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The role of free amino groups of peptides and proteins in the Folin-Lowry and biuret methods

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Abstract. On an equal weight basis polymyxin B and EM 49 which do not contain tyrosine or tryptophan yielded the same colour intensity as proteins in the Folin-Lowry and biuret methods. But, in the absence of reagent C (alkaline copper reagent) polymyxin B and EM 49 yielded no colour in the Folin-Lowry method. Mono-, di- and tri-formyl polymyxins B formed identical amounts of coloured complexes as polymyxin B in the two methods. However, the tetra- and penta-formyl polymyxins B yielded only one-fifth and one-sixth, respectively, of the expected colour in the Folin-Lowry method. Similarly, 40% and 30%, respectively, of the anticipated amount of colour is formed in the biuret method. Formylated and methylated lysozyme and bovine serum albumins form only 70-75% of the expected colour in the Folin-Lowry method. Since formation of colour by reduction of Folin reagent, in the Folin-Lowry method, is at least partly due to complexes of copper, it was inferred that polymyxin B as well as its mono-, di- and tri-formyl derivatives on the one hand and the tetra- and penta-formyl derivatives on the other differ in their ability to complex Cu(II) The former group of compounds was indeed found to complex as many as three Cu(II) ions whereas the tetra- and penta-formyl polymyxins B complexed only one equivalent, under conditions of excess Cu(II). Under conditions of low Cu(II), polymyxin B and all its derivatives complexed only one Cu(II). In proteins, sites other than amino groups which complex Cu(II) probably play a major role in the reduction of the Folin reagent, since methylated lysozyme and bovine serum albumin yield 70-75% of the colour formed by the unmodified proteins in the Folin-Lowry reaction.

Keywords. Polymyxin B; acylation of polypeptides; reductive methylation of proteins; Folin-Lowry and Biuret methods; copper chelate of polymyxin B; formyl polymyxins B-Cu(II) chelates; Gramicidin S.

Introduction

The Folin-Lowry method (Lowry *et al*., 1951) has been widely used for the estimation of proteins owing to its high sensitivity and simplicity. The colour formed in this method has been attributed to the reduction of the Folin reagent by the copper complexes formed by peptides and proteins and the tyrosine and tryptophan residues therein (Layne, 1957). Specific peptide bonds and favourable steric factors (Wu, *et al.*, 1978) have also been invoked to explain colour formation. The less sensitive

Abbreviations: DABA, α,γ, -diaminobutyric acid; Thr, threonine; Phe, phenylalanine; Leu, leucine; Val-valine; Orn, ornithine; Pro, proline. Unless otherwise mentioned configuration of amino acids is of the L-type.

biuret method (Gornall *et al.*, 1949) also finds use in the estimation of proteins and the colour formed in this method is the result of copper complexation with proteins. A variety of substances are known to interfere in the Folin-Lowry (Zondag and Boetzlaer, 1960; Diamont *et al.,* 1967; Reider, 1961; Vallejo and Lagunas, 1970; Chou and Goldstein, 1960; Ramachandran and Fraenkel-Conrat, 1958) and biuret (Parvin *et al.*, 1965; Pandey *et al.*, 1961) reactions. Polymyxin B is a basic peptide antibiotic which can be determined by the Folin-Lowry and biuret methods. However, some of the formyl polymyxins are not as chromogenic as polymyxin B. Difficulties encountered earlier (Srinivasa and Ramachandran, 1979) in the determination of formyl derivatives of polymyxin B had been avoided by deformylation of the derivatives prior to analysis.

We present in this paper data on the role of free amino groups of polymyxin B in complex formation with copper ions and the importance of such complexes in the formation of colour in the Folin-Lowry method. The role of free amino groups of proteins like bovine serum albumin and lysozyme in the reduction of Folin-Lowry reagent is also discussed based on data on chromogenicity of methylated and formylated derivatives of the two proteins.

Materials and methods

Polymyxin B sulphate with biolotical activity of 7531 IU/mg (Calbiochem, Richmond, California, USA) was a gift from Dr. B. Witkop. Bovine serum albumin and lysozyme were commercial samples from Sigma Chemical Co., St. Louis, Missouri, USA. EM 49 was a gift from Squibb Institute for Medical Research, Princeton, New Jersey, USA. All other chemicals and reagents were from different commercial sources. Folin reagent (Folin and Ciocalteu, 1927) was prepared in the laboratory.

Amino groups were estimated by the ninhydrin method (Rosen, 1957). All colorimetric measurements were carried out using a Junior Coleman Spectrophotometer. A Toshniwal Spectrophotometer Type RL 02 or a Beckman DB Spectrophotometer was used for spectrophotometric measurements.

Partially formylated polymyxin B (PF-polymyxin B) was prepared as described earlier (Srinivasa and Ramachandran, 1978). The crude samples of PF-polymyxin B used had an average degree of substitution of 2.8 groups per mole. Characterized samples with discrete degrees of formylation were prepared as described earlier (Srinivasa and Ramachandran, 1979).

The experimental conditions used for the colour development in the two methods are the same as recommended in the original methods. While solutions of mono, di-, tri- and tetra-formyl polymyxins B were prepared in 0.1 M sodium acetate, penta-formyl polymyxin B was dissolved in aqueous methanol (80%).

Determination of copper complex formation by polymyxin B and its derivatives

The ability of polymyxin B and its formyl derivatives to complex with popper was determined by the modified continuous variation method (Vosburgh and Cooper, 1941), as follows. To 0.1 ml of polymyxin B sulphate (formyl polymyxin B) solution (0.3 mM), was added 0.025, 0.05, 0.1, 0.2, 0.3, 0.4 and 0.5 ml of copper sulphate solution (0.3 mM) containing sodium potassium tartrate (0.015%) to obtain molar ratios of 1:0.25; 1:0.5; 1:1; 1:2; 1:3; 1:4 and 1:5 of polymyxins B sulphate to copper sulphate. The volume was made upto 3 ml, with 0.1N NaOH. After 30 min of mixing, the absorbance was measured at 280 nm. The difference (▲) between the observed

absorbance and that obtained in the absence of polymyxin B was plotted against the ratio of the concentration of Cu(II) to total concentrations of Cu(II) and polymyxin B. The maximum in the graph indicated the molar ratio of the metal ion to ligand in the chelate formed. Similarly the chelating ability of polymyxin B or its formyl derivatives under a fixed limiting concentration of Cu(II) was determined by adding to 0.1 ml of copper sulphate solution (0.3 mM), 0.025, 0.05, 0.1, 0.2, 0.3, 0.4 and 0.5 ml of polymyxin B sulphate (formyl polymyxins B) solution (0.3 mM).

Formylation of bovine serum albumin and lysozyme

Bovine serum albumin (25 mg) was formylated (Sheehan and Yang, 1958) for 24 h at room temperature. Formylated bovine serum albumin thus obtained had 20 (of the original 62) amino groups still free. Reformylation of this derivative or formylation of bovine serum albumin at 50°C afforded derivatives still containing 15 free amino groups per mol. Masking of all amino groups in bovine serum albumin was not achieved. Formylated lysozyme was prepared similarly and found to contain 0.1 free amino group per mol.

Reductive methylation of bovine serum albumin

Bovine serum albumin dissolved in 1 ml of 0.25 M borax (pH 9.4) was methylated overnight (Means and Feeney, 1968) using formaldehyde (0.02 ml, 37-40%) and sodium borohydride (4 mg) in the cold overnight. The methylation of all amino groups was rendered possible on addition of additional amounts of sodium boro-hydride (4 mg) and formaldehyde (0.02 ml) and allowing the reaction mixture to stand in the cold for 6 h longer. The methylated bovine serum albumin showed a content of 0.5 free amino group per mol. Methylated lysozyme was prepared under the above experimental conditions and found to contain 0.9 free amino groups per mol.

Results and discussion

On an equal weight basis, polymyxin B and EM 49 (figure 1) which contained neither tyrosine nor tryptophan formed as much, or a little more, colour as lysozyme

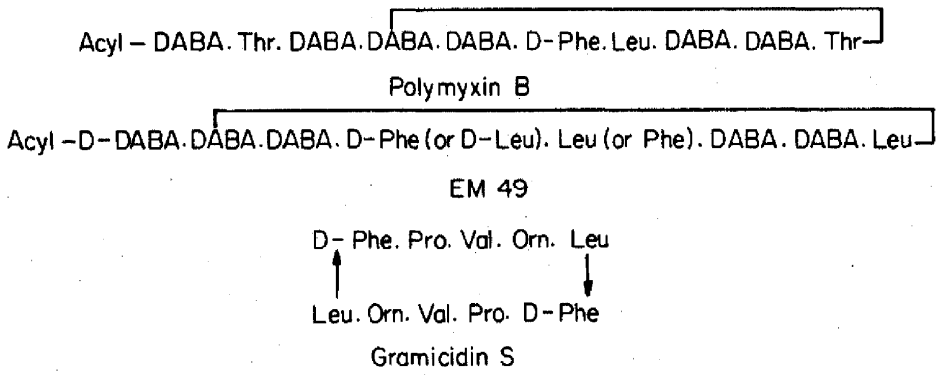

Figure 1. Structures of cyclic peptides.

in the Folin-Lowry and biuret methods (figures 2 and 3). Bovine serum albumin is

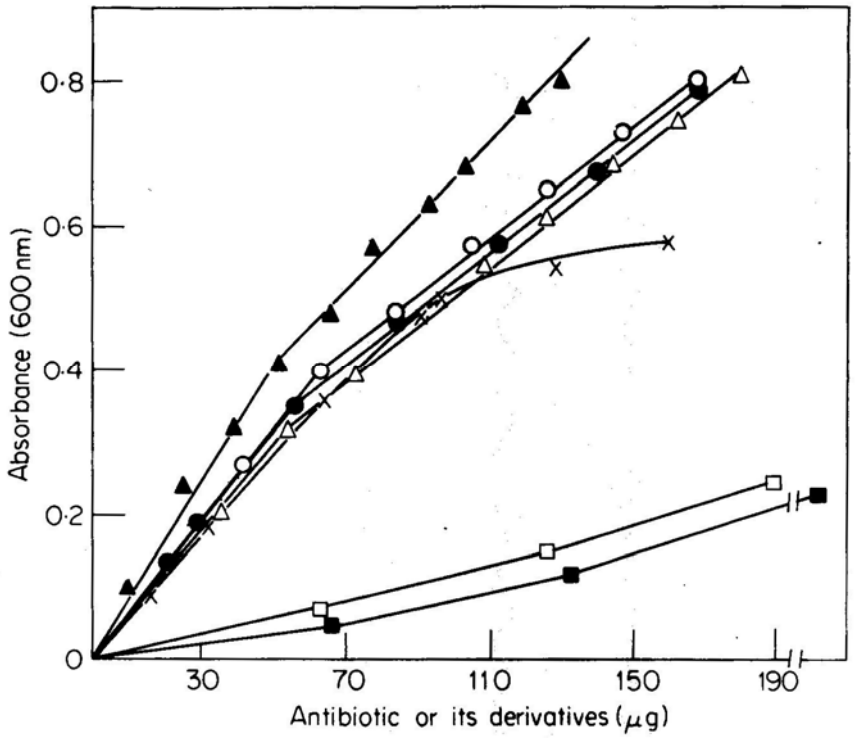

Figure 2. Chromogenicity of EM 49, polymyxin B and derivatives of polymyxin B in the Folin-Lowry method.
EM49, ▲; Polymyxin B,O; monoformyl polymyxin B, ●; diformyl polymyxin B, Δ triformyl polymyxin B, × ; tetraformyl polymyxin B, □; penta formyl polymyxin B, ■.

16% less chromogenic (figure 4) than polymyxin B and lysozyme in the Folin-Lowry method. Unlike proteins, polymyxin B and EM 49 formed little or no colour with the Folin reagent in the absence of alkaline copper reagent (reagent C). It is evident from this that the colour formed by polymyxin B and EM 49 in the Folin-Lowry method is due to the reduction of Folin reagent by the copper-complexes of polymyxin B or EM 49 formed on reaction with reagent C. Conversely, the cyclic peptide antibiotic gramicidin S (figure 1) having two free side chain amino groups of ornithine does not reduce Folin reagent either in the presence or absence of alkaline copper reagent (reagent C) in the Folin-Lowry method. This must necessarily mean that compositional and structural factors may play a significant role in the formation of colour in the Folin-Lowry method.

Crude formyl polymyxin B (a mixture of formyl polymyxins B)containing 2.2 free amino groups per mol formed only 75% of the colour given by polymyxin B. However, the mono-, di- and tri-fomyl polymyxins B were as efficient as polymyxin B in forming the chromogen. In contrast, the tetra- and penta-formyl polymyxins B

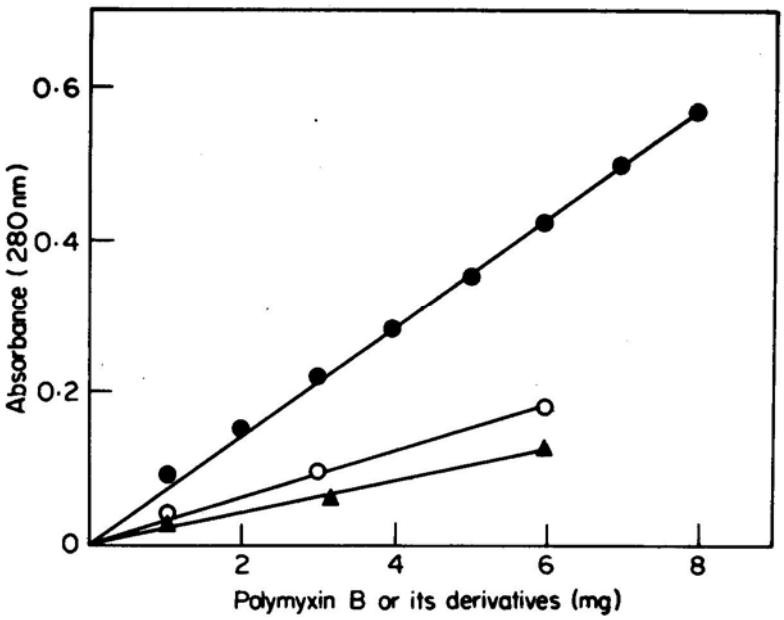

Figure 3. Comparison of the colour formed by polymyxin B and its formyl derivatives.in the biuret method.
Polymyxin B, mono-, di-and tri-formyl polymyxins B, ●; tetraformyl polymyxin B, O; penta-formyl polymyxin B, ▲.

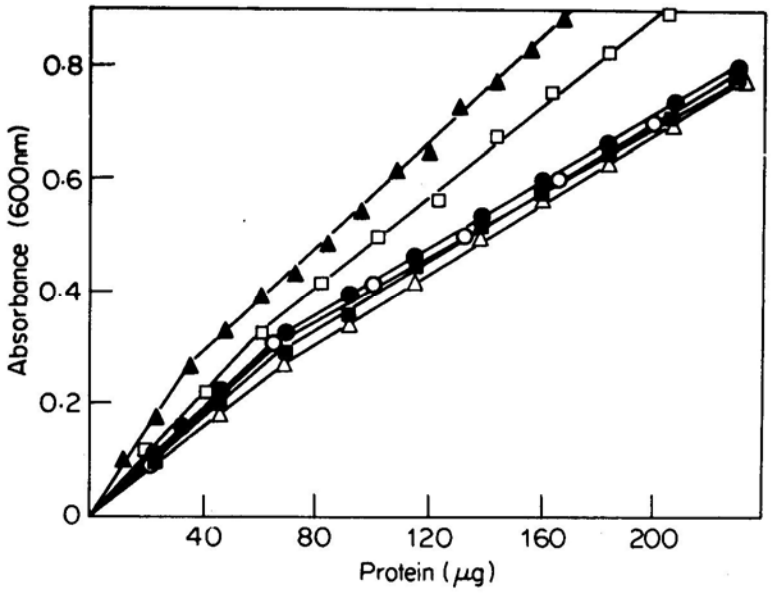

Figure 4. Colour concentration relationships for bovine serum albumin, lysozyme and their derivatives.
Lysozyme, ▲; bovine serum albumin, □; formylated lysozyme, O; methylated lysozyme, ●; formylated bovine serum albumin, M ■ methylated bovine serum albumin, Δ.

yielded one-fifth and one-sixth, respectively, of the colour expected. Unlike polymyxin B or it formyl derivatives with lower degree of substitution, the tetra-and penta-formyl derivatives formed only 40% and 30%, respectively, of the expected colour in the biuret method (figure 3).

It, therefore, appears from the above data that the colour formed in the Folin-Lowry and biuret methods by polymyxin B is largely due to amino groups and their ability to complex Cu(II). In those cases of peptides or proteins containing neither tyrosine nor tryptophan the overwhelming role of amino groups or the sites that can bind copper ions and of characteristic Cu(II) complexes formed by them in the formation of colour through reduction of the Folin reagent is easily recognised. A study (Berg. 1971) in which the N-acetyl derivatives of D-glucosamine and D-galacto-samine were found to form only 20% as much colour as the corresponding free amino sugar points to the importance of amino groups in the reaction given by amino sugars.

The modified continuous variation method of Job (Vosburgh and Cooper, 1941) revealed the correctness of the inference about the importance of amino groups since polymyxin B and mono-, di- and tri-formyl polymyxins B were found to complex with three Cu(II) under conditions of Cu(ll) excess, while the tetra- and penta-formyl polymyxins B complexed only one (figure 5). However, when Cu(II) was limiting polymyxin B and all its formyl derivatives complexed only one Cu(II)

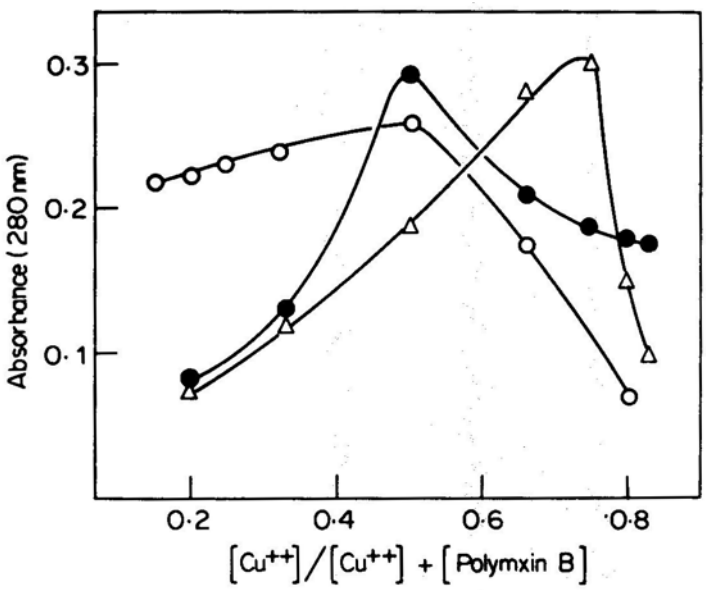

Figure 5. Modified job plot for complex formation of Cu(II) by polymyxin B and formyl polymyxins B.

The chelating ability of polymyxin B and its formyl derivatives under limiting concentration of Cu(II),●; the chelating ability of tetra and pentaformyl polymyxin B under concentrations of Cu(II) excess, O; copper complexing ability of polymyxin B and mono-, di-, and triformyl polymyxins B under concentrations of Cu(II) excess, Δ

(figure 5). The actual conditions of colour formation during routine analysis are such that the ratio of Cu(II): polymyxin B is always greater than 4:1. An earlier

investigation (Brintzinger, 1960) had noted the ability of polymyxin B sulphate to form 1:1 and 1:2 complexes with copper (II), at lower pHs than is realised in the experiments mentioned earlier.

Tyrosine, tryptophan and -SH groups of proteins are known to reduce Folin reagent even in the absence of alkaline copper reagent (reagent C). The colour formed is however enhanced three- to four-fold by reagent C. Modified bovine serum albumin and lysozyme which contain no free amino groups form almost 70-75% of the expected colour (figure 4). Thus, 25-30% of the colour formed in the method as normally used is assignable to free amino groups of the two proteins. It is clear that in such proteins additional sites which can complex Cu(II) possibly play an even greater role relative to free amino groups in colour formation. On the other hand, the hexosaminyl and sialyl residues in the oligosaccharide chains of the Armadillo salivary gland glycoprotein have been shown to exert a marked shielding effect in formation of colour by the protein in the Folin-Lowry reaction (Wu *et al.*, 1978). This glycoprotein contains only non-chromogenic amino acids—glycine, threonine, serine, glutamic acid, alanine and valine, and its higher reactivity with the Folin-Lowry reagent is attributed to both acid-sensitive and acid-resistant peptide linkages involving threonine and valine, since these two amino acids account for two-thirds of the total amino acids in the protein. The absence of proline and cysteine in this protein is considered as favouring better complex formation with copper ions. Therefore, it is to be concluded that compositional, structural and steric factors affect colour formation by peptides and proteins in the Folin-Lowry method. In the biuret reaction, methylation or formylation of the two proteins studied was not found to alter the chromogenicity.

All but the tetra- and penta-formyl polymyxins B are known to be fully biologically active (Srinivasa and Ramachandran, 1978). It is possible that the amino groups involved in complex formation with the extra copper ions under conditions of Cu(II) excess are the two side chain amino groups of residues 1 and 3 (Srinivasa and Ramachandran, 1980) involved in the biological action of triformyl polymyxin B. That some charged/uncharged (Feingold *et al*., 1974) amino groups of polymyxin B may interact with the acidic phospholipids to complex with the divalent cations such as Ca^{2+} and Mg^{2+} (Newton, 1955) which normally stabilise (Lieve, 1974) the outer membrane of Gram negative bacteria to destablize its recognized. It is conceivable that those amino groups of polymyxin B involved in this activity and in the binding of excess Cu(II) encountered in the 3:1 complexes are the same.

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