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*PHOSPHOPROTEIN METABOLISM IN ISOLATED LYMPHOCYTE NUCLEI**

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The experiments to be described are concerned with the synthesis and function of phosphorylated proteins in cell nuclei, and deal specifically with the nature of phosphate incorporation and "exchange" on serine and threonine residues in nuclear proteins.

The existence of phosphoprotein fractions which rapidly incorporate P^{32}-phosphate has been known in a variety of cell types for some time.[1-4] Recently, the occurrence and formation of phosphoproteins in the cell nucleus have come under intensive investigation, notably in the work of T. A. Langan and F. Lipmann.[5] They have presented convincing evidence for the nuclear localization of a protein fraction containing 1.0–1.2 per cent phosphorus, mainly in the form of phosphoserine.

The presence of highly phosphorylated proteins in the cell nucleus is of special interest because it raises the possibility that regions of high negative charge density in phosphoproteins may modify DNA-histone interactions and perhaps influence the template activity of the chromatin in RNA synthesis. Two lines of evidence support this view: (1) recent experiments by Langan and Smith have shown that phosphoproteins can interact with histones *in vitro*, and that complex formation with phosphoprotein diminishes the inhibitory effects of added histones on DNA-dependent RNA synthesis;[6] and (2) several analyses by T. A. Langan of chromatin fractions derived from thymocyte nuclei by the method of Frenster, Allfrey, and Mirsky[7] indicate that "phosphoprotein" concentrations in chromatin fractions which are active in RNA synthesis greatly exceed those observed in chromatin fractions which are relatively inactive in RNA synthesis.[6] Thus, phosphoproteins appear not only to be localized in chromatin but also to be preferentially bound to its "diffuse" or "active" state.[7, 8]

In order to obtain further information on the behavior of nuclear phosphoproteins, we have employed tracer techniques 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

in this paper 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 appears to be itself energy-dependent. Histone fractions purified by chromatographic and electrophoretic techniques show the presence of phosphoserine. When human lymphocytes are stimulated to enlarge and divide by the addition of phytohemagglutinin to tissue cultures, the nuclear response is rapid and it includes an increase in the rate of phosphorylation of nuclear proteins.

Methods.—Isolation of nuclei: Nuclear fractions were isolated from fresh calf thymus tissue by homogenization and differential centrifugation in 0.25 *M* sucrose–3 mM $CaCl_2$ as previously described,[5, 10] using a brief hypotonic shock (0.22 *M* sucrose–3 mM $CaCl_2$) in some cases to facilitate cell breakage.[10] To rule out cell contamination in critical experiments, nuclei were further purified by centrifugation through sucrose density barriers (1.6 *M* sucrose layered over 1.95 *M* sucrose).[10]

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* sodium phosphate buffer (pH 6.8) in 0.25 *M* sucrose; 0.4 ml 0.1 *M* glucose containing 3.75 mg NaCl + 4.2 mg $MgCl_2 \cdot 4H_2O$ per ml; and 0.1 ml H_2O containing either 25 μc $Na_2HP^{32}O_4$ (sp. act. 100 mc/mmole), 4 μc DL-alanine-1-C^{14} (sp. act. 4.4 mc/mmole), 1 μc DL-serine-3-C^{14} (sp. act. 2.0 mc/mmole) or 1 μc guanosine-8-C^{14} (sp. act. 4.6 mc/mmole). The suspensions were shaken at 37° in a water bath. Conditions were aerobic unless otherwise specified.

In isotope retention ("cold chase") experiments, the nuclei were chilled after 15 min incubation and centrifuged. They were washed three times in incubation medium containing an excess of nonradioactive precursor; in tests for C^{14}-serine "turnover," the wash solution contained 2 mg unlabeled serine per ml; in P^{32}-exchange experiments, the phosphate buffer of the incubation medium sufficed for the "cold chase." After washing to remove radioactive precursors, the nuclei were resuspended in incubation medium and reincubated at 37°.

Preparation and analysis of the "phosphoprotein" fraction: Phosphoprotein was determined as recommended by Langan and Lipmann.[5] In this procedure, nucleic acids are removed by hot-acid extraction,[11] followed by the use of acidified chloroform-methanol to remove phospholipids.[12] 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 by spectrophotometry.[13-15]

The detailed procedure used in tracer experiments is as follows: after incubation the nuclei were treated with 16% trichloroacetic acid (TCA), centrifuged, resuspended in 16% TCA, and heated at 90° for 15 min. They were recentrifuged and washed three times with 16% TCA, once with 1:1 chloroform-methanol, once with 2:1 chloroform-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 were corrected for self-absorption.[16] Phosphorylation of the protein was measured by analysis and counting of alkali-labile phosphate. The protein residue was dissolved in 1.0 *N* NaOH, an aliquot taken for biuret protein determination,[17] and the rest of the solution heated at 100° for 15 min. After cooling, the solution was acidified and the protein precipitated with silicotungstic acid. Inorganic phosphate released into the supernatant was analyzed as the phosphomolybdate complex, which was extracted in 1:1 isobutanol-benzene, reduced with $SnCl_2$, and measured at 660 mμ. Aliquots of the isobutanol-benzene extract were also mixed with Bray's scintillation solution[18] for determination of P^{32} activity.

Separation of phosphoserine and phosphothreonine: The method of Schaffer, May, and Summerson[19] was used to identify the phosphorylated amino acids. The protein residues were hydrolyzed in 2 *N* HCl at 110° for 10 hr, and the hydrolysate was chromatographed on Dowex-50 in 0.05 *N* HCl. Excellent resolution of inorganic phosphate, phosphoserine, and phosphothreonine was obtained.

Preparation and purification of histones: Nuclei were washed after incubation with 0.01 *M* "tris"-HCl buffer (pH 7.1) containing 3 mM $MgCl_2$, in order to remove soluble nuclear proteins and nuclear ribosomes.[20] 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 vol of acetone. They were further purified by electrophoresis at pH 9 on cellulose polyacetate strips[21, 22] and by chromatography on carboxymethylcellulose.[23]

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 is indicated by the uppermost curve in Figure 1; the uptake proceeds for up to 2 hr. In order to rule out the possibility that this activity is due to a small fraction of whole cells contaminating the nuclear suspension, the nuclei were further purified by centrifugation through sucrose density barriers[10] after incubation *in vitro* in the presence of P^{32}-orthophosphate. The purified nuclei were found to contain the isotopically labeled phosphoproteins. Further tests for nuclear localization of the activity made use of the fact that the phosphorylation of proteins 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,[24] was found to have no effect on the phosphorylation reaction (Table 1). On the other hand, deoxyribonuclease treatment, which inhibits energy metabolism in the isolated nucleus but has no such effect on the intact cells, caused a marked inhibition of P^{32} incorporation into phosphoproteins (Table 1). Thus, both types of experiment support the conclusion that protein phosphorylation is a nuclear process.

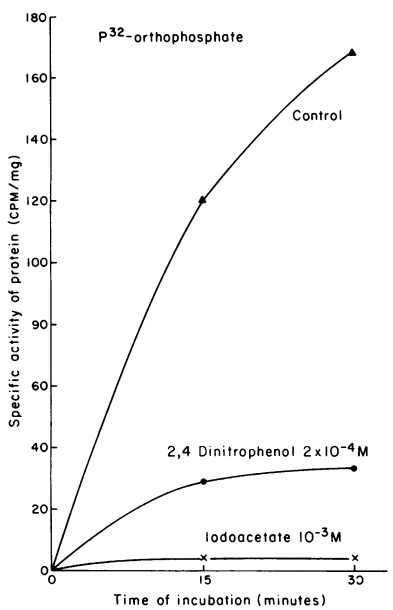

FIG. 1.—Time course of P^{32}-orthophosphate incorporation into the proteins of isolated calf thymus nuclei. The specific activity of the proteins is plotted against the time of incubation at 37°. Note that agents which block glycolysis (iodoacetate) or nuclear phosphorylation (2,4-dinitrophenol) also inhibit P^{32} uptake into the proteins, indicating the energy dependence of the reaction.

TABLE 1

EFFECTS OF INHIBITORS ON PROTEIN PHOSPHORYLATION IN ISOLATED NUCLEI

Conditions of experiment	P^{32}-phosphate incorporation (cpm/mg/min)	Inhibition (%)
Control nuclei	187.2	—
Nuclei incubated in 90% CO–10% O_2 (in dark)	181.0	3.3
Nuclei preincubated with DNase (15 min; 1 mg/ml)	112.3	40.0
Nuclei incubated in 5×10^{-4} *M* DRB*	156.5	16.4†

* DRB: 5,6-dichloro-beta-D-ribofuranosylbenzimidazole.
† This slight inhibition is in accord with the observation that DRB causes a 20% inhibition of ATP synthesis.[42]

Nature of the phosphate linkage: Langan and Lipmann[5] have already demonstrated the presence of phosphoserine and phosphothreonine in the proteins of rat liver. We have observed that the alkali-labile phosphate found in lymphocyte nuclear proteins also occurs in the esterified form. The protein residues prepared from nuclei after incubation with P^{32}-orthophosphate were hydrolyzed in acid and the hydrolysate was chromatographed on Dowex-50. A clear separation of phosphoserine and phosphothreonine was obtained. After taking into account the breakdown of phosphorylated amino acids during hydrolysis, it was calculated that approximately 85 per cent of the radioactivity in the alkali-labile phosphate assay could be accounted for as phosphoserine and an additional 5 per cent could be accounted for as phosphothreonine.

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[24]). Both of these compounds are very effective in reducing the uptake of P^{32}-phosphate into the proteins of isolated nuclei (Fig. 1). This indication of ATP dependence is in accord with the work of Langan and Lipmann[5] on the protein phosphokinase activity of rat liver nuclei, an enzymatic reaction which transfers phosphoryl groups from ATP to the hydroxyl groups of serine residues in the protein.

Failure of puromycin to inhibit 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 uptake. The amino acid selected was serine, since most of the phosphate appears in phosphoserine linkage. The results are summarized in Figure 2. Under conditions in which puromycin markedly inhibited the incorporation of serine-3-C^{14} into the proteins of the 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,[25, 26] and the acetylation of hemoglobin.[27] In all 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 min 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 and P^{31}-phosphate. Aliquots were withdrawn at different times and the nuclear proteins were analyzed for their

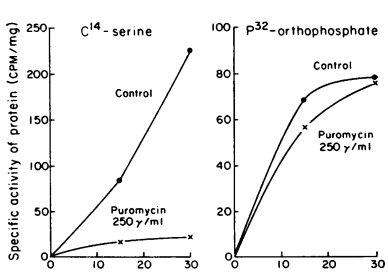

FIG. 2.—Comparison of the kinetics of incorporation of C^{14}-serine and P^{32}-phosphate into the proteins of isolated thymus nuclei. Note that puromycin inhibits serine incorporation without a corresponding inhibition of protein phosphorylation.

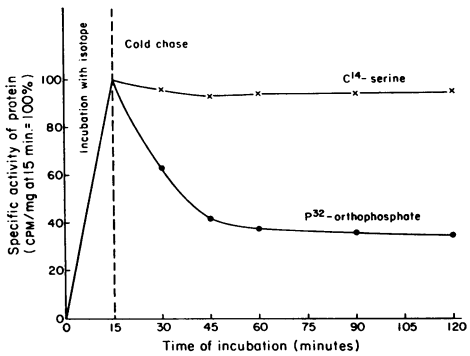

FIG. 3.—"Turnover" of previously incorporated P^{32}-phosphate in nuclear proteins. Nuclei were incubated for 15 min in the presence of C^{14}serine or P^{32}-phosphate. The retention of isotope was measured during a "cold chase" in radioisotope-free media. Note thatC^{14}-serine is retained while P^{32}-phosphate is rapidly lost.

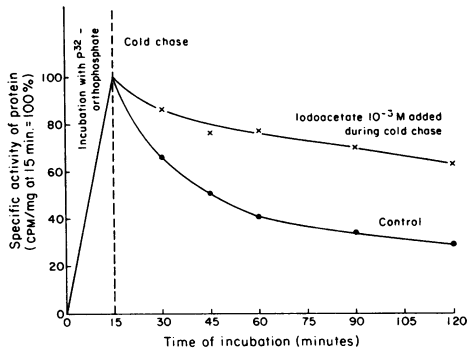

FIG. 4.—Evidence for the energy dependence of P^{32} "turnover" in nuclear phosphoproteins. Nuclei were incubated with P^{32}-phosphate for 15 min, washed to remove the precursor, and then subjected to a "cold chase." Note that the presence of iodoacetate (which blocks nuclear glycolysis and ATP synthesis) reduces the rate of phosphate "turnover."

contents of C^{14}-serine and P^{32}-phosphate. The results are summarized in Figure 3. 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 per cent of the labeled phosphate is lost during a 2-hr "chase."

Energy dependence of phosphate "turnover": It is an interesting fact that if nuclear ATP synthesis is blocked by the addition of iodoacetate, then the "turnover" of protein phosphate is also inhibited (Fig. 4). The inhibition of nuclear ATP synthesis by iodoacetate can be overcome by the addition of pyruvate,[24] an end product of nuclear glycolysis. Under these conditions the "turnover" of phosphate in nuclear proteins resumes. In one experiment, for example, the control nuclei lost 45.4 per cent of their previously incorporated P^{32} during a 75-min "cold chase"; nuclei exposed to iodoacetate during the "chase" lost only 12.1 per cent of their counts in the same interval; but nuclei treated with iodoacetate + pyruvate lost 38.8 per cent of their P^{32} label, nearly as much as was lost from the "controls." *These findings make it very unlikely that the phosphoester linkages in nuclear phosphoproteins are broken by a simple hydrolytic reaction, and they suggest that protein dephosphorylation is coupled to an energy-dependent reaction.*

P^{32}-phosphate distribution in nuclear proteins: A fractionation of the nuclear proteins following P^{32}-phosphate incorporation shows the presence of radioactivity in several of the fractions. Phosphorylated proteins appear in the soluble phase prepared by extracting nuclei with 0.01 *M* "tris" buffer at pH 7.1, in the 0.2 *N* HCl extract containing the histones, and in the residue. By far the greater part of the alkali-labile phosphate and most of the radioactivity remain in the insoluble residue after nuclei are extracted with neutral buffers and dilute acids (Table 2).

The proteins in the "tris" extract contain little phosphorus but have relatively high specific P^{32} activities. Phosphorylated proteins are known to be intermediates in several enzymatic reactions, e.g., phosphoglucomutase,[28] hexokinase,[29] and phosphorylase activities.[30, 31] Since thymus nuclei contain all the enzymes of the gly-

TABLE 2

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} incorporated)	Specific activity of phosphoprotein (cpm/μmole-P)
0.01 *M* "tris" buffer extract	2.2	14.0	44,900
0.01 *M* HCl–80% ethanol extract	1.0	3.2	44,660
0.2 *N* HCl extract (histone fraction)	13.6	14.6	9,460
Insoluble residue	83.2	68.2	8,090
Recovery	100.	100.	

colytic pathway,[24] many of which are soluble, it is likely that phosphoenzyme-P^{32} contributes appreciably to the specific P^{32} activity of the "tris"-extractable protein fraction.

The presence of phosphate in the 0.2 *N* HCl extract containing the histones was not expected, and further purification procedures were carried out in an attempt to rule out contamination of the histones by P^{32}-labeled nonbasic proteins. Chromatography on carboxymethylcellulose showed that the P^{32} label followed the histone peaks (Fig. 5). The f_1 and f_3 histone fractions were hydrolyzed, and the radioactive material was identified as phosphoserine. The f_1 histones contained about three times more phosphoserine per milligram than did the f_3 histones. In other experiments the histones were purified by electrophoresis at pH 9 on cellulose polyacetate strips.[21, 22] Under such conditions only the highly basic proteins migrate toward the cathode; the P^{32} counts were again localized in the histone bands. Stocken and Ord have also detected the presence of phosphoserine in purified histone fractions.[32]

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-beta-D-ribofuranosylbenzimidazole (DRB), a potent inhibitor

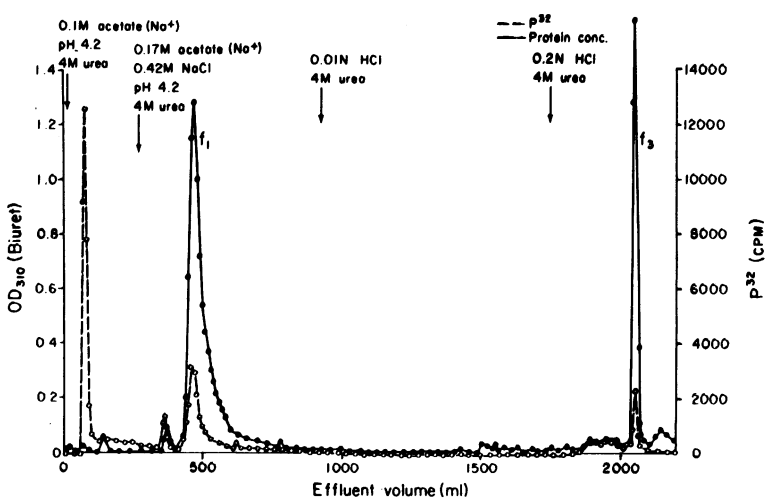

FIG. 5.—Separation of P^{32}-labeled histones on carboxymethylcellulose columns. Nuclei were incubated for 60 min with P^{32}-phosphate, and the histones extracted and precipitated as described in the text. The presence of P^{32}-phosphate in the major histone peaks, f_1 and f_3, is indicated. Protein concentration is indicated by the *solid circles*, radioactivity by the *open circles*.

of RNA synthesis in thymus nuclei.[9] Although the uptake of guanosine-8-C^{14} into RNA was inhibited, P^{32} uptake into proteins was not (Table 1).

A phosphorylation of membrane-bound proteins has been implicated in ion-transport mechanisms.[33–36] Since isolated thymus nuclei do display sodium-dependent "transport" reactions in which potassium ions cannot substitute for sodium,[37] 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. Phosphoprotein "turnover," then, cannot be correlated with membrane activity by this test.

The distribution of phosphoproteins in nuclei suggests other functions. Their presence in isolated chromatin fractions[6] and their solubilization when nuclei are lysed and treated with deoxyribonuclease[5] strongly suggest that phosphoproteins are involved in chromatin structure and may affect its function. Their high phosphate content (1.0–1.2%)[5] corresponds to 4–5 phosphate groups per 100 amino acid residues, and there is good evidence also that the phosphoserines occur in clusters.[5] 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-protein complex. If this is so, one would predict an increase in the extent of nuclear protein phosphorylation during periods of gene activation. This has been found to occur in lymphocytes stimulated by phytohemagglutinin.

Phytohemagglutinin effects on protein phosphorylation in lymphocytes: Human lymphocytes treated with phytohemagglutinin (PHA) increase in size and then divide.[38] Early in this response, they increase their rates of RNA synthesis.[39–41] Recent experiments have shown that this increased genetic activity is signaled by early changes in the chemistry of chromatin, among them an increased rate of histone acetylation.[22] We have now compared protein phosphorylation in control lymphocytes and in lymphocytes stimulated by PHA, and have observed higher rates of phosphorylation in cells responding to the stimulus. In one test, after 60-min incubation with PHA followed by 15-min labeling with radioactive phosphate, the specific activity of the phosphoproteins in PHA-treated cells was 209 per cent that observed in the controls. Moreover, the kinetics of P^{32} uptake suggest that protein phosphorylation is an early event in the course of nuclear activation. These results will be reported in detail in a separate communication.

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. Some evidence relating protein phos-

phorylation to the physical state and genetic activity of the chromatin is presented and discussed.

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