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O-Phosphoserine Content of Intermediate Filament Subunits†

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ABSTRACT: Purified subunits of intermediate filaments obtained from a variety of tissues and cell types contain *O*-phosphoserine and, in some cases, smaller amounts of *O*-phosphothreonine. The *O*-phosphoserine content was estimated by reaction of performic acid oxidized subunits with methylamine in NaOH. Decamin of BHK-21 and CHO fibroblasts contained about 1 mol/mol. Avian and mammalian desmin consists of two subunits, an acidic (α) subunit which contained 2 mol/mol and a more basic (β) nonphosphorylated subunit. The principal (M_r ~60 000) subunit of squid brain neurofilaments contained 5 mol/mol. Most mouse and bovine keratin subunits contained 3–6 mol/mol, although certain bovine subunits of higher molecular weight contained none. The *O*-phosphoserine contents of keratin subunits purified from the viable and stratum corneum layers were the same. The *O*-phosphoserine was located in non-α-helical regions of the subunits which presumably project out from the α-helical wall of the intermediate filaments. Most subunits could be partially dephosphorylated in vitro with alkaline phosphatase. It was found that the capacity of such partially dephosphorylated subunits for assembly into native-type filaments in vitro was independent of their phosphate content.

Intermediate filaments, microfilaments, and microtubules and their associated proteins constitute the three major classes of cytoskeletal proteins of eukaryote cells. Of these three classes, the intermediate filaments of different cells are the least conserved in terms of their subunit complexity and properties (Goldman et al., 1979; Lazarides, 1980; Zackroff et al., 1981). Nevertheless, intermediate filaments appear to be structurally homologous fibrous proteins (Steinert et al., 1978, 1980a).

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Considerable morphological, immunological, and biochemical data suggest that intermediate filaments function in cells coordinately with other cytoskeletal proteins in many fundamental cellular activities, including protein synthesis (Fulton et al., 1980), intracellular organelle support and movement (Goldman & Follet, 1970; Small & Sobieszek, 1977; Goldman et al., 1976; Solomon, 1980), and cellular division and locomotion (Goldman et al., 1973; Starger et al., 1978). It is possible that much of this functional diversity is mediated by the complexity of the intermediate filament component (Lazarides, 1980; Zackroff et al., 1981).

Evidence accumulated in several studies indicates that the intermediate filaments of a variety of tissues and cell types are phosphorylated, presumably by cyclic nucleotide-dependent protein kinases (Pant et al., 1978; Eagles & Gilbert, 1979; Corbett et al., 1980; Gilmartin et al., 1980; O'Connor et al., 1981). In view of the important role of such kinases in the regulation of cellular activity (Green, 1978; Krebs & Beavo, 1979), it seems possible that the phosphorylation of the intermediate filaments may directly or indirectly modulate their function in cells.

As part of an investigation of the role of phosphorylation on the structure and function of intermediate filaments in cells, we report in this paper some basic properties of the phosphorylated intermediate filament subunits, including the nature of the phosphate linkages and the amounts and location of the phosphate on the subunits. Since subunits could be partially dephosphorylated in vitro, we have also explored the possible role of phosphate on the capacity of the subunits to assemble into filaments in vitro.

Materials and Methods

Isolation of Intermediate Filament Subunits. Newborn mouse (BALB/c) epidermal cells were cultivated in 150-mm plastic dishes by established procedures (Yuspa & Harris, 1974). Some cells were cultured in the presence of [^{32}P]-orthophosphate (carrier free) (1 mCi/dish). The attached cells were harvested after 2 days and washed with phosphate-buffered saline. The keratin filament subunits (K_1 and K_2 families) were extracted into a buffer of 8 M urea, 0.05 M 2-amino-2-(hydroxymethyl)-1,3-propanediol hydrochloride (Tris-HCl)[1] (pH 9.0), and 25 mM 2-mercaptoethanol (Steinert & Yuspa, 1978; Steinert et al., 1979). These subunits were also isolated from freshly prepared mouse epidermal stratum corneum and from differentiating (4-h unattached) cells (Steinert & Yuspa, 1978; Steinert et al., 1979). The K_1 and K_2 subunits were then purified from each extract by chromatography on DEAE-cellulose and separated by preparative polyacrylamide gel electrophoresis in the presence of 0.1% $NaDodSO_4$ (Steinert et al., 1979).

The principal keratin filament subunits of bovine epidermis, designated 1c, 1a, 3, 4, and 5, were isolated from the viable and stratum corneum layers and purified by preparative gel electrophoresis (Steinert & Idler, 1975; Steinert et al., 1980b).

The intermediate filament subunit type of fibroblastic cells, variously termed vimentin (Franke et al., 1978) or decamin (Zackroff & Goldman, 1979), was prepared from BHK-21/C13 and CHO pro$^-$[1] cells. BHK-21 cells were also grown in the presence of [^{32}P]orthophosphate (100 μCi/100-mm dish) for 3 h. The subunits were then isolated, enriched by two cycles of assembly/disassembly/reassembly in vitro, and purified to homogeneity by preparative gel electrophoresis (Cabral et al., 1981; Steinert et al., 1981a).

Mammalian desmin was isolated from Golden Syrian hamster stomach smooth muscle (Tuszynski et al., 1979) and BHK-21 fibroblasts (Steinert et al., 1981a). Turkey gizzard desmin, prepared as described by Huiatt et al. (1980), was a gift of Dr. R. M. Robson. In each species, the desmin consists of an acidic α subunit and a more basic β subunit when resolved by two-dimensional gel electrophoresis (Huiatt et al., 1980; Steinert et al., 1981a). These were separated by preparative gel electrophoresis as described before (Steinert et al., 1981a).

Twice repolymerized squid brain neurofilaments were a gift of Dr. R. V. Zackroff. The quantitatively major subunit of M_r ~60 000 (Zackroff & Goldman, 1980) was isolated by preparative gel electrophoresis.

The purified intermediate filament subunits were stored frozen (−70 °C) in $NaDodSO_4$ solution. When necessary, the detergent was removed by ion-pair extraction (Steinert et al., 1981a).

Identification of Phosphorylated Amino Acids in Subunits. Preliminary experiments indicated that the subunits contained phosphate bound only through *O*-phosphate esters. To identify which amino acids were phosphorylated, we hydrolyzed purified labeled or unlabeled subunits in 5.7 M HCl at 105 °C in vacuo for 2 h, and the products were resolved by electrophoresis on thin-layer sheets in pH 3.5 buffer (Pyridine:acetic acid:water, 1:10:289 v/v/v) at 1000 V for 60 min at 10 °C (Corbett et al., 1980). Authentic *O*-phosphorylated amino acids were used as standard markers. When ^{32}P-labeled subunits were studied, the electrophoretogram was examined by autoradiography using Kodak X-RP film. When unlabeled subunits were studied, the electrophoretogram was cut into 0.5-cm-wide strips in the regions where the standard *O*-phosphorylated amino acids migrated and extracted with water. The extracts were dried, subjected to acid hydrolysis (5.7 M HCl at 105 °C in vacuo for 18 h), and analyzed to determine the amino acid content.

Estimation of O-Phosphoserine Content. This was done by modification of the procedure of Kolesnikova et al. (1974). All proteins were freed of $NaDodSO_4$, oxidized with performic acid (Weber, 1972), dried, and reacted with 1.0 M methylamine in 0.25 M NaOH at 37 °C. The optimal conditions of protein concentration and the time of reaction were determined by use of phosvitin (Calbiochem) which contains about 120 mol/mol of *O*-phosphoserine (Allerton & Perlman, 1965). In alkaline conditions, methylamine reacts specifically with *O*-phosphoserine residues to produce an *N*-methylserine derivative. Reactions were dried and then subjected to acid hydrolysis at 105 °C in vacuo for 22 h. The derivatized serine is released as β-*N*-methyldiaminopropionic acid. This compound eluted between lysine and NH_3 when resolved on a Beckman 119CL amino acid analyzer. In order to determine the color yield of the compound to determine the amount of *O*-phosphoserine in subunits, it was prepared in a weighable quantity by reaction of 5 mg (~0.2 μmol) of phosvitin and harvested from the analyzer by use of a stream division system. In the standard Beckman 119CL system utilized, the compound had a color value which was 0.70 ± 0.02 (mean ± SD) that of histidine and 1.09 ± 0.02 that of lysine.

Enzymatic Dephosphorylation of Subunits. ^{32}P-labeled keratin subunits (1 μg/mL) or other unlabeled subunits (100 μg/mL) were equilibrated in a buffer of 0.1 M Tris-HCl (pH 8.9), 0.3 M NaCl, and 0.1 M $MgCl_2$ and reacted with 1 or 100 units, respectively, of alkaline phosphatase (Sigma, type

[1] Abbreviations: Tris-HCl, 2-amino-2-(hydroxymethyl)-1,3-propanediol hydrochloride; $NaDodSO_4$, sodium dodecyl sulfate; EDTA, ethylenediaminetetraacetic acid.

VII) for up to 2 h at 23 °C. Aliquots were removed, and the reaction was terminated with EDTA (0.1 M final concentration). Protein was separated from free [^{32}P]phosphate by chromatography on a 5 × 1 cm column of Sephadex G-25 equilibrated in 5 mM Tris-HCl (pH 7.6) and counted. Aliquots of unlabeled subunits were dialyzed exhaustively against water and reacted with methylamine to estimate the remaining *O*-phosphoserine content. Some subunits dephosphorylated in this way were reequilibrated into $NaDodSO_4$ solution and used for in vitro filament assembly.

Two-Dimensional Gel Electrophoresis. This was done by the following modifications of the O'Farrell (1975) method. Isoelectric focusing was done on 4% acrylamide gels containing BioLytes (Bio-Rad Laboratories) in the pH range of 5–7 (Hearing et al., 1978). Histidine (50 mM) was used as the cathode electrolyte to enhance the separation of the proteins of p*I* 5–6 (Nguyen & Chrambach, 1980). The second dimension was done on 1.5-mm-thick 10–18% acrylamide gradient gels by the method of Douglas et al. (1979).

Filament Assembly in Vitro. Native-type keratin filaments were assembled in vitro from approximately equimolar mixtures of purified subunits (total protein concentration ~0.5–1 mg/mL) (Steinert et al., 1976, 1979). Subsequent to the removal of the $NaDodSO_4$, filaments were assembled in a buffer of 5 mM Tris-HCl (pH 7.6), 1 mM dithiothreitol, and 0.1 mM phenylmethanesulfonyl fluoride. Decamin and desmin subunits were assembled into structurally similar homopolymer filaments in vitro by equilibration into the same buffer modified to contain 0.17 M NaCl (Cabral et al., 1981; Steinert et al., 1981a). Yields of protein in filaments were determined by centrifugation at 100000*g* for 1 h and measurement of the protein concentrations in solution before and after centrifugation either by protein assay (Bramhall et al., 1969) or by spectrophotometric methods. Filaments were also examined in an electron microscope following negative staining with uranyl acetate.

α-Helix-enriched fragments were prepared from some filaments by limited tryptic digestion (Steinert, 1978a,b, 1980a). The citrate-soluble forms of the filaments were prepared by addition of 3 M citric acid to the filament suspensions to give a final concentration of 0.1 M and a pH of about 2.6. The protofilamentous forms of the filaments prepared in this way were then added dropwise to a solution of trypsin (Sigma, type III) and digested for 10 min exactly as described previously (Steinert, 1978a). Subsequently, the products were separated by chromatography on Sepharose 6B (Steinert, 1978a,b, 1980a). α-Helix contents of the fragments obtained were estimated by circular dichroism (Greenfield & Fasman, 1969).

Results

Identification of Phosphorylated Amino Acids of Filament Subunits. The relative stability of [^{32}P]phosphate on labeled mouse epidermal keratin and BHK-21 intermediate filament subunits in solutions of acid but not alkaline pH indicated the presence of *O*-phospho esters of serine, threonine, and/or tyrosine (Anthony & Spector, 1972). The nature of the amino acids labeled was investigated by resolution of the products of a 2-h acid hydrolysis of subunits by high-voltage thin-layer electrophoresis at pH 3.5 (Figure 1). The 2-h hydrolysis was sufficient to release some of the labeled *O*-phospho amino acids intact before complete hydrolysis of the phosphate ester bonds. Electrophoresis at pH 3.5 was chosen to optimize separation of the three *O*-phospho amino acids (Corbett et al., 1980). The labeled mouse epidermal K_1 and K_2 subunits both contained *O*-phosphoserine, but K_1 also contained some *O*-phosphothreonine (Figure 1, lane c). The labeled BHK-21 intermediate filament subunits contained only *O*-phosphoserine (Figure 1, lane e). BHK-21 cells contain both decamin (fibroblastic type) and desmin (muscle type) of intermediate filament subunits (Tuszynski et al., 1979; Gard et al., 1979; Steinert et al., 1981a). These components were resolved by two-dimensional gel electrophoresis (Figure 2), and it is seen that [^{32}P]phosphate was associated with decamin and α-desmin; the more basic β-desmin subunit was not labeled.

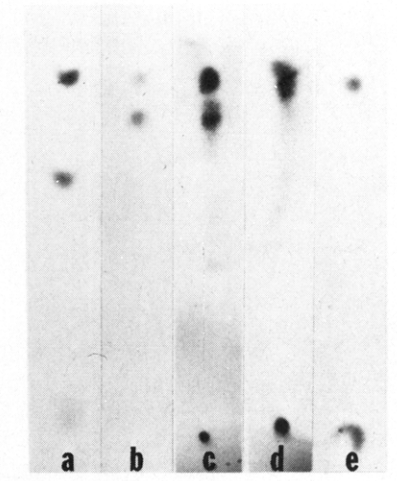

FIGURE 1: Thin-layer electrophoretogram of *O*-phospho amino acids. (a and b) Standards developed with ninhydrin: (a) *O*-phosphoserine (upper mark) and *O*-phosphotyrosine; (b) *O*-phosphoserine (upper mark) and *O*-phosphothreonine. (c–e) Acid hydrolysates of ^{32}P-labeled subunits developed by autoradiography: (c) mouse epidermal K_1; (d) mouse epidermal K_2; (e) BHK-21 subunits.

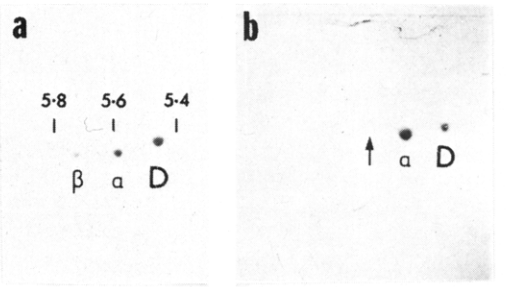

FIGURE 2: Two-dimensional gel electrophoretogram of ^{32}P-labeled BHK-21 subunits. Isoelectric focusing was done in the horizontal dimension (low pH on right) and gel electrophoresis with $NaDodSO_4$ in the vertical dimension. (a) Stained; (b) autoradiogram; the arrow indicates the position of migration of nonphosphorylated β-desmin. D, decamin; α and β indicate the two desmin subunits. The approximate pH values are indicated.

O-Phospho amino acids of other unlabeled subunits were also isolated and separated. In these cases, material eluted from the thin-layer sheets in the vicinity of migration of standard *O*-phospho amino acids was characterized by complete acid hydrolysis and amino acid hydrolysis. Avian and mammalian desmins, the squid brain neurofilament subunit, and the keratin subunits contained only *O*-phosphoserine. Bovine epidermal keratin subunits 1c and 1a did not contain any detectable phosphorylated amino acids.

O-Phosphoserine Content of Filament Subunits. The use of the [^{32}P]phosphate label does not provide any quantitative information on the phosphate content of the subunits, since *O*-phosphoserine and *O*-phosphothreonine have different rates of hydrolysis on partial acid hydrolysis and their rates of release from proteins are also variable (Bylund & Huang, 1976). However, Kolesnikova et al. (1974) have developed a procedure for estimation of *O*-phosphoserine which we have modified to

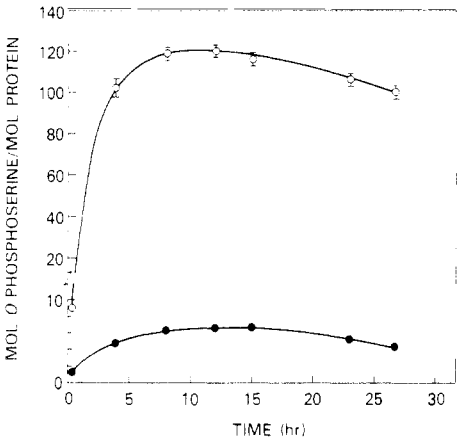

FIGURE 3: Estimation of *O*-phosphoserine content in proteins by reaction with 1.0 M methylamine in 0.25 M NaOH. Experimental details are described in the text. (○) Phosvitin; (●) bovine epidermal keratin subunit 5.

afford a more quantitative assay. This procedure involves reaction of the protein in alkaline solution with methylamine. Presumably the reaction proceeds by addition of methylamine to dehydroalanine that is formed from the β elimination of phosphate from *O*-phosphoserine in alkaline solution. There is negligible reaction of methylamine with *O*-phosphoserine in the absence of added alkali (Kolesnikova et al., 1974). A similar adduct is not formed in significant amounts with *O*-phosphothreonine or *O*-phosphotyrosine since a reduced intermediate is not produced in these amino acids in alkaline conditions. The optimal conditions for the reaction were determined by using chicken egg phosvitin which contains about 120 mol/mol of *O*-phosphoserine (Allerton & Perlman, 1965). The optimal protein concentration was 0.1–0.5 mg/mL and reaction time for 8–12 h (Figure 3). Under these conditions, about 95% of the *O*-phosphoserine, based on the known phosphate content, could be measured. However, when the intermediate filament subunits were studied, more variable results were obtained. This variability was apparently due to reaction of methylamine with dehydroalanine formed from cystine (or cysteine) residues, which are absent from phosvitin (Allerton & Perlman, 1965), since prior performic acid oxidation of the subunits afforded highly reproducible estimates. In addition, some of the dehydroalanine formed transiently from *O*-phosphoserine reacted with adjacent lysine residues instead of methylamine to form lysinoalanine (3–5% of the total serine and lysine contents of the protein). Thus, the estimates of the *O*-phosphoserine contents of the subunits shown in Table I may be slightly low. Most keratin subunits contained 3–6 mol/mol, but the bovine subunits of highest molecular weight (designated subunits 1a and 1c) did not contain any detectable *O*-phosphoserine. Table I also shows that in both mouse and bovine epidermis there were no significant differences in the *O*-phosphoserine contents of the subunits extracted from the living epidermal cell layers and those of fully differentiated stratum corneum. Both avian and mammalian α-desmin contained 2 mol/mol of *O*-phosphoserine, but the more basic β-desmins contained none. Decamin from two fibroblastic cell types contained somewhat less than 1 mol/mol, and the squid neurofilament 60000-dalton subunit contained about 5 mol/mol (Table I).

Dephosphorylation of Filament Subunits in Vitro. Figure 4 shows that 80–85% of the total ^{32}P label on mouse epidermal K_1 could be released within 2 h of digestion with alkaline phosphatase. Analysis of the *O*-phospho amino acid content of the reacted protein as described in Figure 1 indicated that

Table I: *O*-Phosphoserine Content (Mole per Mole) of Filament Subunits

source	subunit	M_r ($\times 10^{-3}$)	living cell layers	stratum corneum
keratin	mouse K_1	68	4.24 ± 0.45 (3)[a]	4.20 ± 0.37 (3)
	K_2	60	5.23 ± 0.08 (5)	5.12 ± 0.09 (3)
	bovine 1c	68	trace[b]	trace
	1a	58	trace	trace
	3	54	3.00 ± 0.30 (2)	3.08 ± 0.42 (3)
	4	52	4.43 ± 0.24 (2)	4.56 ± 0.28 (3)
	5	48	4.75 ± 0.50 (4)	5.12 ± 0.35 (3)
	6	47	5.45 ± 0.28 (2)	5.44 ± 0.10 (2)
desmin	avian muscle α	53	2.08 ± 0.31 (2)	
	β	53	trace	
	mammalian muscle α	54	1.91 ± 0.17 (4)	
	β	54	trace	
	BHK-21 α	54	1.87 ± 0.13 (4)	
decamin	CHO	55	0.87 ± 0.31 (6)	
	BHK-21	55	0.75 ± 0.20 (3)	
neurofilament		60	5.04 ± 0.20 (3)	

[a] The number in parentheses is the number of replicates tested. [b] Trace is defined as <0.5 residue/2000 amino acid residues, or <0.1 mol/mol.

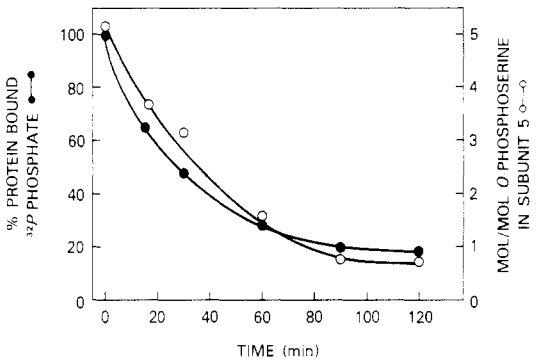

FIGURE 4: Time courses of enzymatic dephosphorylation of epidermal keratin subunits with alkaline phosphatase. Experimental details are described in the text. (●) Loss of ^{32}P label from K_1; (○) loss of *O*-phosphoserine from subunit 5 by estimation with methylamine.

both *O*-phosphoserine and *O*-phosphothreonine residues had been dephosphorylated. The remaining 10–20% of ^{32}P label could not be released by further digestion. Similar degrees of dephosphorylation were achieved with other unlabeled subunits, as determined by chemical estimation of the *O*-phosphoserine content remaining after reaction (Figure 4).

Dephosphorylation was also monitored by two-dimensional gel electrophoresis. For example, freshly prepared bovine epidermal keratin subunit 5, which contains about 5 mol/mol of *O*-phosphoserine (Table I), migrates as a single spot of p*I* ~5.2 (Figure 5a). During reaction with alkaline phosphatase, a series of spots were produced which had similar molecular weights, but their p*I* values varied between 5.2 and 5.8 (Figure 5b). At the maximal dephosphorylation possible (average *O*-phosphoserine content of ~0.8 mol/mol) (Figure 4), three major spots remained, the most basic of which had a p*I* of ~5.8 (Figure 5c). Similarly, purified muscle α-desmin (p*I* ~5.6), which contains 2 mol/mol of *O*-phosphoserine (Table I), could be substantially dephosphorylated to give a basic spot that exactly comigrated with nonphosphorylated β-desmin (p*I*

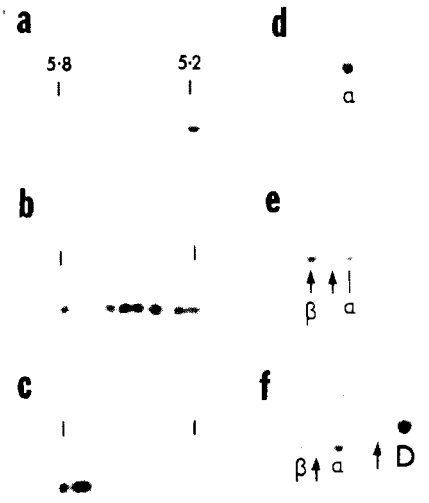

FIGURE 5: Enzymatic dephosphorylation of subunits with alkaline phosphatase assayed by two-dimensional gel electrophoresis. (a–c) Reaction with bovine subunit 5: (a) zero time; (b) 20 min; (c) 90 min. (d and e) Reaction with hamster α-desmin: (d) zero time; (e) 60 min; two more basic spots appear (arrows), the most basic of which exactly comigrated with mammalian β-desmin and another spot of intermediate charge. (f) A 60-min reaction with BHK-21 subunits: α and β indicate desmin subunits; arrow indicates spot of intermediate charge seen in (e); D, decamin; a faint spot (arrow) may be a dephosphorylated form of decamin. The approximate pH values are indicated.

Table II: In Vitro Assembly of Variably Phosphorylated Subunits

line	subunits utilized (*O*-phosphoserine content, mol/mol)	yield (%)[a]
1	3 (3.0) + 5 (4.8)	65–80
2	3 (3.0) + 5 (0.8)	75–80
3	3 (1.1) + 5 (4.8)	70–85
4	3 (1.1) + 5 (0.8)	75–85
5	1c (~0) + 5 (4.8)	45–65
6	1c (~0) + 5 (0.8)	40–65
7	K_1 (4.2) + K_2 (5.2)	60–70
8	K_1 (1.8) + K_2 (1.4)	65–80
9	K_1 (4.2) + 5 (4.8)	60–70
10	K_1 (1.8) + 5 (0.8)	65–85
11	α-desmin (2.0)	70–90
12	β-desmin (~0)	75–90
13	decamin (0.9)	80–90

[a] Determined from two to four replicates.

~5.8) and a spot of intermediate p*I* (~5.7) which possibly corresponds to a molecule containing only 1 residue/mol of *O*-phosphoserine (Figure 5d,e). On the other hand, BHK-21 decamin (p*I* ~5.4) could be only slightly dephosphorylated, yielding a minor spot of p*I* ~5.5 (Figure 5f).

In Vitro Assembly of Filaments from Subunits with Variable O-Phosphoserine Content. Several epidermal keratin subunits that assemble into native-type filaments in high yield in vitro were reacted with alkaline phosphatase as described above to reduce their *O*-phosphoserine content. Subsequently, their facility for assembly was reexamined, using as criteria to adjudicate the results the presence of filaments, filament morphology, and the yields of subunits in filaments (that is, the assembly efficiency) (Table II). In all cases tested, native-type filaments that were 7–10 nm in diameter and 0.5–10 μm in length were obtained as determined by electron microscopy following negative staining. Comparisons of filaments assembled from a mixture of subunits 3 and 5 which were not dephosphorylated (line 1) with partially dephosphorylated

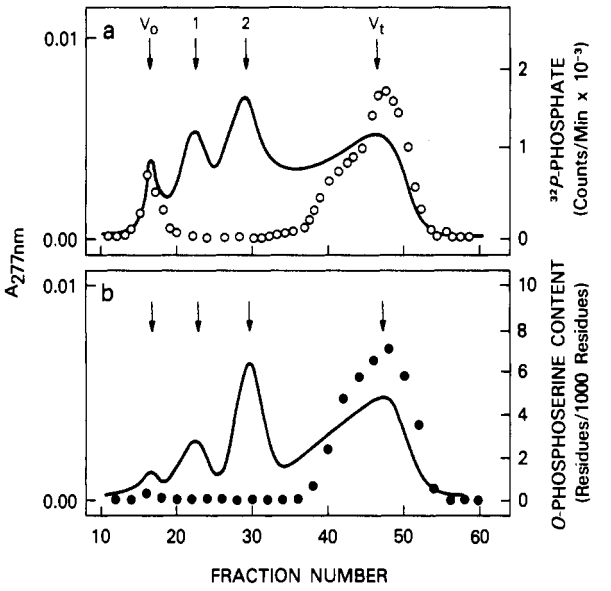

FIGURE 6: Location of *O*-phosphoserine on subunits. The citrate-soluble forms of filaments derived from (a) ^{32}P-labeled mouse keratin filaments or (b) unlabeled bovine keratin filaments containing subunits 3 and 5 were subjected to partial tryptic digestion and the products separated as described in the text. The protein of resolved peaks 1 and 2 (arrows) possessed α-helix contents of 73 ± 4% and 85 ± 5%, respectively. These chromatographic and physicochemical properties indicate the peaks contained the α-helical three-chain coiled-coil segments of the filaments (Steinert, 1978a,b). (—) Absorbance at 277 nm; (○) [^{32}P]phosphate; (●) amount of *O*-phosphoserine.

subunits (lines 2–4) show that there was no detectable difference in filament yields. Likewise, copolymer filaments containing subunits 1c and 5 (lines 5 and 6) and K_1 and K_2 (lines 7 and 8) and heterologous copolymer keratin filaments containing subunits K_1 and 5 (lines 9 and 10) formed filaments in high yields irrespective of their *O*-phosphoserine content. In addition, the nonphosphorylated β-desmin subunit, like α-desmin, assembled with high efficiency (line 12) as observed previously (Steinert et al., 1981a,b).

O-Phosphoserine Is Located in Non-α-helical Regions of the Subunits. The citrate-soluble protofilamentous forms of ^{32}P-labeled mouse epidermal keratin filaments were subjected to limited tryptic digestion, and the products were separated by chromatography on Sepharose 6B as described previously (Steinert, 1978a,b; Steinert et al., 1980a). Typically, two resolved peaks of protein of highly enriched α helix, termed peaks 1 and 2, were obtained, as well as a minor peak at V_0 of undigested protein and non-α-helical protein eluted at V_t (Figure 6). No ^{32}P label appeared in the α-helical fractions; almost all was located in the non-α-helical fraction (Figure 6b).

Similar proteolytic digests were done on unlabeled bovine epidermal keratin filaments containing subunits 3 and 5 (Figure 6b) and homopolymer decamin filaments (data not shown). In both cases, no *O*-phosphoserine was detected in the α-helical fractions of peaks 1 and 2; all of the *O*-phosphoserine content of the original filaments could be accounted for in the non-α-helical fractions eluted at V_t.

Discussion

Characterization of O-Phosphoserine Contents. The present experiments confirm and extend the observations of a number of investigators that the subunits of intermediate filaments of a variety of tissues and cell types contain phosphate, bound mostly as *O*-phosphoserine (Pant et al., 1978; O'Connor et al., 1979, 1981; Corbett et al., 1980; Gilmartin et al., 1980).

By improvement of an existing chemical procedure, we report here more quantitative information for the first time on the amounts of *O*-phosphoserine in intermediate filament subunits. Epidermal keratin filaments have long been known to be heterogeneous with respect to their subunit compositions, and we show here that they also contain variable amounts of phosphate. Whereas the two principal families of subunits of mouse epidermis each contained several moles per mole of *O*-phosphoserine, the subunits of highest molecular weight from bovine epidermis contained none (Table I). Organ culture labeling of normally and abnormally keratinizing human epidermis with [^{32}P]phosphate showed that all keratin subunits of this species could be labeled (Gilmartin et al., 1980). The reason for the absence of phosphate on certain bovine subunits remains unclear at this time. Interestingly, there was no detectable difference in the *O*-phosphoserine content of the keratin subunits extracted from the living or fully differentiated stratum corneum layers. This finding contrasts with the events that occur on the histidine-rich filaggrin protein component of the epidermis. This protein is initially synthesized as a highly phosphorylated (through *O*-phosphoserine) precursor and is then dephosphorylated at terminal differentiation (Lonsdale-Eccles et al., 1980). It is therefore clear that the role of phosphate on these two differentiation products of the epidermis is different.

O'Connor et al. (1979, 1981) have demonstrated and we confirm here that desmin of avian or mammalian muscle or BHK-21 fibroblasts in culture consists of two isoelectric variants, an acidic phosphorylated α form and a more basic nonphosphorylated β form. In each species, the α-desmin contains 2 mol/mol of *O*-phosphoserine (Table I). Partial dephosphorylation of mammalian α-desmin revealed (Figure 4) another isoelectric variant, intermediate in p*I* between α- and β-desmin, which probably corresponds to a species containing only 1 mol of *O*-phosphoserine. Decamin is the major intermediate filament subunit of fibroblasts. Cabral & Gottesman (1979) showed that in CHO cells it could be slightly labeled at one site during a 3-h pulse with [^{32}P]-phosphate. We show here that decamin of BHK-21 and CHO cells contains approximately 1 mol/mol of *O*-phosphoserine (Table I). Since decamin has a long half-life (Cabral & Gottesman, 1979), it would seem that a much longer pulse time may be required to fully label it. Experiments utilizing catalytic subunits of cAMP-dependent protein kinases indicated that both decamin and desmin of isolated cytoskeletal preparations of myotubes could be labeled in vitro at multiple sites (O'Connor et al., 1979, 1981). However, the physiological significance of these in vitro observations has yet to be determined.

Vertebrate and invertebrate neuronal tissues contain an abundance of neurofilaments which are comprised of a number of subunits of molecular weights in the range 60 000–200 000 (Goldman et al., 1976; Pant et al., 1978; Zackroff & Goldman, 1980). We show here that the quantitatively major subunit of squid brain of $M_r \sim 60\,000$, contains about 5 mol/mol of *O*-phosphoserine. While characterization of the several other neurofilament subunits of this and related tissues has yet to be done, it is likely they also contain considerable amounts of *O*-phosphoserine, since Pant et al. (1978) have shown that the neurofilament subunits of highest molecular weight become labeled when whole squid axoplasm is incubated in the presence of [^{32}P]phosphate.

Presence of O-Phosphothreonine in Intermediate Filament Subunits. In contrast with the earlier report of Gilmartin et al. (1980), the data of Figure 1 in this paper indicate that certain keratin subunits contain smaller amounts of *O*-phosphothreonine. While quantitation of it by chemical means or by autoradiography was not possible here, it would seem that the total amount of *O*-phosphothreonine was on the order of 1 mol/mol.

Presence of O-Phosphotyrosine in Intermediate Filament Subunits. Recent reports have indicated that on cellular transformation, cytoskeletal proteins including intermediate filaments acquire traces of *O*-phosphotyrosine. For example, Sefton & Hunter (1981) showed that the decamin of RSV-transformed chick embryo fibroblasts acquires 0.05–0.1 mol/mol of phosphate, of which about 2% (or about 0.002 mol/mol) is *O*-phosphotyrosine. Similarly, Corbett et al. (1980) reported that the virus-transforming protein kinase pp60^{src} introduced traces of *O*-phosphotyrosine onto desmin in a cell-free system. The experiments reported here in Figure 1 suggest that the amount of *O*-phosphotyrosine on the intermediate filament subunits of normal cells is very small.

Possible Location of O-Phosphoserine on Subunits. The intermediate filament subunits so far investigated seem to be structurally similar (Steinert et al., 1980a). About 40–50% of the polypeptide chains consist of α-helical sequences located in two discrete regions. The remaining 50–60% consists of non-α-helical sequences, small amounts of which are located at the amino-terminal end and between the two α-helical regions, and most of it is located at the carboxyl-terminal end (Steinert et al., 1980a). Amino acid analysis data have shown that about 80% of the serine residues of the subunits are located in the non-α-helical sections (P. M. Steinert, unpublished observations; Steinert, 1978b). It is clear from the experiments on the two types of filaments reported in Figure 6 that the *O*-phosphoserine is not distributed evenly along the subunits. The α-helix-enriched fragments contained no detectable *O*-phosphoserine: all of it could be accounted for in the non-α-helical sections. Presumably it was located on the ends of the subunits since the non-α-helical section which separates the two α-helical regions (within α-helical particle 1 of peak 1) contained no *O*-phosphoserine. These observations are of interest with respect to the postulated structure of the filaments (Fraser et al., 1976; Steinert et al., 1980a). It is thought the α-helical regions comprise the basic framework or wall of the filament, and the non-α-helical sections occupy interstitial spaces and/or project out beyond the wall where they may interact with the environment around the filament. However, it remains to be determined how the subunits are incorporated into filaments following synthesis and when the subunits become phosphorylated. Nevertheless, the location of the phosphate on these outer non-α-helical regions seems to be consistent with the accessibility that would be required by the responsible protein kinases and with the possible regulatory role of phosphate on the function of the filaments in cells.

Possible Role of Phosphorylation. The primary role of intermediate filaments in cells is thought to be mechanical and/or structural (Lazarides, 1980; Zackroff et al., 1981). The results of several types of studies support the notion that filaments function coordinately with other cytoskeletal and cytoplasmic constituents (Goldman et al., 1976; Solomon, 1980). Phosphorylation of the intermediate filaments in response to various stimuli may modify the structure and assembly of the filaments and their organization within the cytoskeleton. It seemed to us that one approach to the analysis of this possibility was to examine the capacity of subunits containing variable amounts of phosphate to assemble into native-type filaments in vitro. However, the experiments re-

ported in Table II failed to detect significant differences in the yield or structure of filaments assembled in vitro from such subunits. Very recently it has become clear that the intermediate filaments are attended by a variety of accessory proteins (Granger & Lazarides, 1981; Steinert et al., 1981b; Willard & Simon, 1981). Thus, future experiments will examine the role of phosphate on the interaction of the filaments with their accessory proteins and with other cytoskeletal proteins.

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