

## Purification and Some Characteristics of an ACTH-Sensitive Protein Kinase and Its Substrate Protein in Rat Brain Membranes

H. Zwiers, P. Schotman, and W. H. Gispen

*Division of Molecular Neurobiology, Rudolf Magnus Institute for Pharmacology and Laboratory of Physiological Chemistry, Medical Faculty, Institute of Molecular Biology, State University of Utrecht, Utrecht, The Netherlands*

---

**Abstract:** ACTH inhibits the phosphorylation of a rat brain membrane-bound protein (B-50). Both the protein kinase and the substrate protein could be extracted from the membranes by means of treatment with Triton X-100 in 75 mM-KCl. Using column chromatography over DEAE-cellulose and ammonium sulphate precipitation a protein fraction (ASP 55-80) enriched in endogenous B-50 phosphorylating activity was obtained. The time course of the endogenous phosphorylation of B-50 in this fraction showed a linear incorporation with time for at least 10 min and reached an estimated maximal incorporation of 0.65 mol P/mol B-50 after 60 min. The inhibition by ACTH<sub>1-24</sub> of the B-50 protein kinase in ASP 55-80 was dose-dependent; the half-maximal effective concentration was  $5 \times 10^{-6}$  M, being 10 to 50 times lower as compared with intact synaptic plasma membranes (SPM). cAMP, cGMP and various endorphins had no effect on the B-50 protein kinase. The B-50 protein kinase required both magnesium and calcium for optimal activity. Using two-dimensional electrophoresis on polyacrylamide slab gels the B-50 protein kinase and the B-50 protein could be identified and purified. The isoelectric point (IEP) of the kinase is 5.5 and the apparent molecular weight 70,000, whereas the IEP of the substrate protein B-50 is 4.5 and the apparent molecular weight 48,000. Amino acid analysis on microgram quantities of purified kinase and B-50 protein revealed basic/acidic amino acid ratios in agreement with the respective IEP's. It is speculated that the inhibition of B-50 protein kinase may be related to known modulatory effects of ACTH and related peptides on certain types of neurotransmission and behaviour. **Key words:** ACTH—Kinase—Calcium—Cyclic nucleotides—Endorphins—Phosphorylation.

---

Evidence is accumulating that peptides may modulate the activity of certain protein kinases (Zwiers et al., 1976; Kemp et al., 1976; Davis and Ehrlich, 1978). With respect to the behaviorally active synthetic ACTH-fragments, it was shown that *in vitro* these peptides selectively reduce the endogenous phosphorylation of certain membrane-bound protein bands, as separated by polyacrylamide gel electrophoresis in SDS (SDS-PAGE; Zwiers et al.,

1976; 1978). The structural requirements for inhibiting the phosphorylation of these synaptic plasma membrane (SPM) proteins were strikingly similar to those necessary to induce excessive grooming when administered intraventricularly to the rat (Gispen et al., 1975; 1979; Zwiers et al., 1977, 1978). It could be demonstrated that inhibition of the phosphorylation of one synaptic plasma membrane (SPM) protein band (B-50; 48K) was the result of a direct in-

---

Received July 9, 1979; revised November 15, 1979; accepted December 10, 1979.

Address reprint requests to Dr. W. H. Gispen, Division of Molecular Neurobiology, Institute of Molecular Biology, State University of Utrecht, Padualaan 8, Utrecht, The Netherlands.

Part of this work was reported at the international meeting of the ISN at Jerusalem, 1979.

*Abbreviations used:* IEF, Isoelectric focussing; PAGE, Polyacrylamide gel electrophoresis; SDS, Sodium dodecylsulphate; SPM, Synaptic plasma membranes.

teraction of the peptide with the corresponding protein kinase (Zwiers et al., 1978). Subsequently, we reported that the protein kinase and its substrate could be solubilized from SPM using 0.5% Triton X-100 in 75 mM-KCl (Zwiers et al., 1979). The solubilized ACTH-sensitive B-50 protein kinase was separated from other protein kinases by column chromatography over DEAE-cellulose (Zwiers et al., 1979).

The present paper describes the purification of the B-50 protein kinase and its B-50 substrate protein using DEAE-cellulose column chromatography, ammonium sulphate precipitation, and two-dimensional slab gel electrophoresis.

## MATERIALS AND METHODS

### *Membrane Preparation and Solubilization of Membrane-bound Kinase*

The crude mitochondrial pellet (20 min at 10,000  $g_{av}$ ) was prepared at 0–4°C from 30 rat cerebral cortices (27 g) essentially as described by Whittaker (1969). This fraction was subjected to a hypo-osmotic shock (15 min stirring, 240 ml double-distilled water, 0–4°C), followed by centrifugation for 20 min at 10,000  $g_{av}$  (Sorvall SS 34). The supernatant containing light membrane fragments and soluble proteins (Terenius, 1973) was spun for 30 min at 100,000  $g_{max}$  in an ultracentrifuge (Sorvall OTD-2), using a Beckman SW 27.1 rotor to collect these floating membranes. Inspection of the particulate fraction by electron microscopy indicated the presence of mainly membranous material and small vesicles. The fraction was contaminated by some mitochondria, but hardly any myelin could be detected. The membranes were resuspended in 110 ml 6 mM-Tris, pH 8.1, containing 0.1 mM-dithiothreitol (buffer A). The membrane-bound B-50 protein kinase activity was then solubilized by mixing the membrane suspension with an equal volume of a solution of 1% Triton and 150 mM-KCl in buffer A. This mixture was vigorously shaken and allowed to stand for 15 min at 4°C with continuous stirring. Subsequently, residual membranes were spun down by another centrifugation at 100,000  $g_{max}$  for 30 min.

### *DEAE-Cellulose Column Chromatography*

The Triton extract was diluted to 450 ml with buffer B (10 mM-Tris-HCl, 1 mM- $CaCl_2$ , 0.1 mM-dithiothreitol, pH 7.4) and the resulting pH was adjusted to 7.4 using 2 M-HCl. The extract was then applied overnight to a DEAE-cellulose column (25 × 1.0 cm; DE 52, Whatman) previously equilibrated with buffer B. Subsequently the loaded column was washed with buffer B until the absorbance at 275 nm had reached background level and a linear salt gradient (0–400 mM-NaCl in buffer B, 200 ml total volume) was started. The column eluate was collected in 4-ml fractions and assayed for conductivity, protein content (see below), and protein kinase activity (see below and Zwiers et al., 1979).

### *Ammonium Sulphate Precipitation of DEAE-Eluate Fractions*

Eluate fractions containing ACTH-sensitive B-50 protein kinase activity (see below) were pooled (usually three fractions, i.e. 12 ml) and subjected to 55% ammonium sulphate saturation by slowly adding 350 mg ammonium sulphate per ml and allowing it to stand for 1 h at 0–4°C. Precipitated proteins were removed by centrifugation in a Sorvall OTD-2, Beckman SW 27.1 at 40,000  $g_{av}$  for 20 min. To the supernatant, 175 mg ammonium sulphate per ml was added (80% saturation). This mixture was allowed to sit for 30 min and was then centrifuged again for 20 min at 40,000  $g_{av}$ . The pellet (ASP 55-80) was dissolved in 0.5 ml of buffer B and dialysed overnight against 1 litre of buffer B.

### *Isoelectric Focussing (IEF) of the 55–80% Ammonium Sulphate Precipitate*

IEF was performed by slight modification of the method described by Winter et al. (1977). It was carried out for 5 h at 300 V, followed by 1 h at 450 V on a vertical slab gel of 5% (w/v) polyacrylamide in the presence of 2.5% (w/v) Ampholine (pH 3.5 to 9.5) in a cold room. The dimensions of the gel were 11 cm × 15 cm × 1.3 mm. After casting the gel, a comb was mounted on top resulting in 18 slots, which could contain 50- $\mu$ l sample aliquots. In order to minimize damage to enzyme activity, instead of using TEMED and ammonium persulphate (see Winter et al., 1977), polymerization of the gel was accomplished using riboflavin (0.1 mg/100 ml gel mixture) under TL light exposure. The redissolved, dialysed 55–80% ammonium sulphate protein (ASP 55-80) fraction was mixed with a solution containing Ampholine (pH 3.5–9.5) and sucrose resulting in final concentrations of 1% (w/v) Ampholine and 5% (w/v) sucrose (final sample volume 50  $\mu$ l). Immediately following electrophoresis, a gel track was cut into 5-mm slices which were eluted in 150- $\mu$ l double-distilled water for 2 h, and the pH of the eluates was measured. The other gel tracks were used for separation of the ASP 55-80 in a second dimension and for isolating the B-50 protein and its kinase (see below).

### *SDS-PAGE*

Phosphoproteins were separated on SDS-PA slab gels (acrylamide 11%; methylene bisacrylamide 0.20%) as first dimension essentially as described previously (Zwiers et al., 1976). Using SDS-PAGE as second dimension after IEF, similar SDS-PA gels were cast but now a comb was used which produced only two slots (one at each side) suitable for running reference samples. The space between the two slots was used for mounting an IEF gel track (dimensions: 10 cm × 1 cm × 1.3 mm), which was imbedded in a hot denaturing agarose solution (0.5% agarose, 62.5 mM-Tris-HCl, pH 6.5, 2% SDS, 10% glycerol, 0.001% Bromphenol blue and 5% 2-mercaptoethanol, 70°C). SDS-PAGE was carried out by stacking for 2 h at 15 mA followed by a run of 3 h at 30 mA (see Zwiers et al., 1976). SDS-PA gels were stained for proteins, using Fast Green, destained, dried, and subjected to autoradiography using Kodak Royal-X-Omat X-ray film (type XR-1) as described previously (Zwiers et

al., 1976). Quantitative determination of the radioactivity incorporated into phosphoprotein(s) was achieved, using liquid scintillation counting as described by Wiegant et al. (1978).

### Phosphorylation Assay

#### Endogenous protein kinase activity

Endogenous B-50 protein kinase activity was assayed under the following condition: 7.5  $\mu\text{M}$ -ATP, 1  $\mu\text{Ci}$  [ $\gamma$ - $^{32}\text{P}$ ]-ATP (3000 Ci/mmol, Amersham, U.K.), 15  $\mu\text{l}$  protein sample (Triton/KCl extract, DEAE column fractions, ammonium sulphate fractions), 50 mM-sodium acetate, 10 mM-magnesium acetate, 1 mM-calcium acetate, pH 6.5, in a final volume of 25  $\mu\text{l}$ . Routinely the protein samples were preincubated at 30°C for 5 min. The reaction was started by the addition of 5  $\mu\text{l}$  [ $\gamma$ - $^{32}\text{P}$ ]-ATP and the incubation was continued for 1 min at 30°C. The phosphorylation reaction was terminated by the addition of a denaturing solution as described previously (Zwiers et al., 1976), or by immersion in liquid nitrogen when separation of proteins was performed using IEF.

#### Effect of peptide or cyclic nucleotides

Synthetic ACTH-fragments and endorphins (Organon Int. b.v., Oss, The Netherlands) were added 10 s prior to the addition of ATP, resulting in a final concentration of  $10^{-5}$  M. Similarly in some instances the effect of cAMP or cGMP was studied (final concentration ranging from  $10^{-6}$  to  $10^{-5}$  M).

### Identification and Purification of B-50 Protein Kinase Using Two-Dimensional Gel Electrophoresis

Two hundred micrograms of the dialysed ASP 55-80 fraction was divided over 10 tracks of an IEF slab gel and subjected to IEF as described. After termination of the run, the gel was cut into 5-mm horizontal sections which individually were eluted in 0.5 ml 100 mM-Tris-HCl, pH 7.0, at 0–4°C overnight. Aliquots (15  $\mu\text{l}$ ) of each such slice eluate were subjected to SDS-PAGE as described above in order to locate the position of the B-50 protein by comparison of the slice protein profile with tracks with standard SPM and crude B-50 samples. The remaining portion of the slice eluates was then subjected to dialysis against buffer B for 6 h at 0–4°C. Aliquots (15  $\mu\text{l}$ ) of these dialysed slice eluates were assayed for endogenous phosphorylation, for phosphorylation in the presence of a 15- $\mu\text{l}$  aliquot of the B-50 substrate protein-containing fraction, and for phosphorylation of histon (0.25  $\mu\text{g}/15 \mu\text{l}$ ) in buffer B). These phosphorylation assays were carried out as described for the endogenous protein kinase activity (see above). After termination, proteins were separated, using SDS-PAGE. Radioactive phosphoproteins were detected, using autoradiography and liquid scintillation counting as described above.

### Amino Acid Analysis

The amino acid composition of purified B-50 protein and B-50 protein kinase was determined by automated

amino acid analysis (TSM, Technicon), using fluorescence detection with *o*-phthalaldehyde (Serva, Heidelberg) and 2-mercaptoethanol (Roth and Hampai, 1973). B-50 protein and its kinase were obtained after IEF followed by semi-preparative SDS-PAGE (18 samples per slab) as described in the foregoing section. The relevant slices containing Fast Green stained B-50 or its kinase were thoroughly washed in destaining solution (50% methanol, 10% acetic acid, 40% double-distilled water, v/v). The proteins were extracted from gel slices in 100 mM-Tris-HCl, pH 7.0, containing 0.1% SDS, and subsequently desalted by chromatography on Sephadex G25. The recovery of this extraction procedure was over 90% as checked by the measurement of the recovery of standard, prelabelled B-50 protein. The proteins (2  $\mu\text{g}$ ) were then transferred to test tubes, containing 50  $\mu\text{l}$  of a solution of 6 M-HCl and  $10^{-6}$  M-thioglycolic acid. The samples were evacuated under  $\text{N}_2$  and subsequently sealed. Hydrolysis was carried out at 110°C for 24 or 72 h. After lyophilization, the hydrolysate was dissolved in 40  $\mu\text{l}$  0.01 M-HCl containing thioglycol (0.1% v/v) and methylcellosolve (3.5% v/v). Samples were run in duplicate; each sample was followed by a protein hydrolysate standard (Pierce Chemical Comp., Illinois). Amino acid quantities as low as 50 pmol could be detected. The fluorescence detection procedure did not detect proline and cysteine.

### Determination of Protein

Protein determinations were carried out, using the method of Lowry et al. (1951). The total reaction volume was 110  $\mu\text{l}$ , allowing routine determinations in the range of 0.25–5  $\mu\text{g}$  protein.

## RESULTS

### Solubilization and Isolation of ACTH-Sensitive B-50 Protein Kinase Activity

The ACTH-sensitive phosphorylation of the endogenous B-50 phosphoprotein was used as reference in the solubilization and purification of B-50 protein kinase. In Table 1 the quantitative aspects of the isolation of solubilized B-50 protein kinase are summarized, using the phosphorylation of B-50 protein present in the various fractions without adding exogenous B-50 protein. The endogenous B-50 protein kinase activity was measured using linear incorporation conditions; throughout the purification procedure the B-50 phosphorylating activity could be inhibited by ACTH<sub>1–24</sub> ( $10^{-5}$  M) by 50% or more (data not shown).

Chromatography of the solubilized B-50 protein kinase on DEAE-cellulose led to a substantial loss of endogenous B-50 phosphorylating activity. The pooled fractions (DEAE-pool) exhibiting endogenous B-50 protein kinase activity contained only about 16% of the original solubilized enzyme activity. However, after DEAE-cellulose chromatography an increase in the specific activity (3 $\times$ ) of the B-50 protein kinase was observed.

TABLE 1. Summary of isolation of ACTH-sensitive protein kinase from rat brain membranes

Step <sup>a</sup>	Volume (ml)	Protein (mg)	Specific activity (pmol/mg/min)	Total activity (units) <sup>b</sup>
Triton/KCl extract	220	225	1.8	405
DEAE-pool	24	12	5.5	65
Ammonium sulphate (0–55%)	4	11	0.9	11
Ammonium sulphate (55–80%)	0.8	0.4	148	53

<sup>a</sup> Samples (1 ml) of all purification steps were dialysed overnight against 1 litre of buffer B. Some 15  $\mu$ g total protein of each step (in triplicate) was used in the phosphorylation assay.

<sup>b</sup> One unit is defined as the amount of endogenous B-50 kinase activity transferring 1 pmol phosphate to B-50 in 1 min at 30°C.

Further fractionation using ammonium sulphate hardly affected the total activity. The fraction precipitating between 55 and 80% ammonium sulphate (ASP 55-80) displayed a greatly enhanced specific activity.

Figure 1 summarizes qualitative aspects of the B-50 protein kinase isolation procedure, as visualized by protein patterns and autoradiographs after

one-dimensional separation on SDS-PA gels. The enhancement in specific activity observed in the ASP 55-80 was corroborated by the marked loss of protein bands with a relatively high molecular weight on SDS-PA, and with progressing purification a reduction in number of labelled phosphoproteins was also obtained, yielding one major phosphoprotein band in the ASP 55-80 fraction, B-50.

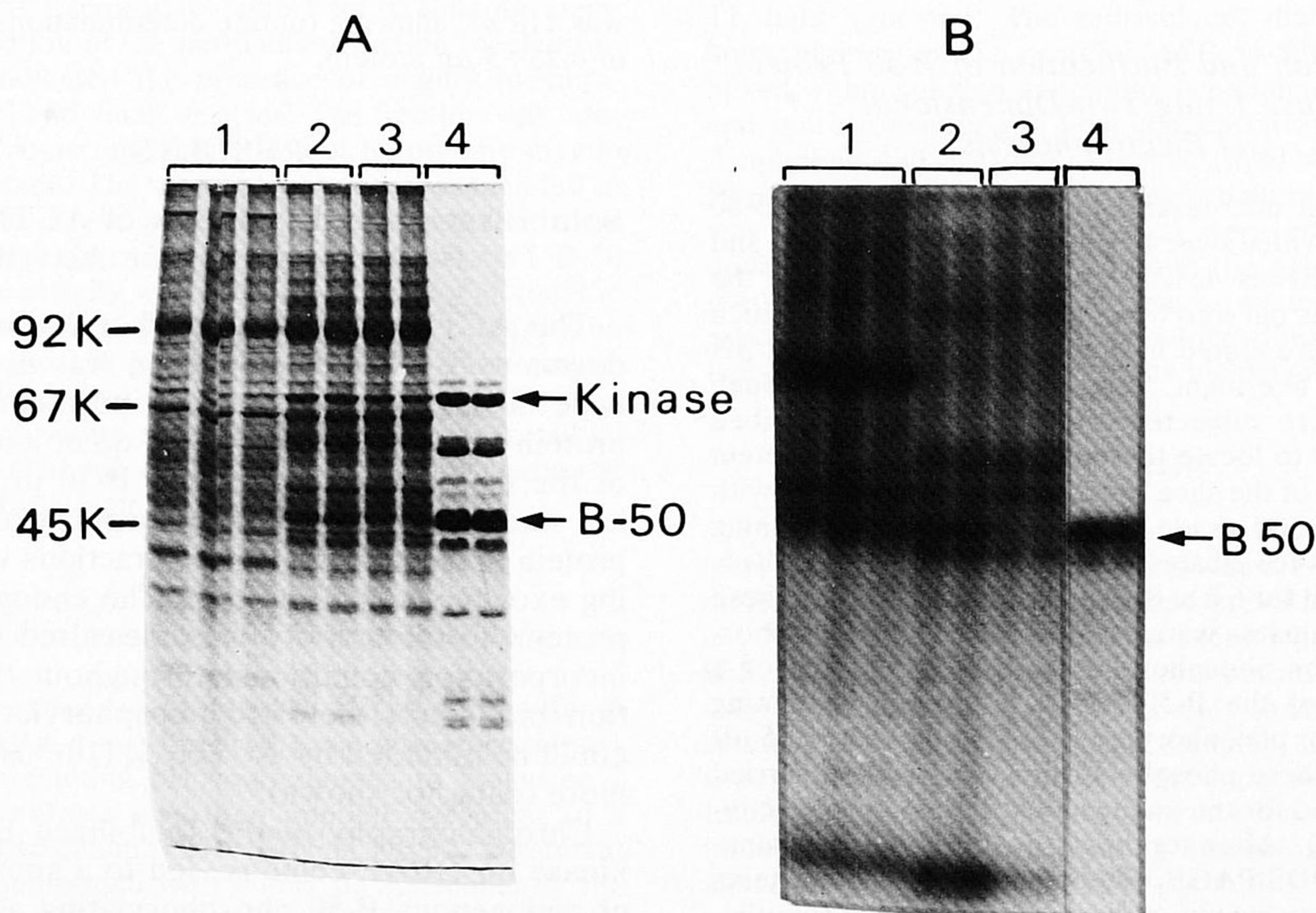


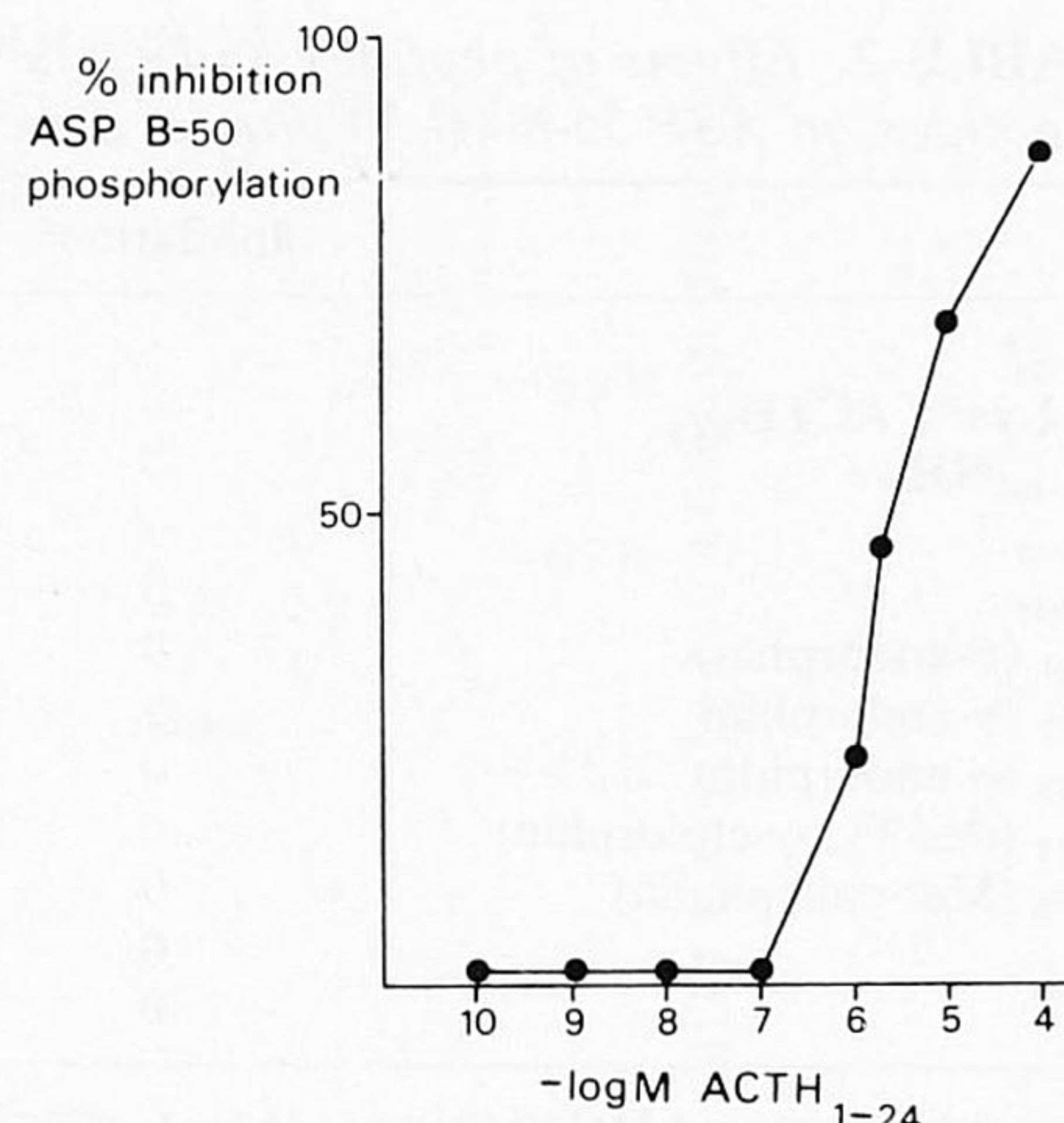
FIG. 1. (A) Protein-staining pattern of different steps of the purification of protein kinase and B-50 protein. The numbers on top refer to the following steps: (1) Triton X-100/KCl extract; (2) DEAE-pooled fractions; (3) 0–55% ammonium sulphate precipitate; (4) 55–80% ammonium sulphate precipitate. Tracks 1–3 contain 12  $\mu$ g of total protein. Track 4 contains 6  $\mu$ g of total protein. At the left, the positions of three molecular weight marker proteins; at the right, the positions of B-50 and kinase are indicated.

(B) Autoradiograph showing the corresponding endogenous phosphorylation profile. Phosphorylation was carried out for 1 min with 7.5  $\mu$ M-ATP (see Methods) using 1  $\mu$ Ci [ $\gamma$ - $^{32}$ P]ATP. Autoradiography took 16 h; because of high incorporation into B-50 (step 4) these tracks are from an autoradiograph which was exposed for 2 h.

### Characteristics of the Endogenous Phosphorylation of B-50 Proteins in the ASP 55-80 Fraction

The time course of endogenous phosphorylation of B-50 protein(s) in the ASP 55-80 fraction is shown in Fig. 2. During the first 10 min the incorporation was linear with time. Then the incorporation rate levelled off, reaching a maximum value between 30 and 60 min. Incubation of 4.5  $\mu$ g ASP 55-80 protein, containing 1  $\mu$ g B-50 protein(s) for 30 min led to an incorporation of 13.6 pmol  $\text{PO}_4$  per  $\mu$ g B-50 protein. In further experiments the endogenous B-50 protein kinase was studied, using 1 min of incubation time.

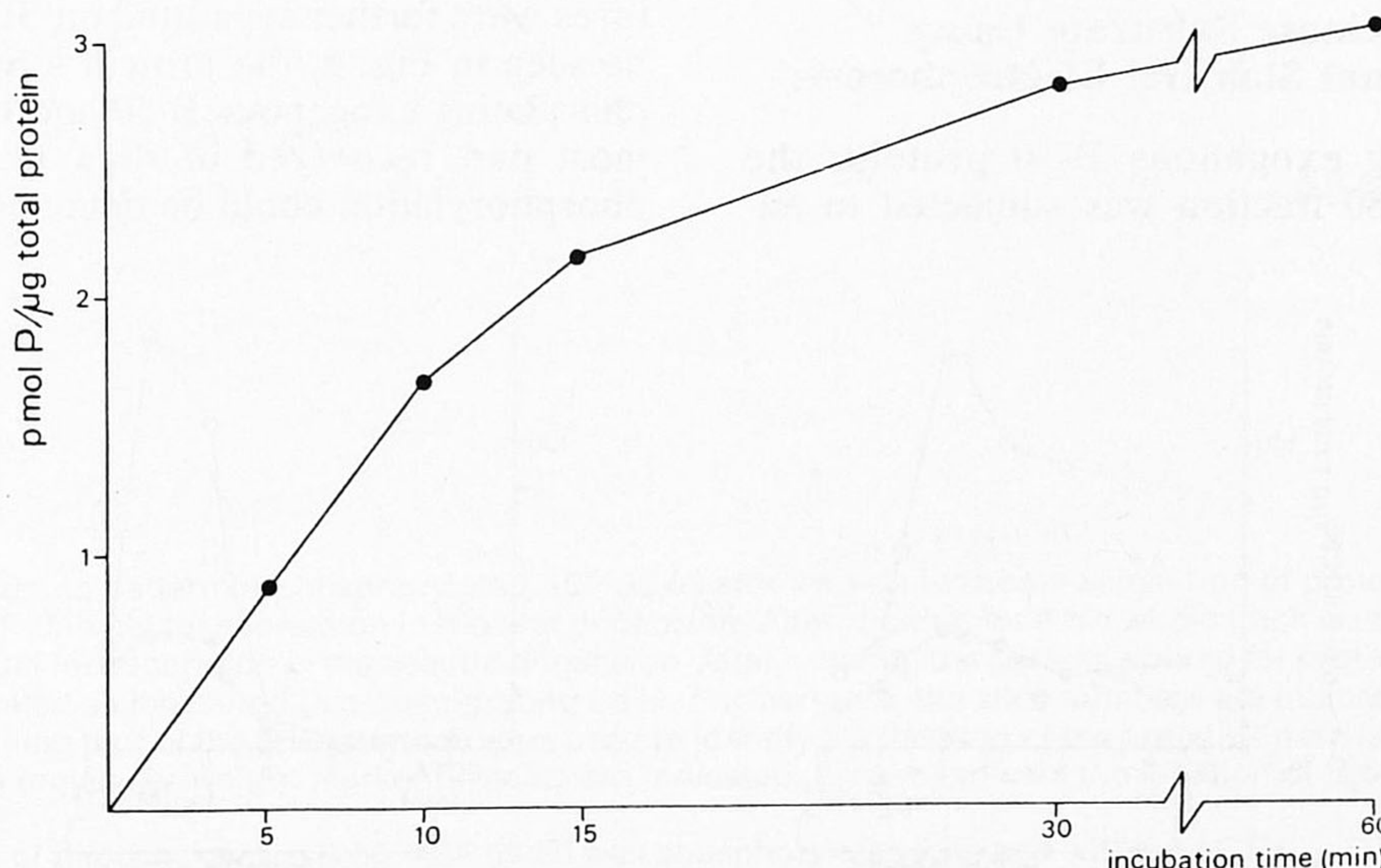
As expected from previous studies (Zwiers et al., 1976; 1978) in the presence of  $\text{ACTH}_{1-24}$  an inhibition of the endogenous B-50 protein kinase was observed (Fig. 3). The half-maximal effective concentration was  $5 \times 10^{-6}$  M- $\text{ACTH}_{1-24}$ . Concentrations equal to or lower than  $10^{-7}$  M were without effect. In further experiments, the ACTH sensitivity of the given preparation was studied, using  $10^{-5}$  M- $\text{ACTH}_{1-24}$ . In simultaneous experiments the effect of cAMP and cGMP (in concentrations of  $10^{-6}$ ,  $5 \times 10^{-6}$ , and  $10^{-5}$  M) on the endogenous B-50 protein kinase in the 55–80% ASP fraction was studied. Neither cyclic nucleotide in any of these concentrations had an effect, confirming previous observations on their ineffectiveness in intact and solubilized SPM material (Zwiers et al., 1976; 1979; Table 2). Synthetic fragments of lipotropin (Met-enkephalin, des-Tyr- $\gamma$ -endorphin,  $\beta$ -endorphin, etc.) were also without appreciable effect on the endogenous phosphorylation of B-50 in the dialysed ASP 55–80% fraction (Table 2). As reported previ-



**FIG. 3.** Dose-response curve for the inhibition of B-50 phosphorylation in ASP 55-80. Ten seconds prior to the  $[\gamma\text{-}^{32}\text{P}]\text{-ATP}$ , the peptide was added to the medium containing 4.5  $\mu$ g of ASP 55-80. Phosphorylation was carried out as described in Materials and Methods. Each point is the mean of two incubations. Similar results were obtained using three batches of ASP 55-80.

ously for intact SPM (Zwiers et al., 1978),  $\text{ACTH}_{1-10}$  and  $\text{ACTH}_{4-10}$  were ineffective, whereas  $[\text{Lys}^{17}, \text{Lys}^{18}]\text{ACTH}_{5-18}$  was as effective as  $\text{ACTH}_{1-24}$  (Table 2).

As can be seen in Fig. 4, the endogenous ASP-B-50 protein kinase required magnesium ions for its activity. If the ASP-fraction was dialysed against buffer B, containing 1 mM-EDTA, addition of  $\text{Mg}^{2+}$  in the presence of 1 mM  $\text{Ca}^{2+}$  resulted in a dose-dependent enhancement of B-50 protein phosphorylation with optimal conditions in the range of  $5 \times 10^{-3}$ – $10^{-2}$  M- $\text{Mg}^{2+}$ . A sharp drop in activity was



**FIG. 2.** Endogenous phosphorylation of protein B-50 in the ASP 55-80 fraction. Phosphorylation was studied as described in Materials and Methods using 4.5  $\mu$ g of total protein. The amount of incorporated radioactive phosphate was determined by liquid scintillation counting of the excised band B-50. Data are expressed as pmol phosphate incorporated per microgram total incubated protein. Each point is the mean of duplicate incubations. Similar results were obtained using three batches of ASP 55-80.

TABLE 2. Effects of peptides and cyclic nucleotides on ASP 55-80 B-50 protein kinase

	Inhibition <sup>a</sup>
ACTH <sub>1-24</sub> <sup>b</sup>	+
(Lys <sup>17</sup> , Lys <sup>18</sup> ) ACTH <sub>5-18</sub>	+
ACTH <sub>1-16</sub> -NH <sub>2</sub>	+
ACTH <sub>1-10</sub>	0
ACTH <sub>4-10</sub>	0
LPH <sub>61-91</sub> (β-endorphin)	0
LPH <sub>61-77</sub> (γ-endorphin)	0
LPH <sub>61-76</sub> (α-endorphin)	0
LPH <sub>62-77</sub> (des-Tyr-γ-endorphin)	0
LPH <sub>61-65</sub> (Met-enkephalin)	0
cAMP	0
cGMP	0

<sup>a</sup> Each substance was tested using at least two different batches of ASP 55-80. Incubations were performed in triplicate. 0 indicates less than 10% difference from control value; + indicates more than 50% decrease in B-50 phosphorylation.

<sup>b</sup> Concentration of 10<sup>-5</sup> M, except for cAMP and cGMP, which were tested in concentrations of 10<sup>-5</sup>, 5 × 10<sup>-6</sup>, and 10<sup>-6</sup> M.

found at 5 × 10<sup>-2</sup> M-Mg<sup>2+</sup>. Similarly, if the ASP-fraction was dialysed against buffer B, containing no Ca<sup>2+</sup> and 1 mM-EGTA, in the presence of 10 mM-Mg<sup>2+</sup>, a clear dependency on Ca<sup>2+</sup> ions could be demonstrated. The effect was biphasic, i.e., stimulation at low concentrations of Ca<sup>2+</sup> ions with a maximum of 10<sup>-3</sup> M, and an inhibiting effect at concentrations over 5 × 10<sup>-2</sup> M-Ca<sup>2+</sup>. Under optimal Mg<sup>2+</sup> and Ca<sup>2+</sup> conditions (10 mM-Mg<sup>2+</sup>, 1 mM-Ca<sup>2+</sup>) the largest effect of ACTH<sub>1-24</sub> (10<sup>-5</sup> M) was observed.

#### Purification of B-50 Protein and the B-50 Protein Kinase Substrate Using Two-Dimensional Slab Gel Electrophoresis

Without adding exogenous B-50 protein, the dialysed ASP 55-80 fraction was subjected to en-

dogenous phosphorylation with or without the presence of ACTH<sub>1-24</sub>. Then the proteins were separated using IEF, and subsequently one track of the IEF gel was mounted on top of a SDS-PA gel and the proteins were separated according to their molecular weight.

In order to identify the location of B-50 protein(s), the protein pattern was visualized by Fast Green staining and compared to that of a standard sample containing total SPM proteins (Fig. 5A). The ACTH sensitivity of the B-50 phosphorylation was established using autoradiography and liquid scintillation counting (Fig. 5, B). After two-dimensional slab gel electrophoresis, more than one protein with an apparent molecular weight in SDS of 48,000 was found. However, these proteins had different isoelectric points (in a pH range of 4.5–8). The corresponding autoradiograph yielded only one labelled phosphoprotein (IEP 4.5) with a molecular weight of 48,000. Phosphorylation in the presence of ACTH<sub>1-24</sub> (10<sup>-5</sup> M) resulted in the same two-dimensional protein pattern, but the incorporation of radioactive phosphate into 48,000 phosphoprotein was reduced by 70% as determined by liquid scintillation counting of the dissected stained protein spot. Thus the purified B-50 protein is an acidic substrate protein with a molecular weight of 48,000 and an IEP of 4.5.

In order to identify the B-50 protein kinase activity, 200 μg of dialysed ASP 55-80 protein was subjected to IEF (10 tracks). After elution of the 5-mm gel slices, the endogenous phosphorylation of each dialysed fraction was studied, as well as its capacity to phosphorylate pure, native B-50 protein, as isolated from IEF gels, and histon (see Methods). After this phosphorylation assay, the protein mixtures were further separated on SDS-PAGE. As can be seen in Fig. 6, the protein kinase activity phosphorylating exogenous B-50 and histon was for the most part recovered in slice 14. No endogenous phosphorylation could be detected in the slice con-

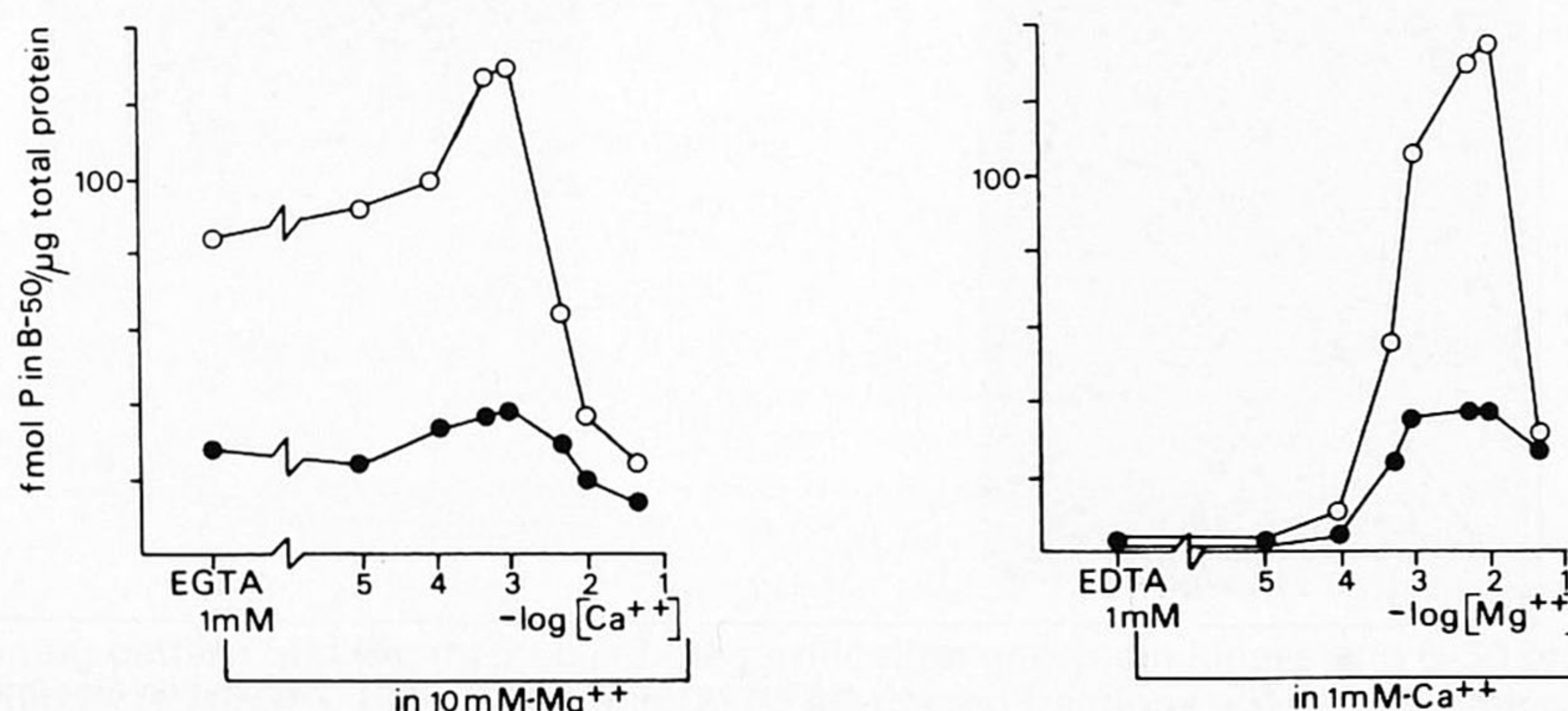
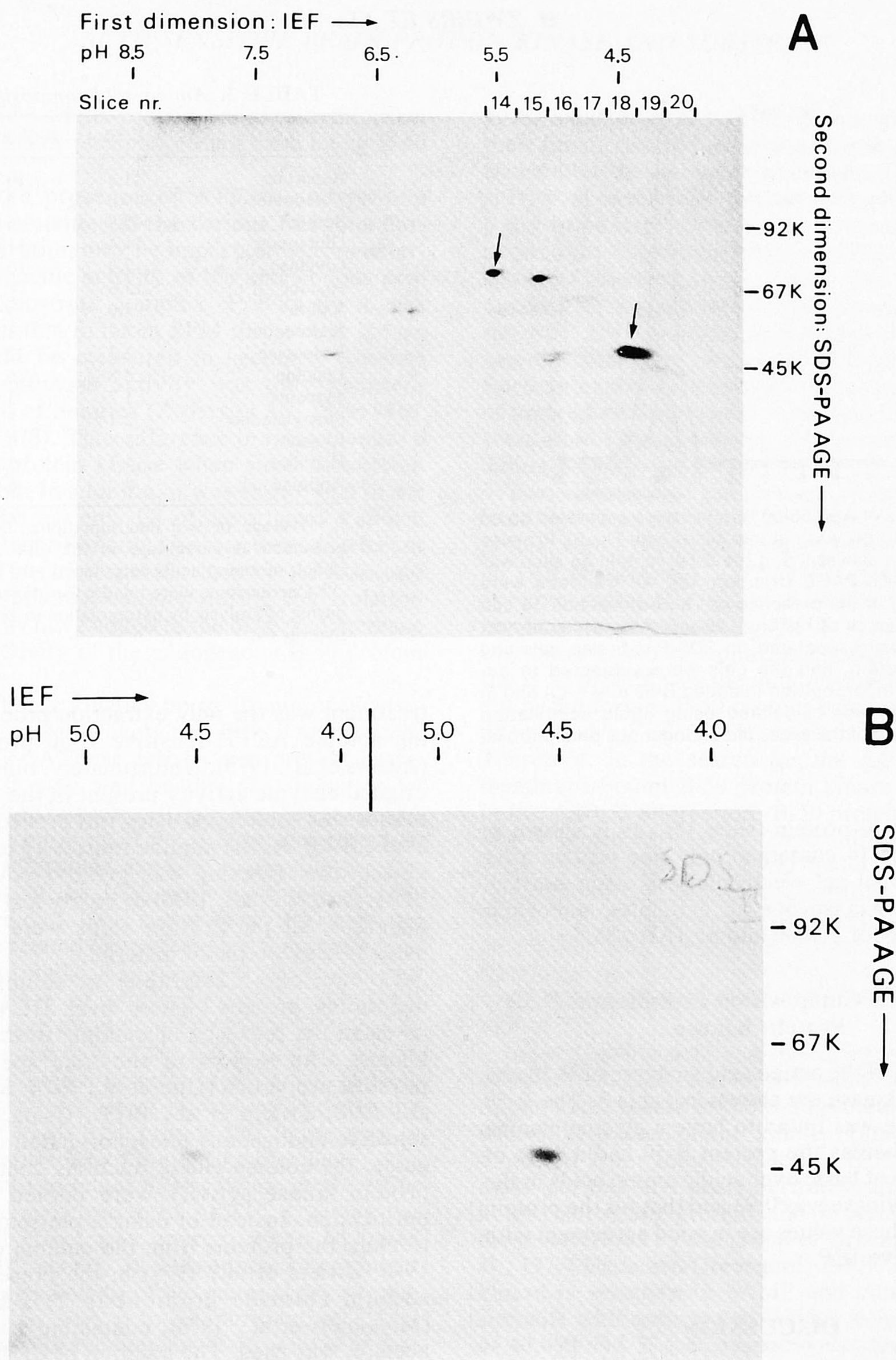
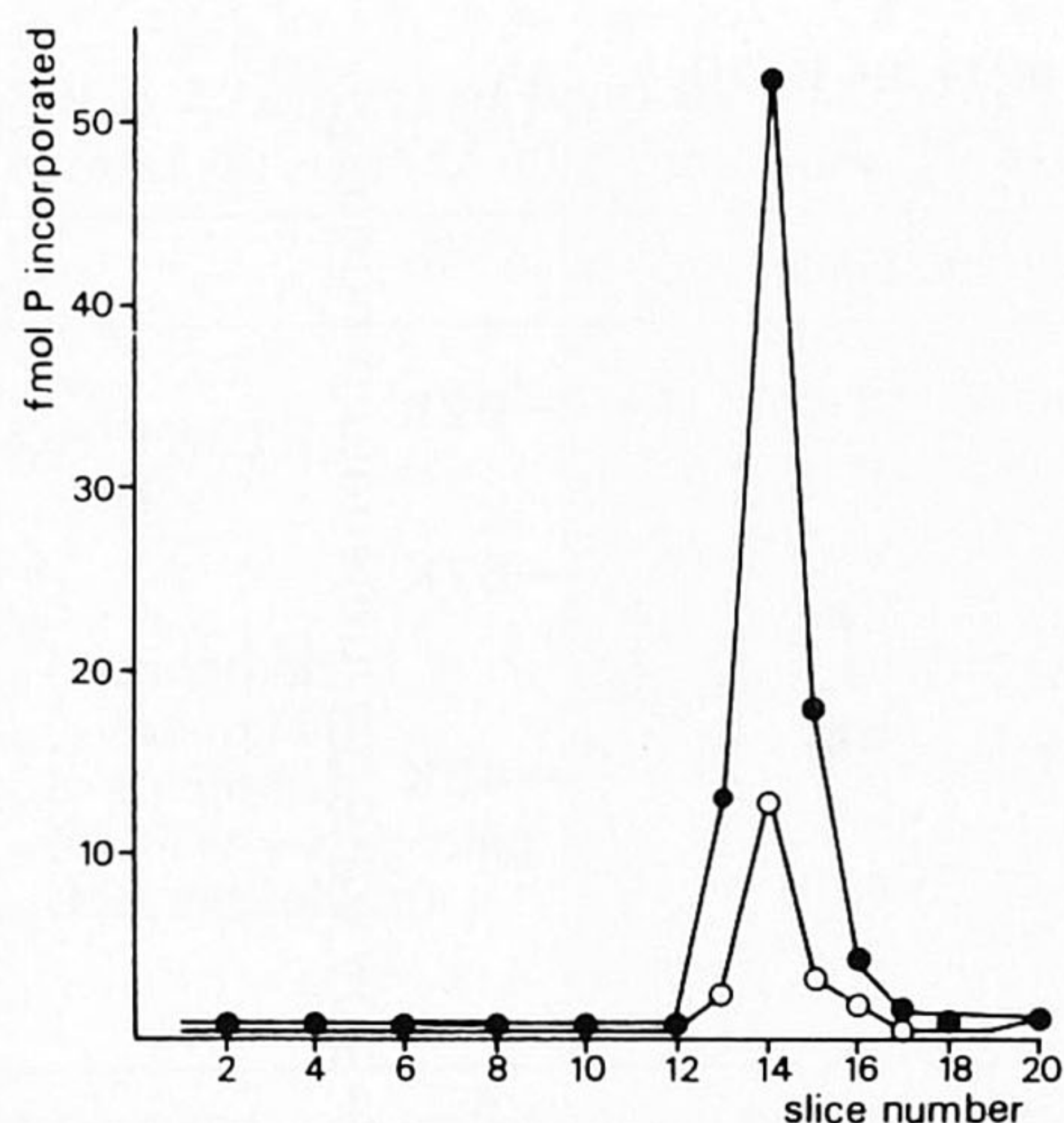


FIG. 4. Effect of Ca<sup>2+</sup> and Mg<sup>2+</sup> on the phosphorylation of B-50 in ASP 55-80. (left) ASP 55-80 was dialysed overnight against 10 mM-Tris-HCl, pH 7.4, containing 1 mM-EGTA and 0.1 mM-dithiothreitol. Phosphorylation was carried out in 1 mM-EGTA, 7.5 μM-ATP, 1 μCi [γ-<sup>32</sup>P]ATP, and 4.5 μg total protein for 1 min. (right) ASP 55-80 was dialysed overnight against buffer B containing 1 mM-EDTA. Phosphorylation was carried out in 1 mM-EDTA (as for left figure). (○—○) incubation without ACTH<sub>1-24</sub>; (●—●) incubation in the presence of ACTH<sub>1-24</sub> (10<sup>-5</sup> M).



**FIG. 5 (A)** Protein-staining pattern of phosphorylated ASP 55-80 after two-dimensional separation of proteins. Samples ( $4.5 \mu\text{g}$ ) were applied to an IEF slab gel for separation in the first dimension. After running for 6 h a whole track was cut out and mounted on top of a SDS slab gel for separation in the second dimension. After running, the gel was stained for proteins. At the top, the pH in the IEF gel is indicated as measured in a co-migrating track. Furthermore, the slice numbers are indicated, corresponding to fractions of the remaining part of the IEF gel which were used to identify the location of the protein kinase (see Fig. 6). At the right the positions of three molecular weight marker proteins are indicated. Arrows indicate the location of B-50 (48,000) and kinase (70,000).

**(B)** Autoradiograph of phosphorylated B-50. ASP 55-80 was phosphorylated with and without  $\text{ACTH}_{1-24}$  ( $10^{-5} \text{ M}$ ) using  $4.5 \mu\text{g}$  of total protein. After incubation at  $30^\circ\text{C}$  for 1 min the reaction mixture was placed in liquid  $\text{N}_2$ . The two samples were thawed and applied as quickly as possible on an IEF slab gel. After running, the lower halves of the two tracks were mounted on a SDS gel for separation in the second dimension. The left half of the autoradiograph shows the incorporation into B-50 when  $\text{ACTH}_{1-24}$  was co-incubated (1368 d.p.m.  $^{32}\text{P}$  in B-50), the right half the control incubation (4589 d.p.m. in B-50). At the top the pH of corresponding IEF gel slices is indicated.



**FIG. 6.** Proteins of ASP 55-80 fraction were separated on an IEF slab gel. The gel was cut into slices (see Fig. 5). Proteins were eluted and dialysed, and the B-50-containing slice was localized by SDS-PAGE (fraction 18). All fractions were phosphorylated in the presence of 15  $\mu$ l of fraction 18 and also in the presence of histon (0.25  $\mu$ g). After phosphorylation, proteins were separated on SDS-PAGE slab gels and stained for protein, and the gels were subjected to autoradiography. Incorporation in added B-50 (○—○) and in histon (●—●) was calculated using liquid scintillation counting. In none of the slices did endogenous phosphorylation occur.

taining the B-50 protein (slice 18). As is shown in fig. 5, A, slice 14 contained only one protein after two-dimensional gel electrophoresis. The purified B-50 protein kinase has an estimated molecular weight in SDS of 70,000 and an IEP of 5.5.

#### Amino Acid Composition of B-50 and B-50 Protein Kinase

The results of the amino acid analyses of B-50 and B-50 protein kinase are shown in Table 3. The B-50 protein kinase was found to have a glycine-alanine ratio of 2, whereas the protein B-50 had a ratio of 0.5. The ratio of basic over acidic amino acids in the B-50 protein kinase was 0.93 and that for the protein B-50, 0.66. These values are in good agreement with their respective IEP.

#### DISCUSSION

In the present paper, the purification and some characteristics of a newly identified rat brain membrane protein kinase and (one) of its substrate protein(s) are reported.

The membrane-bound protein kinase, which is responsible for the phosphorylation of a membrane-bound protein substrate (MW 48,000, B-50) and is modulated by the behaviorally active peptide ACTH<sub>1-24</sub>, was solubilized from rat brain membranes using 0.5% Triton X-100 in 75 mM-KCl. From a variety of detergent procedures used, this

**TABLE 3.** Amino acid composition<sup>a</sup>

	B-50	B-50 kinase
Aspartate	11.8	10.3
Threonine <sup>b</sup>	9.0	5.2
Serine <sup>b</sup>	7.9	6.6
Glutamate	14.4	11.6
Glycine	7.9	16.4
Alanine	15.6	8.3
Valine	5.0	4.0
Methionine	1.4	1.7
Isoleucine	2.3	2.5
Leucine	3.8	6.1
Tyrosine	1.2	3.0
Phenylalanine	2.1	3.9
Lysine	8.8	6.0
Histidine	4.2	7.9
Arginine	4.2	6.5

<sup>a</sup> Average of two determinations. Data are presented as percentage of the total amount (pmol) of amino acids determined.

<sup>b</sup> Corrections were made for degradation during hydrolysis by extrapolation to zero-time hydrolysis.

treatment was the only extraction procedure yielding soluble ACTH-sensitive B-50 protein kinase (Zwiers et al., 1979). Unfortunately, only 16% of the original enzyme activity present in the intact membranes was solubilized using this procedure (Zwiers et al., 1979). As the peptide sensitivity of the protein kinase after freezing was greatly reduced (intact SPM; Zwiers et al., 1976) or nearly lost (solubilized material), all purification steps were carried out using freshly prepared material.

Column chromatography of solubilized brain membrane protein kinases over DEAE-cellulose revealed the presence of multiple forms of protein kinases with respect to substrate specificity and physical properties (Uno et al., 1976; Miyamoto et al., 1978; Zwiers et al., 1979). Using the ACTH-sensitive endogenous phosphorylation of B-50 as a guide, the column eluate fractions containing B-50 protein kinase activity were pooled for further purification. Instead of using a phosphate gradient to elute the proteins from the column (Uno et al., 1976; Zwiers et al., 1979) in the present study, a sodium chloride gradient in Tris-HCl buffer (Miyamoto et al., 1978), containing 1 mM-calcium acetate, was used. The addition of Ca<sup>2+</sup> to this and all other buffers used was based on the finding that in intact SPM the endogenous phosphorylation of B-50 was Ca<sup>2+</sup>-dependent (Gispen et al., 1979).

The endogenous B-50 phosphorylating activity in the DEAE-pool could be isolated by collecting the fraction precipitating between 55 and 80% ammonium sulphate (ASP 55-80). Since the endogenous phosphorylation was measured, most likely enzyme and substrate were still in an aggregated or complexed form. The increased specific activity of the ASP 55-80 fraction was reflected by a marked

elimination of various protein bands and the presence of one major phosphoprotein band being B-50 (Fig. 1).

Clearly, the presence of ATPase and protein phosphatase activities in the various fractions during the purification may be important factors determining the specific activity of the endogenous protein kinase-substrate complex. Previously it was demonstrated that in intact SPM the protein kinase activity could be measured in seconds, whereas protein phosphatase activity was only apparent over a period of minutes (Zwiers et al., 1978; Wiegant et al., 1978). This difference in rates favours a role for the protein kinase when short incubation times are used. In addition, it was shown that in the Triton-KCl extract only 1% of the original ATPase activity of intact SPM could be recovered (Zwiers et al., 1979). Thus the specific loss of ATPase and protein phosphatase activity during the isolation procedure may have added to the observed increase in specific activity of the endogenous B-50 protein kinase.

From the linear kinetics up to 10 min of incubation (Fig. 2), it seems likely that little if any protein phosphatase or ATPase activity was present in the ASP 55-80. It may well be that the reduction in phosphorylation rate after 10 min (Fig. 2) was caused by a gradual saturation of the B-50 protein. If so, at least two assumptions must be made. Firstly, the determination of the B-50 protein content of ASP 55-80 is based on a 100% recovery of B-50 from the gels after the second dimension electrophoresis (see Methods). Secondly, it is assumed that there is one site susceptible of phosphorylation per B-50 molecule. Under these conditions, already after 30 min of incubation 65% of the available sites will be phosphorylated (13.6 pmol/21 pmol B-50). In view of the unknown degree of phosphorylation of the original ASP 55-80 B-50 protein this figure may in fact reflect saturation of the B-50 molecule.

As compared to intact SPM (Zwiers et al., 1976; band 6) the concentration of ACTH<sub>1-24</sub> giving the half-maximal effect was approx. 10 to 50 times lower ( $5 \times 10^{-6}$  M; Fig. 3). This enhancement might be related to the dialysis of the material prior to the phosphorylation assay. In all purification steps, dialysis enhanced endogenous B-50 phosphorylating activity and the percentage inhibitory effect by ACTH (Zwiers et al., *in preparation*). Davis and Ehrlich (1978) have also reported effects of prior dialysis on rat membrane phosphorylation.

The ion sensitivity of the phosphorylation of B-50 in intact SPM was not identical to that in the ASP 55-80 fraction. In SPM, phosphorylation of B-50 was totally dependent on the presence of  $\text{Ca}^{2+}$ ; however, in the ASP fraction only a reduced  $\text{Ca}^{2+}$  sensitivity was observed. Recently, for other cAMP-dependent or non-dependent membrane phosphoproteins (Delorenzo, 1976; Hershkowitz,

1978; Sieghart et al., 1979), it was speculated that their function could involve a role in exocytosis of transmitter at the nerve terminals. The  $\text{Ca}^{2+}$  sensitivity of membrane protein phosphorylation appears to be dependent on the presence of a  $\text{Ca}^{2+}$ -dependent regulator protein (calmodulin, MW 15,000; Delorenzo et al., 1979). The observed reduced  $\text{Ca}^{2+}$  sensitivity of B-50 phosphorylation in the ASP 55-80 fraction is probably caused by the loss of calmodulin during the isolation procedure. Such an explanation may also account for the loss of units of endogenous B-50 protein kinase activity seen after DEAE column chromatography of the Triton-KCl extract (Table 1).

Two-dimensional purification of the phosphorylated substrate protein B-50 yielded one phosphoprotein with a molecular weight in SDS of 48,000 and an IEP of 4.5 (Fig. 5). The other proteins with an apparent molecular weight of 48,000 (Fig. 5) could not be phosphorylated by the B-50 protein kinase, even after exhaustive phosphorylation. Apparently, the B-50 protein was the only phosphoprotein present in the former B-50 protein band and was phosphorylated by an ACTH-sensitive protein kinase. Therefore, in the search for the ACTH-sensitive membrane-bound B-50 protein kinase also present in the ASP 55-80 fraction, B-50 protein isolated by one-dimensional IEF of ASP 55-80 served as substrate. Thus, after IEF of ASP 55-80, to the aliquots of dialysed slice eluates native B-50 protein was added. After subsequent SDS-PAGE it could be established that the protein kinase responsible for the exogenous B-50 protein phosphorylation had an apparent molecular weight in SDS of 70,000 and an IEP of 5.5.

Indeed, amino acid analysis of microgram quantities of the kinase and its B-50 substrate showed a rather acidic nature of the B-50 protein and a rather neutral character of the kinase (Table 3), as was expected from their respective IEP's. The only other purified and characterized brain membrane-bound substrate protein and protein kinase are those described by the group of Greengard (Uno et al., 1977; Ueda and Greengard, 1977). Their protein kinase is sensitive to cAMP and consists of two subunits with apparent molecular weights (in SDS) of 40,000 and 52,000, respectively; the IEP of the native protein is 5.5. The ACTH-sensitive B-50 protein kinase is insensitive to cAMP (Zwiers et al., 1976; this paper) and has a higher molecular weight in SDS. Likewise, the substrate protein I is different from the presently purified B-50 protein as protein I has an IEP of 10.3 and a molecular weight in SDS of 86,000. Thus, the two membrane-associated phosphorylation systems are clearly different.

Using specific antibodies against B-50 protein, immunofluorescent localization of B-50 protein in slices from rat brain structures suggested an accumulation of fluorescence in regions rich in

neuronal projections and a virtual absence of fluorescence in cell bodies (Oestreicher et al., 1979). Although in the present study a less enriched synaptosomal membrane fraction was used as compared to previous studies, the B-50 protein and its kinase can be studied in subcellular fractions enriched in synaptosomal plasma membranes (Zwiers et al., 1976; 1978; 1979). The histochemical observations so far are in line with such cellular localization of B-50 protein.

With respect to the mechanism by which ACTH inhibited the endogenous phosphorylation of B-50, it should be noted that ACTH itself also contains serine residues in positions 1 and 3. Thus, the inhibition of the endogenous phosphorylation could be envisaged to result from a favorable phosphorylation of ACTH<sub>1-24</sub> by the B-50 protein kinase. However, the ineffectiveness of ACTH<sub>1-10</sub> and effectiveness of ACTH<sub>5-18</sub> (lacking Ser<sup>1,3</sup>) to inhibit B-50 protein kinase in intact SPM (Zwiers et al., 1978) or in ASP 55-80 (Table 2) suggests that the phosphorylation of the exogenous peptide cannot be sufficient to explain the mechanism. In support of this notion was the absence of radioactive peptides (running in the bromophenol front) in autoradiographs obtained from SDS-PA slab gels not subjected to the fixation, staining, and destaining procedure (data not shown). Yet, it may be that an amino acid sequence of ACTH, although a basic peptide, resembles part of the sequence of B-50 protein involved in the interaction with the catalytic site of the kinase. Such a peptide-induced inhibition was recently demonstrated to exist for cAMP-dependent protein kinase from beef skeletal muscle (Feramisco and Krebs, 1978).

With respect to the significance of the ACTH sensitivity of the membrane B-50 phosphorylation system, little if any speculation seems possible. Certainly the effect of ACTH on B-50 protein kinase was not shared by C-terminal fragments of  $\beta$ -LPH (endorphins; Table 2). Yet, in recent studies morphine and endorphins were shown to inhibit the endogenous phosphorylation of a variety of SPM protein bands (Davis and Ehrlich, 1978; O'Callaghan et al., 1979).

Structure-activity studies on the *in vitro* inhibition of B-50 protein kinase by ACTH-fragments indicated a remarkable resemblance to the structural requirements of these peptides to induce excessive grooming in the rat after intracerebroventricular (i.c.v.) administration (Zwiers et al., 1978; Gispen et al., 1975; 1979). Furthermore, after i.c.v. injection of ACTH<sub>1-24</sub> into rats, followed by *in vitro* phosphorylation of isolated synaptosomal membranes, the same protein bands were influenced as after addition of the peptide to the *in vitro* phosphorylation assay (Zwiers et al., 1977). These data, combined with the calcium dependency of B-50 phosphorylation *in vitro*, may point to a molecular mechanism

by which ACTH and related peptides may modulate specific neurotransmission by an alteration of the phosphorylated state of certain membrane phosphoproteins (Gispen et al., 1979).

## ACKNOWLEDGMENT

The authors wish to express their gratitude to Marleen Schmidt-Michels and Jan Brakkee for their excellent technical assistance. Prof. John E. Wilson and Thea Frankenhuys-van den Heuvel are herewith thanked for their help with the IEF of membrane proteins.

## REFERENCES

- Davis L. G. and Ehrlich Y. H. (1978) Neuropeptides and protein phosphorylation in brain membranes. *Soc. Neurosci.* **4**, abstract no. 1281.
- Delorenzo R. J. (1976) Calcium-dependent phosphorylation of specific synaptosomal fraction proteins: Possible role of phosphoproteins in mediating neurotransmitter release. *Biochem. Biophys. Res. Commun.* **71**, 590-597.
- Delorenzo R. J., Freedman S. D., Yoke W. B., and Maurer S. C. (1979) Stimulation of Ca<sup>2+</sup>-dependent neurotransmitter release and presynaptic nerve terminal protein phosphorylation by calmodulin and a calmodulin-like protein isolated from synaptic vesicles. *Proc. Natl. Acad. Sci. USA* **76**, 1838-1842.
- Feramisco J. R. and Krebs E. G. (1978) Inhibition of cyclic AMP-dependent protein kinase by analogues of a synthetic peptide substrate. *J. Biol. Chem.* **253**, 8968-8971.
- Gispen W. H., Wiegant V. M., Greven H. M., and De Wied D. (1975) The induction of excessive grooming in the rat by intraventricular application of peptides derived from ACTH: Structure-activity studies. *Life Sci.* **17**, 645-652.
- Gispen W. H., Zwiers H., Wiegant V. M., Schotman P., and Wilson J. E. (1979) The behaviorally active neuropeptide ACTH as neurohormone and neuromodulator: The role of cyclic nucleotides and membrane phosphoproteins, in *Modulators, Mediators and Specifiers in Brain Function* (Ehrlich Y. H. and Davis L. G., eds), pp. 199-224, Plenum Press, New York.
- Hershkowitz M. (1978) Influence of calcium on phosphorylation of a synaptosomal protein. *Biochim. Biophys. Acta* **542**, 274-283.
- Kemp B. E., Benjamini E., and Krebs E. G. (1976) Synthetic hexapeptide substrates and inhibitors of 3':5'-cyclic AMP-dependent protein kinase. *Proc. Natl. Acad. Sci. USA* **73**, 1038-1042.
- Lowry O. H., Rosebrough N. J., Farr A. L., and Randall R. J. (1951) Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* **193**, 265-275.
- Miyamoto E., Miyazaki K., Hirose R., and Kashiba A. (1978) Multiple forms of protein kinases in myelin and microsomal fractions of bovine brain. *J. Neurochem.* **31**, 269-275.
- O'Callaghan J. P., Williams N., and Clouet D. H. (1979) The effect of morphine on the endogenous phosphorylation of synaptic plasma membrane proteins of rat striatum. *J. Pharmacol. Exp. Ther.* **208**, 96-105.
- Oestreicher A. B., Zwiers H., Schotman P., and Gispen W. H. (1979) Immunohistochemical studies of a phosphoprotein (B-50), modulated in rat brain membranes by ACTH. *Abstr. 7th Int. Meet. ISN*, Jerusalem.
- Roth M. and Hampai A. (1973) Column chromatography of amino acids with fluorescence detection. *J. Chromatogr.* **83**, 353-356.

- Sieghart W., Forn J., and Greengard P. (1979)  $\text{Ca}^{2+}$  and cyclic AMP regulate phosphorylation of same two membrane-associated proteins specific to nerve tissue. *Proc. Natl. Acad. Sci. USA* 76, 2475-2479.
- Terenius L. (1973) Stereospecific interaction between narcotic analgesics and a synaptic plasma membrane fraction of rat cerebral cortex. *Acta Pharmacol. Toxicol.* 32, 317-320.
- Ueda T. and Greengard P. (1977). Adenosine 3',5'-monophosphate-regulated phosphoprotein system of neuronal membranes. I. Solubilization, purification and some properties of an endogenous phosphoprotein. *J. Biol. Chem.* 252, 5155-5163.
- Uno I., Ueda T., and Greengard P. (1976) Differences in properties of cytosol and membrane-derived protein kinases. *J. Biol. Chem.* 251, 2192-2195.
- Uno I., Ueda T., and Greengard P. (1977) Adenosine 3',5'-monophosphate-regulated phosphoprotein system of neuronal membranes. II. Solubilization, purification and some properties of an endogenous adenosine 3',5'-monophosphate-dependent protein kinase. *J. Biol. Chem.* 252, 5164-5174.
- Whittaker V. P. (1969) The synaptosome, in *Handbook of Neurochemistry*, (Lajtha A., ed), vol. 2, pp. 327-364. Plenum Press, New York.
- Wiegant V. M., Zwiers H., Schotman P., and Gispen W. H. (1978) Endogenous phosphorylation of rat brain synaptosomal plasma membranes *in vitro*: some methodological aspects. *Neurochem. Res.* 3, 443-453.
- Winter A., Ek K., and Andersson U. B. (1977) Analytical electrofocussing in thin-layer polyacrylamide gels. *L.K.B. Application Note* 250.
- Zwiers H., Veldhuis D., Schotman P., and Gispen W. H. (1976) ACTH, cyclic nucleotides and brain protein phosphorylation *in vitro*. *Neurochem. Res.* 1, 669-677.
- Zwiers H., Wiegant V. M., Schotman P., and Gispen W. H. (1977) Intraventricular administered ACTH and changes in rat brain protein phosphorylation: a preliminary report, in *Mechanisms, Regulation and Special Function of Protein Synthesis in the Brain* (Roberts S., Lajtha A., and Gispen W. H., eds), pp. 267-272. Elsevier-North Holland Biomedical Press, Amsterdam.
- Zwiers H., Wiegant V. M., Schotman P., and Gispen W. H. (1978) ACTH-induced inhibition of endogenous rat brain protein phosphorylation *in vitro*: structure-activity. *Neurochem. Res.* 3, 455-463.
- Zwiers H., Tonnaer J., Wiegant V. M., Schotman P., and Gispen W. H. (1979) ACTH-sensitive protein kinase from rat brain membranes. *J. Neurochem.* 33, 247-256.