

tein **Protein** Protein Protein Prote
Phosphorylation
n Protein **Protein** Protein Protein
Phosphorylation
tein Protein Protein Protein **Prot**
sphorylation **Phos**

M Weller

Protein Phosphorylation

The nature, function, and metabolism of proteins which contain covalently bound phosphorus

p Pion Limited, 207 Brondesbury Park, London NW2 5JN

© 1979 Pion Limited

All rights reserved. No part of this book may be reproduced in any form by photostat microfilm or any other means without written permission from the publishers.

ISBN 0 85086 062 8

Protein Phosphorylation

The nature, function, and metabolism
of proteins which contain covalently
bound phosphorus

Series editor J R Lagnado

- 1 Free radical mechanisms in tissue injury T F Slater
- 2 Biochemical reactors B Atkinson
- 3 Brain function and macromolecular synthesis B Jakoubek
- 4 Infectious multiple drug resistance S Falkow
- 5 Aldehydes in biological systems E Schauenstein, H Esterbauer, H Zollner
- 6 Protein phosphorylation M Weller

It has been known for over a century that certain proteins contain covalently bound phosphorus, and it is equally well-established that, if animals are injected with ^{32}P -labelled inorganic phosphorus, radioactivity is rapidly incorporated into many tissue proteins, showing that protein-bound phosphorus has a rapid turnover. It is, however, only in the last ten years or so that studies on protein phosphorylation have really gained momentum. The reason for the current interest in the topic is that cyclic AMP has been shown to stimulate the phosphorylation of certain proteins, resulting in important changes in their properties. These observations have naturally led many workers to consider the possibility that protein phosphorylation may have as important and widespread a function in regulation as does cyclic AMP itself. Many protein phosphorylation reactions are, however, unaffected by cyclic AMP, and much interesting work on these reactions is in danger of being overshadowed by the current interest in cyclic-AMP-stimulated protein kinase activities. Indeed, the properties of certain phosphorylated proteins have been much more thoroughly investigated than others and there are many gaps in our knowledge.

The literature on protein phosphorylation, and on proteins which contain covalently bound phosphorus, is now very large and continues to increase rapidly. Consequently it seems to me to be a very suitable time to present a book which attempts to summarise, review, and critically discuss the present state of our knowledge on these subjects and to bring together previously unrelated observations, put forward possible new hypotheses, and indicate where gaps occur. I should feel especially rewarded if this book, by indicating some of these gaps, in any way helped to identify and stimulate suitable fields for future research.

At the risk of occasionally being repetitive I have divided the text into a series of chapters each of which is complete in itself and may be read independently, so that the reader who is interested in only one type of phosphoprotein, or one aspect of protein phosphorylation, will not have to read the whole book in order to gain the relevant information about his topic. The individual chapters are further divided into sections for easy reference.

I trust that the book will prove to be of value to anyone carrying out research on protein phosphorylation, or on proteins which contain covalently bound phosphorus, and that it will provide instructive reading to advanced students interested in these subjects.

In conclusion, it is a great pleasure to thank Miss Wilma Laing and Miss Jeanne Rousseau as well as my wife, Jennifer, for their help in the preparation of the manuscript for this book. I am also indebted to those of my colleagues who have provided information about their current work.

M Weller

Tygerberg Hospital, University of Stellenbosch, South Africa

List of abbreviations

The abbreviations used in the text are those accepted by the Biochemical Journal [*Biochem. J.* (1966) **101** 1–7]. In addition the following abbreviations are used:

ACTH adrenocorticotrophic hormone,
DFP diisopropylphosphofluoridate,
EGTA ethyleneglycol-*bis*(β -aminoethyl ether)*N,N'*-tetraacetic acid,
MSH melanocyte stimulating hormone,
NEM *N*-ethylmaleimide,
PCMB *p*-chloromercuribenzoate,
SDS sodium dodecyl sulphate.

In peptide sequences the symbols are joined by hyphens where the sequence is known, or separated by commas if the sequence is not known. Phosphorylated amino acids are represented by the following abbreviations:

aspP phosphoaspartate,
gluP phosphoglutamate,
serP phosphoserine,
thrP phosphothreonine.

1	General aspects and functions of proteins which contain covalently bound phosphorus	
1.1	Classification and nomenclature of proteins which contain covalently bound phosphorus—the phosphoproteins	1
1.2	Phosphorylated amino acids which occur in proteins, and their chemical properties	1
1.3	The occurrence of protein-bound polyphosphate	5
1.4	The turnover and formation of protein-bound phosphate	6
1.5	Possible function of proteins which contain covalently bound phosphorus	7
1.5.1	Enzymic proteins	7
1.5.2	Nonenzymic proteins	8
2	Enzymes which catalyse the phosphorylation of proteins—the protein kinases	
2.1	The classification of protein kinases	10
2.2	Intrinsic protein kinase activities	12
2.3	Protein kinases with limited substrate specificity	12
2.3.1	Type a_1 protein kinases—enzymes which catalyse the phosphorylation of histones and other (unspecified) proteins and which are stimulated by cyclic AMP	12
2.3.2	Type a_2 protein kinases—enzymes which catalyse the phosphorylation of histones and other (unspecified) proteins and which are stimulated by cyclic GMP	37
2.3.3	Type b protein kinases—enzymes which catalyse the phosphorylation of phosvitin and other (unspecified) proteins and which are not affected by cyclic nucleotides	40
2.3.4	Type c protein kinases—enzymes which catalyse the phosphorylation of histones and other (unspecified) proteins and which are not affected by cyclic nucleotides	45
2.3.5	Miscellaneous protein kinases	46
2.4	Protein kinases which are specific to one protein substrate	47
2.4.1	Is there a casein-specific protein kinase?	48
2.4.2	Are there histone-specific protein kinases?	48
3	Enzymes which catalyse the dephosphorylation of proteins	
3.1	Classification of enzymes which catalyse the dephosphorylation of proteins	49
3.2	Enzymes which catalyse the dephosphorylation of phosvitin or casein	50
3.2.1	Occurrence	50
3.2.2	Attempts at purification	50
3.2.3	Substrate specificity	50
3.2.4	Properties	51

3.3	Enzymes which catalyse the dephosphorylation of histones	53
3.3.1	Occurrence	53
3.3.2	Purification and physical properties	53
3.3.3	Substrate specificity	54
3.3.4	Properties of the enzymically catalysed dephosphorylation of histones	55
3.4	Intrinsic protein phosphatase activities	56
3.5	Specific protein phosphatases	57
3.6	Acid phosphatases	57
3.6.1	Occurrence	57
3.6.2	Purification	57
3.6.3	Structure	58
3.6.4	Substrate specificity	58
3.6.5	Properties	59
3.6.6	Mechanisms of reaction	59
3.7	Alkaline phosphatases	60
3.7.1	Structure	60
3.7.2	Substrate specificity	61
3.7.3	Properties	62
3.7.4	Mechanism of reaction	63
3.7.5	Function	63
3.8	Phosphoserine phosphatase	64
4	Cyclic nucleotides, the action of hormones, and protein phosphorylation	
4.1	Some comments on the 'secondary messenger' hypothesis	65
4.2	The structure of cyclic nucleotides	68
4.3	The metabolism of cyclic AMP	68
4.3.1	Adenylate cyclase	68
4.3.2	Cyclic-nucleotide phosphodiesterase	69
4.4	Stimulation of adenylate cyclase activity	72
4.5	The function of cyclic AMP	75
4.5.1	Cyclic AMP and the control of glycogen metabolism	76
4.5.2	Cyclic AMP and the control of steroidogenesis	79
4.5.3	Cyclic AMP and the control of lipolysis	80
4.5.4	Cyclic AMP and the control of secretion	81
4.5.5	Cyclic AMP and the control of mitosis, cell growth, and morphology	84
4.5.6	Cyclic AMP and the control of protein synthesis and enzyme induction	85
4.5.7	Cyclic AMP and the control of muscular contraction in the heart	87
4.5.8	Cyclic AMP and the control of mechanical function in skeletal muscle	90
4.5.9	Cyclic AMP and the control of mechanical function in smooth muscle	91
4.5.10	Cyclic AMP and the control of permeability and ion transport	92
4.5.11	Cyclic AMP and the control of amino acid transport	101
4.5.12	Cyclic AMP and platelet aggregation	101
4.5.13	Cyclic AMP and melanin dispersion	101
4.5.14	The effect of cyclic AMP on the activity of certain enzymes	102
4.6	Are the effects of cyclic AMP mediated by protein phosphorylation?	104

4.7	Cyclic GMP	106
4.7.1	The metabolism of cyclic GMP	107
4.7.2	Stimulation of the production of cyclic GMP	107
4.7.3	The effects of cyclic GMP	108
4.7.4	Is the role of cyclic GMP to antagonise the effect of cyclic AMP?	110
4.7.5	The mechanism of action of cyclic GMP	110
4.8	Cyclic nucleotides other than cyclic AMP and cyclic GMP	110
5	Phosphoproteins of no known enzymic function	
5.1	Phosphoproteins of milk—the caseins	112
5.1.1	Definition and classification of caseins	112
5.1.2	α_s -Caseins	115
5.1.3	β -Casein	118
5.1.4	κ -Casein	119
5.1.5	λ -Casein	121
5.1.6	Other phosphoproteins in milk	121
5.1.7	Nonbovine caseins	122
5.1.8	The structure of casein	123
5.1.9	The casein micelle	124
5.1.10	The binding of ions to casein	126
5.1.11	Modified caseins	126
5.1.12	The phosphorylation of casein	127
5.1.13	The synthesis of casein	128
5.1.14	Functions of casein	129
5.2	The phosphoproteins of egg yolk	129
5.2.1	The lipovitellins	130
5.2.2	Lipovitellenin	133
5.2.3	Phosvitin	134
5.2.4	Formation and phosphorylation of phosvitin and lipovitellin	141
5.2.5	The function of egg yolk phosphoproteins	141
5.3	Ovalbumin	142
5.3.1	Preparation of ovalbumin	142
5.3.2	Composition of ovalbumin	142
5.3.3	Phosphorylation of ovalbumin	145
5.3.4	Synthesis of ovalbumin	145
5.4	Fibrinogen	146
5.4.1	Purification and structure of fibrinogen	146
5.4.2	The phosphorylation of fibrinogen	149
5.4.3	Abnormal fibrinogens	154
5.5	Miscellaneous tissue phosphoproteins	154
5.5.1	Attempts to isolate phosphoproteins from brain tissue	155
5.5.2	Phosphoproteins of bone and dentine	157
5.5.3	A phosphoprotein from the eggshell of the garden cricket	159
5.5.4	Cytoplasmic phosphoproteins	159
5.5.5	Phosphoproteins from human saliva	162

6	Enzymes which contain covalently bound phosphorus	
6.1	Enzymes which are regulated through phosphorylation	163
6.1.1	Enzymes of glycogen metabolism	163
6.1.2	Pyruvate dehydrogenase	183
6.1.3	Fructose 1,6-diphosphatase	186
6.1.4	Adipose tissue lipase	186
6.1.5	Acetyl-CoA carboxylase	186
6.1.6	Long-chain acyl-CoA synthetase (palmityl-CoA synthetase)	187
6.1.7	Polynucleotide phosphorylase	187
6.1.8	Cyclic nucleotide phosphodiesterase	188
6.1.9	Carbonic anhydrase	188
6.1.10	Pyruvate kinase	189
6.1.11	Cyclic-AMP-stimulated protein kinases	190
6.1.12	Cholesterol esterase	190
6.1.13	Cholesterol side-chain cleavage enzyme	191
6.1.14	Tyrosine 3-monooxygenase	191
6.1.15	RNA nucleotidyltransferase	192
6.1.16	6-Phosphofructokinase	195
6.1.17	Nomenclature of enzymes which are regulated by phosphorylation	196
6.2	Enzymes which form phosphorylated reaction intermediates	198
6.2.1	Phosphoglycerate kinase	199
6.2.2	Acetate kinase	199
6.2.3	Inorganic pyrophosphatase	200
6.2.4	Phosphoglucomutase	201
6.2.5	Phosphoglyceromutase	203
6.2.6	Nucleosidediphosphate kinase	204
6.2.7	Succinyl-CoA synthetase (succinic thiokinase)	205
6.2.8	ATP citrate (<i>pro</i> -3 <i>S</i>)-lyase	207
6.2.9	Hexokinase	208
6.2.10	Alkaline phosphatase	208
6.2.11	Acid phosphatase	208
6.2.12	Adenosinetriphosphatase	209
6.2.13	Type a ₁ protein kinase	217
6.2.14	Pyruvate, orthophosphate dikinase	217
6.3	Other enzymes which contain covalently bound phosphorus	218
6.3.1	Pepsin A	219
7	Protein phosphorylation and phosphoproteins in the nucleus	
7.1	The nucleus	221
7.2	Stages in the cell cycle	221
7.3	Histones	223
7.3.1	General characteristics and nomenclature	223
7.3.2	Methods of preparation	224
7.3.3	Primary structure and properties of individual histones	226
7.3.4	Secondary and tertiary structure of the histones	234
7.3.5	The interaction of histones with DNA	235
7.3.6	The function of histones in the nucleus	237
7.3.7	Modifications of histone side chains	239

7.4	Protamines	257
7.4.1	Fish protamines	257
7.4.2	Nonfish protamines	264
7.4.3	The function of the protamines	267
7.4.4	Phosphorylation of protamines	268
7.5	Nonhistone chromatin proteins	269
7.5.1	Isolation of nonhistone chromatin proteins	270
7.5.2	Characteristics of nonhistone chromatin proteins	272
7.5.3	Function of the nonhistone chromatin proteins	273
7.5.4	Phosphorylation of nonhistone chromatin proteins	276
7.6	A unifying hypothesis for the role of histones and nonhistone chromatin proteins in the nucleus	281
7.7	Protein phosphorylation in the nucleolus	284
8	Protein phosphorylation and phosphoproteins in the ribosomes	
8.1	Isolation of ribosomes	286
8.2	Structure of ribosomes	286
8.3	Functions of the ribosomal components	292
8.4	Phosphorylation of ribosomal proteins	293
8.4.1	Some precautions to be taken when studying the phosphorylation of ribosomal proteins	294
8.4.2	Enzymes which catalyse the phosphorylation of ribosomal proteins	294
8.4.3	Dephosphorylation of ribosomal proteins	297
8.4.4	The nature of the phosphorylated ribosomal proteins	297
8.4.5	Stimulation of the phosphorylation of ribosomal proteins	303
8.4.6	The function of the phosphorylation of ribosomal proteins	304
9	Protein phosphorylation and phosphoproteins in the mitochondrion	
9.1	The structure and function of mitochondria	306
9.2	Protein phosphorylation in the mitochondria	306
9.2.1	The nature of the mitochondrial proteins which can be phosphorylated	306
9.2.2	The nature of the phosphate donor for the phosphorylation of mitochondrial proteins	308
9.2.3	Dephosphorylation of mitochondrial proteins	309
9.2.4	Protein kinases which can catalyse the phosphorylation of mitochondrial proteins	310
9.2.5	The function of the phosphorylation of mitochondrial proteins	310
10	Protein phosphorylation in the cell membrane	
10.1	Preparation of cell membrane fragments	312
10.1.1	Preparation of microsomal fragments	315
10.1.2	General considerations of the methods used for the preparation of membrane fragments	316
10.1.3	Consideration of the purity of preparations of membrane fragments	316
10.2	Structure of the cell membrane	317

10.3	Protein phosphorylation in the cell membrane	318
10.3.1	The turnover of protein-bound phosphate in cell membrane fragments	319
10.3.2	Phosphorylation of proteins in membrane fragments from cerebral cortex	321
10.3.3	Protein phosphorylation in membrane fragments prepared from the anterior pituitary gland	344
10.3.4	Phosphorylation of certain myelin proteins	344
10.3.5	Protein phosphorylation in membrane fragments prepared from skeletal muscle	346
10.3.6	Protein phosphorylation in membrane fragments prepared from heart muscle	348
10.3.7	The phosphorylation of proteins in membrane fragments prepared from smooth muscle	349
10.3.8	The phosphorylation of proteins in membrane fragments prepared from liver	350
10.3.9	Phosphorylation of proteins in membrane fragments prepared from kidney	350
10.3.10	Phosphorylation of proteins in plasma membrane prepared from erythrocytes	351
10.3.11	Phosphorylation of proteins in membrane fragments prepared from toad-bladder epithelial cells	355
10.3.12	Lack of phosphorylation of proteins in membrane fragments prepared from adrenal medulla	356
10.3.13	Phosphorylation of proteins in ovary cell membrane preparations	356
10.3.14	Phosphorylation of proteins in membrane fragments prepared from corpus luteum	357
10.3.15	Phosphorylation of proteins in membrane fragments prepared from pancreas	358
10.3.16	Phosphorylation of proteins in membrane fragments prepared from fat cells	358
10.3.17	Phosphorylation of proteins in membrane preparations from gastric and intestinal mucosa	359
10.3.18	Phosphorylation of proteins in the plasma membrane of blood platelets	359
10.3.19	Phosphorylation of proteins in the outer membranes of whole cells	359
10.3.20	General considerations of the phosphorylation of membrane proteins	360
10.3.21	Control of the state of phosphorylation of membrane proteins	361
10.3.22	The function of the phosphorylation of membrane proteins	363

11	Protein phosphorylation, secretion, transport, and permeability	
11.1	Protein phosphorylation and secretion	364
11.2	Protein phosphorylation and the movement of ions	366
11.2.1	Models of active transport	367
11.2.2	Protein phosphorylation and passive permeability	369
11.2.3	Protein phosphorylation and the control of the active transport of Ca^{2+} ions	376
11.2.4	Protein phosphorylation and the control of water permeability	377
12	Cyclic AMP, protein phosphorylation, and the nervous system	
12.1	Organisation of the nervous system and transmission of the nervous impulse	378
12.1.1	The passage of a nervous impulse	379
12.1.2	Transmission of a nervous impulse across a synapse	380
12.1.3	Synaptosomes	382
12.2	Metabolism of cyclic AMP in the nervous system	382
12.3	Determination of the concentration of cyclic nucleotides in brain	384
12.4	Factors which affect the concentration of cyclic AMP in nervous tissue	384
12.4.1	Factors which affect the concentration of cyclic AMP in brain slices	384
12.4.2	The effect of agents on the concentration of cyclic AMP in isolated ganglia	389
12.4.3	The effect of agents on the production of cyclic AMP in tissue cultures	389
12.4.4	The effect of agents and electrical stimulation on the concentration of cyclic AMP in whole brain	390
12.4.5	The effect of agents on the production of cyclic AMP in brain homogenates	391
12.4.6	The sites of regulation of adenylate cyclase activity in brain	391
12.5	The effect of cyclic AMP in nervous tissue	392
12.5.1	The effect of cyclic AMP on whole brain	392
12.5.2	Cyclic AMP and axonal elongation	393
12.5.3	Cyclic AMP, protein synthesis and serotonin metabolism	394
12.5.4	The effect of cyclic AMP on the enzymes of glycogen metabolism in brain	396
12.5.5	The effect of cyclic AMP on synaptic function	396
12.5.6	Possible roles of protein phosphorylation in the nervous system	404
12.6	Cyclic GMP and the nervous system	412
12.6.1	Metabolism of cyclic GMP in nervous tissue	412
12.6.2	Factors which increase the concentration of cyclic GMP in nervous tissue	412
12.6.3	The role of cyclic GMP in the nervous system	414

13	Phosphorylation of proteins in miscellaneous tissues, species, and subcellular fractions	
13.1	Phosphorylation of the contractile proteins of muscle	416
13.1.1	Light chain myosin (preparation, properties, and function)	416
13.1.2	Troponin: preparation, properties, and function	421
13.1.3	Phosphorylation of a Ca^{2+} -binding protein from dogfish skeletal muscle	427
13.2	Phosphorylation of proteins in blood platelets	428
13.2.1	Platelet myosin	428
13.3	Cyclic nucleotides and the phosphorylation of proteins in the outer segments of retinal rods	429
13.3.1	Structure and function of retinal-rod outer segments (ROS)	429
13.3.2	Cyclic nucleotides in rod outer segments	430
13.3.3	Phosphorylation of protein in rod outer segments	432
13.4	Phosphorylation of microtubule proteins	437
13.4.1	Isolation and structure of microtubules	437
13.4.2	The function of microtubules	439
13.4.3	The effect of cyclic AMP on microtubules	440
13.4.4	The phosphorylation of microtubular proteins	440
13.5	The phosphorylation of proteins in viruses	442
13.5.1	The function of the phosphorylation of viral proteins	443
Appendices		
Appendix 1.	A theoretical treatment of the turnover of protein-bound phosphate in the presence of protein kinase and phosphatase activities	445
Appendix 2.	Determination of protein kinase activities	449
Appendix 3.	Estimation of protein-bound phosphate	452
Appendix 4.	Determination of protein phosphatase activity	457
Appendix 5.	Determination of protein kinase activities in polyacrylamide gels	458
Appendix 6.	Detection of phosphorylated proteins on polyacrylamide gels	459
Appendix 7.	The chemical phosphorylation of protein-bound serine residues	460
Appendix 8.	Preparation of phosphorylated amino acids	461
References		463
Index		547

General aspects and functions of proteins which contain covalently bound phosphorus

This chapter is written with the intention of introducing the reader to some general considerations of phosphoproteins and phosphorylated amino acids.

1.1 Classification and nomenclature of proteins which contain covalently bound phosphorus—the phosphoproteins

The first proteins to be discovered which contained covalently bound phosphorus were phosvitin (from egg yolk) and casein (from milk). These proteins, not unnaturally, were called 'phosphoproteins'. It was many years after these initial discoveries that it was found that other proteins could contain covalently bound phosphorus, though often in quite small amounts. This has led to some confusion in nomenclature. The term 'phosphoprotein' has been used for such a long time only to describe casein and phosvitin that people tend to be hesitant about using it to describe a protein which is newly discovered to contain covalently bound phosphorus, particularly if the concentration is low. Few people, for example, would think of describing a histone as a phosphoprotein even though it contains phosphorus, and the same applies to enzymes such as phosphorylase *a* which also contain phosphorus.

The term phosphoprotein, however, means, quite simply, a protein which contains covalently bound phosphorus and I shall use it in this sense even if the protein in question contains only a very low level of phosphorus.

Because of the great diversity of proteins which contain covalently bound phosphorus, and because of our frequent lack of knowledge about them, a proper classification is impossible. The phosphoproteins which have been at least partially purified and about which we know most may be divided into two groups: those which have enzymic activity and those which do not. The former group will be described in chapter 6 and the latter in chapter 5.

The properties and functions of proteins which contain covalently bound phosphorus will also be discussed in relation to their subcellular location in the appropriate chapters.

1.2 Phosphorylated amino acids which occur in proteins, and their chemical properties

In all the phosphoproteins so far investigated phosphorus has been found to be covalently bound only to serine, threonine, histidine, lysine, aspartic acid, or glutamic acid residues. The structures of these phosphorylated amino acids are shown in figure 1.1.

There was some early confusion but protein-bound phosphorylated amino acids are now known to occur almost entirely as phosphomonoesters,

although the presence of serine phosphodiesteres has been reported in certain peptides derived from nervous tissue^{1,2}.

Phosphoserine is by far the most commonly found protein-bound phosphorylated amino acid, though phosphothreonine is also frequently detected. These are the only phosphorylated amino acids which occur in casein and phosvitin and their presence has been recorded in many animal tissues³ as well as in certain bacteria⁴, viruses (chapter 13, page 143), and plant tissues. Many enzymes also contain phosphoserine (chapter 6).

It has been suggested that in certain circumstances the phosphoserine of phosvitin might occur in an enol form which would be of high energy⁵.

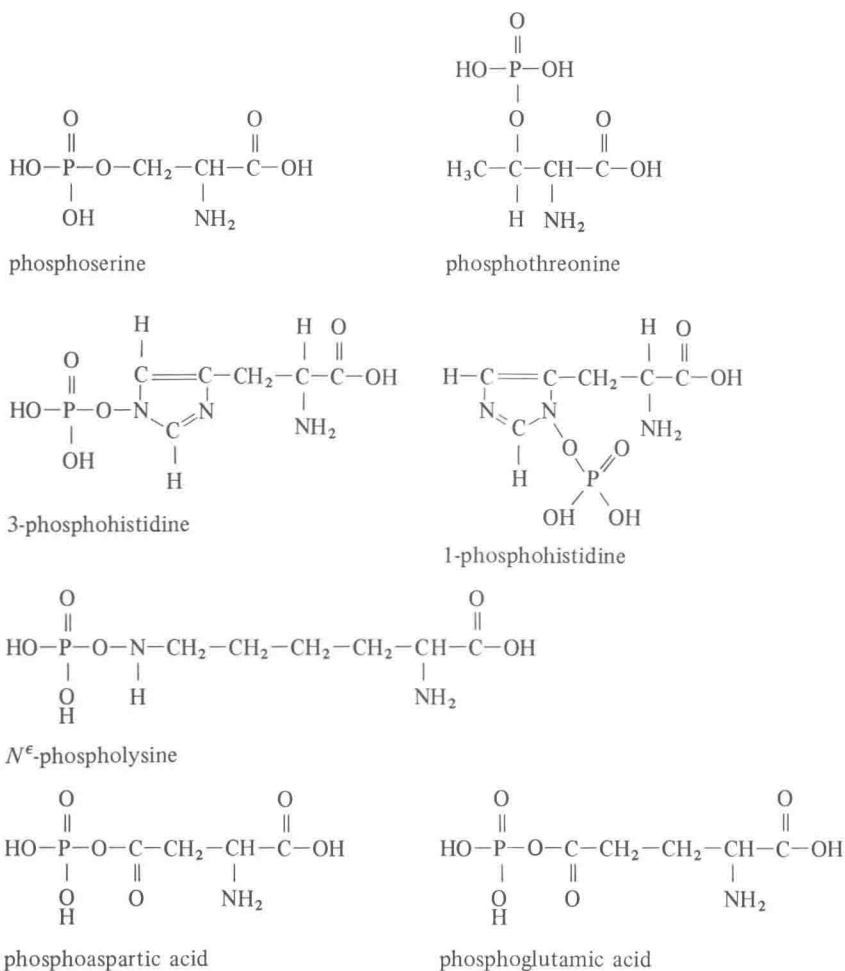


Figure 1.1. The structures of the phosphorylated amino acids.