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Characterization of Infant Rat Cerebral Cortical Membrane Proteins Phosphorylated *In Vivo:* Identification of the ACTH-Sensitive Phosphoprotein B-50

*A. B. Oestreicher, *H. Zwiers, *W. H. Gispen, and †S. Roberts

**Division of Molecular Neurobiology, Rudolf Magnus Institute for Pharmacology, Medical Faculty, Institute of Molecular Biology, State University of Utrecht, Utrecht, The Netherlands; and †Department of Biological Chemistry, School of Medicine, and The Brain Research Institute, UCLA Center for the Health Sciences, Los Angeles, California, U.S.A.*

Abstract: This study on the phosphorylation *in vivo* of membrane proteins in cerebral cortices of infant rats reports the identification of the adrenocorticotropin (ACTH)-sensitive phosphoprotein B-50 as one of the substrate proteins that are rapidly phosphorylated *in vivo* following intracisternal administration of 2 mCi [^{32}P]orthophosphate. Rats were sacrificed 30 min after isotope injection. A fraction enriched in membranes, designated neural membranes (NM), was isolated from the cerebral cortices according to the procedure used for preparation of synaptic plasma membranes (SPM) from adult brain. This NM fraction was characterized by electron microscopy. The proteins of NM were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and autoradiography. Numerous protein bands of NM in infant rat brain were phosphorylated *in vivo*. Attention was focussed on the ^{32}P-labeled protein bands in the molecular weight range of 47K–67K. In this region one phosphoprotein band (MW 48K) was more highly labeled than the other bands. The electrophoretic behavior of three of these labeled bands, designated a, c, and e (MW 48K, 55K, and 62K, respectively) was compared with that of protein bands that were phosphorylated *in vitro* in cerebral membranes isolated from noninjected infant rats. The effects of $ACTH_{1-24}$ and cyclic AMP in the *in vitro* system were also studied to probe for the presence of specific membrane proteins known to be sensitive to these modulators. On incubation of NM with [γ-^{32}P]ATP in the presence and absence of $ACTH_{1-24}$ *in vitro*, phosphorylation of a 48K protein band was inhibited in a dose-dependent fashion by the neuropeptide. Two-dimensional electrophoretic separation of NM proteins labeled *in vivo* indicated that the 48K band had an isoelectric point of 4.5, identical to that of the ACTH-sensitive B-50 protein previously identified. Cyclic AMP stimulated phosphorylation *in vitro* of two protein bands (MW 55K and 59K) in NM preparations. This result indicates that the *in vivo* labeled band c may correspond to the cyclic AMP-sensitive 55K protein, whereas phosphoprotein band e, labeled *in vivo*, appears to be different from the cyclic AMP-sensitive 59K protein band. These observations indicate that neural membranes isolated from infant rat cerebral cortices contain a variety of proteins that can be phosphorylated *in vivo*. Several of these, for example, the 48K protein band, have the properties of synaptic plasma membrane proteins of adult rat brain that have been characterized by their sensitivity to

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Address correspondence and reprint requests to A. B. Oestreicher, Division of Molecular Neurobiology, Rudolf Magnus Institute for Pharmacology, Medical Faculty, Institute of Molecular Biology, State University of Utrecht, Utrecht, The Netherlands.

Abbreviations used: ACTH, Adrenocorticotropin; IEP, Isoelectric point; IMX, 3-Isobutyl-1-methylxanthine; NM, Neural membranes; PAGE, Polyacrylamide gel electrophoresis; SDS, Sodium dodecyl sulfate; SPM, Synaptic plasma membranes; TCA, Trichloroacetic acid.

neuromodulators in endogenous phosphorylating systems *in vitro*. **Key Words:** Phosphorylation *in vivo*—B-50 protein—ACTH—Cyclic nucleotides—Infant rat brain. **Oestreicher A. B. et al.** Characterization of infant rat cerebral cortical membrane proteins phosphorylated *in vivo:* Identification of the ACTH-sensitive phosphoprotein B-50. *J. Neurochem.* **39**, 683–692 (1982).

Phosphorylation of membranes is thought to play a functional role in processes of neurotransmission and transport across membranes (Greengard, 1976, 1979). Evidence for this involvement is derived principally from a growing body of studies *in vitro* with nervous tissue preparations (Zwiers et al., 1976, 1980; Forn and Greengard, 1978; Browning et al., 1979; Bär et al., 1982; see reviews by Greengard, 1976, 1979; Williams and Rodnight, 1977). However, several investigations indicate that alterations in brain membrane phosphoproteins can also be produced *in vivo* and these alterations can be detected in subsequent *in vitro* phosphorylation assays (Ehrlich et al., 1977; Zwiers et al., 1977; Browning et al., 1979; Strombom et al., 1979). Moreover, a direct correlation between behavioral experiences and brain membrane protein phosphorylation *in vivo* was reported by Perumal et al. (1975). Acquisition of a conditioned avoidance response was accompanied by enhanced incorporation *in vivo* of [^{32}P]orthophosphate into total protein of synaptosome-enriched fractions of mouse brain homogenate (Perumal et al., 1977; Gispen et al., 1977). Detailed analysis revealed that the radioactivity of the incorporated phosphate was covalently bound to amino acids in protein (Perumal et al., 1977).

Recent reports indicate that administration of [^{32}P]orthophosphate to adult rats *in vivo* results in labeling patterns of brain membrane phosphoproteins that are very similar to those observed in *in vitro* systems (Mitrius et al., 1981). However, protein bands of synaptic plasma membrane (SPM) preparations that incorporated a large fraction of the total radioactivity in the presence of cyclic AMP *in vitro* contained only a minor fraction after *in vivo* labeling (Berman et al., 1980).

The present report describes the results of electrophoretic analyses of neural membrane proteins phosphorylated *in vivo* in cerebral cortices of 8-day-old infant rats. Certain of these proteins of the molecular weight range of 47K–67K have been characterized by comparison with the results obtained when neural membrane preparations, which were not labeled *in vivo*, were exposed to the neuromodulators adrenocorticotropin ($ACTH_{1-24}$) and cyclic AMP in an *in vitro* phosphorylating system (Zwiers et al., 1976). This comparison demonstrates that the 48K ACTH-sensitive phosphoprotein B-50 is one of the membrane proteins that were rapidly phosphorylated in cerebral cortex of the living infant rat.

EXPERIMENTAL PROCEDURES

Materials

All chemicals were analytical grade. [^{32}P]Orthophosphate in 0.02 *M* HCl, purchased from ICN Pharmaceuticals (Irvine, CA), was neutralized with NaOH and supplemented with NaCl to a final concentration of 0.9% (wt/vol) NaCl just before administration. [γ-^{32}P]ATP was synthesized by the method of Post and Sen (1967) from [^{32}P]phosphate. For some experiments, [γ-^{32}P]ATP was obtained from the Radiochemical Centre (Amersham, U.K.).

Acrylamide and methylene bisacrylamide were obtained from Bio-Rad Laboratories (Richmond, CA) and from Serva (Heidelberg, F.R.G.). Standard proteins used for molecular weight estimation were obtained from Sigma Chemical Co. (St. Louis, MO), which also supplied cyclic AMP. 3-Isobutyl-1-methylxanthine (IMX) was purchased from Mallinckrodt (St. Louis, MO). Scintillation counting solution (3a70B) was obtained from Research Products International (Elk Grove Village, IL). Synthetic $ACTH_{1-24}$ was obtained from Organon Int. BV (Oss, The Netherlands).

Treatment of animals, administration of [^{32}P]orthophosphate *in vivo*

Experiments were performed with young rats of an inbred Sprague-Dawley strain, 8 days old and weighing 16–20 g. Brain proteins were labeled by intracisternal administration of 2 mCi [^{32}P]orthophosphate (carrier-free, 285 Ci/mg), in 20 μl of 0.9% (wt/vol) NaCl at pH 7. The intracisternal route was selected because it favors incorporation of the isotope into proteins of infant rat cerebral cortex in comparison with intraperitoneal or intravenous injections (Roberts and Morelos, 1979). The animals were kept warm in the absence of the mother under an incandescent lamp throughout the experiment. The treated animals showed behaviors (such as sleeping, moving, and shaking) very similar to the untreated littermates kept under identical conditions. Preliminary experiments indicated that the change in incorporation of radioactivity into the membrane proteins of the molecular weight region of our interest was relatively small during the time period of 30–60 min after the injection. Since it was difficult to keep the animals healthy and alive for a long period of time without the mother rat, they were sacrificed by decapitation 30 min after injection of the radioactive isotope. Brains were removed rapidly and dissected in the cold at 4°C. The cerebral cortices (0.4–0.45 g wet weight/rat) were washed with homogenization medium (medium H, containing 0.305 *M* sucrose, 0.5 m*M* EDTA, pH 7.3) to remove adherent radioactivity.

Subcellular fractionation, isolation of neural membranes

Cerebral cortical tissue from three or four infant rats was pooled to provide sufficient material for isolation of

neural membranes (NM). The tissue was homogenized in medium H and the subcellular fractions prepared as described by Zwiers et al. (1976).

In preliminary experiments, the membrane fraction was prepared in the presence of inhibitors of phosphoprotein phosphatase (10 m*M* sodium fluoride or 10 m*M* sodium molybdate). These inhibitors had no detectable effect on ^{32}P incorporation into phosphoprotein bands of the 45K–67K MW region. Moreover, the pattern of ^{32}P labeling *in vivo* of protein bands in a cerebral homogenate, kept in ice during subcellular fractionation of the tissue in the absence of phosphoprotein phosphatase inhibitors, did not change noticeably (see also Mitrius et al., 1981). This also indicates that the incorporation of radioactive phosphate did not continue during storage of the homogenate and the preparation of the membranes at 0–4°C.

NM obtained from *in vivo* ^{32}P incorporation experiments were suspended in 5 volumes of buffer composed of 50 m*M* sodium acetate, 10 m*M* magnesium-acetate, pH 6.5, and 1 volume 40% (vol/vol) glycerol, to yield a suspension of approximately 2 g wet weight/ml. Protein content, determined by the method of Lowry et al. (1951), was 2–4 mg/ml. Prior to electrophoretic analysis, 2 volumes of this suspension were mixed with 1 volume of medium A. Medium A contained 6% (wt/vol) sodium dodecyl sulfate (SDS), 30% (vol/vol) glycerol, 187.5 m*M* Tris-HCl, pH 6.8, 15% (wt/vol) β-mercaptoethanol, and 0.003% (wt/vol) bromophenol blue. Samples were heated for 10 min at 65°C to ensure complete solubilization of the membranes.

Phosphorylation of neural membranes *in vitro*

^{32}P labeling of membrane proteins *in vitro* was carried out with NM isolated from noninjected infant rats as described earlier (Zwiers et al., 1976). NM protein (20–40 μg) was incubated at 30°C in 50 μl of an incubation medium containing final concentrations of 10 μ*M* ATP (1 μCi [γ-^{32}P]ATP, about 8 mCi/mol), 50 m*M* sodium acetate, and 10 m*M* magnesium acetate, pH 6.5. The influence of $ACTH_{1-24}$ on protein phosphorylation was studied at 10^{-4} *M* and 2×10^{-5} *M;* the concentration of cyclic AMP was 5×10^{-6} *M*. In the experiments with cyclic AMP, Ca^{2+}-dependent protein kinase activity was inhibited by addition of 1 m*M* EDTA and 1 m*M* EGTA, pH 6.5. The breakdown of cyclic AMP was prevented by addition of 0.5 m*M* IMX, an inhibitor of cyclic nucleotide phosphodiesterase. Two control samples were also included: one containing the additions IMX, EGTA, and EDTA, and one without these additions.

Incubation media containing NM, plus or minus neuromodulators, were preincubated for 3 min; phosphorylation was initiated by addition of 10 μl of [γ-^{32}P]-ATP. The reaction was terminated 20 s later with 25 μl of medium A. The samples were heated for 10 min at 65°C.

Measurement of protein-bound radioactivity in NM preparation

Proteins in samples (30–50 μl) of NM labeled with [^{32}P]phosphate were precipitated by addition of trichloroacetic acid (TCA) to a final concentration of 10% (wt/vol). Non-protein-bound ^{32}P was removed by heating the precipitate for 20 min at 90°C in 10% (wt/vol) TCA. The suspension was then chilled and the protein precipitate was collected on 25 mm Millipore filters (HAWP pore size, 0.45 μm). After thorough washing with 10% (wt/vol) TCA, the filters were transferred to counting vials, immersed, and vigorously shaken in 5 ml of chloroform-methanol (2:1) for 60 min, then washed twice with chloroform-methanol. The filters were next extracted for 20 min in 3 ml 95% ethanol. Finally, the filters were air-dried, placed in fresh vials, and 10 ml of scintillation fluid was added. ^{32}P radioactivity was counted in a Packard Tri-Carb scintillation spectrophotometer. Efficiency of detection of ^{32}P radioactivity exceeded 90%. (Counts were corrected to the value of the ^{32}P preparation at the reference date).

Separation of phosphoprotein bands by SDS-polyacrylamide gel electrophoresis (PAGE)

Samples (20–50 μg) of the phosphorylated proteins of NM, solubilized in medium A, were analyzed by electrophoresis in an SDS system on 11% (wt/vol) or 9% (wt/vol) polyacrylamide slab gels (11 × 9 cm) as described previously (Oestreicher et al., 1981). Proteins on the gel were stained for 1 h in 0.1% (wt/vol) Coomassie Brilliant Blue in 7.5% (vol/vol) acetic acid, and 40% (vol/vol) methanol and then destained by diffusion overnight. In some experiments, staining was carried out with Fast Green. After destaining, gels were dried and subjected to autoradiography against Kodak No-Screen X-ray or Kodak Royal X-Omat X-ray films (Zwiers et al., 1976). Exposure of a film to a gel with proteins labeled *in vivo* required 5 days or longer. Radioactivity of individual bands was determined by photometric scanning of the autoradiograph at 550 nm with a Zeiss PMQ-II spectrophotometer (slide width: 0.02–0.03 mm) and a linear gel scanner.

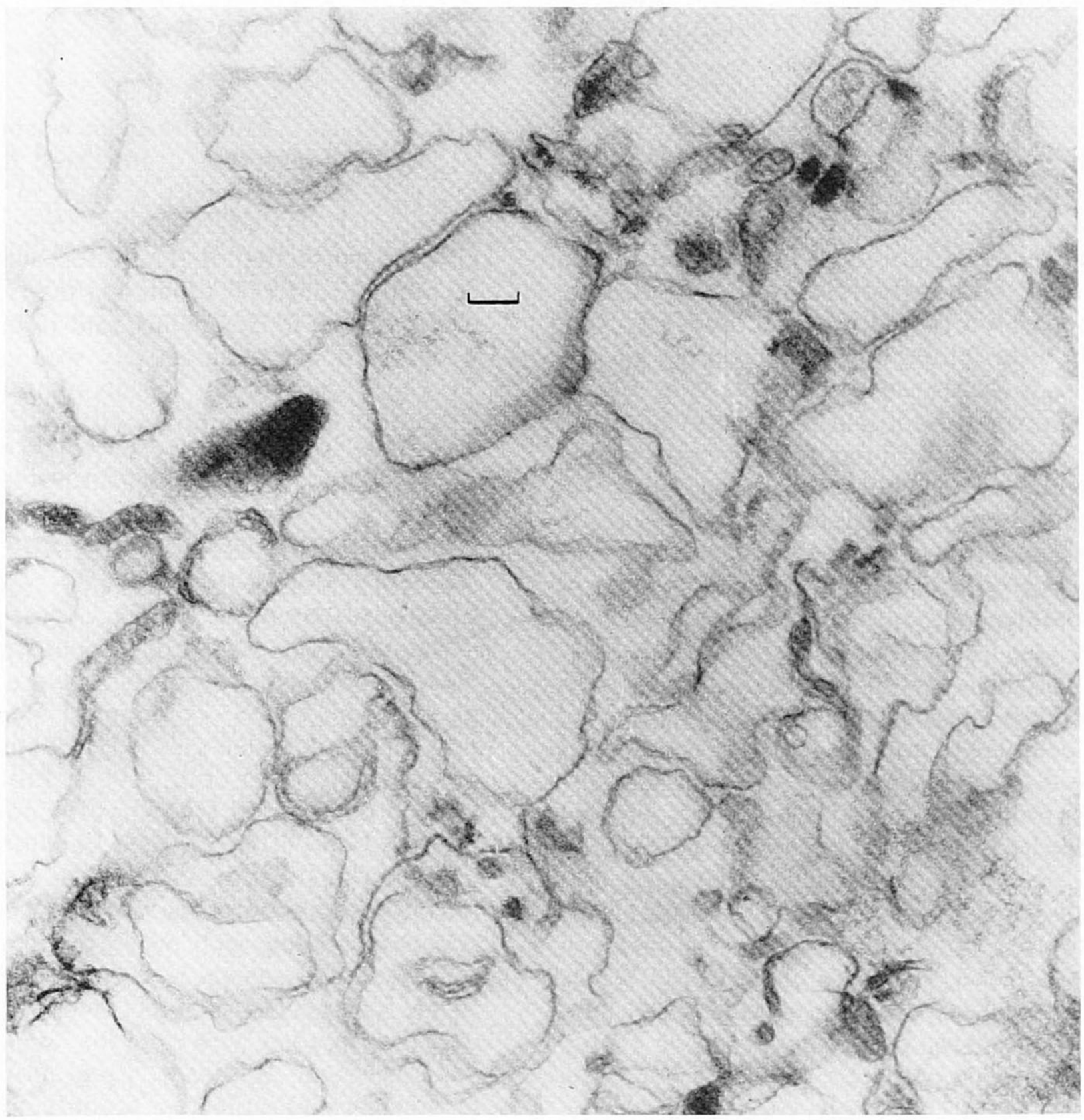
Analysis by two-dimensional slab gel electrophoresis

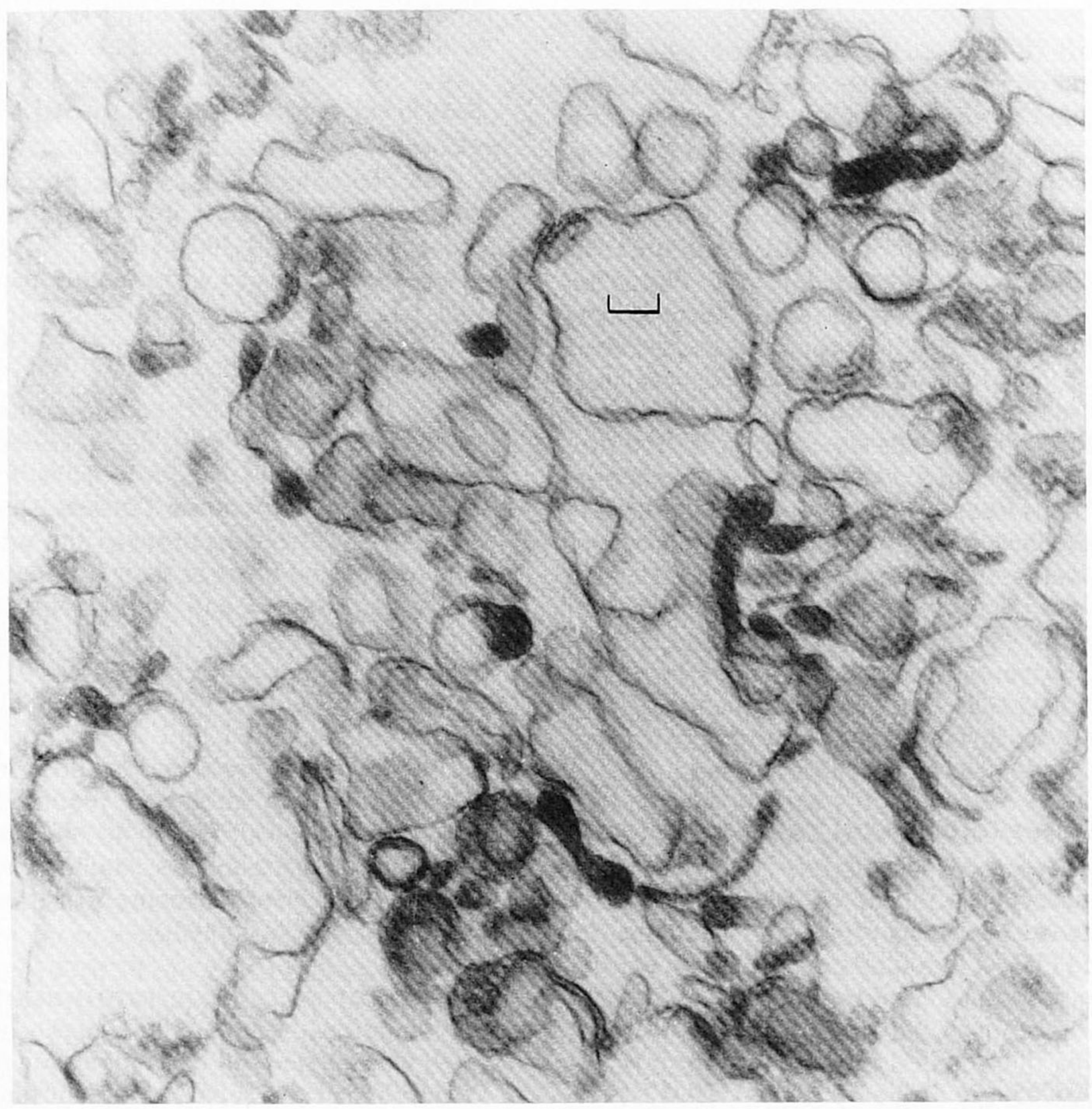
Samples containing 100 μg protein of NM labeled *in vivo* were solubilized in a medium composed of 0.5% (vol/vol) Triton X-100 and 8.5 *M* urea. The solubilized proteins were mixed with a solution containing ampholine (pH 3.5–9.5) and sucrose to produce final concentrations of 1% (wt/vol) ampholine and 5% (wt/vol) sucrose. This mixture (50 μl) was applied to a slot of a vertical slab gel of 5% (wt/vol) polyacrylamide containing 2.5% (wt/vol) ampholine (pH 3.5–9.5), 0.5% (vol/vol) Triton X-100, and 8.5 *M* urea. The vertical isoelectric focussing was carried out at room temperature (Zwiers et al., 1980).

Following the separation of the proteins in the first dimension on the basis of their isoelectric point (IEP), a gel track was excised and soaked for 30 min in a solution containing 2% (wt/vol) SDS, 10% (vol/vol) glycerol, 5% (vol/vol) β-mercaptoethanol, 0.001% (wt/vol) bromophenol blue, and 62.5 m*M* Tris-HCl (pH 6.8). This treatment removes urea and allows the binding of SDS to the proteins. To separate the proteins in the second dimension, the gel track was mounted on top of a SDS-polyacrylamide (11%, wt/vol) slab gel. Mounting of the gel track, electrophoresis, staining, and autoradiography procedures were performed as described previously (Zwiers et al., 1976, 1980).

Molecular weight determination

Estimation of molecular weight (Weber and Osborn, 1969) was carried out by comparison of the electrophoretic mobility of the separated membrane proteins with that





of standard proteins: phosphorylase a (93K), bovine serum albumin (67K), ovalbumin (45K), pepsinogen (35K), trypsinogen (24K), lactoglobulin (18.4K), and egg lysozyme (14.3K).

Electron microscopy

Membranes were pelleted at high speed and fixed in 310 mOs*m* phosphate buffer, pH 7.4, containing 3% (wt/vol) glutaraldehyde. After fixation in 1% (wt/vol) osmium tetroxide, the membranes were dehydrated in a series of 70–100% (vol/vol) acetone-water solutions and embedded in Araldite. Ultrathin sections (600–800 Å) were grid-stained with 4% (wt/vol) uranyl acetate, followed by treatment with 0.4% (wt/vol) lead citrate. The sections were examined in a Philips EM-200 electron microscope.

RESULTS

Characterization of the NM fraction by electron microscopy

Since NM of infant cerebral cortex were isolated by a procedure currently used for the preparation of SPM from adult rat brain (Zwiers et al., 1976) it was necessary to determine whether the NM preparation was indeed composed mainly of membrane fragments. Figure 1 shows an electron microscopic comparison of membranes derived from cerebral cortices of infant rats (A) and adult rats (B). Both membrane preparations contained many membrane profiles and few synaptosomal ghosts. Myelin, mitochondria, and synaptic junctions were not observed in either preparation.

^{32}P incorporation in cerebral cortex membrane proteins *in vivo*

The membrane fraction (1 mg/g wet weight) isolated from cerebral cortices of infant rats given 2 mCi of [^{32}P]orthophosphate 30 min before decapitation contained 1–2 nCi radioactivity per milligram protein. About 94% of the radioactivity incorporated was extracted with hot TCA, and then by chloroform-methanol. Only 5–6% of the total radioactivity remained in the protein residue after treatment by hot TCA and chloroform-methanol. The specific activity in the membranes was 7.5 ± 1.0 cpm/μg membrane protein (n = 3).

Separation of the ^{32}P-labeled membrane proteins by electrophoresis on 9% (wt/vol) polyacrylamide gels containing SDS yielded phosphoprotein patterns (Fig. 2A) with a large number of radioactive protein bands (Fig. 2, B and C). Zwiers et al. (1976) have shown with SPM from adult rat brain that the endogenous phosphorylation *in vitro* of several interesting protein substrates is modulated by ACTH, cyclic AMP, and calcium ions. These proteins can be detected in the molecular weight range of 47K–67K in a polyacrylamide gel. As we are particularly interested in the ACTH-sensitive 48K protein B-50 previously studied in adult rat brain (Zwiers et al., 1976, 1980), we chose to restrict the present investigation to the phosphoprotein bands labeled *in vivo* and detected in the molecular weight range of 47K–67K. Seven major phosphoprotein bands in this molecular weight range were resolved in protein preparations isolated from infant rat cerebral cortical membranes labeled *in vivo*. Three bands (a, c, and e, with MW 48K, 55K and 62K, respectively) were characterized by comparison of the *in vivo* and *in vitro* protein phosphorylation patterns of the NM fraction (see below).

Endogenous phosphorylation of cerebral cortex membranes *in vitro:* Effects of $ACTH_{1-24}$ and cyclic AMP

Membranes isolated from cerebral cortices of untreated infant rats were incubated *in vitro* with [γ-^{32}P]ATP in the presence or absence of $ACTH_{1-24}$ (10^{-4} and 2×10^{-5} *M*) or cyclic AMP (5×10^{-6} *M*) as described in the experimental procedures. Figure 3 shows the protein staining patterns (I) and the autoradiographs (II) of the endogenous protein phosphorylation profile of the membranes. In a dose-dependent fashion, $ACTH_{1-24}$ decreased ^{32}P incorporation into a protein band with apparent molecular weight of 48 K (compare tracks B and C with A). In contrast, cAMP stimulated ^{32}P incorporation into two protein bands (track D, bands 3 and 4) with apparent molecular weights of 55K and 59K, respectively (see also Fig. 4C versus 4B). However, although the phosphorylation of other protein bands in the molecular weight range larger than 67K were not systematically examined, it was noticed that stimulation by cAMP of the endogenous phosphorylation *in vitro* did not reveal enhanced phosphate incorporation in protein bands at the molecular weight positions of proteins Ia and Ib. The absence of phosphorylation of proteins I in cerebral membranes of 8-day-old rats is in agreement with the findings of Lohmann et al. (1978*a*), who demonstrated that the phosphorylation *in vitro* of proteins I started to increase markedly from day 10 on during postnatal development of rats. The presence of 1 m*M* EDTA and 1 m*M* EGTA in the incubation medium resulted in decreased ^{32}P incorporation into protein band 1 (Fig. 3, tracks D and E versus track A). This finding suggested that endogenous phosphorylation of this protein band of 48K may be dependent on the presence of Ca^{2+}.

Comparison of the phosphorylation profiles of membrane proteins labeled *in vivo* or *in vitro* with [^{32}P]phosphate

The phosphoprotein bands labeled *in vivo* were characterized by parallel studies with NM fractions isolated from ^{32}P-treated and untreated rats. The

FIG. 1. Electron micrographs of infant NM (**A**, top) and adult SPM (**B**, bottom) isolated from rat cerebral cortex. ×56,952; bar = 0.1 μm.

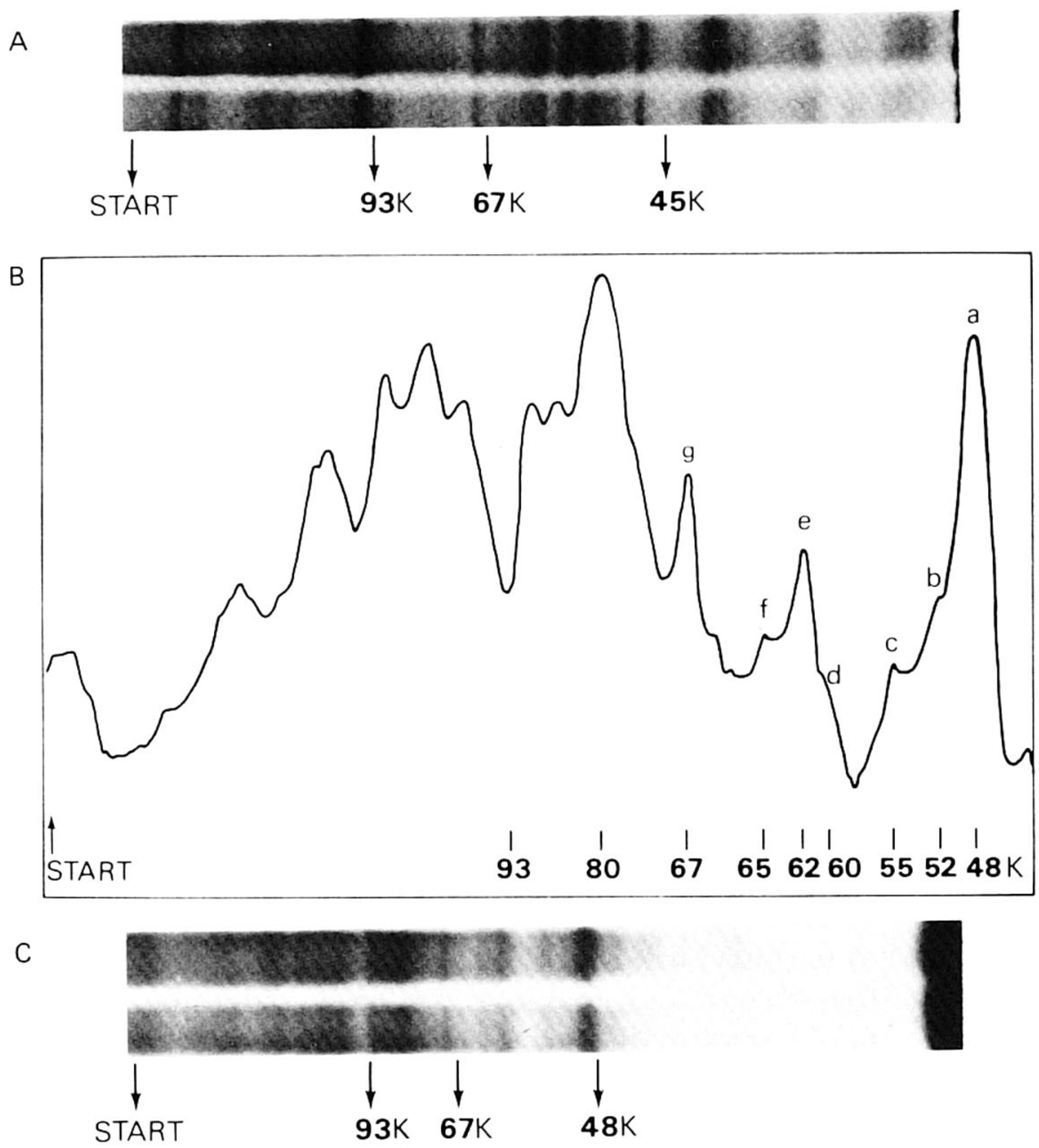


FIG. 2. Protein staining pattern **(A)** and densitometric scan **(B)** of an autoradiograph **(C)** of the ^{32}P radioactivity profile of *in vivo* labeled membrane proteins from infant rat brain separated by SDS-polyacrylamide (9%, wt/vol) slab gel electrophoresis. The position of various protein bands is indicated by the molecular weight markers expressed in K units.

membrane proteins of the untreated rats were phosphorylated *in vitro* in the presence and absence of neuromodulators. After separation of the membrane proteins of both experimental groups by PAGE on the same slab gel, comparisons were made of the apparent molecular weights of the major radioactive protein bands obtained from the *in vivo* and *in vitro* phosphorylated membranes. The patterns of the stained protein bands did not reveal any significant differences between experimental groups.

Densitometric scans of the autoradiographs are shown in Fig. 4. Of the seven major phosphoproteins in the molecular weight range 47K–67K that

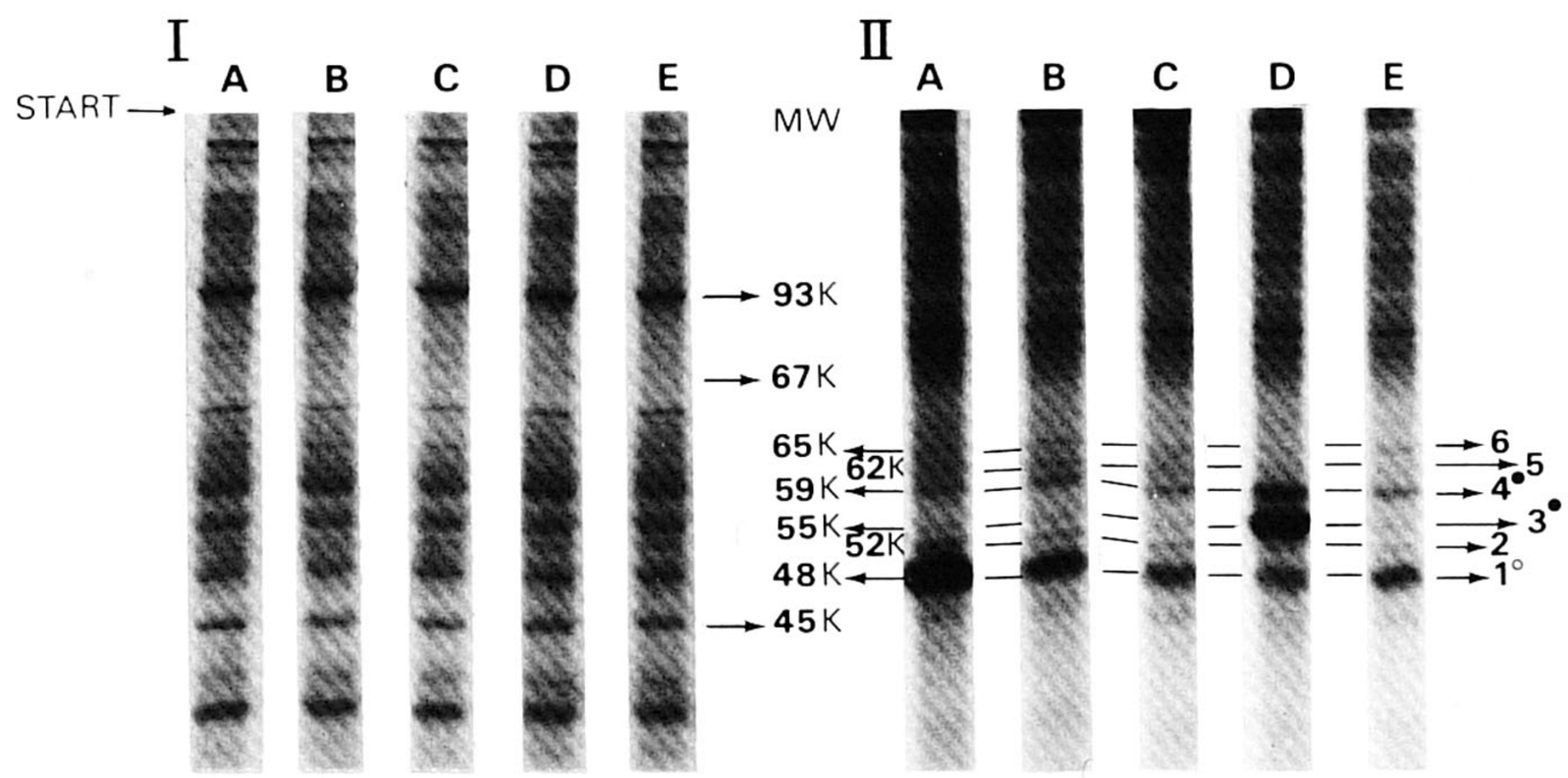


FIG. 3. Protein staining patterns (I) and autoradiographs (II) of an SDS-polyacrylamide (11%, wt/vol) gel electrophoretogram of NM proteins from cerebral cortex of infant rats after phosphorylation *in vitro* by [γ-^{32}P]ATP. (Track A), Control; (track B), 2×10^{-5} *M* $ACTH_{1-24}$ added; (track C), 10^{-4} *M* $ACTH_{1-24}$ added; (track D), 5×10^{-6} *M* cyclic AMP, IMX, EGTA, and EDTA added; (track E), control for D, containing IMX, EGTA, and EDTA (see Experimental Procedures). The numbers 1–6 refer to the major phosphorylated protein bands in the molecular weight region 48K–67K. (○), Phosphorylation inhibited by $ACTH_{1-24}$; (●), phosphorylation stimulated by cyclic AMP.

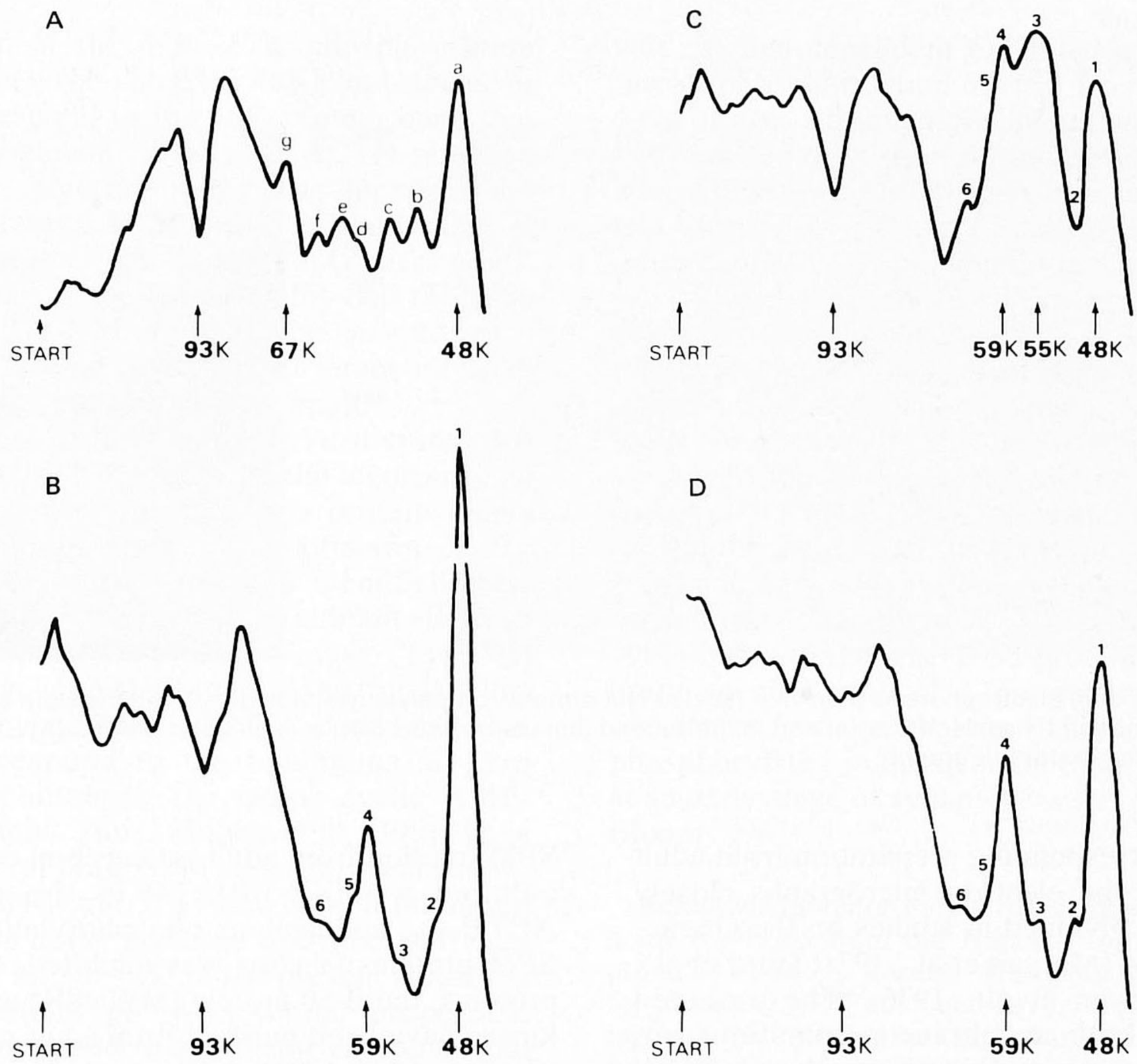


FIG. 4. Densitometric scans of the autoradiographs comparing *in vivo* and *in vitro* phosphorylated membrane proteins separated on the same SDS-polyacrylamide (11%, wt/vol) gel. **(A)** *In vivo* phosphorylation profile. **(B, C, D)** *In vitro* phosphorylation profiles. (B), control; (C), 5×10^{-6} *M* cyclic AMP added; (D), 10^{-4} *M* $ACTH_{1-24}$ added.

were labeled *in vivo* (Fig. 4A) band a (MW 48K) incorporated the most radioactivity. This band seemed to correspond to phosphoprotein band 1 following phosphorylation of cerebral membranes *in vitro* (Fig. 4D versus 4A). Phosphorylation *in vitro* of this band was inhibited by $ACTH_{1-24}$ (Fig. 3, tracks B and C; Fig. 4D) and by the lack of calcium ions (Fig. 4C); both are properties of the B-50 protein of adult rat SPM (Zwiers et al., 1976). The protein band c (MW 55K) and the relatively weaker band d (MW 59K) phosphorylated *in vivo* (Fig. 4A) may correspond to bands 3 and 4, the cAMP-sensitive protein substrates *in vitro* (Fig. 4C versus 4B). Of these two bands, the phosphorylation of protein band 3 (MW 55K) was stimulated the most by cAMP *in vitro*. Band e (MW 62K) in the *in vivo* phosphorylation system (Fig. 4A) may correspond to band 5, which was only weakly phosphorylated under *in vitro* conditions (Fig. 4C versus 4B).

Analysis of NM labeled *in vivo* by two-dimensional slab gel electrophoresis

Various protein stained spots were observed in the two-dimensional electrophoretograms. One relatively large spot, containing at least two proteins (Fig. 5A, arrow a) had a 48K MW and IEP of 4.5 (Fig. 5A). In the autoradiographs of this electrophoretogram (Fig. 5B), only one spot (a′) was detected at the same molecular weight position and IEP. Though the pattern of stained proteins revealed other minor proteins of 48K MW, these were not detected in the autoradiograph. Moreover, analysis of the membrane proteins on an isoelectric focussing gel with a more extended pH range, to 10.5, did not reveal protein spots or radioactivity at 48K MW in the basic region beyond pH 8. Thus, it is likely that spot a alone may represent the 48K band of NM that is phosphorylated *in vivo*. This finding supports the possibility that phosphoprotein band a and the B-50 protein are identical. The autoradiograph also revealed three other phosphorylated spots (b′, c′, and d′). Spot c′, consisting of two phosphorylated components, had a 55K MW (Fig. 5A, arrow c) and IEP of approximately 5.5.

DISCUSSION

The electron microscopic studies revealed that the neural membrane fraction prepared from cerebral cortices of infant rats was morphologically

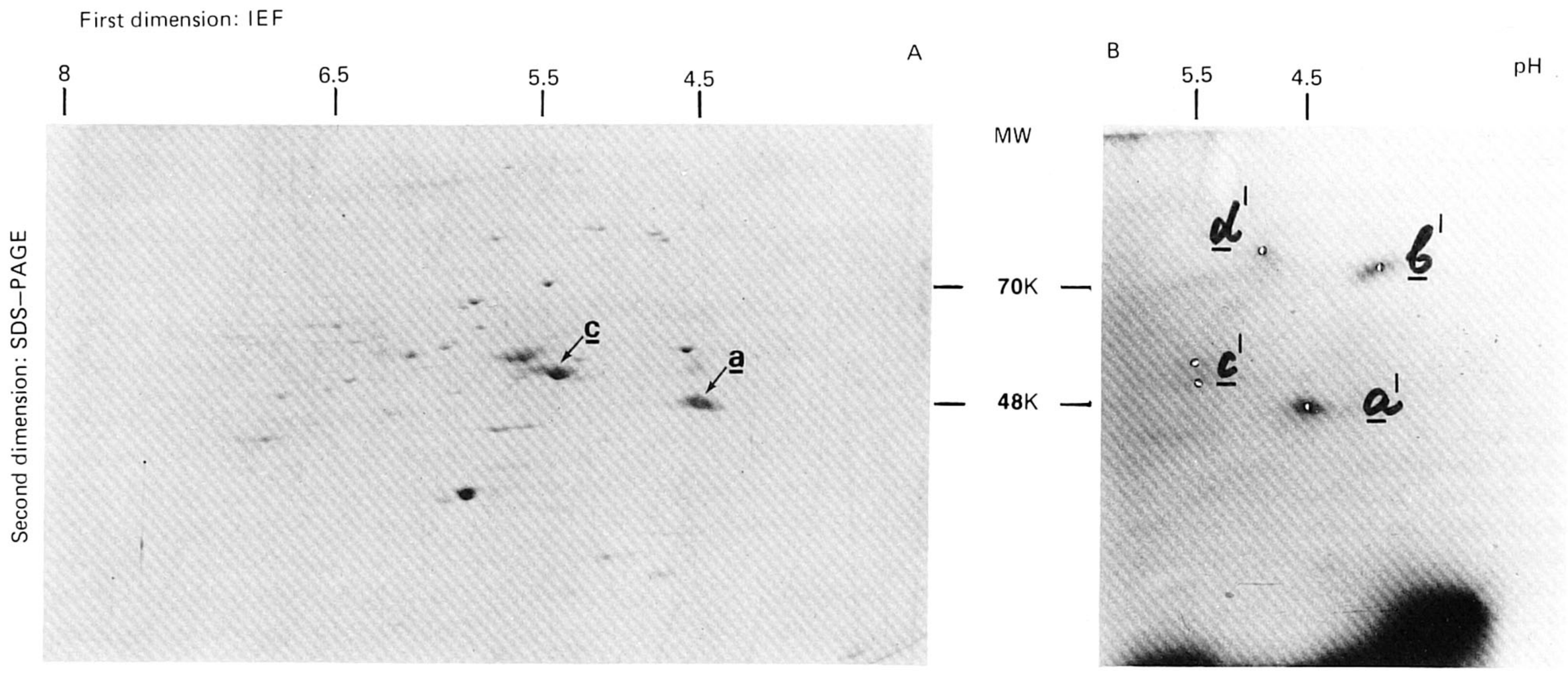


FIG. 5. Two-dimensional electrophoresis of *in vivo* labeled NM proteins on polyacrylamide gels. Proteins were separated in the first dimension according to isoelectric point and in the second dimension according to molecular weight. **(A)** Stained proteins. **(B)** The corresponding autoradiograph.

similar to the corresponding preparation from adult rats. Moreover, the electron micrographs closely resembled those obtained in studies on the characterization of SPM (Morgan et al., 1971; Gurd et al., 1974; van Leeuwen et al., 1976). The observed morphology of both membrane preparations appears to correspond with that of presynaptic membranes. This is in agreement with the conclusion of Sorensen et al. (1981) that in adult rat brain the B-50 protein is localized in light membranes of presynaptic origin. The absence of recognizable synaptic junctions in the membrane preparation of the adult rat may be attributed to loss of structural integrity of the synaptic cleft region during the homogenization in the presence of EDTA (Cotman et al., 1971).

^{32}P incorporation into individual phosphoprotein bands of the isolated cerebral membrane fraction may vary with a number of variables, including (a) physiological state of the animal, (b) experimental conditions; e.g., dose of isotope, route of application, incorporation time, method of sacrifice, and (c) procedures of tissue processing; e.g., brain region, subcellular fractionation (Berman et al., 1980). The present investigations were concerned with only those membrane phosphoprotein bands that retained a considerable degree of radioactivity from [^{32}P]orthophosphate administered 30 min earlier. Moreover, since a number of phosphoproteins in the molecular weight region of 47K–67K have been described in studies *in vitro* of endogenously phosphorylating SPM from rat brain (Zwiers et al., 1976; Greengard, 1976, 1979; Lohmann et al., 1978*a,b;* Mahler, 1979), the present analysis was limited to the major membrane phosphoprotein bands of that molecular weight region that were labeled *in vivo*.

Zwiers et al. (1976) demonstrated that when the SPM fraction from adult rat cerebral cortex was incubated with [γ-^{32}P]ATP in the presence of $ACTH_{1-24}$, endogenous phosphorylation of certain SPM proteins *in vitro* was inhibited. One of these proteins, the B-50 protein (MW 48K) and its protein kinase have been purified from adult rat brain SPM (Zwiers et al., 1980). In the present studies, membranes isolated from cerebral cortex of 8-day-old rat also contained a 48K phosphoprotein band that incorporated a remarkable fraction of the total radioactivity. Moreover, in common with the SPM fraction of adult brain, endogenous phosphorylation of the 48K phosphoprotein band of infant rat cerebral membranes was inhibited by $ACTH_{1-24}$ in a dose-dependent fashion, indicating the presence of an ACTH-sensitive protein kinase in these membranes as well. Zwiers et al. (1977) have reported that intraventricular administration of $ACTH_{1-24}$ into adult rats and subsequent *in vitro* phosphorylation of membranes prepared from brains of these rats resulted in an enhanced phosphorylation of low-molecular-weight SPM phosphoprotein bands including B-50. These phosphoprotein bands were identical to those that are inhibited by addition of $ACTH_{1-24}$ to the phosphorylation assay *in vitro*. Further, the changes observed *in vitro* were dependent on the dose of $ACTH_{1-24}$ *in vivo* and the time between injection and decapitation of the rats (Zwiers et al., 1977). Thus under appropriate conditions of dose, time, brain region, age, etc., it may be possible that *in vivo* administration of $ACTH_{1-24}$ influences B-50 protein phosphorylation (Wiegant et al., 1981).

Comparison of *in vivo* and *in vitro* phosphorylation profiles of NM after electrophoretic separation of the proteins permitted preliminary characteriza-

tion of certain of the 47K–67K phosphoprotein bands labeled *in vivo*. Thus, the 48K band labeled *in vivo* may correspond to the 48K protein band that was phosphorylated *in vitro* by the ACTH-sensitive protein kinase. This possibility was supported by the finding that the 48K band of NM had an IEP of 4.5, identical to that of B-50 protein (Zwiers et al., 1980). Thus, this study demonstrates that the B-50 protein present in NM is one of the acceptor proteins that are labeled in the intact infant rat after administration of radioactive phosphate.

Addition of cyclic AMP to the *in vitro* system for phosphorylation of NM enhanced the incorporation of radioactive phosphate into two protein bands (MW 55K and 59K) (see also Lohmann et al., 1978*a,b*). Lohmann et al. (1978*b*) identified their 55K protein band as the regulatory subunit (R-II) of cyclic AMP-dependent protein kinase (Type II) both by [^{3}H]cyclic AMP binding and by labeling with 8-N_3-[^{32}P]cyclic AMP. The 55K band labeled *in vivo* may correspond to the analogous *in vitro* labeled band containing the major cyclic AMP-stimulated protein. Thus, Mahler et al. (1981) have shown that the rat brain SPM contain two phosphoproteins (MW 54K and 57K) that exhibit increased phosphorylation in the presence of cyclic AMP and possess an IEP of 5.0–5.5. In the present studies, a radioactive protein spot with similar properties, which may correspond to the major component of the 55K band labeled *in vivo*, was separated by two-dimensional electrophoresis. In contrast with the findings of Berman et al. (1980) in adult rat brain, we detected labeling of a 55K protein band in membranes of infant rat brain. This apparent difference might be explained by several of the factors that influence the phosphorylation *in vivo* and the detection of the phosphorylated acceptor proteins as discussed above. However, the use of infant versus adult experimental animals might also have contributed to the observed discrepancy of the two studies.

Mitrius et al. (1981) recently reported that after intracranial injection of [^{32}P]orthophosphate into neostriatum or hippocampus of the adult rat, more than 50 electrophoretically separated protein bands were labeled in a crude synaptosome fraction (P2). A 47K protein band, designated band F, and assumed to be derived from SPM, was most rapidly labeled *in vivo* and was one of the most prominent phosphoproteins in P2 samples obtained at short incorporation times. If this protein band contains the 48K B-50 protein, then it would appear that phosphorylation of this protein in brain *in vivo* is very active during both infancy and adult life in the rat. In this regard, Holmes and Rodnight (1981) examined the ontogeny of the major intrinsic phosphoproteins in membrane fractions isolated from cerebral cortex during postnatal development of the rat. A 47K membrane protein whose phosphorylation was calcium-dependent exhibited a relatively high rate of phosphorylation *in vitro* from birth up to 15 days of age. Although phosphorylation of this protein declined thereafter, it is not known whether this decrease was due to changes in activity of the protein kinase, the amount of substrate protein, or to other factors (e.g., calmodulin).

The function of the B-50 protein is not well understood. There is some indication that its degree of phosphorylation may determine the activity of phosphatidylinositol 4-phosphate kinase in adult brain SPM; thus linking the B-50 phosphorylation system to the turnover of (poly)phosphoinositides (Jolles et al., 1980, 1981). Localization studies using immunohistochemical methods (Oestreicher et al., 1981) and a two-dimensional gel electrophoretic approach (Kristjansson et al., 1982; Sorensen et al., 1981) indicate that the B-50 protein is most likely brain specific, and of a presynaptic origin. The present study shows that the B-50 protein is actively phosphorylated in the neonate brain, just before or at an early stage of synaptogenesis (Aghajanian and Bloom, 1967).

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