

Resolution of Rat Brain Synaptic Phosphoprotein B-50 into Multiple Forms by Two-Dimensional Electrophoresis: Evidence for Multisite Phosphorylation

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Abstract: Phosphoprotein B-50 was extracted from rat brain membranes by alkaline extraction and purified by ammonium sulphate precipitation and flat-bed isoelectric focusing. The purified protein shows microheterogeneity upon isoelectric focusing in a narrow pH gradient (pH 3.5–5.0). As visualized by two-dimensional gel electrophoresis, B-50 resolved into four clearly separated forms which differ slightly in isoelectric point. The forms are in part mutually convertible by exhaustive phosphorylation (using protein kinase C) and dephosphorylation (using *Escherichia coli* alkaline phosphatase). Proteolysis with *Staphylococcus aureus* protease yielded two radioactive peptides. Analysis of their molecular weights and the time

course of their formation suggests that B-50 was cleaved at only one specific site. Our data indicate the presence of more than one phosphorylatable site. The possibility that the heterogeneity of B-50 was in part due to a glycoprotein nature of B-50 was studied extensively. However, none of the six different methods used revealed the presence of glyco-moieties in B-50. **Key Words:** Phosphoprotein B-50—Protein kinase—Phospholipids—Multisite phosphorylation—Glycoproteins. **Zwiers H. et al.** Resolution of rat brain synaptic phosphoprotein B-50 into multiple forms by two-dimensional electrophoresis: Evidence for multisite phosphorylation. *J. Neurochem.* **44**, 1083–1090 (1985).

Although brain membranes are particularly enriched in phosphoproteins (Weller, 1979), only two of them thus far have been isolated and characterized. The first phosphoprotein isolated was protein I, M_r approximately 80 kD, by the group of Greengard (Ueda and Greengard, 1977). This protein, presently called synapsin I (Huttner et al., 1983), appeared to be extremely basic (IEP 10.3). The other protein, called B-50, was isolated in 1980 (Zwiers et al., 1980) and appeared to be a major acidic protein present in synaptic plasma membranes (SPM) (IEP 4.5; Zwiers et al., 1980; Kristjansson et al., 1982). Evidence was obtained suggesting that B-50 kinase and the Ca^{2+} , phospholipid-dependent protein kinase described by the group of Nishizuka (Takai et al., 1979) are similar if not identical (Aloyo et al., 1983). B-50 was exclusively found in the particulate fraction of brain

tissue (Kristjansson et al., 1982). Immunohistochemistry provided evidence that B-50 was distributed throughout the brain, but concentrated in regions rich in synaptic contacts (Oestreicher et al., 1981). Presumably, it is localized in presynaptic membranes (Sörensen et al., 1981; Oestreicher et al., 1983). Recently we studied the interspecies distribution of B-50. A class of B-50 proteins was detected in the brain of mammals and birds; the molecular weight of these proteins ranged from 47 to 53 kD (Oestreicher et al., 1984).

Gower and Rodnight (1982) considered the possibility that the anomalous migration of B-50 in sodium dodecyl sulphate (SDS)-polyacrylamide gels (see also Mahler et al., 1982; Oestreicher et al., 1984) might be related to a microheterogeneity caused by the presence of carbohydrate residues. Indeed it has been shown that differences in sialic

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Abbreviations used: BSA, Bovine serum albumin; Con A,

Concanavalin A; IEP, Isoelectric point; kD, 10^3 Daltons; M_r , Relative molecular weight; PBS, Phosphate buffered saline; SAP, *Staphylococcus aureus* protease; SDS-PAGE, Polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulphate; SPM, Synaptic plasma membranes.

acid content and state of phosphorylation result in considerable heterogeneity in various proteins (Hammann, 1977).

In the present paper we report that B-50 can be resolved into multiple forms by isoelectric focusing, provided a very narrow pH range of ampholines is used. Evidence is presented showing that differences in the phosphorylation state of B-50 underlie at least part of the observed microheterogeneity and that this heterogeneity is not due to the presence of glyco-moieties in B-50.

MATERIALS AND METHODS

Chemicals

All chemicals used were of analytical grade. [γ - 32 P]ATP (specific activity 2900 Ci/mmol) was obtained from New England Nuclear (U.S.A.). L-[1- 3 H]Fucose (specific activity 2 Ci/mmol) was purchased from Amersham (U.K.).

Sephadex IEF, concanavalin A (Con A)-sepharose-4B, PD-10 columns, and molecular weight marker proteins were purchased from Pharmacia Fine Chemicals (Sweden). Ampholines (pH 4.0–6.0 and pH 3.5–5.0) were from LKB Produkter (Sweden); *Staphylococcus aureus* protease (SAP) was from Miles Laboratories (U.K.); transferrin (human), phosphorylase B (rabbit muscle), and serum albumin (bovine) were from Sigma Chemical Co. (U.S.A.).

Alkaline phosphatases were obtained from Boehringer (F.R.G.; calf), Miles (U.K.; calf), and Worthington (U.S.A.; *E. coli*). Acid phosphatase (potato) was from Boehringer (F.R.G.). Purified pig brain phosphatidylserine was a generous gift from Dr. B. Poorthuis (Department of Biochemistry, State University of Utrecht).

Isolation of B-50

A procedure involving alkaline extraction of the protein from membranes was used according to Oestreicher et al. (1983) with slight modifications. In short, the procedure (performed at 0–4°C) consisted of the following steps: (1) rat brain tissue (100 g wet weight) was homogenized in 4 ml of distilled water per gram wet weight, followed by centrifugation for 20 min at 48,000 g (Sorvall SS-34); (2) the pellet was suspended in 200 ml 10 mM Mg acetate and the pH was adjusted to 11.5 with 2 M NaOH. Solubilized proteins were separated from particulate material by centrifugation (see 1); (3) the decanted supernatant was neutralized to pH 5.5 with 1 M Na acetate at pH 5.0. Centrifugation (see 1) yielded a supernatant enriched in B-50; (4) the supernatant was heated for 15 min at 70°C, resulting in precipitation of the bulk of the proteins, except B-50. Centrifugation (see 1) yielded a supernatant that was enriched in B-50; (5) with treatment of this supernatant with ammonium sulphate, B-50 and some other proteins precipitated at between 57% and 82% saturation. Again this precipitate was collected by centrifugation (see 1) for 20 min. The pellet was dissolved in 6 ml 10 mM Mg acetate (pH 6.5); (6) the final purification step consisted of isoelectric focusing on a Sephadex IEF flat bed (for details, see Oestreicher et al., 1983). The B-50-containing fractions were pooled and applied to a prepacked Sephadex G-25 column (Pharmacia PD-10) and eluted with 10 mM NH_4 -formate (pH 7.0). The void volume was collected, lyophilized, and dissolved in 10 mM Na acetate

buffer at pH 6.5 (protein concentration about 0.5 $\mu\text{g}/\mu\text{l}$) and dialyzed overnight against 1 L of the same buffer to remove trace amounts of ampholines. The purity of the B-50 thus obtained was assessed by SDS-polyacrylamide gel electrophoresis (PAGE).

Isolation of kinase C

Kinase C was isolated as described previously by Aloyo et al. (1983). In short, the soluble fraction of whole brains (minus cerebellum) of 20 female Wistar rats (200 g body weight) was fractionated using DEAE-cellulose chromatography and Sephadex G-100 gel filtration. Kinase C activity was monitored by testing fractions for histone kinase activity in the absence and presence of phosphatidylserine (10 $\mu\text{g}/\text{ml}$ final concentration), using a filter paper assay. The peak of the kinase C activity, eluting from the Sephadex G-100 column, was used for all further experiments.

Phosphorylation of B-50

B-50 (0.5 μg) was preincubated for 5 min at 30°C with 2 μg of the kinase C preparation (final volume: 20 μl) in a phosphorylation buffer consisting of 10 mM Na acetate, 10 mM Mg acetate, and 0.1 mM Ca acetate at pH 6.5. Phosphatidylserine was present in a final concentration of 20 $\mu\text{g}/\text{ml}$. The phosphorylation reaction was started by the addition of 5 μl of ATP (containing 2 μCi [γ - 32 P]ATP) dissolved in phosphorylation buffer. The final concentration of ATP was 0.1 mM. The reaction was stopped after different times of phosphorylation by either adding 12.5 μl of a denaturing solution containing SDS (this solution is further referred to as denaturing solution; see Zwiers et al., 1976) or by immersion in liquid nitrogen when B-50 was analyzed by two-dimensional (2D) gel electrophoresis (see below). The samples of B-50 and protein kinase C used were devoid of endogenous B-50 phosphatase activity.

Radioactive B-50 was prepared by incubation of 200 μg of purified B-50 (in 1 ml) with 400 μg kinase C and 100 μCi [γ - 32 P]ATP under conditions described above. After phosphorylation for 90 min at 30°C the total incubate was applied to a flat bed of Sephadex IEF, containing ampholines. Labelled B-50 was separated and recovered as described above.

Dephosphorylation of B-50

Radioactive B-50 (3 μg , containing 20,000 dpm [32 P]phosphate) was incubated at 30°C with 4 μg alkaline phosphatase (*E. coli*, Worthington) in 50 mM Tris (pH 8.0). The final volume was 25 μl , and the incubation was terminated after different incubation times by adding denaturing solution or immersion in liquid nitrogen. Samples were subjected to SDS-PAGE (10% polyacrylamide) or 2D gel electrophoresis and analyzed for the amount of protein and radioactivity.

Separation of proteins by 1D and 2D electrophoresis

For 1D electrophoresis, the denatured proteins were separated by SDS-PAGE (see Zwiers et al., 1976). The 2D separation was performed as described by Kristjansson et al. (1982) with slight modifications. In short, the protein samples (25 μl) were pretreated with urea (8.5 M final concentration) and Triton X-100 (0.5% vol/vol final concentration). A mixture of ampholines (2.5% wt/vol; pH range 3.5–10.0) and sucrose (5% wt/vol final con-

centration) was added to the pretreated samples. The samples were applied to a 5% polyacrylamide gel (0.15% bisacrylamide) containing ampholines (pH range 3.5–5.0; final concentration 2.5% wt/vol) and urea and Triton X-100 (final concentration, respectively, 8.5 M and 0.5% vol/vol).

The IEF gel was run overnight at 200 V at room temperature. Individual tracks were cut and mounted on SDS-polyacrylamide slab gels (11% polyacrylamide; see Kristjansson et al., 1982). Gels were stained for protein with Fast Green, protein was quantified as described below, and radioactivity was determined by autoradiography and liquid scintillation counting.

Proteolysis of B-50

Purified radioactive B-50 (1.5 μ g, containing 10,000 dpm [32 P]phosphate) was incubated at 30°C in the presence of 50 ng SAP in 25 μ l 125 mM Tris (pH 7.4). Proteolysis was stopped after different incubation times by adding denaturing solution, immediately followed by heating for 10 min at 90–100°C and immersion in liquid nitrogen (SAP is not inactivated by SDS treatment). The samples were quickly thawed just prior to SDS-PAGE (15% acrylamide).

Con A-Sepharose affinity chromatography and glycoprotein determinations

Con A-Sepharose 4B (1.2 ml) was washed thoroughly with 20 ml of an equilibration buffer consisting of 50 mM Tris-HCl, 150 mM NaCl, 1 mM CaCl_2 , 1 mM MgCl_2 , and 1 mM MnCl_2 (pH 7.4). The sample (about 40 μ g of a crude B-50 preparation and 85 μ g of transferrin, dissolved in 3 ml of equilibration buffer) was added to the Con A column and mixed by gentle rotation for 1 h at 22°C. The column was eluted stepwise with three times 3 ml of phosphate buffered saline (PBS), followed by four times 3 ml of 0.1 M Na borate (pH 6.5) to elute glycoproteins (Kennedy and Rosevear, 1973). Samples (30 μ l) were analyzed by SDS-PAGE (11% gels) and stained for proteins with a sensitive silver staining procedure (Merril et al., 1981).

Three different methods for the visualization of glycoproteins in polyacrylamide gels were used employing periodic acid Schiffs' base (Zacharius et al., 1969), dansylhydrazin (Eckhardt et al., 1976), and Con A-peroxidase (Pohle et al., 1980), respectively.

Incorporation of [3 H]fucose into glycoproteins was tested according to Popov et al. (1976). Light synaptosomal plasma membranes were prepared from hippocampal tissue and proteins were separated by 2D gel electrophoresis (Kristjansson et al., 1982). Radioactivity incorporated into specific protein spots including B-50 was measured by liquid scintillation counting (Popov et al., 1976).

Quantification of proteins

The method of Lowry et al. (1951) was used to determine the amount of total protein. The amount of pure B-50 was estimated by densitometric scanning of Fast Green-stained gel patterns with known amounts of bovine serum albumin (BSA) as standard. Scanning was performed at 650 nm with a Zeiss PM-QII spectrophotometer with KM3 chromatography attachment.

RESULTS

Exhaustive phosphorylation and dephosphorylation of B-50

B-50 was purified to homogeneity by a modification of the method described by Oestreicher et al. (1983). The yield of B-50 was about 30 μ g/g tissue (wet weight). Figure 1A, lane 1, shows the protein profile of B-50 (0.5 μ g) as separated on an 11% SDS slab gel. In agreement with previously published data, a M_r of 48 kD was calculated. Lane 2 shows the protein staining pattern of the semipurified kinase C. The major band (M_r 70 kD) was identified to possess the kinase activity (Aloyo et al., 1983) and has the same characteristics as the previously characterized B-50 kinase (Zwiers et al.,

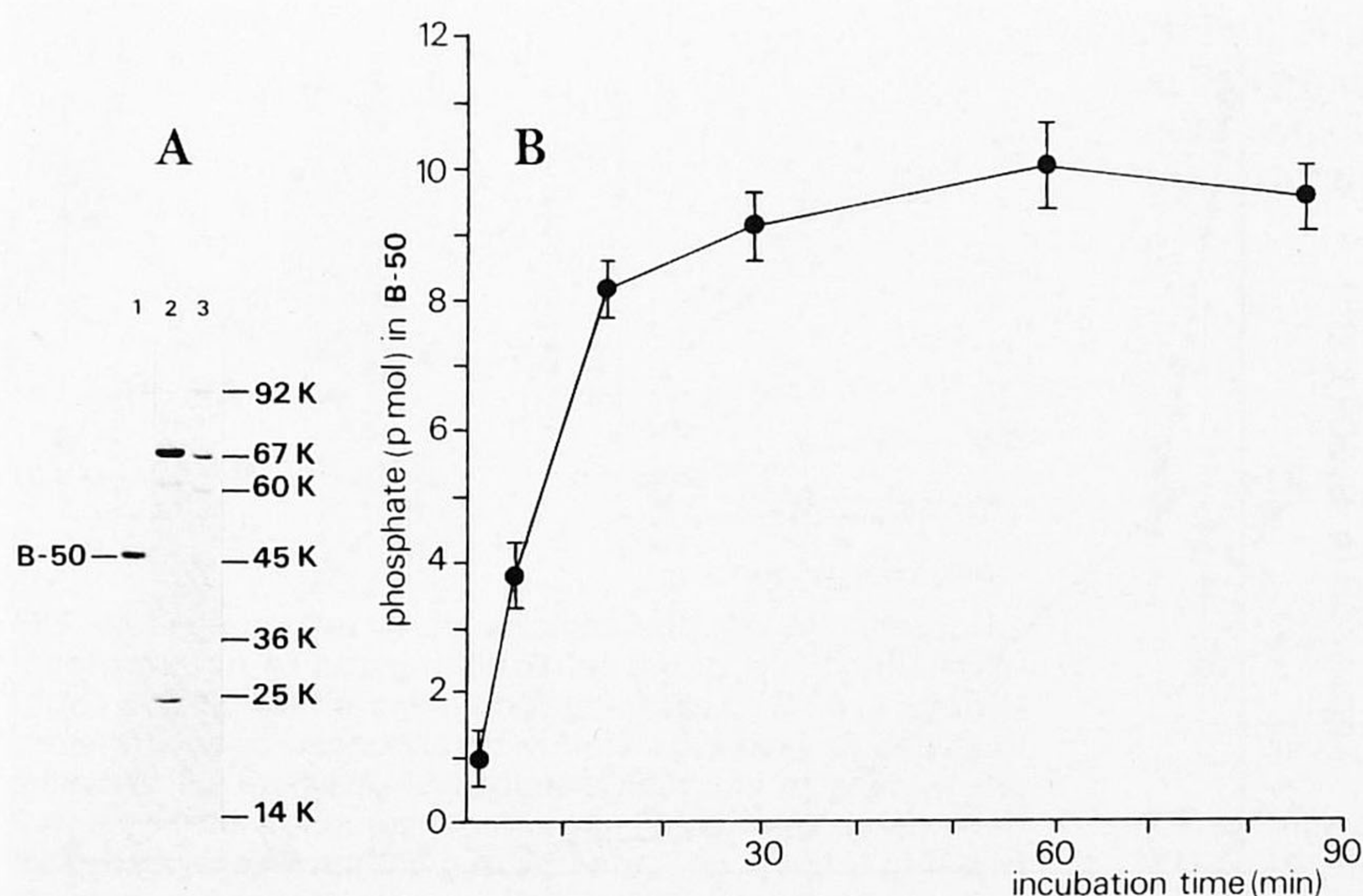


FIG. 1. A: Protein staining pattern after SDS-PAGE. Track 1, 0.5 μ g B-50; track 2, 2 μ g semipurified protein kinase C; track 3, molecular weight marker proteins; phosphorylase (92 kD), bovine serum albumin (67 kD), catalase (60 kD), ovalbumin (45 kD), lactate dehydrogenase (36 kD), trypsin inhibitor (25 kD), and α -lactalbumin (14 kD). **B:** Time course of the incorporation of phosphate in 0.5 μ g B-50 (about 10 pmol protein). Mean of duplicate incubations.

1980). Figure 1B shows the time course of the phosphorylation of purified B-50 by the kinase C preparation. B-50 is phosphorylated to a high level and incorporates up to about 10 pmol of phosphate per 0.5 μg B-50, amounting to about 1 molecule phosphate per molecule B-50. In control experiments we found that the amount of phosphate incorporated was not enhanced in the presence of other ATP concentrations (7.5 μM , 500 μM , or 1000 μM). Also, addition of fresh kinase C after 60 min of incubation was ineffective, suggesting that the amount of empty sites of B-50 was the limiting factor and not the availability of ATP and active protein kinase C.

Radiolabelled B-50 was obtained on a preparative scale (100 μg protein) by phosphorylating purified B-50 with kinase C using [γ - ^{32}P]ATP as phosphate donor. After labelling, B-50 was further purified by isoelectric focusing on a flat bed. B-50 thus obtained had a specific radioactivity of about 10,000 cpm phosphate/ μg B-50 protein (about 0.25 mol phosphate/mol B-50) and was used as substrate for four commercially available phosphatases. The action of the phosphatases on B-50 was visualized by SDS-PAGE and autoradiography (10% gels). It appeared that the amount of B-50 was greatly reduced after treatment with three of the four phosphatases tested, which was most likely due to contaminating protease activity (data not shown). Only alkaline phosphatase from *E. coli* was able to dephosphorylate B-50 without having detectable proteolytic activity.

In Fig. 2 (inset) the protein staining pattern of the phosphatase and B-50 mixture is shown. In the standard 11% gels, B-50 and this phosphatase comigrated; however, in 10% gels (cross-linking 0.1%) they separated completely, enabling us to show that

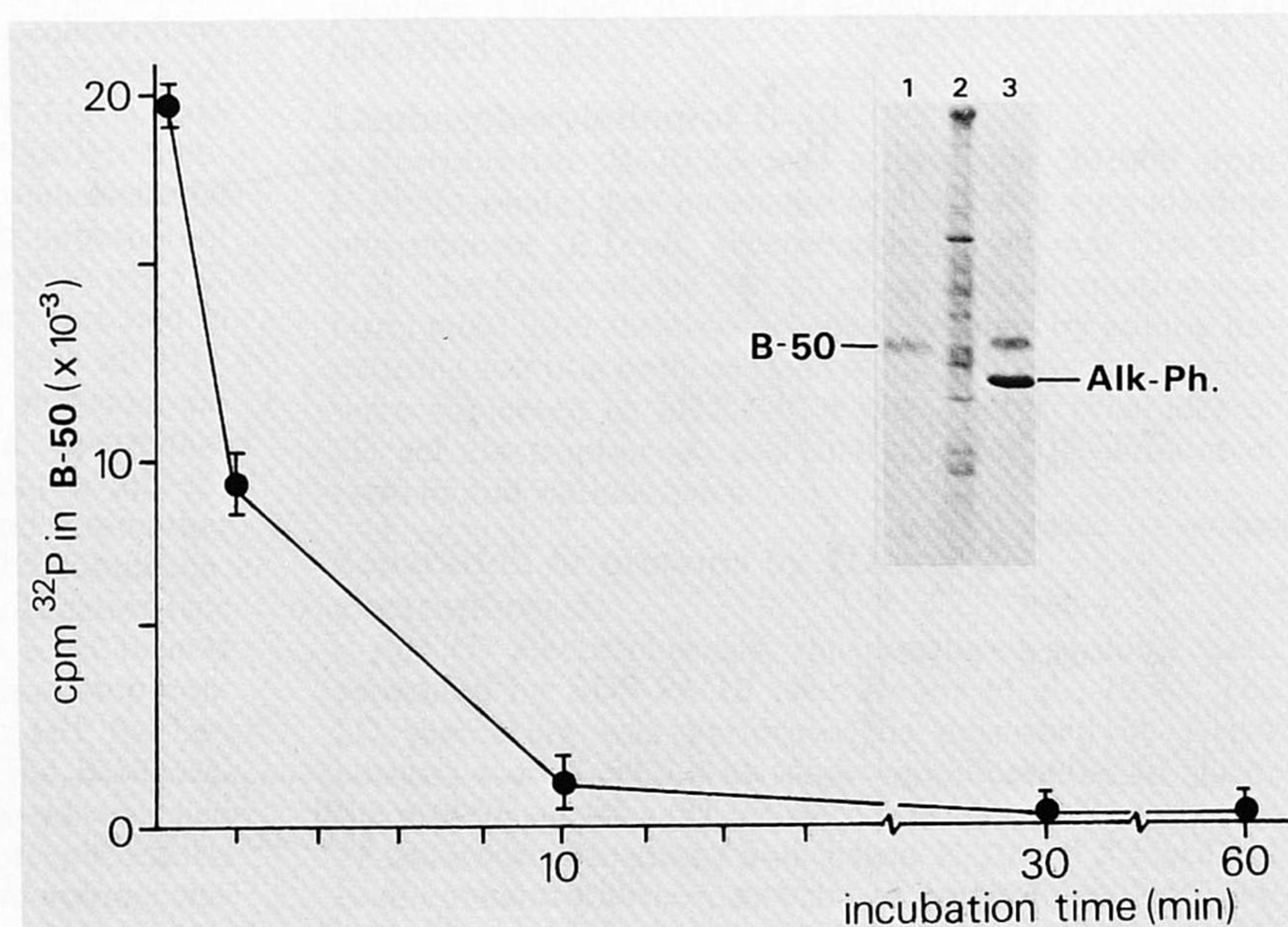
B-50 was not proteolytically degraded. The time course of the dephosphorylation of radioactive B-50 by the alkaline phosphatase shows that after 2 min at 30°C 50% of the label and after 10 min even 95% has been removed. Parallel incubation of B-50 for 60 min at 30°C without phosphatase did not reduce the recovery of radioactivity in B-50.

Microheterogeneity of B-50

Purified B-50 was subjected to isoelectric focusing in polyacrylamide slab gels containing a very narrow pH range (3.5–5.0). Analysis of Fast Green-stained proteins in the 2D gels revealed that B-50 resolved into four individual spots (Fig. 3, part B). Measurement of the pH of relevant gel slices with a microelectrode indicated that the difference between the IEPs of the most basic (form 1) and the most acidic component was approximately 0.17 pH unit. Spots 1 and 4 represent minor components, whereas 2 and 3 are the major forms present in about equal amounts.

Upon phosphorylation of B-50 with kinase C (60 min at 30°C) prior to 2D analysis, we observed that the distribution pattern of the B-50 iso-forms is shifted toward the more acidic forms of B-50. Quantification of stained protein by densitometric scanning indeed showed that the amount of spot 4 is greatly enhanced, whereas those of 1 and 2 are decreased (Fig. 3, part A). On the other hand, incubation of B-50 with *E. coli* alkaline phosphatase prior to 2D analysis (Fig. 3, part C) resulted in a maximal intensity of the relatively basic forms of B-50. Form 1 is greatly enhanced, whereas the staining intensities of 3 and 4 are reduced. To identify the spots unequivocally, labelled B-50 was mixed with cold, phosphatase-treated B-50 and subjected to 2D analysis (data not shown). As ex-

FIG. 2. Time course of the dephosphorylation of radioactive B-50 (3 μg protein) by *E. coli* alkaline phosphatase (4 μg protein). Mean of duplicate incubations. **Inset:** Protein staining patterns after SDS-PAGE of B-50 (lane 1, 1 μg protein), SPM proteins purified according to Kristjansson et al. (1982) (lane 2, 15 μg protein), and B-50 (1 μg protein) and *E. coli* alkaline phosphatase (2 μg protein) together (lane 3).



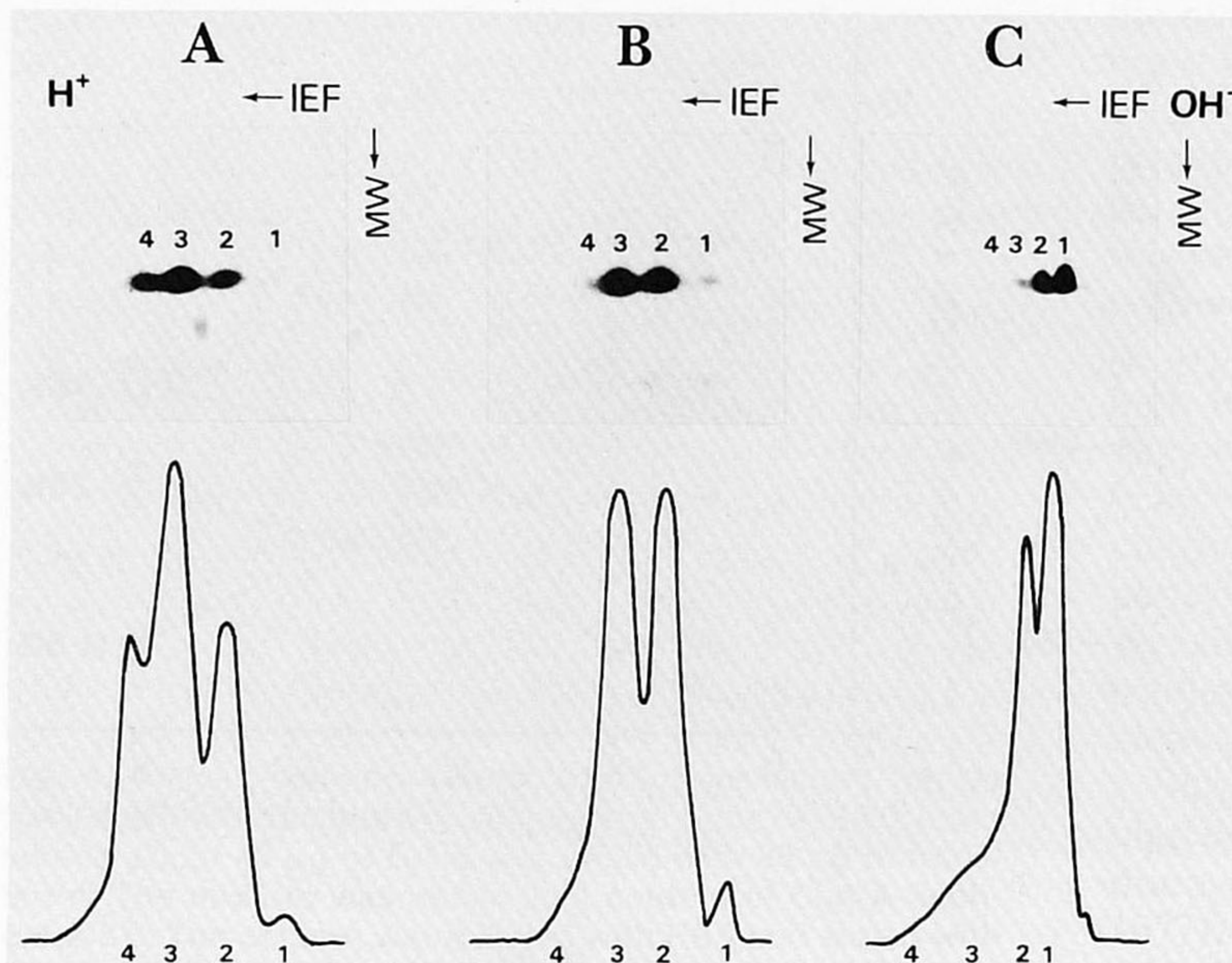


FIG. 3. Upper part: Protein staining pattern of B-50 (3 μ g) after 2D separation. Only the B-50 containing part of the 2D gels is shown. The direction of isoelectric focusing was from right to left and the separation in SDS gels from top to bottom. **A:** Incubation of B-50 at 30°C with 5 μ g protein kinase C for 60 min. **B:** Incubation with buffer. **C:** Incubation with 4 μ g of alkaline phosphatase (*E. coli*) for 60 min. **Lower part:** Densitometric tracings (at 650 nm) of protein staining patterns shown in the upper part of the figure.

pected, the spots with maximal protein staining (1 and 2) did not coincide with the spots having maximal radioactivity (3 and 4). Furthermore, all four protein spots could be stained with the specific anti-B-50 immunoglobulin Gs prepared by Oestreicher et al. (1983) (data not shown).

The relationship between the observed shifts in iso-forms was further analyzed by monitoring the changes in Fast Green staining after various times of incubation with kinase C. Figure 4 (part A) shows that there is a time-dependent decrease in the amounts of protein of forms 1 and 2, concomitant with an increase in form 4. Analysis of radioactivity in the individual iso-forms (Fig. 4, part B) shows that form 1 does not contain label, whereas 3 and 4 have incorporated a substantial amount. In Fig. 5

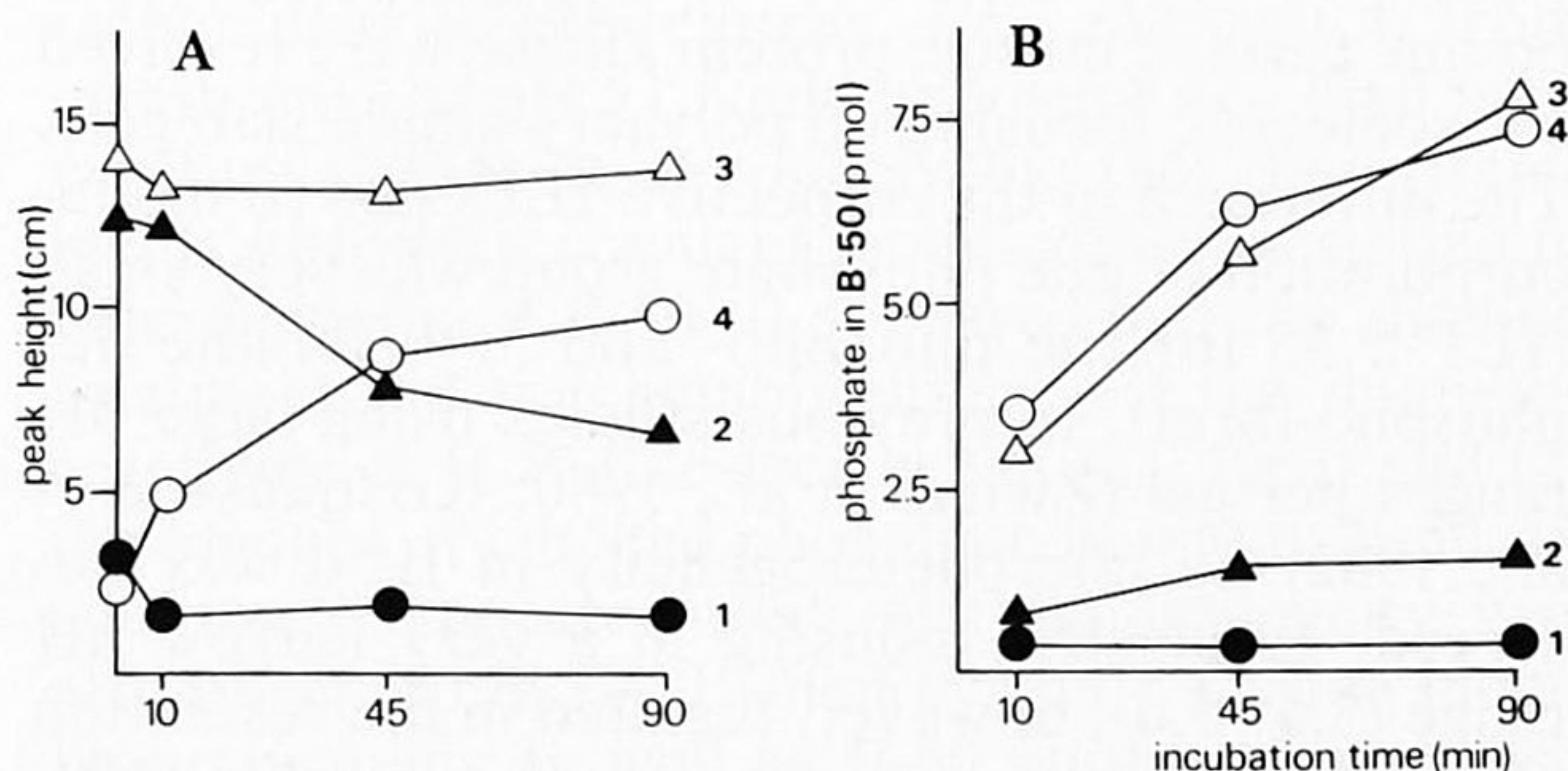


FIG. 4. Time course of the relative amounts (A) and of the incorporation of phosphate (B) in the four individual iso-forms of B-50 after incubation with kinase C. B-50 (2 μ g total protein) was phosphorylated with 3 μ g kinase C and subjected to 2D analysis. The relative amounts of each of the four iso-forms (for nomenclature see Fig. 3) were determined by densitometric scanning at 650 nm. The amount of incorporated phosphate was determined by liquid scintillation counting of the excised spots. This experiment was repeated twice with similar results.

(part A) the time dependency of the action of alkaline phosphatase on the amount of protein in the individual B-50 iso-forms is given. After 2 min of incubation form 1 is already the major form, whereas the amounts of forms 3 and 4 are substantially reduced. When labelled B-50 is used as substrate of *E. coli* alkaline phosphatase, the label originally present in forms 3 and 4 is rapidly (within 4 min) eliminated, and, interestingly, a small increase in the amount of label in form 2 is observed after 4 min. After a much longer time of incubation (30 min) this form also has a reduced amount of radioactivity. This suggests that form 2 is formed from either form 3 or form 4.

Proteolysis of B-50

As is shown in Fig. 6A, incubation of 32 P-labelled B-50 (2.5 μ g) with 50 ng of SAP yields two radioactive products. Protein staining revealed two products coinciding with the two radioactive spots on the autoradiogram; no other protein spots were de-

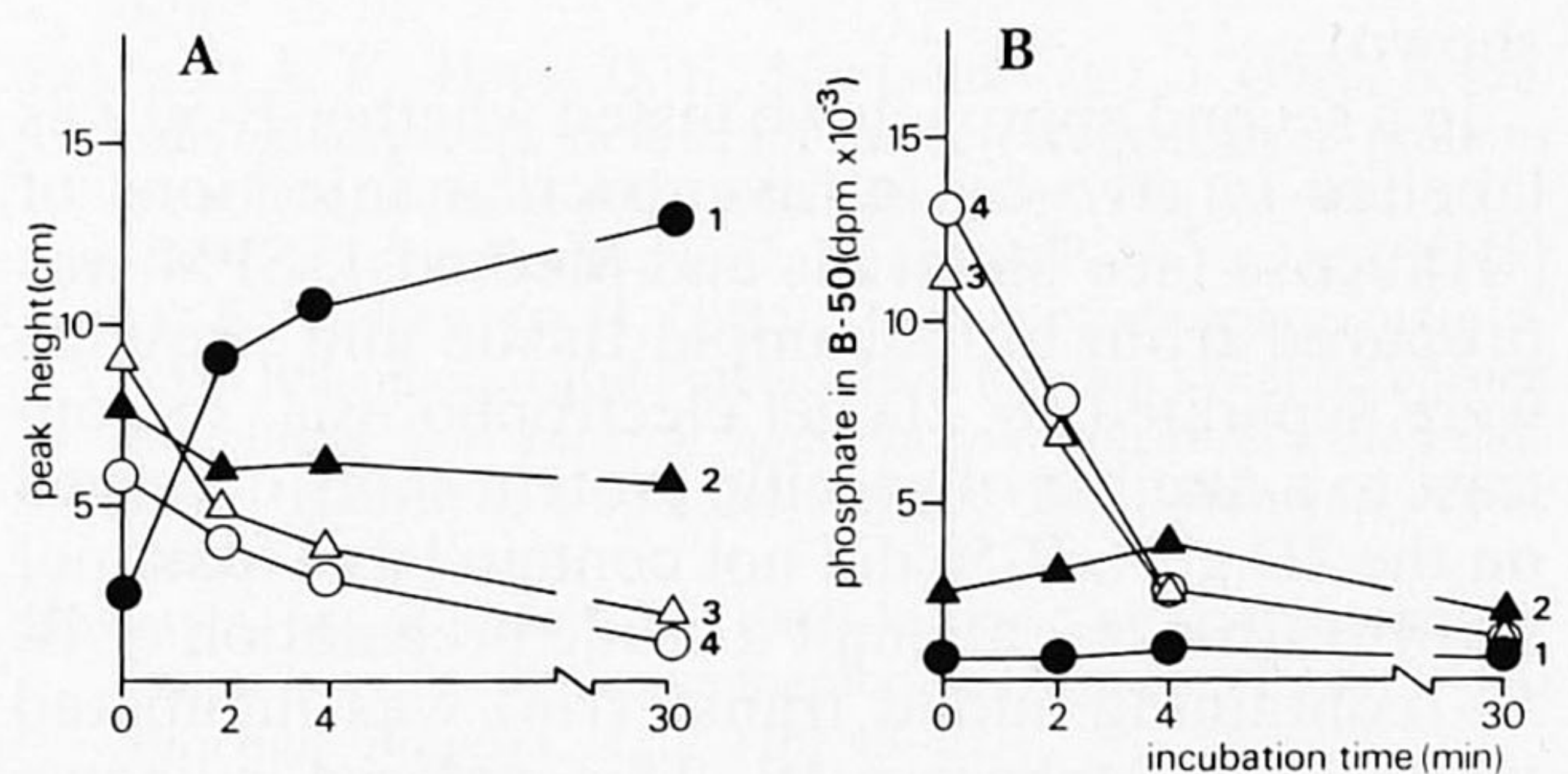
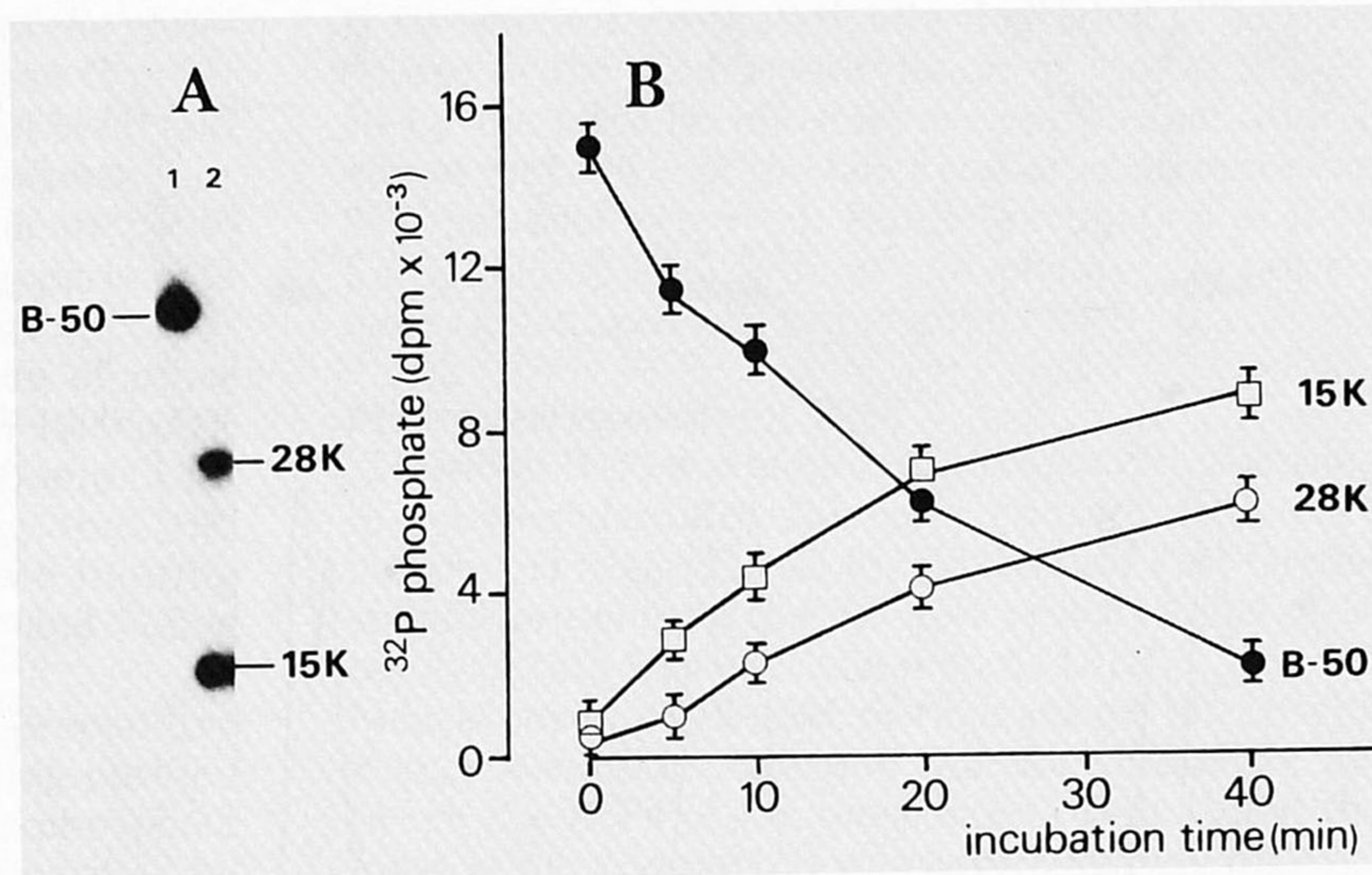


FIG. 5. Time course of the relative amounts of each iso-form of B-50 (A) and the radioactivity recovered in each iso-form of B-50 (B) after incubation of radioactive B-50 (3 μ g total protein, 30,000 dpm) with *E. coli* alkaline phosphatase (4 μ g protein).

FIG. 6. Proteolysis of radioactive B-50 with SAP. **A:** Autoradiograph showing radioactive B-50 as separated on a 15% SDS gel. Lane 1, control incubation of 1.5 μ g B-50 (10,000 dpm); lane 2, incubation of B-50 for 50 min in the presence of 50 ng SAP. **B:** Time course of the fragmentation of radioactive B-50 by SAP to yield the two radioactive fragments of 28 kD and 15 kD, respectively. Amount of radioactivity was determined by liquid scintillation counting. Mean of duplicate incubations.



tected (data not shown). The analysis was performed in 15% gels and with this system (using Pharmacia's low-molecular-weight calibration kit as reference) the M_r of B-50 was calculated to be 42.3 ± 0.7 kD (mean \pm SEM, $n = 4$) and that of the two fragments 28.2 ± 0.5 and 15.7 ± 0.4 kD, respectively.

The time course of the degradation of radioactive B-50 and the formation of the two fragments is indicated in Fig. 6B. Although the absolute levels of label in the two fragments differ, the time course of formation is similar. In addition, at any time of proteolysis, the sum of the label recovered in the fragments equals the loss of label from B-50. Even after 40 min of incubation no degradation of the two fragments was detected.

Con A-Sepharose 4B affinity chromatography and other tests for the detection of glycoproteins

In a series of tests we investigated the possibility that B-50 was a glycoprotein. Staining for glycoproteins in SDS gels (see Materials and Methods) did not give staining of B-50, whereas transferrin, used as glyco-marker protein (Hamann, 1977) was stained with all the three methods used (data not shown).

In a second approach we tested whether B-50 was labelled *in vivo* by intraventricular injections of [3 H]fucose (see Materials and Methods). SPM was prepared from hippocampal tissue and proteins were separated by 2D gel electrophoresis. In contrast to a number of specific protein spots observed on the 2D gels, B-50 did not contain label (data not shown). In a last attempt a crude preparation of B-50 (containing added transferrin) was incubated with Con A-Sepharose 4B. This material is known for its capacity to bind glycoproteins. Figure 7 shows the protein staining pattern of the fractions obtained after elution of the column. Transferrin was eluted with a borate buffer, known to elute gly-

coproteins, whereas B-50 was not. In addition, no elution of B-50 was obtained with 0.1 and 0.5 mM α -methylmannoside.

DISCUSSION

In the present paper the microheterogeneity of the brain-specific phosphoprotein B-50 was studied in relation to a different degree of phosphorylation. Knowledge of this protein is important because we have presented evidence that the degree of phosphorylation of this protein influences the activity of the lipid kinase that phosphorylates phosphoinositol 4-phosphate to phosphoinositol 4,5-bisphosphate (Jolles et al., 1980, 1981; Oestreicher et al., 1983; Gispén et al., 1985).

Isoelectric focusing has been used to separate the phosphorylated and nonphosphorylated forms of protein B-50. Rangel-Aldao et al. (1979) have shown that the phospho- and dephospho-cyclic AMP binding components (about 55 kD) from the purified bovine cardiac muscle protein kinase were resolved by isoelectric focusing on polyacrylamide slab gels. The difference in the respective IEPs due to the incorporation of one phosphate group was very small (IEP 5.35 for the phospho- and 5.40 for the dephospho-form). In previous studies using large pH ranges per gel (Zwiers et al., 1980; Kristjansson et al., 1982) no microheterogeneity in B-50 was observed. Isoelectric focusing in a very narrow pH range (3.5–5.0), however, resulted in the resolution of B-50 in four clearly separated spots (Fig. 3B). Interestingly, the magnitude of the difference in the IEP of the various iso-forms of B-50 are in the same order of magnitude as observed by Rangel-Aldao et al. (1979) for the muscle protein kinase regulating subunit. Further evidence to support the suggestion that differences in the state of phosphorylation underlie the observed heterogeneity in B-50 was obtained from the results shown in Figs. 3 and 4. Ex-

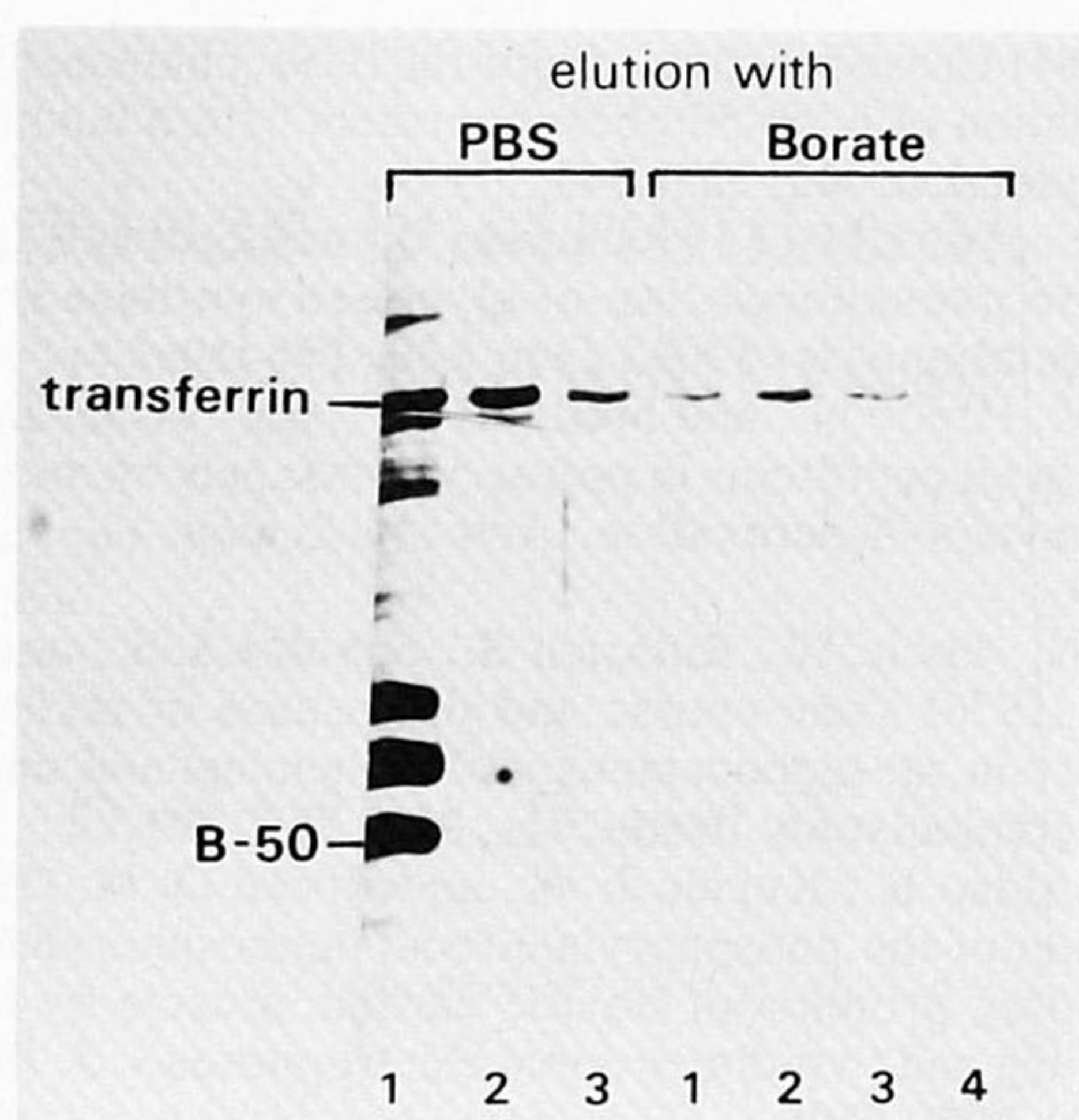


FIG. 7. Protein staining pattern of fractions eluted from a Con A affinity column. A crude preparation of B-50 (containing about 40 μ g of B-50) was mixed with 85 μ g of transferrin. The mixture was added to a column of Con A-Sepharose 4B. The column was washed with PBS and eluted with 0.1 M Na borate (pH 6.5) to release glycoproteins. Samples of the eluate fractions were subjected to SDS-PAGE and stained for protein with a silver staining procedure.

haustive phosphorylation (with kinase C) resulted in higher amounts of the acidic forms of B-50 (spots 3 and 4). In contrast to this, exhaustive dephosphorylation (with alkaline phosphatase) resulted in a predominant appearance of the more basic forms of B-50 (spots 1 and 2). The apparent discrepancies between amount of protein and level of radioactivity of a given spot result from the fact that these parameters are determined by different processes. For instance the amount of 3 (Fig. 4A) is determined by conversion of 2 \rightarrow 3 and 3 \rightarrow 4, whereas the labelling of 3 is only determined by the incorporation of phosphate into 2. Therefore, one may expect to find an increased labelling with a constant amount of 3 in time (Fig. 4A and B).

The procedure used to isolate B-50 involved rather drastic procedures, i.e., extraction from membranes at pH 11.5 and exposure to a high temperature (70°C). It is conceivable that these treatments introduce covalent modifications resulting in heterogeneity of B-50. It appeared that the alkaline treatment did not notably affect: (1) the immunoreactivity of B-50 (Oestreicher et al., 1983) and (2) the amount of labelled phosphate bound to B-50. In addition it appeared that microheterogeneity was also observed in B-50 when synaptic plasma membrane proteins as well as B-50 purified from these membranes after mild conditions of extraction (Zwiers et al., 1980) were subjected to the narrow-range isoelectric focusing (J. Verhaagen, unpublished observations.) Therefore, the observed microheterogeneity in B-50 is certainly not due to an artifact introduced by the isolation procedure.

Multisite phosphorylation of proteins seems to be a rather general phenomenon (Cohen, 1982). From

the numerous examples, we mention glycogen synthase that is phosphorylated on seven different sites (Picton et al., 1982) and the brain synaptic vesicle protein "synapsin," studied by the group of Greengard (Huttner et al., 1981). The data presented in this paper suggest that the same holds for B-50. The arguments for this can be listed as follows: (1) the incorporation of 1 mol phosphate/mol B-50 is too high to saturate only one site, since isolated B-50 does not appear to be completely dephosphorylated (Fig. 3B); (2) we observed four spots with regular charge differences, whereas with one-site phosphorylation we could expect only two iso-forms; (3) digestion of B-50 with SAP resulted in only two (radioactive) fragments suggesting only one specific cleavage site.

Since it is known that glyco-moieties, in particular negatively charged sialic acid residues, introduce charge differences in proteins (Hamann, 1977), we tried with several different procedures to detect glyco-residues attached to B-50. Although none of the six procedures used would by itself give convincing evidence, we tend to conclude from the combined results that B-50 is not a glycoprotein. However, from the results presented in this paper we cannot completely exclude the possibility that other posttranslational factors also contribute to the heterogeneity observed in B-50.

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