The possible occurrence of phosphorylation of pro-adrenocorticotropin (ACTH)/endorphin-derived peptides in the rat was examined by incubating dissociated anterior and intermediate pituitary cells with a 3H-amino acid plus 32P. In both lobes of the pituitary newly synthesized pro-ACTH/endorphin was labeled with 32P. In the intermediate pituitary, corticotropin-like intermediate lobe peptide (CLIP) and glycosylated CLIP were the major phosphorylated product peptides; phosphorylated ACTH and glycosylated ACTH were also detected. In the anterior pituitary ACTH and glycosylated ACTH were the major phosphorylated product peptides; phosphorylated ACTH biosynthetic intermediate was also detected. Glycosylated and nonglycosylated ACTH and CLIP were phosphorylated to approximately the same extent. In both lobes of the pituitary, 18,000 daltons fragment-related peptides were phosphorylated, but to an approximately 10-fold lesser extent than CLIP or ACTH. No detectable phosphorylation of β-lipotropin, γ-lipotropin, or β-endorphin occurred.

Based on isoelectric focusing of double labeled samples, approximately two-thirds of the CLIP in the rat intermediate lobe gets phosphorylated; based on COOH-terminal ACTH immunoassay a similar fraction of the CLIP in fresh intermediate lobe extracts is phosphorylated. Alkaline phosphatase treatment converts phosphorylated CLIP into CLIP. In anterior pituitary extracts roughly half of the ACTH is phosphorylated. In the mouse intermediate pituitary the phosphorylation pattern is qualitatively similar to that described for rat intermediate pituitary but only approximately a fifth of the CLIP is phosphorylated; no phosphorylation of β-lipotropin, γ-lipotropin, or β-endorphin was observed. In bovine intermediate pituitary no phosphorylation of CLIP was detected in double label incubations or in tissue extracts.

The serine residue phosphorylated in rat and mouse ACTH and CLIP (Ser31) could be phosphorylated by a protein kinase with specificity similar to that of "physiological casein kinases," which require the sequence Ser-X-Acidic.

There have been two series of reports on the phosphorylation of pro-adrenocorticotropin/endorphin-derived peptides. Raese et al. (1, 2) and Boarder et al. (3) reported phosphorylation of rat pituitary and hypothalamic β-lipotropin using tissue slices incubated in saline with 32P. It was suggested that phosphorylation occurred at serine residues near the paired basic amino acids that mark several of the proteolytic cleavage sites in pro-ACTH/endorphin and that phosphorylation directed proteolytic processing (1-3). In a separate series of studies, Browne et al. (4) and Bennett et al. (5, 6) reported that about half of the ACTH(1-39) in the rat anterior pituitary and two-thirds of the corticotropin-like intermediate lobe peptide (ACTH(18-39)) in the rat intermediate pituitary are phosphorylated on Ser31; they did not investigate the occurrence of phosphorylation of βLPH-related peptides. Bennett et al. (6) and Browne et al. (4) proposed that phosphorylation of Ser31 in ACTH might regulate glycosylation by masking the recognition site for glycosylation at Asn26 of ACTH.

Phosphorylation of proteins by cAMP-dependent and cAMP-independent protein kinases regulates many biological processes (7-10). In many cases phosphorylation is a post-translational modification, but co-translational phosphorylation of nascent actin polypeptides has been reported (11). The role of phosphorylation can vary in different tissues: phosphorylation of smooth muscle myosin is crucial to the initiation of smooth muscle contraction; in skeletal muscle phosphorylation of myosin is thought to play only a modulatory role in contraction (12-15). Given the various reports on phosphorylation of pro-ACTH/endorphin-derived peptides and the potential biological importance of such modifications, we decided to investigate further the phosphorylation of pro-ACTH/endorphin-derived peptides in vivo and in vitro.

**MATERIALS AND METHODS**

**Incubations with 32P**—Rat, mouse, mouse tumor, and beef pituitary cells were prepared as described (16) and incubated in medium containing high specific activity 32P (6.9 µCi/µmol) and either [3H]tyrosine (150 µCi/µmol) or [3H]phenylalanine (150 µCi/µmol). For some incubations, cells were preincubated for 1 h in complete medium containing 45 µmol inorganic phosphate (5% of the normal inorganic phosphate level). Mouse anterior pituitary tumor cells (AT(T-20/D-16)) were grown for 1 week in medium containing 45 µmol inorganic phosphate with no significant alteration in growth rate or ACTH secretion rate. All radiolabeled compounds were from New England Nuclear.

**Immunoprecipitations**—Pro-ACTH/endorphin-derived peptides were immunoprecipitated from radiolabeled cell extracts with affinity-purified rabbit antisera to β-endorphin (antiserum Melinos; 17), mouse γ-lipotropin (antiserum Bertha; 18), ACTH(1-16) (antiserum Amanda, raised against an ACTH(1-24)-bovine serum albumin conjugate), or ACTH(18-39) (antiserum Edwina, raised against a porcine ACTH(1-39)-hemocyanin conjugate). Immunoprecipitation of 14K fragment using antiserum Georgie has been described (16). Anterior

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The abbreviations used are: pro-ACTH/endorphin, pro-adrenocorticotropin/endorphin (also referred to as proopiomelanocortin); βLPH, β-lipotropin; CLIP, corticotropin-like intermediate lobe peptide (ACTH(18-39)); γLPH, γ-lipotropin; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; hACTH, human ACTH; 4.5K, 13K, 16K, 4,500, 13,000, 16,000 apparent molecular weight during SDS-PAGE.
Phosphorylation of Pro-ACTH/Endorphin-derived Peptides

pituitary extracts were immunoadsorbed onto Affi-Gel 10 (Bio-Rad Laboratories) resin to which β-endorphin antisera Melinda, affinity-purified mouse γ-lipotropin antiserum Bertha, or N-terminal ACTH antisera Amanda had been linked; resins were washed extensively with 0.5 M NaCl, 1.5% Nonidet P-40, 1% Tyloxapol, 3.75 mM EDTA, 50 mM NaF, pH 7.6, eluted with 10% formic acid, and dried for analysis on sodium dodecyl sulfate-polyacrylamide gels. Binding of [3H]-hACTH(1-16) to antisera Edwina is fully competed on that peptide at a given trial pH, adjust the trial pH, and repeat the calculation until the magnitude of the charge on the peptide was less than 0.001. The pH at which the program terminates is called the theoretical isoelectric point (PI) of the peptide. Input pKₐ values were: Glu, 4.40; Asp, 4.35; His, 4.29; Arg, 12.40; Lys, 10.58; Tyr(OH), 10.60; NH₂-terminal, 7.37; COOH-terminal, 3.47; sialic acid, 4.00; phosphate, 3.30 and 6.20. These values are all in the range of published values (21, 27); values for Glu, Asp, COOH-terminal, and phosphate have been selected to maximize agreement between theoretical and experimental data in the range of interest. Peptides used to plot Fig. 1 are listed in the figure legend.

Enzymatic Digestions—Carrier bovine serum albumin was always added to samples before enzyme treatment (17). Digestions with alkaline phosphatase were performed at 37 °C in 0.1 M NaH₂CO₃ containing 0.5 mg/ml of bovine serum albumin and enzyme (Sigma type III or IIIR); the reaction was stopped by lyophilization. Digestions with neuraminidase were carried out at 37 °C in 0.05 M ammonium acetate, pH 5.0, containing 5 mg/ml of bovine serum albumin and 0.5 mg/ml of enzyme (Sigma type X) and terminated by lyophilization. Ligation of tosylphenylalanyl chloromethyl ketone

FIG. 1. Comparison of theoretical and experimental isoelectric points of some peptides. The isoelectric points calculated for various peptides subjected to focusing are listed; hACTH(22-39) (3.50) (the multiple experimental determinations are for tryptic peptides derived from hACTH(1-39), hACTH(17-39), and hACTH(18-39)); bovine ACTH(22-39) (3.52); rat and mouse ACTH(25-39) (3.52) (the multiple experimental determinations are for tryptic peptides derived from rat ACTH(1-39), rat ACTH(18-39), and mouse ACTH(18-39)); hACTH(18-39) (3.48); mouse γLPH(4.28); bovine ACTH(18-39) (4.42); rat ACTH(18-39) (4.43); hACTH(17-39) (4.61); mouse βLPH(6.69). Isoelectric points calculated for peptides with published pIs are listed: methionine enkephalin (5.41; Ref. 22); thymosin α₁ (4.12), β₄ (4.82), and polypeptide PI (7.10; Refs. 23 and 24); rat pro-ACTH/endorphin (8.37; Ref. 25); nerve growth factor subunits γ₁, γ₂, and γ₃ (5.93, 5.66, and 5.93; Ref. 26).

Polyacrylamide Gel Electrophoresis—SDS-PAGE was performed after boiling samples into 1% sodium dodecyl sulfate, 8 M urea, and 5% 2-mercaptoethanol with the appropriate gel buffer. Two different SDS-PAGE systems were used: borate-buffered gels (11.25% acrylamide, 0.6% N,N'-methylenebisacrylamide; 16) or phosphate-buffered gels (14% acrylamide, 0.4% N,N'-methylenebisacrylamide, 6 M urea, 0.1% sodium dodecyl sulfate, 100 mM sodium phosphate; pH 7.2, as described in the Bethesda Research Laboratories catalog). Gels were sliced, eluted, and counted in a liquid scintillation counter (17).

Unlabeled ACTH and CLIP—Rat or beef pituitarys were separated into anterior lobe tissue and intermediate (beef) or intermediate plus neural (rat) tissue and homogenized in 3 N acetic acid with protease inhibitors (16). Lyophilized extracts were dissolved and fractionated on a column of Sephadex G-75 equilibrated with 10% formic acid, 0.1 mg/ml of bovine serum albumin, 0.1% 2-mercaptoethanol. The elution positions of ACTH and CLIP were determined with a COOH-terminal ACTH immunoassay, using antisera 220 kindly provided by Drs. C. Rivier and J. Rivier, The Salk Institute (19). Binding of [3H]hACTH(1-39) to antisera 220 is fully competed by ACTH(34-39) at about 25% molar cross-reactivity compared to ACTH(25-39), ACTH(18-39), and hACTH(1-39); thus this antisem is capable of immunoprecipitating molecules phosphorylated at Ser31 of ACTH.

Anti-Amanda and Melinda are directed against peptide 

FIG. 2. Incorporation of 32P into pro-ACTH/endorphin. Rat intermediate pituitary cells were incubated in medium containing [32P]phosphatidylserine plus 32P for 24 h and extracted. The extract was immunoprecipitated with affinity-purified ACTH(1-16) antisem, dissociated, and analyzed by gel filtration on Sephadex G-75 in formic acid. The peak eluting at the position of mouse pro-ACTH/endorphin (Kᵅ = 0.06) was dried and analyzed by borate SDS-PAGE with dithioerythritol-cytochrome c (Cytc) as an internal marker. The mobility of mouse pro-ACTH/endorphin was determined on a parallel gel.
Phosphorylation of Pro-ACTH/Endorphin-derived Peptides

RESULTS

Incorporation of pro-ACTH/endorphin-derived peptides by rat intermediate pituitary cells were incubated for 24 h in complete medium containing high specific activity \(^{32}P\), plus \(^{3}H\)phenylalanine, and pro-ACTH/endorphin was purified by NH\(_2\)-terminal ACTH immunoprecipitation and gel filtration. The labeled pro-ACTH/endorphin was examined by SDS-PAGE, it could be seen that \(^{3}H\)phenylalanine and \(^{32}P\) were both incorporated into a molecule comigrating with mouse tumor cell pro-ACTH/endorphin (Fig. 2). Similar results were obtained with double labeled pro-ACTH/endorphin from both intermediate and anterior pituitary cell incubations obtained by immunoprecipitation with a \(\beta\)-endorphin antiserum. Thus, \(^{32}P\), is incorporated into pro-ACTH/endorphin in primary rat anterior and intermediate pituitary cells in culture.

In order to determine which product peptides derived from pro-ACTH/endorphin are phosphorylated, a series of immunoprecipitations with antisera to \(\beta\)-endorphin, \(\gamma\)-lipotropin, NH\(_2\)-terminal ACTH, COOH-terminal ACTH, and 16K fragment were analyzed by SDS-PAGE (Figs. 3 and 4). After a 24-h incubation in \(^{32}P\), and \(^{3}H\)phenylalanine there was no detectable \(^{32}P\) incorporated into \(\beta\)LPH, \(\beta\)-endorphin, or \(\gamma\)LPH in rat intermediate pituitary (Fig. 3, A and B); plotted on the scale in Fig. 3, incorporation of labeled phosphate into pro-ACTH/endorphin is not obvious, but the ratio of \(^{32}P/^{3}H\) is the same as in Fig. 2. When COOH-terminal ACTH immunoprecipitation was performed after NH\(_2\)-terminal ACTH immunoprecipitation, intermediate pituitary cells were found to incorporate both \(^{32}P\) and \(^{3}H\) into glycosylated CLIP (slices 22–23) and CLIP (slices 27–28) (Fig. 3C). After a similar incubation of rat anterior pituitary cells, glycosylated ACTH(13K) and ACTH(4.5K) were labeled with both \(^{32}P\) and \(^{3}H\) (Fig. 4C), while \(\beta\)LPH-related molecules were not measurably labeled with \(^{32}P\) (Fig. 4, A and B). Thus, using both rat anterior and intermediate pituitary cells in culture, no incorporation of labeled phosphate into \(\beta\)LPH-related peptides was seen. In the anterior lobe, pro-ACTH/endorphin, ACTH biosynthetic intermediate, glycosylated ACTH, and ACTH were found to

![Figure 3](http://www.jbc.org/)

![Figure 4](http://www.jbc.org/)
In order to examine the phosphorylation of ACTH, CLIP, and 16K fragment in more detail and to verify the coexistence of phosphorylated and nonphosphorylated molecules, we utilized isoelectric focusing. The purified molecules were fractionated on a pH 2.5-5.0 isoelectric focusing gradient, with blue dextran (V₀), cytochrome c (Cytc), glucagon, and 2-mercaptoethanol (V₆) as internal markers (17). In A, synthetic CLIP was included in the sample and detected by radioimmunoassay; synthetic CLIP eluted at the peak of CLIP-sized COOH-terminal ACTH immunoactivity. In B, synthetic hACTH(1-39) eluted in fraction 6, and synthetic hACTH(1-16) eluted in fraction 56. Similarly in C, synthetic hACTH(1-16) eluted in fraction 48. Apparent molecular weights for each peak are indicated. Note the 12.5-fold expanded scale for ³²⁵P in C.

In order to examine the phosphorylation of ACTH, CLIP, and 16K fragment in more detail and to verify the coexistence of ³²⁵P and ²H in the labeled peptides, the various peptides were purified by immunoprecipitation and gel filtration. The purified molecules were examined by gel filtration in 6 M guanidine HCl (Fig. 5). The two radiolabels (³²⁵P and ²H) comigrated when samples of CLIP (Fig. 5A), glycosylated CLIP (Fig. 5B), 16K fragment (Fig. 5C), and ACTH (not shown) were analyzed. Fig. 5 shows that, while intermediate lobe 16K fragment is clearly phosphorylated, the extent of phosphorylation (³²⁵P/²H) of rat 16K fragment is only about 10% of the extent of phosphorylation of the two forms of CLIP.

Quantitation of the Extent of Phosphorylation of CLIP and ACTH—Knowing the percentage of molecules phosphorylated is important to any consideration of possible physiological roles for phosphorylation. The specific activity of the ³²⁵P, in the medium vastly exceeded that of the ²H-labeled amino acids; if the ³²⁵P/²H ratios observed for CLIP, ACTH, and pro-ACTH/endorphin simply reflected the specific activity ratios of the labels in the medium, then the results shown in Figs. 2-5 would indicate that less than 1% of the ACTH or CLIP molecules was phosphorylated. However, while amino acids equilibrate into cells and with cellular tRNA within minutes (16, 29, 30), [³²⁵P]inorganic phosphate shows a steady rate of increase of labeling of intracellular pools for many hours (31-35). Our experiments on ³²⁵P, equilibration with mouse AT-20 pituitary tumor, beef intermediate pituitary, and rat anterior pituitary cells in culture were in agreement with the literature, showing linear labeling of the intracellular ³²⁵P pool for up to 8 h, the longest time examined.

A method was needed to separate phosphorylated from nonphosphorylated molecules; based on predicted isoelectric points, isoelectric focusing should resolve phosphorylated label with ³²⁵P. In the intermediate lobe, pro-ACTH/endorphin, glycosylated ACTH, ACTH, glycosylated CLIP, and CLIP were found to label with ³²⁵P. Analysis of 16K fragment immunoprecipitates from rat anterior and intermediate pituitaries indicated that ³²⁵P was incorporated into 16K fragment, but to a much lesser extent than it was into ACTH and glycosylated ACTH, or CLIP and glycosylated CLIP (see below).

In order to determine which residue gets phosphorylated, radiolabeled phosphorylated CLIP, glycosylated CLIP, 16K fragment, and ACTH were subjected to mild acid hydrolysis and paper electrophoresis. All the data were consistent with the presence of phosphoserine, as originally reported for CLIP by Browne et al. (4, 6).

Phosphorylation of Pro-ACTH/Endorphin-derived Peptides

Fig. 5. Analysis of purified phosphoproteins by gel filtration. Rat intermediate pituitary cells were incubated in medium containing [³H]tyrosine plus ³²⁵P, for 21 h, extracted, immunoprecipitated with antiserum to ACTH(1-16) followed by antiserum to ACTH(18-39) followed by antiserum to 16K fragment, and dissociated immunoprecipitates were fractionated by gel filtration. The various peptides and their respective ³²⁵P/³H incorporation are shown. A. CLIP; B. Glyco CLIP; C. 16K fragment. Primer: V₀, V₆, glucagon. 

Fig. 6. Isoelectric focusing of rat intermediate pituitary CLIP COOH-terminal tryptic peptides. A. Cells in culture. B. Tissue extract.
focusing, and analyzed with a COOH-terminal ACTH immunoassay, it is possible to determine the relative amounts of phosphorylated and nonphosphorylated CLIP in tissue extracts. CLIP from fresh rat intermediate pituitary extracts was treated with trypsin, subjected to isoelectric focusing, and analyzed by isoelectric focusing (Fig. 6B). The distribution between phosphorylated and nonphosphorylated CLIP in tissue extracts was very close to the predicted position for phosphoserine-containing rat ACTH(22-39). Thus the two forms of COOH-terminal tryptic peptide derived from CLIP are phosphorylated (pI = 3.2) and nonphosphorylated (pI = 3.6) ACTH(22-39).

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FIG. 7. Alkaline phosphatase digestion of rat CLIP. Biosynthetically labeled CLIP (from the incubation described in Figs. 2 and 3) was incubated at 37 °C for 30 min or 4 h with 0.2 units/ml of alkaline phosphatase (Sigma type III) (B and C) or for 4 h without enzyme (enz) (A). Samples were separated by isoelectric focusing on a gradient formed with 2 parts of pH 3.5-5.0 ampholytes and 1 part of pH 5-8 ampholytes. Recovery of 3H-label averaged 107%; recovery of 32P-label was 104% in A and declined to 3% in C as released inorganic phosphate focused off of the gel. Samples were applied in the region of slice 25.

from nonphosphorylated ACTH, CLIP, or ACTH(22-39) (Fig. 1). The results of Browne et al. (4) indicated that the COOH-terminal tryptic peptide of ACTH and CLIP (ACTH(22-39)) was the site of phosphorylation; this tryptic peptide contains phenylalanine and tyrosine and can be detected with a COOH-terminal ACTH immunoassay. Based on the calculations shown for Fig. 1, replacement of serine with phosphoserine in rat ACTH(22-39) should shift the isoelectric point of the peptide from 3.52 to 3.18. Rat intermediate pituitary CLIP labeled with [3H]tyrosine and 32P, was digested with trypsin and analyzed by isoelectric focusing (Fig. 6A). Tritium radioactivity without 32P was focused at pH 3.62 near the theoretical position for the nonphosphorylated form of rat ACTH(22-39). Both 3H and 32P were focused at pH 3.20, which is very close to the predicted position for phosphoserine-containing rat ACTH(22-39). Thus the two forms of COOH-terminal tryptic peptide derived from CLIP are phosphorylated (pI = 3.2) and nonphosphorylated (pI = 3.6) ACTH(22-39).

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Phosphorylation of Pro-ACTH/Endorphin-derived Peptides

Fig. 9. Isoelectric focusing of rat intermediate pituitary glycosylated CLIP COOH-terminal tryptic peptides. A, cells were incubated as in Fig. 5; purified double labeled glycosylated CLIP was digested with trypsin, fractionated or a pH 2.5-5 isolectric focusing gradient, and localized by liquid scintillation counting. B, glycosylated CLIP was prepared from acid extracts of rat intermediate pituitary and analyzed as described in Fig. 6B. Samples were applied in the region of slice 24. RIA, radioimmunoassay.

A peak of material focusing at about pH 3.6 was found in biosynthetically labeled cultures (Fig. 9A) and tissue extracts (Fig. 9B). Based on analysis of the double labeled material, the peak of material at pH 3.6 did not label with $^{32}$P; this peak of material focused at the same position as nonglycosylated nonphosphorylated ACTH(22-39) (Figs. 6 and 8), as expected for those glycosylated CLIP molecules in which the carbohydrate chains have no net charge. Two peaks of $[^3H]$ tyrosine-labeled tryptic peptides from glycosylated CLIP were found to be phosphorylated (focusing at pH 3.2 and 3.0); the ratio of $^{32}$P counts per min to $^3$H counts per min in these two peaks was not constant (Fig. 9A). Peaks of COOH-terminal ACTH immunoactivity with comparable isolectric points were also found in tryptic digests of glycosylated CLIP from tissue extracts (Fig. 9B). The most acidic peak (Fig. 9A) had a $^{32}$P to $^3$H ratio that matched the ratio for phosphorylated nonglycosylated ACTH(22-39) from the same incubation (Fig. 6A, pI = 3.20), while the pH 3.2 peak had a substantially lower $^{32}$P to $^3$H ratio. The presence of sialic acid residues on some of the carbohydrate chains would be expected to generate a phosphorylated and sialylated peak focusing at or below pH 3.0 and to produce some sialylated but nonphosphorylated material focusing near pH 3.2 (Fig. 1). Neuraminidase treatment of glycosylated CLIP supports these suggestions.

Phosphorylation of Pro-ACTH/Endorphin-derived Peptides in Mouse and Beef Pituitary—Evidence for the physiological importance of certain elements of peptide structure can often be discerned by determining how well conserved these features are in different species. Therefore the possibility that phosphorylation of ACTH/endorphin-derived peptides

Fig. 10. Incorporation of $[^32P]$Phosphate into pro-ACTH/endorphin-derived peptides in mouse intermediate pituitary. Mouse intermediate pituitary cells were incubated in medium containing $[^3]H$phenylalanine and $[^32P]$Phosphate for 24 h, extracted, and immunoprecipitated sequentially with antisera to NH$_2$-terminal ACTH, COOH-terminal ACTH, and 16K fragment. A, purified immunoprecipitated CLIP was digested with trypsin and analyzed on a pH 2.5-5.0 isolectric focusing (IEF) gradient; the sample was applied in the region of slice 25. B, purified immunoprecipitated 16K fragment was analyzed by SDS-PAGE in phosphate buffer.
Phosphorylation of Pro-ACTH/Endorphin-derived Peptides

Bovine pituitaries did not contain or produce measurable amounts of phosphorylated CLIP or ACTH, either in vivo or in culture. Upon isoelectric focusing, intact bovine CLIP exhibited a single peak of COOH-terminal ACTH immunoreactivity at pH 4.10 (Fig. 11A). The isoelectric focusing pattern of bovine CLIP was not affected by alkaline phosphatase treatment that was effective at converting phosphorylated rat CLIP into CLIP (Fig. 11A; Fig. 7). Similarly the COOH-terminal tryptic peptide from bovine anterior pituitary ACTH exhibited a single peak on isoelectric focusing (at pH 3.43); no immunoreactivity focused at the calculated position of a phosphoserine-containing bovine ACTH(22-39) peptide (pH 3.18; Fig. 1). When bovine intermediate pituitary cells were incubated under the same conditions used to label rat anterior pituitary ACTH and glycosylated ACTH with both $[^{3}H]tyrosine$ and $[^{32}P]inorganic phosphate$ (Fig. 4), no detectable $[^{32}P]$ was incorporated into $[^{3}H]tyrosine$-labeled $\beta$LPH, $\beta$-endorphin, or CLIP (Fig. 11, B and C). Our five antibodies to mouse 16K fragment do not react well with bovine 16K fragment, so phosphorylation of that peptide was not examined.

**DISCUSSION**

In this work we have studied the phosphorylation of pro-ACTH/endorphin-derived peptides in tissue extracts and in tissue culture. Roughly half of the ACTH in rat anterior pituitary and two-thirds of the CLIP in rat intermediate pituitary is phosphorylated on a serine residue (presumably Ser$^{31}$), as originally reported by Bennett et al. (5, 6) and Browne et al. (4). In addition, about 5% of the 16K fragment molecules in rat pituitary contain phosphoserine. Contrary to previous reports (1-3), no phosphorylation of PLPH, $\beta$-endorphin, or $\gamma$LPH was detected. The level of phosphorylation of pro-ACTH/endorphin in vitro was very similar to the level of phosphorylation of ACTH or CLIP; this observation is consistent with the suggestion that phosphorylation only occurs onto pro-ACTH/endorphin, not onto smaller peptides (Figs. 2-4 and 12). For example, in Fig. 2 the experimental ratio of $[^{32}P]$ to $[^{3}H]$ counts per min incorporated into trichloroacetic acid-precipitable material in this incubation was the same as the ratio using rat anterior pituitary cells (Fig. 4). RIA, radioimmunoassay.

occurs in mouse and beef pituitary tissue was examined. Mouse intermediate pituitary cells incorporated $[^{32}P]$ into pro-ACTH/endorphin, ACTH biosynthetic intermediate, glycosylated CLIP, nonglycosylated CLIP, and 16K fragment. When double labeled mouse CLIP was digested with trypsin and analyzed by isoelectric focusing (Fig. 10A), two peaks of $[^{3}H]phenylalanine$ were found at pH 3.12 and pH 3.58; these tryptic peptides correspond to the two COOH-terminal tryptic peptides observed in rat CLIP samples (Fig. 6). The mouse CLIP COOH-terminal tryptic peptide focusing at pH 3.58 was not detectably labeled with $[^{32}P]$ while the peptide focusing at pH 3.12 was clearly labeled with $[^{32}P]$. In mouse intermediate pituitary roughly one-fifth of the CLIP was phosphorylated. When mouse intermediate pituitary 16K fragment was purified by immunoprecipitation and gel filtration and then examined by SDS-PAGE (Fig. 10E), 16K fragment was found to be phosphorylated but to a far lesser extent than CLIP or glycosylated CLIP. No measurable phosphorylation of mouse $\beta$LPH, $\gamma$LPH, or $\beta$-endorphin was observed when immunoprecipitates were analyzed by SDS-PAGE (not shown). Mouse anterior pituitary tumor cell pro-ACTH/endorphin, ACTH biosynthetic intermediate, glycosylated ACTH, nonglycosylated ACTH, and 16K fragment were also found to be phosphorylated, while $\beta$LPH-related peptides were not phosphorylated (not shown).
Phosphorylation of Pro-ACTH/Endorphin-derived Peptides

The major phosphorylated peptides derived from pro-ACTH/endorphin are identified; the minor amount of phosphorylation in the 16K fragment region is not indicated. It is not yet clear whether phosphorylation of prepro-ACTH/endorphin occurs; it is also not clear whether phosphorylation and/or dephosphorylation of small product peptides occur. mMSH, α-melatonopin [N-acetyl-
ACTH(1-13)NH₂].

βLPH. However, rat βLPH is significantly shorter than sheep βLPH (17, 18, 37, 38), consistently migrates faster than sheep βLPH during SDS-PAGE (18, 38), and differs greatly from sheep βLPH in amino acid sequence (37, 38); therefore, the phosphorylated peptide examined in these earlier studies (1–3) was not rat βLPH.

Many different protein kinases have been characterized in recent years. Cyclic nucleotide-dependent and calcium-de-
pendent protein kinases phosphorylate serine residues near groupings of basic amino acids (7, 39); this type of specificity cannot explain the phosphorylation of Ser31 in ACTH. There are several other types of protein kinases, and one type in particular (called “physiological casein kinases”; Ref. 39) phosphorylates seryl or threoninyl residues with an acidic
ring phosphoserine residues in two well studied secreted mol-
ecules (37, 38), therefore, the phosphorylated peptide examined in these earlier studies (1–3) was not rat βLPH.

Fig. 12. Post-translational processing of rat pro-ACTH/endo-
phin. The major phosphorylated peptides derived from pro-
ACTH/endorphin are identified; the minor amount of phosphorylation in the 16K fragment region is not indicated. It is not yet clear whether phosphorylation of prepro-ACTH/endorphin occurs; it is also not clear whether phosphorylation and/or dephosphorylation of small product peptides occur. aMSH, α-melatonopin [N-acetyl-
ACTH(1-13)NH₂].

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