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Phosphoproteins of the Cell Nucleus: Metabolism and Characterization

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PHOSPHOPROTEINS OF THE CELL NUCLEUS:

METABOLISM AND CHARACTERIZATION

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ABSTRACT

This thesis is concerned with the chemistry and metabolism of the phosphorylated proteins which are found localized in cell nuclei. The studies focus first on phosphoprotein metabolism as it occurs in the intact nucleus under varying conditions, and then on the chemistry and enzymology of a phosphoprotein fraction which has been extracted and purified from isolated nuclei.

The metabolism of phosphoproteins was studied in isolated calf thymus nuclei employing P^{32}-orthophosphate as a tracer. These isolated nuclei incorporate this isotope into nuclear proteins, which yield phosphoserine and phosphothreonine on hydrolysis. Protein phosphorylation is energy dependent, and occurs after the polypeptide chain has been completed. The phosphate groups, once incorporated into the protein, are not stable, but are subject to a very rapid "turnover" reaction. This "turnover" reaction in the nuclei also appears to be energy dependent. The bulk of phosphate incorporation in these nuclei is into non-histone protein, but small amounts of radioactive serine phosphate can be detected in highly purified histone fractions.

Since nuclear phosphoproteins have been suggested to play a role in regulating RNA synthesis, experiments were performed to determine whether the rate of protein phosphorylation in nuclei increases when gene activity is stimulated. Human lymphocytes treated with phytohemagglutinin undergo extensive gene activation, as evidenced by augmented synthesis of RNA. This activation is preceded by an early stimulation in the rate of phosphorylation and dephosphorylation of nuclear proteins. This finding is consistent with the hypothesized role of phosphoproteins in the modification of chromatin structure and in modulation of the template activity of DNA *in vivo*.

The protein fraction which is phosphorylated in the intact calf thymus nuclei has been extracted and extensively purified. This protein fraction contains about 1.3% alkali-labile phosphorus by weight,

which is the equivalent of 4-5 phosphorylated amino acids per 100 residues. The enzymatic reaction in which serine and threonine residues in the protein are phosphorylated by the terminal phosphate of ATP (and other ribonucleoside and deoxyribonucleoside triphosphates) has been extensively studied in vitro. The purified protein fraction contains endogenous kinase activity, so that protein phosphorylation proceeds without the need for any added enzyme. Unlike the case of phosvitin, the reaction between ATP and the nuclear phosphoprotein is only slightly reversible. Radioactive phosphate groups incorporated in this reaction are stable to incubation under "cold-chase" conditions, indicating that the enzyme activity responsible for the rapid "turnover" of protein-phosphate groups seen in intact nuclei is not an inherent part of the phosphoprotein.

However, if P^{32}-labeled phosphoprotein is prepared and added back to an unlabeled nuclear suspension, "turnover" is reestablished and the P^{32} appears as inorganic phosphate. Thus, there seem to be two distinct reactions for modifying the phosphoprotein, a kinase reaction for phosphorylating the protein and a "phosphatase"-like reaction for dephosphorylating it.

The results of these experiments are discussed in terms of the various biological functions in which phosphoproteins are thought to be involved. Arguments are presented against the participation of the nuclear phosphoproteins in phosphate storage, phosphoryl-group transfer, active transport, and oxidative phosphorylation. It is suggested that the phosphorylation and dephosphorylation reactions may be functioning to induce structural changes in the phosphoprotein which lead to modification of the structure and metabolic activity of the chromatin.

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CHAPTER ONE: GENERAL CONSIDERATION OF PHOSPHOPROTEINS

The experiments to be described in this dissertation are concerned with a study of the nature of the phosphorylated proteins which are found localized in cell nuclei. These studies will focus first on the metabolism of phosphoproteins as it occurs in the intact nucleus, and then on the chemical and enzymological characterization of a phosphoprotein fraction which has been extracted and purified from isolated nuclei.

A phosphoprotein is formally defined as any protein containing a phosphate group in direct covalent linkage with one of the amino acid residues in the polypeptide chain. There are relatively few phosphoproteins known, but within this small group there is a wide structural and functional diversity. It includes proteins as strikingly different as phosvitin, phosphorylase, and phosphoglucomutase. Since phosphoproteins are of relatively rare occurrence, the recent discovery by Langan and Lipmann (Langan, 1967) that a phosphoprotein fraction is localized in the nuclei of higher cell types was of immediate interest to people concerned with problems of synthesis and regulation in the cell nucleus. Before dealing specifically, however, with the detailed nature of the nuclear phosphoproteins and the speculations on their possible importance

in nuclear function, it will be useful to our ultimate understanding if we first briefly review what is known about the chemistry and metabolism of the other phosphorylated proteins which have been more thoroughly studied.

CHARACTERISTICS OF PHOSPHORYLATED PROTEINS

Since the designation "phosphoprotein" brings together under one heading such a diverse group of proteins, it seems desirable at the outset to subdivide this group on the basis of some rational criteria. Three characteristics are especially useful in pointing out the similarities and differences between the various types of phosphorylated proteins: 1) biological function of the phosphoprotein; 2) quantity of phosphorus contained in the protein; and 3) nature of the protein-phosphate linkage.

A complete separation of phosphoproteins into functional categories would be highly desirable, but unfortunately this is not feasible because the biological function of many of the phosphoproteins is still unknown. Nonetheless, a few functional subdivisions are obvious and useful. One such category is that of the phosphate transfer enzymes, in which case the active site of the protein is phosphorylated as an intermediate during the metabolic transfer of a phosphate group. Phosphoglucomutase, alkaline phosphatase, and *E. coli* phosphotransferase are good examples of phosphoproteins in this category. On the other hand, certain enzymes like pepsin, phosphorylase, and Na^+-K^+ ATPase are also phosphorylated, although the primary function of these enzymes is not the transfer of phosphate groups. Thus, just because an enzyme is phosphorylated does not in itself place it in the category of a phosphate transfer enzyme.

A second category of phosphoproteins which are often grouped together functionally are the so-called "classical" phosphoproteins, phosvitin and casein, which contain relatively large amounts of phosphorus and are thought to function as a storage mechanism for inorganic phosphate. A problem with this particular category is that some recently discovered phosphoproteins, like those present in cell nuclei and the phosphoprotein component of dentin and enamel, bear a striking resemblance to the classical phosphoproteins in certain aspects of structure and enzymology, but yet are probably not involved functionally in phosphate storage.

Other functional categories which can be considered are phosphoproteins involved in active transport, oxidative phosphorylation, and proteins in which the phosphate group functions to modify the protein's structure. Possible examples of such instances will be discussed subsequently when the individual phosphoproteins are described in detail.

Since division into functional categories would leave too many phosphoproteins unclassified due to the uncertainty about their biological function, let us consider the possibility of categorizing the phosphoproteins on the basis of how much phosphorus they contain. For those proteins which contain relatively large amounts of phosphorus (>1%), this would work quite well. Phosvitin, casein, phosphoproteins of dentin and enamel, and nuclear phosphoproteins would all fit in this category, which seems reasonable on the basis of some of the similarities in their chemistry and enzymology. However, this type of classification would be quite unsatisfactory for the proteins containing relatively small amounts of phosphorus. Thus, pepsin, ovalbumin, phosphoglucomutase, and the E. coli phosphotransferase would fall into the same category of proteins with low phosphorus content. Yet it is obvious that these four proteins differ markedly in terms of structure and function and especially in the chemical properties and functional nature of the phosphate bonds involved.

The final possible way of classifying phosphoproteins is according to the way in which the phosphate group is linked to the protein. There are several types of amino acid side-chain groups which are capable of forming covalent linkages with phosphate:

1) Hydroxyl group – serine, threonine, tyrosine, hydroxyproline, hydroxylysine

2) Nitrogen-containing groups – lysine, arginine, histidine, N-terminal amino

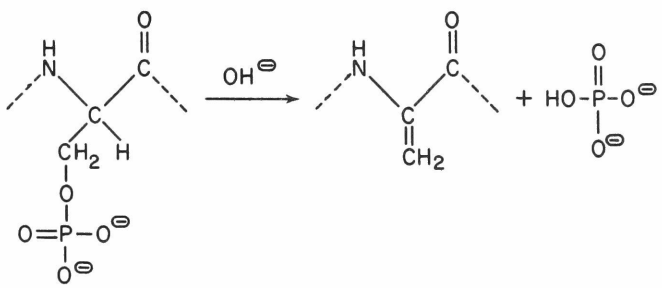
3) Carboxyl group – glutamic acid, aspartic acid, C-terminal carboxyl

4) Sulfhydryl group – cysteine

Of these 13 different possible sites of phosphate linkage, only four have been definitely identified in naturally-occurring phosphoproteins: serine, threonine, histidine, and glutamic acid. However, it is quite conceivable that future study of phosphoproteins will bring to light the natural occurrence of additional types of protein-phosphate linkages, so it will be useful to briefly consider all the possibilities.

The most common mode of phosphate binding in phosphoproteins is esterification to the hydroxyl group of a serine residue. Phosphoserine was first discovered in the egg yolk phosphoprotein, vitellin, by Lipmann and Levene in 1932. Subsequently this amino acid has also been identified in casein, ovalbumin, pepsin, phosphoglucomutase, phosphorylase a, alkaline phosphatase, fibrinogen, acyl carrier protein, and phosphoproteins from enamel, dentin, mitochondria, and cell nuclei. The other β-hydroxy amino acid, threonine, can also be phosphorylated in proteins, although it occurs much less frequently than does phosphoserine. Phosphothreonine was first isolated by de Verdier in 1953 from an acid hydrolysate of casein. It has subsequently also been discovered in phosvitin and in nuclear phosphoproteins.

A distinguishing characteristic of proteins containing phosphoserine and phosphothreonine is that although they are quite stable in acid solution, they are readily dephosphorylated by treatment with dilute alkali (Plimmer and Bayliss, 1906). This observation is of special interest because the isolated phosphorylated amino acids themselves are quite stable in alkali (Plimmer, 1941). The rapid removal of phosphate from these phosphoproteins by mild alkali is not caused by hydrolysis of the phosphate esters, but is rather due to a β-elimination reaction:



(phosphoseryl residue) (dehydroalanyl residue)

Evidence for this mechanism was provided by Anderson and Kelley (1959), who showed that when alkaline dephosphorylation of casein is allowed to take place in water enriched with O^{18}, none of the isotope appears in the released inorganic phosphate. Spectroscopic evidence also indicates that during dephosphorylation of phosvitin with alkali, the phosphoserine residues are converted to dehydroalanine (Mecham and Olcott, 1949). Further degradation of these alkali-treated proteins with concentrated acid rapidly converts the dehydroalanine to pyruvate and ammonia (Posternak and Posternak, 1928; Allerton and Perlmann, 1965).

It should be pointed out that for a long time the alkali lability and acid stability of these phosphate-protein linkages were considered as identifying characteristics of all phosphoproteins, and were often employed as the bases of assay systems for phosphoproteins. However, it has subsequently been discovered that other protein-phosphate linkages exist which do not exhibit these same properties. Therefore acid stability and alkali lability apply only to the group of phosphoserine- and phosphothreonine- containing proteins.

The other amino acids which contain -OH groups, namely tyrosine, hydroxyproline, and hydroxylysine, have not been observed to be phosphorylated in natural proteins. However, phosphotyrosine is thought to occur in serum albumin which has been chemically phosphorylated with phosphorus oxychloride (Mayer and Heidelberger, 1946), and phosphohydroxylysine has been identified in calf embryo extracts (Gordon, 1948). Thus investigators should be on the lookout for the possible occurrence of these derivatives in phosphoproteins.

The first example of an N-phosphorylated amino acid to be discovered in biological material was phosphoarginine (Meyerhof and Lohmann, 1928). It occurs in the muscles of invertebrates and functions as a reservoir of high energy phosphate, analagous to the role of creatine phosphate in vertebrates (Lipmann, 1941). Thus far phosphoarginine has not been observed in peptides or intact proteins.

The only N-phosphorylated amino acid which has definitely been identified in protein molecules is phosphohistidine. It was first discovered by Boyer *et al.* (1962) in a mitochondrial protein, and subsequently has also been found to occur in a protein component of an

E. coli phosphotransferase system (Kundig et al., 1964). N-ε-phosphoryl-lysine has been observed in a high molecular weight material obtained from rat liver cell sap, but it has not yet been definitely identified as protein (Zetterqvist and Engström, 1967). There are no known examples of N-terminal phosphorylation of a protein molecule.

Phosphoproteins which contain N-phosphoryl bonds behave quite differently from those containing phosphoserine or phosphothreonine (De Luca et al., 1963; Kundig et al., 1964). They are quite stable in alkali, and in fact phosphohistidine has been isolated and identified in these proteins after alkaline degradation. On the other hand, they are readily dephosphorylated in dilute acid.

The only well-established case of phosphorylation of a protein carboxyl group is in Na^+-K^+ ATPase, where γ-phosphoryl glutamic acid has been identified as an intermediate in the enzymatic reaction (Kahlenberg et al., 1967). The acyl-phosphate linkage differs from the previous protein-phosphate linkages, in that it is extremely labile in both acid and alkaline solution, and in addition is cleaved by hydroxylamine (Nagano et al., 1965; Hokin et al., 1965). Phosphorylated carboxyl derivatives of aspartic acid or C-terminal amino acids have not been observed in phosphoproteins.

The final amino acid side chain to be considered which is potentially capable of forming a covalent linkage to a phosphate group is the sulfhydryl group of cysteine. The possibility that phosphorylated thiols may play a role in biological reactions has been suggested by several investigators (Green, 1952; Feuer and Wollemann, 1955; Smith et al., 1957; Kaufman, 1955), but there is as of yet no conclusive evidence for the natural occurrence of thiolphosphates. It is possible, however, that their extreme acid lability has prevented their isolation from biological material (Akerfeldt, 1963). An enzyme has been isolated from bacteria which is capable of utilizing thiolphosphates in the phosphorylation of various hexoses (Korman et al., 1965). Although it is quite possible that this does not represent the normal function of the enzyme within the cell, it nonetheless supports the possibility that the sulfur-phosphorus bond may exist as an important intermediate in biological reactions.

Having thus considered the three possible ways of classifying and describing phosphoproteins, it is apparent that no single criterion by itself is adequate. Classification into functional categories is not possible because the function of too many phosphoproteins is unknown. Classification according to phosphate content ignores the large chemical and functional diversity among the proteins containing small amounts of phosphorus. Finally, classification according to the nature of the protein-phosphate bond by itself is also inadequate, because most of the phosphoproteins contain phosphoserine, but differ markedly in many other aspects.

Since the use of any single criterion for classification would thus tend to be misleading, no attempt will be made to divide the subsequent detailed discussions of the specific phosphoproteins into any of these categories. Instead, the phosphoproteins have simply been put into very general categories according to the type of biological material from which they were first obtained. This is not meant to imply anything about the similarities or differences of the phosphoproteins involved, but is merely done to avoid forcing these proteins into artificial categories. One incidental advantage of this type of grouping is that it tends to parallel very closely the historical sequence in which these phosphoproteins have been discovered.

PHOSPHOPROTEINS OF MILK AND EGGS

Historically the first phosphoproteins to be studied were those present in milk and eggs. An obvious reason for this is that these materials could be easily obtained in large quantities, and the phosphoproteins, being major bulk components, could be prepared relatively simply. The term "classical" phosphoproteins is usually employed in reference to the caseins and the egg yolk phosphoproteins, and does not include ovalbumin, which contains much less phosphorus than the former proteins.

Milk phosphoproteins: caseins

One of the most thoroughly studied of all phosphoproteins

is casein, the principal protein component of milk. Casein, which contains about 0.85% bound phosphorus by weight, accounts for almost 80% of the total protein nitrogen of cow's milk (Menefee et al., 1941). It is thus obviously of great nutritive importance and was isolated and studied as early as the first part of the 19th century (Braconnot, 1830; Dumas and Cahours, 1842; Millon and Commaille, 1865a,b). Perhaps a reason for the great attention paid this protein by early investigators is to be found in the relative ease with which large quantities could be purified at a time when relatively few sophisticated techniques for protein fractionation were available. In the earliest procedures, fresh milk was simply skimmed to remove the cream and then acidified to pH 4.6, at which point the casein precipitates out, leaving the remaining milk proteins in solution (Hammersten, 1883, 1885).

Due to the early contentions of Hammersten, casein was long considered to be a pure protein. However, it was conclusively demonstrated as early as 1925 by the solubility studies of Linderstrøm-Lang and Kodama that casein is actually a heterogeneous mixture of proteins. This discovery led to numerous attempts to fractionate casein, and in 1929, Linderstrøm-Lang, utilizing fractionation in acid-alcohol mixtures, reported the isolation of seven casein fractions which varied in their phosphorus contents from 0.1% to 0.9%. Perhaps the most important fractionation historically was accomplished subsequently by Mellander (1939), who employed moving boundary electrophoresis to distinguish between three major fractions of casein, which he designated α, β, and γ in order of decreasing mobility at neutral pH.

The earliest methods for the preparative scale isolation of these components, which involved isoelectric precipitation and differential solubility in alcohol and urea solutions, were developed by Warner (1944) and by Hipp and co-workers (1950, 1952). The development of these procedures allowed the α, β, and γ fractions to be chemically characterized in terms of phosphorus content and amino acid composition (Gordon et al., 1949, 1950, 1953). From these studies it was concluded that α-casein represents about 70% of the casein complex, β-casein 27%, and γ-casein about 3%; the respective phosphorus contents were found to be α=1.0% P, β=0.6% P, and γ=0.1% P.

Unfortunately it was soon realized by these workers that the α, β, and γ fractions themselves are not homogeneous, and it wasn't long before continuing investigation led to descriptions of an ever-increasing number of casein components. The α-casein fraction was soon resolved into two main components, the α_s fraction which is insoluble in the presence of Ca^{++}, and the k-fraction, which is soluble under these conditions (von Hippel and Waugh, 1955; Waugh and von Hippel, 1956; Waugh, 1958). As more and more modern techniques of protein fractionation were applied to casein, more and more new fractions were described, so that the maximum estimate of the number of different casein fractions has now gone as high as 20 (Wake and Baldwin, 1961).

A relatively recent development has been the discovery of additional protein heterogeneity based on inheritable variations in the structure of α_s-casein (Thompson et al., 1962; Kiddy et al., 1964; Thompson and Kiddy, 1964) and β-casein (Aschaffenberg, 1961, 1963; Thompson et al., 1964; Thompson and Pepper, 1964). Although this additional complexity of genetic polymorphism increased the immediate confusion surrounding the classification of casein components, it might hopefully lead to an eventual clarification of the great variability seen in casein fractionations. Although the present state of casein nomenclature is still one of great confusion, several recent reviews are available which attempt to clarify the relationships between the many different reported casein fractions (Thompson et al., 1965; Jollès, 1966).

Due to the complex nature of casein heterogeneity, it is not too surprising to find a lack of general agreement on the nature of the phosphorus linkages in casein. Although phosphoserine (Lipmann, 1933a,b) and phosphothreonine (de Verdier, 1953) have both been isolated from casein, considerable controversy exists as to whether this type of phosphate monoester linkage is the only type of linkage present. On the basis of the dephosphorylation of casein with specific enzymes, Perlmann (1954, 1955) concluded that α-casein contained 40% phosphate monoester linkages, 20% pyrophosphate linkages, and 40% phosphate diester linkages of the type -O-PO(OH)-NH-. Although there was some independent support for this conclusion (Thoai et al., 1954), it has been questioned by several other investigators on the basis of

additional enzymatic (Sundararajan and Sarma, 1957; Hofman, 1958; Kalan and Telka, 1959), chemical (Peterson et al., 1958), and calorimetric data (Belec and Jeness, 1962). One must be extremely cautious in interpreting the enzymatic arguments, since they are all based on the tenuous assumption that enzymes show the same specificity for phosphate bonds in naturally-occurring proteins as they do for phosphate bonds in low molecular weight substrates. It is interesting to note that more recent experiments employing nuclear magnetic resonance spectroscopy (Ho and Kurland, 1966) have tended to support Perlmann's claim of the existence of diester and/or pyrophosphate bonds in casein, but the issue is certainly far from settled at present.

Egg yolk phosphoproteins: vitellin, vitellenin, phosvitin

It was known as early as 1900 that a large quantity of phosphoprotein is contained in the yolk of hen's eggs (Osborne and Campbell, 1900; Levene and Alsberg, 1901). At that time, yolk protein was thought to be composed of two major components, vitellin and livetin, which were the fractions soluble and insoluble in water respectively. Vitellin, which accounts for almost 80% of the total yolk protein, is found in association with phospholipids and contains about 1% protein-bound phosphorus by weight; livetin, on the other hand, contains neither lipid nor phosphorus.

Attempts were made at that early stage to fractionate vitellin by exposure to 12% ammonium hydroxide (Levene and Alsberg, 1901). Such treatment yielded a phosphorus-enriched protein fraction which contained about 10% phosphorus by weight, and which was designated vitellinic acid. Somewhat later Posternak and Posternak (1927) employed proteolytic digestion of vitellin with trypsin to obtain similarly enriched phospho-peptide fractions which they called ovotyrines. However, due to the degradative nature of both these approaches, the actual significance of these phosphorus-rich fractions was not realized at the time, and for many years the phosphoprotein of egg yolk was thought to contain only about 1% phosphorus.

However, in 1949 Mecham and Olcott developed a non-degradative procedure based on magnesium sulfate precipitation which yielded a

lipid-free protein fraction containing about 10% phosphorus. This protein fraction accounted for 7% of the total yolk protein and at least 60% of the yolk's protein phosphorus. It closely resembled vitellinic acid and β-ovotyrine in composition, and was named "phosvitin" because of its high phosphorus content and its origin in egg yolk. Since phosvitin displayed a great affinity for binding to other proteins, it was suggested that the phosphorus content of vitellin, which varied considerably from preparation to preparation, was actually due to a varying contamination of this fraction with the phosphorus-rich phosvitin.

However, Fevold (1951) has pointed out several observations which are not consistent with this interpretation. For example, vitellin can be digested with pepsin or trypsin to yield peptides containing 3-4% phosphorus; yet phosvitin cannot be responsible for these peptides because phosvitin is resistant to these enzymes. Fevold claims that crude vitellin is actually composed of at least two phosphoproteins distinct from phosvitin, which he has referred to as vitellin and vitellenin, which contain 1.0% and 0.29% phosphorus respectively. Thus it appears most likely that there are several different phosphoproteins present in egg yolk, among which phosvitin contains the highest concentration of phosphorus and has been the most thoroughly studied.

Phosvitin is a very unique protein in many ways other than its high concentration of phosphorus. For example, the amino acid composition is very unusual, in that about half of the total residues are serine. Cysteine is totally lacking, and the content of methionine and the aromatic amino acids is very low (Mecham and Olcott, 1949; Lewis et al., 1950; Allerton and Perlmann, 1965). Phosphoserine was isolated from egg yolk phosphoproteins as early as 1932 (Lipmann and Levene), and the subsequent finding that the molar phosphorus content of phosvitin was essentially identical to the molar serine content led to the conclusion that phosphoserine could account for all the phosphate present in phosvitin (Mecham and Olcott, 1949). However, recently it has been discovered that at least one residue of phosphothreonine is present per molecule of phosvitin (Allerton and Perlmann, 1965).

Since better than one out of every two residues in phosvitin is phosphoserine, it is not surprising to find that peptides have been isolated from phosvitin which contain up to six adjacent phosphoserine residues (Williams and Sanger, 1959). (However, similar clusters of up to three phosphoserines have been obtained from casein where only one-tenth as much phosphate is present, so it is possible that the tendency for phosphoserine to occur in clusters is characteristic of these types of phosphoproteins.) Evidence from base titrations (Mecham and Olcott, 1949) indicate that all the phosphate groups are present in the monoester form.

Phosvitin as prepared by the procedure of Mecham and Olcott is heterogeneous by several criteria, including free-boundary electrophoresis (Mecham and Olcott, 1949; Sugano, 1957) and paper electrophoresis (McCully et al., 1959; Sundararajan et al., 1960). Both ion exchange chromatography (Connelly and Taborsky, 1961; Pinna et al., 1963; Heald and McLachlan, 1963, 1964) and countercurrent distribution (Mok et al., 1966) have been employed in preparative scale procedures for the fractionation of phosvitin into two major fractions, which occur in the approximate ratio of 2 to 1. These two components are very similar in terms of phosphorus content, amino acid composition, and molecular size and shape (Taborsky and Mok, 1967). There is a small significant difference in molecular weights (major component= 36,000 and minor component = 40,000), but the main difference between the two fractions seems to be the capacity to bind metals, since the minor component contains a 10-fold higher concentration of tightly bound non-heme iron.

Recently the study of the classical yolk phosphoproteins has been extended to several species of fish. One of the more striking findings is the extreme deficiency of amino acids which occurs in some of these fish roe phosvitins. For example, only six amino acids could be detected in a phosvitin fraction isolated from rainbow trout containing 4% phosphorus (Ito et al., 1963), and only five amino acids in a herring phosvitin containing 12% phosphorus (Barman et al., 1964). The three amono acids, serine, aspartic acid, and glutamic acid were common to both these phosphoproteins; in addition, the herring phosvitin

contained valine and threonine, and the trout protein contained glycine, alanine, and minute quantities of an unidentified amino acid.

Another striking finding from the fish roe phosphoproteins is that in some cases several distinct phosvitin fractions with differing phosphorus contents can be isolated. In the most thoroughly studied case, Mano and Lipmann (1966a) found that ling roe phosvitin could be fractionated by ion exchange chromatography into four distinct fractions containing 3.2, 5.7, 7.2, and 9.5% alkali-labile phosphorus respectively. When the phosphorylation of these fractions was studied in an enzymatic system employing purified phosphoprotein kinase and ATP, it was found that enzymatic phosphorylation proceeded in discrete discontinuous steps to yield fractions with distinct new levels of phosphorylation (Mano and Lipmann, 1966b). The existence of these distinct levels of phosphorylation may be related to the previously mentioned existence of phosphoserine clusters in these types of phosphoproteins.

In addition to the rather unique chemical properties of phosvitin, the biological synthesis of this protein class also shows some rather unusual features. In 1935 Laskowski discovered that a phosphoprotein, which he designated serum vitellin, is present in the serum of laying hens which cannot be found in the serum of non-laying hens. Isolation and characterization of the serum phosphoprotein showed it to be very similar in composition to the phosphoprotein occurring in egg yolk (Schjeide and Urist, 1956, 1959). When the synthesis of this phosphoprotein was followed using P^{32} injected into laying hens, it was found that the phosphoprotein was labeled first in the liver, subsequently appeared in the serum, and ultimately ended up in the egg yolk (Flickinger and Rounds, 1956). These data, along with the antigenic similarity found between serum and yolk phosphoprotein, provide convincing evidence for the theory that the phosphoprotein of the hen's egg is actually synthesized in the liver and is subsequently transported to the egg via the bloodstream.

Since the yolk mass must provide all the nutritional requirements necessary to support the developing organism up until the late stage when oral feeding becomes possible, it is apparent why the yolk phosphoproteins have long been thought to play an important nutritional

role. It is obvious that large quantities of phosphate are stored in phosvitin, and the relatively high phosphoryl potential of these groups implies that they may be serving as a high energy phosphate pool (Rabinowitz and Lipmann, 1960). Grant and Taborsky (1966) have recently suggested that phosvitin can be oxidized via an α-β-dehydrogenation of phosphoserine to the corresponding enol derivative. This would result in an even higher level of phosphoryl potential. Phosvitin has also been suggested to simultaneously function as an iron-carrier protein (Greengard et al., 1964; Grant and Taborsky, 1966).

Egg white phosphoproteins: ovalbumin, plakalbumin

Ovalbumin, a glycoprotein which accounts for about 60% of the total protein of egg white, has long been known to contain small amounts of phosphorus (Osborne and Campbell, 1900b). However, early investigators were puzzled by the finding that in spite of extensive recrystallization, the number of moles of phosphorus per molecule of ovalbumin did not come out to an integral number. The values were always around 0.12% phosphorus, or 1.7 moles per molecular weight of 45,000. A further unusual observation was that the phosphorus content decreased upon prolonged storage, and values from 0.12% phosphorus to as low as 0.073% were reported (Sørensen, 1930).

These data strongly suggested that in spite of repeated recrystallizations, ovalbumin was still a heterogeneous preparation. Finally in 1940, Longsworth and co-workers demonstrated that two distinct components of ovalbumin could be detected in free boundary electrophoresis. The faster moving, more acidic component accounted for about 80% of the material and was designated A_1; the component present in smaller amounts was designated A_2. Later Cann (1949) described the presence of a third component, A_3, which was even less acidic than A_2 and which accounted for about 5% of the total material.

On the basis of this electrophoretic heterogeneity, Linderstrøm-Lang and Ottesen (1949) proposed a possible explanation of the lack of an integral number of phosphorus atoms in ovalbumin. If A_1 were to contain two moles of phosphorus per molecule and A_2 only one mole of phosphorus, then a mixture containing about 80% A_1 and 20%

A_2 would be expected to have a total phosphorus content very close to the observed value of 1.7 mole %. Furthermore, the loss of one negatively-charged phosphate group in the conversion of A_1 to A_2 would explain the lowered electrophoretic mobility of the latter component. This conversion of A_1 to A_2 would also explain the decrease in phosphorus content observed in ovalbumin preparations during prolonged storage.

This proposed model was confirmed by the elegant experiments of Perlmann (1950, 1952a), who studied the effects of the enzymatic removal of phosphate groups on the electrophoretic behavior of ovalbumin. Treatment of ovalbumin with prostatic phosphatase was found to release 46% of the phosphorus, with simultaneous conversion of electrophoretic component A_1 to A_2. Further digestion with intestinal or potato phosphatase was capable of removing the remaining phosphorus, while electrophoretically A_2 was replaced by the slowest moving component, A_3.

Therefore, it has been conclusively established that ovalbumin can exist in three distinct forms, which contain either two, one or no phosphate groups per molecule. Quite recently it has been discovered that during normal biosynthesis of the protein, ovalbumin is first completed in the totally unphosphorylated form (A_3), and the two phosphate groups are added afterwards (Sanger and Hocquard, 1962). Results from electrophoretic and enzymatic studies indicate that the phosphate is present totally in the monoester form (Perlmann, 1955).

The nature of the linkage of the two phosphate groups within the protein has not yet been completely worked out. At least one of the phosphate groups is known to be present as phosphoserine (Perlmann, 1952b; Flavin, 1954), but the nature of the second phosphate is a subject of some controversy. Flavin (1954) thought the second phosphate was also present as phosphoserine, because he was able to isolate two different peptides from ovalbumin, both containing phosphoserine. However, the yield of the second peptide was too low to rule out the possibility of rearrangement during acid hydrolysis. Perlmann (1955) has observed that the susceptibility of the two phosphate groups to enzymatic cleavage is quite different, and although this could indicate different phosphate linkages, it could also be due to differ-

ent molecular environments of the two phosphate groups. Although the nature of the second phosphate group has thus not been elucidated, it is known that it cannot be esterified to the polysaccharide component of this glycoprotein, since it is possible to recover all of the carbohydrate of ovalbumin in a fraction which is completely free of phosphorus (Perlmann, 1955).

Ovalbumin can be converted in high yield to another crystalline form, designated "plakalbumin", by treatment with the proteolytic enzyme subtilisin (Linderstrøm-Lang and Ottesen, 1947, 1949). The transformation involves the release of three different peptides, containing a total of 14 amino acid residues (Guntelberg and Ottesen, 1954; Ottesen, 1958). Plakalbumin, like ovalbumin, exists in three distinct forms, P_1, P_2, and P_3, which contain two, one and no moles of phosphorus respectively (Perlmann, 1950, 1952a). The electrophoretic mobilities of these components are somewhat less than the corresponding forms of ovalbumin, due to the loss of some acidic residues in the peptides released by subtilisin.

TISSUE PHOSPHOPROTEINS

The early descriptions of phosphoproteins in mammalian tissues were mainly based on the chemical fractionation procedures developed by Schmidt and Thannhauser (1945) and by Schneider (1945). In the Schmidt-Thannhauser procedure, tissue homogenates are treated with cold TCA followed by ethanol-ether to remove small molecules and lipids. The insoluble residue is then incubated with warm alkali, and any inorganic phosphate which is released is considered to be phosphoprotein phosphorus. In the Schneider procedure, a similar acid-insoluble, lipid-free residue is extracted with hot TCA to remove the nucleic acids, and any phosphorus remaining in the residue is considered to be from phosphoprotein.

Utilizing these fractionation schemes, early investigators identified phosphoprotein fractions in a wide variety of tissue types (Marshak and Calvet, 1949; Friedkin and Lehninger, 1949; Davidson et al., 1951; Albert et al., 1951; Johnson and Albert, 1952, 1953; Williams-Ashman and Kennedy, 1952). These tissue phosphoproteins were character-

ized by an extremely high rate of phosphate group turnover when P^{32}-orthophosphate was employed as a tracer. However, the significance of these findings is difficult to determine, since the so-called "phosphoprotein" fractions obtained by these procedures are obviously contaminated by other components.

Subsequently, more convincing evidence for the existence of tissue phosphoproteins exhibiting high rates of phosphate turnover was provided by the actual isolation of phosphoserine from the Schneider residue (Kennedy and Smith, 1954; Ågren et al., 1954). Kennedy and Smith (1954) suggested that the high rate of turnover of these tissue phosphoproteins reflected the activity of phosphotransferases, a speculation which was shown to be at least partially valid by the discovery that phosphoglucomutase was a component of this fraction (Kennedy and Koshland, 1957).

One tissue in which phosphoprotein metabolism has attracted considerable attention is brain. The interesting aspect of the phosphoserine-containing brain proteins is that their rate of phosphate group turnover increases markedly when the nervous tissue is stimulated electrically (Heald, 1957, 1958; Ahmed et al., 1963). This phosphoprotein seems to be localized in the microsome fraction (Trevor and Rodnight, 1965), is closely associated with lipid, and has been suggested to play a role in active transport reactions (Heald, 1962). Similar phosphoprotein fractions have been suggested to play a role in active transport reactions in liver (Ahmed and Judah, 1962; Judah and Ahmed, 1962) and in erythrocytes (Judah et al., 1962a,b).

It is apparent that studies on the "phosphoprotein fraction" of various tissues are complicated by the fact that these fractions are actually composed of a mixture of many different phosphoproteins. Thus a meaningful characterization of tissue phosphoproteins requires that the individual components of this fraction be isolated and studied individually. Thus far seven distinct phosphoproteins have been isolated from mammalian tissues: pepsin, phosphoglucomutase, phosphorylase a, phosphorylase kinase, alkaline phosphatase, a sodium-potassium dependent adenosine triphosphatase, and fibrinogen. In addition, phosphoproteins have been partially purified from enamel,

dentin, mitochondria, and cell nuclei. The enzymes phosphoglycero-mutase (Pizer, 1960) and hexokinase (Ågren and Engström, 1956) were once thought to be phosphoproteins, but more recent evidence has indicated that this is not the case (Pizer, 1962; Hass et al., 1961).

Pepsin

It has long been known that the proteolytic enzyme pepsin, as well as its inactive precursor, pepsinogen, contain one atom of phosphorus per molecule (Northrop, 1930; Herriott, 1938). The point of attachment of the phosphate group once again appears to be the hydroxyl group of a serine residue (Flavin, 1954; de Verdier, 1954). This phosphate linkage, however, does not behave like a simple phosphate monoester when treated with various phosphatases. For example prostatic phosphatase, which normally cleaves phosphate monoesters, will not release the phosphate from pepsin unless the pepsin is first pretreated with venom phosphodiesterase (Perlmann, 1955, 1958). This suggests the possible existence of a phosphodiester bond, a speculation which is given further support by the failure to observe a dissociable proton in the pH range from 6.0 to 8.0, as would be expected if we were dealing with a simple phosphate monoester. However, we must again use caution in interpreting such data, since similar effects might be caused by the particular environment of the phosphoserine within the protein chain. The actual existence of a phosphodiester bond within the protein chain can be conclusively proven only by the physical isolation of two peptides joined by a phosphodiester linkage.

Since the enzymatic activity of pepsin has been so thoroughly investigated, this was the first case where the functional role of a phosphate group within a polypeptide chain could be easily studied. Using enzymatic dephosphorylation, it was found that neither proteolytic activity nor the transformation of pepsinogen to pepsin are affected by the removal of the phosphate group (Perlmann, 1952c). However, an increased rate of autodigestion was observed after phosphate removal, suggesting that the phosphate group may be involved in a linkage which functions to stabilize the molecule and thus protect pepsin from its own proteolytic activity (Perlmann, 1958).

Phosphoglucomutase

Phosphoglucomutase is the enzyme responsible for the reversible conversion of glucose 1-phosphate to glucose 6-phosphate (Cori et al., 1938). The participation of a phosphorylated enzyme intermediate in this reaction was first proposed by Jagannathan and Luck in 1949 on the basis of P^{32}-exchange experiments. The apparent participation of glucose 1,6-diphosphate in the reaction led Najjar and Pullman (1954) to propose the following reaction scheme:

glucose 1-phosphate + phosphoenzyme

glucose 1,6-diphosphate + dephosphoenzyme

glucose 6-phosphate + phosphoenzyme

The phosphorylated form of the enzyme contains one mole of phosphate per molecule (Sidbury and Najjar, 1957), which is present as phosphoserine (Anderson and Jolles, 1957; Kennedy and Koshland, 1957). Early attempts to map the amino acid sequence around this active serine residue led to the proposal to two totally different sequences (Milstein and Sanger, 1961; Harshman and Najjar, 1962). This apparent discrepancy was subsequently resolved by the discovery that phosphoglucomutase actually contains two active serine residues within its catalytic site, separated by four amino acids (Harshman and Najjar, 1965). Either one of these serine residues can be phosphorylated, but not both at the same time. On the basis of these data, Harshman and Najjar have proposed the scheme depicted in Figure 1. According to this model, one of the active serine residues is specific for phosphorylating the glucosyl carbon (C-1), while the other serine is specific for C-6. It is obvious that in order for this model to maintain catalytic activity, there must be a reversible transfer of phosphate between the two serines.

This model, based on the intermediate formation of glucose 1,6-diphosphate, has recently run into some significant criticism. On the basis of extensive examination of the reaction kinetics under many different conditions, several investigators have come to the conclusion that glucose 1,6-diphosphate is not an obligatory intermediate of the

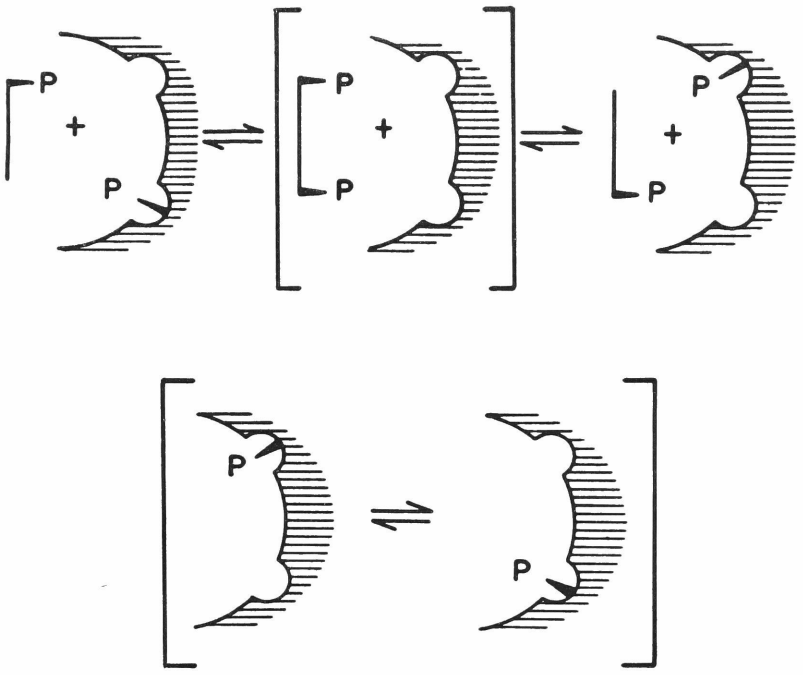


Figure 1. Model of mechanism of phosphoglucomutase reaction proposed by Harshman and Najjar (1965). Glucose 1-phosphate is shown reacting with the phosphorylated serine residue to form glucose 1,6-diphosphate. The phosphate on the C-1 position is then transferred to the other active serine residue in the enzyme. The bottom reaction represents the equilibrium between the two phosphorylated serine residues, which must be postulated if each of the active serines is specific for one of the sites on the glucose molecule. The parts of the model in brackets have been questioned recently on the basis of new kinetic data (Ray and Roscelli, 1964a,b; Gounaris et al., 1967).

reaction, but is merely an abortive side-product whose function is to rephosphorylate any dephosphoenzyme which may accumulate (Ray and Roscelli, 1964a,b; Gounaris et al., 1967). According to this view, the phosphoenzyme would phosphorylate one position on the glucose while simultaneously dephosphorylating the other, without the intermediate formation of free glucose 1,6-diphosphate. Another problem with the Harshman-Najjar model is that kinetic data make it appear extremely unlikely that there can be a reversible transfer of phosphate between the two active serines (Ray and Roscelli, 1964b), so one must seriously consider the possibility that each active serine is capable of phosphorylating both the C-1 and C-6 positions.

Phosphorylase and phosphorylase kinase

The skeletal muscle enzyme, glycogen phosphorylase, has long been known to exist in two interconvertible forms designated phosphorylase a and phosphorylase b (Green and Cori, 1943; Cori and Green, 1943; Cori and Cori, 1945; Cori, 1945; Krebs and Fischer, 1962). Phosphorylase b is usually referred to as the inactive form of the enzyme, although in the presence of AMP it does exhibit some activity; phosphorylase a, on the other hand, is considered to be the more active form physiologically, and maintains most of its enzyme activity in the absence of AMP.

The chemical nature of the interconversion of these two forms of the enzyme involves both phosphorylation and dimerization reactions, with separate enzymes involved in the forward and reverse pathways. The conversion of phosphorylase b to a, commonly referred to as phosphorylase activation, requires ATP, Mg^{++}, and the enzyme phosphorylase b kinase. During the reaction, four moles of terminal phosphate are esterified to serine residues in phosphorylase, and the molecular weight of the protein doubles (Krebs and Fischer, 1956; Krebs et al., 1958). The reaction can be summarized:

$$2 \text{ phosphorylase b} + 4 \text{ ATP} \xrightarrow[Mg^{++}]{\text{phosphorylase b kinase}} \text{phosphorylase a} + 4 \text{ ADP}$$

This reaction is essentially irreversible, and the conversion of phosphorylase a back to the b form requires a different enzyme, phosphorylase phosphatase, which cleaves the four phosphoserine linkages with simultaneous halving of the molecular weight (Keller and Cori, 1953; Graves et al., 1960):

$$\text{phosphorylase } a + 4\ H_2O \xrightarrow[\text{phosphatase}]{\text{phosphorylase}} 2 \text{ phosphorylase } b + 4\ P_i$$

The molecular relationships between the various forms of phosphorylase were worked out primarily by the extensive studies of the Coris and their collaborators (Keller and Cori, 1953; Keller, 1955; Madsen and Cori, 1956; Madsen and Gurd, 1956). Phosphorylase a (495,000) has a molecular weight twice that of phosphorylase b (242,000), and both these proteins can be further dissociated into subunits of molecular weight 125,000. Thus it appears as if the active form of phosphorylase is a tetramer, a conclusion which is further supported by the observed uptake of four moles of phosphate during activation, and by the finding of four moles of pyridoxal phosphate bound per mole of phosphorylase a (Cori and Illingworth, 1957; Kent et al., 1958). From studies on tryptic hydrolysates of phosphorylase a, it has been concluded that the same specific serine residue in each of the four subunits is phosphorylated during activation (Fischer et al., 1959).

This rather complex set of reactions for the interconversion of phosphorylase is further complicated by the fact that the enzyme responsible for activation, namely phosphorylase b kinase, can itself exist in both active and inactive forms. The activation of phosphorylase b kinase also involves phosphorylation of the protein by ATP, although it differs in several ways from the activation of phosphorylase (Krebs et al., 1959; Krebs and Fischer, 1960; Krebs et al., 1964, 1966). First of all, the reaction is strongly accelerated in the presence of cyclic 3',5'-AMP, although this nucleotide is not absolutely essential for the reaction. Secondly, more than one site in the protein is phosphorylated during the activation reaction. Finally, the

reaction is autocatalytic, i.e., it does not require any additional enzyme other than phosphorylase kinase itself for this reaction to occur. The reaction can be summarized:

$$\text{inactive phosphorylase } \underline{b} \text{ kinase} + \text{ATP} \xrightarrow[Mg^{++}]{\text{cyclic 3',5'-AMP}} \text{active phosphorylase } \underline{b} \text{ kinase} + \text{ADP}$$

Although more than one site appears to be phosphorylated during this activation reaction, 60% of the bound phosphate can be removed enzymatically without inactivating the phosphorylase kinase (Krebs et al., 1966). However, a specific phosphatase activity was found in muscle which selectively removes some of the phosphate and simultaneously inactivates the enzyme (Riley and Krebs, 1966). Thus it appears that some, but not all, of the bound phosphate is required for the activation of phosphorylase b kinase. Although the specific nature of this phosphate linkage has not yet been determined, its acid-stability and alkali-lability suggest a phosphoserine (or phosphothreonine) linkage.

Glycogen phosphorylase from liver, which shows many similarities to the muscle system, has been extensively studied by Sutherland and co-workers (Wosilait and Sutherland, 1956; Rall et al., 1956a,b; Sutherland and Wosilait, 1955; Wosilait, 1958). Liver phosphorylase also exists in an inactive form which can be activated in a specific kinase reaction involving phosphorylation of serine residues in the protein. However, an interesting difference from the muscle phosphorylase is that activation in this preparation does not involve a simultaneous change in molecular weight. Thus it appears that phosphorylation of liver phosphorylase does not increase enzyme activity via its effect on promoting the association of subunits, but that the phosphorylation reaction itself is directly responsible for the observed increase in enzyme activity. In this regard it is interesting to note that although phosphorylation of muscle phosphorylase does induce a dimer to tetramer transition, recent evidence suggests that the phosphorylated dimer is actually the active enzyme form with glycogen as substrate (Metzger et al., 1967).

Thus the activation of phosphorylase and phosphorylase kinase are extremely interesting reactions, because they represent the first clear-cut case where the function of the phosphate group is to act as an agent for the modification of protein structure. Unlike phosphoglucomutase, the phosphate group is not being bound to the protein as an intermediate step in its metabolic transfer. On the contrary, the phosphate groups in phosphorylase and phosphorylase kinase do not participate directly in the reactions which these enzymes catalyze. The net effect is that the energy stored in the terminal phosphate bond of ATP is utilized in a reaction for modifying protein structure and thereby activating these enzymes.

It is not exactly clear why the phosphorylated form of the enzyme should be active while the non-phosphorylated form is inactive, but an interesting model has recently been proposed by Sealock and Graves (1967). On the basis of the effects of various salt solutions on the enzymatic activity and sedimentation properties of phosphorylase, they postulated the existence of a specific site on the enzyme which is particularly sensitive to the ionic character of its environment. The covalently bound phosphate group, which is bound at another position in the protein, is thought to interact with this site in inducing enzyme activity. Interactions at this site are thought to explain why phosphorylase b is active in the presence of AMP; on the other hand, in phosphorylase a, where a phosphate group is already interacting with this site, AMP has no effect.

Alkaline phosphatase

Alkaline phosphatase from a number of sources has been shown to contain one reactive serine residue per molecule which is capable of being phosphorylated. This reaction was first described for calf-intestine alkaline phosphatase (Engström and Ågren, 1958; Engström, 1961a), where radioactive phosphoserine was isolated from enzyme preparations which had previously been incubated in the presence of P_i^{32} at acid pH. Similar reactions have since been discovered and studied in alkaline phosphatase from bone (Ågren et al., 1959), milk (Barman and Gutfreund, 1966), and E. coli (Engström, 1962; Schwartz and Lipmann, 1961; Schwartz, 1963).

There are several reasons for believing that the reaction with P_i^{32} is analagous to the reaction of the enzyme when cleaving a phosphate monoester. For example a normal substrate of the enzyme, glucose 6-phosphate, competitively inhibits the labeling of the protein with P_i^{32} (Engström, 1961b; Schwartz and Lipmann, 1961). Furthermore, during hydrolysis of glucose 6-P^{32} by alkaline phosphatase, the serine residue is found to be heavily labeled with P^{32} early in the course of the reaction, but later the radioactivity is found mainly in released inorganic phosphate (Engström, 1963). Finally, study of the reaction kinetics is also consistent with the hypothesis that the phosphorylated enzyme is an intermediate in the phosphatase reaction (Wilson and Dayan, 1965).

A striking feature of the phosphorylation of alkaline phosphatase by P_i^{32} is that it occurs best under acid conditions, whereas the phosphatase activity of the enzyme is optimal under alkaline conditions. Recent experiments (Aldridge et al., 1964; Fernley and Walker, 1966, 1967) have indicated that the reason for this apparent discrepancy is that under acid conditions the substrate (P_i^{32} or a phosphate ester) is rapidly bound to the enzyme, but the dissociation of the phosphoenzyme is very slow and so the net rate of hydrolysis is low. Under alkaline conditions, on the other hand, the dissociation of the phosphoenzyme becomes very rapid, thus explaining both why the enzyme is active in this pH range and why less P^{32}-labeling of the active serine can be detected.

The exact details of the nature of the formation and dissociation of the phosphorylated enzyme have not yet been worked out, and several aspects of the enzyme's behavior are still to be explained. For instance, the situation is complicated by the fact that although there is only one serine residue phosphorylated during the enzymatic reaction, the existence of two binding sites for phosphate can be detected (Fife, 1967; Levinthal et al., 1962). It is apparent that much more work will be needed before the complete mechanism of the active site of this interesting enzyme will be understood.

Phosphorylation of the enzyme acid phosphatase from human prostate has also been observed with P_i^{32}. Although the phosphorylated

amino acid has not yet been identified, it does not appear to be serine (Greenberg and Nachmansohn, 1965).

Sodium-potassium adenosine triphosphatase

There is considerable evidence that a (Na^+-K^+)-activated, ouabain-inhibitable, adenosine triphosphatase found in membrane fractions of various animal tissues is involved in the transport of Na^+ and K^+ (Skou, 1965). Although the mechanism by which this transport is accomplished is unknown, the hydrolysis of ATP by this enzyme appears to involve formation of a phosphorylated enzyme intermediate (Charnock and Post, 1963; Albers et al., 1966; Charnock et al., 1967). The terminal phosphate of ATP is transferred to the enzyme in the presence of Na^+, and the protein is subsequently dephosphorylated in a reaction which is activated by K^+. Both of these reactions are inhibitable by ouabain, and in general the behavior of this phosphorylated intermediate under different conditions parallels the behavior of active cation transport.

Due to the extreme lability of the phospho-enzyme linkage in aqueous solution, as well as its cleavage by hydroxylamine and acyl phosphatase, early workers on this problem concluded that phosphorylation of this protein probably involved formation of a high-energy acyl phosphate bond (Charnock et al., 1963; Nagano et al., 1965; Hokin et al., 1965; Bader et al., 1966; Hems and Rodnight, 1966). This conclusion has been confirmed quite recently by Kahlenberg et al. (1967), who identified the site of phosphorylation as the γ-carboxyl group of a glutamic acid residue in the enzyme.

Detailed knowledge of the structure and activity of the membrane-associated ATPase has been hampered by difficulties in solubilization and stabilization of the enzyme during purification. However, by carefully choosing the extraction conditions, Medzihradsky and co-workers (1967) have recently been able to solubilize and partially purify a Na^+-K^+ transport ATPase from guinea pig brain microsomes which has an apparent molecular weight of 670,000. These results are quite promising and provide hope that a considerable degree of purification of this enzyme can eventually be effected.

Fibrinogen

The occurrence of covalently bound phosphorus in human fibrinogen has been extensively studied by Blombäck and collaborators (Blombäck et al., 1962, 1963, 1966). This molecule contains about one mole of phosphate per molecular weight of 340,000. This phosphate is at least partially present as a phosphoserine residue in one of the peptides which is released during the thrombin-induced conversion of fibrinogen to fibrin. However, about 60-75% of the fibrinogen phosphorus is not released from fibrin during clot formation, and present evidence suggests that this phosphate is present in a different peptide.

The occurrence of the strongly acidic phosphate group in human fibrinogen bears a striking resemblance to the occurrence of the strongly acidic sulfate group found in fibrinopeptides of other species (Bettelheim, 1954; Blombäck et al., 1960; Blombäck and Doolittle, 1963). This general occurrence of strongly acidic groups in various fibrinogens may indicate an important functional role in the proteins. However, it is known that the phosphate group is not essential for thrombin-induced clot formation, since dephosphorylated fibrinogen also works in this system (Blombäck et al., 1963). However, the clotting rate of the dephosphorylated fibrinogen is significantly slower than normal.

Phosphoproteins of enamel and dentin

Organically-bound phosphate groups have been proposed to play a critical role in initiating the deposition of calcium phosphate crystals of apatite during the process of calcification (Glimcher and Krane, 1964a; Glimcher et al., 1964). If this hypothesis is valid, then one would expect to find matrix-bound phosphate groups in the dentin and enamel of developing teeth, since enamel is the most highly mineralized vertebrate tissue known. Careful examination of bovine teeth has shown that serine phosphate can be identified in protein both from enamel and dentin (Glimcher and Krane, 1964b; Veis and Perry, 1967). Furthermore, connective tissues such as bone, cartilage, and enamel have been found to contain a phosphoprotein kinase which catalyzes the transfer of the terminal phosphate of ATP to serine

phosphate in enamel protein (Krane et al., 1965). This reaction appears to be fairly specific, since the phosphoprotein kinase from brewer's yeast was not active with enamel protein as substrate.

The phosphoprotein component of dentin matrix has been partially characterized by Veis and Perry (1967). This protein has a molecular weight of about 38,000, appears homogeneous on electrophoresis, and contains 5.9% phosphorus by weight. The amino acid composition is quite unusual, consisting primarily of serine (37.5 mole %) and aspartic acid (36.5 mole %). Although phosphoserine has been isolated from this protein, the possibility of other phosphate-amino acid linkages has not been ruled out.

Although phosphoproteins have thus been implicated in the calcification of teeth, similar components have not as yet been discovered in bone. Highly purified collagens are known to contain tightly-bound organic phosphate groups, but these appear to be linked as sugar phosphates (Glimcher and Krane, 1964a).

Mitochondrial phosphoproteins

Of all the various types of tissue phosphoproteins which have been studied, there is little doubt that the greatest complexity and confusion lies in the field of mitochondrial phosphoproteins. As Pullman and Schatz (1967) have recently pointed out, at least five different categories of phosphoproteins have thus far been described in mitochondria: phosphoserine-containing proteins (Kennedy and Smith, 1954), phosphohistidine-containing proteins (Boyer et al., 1962), a Ca^{++}-induced protein-bound phosphate fraction (Norman et al., 1964), an unidentified phosphoprotein fraction of aged mitochondria (Lindberg et al., 1965), and phosphoproteins catalyzing ADP-ATP exchange reactions (Beyer, 1964a,b; Colomb et al., 1966). In addition, the unusual amino acid phosphoroiodohistidine has recently been identified in mitochondrial extracts, although there is no evidence that this is protein-bound (Perlgut and Wainio, 1967).

The phosphoserine-containing mitochondrial protein(s) appear to be part of the structural protein of the membrane (Pinna and Wadkins, 1967). Several investigators have studied the reaction in which P_i^{32} is

incorporated into serine phosphate of this mitochondrial protein fraction during coupled respiration of intact mitochondria nad phosphorylating sub-mitochondrial particles (Ahmed and Judah, 1963; Moret et al., 1963; Wadkins, 1963; Sperti et al., 1964). The physiological significance of this reaction is not clear, but several interesting correlations have been observed. Judah (1961) has pointed out that phosphoprotein turnover correlates with mitochondrial swelling and contraction, and has suggested that these proteins are involved in water transport. Later studies employing various metabolic inhibitors indicated a relationship between these phosphoserine-containing proteins and oxidative phosphorylation. These investigations led to the conclusion that in addition to phosphorylation by ATP, these proteins could also be phosphorylated by intermediates of substrate-linked and respiratory chain phosphorylation (Ahmed and Judah, 1963; Sperti et al., 1964; Siliprandi et al., 1966). However, it does not appear possible that these phosphoproteins are actual direct intermediates in oxidative phosphorylation, because among other reasons, they reach isotopic equilibrium much more slowly than ATP.

The occurrence of 3-phosphohistidine in mitochondrial protein was first reported by Boyer and co-workers (Boyer et al., 1962; DeLuca et al., 1963; Hultquist et al., 1966). The mode of formation of phosphohistidine from P_i and ATP in both intact mitochondria and in soluble enzyme preparations led Boyer (1963) to suggest that the phosphorylation of histidine is an intermediate step in oxidative phosphorylation. However, it is now generally agreed that the labeling kinetics of phosphohistidine are not consistent with this hypothesis, and it appears more likely that the bulk of the phosphohistidine is associated with substrate-level phosphorylation in the succinate thiokinase reaction (Mitchell et al., 1964; Slater and Kemp, 1964; Lindberg et al., 1965; Kemp, 1966). However, the possible association of a small amount of phosphohistidine with other mitochondrial reactions has not been completely ruled out.

The possibility that histidine phosphorylation might be involved in supplying energy for active ion transport was suggested by Pressman (1964) on the basis of the observation that calcium ions

induced an increase in bound phosphohistidine in mitochondria. However, when this phenomenon was reexamined by Norman et al. (1964, 1965), they found that the apparent increase in phosphohistidine really was an increase in phosphate bound in an unidentified protein fraction, while the level of bound phosphohistidine actually decreased. The unidentified phosphate fraction exhibited very unusual properties, and did not behave like any of the routinely-encountered phosphorylated amino acids. The phosphate in this protein fraction was non-dialyzable, was retained during ultrafiltration, and did not stick to a Dowex-1 column. However, it did appear in the inorganic phosphate fraction upon gel filtration with Sephadex G-25. It was suggested that this represents a specifically occluded form of inorganic phosphate or some metal phosphate, although the possibility of an extremely labile covalent linkage to protein could not be excluded.

Lindberg and co-workers (1965) have discovered another phosphoprotein component in mitochondria which has eluded precise identification. This phosphoprotein fraction appears only in aged mitochondria, and is relatively inert metabolically. The phosphate-protein linkage is more stable to alkali that phosphoserine, and more stable to acid than phosphohistidine. Its significance in mitochondrial function is unknown.

The last category of mitochondrial phosphoprotein to be considered is enzymes which catalyze ADP-ATP exchange reactions. Both Beyer (1964a,b) and Colomb et al. (1966) have isolated phosphorylated enzymes from mitochondria which catalyze the transfer of the terminal phosphate of ATP to ADP. The exact relationship between these two phosphoproteins is not clear, due to the lack of comparative studies. Beyer's phosphoprotein appears to be specific for ATP synthesis at site II in the respiratory chain. The nature of the protein-phosphate linkage which occurs as an intermediate during catalysis has not been elucidated, but in both cases the bond appears to be much more labile than phosphoserine or phosphohistidine, suggesting the possibility of an acyl phosphate.

Nuclear phosphoproteins

The occurrence of a phosphoprotein fraction localized in

cell nuclei was first demonstrated quite recently by Langan and Lipmann (Langan, 1967). Working with a highly purified preparation of rat liver nuclei, they were able to isolate a protein fraction containing about 1.1% phosphorus by weight. The majority of the phosphate was bound as phosphoserine, but some phosphothreonine was also present. This protein fraction was not extractable in isotonic saline, but it was soluble in 1.0 M sodium chloride, making it part of the "nucleoprotein" fraction. This phosphoprotein could also be solubilized by treatment with DNAse (Langan, 1965), again suggesting a close relationship with the nucleoprotein. The isolated phosphoprotein fraction was capable of being phosphorylated in vitro with ATP, indicating the presence of protein phosphokinase activity.

Langan quickly established two lines of evidence which suggested the interesting possibility that the nuclear phosphoproteins might play a role in the regulation of gene activity. First, he studied the distribution of phosphoproteins in chromatin fractions derived from the calf thymus nucleus. Frenster et al. (1963) had previously shown that the thymocyte chromatin could be fractionated by relatively simple centrifugal techniques into the so-called "dense" and "diffuse" chromatin fractions, the latter being several-fold more active in RNA synthesis than the former. Although these chromatin fractions contain roughly the same ratio of histone to DNA, Langan (1965) found that the diffuse chromatin contained up to four times as much phosphoprotein per mg. DNA as did the dense chromatin. Thus, not only is the phosphoprotein localized in the chromatin, but it seems to be preferentially located in the fractions active in RNA synthesis.

Next, Langan studied the same problem in vitro, utilizing isolated DNA as a template with purified RNA polymerase. In such a system, histones are known to be strongly inhibitory on the ability of DNA to serve as a template for RNA synthesis (Huang and Bonner, 1962; Barr and Butler, 1963; Hindley, 1963; Sonnenberg and Zubay, 1965; Butler and Chipperfield, 1967). However, if the histones are first complexed with the nuclear phosphoproteins, the inhibitory effect of the complex is much less than that of the histone alone.

Thus, the nuclear phosphoproteins are not only capable of forming complexes with the histones, but the formation of such a complex can lead to an increased rate of RNA synthesis (Langan and Smith, 1966).

Although both types of experiments are obviously subject to alternative interpretations, the results are nonetheless suggestive and indicate the need for further study on this interesting new class of nuclear protein. My studies on the chemistry and metabolism of these proteins will be described in subsequent chapters.

BACTERIAL PHOSPHOPROTEINS

The occurrence of phosphoserine in alkaline phosphatase derived from E. coli has already been described. Likewise, other generally-occurring enzymes with phosphorylated intermediates, like phosphoglucomutase (Joshi and Handler, 1964), would also be expected to be found in bacteria. However, in this section, I would like to restrict the discussion to phosphoproteins which are either unique to bacteria, or whose discovery and study has been primarily in bacterial systems.

The existence of phosphoserine or phosphopeptides has been described in a variety of microorganisms (Ågren et al., 1955; Ågren, 1959; Rafter and Lane, 1962; Walker and Rafter, 1967). However, the only two well-characterized phosphoproteins whose study has been primarily in bacteria are: 1) acyl carrier protein, and 2) a heat stable protein component of a bacterial phosphotransferase system.

Acyl carrier protein

Recent work on the mechanism of biosynthesis of long chain fatty acids has led to the discovery of a rather unusual protein which functions as a coenzyme during chain elongation (Goldman et al., 1963; Majerus et al., 1964, 1965a; Wakil et al., 1964). This protein, designated the acyl carrier protein, contains one sulfhydryl group per molecule to which the acyl group of the growing fatty acid chain is linked through a thioester linkage. This active sulfhydryl group does not originate in a cysteine residue, but is rather located at the terminal end of the prosthetic group, 4'-phosphopantetheine (Majerus

et al., 1965b). This prosthetic group, which is actually a fragment of the coenzyme A molecule, is linked to the acyl carrier protein by an ester linkage between its 4'-phosphate group and the hydroxyl group of a serine in the protein chain (Majerus et al., 1965c; Pugh and Wakil, 1965).

Thus this represents the first well-established case of a phosphodiester linkage in a naturally-occurring protein. Although this protein was discovered and studied in bacterial systems, some evidence exists that a similar protein is involved in mammalian fatty acid biosynthesis (Larrabee et al., 1965). Considering the relative ease with which a phosphate group can form a diester linkage, it seems a logical candidate to serve as a covalent bridge between proteins and other molecules, and it would not be surprising if further examples of such linkages were discovered in the future.

Bacterial phosphotransferase

A novel phosphotransferase system has been isolated from bacteria which catalyzes the transfer of phosphate from phosphoenol-pyruvate to certain monosaccharides (Kundig et al., 1964). The system is composed of two distinct enzymes, plus a heat-stable protein (HPr) which acts as an intermediate carrier of the phosphate groups:

$$\text{phosphoenolpyruvate} + \text{HPr} \xrightleftharpoons{\text{Enzyme I}} \text{pyruvate} + \text{phospho-HPr}$$

$$\text{phospho-HPr} + \text{sugar} \xrightarrow{\text{Enzyme II}} \text{HPr} + \text{sugar-P}$$

The phosphorylated form of the heat-stable protein was identified as phosphohistidine. Recent evidence suggests that this phosphotransferase system may be involved in the active transport of glycosides across the bacterial cell membrane (Kundig et al., 1966; Simoni et al., 1967).

ENZYMES OF PHOSPHOPROTEIN METABOLISM

A common feature of many of the phosphoproteins which have been discussed is that the phosphate groups are metabolized independent

of the metabolism of the protein itself. Two types of enzymes have been described which are involved in the phosphate group metabolism of those phosphoprotiens which are not involved in specific phosphate-transfer reactions. One is a phosphoprotein phosphatase, which is involved in the hydrolytic cleavage of protein-phosphate bonds. The other is phosphoprotein kinase, which catalyzes the phosphorylation of phosphoproteins by the terminal phosphate of ATP.

An alternative possibility for phosphoprotein biosynthesis is the incorporation of a pre-existing phosphorylated amino acid directly into the growing polypeptide chain. Although some evidence does exist for the occurrence of a transfer RNA which binds phosphoserine (Carlsen et al., 1964), the direct incorporation of preformed phosphoserine into phosphoproteins has not yet been demonstrated. Phosphorylation of the protein seems to occur in general after the polypeptide chain has been completed (Sperti et al., 1961; Olivo et al., 1961; Sanger and Hocquard, 1962; Turkington and Topper, 1966; Singh et al., 1967).

Phosphoprotein kinase

Burnett and Kennedy (1954) were the first to describe the occurrence of a Mg^{++}-requiring kinase capable of catalyzing the transfer of the terminal phosphate of ATP to the serine hydroxyl group of phosphoproteins. This enzyme, which was discovered in rat liver mitochondria, was found to be capable of phosphorylating casein and ovalbumin, as well as mitochondrial proteins. Since then, phosphoprotein kinase activity has been demonstrated in yeast, brain, mammary gland, erythrocytes, kidney, and rat liver nuclei (Rabinowitz and Lipmann, 1960; Schmidt and Davidson, 1956; Sundararajan et al., 1958; Judah et al., 1962; Jackson et al., 1965; Langan, 1967).

The phosphoprotein kinase from yeast has been extensively purified and studied by Rabinowitz and Lipmann (1960). This enzyme is capable of catalyzing the phosphorylation of both casein and phosvitin by ATP. On the other hand, the active serine residues in phosphorylase b, which are normally phosphorylated by the enzyme phosphorylase kinase, could not be phosphorylated by this phospho-

protein kinase. Conversely, phosvitin was not phosphorylated by phosphorylase kinase. Thus there appears to be a strict specificity of phosphoprotein kinase for phosphoproteins like phosvitin and casein. Rabinowitz and Lipmann have suggested that the clusters of adjacent phosphoserine residues which occur in these phosphoproteins (Williams and Sanger, 1959) may be responsible for this specificity.

Another interesting finding which arose from the studies on yeast phosphoprotein kinase was that the reaction between ATP and phosvitin is reversible. When the equilibrium conditions of the reaction were investigated, it was found that the phosphoryl potential of phosphoserine in phosvitin is almost as high as the terminal phosphate in ATP. The large amount of free energy stored in this manner in phosvitin suggests the possibility that this protein might serve as a reservoir of high energy phosphate.

Phosphoprotein phosphatase

Phosphoprotein phosphatase activity was first discovered in 1946 by Harris, who described an enzyme in frog eggs which readily split phosphate groups from casein and vitellin, but reacted only very slowly with nucleic acid or glycerophosphate. Similar enzyme activity has since been found in chick embryos, and in mammalian spleen, liver, kidney, and brain (Foote and Kind, 1953; Paigen, 1958; Norberg, 1950; Sundararajan and Sarma, 1954, 1957; Rose and Heald, 1961). The various enzyme preparations do not all exhibit the same patterns of specificity for phosphate esters, and so the term "phosphoprotein phosphatase" actually refers to a heterogeneous group of several different enzymes.

The particular enzyme which has thus far shown the greatest degree of specificity is the phosphoprotein phosphatase from brain (Rose and Heald, 1961). This enzyme has been found to be inactive toward a wide spectrum of phosphate esters, including aliphatic and aromatic phosphomonoesters, phosphoamides, phosphodiesters, polyphosphates, pyrophosphates, polyseryl phosphates and mixed phospholipids. It acts rapidly to release inorganic phosphate from phosvitin and casein, but an interesting aspect of this reaction is

that it stops short of completion. A considerable amount of phosphate remains bound to these phosphoproteins, apparently resistant to the enzyme's action.

The phosphoprotein phosphatase which has been purified from spleen behaves quite differently (Sundararajan and Sarma, 1957). It is relatively non-specific in nature, since in addition to phosphoserine bonds in phosphoproteins, it can also hydrolyze pyrophosphate, phenylphosphate, and phosphoamide linkages. Various types of evidence, including specific activities during purification, effects of inhibitors and activators, heat denaturation effects and the study of adsorption data all indicate that a single enzyme is responsible for these various activities, and that they are not the result of contamination by other enzymes. This phosphatase also differs from the brain enzyme in that it is capable of completely dephosphorylating casein and phosvitin.

The enzyme from frog egg also hydrolyzes a broad spectrum of phosphate esters, although there are some critical differences between its behavior and that of the spleen enzyme (Harris et al., 1966). Liver seems to contain two separable phosphoprotein phosphatases, one very similar to the spleen enzyme and one which differs in several respects (Paigen and Griffiths, 1959). Harris and co-workers (1966) have recently pointed out that since these phosphoprotein phosphatases from eggs, spleen, and liver seem to work in general on high energy O-P bonds in acid anhydrides, the official designation of "phosphoprotein phosphatase" is really a misnomer. They have proposed that these enzymes be classified as nonspecific phosphoanhydride hydrolases. Their activity on phosphoproteins appears to be incidental, reflecting the relatively energy-rich nature of the phosphoserine bond in these proteins. The enzyme preparation from brain seems to be the only one which justifies the designation of phosphoprotein phosphatase.

ARTIFICIALLY PHOSPHORYLATED PROTEINS

In addition to the natural phosphoproteins, many investigators have studied the chemical and immunological properties of proteins which have been artificially phosphorylated by treatment with

phosphorus oxychloride (Bechhold, 1901; Neuberg and Pollak, 1910; Neuberg and Oertel, 1914; Rimington, 1927; Mayer and Heidelberger, 1946). This is a rather drastic procedure and causes pronounced changes in the proteins' behavior. In contrast to the natural phosphoproteins, the phosphate groups tend to be unstable and are lost spontaneously. Phosphorylated bovine serum albumin has been used to form complexes with basic polypeptides in order to make the later antigenic (Van Vunakis et al., 1966).

A totally different mode of chemical phosphorylation is possible in the group of esterases which are characterized by their susceptibility to inhibition by organophosphorus compounds, such as di-isopropyl phosphorofluoridate (DFP). Schaffer, May, and Summerson (1954) were the first to show that in the case of one of these enzymes, chymotrypsin, inhibition was caused by phosphorylation of a single specific serine residue in the active site by DFP. Since then, similar reactions have been observed in carboxylesterase, cholinesterase, trypsin, pancreatopeptidase E, thrombin, and subtilopeptidase A (Jansz et al., 1959a,b; Dixon et al., 1958; Naughton et al., 1960; Gladner and Laki, 1958; Sanger and Shaw, 1960). Thus organophosphorus compounds are capable of specifically phosphorylating serine residues involved in the active centers of a wide variety of enzymes.

BIOLOGICAL FUNCTIONS OF PHOSPHOPROTEINS

Some of the general categories of phosphoprotein function have already been mentioned in the earlier section on characteristics of phosphorylated proteins. The two main functions which are usually considered for phosphoproteins are phosphate group transfer (phosphoglucomutase, alkaline phosphatase, E. coli phosphotransferase) and phosphate storage (phosvitin, casein). Having considered all the phosphoproteins in some detail, two other general functional possibilities for the phosphate group become apparent.

One is the utilization of phosphate groups for modifying pre-existing protein structure. The most obvious example of this phenomenon occurs in the case of phosphorylase, where the introduction of phosphate groups into the polypeptide chain converts a relatively

inactive enzyme to a more active form. A similar transformation seems to take place in the case of phosphorylase kinase. It is possible that a similar mechanism is involved in the case of other phosphoproteins, where the function of the phosphate group is not so readily apparent.

A final general function for phosphate groups is demonstrated in the recently discovered example of the acyl carrier protein, where the phosphate serves as a bridge between a protein and a prosthetic group. Since the phosphate group readily forms diesters, it is a good candidate to serve as a link between proteins and other molecules.

Other possible functions of phosphoproteins have been alluded to, such as involvement in oxidative phosphorylation and active transport reactions. However, these cannot be considered as general functions of phosphoproteins until more specific evidence is available in support of their validity.

CHAPTER TWO: PHOSPHOPROTEIN METABOLISM IN ISOLATED LYMPHOCYTE NUCLEI

The studies of Langan and Lipmann, described in the previous chapter, indicated the possibility that the nuclear phosphoproteins may play an important role in the regulation of nuclear metabolism. In order to obtain a clearer understanding of the possible functional roles of these proteins, detailed information is required about their normal metabolism in the intact nucleus. In this chapter experiments on the metabolism of phosphoproteins in the isolated calf thymus nucleus will be described (Kleinsmith et al., 1966a). Tracer techniques were employed to study the pathways of phosphate incorporation, the nature of the linkage between phosphate and protein, and the metabolic stability of the phosphate previously incorporated. It will be shown that P^{32}-orthophosphate incorporation into protein occurs in isolated lymphocyte nuclei, that it is energy-dependent, and that it proceeds independently of protein synthesis. Phosphate so incorporated is esterified to the hydroxyl groups of serine and threonine. In this form it is subject to a rapid exchange or "turnover" reaction, and this "turnover" as observed in isolated nuclei appears to be itself energy-dependent. The bulk of phosphate incorporation is into non-histone protein, but small amounts of radioactive serine phosphate can be detected in highly

purified histone fractions.

METHODS

Isolation of nuclei: Nuclear fractions were isolated from fresh calf thymus tissue by homogenization and differential centrifugation in 0.25 M sucrose - 3mM $CaCl_2$ as described by Allfrey et al. (1957, 1964b). In some cases a brief hypotonic shock (0.22 M sucrose - 3 mM $CaCl_2$) was employed to facilitate cell breakage. To rule out contamination of nuclei by intact cells in critical experiments, nuclei were further purified by centrifugation through sucrose density-barriers (1.6 M sucrose layered over 1.95 M sucrose).

Incubation procedures: Nuclear suspensions in 0.25 M sucrose - 3 mM $CaCl_2$ were added to a buffered sucrose medium containing isotopic precursors, glucose and salts, in the following proportions: 1.0 ml of nuclear suspension, containing about 40 mg nuclei (dry weight); 0.5 ml 0.1 M tris-HCl buffer (pH 7.4) in 0.25 M sucrose; 0.4 ml 0.1 M glucose containing 8.0 mg NaCl + 4.2 mg $MgCl_2:4H_2O$ per ml; and 0.1 ml H_2O containing either 25 μc $Na_2HP^{32}O_4$ (specific activity 100 mc/mmole), 4 μc DL-alanine-1-C^{14} (specific activity 4.4 mc/mmole), 1 μc DL-serine-3-C^{14} (specific activity 2.0 mc/mmole), 1 μc guanosine-8-C^{14} (specific activity 4.6 mc/mmole), or 1 μc uridine-2-C^{14} (specific activity 25 mc/mmole). The suspensions were shaken at 37°C in a water bath. Conditions were aerobic unless otherwise specified.

In isotope retention ("cold chase") experiments, the nuclei were chilled after 15 minutes incubation and centrifuged at 1000 g. They were washed three times in incubation medium containing an excess of non-radioactive precursor: in tests for C^{14}-serine "turnover", the wash solution contained 2 mg unlabeled serine per ml; in P^{32}-exchange experiments, sodium phosphate buffer (0.1 M, pH 6.8) was used instead of tris-HCl in the incubation medium (when using sodium phosphate buffer, the NaCl concentration must be decreased from 8.0 mg to 3.75 mg per 2 ml of final incubation medium). After washing to remove radioactive precursors, the nuclei were resuspended in incubation medium and reincubated at 37°C.

Preparation and analysis of the "phosphoprotein" fraction:

Phosphoprotein was determined as recommended by Langan (1965). In this procedure, nucleic acids are removed by hot-acid extractions (Schneider, 1945), followed by the use of acidified chloroform-methanol to remove phospholipids (LeBaron and Folch, 1956). The protein residue is treated with alkali to hydrolyze phosphoester linkages and the inorganic phosphate is assayed as the phosphomolybdate complex after extraction in isobutanol-benzene (Berenblum and Chain, 1938; Martin and Doty, 1949; Ernster et al., 1950).

The detailed procedure used in tracer experiments is as follows: after incubation the nuclei were treated with 16% trichloro-acetic acid (TCA), centrifuged, resuspended in 16% TCA, and heated to 90°C for 15 minutes. They were recentrifuged and washed three times with 16% TCA, once with 1:1 chloroform-methanol, once with 2:1 chloro-form-methanol containing 1 ml concentrated HCl per 300 ml, and once with ether. The protein residues were dried under vacuum. In amino acid incorporation studies, the radioactivity of the residue was measured directly in a thin-window, gas-flow G-M counter, and the counts corrected for C^{14}-self-absorption (Schweitzer and Stein, 1950).

Phosphorylation of the protein was measured by analysis and counting of alkali-labile phosphate. The protein residue (5-10 mg) was dissolved in 2.0 ml 1.0 N NaOH, an aliquot (0.1 ml) taken for biuret protein determination (Crampton et al., 1954), and the rest of the solution heated at 100°C for 15 minutes. After cooling, the solu-tion was acidified by the addition of 0.5 ml of 4 N HCl-1 N H_2SO_4, and the protein precipitated with 0.1 ml of 0.1 M silicotungstic acid (made up in 0.1 N H_2SO_4). The suspension was centrifuged for five minutes, and 2.0 ml of the supernatant transferred to a clean glass-stoppered tube. Then 0.5 ml 5% ammonium molybdate in 4 N H_2SO_4 was added, followed by 2.5 ml 1:1 isobutanol-benzene. The tubes were shaken for 10 seconds, and then centrifuged to cleanly separate the layers. When determining P^{32}-activity, a 1.0 ml aliquot of the upper phase was counted directly in Bray's scintillation solution (Bray, 1960). To determine total phosphoprotein-phosphate, another 1.0 ml aliquot of the upper phase was taken and mixed with 0.45 ml of ethanol-H_2SO_4 (100% ethanol/conc. H_2SO_4 in ratio of 490/10 by volume). The phospho-molybdate complex was then reduced by adding 0.05 ml $SnCl_2$, and the resulting blue color read at

660 mμ. The $SnCl_2$ was prepared fresh by diluting a stock solution 1:200 with 1.0 N H_2SO_4. The stock solution was made by dissolving 10 g $SnCl_2:2H_2O$ in 25 ml conc. HCl, and was stored cold in the dark.

Measurement of RNA synthesis: In experiments on the incorporation of radioactive precursors into RNA, the reaction was stopped with cold 2% perchloric acid (PCA). The precipitates were washed three times with cold 2% PCA, once with 3:1 ethanol-ether, and once with ether. The dried residue was plated and counted directly in a gas-flow G-M counter as described above.

Analysis of phosphoserine and phosphothreonine: The method of Schaffer, May, and Summerson (1953) was used to identify the phosphorylated amino acids. The protein residues were hydrolyzed in 2 N HCl at 110°C for 10 hours, the HCl removed under vacuum, the hydrolysate dissolved in 0.05 N HCl and then chromatographed on a column of Dowex-50 (0.9 cm x 37 cm) eluted with 0.05 N HCl.

Preparation and purification of histones: Nuclei were washed after incubation with 0.01 M tris-HCl (pH 7.1) containing 3 mM $MgCl_2$, in order to remove soluble nuclear proteins and nuclear ribosomes (Pogo et al., 1962). This was followed by a wash in 80% ethanol-0.01 N HCl to remove a tryptophan-containing protein fraction, after which the histones were extracted in 0.2 N HCl. Histones were precipitated from the acid extract by the addition of 10 volumes of acetone. They were further purified by electrophoresis at pH 9 on cellulose polyacetate strips (Poge et al., 1966) and by chromatography on carboxymethylcellulose (Johns et al., 1960).

RESULTS

Phosphorylation of proteins in isolated nuclei: Suspensions of isolated thymus nuclei are capable of incorporating P^{32}-labeled orthophosphate into nuclear proteins. The early time course of P^{32}-incorporation into alkali-labile phosphate is indicated in Figure 2; the uptake is seen to proceed for up to two hours. In order to rule out the possibility that this activity is due to the small fraction of whole cells known to contaminate these nuclear suspensions, the nuclei were further purified by centrifugation through sucrose density barriers (Allfrey et al., 1964b) after incubation in vitro in the presence of

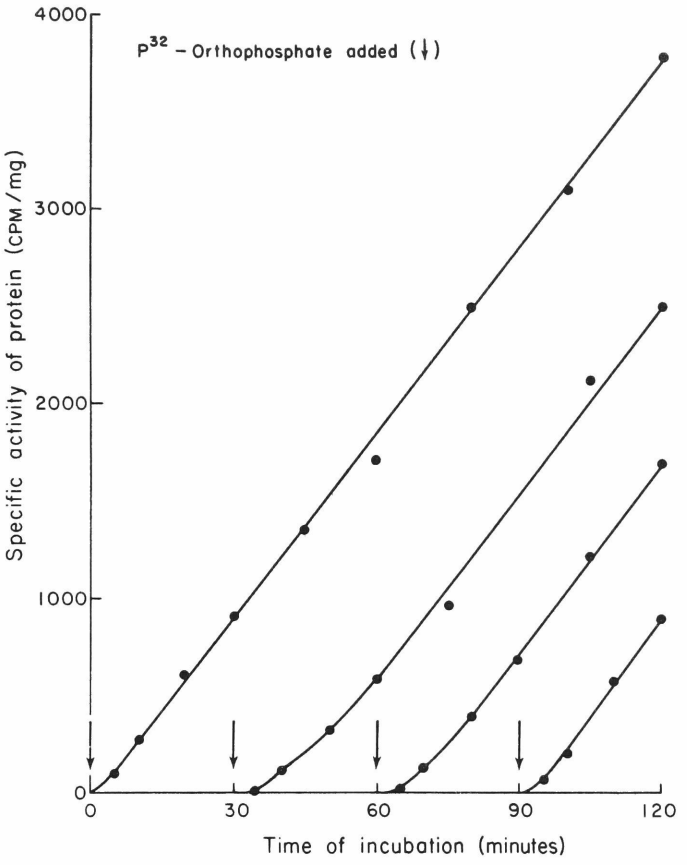


Figure 2. Time course of P^{32}-orthophosphate incorporation into alkali-labile phosphate in proteins of isolated calf thymus nuclei. The specific activity of the proteins is plotted against the time of incubation at 37°C. Separate batches of nuclei were preincubated for 30, 60, or 90 minutes before the addition of radioisotope. Note that the reaction proceeds at the same rate for up to two hours.

P^{32}-orthophosphate. These highly purified nuclei were still found to contain isotopically-labeled phosphoproteins (Figure 3).

Further tests for nuclear localization of the activity made use of the fact that the phosphorylation of nuclear phosphoproteins is an energy-dependent reaction (as will be shown below). This made it possible to discriminate between nuclear activity and that due to cytoplasmic or whole cell contamination by the use of selective inhibitors. For example, carbon monoxide, which inhibits mitochondrial energy metabolism without affecting energy-yielding reactions in free thymus nuclei (McEwen et al., 1963a), was found to have no effect on the phosphorylation reaction (Figure 4). On the other hand, deoxyribonuclease treatment, which inhibits energy metabolism in the isolated nucleus but has no such effect on intact cells, caused a marked inhibition of P^{32}-incorporation into phosphoproteins (Figure 5). Thus both types of experiment support the conclusion that protein phosphorylation is a nuclear process.

Nature of the phosphate linkage: Langan and Lipmann have already demonstrated the presence of phosphoserine and phosphothreonine in the proteins of rat liver nuclei. Experiments were performed to determine whether the alkali-labile phosphate found in the protein of calf lymphocyte nuclei was also derived from this type of ester linkage. Protein residues prepared from nuclei after incubation with P^{32}-orthophosphate were hydrolyzed in 2 N HCl and the hydrolysate chromatographed on Dowex-50 in 0.05 N HCl. A clear separation of inorganic phosphate, phosphoserine, and phosphothreonine is obtained on such a column (Figure 6).

An exact calculation of the amount of radioactivity present in phosphoserine and phosphothreonine is not possible due to the large degree of breakdown of these amino acid esters during acid hydrolysis. However, approximate figures can be obtained by correcting for the average amount of hydrolysis which occurs when standards of phosphoserine and phosphothreonine are subjected to the same conditions. These figures can only be considered a general approximation, since phosphoserine and phosphothreonine within the protein would not necessarily be expected to be hydrolyzed at the same rate. Using such

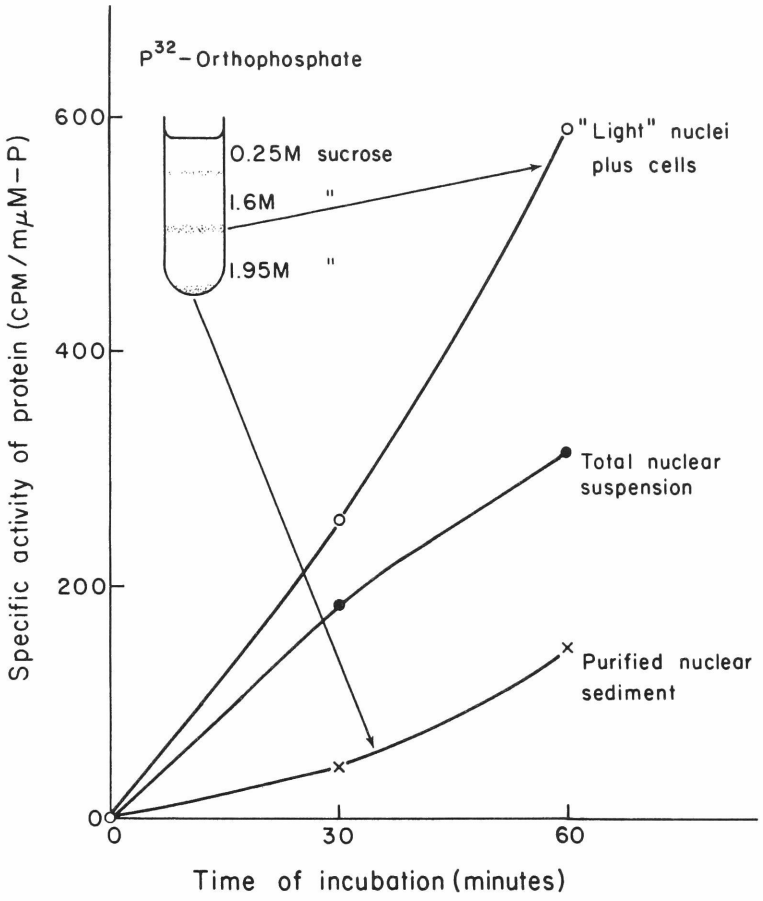


Figure 3. Uptake of P^{32}- orthophosphate into highly purified nuclei. After incubation in the presence of radioisotope, the crude nuclear suspension was further purified by centrifuging through sucrose density barriers. Counts are still present in the purified nuclear sediment.

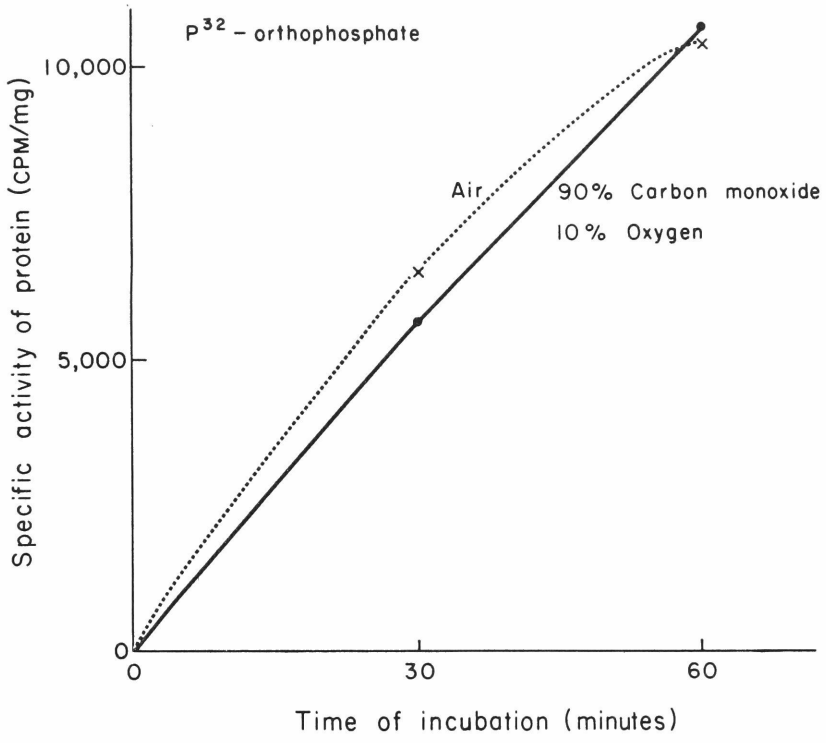


Figure 4. Effects of carbon monoxide on phosphorylation of proteins in isolated thymus nuclei. This potent inhibitor of mitochondrial oxidative phosphorylation is seen to have no effect on the rate of incorporation of P^{32}-orthophosphate into alkali-labile phosphate.

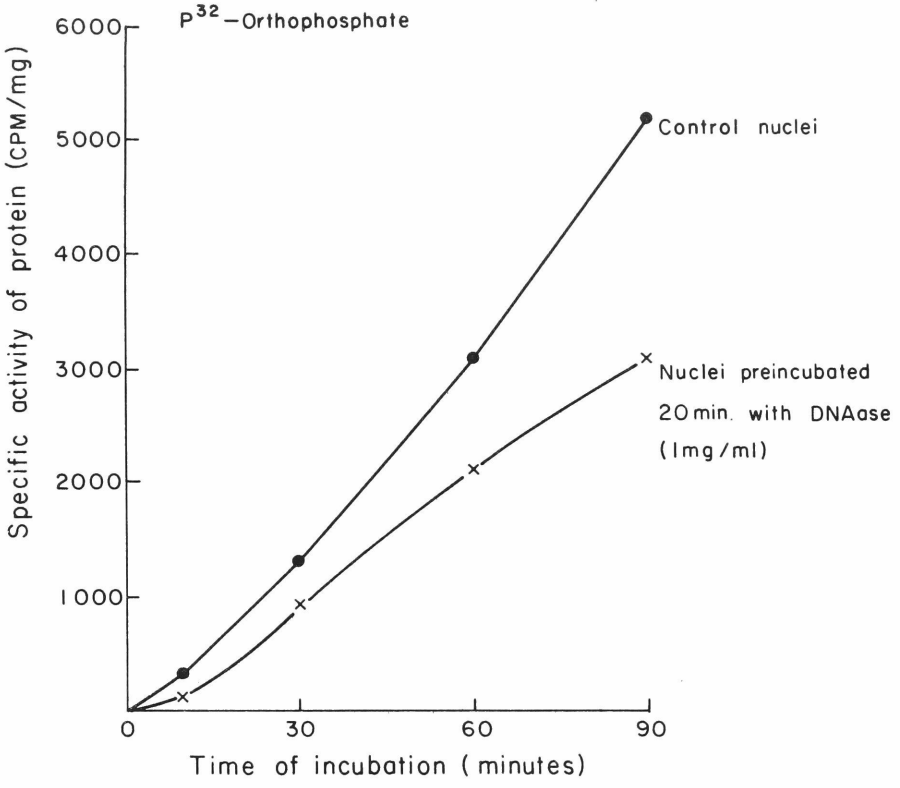


Figure 5. Effect of deoxyribonuclease treatment on phosphorylation of nuclear protein. This enzyme, which is known to inhibit energy metabolism in isolated thymus nuclei but not in intact cells, causes a decrease in the rate of P^{32}-orthophosphate incorporation into alkali-labile phosphate. Although the DNAase treatment could also be affecting the phosphorylation reaction directly, the results still support the conclusion that protein phosphorylation is a nuclear process.

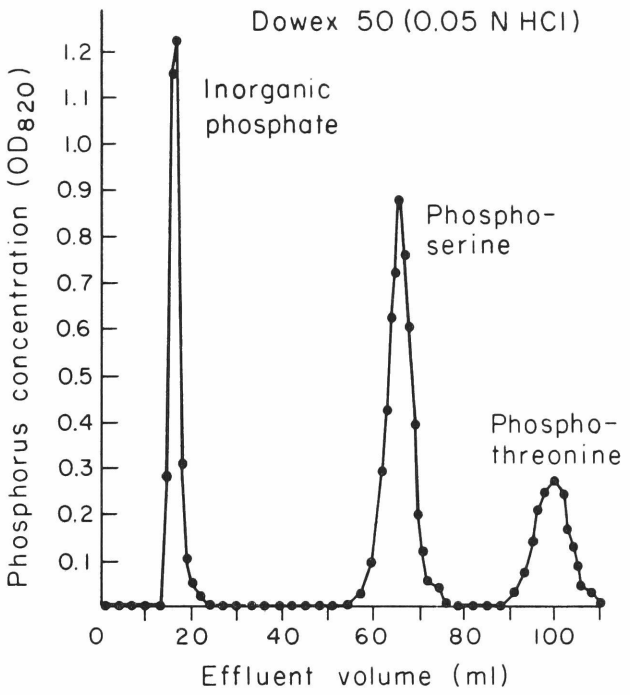


Figure 6. Separation of inorganic phosphate, phosphoserine, and phosphothreonine markers on a column of Dowex-50 eluted with 0.05 N HCl. Total phosphate concentration was determined by the method of Ames and Dubin (1960).

corrections, it was calculated that about 85% of the radioactivity present in the alkali-labile phosphate fraction could be accounted for as phosphoserine, and an additional 7% could be accounted for as phosphothreonine (Table I).

Since the possibility exists that phosphoserine and phosphothreonine can be formed from inorganic phosphate and the respective amino acids in the presence of HCl (Moore, 1963), experiments were performed to determine whether the radioactivity appearing in the phosphoserine and phosphothreonine peaks could have arisen in this manner. Unlabeled nuclear protein was hydrolyzed as described above and then a small amount of P_i^{32} added. The HCl was removed under vacuum and the hydrolysate chromatographed on Dowex-50. The inorganic phosphate peak contained 65,000 cpm, while no counts above background could be detected in either the phosphoserine or phosphothreonine peaks. Therefore it can be concluded that formation of these phosphorylated amino acids does not occur to a significant degree under these conditions.

Energy-dependence of protein phosphorylation: The uptake of P^{32} into nuclear proteins is energy-dependent. This can be shown by the use of inhibitors of nuclear ATP synthesis such as iodoacetate, which blocks glycolysis, or 2,4-dinitrophenol, which inhibits aerobic phosphorylation (McEwen et al., 1963a,b). Both of these compounds are very effective in reducing the uptake of P^{32}-phosphate into the proteins of isolated nuclei (Figure 7). This indication of ATP dependence is in accord with the work of Langan and Lipmann on the protein phosphokinase activity of rat liver nuclei, an enzymatic reaction which transfers phosphoryl groups from ATP to the hydroxyl groups of serine and threonine residues in the protein.

Effect of puromycin on P^{32}-phosphate uptake into nuclear proteins: The phosphorylation of nuclear proteins does not appear to be tightly coupled to protein synthesis. The independent nature of phosphorylation is indicated by experiments in which puromycin was used to inhibit amino acid incorporation. The amino acid selected was serine, since most of the phosphate appears in phosphoserine linkage. The results are summarized in Figure 8. Under conditions in which puromycin markedly inhibited the incorporation of serine-3-C^{14} into the proteins of the

TABLE I

Calculation of phosphoserine and phosphothreonine contents of alkali-labile phosphate fraction

Material	Total P^{32} put on Dowex-50 column (cpm)	Total P^{32} recovered (cpm)	Recovery of standards after hydrolysis (per cent)	Total P^{32} corrected for loss during hydrolysis (cpm)	Per cent recovery $\frac{\text{cpm: amino acid}}{\text{cpm: alkali-labile P}}$
Alkali-labile P	26,330				
Phosphoserine		4,890	22%	22,230	84.4%
Phosphothreonine		720	40%	1,800	6.8%

Nuclei were incubated 30 minutes in the presence of P^{32}-orthophosphate. The "phosphoprotein" residue was prepared as described in the text. One portion of the residue was analyzed for alkali-labile P^{32}, while a similar portion was hydrolyzed in 2.0 N HCl and chromatographed on Dowex-50. The P^{32} contents of the phosphoserine and phosphothreonine peaks were determined, and were corrected for the breakdown of these amino acids during hydrolysis.

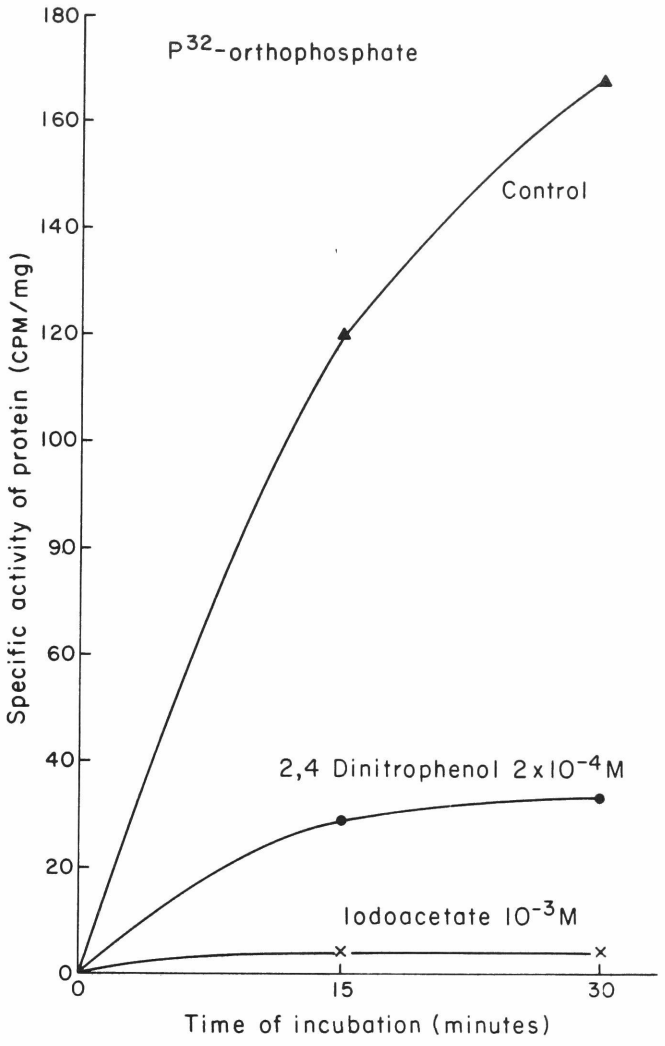


Figure 7. Energy-dependence of P^{32}-orthophosphate incorporation into the proteins of isolated calf thymus nuclei. Note that agents which block glycolysis (iodoacetate) or nuclear phosphorylation (2,4-dinitrophenol) also inhibit P^{32} uptake into alkali-labile phosphate.

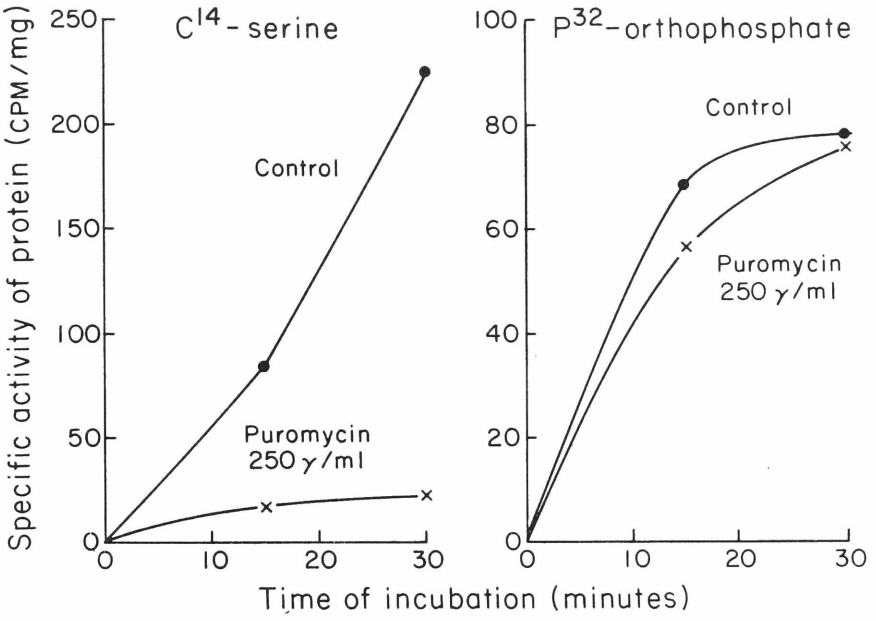


Figure 8. Effects of puromycin on protein synthesis and protein phosphorylation in isolated calf thymus nuclei. Note that puromycin strongly inhibits the incorporation of C^{14}-serine into proteins without a corresponding inhibition of P^{32}-orthophosphate incorporation.

nucleus, the phosphorylation reaction was hardly affected. In this respect the phosphorylation of nuclear proteins resembles some other reactions in which protein structure is chemically modified, such as acetylation and methylation of the histones (Allfrey et al., 1964a; Allfrey, 1964), and the acetylation of hemoglobin (Marchis-Mouren and Lipmann, 1965). In all of these cases the modification of a previously existing polypeptide chain is catalyzed by specific enzymatic transfer reactions.

Evidence for protein-phosphate "turnover": The distinction between protein synthesis and protein phosphorylation is also evident in "cold chase" experiments in which the retention of previously incorporated P^{32}-phosphate is compared with that of serine-3-C^{14}. In these tests, nuclei were incubated for 15 minutes in the presence of either C^{14}-serine or P^{32}-phosphate. They were then washed to remove the radioactive precursors and subsequently incubated in a radioisotope-free medium containing an excess of C^{12}-serine or P^{31}-phosphate. Aliquots were withdrawn at different times and the nuclear proteins were analyzed for their contents of C^{14}-serine and P^{32}-phosphate. The results are summarized in Figure 9. It is clear that serine, once incorporated into the proteins of the nucleus, remains stable for the duration of the experiment. On the other hand, the protein-bound phosphate groups "turn over" very rapidly, and more than 75% of the labeled phosphate is lost during a two hour "chase".

Energy-dependence of phosphate "turnover": Since the uptake of P^{32} into nuclear phosphoprotein was found to be an energy-dependent reaction, experiments were performed to determine whether phosphate "turnover" is also energy-dependent. When iodoacetate was added to inhibit nuclear ATP synthesis during the period of the "cold chase", it was found that the turnover of previously incorporated P^{32} was inhibited (Figure 10).

Although this suggests that phosphate "turnover" is energy-dependent, one must be cautious in interpreting the effects of an inhibitor such as iodoacetate. It is true that its primary effect is to block glycolysis and hence ATP formation, but it is also a general reagent for protein sulfhydryl groups, and could conceivably

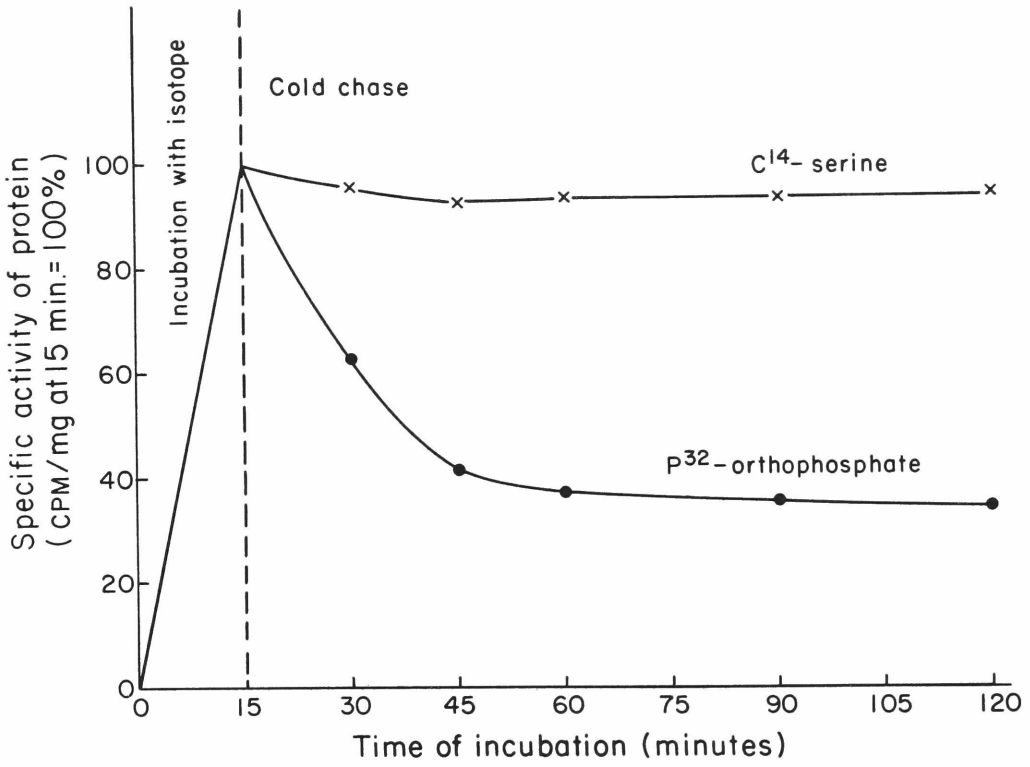


Figure 9. "Turnover" of previously-incorporated P^{32}-phosphate in nuclear proteins. Nuclei were incubated for 15 minutes in the presence of C^{14}-serine or P^{32}-orthophosphate. The nuclei were then washed and reincubated in radioisotope-free media. Note that C^{14}-serine is retained while P^{32}-phosphate is rapidly lost.

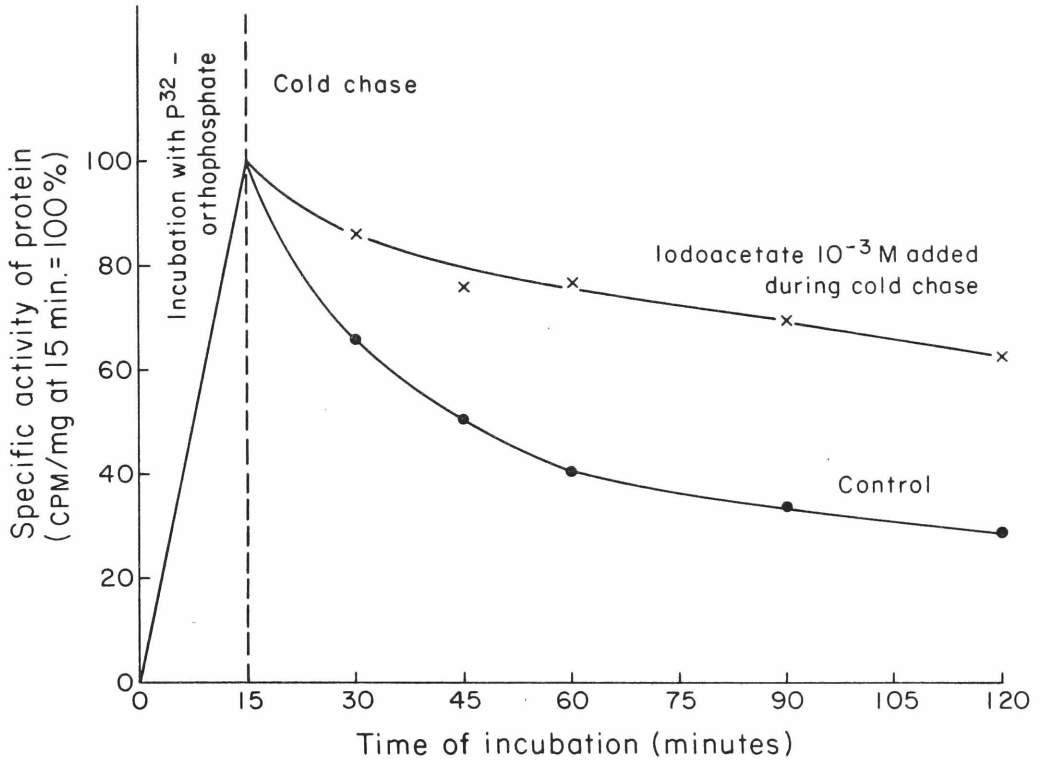


Figure 10. Evidence for the energy-dependence of P^{32}-"turnover" in nuclear phosphoproteins. Nuclei were incubated with P^{32}-orthophosphate for 15 minutes, washed to remove the precursor, and then subjected to a "cold chase". Note that the presence of iodoacetate (which blocks nuclear glycolysis and ATP synthesis) reduces the rate of phosphate "turnover".

be inhibiting phosphate "turnover" via a direct effect on the enzyme(s) involved in the cleavage of the protein-phosphate linkages. In order to overcome this objection, pyruvate was added back to iodoacetate-treated nuclei to by-pass the glycolytic pathway and reestablish ATP synthesis (McEwen et al., 1963b). When ATP formation was reestablished in this manner, the "turnover" of protein phosphate groups resumed at its normal rate, even though iodoacetate was still present (Figure 11). Thus it can be concluded that the "turnover" of phosphoprotein phosphate is inhibited in the absence of ATP synthesis.

Alkali-labile phosphate distribution in nuclear proteins: A fractionation of the nuclear proteins following P^{32}-phosphate incorporation shows the presence of alkali-labile phosphate and radioactivity in several of the fractions. Phosphorylated proteins appear in the soluble phase prepared by extracting nuclei with 0.01 M tris-HCl buffer at pH 7.1, in the 0.2 N HCl extract containing the histones, and in the residue (Table II). By far the greatest part of the total alkali-labile phosphate and most of the radioactivity remain in the insoluble residue. The proteins of the soluble phase contain a very small amount of alkali-labile phosphate of very high specific activity. The histone fraction contains about 13-15% of the total phosphoprotein phosphate and radioactivity.

The presence of phosphate in the 0.2 N HCl extract containing the histones was not expected, so further purification procedures were carried out on this relatively crude fraction in an attempt to determine whether this represented contamination by non-histone protein. In one set of experiments, the 0.2 N HCl extract was electrophoresed at pH 9 on cellulose polyacetate strips. Under these conditions, only the most highly basic proteins migrate toward the cathode; P^{32} counts were found to be localized in the histone bands (Figure 12). Chromatography on carboxymethylcellulose in 4 M urea also showed the presence of P^{32} in the histone peaks (Figure 13). In order to determine the exact nature of these P^{32} counts, the f_1 and f_3 histone peaks were hydrolyzed and analyzed on Dowex-50 columns. The radioactivity was identified as phosphoserine, with the f_1 fraction containing about three times as much radioactivity per mg histone as the f_3 fraction (Figure 14).

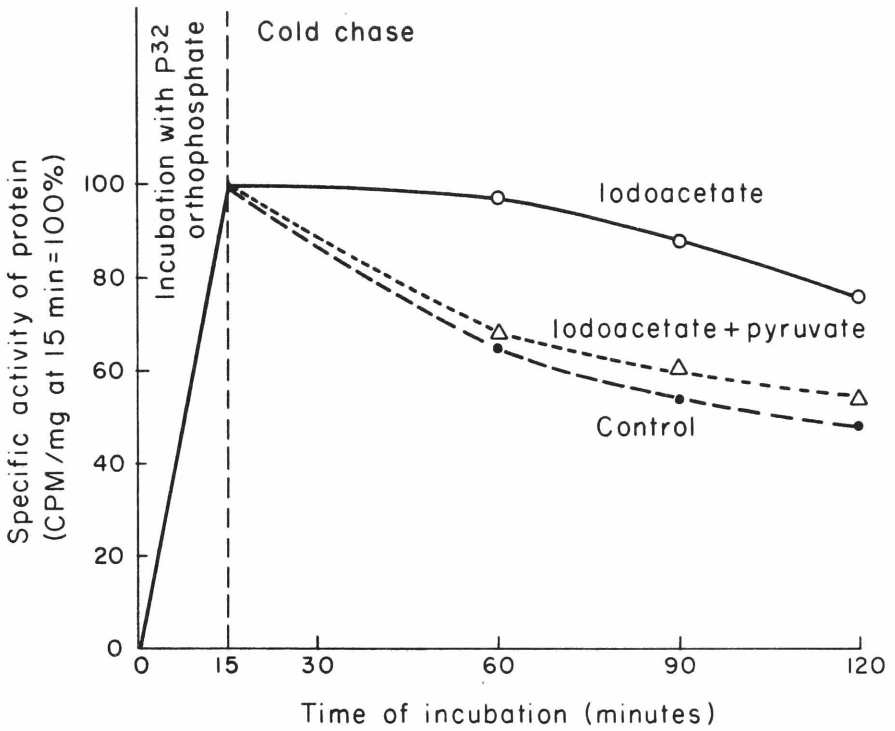


Figure 11. Effects of pyruvate on phosphate "turnover" which has been inhibited with iodoacetate (10^{-3} M). Nuclei were incubated for 15 minutes in the presence of P^{32}-orthophosphate, washed to remove the precursor, and then subjected to a "cold chase". Note that the inhibition of P^{32}-"turnover" caused by iodoacetate is reversible with pyruvate (.02 M).

TABLE II

Distribution of P^{32}-labeled phosphoproteins in thymus nuclear subfractions

Fraction analyzed	Phosphoprotein content (% of total alkali-labile P)	P^{32}-distribution (% of total P^{32} incorporation)	Specific activity of phosphoprotein (cpm/μmole-P)
0.01 M "tris" buffer extract	2.2	14.0	44,900
0.01 N HCl-80% ethanol extract	1.0	3.2	44,660
0.2 N HCl extract (histones)	13.6	14.6	9,460
Insoluble residue	83.2	68.2	8,090
Recovery	100.	100.	

A nuclear suspension was incubated 30 minutes in the presence of P^{32}-orthophosphate. The nuclei were then extracted as described in the text, and the different protein subfractions analyzed for total alkali-labile phosphate and for alkali-labile P^{32}. The figures for each subfraction are expressed as percentages of the total recovery.

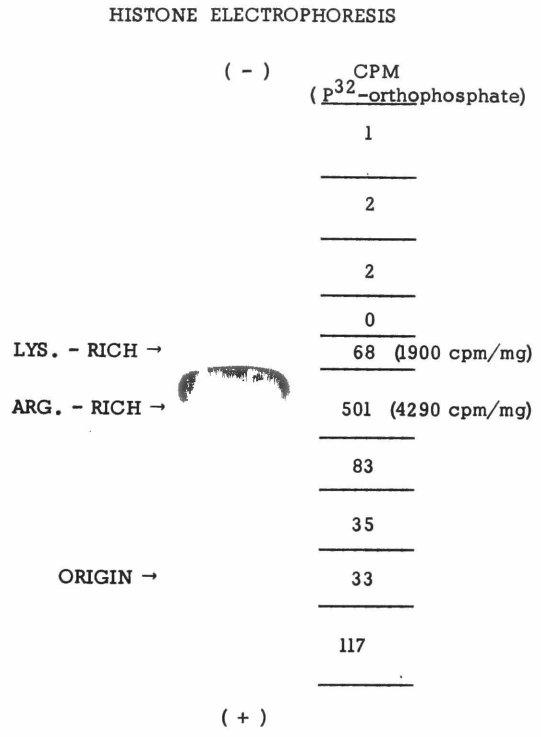


Figure 12. Electrophoresis of P^{32}-labeled histones on cellulose polyacetate strips. After electrophoresis, the strips were cut into sections and counted by dissolving in Bray's scintillation solution. Note that P^{32} counts are found localized in the lysine-rich and arginine-rich histone bands.

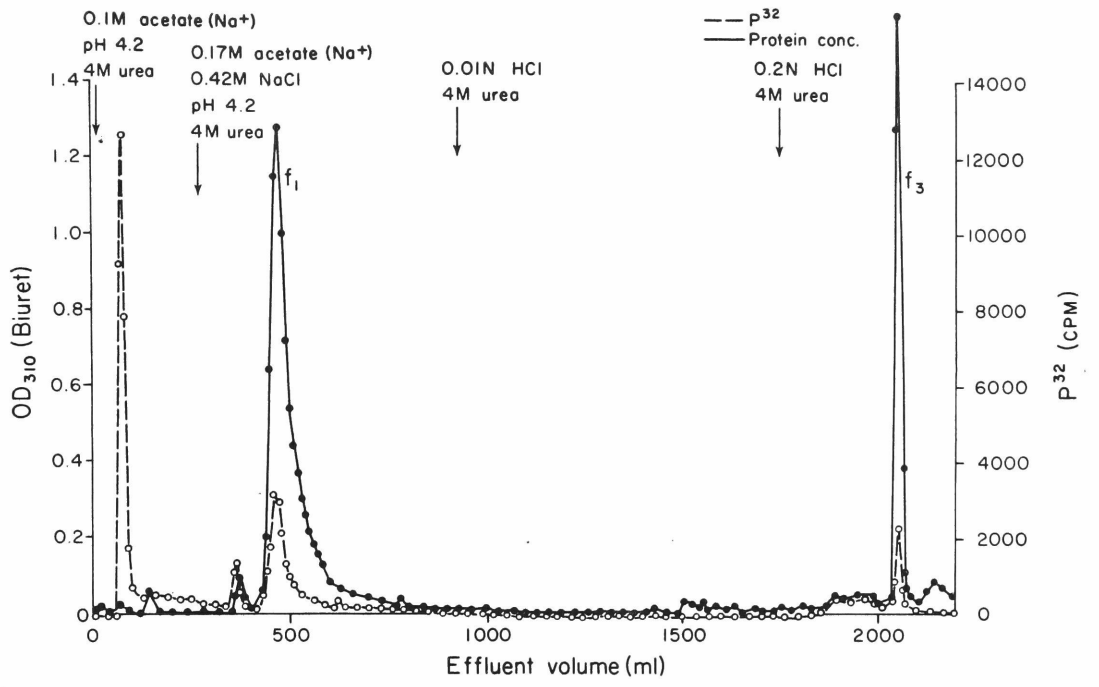


Figure 13. Separation of P^{32}-labeled histones on carboxymethylcellulose columns. Nuclei were incubated for 60 minutes with P^{32}-orthophosphate and the histones extracted and precipitated as described in the text. The presence of P^{32} in the major histone peaks, f_1 and f_3, is indicated. Protein concentration is indicated by the solid circles; radioactivity by the open circles.

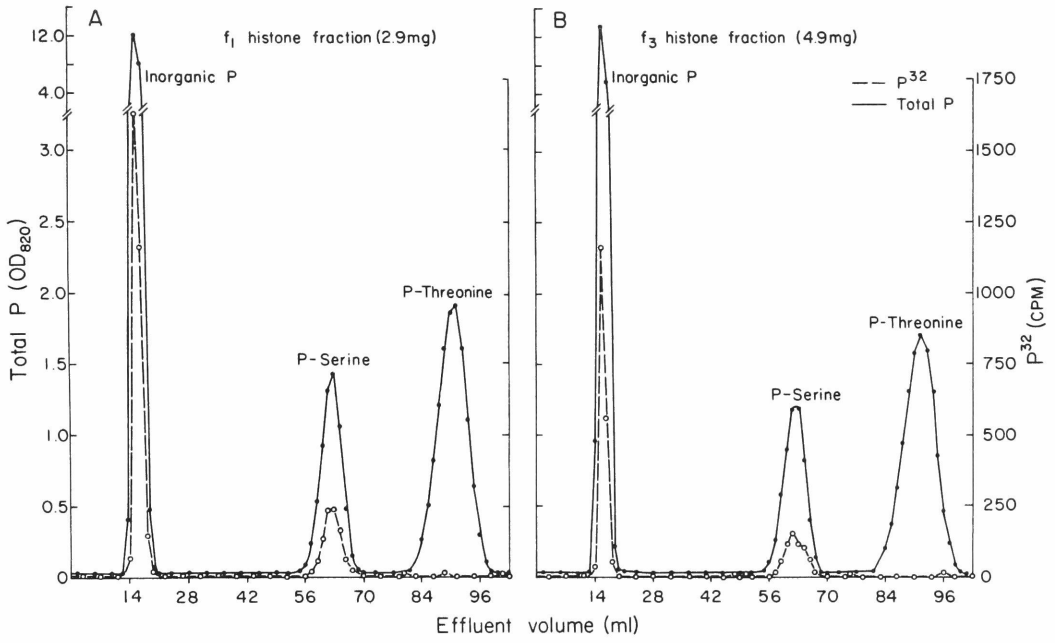


Figure 14. Isolation of radioactive phosphoserine from acid hydrolysates of P^{32}-labeled f_1 and f_3 histone fractions. Chromatography was on Dowex-50 columns as described in the text. Phosphoserine and phosphothreonine (2 mg each) were added before hydrolysis as markers. Total phosphorus is indicated by the solid circles; radioactivity by the open circles.

Since the results on nuclear protein fractionation indicated phosphoprotein heterogeneity, the "turnover" rates of the various fractions were studied to determine whether they all metabolize their phosphate at the same rate. The results, summarized in Figure 15, show that all the phosphoprotein fractions are subject to rapid "turnover" reactions. Some significant differences do occur, however, which are more apparent when the reactions are slowed down by incubating the nuclei at 25°C instead of 37°C. In this case, it is seen that the phosphoproteins of the soluble phase of the nucleus "turn over" their phosphate more rapidly than do the histones or residual proteins (Figure 16).

Tests for phosphoprotein function: In an attempt to relate phosphate "turnover" in nuclear proteins to RNA synthesis, the latter process was inhibited by the addition of 5,6-dichloro-β-D-ribofuranosylbenzimidazole (DRB), a potent inhibitor of RNA synthesis in thymus nuclei (Allfrey et al., 1957). Although the uptake of guanosine-8-C^{14} into RNA was inhibited, P^{32} uptake into nuclear proteins was not (Figure 17). Similar experiments where actinomycin-D was employed to inhibit RNA synthesis showed a similar lack of effect on protein phosphorylation (Figure 18).

The possibility that phosphorylated proteins may be involved in active transport mechanisms has already been discussed. Since isolated thymus nuclei do display sodium-dependent "transport" reactions in which potassium ions cannot substitute for sodium (Allfrey et al., 1961), tests have been carried out comparing protein phosphorylation in sodium- or potassium-containing incubation media. The phosphorylation of nuclear proteins shows no sign of sodium dependence, and over a wide range of salt concentrations, the results in potassium-containing media are indistinguishable from those obtained in the presence of sodium ions (Figure 19).

DISCUSSION

The present experiments indicate that phosphorylation and dephosphorylation of several different protein fractions occur in the isolated calf thymocyte nucleus. The proteins in the "tris" extract

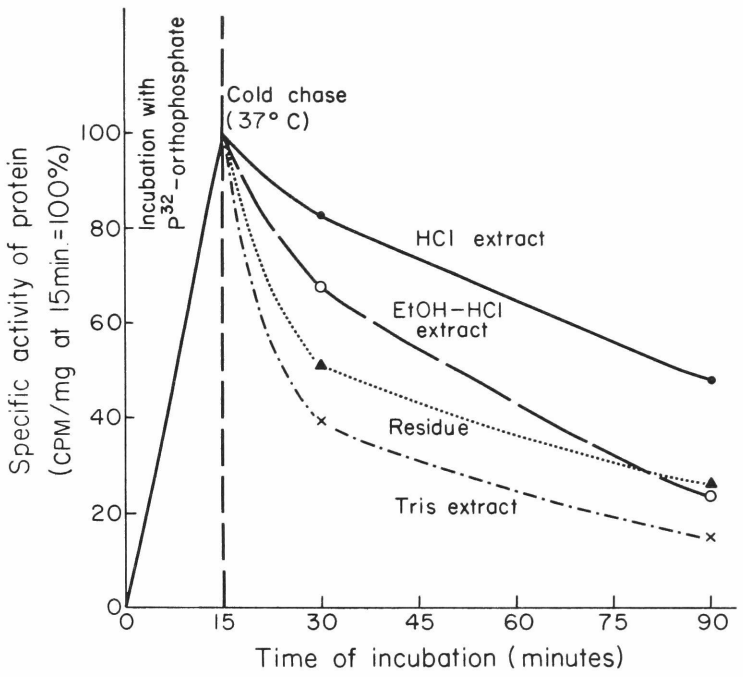


Figure 15. "Turnover" of previously incorporated P^{32} in nuclear protein fractions. Nuclei were incubated for 15 minutes in the presence of P^{32}-orthophosphate, washed to remove the precursor, and then reincubated at 37°C in radioisotope-free media. At various time intervals, aliquots were withdrawn and the nuclear proteins fractionated as described in the text. Note that the P^{32} in all four protein fractions is subject to a rapid "turnover" reaction.

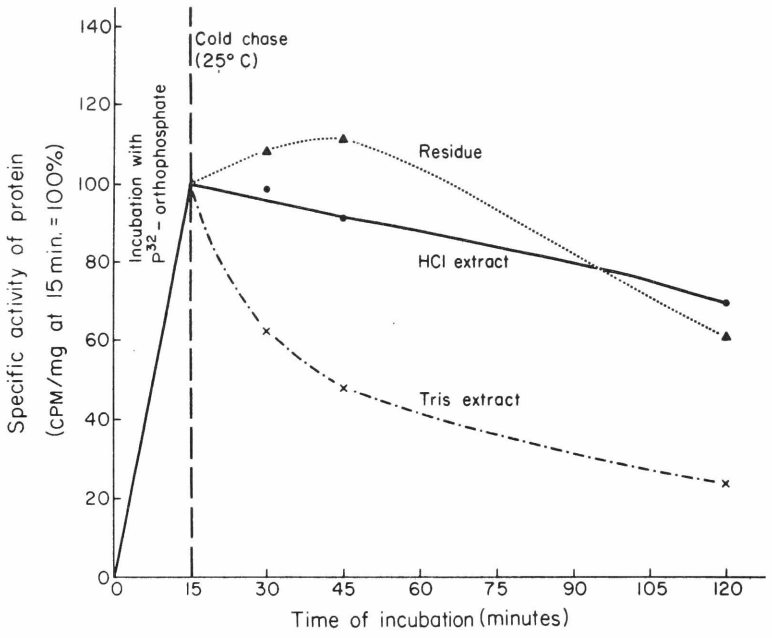


Figure 16. Methods for studying P^{32} "turnover" in nuclear protein fractions were the same as in Figure 15, except reincubation was at 25°C instead of 37°C. Note that the P^{32} in the "tris" extract "turns over" more rapidly than the P^{32} in the HCl extract or the residue. An ethanol-HCl extraction was performed in this experiment, but the yields were too small to permit analysis.

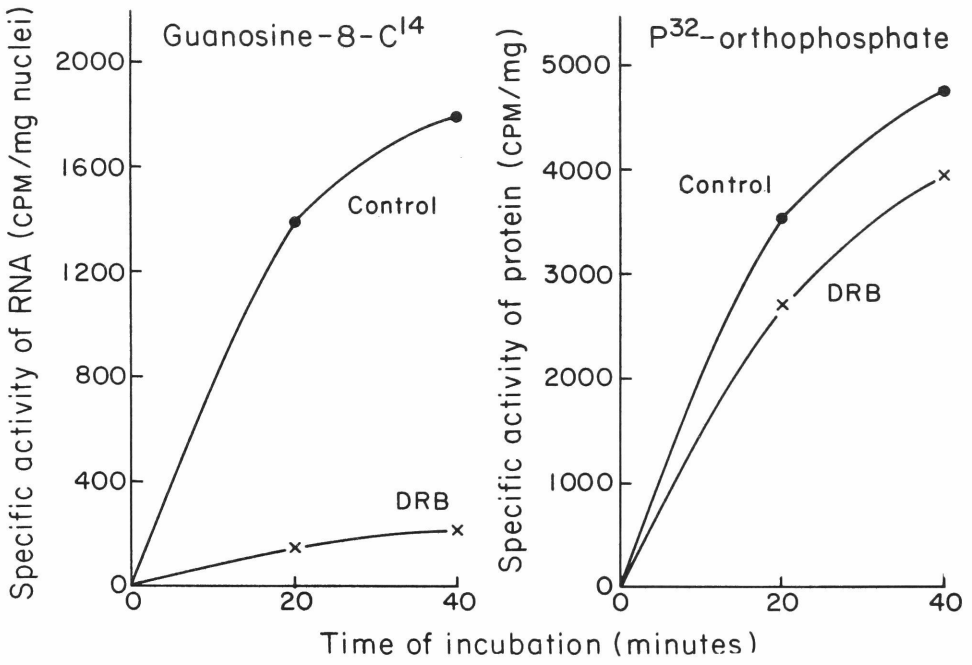


Figure 17. Effects of DRB on RNA synthesis and protein phosphorylation in isolated calf thymus nuclei. Note that DRB strongly inhibits the incorporation of guanosine-8-C^{14} into RNA while only causing a slight inhibition of P^{32}-incorporation into protein. This slight inhibition is in accord with the observation that DRB causes a 20% inhibition of ATP synthesis (Allfrey, 1965).

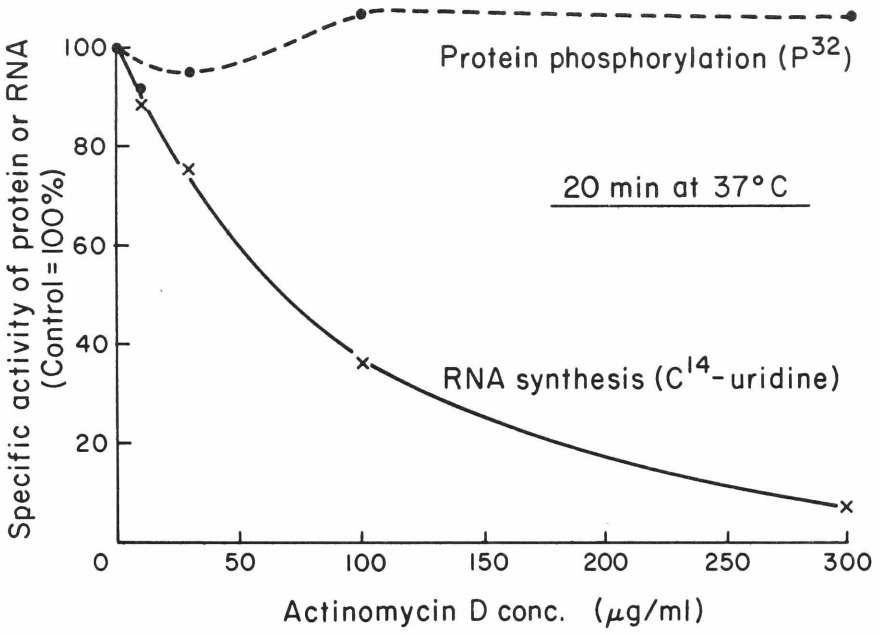


Figure 18. Effects of actinomycin D on RNA synthesis and protein phosphorylation in isolated calf thymus nuclei. The uptake of P^{32}-orthophosphate into protein and C^{14}-uridine into RNA during a 20 minute incubation is plotted as a function of actinomycin D concentration. Note that RNA synthesis is strongly inhibited without any corresponding inhibition of protein phosphorylation. (The final concentration of nuclei in these experiments was 10 mg per ml instead of the usual 20 mg per ml.)

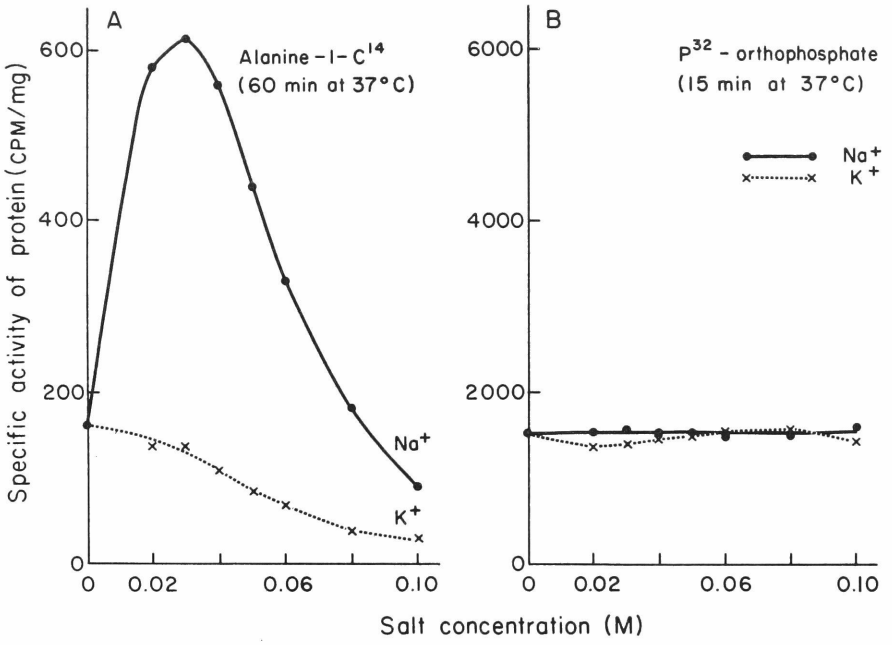


Figure 19. Comparative effects of Na^+ and K^+ on amino acid uptake and protein phosphorylation in isolated calf thymus nuclei. (A) Effects of varying ion concentration on alanine-1-C^{14} incorporation. The specific activity of the nuclear proteins after 60 minutes' incubation is plotted against the salt concentration of the medium. The existence of the sharp Na^+ optimum is known to reflect the sodium-dependence of the "transport" of the amino acid into the nuclear "pool" (Allfrey et al., 1961). (B) Effects of varying ion concentration on P^{32}-incorporation into nuclear proteins during a 15 minute incubation. Note that there is no sign of a Na^+ or K^+ dependence.

contain little phosphorus but have relatively high specific P^{32} activities. Although this fraction has not been extensively investigated, it would be expected to contain the soluble phosphoenzymes, like phosphoglucomutase and alkaline phosphatase.

The significance of histone phosphorylation is not clear, but it has been observed by other investigators (Langan, 1965; Ord and Stocken, 1966). The phosphorylation of histones is increased during liver regeneration (Ord and Stocken, 1967), and there is some evidence which suggests that phosphorylation of histones is tissue specific (Gutierrez and Hnilica, 1967). Ingles and Dixon (1967) have recently demonstrated that phosphorylation of protamines occurs in trout testis and that the degree of phosphorylation seems to correlate with the state of genetic activity.

The bulk of the nuclear phosphoprotein is in the non-histone, acid-insoluble protein fraction. The present studies make it possible to eliminate several of the possible functions which could be considered for this phosphoprotein fraction. Participation in a Na^+-K^+ dependent transport mechanism is ruled out by the lack of effect of Na and K ions. Involvement in a phosphate storage mechanism seems very unlikely because of the rapid rate of phosphate group "turnover". The possibility that this phosphoprotein is a high-energy intermediate in oxidative phosphorylation seems unlikely on the basis of the experiments demonstrating the energy-dependence of phosphate "turnover"; if we were dealing with a high-energy intermediate, it would not be expected to be discharged more slowly in the presence of iodoacetate when the nucleus is being starved for energy.

The distribution of phosphoproteins in nuclei suggests other functions. Their presence in isolated chromatin fractions and their solubilization when nuclei are lysed and treated with DNAse strongly suggests that phosphoproteins are involved in chromatin structure and may affect its function (Langan, 1965). Their high phosphate content corresponds to 4-5 phosphate groups per 100 amino acid residues, and there is good evidence also that the phosphoserines occur in clusters (Langan, 1965). Such regions of high negative charge density might be expected to influence DNA-histone interactions and so modify the

structure of the chromatin. Since the phosphoprotein concentrations of the "diffuse" or "active" chromatin fractions greatly exceed those in the relatively inactive chromatin "clumps", and since the phosphate groups of these proteins do "turn over" rapidly, one can envision a mechanism in which phosphorylation of chromosome-associated proteins influences DNA-histone interactions and leads to a shift from the "condensed" inactive state of the chromatin to the "diffuse" state, while dephosphorylation could again lead to tighter coiling of the DNA-histone-phosphoprotein complex. If this is so, one would predict an increase in the extent of nuclear protein phosphorylation during periods of gene activation. Experiments designed to test this hypothesis will be described in the next chapter.

SUMMARY

Isolated lymphocyte nuclei incorporate P^{32}-phosphate into nuclear proteins, which yield phosphoserine and phosphothreonine on hydrolysis. Protein phosphorylation is energy-dependent and proceeds independently of protein synthesis. Phosphate once incorporated is not stable, but "turns over" rapidly. This "turnover" is also energy-dependent. The bulk of phosphate incorporation is into non-histone protein, but small amounts of radioactive serine phosphate can be detected in highly purified histone fractions. Some evidence relating protein phosphorylation to the physical state and genetic activity of the chromatin is presented and discussed.

CHAPTER THREE: STIMULATION OF NUCLEAR PROTEIN PHOSPHORYLATION WITH PHYTOHEMAGGLUTININ

If phosphoproteins are actually involved in the modification of chromatin structure and the regulation of its metabolic activity, then one would expect to find an increased rate of phosphorylation of these proteins during periods of intense gene activity. The present experiments on gene activation in lymphocytes stimulated by phytohemagglutinin (PHA) confirm this prediction (Kleinsmith et al., 1966b).

Human lymphocytes treated with PHA, a protein extract of the red kidney bean, are known to undergo a striking transformation which includes a marked stimulation of metabolic activity, an increase in cell size, and other changes which eventually lead to cell division (Robbins, 1965). It is now clear that an early step in this transformation is an increased capacity for RNA and protein synthesis (McIntyre and Ebaugh, 1962; Tanaka et al., 1963; Rubin and Cooper, 1965; Bach and Hirschhorn, 1963), and Pogo et al. (1966) have provided evidence that this process may be regarded as a case of extensive gene activation. Although cell division does not occur for several days, an increase in RNA synthesis can be detected within a few minutes after the addition of PHA (Pogo et al., 1966). Even before the increase in RNA synthesis, there is an increase in the rate of histone

acetylation (Pogo et al., 1966). We therefore decided to study the behavior of nuclear phosphoproteins during this critical time period, the first few minutes after the addition of PHA.

METHODS

Preparation and incubation of lymphocytes: Lymphocytes were prepared from fresh heparinized human blood according to the method of Pogo et al. (1966). The blood was allowed to stand for 2 hours at 37°C, after which the upper layer of plasma and white blood cells was transferred to sterile conical centrifuge tubes and spun at 1000 g for 10 minutes. The pellets were resuspended in Eagle's minimal essential medium (MEM) containing 1 mg/ml heparin. The cell suspensions were centrifuged for two minutes at 100 g to sediment the polymorphonuclear leukocytes. The supernatant was carefully withdrawn and dispersed in MEM containing 20% inactivated newborn calf serum, glutamine, and antibiotics, to give a final concentration of 2-5 x 10^6 cells/ml. The cells were then incubated in culture bottles for 18 hours at 37°C. At this time the cultures consisted of about 90% lymphocytes, with the contaminating cells being mainly monocytes. It is assumed that the results of the present studies represent changes which have occurred in the lymphocytes, but there has been no way of evaluating the possible contributions of the contaminating cells.

After 18 hours of culture, 0.1 ml of PHA (Difco preparation P) was added to 5 ml of cell suspension containing 10 to 20 x 10^6 cells/ml, and the cells were reincubated at 37°C. At various times during incubation either $Na_2HP^{32}O_4$ (100 mc/mmole, 25 μc/ml) or uridine-2-C^{14} (26.7 mc/mmole, 0.5 μc/ml) was added for a 15 minute pulse-labeling. At the end of the pulse, the tubes were chilled in ice and the cells subsequently washed two times with cold MEM.

Isolation of nuclei: The washed cells were resuspended in cold 0.01 M citric acid and homogenized at 6000 rpm for two minutes in a Micro-Omnimixer (Ivan Sorvall, Inc.). The nuclei were sedimented at 1000 g for six minutes, and washed three times with 0.01 M citric acid. Preparations were monitored for purity by light microscopy.

Measurement of protein phosphorylation and RNA synthesis: In

experiments employing P^{32}-orthophosphate, nuclei were precipitated with 16% TCA and incorporation into alkali-labile phosphate determined as described in Chapter Two. When measuring RNA synthesis with uridine-2-C^{14}, nuclei were washed three times with cold 2% PCA, once with 3/1 ethanol:ether, and once with ether. RNA was hydrolyzed and extracted from the residue by incubating in 0.3 N KOH at 37°C for three hours. The extract was neutralized with 1.0 N PCA, centrifuged, and RNA concentration determined by the UV absorption method of Fleck and Munro (1963). Aliquots were counted for C^{14}-activity in Bray's scintillation solution.

Determination of ATP "pool": In one set of experiments, the incorporation of P^{32} into charcoal-adsorbable, easily-hydrolyzable nucleotide phosphate was measured as an approximation of the ATP "pool" (Crane and Lipmann, 1953). The supernatant from the 0.01 M citric acid homogenate was brought to a final concentration of 10% TCA and centrifuged in the cold. The supernatant was removed and 200 mg Norit A added to it. The Norit was washed four times with cold water, and then heated for 10 minutes at 100°C in 2.0 ml 1.0 N HCl. The charcoal was then removed by centrifugation and the supernatant clarified by filtration. Aliquots were dried and counted in a gas-flow G-M counter. Inorganic phosphate was determined by the method of Chen et al. (1956), as modified by Ames and Dubin (1960).

ATP was also isolated directly by chromatography on Dowex-1-formate (Siekevitz and Potter, 1955; McEwen et al., 1963a). The supernatant from the citric acid homogenate was brought to a final concentration of 2% PCA and the resulting precipitate removed by centrifugation. The extract was neutralized to pH 6-7 with KOH and centrifuged again to remove the potassium perchlorate formed. The supernatant was then washed onto a column of Dowex-1-formate (0.9 cm x 10 cm) with 10 ml of water. After washing the column with 75 ml 4.0 N formic acid, the ATP was eluted with 15 ml of 4.0 N formic acid:0.4 M ammonium formate.

RESULTS

Stimulation of protein phosphorylation and RNA synthesis: Although a certain variability in experimental results was encountered

owing to the need for employing different blood donors for each pint of blood, all lymphocytes tested displayed a stimulation of nuclear protein phosphorylation and RNA synthesis following the addition of PHA (Table III). An examination of the early kinetics of the process indicates that this increase in protein phosphorylation can be detected within the first 15 minutes after the addition of PHA (Figure 20). Furthermore, comparison of the kinetics of the stimulation of protein phosphorylation with those of RNA synthesis indicates that protein phosphorylation does not exhibit the early lag in stimulation characteristic of RNA synthesis under the same conditions (Pogo et al., 1966); these kinetics suggest the possibility that the stimulation in protein phosphorylation precedes the major stimulation in RNA synthesis. In this respect, the results are similar to those indicating that histones are acetylated before lymphocyte nuclei increase their capacity for RNA synthesis (Pogo et al., 1966).

Labeling of ATP "pool": Although it is clear that the rate of P^{32} uptake into nuclear proteins is increased in PHA-treated cells, a possible objection is that this change might merely reflect a change in the specific activity of the high-energy phosphate "pool", rather than an actual increase in the rate of protein phosphorylation. Therefore it was decided to measure the uptake of P^{32} in the ATP pool during the first hour after the addition of PHA in order to determine whether it increased enough to account for the observed increase in labeling of the phosphoproteins.

In the first experimental approach, the incorporation of P^{32} into charcoal-adsorbable, easily-hydrolyzable nucleotide phosphate was measured as an approximation of the ATP pool (Crane and Lipmann, 1953). Although some increase in the specific activity of this phosphate pool was observed, the increase was not sufficient to account for the increase in specific activity of the phosphoprotein fraction (Figure 21). In the second approach, ATP was isolated directly by chromatography on Dowex-1-formate. In this experiment a 15-minute pulse of P^{32}-orthophosphate was given one hour after the addition of PHA, and then the specific activities of the phosphoprotein fraction and ATP were compared. The specific activity of the phosphoprotein

TABLE III

Nuclear protein phosphorylation and RNA synthesis in PHA-treated lymphocytes

Expt.	Condition	Specific activity of phosphoprotein (cpm/mg)	(% control)	Specific activity of RNA (cpm/μg RNA-P)	(% control)
1	Control	18		16	
	PHA	38	211	23	144
2	Control	65		77	
	PHA	99	152	114	148
3	Control	43		67	
	PHA	90	209	131	196
4	Control	402			
	PHA	1014	252		
5	Control	538		16	
	PHA	655	122	40	250
6	Control	3495			
	PHA	4517	129		

Human lymphocytes were cultured in the presence of PHA as described in the text. One hour after the addition of PHA, the desired isotopic precursor (P^{32}-orthophosphate for phosphoprotein, 2-C^{14}-uridine for RNA was added for a 15 minute pulse-labeling).

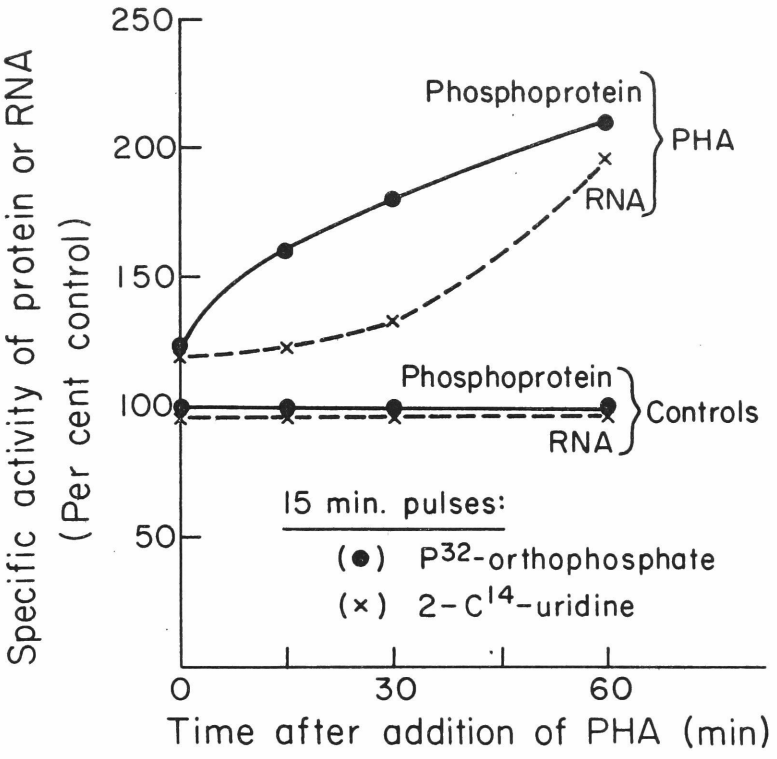


Figure 20. Comparative effects of PHA on nuclear protein phosphorylation and RNA synthesis in human lymphocytes. Cells were exposed to PHA and at the indicated times were pulse-labeled for 15 minutes in the presence of P^{32}-orthophosphate or 2-C^{14}-uridine. The specific activities of the phosphoprotein and RNA are plotted as percentages of control values. Note that the stimulation of protein phosphorylation seems to precede that of RNA synthesis.

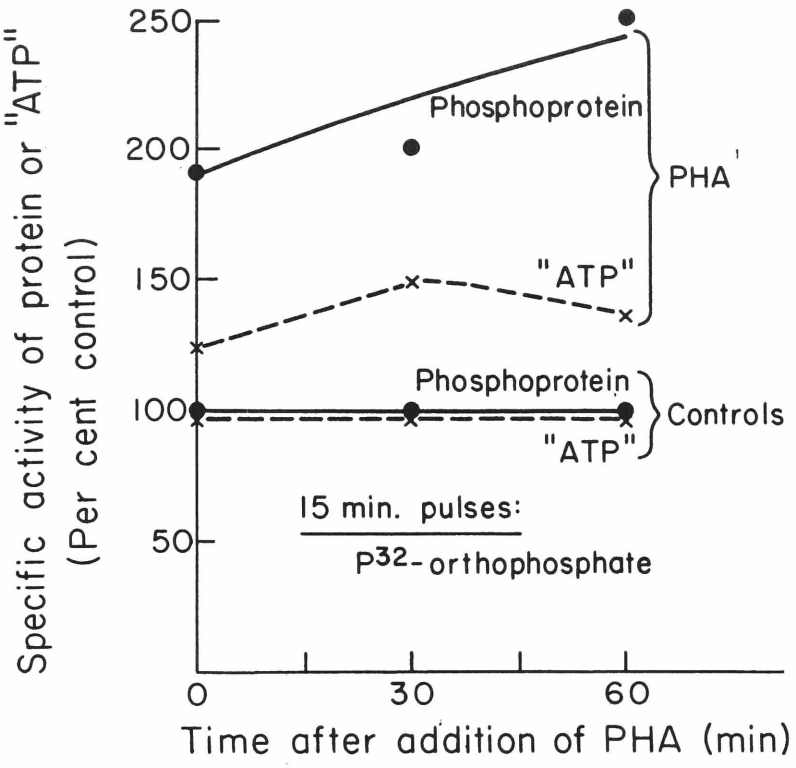


Figure 21. Comparative effects of PHA on protein phosphorylation and ATP phosphorylation. Cells were pulse-labeled for 15 minutes with P^{32}-orthophosphate at the indicated times. The specific activities of the nuclear phosphoprotein and "ATP" (measured as easily-hydrolyzable nucleotide phosphate) are expressed as percentages of control values. Note that the stimulation in protein phosphorylation exceeds the increase in specific activity of the ATP pool.

fraction of the PHA-treated cells was found to be 129% that of the controls, while the specific activity of the ATP in these PHA-treated cells was actually depressed slightly to 86% of control values. Thus in both types of experiment it does not appear that the increase in specific activity of the phosphoprotein fraction can be explained in terms of an increased specific activity of the high-energy phosphate pool.

"Turnover" of protein phosphate groups: Since the phosphorylation of nuclear protein appears to involve a dynamic equilibrium in which previously incorporated phosphate groups are "turned over", a comparison was made of the retention of P^{32}-phosphate in the phosphoproteins of PHA-treated and control cells. Lymphocytes were incubated for 15 minutes in the presence of P^{32}-orthophosphate to label the phosphoproteins, and the cells were subsequently washed and resuspended in radioisotope-free media; PHA was added to one group of cells after the washing procedure. Samples were withdrawn at later times for measurement of the specific activity of the nuclear phosphoproteins. The results are shown in Figure 22. There is a striking difference in the extent of loss of previously incorporated phosphate groups between control and PHA-treated lymphocytes. Within two hours the PHA-treated cells have lost 75% of the P^{32} label, while the controls, after an initial period in which P^{32}-uptake continues, lose only about 40% of their maximal specific activity in the same time interval. It is not known whether these differences reflect only a difference in phosphate "turnover", since the situation could be complicated by a degradation of the protein molecules themselves. The finding that the cold chase is effective more quickly in the PHA-treated cells also suggests the possibility that PHA might be affecting the transport of phosphate into the cells.

Effects of puromycin: Since it was shown in the previous chapter that phosphorylation of nuclear proteins in thymus nuclei proceeds independently of protein synthesis, the effects of puromycin were tested in the human lymphocyte system in an attempt to determine whether the increase in protein phosphorylation following PHA is entirely independent of new protein synthesis. When lymphocytes were examined one hour after the addition of PHA with a 15-minute pulse of

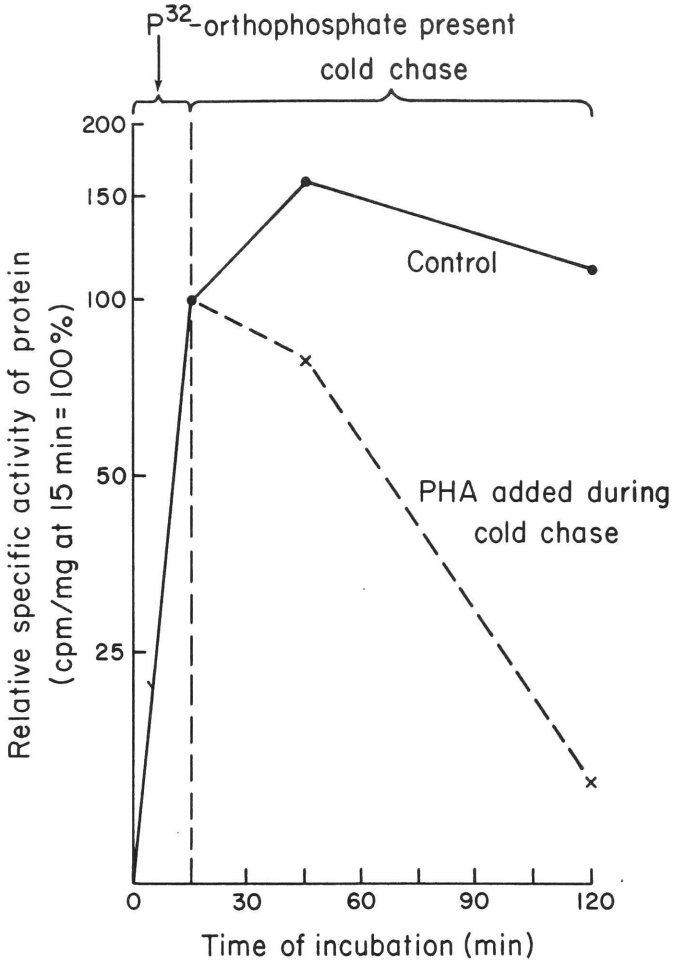


Figure 22. Effects of PHA on turnover of previously incorporated P^{32}-phosphate in nuclear phosphoprotein. Cells were incubated for 15 minutes with P^{32}-orthophosphate, after which they were washed and resuspended in radioisotope-free media, and PHA was added. The retention of isotope was measured as a function of time. Note the rapid loss of P^{32} from the PHA-treated cells.

P^{32}-phosphate, it was found that protein phosphorylation was stimulated even while protein synthesis was being blocked with puromycin (Table IV). However, the stimulation was not as great as that seen in the absence of puromycin, so that it is possible that some new synthesis of phosphoprotein is occurring. Indirect toxic effects of puromycin, however, might also be responsible for the slight inhibition of protein phosphorylation which was observed.

DISCUSSION

It can be concluded from the present experiments that the rate of phosphorylation and dephosphorylation of nuclear protein increases within the first few minutes of PHA-induced gene activation in human lymphocytes. Since it has been suggested that the phosphorylation of these nuclear proteins is involved in the modification of chromatin structure and DNA template activity, it is interesting to note that some independent evidence exists which indicates that the physical state of the DNA changes shortly after the addition of PHA. Killander and Rigler (1965) have observed that lymphocytes which have been treated with PHA rapidly increase their ability to bind acridine orange to DNA; the kinetics of this increase are similar to those found for the increase in protein phosphorylation, as well as to those reported for histone acetylation by Pogo et al. (1966).

Although the results of the present experiments are consistent with the proposed role of phosphoproteins in the regulation of DNA template activity, alternative explanations, such as the possibility that these phosphoproteins are involved in phosphate transfer reactions, could not be excluded. Therefore a somewhat different experimental approach was turned to in an attempt to clarify the situation. Up until this point, we have been considering only the phosphorylation reaction as it occurs in the intact nucleus. In the next two chapters, experiments on the physical isolation and chemical characterization of the protein moiety itself will be described. Through this approach, additional valuable information about the functional characteristics of the nuclear phosphoproteins has been obtained.

TABLE IV

Effects of puromycin on protein synthesis and phosphorylation in PHA-treated lymphocytes

PHA	Puromycin	Specific activity of protein: P^{32}-phosphate (cpm/mg)	(P^{32}-phosphate % control)	C^{14}-serine (cpm/mg)	(C^{14}-serine % control)
−	0	2569	100.0	198	100.0
	10 μg/ml	2273	89.3	27	13.6
+	0	4290	166.9		
	10 μg/ml	3129	124.9		

Human lymphocytes were cultured in the presence of PHA and puromycin. After one hour of incubation either P^{32}-orthophosphate or C^{14}-serine was added for a 15 minute pulse-labeling.

SUMMARY

Human lymphocytes treated with phytohemagglutinin undergo extensive gene activation, as evidenced by augmented synthesis of ribonucleic acids. This activation is preceded by an early stimulation in the rate of phosphorylation and dephosphorylation of nuclear proteins. This finding is consistent with a hypothesized role of phosphoproteins in the modification of chromatin structure and in modulation of the template activity of DNA *in vivo*.

CHAPTER FOUR: ISOLATION AND CHARACTERIZATION OF A PHOSPHOPROTEIN FRACTION FROM CALF THYMUS NUCLEI

In the experiments described thus far on protein phosphorylation in intact nuclei, the general approach has been to measure the incorporation of P^{32}-orthophosphate into the total nuclear protein. In Chapter Two it was pointed out that the majority of this incorporation is into the non-histone, "residual" protein fraction. In this chapter the isolation and purification of the phosphoprotein component(s) of this fraction will be described, together with the results of studies of the enzymatic phosphorylation of this protein fraction in vitro.

It has been found that this purified phosphoprotein fraction from calf thymus nuclei is similar in several aspects to the one isolated from rat liver nuclei (Langan, 1967). It contains about 1.3% phosphorus by weight, or the equivalent of 4-5 phosphorylated amino acids per 100 residues. The protein fraction retains its own phosphoprotein kinase activity, so that it can be phosphorylated directly in vitro by the addition of ATP and Mg^{++} without the need for any other added enzyme. It has been discovered that in addition to ATP, several other ribonucleoside- and deoxyribonucleoside triphosphates are capable of phosphorylating the protein in vitro. Unlike the case of phosvitin (Rabinowitz and Lipmann, 1960), the enzymatic reaction between ATP and

the nuclear phosphoprotein appears to be almost irreversible. Furthermore, phosphate groups which have been incorporated into the nuclear phosphoprotein *in vitro* are stable to incubation, indicating the absence of the enzyme activity which is responsible for the rapid "turnover" of phosphate groups seen in the phosphoproteins of intact nuclei.

METHODS

Purification of phosphoprotein from calf thymus nuclei: The procedure employed for purification of the calf thymus nuclear phosphoprotein was a slight modification of the method developed by Langan and Lipmann for the isolation of phosphoprotein from rat liver nuclei (Langan, 1967). The rationale of the procedure is as follows. Nuclei are first isolated and are then washed with dilute salt solutions to remove the soluble nuclear proteins and ribosomes. The phosphoprotein is solubilized along with the DNA and histones by homogenizing in 1.0 M NaCl. The DNA and histones are then precipitated from this extract by lowering the salt concentration to 0.4 M. Any basic protein remaining in the supernatant is removed by a bulk adsorption with a cation-exchange resin. The phosphoprotein is then removed from the solution by adsorption on a calcium phosphate gel. The gel is washed and then dissolved in 0.2 M EDTA, bringing the phosphoprotein into solution. The salts and EDTA are finally removed by gel filtration on Bio-Gel P-10. The details of the procedure are described in the following paragraphs.

All steps of the purification were carried out at 4°C. Nuclei were first prepared from 200 grams fresh calf thymus according to the procedure of Allfrey *et al.* (1957). The nuclear suspension was sedimented at 1000 *g*, and was then washed first with 1000 ml of 0.01 M Tris (pH 7.4), 3 mM $MgCl_2$, and then with 500 ml of 0.14 M NaCl. After each wash the nuclei were collected by centrifugation at 1000 *g* for seven minutes. The sediment was resuspended in 270 ml of 0.14 M NaCl and was then added to 270 ml of 2.0 M NaCl, 0.03 M Tris (pH 7.4). The resulting gel was homogenized for five minutes at 1000 rpm in a Waring blendor, and was then centrifuged for 15 minutes at 33,000 *g*. The supernatant was collected and slowly diluted with 1.5 volumes of 0.02 M Tris (pH 7.4); the nucleoprotein aggregate which formed was dispersed by blending for two minutes at 1000 rpm in the Waring blendor. The resulting suspension

was centrifuged for 75 minutes at 105,000 g.

To the supernatant was added 10 grams of Bio-Rex 70 (Na^+) which had been equilibrated with 0.4 M NaCl, 0.02 M Tris (pH 7.4). After stirring 10 minutes, the suspension was centrifuged 10 minutes at 6000 g and the resin washed with 50 ml 0.4 M NaCl, 0.02 M Tris (pH 7.4). The two supernatants were combined and 66 mg of calcium phosphate gel added (Keilin and Hartree, 1938). After stirring 10 minutes, the gel was collected by centrifuging five minutes at 6000 g. It was washed by resuspending in 100 ml of 1.0 M $(NH_4)_2SO_4$, 0.05 M Tris (pH 7.4), and was again collected by centrifugation. The gel was then dissolved in 32 ml of 0.2 M EDTA (pH 7.4), 0.33 M $(NH_4)_2SO_4$ by gentle stirring for 45 minutes. A small insoluble residue was removed by centrifuging 10 minutes at 33,000 g, and the supernatant desalted by passing over a column of Bio-Gel P-10 (2.5 cm x 44 cm) equilibrated with 0.05 M Tris (pH 7.4). The exclusion peak, containing 10-25 mg phosphoprotein, was collected and stored at -90°C. At this temperature the preparation is stable for at least a year. This procedure for the purification of the phosphoprotein fraction from calf thymus nuclei is summarized in the flow-diagram in Figure 23.

Analytical methods: Alkali-labile phosphate, phosphoserine, and phosphothreonine were determined as described in Chapter Two. Amino acid analyses were performed on a Beckman automatic analyzer (Spackman et al., 1958).

Tryptophan was determined by a micro-scale modification of a method described by Spies and Chambers (1949). Approximately 500 μg of phosphoprotein was precipitated with 10 volumes of acetone and resuspended in 0.5 ml of a solution of dimethylaminobenzaldehyde (3 mg/ml in 19 N H_2SO_4). After standing in the dark for 19 hours with occasional stirring, 0.025 ml of 0.009% $NaNO_2$ was added, and the resulting color read 30 minutes later at 610 mμ. A standard curve was run with tubes containing 2, 4, and 8 μg tryptophan in the same volume.

Assay of enzymatic phosphorylation of nuclear phosphoprotein: In the standard assay procedure, 0.5 ml of final incubation mixture was made by combining the following components: 0.4 ml of purified phosphoprotein in 0.05 M Tris, pH 7.4 (100-150 μg/ml); 0.05 ml of 0.05 M $MgCl_2$;

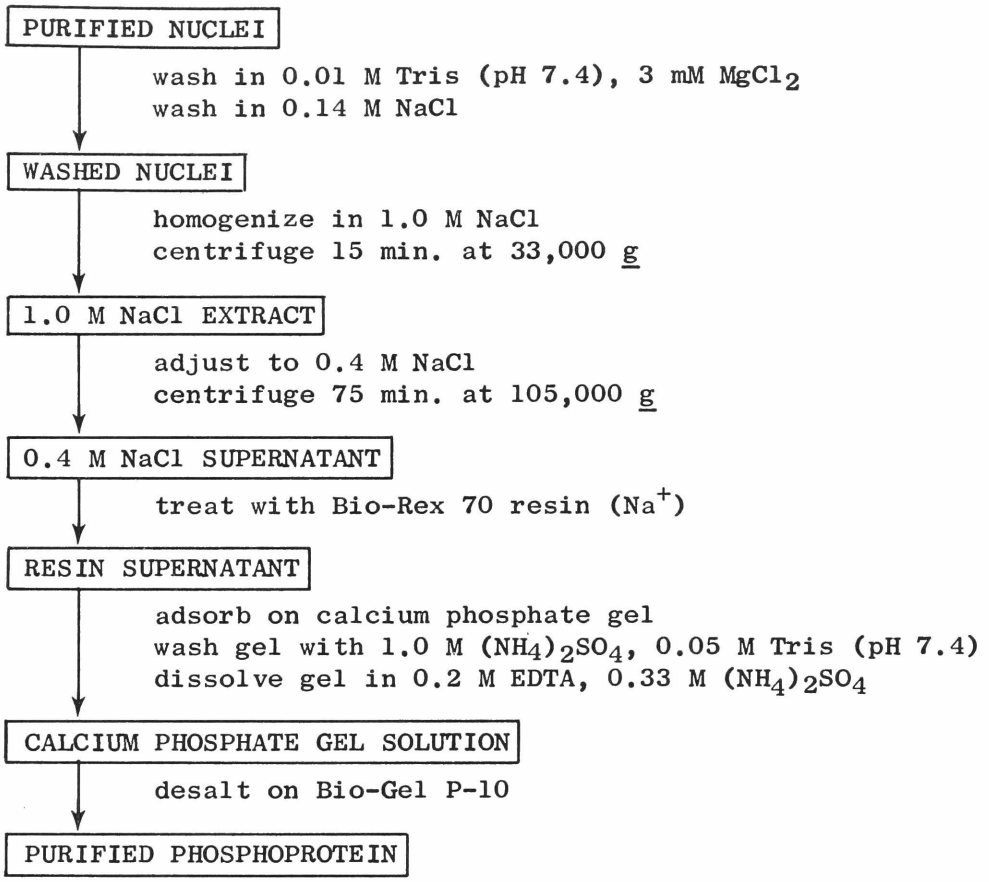


Figure 23. Flow-diagram for phosphoprotein purification.

and 0.05 ml of ATP-γ-P^{32} (1 mμmole/ml, 300-3000 mc/mmole). Tubes were incubated for 10 minutes at 37°C, and the reaction stopped with cold 25% TCA. To each tube 0.5 mg phosvitin was added as carrier, and the precipitates were washed three times with cold 25% TCA and once with 20% ethanol in ether. Incorporation of P^{32} into alkali-labile phosphate was determined as described in Chapter Two. About 90% of the counts in the alkali-labile phosphate fraction originate from phosphoserine, and the remaining 10% are from phosphothreonine.

P^{32}-labeled nucleoside triphosphates were obtained from International Chemical and Nuclear Corporation. ADP-β-P^{32} was prepared from ATP-β,γ-P^{32} obtained from Schwartz Bioresearch. The β,γ-labeled ATP was incubated for 20 minutes with the phosphoprotein as described above in order to cleave the terminal phosphate linkage and form ADP-β-P^{32}. The material was then placed on a column of Bio-Gel TE-2 (0.9 cm x 7.5 cm) equilibrated with 0.08 M glycine-HCl (pH 3.2), 0.15 M KCl, and the column eluted with the same buffer. The ADP-β-P^{32} passed through the column quickly and was collected, leaving the ATP-β,γ-P^{32} behind.

Preparation of P^{32}-labeled phosphoprotein: In order to study the reversibility of the phosphorylation reaction, phosphoprotein was labeled with P^{32} by incubating it in the presence of ATP-γ-P^{32} as described above. After 20 minutes of incubation, the mixture was extensively dialyzed in the cold, first against 0.01 M EDTA (pH 7.4), and then against 0.05 M Tris (pH 7.4), 5 mM $MgCl_2$. After dialysis the phosphoprotein was diluted to a final concentration of 100,000 cpm/ml, and was incubated with excess ADP as described by Rabinowitz and Lipmann (1960).

RESULTS

Composition of purified phosphoprotein fraction: The alkali-labile phosphorus content of the phosphoprotein fraction purified from calf thymus nuclei was found to be 1.28%, compared to 1.14% obtained by Langan (1967) for the rat liver phosphoprotein (Table V). In both cases, this phosphorus occurs primarily as phosphoserine, with smaller amounts (about 10%) of phosphothreonine. The amino acid compositions

TABLE V

Phosphorus content of nuclear protein

	Calf thymus	Rat liver
Total nuclear protein	0.07% P	0.14% P
Purified phosphoprotein	1.28% P	1.14% P
Purification	19 x	8 x
Yield	12%	25%
Maximal estimate of:		
$\frac{\text{phosphoprotein}}{\text{total dry wt. nucleus}}$	.04	.09
$\frac{\text{phosphoprotein}}{\text{DNA}}$	.14	.49

Data for the composition of rat liver nuclear phosphoprotein was obtained from Langan (1965, 1967).

of the phosphoproteins from these two different sources are also quite similar (Table VI).

As has been pointed out by Langan (1967), the amino acid composition of the phosphoprotein fraction bears some resemblance to that of certain histone subfractions (see Table VI). One difference between these two protein classes is the occurrence of tryptophan in the phosphoprotein fraction and its absence from the histones, but the major distinction between the amino acid compositions of these two types of protein is in the ratio of basic to acidic residues, which is much lower in the phosphoproteins than in the histones. This difference is reflected in a large difference in isoelectric points. At pH 7.2, the histones carry a heavy positive charge and migrate rapidly toward the cathode during electrophoresis, while the phosphoprotein fraction carries a net negative charge and moves toward the anode (Figure 24). Although the phosphoproteins appear to be moving as one band during electrophoresis on cellulose polyacetate, preliminary experiments employing discontinuous polyacrylamide gel electrophoresis have indicated the presence of at least five separate components in this fraction.

The ultraviolet spectra of phosphoprotein preparations indicate the presence of another form of heterogeneity (Figure 25). Calculations based on the ratio of absorbancy at 280 mμ:260 mμ (Layne, 1957) indicate the occurrence of from 5-12% nucleic acid by weight in various phosphoprotein preparations. Thus far it has not been possible to completely separate the nucleic acid from the phosphoprotein by physical means, indicating the possibility that some type of binding exists between the two species. This observation is of possible interest in relation to the proposed role of the phosphoproteins in modifying chromatin structure and DNA template activity.

Enzymatic phosphorylation of nuclear phosphoprotein: The purified phosphoprotein fraction can be phosphorylated in vitro by incubating it in the presence of ATP-γ-P^{32} and Mg^{++} (Table VII). This reaction is different from similar reactions involving phosphoproteins such as casein and phosvitin in that it does not require the addition of the enzyme phosphoprotein kinase. That the reaction between ATP and

TABLE VI

Comparison of amino acid composition of nuclear phosphoprotein and histone fractions

	Nuclear phosphoprotein (Calf thymus)	Nuclear phosphoprotein (Rat liver)	Histone (F2b)
Lysine	9.4	8.0	14.5
Histidine	1.9	2.2	2.5
Arginine	8.5	9.8	7.5
Aspartic acid	10.5	9.5	5.5
Glutamic acid	14.9	13.5	9.0
Threonine	3.8	4.3	6.5
Serine	10.3	10.3	9.0
Proline	6.2	7.1	4.5
Glycine	6.8	7.6	7.0
Alanine	6.2	6.4	10.5
Cysteine	0.6	0.3	---
Valine	4.5	5.1	6.8
Methionine	1.9	1.6	0.7
Isoleucine	3.0	3.2	5.0
Leucine	6.2	6.5	6.0
Tyrosine	2.4	2.1	3.1
Phenylalanine	2.8	2.7	2.0
Tryptophan	0.9%		---
Phosphorus (alkali-labile)	1.28%	1.14%	0.02%
Basics:Acidics	0.78	0.87	1.69

Amino acid compositions are expressed in terms of moles per 100 moles of amino acids recovered (except for tryptophan which is expressed in terms of percent by weight). Composition of rat liver nuclear phosphoprotein is from Langan (1967), and data for the F2b histone is from Busch (1965

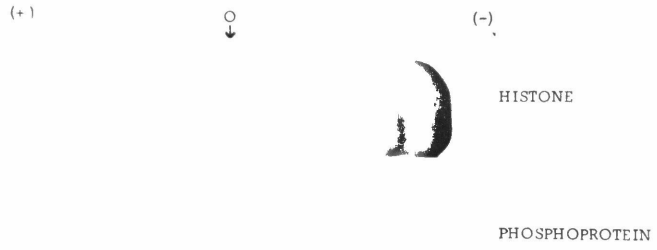


Figure 24. Electrophoresis of phosphoprotein and histone preparations from calf thymus nuclei on cellulose polyacetate strips (0.01 M Tris buffer, pH 7.2, 40 minutes at 200V).

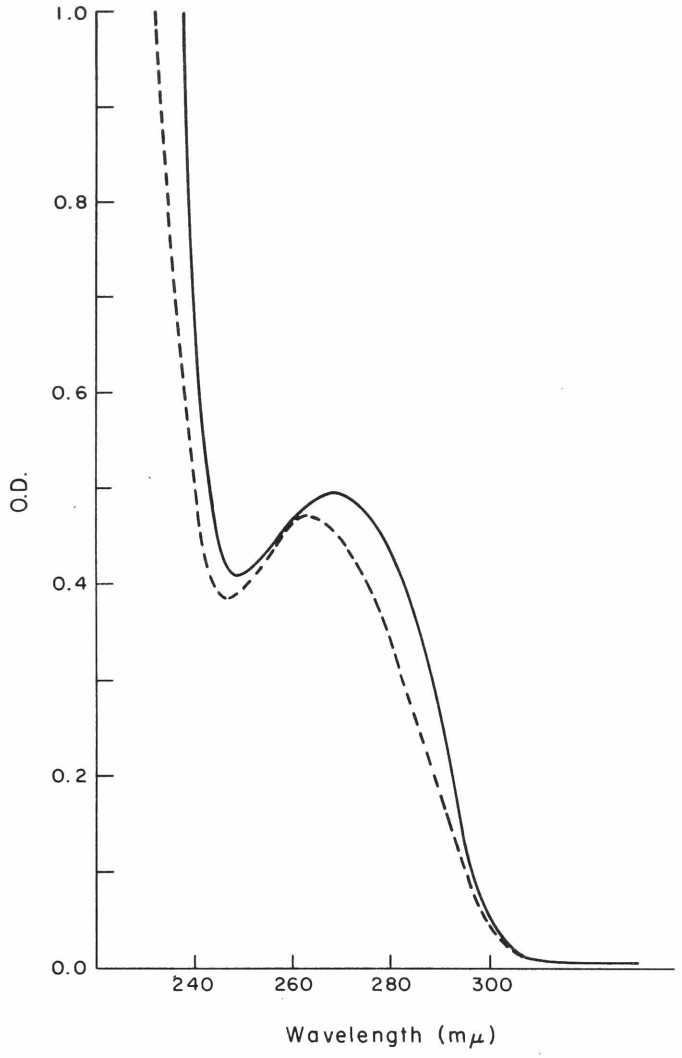


Figure 25. Ultraviolet spectra of two phosphoprotein preparations with different nucleic acid contents. Note that the spectra are considerably shifted away from 280 mμ and toward 260 mμ, as would be expected if nucleic acids were present.

TABLE VII

Enzymatic phosphorylation of nuclear phosphoprotein

	Alkali-labile P^{32} formed (μμmoles)
Complete system	10.84
zero time	0.01
minus $MgCl_2$	0.03
preheat 3 min at 60°C	0.08

The purified phosphoprotein fraction was incubated for 10 minutes at 37°C in the presence of 100 μμmoles ATP-γ-P^{32} and 5 mM Mg^{++} as described in the text. Incorporation of P^{32} into alkali-labile phosphate was then determined.

the nuclear phosphoprotein is enzymatic is indicated by its thermal lability; it can be completely abolished by pre-heating the phosphoprotein for three minutes at 60°C. Either the nuclear phosphoprotein fraction carries along some phsophoprotein kinase as a contaminant, or else the phosphoprotein has its own inherent kinase activity.

The standard incubation mixture used in the initial studies was made with a final concentration of 5 mM $MgCl_2$ because this is the concentration employed in the past with phosphoprotein kinase systems (Burnett and Kennedy, 1954; Rabinowitz and Lipmann, 1960; Langan, 1967). When a Mg^{++}-concentration curve was run, however, it was found that the optimum for the nuclear phosphorylation reaction actually occurred closer to 25 mM $MgCl_2$ (Figure 26). The reaction exhibited a specific requirement for Mg^{++}; of 11 other divalent cations tested, only Fe^{++}, Mn^{++}, and Co^{++} showed significant activity, and these were only about half as active as Mg^{++} (Table VIII). Many of the divalent cations tested were highly inhibitory. In the presence of 5 mM Mg^{++}, small amounts (1 mM) of Be^{++}, Zn^{++}, and Pd^{++} inhibited the phosphorylation reaction more than 95%; Fe^{++}, Cu^{++}, Cd^{++}, and Ni^{++} were inhibitory to a lesser extent. The phosphorylation of nuclear phosphoprotein in vitro does not exhibit a sharp pH optimum, but the reaction strongly favors the alkaline pH range (Figure 27).

In an attempt to determine the substrate specificity of the phosphorylation reaction, various unlabeled nucleoside triphosphates were tested as competitive inhibitors of the reaction between ATP^{32} and the phosphoprotein. It was found that in addition to nonradioactive ATP, the nucleotides GTP, CTP, and UTP were also effective as competitive inhibitors of the reaction (Figure 28). Although none of the latter were as effective as ATP, the results indicated the possibility that these nucleoside triphosphates might be active as phosphoryl group donors. When this possibility was tested by substituting the different P^{32}-labeled nucleoside triphosphates for ATP-γ-P^{32} in the incubation mixture, it was found that GTP-γ-P^{32}, ITP-γ-P^{32}, CTP-γ-P^{32}, UTP-γ-P^{32}, and dATP-γ-P^{32} were all capable, to varying degrees, of labeling the phosphoprotein (Table IX). On the other hand, ADP-β-P^{32}, P^{32}-pyrophosphate, and P^{32}-orthophosphate were inactive as phosphorylating agents.

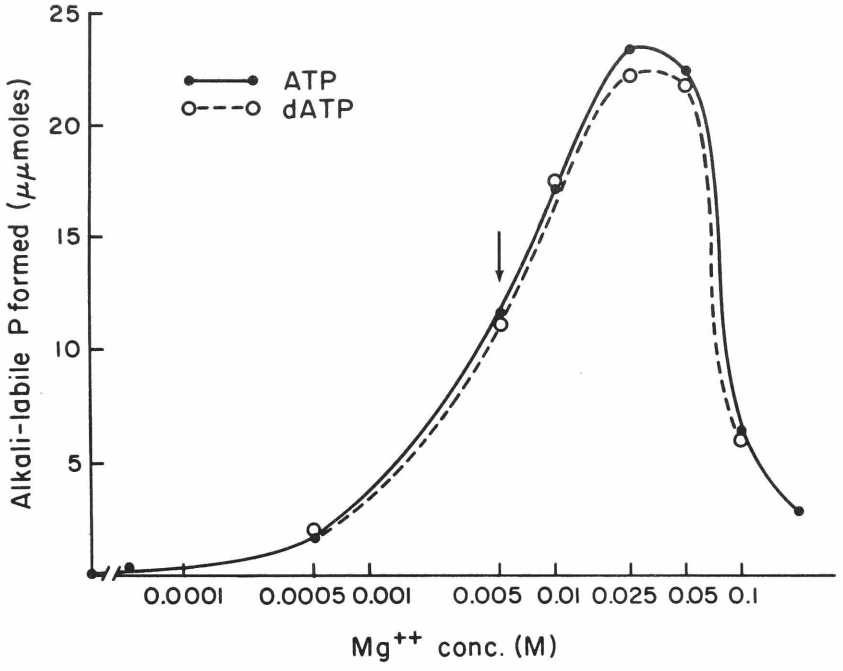


Figure 26. Effect of varying Mg^{++} concentration on the phosphorylation of nuclear phosphoprotein by ATP-γ-P^{32} and dATP-γ-P^{32}. After 10 minutes of incubation with 100 μμmoles of substrate, the incorporation of P^{32} into alkali-labile phosphate was determined. The arrow indicates the concentration of Mg^{++} (5 mM) usually employed in phosphoprotein kinase assay systems.

TABLE VIII

Effects of divalent cations on the enzymatic phosphorylation of nuclear phosphoprotein

Divalent cation tested:	No Mg^{++} 5 mM X^{++}	5 mM Mg^{++} 1 mM X^{++}
Mg^{++}	100.0	100.0
Fe^{++}	60.3	7.5
Mn^{++}	51.2	100.5
Co^{++}	36.8	77.0
Ca^{++}	0.7	115.0
Ba^{++}	1.5	103.7
Cd^{++}	3.1	20.7
Ni^{++}	1.8	23.3
Cu^{++}	0.7	9.5
Pd^{++}	3.2	3.1
Zn^{++}	2.4	2.2
Be^{++}	0.2	1.6

Nuclear phosphoprotein was incubated with ATP-γ-P^{32} for 10 minutes with varying divalent cations present. Incorporation of P^{32} into alkali-labile phosphate is expressed as percentage of value obtained when incubation was run with Mg^{++}.

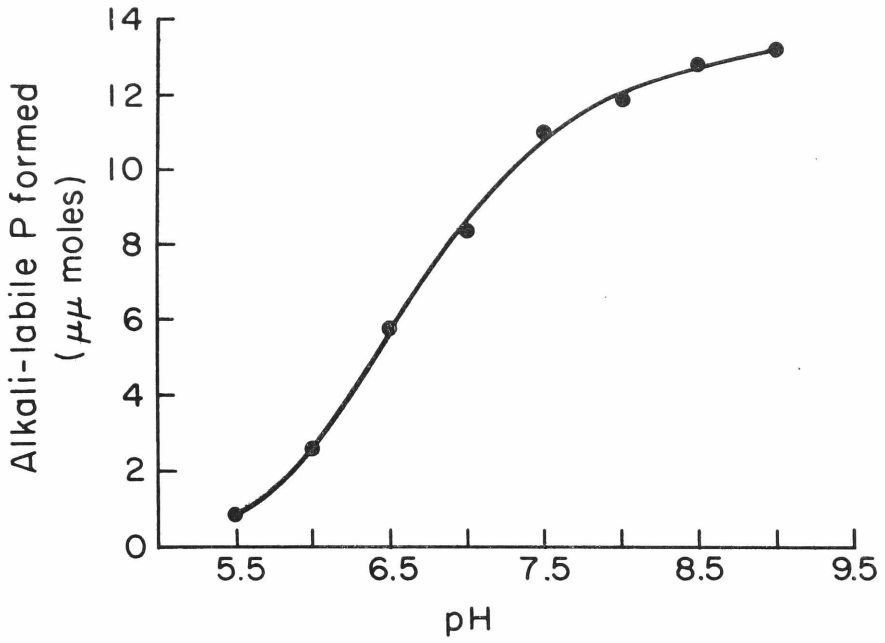


Figure 27. Effect of pH on the phosphorylation of nuclear phosphoprotein by ATP-γ-P^{32} After 10 minutes of incubation, the incorporation of P^{32} into alkali-labile phosphate was determined. Tris-maleate buffer (.04 M) was used to extend the pH range.

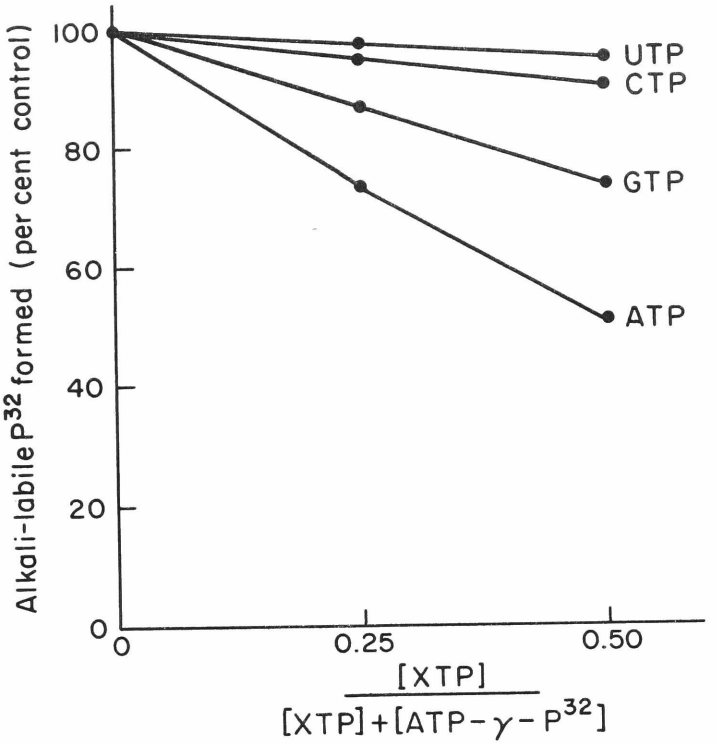


Figure 28. Effects of different unlabeled nucleoside triphosphates on the phosphorylation of nuclear phosphoprotein by ATP-γ-P^{32}. Incorporation of P^{32} into alkali-labile phosphate is plotted as a function of the percent of unlabeled nucleotide.

TABLE IX

Enzymatic phosphorylation of nuclear phosphoprotein by different substrates

Substrate	Alkali-labile P^{32} formed: (μμmoles)	(% of ATP activity)
ATP-γ-P^{32}	11.26	100.0
GTP-γ-P^{32}	6.46	57.4
ITP-γ-P^{32}	4.22	37.5
CTP-γ-P^{32}	2.42	21.5
UTP-γ-P^{32}	0.68	6.0
dATP-γ-P^{32}	11.33	100.6
ADP-β-P^{32}	0.25	2.2
P^{32}_i	0.06	0.5
PP^{32}	0.002	0.02

The purified phosphoprotein fraction was incubated for 10 minutes at 37°C in the presence of 100 μμmoles of substrate and 5 mM Mg^{++}. Incorporation of P^{32} into alkali-labile phosphate was determined as described in the text.

Of all the nucleoside triphosphates tested as possible phosphate donors, only dATP appeared to be as active as ATP in phosphorylating the phosphoprotein (Table IX). However, when the reaction was studied at varying concentrations of substrate, it was discovered that dATP is as active as ATP only at low substrate concentrations; at higher concentrations, dATP is much less effective than ATP as a phosphorylating agent (Figure 29). When the change in reaction velocity is plotted as a function of substrate concentration, it is seen that all the ribonucleoside triphosphates (ATP, GTP, ITP, CTP, UTP) follow the same curve, while the dATP exhibits a much lower rate of increase in reaction velocity with increasing substrate concentration (Figure 30). The meaning of this relationship becomes apparent when the data are replotted according to the method of Lineweaver and Burk (1934), where it is discovered that the various ribonucleoside triphosphates share a common Michaelis constant (K_m), which is considerably higher than the K_m for dATP (Figure 31).

Reversibility of the phosphorylation reaction: Since the phosphorylation of phosvitin by ATP is known to be a reversible reaction (Rabinowitz and Lipmann, 1960), experiments were performed to determine whether the phosphorylation of the nuclear phosphoprotein by ATP is also reversible. P^{32}-labeled phosphoprotein was incubated for two hours with excess ADP, after which the reaction mixture was assayed to see if any radioactive ATP had been formed. The results, summarized in Table X, indicate that less than 1% of the counts from the phosphoprotein are transferred to ADP during this time. This small amount of reversibility does not appear to be an artifact, since the reaction shows a pH optimum at pH 6.5, the same point as the optimum for the reaction in the phosvitin-ATP system. However, the amount of reversibility is less than one-tenth that observed with phosvitin.

Lack of phosphoprotein-phosphate "turnover" in vitro: Since phosphoproteins labeled with P^{32} in intact nuclei have been shown to rapidly "turn over" their phosphate groups in cold chase experiments, the question arose as to whether a similar phenomenon takes place in the isolated phosphoprotein. Purified phosphoprotein was incubated for six minutes in the presence of Mg^{++} and ATP-γ-P^{32}, and was then "chased" by the sudden addition of a thousand-fold excess of unlabeled ATP.

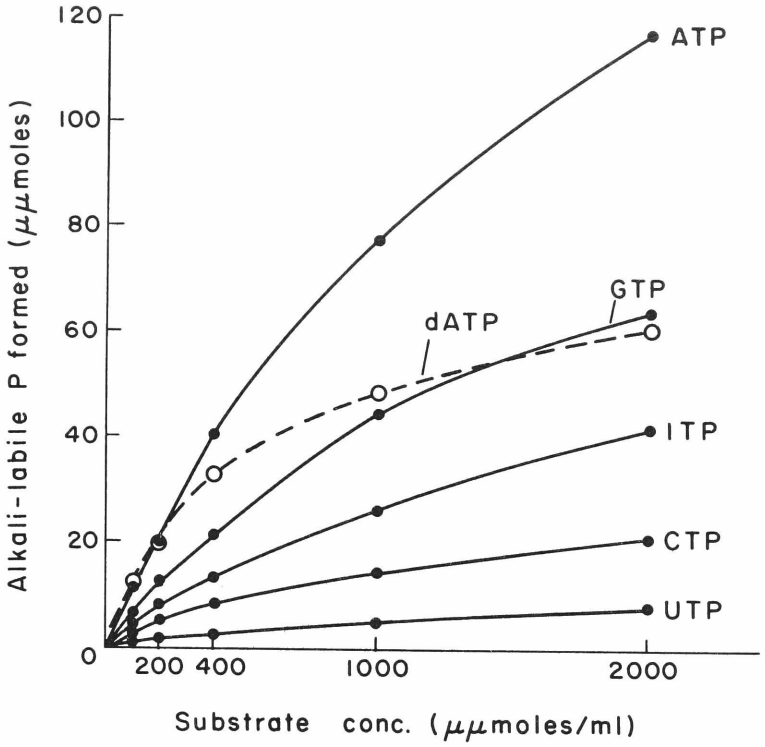


Figure 29. Rate of phosphorylation of nuclear phosphoprotein is plotted as a function of substrate concentration. All substrates were labeled in the γ-position with P^{32}. Note that dATP is as active as ATP in phosphorylating the protein only at low concentrations of substrate.

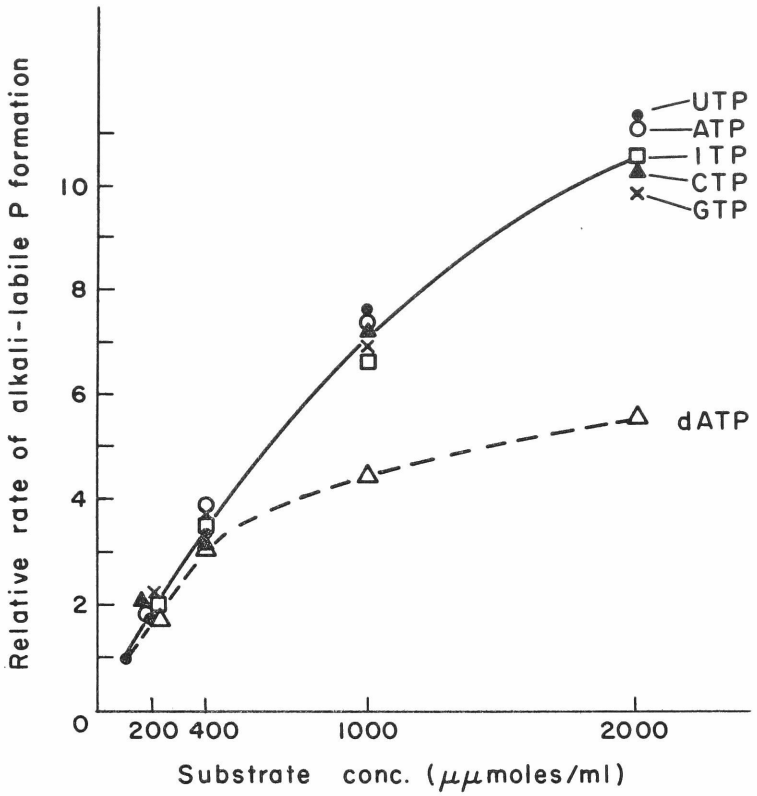


Figure 30. Data from Figure 29 are replotted by setting all the reaction rates at lowest substrate concentration equal to 1. In this way, the relative increase in reaction rates with increasing substrate concentration can be compared. Note that all the ribonucleoside triphosphates exhibit a similar increase in reaction velocity with increasing substrate concentration, and that the rate of this increase is considerably greater than that seen with dATP.

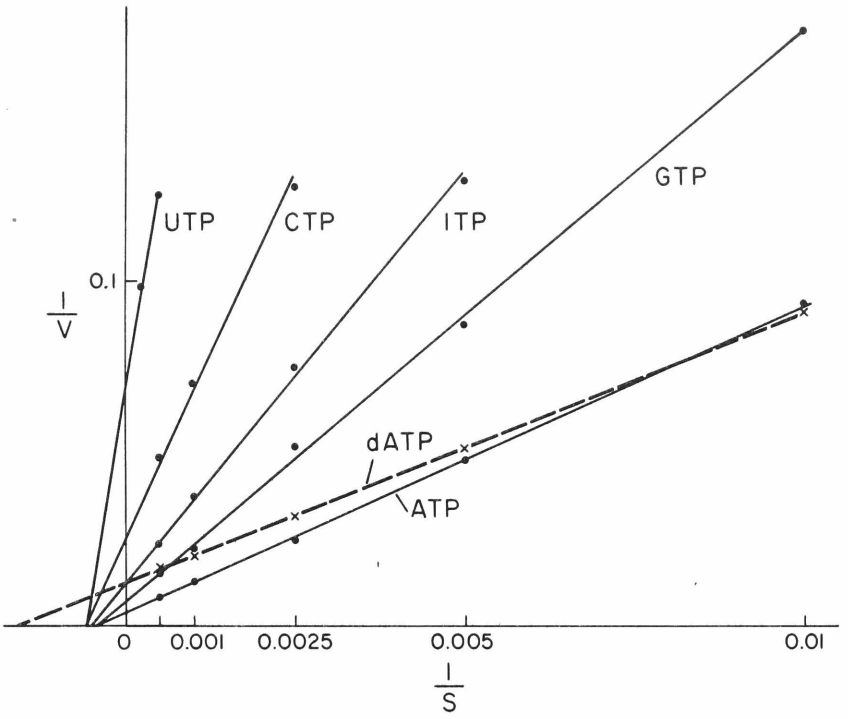


Figure 31. The data of Figure 29 are replotted according to the method of Lineweaver and Burk (1934). The intercept with the x axis equals the negative reciprocal of the Michaelis constant (K_m). Note that the ribonucleoside triphosphates share a similar K_m, which is considerably higher than that of dATP in the reaction.

TABLE X

Reversibility of reaction between ATP and nuclear phosphoprotein

pH	ATP^{32} formed (cpm)
7.5	140
7.0	202
6.5	242
6.0	162

P^{32}-labeled phosphoprotein was prepared as described in the text, and was incubated at a final concentration of 400 µg/ml (55,400 cpm/ml) with ADP (100 mµmole/ml) and $MgCl_2$ (5 µmole/ml). After two hours of incubation, formation of ATP^{32} was determined by adsorption on charcoal as described by Rabinowitz and Lipmann (1960).

The results, summarized in Figure 32, show that the phosphate groups, once incorporated into the protein, do not "turn over" under cold chase conditions but remain stably bound to the protein.

DISCUSSION

The phosphoprotein fraction purified from calf thymus nuclei is similar in many of its chemical and physical properties to the preparation obtained from rat liver nuclei by Langan (1967). It should be emphasized that these protein fractions are still heterogeneous, so that the value of 1.1-1.3% phosphorus for these proteins represents a minimal estimate. If it is assumed for the sake of discussion that this value is approximately correct, then some interesting theoretical calculations can be made (see Table V). For instance, it is found that the phosphoproteins account for 4% of the total dry weight of the thymus nucleus, and 9% of the total dry weight of the liver nucleus. Even if the value of 1.2% phosphorus is low by a factor of ten, which seems exceedingly unlikely on the basis of the properties of the phosphoprotein fraction, then the phosphoproteins would still account for 0.4% and 0.9% of the total dry weight of the thymus and liver nucleus respectively. These values are much higher than would be expected of an enzyme present in catalytic amounts, and point to the conclusion that the phosphoproteins are a major structural component of the cell nucleus.

Another interesting relationship which emerges from such theoretical calculations is the difference between liver and thymus nuclei in regard to their concentration of phosphoprotein per mg DNA. The maximal estimates for the phosphoprotein:DNA ratio are 0.49 in liver and 0.14 in thymus. Although these are only estimates based on the assumption that the phosphoprotein contains 1.2% phosphorus, the conclusion that the liver nucleus contains more than three times as much phosphoprotein per mg DNA as the thymus nucleus is independent of this assumption. This is noteworthy because it is another example of a correlation between phosphoprotein content and the capacity for RNA synthesis; liver nuclei, which are largely derived from the metabolically active parenchymal cells and are very active in RNA synthesis, contain much more phosphoprotein in association with their DNA than do calf

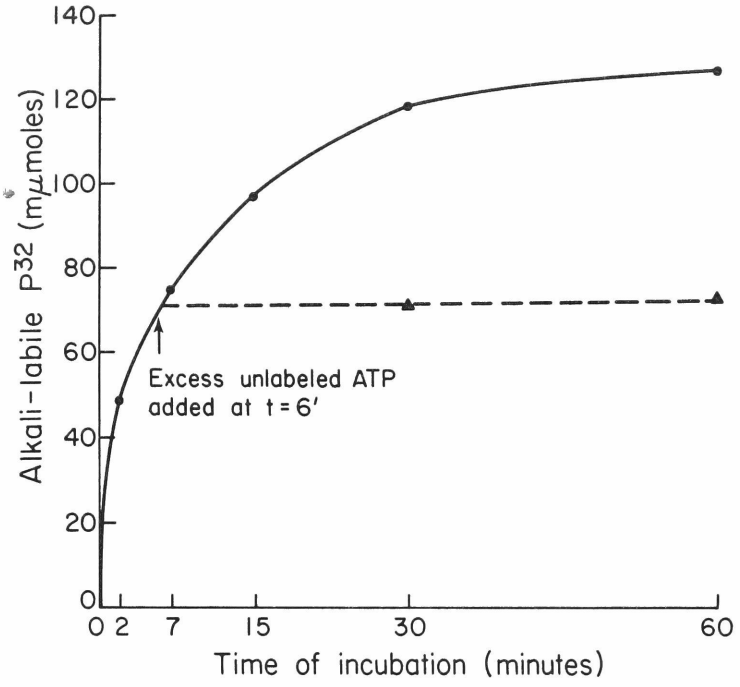


Figure 32. Retention of P^{32} by nuclear phosphoprotein during "cold chase" conditions. After six minutes of incubation with 100 μμmoles of ATP-γ-P^{32}, excess unlabeled ATP (100 μmoles) was added to several of the tubes. The dashed line shows that the P^{32} counts already incorporated into the protein in these tubes are stable to further incubation.

thymocyte nuclei, which are derived from small lymphocytes which are relatively inactive in RNA synthesis.

The similarities in amino acid composition between some histone fractions and nuclear phosphoproteins, as well as the occurrence of phosphoserine in some histone preparations, suggest the possibility that these may be related proteins. Although the phosphoserine has been obtained from highly purified histone fractions, the possibility exists that this might still represent contamination of the histone fraction with other proteins or adsorbed peptides. However, Langan and Smith (1967) have recently reported the isolation of a protein phosphokinase from rat liver which catalyzes the phosphorylation of histones by ATP, but is not active with other phosphoproteins. The existence of such an enzyme supports the conclusion that the phosphoserine found in histone fractions does not originate from contamination by other proteins.

An interesting finding in the current studies is that nucleoside triphosphates other than ATP are capable of donating their terminal phosphate groups to nuclear phosphoproteins. In order to interpret the functional significance of this finding, it will be necessary to determine whether these different precursors are phosphorylating the same or different sites in the phosphoprotein. The fact that dATP has a higher affinity (lower K_m) for the nuclear phosphoprotein-kinase system than any of the ribonucleoside triphosphates is noteworthy, and may have some bearing on the proposed role of phosphoproteins in modifying chromatin structure. The deoxyribonucleoside triphosphates, such as dATP, are normal constituents of the cell nucleus whose concentrations would be expected to change during the cell cycle, and could conceivably be affecting the structural state of the chromatin through their reactivity with the phosphoprotein system.

The failure to find a large degree of reversibility in the reaction between ATP and the nuclear phosphoprotein indicates that the phosphoryl bonds in these proteins are not of high free energy like those in phosvitin. This is further evidence against the possibility that nuclear phosphoprotein functions as either a high-energy phosphate reservoir or as a high-energy intermediate in oxidative phosphorylation.

Since the purified phosphoprotein is capable of being phosphorylated *in vitro* without the addition of any exogenous kinase, it can be concluded that this protein fraction contains its own kinase activity. It has not yet been possible to determine whether this kinase activity is an inherent part of the phosphoprotein, or whether it merely represents contamination of the phosphoprotein fraction with some phosphoprotein kinase. It has been possible to determine, however, that the enzyme activity responsible for the "turnover" of phosphate groups from the protein is not an inherent part of the phosphoprotein, since the rapid loss of P^{32} from nuclear proteins seen in intact nuclei does not occur in the isolated system.

One of the major categories of phosphoprotein function which has not yet been ruled out in regard to the nuclear phosphoproteins is involvement in the enzymatic transfer of phosphate groups. From the current studies it is known that the phosphoprotein-phosphate group originates in the terminal phosphate of various nucleoside and deoxynucleoside triphosphates. It is also known that these phosphoprotein-phosphate groups are rapidly removed from the protein in intact nuclei, but their metabolic fate is unknown. In order to determine whether we are dealing with a phosphoryl group transfer reaction, we must know what happens to these phosphoryl groups which are being continually "turned over". Experiments designed to answer this question will be described in the next chapter.

SUMMARY

A phosphoprotein fraction has been isolated from calf thymus nuclei which contains about 1.3% alkali-labile phosphorus by weight. The enzymatic reaction in which serine and threonine residues in the protein are phosphorylated by the terminal phosphate of ATP (and other nucleoside triphosphates) has been extensively studied *in vitro*. The purified protein fraction contains endogenous kinase activity, so that protein phosphorylation proceeds without the need for any added enzyme. Radioactive phosphate groups incorporated in this reaction are stable to incubation in a medium containing an excess of unlabeled ATP, indicating that the enzyme activity responsible for the rapid "turnover" of protein-phosphate groups seen in intact nuclei is not an

inherent part of the phosphoprotein. The reaction between ATP and the nuclear phosphoprotein is only slightly reversible, making it appear unlikely that this phosphoprotein functions as a high-energy phosphate reservoir or as a high-energy intermediate in oxidative phosphorylation.

CHAPTER FIVE: METABOLISM OF EXOGENOUS PHOSPHOPROTEIN BY INTACT NUCLEI

In view of the data available at this point, the nuclear phosphoprotein could fit into one of two possible general functional categories. On one hand, it might be serving as an intermediate in some type of phosphoryl group transfer reaction, in which case the phosphate groups would be the primary focus of interest. On the other hand, the addition and removal of phosphate groups might serve the sole function of altering the structural and functional characteristics of the protein moiety itself. In this case, changes in the protein would be the main focus of interest, with the phosphate groups merely serving as agents which modify the protein.

In order to distinguish between these two possibilities, it is necessary to determine the metabolic origin and fate of the phosphate groups. It is already known that the phosphoprotein-phosphate groups are derived from the terminal phosphate of various nucleoside and deoxynucleoside triphosphates, but it is not known to what substrate, if any, these phosphoprotein-phosphate groups are transferred during phosphate "turnover". This question could not be answered by studying the "turnover" of P^{32}-phosphoprotein which had been labeled in the intact nucleus, because there is no known way

of specifically labeling the phosphoprotein with P^{32} without also simultaneously labeling the nucleic acids, phospholipids, and low molecular weight phosphate compounds. However, by employing the purified phosphoprotein fraction which had been previously labeled with P^{32} and then adding it to fresh unlabeled nuclei, it was possible to specifically follow the subsequent metabolism of the phosphoprotein-phosphate groups.

In the present chapter, it will be shown that when P^{32}-labeled phosphoprotein is prepared and added back to fresh thymus nuclei, the metabolic "turnover" of the protein phosphate groups is reestablished. The P^{32} which is "turned over" from this labeled phosphoprotein is not transferred to any other stable molecule, but rather appears entirely in the form of inorganic phosphate.

METHODS

Preparation of radioactive phosphoprotein: Thymus nuclei were prepared from 100 grams fresh calf thymus according to the procedure of Allfrey et al. (1957), and were incubated for 30 minutes as described in Chapter Two. Isotopic precursors employed were either $Na_2HP^{32}O_4$ (100 mc/mmole; 0.1 mc/ml final concentration) or 3-H^3-serine (4.2 c/mmole; 0.1 mc/ml final concentration). After incubation the nuclei were chilled and collected via centrifugation at 1000 g for five minutes, and the phosphoprotein fraction then prepared as described in Chapter Four. The final preparations were diluted to contain 200 μg/ml phosphoprotein (H^3-serine-labeled phosphoprotein = 10,000 cpm/ml; P^{32}-labeled phosphoprotein = 120,000 cpm/ml).

Incubation of nuclei with radioactive phosphoprotein: To every 9 ml of phosphoprotein solution was added 1 ml of 1.25 M sucrose containing 40 mg/ml NaCl and 90 mg/ml glucose. Fresh thymus nuclei were prepared as described above and 1 ml of nuclei in 0.25 M sucrose - 3 mM $CaCl_2$ added to 1 ml of the phosphoprotein mixture. Incubation was carried out at 37°C in a shaking water-bath, and the reaction stopped with cold 16% TCA. Alkali-labile P^{32} was measured as described in Chapter Two. H^3-serine-labeled protein was dissolved in 0.3 N NaOH and counted in Bray's scintillation solution.

Identification of P^{32}-labeled nuclear components: Low molecular weight phosphate compounds were fractionated on a column of G-15 Sephadex (1.5 cm x 83 cm) and eluted with 0.14 M NaCl. Nuclei were incubated for one hour with P^{32}-labeled phosphoprotein, after which the reaction was stopped with cold 16% TCA. The precipitate was removed by centrifugation and the TCA extract neutralized with NaOH. An aliquot was placed on a G-15 Sephadex column which had been calibrated with markers of ATP, glucose-6-phosphate, pyrophosphate, and inorganic phosphate. Total phosphate in the fractions was determined by the procedure of Ames and Dubin (1960).

RESULTS

"Turnover" of exogenous phosphoprotein phosphate groups by intact nuclei: When purified radioactive phosphoprotein is added to suspensions of freshly prepared calf thymus nuclei, the phosphoprotein is readily taken up by the nuclei. The kinetics of uptake of H^3-serine-labeled phosphoprotein are summarized in Figure 33. Maximum labeling of the nuclei occurs within five minutes, after which the level of radioactive phosphoprotein reaches a steady plateau.

Since the radioactive phosphoprotein was found to be taken up by the nuclei, the next question which arose was whether the phosphate groups of this exogenous phosphoprotein are "turned over" under these conditions. When P^{32}-labeled phosphoprotein was prepared and added back to fresh unlabeled nuclei, it was found that a rapid loss of the P^{32} counts from the phosphoprotein occurred (Figure 34). The rate of this P^{32} "turnover" is of the same order of magnitude as that seen when the phosphoprotein is labeled and subjected to a cold chase directly in the intact nuclei (Chapter Two). Furthermore, during the rapid "turnover" of the phosphate groups from the exogenous phosphoprotein, H^3-serine counts in the phosphoprotein were found to be completely stable (Figure 34). This indicates that the rapid loss of P^{32} counts from the phosphoprotein is not due to proteolysis and release of phosphorylated peptides.

When ATP synthesis is blocked in these nuclei by the addition

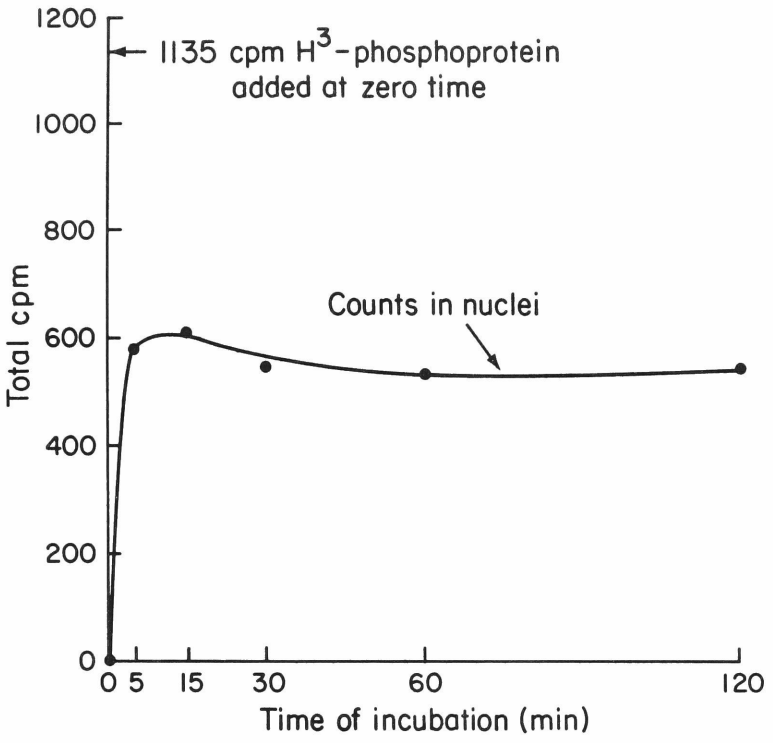


Figure 33. Time course of uptake of H^3-serine-labeled phosphoprotein by isolated calf thymus nuclei. After incubation with the radioactive phosphoprotein, nuclei were chilled, sedimented, and washed 5 times with cold incubation medium. The sediment was precipitated with 16% TCA and washed as described in Chapter Two. The dried protein residue was dissolved in 0.3 N NaOH and counted in Bray's scintillation solution.

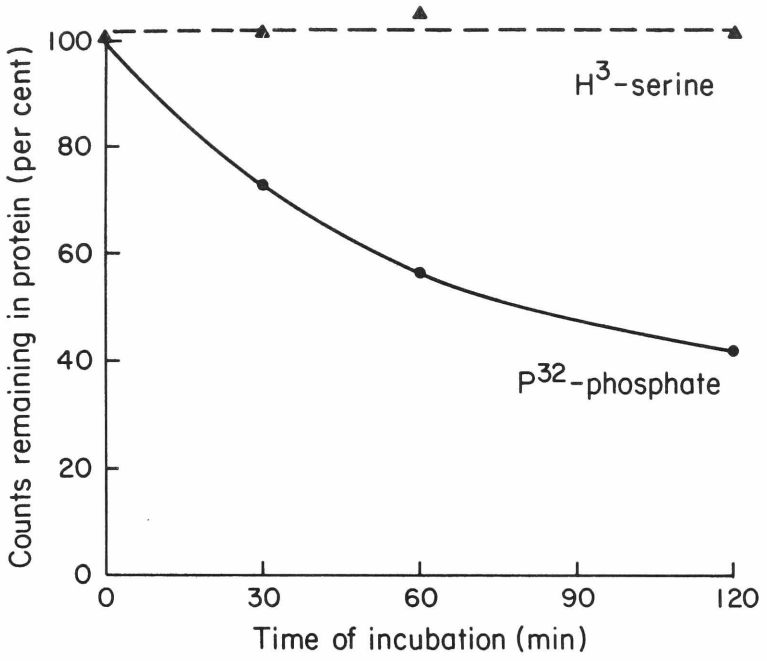


Figure 34. Metabolic "turnover" of phosphate groups from P^{32}-labeled phosphoprotein which has been added to suspensions of isolated calf thymus nuclei. Nuclei were incubated at 37°C in the presence of phosphoprotein labeled with either H^3-serine or P^{32}-phosphate. The retention of label is plotted as a function of time of incubation. Note that H^3-serine is retained while P^{32}-phosphate is rapidly lost.

of iodoacetate, the "turnover" of these phosphoprotein-phosphate groups is inhibited (Figure 35). This dependence of the "turnover" reaction on energy metabolism is similar to that observed when phosphoproteins are labeled directly in the intact nucleus (Chapter Two; Figure 11), although the degree of inhibition by iodoacetate is considerably less in the present case. In both systems the inhibition caused by iodoacetate is reversible by the addition of pyruvate, which bypasses the block in glycolysis and reestablishes ATP formation via its direct metabolism in the Kreb's cycle (McEwen et al., 1963b).

Metabolic fate of phosphoprotein phosphate: Since the "turnover" of P^{32} is reestablished when radioactive phosphoprotein is added back to fresh nuclei, it was possible to determine the fate of these phosphoprotein-phosphate groups by simply examining the various nuclear fractions for the appearance of P^{32} counts. Of all the fractions examined, the only one in which counts were found to appear was in a cold TCA extract of the nuclei (Figure 36).

Since this acid soluble fraction contains most of the low molecular weight phosphate compounds of the nucleus, a G-15 Sephadex column was employed to fractionate the components of the extract. Nuclei were first incubated for one hour in the presence of P^{32}-labeled phosphoprotein in order to allow "turnover" of the phosphate groups to occur. The nuclei were then extracted with cold TCA and the extract fractionated by gel filtration on Sephadex G-15. The results, summarized in Figure 37, indicate that all the P^{32} counts are present in the form of inorganic phosphate.

DISCUSSION

The present experiments indicate that during the "turnover" of phosphoprotein-phosphate groups, the end product which is detectable is inorganic phosphate. Although it is conceivable that some highly unstable phosphate linkage is formed as an intermediate which would be subsequently destroyed during TCA extraction, this alternative seems unlikely in view of the fact that even the N-P bond, which is very labile in acid solution, would not be completely hydrolyzed under these conditions (Morrison and Ennor, 1960). The fact that the

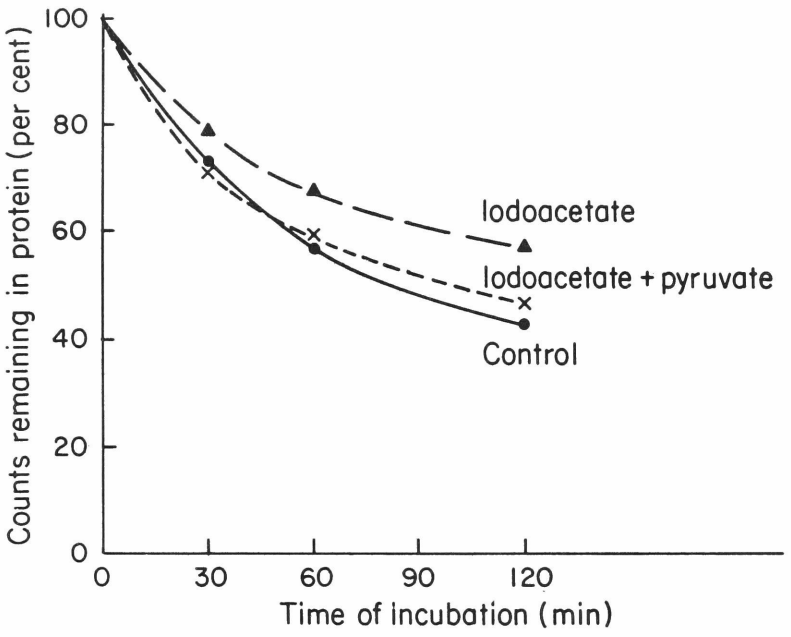


Figure 35. Effect of iodoacetate (10^{-3} M) on "turnover" of P^{32}-phosphate groups from phosphoprotein which has been added back to calf thymus nuclei. Note that iodoacetate causes an inhibition of the "turnover" reaction which is reversible by the addition of pyruvate (.02 M).

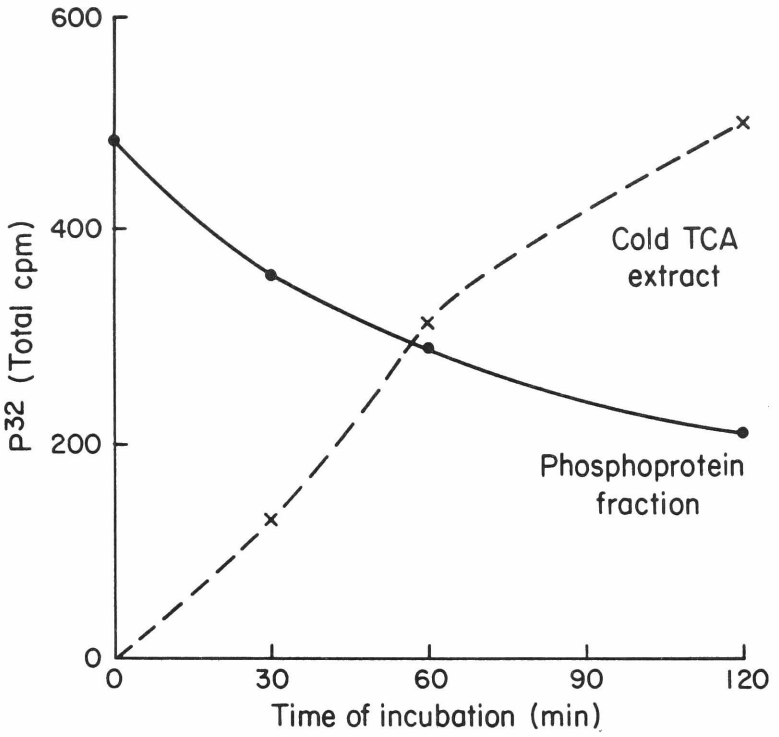


Figure 36. When P^{32}-labeled phosphoprotein is added back to a fresh nuclear suspension, the loss of P^{32} counts from the phosphoprotein fraction coincides with the appearance of counts in a cold TCA extract of the nuclei. The total number of counts in each fraction is plotted as a function of time of incubation.

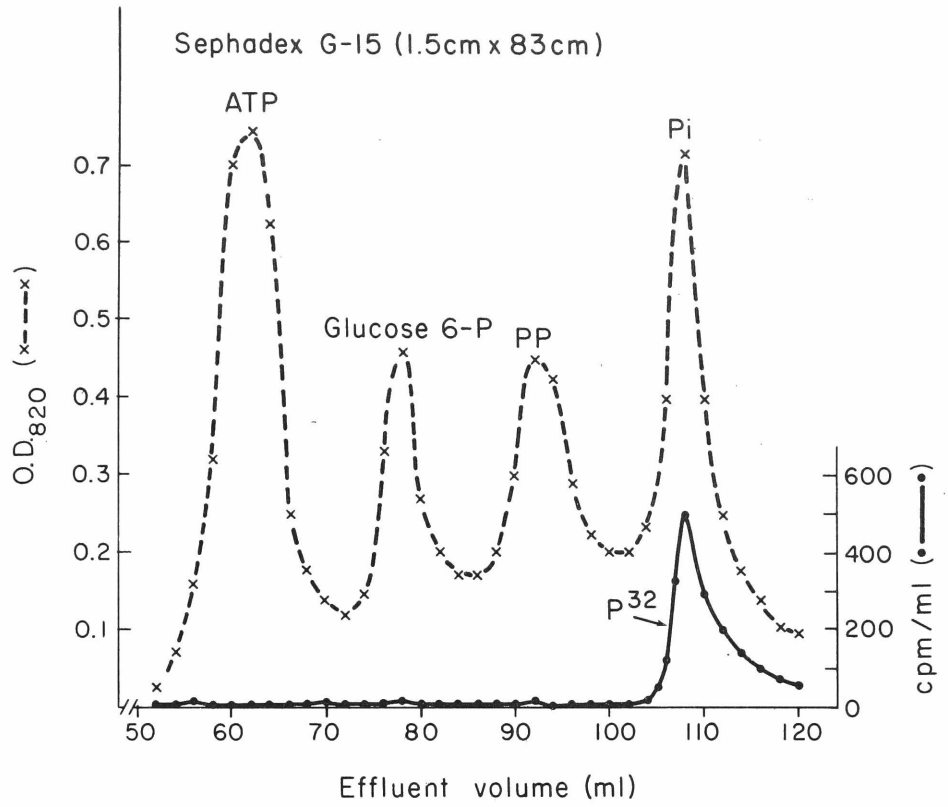


Figure 37. The cold TCA extract from the experiments summarized in Figure 36 was analyzed on a column of Sephadex G-15 which had been calibrated with markers of ATP, glucose-6-phosphate, pyrophosphate, and inorganic orthophosphate. Note that all the P^{32} counts appear in the inorganic phosphate peak.

protein-phosphate bonds do not appear to be of very high free energy (Chapter Four) also argues against the formation of a highly unstable product.

Since the P^{32} is apparently released from the phosphoprotein as inorganic phosphate, it does not appear that this phosphoprotein fraction is involved in the metabolic transfer of phosphoryl groups to other acceptors. Thus the net effect of the metabolic reactions in which these phosphoproteins are involved is that the free energy stored in the terminal phosphate bonds of nucleoside triphosphates is used in the process of phosphorylating the protein; this energy is apparently not recovered in the second step of the reaction in which the phosphate group is released in the relatively low energy state of inorganic phosphate. One possible interpretation of this phenomenon is that the phosphorylation of the protein causes structural and functional changes in the state of the phosphoprotein, and that the energy stored in the terminal pyrophosphate bonds of nucleoside triphosphates is utilized to induce these changes. An example of such a phenomenon is known to occur in the case of phosphorylase and phosphorylase kinase, where the function of the phosphorylation reaction is to induce structural changes leading to the activation of enzyme activity (Krebs et al., 1966). In the case of the nuclear phosphoproteins, the effect of the structrual changes induced by protein phosphorylation might be to modify the structure of the chromatin and the physical interaction between histones and DNA.

SUMMARY

When P^{32}-labeled phosphoprotein is added to a suspension of calf thymus nuclei, the protein enters the nuclei and a rapid metabolic "turnover" of the previously incorporated phosphate groups occurs. The only P^{32}-labeled product which can be detected in these nuclei is inorganic phosphate. The results indicate that the phosphoprotein is probably not involved in the metabolic transfer of phosphate groups from one molecule to another, but favors the hypothesis that the phosphorylation and dephosphorylation of the nuclear phosphoproteins serve the purpose of modifying the structural and functional characteristics of the protein.

CHAPTER SIX: CONCLUDING REMARKS

The overall relationships of the metabolic reactions in which the nuclear phosphoproteins are involved are summarized in the model proposed in Figure 38. Two separate enzymatic reactions appear to be involved, one for the phosphorylation of the protein, and one for its dephosphorylation. In the former reaction, the terminal phosphate group of a nucleoside triphosphate is donated to the phosprotein in a Mg^{++}-dependent kinase reaction. In the second reaction, protein-bound phosphate groups are cleaved from the phosphoprotein as inorganic phosphate in a "phosphatase"-type reaction. In concluding our consideration of these proteins, let us briefly review the implications of this model for the possible biological functions which have been suggested for the nuclear phosphoproteins:

1) There are several reasons for believing that the nuclear phosphoprotein does not function as a high-energy reservoir of phosphate groups. For one, the phosphoprotein-phosphate groups "turn over" very rapidly in the intact nucleus, with up to an 80% loss of previously incorporated P^{32} occurring during a two-hour cold chase. Thus a long-term storage of phosphate groups does not occur. Furthermore, the relatively

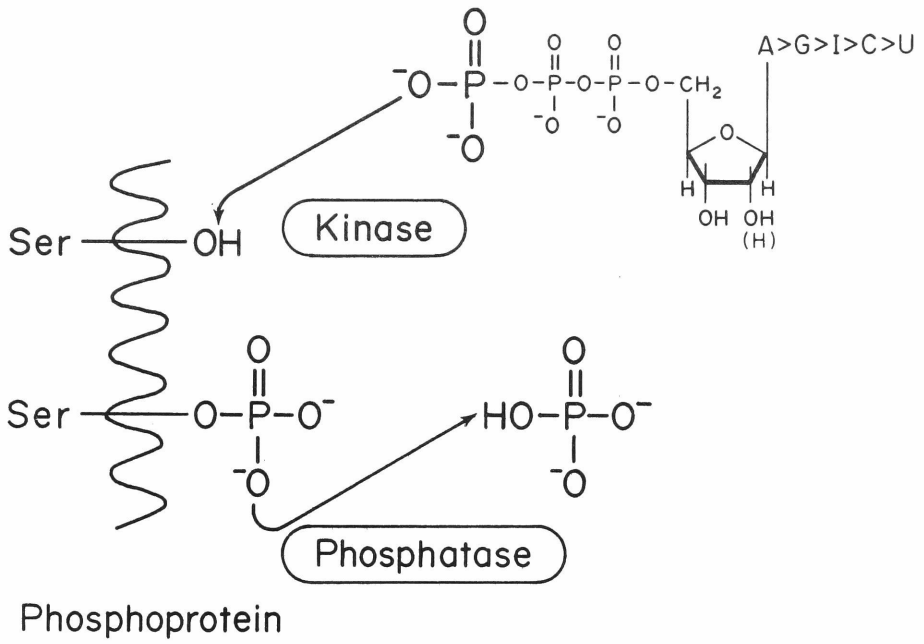


Figure 38. Model summarizing the metabolic relationships of the nuclear phosphoproteins. Serine (and threonine) residues in the protein are phosphorylated in a kinase reaction utilizing the terminal phosphate group of various nucleoside and deoxynucleoside triphosphates. In a separate enzymatic reaction, phosphate groups are cleaved from the protein and released in the form of inorganic phosphate.

low phosphoryl-transfer potential of the protein phosphate groups indicates that we are not dealing with a high-energy phosphate bond like that which occurs in phosvitin.

2) Another possible function which has been considered is that the nuclear phosphoproteins are involved in the metabolic transfer of phosphate groups from one substrate to another. However, the finding that the protein-bound phosphate groups are released in the form of inorganic phosphate makes this type of transfer unlikely.

3) The possible involvement of nuclear phosphoproteins in Na^+-K^+-dependent "active transport" reactions seems unlikely in view of the failure to detect any sign of a Na^+ or K^+ requirement for either the phosphorylation or dephosphorylation reaction.

4) The phosphate group does not seem to be acting as a covalent link between the protein and another molecule, as occurs in the acyl carrier protein, because the release of inorganic phosphate after alkali treatment rules against the existence of this type of phosphodiester linkage.

5) The possibility that we are dealing with a high-energy intermediate in oxidative phosphorylation has been considered, but this seems unlikely for several reasons. First, it was found that the phosphoprotein-phosphate groups are not readily transferred to ADP, indicating the relatively low phosphoryl potential of these phosphate bonds. In addition, nuclei which have been starved for ATP by adding iodoacetate to block glycolysis show a slower release of phosphoprotein phosphate, an observation which would not be expected if we were dealing with a high-energy intermediate which functions in ATP-formation.

6) One remaining possible functional category which has not yet been ruled out is that the incorporation of the phosphate groups functions to modify protein structure

and function. If the nuclear phosproproteins do fit in this category and the function of protein phosphorylation is to modify the structure of the nuclear phosphoproteins, then this might provide a mechanism by which the phosphoproteins can affect the structure and metabolism of the chromatin. The hypothesis that the nuclear phosphoproteins are involved in the modification of chromatin activity has been referred to several times already, but it should be emphasized at this point that the only evidence which supports this theory is of a correlational nature. Among the various positive correlations which have been observed between the occurrence of phosphoproteins and the capacity of the chromatin to synthesize RNA are the following: a) phosphoproteins are preferentially localized in chromatin fractions which are most active in RNA synthesis (Langan, 1965); b) phosphoproteins inhibit the ability of histones to depress RNA polymerase activity in vitro (Langan, 1967); c) phosphorylation of nuclear proteins increases during gene activation in lymphocytes (Chapter Three); d) phosphorylation of protamines decreases with decreased gene activity during spermatogenesis (Ingles and Dixon, 1967); e) histone phosphorylation increases during liver regeneration (Ord and Stocken, 1967); and f) the liver cell nucleus, which is more active in RNA synthesis than the thymocyte nucleus, contains more than three time as much phosphoprotein per mg DNA (Chapter Four). Although there is no direct positive proof for the hypothesis that phosphoproteins are involved in the modification of chromatin structure and the regulation of its metabolic activity, this possibility has received serious attention because of the fact that the other possible functions which have been considered for the nuclear phosphoproteins all have strong arguments against them. However, the possibility should not be overlooked that the nuclear phosphoproteins belong to a new functional

category other than any of those previously described.

The discovery that phosphorylated proteins are localized and actively metabolized in cell nuclei has opened a new and important area for investigation to those interested in the problems of metabolism and regulation in the cell nucleus. In view of their heavy concentration in nuclei, it seems likely that these nuclear phosphoproteins will be found to play an important functional role. Although promising results have been obtained in early investigations in this area, much additional work will be needed before an understanding of the specific nature of their function(s) will be achieved.

REFERENCES

Ågren, G. (1959). Acta Soc. Med. Upsalien. 64, 379.

Ågren, G., de Verdier, C.-H., and Glomset, J. (1954). Acta Chem. Scand. 8, 503

Ågren, G., de Verdier, C.-H., and Glomset, J. (1955). Acta Chem. Scand. 9, 196

Ågren, G., and Engström, L. (1956). Acta Chem. Scand. 10, 489.

Ågren, G., Zetterqvist, Ö., and Ojamae, M. (1959). Acta Chem. Scand. 13, 1047.

Ahmed, K., and Judah, J. D. (1962). Biochim. Biophys. Acta 57, 245.

Ahmed, K., and Judah, J. D. (1963). Biochim. Biophys. Acta 71, 295.

Ahmed, K., Judah, J. D., and Wallgren, H. (1963). Biochim. Biophys. Acta 69, 428.

Akerfeldt, S. (1963). Svensk Kem. Tidskr. 75, 231.

Albers, R. W., Fahn, S., and Koval, G. J. (1963). Proc. Natl. Acad. Sci. U. S. 50, 474.

Albert, S., Johnson, R. M., and Cohan, M. S. (1951). Cancer Res. 11, 772.

Aldridge, W. N., Barman, T. E., and Gutfreund, H. (1964). Biochem. J. 92, 23C.

Allerton, S. E., and Perlmann, G. E. (1965). J. Biol. Chem. 240, 3892.

Allfrey, V. G. (1964). Can. Cancer Conf. 6, 313.

Allfrey, V. G. (1965). Personal communication.

Allfrey, V. G., Faulkner, R., and Mirsky, A. E. (1964a). Proc. Natl. Acad. Sci. U. S. 51, 786.

Allfrey, V. G., Littau, V. C., and Mirsky, A. E. (1964b). J. Cell Biol. 21, 213.

Allfrey, V. G., Meudt, R., Hopkins, J. W., and Mirsky, A. E. (1961). Proc. Natl. Acad. Sci. U. S. 47, 907.

Allfrey, V. G., Mirsky, A. E., and Osawa, S. (1957). J. Gen. Physiol. 40, 451.

Ames, B. N., and Dubin, D. T. (1960). J. Biol. Chem. 235, 769.

Anderson, L., and Jolles, G. R. (1957). Arch. Biochem. Biophys. 70, 121.

Anderson, L., and Kelley, J. J. (1959). J. Am. Chem. Soc. 81, 2275.

Aschaffenburg, R. (1961). Nature 192, 431.

Aschaffenburg, R. (1963). J. Dairy Res. 30, 251.

Bach, F., and Hirschhorn, K. (1963). Exptl. Cell Res. 32, 592.

Bader, H., Sen, A. K., and Post, R. L. (1966). Biochim. Biophys. Acta 118, 106.

Barman, T. E., Bai, N.-K., and Thoai, N.-V. (1964). Biochem. J. 90, 555.

Barman, T. E., and Gutfreund, H. (1966). Biochem. J. 101, 460.

Barr, G. C., and Butler, J. A. V. (1963). Nature 199, 1170.

Bechhold, H. (1901). Z. Physiol. Chem. 34, 122.

Belec, J., and Jenness, R. (1962). Biochim. Biophys. Acta 63, 512.

Berenblum, I., and Chain, E. (1938). Biochem. J. 32, 295.

Bettelheim, F. R. (1954). J. Am. Chem. Soc. 76, 2838.

Beyer, R. E. (1964a). Biochem. Biophys. Res. Commun. 17, 184.

Beyer, R. E. (1964b). Biochem. Biophys. Res. Commun. 17, 764.

Blombäck, B., Blombäck, M., Edman, P., and Hessel, B. (1962). Nature 193, 883.

Blombäck, B., Blombäck, M., Edman, P., and Hessel, B. (1966). Biochim. Biophys. Acta 115, 371.

Blombäck, B., Blombäck, M., and Searle, J. (1963). Biochim. Biophys. Acta 74, 148.

Blombäck, B., Boström, H., and Vestermark, A. (1960). Biochim. Biophys. Acta 38, 502.

Blombäck, B., and Doolittle, R. F. (1963). Acta Chem. Scand. 17, 1819.

Boyer, P. D. (1963). Science 141, 1147.

Boyer, P. D., DeLuca, M., Ebner, K. E., Hultquist, D. E., and Peter, J. B. (1962). J. Biol. Chem. 237, PC3306.

Braconnot, H. (1830). Ann. Chim. Phys. 43, 337.

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

Busch, H. (1965). "Histones and Other Nuclear Proteins," Academic Press, New York.

Cann, J. R. (1949). J. Am. Chem. Soc. 71, 907.

Carlsen, E. N., Trelle, G. J., and Schjeide, O. A. (1964). Nature 202, 984.

Charnock, J. S., Opit, L. J., and Potter, H. A. (1967). Biochem. J. 104, 17C.

Charnock, J. S., and Post, R. L. (1963). Nature 199, 910.

Charnock. J. S., Rosenthal. A. L., and Post, R. L. (1963). Australian J. Exptl. Biol. Med. Sci. 41, 675.

Chen, P. S., Jr., Toribara, T. Y., and Warner, H. (1956). Anal. Chem. 28, 1756.

Colomb, M. G., Laturaze, J. G., and Vignais, P. V. (1966). Biochem. Biophys. Res. Commun. 24, 909.

Connelly, C., and Taborsky, G. (1961). J. Biol. Chem. 236, 1364.

Cori, C. F., and Illingworth, B. (1957). Proc. Natl. Acad. Sci. U. S. 43, 547.

Cori, G. T. (1945). J. Biol. Chem. 158, 333.

Cori, G. T., Colowick, S. P., and Cori, C. F. (1938). J. Biol. Chem. 124, 543.

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

Cori, G. T., and Green, A. A. (1943). J. Biol. Chem. 151, 31.

Crampton, C. F., Lipschitz, R., and Chargaff, E. (1954). J. Biol. Chem. 206, 449.

Crane, R. K., and Lipmann, F. (1953). J. Biol. Chem. 201, 235.

Davidson, J. N., Frazer, S. C., and Hutchison, W. C. (1951). Biochem. J. 49, 311.

DeLuca, M., Ebner, K. E., Hultquist, D. E., Kreil, G., Peter, J. B., Moyer, R. W., and Boyer, P. D. (1963). Biochem. Z. 338, 512.

de Verdier, C.-H. (1953). Acta Chem. Scand. 7, 196.

de Verdier, C.-H. (1954). Acta Chem. Scand. 8, 1302.

Dixon, G. H., Kauffman, D. L., and Neurath, H. (1958). J. Biol. Chem. 233, 1373.

Dumas, J. B., and Cahours, C. (1842). Compt. Rend. 15, 976.

Engström, L. (1961a). Biochim. Biophys. Acta 52, 49.

Engström, L. (1961b). Biochim. Biophys. Acta 54, 179.

Engström. L. (1962). Biochim. Biophys. Acta 56, 606.

Engström. L. (1963). Arkiv Kemi 19, 129.

Engström, L., and Ågren, G. (1958). Acta Chem. Scand. 12, 357.

Ernster, L., Zetterstrom, R., and Lindberg, O. (1950). Acta Chem. Scand. 4, 942.

Fernley, H. N., and Walker, P. G. (1966). Nature 212, 1435.

Fernley, H. N., and Walker, P. G. (1967). Biochem. J. 102, 48P.

Feuer, G., and Wollemann, M. (1955). Acta Physiol. Acad. Sci. Hung. 7, 343.

Fevold, H. L. (1951). Advan. Protein Chem. 6, 187.

Fife, W. K. (1967). Biochem. Biophys. Res. Commun. 28, 309.

Fischer, E. H., Graves, D. G., Crittenden, E. R. S., and Krebs, E. G. (1959). J. Biol. Chem. 234, 1698.

Flavin, M. (1954) J. Biol. Chem. 210, 771.

Fleck, A., and Munro, H. N. (1963). Biochem. Biophys. Acta 55, 571.

Flickinger, R. A., and Rounds, D. E. (1956). Biochim. Biophys. Acta 22, 38.

Foote, M., and Kind, C. A. (1953). Arch. Biochem. Biophys. 46, 254.

Frenster, J. F., Allfrey, V. G., and Mirsky, A. E. (1963). Proc. Natl. Acad. Sci. U. S. 50, 1026.

Friedkin, M., and Lehninger, A. L. (1949). J. Biol. Chem. 177, 775.

Gibbs, R., Roddy, P. M., and Titus, E. (1965). J. Biol. Chem. 240, 2181.

Gladner, J. A., and Laki, K. (1958). J. Am. Chem. Soc. 80, 1263.

Glimcher, M. J., Francois, C. J., Richards, L., and Krane, S. M. (1964). Biochim. Biophys. Acta 93, 585.

Glimcher, M. J., and Krane, S. M. (1964a). Biochemistry 3, 195.

Glimcher, M. J., and Krane, S. M. (1964b). Biochim. Biophys. Acta 90, 477

Goldman, P., Alberts, A. W., and Vagelos, P. R. (1963). J. Biol. Chem. 238, 3579.

Gordan, A. H. (1948). Nature 162, 778.

Gordon, W. G., Semmett, W. F., and Bender, M. (1950). J. Am. Chem. Soc. 72, 4282.

Gordon, W. G., Semmett, W. F., and Bender, M. (1953). J. Am. Chem. Soc. 75, 1678.

Gordon, W. G., Semmett, W. F., Cable, R. S., and Morris, M. (1949). J. Am. Chem. Soc. 71, 3293.

Gounaris, A. D., Horton, H. R., and Koshland, D. E., Jr. (1967). Biochim. Biophys. Acta 132, 41.

Grant, C. T., and Taborsky, G. (1966). Biochemistry 5, 544.

Graves, D. J., Fischer, E. H., and Krebs, E. G. (1960). J. Biol. Chem. 235, 805.

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

Green, D. E. (1952). Science 115, 661.

Greenberg, H., and Nachmansohn, D. (1965). J. Biol. Chem. 240, 1639

Greengard, O., Sentenac, A., and Mendelsohn, N. (1964). Biochim. Biophys. Acta 90, 406.

Güntelberg, A. V., and Ottesen, M. (1954). Compt. Rend. Trav. Lab. Carlsberg, Ser. Chim. 29, 36.

Gutierrez, R. M., and Hnilica, L. S. (1967). Science 157, 1324.

Hammarsten, O. (1883). Z. Physiol. Chem. 7, 227.

Hammarsten, O. (1885). Z. Physiol. Chem. 9, 273.

Harris, D. L. (1946). J. Biol. Chem. 165, 541.

Harris, D. L., Mizock, B. J., and Pilkis, S. J. (1966). J. Biol. Chem. 241, 707.

Harshman, S., and Najjar, V. A. (1962). Federation Proc. 21, 233.

Harshman, S., and Najjar, V. A. (1965). Biochemistry 4, 2526.

Hass, L. F., Boyer, P. D., and Reynard, A. M. (1961). J. Biol. Chem. 236, 2284.

Heald, P. J. (1957). Biochem. J. 66, 659.

Heald, P. J. (1958). Biochem. J. 68, 580.

Heald, P. J. (1962). Nature 193, 451.

Heald, P. J., and McLachlan, P. M. (1963). Biochem. J. 87, 571.

Heald, P. J., and McLachlan, P. M. (1964). Biochem. J. 92, 51.

Hems, D. A., and Rodnight, R. (1966). Biochem. J. 101, 516.

Herriott, R. M. (1938). J. Gen. Physiol. 21, 501.

Hindley, J. (1963). Biochem. Biophys. Res. Commun. 12, 175.

Hipp, N. J., Groves, M. L., Custer, J. H., and McMeekin, T. L. (1950). J. Am. Chem. Soc. 72, 4928.

Hipp, N. J., Groves, M. L., Custer, J. H., and McMeekin, T. L. (1952). J. Dairy Sci. 35, 272.

Ho, C., and Kurland, R. J. (1966). J. Biol. Chem. 241, 3002.

Hofman, T. (1958). Biochem. J. 69, 139.

Hokin, L. E., Sastry, P. S., Galsworthy, P. R., and Yoda, A. (1965). Proc. Natl. Acad. Sci. U. S. 54, 177.

Hultquist, D. E., Moyer, R. W., and Boyer, P. D. (1966). Biochemistry 5, 322.

Ingles, C. J., and Dixon, G. H. (1967). Proc. Natl. Acad. Sci. U. S. 58, 1011.

Ito, Y., Fujii, T., and Yoshioka, R. (1963). J. Biochem. (Tokyo) 53, 242.

Jackson, J. E., Jackson, E. M., and Freeman, S. (1965). Biochim. Biophys. Acta 105, 483.

Jagannathan, V., and Luck, J. M. (1949). J. Biol. Chem. 179, 569.

Jansz, H. S., Brons, D., and Warringa, M. G. P. (1959a). Biochim. Biophys. Acta 34, 573.

Jansz, H. S., Posthumus, C. H., and Cohen, J. A. (1959b). Biochim. Biophys. Acta 33, 387 and 396.

Johns, E. W., Phillips, D. M. P., Simson, P., and Butler, J. A. V. (1960). Biochem. J. 77, 631.

Johnson, R. M., and Albert, S. (1952). Arch. Biochem. Biophys. 35, 340.

Johnson, R. M., and Albert, S. (1953). J. Biol. Chem. 200, 335.

Jollès, P. (1966). Angew. Chem. Intern. Ed. Engl. 5, 558.

Joshi, J. G., and Handler, P. (1964). J. Biol. Chem. 239, 2741.

Judah, J. D. (1961). Biochim. Biophys. Acta 53, 375.

Judah, J. D., and Ahmed, K. (1962). Nature 194, 382.

Judah, J. D., Ahmed, K., and McLean, A. E. M. (1962a). Biochim. Biophys. Acta 65, 472.

Judah, J. D., Ahmed, K., and McLean, A. E. M. (1962b). Nature 196, 484.

Kahlenberg, A., Galsworthy, P. R., and Hokin, L. E. (1967). Science 157, 434.

Kalan, E. B., and Telka, M. (1959). Arch. Biochem. Biophys. 79, 275.

Kaufman, S. (1955). J. Biol. Chem. 216, 153.

Keilin, D., and Hartree, E. F. (1938). Proc. Roy. Soc. (London), Ser. B 124, 397.

Keller, P. J. (1955). J. Biol. Chem. 214, 135.

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

Kemp, A., Jr. (1966). In "Regulation of Metabolic Processes in Mitochondria" (J. M. Tager, S. Papa, E. Quadliariello, and E. C. Slater, eds.), p. 264. Elsevier, Amsterdam.

Kennedy, E. P., and Koshland, D. E., Jr. (1957). J. Biol. Chem. 228, 419.

Kennedy, E. P., and Smith, S. W. (1954). J. Biol. Chem. 207, 153.

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

Kiddy, C. A., Johnston, J. O., and Thompson, M. P. (1964). J. Dairy Sci. 47, 147.

Killander, D., and Rigler, R. (1965). Exptl. Cell Res. 39, 701.

Kleinsmith, L. J., Allfrey, V. G., and Mirsky, A. E. (1966a). Proc. Natl. Acad. Sci. U. S. 55, 1182.

Kleinsmith, L. J., Allfrey, V. G., and Mirsky, A. E. (1966b). Science 154, 780.

Korman, E. F., Shaper, J. H., Cernichiari, O., and Smith, R. A. (1965). Arch. Biochem. Biophys. 109, 284.

Krane, S. M., Stone, M. J., and Glimcher, M. J. (1965). Biochim. Biophys. Acta 97, 77.

Krebs, E. G., DeLange, R. J., Kemp, R. G., and Riley, W. D. (1966). Pharmacol. Rev. 18, 163.

Krebs, E. G., and Fischer, E. H. (1956). Biochim. Biophys. Acta 20, 150.

Krebs, E. G., and Fischer, E. H. (1960). Ann. N. Y. Acad. Sci. 88, 378.

Krebs, E. G., and Fischer, E. H. (1962). Advan. Enzymol. 24, 263.

Krebs, E. G., Graves, D. J., and Fischer, E. H. (1959). J. Biol. Chem. 234, 2867.

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

Krebs, E. G., Love, D. S., Bratvold, G. E., Trayser, K. A., Meyer, W. L., and Fischer, E. H. (1964). Biochemistry 3, 1022.

Kundig, W., Ghosh, S., and Roseman, S. (1964). Proc. Natl. Acad. Sci. U. S. 52, 1067.

Kundig, W., Kundig, F. D., Anderson, B., and Roseman, S. (1966). J. Biol. Chem. 241, 3243.

Langan, T. A. (1965). Personal communication.

Langan, T. A. (1967). In "Regulation of Nucleic Acid and Protein Biosynthesis" (V. V. Koningsberger and L. Bosch, eds.), p. 233. Elsevier, Amsterdam.

Langan, T. A., and Smith, L. K. (1966). Federation Proc. 25, 778.

Langan, T. A., and Smith, L. K. (1967). Federation Proc. 26, 603.

Larrabee, A. R., McDaniel, E. G., Bakerman, H. A., and Vagelos, P. R. (1965). Proc. Natl. Acad. Sci. U. S. 54, 267.

Laskowski, M. (1935). Biochem. Z. 278, 345.

Layne, E. (1957). In "Methods in Enzymology" (S. P. Colowick and N. O. Kaplan, eds.), Vol. III, p. 447. Academic Press, New York.

LeBaron, F. N., and Folch, J. (1956). J. Neurochem. 1, 101.

Levene, P. A., and Alsberg, C. L. (1901). Z. Physiol. Chem. 31, 543.

Levinthal, C., Singer, E. R., and Fetherolf, K. (1962). Proc. Natl. Acad. Sci. U. S. 48, 1230.

Lewis, J. C., Snell, N. S., Hirschmann, D. J., and Fraenkel-Conrat, H. (1950). J. Biol. Chem. 186, 23.

Lindberg, O., Duffy, J. J., Norman, A. W., and Boyer, P. D. (1965). J. Biol. Chem. 240, 2850.

Linderstrøm-Lang, K. (1925). Compt. Rend. Trav. Lab. Carlsberg 16, No. 1, 48.

Linderstrøm-Lang, K. (1929). Compt. Rend. Trav. Lab. Carlsberg 17, No. 9.

Linderstrøm-Lang, K., and Kodama, S. (1925). Compt. Rend. Trav. Lab. Carlsberg 16, No. 1, 1.

Linderstrøm-Lang, K., and Ottesen, M. (1947). Nature 159, 807.

Linderstrøm-Lang, K., and Ottesen, M. (1949). Compt. Rend. Trav. Lab. Carlsberg, Ser. Chim. 26, 403.

Lineweaver, H., and Burk, D. (1934). J. Am. Chem. Soc. 56, 658.

Lipmann, F. (1933a). Naturwissenschaften 21, 236.

Lipmann, F. (1933b). Biochem. Z. 262, 3.

Lipmann, F. (1941). Advan. Enzymol. 1, 99.

Lipmann, F. A., and Levene, P. A. (1932). J. Biol. Chem. 98, 109.

Longsworth, L. G., Cannan, R. K., and MacInnes, D. A. (1940). J. Am. Chem. Soc. 62, 2580.

McCully, K. A., Maw, W. A., and Common, R. H. (1959). Can. J. Biochem. Physiol. 37, 1457.

McEwen, B. S., Allfrey, V. G., and Mirsky, A. E. (1963a). J. Biol. Chem. 238, 758.

McEwen, B. S., Allfrey, V. G., and Mirsky, A. E. (1963b). J. Biol. Chem. 238, 2579.

McIntyre, O. R., and Ebaugh, F. G., Jr. (1962). Blood 19, 443.

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

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

Majerus, P. W., Alberts, A. W., and Vagelos, P. R. (1964). Proc. Natl. Acad. Sci. U. S. 51, 1231.

Majerus, P. W., Alberts, A. W., and Vagelos, P. R. (1965a). J. Biol. Chem. 240, 618.

Majerus, P. W., Alberts, A. W., and Vagelos, P. R. (1965b). Proc. Natl. Acad. Sci. U. S. 53, 410.

Majerus, P. W., Alberts, A. W., and Vagelos, P. R. (1965c). J. Biol. Chem. 240, 4723.

Mano, Y., and Lipmann, F. (1966a). J. Biol. Chem. 241, 3822.

Mano, Y., and Lipmann, F. (1966b). J. Biol. Chem. 241, 3834.

Marchis-Mouren, G., and Lipmann, F. (1965). Proc. Natl. Acad. Sci. U. S. 53, 1147.

Marshak, A., and Calvet, F. (1949). J. Cellular Comp. Physiol. 34, 451.

Martin, J. B., and Doty, P. M. (1949). Anal. Chem. 21, 965.

Mayer, M., and Heidelberger, M. (1946). J. Am. Chem. Soc. 68, 18.

Mecham, D. K., and Olcott, H. S. (1949). J. Am. Chem. Soc. 71, 3670.

Medzihradsky, F., Kline, M. H., and Hokin, L. E. (1967). Arch. Biochem. Biophys. 121, 311.

Mellander, O. (1939). Biochem. Z. 300, 240.

Menefee, S. G., Overman, O. R., and Tracy, P. H. (1941). J. Dairy Sci. 24, 953.

Metzger, B., Hilmreich, E., and Glaser, L. (1967). Proc. Natl. Acad. Sci. U. S. 57, 994.

Meyerhof, O., and Lohmann, K. (1928). Biochem. Z. 196, 49.

Millon, E., and Commaille, A. (1865). Compt. Rend. 60, 118 and 859.

Milstein, C. and Sanger, F. (1961). Biochem. J. 79, 456.

Mitchell, R. A., Butler, L. G., and Boyer, P. D. (1964). Biochem. Biophys. Res. Commun. 16, 545.

Mok, C.-C., Grant, C. T., and Taborsky, G. (1966). Biochemistry 5, 2517.

Moore, S. (1963). J. Biol. Chem. 238, 235.

Moret, V., Pinna, L. A., Sperti, S., Lorini, M., and Siliprandi, N. (1963). Biochim. Biophys. Acta 78, 547.

Morrison, J. F., and Ennor, A. H. (1960). In "The Enzymes" (P. D. Boyer, H. Lardy, and K. Myrbäck, eds.), 2nd edition, Vol. II, p. 89. Academic Press, New York.

Nagano, K., Kanazawa, T., Mizuno, N., Tashima, Y., Nakao, T., and Nakao, M. (1965). Biochem. Biophys. Res. Commun. 19, 759.

Najjar, V. A., and Pullman, M. E. (1954). Science 119, 631.

Naughton, M. A., Sanger, F., Hartley, B. S., and Shaw, D. C. (1960). Biochem. J. 77, 149.

Neuberg, C., and Oertel, W. (1914). Biochem. Z. 60, 491.

Neuberg, C., and Pollak, H. (1910). Biochem. Z. 26, 529.

Norberg, B. (1950). Acta Chem. Scand. 4, 1206.

Norman, A. W., Bieber, L. L., Lindberg, O., and Boyer, P. D. (1964). Biochem. Biophys. Res. Commun. 17, 108.

Norman, A. W., Bieber, L. L., Lindberg, O., and Boyer, P. D. (1965) J. Biol. Chem. 240, 2855.

Northrop, J. H. (1930). J. Gen. Physiol. 13, 739.

Olivo, F., Sperti, S., Rossi, C. S., and Moret, V. (1961). Acta Isotopica 1, 247.

Ord, M. G., and Stocken, L. A. (1966). Biochem. J. 98, 888.

Ord, M. G., and Stocken, L. A. (1967). Biochem. J. 103, 5P.

Osborne, T. B., and Campbell, G. F. (1900a). J. Am. Chem. Soc. 22, 413.

Osborne, T. B., and Campbell, G. F. (1900b). J. Am. Chem. Soc. 22, 422.

Ottesen, M. (1958). Compt. Rend. Trav. Lab. Carlsberg, Ser. Chim. 30, 211.

Paigen, K. (1958). J. Biol. Chem. 233, 388.

Paigen, K., and Griffiths, S. K. (1959). J. Biol. Chem. 234, 299.

Perlgut, L. E., and Wainio, W. W. (1967). Biochemistry 6, 15.

Perlmann, G. E. (1950). Nature 166, 870.

Perlmann, G. E. (1952a). J. Gen. Physiol. 35, 711.

Perlmann, G. E. (1952b). In "Phosphorus Metabolism" (W. D. McElroy and B. Glass, eds.), Vol. II, p. 167. Johns Hopkins Press, Baltimore.

Perlmann, G. E. (1952c). J. Am. Chem. Soc. 74, 6308.

Perlmann, G. E. (1954). Nature 174, 273.

Perlmann, G. E. (1955). Advan. Protein Chem. 10, 1.

Perlmann, G. E. (1958). J. Gen. Physiol. 41, 441.

Peterson, R. F., Nauman, L. W., and McMeekin, T. L. (1958). J. Am. Chem. Soc. 80, 95.

Pinna, L. A., Lorini, M., Sperti, S., and Moret, V. (1963). Ital. J. Biochem. 12, 227.

Pinna, L. A., and Wadkins, C. L. (1967). Biochem. Biophys. Res. Commun. 28, 400.

Pizer, L. I. (1960). J. Biol. Chem. 235, 895.

Pizer, L. I. (1962). In "The Enzymes" (P. D. Boyer, H. Lardy, and K. Myrbäck, eds.), 2nd edition, Vol. VI, p. 179. Academic Press, New York.

Plimmer, R. H. A. (1941). Biochem. J. 35, 461.

Plimmer, R. H. A., and Bayliss, W. M. (1906). J. Physiol. (London) 33, 439.

Pogo, A. O., Pogo, B. G. T., Littau, V. C., Allfrey, V. G., Mirsky, A. E., and Hamilton, M. G. (1962). Biochim. Bicphys. Acta 55, 849.

Pogo, B. G. T., Allfrey, V. G., and Mirsky, A. E. (1966). Proc. Natl. Acad. Sci. U. S. 55, 805.

Post, R. L., Sen, A. K., and Rosenthal, A. L. (1965). J. Biol. Chem. 240, 1437.

Posternak, S., and Posternak, T. (1927). Compt. Rend. 184, 909.

Posternak, S., and Posternak, T. (1928). Compt. Rend. 187, 313.

Pressman, B. C. (1964). Biochem. Biophys. Res. Commun. 15, 556.

Pugh, E. L., and Wakil, S. J. (1965). J. Biol. Chem. 240, 4727.

Pullman, M. E., and Schatz, G. (1967). Ann. Rev. Biochem. 36, 539.

Rabinowitz, M., and Lipmann, F. (1960). J. Biol. Chem. 235, 1043.

Rafter, G. W., and Lane, W. C. (1962). J. Bacteriol. 83, 1077.

Rall, T. W., Sutherland, E. W., and Wosilait, W. D. (1956a). J. Biol. Chem. 218, 483.

Rall, T. W., Wosilait, W. D., and Sutherland, E. W. (1956b). Biochem. Biophys. Acta 20, 69.

Ray, W. J., Jr., and Roscelli, G. A. (1964a). J. Biol. Chem. 239, 1228.

Ray, W. J., Jr., and Roscelli, G. A. (1964b). J. Biol. Chem. 239, 3935.

Riley, W. D., and Krebs, E. G. (1966). Federation Proc. 25, 220.

Rimington, C. (1927). Biochem. J. 21, 272.

Robbins, J. H. (1965). Science 146, 1648.

Rodnight, R., Hems, D. A., and Lavin, B. E. (1966). Biochem. J. 101, 502.

Rose, S. P. R., and Heald, P. J. (1961). Biochem. J. 81, 339.

Rubin, A. D., and Cooper, H. L. (1965). Proc. Natl. Acad. Sci. U. S. 54, 469.

Sanger, F., and Hocquard, E. (1962). Biochim. Biophys. Acta 62, 606.

Sanger, F., and Shaw, D. C. (1960). Nature 187, 872.

Schaffer, N. K., May, S. C., and Summerson, W. H. (1953). J. Biol. Chem. 202, 67.

Schaffer, N. K., May, S. C., and Summerson, W. H. (1954). J. Biol. Chem. 206, 201.

Schjeide, O. A., and Urist, M. R. (1956). Science 124, 1242.

Schjeide, O. A., and Urist, M. R. (1959). Exptl. Cell Res. 17, 84.

Schmidt, G., and Davidson, H. M. (1956). Biochim. Biophys. Acta 19, 116.

Schmidt, G., and Thannhauser, S. J. (1945). J. Biol. Chem. 161, 83.

Schneider, W. C. (1945). J. Biol. Chem. 161, 293.

Schwartz, J. H. (1963). Proc. Natl. Acad. Sci. U. S. 49, 871.

Schwartz, J. H., and Lipmann, F. (1961). Proc. Natl. Acad. Sci. U. S. 47, 1996.

Schweitzer, G. K., and Stein, B. R. (1950). Nucleonics 7, 65.

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

Sidbury, J. B., Jr., and Najjar, V. A. (1957). J. Biol. Chem. 227, 517.

Siekevitz, P., and Potter, V. R. (1955). J. Biol. Chem. 215, 221.

Siliprandi, N., Moret, V., Pinna, L. A., and Lorini, M. (1966). In "Regulation of Metabolic Processes in Mitochondria" (J. M. Tager, S. Papa, E. Quadliariello, and E. C. Slater, eds.), p. 247. Elsevier, Amsterdam.

Simoni, R. D., Levinthal, M., Kundig, F. D., Kundig, W., Anderson, B., Hartman, P. E., and Roseman, S. (1967). Proc. Natl. Acad. Sci. U. S. 58, 1963.

Singh, V. N., Dave, S. S., and Venkitasubramanian, T. A. (1967). Biochem. J. 104, 48C.

Skou, J. C. (1965). Physiol. Rev. 45, 596.

Slater, E. C., and Kemp, A., Jr. (1964). Nature 204, 1268.

Slater, E. C., Kemp, A., and Tager, J. M. (1964). Nature 201, 781.

Smith, R. A., Frank, I., and Gunsalus, I. C. (1957). Federation Proc. 16, 251.

Sonnenberg, B. P., and Zubay, G. (1965). Proc. Natl. Acad. Sci. U. S. 54, 415.

Sørensen, S. P. L. (1930). Compt. Rend. Trav. Lab. Carlsberg 18, No. 5.

Spackman, D. H., Stein, W. J., and Moore, S. (1958). Anal. Chem. 30, 1190.

Sperti, S., Olivo, F., Moret, V., and Rossi, C. S. (1961). Acta Isotopica 1, 239.

Sperti, S., Pinna, L. A., Lorini, M., Moret, V., and Siliprandi, N. (1964). Biochim. Biophys. Acta 93, 284.

Spies, J. R., and Chambers, D. C. (1949). Anal. Chem. 21, 1249.

Sugano, H. (1957). J. Biochem. (Tokyo) 44, 205.

Sundararajan, T. A., Sampath Kumar, K. S. V., and Sarma, P. S. (1958). Biochim. Biophys. Acta 29, 449.

Sundararajan, T. A., Sampath Kumar, K. S. V., and Sarma, P. S. (1960). Biochim. Biophys. Acta 38, 360.

Sundararajan, T. A., and Sarma, P. S. (1954). Biochem. J. 56, 125.

Sundararajan, T. A., and Sarma, P. S. (1957). Enzymologia 18, 234.

Sutherland, F. W., and Wosilait, W. D. (1955). Nature 175, 169.

Taborsky, G., and Mok, C.-C. (1967). J. Biol. Chem. 242, 1495.

Tanaka, Y., Epstein, L. B., Brecher, G., and Stohlman, F., Jr. (1963). Blood 22, 614.

Thoai, N., Roche, J., and Pin, P. (1954). Bull. Soc. Chim. Biol. 36, 483.

Thompson, M. P. and Kiddy, C. A. (1964). J. Dairy Sci. 47, 626.

Thompson, M. P., Kiddy, C. A., Johnston, J. O., and Weinberg, R. M. (1964). J. Dairy Sci. 47, 378.

Thompson, M. P., Kiddy, C. A., Pepper, L., and Zittle, C. A. (1962a). Nature 195, 1001.

Thompson, M. P., Kiddy, C. A., Pepper, L., and Zittle, C. A. (1962b). J. Dairy Sci. 45, 650.

Thompson, M. P., and Pepper, L. (1964). J. Dairy Sci. 47, 633.

Thompson, M. P., Tarassuk, N. P., Jenness, R., Lillevick, H. A., Ashworth, V. S., and Rose, D. (1965). J. Dairy Sci. 48, 159.

Trevor, A. J., and Rodnight, R. (1965). Biochem. J. 95, 889.

Turkington, R. W., and Topper, Y. J. (1966). Biochim. Biophys. Acta 127, 366.

Van Vunakis, H., Kaplan, J., Lehrer, H., and Levine, L. (1966). Immunochemistry 3, 393.

Veis, A., and Perry, A. (1967). Biochemistry 6, 2409.

von Hippel, P. H., and Waugh, D. F. (1955). J. Am. Chem. Soc. 77, 4311.

Wadkins, C. L. (1963). Biochem. Biophys. Res. Commun. 13, 411.

Wake, R. G., and Baldwin, R. L. (1961). Biochim. Biophys. Acta 47, 225.

Wakil, S. J., Pugh, E. L., and Sauer, F. (1964). Proc. Natl. Acad. Sci. U. S. 52, 106.

Walker, C. S., and Rafter, G. W. (1967). Arch. Biochem. Biophys. 120, 589.

Warner, R. C. (1944). J. Am. Chem. Soc. 66, 1725.

Waugh, D. F. (1958). Discussions Faraday Soc. 25, 186.

Waugh, D. F., and von Hippel, P. H. (1956). J. Am. Chem. Soc. 78, 4576.

Williams, J., and Sanger, F. (1959). Biochim. Biophys. Acta 33, 294.

Williams-Ashman, H. G., and Kennedy, E. P. (1952). Cancer Res. 12, 415.

Wilson, I. B., and Dayan, J. (1965). Biochemistry 4, 645.

Wosilait, W. D. (1958). J. Biol. Chem. 233, 597.

Wosilait, W. D., and Sutherland, E. W. (1956). J. Biol. Chem. 218, 469.

Zetterqvist, Ö., and Engström, L. (1967). Biochim. Biophys. Acta 141, 523.

End