The Number of Neurophysins in the Rat

INFLUENCE OF THE CONCENTRATION OF BROMOPHENOL BLUE, USED AS A TRACKING DYE, ON THE RESOLUTION OF PROTEINS BY POLYACRYLAMIDE-GEL ELECTROPHORESIS

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1. The concentration of Bromophenol Blue used as tracking dye in polyacrylamide-gel electrophoresis affected the resolution of rat neurophysins. 2. A final dye concentration of 1 µg/ml in the tris-glycine running buffer was found to give the best results. 3. The presence of two major and one minor neurophysin(s) in the rat was confirmed. 4. The two major proteins were found to re-run as single discrete bands, which had the same mobilities in the absence of dye and different mobilities in its presence.

The neurohypophysial hormones, oxytocin and vasopressin, are present in the posterior pituitary gland in association with a family of proteins, the neurophysins. Bovine (Rauch *et al.*, 1969) and porcine (Uttenthal & Hope, 1970; Burford *et al.*, 1971a) neurophysins have been studied extensively and, in each case, have been shown to consist of three closely related proteins, two of which are present in much greater amounts than the third.

We reported (Burford *et al.*, 1971b) that the rat also elaborates two major ('A' and 'B') and one minor ('C') neurophysin(s), which can be resolved by electrophoresis on polyacrylamide gel at a running pH of 9.5. The major neurophysin with the higher mobility ('A') appeared to be biosynthetically associated with vasopressin and the other major component ('B') with oxytocin (Burford *et al.*, 1971b). Other investigators, using essentially the same electrophoretic system as we have used, have been unable to resolve the two major neurophysins from rat glands (Norström & Sjöstrand, 1971; Norström *et al.*, 1971; Coy & Wu, 1972), and, as we reported (Burford *et al.*, 1971b), there was also a short period when we were unable to resolve them ourselves. We now report how variations in the concentration of the tracking dye Bromophenol Blue, hitherto considered unimportant, were the cause of these discrepancies.

Materials and Methods

Animals

Male rats (150-200 g) were used from the departmental colony of Wistar animals (Porton strain, derived M.R.C. Carshalton).

Chemicals

All chemicals used for preparation and staining of polyacrylamide gels were obtained from BDH Chemicals Ltd., Poole, Dorset, U.K. Sucrose, tris, HCl, glycine and ammonium persulphate of AnalaR grade, and Bromophenol Blue of pH-indicator grade, were used without further purification. Acrylamide and methylenebisacrylamide were recrystallized from chloroform and acetone respectively (Loening, 1967). *NNN'*-Tetramethylethylenediamine was redistilled *in vacuo*. The magnesium salt of 8-anilino-1-naphthalenesulphonate was obtained from Eastman-Kodak Ltd., Rochester, N.Y., U.S.A.

Preparation of gland extracts

Rat neural lobes (10 or 16) were pooled and homogenized in 0.5 ml of 0.1 M-HCl. The homogenate was left for 18 h at 4°C and then tris (2 mg/0.1 ml of 0.1 M-HCl used for extraction) was added. The precipitate that formed was removed, together with the insoluble residue, by centrifugation, and the supernatant liquid loaded on polyacrylamide gels for electrophoresis.

Polyacrylamide-gel electrophoresis

Gels [7.5% (w/v) acrylamide, 0.2% (w/v) methylenebisacrylamide] were prepared by the method of Davis (1964), the sample and spacer gels being omitted (Barka, 1961; Clarke, 1964). The tris-glycine buffer used for the electrophoretic separation contained various concentrations of Bromophenol Blue, ostensibly as a marker for the ion-front. In gels run in the absence of dye, the ion-front could be seen

as an interface of refractive-index change. Electrophoresis was performed and the gels were stained with Amido Black and destained by washing in 7% (w/v) acetic acid, as described previously (Burford *et al.*, 1971b). Stained gels were scanned by using a Canalcro Model G microdensitometer fitted with a Kodak-Wratten 29 filter.

Removal of protein bands and re-electrophoresis

After electrophoresis of a neural-lobe extract, the polyacrylamide gels were stained with 8-anilino-1-naphthalenesulphonate (Hartman & Udenfriend, 1969) and the neurophysin bands were located, cut out and placed on fresh gels for electrophoresis as described previously (Burford *et al.*, 1971b).

Results

Effects of tracking dye on the resolution of rat neurophysins

In an attempt to determine the cause of the variability of separation of rat neurophysins, the concentration of each of the components of the polyacrylamide system was changed in turn. These experiments were conducted during a period when we were unable to resolve the two major neurophysins and, although changes in the concentration of the acrylamide monomers, of the tris and of the glycine affected the mobilities of all the protein bands, in no case were the two neurophysins resolved. Bromophenol Blue is generally used in polyacrylamide-gel electrophoresis at alkaline pH values as a marker to show the position of the ion-front (Davis, 1964). Until now, the dye has not been considered as an important factor in the electrophoretic separation but, for completeness, different dye concentrations were included in the present series of experiments, and their effects on the resolution of the proteins of rat neurohypophysial extracts are shown in Plate 1. The position of the protein band corresponding to rat serum albumin (SA) was affected little, if at all, by the increasing dye concentration. However, there were profound differences in the positions of those bands that had greater mobilities than albumin and that corresponded to the neurophysins. In the absence of Bromophenol Blue there was no resolution of the two major neurophysins, and the resulting electrophoretogram was almost identical with that published by other workers (e.g. Norström & Sjöstrand, 1971). At the lowest dye concentration (0.5 $\mu\text{g/ml}$) used in the present experiments, neurophysin 'A' began to be resolved from neurophysin 'B' and separation improved as the dye concentration increased. At concentrations greater than 5 $\mu\text{g/ml}$, however, the bands became diffuse and it appeared that all three proteins were eventually running

together just behind the ion-front. These latter effects are better shown by the densitometric scans of the gels (Fig. 1). All of the gels were loaded with the same amount of the same neurohypophysial extract and were subjected to electrophoresis, stained and destained at the same time. Thus the relative absorbancies of analogous stained bands at the different Bromophenol Blue concentrations may be considered to be proportional to the relative amounts of protein contained in them. The sum of the absorbancies of the protein bands was constant for dye concentrations up to 5 $\mu\text{g/ml}$, suggesting that within the concentration range 0.5–5 $\mu\text{g/ml}$ the three neurophysins had discrete mobilities. At very high concentrations of Bromophenol Blue, not only did the bands become diffuse, resulting in a high background stain, but there was evidence that some of component 'B' and/or 'C' were running together with component 'A' just behind the ion-front. On the basis of these findings, the tris-glycine buffer, used in the electrode compartments in all future polyacrylamide runs, contained 1 μg of Bromophenol Blue/ml.

Mobilities of isolated components in the presence and the absence of dye

In view of the results of the experiments at high dye concentrations, it seemed possible that a single neurophysin component might give rise to two protein bands in the presence of low concentrations of dye. To test this possibility, an extract of 16 rat neurohypophyses was evenly distributed among eight polyacrylamide gels, and electrophoresis was performed with the optimum dye concentration (1 $\mu\text{g/ml}$). Two of the gels were then stained with Amido Black as records of this stage of the experiment. The other six gels were stained with 8-anilino-1-naphthalenesulphonate to detect the individual neurophysins, which were then sliced out and placed on the top of new gel columns. Three of the six samples of each neurophysin were then subjected to electrophoresis in the absence of tracking dye, and the other three in the presence of Bromophenol Blue at a concentration of 1 $\mu\text{g/ml}$. Plate 2 shows that component 'A' re-ran as a single distinct band, which had a greater mobility than component 'B' in the presence of dye, and the same mobility as component 'B' in the absence of dye. The mobilities of components 'B' and 'C' were unaffected by the dye.

Discussion

The resolution of rat neurophysins by polyacrylamide-gel electrophoresis is highly dependent on the concentration of Bromophenol Blue in the electrophoretic buffer. In our previous work on rat neurophysins (Burford *et al.*, 1971b), it had been the

EXPLANATION OF PLATE I

Effect of concentration of tracking dye (Bromophenol Blue) on the resolution of rat neurophysins by polyacrylamide-gel electrophoresis

Neural lobes from 10 rats were homogenized in 0.5 ml of 0.1 M-HCl and 40 μl of the extract was applied to each gel, which was subjected to electrophoresis in tris-glycine buffer containing Bromophenol Blue at the appropriate concentrations. SA is the band corresponding to rat serum albumin; F is the ion-front.

EXPLANATION OF PLATE 2

Re-electrophoresis of separated rat neurophysins in the absence or the presence of Bromophenol Blue

Rat neurohypophyseal extract was separated by polyacrylamide-gel electrophoresis as shown in gel (a). Slices of gel corresponding to the separated neurophysins were then cut out and placed on the top of fresh gels. One series of these was subjected to electrophoresis in a tris-glycine buffer containing no Bromophenol Blue (b) and the other series in a buffer containing the dye at a concentration of $1 \mu\text{g/ml}$ (c). SA is the serum albumin band; A, B and C are the rat neurophysins; F is the ion-front.

Fig. 1. Densitometric scans of gels run with buffers containing various concentrations of Bromophenol Blue

These are the gels shown in Plate 1 and Bromophenol Blue was either absent (a) or present at concentrations of: (b) 0.5 µg/ml; (c) 1 µg/ml; (d) 2 µg/ml; (e) 5 µg/ml; (f) 10 µg/ml; (g) 20 µg/ml; (h) 50 µg/ml. The densitometer was fitted with a Kodak-Wratten filter which cut out light with wavelengths below 620nm. The values under each neurophysin peak represent the peak area, and the sum (Σ) of the areas for the neurophysin peaks from each gel are given above each scan. The base lines from which the area of each peak was calculated are shown by the dotted lines.

practice to add sufficient solid dye to the stock tris-glycine buffer to produce a deep blue colour, and it would now seem that the periods when we could not obtain resolution resulted from insufficient dye, because of the variability of this subjective assessment.

How can we be sure that the two components ('A' and 'B'), which can be resolved in the presence of dye, really are separate entities? If the two bands resulted from a single protein, because of partial interaction with a component of the electrophoretic

system (e.g. Bromophenol Blue), then the constituents of these bands would each be expected to show two bands when re-run in the presence of dye. This phenomenon is seen when albumin is examined by electrophoresis in carboxylic acid buffers (Cann & Goad, 1964). In the present experiments each of the components re-ran as a single band, both when dye was absent and when it was present at the optimum concentration. In the re-runs the bands originating from components 'A' and 'B' had the same mobilities as each other in the absence of dye and different ones in its presence. Our previous evidence (Burford *et al.*, 1971b) associating neurophysin 'A' with vasopressin and neurophysin 'B' with oxytocin, further supports the contention that they are separate proteins. The ratio of radioactivity in protein 'A' to that in protein 'B', after polyacrylamide-gel electrophoresis of neurohypophysial extracts prepared from rats that had received intracisternal injections of [35 S]cysteine 5–24 h before death, is 1.50 ± 0.05 (S.E.M., $n=13$), but it is lower [0.76 ± 0.03 (S.E.M., $n=6$)] when the label is injected directly into the paraventricular nucleus, and higher (between 2 and 3) when it is given intracisternally to animals whose paraventricular nuclei have been destroyed (Burford *et al.*, 1972). These changes of the relative labelling of the two major neurophysins, depending on the site of injection of radioactive amino acid and on the state of the animal, also support the presence of two separate proteins. Thus it now seems certain that the rat, in common with the domestic animals, elaborates two major and one minor neurophysin(s); allowing the possibility that one protein is biosynthetically linked with vasopressin and the other with oxytocin, perhaps as part of a common pro-hormone precursor (Sachs *et al.*, 1969; Pickering *et al.*, 1971). The final proof will depend on the isolation and characterization of the individual proteins. Preliminary amino acid analyses of the three neurophysins recovered from polyacrylamide gels show that neurophysins 'A' and 'B' have very similar amino acid compositions, but that neurophysin 'A' may have rather more (2 residues/12000 mol.wt.) aspartic acid than neurophysin 'B' (L. Moens, G. D. Burford & B. T. Pickering, unpublished work).

Although we are primarily interested in the resolution of rat neurophysins, these results have wider implications for the separation of proteins by electrophoresis. In our system it would appear that one member of a pair of closely related proteins has a higher affinity for Bromophenol Blue such that it forms a more negatively charged protein-dye complex when the dye concentration is greater than $0.5 \mu\text{g/ml}$. In fact this component ('A') can be seen as a blue band during the actual electrophoretic run. It may be that modification of dye concentration in similar electrophoretic systems will be an aid to the resolution of other mixtures of proteins with high affinities for dyes.

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