Multiple phosphorylation of pp30, a rat brain polyribosomal protein, sensitive to polyamines and corticotropin

Louise H. SCHRAMA,* Geert WEEDA, Philippa M. EDWARDS, A. Beate OESTREICHER and Peter SCHOTMAN

Division of Molecular Neurobiology, Institute of Molecular Biology, Rudolf Magnus Institute for Pharmacology and Laboratory of Physiological Chemistry, State University of Utrecht, Padualaan 8, 3584 CH Utrecht, The Netherlands

(Received 11 June 1984/Accepted 28 August 1984)

A rat brain polyribosomal protein with an apparent M_r of 30000, designated pp30, was further characterized. The protein was identified by its phosphorylation by an endogenous protein kinase sensitive to both corticotropin and spermine. Two-dimensional separation of a polyribosomal fraction was applied, combining non-equilibrium pH-gradient-gel electrophoresis in the first and sodium dodecyl sulphate/polyacrylamide-gel electrophoresis in the second dimension. In this system, pp30 was separated into at least five defined phosphoprotein spots. Pulse-labelling with $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ followed by a chase for various time periods with excess unlabelled ATP resulted in a shift of the distribution of radioactivity and protein staining along the spots towards the anode. This suggests that the various spots of pp30 may represent multiple phosphorylation states. Limited proteolysis of the five spots with three different proteinases resulted in the same one-dimensional peptide maps with a given proteinase, indicating that all five spots represent different forms of a single phosphoprotein. Inhibition of the overall phosphorylation of pp30 by corticotropin or spermine was accompanied by a shift in the recovery of labelled phosphate towards spots nearer the cathode. Immunoblotting with monoclonal antibodies directed against ribosomal protein S6 stained only one band, a protein that had an apparent M_r of 34000 and was clearly distinct from pp30.

Incubation of a free polyribosomal fraction from rat cerebrum with $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ revealed the phosphorylation of eight phosphoproteins in the M_r range 20000–80000 (Schrama *et al.*, 1984b). The protein kinases phosphorylating these proteins were not sensitive to either cyclic nucleotides or Ca^{2+} . The phosphorylation of one of these proteins, of M_r 30000 (pp30), was selectively affected by the polyamine spermine and by ACTH (Schrama *et al.*, 1984b,c). The ACTH sequences inhibiting pp30 phosphorylation all contained basic residues (Schrama *et al.*, 1984c). A possible relationship between the modulation of pp30 phosphorylation and of protein synthesis has been suggested because of the similarities in sensitivities

to Mg^{2+} and spermine and in structure–activity relationships for ACTH analogues (Schrama *et al.*, 1984a,c).

The M_r values of ribosomal proteins S6 and pp30 are in the same range. S6 has been proposed to play a role in the translation of mRNA (for reviews see Leader, 1980; Traugh, 1981).

The present paper describes the characterization of pp30 as a phosphoprotein with multiple phosphorylation sites and distinct from ribosomal protein S6.

Materials and methods

Chemicals

All chemicals used were of analytical grade. Ribonuclease-free sucrose and papain were obtained from Serva (Heidelberg, Germany), spermine was from Sigma Chemical Co. (St. Louis, MO, U.S.A.), low- M_r protein calibration kit was

Abbreviations used: SDS, sodium dodecyl sulphate; ACTH, adrenocorticotrophic hormone (corticotropin); eIF-2, eukaryotic initiation factor 2.

* To whom correspondence and requests for reprints should be addressed.

from Pharmacia (Uppsala, Sweden), Ampholines were from LKB Produkter (Bromma, Sweden), *Staphylococcus aureus* proteinase V8 was from Miles Laboratories (Stoke Poges, Slough, Bucks., U.K.), chymotrypsin A₄ was from Boehringer (Mannheim, Germany), dioctyl sodium sulphosuccinate and 3,3',5,5'-tetramethylbenzidine were from Merck (Darmstadt, Germany), ACTH-(15-24)-peptide was from Organon International (Oss, The Netherlands), and [γ -³²P]ATP (2900 Ci/mmol) was from New England Nuclear (Boston, MA, U.S.A.). Monoclonal anti-(ribosomal protein S6) antibodies, which react with rat brain S6 (Nielsen *et al.*, 1982), were generously given by Dr. H. Towbin (Ciba-Geigy, Basel, Switzerland), rabbit anti-mouse immunoglobulins coupled with horseradish peroxidase were from Nordic (Tilburg, The Netherlands), and pig serum was from the local slaughterhouse.

Preparation of a polyribosomal fraction

All solutions and equipment were sterilized before use to minimize ribonuclease contamination. Polyribosomes were isolated from a postmitochondrial supernatant fraction (S₂₀) (Schrama *et al.*, 1984b) from rat cerebrum in a Beckman SW 27.1 rotor. Then 11 ml of S₂₀ in 50 mM-Tris/acetic acid (pH 7.4)/25 mM-potassium acetate/2 mM-magnesium acetate/2 mM-dithiothreitol was layered over 2 ml of 1.5 M-sucrose and 4 ml of 2 M-sucrose. Both sucrose solutions were made in 50 mM-Tris/acetic acid (pH 7.4)/70 mM-potassium acetate/5 mM-magnesium acetate/2 mM-dithiothreitol. The discontinuous gradient was centrifuged in a Sorvall OTD-2 centrifuge at 85000g for 20 h at 4°C. The supernatant and sucrose gradient were discarded and the tube wall was cleaned with a cotton-wool tip. The pellet was rinsed with 50 mM-Tris/acetic acid (pH 7.4)/25 mM-potassium acetate/2 mM-magnesium acetate/2 mM-dithiothreitol and carefully resuspended with a glass rod in the same buffer containing 250 mM-sucrose, frozen in small portions in liquid N₂ and stored at -80°C.

Non-equilibrium pH-gradient-gel electrophoresis

This was performed by the method of O'Farrell *et al.* (1977). The composition of the gel was essentially the same as for an isoelectric-focusing slab gel (Zwiers *et al.*, 1980), but it contained a different Ampholine mixture: 0.8% (w/v) pH 6-8, 0.8% (w/v) 8-9.5 and 0.2% (w/v) pH 9-11. Polymerization and sample preparation were as described by Zwiers *et al.* (1980). The upper (anode) reservoir was filled with 1 mM-H₂SO₄ and the lower (cathode) reservoir was filled with 0.1 M-NaOH. The gel was run for 16 h at 200 V, followed by 1 h at 400 V.

SDS/polyacrylamide-gel electrophoresis

Phosphoproteins were separated by electrophoresis on SDS/12%-polyacrylamide gels; further processing and autoradiography were performed as described by Van Dijk *et al.* (1981). Non-equilibrium pH-gradient-gel electrophoresis followed by SDS/polyacrylamide-gel electrophoresis in the second dimension were performed with similar gels except that a comb without slots was used. Further processing was performed as described by Zwiers *et al.* (1980).

Phosphorylation of polyribosomes

Incubations were carried out at 30°C in polypropylene tubes in a total volume of 25 μ l. Phosphorylation was performed in 50 mM-Tris/acetic acid (pH 7.4)/100 mM-potassium acetate/1 mM-magnesium acetate/2 mM-dithiothreitol with various MgATP and [γ -³²P]ATP concentrations. The phosphorylation reaction was terminated by addition of a denaturing solution to give final concentrations of 62.5 mM-Tris/acetic acid, pH 6.8, 2% (w/v) SDS, 10% (v/v) glycerol, 0.001% (w/v) Bromophenol Blue and 5% (v/v) 2-mercaptoethanol. These samples were applied directly to gels for SDS/polyacrylamide-gel electrophoresis. For application to gels for non-equilibrium pH-gradient-gel electrophoresis the reaction was terminated by the addition of EDTA (5 mM final concn.) and immersion in liquid N₂.

Silver staining of proteins

This was performed by a combination of the methods described by Oackley *et al.* (1980) and Poehling & Neuhoff (1981). All solutions were made in double-distilled water. Gels were fixed with 50% (v/v) methanol/7% (v/v) acetic acid for 10 min, then transferred to 10% (v/v) methanol/5% (v/v) acetic acid and incubated for 1-24 h, and finally rinsed for 3 $\times$ 10 min in 15% (v/v) methanol to remove the acetic acid. After 30 min incubation in 5% (v/v) glutaraldehyde, gels were rinsed for 20 min in 15% (v/v) methanol and then for 10 min in water; this methanol/water procedure was repeated three times. Silver staining was performed for 20 min in 1.4% (v/v) NH₃/0.04 M-NaOH/0.3% (w/v) AgNO₃. The gel was rinsed three times in water. Gels were developed in 0.00075% (w/v) citric acid/0.015% (w/v) formaldehyde. The reaction was stopped in 10% (v/v) methanol/5% (v/v) acetic acid.

Peptide mapping of pp30

After two-dimensional separation of the polyribosomal proteins, the gel was subjected to autoradiography at -20°C. The phosphoprotein spots of pp30 were excised on the basis of the autoradio-

gram, and the accuracy of excision was verified by a second autoradiogram of the gel.

The excised protein spots were subjected to limited proteolysis essentially as described by Cleveland *et al.* (1977), but with the following modifications. The gel sections were soaked in 125 mM-Tris/HCl buffer, pH 6.8, for 30 min and put on the bottom of a slot of a 5%-polyacrylamide stacking and 15%-polyacrylamide running gel (2 mm thick). Spaces around the gel sections were filled with 10 μ l of 20% (v/v) glycerol. Finally, the gel sections were overlaid with 25 μ l of buffer containing 5 mM-Tris/HCl, pH 6.8, 3% (w/v) SDS, 10% (v/v) glycerol, 0.003% (w/v) Bromophenol Blue and a given amount of proteinase. The gel was

run at 10 mA for 2 h and subsequently at 35–40 mA until the dye front had reached the bottom of the gel.

Immunochemical staining of ribosomal protein S6

Electrophoretic transfer of protein from gels to nitrocellulose filters (0.45 μ m pore size) was performed as described by Nielsen *et al.* (1982). One half of a symmetrical blot was stained for protein with 0.1% (w/v) Amido Black in 45% (v/v) methanol/10% (v/v) acetic acid and destained in 90% (v/v) methanol/2% (v/v) acetic acid; the other half of the blot was stained by using immunochemical techniques for protein S6 (see below).

The incubation with the primary anti-(protein

Fig. 1. Two-dimensional separation of polyribosomal proteins

Polyribosomes (140 μ g of protein) were phosphorylated with 100 μ M-ATP for 15 s (a and b) or 120 s (c and d). The autoradiograms after separation on non-equilibrium pH-gradient-gel electrophoresis (NEPHGE)-SDS/polyacrylamide-gel electrophoresis (SDS/PAGE) after 15 and 120 s are shown in (a) and (c) respectively. Corresponding protein patterns after silver staining are shown in (b) (15 s) and (d) (120 s). Identification of the positions of the labelled spots 1–5 with corresponding spots in the protein-staining pattern, as indicated by the numbered arrows, was checked by developing a second autoradiogram of the gel after selective excision of the protein spots shown in (b) and (d).

S6) antibody and the secondary antibody (rabbit anti-mouse immunoglobulins) coupled with horseradish peroxidase was performed as described by Nielsen *et al.* (1982). The peroxidase was detected by the staining method of Buckel & Zehelein (1981) as follows: 30 ml of phosphate/citrate buffer (7.6 mM-citric acid/22.5 mM- NaH_2PO_4 adjusted with HCl to pH 5.0) was mixed with 10 ml of ethanol containing 24 mg of tetramethylbenzidine and 80 mg of dioctyl sodium sulphasuccinate; H_2O_2 (final concn. 0.01%) was added to the mixture immediately before use. The reaction was stopped by transferring the blot to water.

Results

Two-dimensional separation

Polyribosomes were phosphorylated for 15 or 120 s under conditions described in the Materials and methods section and in the Figure legends. Two-dimensional separation by conventional isoelectric focusing-SDS/polyacrylamide-gel electrophoresis did not give a clear separation of pp30 (results not shown). The protein pp30 appeared at M_r 30000 as a heterogeneous smear from pH 8.5 to pH 5.2. The same result was obtained when the ribosomal proteins were first extracted by the method of Roberts & Ashby (1978).

After separation by non-equilibrium pH-gradient-gel electrophoresis-SDS/polyacrylamide-gel electrophoresis, with a pH gradient from 6.0 to 9.0, pp30 appeared as several clearly separated spots on the autoradiograms (Figs. 1a and 1c). Five spots coincided with the protein-staining pattern (Fig. 1d) and were numbered 1–5 from cathode to anode. The two most anodic spots (4 and 5) were only visible on the protein-staining pattern after prolonged incubation with ATP (cf. Figs. 1b and 1d).

Effects of ACTH and spermine

In the presence of ACTH-(15–24)-peptide or spermine, total phosphorylation of pp30 was decreased (Fig. 2, inset). Moreover, a shift in the distribution of label between the spots was observed, as quantified by densitometric scanning of the autoradiogram (Fig. 2). Inhibition of phosphorylation resulted in a decreased amount of label in spots 4 and 5, accompanied by an increased amount in spot 1. The phosphorylation of spots 2 and 3 was decreased by spermine, but apparently not affected by ACTH-(15–24)-peptide.

Pulse-chase-labelling of pp30

After incubation of polyribosomes with $[\gamma\text{-}^{32}\text{P}]\text{-ATP}$ for 15 s, 1 mM-ATP (including 1 mM- Mg^{2+}) was added and incubation was continued for a further 0, 30, 60 or 120 s. The various phospho-

Fig. 2. Sensitivity of pp30 phosphorylation to ACTH-(15–24)-peptide and spermine

Polyribosomes (100 μg of protein) were phosphorylated with 7.5 μM -ATP for 75 s in the presence of 100 μM -ACTH-(15–24)-peptide, 1 mM-spermine or buffer. The autoradiogram obtained after two-dimensional separation of the proteins was scanned by densitometry to quantify incorporation of labelled phosphate into each of the five spots of pp30. The inset shows the total incorporation into pp30 after a control incubation and after incubation with ACTH-(15–24)-peptide or spermine.

protein spots were excised and quantified by liquid-scintillation counting of radioactivity (Fig. 3). The total amount of incorporated ^{32}P remained equal during the chase period up to 120 s (Fig. 3, inset), indicating negligible phosphatase activity and the adequacy of the chase procedure (see also Schrama *et al.*, 1984b). However, increasing the incubation time with non-radioactive ATP shifted the label from the more cathodic spots 1 and 3 towards the anodic spots 4 and 5. The amount of label decreased gradually with time in spots 1 and 2, whereas a continuous increase was observed in spot 5. A similar shift from the cathode to the anode was also observed in the protein-staining pattern (results not shown).

Peptide mapping of pp30

The five spots of pp30 were subjected to limited proteolysis with *Staphylococcus aureus* proteinase, papain or chymotrypsin. Incubation of the radioactively labelled spots with a given proteinase resulted in similar breakdown products for all the phosphoprotein spots. The results of proteolysis of spots 1 and 5 are shown in Fig. 4. *Staphylococcus aureus* proteinase digested all the phosphorylated

Fig. 3. Pulse-chase-labelling of pp30

Polyribosomes (140 μ g of protein) were labelled for 15s with [γ - 32 P]ATP, followed by the addition of 1mM-ATP, and incubation was continued for a further 0, 30, 60 or 120s. After non-equilibrium pH-gradient-gel electrophoresis-SDS/polyacrylamide-gel electrophoresis the five pp30 spots were excised and the incorporation of labelled phosphate was quantified by liquid-scintillation counting of radioactivity. The inset shows the total amount of 32 P incorporated in pp30 after the various incubation times.

Fig. 4. Peptide mapping of phosphorylated pp30

Phosphorylation and separation of pp30 were performed as described in Fig. 1 legend for 120s. The five spots of pp30 were excised and subjected to limited proteolysis on a 5%-polyacrylamide stacking and 15%-polyacrylamide running gel. Peptide maps are shown of spots 1 and 5 after digestion with *Staphylococcus aureus* proteinase (1.6 μ g/slot, a), with papain (0.8 μ g/slot, b) and with chymotrypsin (1.6 μ g/slot, c). The position of the original pp30 is indicated by the arrow. The M_r values on the right of the Figure indicate the positions of the M_r markers.

pp30, of which the original position is indicated by an arrow (Fig. 4a). The protein pp30 was digested to give three major peptides (25.4, 23.2 and 21.5kDa) and two minor peptides (17.5 and 16.2kDa), which are not visible on the photographic reproduction of the autoradiogram. Papain digested most of pp30 into two major (20.0 and 16.5kDa) and two minor peptides (28.7 and 23.6kDa) (Fig. 4b). Exposure to chymotrypsin left most of the pp30 unhydrolysed (Fig. 4c), but some was digested, giving three fragments of 28.2, 25.7 and 21.0kDa.

Immunochemical detection of ribosomal protein S6

Detection of ribosomal protein S6 was performed with a monoclonal antibody. Phosphorylated polyribosomes were separated on an SDS/polyacrylamide-gel-electrophoresis gel. A nitrocellulose blot from this gel was used for both protein staining with Amido Black (Fig. 5a) and immunochemical detection of S6 (Fig. 5d). The monoclonal antibody specifically detected S6, as shown by the staining of a single band in Fig. 5(d).

Autoradiography of the immunostained blot (Fig. 5c) and of the protein-stained blot (Fig. 5b) revealed pp30 just above the M_r marker carbonic anhydrase (M_r 30000), whereas S6 had an M_r of approx. 34000.

Immunochemical detection of S6 was also performed on a blot from a non-equilibrium-pH-gradient-gel-electrophoresis-SDS/polyacrylamide-gel-electrophoresis gel of phosphorylated polyribosomes (cf. Fig. 1). The autoradiogram of the blot revealed the spots 1-5 of pp30, whereas no positive staining of S6 was observed.

Discussion

This paper describes the characterization of a 30000- M_r phosphoprotein (pp30) from free polyribosomes of rat brain, sensitive in its phosphorylation to spermine and ACTH-(15-24)-peptide, but not to cyclic nucleotides or Ca^{2+} /calmodulin (Schrama *et al.*, 1984b,c). Initial attempts to identify this phosphoprotein further according to its isoelectric point on isoelectric focusing-SDS/poly-

Fig. 5. Immunochemical detection of S6

Polyribosomal phosphorylation was performed as described in Fig. 2. After SDS/polyacrylamide-gel electrophoresis, the protein was blotted on nitrocellulose. The protein-staining pattern and corresponding autoradiogram are shown in lanes (a) and (b) respectively. The immunochemical staining of S6 indicated by an arrow and corresponding autoradiogram are shown in lanes (d) and (c) respectively. The M_r value on the left of the Figure indicates the position of carbonic anhydrase.

acrylamide-gel electrophoresis were unsuccessful, owing to the low resolution power of the basic part of the isoelectric-focusing gel (O'Farrell, 1975). Neither removal of nucleic acids nor addition of SDS improved the separation.

Resolution of the phosphorylated polyribosomal fraction on non-equilibrium pH-gradient-gel electrophoresis-SDS/polyacrylamide-gel electrophoresis resulted in a clear separation of pp30 into five discrete spots (Figs. 1a and 1c). The same spots were also observed by silver stain after prolonged phosphorylation (Fig. 1d). The observed heterogeneity is not due to the presence of nucleic acids, as described by O'Farrell (1975), since removal of RNA before non-equilibrium pH-gradient-gel electrophoresis gave the same separation pattern (results not shown).

Heterogeneity of phosphoproteins in a charge-dependent separation system has been described by others. Phosphorylation of a cyclic AMP-binding protein (Rangel-Aldao *et al.*, 1979) or eIF-2 (Farrell *et al.*, 1978) followed by separation on isoelectric focusing-SDS/polyacrylamide-gel electrophoresis resulted in a shift of the pI. A different charge-dependent system, using urea/polyacrylamide-gel electrophoresis, run at pH 8.6 in the first and pH 4.05 in the second dimension

(Lastick & McConkey, 1976), is used for the separation of basic ribosomal proteins. In this system S6, the major phosphorylated protein *in vivo* in the 40S ribosomal subunit, appears heterogeneous (see, e.g., Leader & Coia, 1978; Roberts & Morelos, 1979; Nielsen *et al.*, 1982; Nishimura & Deuel, 1983). In rabbit reticulocytes, phosphorylated *in vivo*, up to five phosphoprotein spots of S6 can be detected, depending on the pH during incubation with [32 P]P_i (Perisic & Traugh, 1983).

The heterogeneity seen after two-dimensional separation of S6 is caused by multiple-site phosphorylation of this protein. Multiple-site phosphorylation has also been described for the α -subunit of eIF-2 (Tuazon *et al.*, 1980) and for the brain-specific ACTH-sensitive membrane protein B-50 (H. Zwiers, J. Verhaagen, C. J. van Dongen, P. N. E. de Graan & W. H. Gispen, unpublished work).

In both separation systems described above, phosphorylation of proteins produced a shift of the phosphoprotein towards the anode, owing to negative charging of the protein with phosphate.

The heterogeneity of pp30 described in this paper is, at least partly, caused by the degree of phosphorylation of the protein. The evidence for this is as follows: (i) the inhibition of total

phosphorylation with either ACTH-(15–24)-peptide or spermine appeared to be associated with a lower phosphate labelling of the anodic spots 4 and 5, accompanied by an increased labelling of spot 1 (Fig. 2); (ii) increasing the degree of phosphorylation by prolonged incubation with ATP resulted in a shift of the distribution of label towards the anodic spot 5, with a concomitant appearance of protein staining of spots 4 and 5 (Fig. 1d); (iii) pulse-chase-labelling of pp30 clearly demonstrated the shift of radioactive phosphate towards the anode with time, in relation to the increased degree of phosphorylation of pp30 (Fig. 3); (iv) the five phosphoprotein spots detected by non-equilibrium pH-gradient-gel electrophoresis-SDS/polyacrylamide-gel electrophoresis are derived from different forms of a single protein, since proteolysis with three different proteinases in each case resulted in the same breakdown products for all spots.

Partial proteolysis of pp30 with three different enzymes resulted in no detectable qualitative differences between the breakdown products of spots 1 and 5, indicating at least partially random phosphorylation of pp30. Extensive proteolysis of ribosomal protein S6 with trypsin after phosphorylation of five sites by proteinase-activated kinase II indicated partially random and partially sequential phosphorylation (Perisic & Traugh, 1983). However, under physiological conditions, ordered phosphorylation may be more important, as shown by Martin-Perez & Thomas (1983), after serum stimulation of quiescent 3T3 cells.

Incubation of polyribosomal proteins, after separation on SDS/polyacrylamide-gel electrophoresis, with monoclonal S6 antibodies, clearly showed pp30 to be different from S6. S6 had M_r approx. 34000 (Fig. 5) in our separation system. It could not be detected on our non-equilibrium pH-gradient-gel electrophoresis-SDS/polyacrylamide-gel electrophoresis system. This could be because S6 ran off the gel under the electrophoretic conditions used, implying a high pI , as is typical of ribosomal proteins.

We describe in this paper a first characterization of a novel polyribosomal phosphoprotein, pp30. Its phosphorylation is sensitive to polyamines, but not to cyclic nucleotides and Ca^{2+} /calmodulin. It contains multiple phosphorylation sites, but is not identical with ribosomal protein S6.

We express our gratitude to Dr. H. M. Greven and Dr. J. W. F. M. Van Nispen (Organon International BV, Oss, The Netherlands) for the gift of ACTH-(15–24)-peptide, to Dr. H. Towbin (Ciba-Geigy, Basel, Switzerland) for

the gift of the monoclonal S6 antibody, to Professor Dr. W. H. Gispen and Dr. H. Zwiers for stimulating discussions, to Ms. Frankena and Mr. J. H. Brakkee for technical assistance and to Mr. E. D. Kluis for drawing the Figures. This research was supported by FUNGO Grant no. 13-31-43 of the Netherlands Organization for the Advancement of Pure Research (ZWO).

References

- Buckel, P. & Zehelein, E. (1981) *Gene* **16**, 149–159
Cleveland, D. W., Fischer, S. G., Kirschner, M. W. & Laemmli, U. K. (1977) *J. Biol. Chem.* **252**, 1102–1106
Farrell, P. J., Hunt, T. & Jackson, R. J. (1978) *Eur. J. Biochem.* **89**, 517–521
Lastick, S. M. & McConkey, E. H. (1976) *J. Biol. Chem.* **251**, 2867–2875
Leader, D. P. (1980) in *Recently Discovered Systems of Enzyme Regulation by Reversible Phosphorylation* (Cohen, P., ed.), pp. 201–233, Elsevier/North-Holland, Amsterdam
Leader, D. P. & Coia, A. A. (1978) *FEBS Lett.* **90**, 270–274
Martin-Perez, J. & Thomas, G. (1983) *Proc. Natl. Acad. Sci. U.S.A.* **80**, 926–930
Nielsen, P. J., Manchester, K. L., Towbin, H., Gordon, J. & Thomas, G. (1982) *J. Biol. Chem.* **257**, 12316–12321
Nishimura, J. & Deuel, T. F. (1983) *FEBS Lett.* **156**, 130–133
Oackley, B. R., Kirsch, D. R. & Morris, N. R. (1980) *Anal. Biochem.* **105**, 361–363
O'Farrell, P. H. (1975) *J. Biol. Chem.* **250**, 4007–4021
O'Farrell, P. Z., Goodman, H. M. & O'Farrell, P. H. (1977) *Cell* **12**, 1133–1142
Perisic, O. & Traugh, J. A. (1983) *J. Biol. Chem.* **258**, 13998–14002
Poehling, H. M. & Neuhoff, V. (1981) *Electrophoresis* **2**, 141–147
Rangel-Aldao, R., Kupiec, J. W. & Rosen, O. M. (1979) *J. Biol. Chem.* **254**, 2499–2508
Roberts, S. & Ashby, C. D. (1978) *J. Biol. Chem.* **253**, 288–296
Roberts, S. & Morelos, B. S. (1979) *Biochem. J.* **184**, 233–244
Schrama, L. H., Edwards, P. M. & Schotman, P. (1984a) *J. Neurosci. Res.* **11**, 67–77
Schrama, L. H., Frankena, H., Edwards, P. M. & Schotman, P. (1984b) *Neurochem. Res.* **9**, 1265–1278
Schrama, L. H., Frankena, H., Edwards, P. M. & Schotman, P. (1984c) *J. Neurochem.* in the press
Traugh, J. A. (1981) *Biochem. Actions Horm.* **8**, 167–208
Tuazon, P. T., Merrick, W. C. & Traugh, J. A. (1980) *J. Biol. Chem.* **255**, 10954–10958
Van Dijk, A. M. A., Benitez-King, G., Schotman, P. & Gispen, W. H. (1981) *Neurochem. Res.* **6**, 847–861
Zwiers, H., Schotman, P. & Gispen, W. H. (1980) *J. Neurochem.* **34**, 1689–1699