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ACTH-SENSITIVE PROTEIN KINASE FROM RAT BRAIN MEMBRANES

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Abstract—The protein kinase which in rat brain synaptosomal plasma membranes is responsible for the phosphorylation of a protein band B-50 (MW 48,000) was inhibited by the behaviorally active peptide $ACTH_{1-24}$ and not stimulated by cAMP. Treatment with 0.5% Triton X-100 in 75 mM-KCl solubilized 15% of the total B-50 protein kinase activity and preserved the sensitivity of the enzyme to $ACTH_{1-24}$. The rate of endogenous phosphorylation of protein band B-50 was different in intact SPM, solubilized fraction and residue. cAMP stimulated the endogenous phosphorylation of the solubilized fraction in a rather general manner. The solubilized membrane material also phosphorylated B-50 proteins which were previously extracted from membranes. Column chromatography of the solubilized material over DEAE–cellulose pointed to the presence of multiple protein kinase activities from rat brain synaptosomal plasma membranes, one of which was the ACTH-sensitive B-50 protein kinase.

ACTH AND some of its N-terminal sequences are known to affect brain metabolism and behaviour (SCHOTMAN *et al.*, 1976; DE WIED & GISPEN, 1977). Neurochemical studies revealed that such peptides can interfere with RNA and protein metabolism, polyamine metabolism, cyclic nucleotide levels, catecholamine turnover and phosphorylation of membrane proteins (SCHOTMAN *et al.*, 1976; DUNN & GISPEN, 1977).

With regard to the phosphorylation of synaptic plasma membranes (SPM) it was found that *in vitro* synthetic $ACTH_{1-24}$ selectively reduced the endogenous phosphorylation of certain protein bands, as separated on SDS–polyacrylamide slab gels (ZWIERS *et al.*, 1976). The endogenous SPM protein phosphorylation and its modulation by the various peptides were dependent on the ratio of the concentrations of ATP to SPM protein (WIEGANT *et al.*, 1978; ZWIERS *et al.*, 1978).

Further experiments revealed a great similarity in structure–activity relationship between ACTH-induced inhibition of SPM phosphorylation and ACTH-induced excessive grooming in the rat (ZWIERS *et al.*, 1977, 1978). At least for one SPM protein band (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). In the present study, we report that the protein kinase and its substrate can be solubilized from synaptic plasma membranes and that its activity in solution can still be modulated by $ACTH_{1-24}$.

Abbreviations used: SPM, synaptic plasma membranes; PAGE, polyacrylamide gel electrophoresis; DOC, deoxycholate; SDS, sodium dodecyl sulphate.

MATERIALS AND METHODS

Membrane preparation and solubilization of membrane-bound kinase. Synaptic plasma membranes (SPM) were prepared from rat cerebral cortex (12 g) as described previously (ZWIERS *et al.*, 1976). SPM material was suspended (4 mg protein/ml) in 10 ml 6 mM-Tris–HCl, pH 8.1, containing 0.1 mM-dithiothreitol. Aliquots of 2 ml from freshly prepared SPM were treated with 2 ml of one of the following solutions (final concentration) (1) 0.5% Na-deoxycholate; (2) 0.5% Na-deoxycholate + 0.5% Triton X-100; (3) 0.5% Triton X-100; (4) 0.5% Triton X-100 + 75 mM-KCl; (5) 6 mM-Tris–HCl, pH 8.1. The mixtures were vigorously whirled on a Vortex mixer for 1 min and allowed to stand at 4°C for another 15 min. Subsequently, residual membranes were centrifuged at 100,000 *g* for 20 min in an ultracentrifuge (Sorval OTD-2) using a fixed-angle rotor. The supernates containing solubilized SPM material were saved. The extract obtained with Triton–KCl (solubilization procedure 4) is referred to as T-SPM.

DEAE–cellulose column chromatography. Sixteen millilitres T-SPM (see above), containing 25 mg of protein, was diluted to 56 ml with 50 mM-potassium phosphate, pH 7.0, and applied to a DEAE–cellulose column (15 × 0.9 cm; DE52, Whatman) (UNO *et al.*, 1976) previously equilibrated with 50 mM-potassium phosphate buffer, pH 7.0. Proteins were eluted from the column with 35 ml of phosphate buffer and, subsequently, 75 ml of a linear potassium phosphate gradient (50–500 mM-potassium phosphate, pH 7.0). The absorption at 275 nm was monitored continuously using a Uvicord III (LKB, Sweden); fractions of 2.2 ml were collected and assayed for conductivity, protein content (see below) and protein kinase activity (see below).

Determination of protein kinase activity with endogenous substrates. In general, two conditions (A and B) were used

in a standard assay for membrane-bound or solubilized protein kinase-activity: (A) 7.5 μM-ATP, 2–4 μCi [γ-^{32}P]ATP, 2.2 μg SPM-protein per μl and (B) 200 μM-ATP, 4–5 μCi [γ-^{32}P]ATP, 0.4 μg SPM protein per μl (WIEGANT *et al.*, 1978). 55 μg (A) or 10 μg (B) of SPM protein were preincubated at 30°C for 5 min, the phosphorylation reaction was started by addition of the ATP. The final volume was 25 μl, containing: 10 mM-Mg-acetate; 50 mM-Na-acetate, pH 6.5; 7.5 μM-ATP, (A) or 200 μM-ATP (B). Synthetic $ACTH_{1-24}$ (Organon International B.V., Oss, The Netherlands) was added 10 s prior to the addition of ATP, resulting in a final concentration of 10^{-4} M.

After a standard incubation time of 15 s (unless specified otherwise) the reaction was terminated by addition of 12.5 μl of a denaturating solution resulting in final concentrations of: 62.5 mM-Tris–HCl, pH 6.5; 2% SDS; 10% glycerol; 0.001% Bromphenol Blue and 5% 2-mercaptoethanol. Aliquots of 30 μl were subjected to SDS–polyacrylamide gel electrophoresis (PAGE) on slab gels, as previously described (ZWIERS *et al.*, 1976). The gels were stained for proteins using Fast Green, destained and dried, and subjected to autoradiography using Kodak Royal X-Omat X-ray film as described previously (ZWIERS *et al.*, 1976; WIEGANT *et al.*, 1978). The radioactivity of individual bands was measured by photometric scanning of the autoradiographs using a Zeiss PMQII spectrophotometer and a linear gel scanner (slide width 0.1 mm). In some experiments the radioactivity of the individual bands was also determined by cutting the bands from the gels and counting them in a liquid scintillation counter (WIEGANT *et al.*, 1978). Fractions from the DEAE–cellulose column were assayed for endogenous phosphorylating activity under condition A, and for histon kinase activity (see below). Aliquots of 20 μl of the fractions containing variable amounts of protein were taken.

Extraction and phosphorylation of protein band B-50. Freshly prepared SPM was mixed with the denaturing solution (see above, 4 mg protein per 1.4 ml) and layered on top of a SDS–PAGE slab using 24 of 27 available slots. The remaining slots were filled with a similar suspension of SPM previously incubated with [γ-^{32}P]ATP. After electrophoresis the protein bands were stained with Coomassie Brilliant Blue (0.1% w/v in 50% methanol–10% acetic acid, v/v) and phosphorylated proteins were identified by autoradiography of the reference tracks containing phosphorylated SPM. On guidance of the radioactivity pattern, the coloured band B-50 (estimated MW 48,000; WIEGANT *et al.*, 1978) was excised. From the tracks the proteins were extracted subsequently from excised material with a solution of 0.1% SDS in 0.1 M-Tris–HCl, pH 8.0, under continuous shaking at room temperature. After 1 h, the pH was adjusted to 5.5 and 24 h later the gel was further extracted with a fresh portion of the extraction solution. The combined extracts were lyophilized, taken up in bidistilled water, desalted using Sephadex G-25 column, dried and the residue was taken up in bidistilled water. Protein-bound SDS was removed by precipitation of the protein in acetone at −20°C and washing of the protein pellet with acetone. The acetone was evaporated under nitrogen and the proteins were dissolved in 0.005 N-NaOH (1 μg protein/μl). The extracted proteins (1 μg) were incubated as described above, measuring protein kinase activity of T–SPM and DEAE–cellulose fractions under the phosphorylation condition A. After incubation, the phosphoproteins were separated by SDS–PAGE, the slabs were subjected to autoradiography and the protein band B-50 was cut out and counted for incorporated ^{32}P in a liquid scintillation counter.

Determination of histon kinase activity. Protein kinase activity, using histon as substrate, was measured by minor modification of the method described by UNO *et al.* (1976). The standard assay mixture consisted of: 50 mM-Na-acetate, 10 mM-Mg-acetate, pH 6.50; 40 μg histon (type II-A, Sigma Chemical Company); 5 μM-[γ-^{32}P]ATP (2–4 × 10^6 c.p.m.); T-SPM (20 μg) or the DEAE–cellulose column fractions (20 μl), final volume 200 μl. The protein kinase activity of the column fractions was determined in the presence of 5 μM-cAMP. After preincubation for 5 min at 30°C, the reaction was initiated by the addition of 10 μl [γ-^{32}P]ATP and terminated 10 min later by the addition of 1 ml ice-cold 0.6 M-perchloric acid containing 1 mM-ATP and 10 mM-H_3PO_4. Carrier protein (10 mg bovine serum albumin) was added, and the mixture was allowed to stand for 10 min at 0°C. The precipitate was collected by centrifugation and washed 4 times with 0.3 M-perchloric acid. The final pellet was dissolved in 1 ml Soluene (Packard). After addition of a scintillator–xylene mixture (Lipo Luma, Lumac) the glass vials were counted for ^{32}P radioactivity in a liquid scintillation counter (Nuclear Chicago, MK II). The amount of phosphate incorporated into histon was corrected for the phosphorylation of endogenous SPM proteins (<5%) by subtracting the counts recovered in samples incubated without histon. Under the conditions used the histon kinase activity was studied under linear kinetics with respect to time and enzyme concentration.

Determination of free phosphate. Production of free phosphate during the phosphorylation assay as a result of endogenous ATP-hydrolysing activity was studied in the various fractions (SPM, T-SPM, residue) by adding non-labelled ATP (7.5 or 200 μM) to the incubation mixture (see above) and stopping the reaction with TCA (final concentration 5%) after various incubation periods. After centrifugation, free phosphate was measured in the supernatant using the method of HESS & DERR (1975).

Determination of protein. Protein determination was performed according to the method of LOWRY *et al.* (1951). The protein content in column eluate fractions and extracted B-50 preparations was determined in 110 μl total volume allowing routine determinations in the range of 0–5 μg. The detergent procedures used to solubilize the membrane proteins only to a minor degree interfered with the protein determinations (<5%).

RESULTS

Solubilization of membrane-bound protein kinase

In order to solubilize protein kinase from synaptosomal plasma membrane, an ionic (sodium deoxycholate) and a non-ionic (Triton X-100) detergent were used, alone or combined. The non-ionic detergent was also applied in combination with salt (75 mM-KCl). The various treatments all resulted in the solubilization of 75–88% of the SPM protein (Table 1, first column). Thus, when the protein patterns after SDS–PAGE of the various SPM extracts were compared, a great similarity to that of original SPM was seen (Fig. 1). However, after extraction with T/KCl the protein composition of the residue differed from that of the original SPM, indicating that some selective solubilization had occurred. This was clearly

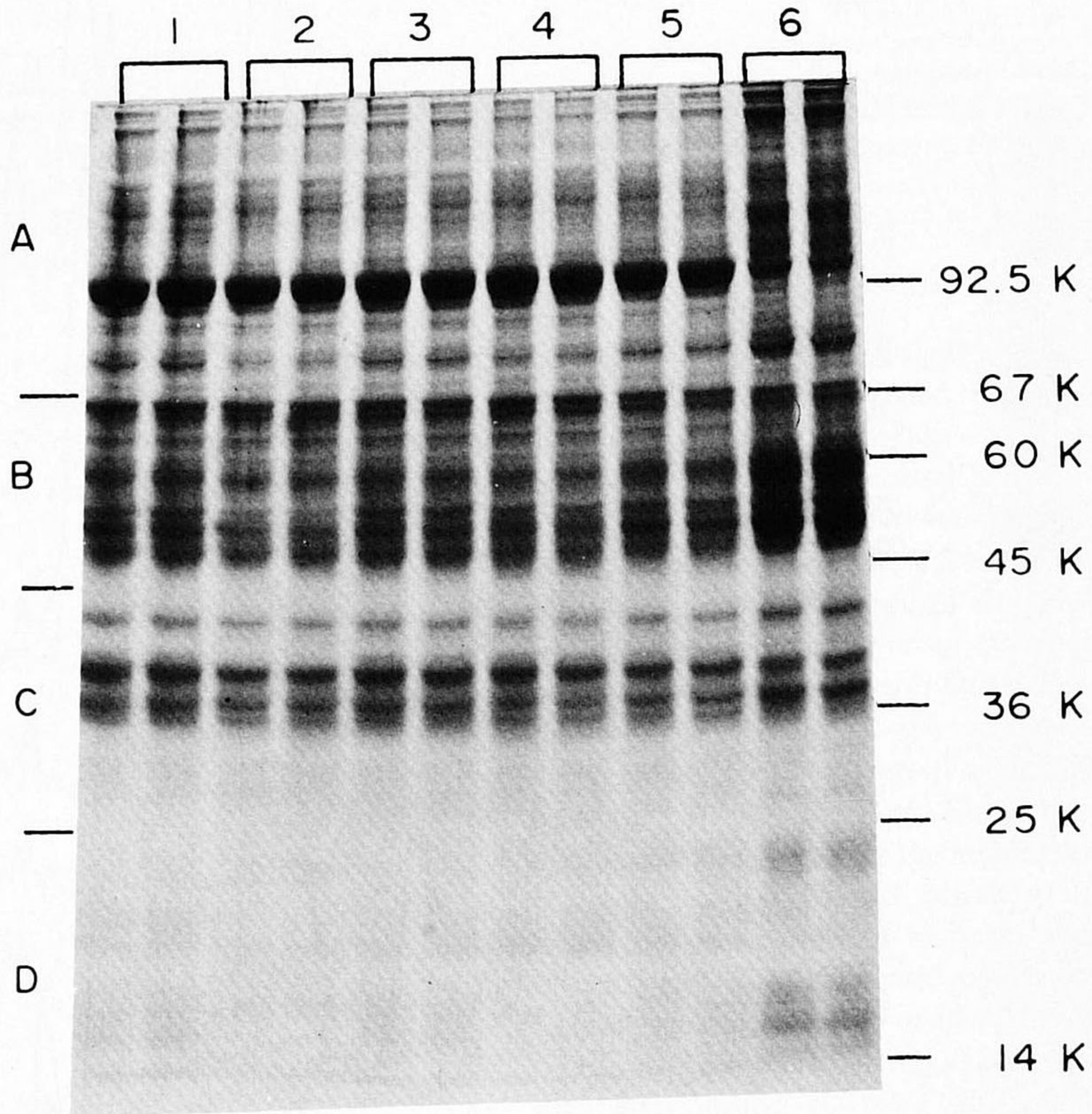
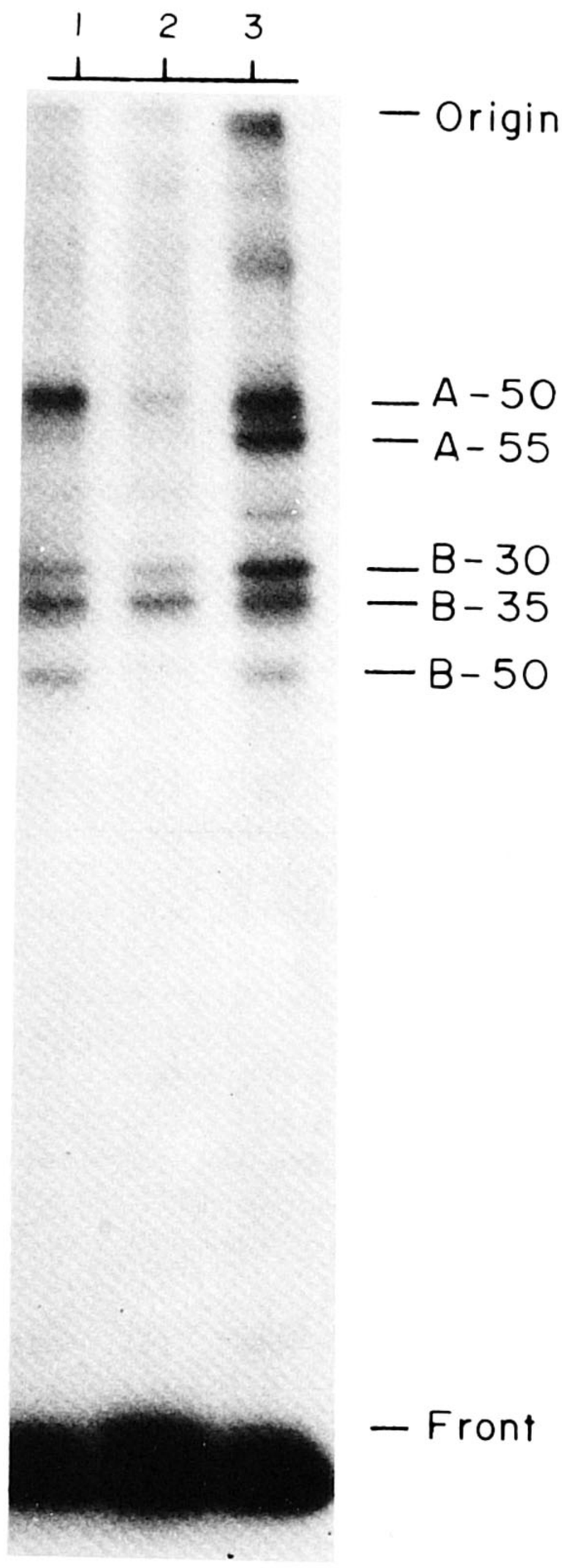
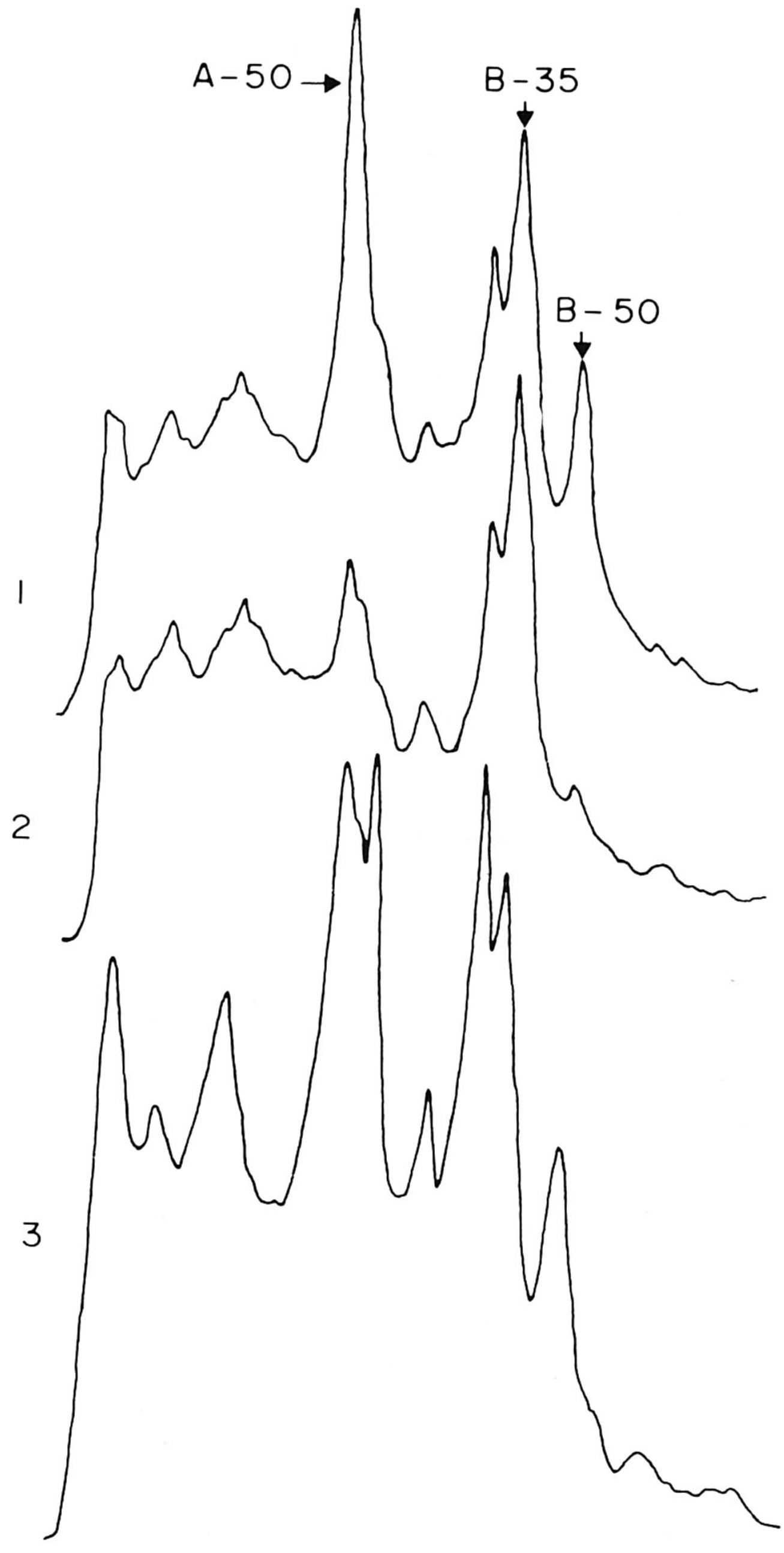


FIG. 1. SDS-gel electrophoresis of solubilized rat brain SPM proteins. The numbers at the top refer to the following treatments: 1, 0.5% Na-DOC; 2, 0.5% Triton X-100; 3, 0.5% Na-DOC + 0.5% Triton X-100; 4, 0.5% Triton X-100 + 75 mM-KCl; 5, control SPM; 6, residue after extraction 4. Twenty μg of protein from each sample was analysed in duplicate by electrophoresis on 11% SDS-gels. The letters on the left side mark the regions referred to in the text. On the right side the position of molecular weight marker proteins is indicated.



(a)



(b)

FIG. 4. A. Autoradiogram showing the endogenous phosphorylation and separation of T-SPM proteins after SDS-PAGE. Phosphorylation was carried out for 15 s with 200 μM-ATP (see Materials and Methods). Track 1, control T-SPM; 2, $+10^{-4}$ M-$ACTH_{1-24}$; 3, $+5 \times 10^{-6}$ M-cAMP. The position of some phosphoprotein bands is indicated at the right. B. Densitometric scans of tracks 1–3 shown in Fig. 4A.

TABLE 1. 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)* A	B	Inhibition by $ACTH_{1-24}$† %
SPM	8.0 ± 0.1‡	—	102.0 ± 5.2	−52
Triton X-100–KCl extract	6.0 ± 0.1	6.2 ± 0.3	16.0 ± 0.4	−52
Triton X-100–KCl residue	2.2 ± 0.1	29.3 ± 1.2	86.4 ± 3.2	−51
Triton X-100 extract	6.4 ± 0.2	n.d.	n.d.	−5
Deoxycholate (DOC) extract	7.0 ± 0.1	n.d.	n.d.	+5
Triton X-100–DOC extract	7.0 ± 0.1	n.d.	n.d.	−2

* The total B-50 protein kinase activity was determined using linear incorporation conditions (15 s, low ATP/SPM ratio (A) and high ATP/SPM ratio (B)). No linear incorporation could be detected in SPM using condition A (see also Fig. 3). One unit of B-50 protein kinase activity is defined as the amount of enzyme transferring 1 pmol phosphate to B-50 in 1 min at 30°C.

† The inhibition by $ACTH_{1-24}$ (10^{-4} M) was studied under a high ATP/SPM ratio (B) and is expressed as the percentage difference from control.

‡ Mean ± S.E.M.; n = 3.

the case in regions A and B (see, for nomenclature of phosphoprotein regions, WIEGANT *et al.*, 1978; Fig. 1).

In contrast to the similarity in protein staining pattern of the extracts, the pattern of phosphorylated proteins per extract depended on the type of detergent used (Fig. 2). Of the various SPM fractions, equal amounts of protein were used in both incubation and SDS–PAGE and comparisons were only made between densitometric scans obtained from tracks on one single autoradiograph. In all the solubilized fractions, the endogenous phosphorylation of the lower molecular weight protein bands (region D) was impaired. When Triton X-100 was used an enhanced endogenous phosphorylation of some protein bands in region B was observed. Furthermore, in T-SPM and Triton X-100 extract the endogenous phosphorylation of band A-50 was enhanced (region A). The deoxycholate solubilized material showed nearly an overall reduction in endogenous phosphorylation although again a small stimulatory effect on some of the protein bands in region B was observed (see also after Triton X-100). The T-SPM residue displayed an overall enhancement of endogenous phosphorylation as compared to intact SPM (Fig. 2).

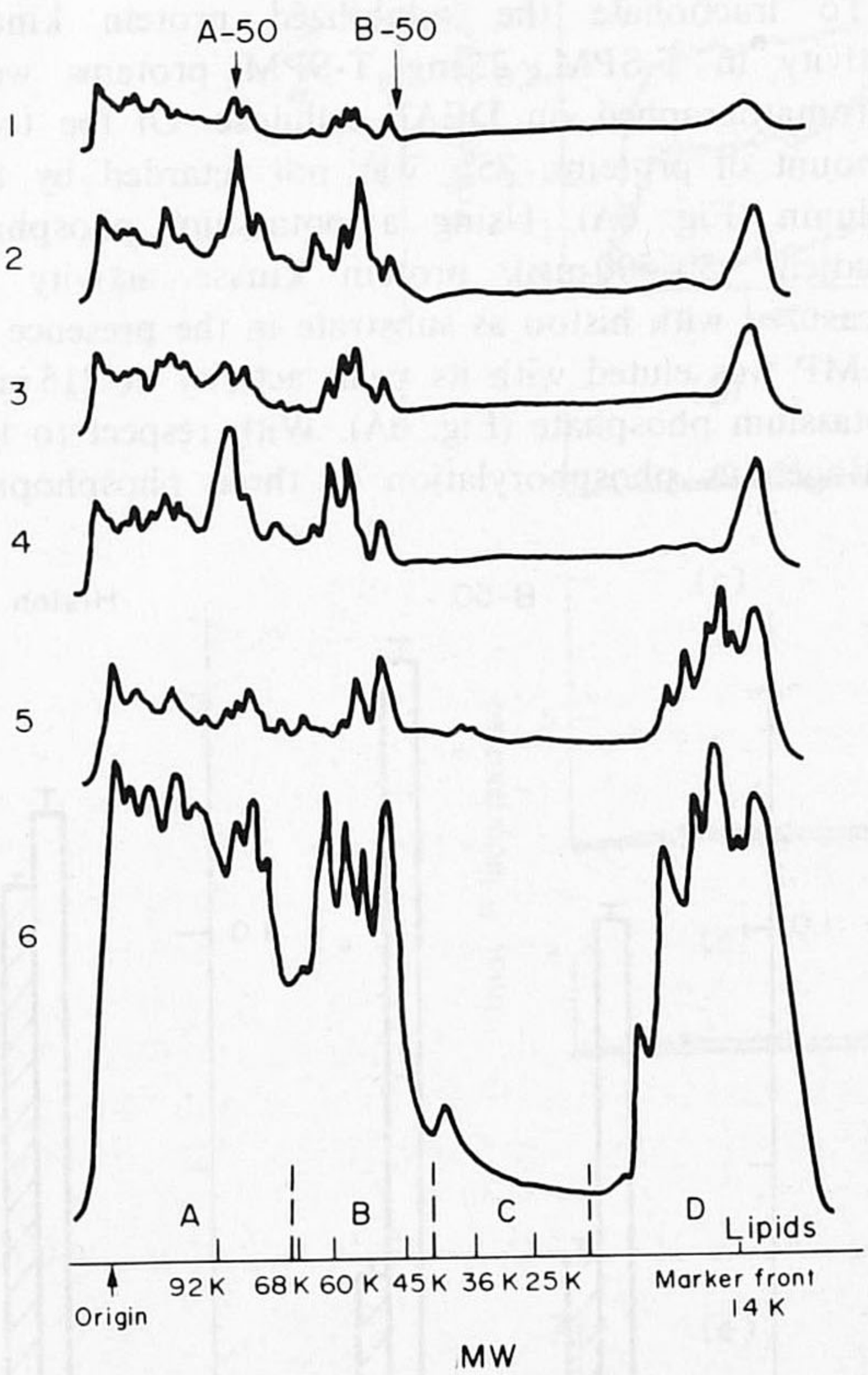


FIG. 2. Densitometric tracing of autoradiograms obtained after endogenous phosphorylation and SDS–PAGE electrophoresis of solubilized rat brain SPM proteins. The numbers refer to the various extraction procedures used (see legend to Fig. 1). On the bottom, the letters mark the regions referred to in the text and the position of the Bromphenol Blue front and molecular weight marker proteins is indicated. The labelled material running ahead of the marker front is lipid extractable and is not stained by Fast Green. The phosphorylation was carried out for 15 s under a high ATP/SPM ratio (condition B, see text).

The rate of ATP hydrolysis, determining the availability of ATP in endogenous phosphorylation reaction, was measured in intact SPM, T-SPM and T-SPM residue. In both T-SPM and residue only 1% of the original ATP hydrolysing activity of the original SPM (250 pmol/μg protein/min, condition B) could be detected, indicating that the Triton X-100–KCl treatment almost totally suppressed the endogenous ATP-ase activity.

The rate of endogenous phosphorylation of protein band B-50 was different when studied in SPM, T-SPM or T-SPM-residue. As reported before (WIEGANT *et al.*, 1978) in intact SPM under phosphorylation condition A (7.5 μM-ATP) no linear incorporation of ^{32}P into band B-50 was observed (Fig. 3). Under the same conditions, the phosphorylation of this band in T-SPM and T-SPM-residue appeared to be linear for at least 60 and 30 s, respectively. If a higher ATP/SPM ratio was used (condition B) linear incorporation during at least 15–20 s was found in all three fractions (Fig. 3). Thus for estimating the

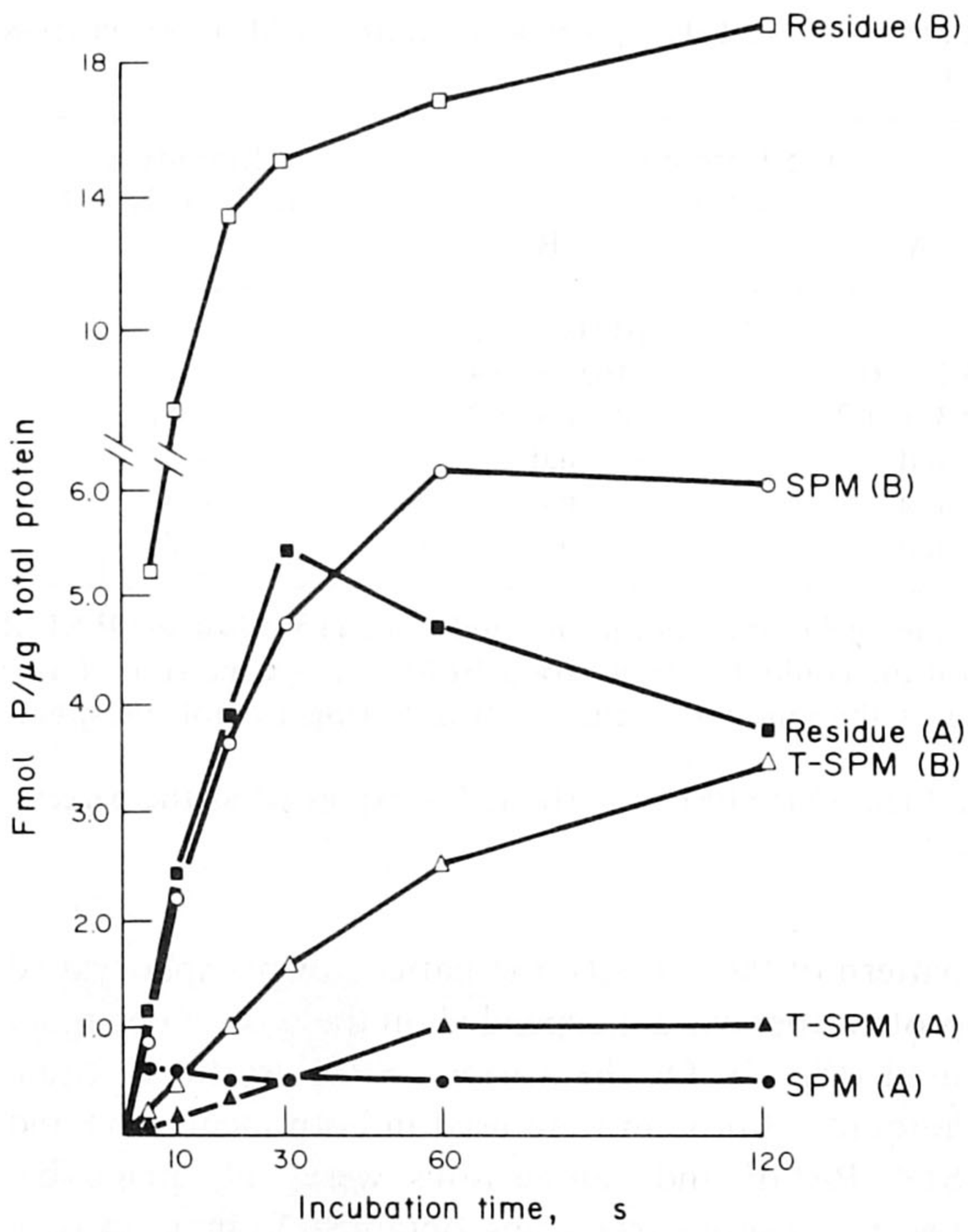


FIG. 3. Endogenous phosphorylation of protein band B-50 in SPM, T-SPM and residue. The phosphorylation was studied under a low (condition A) and high (condition B) ATP/SPM ratio (see text). The amount of incorporated radioactive phosphate was determined by liquid scintillation counting of the excised band B-50. The data are expressed as fmol phosphate incorporated per μg total incubated protein.

amount of B-50 protein kinase activity solubilized by the Triton–KCl procedure, only the high ATP/SPM ratio condition could be used. As is shown in Table 1, only 15% of the original B-50 protein kinase activity was solubilized by Triton X-100–KCl. However, the criterion used to select the optimal solubilization procedure was whether or not the sensitivity of the B-50 protein kinase to ACTH was preserved under solubilized conditions. As can be seen in Table 1, only after extraction with Triton X-100–KCl was the inhibition of B-50 protein kinase by $ACTH_{1-24}$ preserved both in T-SPM and in its residue. Therefore, this solubilization procedure, despite its low efficiency, was used for further study of the membrane-bound ACTH-sensitive B-50 protein kinase.

Effects of $ACTH_{1-24}$ and cAMP on solubilized B-50 protein kinase

In Fig. 4 the autoradiograph from T-SPM proteins after incubation with 200 μM-ATP is shown. Addition of 5×10^{-6} M-cAMP enhanced the endogenous phosphorylation of a number of high molecular weight phosphoprotein bands (Fig. 4, track 3, scan 3). The phosphorylation of the band A-50, B-35 and B-50 was not affected by cAMP under these circumstances. Incubation with 10^{-4} M-$ACTH_{1-24}$ led to a selective inhibition of the endogenous phosphorylation of bands B-50 and A-50 (Fig. 4, track 2, scan 2). The bands D-10, D-20, D-25 and D-30, in which the endogenous phosphorylation in intact SPM is inhibited by $ACTH_{1-24}$ (ZWIERS *et al.*, 1976, 1978) were not phosphorylated in T-SPM (Figs 1 and 4). The protein band B-50 was extracted from SDS–PAGE slabs and added as exogenous substrate to T-SPM (see Materials and Methods). As can be seen in Fig. 5, the exogenously added B-50 proteins could serve as substrate for endogenous T-SPM kinase(s). The addition of 1 μg B-50 protein resulted in an increased radioactivity in band B-50 as determined by liquid scintillation counting of radioactivity present in that band after SDS–PAGE. The phosphorylation of exogenously added B-50 was completely inhibited by $ACTH_{1-24}$, for the residual amount of radioactivity in the presence of $ACTH_{1-24}$ was not affected by the addition of exogenous B-50. T-SPM protein kinase(s) also phosphorylated histon added to the incubation mixture (Fig. 5). However, phosphorylation of this substrate was only slightly affected by $ACTH_{1-24}$ (−15%).

Multiple protein kinase present in T-SPM

To fractionate the solubilized protein kinase activity in T-SPM, 25 mg T-SPM proteins were chromatographed on DEAE–cellulose. Of the total amount of proteins, 75% was not retarded by the column (Fig. 6A). Using a potassium phosphate gradient (50–500 mM), protein kinase activity as measured with histon as substrate in the presence of cAMP was eluted with its peak activity at 215 mM-potassium phosphate (Fig. 6A). With respect to the endogenous phosphorylation of three phosphopro-

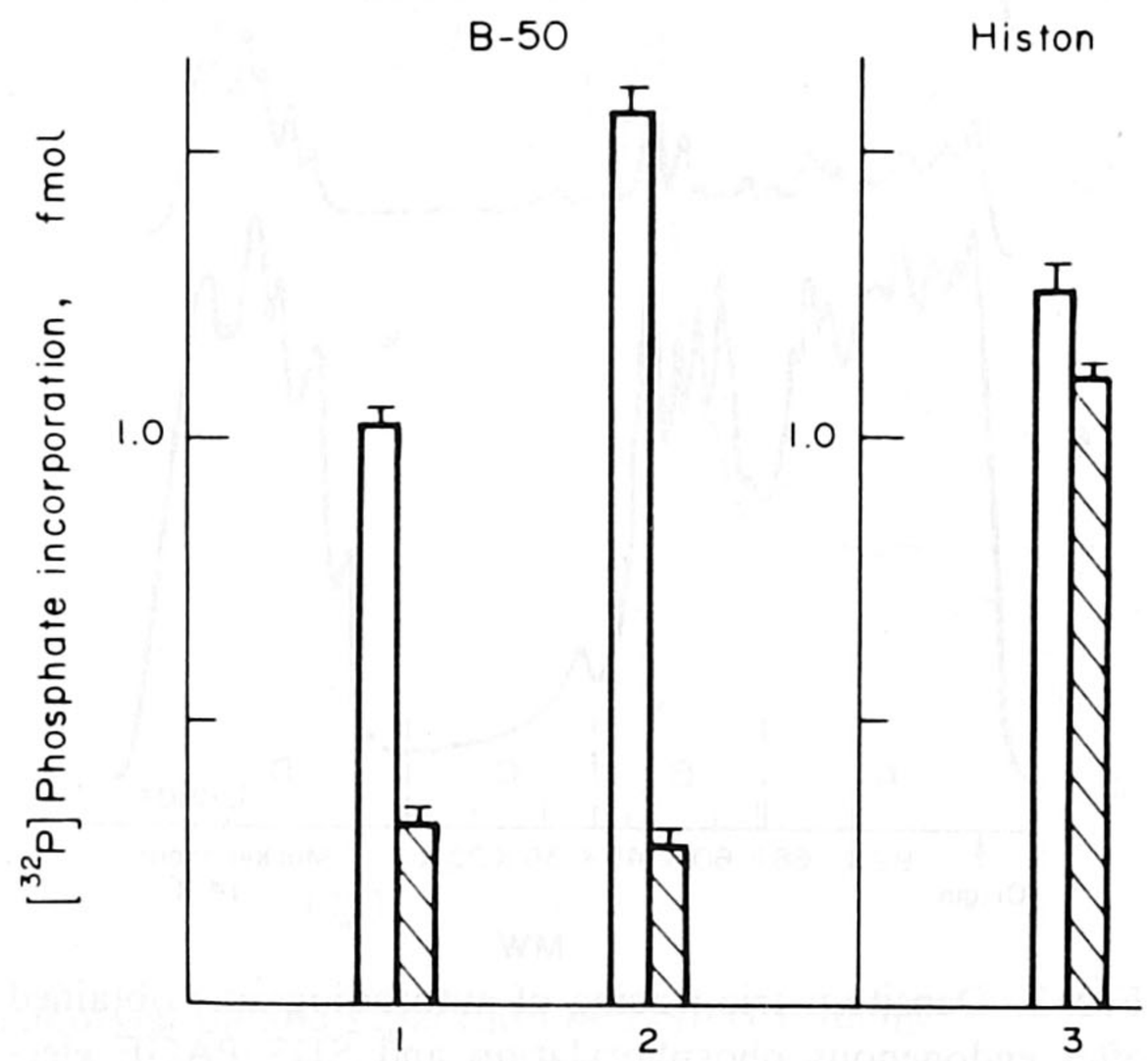


FIG. 5. The effect of $ACTH_{1-24}$ on the phosphorylation of endogenous (1) and exogenous (2) B-50 and histon (3) by T-SPM protein kinases. The phosphorylation of B-50 was carried out under condition A for 1 min. Histon was phosphorylated as described in Materials and Methods. Open bars represent the phosphorylation in the absence of $ACTH_{1-24}$; hatched bars represent the phosphorylation in the presence of 10^{-4} M-$ACTH_{1-24}$. 1, B-50 in T-SPM; 2, T-SPM + 1 μg exogenous B-50; 3, T-SPM + histon.

teins present in T-SPM (A-50, B-50 and B-30), the protein kinase activity was found to be eluted in three overlapping peaks (Fig. 6B, C, D). At 130 mM-potassium phosphate, endogenous phosphorylation of band A-50 was found (Fig. 6B), at 200 mM that of band B-50 (Fig. 6D) and at 260 mM that of band B-30 (Fig. 6C).

The endogenous phosphorylation of bands B-50 and A-50 as studied in eluate fractions of the DEAE–cellulose column was still sensitive to $ACTH_{1-24}$, as was true for the original T-SPM (Fig. 6C, D). Likewise, the endogenous phosphorylation of B-30 was insensitive to $ACTH_{1-24}$ both in the eluate and in the T-SPM. Exogenous B-50 proteins added to the column eluate fractions were only phosphorylated in those fractions which displayed endogenous phosphorylation of protein band B-50 (Fig. 6E).

DISCUSSION

An attempt was made to solubilize protein kinase from rat brain synaptosomal plasma membranes, 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 $ACTH_{1-24}$.

In pilot experiments it was found that the extraction procedure (0.1% Triton) as described by Uno *et al.* (1976, 1977) did yield some solubilized B-50 protein kinase activity but the sensitivity to $ACTH_{1-24}$ was not preserved in the soluble SPM extract. Therefore alternative extraction procedures were studied.

The detergent procedures used, all solubilized between 75 and 88% of the membrane protein, yielding extracts of a rather similar protein composition.

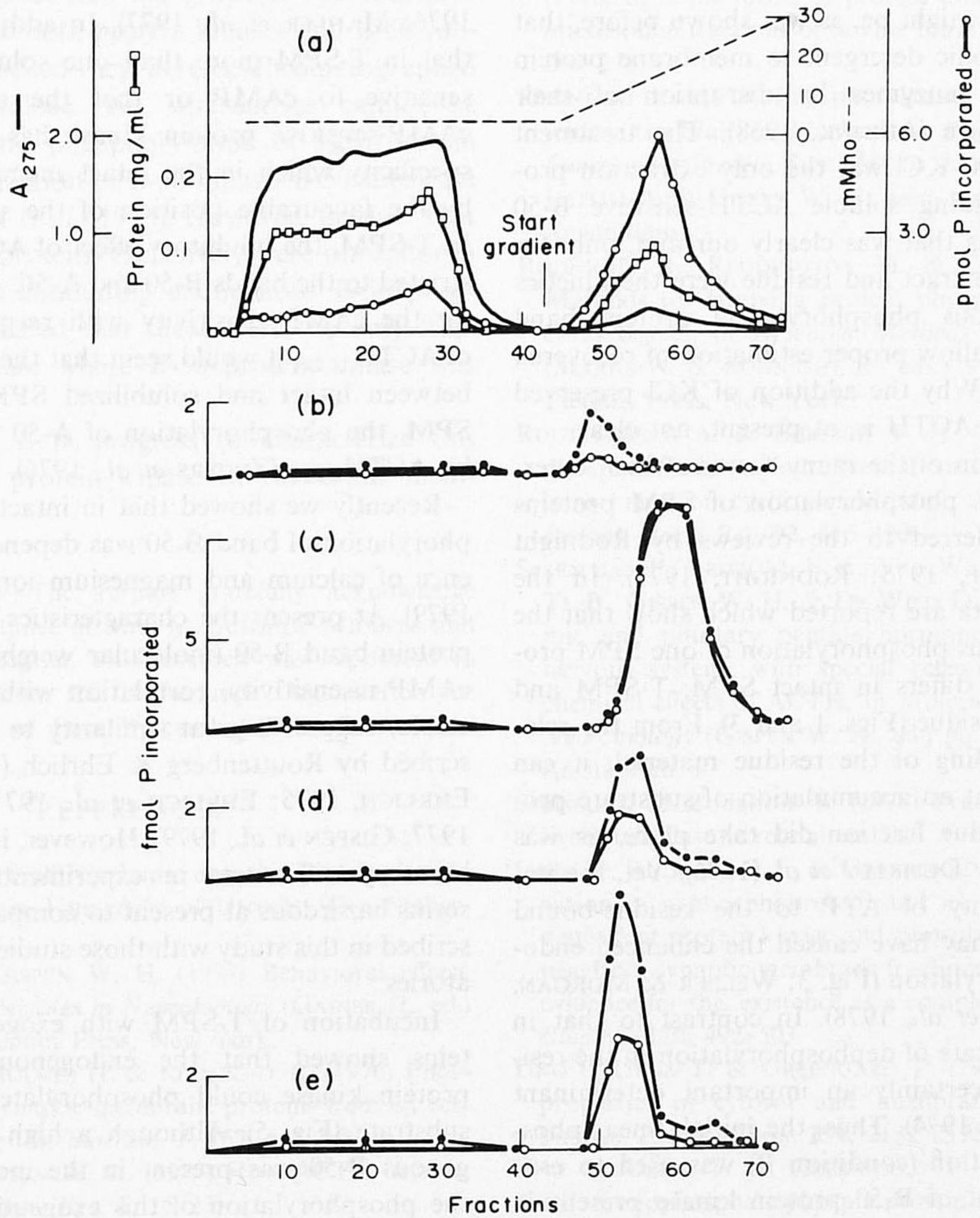


FIG. 6. DEAE–cellulose column chromatography of rat brain SPM protein kinases solubilized by 0.5% Triton X-100–75 mM-KCl. The proteins were eluted using a potassium phosphate gradient (50–500 mM) at pH 7.0. A. Extinction at 275 nm was monitored continuously (——). Fractions of 2.2 ml were collected and assayed for protein content (□——□), histon-kinase activity (○——○) and conductivity in mMho/cm (– – –). B, C, D. Endogenous phosphorylation of protein bands in the absence (●——●) or presence (○——○) of $ACTH_{1-24}$. A-50 (B), B-30 (C) and B-50 (D). E. Endogenous phosphorylation of B-50 in the presence of exogenous B-50 (1 μg) with (○——○) or without (●——●) $ACTH_{1-24}$.

From the PAGE patterns of the small amount of proteins that resided in the particulate fractions after extraction it was concluded that some selectivity of extraction must have occurred (see also DUNKLEY *et al.*, 1976). The degree of selectivity was small and therefore was difficult to detect in the bulk, solubilized proteins. However, the pattern of endogenous protein phosphorylation varied among the extracts obtained. In Triton extracts an enhanced endogenous phosphorylation of proteins of higher molecular weight was observed, as was also reported by others (UEDA *et al.*, 1975; MAENO *et al.*, 1971). Improved accessibility of precursor and substrate protein to the corresponding protein kinase(s) may be factors determining this enhanced endogenous phosphorylation (MIYAMOTO, 1976; SOMMARIN & JERGIL, 1978). Since after extraction with DOC the bulk of proteins was also solubilized, the impairment of endogenous phosphorylation in DOC extracts seems to be not related to a differential extraction of protein kinases and substrate proteins. It might be, as was shown before, that a high ratio of ionic detergent to membrane protein ratio inactivated enzymes by disruption of their secondary structure (ADDINK, 1968). The treatment with Triton X-100–KCl was the only extraction procedure used, yielding soluble ACTH-sensitive B-50 protein kinase. As that was clearly our aim, only for the Triton–KCl extract and residue were the kinetics of the endogenous phosphorylation protein band B-50 studied, to allow proper estimation of recovered enzyme activity. Why the addition of KCl preserved the sensitivity to ACTH is, at present, not clear.

For a discussion of the many factors which determine endogenous phosphorylation of SPM proteins the reader is referred to the reviews by Rodnight (RODNIGHT *et al.*, 1975; RODNIGHT, 1977). In the present paper data are reported which show that the rate of endogenous phosphorylation of one SPM protein band (B-50) differs in intact SPM, T-SPM and corresponding residue (Figs. 1 and 3). From the relatively high labelling of the residue material, it can be concluded that an accumulation of substrate proteins in the residue fraction did take place, as was also suggested by DUNKLEY *et al.* (1976). Yet, the improved availability of ATP to the residue-bound kinase by itself may have caused the enhanced endogenous phosphorylation (Fig. 3; WELLER & MORGAN, 1976; WIEGANT *et al.*, 1978). In contrast to that in intact SPM, the rate of dephosphorylation in the residue fraction is certainly an important determinant (Fig. 3; WELLER, 1974). Thus, the initial, linear phosphorylation reaction (condition B) was used to estimate the amount of B-50 protein kinase present in the various fractions. It was found that only 15% was solubilized by Triton X-100–KCl treatment and that the remaining 85% was recovered in the residue (Table 1). Thus the extraction procedure *per se* did not affect the endogenous B-50 protein kinase activity. The low recovery in the soluble fraction of membrane-bound protein kinase was in line with the data obtained by WELLER & MORGAN (1976). When the units of enzyme activity obtained under low and high ATP/SPM ratio conditions were compared (Table 1), a clear stimulation by ATP of protein kinase activity was observed, as was reported before for membrane-bound protein kinase(s) (RODNIGHT *et al.*, 1975; WIEGANT *et al.*, 1978).

In T-SPM, using a low ATP/protein ratio (condition A) addition of cAMP resulted in a rather general increase in phosphorylation of nearly all phosphoprotein bands (data not shown). Using the high ATP/protein ratio condition (Fig. 4), a pronounced stimulation of the phosphorylation of a number of bands in the high molecular region was observed. In contrast, in intact SPM at high ATP concentrations, the effect of cAMP was greatly reduced or even absent (ROUTTENBERG & EHRLICH, 1975; ZWIERS, unpublished). In addition, others have reported that the regulation of cAMP sensitive phosphorylation is susceptible to a number of experimental variables (DUNKLEY *et al.*, 1976; MAHLER *et al.*, 1977). In addition, it might be that in T-SPM more than one solubilized kinase is sensitive to cAMP or that the membrane-bound cAMP-sensitive protein kinase has a low substrate specificity which in the intact membrane is masked by the favourable position of the substrate protein. In T-SPM, the inhibitory effect of $ACTH_{1-24}$ was restricted to the bands B-50 and A-50. As also discussed for the cAMP sensitivity with respect to the effect of $ACTH_{1-24}$, it would seem that there are differences between intact and solubilized SPM, for, in intact SPM, the phosphorylation of A-50 was not affected by $ACTH_{1-24}$ (ZWIERS *et al.*, 1976).

Recently we showed that in intact SPM the phosphorylation of band B-50 was dependent on the presence of calcium and magnesium ions (GISPEN *et al.*, 1979). At present the characteristics of the phosphoprotein band B-50 (molecular weight, ion sensitivity, cAMP-insensitivity, correlation with behavioral processes) suggest a great similarity to the band H described by Routtenberg & Ehrlich (ROUTTENBERG & EHRLICH, 1975; EHRLICH *et al.*, 1977; ZWIERS *et al.*, 1977; GISPEN *et al.*, 1979). However, in view of interlaboratory differences in experimental procedure, it seems hazardous at present to compare the bands described in this study with those studied in other laboratories.

Incubation of T-SPM with exogenous B-50 proteins showed that the endogenous T-SPM B-50 protein kinase could phosphorylate this exogenous substrate (Fig. 5). Although a high amount of exogenous B-50 was present in the incubation mixture the phosphorylation of this exogenous protein band was rather inefficient (0.5 fmol phosphate/21 pmol B-50). However, $ACTH_{1-24}$ completely inhibited the phosphorylation of exogenous B-50, providing a valuable criterion to establish, in further experiments on fractionation of protein kinase(s) in T-SPM, the fate of the B-50 protein kinase.

DEAE-column chromatography of T-SPM yielded

a single peak of protein kinase activity assayed with histon as exogenous substrate (Fig. 6A). When the phosphorylation of endogenous substrate (Fig. 6B, C, D) was measured, evidence for the presence of more than one solubilized protein kinase was obtained, for the phosphorylation of three endogenous proteins took place in different fractions of the eluate. Furthermore, the phosphorylation of two of those (A-50 and B-50) was inhibited by $ACTH_{1-24}$, whereas that of B-30 was not affected by the peptide. Finally, exogenous B-50 proteins were only phosphorylated in fractions displaying the endogenous B-50 protein kinase activity and not in those displaying only endogenous A-50 or B-30 protein kinase activity. Recently, MIYAMOTO *et al.* (1978) also described the occurrence of multiple forms of protein kinases in brain membrane fractions with respect to substrate specificity and physical property.

At this stage of the fractionation of B-50 protein kinase it may well be that the Triton X-100–KCl procedure solubilized both protein kinases and B-50 substrate and that indeed they were co-chromatographed over DEAE–cellulose. Yet, alternatives cannot be excluded, e.g. auto phosphorylation of B-50 protein kinase or solubilization of B-50 kinase–B-50 substrate complexes (UEDA *et al.*, 1975). However, the fact that exogenous B-50 proteins exclusively were phosphorylated in fractions containing endogenous B-50 phosphorylation indicated that those were the only fractions of the eluate where B-50 protein kinase was present.

Further work is in progress to characterize this ACTH-sensitive protein kinase in rat brain membranes.

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REFERENCES

ADDINK A. D. F. (1968) Carbonic anhydrase of *Sepia officinalis* (L.). Thesis, University of Utrecht, The Netherlands.

DE WIED D. & GISPEN W. H. (1977) Behavioral effects of peptides, in *Peptides in Neurobiology* (GAINER H., ed.) pp. 397–448. Plenum Press, New York.

DUNKLEY P. R., HOLMES H. & RODNIGHT R. (1976) Phosphorylation of synaptic-membrane proteins from ox cerebral cortex *in vitro*. *Biochem. J.* **157**, 661–666.

DUNN A. J. & GISPEN W. H. (1977) How ACTH acts on the brain. *Biobehav. Rev.* **1**, 15–23.

EHRLICH Y. H., RABJOHNS R. H. & ROUTTENBERG A. (1977) Experimental input alters the phosphorylation of specific proteins in brain membranes. *Pharmac. Biochem. Behav.* **6**, 169–175.

GISPEN W. H., ZWIERS H., WIEGANT V. M., SCHOTMAN P. & WILSON J. E. (1979) The behaviorally active neuropeptide ACTH as neurohormone and neuromodulator: the role of cyclic nucleotides and membrane phosphoprotein, in *Modulators, Mediators and Specifiers in Brain Function* (EHRLICH Y. H. & DAVIS L. G.) pp. 199–224. Plenum Press, New York.

HESS H. H. & DERR J. E. (1975) Assay of inorganic and organic phosphorus in the 0.1–5 nanomole range. *Analyt. Biochem.* **63**, 607–613.

LOWRY O. H., ROSEBROUGH N. J., FARR A. L. & RANDALL R. J. (1951) Protein measurement with the Folin phenol reagent. *J. biol. Chem.* **193**, 265–275.

MAENO H., JOHNSON E. M. & GREENGARD P. (1971) Subcellular distribution of cAMP dependent protein kinase in rat brain. *J. biol. Chem.* **246**, 134–142.

MAHLER H., WANG Y. J., DE BLAS A. & CRAWFORD G. (1977) Topography of membrane proteins at vertebrate synapses, in *Mechanisms, Regulation and Special Function of Protein Synthesis in the Brain* (ROBERTS S., LAJTHA A. & GISPEN W. H., eds.) pp. 205–220. Elsevier, Amsterdam.

MIYAMOTO E. (1976) Phosphorylation of endogenous proteins in myelin of rat brain. *J. Neurochem.* **26**, 573–577.

MIYAMOTO E., MIYAZAKI K., HIROSE R. & KASHIBA A. (1978) Multiple forms of protein kinases in myelin and microsomal fractions of bovine brain. *J. Neurochem.* **31**, 269–275.

RODNIGHT R. (1977) Phosphorylation of membrane proteins in the brain, in *Mechanisms, Regulation and Special Function of Protein Synthesis in the Brain* (ROBERTS S., LAJTHA A. & GISPEN W. H., eds.) pp. 255–277. Elsevier, Amsterdam.

RODNIGHT R., REDDINGTON M. & GORDON M. (1975) Methods for studying protein phosphorylation in cerebral tissues, in *Research Methods in Neurochemistry* (MARKS N. & RODNIGHT R., eds.) Vol. **3**, pp. 324–367. Plenum Press, New York.

ROUTTENBERG A. & EHRLICH Y. H. (1975) Endogenous phosphorylation of four cerebral cortical membrane proteins: role of cyclic nucleotides, ATP and divalent cations. *Brain Res.* **92**, 415–430.

SCHOTMAN P., REITH M. E. A., VAN WIMERSMA GREIDANUS TJ. B., GISPEN W. H. & DE WIED D. (1976) Hypothalamic and pituitary peptide hormones and the central nervous system: with special reference to the neurochemical effects of ACTH, in *Molecular and Functional Neurobiology* (GISPEN W. H., ed.) pp. 309–344. Elsevier, Amsterdam.

SOMMARIN M. & JERGIL B. (1978) Protein kinases of rat liver endoplasmic reticulum. *Eur. J. Biochem.* **88**, 49–60.

UEDA T., RUDOLPH S. A. & GREENGARD P. (1975) Solubilization of a phosphoprotein and its associated cAMP-dependent protein kinase and phosphoprotein phosphatase from synaptic membrane fractions, and some kinetic evidence for this existence as a complex. *Archs Biochem. Biophys.* **170**, 492–503.

UNO I., UEDA T. & GREENGARD P. (1976) Differences in properties of cytosol and membrane-derived protein kinases. *J. biol. Chem.* **251**, 2192–2195.

UNO I., UEDA T. & GREENGARD P. (1977) Adenosine 3′,5′-monophosphate 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.

WELLER M. (1974) A theoretical treatment of the turnover of protein-bound phosphate in the presence of both protein kinase and phosphatase activities. *Biochim. biophys. Acta* **343**, 565–583.

WELLER M. & MORGAN I. (1976) Distribution of protein kinase activities in subcellular fractions of rat brain. *Biochim. biophys. Acta* **436,** 675–686.

WIEGANT V. M., ZWIERS H., SCHOTMAN P. & GISPEN W. H. (1978) Endogenous phosphorylation of rat brain synaptosomal plasma membranes *in vitro*: methodological aspects. *Neurochem. Res.* **3,** 443–453.

ZWIERS H., VELDHUIS D., SCHOTMAN P. & 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. & GISPEN W. H. (1977) Intraventricular administered ACTH and changes in rat brain protein phosphorylation: a preliminary report, in *Mechanism, Regulation and Special Functions of Protein Synthesis in the Brain* (ROBERTS S., LAJTHA A. & GISPEN W. H., eds.) pp. 267–272. Elsevier/North Holland Biomedical Press, Amsterdam.

ZWIERS H., WIEGANT V. M., SCHOTMAN P. & GISPEN W. H. (1978) ACTH-induced inhibition of endogenous rat brain protein phosphorylation *in vitro*: structure–activity. *Neurochem. Res.* **3,** 455–463.