B-50 Protein Kinase and Kinase C in Rat Brain

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INTRODUCTION

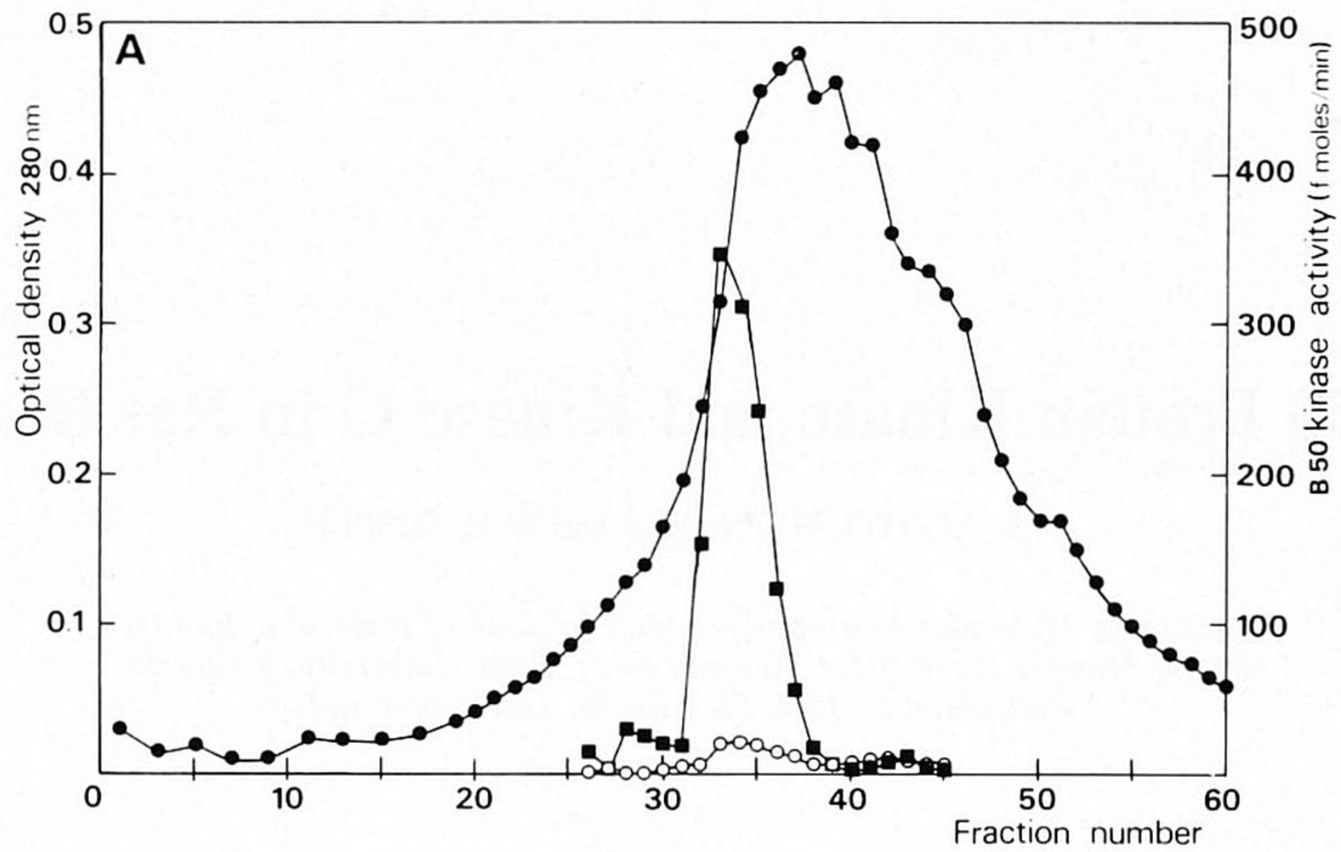
ACTH and some of its fragments are known to affect both brain metabolism and behavior (Schotman et al., 1976; De Wied and Gispen, 1977; Gispen 1981). Neurochemical studies revealed that such peptides can modify RNA and protein metabolism, polyamine metabolism, cyclic nucleotide levels, catecholamine turnover and phosphorylation of both membrane proteins and lipids (Schotman et al., 1976; Dunn and Gispen, 1977; Jolles et al., 1981).

With regard to the in vitro phosphorylation of synaptic plasma membranes (SPM) it was found that synthetic $ACTH_{1-24}$ selectively inhibited the endogenous phosphorylation of certain protein bands as separated by SDS–PAGE (Zwiers et al., 1976). Further experiments revealed that the structure–activity relationship between ACTH-induced inhibition of SPM phosphorylation and ACTH-induced excessive grooming in the rat are very similar (Gispen et al., 1979; Zwiers et al., 1977, 1978, 1981). For at least one SPM protein (B-50) the inhibition of the phosphorylation by ACTH is the result of a direct action of the peptide on the protein kinase activity in the membrane (Zwiers et al., 1978).

Further characterization of the endogenous phosphorylation of the B-50 protein in SPM revealed that it is insensitive to cyclic AMP (cAMP) or cyclic GMP (cGMP) (Zwiers et al., 1976). Furthermore the endogenous phosphorylation of B-50 requires both Ca^{2+} and Mg^{2+} (Gispen et al., 1979).

Another cyclic nucleotide–insensitive, calcium–requiring protein kinase, kinase C, has been isolated from rat brain (Inoue et al., 1977). Although this kinase was originally isolated from the soluble fraction of rat brain homogenate (Inoue et al., 1977) it has recently been found in the particulate fraction as well (Kuo et al., 1980). The activity of kinase C is stimulated by either partial proteolysis by trypsin or a calcium-dependent protease isolated from rat brain (Inoue et al., 1977) or by any of several phospholipids, such as phosphatidylserine (PS) (Takai et al., 1979a). It would appear that in the presence of calcium the soluble kinase binds to membranes resulting in its activation (Takai et al., 1979b).

During our studies on the B-50 protein kinase, we noted several apparent similarities between this kinase and kinase C. Our present paper details the experiments which lead us to conclude that B-50 kinase is indeed very similar if not identical to kinase C. The implication of this finding with regard to the role of ACTH and of lipid metabolism in brain cell function is discussed.



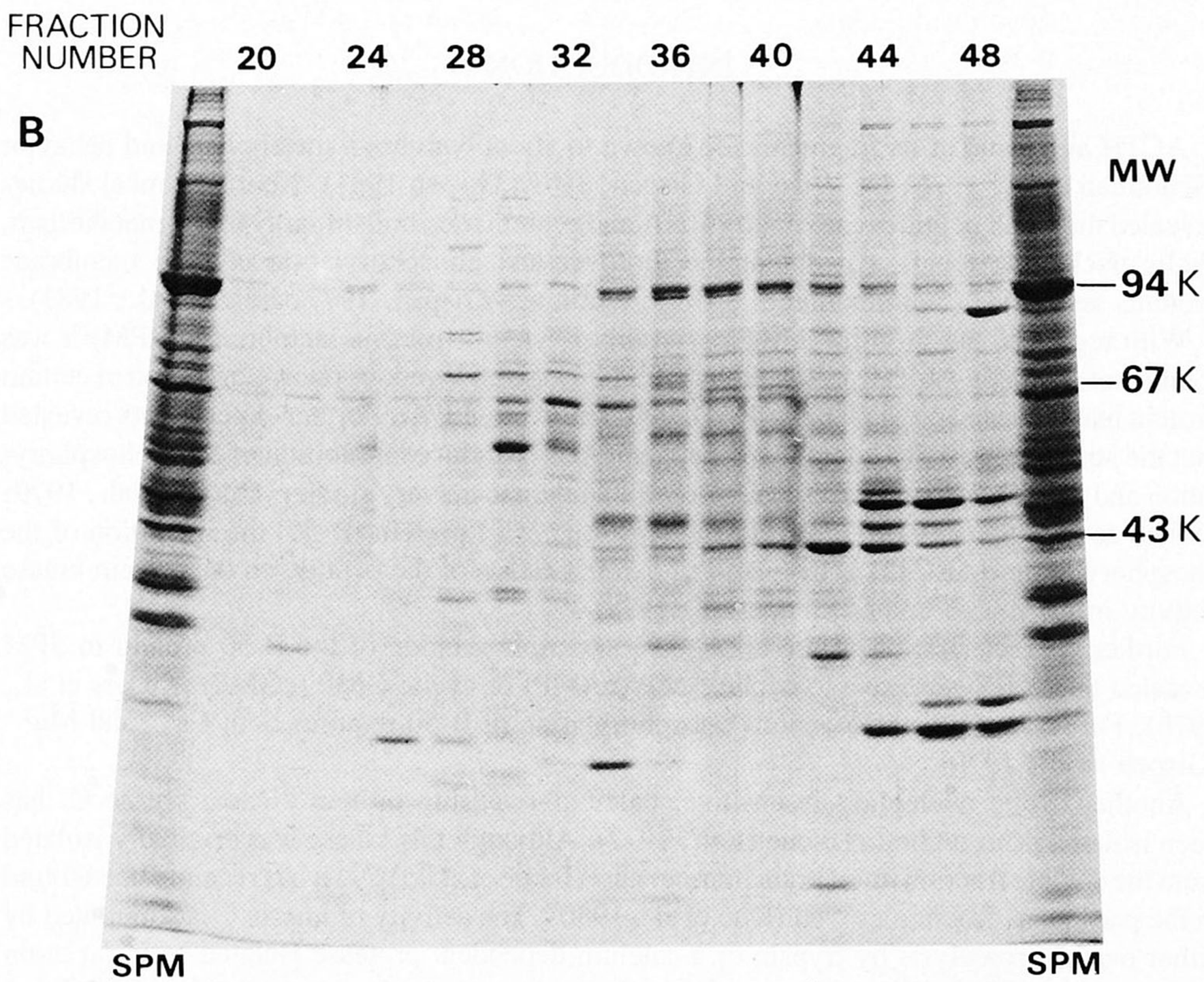


Fig. 1. Purification of B-50 protein and B-50 kinase by DEAE-cellulose chromatography. The Triton X-100/KCl-solubilized material was mixed with DEAE-cellulose and stirred for 15 min to allow the proteins to bind. The unbound proteins were removed by filtration through a sintered glass funnel followed by 5 washes of buffer A (10 mM Tris–HCl, 0.1 mM dithiothreitol, pH 7.4) without detergent (200 ml per wash). The washed DEAE-cellulose was poured into a column and eluted with a linear NaCl gradient (0–0.4 M NaCl in buffer A). A: Optical density and activity profile. The optical density at 280 nm was determined for each fraction (●). The fractions were assayed for endogenous phosphorylation of B-50 protein under the following conditions: 10 μl of each fraction, 10 mM Na^+-acetate, 10 mM Mg^{2+}-acetate, 0.1 mM Ca^{2+}-acetate, 6 mM Tris–HCl, 10 μM ATP, 2μCi [γ-^{32}P]ATP, with (■) or without (○) 0.5 μg PS, pH 7.4, final volume 25 μl. The mixture was prewarmed at 30°C for 5 min and then the reaction was initiated by the addition of ATP. After incubating at 30°C for 10 min the reaction was terminated by the addition of a denaturing solution giving a final concentration of 2% SDS. The proteins were separated by SDS–PAGE and the incorporation of ^{32}P into B-50 protein was determined. B: Protein-staining pattern of fractions. An aliquot of each fraction was applied to an SDS–PAGE slab gel. After electrophoresis the gel was fixed and stained with Fast Green.

PURIFICATION OF B-50 AND B-50 PROTEIN KINASE

An attempt was made to solubilize the protein kinase from rat brain synaptosomal plasma membranes which is responsible for the phosphorylation of a membrane-bound protein substrate (MW 48 K; B-50) and is modulated by the behaviorally active peptide $ACTH_{1-24}$.

In pilot experiments it was found that the extraction procedure (0.1% Triton) described by Uno et al. (1976, 1977) did yield some solubilized B-50 protein kinase activity but its sensitivity to $ACTH_{1-24}$ was not preserved. Therefore alternative extraction procedures were studied. The detergent procedures used (Table I) all solubilized between 75 and 88% of the membrane protein, yielding extracts of a rather similar protein composition. However, only the Triton X-100/KCl treatment yielded soluble ACTH-sensitive, B-50 protein kinase activity. Due to our interest in ACTH-sensitive protein kinase, the recovery of enzyme activity was determined only in the Triton/KCl extract and residue. It was found that only 15% of the membrane-bound B-50 protein kinase activity was solubilized by the Triton X-100/KCl treatment while 85% of the activity remained in the residue (Table I). Why the addition of KCl preserved the sensitivity of the kinases to ACTH is not clear.

The Triton/KCl-solubilized ACTH-sensitive, B-50 protein kinase was further purified by DEAE-cellulose chromatography (Fig. 1). The endogenous phosphorylation of B-50 in the eluate fractions was assayed with or without PS. This activity was markedly stimulated by PS and the peak of activity eluated at about 0.2 M NaCl (Fig. 1). The fractions containing both the B-50 kinase and the B-50 protein were combined and subjected to ammonium sulfate fractionation. As discussed in detail previously (Zwiers et al., 1980) the B-50 kinase and B-50 protein were obtained in the material precipitating between 57 and 82% saturation.

Table II shows the quantitative aspects of the isolation of the B-50 protein kinase while Fig. 2 summarizes the qualitative aspects as visualized by protein patterns and autoradiographs after separation by SDS–PAGE. Throughout the purification procedure, the B-50 phosphorylating activity could be inhibited by $ACTH_{1-24}$.

In order to identify the B-50 protein kinase as well as to obtain purified B-50 protein,

TABLE I

THE EFFECT OF DIFFERENT SOLUBILIZATION PROCEDURES AND $ACTH_{1-24}$ ON RAT BRAIN SPM B-50 PROTEIN KINASE

*Fraction**	*Total protein †* *(mg)*	*B-50 protein kinase (units)***	*Inhibition by* $ACTH_{1-24}$ *(%)****
SPM	8.0 ± 0.1	102.0 ± 5.2	− 52
Triton X-100/KCl extract	6.0 ± 0.1	16.0 ± 0.4	− 52
Triton X-100/KCl residue	2.2 ± 0.1	86.4 ± 3.2	− 51
Triton X-100 extract	6.4 ± 0.2	n.d.	− 5
Deoxycholate (DOC) extract	7.0 ± 0.1	n.d.	+ 5
Triton X-100/DOC extract	7.0 ± 0.1	n.d.	− 2

* SPM was extracted with the following reagents: 0.5% Triton X-100 plus 75 mM KCl; 0.5% Triton X-100; 0.5% DOC; 0.5% Triton X-100 plus 0.5% DOC.

** The total B-50 protein kinase activity was determined using linear incorporation conditions (10 μg protein, 200 μM ATP, 4–5 μCi [γ-^{32}P]ATP, 10 mM Mg^{2+}-acetate, 50 mM Na^{+}-acetate, pH 6.5, total volume 25 μl) with a phosphorylation time of 15 sec.

*** The inhibition by $ACTH_{1-24}$ (10^{-4} M, final concentration) is expressed as the percentage difference from control.

† Protein was determined by the method of Lowry et al. (1951). Each value is the mean (± S.E.M.) for three determinations.

TABLE II

SUMMARY OF ISOLATION OF ACTH-SENSITIVE PROTEIN KINASE FROM RAT BRAIN MEMBRANES

*Step**	*Volume (ml)*	*Protein (mg)*	*Specific activity (pmoles/mg/min)*	*Total activity (units**)*
Triton/KCl extract	220	225	1.8	405
DEAE-pool	24	12	5.5	65
Ammonium sulfate (0–57)	4	11	0.9	11
Ammonium sulfate (57–82)	0.8	0.4	148	53

* Samples (1 ml) of all purification steps were dialyzed overnight against 1 l of buffer A. 15 μg total protein from each step (in triplicate) was used in the phosphorylation assay (as in Fig. 1).

** One unit is defined as the amount of endogenous B-50 kinase activity transfering 1 pmole phosphate to B-50 in 1 min at 30°C.

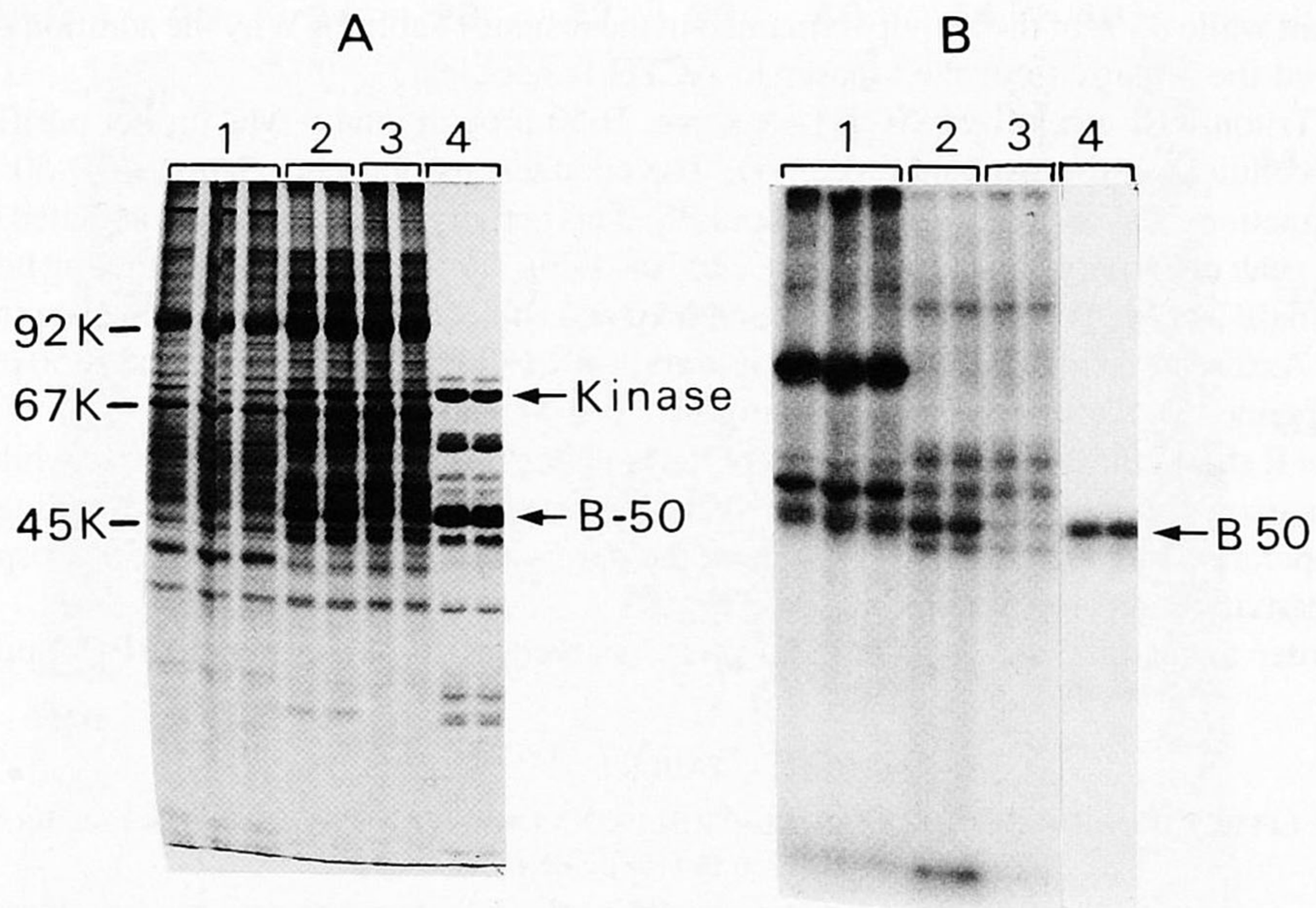
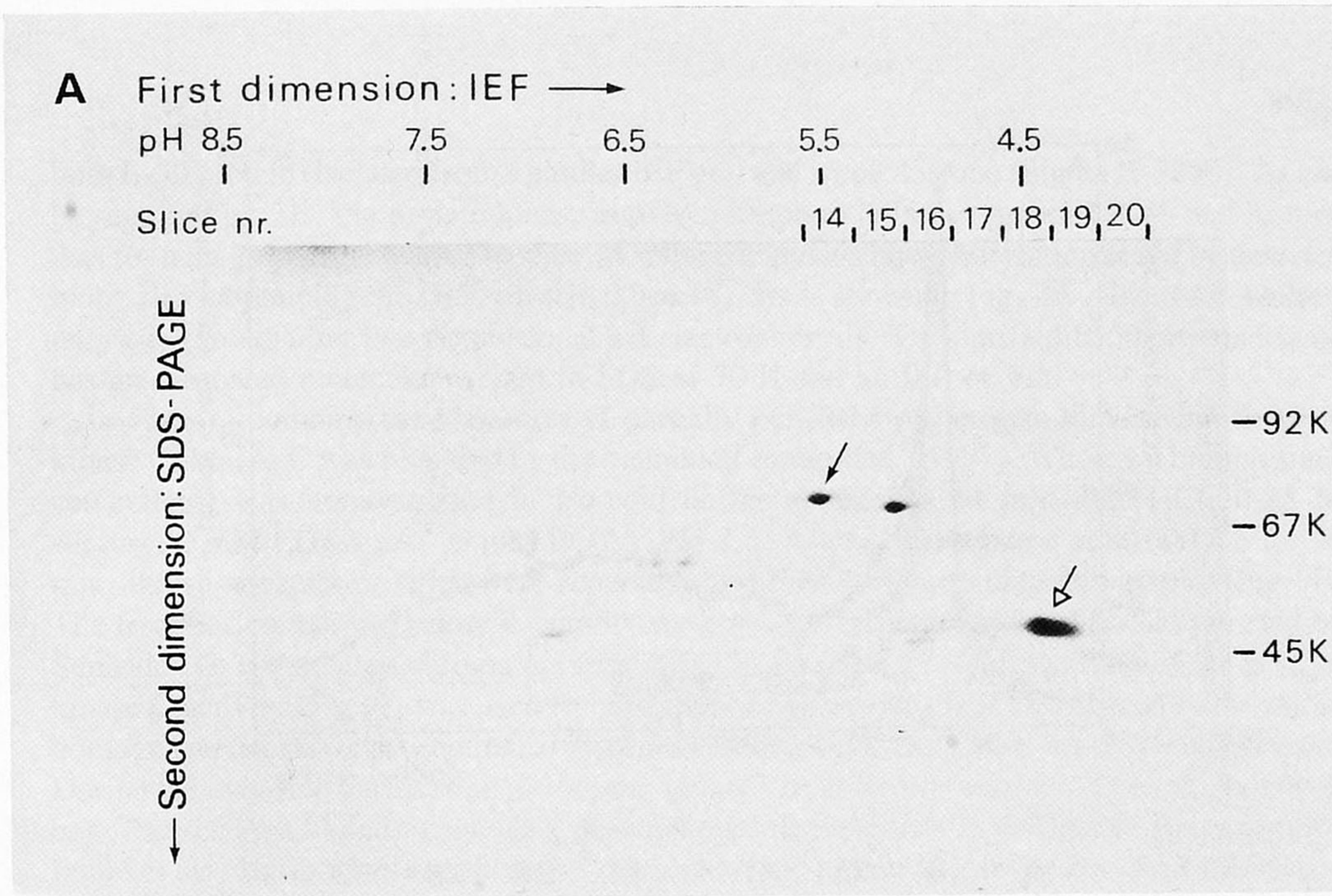


Fig. 2. A: Protein-staining pattern of different steps of the purification of protein kinase and B-50 protein. The numbers on top refer to the following steps: (1) Triton X-100/KCl extract; (2) DEAE-pooled fractions; (3) 0–57% ammonium sulfate precipitate; (4) 57–82% ammonium sulfate precipitate. Tracks 1–3 contain 12 μg of total protein. Track 4 contains 6 μg of total protein. At the left the position of three molecular weight marker proteins; at the right the position of B-50 and kinase is indicated. B: Autoradiogram showing the corresponding endogenous phosphorylation profile. Phosphorylation was carried out for 1 min with 7.5 μM ATP, using 1 μCi [γ-^{32}P]ATP. Autoradiography took 16 h; because of high incorporation into B-50 (step 4) these tracks are from an autoradiogram which was exposed for 2 h.

samples of ASP_{57-82} were subjected to IEF on a 5% acrylamide slab gel as described by Zwiers et al. (1980). After focusing the gel was cut into several lanes. One lane was used for pH determination, another was used for two-dimension SDS–PAGE (Fig. 3A). The remaining lanes were cut into 5 mm slices, eluted and dialyzed against buffer A. Each fraction was studied for endogenous phosphorylating activity as well as for its capacity to phosphorylate



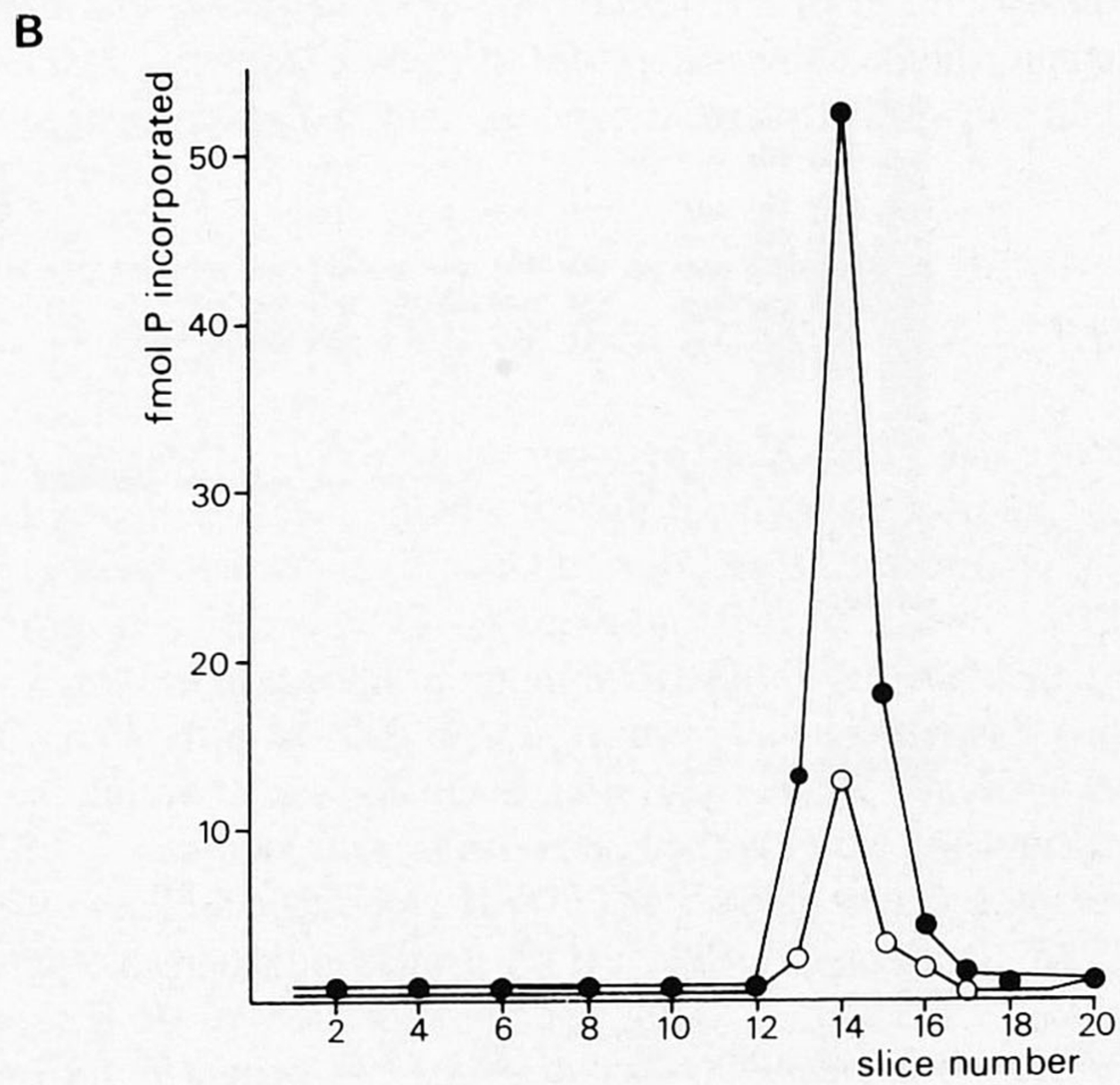
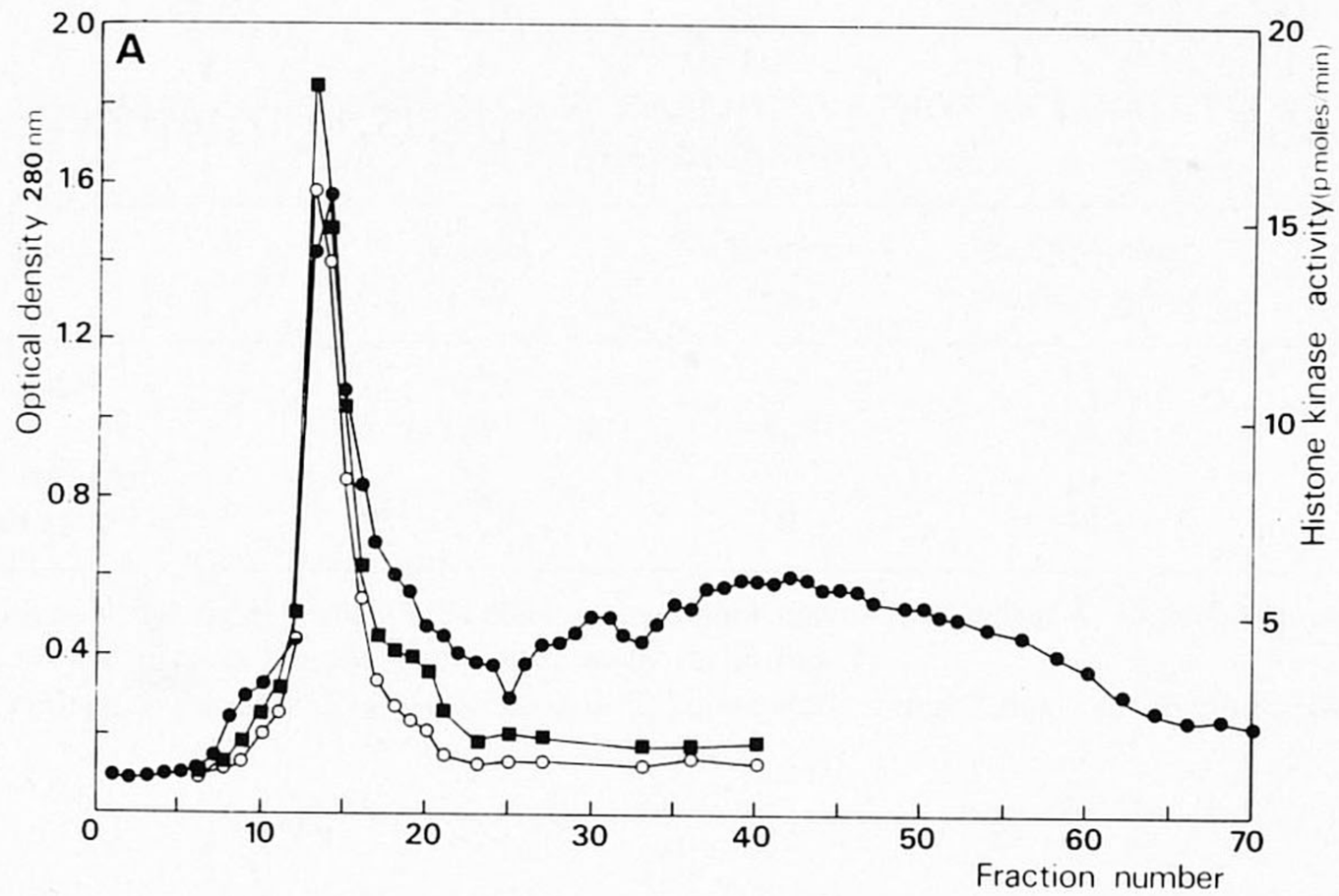


Fig. 3. A: Protein-staining pattern of ASP_{57-82} after two-dimensional separation of proteins. Samples were applicated on an IEF slab gel for separation in the first dimension. After running a whole track was cut out and mounted on top of an SDS slab gel for separation in the second dimension. After running, the gel was stained for proteins. At the top, the pH in the IEF gel is indicated as measured in a corresponding track of the same gel. 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 below). At the right the position of three molecular weight marker proteins are indicated. Arrows indicate the location of B-50 (48 K; open arrow) and kinase (70 K; closed arrow). B: Detection of B-50 kinase by IEF–PAGE. Proteins of ASP_{57-82} were separated on an IEF slab gel. The gel was cut into slices and the proteins were eluted. All fractions were phosphorylated with added B-50 or histone. After phosphorylation, the proteins were separated on an SDS–PAGE slab gel and stained for protein. Incorporation of ^{32}P into B-50 (o——o) and into histone (●——●) was quantified by liquid scintillation counting of the excised gel band. No endogenous phosphorylation was observed in any slice.



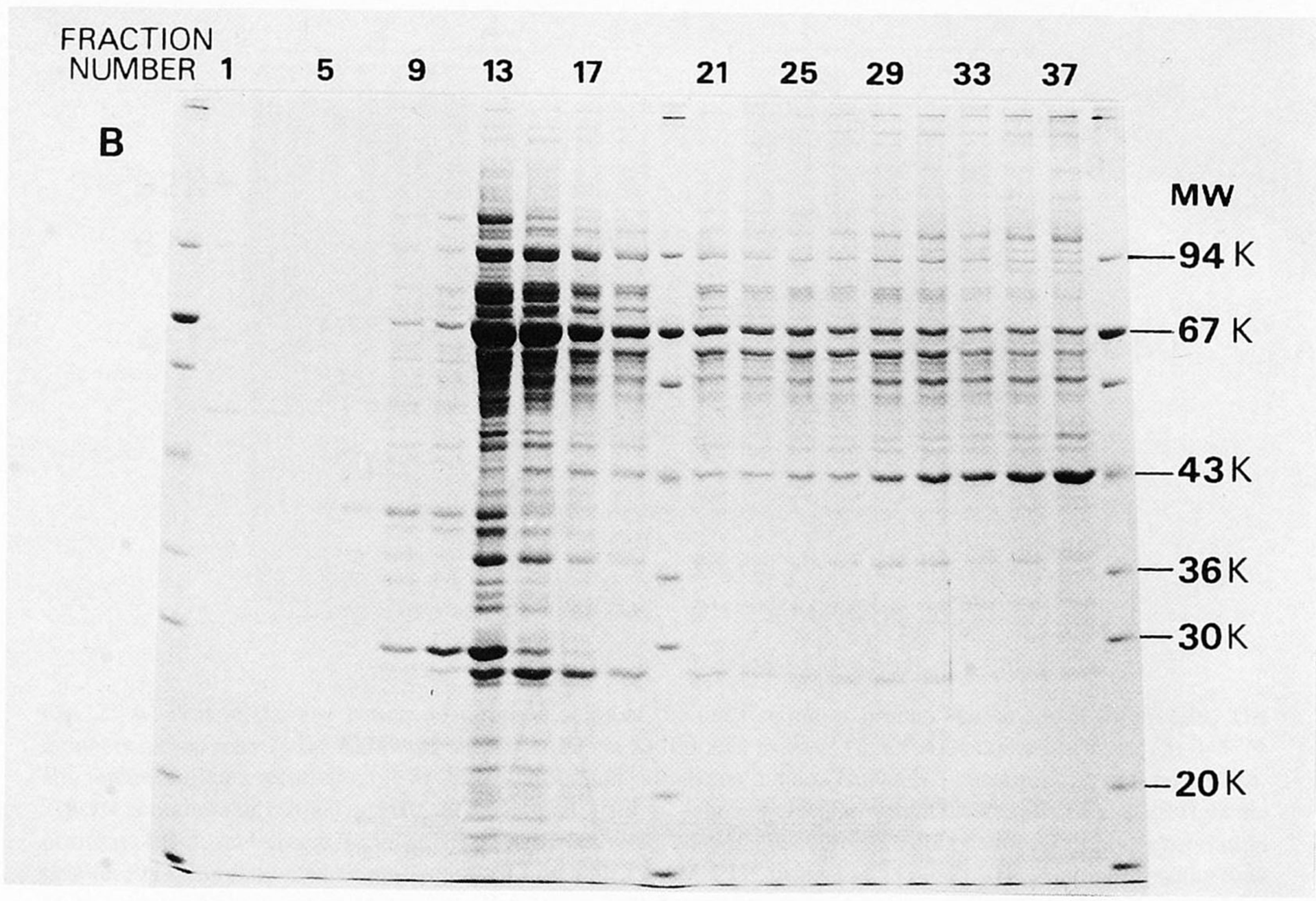


Fig. 4. Purification of kinase C by DEAE-cellulose chromatography. The 80 000 × *g* supernatant was mixed with 80 ml of DEAE-cellulose, pre-equilibrated with buffer B (20 mM Tris–HCl, pH 7.5, 50 mM 2-mercaptoethanol, 2 mM EDTA and 5 mM EGTA) and allowed to stir for 15 min. The unbound proteins were removed by filtration through a sintered glass funnel followed by 5 washes of buffer B (200 ml per wash). The DEAE-cellulose was poured into a column and the bound proteins were eluted with a linear NaCl gradient (0–400 mM NaCl in buffer B, 1 l total volume). A: Optical density and activity profile of eluate fractions. The optical density at 280 nm was determined for each fraction (●). The fractions were assayed for phospholipid-stimulated histone kinase activity under the following conditions: 10 μl of each fraction was added to 50 μl of a mixture containing 30 μg of whole histone (Sigma H-9250) and giving a final concentration of 40 mM Tris–HCl pH 7.5, 10 mM Mg^{2+}-acetate, 1 mM Ca^{2+}-acetate, 10 μM ATP, 0.5 μCi [^{32}P]ATP with (■) or without (○) 0.6 μg of phosphatidylserine. The mixture was prewarmed at 30°C for 5 min before the reaction was initiated by the addition of ^{32}P and was assayed by the filter paper method (Corbin and Reimann, 1974). B: Protein-staining pattern of fractions. An aliquot of each fraction was applied to an SDS–PAGE slag gel. After electrophoresis the gel was fixed and stained with Fast Green.

both B-50 protein (isolated from a similar IEF gel) and whole histone (Sigma H-9250). As can be seen in Fig. 3B, the protein kinase activity phosphorylating exogenous B-50 and histone was for a major part recovered in slice 14. No endogenous phosphorylation could be detected in the slice containing the B-50 protein (slice 18). As is shown in Fig. 3A, 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 K and an IEP of 5.5.

In order to compare the properties of partially purified B-50 protein kinase with those of kinase C, kinase C was prepared by the method of Inoue et al. (1977). Whole rat brain (minus cerebellum) was homogenized in ice-cold buffer containing 20 mM Tris–HCl, 0.25 M sucrose, 2 mM EDTA and 10 mM EGTA, pH 7.5. After centrifugation at $80\,000 \times g$ for 90 min, the supernatant proteins were separated by DEAE-cellulose chromatography (Fig. 4). The fractions containing kinase C activity were pooled, concentrated and further purified by Sephadex G-100 column chromatography (Fig. 5). Fraction 27, which contained the peak of kinase C activity (Fig. 5) was used for all further experiments. When compared to the initial homogenate the above procedure led to an enhancement of specific activity of about 200-fold. During the two–step purification procedure, kinase C activity was assayed after activation with both PS (see Figs. 4 and 5) and Ca^{2+}-dependent protease (partially purified by the method of Inoue et al. (1977) (data not shown). The peak of the kinase C activity after both activation procedures coincided with that of the non-activated kinase activity for both the eluates of the DEAE column and the Sephadex G-100 column. Furthermore, we have confirmed that neither cAMP nor cGMP affect the activity of this kinase while chlorpromazine is an inhibitor. Based on these criteria we conclude that we have indeed partially purified the kinase specified by Inoue et al. (1977).

COMPARISON OF B-50 KINASE AND KINASE C

Previously, Inoue et al. (1977) have reported that kinase C has a molecular weight of 77 K as determined by sucrose density gradient centrifugation analysis and an isoelectric point of 5.6 obtained by isoelectric focusing electrophoresis. An analysis of the elution profile of the kinase C activity from the Sephadex G-100 column (Fig. 5) shows that the peak of activity corresponds to a molecular weight of about 80 K. However, molecular weight determination of the kinase C preparation by SDS–PAGE results in a value of 70 K (Fig. 6). B-50 kinase (from ASP_{57-82}) and kinase C have identical molecular weights by SDS–PAGE. Fig. 6 also shows that the ASP_{57-82} contains several proteins, including the endogenous substrate protein B-50 and the B-50 breakdown product B-60. The corresponding autoradiogram shows that both kinases are able to phosphorylate B-50 (Fig. 6B). In addition, $ACTH_{1-24}$ inhibits the phosphorylation of B-50 by both kinases (Fig. 6B).

A comparison of these two kinases by two-dimensional PAGE, using IEF–PAGE for the first dimension and SDS–PAGE in the second dimension, showed that they have identical mobilities (see Fig. 7). These kinases were further characterized by peptide mapping analysis. The proteins of the two preparations were first separated by IEF–PAGE before being subjected to limited proteolysis. The resulting peptides were separated by SDS–PAGE, thus allowing a comparison of the molecular weights of the peptides. Fig. 7 shows that the protein staining pattern of the digestion fragments of B-50 kinase and kinase C are identical.

B-50 protein kinase as first described by Zwiers et al. (1980) is characterized by its ability to phosphorylate B-50 protein. Therefore, kinase C and three preparations of cAMP-dependent protein kinase (type I, rabbit muscle, Sigma P4890; type II, beef heart, Sigma P5511; catalytic

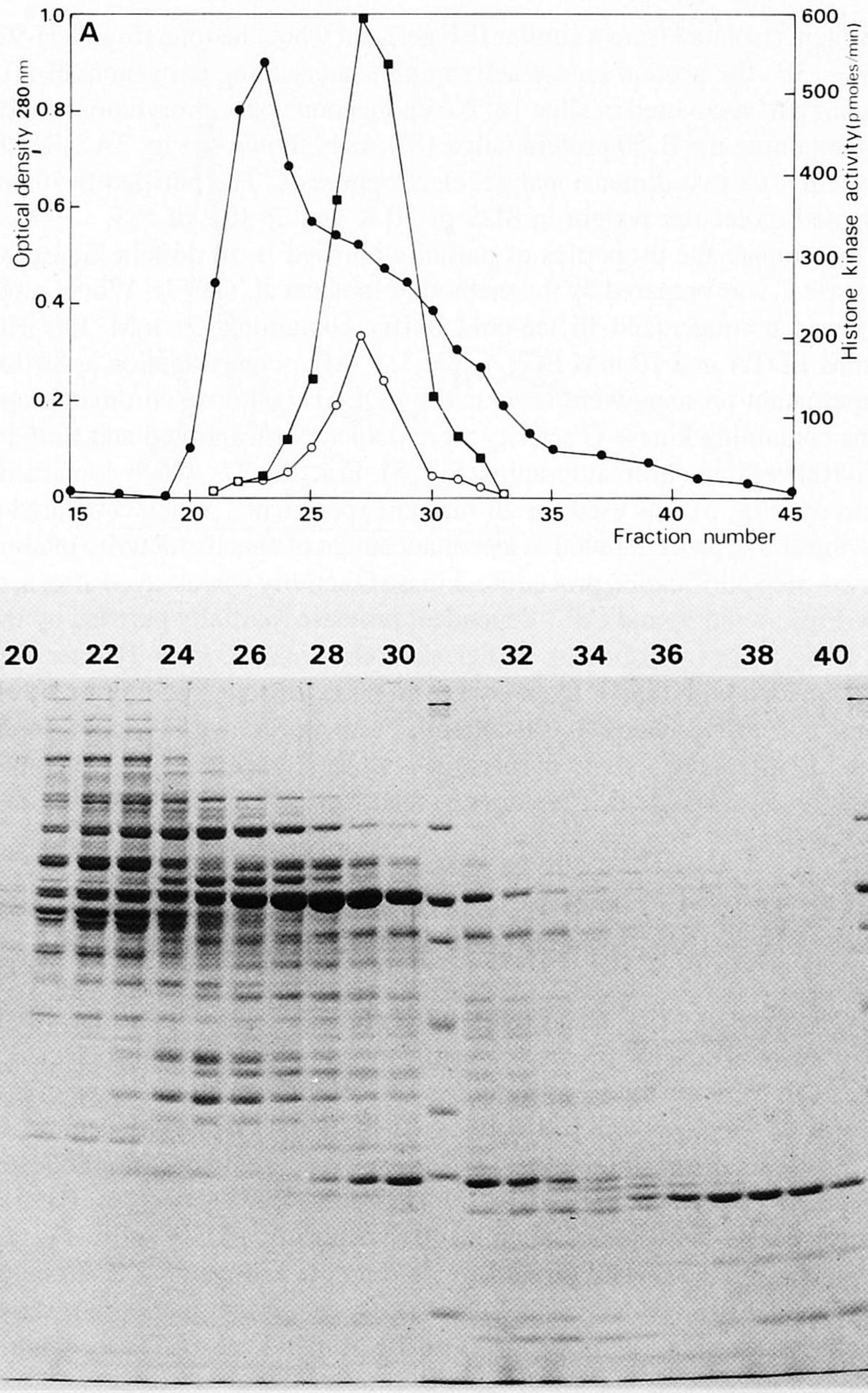


Fig. 5. Purification of kinase C by Sephadex G-100 chromatography. The DEAE-cellulose fractions containing kinase C activity (see Fig. 4) were pooled and concentrated by ultrafiltration (Amicon PM10). The concentrate was applied to a Sephadex G-100 column (100 cm × 2 cm) pre-equilibrated with buffer C (20 mM Tris–HCl, pH 7.5, 50 mM 2-mercaptoethanol and 0.5 mM EGTA). The column was eluted at 20 ml/h with buffer C and 5.4 ml fractions were collected. A: Optical density and activity profile. The optical density at 280 nm was determined for each fraction (●). The fractions were assayed for phospholipid-stimulated histone kinase activity under the following conditions: 1 μl of each fraction was added to 59 μl of a mixture containing 30 μg whole histone and giving a final concentration of 5 mM Tris–HCl pH 7.5, 10 mM Na^+-acetate, 10 mM Mg^{2+}-acetate, 1 mM Ca^{2+}-acetate, 10 μM ATP, 0.5 μCi [^{32}P]ATP with (■) or without (○) 0.6 μg of phosphatidylserine. The mixture was prewarmed at 30°C for 5 min and then the reaction was initiated by the addition of ATP. After incubation for 10 min at 30°C the reaction was terminated and the incorporated ^{32}P was assayed by the filter paper method. B: Protein-staining pattern of fractions. An aliquot of each fraction was applied to an SDS–PAGE slab gel. After electrophoresis the gel was fixed and stained with Fast Green.

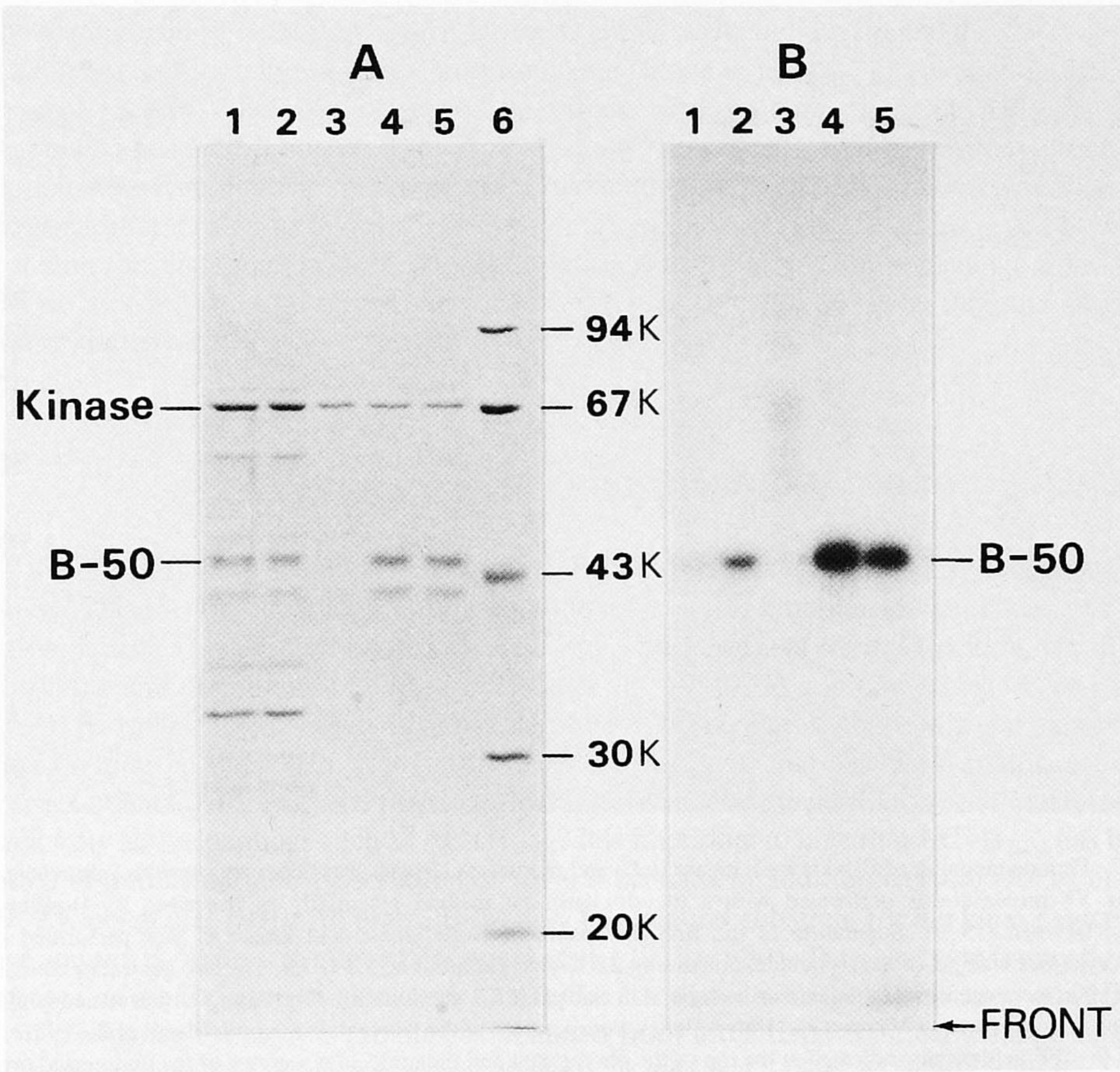


Fig. 6. Phosphorylation of B-50 by B-50 kinase and kinase C. ASP_{57-82} proteins (2.5 μg total protein) plus 10^{-5} M $ACTH_{1-24}$ (track 1); ASP_{57-82} proteins (as in track 1) (track 2); kinase C (track 3, 0.13 μg total protein); kinase C (as in track 2) plus B-50 (0.5 μg protein, including the breakdown product B-60; track 4); kinase C plus B-50 (as in track 4) plus 10^{-5} M $ACTH_{1-24}$ (track 5) were incubated with [^{32}P]ATP for 10 min. Phosphatidylserine (20 μg/ml) was present in the incubation mixtures (for details see legend Fig. 1). Part A shows the protein-staining pattern after SDS–PAGE. The position of the kinases (70 K) and of B-50 (48 K) are indicated. Track 6 shows the molecular weight of several standard proteins. The corresponding autoradiography (part B) shows the phosphorylation of B-50 and its inhibition by $ACTH_{1-24}$.

subunit from beef heart, Sigma P2645) were compared for their ability to use purified B-50 protein as a substrate. Table III shows that the B-50 protein is poorly phosphorylated by the cAMP-dependent kinases, whereas kinase C phosphorylates B-50. A further characteristic of B-50 protein kinase is the inhibition of its activity by ACTH and other behaviorally active neuropeptides (Zwiers et al., 1980, 1981). Similar to B-50 protein kinase, kinase C (using B-50 protein as a substrate) was inhibited 79 % by $ACTH_{1-24}$ and 63 % by $ACTH_{5-18}$ but not by $ACTH_{1-10}$. The same structure–activity relationship was found when endogenous B-50 phosphorylation was assayed in SPM (Zwiers et al., 1978). The phosphorylation of B-50 by kinase C is not affected by 10^{-5} M cAMP or cGMP. Chlorpromazine (10^{-4} M) inhibited the phosphorylation of B-50 by about 85 %.

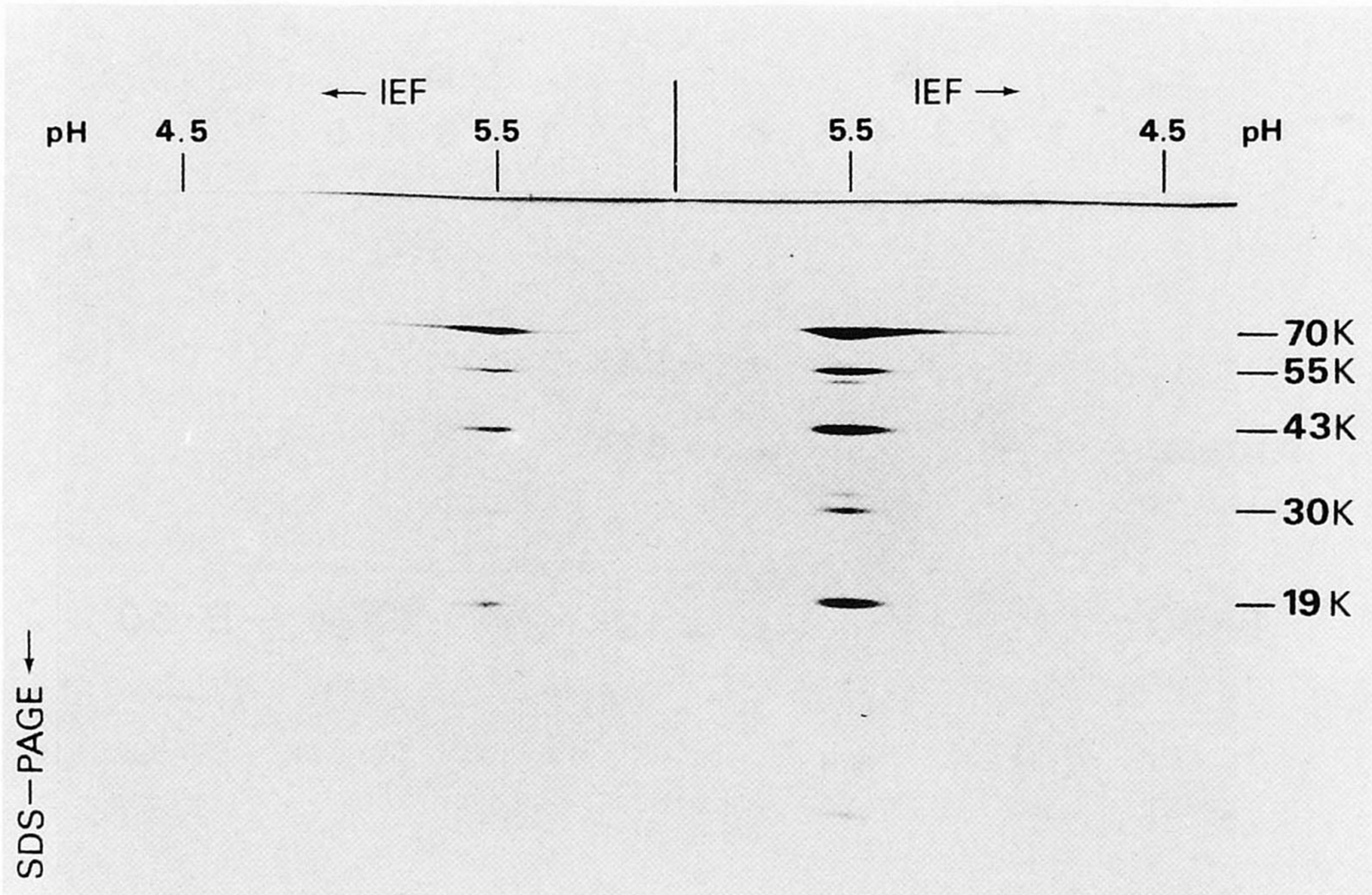


Fig. 7. Peptide mapping of B-50 protein kinase (left) and of kinase C (right). Peptide mapping with *Staphylococcus aureus* V8 protease was performed with a two-domensional method essentially as described by Bordier and Crettol-Järvinen (1979). Separation in the first dimension of B-50 kinase and kinase C was performed on a polyacrylamide slab gel (5% acrylamide) containing 2.5% (w/v) ampholine 3.5–10.0. The two gel tracks containing the samples were cut out and mounted on a single SDS slab gel (15% acrylamide). Digestion was performed with 3 μg of *Staphylococcus aureus* V8 protease. Polypeptides were detected with a silver stain method (Merril et al., 1981). The pH of the IEF gel lanes is indicated at the top of the photograph and the molecular weights of the undigested protein kinases (70 K) and of four major digestion products are indicated on the right.

TABLE III

ABILITY OF SEVERAL KINASES TO PHOSPHORYLATE B-50 PROTEIN

Kinase	*Activity (fmoles/min)*
cAMP-Dependent (beef heart) (0.607 μg)	0.36 ± 0.13
cAMP-Dependent (rabbit muscle) (2.33 μg)	0.10 ± 0.20
Catalytic subunit of cAMP-dependent kinase (beef heart) (0.056 μg)	0.06 ± 0.21
Kinase C (1.56 μg)	37.90 ± 0.81

The kinases were assayed using 0.1 μg B-50 as the substrate under the conditions used in Fig. 1, except 5 μM cAMP (final concentration) was added to the cAMP-dependent protein kinases and 20 μg PS/ml (final concentration) was added to kinase C. Under identical conditions the amounts of kinase used incorporated 5–6 pmoles of phosphate/min into 30 μg histone.

The distinguishing characteristics of kinase C are its activation by PS and the Ca^{2+}-dependent protease and its inhibition by chlorpromazine (Inoue et al., 1977; Takai et al., 1979a; Mori et al., 1980). Therefore we tested the effects of these compounds on ASP_{57-82}. The endogenous phosphorylation of B-50 in ASP_{57-82} is stimulated 4-fold by treatment with the Ca_{2+}-dependent protease. A similar 4-fold stimulation of B-50 phosphorylation was observed after the addition of PS (20 μg/ml, final concentration) to ASP_{57-82}. The addition of PS, while stimulating the endogenous B-50 phosphorylation did not affect the percentage inhibition observed after the addition of $ACTH_{1-24}$. Chlorpromazine (10^{-4} M) is an inhibitor of B-50 phosphorylation in ASP_{57-82} resulting in 80% inhibition.

CONCLUSIONS AND DISCUSSION

By the physical and enzymatic criteria used, B-50 protein kinase and kinase C appear to be identical. This identity leads to questions about the role of this kinase in the regulation of brain function. Kinase C has been reported to occur in a wide variety of tissues (including brain) in both soluble and membrane-bound form (Kuo et al., 1980). In addition, kinase C has been shown to phosphorylate a variety of substrate proteins of nuclear, cytoplasmic and membrane origins (Wrenn et al., 1980; Nishizuka, 1980). In view of this enzyme's multifunctional properties, what then may be the physiological significance of the inhibition of this enzyme by behaviorally active peptides such as $ACTH_{1-24}$? It is important to note that $ACTH_{1-24}$ has been reported to inhibit the phosphorylation of several proteins in addition to B-50 (Jolles et al., 1979; Zwiers et al., 1979). In brain, B-50 may be a natural substrate for the kinase since the kinase and the B-50 protein copurify (Zwiers et al., 1979, 1980). Thus, in brain it may be that at least part of the B-50 protein kinase or kinase C is associated with the membrane-bound B-50 protein. Evidence for such a complex is obtained from the DEAE-cellulose column elution profiles (Figs. 1 and 4). Kinase C elutes at about 70 mM NaCl (Fig. 4), while both the B-50 protein and the B-50 protein kinase elute at about 200 mM NaCl (Fig. 1). This retarded elution may be due to the interaction of the kinase with the B-50 protein. Furthermore, the B-50 protein appears to be a brain–specific protein (Kristjansson et al., 1982), which is presynaptically located (Sorensen et al., 1981). Therefore, the interaction of $ACTH_{1-24}$ and kinase C (B-50 protein kinase) may only be of importance in those brain subcellular areas where the B-50 protein is located.

Another question which arises from the proposed identity of B-50 protein kinase and kinase C is the role of lipids in the regulation of protein phosphorylation. Nishizuka (1980) has suggested that kinase C is activated by the breakdown of phosphoinositides (PI) to diglyceride. The proposed scheme is that hormones interact with a receptor to stimulate a phospholipase C. This phospholipase C then cleaves PI to give a diglyceride which activates membrane-bound kinase C, which then inturn phosphorylates its substrate protein(s). On the other hand, Jolles et al. (1979, 1980) have reported that the phosphorylation of DPI to TPI by DPI kinase is dependent upon the state of B-50 phosphorylation due to the activity of B-50 kinase. Thus, it was concluded that the phosphorylation state of B-50 protein may effect the metabolism of polyphosphoinositides in brain cell membranes (Zwiers et al., this volume). An important avenue of future research will involve what the actual sequence of events is: is the breakdown of PI followed by the activation of kinase C/B-50 kinase (Nishizuka, 1980), or is the change in the phosphorylation state of B-50 a regulating event in polyphosphoinositide metabolism (Jolles et al., 1979, 1980).

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