Phosphorylation of Synaptic-Membrane Proteins from Ox Cerebral Cortex *in vitro*

PREPARATION OF FRACTIONS ENRICHED IN PHOSPHORYLATED PROTEINS BY USING EXTRACTION WITH DETERGENTS AND UREA, AND GEL FILTRATION

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Synaptic-membrane fragments from ox cerebral cortex contain basal and cyclic AMP-stimulated protein kinase(s) that transfer ^{32}P from $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ to hydroxyl groups of serine and threonine residues in membrane-protein substrates. In the present work, labelled membrane fragments were partitioned into soluble and insoluble fractions with Triton X-100, Nonidet P. 40, sodium deoxycholate and urea, and the distribution of ^{32}P -labelled protein in the fractions was determined by polyacrylamide-gel electrophoresis and radioautography. A high percentage of phosphorylated protein substrates remained insoluble, including those whose phosphorylation was most highly stimulated by cyclic AMP. Whole membrane fragments and samples prepared by detergent extraction were fractionated on Sepharose 6B columns in the presence of low concentrations of sodium dodecyl sulphate and pooled fractions were analysed by polyacrylamide-gel electrophoresis and radioautography. Phosphorylated proteins were fractionated on the basis of their molecular weight, but homogeneous protein was not obtained. The results are discussed in relation to the techniques used and the results obtained in other laboratories.

The occurrence in synaptic-membrane fragments of intrinsic protein kinase and phosphatase activities modulated by cyclic AMP (Rodnight & Lavin, 1966; Johnson *et al.*, 1971; Maeno & Greengard, 1972; Weller & Rodnight, 1973; Ueda *et al.*, 1973, 1975; Dunkley *et al.*, 1976; Weller & Morgan, 1976a) has led to suggestions that protein phosphorylation may play a role in some aspects of synaptic transmission (Greengard, 1975, 1976; Rodnight, 1975). In investigating this hypothesis, localization and characterization of the membrane-bound substrates is necessary. To this end extensive use has been made of polyacrylamide-gel electrophoresis of membrane proteins labelled *in vitro* with $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ and solubilized with sodium dodecyl sulphate. The number of phosphorylated proteins detected with or without cyclic AMP present depends on many factors, such as the source of material, the method of membrane preparation, the conditions of phosphorylation and gel electrophoresis, as well as the method for determining ^{32}P distribution in the gels (Ueda *et al.*, 1973, 1975; Routtenberg & Ehrlich, 1975; Rodnight *et al.*, 1975; Dunkley *et al.*, 1976; Weller & Morgan, 1976b). However, in all studies to date the

phosphorylation of two proteins, of approx. mol.wts. 50000–60000 and 80000–90000, has been found to be markedly stimulated by cyclic AMP.

In other work the detergent Triton X-100 has been used to fractionate membrane components both before and after labelling the proteins with $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ (Weller & Rodnight, 1973; Dunkley *et al.*, 1976; Weller & Morgan, 1976a,b). These studies showed that most of the total protein kinase activity occurs in the fraction insoluble in Triton X-100; moreover, Dunkley *et al.* (1976) found that a protein of approx. mol.wt. 76000–85000, whose phosphorylation was virtually dependent on cyclic AMP, was not solubilized by the detergent. Of particular interest also are observations of Weller & Morgan (1976b), who subfractionated the Triton-insoluble fraction from rat brain and showed that most of the cyclic AMP-stimulated activity was concentrated in a membrane fraction highly enriched in synaptic-junctional complexes. However, the pattern of phosphorylated proteins in this fraction, as determined by polyacrylamide-gel electrophoresis, was not markedly different from that of the whole Triton-insoluble fraction.

In the present study we have used radioautography to compare the effectiveness of Triton X-100, Nonidet P. 40, sodium deoxycholate and urea in fractionating ^{32}P -labelled proteins in our standard preparation of

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ox brain synaptic-membrane fragments. The emphasis is on the last three agents, since the use of Triton X-100 was examined in a previous paper (Dunkley *et al.*, 1976), where, however, a different and less discriminating method was used for determining the distribution of radioactivity in the gels. Further fractionation of phosphorylated membrane proteins was attempted by gel filtration on columns of Sepharose 6B in the presence of low concentrations of sodium dodecyl sulphate.

Materials and Methods

Materials

Sodium dodecyl sulphate and cyclic AMP were from Sigma (London) Chemical Co., Kingston-upon-Thames, Surrey, U.K.; Nonidet P. 40 (sample BO 8135) was from Shell Chemical Co., Down Stream Building, London S.E.1, U.K.; Triton X-100 was from Koch-Light Laboratories, Colnbrook, Bucks., U.K.; urea and sodium deoxycholate were from BDH Chemicals, Poole, Dorset, U.K.; acrylamide and *NN'*-methylenebisacrylamide were from Kodak, Kirkby, Liverpool, U.K.; Sepharose 6B was from Pharmacia (G.B.), London W.5, U.K. Carrier-free [γ - 32 P]ATP (ammonium salt; 15.8–16.2 Ci/mmol) was from The Radiochemical Centre, Amersham, Bucks., U.K. Centrifo filters (224-cf-50) were from Amicon Co., Lexington, MA 02173, U.S.A.

Synaptic-membrane preparation

Large-scale preparations rich in synaptic-membrane fragments were made from cerebral-cortex grey matter of ox brain by a procedure modified from that of Rodnight *et al.* (1969). After osmotic shock the suspension was agitated by a Vibromixer for 30 min at 4°C, readjusted to 0.32 M-sucrose and centrifuged at 44000g for 1 h. The loose pellet which formed over a tightly packed tan pellet was resuspended in 0.32 M-sucrose and 15 ml samples were layered over a discontinuous gradient consisting of 10 ml of 1.2 M-sucrose and 10 ml of 0.8 M-sucrose. The gradient was centrifuged at 94000g and 4°C for 3 h in a swing-out rotor. The fraction floating at the 1.2 M-/0.8 M-sucrose interface was collected, adjusted to 0.32 M-sucrose and centrifuged at 78500g for 1 h at 4°C. The pellet was washed in 4 mM-imidazole/HCl buffer, pH 7.4, and resuspended in this buffer at a concentration of 10 mg of protein/ml. These synaptic-membrane fragments were stored frozen at –20°C and were used within 3 months.

Protein concentrations were determined by the method of Miller (1959), with bovine plasma albumin as standard.

Phosphorylation of membrane proteins

Incubations were performed for 1 min at 37°C under the same general conditions, mode of stirring etc. recommended by Rodnight *et al.* (1975). The

reaction mixture contained 30 mM-Tris/HCl, pH 7.4 at 37°C, 1 mM-MgSO₄, 12.5 μ M-[γ - 32 P]ATP (2×10^6 c.p.m./nmol), with or without 50 μ M-cyclic AMP, in a final volume of 1.0 ml. Reaction was started by addition to the rapidly stirred medium of 0.2 ml of synaptic-membrane fragments (2 mg of protein). The methods used to terminate the reaction depended on the aim of the experiments and are described below. Larger-scale labelling of protein (5 mg) was performed under the same conditions as described above except that the ATP concentration was 32.5 μ M (1.2×10^6 c.p.m./nmol) and the final volume was 2 ml.

Fractionation of membrane protein with detergents

When labelled membrane fragments (2 mg) were to be partitioned with detergent the phosphorylation reaction was terminated as previously described (Dunkley *et al.*, 1976). The pellets, comprising over 90% of the original protein, were suspended in 2 ml of the appropriate detergent, which was made up to the following concentrations in imidazole buffer; Nonidet P. 40 (0.2%, v/v), Triton X-100 (0.5%, v/v), sodium deoxycholate (1.5%, w/v), or urea (48%, w/v). In experiments with detergents, suspensions were homogenized by hand in an all-glass test-tube homogenizer at 4°C; in those with urea, samples were homogenized at 15°C to prevent precipitation. Homogenates were immediately centrifuged at 106000g for 30 min at the same temperature as that used for the homogenization. The supernatants were washed twice with 4 ml of imidazole buffer and concentrated in Centrifo filters (see Dunkley *et al.*, 1976).

Fractionation of membrane protein by gel filtration

When labelled membrane fragments (5 mg) were to be fractionated by gel filtration, two procedures were used to terminate the reaction. The first was to add a solution of sodium dodecyl sulphate directly to the reaction tube such that the final volume was 3 ml and the detergent:protein ratio (in mg) was 20:1; the membrane protein was completely solubilized and could be applied directly to the gel-filtration column. The second procedure was introduced to decrease the total radioactivity applied to the column, to decrease the amount of 'salts' present in the applied sample and to diminish the possibility of non-protein-bound radioactivity associating with high-molecular-weight sodium dodecyl sulphate micelles. The reaction was terminated by addition of 10 ml of ice-cold 10% (w/v) trichloroacetic acid containing 1 M-H₃PO₄, and the suspension was centrifuged at 2600g for 15 min at 4°C. The pellet was resuspended in 10 ml of 10% trichloroacetic acid (containing phosphate) and re-centrifuged. The pellet was then washed three times with 10 ml of imidazole buffer and the final pellet was completely solubilized by addition of 3 ml of sodium dodecyl sulphate in imidazole buffer such that the detergent:protein ratio (in mg) was 20:1.

The first procedure was used to dissolve the soluble and insoluble membrane fractions prepared by extraction as described above. Both procedures were used for solubilizing unlabelled protein, which was first diluted to the appropriate protein concentration with imidazole buffer.

A column of Sepharose 6B gel (2.5 cm × 90 cm) was operated at room temperature by upward displacement with a flow rate of 15 ml/h. The standard eluent contained 50 mM-Tris/HCl, pH 7.0, 0.1% sodium dodecyl sulphate and 0.01% NaN₃ (to inhibit bacterial growth) and columns were washed with at least 2 column-volumes of eluent between sample applications. The *A*₂₈₀ of the effluent was monitored and 6–8 ml fractions were collected. Radioactivity in individual fractions was determined by Čerenkov radiation. Fractions were pooled on the basis of the protein and radioactivity profiles and concentrated to approx. 500 µl by rapid filtration through Centriflo membrane cones. After protein determination the fractions were analysed by polyacrylamide-gel electrophoresis.

Polyacrylamide-gel electrophoresis

The discontinuous system used previously by Dunkley *et al.* (1976) was modified to increase the resolution and staining of the protein bands. All operations were at room temperature (18–24°C). The buffers were the same as used by Dunkley *et al.* (1976) except that the upper buffer was adjusted to pH 8.7 and the lower buffer to pH 8.0, both adjustments being made at the temperature of the experiment. The resolving gel mixture consisted of 9.7% (w/v) acrylamide, 0.3% (w/v) *NN'*-methylenebisacrylamide, 0.1% sodium dodecyl sulphate, 0.05% ammonium persulphate, 0.025% (v/v) *NNN'*-tetramethylethylenediamine and 38 mM-Tris/HCl, pH 8.7. Gels were overlaid with 0.1% sodium dodecyl sulphate and left to set for 2 h at room temperature. The resolving gel (9 cm × 0.6 cm) was dried and overlaid with a stacking-gel mixture (1 cm) of the same composition, except that the concentrations of acrylamide and *NN'*-methylenebisacrylamide were 3% (w/v) and 0.07% (w/v) respectively. After setting at room temperature gels were stored at 4°C for up to 1 week, but they were equilibrated at room temperature before use.

Preparations of membrane fragments (100 µg of protein in 50–100 µl) were solubilized by using 17.5 µl of a buffer (pH 8.7) containing 286 mM-Tris/HCl, 266 mM-glycine, 5.7% (w/v) sodium dodecyl sulphate, 5.7% (v/v) 2-mercaptoethanol, 28.5% (w/v) sucrose and 0.03% Bromophenol Blue. Samples were applied to the gels and a starting current of 0.25 mA/gel was gradually increased to 2.0 mA/gel over the next 2 h. When the dye front reached the bottom of the gels (4–5 h) they were removed from the tubes and stained in 0.2% Coomassie Blue in methanol/acetic

acid/water (55:7:43, by vol.) overnight. Gels were destained in methanol/acetic acid/water (4:1:6, by vol.).

Protein distribution in the gels was recorded with a Chromoscan double-beam densitometer (Joyce Loebel, Team Valley, Gateshead, U.K.) by using a green filter (5-040) and an aperture of 5 mm × 0.3 mm.

The [³²P]phosphate distribution in gels was determined by radioautography of half gels, which had been dried under vacuum essentially as described by Fairbanks *et al.* (1965). The procedure involved slicing gels into equal halves by passing a thin wire through the length of a gel, which was held fast in a tightly fitted Perspex mould (Fairbanks *et al.*, 1965). Gel halves were dried, round side up, on to the smooth surface of sheets of porous polythene in a Perspex block which was covered with two layers of Cling-Film to ensure a good vacuum. Gels were initially flattened for 1–2 h under low vacuum pressure and full pressure was applied overnight until the gels were dry. An i.r. lamp was often used on the bottom of the block to assist in drying, but caution was required, as too much heat cracked the gels. Some shrinkage of gels occurred, but never more than 10% in the long axis. Kodirex EP X-ray film (Kodak) was exposed to the bottom surface of the dried gels, which were inverted and covered with a layer of Cling-Film. After a standard 24 h exposure at 4°C, and development (in Kodak DX-80 developer at 22°C for 3.5 min in absolute darkness), the emulsion on the side away from the gel was removed from the film with 1 M-NaOH to decrease the background (single-coated Kodirex would have been preferable, but was not available in the U.K.).

Calculation and expression of results

In our previous paper, when the radioactivity distribution in gels was determined by counting individual gel segments, it was convenient (see Dunkley *et al.*, 1976) to divide the gels into regions of high phosphate content termed α , β , γ , δ and ϵ ; the approx. mol.wts. of these regions were determined to be 98 000–110 000, 76 000–85 000, 56 000–67 000, 33 000–37 000 and 12 500–13 500 respectively. Radioautography of gels, as was used here, allowed a more direct comparison to be made between the protein and [³²P]phosphate profiles of a gel (e.g. see Figs. 1 and 2), but a quantitative measure of the differences in phosphate content of the various samples prepared by extraction of ³²P-labelled membranes was still required. Gels were therefore again divided into regions α , β , γ , δ and ϵ on the basis of the [³²P]-phosphate profile for the whole membrane labelled in the presence of cyclic AMP (see Fig. 1). However, for the radioautographs the [³²P]phosphate, which was just included into the gel, was also measured (region O, Fig. 1) and the six gel regions together accounted for the entire length of the gel. For some

samples, such as the Nonidet P. 40-soluble fraction labelled in the absence of cyclic AMP (Fig. 2a), division of the gel into the six regions was especially difficult and was generally based on the [32 P]phosphate distribution for the corresponding insoluble fraction, labelled in the presence of cyclic AMP. The large number of gels studied and the low values for the s.e.m. obtained (Table 2) seems to justify this somewhat arbitrary approach. The area in each region of the Chromoscan recording was determined by using a planimeter set to measure in cm^2 . After correction for background and radioactive decay the arbitrary values for [32 P]phosphate content could be averaged and compared (Table 2). An estimate, in arbitrary units, of the total [32 P]phosphate content of any sample was obtained by summing the mean values (Table 2) for [32 P]phosphate for gel regions O, α , β , γ , δ and ϵ . These total values could then be adjusted to account for the difference in protein distribution in the various fractions (Table 1), as each polyacrylamide gel contained 100 μg of protein whether it was a soluble or insoluble fraction.

Results

Intact membrane fragments

Incubation of synaptic-membrane fragments with ATP induced the phosphorylation of a number of proteins that could be resolved on polyacrylamide gels (Fig. 1). In earlier work five regions (referred to as α , β , γ , δ and ϵ) of a gel of labelled protein showed obvious peaks of radioactivity (Dunkley *et al.*, 1976). These regions are also clearly distinguishable in Fig. 1, where entirely different techniques were used for detecting the distribution of radioactivity in gels.

Cyclic AMP stimulated the phosphorylation of a number of protein bands; stimulation was most obvious in region β , but there was also a marked increase in radioactivity in regions O, α and γ (Fig. 1). The peak of radioactivity in region O was included in the resolving gel and corresponded to a sharply defined, but minor protein, peak. Region α contained a minor and a major peak of radioactivity, each of which was a composite of incompletely resolved

peaks; the major peak was clearly stimulated by cyclic AMP. The major protein peak between regions α and β [no. 4 in Dunkley *et al.* (1976)] had label associated with it, but it did not correspond to any major [32 P]phosphate peak. Region β contained two minor [32 P]phosphate peaks and one major peak which was apparently dependent for its appearance on cyclic AMP. This major peak clearly did not correspond to an obvious protein band. Region γ contained the major [32 P]phosphate peak, but shoulders on the peak (Fig. 1) suggested that more than one phosphorylated protein was present. Three of the proteins in this region are major membrane components, but often the two lower-molecular-weight proteins were not resolved (as in Fig. 1). Region δ contained two [32 P]phosphate peaks which corresponded to protein bands. As before (Dunkley *et al.*, 1976), region ϵ always contained a very sharply defined peak which included the anodal front up to the position of the dye marker.

Partition of ^{32}P -labelled membrane fragments with detergents and urea

At the detergent:protein ratios used sodium deoxycholate was the most effective protein-solubilizing agent, whereas Triton X-100, Nonidet P. 40 and urea were about equal (Table 1). The low recovery of total protein reflects the number of centrifugation and washing steps required to obtain the final soluble and insoluble fractions.

Calculation of the ratio of phosphorylated protein:total protein in the various fractions showed that the efficiency of the detergents was greater than that of urea in solubilizing unlabelled protein. The ratio of phosphorylated protein:total protein was very similar whether membranes were labelled in the presence or absence of cyclic AMP.

Typical protein and [32 P]phosphate profiles for the soluble and insoluble fractions of synaptic membranes after extraction with Nonidet P. 40, sodium deoxycholate and urea are shown in Figs. 2(a), 2(b) and 2(c) respectively, and the distribution of [32 P]phosphate in the six regions for each fraction is given in Table 2. The results for Triton X-100 were

Table 1. Distribution of protein after partial solubilization of synaptic-membrane fragments

The values quoted are means $\pm$ s.e.m. The number of determinations from at least two separate experiments are indicated in parentheses. The concentrations of agents used were as follows: urea, 48%, w/v; Triton X-100, 0.5%, v/v; Nonidet P. 40, 0.2%, v/v; sodium deoxycholate, 1.5%, w/v.

Solubilizing agent	Recovered protein (%)		Recovery (%)
	Soluble	Insoluble	
Urea (6)	28.3 $\pm$ 3.1	71.7 $\pm$ 3.1	64.2 $\pm$ 4.9
Triton X-100 (4)	31.3 $\pm$ 2.8	68.8 $\pm$ 2.8	79.3 $\pm$ 2.1
Nonidet P. 40 (17)	31.8 $\pm$ 2.5	68.2 $\pm$ 2.5	73.0 $\pm$ 2.5
Sodium deoxycholate (11)	52.0 $\pm$ 2.3	47.9 $\pm$ 2.3	69.5 $\pm$ 2.3

Fig. 1. Polyacrylamide-gel electrophoresis of ^{32}P -labelled synaptic-membrane fragments

Typical protein profiles and corresponding ^{32}P phosphate profiles from gels of whole synaptic-membrane fragments are shown. For details of experimental procedures see the Materials and Methods section. Mean values for the areas under the six regions of the ^{32}P phosphate profiles from a range of experiments are quoted in Table 2.

very similar to those obtained earlier by the different procedure (see Dunkley *et al.*, 1976). Further, the general shape of the protein profiles for soluble and insoluble fractions extracted with Nonidet P. 40, Triton X-100 and sodium deoxycholate were overall very similar despite the differences in the percentage of protein solubilized, although the exact proportions of individual protein peaks varied depending on which

detergent was used, suggesting some degree of specificity. Urea appeared to solubilize a greater proportion of protein from region γ and less from region α than did the detergents. On the whole, except for regions γ and ϵ , it appeared that most of the substrate proteins for the protein kinases of synaptic membrane fragments were associated with the insoluble fractions.

Fig. 2. Protein and $[^{32}\text{P}]$ phosphate profiles of soluble and insoluble fractions of synaptic-membrane fragments after extraction with Nonidet P. 40, sodium deoxycholate and urea
Typical protein profiles and $[^{32}\text{P}]$ phosphate profiles of soluble and insoluble membrane fractions labelled in the absence or presence of $50\text{ }\mu\text{M}$ -cyclic AMP are shown. For details of experimental procedures see the Materials and Methods section. (a) Nonidet P. 40; (b) sodium deoxycholate; (c) urea.

Table 2. *Distribution of ^{32}P phosphate in polyacrylamide gels of synaptic-membrane fragments*
 The values quoted are the means $\pm$ s.e.m. The number of gels on which determinations were made is indicated in parentheses, with the first value referring to results with cyclic AMP and the second to results without cyclic AMP. Regions O-e are indicated on scans in Fig. 1.

Region ...	Cyclic AMP present						Cyclic AMP absent					
	O	α	β	γ	δ	ϵ	O	α	β	γ	δ	ϵ
Whole membrane (2, 2)	38	81	52	77	37	41	20	52	30	56	23	31
Urea												
Soluble (5, 4)	22 $\pm$ 4	27 $\pm$ 2	18 $\pm$ 3	33 $\pm$ 3	15 $\pm$ 1	27 $\pm$ 2	13 $\pm$ 3	20 $\pm$ 2	13 $\pm$ 3	24 $\pm$ 2	11 $\pm$ 2	29 $\pm$ 8
Insoluble (8, 6)	24 $\pm$ 2	36 $\pm$ 4	22 $\pm$ 2	30 $\pm$ 3	14 $\pm$ 2	34 $\pm$ 4	16 $\pm$ 2	16 $\pm$ 4	10 $\pm$ 2	13 $\pm$ 2	6 $\pm$ 2	17 $\pm$ 2
Triton X-100												
Soluble (8, 6)	9 $\pm$ 2	13 $\pm$ 1	8 $\pm$ 1	14 $\pm$ 1	9 $\pm$ 1	16 $\pm$ 3	7 $\pm$ 1	12 $\pm$ 1	5 $\pm$ 1	10 $\pm$ 1	4 $\pm$ 1	13 $\pm$ 2
Insoluble (4, 6)	69 $\pm$ 2	66 $\pm$ 10	41 $\pm$ 4	53 $\pm$ 7	20 $\pm$ 3	52 $\pm$ 6	30 $\pm$ 4	39 $\pm$ 2	14 $\pm$ 2	30 $\pm$ 3	14 $\pm$ 2	22 $\pm$ 5
Nonidet P. 40												
Soluble (8, 12)	10 $\pm$ 2	22 $\pm$ 3	15 $\pm$ 2	27 $\pm$ 4	14 $\pm$ 2	16 $\pm$ 3	4 $\pm$ 1	14 $\pm$ 2	10 $\pm$ 2	20 $\pm$ 2	7 $\pm$ 1	12 $\pm$ 2
Insoluble (14, 14)	41 $\pm$ 6	76 $\pm$ 6	52 $\pm$ 6	57 $\pm$ 5	21 $\pm$ 3	27 $\pm$ 2	27 $\pm$ 2	60 $\pm$ 2	17 $\pm$ 2	40 $\pm$ 2	19 $\pm$ 2	22 $\pm$ 2
Sodium deoxycholate												
Soluble (12, 12)	12 $\pm$ 2	22 $\pm$ 2	18 $\pm$ 2	34 $\pm$ 2	18 $\pm$ 2	28 $\pm$ 2	4 $\pm$ 1	10 $\pm$ 1	10 $\pm$ 1	21 $\pm$ 3	13 $\pm$ 2	22 $\pm$ 3
Insoluble (12, 12)	41 $\pm$ 6	64 $\pm$ 5	48 $\pm$ 4	54 $\pm$ 4	17 $\pm$ 2	26 $\pm$ 5	30 $\pm$ 4	55 $\pm$ 5	22 $\pm$ 2	33 $\pm$ 3	15 $\pm$ 2	22 $\pm$ 4

Fractionation of ^{32}P -labelled membrane fragments by gel filtration on Sepharose 6B

Polyacrylamide-gel electrophoresis of ^{32}P -labelled synaptic-membrane proteins in the presence of sodium dodecyl sulphate indicated that the phosphorylated proteins had a wide range of molecular weights, ranging from approx. 12000 to well over 100000 (Dunkley *et al.*, 1976). It therefore appeared possible that some fractionation of phosphorylated proteins might be achieved by gel filtration. For all experiments a sodium dodecyl sulphate:protein ratio of 20:1 (w/w) was used in the solubilizing buffer, and the elution buffer contained 0.1% sodium dodecyl sulphate and no NaCl.

Fig. 3. *Gel filtration of ^{32}P -labelled membrane fragments*
 Membrane fragments were labelled in the presence of 50 μM -cyclic AMP and applied to a column (2.5 cm $\times$ 90 cm) of Sepharose 6B. Protein was eluted with a buffer containing 50 mM-Tris/HCl, pH 7.0 at 20°C, and 0.1% sodium dodecyl sulphate and the A_{280} (—) and the radioactivity (----) were determined for each sample (6–8 ml). (a) Unfractionated membrane fragments. After labelling, the fragments were precipitated and washed with trichloroacetic acid before solubilization and application to the column. (b) Membrane fraction soluble in sodium deoxycholate. Precipitation with trichloroacetic acid was omitted in this case and in (c) below. (c) Membrane fraction insoluble in sodium deoxycholate. For further details see the Materials and Methods section.

Fig. 4. Polyacrylamide-gel electrophoresis of fractions from a gel-filtration column of sodium deoxycholate-soluble material. ³²P-labelled synaptic-membrane fragments phosphorylated in the presence of 50 μ M-cyclic AMP were extracted with sodium deoxycholate (1.5%, w/v) and the soluble material was applied to a column (2.5 cm $\times$ 90 cm) of Sepharose 6B (see Fig. 3b). Fractions A (128–204 ml), B (205–236 ml), C (237–267 ml), D (268–297 ml) and E (298–330 ml) from the column were pooled, concentrated and applied to polyacrylamide gel. Protein and [³²P]phosphate profiles for each fraction were prepared as described in the Materials and Methods section.

Phosphorylation of the membrane fragments made no difference to the protein elution profiles.

To decrease the amount of radioactivity applied to the column and to avoid the possibility of non-specific adherence of P_i to the sodium dodecyl sulphate-protein complexes, labelled protein was first precipitated with trichloroacetic acid and washed before solubilization. Only minor alterations in the protein and [³²P]phosphate profiles were observed as a result of this treatment, but the huge peak of radioactivity previously eluted at the end of the profile almost disappeared. Polyacrylamide-gel electrophoresis of pooled samples from these columns were very similar with respect to both protein and [³²P]phosphate profiles, except for the void-volume peak; when trichloroacetic acid precipitation was used more of the label was associated

with region ϵ , not region O as was the case when no precipitation was used. The explanation for this phenomenon is unknown. It is clear from the results, however, that phosphorylated proteins were being fractionated on the basis of their molecular weights.

To increase the resolution obtained with samples of whole membrane fragments, it was decided to fractionate the soluble and insoluble fractions obtained by treatment with sodium deoxycholate (Fig. 3). Typical protein and [³²P]phosphate profiles for column fractions derived from a deoxycholate-soluble sample are shown in Fig. 4. This shows that some enrichment of protein from region γ was obtained in fraction C and fraction B contained phosphorylated proteins with similar migration rates to proteins in regions α and β , although these

were not apparent in the original unfractionated material (see Fig. 2b). Inhomogeneity in region γ was also evident after column fractionation. It should be noted that the deoxycholate-soluble and -insoluble fractions were not precipitated with trichloroacetic acid before application to the gel-filtration column, as most of the ATP and P_i was washed away during the preparation of these samples, but some remained, as indicated by the larger trailing peaks. Clearly the combination of detergent extraction followed by gel filtration offers the potential for separating phosphorylated proteins from synaptic-membrane fragments, but further work will be required to understand the exact basis for the separations obtained in the presence of sodium dodecyl sulphate.

Discussion

In our previous paper (Dunkley *et al.*, 1976), ^{32}P -labelled protein was fractionated on polyacrylamide gels, the gels were sliced into segments and the $[^{32}P]$ phosphate content of individual segments was determined after liquid-scintillation counting. Since 2.5 mm slices were used it was impossible to ensure that single sections did not contain more than one phosphoprotein. The radioautographic technique used here did enable an exact correspondence to be made between the protein and $[^{32}P]$ phosphate peaks detected on the gels (see Figs. 1, 2 and 4). The latter technique, however, suffered the disadvantages that only arbitrary values could be conveniently determined for the phosphate contents of any particular gel region, and the Chromoscan recordings did not resolve all the protein or $[^{32}P]$ phosphate peaks seen by eye on the gels and radioautographs respectively. Both techniques have advantages, and it depends on whether qualitative or quantitative data are required as to which would be the most appropriate method for use.

The identity of the major phosphorylated proteins associated with synaptic-membrane fragments is unknown. The major phosphorylated protein in region γ approximately corresponds in molecular weight to tubulin (Lagnado *et al.*, 1975) and the regulatory subunit of a cyclic AMP-stimulated protein kinase (Malkinson *et al.*, 1975), both of which have been found in a phosphorylated form. Tubulin has been detected in synaptic-membrane fragments (Feit & Barondes, 1970; Walters & Matus, 1975; Matus *et al.*, 1975; Kornguth & Sunderland, 1975); however, even though cyclic AMP-stimulated protein kinase activity has been repeatedly shown to be present in synaptic membranes, no evidence exists for the presence in membranes of a regulatory subunit similar to the subunit found with soluble protein kinases. The low-molecular-weight region is invariably present and contains $[^{32}P]$ phosphate material

which stains with Coomassie Blue; this region also comprises the anodal front, which contains sodium dodecyl sulphate micelles, lipids and any other small molecules. Although we do not know of any reports of proteinase activity in synaptic-membrane fragments, it is possible that the phosphorylated protein(s) in this region are actually phosphopeptides derived from higher-molecular-weight proteins. It is also likely that region ϵ contains some $^{32}P_i$ which was originally tightly, but not covalently, bound and becomes dissociated from membranes only under the conditions of solubilization and electrophoresis. The relationship between the brain phosphopeptides described by Ledig *et al.* (1975) and the phosphorylated proteins described here is unknown.

In this field comparison of data obtained from different laboratories is difficult, since the membrane preparations and the conditions used for phosphorylation and gel electrophoresis differ greatly. It is also noteworthy that to observe a phosphorylated protein on a polyacrylamide gel the membrane fragments must contain active protein kinase as well as substrate, and protein phosphatase activity should be minimal. In our present studies we have been concerned with synaptic-membrane fragments prepared from bovine cerebral cortex by the modified method of Rodnight *et al.* (1969), which is described in the Materials and Methods section. This procedure is especially convenient for large-scale preparation of membranes from a restricted area of the brain, the cerebral cortex, but it does not provide highly purified membrane fragments. Most other studies to date have used rat brain material, and only in the work of Weller & Morgan (1976b) has highly purified membrane been used to study phosphorylation; however, these authors used whole rat brain as starting material, not cerebral cortex. The extent to which membrane-bound protein kinase retains its activity, its specificity and its ability to be stimulated by cyclic AMP is likely to be altered by minor changes in procedures used for preparing membrane fragments, as for example addition of EDTA or Ca^{2+} to solutions.

It is clear from the data presented in Fig. 2 and Table 2 that except for the phosphorylated protein in region γ , most of the acceptor substrates for kinase activity are not extracted with detergents under the conditions used. This suggests that the acceptors are intimately associated with the membranes, as is the cyclic AMP-stimulated protein kinase activity (Weller & Morgan, 1976a; Dunkley *et al.*, 1976). They can only be dissociated by the denaturing conditions in sodium dodecyl sulphate. As yet it has not been possible to determine whether the acceptor proteins are located in the pre- or the post-synaptic membranes, but it is now certain (Weller & Morgan, 1976a) that most are associated with the junctional

region and not the presynaptic extra-junctional protein.

Preparation of individual components from complex membrane systems in yields sufficient to perform detailed chemical analysis remains a challenge (Maddy & Dunn, 1976). Gel filtration in the presence of sodium dodecyl sulphate has been used on a number of occasions with variable success to fractionate membrane bound proteins [see Dunn & Maddy (1976) for discussion]. We found (Figs. 3 and 4) that the phosphorylated proteins of the synaptic membrane can be partially fractionated on the basis of their molecular weight, but no homogeneous phosphorylated protein was obtained. Longer columns and smaller fractions would help to enrich further individual proteins, but there will remain the problem of eliminating all non- ^{32}P -labelled proteins from the phosphoprotein fraction. New and more specific approaches, such as the use of affinity columns (Zanetta *et al.*, 1975), must be used in future work if progress is to be made in definitively characterizing the synaptic-membrane phosphorylated proteins. A specific affinity reagent which reacts with phosphoserine in proteins would be of considerable use for such work.

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