Colorimetric Method for Estimating Polylysine and Polyarginine

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In the course of studies aimed at determining the percentage of free DNA-phosphate groups in chromatin (1,2) by their binding to polylysine (PL), a method was developed for estimating excess unbound lysyl residues. The method was found to be usable also for polyarginine. It involves reaction of the anionic dye methyl orange (MeO) with the lysyl or arginyl residues under conditions such that the complex precipitates: the unbound MeO is then estimated from the absorbance of the supernatant. This procedure was adapted from a study by Wetlaufer and Stahmann (3) of the interaction of MeO and PL.

EXPERIMENTAL

Materials. Poly-L-lysine hydrobromide, L-lysyl-L-lysine hydrochloride, and poly-L-arginine hydrochloride were obtained from Miles-Seravac Ltd., England, who kindly provided free samples of tetra-L-lysine hydrochloride and tri-L-lysine hydrochloride. The average molecular weight of the PL samples was determined by the suppliers from ultracentrifugation measurements of the parent compound poly-L-carbobenzoxy-L-lysine. L-Lysine hydrochloride was obtained from Sigma Chemical Co. Methyl orange was recrystallized from distilled water, washed with alcohol and ether, and dried to constant weight. In initial experiments the above reagents were dissolved in 0.7 mM sodium phosphate buffer, pH 6.9 (Buffer A), and in later work in water; similar results were obtained with the two solvents.

The spectrum of MeO (at 25 μM) has a maximum at 465 nm and minima at 340 nm and 575 nm (Fig. 1, curve A). The absorbance at 465 nm varied linearly with MeO concentration up to at least 35 μM.

Sephadex Chromatography. 4–10 mg PL dissolved in 0.5 ml Buffer A

1 These compounds will be referred to subsequently as PL, polyarginine, etc., i.e., omitting HBr or HCl. Also, all concentrations will be given in terms of molarity of lysyl or arginyl residues.

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Fig. 1. Absorption spectra of 25 μM MeO (○), of supernatants diluted 1 in 20 from mixtures containing 500 μM MeO, 100 μM PL (●), and 500 μM MeO, 280 μM PL (×).

was applied to a G-25 fine Sephadex column (20 × 1.5 cm) and 2 ml fractions were collected using Buffer A as eluant. A rapid test for the presence of polylysine or lysine in the eluate tubes was developed. A strip of paper was dipped into a solution of 0.2% ninhydrin in acetone and was then dried at about 80°. A drop from each tube was spotted on to the paper and the presence of polylysine or lysine was revealed by the formation of a purple color on drying. The intensity of the ninhydrin color varied directly with concentration of PL and inversely with molecular weight of PL. The presence of PL was confirmed by addition of 0.5 ml samples of 1 mM MeO to 0.5 ml aliquots from each tube: PL at low concentrations formed orange-colored suspensions with MeO and, at high concentrations, yellow solutions.

General Procedure. Optimal experimental conditions were found as described below but in all cases 2 ml PL or polyarginine at a range of concentrations was mixed with 2 ml MeO at a given concentration, the mixtures were shaken for a given time, and the resulting suspensions were centrifuged. The absorbance of the supernatants was then read (after dilution if necessary with water). Figure 1, curves B and C, shows the spectra of two supernatants (diluted 20-fold) obtained from mixtures of MeO at 500 μM with PL at 100 and 280 μM, respectively. The shape of the curves is similar to that of MeO alone, showing that under these conditions there is no metachromasia.

Effect of MeO Concentration. In initial experiment, MeO was used to a final concentration of 500 μM and PL at concentrations of about 50–500 μM. Since the MeO concentration in the final supernatants was
about 20 times that required for reading the absorbance, the use of MeO at lower concentrations was studied to find if lower concentration ranges of PL could be estimated thus. Sets of mixtures were made using MeO at final concentrations of 25, 50, 100, and 250 μM, respectively, with corresponding reductions in PL concentration. It was found that little precipitate formed at low concentrations of the reagents and the absorbance of the supernatants decreased only to a limited extent with increasing PL concentration and then flattened off. In contrast, with MeO at 500 μM, the absorbance of the supernatants decreased linearly with increasing PL concentration up to 400 μM. Therefore, in subsequent experiments, MeO was used at a final concentration of 500 μM.

**Experimental Conditions.** The absorbance of the supernatants of PL-MeO mixtures was found to be independent of (a) shaking time above a minimum of about 30 min, (b) temperature of shaking and centrifuging between 4°C and room temperature, and (c) speed of centrifugation of the mixture above about 400g.

**Method Finally Adopted.** 2 ml samples of PL at concentrations of about 50–400 μM were each mixed with 2 ml of 1 mM MeO. The mixtures were shaken for 30 min. After centrifugation for 15 min at 400g, the absorbance of the supernatants diluted 20-fold was read at 465 nm. Also, values of absorbance at 445 and 485 nm were read to check that no spectral shift had occurred (due, e.g., to formation of a soluble PL-MeO complex).

**RESULTS AND DISCUSSION**

The standard deviation of $A_{445}$ for two sets of five supernatants from separate PL-MeO mixtures (with MeO at 500 μM and PL at 125 and 250 μM, respectively) was found to be ±1% of the mean in each case.

**Molecular Weight of PL.** Calibration curves for various PL samples were obtained by preparing serial dilutions of PL from a stock solution containing a known weight of the material and mixing 2 ml samples with MeO as described above. Figure 2 shows the curve of $A_{445}$ of the MeO-PL supernatants versus PL concentration, for PL of molecular weight 8000. It is seen to decrease linearly with increasing PL concentration up to about 400 μM. Extrapolation to zero absorbance, corresponding to total precipitation of the MeO, gave a value of 480 μM PL. This indicates that the reaction of MeO with the PL is stoichiometric, at least in the presence of excess MeO, one MeO anion being bound per lysyl residue. Identical results were obtained with PL of molecular weight 14,000, the calibration curve being linear to a PL concentration of about 400 μM and extrapolation to zero $A_{445}$ giving a value of 480 μM PL.

Certain samples of polylysine (PLL) were found to give calibration
curves with methyl orange which were concave upwards. It was sus-
ppected that this might be due to the presence of components of much
lower molecular weight than the value quoted. To check this, samples
were filtered through Sephadex. It was found that there were two main
peaks, one in the void volume, the second eluting later. Groups of tubes
found to contain PL were pooled, diluted 1 in 2 with buffer A, and
mixed with aliquots of MeO (1 mM), shaken, and centrifuged in the
usual way. With PL eluting in the void volume, measurement of $A_{405}$ of
the MeO supernatants showed a linear decrease down to at least 0.2
unit of absorbancy with increasing PL concentration. However, PL elut-
ing later gave a curve that was very concave upwards. By extrapolation
of the linear part of each graph, a rough calculation of the relative
amounts of PL in each fraction was obtained and showed that the high
molecular weight material comprised 20% or less of the total.

In the case of PL samples giving linear calibration curves with MeO,
gel filtration and the ninhydrin test showed that almost all the material
was eluted in the void volume. These preparations lacking low molecular
weight fractions were used subsequently as they were clearly more
suitable for the present study and also for the studies on chromatin. It
is of interest that Stewart and Stahmann (4), who studied the chromato-
graphic analysis of PL on carboxymethylcellulose, found similarly that
certain preparations of PL contained low molecular weight as well as
high molecular weight species.

Effect of Various Salts. The presence of NaCl at 75 mM, Na$_2$SO$_4$ at
5.0 mM and MgCl₂ at 20 μM in the PL-MeO mixtures did not affect the calibration curve of 14,000 molecular weight PL. Higher concentrations of MgCl₂ caused precipitation of MeO in the absence of PL. 4 mM potassium phosphate buffer (pH 7.4) caused precipitation of PL in the absence of MeO.

Lysine and Lysine Peptides. No precipitate formed in mixtures of MeO and lysine and of MeO and lysine up to a final concentration of 25 mM. Trilysine gave no precipitate at 5 mM but a precipitate formed at 25 mM. With tetralysine the absorbance of the supernatants decreased linearly up to a peptide concentration of 250 μM but extrapolation to zero absorbance gave a value of 650 μM tetralysine, corresponding to 500 μM MeO; this suggests that precipitation of MeO (and probably of tetralysine) was incomplete. Similarly, Wetlaufer and Stahmann (3), who studied the interaction of PL and MeO with the former present at excess, found that the extent of precipitation of PL depended on its degree of polymerization.

Polyarginine. Figure 3 shows the calibration curve for polyarginine of molecular weight 16,000. It is seen to be linear to 400 μM and extrapolation to zero absorbance, corresponding to complete precipitation of MeO, gives a value of 500 μM polyarginine. Thus, as with polylysine, the reaction is stoichiometric, with one methyl orange anion bound per arginyl residue.

The results show that PL and polyarginine can be estimated simply and rapidly in amounts down to at least 50 μmoles (10.5 μg and 9.6 μg, Fig. 3. Variation of A₄₈₅ of supernatants from polyarginine-MeO mixtures with polyarginine concentration.
respectively, of the hydrobromide and the hydrochloride). Other methods that can be used for estimating lysyl or arginyl residues include the colorimetric ninhydrin (5), the Sakaguchi procedure (6), and the reaction with 1-fluoro-2,4-dinitrobenzene (7) and with 2,4,6-trinitrobenzenesulfonic acid (7). However, the present method is much quicker and simpler (though less sensitive) than the above methods, mainly because hydrolysis of the polyamino acid is not required. Further, solutions of MeO, unlike reagents in the above methods, are stable for several months at least.

SUMMARY

A rapid and sensitive method is described for estimating polylysine and polyarginine. The method involves the stoichiometric precipitation of these polyamino acids by excess of the anionic dye methyl orange, followed by spectrophotometric determination of unbound dye. 10 μg or less of polylysine or polyarginine can thus be estimated. The effects of various salts on the estimation are described.

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REFERENCES
