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## Note

# Quantitative staining of submicrogram amounts of histone and high-mobility group proteins on sodium dodecylsulphate–polyacrylamide gels

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The high-mobility group (HMG) proteins are amongst the best characterised of nuclear non-histone proteins[1,2]. It was of interest to us to quantify accurately some of these proteins, in order to gain insight to their role in chromatin function. To avoid errors which can arise because of loss of protein during purification it was decided to use simple extracts of tissues. For resolution of such complex mixtures of proteins one of the best systems currently available is that of polyacrylamide gel electrophoresis in the presence of sodium dodecylsulphate (SDS).

Visualisation of proteins in SDS gels for qualitative purposes in usually achieved by staining with Coomassie Brilliant Blue R (or R250), but we have found this stain to be unsuitable for quantitative work for the following reasons: its sensitivity varies from batch to batch; its ability to stain proteins varies according to the order in which constituents of the staining solution are mixed; it shows metachromatic effects; for the proteins in which we were interested, the relationship between Coomassie Brilliant Blue R staining and protein concentration was not linear. Descriptions of methods for quantitative staining proteins in gels are frequently inadequate, as Wilson[3] has remarked — even details such as the precise name and source of the dye are omitted. We therefore devised a method for quantitatively staining submicrogram amounts of proteins on SDS gels, using the dye Procion Navy, which has previously been used in another, non-detergent gel system[4]. The efficacy of the method was tested by quantifying histone H1, and it was used to quantify accurately HMG proteins 1 and 2.

## METHODS

### *Protein preparation*

Proteins used in this study were extracted from whole thymus glands of CBA/LAC mice. After removal from the mice the glands were frozen and stored in liquid nitrogen.

Standard HMG1 and HMG2 were prepared by the method of extraction with NaCl (0.35 *M*) followed by chromatography on CM Sephadex[5]. Standard H1 was prepared by extraction of the HMG-depleted pellet (left after extraction with 0.35 *M* NaCl with $HClO_4$ (0.74 *M*)[6]. The final precipitation of H1 was brought about by addition of acetone (six volumes) to the extract made 0.3 *M* HCl. The identity of

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these standards was confirmed by electrophoretic and total amino acid analyses (which were similar to those of their counterparts in calf thymus[2], as was their purity.

A standard of combined core histones (H2A, H2B, H3 and H4) was prepared from the pellet remaining after extraction of whole tissue with $HClO_4$ (0.74 *M*). The pellet was extracted in the cold with HCl (0.25 *M*)[5], four times (to ensure thorough extraction), and core histones precipitated by addition of acetone (ten volumes, to ensure precipitation of all of the histones).

The protein content of stock solution of standards was accurately determined by total amino acid analysis on a Rank-Hilger Chromospek analyser.

Samples for study were made by thorough extraction of whole tissue with either (a) HCl (0.25 *M*), four times with precipitation with ten volumes of acetone, or (b) $HClO_4$ (0.74 *M*) four times, with precipitation with six volumes of acetone.

Extracts were made of rapidly thawed whole tissue, not of purified nuclei, so as to avoid the possibility of protein degradation which may occur during nuclei isolation (even in the presence of specific protease inhibitors in the case of some proteins, *e.g.* $H1^0$ (ref. 7)).

*Electrophoresis*

SDS–polyacrylamide gel slabs of *ca.* 0.5 mm thickness were made and run according to the method of Laemli[8]. Samples were loaded into 2 mm-wide wells made in the stacking gel, but during electrophoresis the width of protein bands increased to not more than 6 mm.

*Staining*

The stain used was: 0.2% (w/v) Procion Navy MXRB (I.C.I., Organics Division, Manchester, Great Britain) in methanol–acetic acid–water (50:10:40, v/v/v). The dye was dissolved in the methanol before the addition of acetic acid and water. The stain was made up just before use.

When electrophoresis had been completed, gels were immersed in stain, with gentle shaking at room temperature for 1.5 h. Excess dye was then removed from the gel by gentle shaking in methanol–acetic acid–water (10:10:80, v/v/v) at room temperature, with several changes of the destaining mixture until the background was colourless (2 days or so).

*Scanning*

Gels for scanning were cut into vertical strips, each bearing one sample, and immersed in ethanol–water (40:60, v/v) for at least 2 h, in order to shrink the gel. This was to ensure inclusion of the whole width of the sample in the light beam of the spectrophotometer.

Gel strips were scanned (whilst still in ethanol–water (40:60, v/v) at 580 nm in the linear transport system of a Gilford 250 spectrophotometer, with a 0.1 × 7.0 mm slit, and set to maximum sensitivity for absorbance of each sample.

Each scan was recorded on a chart, and the amount of stain bound in each protein band was estimated from the weight of paper in the corresponding peak of the scan profile.

Standard curves of units of dye bound *vs.* weight of protein were constructed by calculating the lines of best fit.

## RESULTS AND DISCUSSION

It was found that the Procion Navy stain showed no metachromatic effect and could visibly stain as little as 0.2 $\mu$g or less of protein in a band of *ca.* 5-mm width on an SDS gel (*e.g.* see Fig. 1i). This is probably not less than a half or a third as sensitive as Coomassie Brilliant Blue R. The relationships of stain binding to protein concentration were linear in the following ranges: up to 3–4 $\mu$g of HMG 1 or 2; up to at least 6 $\mu$g of H1; up to 25–30 $\mu$g of core histones (combined). Mouse globin and pig thymus HMG 1 and 2 also gave linear responses up to at least 2–3 $\mu$g/band. Fig. 1 shows examples of standard curves. On gels such as those used here a loading of 1 $\mu$g/band is adequate for quantification.

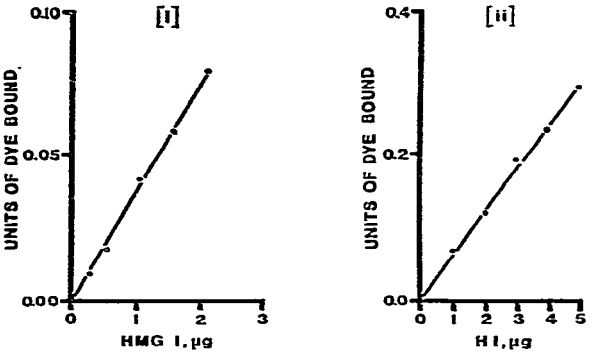


Fig. 1. Procion Navy stain binding by (i) mouse thymus HMG 1, and (ii) mouse thymus H1; calculated lines of best fit.

The staining responses of pig thymus HMG 1 and 2 were exactly alike, which is not surprising for they are very similar proteins, as are their counterparts in other species, *e.g.* calf[9]. However, it was found that proteins with markedly different amino acid contents bound different amounts of Procion Navy, *e.g.* HMG 1 or 2 bound only *ca.* 65% of the amounts of dye bound by the same weight of H1. This observation probably reflects the covalent nature of the Procion dye–protein interaction[10], and means that a pure standard is required for absolute quantification of any particular protein.

Calculations of coefficients of staining for these proteins (units of dye bound/$\mu$g protein) were not completely consistent. This experimental variation makes it advisable to run standards every time samples for study are run. This is not inconvenient if slab gels are used, for one gel can bear many samples which undergo identical electrophoresis and staining.

From scans of HCl extracts of mouse thymus run on SDS gels (*e.g.* Fig. 2) and by use of standard curves H1 was quantified and related to the core histone content of the same sample. The results are given in Table I. The mean of the molar H1: core histone ratios is 1.08, *i.e.* an average of 1.08 molecules of H1 per octamer of core histones (or per nucleosome). The value agrees with previous estimates for H1 of about one H1 molecule per nucleosome (*e.g.* ref. 4), and confirms the efficacy of the method.


Fig. 2. Profile of scan at 580 nm of an HCl extract of mouse thymus run on an SDS–polyacrylamide gel and stained by Procion Navy. Electrophoresis was from left to right.

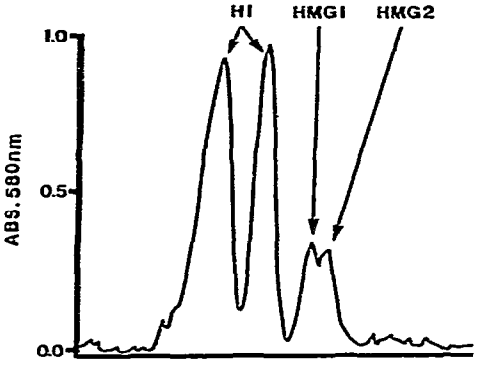


Fig. 3. Profile of scan at 580 nm of an $HClO_4$ extract of mouse thymus run on an SDS–polyacrylamide gel and stained by Procion Navy. Electrophoresis was from left to right.

HMG 1 and HMG 2 may be seen in gels of HCl extract, as Fig. 2 shows, but the presence of other proteins in the sample can sometimes make the baseline in the chart recordings of such scans less obvious. This may introduce error, especially when studying small peaks. For HMG 1 and 2 this problem was avoided by extraction by $HClO_4$, which yields simpler mixtures of proteins (which lack core histones for example). Fig. 3 shows a scan of an $HClO_4$ extract. From this and other scans, and using the standard curves from Fig. 1, HMG 1 and 2 were quantified (see Table I). They were related to H1 in the same sample, and then could be related to core histones. In our system HMG 1 and 2 were not completely resolved and so were quantified as a pair. It may be said, however, that there are approximately equal amounts of these proteins in mouse thymocytes. It may be estimated from preparative column runs that HMG 1 and 2 together represent *ca.* 2% of the mass of the histones. This agrees with the present data, for from Table I it can be calculated that HMG 1 and 2 represent 3.79% (w/w) of total histone in thymocytes. Whole tissue was used in these experiments (in order to minimise loss of protein by enzymatic degradation) but although HMG proteins occur in the cytoplasm[11], the contribution of HMGs from this source is expected to be small in the present case, for thymocytes have little cytoplasm. It may be calculated, then, that in the nucleus there is an average of one HMG 1 or HMG 2 molecule for every five or six nucleosomes. This ratio may have some significance for models of chromatin structure, for it is thought that the basic nucleosomal thread is coiled up, with between six and ten nucleosomes per coil (as reviewed in ref. 12).

TABLE I

QUANTIFICATION OF MOUSE THYMUS H1 AND HMG1 AND HMG2

Molecular weights of 21,000 for H1, 27,000 for each of HMG1 and HMG2, and 109,400 for nucleosomal histone octamer (H2A, H2B, H3 and H4) were used. Moles of HMG1 and HMG2 per mole of octamer were calculated by multiplying calculated molar ratio (HMG1 and 2):H1 (0.17) by mean of molar ratios H1:octamer (1.08).

| *For estimation of protein x* | *Sample* | *Approximate loading (μg)* | *Ratio of H1 to core histone (w/w)* | | *Ratio of (HMG1 + 2) to H1 (w/w)* | | *Ratio of x to octamer (mol/mol)* |
|---|---|---|---|---|---|---|---|
| | | | *Observed* | *Mean* | *Observed* | *Mean* | |
| H1 | HCl extract no. 1 | 28 | 0.19 | 0.19 | – | – | 0.98 |
| | | 32 | 0.18 | | – | | |
| H1 | HCl extract no. 2 | 28 | 0.26 | 0.23 | – | – | 1.19 |
| | | 32 | 0.19 | | – | | |
| HMG1 and HMG2 | $HClO_4$ extract | 2 | – | – | 0.24 | 0.22 | 0.19 |
| | | 4 | – | | 0.24 | | |
| | | 8 | – | | 0.18 | | |

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