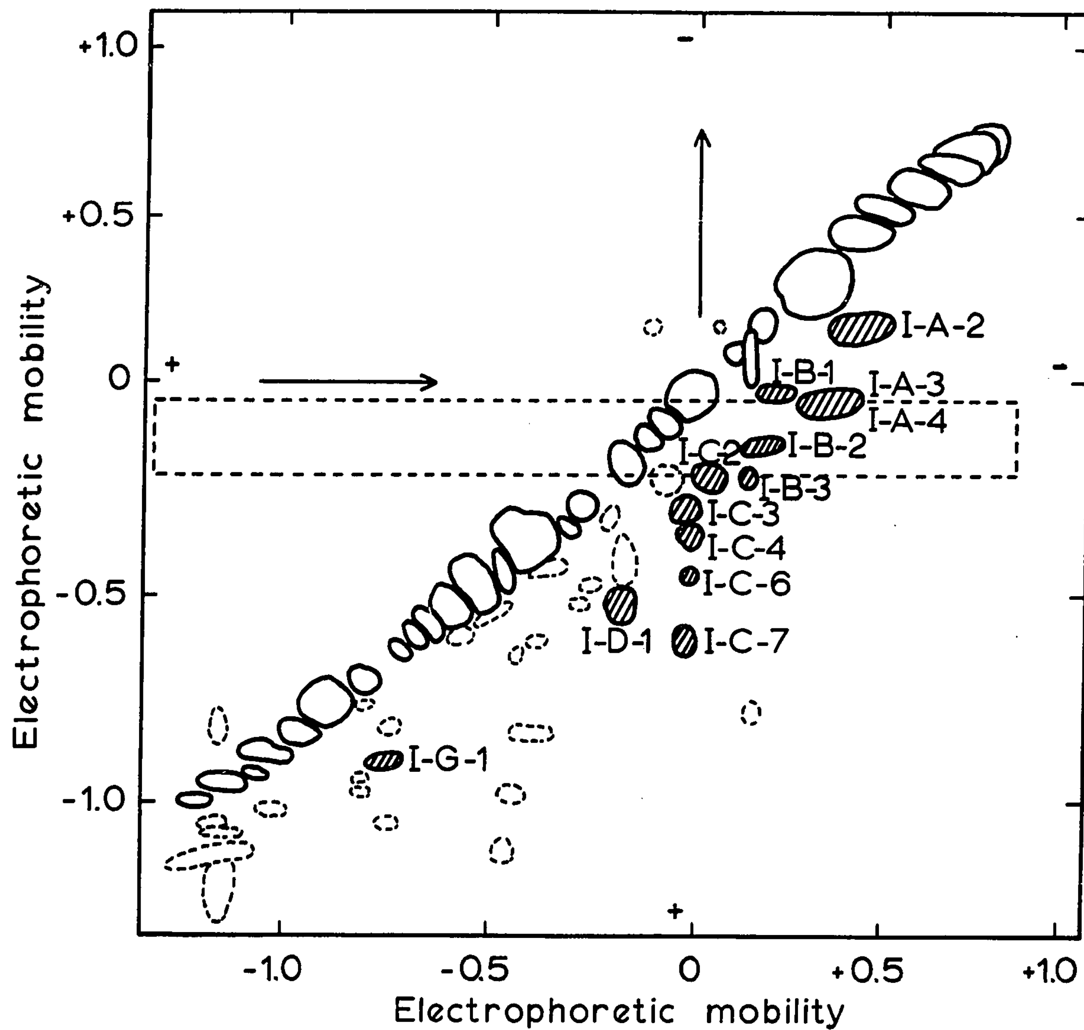


Figure 7. Diagonal Peptide "Map" of Fraction I Diethanol Disulfide Peptic Digest of Phosphorylase *b*.
Electrophoresis was at pH 6.5 in both dimensions. The major cysteic acid peptides are hatched. The conditions and nomenclature are as described in the text, and in Tables IV and X, pages 51 and 60.

TABLE IV

AMINO ACID COMPOSITION OF BASIC HALF-CYSTINE PEPTIC PEPTIDES

ISOLATED FROM FRACTION I

(The Values are Expressed as Mole Ratios)

Amino Acid	Constituent Amino Acid Residues Per Molecule of Peptide					
	I-A-1	I-A-2	I-A-3	I-A-4	I-B-2	I-B-2
Lysine	1.05	0.92	0.97	0.85	0.92	
Histidine.						0.97
Arginine	1.05	0.93				
Cysteic Acid	0.90	0.93	0.99	1.02	1.00	0.97
Aspartic Acid.	0.90	1.19			0.97	0.97
Threonine.	1.21		0.89	1.71		
Serine	0.90					1.06
Glutamic Acid.		1.19			1.00	
Proline.	0.75					
Glycine.	1.96				2.00	2.11
Alanine.	1.21		1.07	1.53		2.03
Valine	1.81		0.96	1.88		0.89
Methionine Sulfone . .	1.21					
Isoleucine					0.95	1.06
Leucine.		1.84				
Tyrosine		0.79				
Phenylalanine.	0.90					
Number of Residues	13	8	5	7	7	10
Mobility at pH 6.5 (m)	+0.30	+0.25	0.00	0.00	-0.14	-0.25
Mobility at pH 1.8 (m')	0.95	0.81	0.63	0.54	0.83	0.67
N-terminal		Tyr	Val	Val	Asn	Cya
Percentage Yield	0.5	6.3	24.4	3.2	14.0	9.0

presented below for Fractions II to V are therefore derived from peptic digests interchanged with diethanol disulfide.

(1) Fraction I

a. Diagonal Peptide "Maps" of Fraction I

i. Cystine-3-^{14}C interchanged peptic digest of phosphorylase *b*:-

Only Fraction I from cystine-3-^{14}C interchanged peptic digest of phosphorylase *b* was submitted to the diagonal fingerprint procedure outlined in the Methods (page 38). The pH 6.5/pH 6.5 diagonal peptide "map" of Fraction I is shown in Figure 6, and illustrates the nomenclature of the unique cysteic acid peptides adopted in this chapter and summarized in Table IV. On such a diagonal peptide "map", each of the unique half-cystine peptides of the original protein was found off the diagonal of Fraction I as a cysteic acid peptide (hatched) and associated with a radioactive cysteic acid residue vertically in line with it. Each cysteic acid peptide thus located was isolated from the preparative sheets and was further purified by electrophoresis at pH 6.5 or 1.8 and 3.5.

ii. Diethanol disulfide interchange peptic digest of phosphorylase *b*:-

The pH 6.5/pH 6.5 diagonal "map" of Fraction I that originated from the diethanol disulfide interchanged peptic digest of phosphorylase *b* is shown in Figure 7. The cysteic acid peptide spots in this diagonal "map" are hatched. Although it differs in some respects from the corresponding Fraction I diagonal peptide "map" shown in Figure 6 that was derived from the cystine-3-^{14}C interchanged

peptic digest of phosphorylase *b*, the cysteic acid peptides present are found in similar positions in both diagonals.

b. Peptides from Band I-A

i. Purification of peptides:-

The half-cystine peptides were further purified by cutting out band I-A from the original preparative pH 6.5 electrophoresis and re-run at pH 1.8. The cysteic acid peptides I-A-1 to I-A-4 were thus separated and their mobilities at pH 6.5 and 1.8 are shown in Table IV. The peptides were still impure at this stage of purification. They were finally isolated by eluting the corresponding bands from the pH 1.8 electrophoresis and re-running on Whatman No. 1 paper at pH 3.5 (80 v./cm., 50 min.). In the case of peptide I-A-4, however, electrophoresis was for 2.5 hours at 80 v./cm. The final amino acid composition of these peptides is shown in Table IV.

ii. Sequence determinations:-

Peptide I-A-1

This peptide was located practically on the diagonal and was still impure, being contaminated with peptides lacking cysteic acid because of its proximity to the diagonal. After tryptic digestion of band I-A-1, a peptide fragment was isolated and found to contain methionine sulfone and a trace of cysteic acid. Hence it is possible that peptide I-A-1 is either a methionine-containing peptide or a minor variety of sequence represented in the diagonal "map", as judged by its low recovery(0.5 per cent), and it was ignored in the present study.

TABLE V

AMINO ACID COMPOSITION AND SEQUENCE OF TRYPTIC PEPTIDES FORMED BY TRYPSIN DIGESTION OF PEPTIDE I-A-2

(Values are Expressed as Mole Ratios)

Peptide	Electrophoretic mobility at pH 6.5 (m)	Amino Acid Composition and Sequence	Cadmium-Ninhydrin Colour	Molecular Weight
I-A-2	+ 0.25	Tyr-(Lys, Arg, Gln, Leu, Leu, Asn, Cya) 0.79⟶ 0.92 0.92 1.19 0.92 0.92 1.19 0.92	orange slow red	1074.2
I-A-2T1	+ 0.96	Arg 1.00⟶	red	174.2
I-A-2T2	+ 0.88	Tyr- Lys- Arg 0.59⟶ 1.00⟶ 1.00⟶	orange slow red	467.6
I-A-2T4	+ 0.59	Tyr- Lys 0.50⟶ 1.00⟶	orange slow red	302.4
I-A-2T11	- 0.49	Gln- Leu- Leu- Asn- Cya 0.86⟶ 1.05⟶ 1.05⟶ 1.03⟶ 1.09⟶	yellow turned red	627.6

Definition of symbols: ⟶, represents N-terminal analyses by the Dansyl-Edman procedure. Cya, represents cysteic acid.

Peptide I-A-2

This peptide was pure as judged by its amino acid composition shown in Table IV. However, the total recovery was low (6.3 per cent) indicating considerable losses, probably mainly during elution of the peptide from the filter paper and running again at pH 3.5. The orange colour produced with the cadmium-ninhydrin stain that turned red upon standing, is consistent with N-terminal tyrosine as determined by the "dansyl" method. The 1-nitroso-2-naphthol (Jepson and Smith, 1953) reagent gave a positive colour confirming the presence of tyrosine. C-terminal analysis with carboxypeptidase A gave inconclusive results. Its electrophoretic mobility at pH 6.5 (m = + 0.26) strongly suggested that both acidic amino acid residues must be amidated. This peptide was therefore tentatively assigned the sequence:

Tyr-(Lys,Arg,Gln,Leu,Leu,Asn,Cya).

Additional evidence for the N-terminus residue tyrosine and for the sequence of this peptide was obtained from tryptic digestions. The peptide (0.40 µmole) was digested with TPCK-trypsin (0.008 µmole) (ratio of trypsin to I-A-2, 1:50). The digest (1.2 ml.) was spotted as an 11 cm. band (approximately 0.035 µmole per cm.) at a distance of 28 cm. from the anode on Whatman No. 1 paper. After electrophoresis at pH 6.5, the digestion products were separated into four major cadmium-ninhydrin positive bands. The results of amino acid composition, N-terminal analysis by the "dansyl" method, and electrophoretic mobilities are shown in Table V.

After the fourth Edman step of peptide I-A-2T11, difficulties were encountered in determining unequivocally the N-terminal

cysteic acid. Weak aspartic and cysteic acids were shown on the paper electropherogram at pH 4.38 (400 v./cm.; 2½ hours). Paper electrophoresis of this peptide at pH 6.5 (80 v./cm.; 30 min.) showed it to travel with the same mobility as the standard cysteic acid. Amino acid analysis of the peptide showed it to contain only cysteic acid (0.029 µmole). It was thus concluded that cysteic acid was the C-terminal of peptide I-A-2T11.

From the data presented, peptides I-A-2T1 to I-A-2T11 are clearly smaller varieties of peptide I-A-2 (Table V), arising from multiple tryptic cleavages between the lysine and arginine residues of the latter peptide. The low positive mobility of peptide I-A-2, and low negative mobility of peptide I-A-2T11 are consistent with a positive and negative charge of 1, respectively, and it can be concluded that both acidic amino acid residues are amidated. It is thus possible to write the following sequence for peptide I-A-2:

Tyr-Lys-Arg-Gln-Leu-Leu-Asn-Cya.

Peptides I-A-3 and I-A-4

The separation of these peptides, shown in the diagonal "maps" (Figures 6 and 7, page 51), suggested that these two neutral peptides were appreciably cross contaminated. Upon further purification by electrophoresis at pH 1.8 and 3.5 they were obtained in pure form and had the composition shown in Table IV (page 51). The "dansyl" method yielded N-terminal valine for both peptides (Table IV). The amino acid composition, given in Table IV, suggested that peptide I-A-4 was a larger variety of I-A-3 in that it contained one

TABLE VI

AMINO ACID COMPOSITION AND SEQUENCE OF TRYPTIC PEPTIDES
FORMED BY TRYPSIN DIGESTION OF PEPTIDE I-A-3

(Values are Expressed as Mole Ratios)

Peptide	Electrophoretic mobility at pH 6.5 (m)	Amino Acid Composition and Sequence	Cadmium Ninhydrin Colour	Molecular Weight
I-A-3	0.00	Val-(Lys, Thr, Cya, Ala) 0.96→ 0.97 0.89 0.99 1.07	red	556.55
I-A-3T1	+ 0.70	Val- Lys 0.95→ 1.05→	red	245.34
I-A-3T2	- 0.69	Thr- Cya- Ala 0.95→ 1.02→ 1.02→	yellow to slow red	329.21

Definition of symbols: same as in Table V, page 53.

TABLE VII

AMINO ACID COMPOSITION AND SEQUENCE OF TRYPTIC PEPTIDES
FORMED BY TRYPSIN DIGESTION OF PEPTIDE I-A-4

(Values are Expressed as Mole Ratios)

Peptide	Electrophoretic mobility at pH 6.5 (m)	Amino Acid Composition and Sequence	Cadmium-Ninhydrin Colour	Molecular Weight
I-A-4	0.00	Val,(Thr, Val, Lys, Thr, Cya, Ala) 0.94 0.86 0.94 0.85 0.86 1.02 1.53	red	756.82
I-A-4T2	+ 0.44	Val - Thr - Val - Lys 1.05 0.90 1.05 1.00	red	545.60
I-A-4T6	- 0.67	Thr - Cya - Ala 0.95 1.02 1.02	yellow slow red	329.21

Definition of symbols: same as in Table V, page 53.

additional residue of valine and threonine. The partial sequence of these peptides can be written as:

I-A-3 Val-(Lys,Thr,Cya,Ala)

I-A-4 Val-(Thr,Val,Lys,Thr,Cya,Ala).

Additional evidence for the sequence of both of these peptides was obtained from tryptic digestion as follows: Peptide I-A-3 (0.70 μmole) was digested with TPCK-trypsin (0.014 μmole) in 2.2 ml. 0.05 M *N*-Ethylmorpholine formate buffer, pH 8.0, at 37° for 5 hours, and the reaction products were separated by high voltage paper electrophoresis at pH 6.5. Two major cadmium-ninhydrin positive bands were separated. The results of the electrophoretic mobilities, amino acid compositions and N-terminal determination by the Dansyl-Edman procedure of the tryptic peptides obtained are summarized in Table VI. From the data presented, it is possible to write the following structure for the I-A-3 peptide:

Val-Lys-Thr-Cya-Ala.

Similarly peptide I-A-4 (0.20 μmole) was degraded with trypsin (0.004 μmole)(ratio of trypsin to I-A-4, 1:50) and the reaction products were separated by high voltage paper electrophoresis at pH 6.5, as described *vide supra*. The two major cadmium-ninhydrin positive tryptic peptides I-A-4T2 and I-A-4T6 were isolated and Table VII summarizes their composition, electrophoretic mobilities, and sequence by the Dansyl-Edman procedure. Since peptides I-A-4T6 and I-A-3T5 had identical compositions, mobilities and N-terminal

sequence by the Dansyl-Edman method (Tables VI and VII), it was clear that peptide I-A-4T6 corresponded to the tryptic peptide I-A-3T2, namely:

Thr-Cya-Ala.

These results suggest that the heptapeptide I-A-4 may well be a larger variety of the pentapeptide I-A-3 and it contained two additional amino acid residues at the N-terminal end of peptide I-A-3, i.e. valine and threonine. To confirm the postulated N-terminal extension of this unique half-cystine sequence, the complete sequence of peptide I-A-4T6 by the Dansyl-Edman procedure was undertaken and the results are shown in Table VII. The sequence of peptide I-A-4 is therefore:

Val-Thr-Val-Lys-Thr-Cya-Ala,

and it was concluded that it was a larger variety of peptide I-A-3.

c. Peptides from Band I-B

Although there are three cysteic acid peptides in this band, it was difficult to isolate peptide I-B-1 in sufficient amounts because of its proximity to the diagonal and its large molecular weight. Its amino acid composition was equivocal, being contaminated with peptides lacking cysteic acid from the "diagonal" (Figures 6 and 7, page 51). Hence it is possible that peptide I-B-1 is a minor variety of the sequence represented in the diagonal "map" and it was ignored in the present study. Peptides I-B-2 and I-B-3 were isolated by cutting out band I-B from the original pH 6.5 electrophoresis, oxidizing the strips with performic acid vapour and repeating

TABLE VIII

AMINO ACID COMPOSITION AND SEQUENCE OF TRYPTIC PEPTIDES FORMED BY TRYPSIN DIGESTION OF PEPTIDE I-B-2

(Values are Expressed as Mole Ratios)

Peptide	Electrophoretic Mobility at pH 6.5 (m)	Amino Acid Composition and Sequence	Cadmium-Ninhydrin Colour	Mol. Wt.
I-B-2	- 0.14	Asn, (Gln , Lys , Ile , Cya , Gly , Gly) 0.97 1.00 0.92 0.95 1.00 1.00 1.00	orange	754.77
I-B-2T1	+ 0.47	Asn , Gln , Lys 0.96 1.07 0.96	orange	388.46
I-B-2T2	- 0.57	Ile , Cya , Gly , Gly 0.94 0.99 1.03 1.04	orange slow red	266.27

Definition of symbols: same as in Table V, page 53.

electrophoresis at pH 6.5 (80 v./cm.; 45 min.). The slightly acidic peptide I-B-2 was still impure at this stage, but it was further purified by electrophoresis at pH 1.8 (80 v./cm.; 45 min.). Table IV (page 51) shows the amino acid composition of these peptides.

Peptide I-B-2

After purification by electrophoresis at pH 6.5 and 1.8, samples were taken for N-terminal and amino acid analyses and the results are shown in Table IV. Since the mobility of I-B-2 was m = - 0.14, asparagine and not aspartic acid must be the N-terminal residue and the additional glutamic acid residue must be amidated. The composition, mobility, and N-terminus permit the assignment of the following structure to this peptide:

Asn-(Gln,Lys,Ile,Cya,Gly,Gly).

Further evidence for the sequence of I-B-2 was obtained from tryptic digestions. Digestion of I-B-2 (0.50 μmole) with TPCK-trypsin (0.01 μmole) in 1.0 ml. *N*-Ethylmorpholine formate buffer, pH 8.0, was carried out for 5 hours at 37°. The digestion products were applied as a 14 cm. wide band (0.04 μmole per cm.) to Whatman No. 1 filter paper. Separation of the digestion products by electrophoresis at pH 6.5 gave two cadmium-ninhydrin positive bands. The results of the electrophoretic mobilities, Dansyl-Edman degradation, and amino acid compositions of the tryptic peptides formed by trypsin digestion of peptide I-B-2 are summarized in Table VIII. From the data presented, the following sequence may be written for I-B-2:

Asn-Gln-Lys-Ile-Cya-Gly-Gly.

TABLE IX

AMINO ACID COMPOSITION AND SEQUENCE OF α-LYTIC PROTEASE PEPTIDES
FORMED BY α-LYTIC PROTEASE DIGESTION OF PEPTIDE I-B-3

(Values are Expressed as Mole Ratios)

Peptide	Electrophoretic mobility at pH 6.5 (m)	Amino Acid Composition and Sequence	Cadmium-Ninhydrin Colour	Mol. Wt.
I-B-3	- 0.25	Cya, Ile, Ala, Gly,(Ser, His, Ala, Val, Asn, Gly) 0.97→ 1.06→ 1.01→ 1.05→ 1.06 0.97 1.01 0.89 0.97 1.05	orange	954.90
I-B-3αLP1	+ 0.20	Ser, His, Ala, Val 0.88→ 1.04→ 1.04→ 1.04→	yellow	412.50
I-B-3αLP2	+ 0.07	His, Ala, Val 0.75→ 1.23→ 1.04→	orange	325.40
I-B-3αLP4	- 0.18	Asn, Gly 0.99→ 1.01→	yellow	189.19
I-B-3αLP5	- 0.67	Cya, Ile, Ala, Gly 1.09→ 0.73→ 1.09→ 1.09→	orange	398.34
I-B-3αLP6	- 0.70	Cya- Ile- Ala- Gly 0.98→ 0.98→ 1.07→ 0.98→	orange	398.34
I-B-3αLP7	- 0.74	Cya-(Ile- Ala- Gly, Ser) 0.73→ 1.28 1.54 0.76 0.41	orange	481.43

Definitions of symbols: same as in Table V, page 53.

Peptide I-B-3

The decapeptide I-B-3 gave a distinct yellow to orange colour with cadmium-ninhydrin stain, which is consistent with cysteic acid as the N-terminal residue (Table IV, page 51) found by the "dansyl" method. Its low mobility with respect to aspartic acid was m = - 0.25, suggesting that there was a net negative charge of 1 (Offord, 1966) and that asparagine rather than aspartic acid was present. The N-terminal sequence was shown, by three steps of the Dansyl-Edman procedure, to be:

Cya-Ile-Ala-Gly

and these results are consistent with the following sequence as shown in Table IX:

Cya-Ile-Ala-Gly(Ser,His,Ala,Val,Asn,Gly).

The sequence of peptide I-B-3 was also studied by digesting the peptic fragment (0.38 μmole) with α-lytic protease (generously supplied by Dr. D. R. Whitaker) in 1.1 ml. of 0.05 M *N*-Ethylmorpholine formate buffer, pH 8.0, at 37° for 5 hours (ratio of α-lytic protease to I-B-3, 1:50). The peptides produced were purified by electrophoresis at pH 6.5 (80 v./cm.; 45 min.). The relatively low positive mobility of I-B-3αLP2 compared to I-B-3αLP1 is attributable to a lower pK for imidazole when histidine is in the N-terminal position of a peptide. Thus at pH 6.5, the former peptide will bear a lesser positive net charge than the latter. The low negative mobility of I-B-3αLP4 is inconsistent with the presence of aspartic acid and can be attributed to an abnormally low pK for the α-amino

group of this peptide. This is a common observation with peptides containing N-terminal asparagine. An additional observation, as yet unexplained, is the slightly different electrophoretic mobilities measured for peptides I-B-3αLP5 and I-B-3αLP6. These two peptides had identical amino acid compositions and sequences as determined by the Dansyl-Edman procedure.

Since it was not possible to obtain the overlapping peptide I-B-3αLP7 in sufficient quantity for sequence determinations and because its amino acid composition was equivocal, it was difficult to determine the C-terminus peptide and to align I-B-3αLP1 and I-B-3αLP4 in order. C-terminal analysis of I-B-3 with carboxypeptidase A gave inconclusive results.

From the data presented in Table IX and the complete sequences of peptide fragments I-B-3αLP1, I-B-3αLP2, I-B-3αLP4, and I-B-3αLP5, the partial sequence of I-B-3 is therefore concluded to be:

Cya-Ile-Ala-Gly,(Ser-His-Ala-Val),(Asn-Gly).

d. Peptides of Band I-C

The neutral band was further purified by cutting out band I-C from the original preparative pH 6.5 electrophoresis, exposing the strip to performic acid vapours, and re-running at pH 6.5 (80 v./cm.; 45 min.). An alternative method of purification was by cutting out band I-C and re-running it at pH 6.5 for 5 hours (80 v./cm.), then oxidizing the strip with performic acid vapour and repeating the electrophoresis at pH 6.5 (80 v./cm.; 45 min.). However, during this alternative purification procedure heavy losses were encountered. The

TABLE X

AMINO ACID COMPOSITION OF NEUTRAL AND ACIDIC HALF-CYSTINE PEPTIC PEPTIDES ISOLATED FROM FRACTION I

(Values are Expressed as Mole Ratios)

Amino Acid	Constituent Amino Acid Residues Per Molecule of Peptide							
	I-C-2	I-C-3	I-C-4	I-C-6	I-C-7	I-D-1	I-G-1	I-I-3
Lysine	0.79	1.00	1.00					
Histidine.	1.05							
Arginine	0.66			0.98				
Cysteic Acid . . .	1.00	0.95	0.99	1.02	0.98	0.95	1.05	0.87
Aspartic Acid. . .	3.11			1.19		1.07	1.98	1.90
Threonine.	0.74							
Serine	1.69							
Glutamic Acid. . .	0.79	2.20	1.98					1.91
Proline.	1.85					0.93		
Glycine.	3.59			1.02		1.05		
Alanine.	3.17				2.02		0.97	1.03
Valine	2.27	1.20	1.03					
Isoleucine	1.27							
Leucine.						1.00		
Tyrosine		0.75						
No. of Residues	22	6	5	4	3	5	4	6
Mobility at pH 6.5	-0.25	-0.35	-0.40	-0.52	-0.69	-0.63	-0.91	-1.05
N-terminal		Tyr	Val	Gly	Ala	Cya	Asn	Asn
Percentage Yield	3.3	14.0	18.9	16.8	36.4	24.7	8.1	1.4

neutral band I-C gave five acid peptides I-C-2, I-C-3, I-C-4, I-C-6, and I-C-7 after oxidation. All of these were yellow with cadmium-ninhydrin reagent, and their composition, N-terminal analyses by the "dansyl" method, electrophoretic mobilities and percentage yields are shown in Table X. Peptide I-C-3 clearly is a larger variety of I-C-4, and peptides I-C-6 and I-C-7 must come from different sequences of the polypeptide chain of the protein.

Peptide I-C-2

Although this peptide was separated from the diagonal (Figure 7, page 51), it was difficult to isolate peptide I-C-2 in sufficient amounts because of its large molecular weight and its proximity to peptides just off the diagonal. Its amino acid composition was equivocal, being contaminated with peptides lacking cysteic acid (Figure 7) and it was ignored in the present study. The composition of this peptide taken together with its electrophoretic mobility suggest that peptide I-C-2 may be a larger variety of I-B-3 (*supra*, page 58).

Peptides I-C-3 and I-C-4

These two peptides had similar amino acid compositions except that peptide I-C-3 had an additional tyrosine residue. From their low electrophoretic mobility it was concluded that one of the two glutamic acid residues must be amidated (Offord, 1966). Since N-terminal analyses by the "dansyl" method gave tyrosine for I-C-3 and valine for I-C-4, the following partial sequences may be written for each of the peptic peptides:

TABLE XI

AMINO ACID COMPOSITION AND SEQUENCE OF TRYPTIC PEPTIDES FORMED BY TRYPSIN DIGESTION OF PEPTIDE I-C-4

(Values are Expressed as Mole Ratios)

Peptide	Electrophoretic mobility at pH 6.5 (m)	Amino Acid Composition and Sequence	Cadmium Ninhydrin Colour	Mol. Wt.
I-C-4	- 0.40	Val-(Lys , Cya , Glx , Glx) 1.03 1.01 0.99 0.99 0.99	orange slow red	641.62
I-C-4T1	+ 0.69	Val- Lys 1.00 1.00	orange red	245.34
I-C-4T5	- 1.03	Cya - Gln - Glu 0.96 1.02 1.02	yellow slow red	412.28

Definitions of symbols: same as in Table V, page 53.

I-C-3 Tyr-(Val,Lys,Cys,Glx,Glx)

I-C-4 Val-(Lys,Cys,Glx,Glx)

Tryptic digestion of these two peptides provided additional evidence for their amino acid sequences. Peptide I-C-4 (0.76 μmole) was digested with TPCK-trypsin (0.015 μmole) in 1.0 ml. of 0.05 M *N*-Ethylmorpholine formate buffer, pH 8.0, at 37° for 5 hours. The digest was separated by high voltage paper electrophoresis at pH 6.5. The results of the electrophoretic mobilities and amino acid compositions of the tryptic peptides so obtained are summarized in Table XI. N-terminal analyses showed that the partial sequences of these two peptides were as follows:

I-C-4T1 Val-Lys

I-C-4T5 Cya-(Glx,Glx).

The mobility of peptide I-C-4T5 with respect to aspartic acid was m = - 1.03, suggesting that there was a net negative charge of 2 (Offord, 1966), and that there was only one acetic acid residue amidated. After the first Edman step, the dipeptide was submitted to electrophoresis at pH 6.5, and its mobility (m = - 0.67) and molecular weight (258.26) were consistent with a net negative charge of 1. This material was subjected to a second Edman step and the degradation product was subjected to electrophoresis at pH 6.5, and the neutral band was further submitted to an additional pH 1.8 electrophoretic run. Glutamine standards were included during both electrophoretic purification steps. Cadmium-ninhydrin staining of both electropherograms revealed that the C-terminal residue had run in parallel with the glutamic acid standard at pH 6.5 electropherogram,

TABLE XII

AMINO ACID COMPOSITION AND SEQUENCE OF TRYPTIC PEPTIDES FORMED BY TRYPSIN DIGESTION OF PEPTIDE I-C-3

(Values are Expressed as Mole Ratios)

Peptide	Electrophoretic mobility at pH 6.5 (m)	Amino Acid Composition and Sequence	Cadmium-Ninhydrin Colour	Mol. Wt.
I-C-3	- 0.36	Tyr-(Val , Lys , Cys , Glx , Glx) 0.75 1.20 1.00 0.95 1.10 1.10	orange slow red	804.80
I-C-3T1	+ 0.50	Tyr- Val - Lys 0.65 1.30 1.04	orange slow red	441.98
I-C-3T5	- 1.03	Cys - Gln - Glu 0.93 1.03 1.03	yellow slow red	412.28

Definition of symbols: same as in Table V, page 53.

and there was no trace of glutamine at the pH 1.8 electropherogram. From the data presented the following sequence may be written for I-C-4T5 tryptic peptide:

Cya-Gln-Glu

and the sequence of I-C-4 is therefore concluded to be:

Val-Lys-Cya-Gln-Glu.

Peptide I-C-3 (0.56 μmole) was similarly digested with TPCK-trypsin (0.016 μmole) and the digestion products were separated into two cadmium-ninhydrin positive tryptic peptides, I-C-3T1 and I-C-3T5, on electrophoresis at pH 6.5. The results of the analyses of the amino acid composition, N-terminal determinations, and electrophoretic mobilities (Table XII) permit the assignment of the following structure to these tryptic peptides:

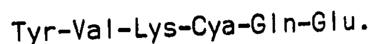
I-C-3T1 Tyr-(Val,Lys)

I-C-3T5 Cya-(Glx,Glx).

A molecular weight of 414.28 and the low electrophoretic mobility (m = - 1.03) of peptide I-C-3T5 indicated that there was a net negative charge of 2 (Offord, 1966). It was therefore concluded that only one glutamic acid was present as glutamine. These results were identical with those obtained for the tryptic peptide I-C-4T5 (Table XI, page 61), and the sequence of I-C-3T5 is therefore concluded to be:

Cya-Gln-Glu.

In summary it may be concluded that peptide I-C-3 is a larger variety of peptic peptide I-C-4 whose sequence has been previously elucidated. This sequence has now been extended and may be written as:

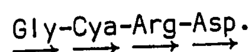


Tyr-Val-Lys-Cya-Gln-Glu.

Peptide I-C-6

This peptide, originating from the neutral region in the first dimension electrophoresis at pH 6.5, was purified as described above. From its amino acid analysis, electrophoretic mobility (Table X, page 60), and molecular weight (485.4) it was concluded that there was a net negative charge of 1 (Offord, 1966) and that aspartic acid was present rather than asparagine. This peptide (0.67 µmole) was not attacked by trypsin (ratio of trypsin to I-C-6, 1:50) and it was recovered from the paper electrophoresis, pH 6.5, intact.

The sequence of this peptide was determined by the Dansyl-Edman method, as shown below. Hence it was concluded that peptide I-C-6 had the unique half-cystine sequence:



Gly-Cya-Arg-Asp.

Peptide I-C-7

This peptide was purified as described above. Staining of the band with cadmium-ninhydrin reagent developed an orange colour that turned red. Because of its position in the diagonal (Figures 6 and 7, page 51) and small molecular weight (299.2), peptide I-C-7 was isolated in high purity and good yield, which on analysis of amino acid composition and N-terminus by the "dansyl" method gave the results shown in Table X (page 60). The sequence of this tripeptide

was determined by the Dansyl-Edman method and the final sequence may therefore be written as:

e. Peptides of Band I-D

This acidic band in the first dimension electrophoresis, pH 6.5, contained only one cysteic acid sequence and was purified by cutting out band I-D, exposing it to performic acid vapours, and re-running it at pH 6.5 electrophoresis (80 v./cm.; 45 min.).

Peptide I-D-1

The amino acid composition and electrophoretic mobility of this peptide are given in Table X (page 60). Staining of the band with cadmium-ninhydrin reagent developed a stable yellow colour, consistent with N-terminal cysteic acid as demonstrated by N-terminal analysis. Its position in the first dimension electrophoresis, pH 6.5, and mobility after oxidation (mol. wt. 533.0) indicated that there were two negative charges (Offord, 1966), consistent with one aspartic acid residue and one cysteic acid residue described in Table X. Sequence analysis by the Dansyl-Edman method showed that this peptide had the following structure:

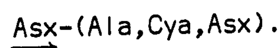
f. Peptides of Band I-G

This band, I-G, was cut from the preparative electropherograms, exposed to the vapour of performic acid, and the oxidized strips were stitched to full sheets of 3MM Whatman paper and electrophoresis was

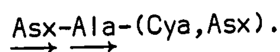
carried out at pH 6.5 (80 v./cm.; 45 min.) perpendicular to the original direction. Amino acid analysis of the single yellow acidic peptide showed it to be contaminated with glutamic acid, so it was subjected to an additional purification step at pH 3.5 (80 v./cm.; 50 min.).

Peptide I-G-1

The composition of this peptide (Table X, page 60) taken together with its mobility and molecular weight (458.3) suggested that there was a net negative charge of 2 (Offord, 1966) and that one acidic residue was amidated. It gave a yellow colour with the cadmium-ninhydrin reagent and yielded aspartic acid or asparagine as its N-terminal residue. It may therefore be written as:


Asx-(Ala,Cya,Asx).

The sequence of Peptide I-G-1 (0.22 µmole) was partially determined by the Dansyl-Edman procedure, and the results obtained are consistent with the sequence:

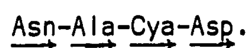

Asx-Ala-(Cya,Asx).

After the second Edman degradation step the material was applied as a 5 cm. band to Whatman No. 1 filter paper at a distance of 28 cm. from the cathode and was then purified by electrophoresis at pH 6.5 (80 v./cm.; 20 min.). Amino acid analysis gave:

(Cya,Asx)
1.00 1.00

Its electrophoretic mobility (m = - 1.25) and composition (mol. wt. = 272.1) suggested two negative charges (Offord, 1966) and

that the N-terminal of I-G-1 was asparagine rather than aspartic acid. The yellow colour of this dipeptide was consistent with an N-terminus cysteic acid which was confirmed by the "dansyl" method. After a single Edman degradation of this dipeptide, aspartic acid was demonstrated to be the only product by electrophoresis at pH 6.5. It was concluded that peptide I-G-1 had the sequence:



Asn-Ala-Cya-Asp.

g. Peptides of Band I-I

The acidic band, I-I, was cut from the preparative sheets and purified by paper electrophoresis under essentially the same conditions as Band I-G. This band was shown to contain only one cysteic acid peptide which was further purified by electrophoresis at pH 3.5 (80 v./cm.; 50 min.).

Peptide I-I-3

From its amino acid composition and electrophoretic mobility it was concluded that peptide I-I-3 was a larger variety of I-G-1 in that it contained one additional glutamic acid residue. As the peptide had an electrophoretic mobility, m = - 1.05 at pH 6.5, and a molecular weight of 587.4, it was concluded that peptide I-I-3 must contain one asparagine, one aspartic acid and one glutamic acid. The N-terminal group was shown to be either aspartic acid or asparagine, and the partial sequence could thus be written as:

Asn-(Ala,Cya,Asp,Glu).

The peptide I-I-3 (0.08 µmole) was subjected to one stage of the Dansyl-Edman degradation and the product was purified by paper

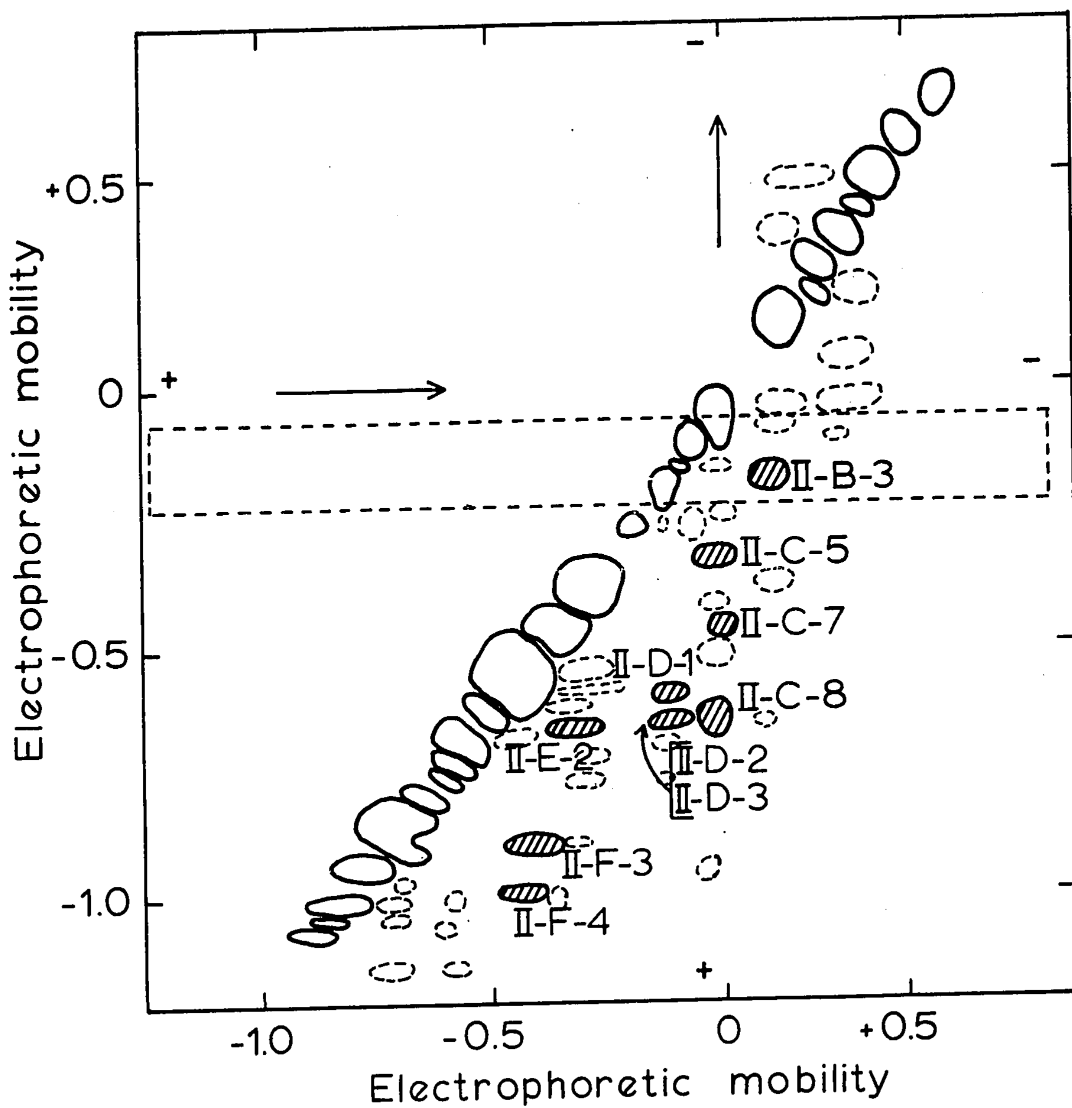


Figure 8. Diagonal Peptide "Map" of Peptic Digest of Phosphorylast *b* Fraction II.
Electrophoresis was at pH 6.5 in both dimensions. The numbered hatched spots correspond to the designation used in Table XIII.

electrophoresis at pH 6.5 (80 v./cm.; 20 min.). The composition of this peptide,

(Ala, Cya, Asp, Glu)
1.00 1.00 0.82 1.18

taken together with its mobility at pH 6.5 (m = - 1.2; mol. wt. = 472.33), suggest that there is a net negative charge of 3, indicating the presence of one aspartic acid and one glutamic acid.

Because of the small amount of peptide I-I-3 left after Stage 1, the remaining material was submitted to partial sequence determination by the Dansyl-Edman procedure. The partial sequence of I-I-3 could thus be written as:

$\underrightarrow{\text{Asn}}$-$\underrightarrow{\text{Ala}}$-$\underrightarrow{\text{Cya}}$-(Asp,Glu).

Since asparagine was shown to be the N-terminal to both peptides, I-G-1 and I-I-3, this extra glutamic acid residue must be at the C-terminal end of I-I-3. This unique half-cystine sequence has now been extended by the data presented and may be written as:

Asn-Ala-Cya-Asp-Glu.

(2) Fraction II

This fraction emerged as a well separated peak from the gel-filtration column (Figure 5, page 50). A typical pH 6.5/pH 6.5 diagonal "map" of Fraction II that originated from the diethanol disulfide interchanged peptic digest (*supra*, page 34) of phosphorylase *b* is shown in Figure 8. On such a diagonal fingerprint the cysteic acid peptide spots found off the diagonal are hatched.

TABLE XIII

AMINO ACID COMPOSITION OF HALF-CYSTINE PEPTIC PEPTIDES
ISOLATED FROM FRACTION II

(Values are Expressed as Mole Ratios)

Amino Acid	Constituent Amino Acid Residues Per Molecule of Peptide									
	II-B-3	II-C-5	II-C-7	II-C-8	II-D-1	II-D-2	II-D-3	II-E-2	II-F-3	II-F-4
Lysine	0.96	1.19								
Arginine			0.86							
Cysteic Acid	0.88	0.82	0.94	1.06	0.61	1.06	0.89	1.03	0.95	0.81
Aspartic Acid. . . .	1.05	1.19	1.15		1.19	1.13	0.91		2.02	1.37
Serine							0.91			1.00
Glutamic Acid. . . .	2.10	2.76								
Glycine.	2.01	0.82	1.05		1.19					
Alanine.		0.82		1.94		0.81	1.28	1.97	1.03	0.81
Proline.					1.02					
Valine		0.90								
Isoleucine	0.99									
Leucine.					0.94					
Tyrosine		0.52								
Tryptophan	+									
No. of Residues	9	10	4	3	5	3	4	3	4	4
Mobility at pH 6.5	-0.18	-0.37	-0.48	-0.67	-0.63	-0.68	-0.71	-0.72	-0.94	-1.03
Molecular Weight	1072.1	1178.2	804.8	299.2	533.0	343.2	431.2	299.2	464	546.4
N-terminal	Asx	Tyr	Gly	Ala				Ala	Asx	
Percentage Yield	0.8	0.4	2.5	9.6	0.2	0.14	0.9	0.4	6.2	1.5

Certain resemblances immediately become apparent, and therefore the nomenclature of cysteic acid peptides and the corresponding peptide bands in the first dimension electrophoresis at pH 6.5 are similar to that used for Fraction I (Figures 6 and 7, page 51). Some bands, i.e., II-A, II-G, II-H, and II-I are missing because the corresponding cysteic acid peptides were either present in insufficient quantity or were minor varieties of sequences represented in the diagonal "map" and they were ignored in the present study.

a. Peptides of Band II-B

Band II-B was cut from the original preparative pH 6.5 electrophoresis sheets, exposed to the vapour of performic acid, and the oxidized strips were re-run at pH 6.5. The only cysteic acid peptide, II-B-3, that was derived from the basic region stained orange with cadmium-ninhydrin reagent that developed slowly to a red colour. The other peptides of Band II-B contained no cysteic acid upon analysis.

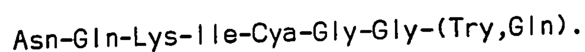
Peptide II-B-3

This peptide was eluted from the preparative sheets and was finally purified by electrophoresis at pH 1.8 (80 v./cm.; 45 min.). The resulting recoveries, amino acid composition, and electrophoretic mobility are shown in Table XIII. The amino acid composition indicated that it contained eight amino acid residues. It also appeared to contain an oxidized tryptophan as judged by its strong fluorescence on paper. Its electrophoretic mobility suggested that there were one asparagine and two glutamines present, rather than

the corresponding acids. Its slight anionic mobility at pH 6.5 (m = - 0.18) after oxidation was not unexpected for peptide II-B-3 with N-terminal asparagine. This peptide was therefore tentatively assigned the sequence:

Asn-(Gln,Lys,Ile,Cya,Gly,Gly,Try,Gln).

It was also evident from the data presented in Table XIII that the peptide II-B-3 is a larger variety of peptide I-B-2 (Table VIII, page 57) arising from multiple pepsin cleavages of the same half-cystine sequence. It is thus possible to write the following structure for II-B-3:


Asn-Gln-Lys-Ile-Cya-Gly-Gly-(Try,Gln).

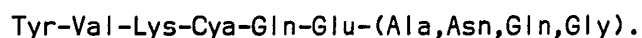
Additional evidence for the complete sequence of this peptide was obtained in subsequent work and is presented in Chapter III (Table XXVIII, page 113).

b. Peptides of Band II-C

This neutral band was cut from the original preparative pH 6.5 electrophoresis sheets and re-run at pH 6.5 (80 v./cm.; 5 hours). The sub-bands thus separated were exposed to vapours of performic acid and the oxidized strips were further purified by electrophoresis at pH 6.5. Thus, band II-C separated into a number of cadmium-ninhydrin positive spots. Of these, only peptides II-C-5, II-C-7, and II-C-8 were recovered in amounts adequate for further characterization (Table XIII, page 68).

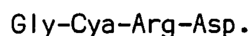
Peptide II-C-5

From its amino acid analysis, N-terminal and electrophoretic mobility (m = - 0.37) (Table XIII, page 68), it was concluded that peptide II-C-5 was a larger variety of I-C-3 and I-C-4 in that it contained one additional residue of each of asparagine, glutamine, alanine, and glycine. Since tyrosine was shown to be the N-terminal for I-C-3 and II-C-5, these three extra residues must be at the C-terminal end of II-C-5, and the partial sequence of this peptide may be written as:


Tyr-Val-Lys-Cya-Gln-Glu-(Ala,Asn,Gln,Gly).

Peptide II-C-7

This peptide had the composition, electrophoretic mobility, and N-terminal summarized in Table XIII (page 68) and these properties of II-C-7 are identical to those reported for peptide I-C-6 (Table X, page 60). Since glycine was shown to be the N-terminal residue for both of these peptides it is evident that they are identical. It is thus possible to write the following structure for the tetrapeptide II-C-7:


Gly-Cya-Arg-Asp.

From its small recovery shown in Table XIII, it is apparent that Fraction I cross-contaminated Fraction II during chromatography of the peptic digest of phosphorylase on Sephadex G-25 columns.

Peptide II-C-8

Peptides II-C-8 and I-C-7 are clearly identical by all criteria. The apparent identity of these two peptides, shown in

Tables X and XIII (pages 60 and 68), and the presence of peptide II-C-8 in Fraction II, suggests that cross contamination between Fractions I and II (Figure 5, page 50) does occur, as a result of incomplete separation of these two fractions by gel-filtration.

c. Peptides from Band II-D

In the diagonal "map" of Fraction II (Figure 8, page 67) the cysteic acid peptides were well resolved. For this reason this band from the original electrophoresis, pH 6.5, was cut out, subjected to vapours of performic acid, and the band was then re-run at pH 6.5. Each of the three cysteic acid peptides indicated (Figure 8) was isolated as described above, and Table XIII (page 68) summarizes the results obtained for their composition, mobilities, and percentage yields.

Peptide II-D-1

This peptide is clearly identical to the corresponding peptide I-D-1 of Fraction I. Peptide II-D-1 was recovered in low yields from Fraction II and it might be possible that these two fractions were cross-contaminated as described previously.

Peptides II-D-2 and II-D-3

The properties of these two peptides are summarized in Table XIII (page 68). No further analysis of these peptides was attempted since they were recovered in too low yields for full characterization. It is possible that peptide II-D-2 is a smaller variety of I-G-1 (Table X, page 60). However, peptide II-D-3 may have arisen from a minor contaminating protein of this phosphorylase preparation.

d. Peptides from Band II-E

This band contained only one cysteic acid peptide and it was purified from this band as shown in the diagonal "map" of Fraction II (Figure 8, page 67) and the data obtained is shown in Table XIII.

Peptide II-E-2

This tripeptide, present on the pH 6.5/pH 6.5 diagonal "map" of one peptic digest experiment, was not observed when similar digests of other enzyme preparations were performed. It appeared from its amino acid composition that it had an identical structure with that of peptide I-C-6 (Table X, page 60). It is possible that this peptide was oxidized to cysteic acid during the preparation of phosphorylase and in this form it separated with Fraction II.

e. Peptides from Band II-F

This band was cut from the original electropherograms pH 6.5, oxidized by performic acid and re-run at pH 6.5, isolated, and the results are shown in Table XIII (page 68).

Peptide II-F-3

This peptide gave a transient yellow colour which turned red when treated with cadmium-ninhydrin reagent on paper. Its N-terminal residue, amino acid composition, and mobility, suggest that this peptide is identical with peptide I-G-1. Hence the sequence of the peptide is assumed to be:

Asn-Ala-Cya-Asp.

TABLE XIV

AMINO ACID COMPOSITION OF HALF-CYSTINE PEPTIC PEPTIDES
ISOLATED FROM FRACTIONS III, IV, AND V

(Values are Expressed as Mole Ratios)

Amino Acid	Constituent Amino Acid Residues Per Molecule of Peptide									
	III-B-2	III-B-3	III-C-7	III-C-9	III-D-2	III-G-3	III-H-2	III-I-2	IV-C-2	V-C-1
Lysine	1.15									
Histidine		1.35								
Cysteic Acid	0.88	1.20	1.05	1.00	1.03	0.82	1.02	1.14	0.97	1.40
Aspartic Acid	1.10	1.12			0.96	2.35	2.03	1.96		
Serine		0.98					0.88	0.78	0.81	0.85
Glutamic Acid	1.51	2.92				1.63	0.98	3.02	0.97	0.72
Glycine	2.41	3.52					1.07	1.10	1.12	0.98
Alanine			1.95			1.29				
Isoleucine	0.95	0.90								
Tryptophan	+									
Phenylalanine						0.91			1.12	
No. of Residues	8	11	3	1	2	7	6	8	5	4
Mobility	-0.15	-0.16	-0.67	-1.06	-0.78	-1.10	-1.26	-1.12	-0.67	-0.80
Molecular Weight	943.0	765.7	299.2	157	272.1	864.7	661.5	919.8	577.5	430.3
N-terminal					Asx	Glx				
Percentage Yield	2.7	0.5	0.9	2.9	3.5	0.9	1.1	0.5	0.01	0.02

TABLE XV

SUMMARY OF THE NUMBER AND AMINO ACID SEQUENCE
OF HALF-CYSTINE PEPTIDES FROM RABBIT MUSCLE PHOSPHORYLASE *b*

No. of Unique Sequence	Peptide	Sequence	Percentage Yield
1	I-A-3 I-A-4	Val-Thr-Val-Lys-Thr-Cya-Ala	27.6
2	I-B-2,II-B-3 III-B-2	Asn-Gln-Lys-Ile-Cya-Gly-Gly(Try,Gln)	14.0
3	I-C-3,I-C-4 II-C-5	Tyr-Val-Lys-Cya-Gln-Glu	32.9
4	I-C-7,II-C-8 II-E-2 III-C-7	Ala-Cya-Ala	36.4
5	I-C-6,II-C-7	Gly-Cya-Arg-Asp	16.8
6	I-G-1,I-I-3 II-F-3	Asn-Ala-Cya-Asp-Glu	9.5
7	I-A-2	Try-Lys-Arg-Gln-Leu-Leu-Asn-Cya	6.3
8	I-B-3,I-C-2	Cya-Ile-Ala-Gly,(Ser-His-Ala-Val),(Asn-Gly)	12.3
9	I-D-1,II-D-1	Cya-Asp-Pro-Gly-Leu	24.7

Definition of symbols: Cya, represents cysteic acid.

Peptide II-F-4

This peptide was difficult to match with any of the unique half-cystine peptides whose sequence was previously elucidated because its amino acid composition showed it to contain one extra serine residue. It was distinguishable on the diagonal "map" (Figure 8, page 67) by the orange colour with cadmium-ninhydrin reagent. Attempts to determine the N-terminal residue of II-F-4 were unsuccessful. The peptide was subjected to one subtractive Edman degradation step, and the product was purified by electrophoresis at pH 6.5. Analysis of its amino acid composition was equivocal and no further attempts were made to characterize it.

(3) Fractions III, IV, and V

Fractions III, IV, and V did not contain any half-cystine peptides that could be recovered in amounts adequate for full characterization. Fraction III gave seven minor peptides, III-B-2, III-B-3, III-C-7, III-D-2, III-G-3, III-H-2, and III-I-2, and Fractions IV and V gave one each of IV-C-2 and V-C-1. Table XIV summarizes the results obtained on their amino acid compositions, percentage yields, and electrophoretic mobilities. Some of these minor peptides corresponded to unique half-cystine sequences previously elucidated (Table XV). However, peptides III-H-2, III-I-2, IV-C-2, and V-C-1, recovered in very low yields, had an amino acid composition that was incompatible with the known cysteic acid sequences and these results are discussed below.

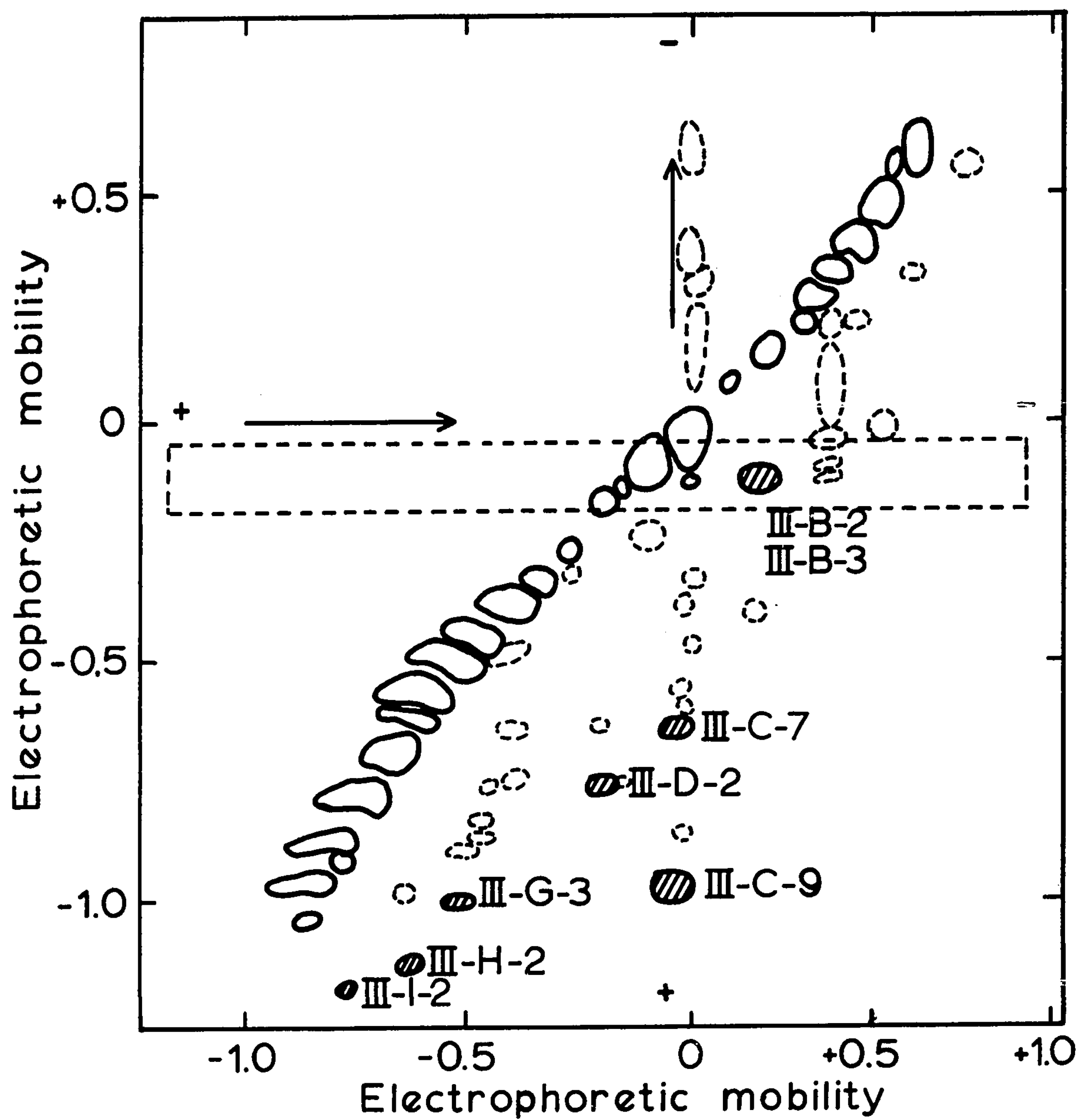


Figure 9. Diagonal Peptide "Map" of Peptic Digest of Phosphorylase *b* Fraction III.
Electrophoresis was at pH 6.5 in both dimensions. The cysteic acid peptides are indicated by the hatched spots and the nomenclature corresponds to the designations used in Table XIV, page 73.

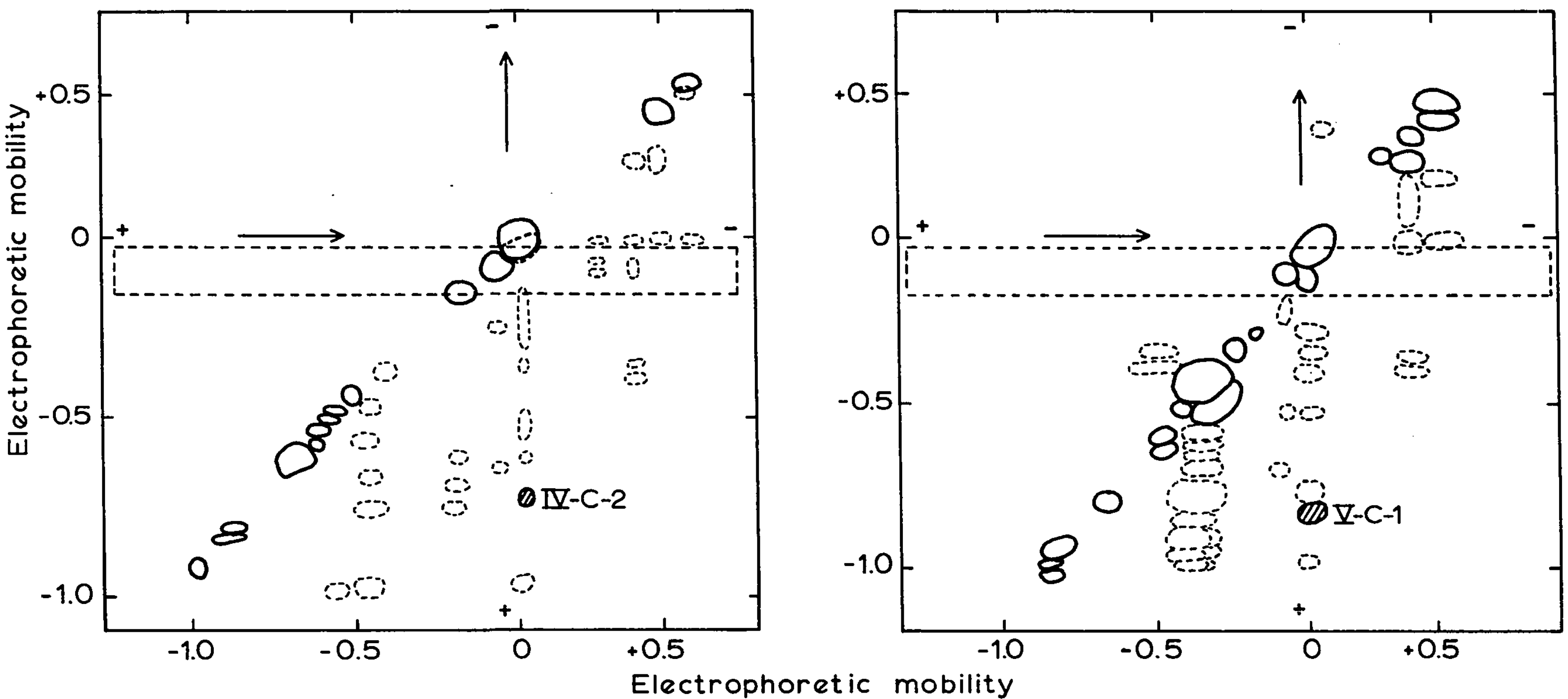


Figure 10. Diagonal Peptide "Maps" of Peptic Digest of Phosphorylase *b* of Fractions **IV** (*left*) and **V** (*right*).
Electrophoresis was at pH 6.5 in both dimensions. Conditions, abbreviations and definition of symbols are given in the text, and in Table XIV, page 73.

a. Minor Components in Fractions III, IV, and V

The pH 6.5/pH 6.5 diagonal "map" of Fraction III is shown in Figure 9, and those of Fractions IV and V are shown in Figure 10. In addition to the cysteic acid peptides III-B-2, III-B-3, III-C-7, III-C-9, III-D-2, III-G-3, III-H-2, and III-I-2 lying off the diagonal (Figure 10) there were several fluorescent peptides off these diagonals that appeared to contain an oxidized tryptophan residue which altered their mobility characteristics. These fluorescent cadmium-ninhydrin positive peptides were most pronounced in the diagonals of Fraction IV and in particular of Fraction V. Where amino acid analyses indicated the absence of cysteic acid, these peptides were not studied further.

Peptide III-B-2

This peptide was purified by a second pH 6.5 electrophoresis after oxidation, followed by electrophoresis at pH 3.5 and 1.8. From its amino acid analysis (Table XIV) and its essentially zero mobility, it is suggested that peptide III-B-2 had one asparagine and one glutamine residue present rather than the corresponding acids. It is also possible that an additional glutamine may be present. Its strong fluorescence suggested that this peptide contained an oxidized tryptophan. The yellow colour developed with cadmium-ninhydrin and the low anionic mobility at pH 6.5 (m = - 0.15) after oxidation indicated an asparagine as its N-terminal residue. It was thus concluded that peptide III-B-2 was a larger variety of I-B-2 in that it contained an additional tryptophan and glutamine

residue at the C-terminus. These results are consistent with the sequence:

Asn-(Gln,Lys,Ile,Cys,Gly,Gly,Try,Gln).

Peptide III-B-3

This peptide had a practically identical mobility with that of III-B-2 and they were appreciably cross contaminated. For this reason bands containing III-B, after oxidation, were subjected to electrophoresis at pH 6.5, 3.5, and 1.8, in which case the peptide was obtained in adequate amounts for amino acid analysis (Table XIV, page 73). From its electrophoretic mobility and amino acid composition, it is possible that III-B-3 is a larger variety of peptide I-B-3 (Tables IV and IX, pages 51 and 58).

Peptide III-C-7

The tripeptide III-C-7 had the same amino acid composition (Table XIV, page 73), mobility, and colour as those reported for peptides I-C-7 (Table X, page 60) and II-C-8 (Table XIII, page 68). It was concluded that peptide III-C-7 had the sequence:

Ala-Cya-Ala.

Although these peptides, I-C-7, II-C-8, and III-C-7 were found to have an identical structure, their separation in different distinct peaks on Sephadex G-25 chromatography (Figure 5, page 50) is as yet unexplained. The reason for this different separation can be of considerable interest. Ideally gel-filtration is considered to separate on the basis of size, and the method is regarded to be a countercurrent diffusional process. It might be possible that the

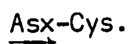
apparent distribution of peptides I-C-7, II-C-8, and III-C-7 in three distinct peaks (Figure 5, page 53) may be due to adsorption or other unexplained effects. For instance, the presence of peptide II-C-8 in Fraction II could be expected as a result of incomplete resolution between Fractions I and II (Figure 5). However, Fraction III is well resolved from both of these fractions. An alternative explanation may be that interactions of III-C-7 with components of the system greatly complicate the separation of these otherwise identical peptides.

Peptide III-C-9

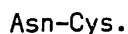
Paper electrophoresis of this fragment at pH 6.5 showed that it migrated with the same mobility as the standard cysteic acid. Amino acid analysis showed it to contain only cysteic acid. It is possible that this may have risen by peptic cleavage of any of the unique sequences, since it was isolated from diethanol disulfide interchange experiments.

Peptide III-D-2

From its amino acid analysis (Table XIV) and the identification of its N-terminal aspartic acid or asparagine, it was concluded that peptide III-D-2 had the sequence:



The yellow colour developed with cadmium-ninhydrin on paper and its electrophoretic mobility (m = - 0.78) is consistent with N-terminal asparagine and therefore the sequence is:



This dipeptide is a smaller variety of peptide I-A-2 (Tables IV and V) or alternatively may be an N-terminal extension of I-B-3 and I-D-1.

Peptide III-G-3

From the data shown in Table XIV (page 73) this peptide may be a larger variety of I-G-1. However, its electrophoretic mobility suggests that its acidic amino acid residues are fully charged. The peptide had a net negative charge of 4 (Offord, 1966), inconsistent with the sequence around I-G-1. After one stage subtractive Edman degradation two peptides were separated by high voltage electrophoresis, which on amino acid analysis gave equivocal results. The peptide was recovered in very low yield and was not investigated further.

Peptides III-H-2, III-I-2, IV-C-2, and V-C-1

These peptides were purified as shown in Figures 9 and 10 (page 74) and their properties are summarized in Table XIV (page 73). They were isolated in very low yields and had amino acid compositions that could not match with any of the unique sequences reported in this chapter (Table XV, page 73). Because they were isolated in low yields, these peptides provided ambiguous evidence for the presence of unique half-cystine sequences in addition to those reported and summarized in Table XV. However, because of the presence of two unique amino acid residues in these four peptides (serine and glycine), and their variable molecular weights (Table XIV) it is suspected that these may be varieties of the same sequence. They may arise from the N-terminal ends of either peptides I-B-3 or I-D-1,

owing to a minor peptic cleavage between an as yet unknown residue and cysteic acid. In fact, in Fraction I, a minor anionic cysteic acid peptide (m = - 1.22; mol. wt. = 530.4), I-J-2, was isolated in very low yields (0.5 per cent) having the following composition:

(Glx, Asx, Glx, Cya)
1.01 1.17 1.01 0.78

Partial amino acid sequence by the Dansyl-Edman procedure suggests the sequence:

Glx-Asx-Glx-(Cya)

As this peptide had a mobility of m = - 1.22, and net negative charge of 3, one of the acidic acid residues must have been present as the amide. This peptide, I-J-2, was never again isolated in quantities large enough that its structure could be fully characterized. An alternative explanation is that these minor fragments may arise from peptic cleavages of proteins contaminating certain phosphorylase *b* preparations.

4. DISCUSSION

The elucidation by the Cori's (Keller and Cori, 1955; Madsen and Cori, 1956) that skeletal muscle phosphorylase *a* and *b* consist of four and two subunits, respectively, has produced an increased interest in the enzyme in recent years. Physicochemical studies leading to the suggestion that the subunits of phosphorylase are identical (Table II, page 19) have already been mentioned. Further evidence in support of this suggestion has come from several lines

of investigation including the isolation of the unique peptides involved in the binding of phosphate, Pyridoxal-5-phosphate and AMP.

However, the possibility of non-identity of subunits has been suggested by the results of Seery *et al.* (1967) and Valentine and Chignell (1968). Furthermore, much concerning the chemistry of this protein is still unknown and evidence, for example, on the end terminal residues of the protein is not available. A preliminary search for the C-terminal and N-terminal residues by Appleman *et al.* (1963) proved negative, suggesting that each peptide chain is circular or blocked at either of the ends.

From the work of Madsen and Cori (1956) it was indicated that the thiol groups of phosphorylase are of considerable importance to the structure and biological properties of the enzyme, and its cysteine content is relatively low. Following these investigations, the number of half-cystine residues, measured as cysteic acid, has been estimated to be 9 per molecular weight of 92,500 (Appleman *et al.*, 1963; Battell *et al.*, 1968a).

Thus, the experiments for the isolation and determination of the number of unique half-cystine peptides and their sequence in the phosphorylase molecule were designed to derive information on the basic structural subunit of the enzyme. For the isolation of peptic half-cystine peptides, the diagonal electrophoretic technique was originally employed. This method was not directly applicable to the elucidation of sulfhydryl sequences of phosphorylase because of its high molecular weight and the instability of sulfhydryl residues during the electrophoretic separation procedures.

It was therefore decided to disulfide interchange the peptic digest of phosphorylase b with cystine-3-^{14}C. This method had the advantage that the thiol groups were protected from oxidation during the isolation procedures of half-cystine peptides, although it was necessary to dilute the reaction mixture so that cystine-3-^{14}C, in the concentrations used for complete substitution, would remain in solution. The disulfide diethanol reagent had the advantage over cystine-3-^{14}C in that it was completely soluble and did not interfere with subsequent purification procedures. A disadvantage in the diethanol disulfide reaction was that the final yields of purified peptides were low.

Peptic digestions of phosphorylase have been performed successfully throughout this work and the peptic peptides thus produced were completely soluble. However, pepsin in its action on the protein has been found to produce families of peptides arising from the same sequence. Although this complicated the diagonal electrophoretic procedure for the isolation of all cysteic acid peptides in good yields, it did not hinder the purification procedures. Further complications were observed in situations where pepsin cleaved the susceptible peptide bond formed by tryptophan at the amino group. The N-terminal tryptophan containing peptides, upon oxidation, acquired one or two negative charges. These ninhydrin-negative fluorescent peptides were found off the pH 6.5 diagonal, and in many cases they were contaminating to a considerable extent the cysteic acid peptides. Similar observations with N-terminal tryptophan peptides were reported by Brown and Hartley (1966).

For these reasons, the peptic digests, after disulfide interchange reactions, were subjected to a preliminary sub-fractionation on a Sephadex G-25 column. The results showed that most of the cysteic acid peptides were recovered from Fraction I by this procedure. As is evident from Figures 4 and 5 (pages 49 and 50), the remaining Fractions II to V in the chromatographic profile showed high absorbancies at 280 mμ and 260 mμ indicating that most of the aromatic-containing peptides were retarded by the column. Thus, after the preliminary sub-fractionation on the Sephadex G-25 column, each fraction was then submitted to the diagonal electrophoretic procedure and the resulting "maps" were simplified considerably. The results of the diagonal "maps" are given in the text and in Figures 6 to 10 (pages 51, 67, and 74). Each cysteic acid peptide, thus located, was isolated and further purified by electrophoresis at pH 6.5 or 1.8 and 3.5, in which case very pure peptides were obtained. Careful characterization of these half-cystine peptides was made by the Dansyl-Edman procedure as described by Gray and Hartley (1963a; 1963b). This method was extremely valuable for determining the sequences of the peptides isolated in this work.

Table XV (page 73) summarizes the number and amino acid sequence of the unique half-cystine peptides isolated from Fractions I to V of the peptic digests of skeletal rabbit muscle phosphorylase *b*. As previously described and as is evident from Table XV, these sequences were labelled from number 1 to number 9 unique sequences in the publication of Zarkadas *et al.* (1968) for simplicity of presentation.

For example, the peptide designated I-A-4 is the same peptide as that designated I-A-3, except that it is a larger variety of number 1 unique half-cystine sequence. Similarly peptides II-B-3 and II-B-2 are larger varieties of peptide I-B-2 and the evidence for extending number 2 unique half-cystine sequence is presented in Chapter III (Table XXVIII, page 113). Peptides I-C-3 and I-C-5 are clearly larger varieties of I-C-4 all arising from number 3 unique sequence. It is also evident that peptides I-C-7, II-C-8, II-E-8, and III-C-7, that belong to number 4 unique sequence, are clearly identical by all criteria. Peptides I-C-6 and II-C-7 are identical and correspond to number 5 unique sequence which has now been extended by the work presented in Chapter III (Table XXIX, page 113).

It may be noted that in the unique half-cystine sequences, numbers 7 to 9, cysteic acid is situated either in the N-terminal or C-terminal position. These results are consistent with the broad specificity of pepsin and the susceptibility of peptide bonds formed by the amino or carboxyl groups of cysteine. Pepsin can split the bond on either side of this residue, an observation which was further confirmed by the isolation of cysteic acid (III-C-9, page 76) from diethanol disulfide interchanged peptic digests of phosphorylase. The low recoveries of the unique half-cystine sequences, ranging from 6.0 to 36.0 per cent of the theoretical are accounted for by the low specificity of peptic cleavage and by the large number of purification steps by paper electrophoresis. It is also due to the losses associated with adsorption to and elution from paper.

From the data presented previously it was shown that all of the major cysteic acid peptides were located and fully characterized, accounting for all nine unique half-cystine sequences in phosphorylase (Table XV, page 73) from Fraction I. At this stage of the investigation it was easy to conclude that the remaining cysteic acid peptide fragments should be minor varieties of the sequences described above and should be found in Fractions II, III, IV, and V. Nonetheless, extensive investigations were conducted, and approximately 140 peptides were isolated and analyzed. Some of these minor peptides corresponding to the 9 unique half-cystine sequences have been reported in this thesis to illustrate the extent of these investigations and to show that no cysteic acid peptide has been overlooked.

However, it should also be noted that peptides I-J-2, II-F-4, III-H-2, III-I-2, IV-C-2, and V-C-1 had an amino acid composition that did not match the known cysteic acid sequences. Two reasons were given for the presence of these peptides. First, they were attributable to minor fragments which may have arisen from contaminating proteins. Second, from the results reported on the partial sequence of peptide I-J-2, it is suspected that these peptides may arise from the N-terminal ends of either peptide I-B-3 or I-D-1. It was also suspected that peptide I-C-2 may be a larger variety of I-B-3. Since these peptides were recovered in very low yields (Tables XIII and XIV, pages 68 and 73) and did not match any of the sequences reported, they were ignored in this study.

From the experimental evidence described and the results presented in Table XV (page 73), it can be concluded that there is a minimum of 8 and a maximum of 9 unique half-cystine sequences in phosphorylase. These results provide additional evidence that the subunits of phosphorylase are identical. The possibility remains, of course, that there exist minor differences in the subunits which do not involve residues in the immediate vicinity of the half-cystines, the ε-*N*-pyridoxylphosphatelysine moiety or the phosphorylated serine. Nor do the present results exclude the existence of identical subunits composed of non-identical polypeptide chains of molecular weight of less than 92,500. However, there is no reliable evidence for such a possibility.

CHAPTER III

THE REACTIVE SULFHYDRYL PEPTIDES

1. INTRODUCTION

In the previous chapter it was shown that there is a minimum of eight and a maximum of nine unique sulfhydryl sequences in each of the apparently identical subunits of rabbit skeletal muscle glycogen phosphorylase (Zarkadas *et al.*, 1968).

The reactivity of these thiol groups has been investigated with a variety of reagents (Battell *et al.*, 1968a). On the basis of the extent and rate of incorporation of iodoacetamide-1-^{14}C into the protein, it was concluded that, of these nine sulfhydryl groups, one reacts at a rate which is at least as fast as that of model compounds and without loss of enzymic activity. The next two sulfhydryl groups in each monomer react much more slowly with iodoacetamide, and their alkylation is correlated with a loss of enzymatic activity and a dissociation of the original dimer of phosphorylase *b* or tetramer of phosphorylase *a*. The remaining six sulfhydryl groups are not available for reaction until the protein is denatured.

From the known sequences of the nine sulfhydryl groups (Zarkadas *et al.*, 1968) it was possible to isolate and identify the two cysteinyl peptides which are alkylated concomitantly with the loss of activity. Thus, the two radioactive peptides were isolated from pepsin digests and were characterized sufficiently

to identify them as Ala-Cya-Ala (peptide N) and Asn-Ala-Cya-Asp (peptide A)(Battell *et al.*, 1968b). The radioactive area on the chromatograms corresponding to the basic peptides (B_1 and B_2) whose alkylation did not affect activity, was not readily amenable to purification on paper by high-voltage electrophoresis. However, from a tryptic digest of the partially purified peptides, a basic radioactive fragment (Gly,CMC,Arg) was isolated in low yields having a composition and electrophoretic mobility consistent with a sequence previously elucidated (Zarkadas *et al.*, 1968), namely Gly-Cya-Arg-Asp (peptide I-C-6). Thus, it was tentatively concluded that the rapid incorporation of approximately one mole of iodoacetamide-1-^{14}C per mole of phosphorylase subunit, without loss of enzymic activity, was associated with the alkylation of this single sulfhydryl sequence (Battell *et al.*, 1968b).

However, careful purification and characterization of the basic peptides has now demonstrated, by the results of experiments to be discussed in this chapter, that these peptides are a mixture of two unique half-cystine sequences--Gly-Cya-Arg-Asp-(Val,Pro)-Arg-Thr-(Asn,Phe); and Asn-Gln-Lys-Ile-Cya-Gly-Gly-Try-Gln-Ser--both of which are extended sequences of peptides I-C-6 and I-B-2 elucidated in the previous chapter.

2. MATERIALS AND METHODS

A. Materials

Iodoacetamide was purchased from Sigma Chemical Company, St. Louis, Missouri, U. S. A. Iodoacetamide-1-^{14}C (26.8 mc. per

mmole) was obtained in 0.1 to 0.2 mc. quantities from the Radiochemical Center, Amersham, England, and it was recrystallized with an excess of non-radioactive iodoacetamide from hot water, prior to use, as follows: Unlabelled iodoacetamide (0.2 mg.) carrier was dissolved in 0.2 to 0.3 ml. of hot water and was then transferred to the vial containing 0.1 to 0.2 mc. iodoacetamide-1-^{14}C. The dissolved materials were then diluted with hot water, the reagent mixture was allowed to crystallize in an ice bath, and the crystals were filtered out and dried in a dessicator under vacuum. The purified material gave about 5.0 µc. per mmole. The cation exchange resin, Chromobeads Type P, equivalent to Dowex 50-X4, is a product of Technicon Corporation, Ardsley, New York, U. S. A. The anion exchange resin, Cellex D, equivalent to DEAE-cellulose (0.9 ± 0.05 m equiv. per g.), was obtained from Bio-Rad Laboratories, Richmond, California, U. S. A. All other chemicals and enzymes have been described in this thesis (Chapter II, page 28) or were reagent grade and were used without further purification.

B. Methods

(1) Preparation of Muscle Phosphorylase *b*

Crystalline phosphorylase *b* was prepared from the back and hind leg muscles of adult female rabbits of the New Zealand white strain by the method of Fischer and Krebs (1962) as described in this thesis (Chapter II, page 30) except that in the final purification procedure the following modification was made. Prior to use,

the crystals were centrifuged out of suspension, the supernatant was removed and the crystals were then dissolved in 0.2 M sodium β-glycerophosphate, 0.0015 M EDTA, 0.135 M KCl, and 0.0015 M 2-mercaptoethanol buffer pH 7.5. The concentrated protein solution was then passed through a Sephadex G-25 column (40 cm. x 2.5 cm.) pre-equilibrated with the same buffer. Protein concentration was determined spectrophotometrically using a value for $E^{1\%}_{1cm.}$ of 13.2 calculated by Buc and Buc (1967) from the data of Appleman *et al.* (1963) as described in this thesis (Chapter II, page 31) except that the buffer described in this section was used. Phosphorylase *b* activity was determined by the method of Cori *et al.* (1943) and Illingworth and Cori (1953) as described in this thesis (Chapter II, page 31).

(2) Alkylation of Protein with Iodoacetamide-1-^{14}C

The procedure for the alkylation of phosphorylase *b* with iodoacetamide-1-^{14}C was the following. A sample of recrystallized iodoacetamide-1-^{14}C (28.67 mg.; 1.55 mM) was dissolved in 15.0 ml. buffer and added to the protein (1.65 g.; 9.03 μmole) dissolved in 135.0 ml. of the same buffer (pH 7.5). The reaction was carried out under constant temperature (30°) and pH 7.5, the final concentrations being:

Phosphorylase *b*	11.1 mg./ml.	= 1.08 mM phosphorylase - SH groups
Iodoacetamide-1-^{14}C	0.19 mg./ml.	= 1.32 mM
2-mercaptoethanol		0.04 mM

EDTA	1.5 mM
KCl	0.135 M
sodium β-glycerophosphate	0.20 M

The molar concentrations of phosphorylase *b* were calculated on the basis of a molecular weight of 185,000 daltons (Seery *et al.*, 1967; De Vincenzi and Hedrick, 1967; Ullmann *et al.*, 1958; Buc and Buc, 1967). To terminate the reaction, a small volume of the buffer containing a fifty-fold molar excess of 2-mercaptoethanol over iodoacetamide, was added to the reaction mixture. Enzyme samples were withdrawn at 0, 5, 20, 40, 60, 100, and 120 minute intervals and their activity was determined as previously described (Chapter II, page 31).

The incorporation of radioactivity into the protein was measured in three ways. Samples of protein were precipitated with trichloroacetic acid and collected on either Gilman trioacetate or Millipore filters as described by Battell *et al.* (1968a). After four washes with 5 per cent TCA, each filter was sucked dry, transferred to a scintillation vial and the radioactivity measured in the usual way (*vide infra*). Radioactivity was also measured after dialyzing the samples exhaustively against distilled water. The formation of S-carboxamidomethyl cysteine was also estimated by determination of the S-carboxymethyl cysteine on the amino acid analyzer after acid hydrolysis.

(3) Pepsin Digestion of Protein

After alkylation, the solution containing the protein in pH 7.5 buffer was adjusted to pH 2.0 with formic acid (99.8 per

cent v/v). The entire reaction mixture was transferred to 8/32 Visking tubing and dialyzed against three changes of 5 per cent (v/v) formic acid for 24 hours at room temperature (21°). Following dialysis, the protein solution was digested with pepsin (ratio of pepsin to protein 1:10) for 24 hours at 37°. The digest was then freeze-dried and redissolved in 5 per cent (v/v) formic acid at a concentration of approximately 16 mg. per ml.

(4) Peptide Purification Procedures

a. Gel-Filtration

The column (250 cm. x 5 cm.) was prepared in two sections, from two Sephadex precision borosilicate glass columns 100 and 150 cm. long, fitted with adapted end pieces, nylon nets and bed support screens, and mounted at the same level and connected in series with polyethylene tubing (1.0 to 1.4 mm. I.D.). The Sephadex G-25 (200 mesh "fine" beads) was allowed to swell in 0.05 M acetic acid solution, pH 2.0, the very fine particles were removed, the gel slurry and solutions were de-aerated and then packed and equilibrated with the same solution by the procedure recommended by Flodin (1961). A 25 ml. aliquot of the pepsin digest sample containing about 400 mg. of the peptides was applied to the column, and was then eluted with 0.05 M acetic acid at a flow rate of about 204 ml. per hour and at room temperature (21°). The eluate was collected in 17.0 ml. fractions. The fractionation was monitored by radioactive counting on 200 µl. aliquots of the effluent fractions, by absorbancy measurements at 280 mµ, and by electrophoretic separation of portions of

the effluent on paper at pH 6.5. The effluent fractions containing radioactive peptides were combined and recovered by lyophilization, transferred to vials, and stored at -10° for further separation by ion exchange chromatography.

b. Cellex D Chromatography

The general procedure described by Peterson and Sober (1956) for the separation of proteins by ion exchangers was adapted for peptide purification with volatile developers on a preparative scale. The anionic resin, Cellex D, was purified according to Peterson and Sober (1956) and equilibrated with a 0.05 M *N*-Ethylmorpholine-formic acid buffer, pH 8.0. The slurry was de-aerated and packed into a 100 cm. x 2 cm. (Chromaflex 20, Kontes Glass Company, Vineland, New Jersey, U. S. A.) column. Fractions containing between 2.0 to 2.5 μmole radioactive peptides from Sephadex G-25 gel-filtration columns were dissolved in 5.0 ml. of the starting buffer and applied to the column. After application, a linear gradient elution was generated by an admixture of 250 ml. volumes of equilibration buffer and 0.5 M *N*-Ethylmorpholine-formate buffer, pH 8.0, contained in a two-chambered apparatus of identical cross section. The peptides were eluted at a flow rate of 24 ml. per hour, at room temperature, and fractions of 4.0 ml. were collected in a Beckman automatic fraction collector.

c. Chromobead Type P Chromatography

The general procedure recommended by Schroeder *et al.* (1962) was used. The cation exchange resin, Chromobead Type P (Dowex 50-X4),

equilibrated with 0.2 M pyridine-acetic acid buffer, pH 3.1, was packed in a 0.6 cm. x 110 cm. column. The appropriate peptide fractions, cut from the Cellex D fractionation step, were dissolved in 2 ml. of the equilibration buffer and the solution was applied to the column. The column was connected to a two-chambered gradient elution apparatus in which both chambers were of identical cross-section and contained equal volumes (335 ml.) of the equilibration buffer and 2 M pyridine-acetic acid buffer, pH 5.0 (Schroeder *et al.*, 1962). The peptides were then eluted at 40° by application of the linear gradient at a flow rate of 18 ml. per hour. Fractions of 3.0 ml. were collected. The fractionation was monitored by radioactivity counting, and whenever economy of materials permitted, by ninhydrin analysis after alkaline hydrolysis of suitable aliquots by the method recommended by Schroeder *et al.* (1962). The radioactive fractions from ion exchange column chromatography were concentrated by freeze-drying as described in this thesis (Chapter II, page 37).

(5) Counting of Radioactivity

For routine determinations of radioactive elution profiles, 200 µl. aliquots of the effluent fractions were transferred to a scintillation vial and the samples were counted in a Nuclear-Chicago Mark I liquid-scintillation counter as previously described.

Where incorporation of iodoacetamide-1-^{14}C into the protein was being determined from radioactivity, 100 µl. aliquots of the modified protein, after dialysis, were transferred to a scintillation

vial. The radioactivity was determined in the usual way (*supra*, page 31) and the amount of radioactivity incorporated into the protein was then calculated.

When the radioactive peptides were purified by paper electrophoresis on Whatman No. 1 or 3 MM filter paper, they were located by the usual staining procedures as described in the Methods (page 37) and were also analyzed for radioactivity as follows: Prior to or after staining, the side strips were evenly cut 3 cm. wide across the length of the paper. Two aliquots (1 to 2 µl.) of radioactive India ink (markers) were spotted as a 2 mm. spot at the center of the filter paper and at a distance of 3 to 5 cm. from either end of the side strip. After drying, several such strips were joined end to end with adhesive tape (Scotch Brand Transparent Tape), and the strips were analyzed for radioactivity with a Nuclear-Chicago Actigraph III strip scanner with 4π geometry, the final settings being:

950 volts
50 seconds
300 counts per minute
3 mm. slit width
15 to 30 cm. per hour.

The radioactive peptide was eluted with water and the solution was adjusted to 1.0 ml. An aliquot (10 to 20 µl.) was transferred to a scintillation vial and the radioactivity was counted as previously described (*supra*, page 31). Thus, knowing the specific activity of iodoacetamide-1-^{14}C (6.07×10^5 d.p.m./µmole) and from measurements

of radioactivity of any given sample, the amount of S-carboxamidomethylated cysteine was calculated. Hence, the concentration of any given peptide sample could be quantitatively determined.

(6) Amino Acid Analysis

Quantitative determination of the amino acid composition of radioactive peptides was made on a Beckman Spinco Model 120C automatic amino acid analyzer as described in this thesis (Chapter II, page 41). Since recoveries of S-carboxymethylcysteine were often low in peptides eluted from paper electropherograms, the yield of this derivative was also calculated from measurement of the ^{14}C content of the peptide. S-carboxymethylcysteine emerged at 96 ml. off the 55 cm. column near aspartic acid and was quantitatively determined by the procedure recommended by Moore and Stein (1963).

Where incorporation of iodoacetamide-1-^{14}C into the protein was being determined, the modified protein samples were transferred to 8/32 Visking tubing for dialysis against 5 per cent formic acid for 36 hours, to remove excess iodoacetamide, 2-mercaptoethanol and buffers. Samples of the enzyme containing 3.0 to 4.0 mg. were transferred into Pyrex glass tubes (15 mm. x 150 mm.) and freeze-dried. The protein samples were then hydrolyzed in vacuo at 110° for 22 hours with 3 to 4 ml. of constant boiling HCl. S-carboxamidomethylcysteine, the product of the reaction of sulfhydryl with iodoacetamide-1-^{14}C, which is completely converted to S-carboxymethylcysteine by hydrolysis, is sensitive to oxygen and the usual precautions during evacuation were observed as described by Moore and Stein (1963).

The hydrolysates, concentrated in the hydrolysate tube on a rotary Evapo-Mix (Buchler Instruments, Inc., Fort Lee, New Jersey, U. S. A.), were analyzed on a Beckman Spinco Model 120B automatic amino acid analyzer equipped with the accelerated system. S-carboxymethylcysteine emerged at 96 ml. off the 55 cm. column near aspartic acid and was quantitatively determined from an analysis of an aliquot of the concentrated hydrolysate sample (1.5 to 2.0 mg.). Alanine and leucine however were determined from a second analysis using a diluted hydrolysate aliquot (0.2 to 0.3 mg.). The recovery of S-carboxymethylcysteine was calculated relative to 58 and 75 residues of alanine and leucine respectively (Appleman *et al.* (1963). To obtain values for phosphorylase *b* dimer, the reported values for the monomer unit must be multiplied by two.

(7) The Sequence Methods

N-terminal residues were determined by their reaction with DNS chloride as described by Gray and Hartley (1963). This method, coupled with the Edman procedure, developed by Gray and Hartley (1963a;1963b)was used (*supra*, page 41) for sequence analysis of the purified peptides.

3. RESULTS

A. Nomenclature of Peptides

The sequence of purification steps for the isolation of the S-carboxamidomethylated peptides were the following: (1) Gel-filtration on Sephadex G-25. Four fractions were designated A to D.

TABLE XVI

EXTENT OF S-CARBOXAMIDOMETHYLATION OF PHOSPHORYLASE *b*

Time of incubation (minutes)	Specific activity units/mg. protein	Moles of iodoacetamide-1-^{14}C incorporated per mole of phosphorylase *b* (mol. wt. 185,000)	
		Trichloroacetic acid method of Battell *et al.* (1968a)	After 24 hours dialysis of the protein
0.5	1860	0.104	
5.0	2020	0.843	
20.0	2240	1.515	
40.0	2090	1.610	
60.0	2200	1.600	
80.0	-	1.600	
100.0	2080	1.680	
120.0	2130	1.580	
120.0			3.089

(2) Chromatography on Cellex D. Each radioactive peak in order of elution was assigned a Roman numeral. (3) Chromatography on Chromobeads Type P. Each radioactive peak in order of elution was designated by an Arabic number. The products of peptides subsequently digested with trypsin have been designated by the letter T. An Arabic number is assigned to each of the products to indicate the relative mobility of the peptide on electrophoresis at pH 6.5, the most basic peptide of the digest having the lowest number. As an example, peptide D-II-4T2 was obtained from fraction D of the Sephadex G-25 gel-filtration step, was the second peak upon chromatography on Cellex D, and was eluted as the fourth radioactive peak on Chromobeads Type P chromatography. After trypsin digestion of this fraction it was recovered as the second most basic component upon electrophoresis at pH 6.5.

B. Extent of Incorporation of Iodoacetamide-1-^{14}C into Phosphorylase *b*

Battell *et al.* (1968a) have previously reported the incorporation of 1.6 moles of iodoacetamide per mole of phosphorylase *b* without inactivation of the enzyme. Radioactivity was measured by the TCA precipitation method and the results are presented in Table XVI. However when the bulk of the material upon the termination of the experiment was dialysed exhaustively against distilled water and the radioactivity measured, the extent of incorporation was almost double that previously reported (Table XVI). It thus appeared that the TCA precipitation method gave low results for estimations of

TABLE XVII

SUMMARY OF THE EXTENT OF S-CARBOXAMIDOMETHYLATION AND AMINO ACID ANALYSES OF THE S-CARBOXYMETHYL CYSTEINE OF PHOSPHORYLASE *b*

Time of incubation	Specific activity units/mg. protein	Residues per dimer of phosphorylase *b* (mol. wt. 185,000)		
		Moles of S-carboxymethyl cysteine residues per mole dimer	Moles of iodoacetamide-1-^{14}C incorporated per mole dimer	
			After dialysis	TCA Method
5	2,124[a]	1.062	0.870[a]	
20	2,058	1.644	1.655	
60	1,974	2.128	1.880	
120	2,010	2.180	2.020	1.17[a]

[a]The author is indebted to Dr. N. B. Madsen and Dr. Olga Avramovic-Zikik for permission to quote the unpublished observations in this Table.

of iodoacetamide-1-^{14}C incorporated and that the extent of incorporation was in fact greater than 1.6 moles per mole of phosphorylase *b* previously reported.

To investigate this question further, an additional experiment was performed in collaboration with Dr. N. B. Madsen and Dr. O. Avramovic-Zikik. Enzyme samples from a different phosphorylase *b* preparation were allowed to react with iodoacetamide-1-^{14}C for 5, 10, 60, and 120 minutes under the same conditions as previously described (page 88). The extent of incorporation was measured by the TCA precipitation method, by amino acid analysis of the protein, and by measurement of the radioactivity after thorough dialysis. The results presented in Table XVII show that the TCA precipitation method gives low results as compared to the other two methods which are in reasonable agreement. The lower incorporation of slightly in excess of 2.0 moles of iodoacetamide-1-^{14}C per mole of phosphorylase *b* as compared to 3.1 moles in the previous experiment can probably be accounted for by the ages of the two phosphorylase *b* preparations (4 months and 1 week respectively). The results from these two experiments, taken together, indicate that there is more than one highly reactive cysteine residue per monomer unit of phosphorylase *b* which can be alkylated without affecting the enzymic activity of the protein.

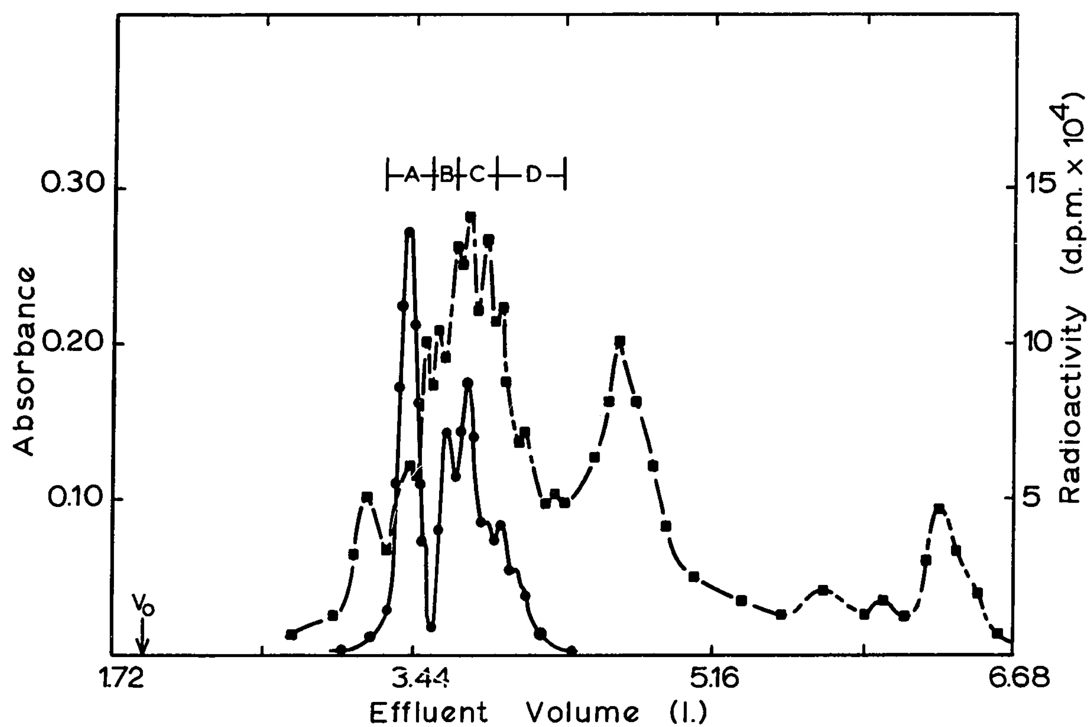


Figure 11. Fractionation of Peptic Digest of S-carboxamidomethylated Phosphorylase *b* (0.4 gm.) on a Sephadex G-25 Column. The effluent was monitored for radioactivity (●——●) and absorbancy measurements at 280 mμ (■— —■). Fractions A to D were taken as shown by the vertical.

TABLE XVIII

QUANTITATIVE RECOVERIES OF S-CARBOXAMIDOMETHYLATED PEPTIC PEPTIDES BY GEL-FILTRATION

(Total radioactivity in the peptic digest: $17,025 \times 10^3$ disintegrations per minute; 28.05 μmole of sulfhydryl groups per 9.08 μmole protein)

Radioactive fractions from Sephadex G-25 columns	Recoveries		Percentage recovery
	disintegrations per minute ($\times 10^6$)	μmole calculated from radioactivity	
A	5.31	8.75	31.3
B	2.16	3.56	12.7
C	3.83	6.31	22.6
D	1.98	3.26	11.7
Total	13.28	21.88	78.3

C. Gel-Filtration of the Peptic Digest of ^{14}C-Carboxamidomethylated Phosphorylase *b*

After dialysis, the ^{14}C-carboxamidomethylated protein was digested with pepsin as described in the Methods (page 89). Group fractionation of the soluble peptic peptides was carried out by gel-filtration on a Sephadex G-25 column (250 cm. x 5 cm.) as described in the Methods (page 90). Although the maximal load capacity of such a column was never determined, up to 400 mg. of peptic peptides were separated, and the elution profile curves of four such separations showed remarkable reproducibility (Figure 11). Four fractions labelled A to D were prepared by pooling the column eluates as indicated by the vertical lines in Figure 11. The pooled fractions were concentrated, and the radioactivity incorporated in each fraction was measured as described in the Methods (page 92). The results of the gel-filtration step, summarized in Table XVIII, indicated that the overall recovery of radioactivity was 78 per cent. Further purification and characterization of the S-carboxamidomethylated peptic peptides were carried out and the results are presented in the succeeding sections.

D. Purification and Characterization of the C^{14}-Labelled Peptides

Preliminary high-voltage paper electrophoretic separation showed that each fraction contained more than one radioactive peptide, some of which overlapped and in nearly all instances required further

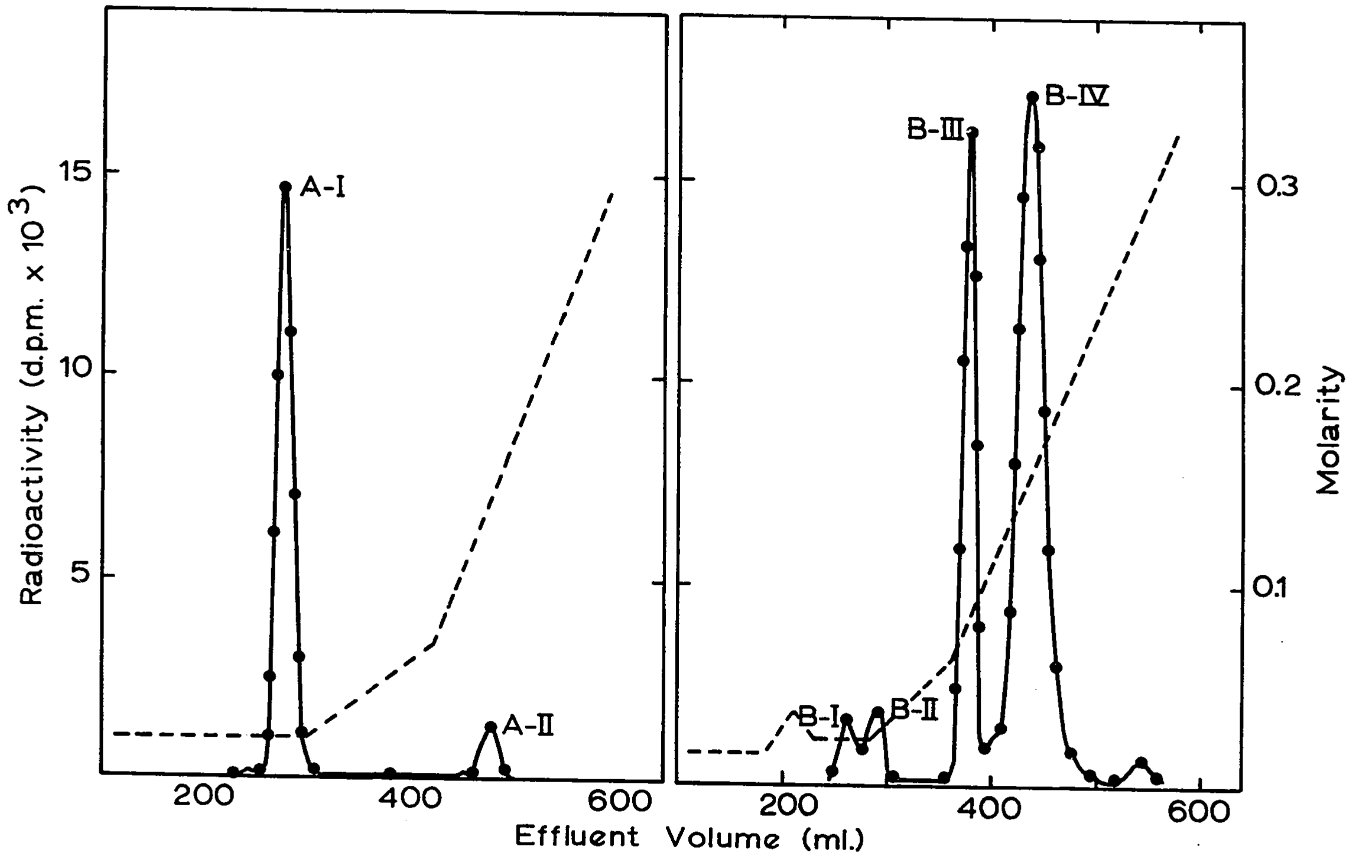


Figure 12. Chromatography of Radioactive Peptide Fractions A (*left*) and B (*right*) on 100 cm. x 2 cm. Cellex D Column Developed by *N*-Ethylmorpholine Gradient of Increasing Ionic Strength, pH 8.0.
The effluent was monitored for radioactivity (●——●). The gradient is indicated by the broken line, the numbered peaks corresponding to the designations used in the text.

TABLE XIX

QUANTITATIVE RECOVERIES OF S-CARBOXAMIDOMETHYLATED PEPTIC PEPTIDES BY CELLEX D CHROMATOGRAPHY

Radioactive Fractions	Percentage Recovery
A-I	24.4
A-II	2.2
B-III	2.8
B-IV	6.3
C-I	14.8
C-II	4.3
D-II	8.8
Total	63.6

purification. Therefore, after lyophilization, each of the four fractions so obtained was further purified by Cellex D and Chromobead Type P Chromatography in succession employing volatile buffers, and satisfactory results have been obtained by these procedures. This method takes advantage of the different conditions prevailing in the two types of chromatography, so that peptides that were coincident on the anion exchange column were well separated on the cation exchanger. In all instances final purification of the radioactive peptic peptides was attained by high-voltage electrophoresis. By this means pure peptides were obtained and the results are presented separately below.

(1) Fraction A

This fraction emerged as a well separated symmetrical peak from the gel-filtration column (Figure 11) and when taken to dryness (646.1 mg.) it contained 8.75 µmole of radioactive peptides. Of the material recovered a 6.45 µmole sample was rechromatographed on Cellex D columns by means of *N*-Ethylmorpholine gradient of increasing ionic strength, pH 8.0. In order to accommodate the large quantity of sample used in this study, it was necessary to conduct three such column chromatographic separations, as described in the Methods (page 91). A typical chromatogram is shown in Figure 12. By monitoring the radioactivity, ninhydrin reaction, and absorption at 280 mµ of the effluent, it was found that Fraction A was resolved into two radioactive peaks labelled Fraction A-I and Fraction A-II. On the basis of radioactivity the resulting recoveries are shown in Table XIX.

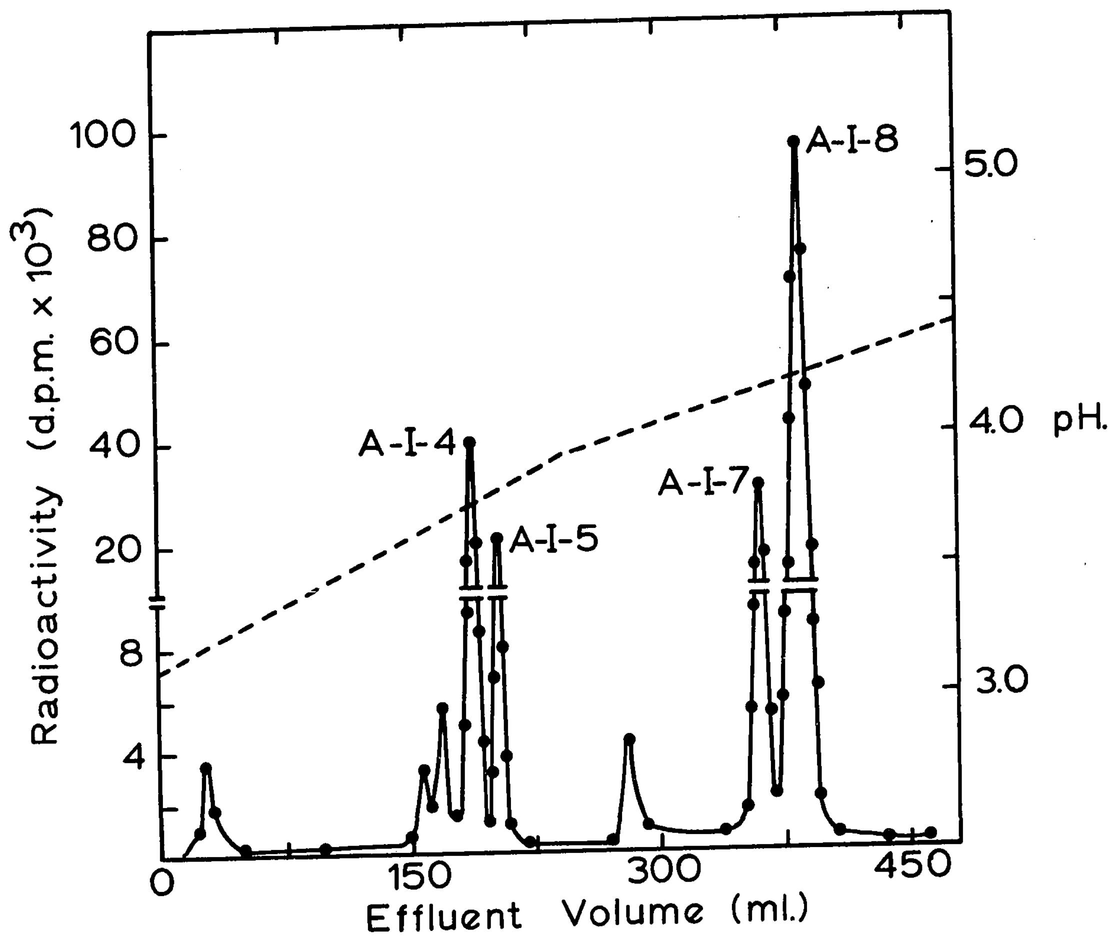


Figure 13. Chromatography of Peptides of Fraction A-I on Chromobead Type P Column.
The effluent was monitored for radioactivity (●——●). The pH gradient is indicated by the broken line, the numbered peaks corresponding to the designations used in Table XX.

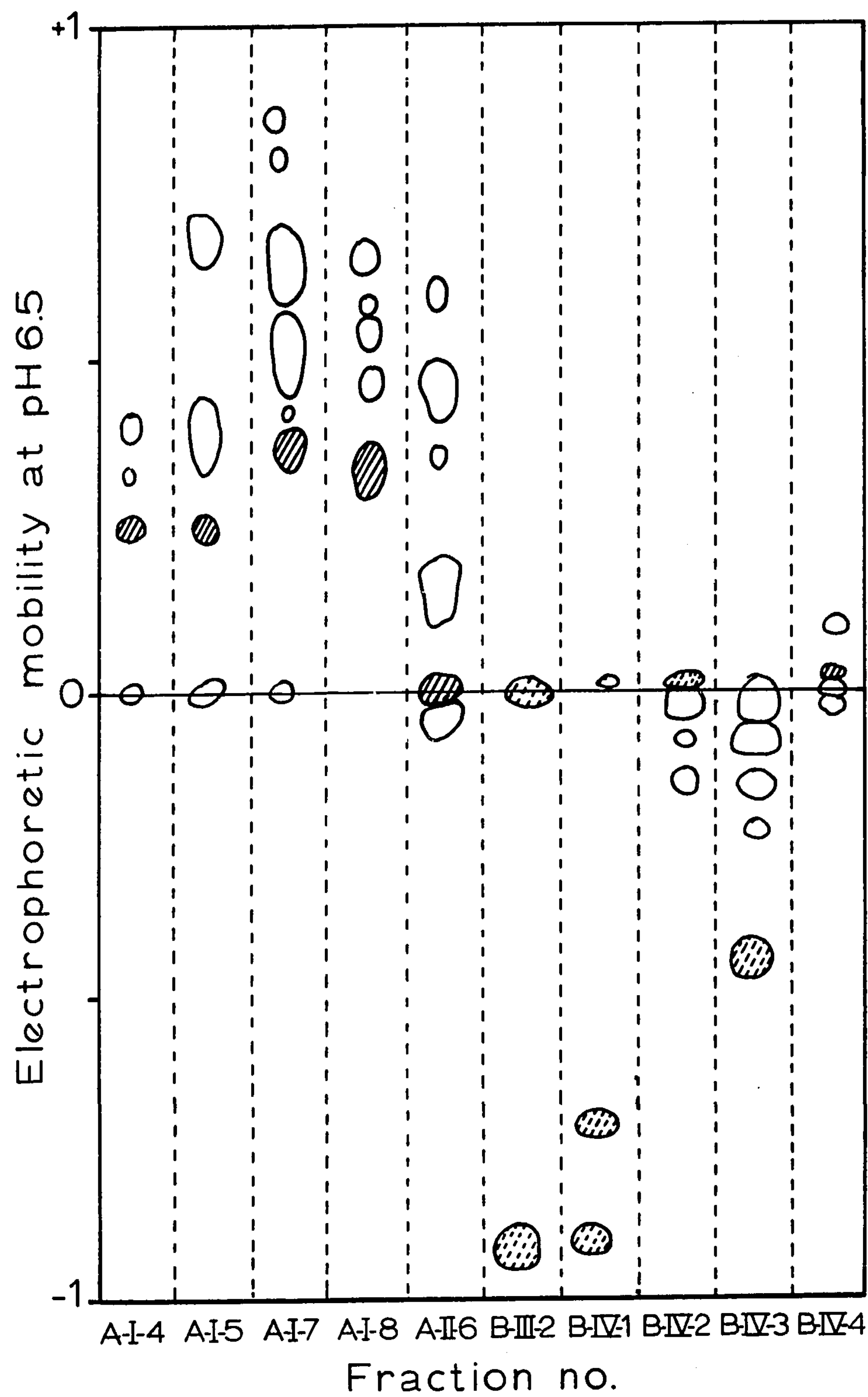


Figure 14. Radioactive Peptide Bands Arising From Fractions A and B Obtained by Electrophoresis at pH 6.5. The major peptides are hatched and the minor components in broken hatching.

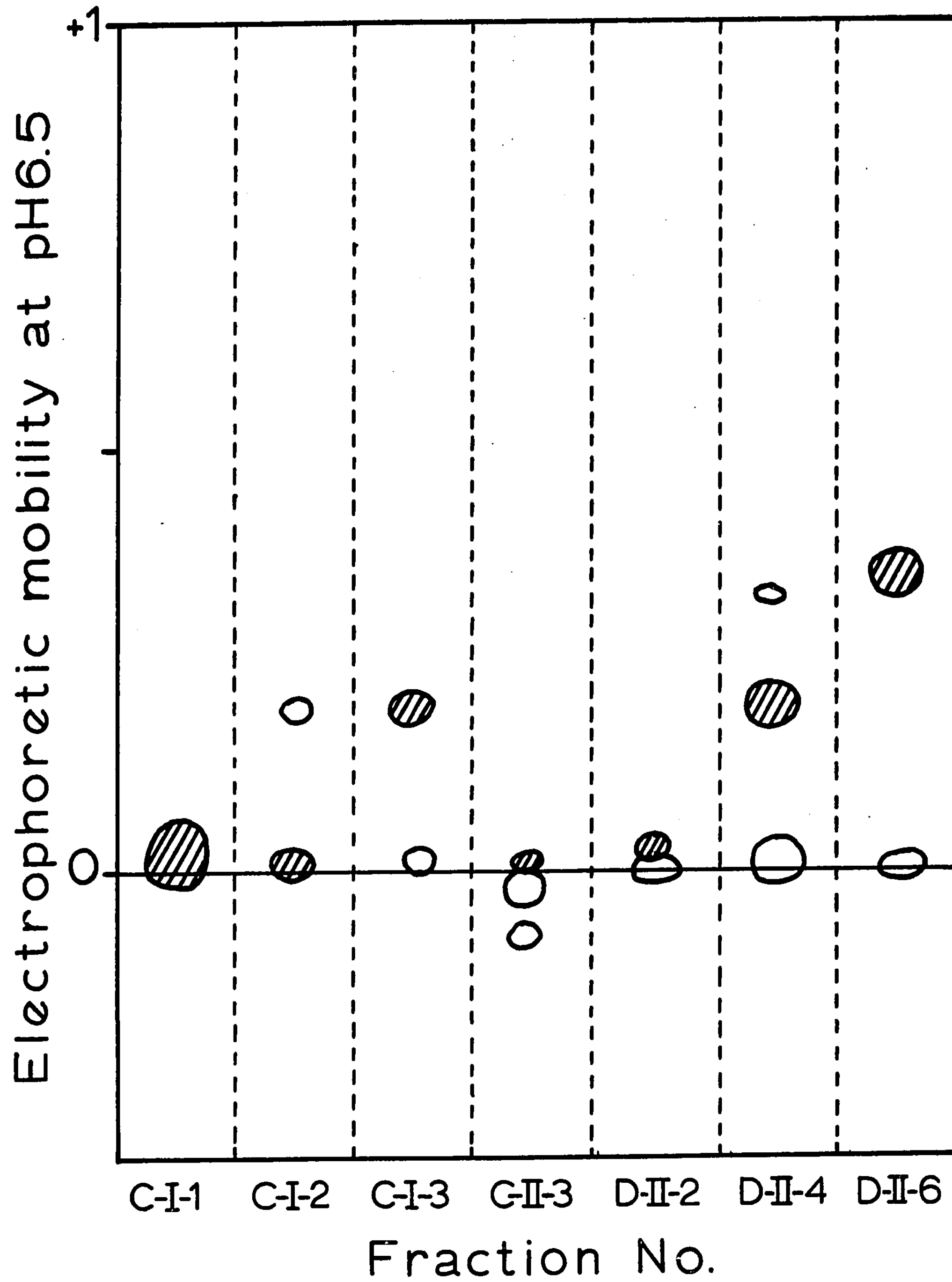


Figure 15. Radioactive Peptide Bands Arising From Fractions C and D Obtained by Electrophoresis at pH 6.5. The major peptides are hatched and the minor components in broken hatching.

TABLE XX

AMINO ACID COMPOSITION OF S-CARBOXAMIDOMETHYLATED PEPTIC PEPTIDES FROM FRACTION A

(Values are Expressed as Mole Ratios)

Amino Acid	Constituent Amino Acid Residues Per Molecule of Peptide				
	A-I-4	A-I-5	A-I-7	A-I-8	A-II-6
Lysine	1.02	1.18			
Arginine			2.10	2.02	0.97
Carboxymethyl-cysteine	0.83	0.91	0.71	0.92	0.78
Aspartic Acid	0.94	0.90	1.04	2.00	1.10
Threonine			0.95	1.01	
Glutamic Acid	1.04	1.29			
Proline			0.94	1.08	
Glycine	2.02	1.56	0.87	1.03	0.93
Valine			1.10	0.99	
Isoleucine	0.99	1.08			
Phenylalanine				0.97	
Number of residues	7	7	8	10	4
Mobility at pH 6.5	+0.24	+0.24	+0.34	+0.32	+0.02
N-terminal	Asx	Asx	Gly	Gly	Gly
Percentage Recovery	2.64	0.78	2.13	7.96	0.41

a. Fraction A-I

This fraction (5.9 µmoles) separated into a number of peaks by chromatography on Chromobead Type P resin (Figure 13). Of these, only fractions A-I-4, A-I-5, A-I-7, and A-I-8 were recovered in amounts adequate for further characterization (Table XX). These were further purified by high-voltage electrophoresis at pH 6.5 (Figure 14), their positions located by scanning side strips in the radioactive strip scanner and by staining with ninhydrin. The corresponding bands were eluted from the paper with water and characterized. For comparison, Figures 14 and 15 show drawings of all radioactive bands separated at pH 6.5.

<u>Peptides A-I-4 and A-I-5</u>

These two peptides had the same mobilities upon purification by electrophoresis at pH 6.5 (80 v./cm.; 90 min.) and had identical compositions (Table XX). N-terminal analyses indicated that these residues were either aspartic acid or asparagine in both peptides. From their positive mobilities it was concluded that asparagine and glutamine were present rather than the corresponding acids. The partial sequence of these two peptides was therefore concluded to be:

Asn-(Gln,Lys,Ile,CMC,Gly,Gly).

The apparent identity of these two peptides by all criteria raises the question of why they separated as two well-defined peaks on Chromobead Type P chromatography (Figure 13). Similar behaviour for peptides of apparently identical sequence has previously been observed by Schroeder (1948). It has been suggested that double zoning at the beginning of the Chromobead Type P chromatograms may be caused

by a variety of ionizable groups in peptides or by abrupt local changes in the ionic environment of the cation column. The phenomenon has not been observed so frequently that its appearance can be correlated definitely with the structure of a peptide. It might be possible that the ionic strength of the starting buffer changes locally by the addition of the sample to the column in which the peptides of identical sequence travel as distinct peaks. Since the N-terminal of this sequence is asparagine and because both acidic acid residues are amidated, it is not likely that this amino acid residue could undergo an α to β rearrangement (Naughton *et al.*, 1960). Schroeder *et al.* (1962) have suggested that this phenomenon is not restricted to peptides which contain aspartic acid or to those which contain histidine. An alternative explanation is that the S-carboxamidomethyl cysteine has been, in part, oxidized to the sulfoxide during the preparative procedures. Such a modification would not be detected in the amino acid analyses, nor would it be expected to affect the electrophoretic mobility or staining properties of the peptide. However, it could lead to differences in the properties of the two varieties upon chromatography on Chromobeads Type P resin.

Peptide A-I-7

Upon further purification by paper electrophoresis at pH 6.5 (80 v./cm., 90 min.) Fraction A-I-7 was resolved into six ninhydrin-positive bands. The radioactive band corresponding with a peptide staining yellow with cadmium-ninhydrin was cut from the original pH 6.5 electropherogram and was then subjected to further purification

by electrophoresis at pH 1.8 (80 v./cm., 45 min.). The amino acid composition, given in Table XX (page 100), indicated that it contained eight amino acid residues including two arginines and one aspartic acid residue. Its electrophoretic mobility at pH 6.5 (m = + 0.34) showed it to be a definitely basic peptide running just ahead of peptide A-I-8. The "dansyl" method yielded N-terminal glycine. The partial sequence of A-I-7 could thus be written as:

Gly-(CMC,Arg,Asx,Val,Pro,Arg,Thr).

Peptide A-I-8

In the Chromobead Type P elution profile shown in Figure 13 (page 100), Fraction A-I-8 was the major fraction. When this fraction was subjected to high-voltage electrophoresis at pH 6.5 (80 v./cm.; 90 min.) it was resolved into four well-separated ninhydrin-positive bands. A side strip, scanned for radioactivity, showed a single radioactive peak matching with a well separated peptide that stained yellow with the cadmium-ninhydrin reagent. The radioactive peptide A-I-8 was eluted from the paper and the concentration was determined as usual from its radioactivity. Samples (0.03 μmole) were taken for amino acid analysis and the results, given in Table XX (page 100), showed a content of ten amino acid residues. From its amino acid analysis and electrophoretic mobility (m = + 0.32), it was concluded that peptide A-I-8 was a larger variety of A-I-7 in that it contained one additional aspartic acid and one phenylalanine residue. Since glycine was shown to be N-terminal for both

TABLE XXI

PEPTIDES FORMED BY TRYPSIN DIGESTION OF PEPTIDE A-I-8
WITH VALUES EXPRESSED AS MOLE RATIOS

(Molar ratios are given in parentheses)

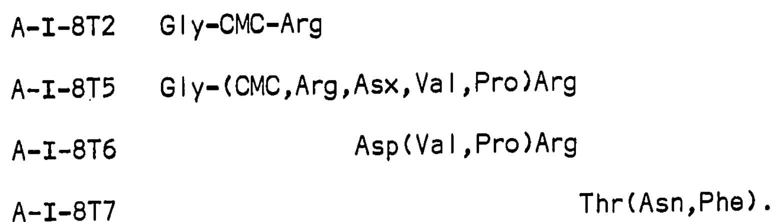
Peptide	Electrophoretic Mobility at pH 6.5 (m)	Amino Acid Composition	Cadmium-Ninhydrin Colour	N-terminal
A-I-8T2	+ 0.64	Gly(0.96), Arg(1.04), CMC(0.60)	yellow	Gly
A-I-8T5	+ 0.41	Gly(1.15), Arg (1.89), CMC (0.81), Asp(0.93), Val(1.16), Pro(0.89)	yellow	Gly
A-I-8T6	0.00	Asp(0.98), Val(0.99), Pro(1.08), Arg(0.95)	yellow	Asp
A-I-8T7	- 0.11	Thr(0.98), Asn(1.00), Phe(1.01)	yellow slow red	Thr

peptides, these two extra residues must be at the C-terminal end of A-I-8. This peptide was therefore tentatively assigned the sequence:

Gly-(CMC,Arg,Asx,Val,Pro,Arg,Thr)(Asx,Phe).

Additional evidence for the sequence of this peptide was obtained from tryptic digestions. The peptide (0.79 µmole) was digested with trypsin (0.016 µmole) in 1.2 ml. of 0.05 M *N*-Ethylmorpholine formate buffer, pH 8.0, at 37°C for 18 hours, and the reaction products were separated by high-voltage paper electrophoresis, pH 6.5. Four major cadmium-ninhydrin positive bands were separated, two of which showed radioactivity. The neutral band was further purified by electrophoresis at pH 1.8, and stained yellow with the cadmium-ninhydrin reagent. The results of the electrophoretic mobilities, N-terminal, and amino acid compositions of the tryptic peptides obtained are summarized in Table XXI.

From the data presented, the following partial sequences may be written for each of the tryptic peptides:


A-I-8T2 Gly-CMC-Arg
A-I-8T5 Gly-(CMC,Arg,Asx,Val,Pro)Arg
A-I-8T6 Asp(Val,Pro)Arg
A-I-8T7 Thr(Asn,Phe).

Since the mobility of A-I-8T6 was zero, aspartic acid and not asparagine must be the N-terminal residue. Peptide A-I-8T7 is assigned to the C-terminus of A-I-8 since it is the only tryptic fragment which does not contain arginine. The low negative mobility of this peptide is not consistent with a full negative charge of 1

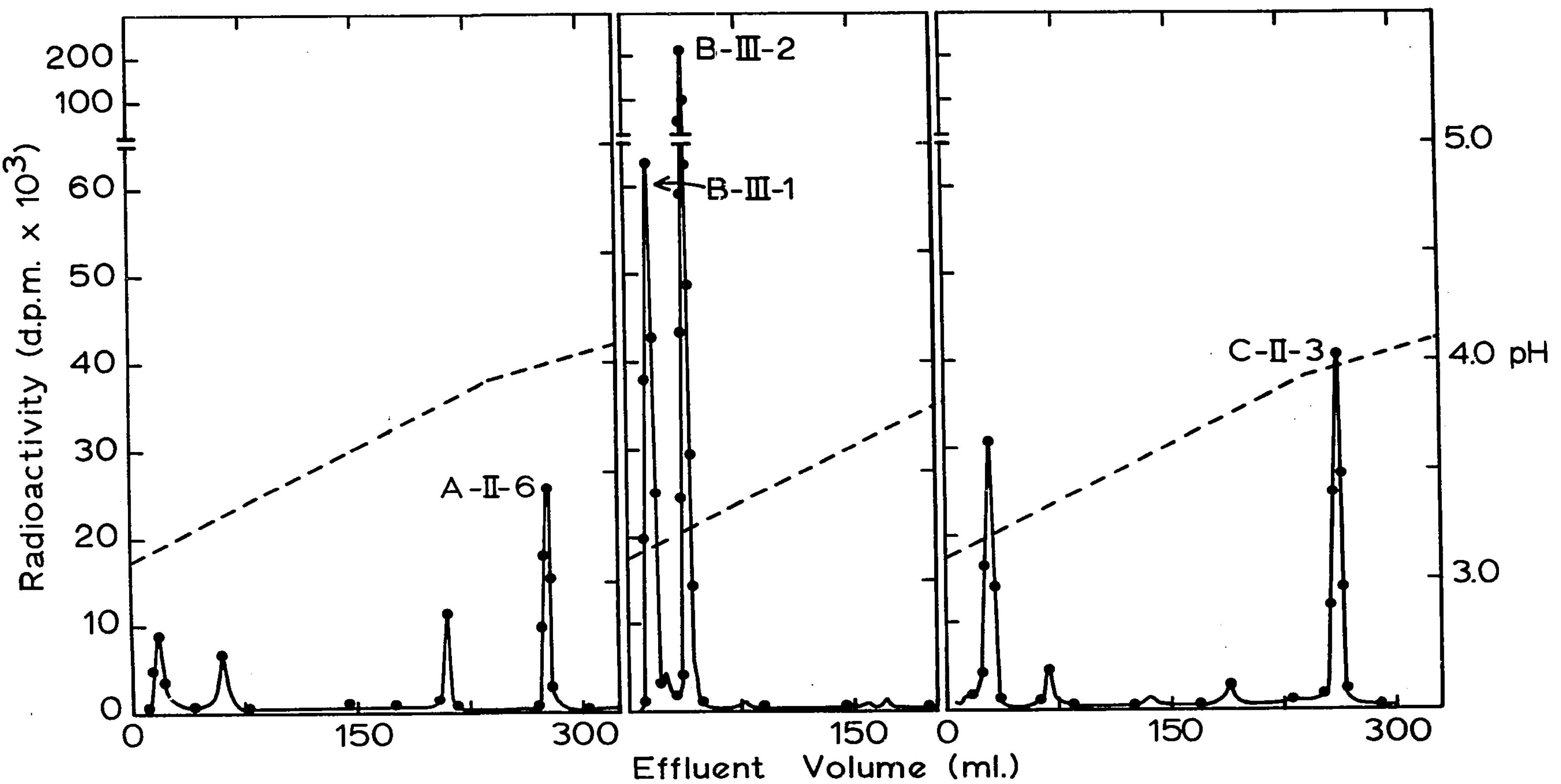


Figure 16. Chromatography of Fractions A-II (*left*), B-III (*center*), and C-II (*right*) on Chromobead Type P Columns.
Buffers, conditions, and abbreviations are the same as described in the text. The effluent was monitored for radioactivity (●——●) and the gradient is indicated by the broken line.

and is attributable to a low pK for the α-amino group of N-terminal threonine normally observed with such a peptide. The sequence of A-I-8 is therefore concluded to be:

Gly-CMC-Arg-Asp(Val,Pro)Arg-Thr(Asn,Phe).

b. Fraction A-II

This fraction was purified chromatographically essentially as described for Fraction A-I and the resulting chromatogram is shown in Figure 16. Only one radioactive peptic peptide, designated A-II-6, was obtained in reasonable yields. The remaining peaks were present in insufficient quantity to be considered significant or useful. Fraction A-II-6 was further purified by high-voltage electrophoresis at pH 6.5. The neutral band from pH 6.5 electrophoresis containing the radioactive peptide A-II-6 was submitted to further purification by electrophoresis at pH 1.8, and Figure 14 (page 100) shows a drawing of the radioactive band thus separated at pH 6.5.

Peptide A-II-6

Table XX (page 100) shows the amino acid composition, electrophoretic mobility, and percentage recovery from Chromobead Type P column chromatography of the pure radioactive peptide A-II-6. N-terminal analysis by the "dansyl" method gave glycine. The composition, mobility, and N-terminus permit the assignment of the following structure to this peptide:

Gly-(CMC,Arg,Asp).

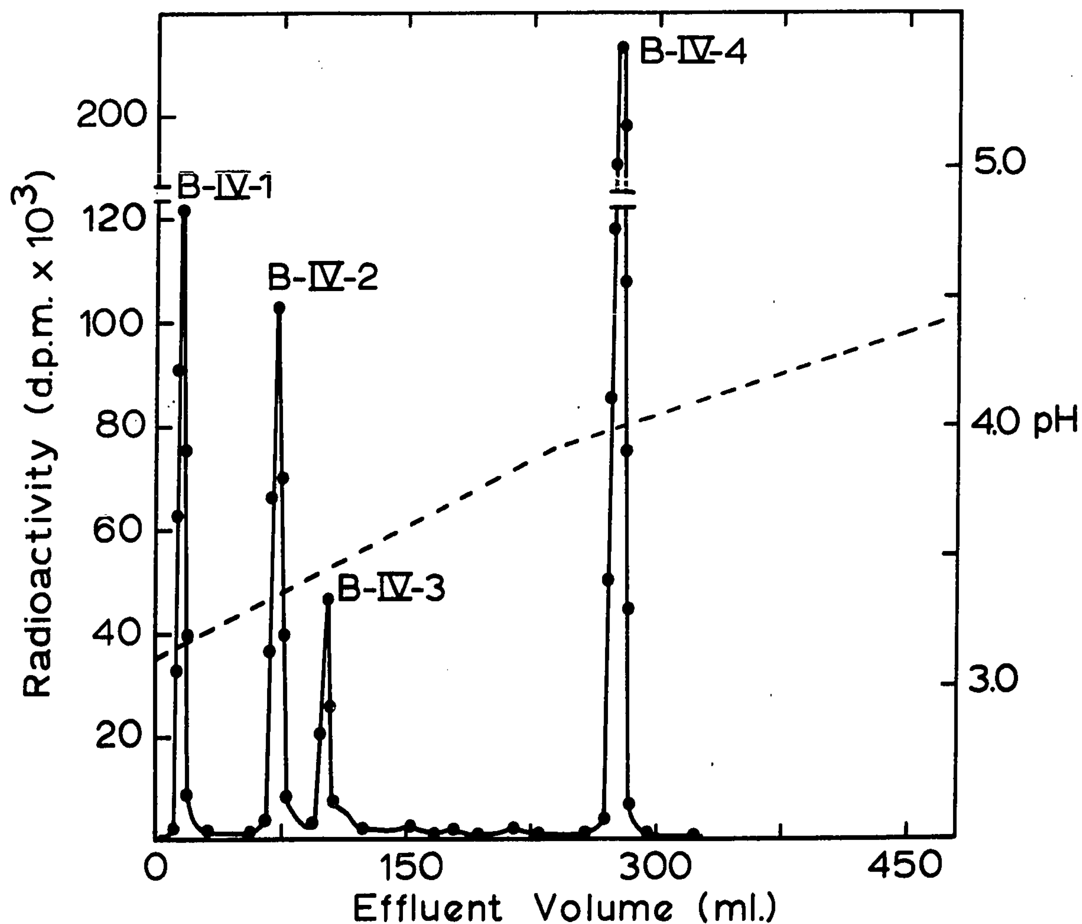


Figure 17. Chromatography of Radioactive Peptides of Fraction B-IV on Chromobead Type P Column.
The effluent was monitored for radioactivity (●——●) and the pH gradient is indicated by the broken line. The numbered peaks correspond to the designations used in Table XXII.

TABLE XXII

QUANTITATIVE RECOVERIES OF S-CARBOXAMIDOMETHYLATED PEPTIC PEPTIDES OF FRACTION B BY CHROMOBEAD TYPE P CHROMATOGRAPHY

Radioactive Peptide	Percentage Recovery
B-III-1	0.09
B-III-2	0.25
B-IV-1	0.34
B-IV-2	0.43
B-IV-3	0.22
B-IV-4	1.27

TABLE XXIII

AMINO ACID COMPOSITION OF B-IV-4 PEPTIDE

(Values are Expressed as Mole Ratios)

Amino Acid	Mole Ratios
Arginine	0.91
^{14}C-S-Carboxymethyl cysteine	0.77
Aspartic Acid	1.05
Glycine	1.04
Mobility at pH 6.5 (m)	0.0
Mobility at pH 1.8 (m')	1.24
N-terminal	Gly
Percentage Recovery	1.6

(2) Fraction B

This fraction (2.28 µmole) was chromatographed on a Cellex D column under essentially the same conditions as Fraction A. The resulting chromatogram is shown in Figure 12 (page 99). Fraction B was resolved into two major and two minor peaks labelled Fraction B-I, B-II, B-III, and B-IV. On the basis of radioactivity, the resulting recoveries are shown in Table XIX (page 99). Each of the two major peaks from the Cellex D column was rechromatographed on a Chromobead Type P column (0.6 cm. x 110 cm.) as described in the Methods (page 38) and the percentage recoveries of the major radioactive peptides are shown in Table XXII. Figure 17 shows the typical chromatographic profiles obtained. Since only one major radioactive peptide was present in sufficient quantity, the remaining radioactive peptides were not further chromatographed. The final purification of this neutral radioactive peptide, B-IV-4, was achieved by high-voltage paper electrophoresis at pH 6.5 and 1.8, and Figure 14 (page 100) shows the radioactive bands thus obtained.

Peptide B-IV-4

The evidence presented in Table XXIII, on the amino acid composition, electrophoretic mobility, N-terminal analysis, and colour when stained with cadmium-ninhydrin reagent for peptide B-IV-4 are consistent with the structure previously assigned to peptide A-II-6, namely:

Gly-(CMC,Arg,Asp).

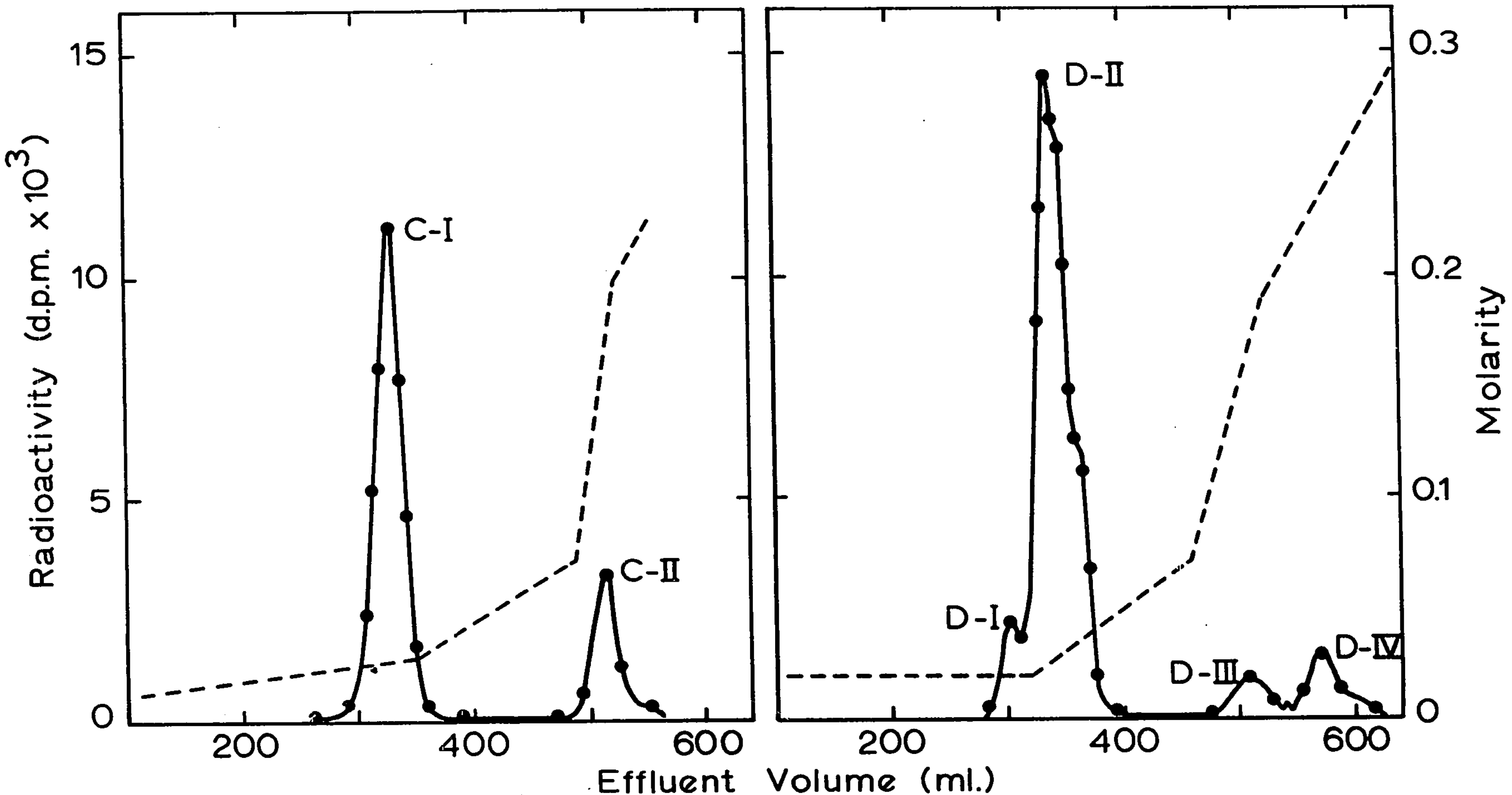


Figure 18. Chromatography of Fractions C (*left*) and D (*right*) on Cellex D Columns. Buffers and conditions are given in the text. The effluent was monitored for radioactivity (●——●). The gradient is indicated by a broken line, and the numbered peaks correspond to the designations used in the text.

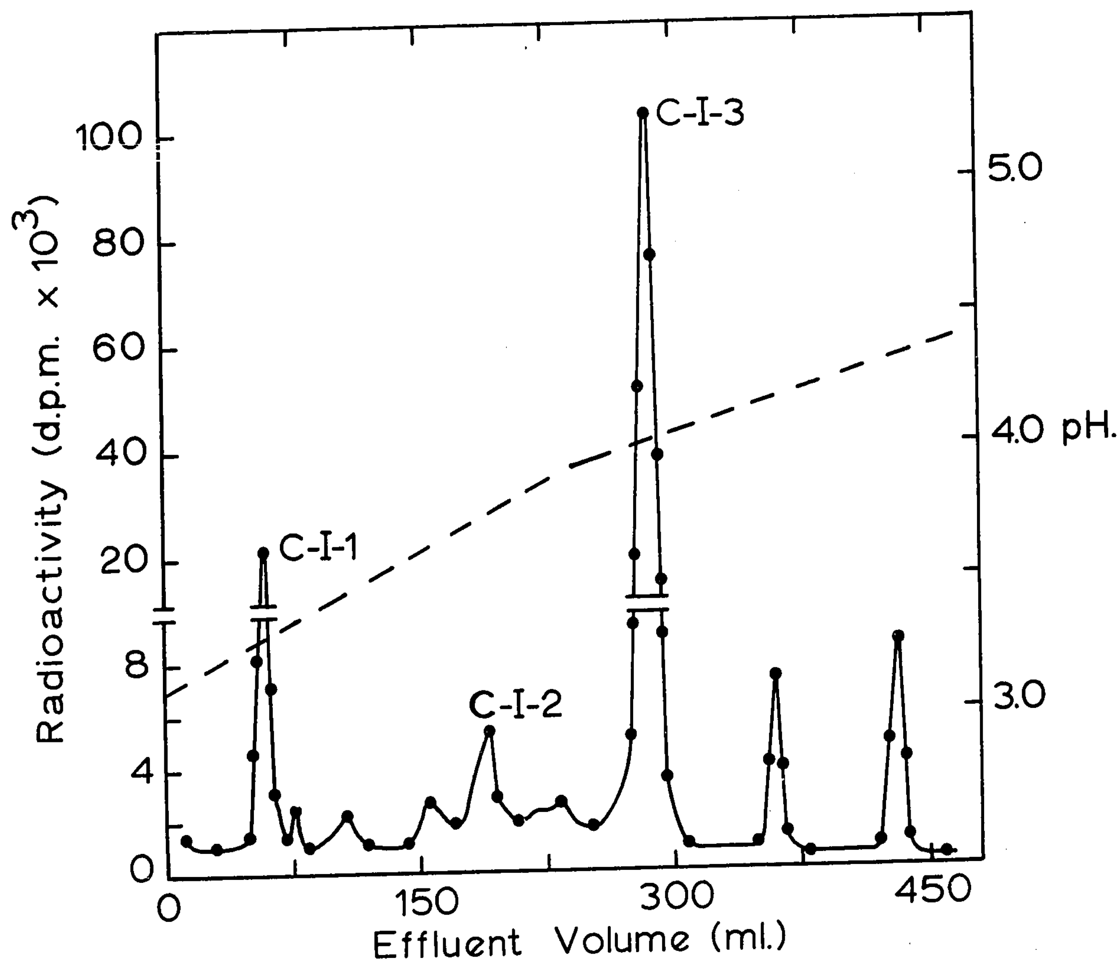


Figure 19. Chromatography of Radioactive Peptides of Fraction C-I on Chromobead Type P Column.
The effluent was monitored for radioactivity (●——●) and the pH gradient is indicated by the broken line. The numbered peaks correspond to the designations used in Table XXIV.

(3) Fraction C

This fraction (4.51 μmole) was chromatographed on Cellex D columns under essentially the same conditions as Fraction A, and the resulting chromatographic pattern is shown in Figure 18. Fraction C was resolved into two well-separated peaks, labelled Fraction C-I and C-II, as judged by radioactivity, and the recoveries calculated are shown in Table XIX (page 99).

a. Fraction C-I

This fraction was rechromatographed on a Chromobead Type P column as described in the Methods (page 38) and the resulting elution profile (Figure 19) showed five radioactive peptides. The percentage recoveries are summarized in Table XXIV. The final purification of the radioactive peptides C-I-1, C-I-2, and C-I-3, was obtained by high-voltage electrophoresis as is illustrated in Figure 15 (page 100).

Peptide C-I-1

The peptide was purified by high-voltage electrophoresis at pH 6.5 (80 v./cm., 90 min.) (Figure 15, page 100) and the neutral radioactive band was re-run by electrophoresis at pH 1.8 (80 v./cm., 40 min.). Staining of the band with cadmium-ninhydrin reagent developed a yellow colour that slowly turned red, consistent with N-terminal aspartic acid as demonstrated by N-terminal analysis. The partial sequence may therefore be written as:

Asx(Glx,Lys,Ile,CMC,Gly,Gly,Try,Glx).

TABLE XXIV

AMINO ACID COMPOSITION OF RADIOACTIVE PEPTIC PEPTIDES FROM FRACTION C

(Values are Expressed as Mole Ratios)

Amino Acid	Constituent Amino Acid Residues Per Molecule of Peptide			
	C-I-1	C-I-2	C-I-3	C-II-3
Lysine	1.00	0.92	0.98	
Arginine				0.91
Carboxymethyl cysteine	0.84	0.76	0.84	0.77
Aspartic acid	0.98	0.87	0.96	1.05
Serine		0.60		
Glutamic acid	2.03	2.09	2.05	
Glycine	1.99	2.25	2.04	1.04
Isoleucine	0.99	0.83	0.94	
Tryptophan	+	+	+	
Number of residues	9	10	9	
Mobility at pH 6.5	+0.06	0.0	+0.21	+0.02
N-terminal	Asx	Asx	Asn	Gly
Percentage Recovery	0.80	0.46	4.32	0.26

The neutrality of the peptide upon electrophoresis at pH 6.5 indicates that there is one aspartic or glutamic acid present.

Peptide C-I-2

This peptide was purified as described above for peptide C-I-1, to give the pattern shown in Figure 15 (page 100) by high-voltage electrophoresis at pH 6.5. Peptide C-I-2 was both ninhydrin-positive and Ehrlich-positive indicating the presence of tryptophan. The amino acid composition shown in Table XXIV is very similar to that found for peptide C-I-1 except that it contains one additional amino acid residue (serine). These results suggest that the decapeptide may well be a larger variety of the latter:

(Asx,Glx,Lys,Ile,CMC,Gly,Gly,Try,Glx,Ser)

As in the case of peptide C-I-1, the electrophoretic mobility at pH 6.5 (m = 0.00) of C-I-2 is indicative of the presence of one aspartic acid or one glutamic acid.

Peptide C-I-3

The major peak, C-I-3, that originated from Fraction C was shown to resolve into several ninhydrin-positive peptide bands on paper electrophoresis, pH 6.5. Peptide C-I-3 was both ninhydrin-positive and Ehrlich-positive. It was located on the electropherogram by its fluorescent band under the ultraviolet light, and by its radioactivity, traced in the radioactive strip scanner. After purification by electrophoresis at pH 6.5 and 1.8, as described for peptide C-I-1 (Figure 15, page 100), samples were taken

TABLE XXV

PEPTIDES FORMED BY TRYPSIN DIGESTION OF PEPTIDE C-I-3

(The Values are Expressed as Mole Ratios)

Peptide	Electrophoretic Mobility at pH 6.5 (m)	Amino Acid Composition	Cadmium-Ninhydrin Reagent	Ehrlich's Reagent	N-terminal
C-I-3T1	+ 0.42	Asn(0.94), Gln(1.03), Lys(1.01)	yellow brown slow red		Asp
C-I-3T2	0.0	Ile(0.94), CMC(0.81), Gly(2.01), Try(+)[a], Gln(1.03)	orange slow red	+	Ile

[a]Tryptophan detected by the staining reaction of the peptide on paper.

for amino acid analysis, and the results are shown in Table XXIV. The presence of tryptophan was indicated by Ehrlich's positive staining and from hydrolysates of C-I-3 which gave rise to two small peaks on the chromatogram (10 cm. column) just ahead of lysine. Neither Dreze's procedure (Dreze, 1956), nor the spectrophotometric method for the determination of tryptophan were employed for quantitating tryptophan in this case. As the peptide had an electrophoretic mobility, m = + 0.21 at pH 6.5, it must contain one asparagine and two glutamines rather than aspartic and glutamic acids. The N-terminal group was shown to be either aspartic acid or asparagine and its partial sequence could thus be written as:

Asn(Gln,Lys,Ile,CMC,Gly,Gly,Try,Gln).

The S-carboxamidomethylated peptide C-I-3 (0.40 µmoles) was digested with 0.008 µmoles of trypsin (ratio of peptide to trypsin 50:1) in 1.0 ml. of 0.05 M *N*-Ethylmorpholine acetate buffer, pH 8.0, at 37° for 5 hours. The digest was separated by high-voltage paper electrophoresis at pH 6.5, and the neutral band re-run by electrophoresis at pH 1.8. In each case a single band was observed after staining with cadmium-ninhydrin reagent. The neutral band C-I-3T2 stained with Ehrlich's reagent. The results of the electrophoretic mobilities and amino acid compositions of the tryptic peptides so obtained are summarized in Table XXV. N-terminal analysis showed that the sequences of these two peptides were as follows:

C-I-3T1 Asn-Gln-Lys

C-I-3T2 Ile(CMC,Gly,Gly,Try,Gln)

The partial sequence of C-I-3 could thus be written as:

Asn-Gln-Lys-Ile-(CMC-Gly-Gly-Try-Gln).

b. Fraction C-II

This fraction was chromatographed as described for Fraction C-I to give one major peak labelled C-II-3. After purification by high-voltage electrophoresis at pH 6.5 and 1.8, this peptide had the same amino acid composition (Table XXIV, page 107) and N-terminal as those of the major tetrapeptide B-IV-4 of Fraction B. It was therefore assigned the structure:

Gly-(CMC,Arg,Asp).

This peptide was presumably cross-contaminating Fraction C as a result of incomplete separation of the two peaks in the gel-filtration chromatogram (Figure 11, page 98) and was not examined further.

(4) Fraction D

This fraction (3.79 μmole) was chromatographed as described for Fraction C to give the pattern shown in Figure 18 (page 106). Four peaks (D-I, D-II, D-III, and D-IV) were detected. Each contained impure peptides as judged by paper electrophoresis, and the resulting recoveries are shown in Table XIX (page 99). Fractions D-I, D-III, and D-IV were found to contain low radioactivity, and were not investigated further.

a. Fraction D-II

The major fraction (2.07 μmole) from the Cellex D column was rechromatographed on a Chromobead Type P column, as shown in Figure

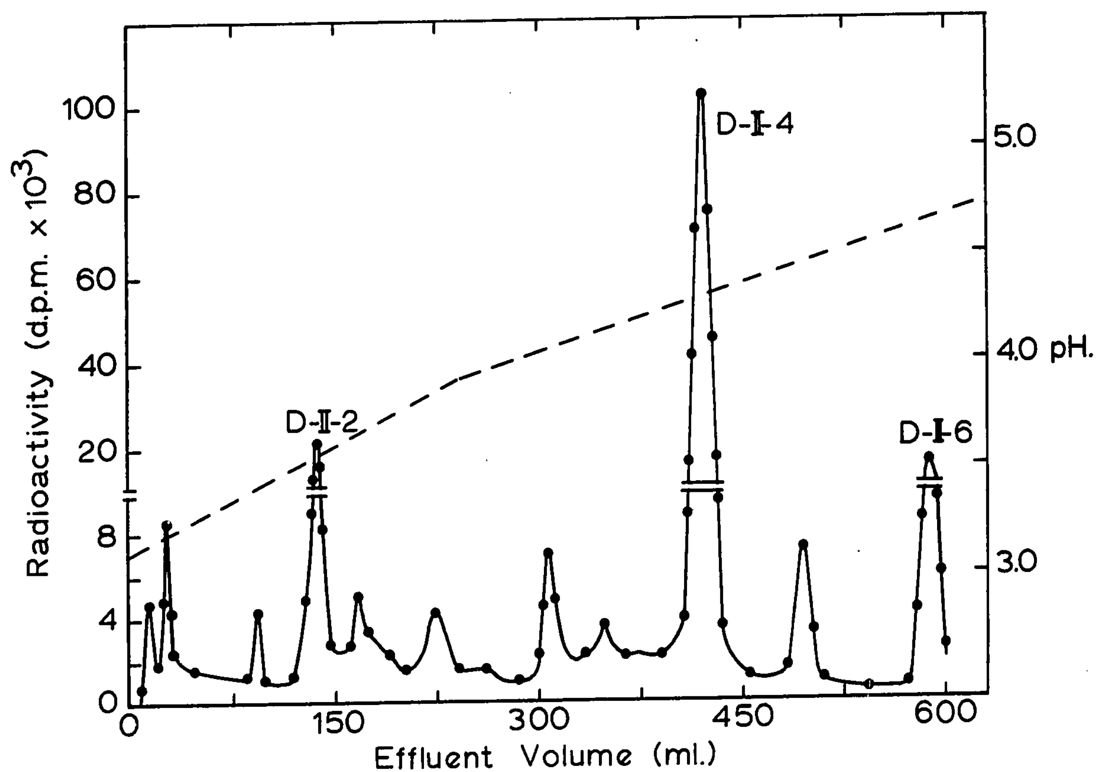


Figure 20. Chromatography of Radioactive Peptides of Fraction D-II on Chromobead Type P Column.
The effluent was monitored for radioactivity (●——●) and pH gradient is indicated by the broken line. The numbered peaks correspond to the designations used in Table XXVI.

TABLE XXVI

AMINO ACID COMPOSITION OF RATIOACTIVE PEPTIC PEPTIDES FROM FRACTION D

(Values are Expressed as Mole Ratios)

Amino Acid	Constituent Amino Acid Residues Per Molecule of Peptide		
	D-II-2	D-II-4	D-II-6
Lysine	1.10	1.00	+
Carboxymethyl cysteine	0.63	0.69	0.76
Aspartic Acid	0.81	0.98	-
Serine	0.70		
Glutamic Acid	1.26	1.00	-
Glycine	2.31	2.00	2.14
Isoleucine	0.81	1.01	0.86
Tryptophan	+	+	
Number of residues	9	8	5
Mobility at pH 6.5	+0.04	+0.23	+0.37
N-terminal	Asx	Asn	Lys
Percentage Recovery	0.37	2.34	0.39

20. The major peaks from this column contained peptides D-II-2 D-II-4, and D-II-6, the composition and recoveries of which are given in Table XXVI.

Peptide D-II-2

The composition of this peptide (Table XXVI) taken together with its essentially zero mobility suggest that there is either one aspartic or glutamic acid present. It gave a positive test for tryptophan and yielded aspartic acid or asparagine as its N-terminal residue. It may therefore be written as:

Asx(Glx,Lys,Ile,CMC,Gly,Gly,Try,Glx,Ser).

Peptide D-II-4

The major radioactive peptide from Fraction D-II was separated by column chromatography and further purified by paper electrophoresis at pH 6.5 (80 v./cm.; 90 min.). The separation patterns obtained are shown in Figure 15 (page 100). The D-II-4 peptide band was fluorescent and gave both Ehrlich- and ninhydrin-positive colours. The N-terminal residue was asparagine or aspartic acid. The amino acid composition and electrophoretic mobility of the pure octapeptide is given in Table XXVI. Its mobility with respect to lysine was m = + 0.23, suggesting that there was a net positive charge of 1 (Offord, 1966) and that both acidic residues were amidated. These results are consistent with the sequence:

Asn-(Gln,Lys,Ile,Cys,Gly,Gly,Try).

TABLE XXVII

PEPTIDES FORMED BY TRYPSIN DIGESTION OF PEPTIDE D-II-4[a]

(The Values are Expressed as Mole Ratios)

Peptide	Electrophoretic Mobility at pH 6.5 (m)	Amino Acid Composition	Cadmium-ninhydrin Reagent	Ehrlich's Reagent	N-terminal
D-II-4T2	+ 0.43	Asn(0.94),Gln(1.02),Lys(1.04)	yellow slow red		Asn
D-II-4T3	0.0	Ile(1.00),CMC(0.59),Gly(2.00) Try (+)[b]	orange slow red	+	Ile

[a]Experimental details and definition of the symbols are given in the text.
[b]Tryptophan detected by the staining reaction of the peptide on paper.

The sequence of peptide D-II-4 was also studied by digesting the peptide fragment (0.32 μmole) with trypsin in 1.0 ml. of 0.05 M *N*-Ethylmorpholine acetate buffer, pH 8.0, at 37° for 5 hours. The peptides produced were purified by electrophoresis at pH 6.5, and the neutral band D-II-4T3 was further purified by an additional pH 1.8 electrophoretic run. The properties of these peptides are given in Table **XXVII**. The partial sequence of D-II-4 could thus be written as:

Asn-Gln-Lys-Ile(CMC,Gly,Gly,Try).

Peptide D-II-6

Amino acid analysis of this peptide (Table **XXVI**, page 110) suggests the composition (Lys,Ile-CMC,Gly,Gly). Lysine was found to be the N-terminal by the "dansyl" method. From its N-terminal analysis, mobility at pH 6.5 (Figure 15, page 100), and amino acid composition, it was concluded that peptide D-II-6 had the sequence:

Lys-(Ile,CMC,Gly,Gly).

4. DISCUSSION

Previous studies on the reactivity of the sulfhydryl groups of phosphorylase have indicated that there were several classes of such residues differing in their rates of reaction with a variety of sulfhydryl reagents (Battell *et al.*, 1968a; 1968b). It was concluded that of the nine sulfhydryl residues present in each monomer of phosphorylase, one was particularly reactive and could be alkylated with iodoacetamide-1-^{14}C at a rate which is at least as fast as that

of model compounds. This alkylation occurred without significant change in the enzymic properties of the protein. The extent of incorporation in these experiments had been measured by TCA precipitation and measurement of the amount of radioactivity in the precipitated protein. A tentative identification of the alkylated cysteine was made by isolation of a tryptic fragment, Gly-CMC-Arg, from the partially purified radioactive peptides, B_1 and B_2, previously observed to be those most rapidly alkylated. However, the yield of this peptide was very low and attempts to purify the intact B_1 and B_2 peptides by paper electrophoretic methods were unsuccessful. For the purpose of more fully characterizing these peptides, the work described in this chapter was undertaken.

The alkylation experiments were designed to limit the reaction with iodoacetamide to the single sulfhydryl residue whose alkylation was believed not to affect activity. Thus a low concentration of ^{14}C-labelled reagent was used and the extent of incorporation was followed by three methods: namely, (1) TCA precipitation and measurement of the radioactivity in the washed precipitate, (2) measurement of the radioactivity in samples of thoroughly dialysed protein, and (3) measurement of the amount of formation of S-carboxyamidomethyl cysteine by amino acid analysis of samples of the modified protein. These results showed that the TCA precipitation method was not a reliable procedure and gave low values for the extent of the reaction. This observation has been confirmed by Dr. N. B. Madsen and Dr. O. Avramovic-Zikik in independent experiments. However, the other two methods gave results in reasonable agreement.

TABLE XXVIII

SUMMARY OF ^{14}C-S-CARBOXAMIDOMETHYL CYSTEINE PEPTIDES CORRESPONDING TO UNIQUE HALF-CYSTINE SEQUENCE No. 2

Peptide	Sequence	Percentage Recovery
Unique Sequence No. 2	Asn-Gln-Lys-Ile-Cya-Gly-Gly	
A-I-4	Asn-(Gln,Lys,Ile,CMC,Gly,Gly)	2.64
A-I-5	Asn-(Gln,Lys,Ile,CMC,Gly,Gly)	0.78
C-I-1	Asn(Glx,Lys,Ile,CMC,Gly,Gly,Try,Glx)	0.80
C-I-2	Asx(Glx,Lys,Ile,CMC,Gly,Gly,Try,Glx,Ser)	0.46
C-I-3	Asn-Gln-Lys-Ile-(CMC,Gly,Gly,Try,Gln)	4.32
D-II-2	Asx(Glx,Lys,Ile,CMC,Gly,Gly,Try,Glx,Ser)	0.37
D-II-4	Asn-Gln-Lys-Ile-(CMC,Gly,Gly,Try)	2.34
D-II-6	Lys-(Ile,CMC,Gly,Gly)	0.39
Extended Final Sequence	Asn-Gln-Lys-Ile-CMC-Gly-Gly-Try-Gln-Ser	12.1

TABLE XXIX

SUMMARY OF ^{14}C-S-CARBOXAMIDOMETHYL-CYSTEINE PEPTIDES

CORRESPONDING TO UNIQUE HALF-CYSTINE SEQUENCE No. 5

Peptide	Sequence	Percentage Recovery
Unique Sequence No. 5	Gly-Cya-Arg-Asp	
A-I-7	Gly-(CMC,Arg,Asp,Val,Pro,Arg,Thr)	2.12
A-I-8	Gly-CMC-Arg-Asp(Val,Pro)-Arg-Thr-(Asn,Phe)	7.96
A-II-6	Gly-(CMC,Arg,Asp)	0.41
B-IV-4	Gly-(CMC,Arg,Asp)	1.27
C-II-3	Gly-(CMC,Arg,Asp)	0.26
Extended Sequence	Gly-CMC-Arg-Asp(Val,Pro)Arg-Thr-(Asn,Phe)	12.0

With two separate preparations of phosphorylase *b* (1 week and 4 months old) the extent of incorporation was estimated to be 3.1 residues and 2.0 residues of iodoacetamide-1-^{14}C incorporated per mole of phosphorylase *b*, indicating that the extent of reaction was dependent on the age and history of the enzyme preparation. In sum, these results indicated that there was more than a single cysteine per monomer of phosphorylase *b* which could be rapidly alkylated without loss of enzymic activity.

Careful purification and characterization of the radioactive peptides produced by peptic digestion of the modified protein has confirmed this conclusion. Upon examination of the partial and complete sequences reported above and summarized in Tables XXVIII and XXIX, it is clear that these correspond to *two* unique half-cystine sequences previously isolated and characterized. These sequences were labelled number 2 and number 5 unique sequences in the publication of Zarkadas *et al.* (1968) and in Table XV (page 73) of Chapter II. It is also pertinent that although the overall recovery of radioactivity found in these peptides accounted for only about 24 per cent of the radioactivity incorporated into the protein and present in the original peptic digest, the recoveries of peptides corresponding to sequence number 2 (12.1 per cent) was also almost exactly equal to the recovery of radioactivity in peptides corresponding to sequence number 5 (12.0 per cent).

The low recoveries of total radioactivity is accounted for by the large number of purification and manipulative steps required for the purification of these peptides. It is also due to the losses of

peptides arising from the degradation and/or oxidation of the peptide during purification. Evidence for the latter came from several observations. Peptides A-I-4 and A-I-5, for example, showed identical electrophoretic mobilities, amino acid compositions, N-terminal analyses, and staining reactions with cadmium-ninhydrin reagent but were separable by chromatography on Chromobeads Type P resin. A possible explanation for the observation is that partial oxidation of the S-carboxamidomethyl cysteine had occurred leading to the corresponding sulfoxide. Such a chemical change would not be detected by any of the criteria mentioned above but could lead to differences in the chromatographic properties on the cation exchange resin. Other observations have indicated the partial deamidation of either asparagine or glutamine during the purification steps. Thus peptides C-I-1, D-II-2, and C-I-3 all had similar amino acid compositions and identical N-terminal analyses but differed in their electrophoretic mobilities on paper at pH 6.5. Both peptides C-I-1, D-II-2 were neutral peptides indicating the presence of one acidic residue in addition to a single lysine residue. However, peptide C-I-3 was basic indicating the absence of any acidic residue. Presumably the former two peptides had arisen from deamidation of one of the two glutamines and one asparagine present in this sequence. It is clear that the rather large number of minor radioactive fractions, present in inadequate amounts for further characterization and observed during the purification procedures, arose from such modifications of the two parent sequences.

In summary, it may be concluded that there are two cysteine residues per monomer of phosphorylase *b* whose rate of reaction with iodoacetamide approaches that of model compounds and whose alkylation does not affect significantly the enzymic properties of the protein. These two cysteines have been identified in sequences corresponding to numbers 2 and 5 previously elucidated. These sequences have now been extended in the present work and may be written as:

No. 2 Asn-Gln-Lys-Ile-CMC-Gly-Gly-Try-Gln-Ser

No. 5 Gly-CMC-Arg-Asp-(Val,Pro)-Arg-Thr-(Asn,Phe).

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