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Why Does Coomassie Brilliant Blue R Interact Differently with Different Proteins?

A PARTIAL ANSWER*

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Dimethyl sulfoxide was found to be effective for extraction of Coomassie Brilliant Blue R-250 (Coomassie R) from stained proteins on polyacrylamide gel slices. A good correlation was found between the ability of different proteins to bind Coomassie R and their capacity for interaction with Coomassie Brilliant Blue G-250 (Coomassie G) in solution. Scatchard analysis showed that the number of Coomassie R ligands bound to each protein molecule is approximately proportional to the number of positive charges on the protein, about 1.5–3 dye molecules/charge.

A large number of papers have appeared dealing with the quantitation of total protein concentration with Coomassie Blue compounds. The simplicity and sensitivity of the method and the variety of environments in which the determination can be carried out (in solution, on polyacrylamide gel, on cellulose acetate strips, etc.) encourage attempts to adapt it to different needs. At present, one important aspect is still unresolved: the variation of the response of different proteins with Coomassie G.

Contrary to the report of Bradford (1) who claimed similar responses for a series of proteins, Read and Northcote (2), in agreement with Pierce and Suelter (3) and Van Kley and Hale (4), reported significant differences. Seeking to minimize these variations, they established that either an increase of the dye concentration or a decrease of the phosphoric acid content of the reagent will enhance the color yield of the protein-Coomassie G complex. These modifications also bring the response curves of other proteins closer to that of the bovine serum albumin standard. Efforts invested in improving reaction conditions are undoubtedly justified, but there remains the problem of the different response curves of the various proteins. Is this due to the inherent properties of the protein itself, and does Coomassie R interact with proteins similar to Coomassie G?

These questions were investigated by means of Scatchard analysis of the binding of Coomassie R to the following proteins: cytochrome *c*, lysozyme, RNase A, trypsin, pepsin, and pepsinogen, as well as of gramicidin S and poly(L-lysine). The results indicate that a different number of dye molecules are bound to each of these proteins and that the values are dependent on their basic amino acid content. In fact, apart from poly(L-lysine), lysozyme and cytochrome *c* have the highest response curves with Coomassie G, as well as with Coomassie R, whereas pepsin has the lowest with both ligands; the curves of the other proteins fall in between.

MATERIALS AND METHODS[1]

RESULTS

In the absence of a satisfactory explanation for the variations in response to Coomassie G (2–4), in spite of the improvement achieved by Read and Northcote (2), it was of interest to compare the response of a series of proteins to Coomassie G and Coomassie R. Results are summarized in Fig. 1. The Coomassie G curves for cytochrome *c*, lysozyme, trypsin, RNase A, gelatin, and pepsin are in good agreement with the results of Read and Northcote (2). These proteins were chosen because of their wide range of response to Coomassie G. In a parallel experiment, the same proteins were subjected to electrophoresis on gels and stained with Coomassie R (all except gelatin were homogeneous in electrophoresis). The bound dye was extracted with dimethyl sulfoxide and its concentration determined spectrophotometrically. The results (Fig. 1*b*) are quite similar to those obtained for the Coomassie G (Fig. 1*a*). Each protein has its own specific response curve, cytochrome *c* being the steepest and pepsin the flattest. RNase and lysozyme are close to cytochrome *c*, while trypsin and gelatin are close to pepsin (although not in the same order as in the Coomassie G analysis). Moreover, the absolute absorbance of dimethyl sulfoxide-extracted Coomassie R associated with a given amount of protein is about double that obtained with Coomassie G. This increase is probably due to a solvent effect.[2]

An attempt was made using Scatchard plots to find the cause for the dissimilar response curves in Fig. 1 by determination of the number of Coomassie R molecules bound per molecule of protein. Constant amounts of cytochrome *c*, RNase A, lysozyme, trypsin, pepsin, and pepsinogen, as well as gramicidin S, were subjected to polyacrylamide gel electrophoresis and stained in a series of decreasing concentrations of Coomassie R solutions. The results summarized in Fig. 2 show that each molecule of lysozyme, cytochrome, and pepsin binds 48, 45, and 11 dye molecules, respectively. The other proteins showed intermediate values. Similar analysis of

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[1] Portions of this paper (including "Materials and Methods," Figs. 1–3, and Tables I and II) are presented in miniprint at the end of this paper. Miniprint is easily read with the aid of a standard magnifying glass. Full size photocopies are available from the Journal of Biological Chemistry, 9650 Rockville Pike, Bethesda, MD 20814. Request Document No. 84M-1968, cite the authors, and include a check or money order for $4.00 per set of photocopies. Full size photocopies are also included in the microfilm edition of the Journal that is available from Waverly Press.

[2] M. Tal, A. Silberstein, and E. Nusser, unpublished experiments.

gramicidin S showed that it binds 4.4 Coomassie R molecules. The highest concentration of the Coomassie R staining solution was 0.5 mg/ml. Above this concentration there is some cystallization of the dye during the staining period, even at 37 °C. The lowest concentration used, 0.025 mg/ml, proved the minimum possible, as after the staining period the solution outside the gel had a very faint color, while the protein band was loaded with dye. At this lowest concentration the point in the Scatchard plot always deviated downwards from linearity and was not used. No upward curvature like that mentioned by Nørby *et al.* (6) was observed. Our system does not pertain to different affinity sites for Coomassie R in a protein molecule but rather indicates the number of bound ligands and their average association constant.

There is a striking correlation between intensity of response to Coomassie dyes and the basicity of a protein which depends on the number of lysine, histidine, and arginine residues, and the NH_2-terminal amino group. The Coomassie R molecule has two sulfonic groups and three nitrogens, two of which are in all likelihood positively charged under the staining conditions. The electrostatic association between Coomassie R and a protein must involve the sulfonic groups and basic amino acids, rather than the positively charged nitrogens on the ligand with the carboxylates of the protein, since the latter are fully protonated in 14% trichloroacetic acid. Moreover, there is no correlation between the number of ligand molecules bound and aspartic and glutamic acid content. As the interaction of Coomassie R with the protein is enhanced by hydrophobic bonding (7), we feel that it is too much to expect perfect proportionality between the number of positive charges on a protein and Coomassie R molecules bound. The distribution of positive charges on the protein (separated as in trypsin or partially clustered as in cytochrome *c* (8)), in addition to the hydrophobicity of the amino acid residues adjoining the positively charged sites, probably affects the number of ligand molecules bound. The results depict in Table I show that in the proteins tested, about 1.5–3 molecules of Coomassie R are bound per each positive charge. The association constants (k_o) may also affect the number of bound dye molecules, albeit to a lesser extent, and the calculated number of Coomassie R molecules bound per M_r 10,000 of each protein are also given.

Our conjecture that Coomassie molecules are bound to basic amino acids in the protein is supported by additional findings. Poly-(L-lysine), as well as poly-(L-arginine), poly-(L-histidine), and poly-(L-ornithine) have the steepest response curves with Coomassie G and Coomassie R, while poly-(L-glutamic) acid has the flattest (Fig. 3, *a* and *b*). The positive, though very weak, effect in the latter case is probably due to the NH_2-terminal group. Analysis of binding of Coomassie R to poly-(L-lysine) by Scatchard plots is illustrated in Fig. 3*c*. The results show that in this highly charged polymer 3.6 Coomassie R molecules are bound per lysine.

The response of pepsinogen to Coomassie G and Coomassie R deviates significantly from that of pepsin (Figs. 1*a*, 1*b*, 2, and Table I); its NH_2-terminal 42-residue segment released during conversion to pepsin contains 13 positive charges. This positively charged polypeptide accounts for 13% of the molecular weight of pepsinogen, while its binding capacity is 136% $((26 - 11) \times 100)/11$ that of pepsin.

The fact that poly-(L-lysine) interacts more tightly than any other polypeptide with Coomassie R or Coomassie C may provide a means for determining the minimal size of a positively charged peptide able to react with Coomassie G. Partial tryptic digestion of poly-(L-lysine) results in accumulation of di-, tri-, and tetralysine (9). We have thus isolated the di, tri, and tetra compounds by paper chromatography. When used in quantities of 40, 50, and 16 μg, respectively, they failed to show any reaction with Coomassie G, while 3 μg of poly-(L-lysine) (Fig. 3*a*) sufficed for an intense reaction.

In an additional experiment, a series of short polypeptides was examined in the same context. Results are summarized in Table II. It is seen that L-carnosine does not react with Coomassie G at all. The polyamines, spermidine and spermine, and also streptomycin did not react with Coomassie G at the 100-μg level. Oxytocin, vasopressin, and bradykinin were just at the threshold of interaction with Coomassie G. By contrast, gramicidin S (with two positive charges and six hydrophobic residues) interacts effectively. It could be that here the hyrophobic interaction makes a major contribution. A noteworthy effect is observed when comparing the A and B chains of insulin; the A chain has a single positive charge, the terminal amino group, while the B chain has five. Coomassie G binding is poor in the first case but relatively strong in the second.

Although positive charges seem to determine the number of Coomassie R dye molecules bound, it is clear that electrostatic interactions do not in themselves suffice for tight binding. NaCl in concentration as high as 2.8 M at 37 °C over a period of 24 h does not liberate even a minute amount of bound Coomassie R ligand. The association of Coomassie G with proteins is not affected by 1 M salt (1), irrespective of whether the salt is added before or after Coomassie G reagent.

Finally, we address the question whether hydrophobic interactions alone will facilitate binding of Coomassie dye molecules to the polypeptide. For this purpose we chose two systems: poly-N^5-(2-hydroxyethyl)-L-glutamine, poly-N^5-(3-hydroxypropyl)-L-glutamine, and poly-N^5-4-hydroxybutyl)-L-glutamine (generously provided by Prof. N. Lotan) and the succinyl derivative of poly-(L-lysine). These polymers failed to react with Coomassie G to any detectable extent, in spite of the extended side chains which permit hydrophobic interactions to take place. Thus, hydrophobic interaction of the dye molecule with the polypeptide backbone adjoining the positively charged amino acid in the protein enhances the binding effect. In the absence of quantitative measurements of these hydrophobic bondings, we regard our results as a partial answer only to the question posed at the beginning of the paper. The association constants shown in Table I are probably related to both electrostatic and hydrophobic interactions.

DISCUSSION

Determination of the amount of protein-associated dye in a polyacrylamide gel permits correlation of the binding capacity of Coomassie R for a series of proteins and comparison with the absorbance of the same proteins in complexes with Coomassie G. The correlation is good, as demonstrated in Fig. 1, *a* and *b*, and in agreement with Read and Northcote (2). It may well be that the binding of Coomassie R and G to proteins is similar since both dyes differ only in a pair of methyl groups attached to the triphenylmethane skeleton.

Fazekas de St. Groth *et al.* (7) suggest that Coomassie dye molecules are bound to proteins by electrostatic attraction enhanced by hydrophobic bonding. Righetti and Chillemi (10) observed that Coomassie G caused aggregation of short polypeptides rich in lysine and arginine residues. They hypothesized that the dye binds to the basic groups via its $-SO_3^-$ groups. Their hypothesis is fully supported by our results. In fact, the same conclusion follows from the specific color yields of a series of proteins listed by Read and Northcote (2).

Highly relevant is a report on the binding of Cibacron, the

dye component of dextran blue (11). On the basis of spectrophotometric analysis, it was suggested that this dye interacts through its negatively charged sulfonic groups with positive charges on the protein.

Binding of Coomassie dyes to proteins may be usefully compared to that of sodium dodecyl sulfate (SDS[3]) as both ligands have negatively charged groups and hydrophobic moieties. A number of workers (12–16) observed that plasma albumin interacts with alkyl sulfates or sulfonates (mostly SDS) to form discrete complexes. In the pH range 5.0–9.0 detergent-induced conformational changes take place which increase the binding capacity of the protein. The maximum value of 110 ligands/molecule fits quite well with the total number of positive changes in serum albumin (12). On other hand, the interaction of any proteins with SDS micelles under conditions suitable for molecular weight estimation in gel electrophoresis (17) results in excess loading of the ligand by hydrophobic interaction.

We suggest that in the binding of Coomassie R by proteins, concurrently with the electrostatic interaction between the dye and basic amino acids, hydrophobic interactions with adjacent amino acid residues and the peptide backbone probably take place. The number of Coomassie R molecules bound is approximately proportional to the number of positively charged groups in the protein. Why the number of bound molecules/positive charge varies from approximately 1.5 to 3 is unclear. The simplest explanation is that in addition to the first dye molecule bound to a positive charge, at most two more may be hydrophobically bound on either side of it. Factors which determine whether additional molecules are bound may include the spacing of positive charges and the hydrophobicity of the regions near the basic amino acid residues.

The lowest binding value of Coomassie R/positive charge (1.4) was observed with trypsin (Table I), in which the basic amino acids are fairly widely separated (8). After a longer staining period (48 h), the extent of binding remained the same. It may be concluded that our staining conditions suffice for maximal binding and that the different binding capacities observed are intrinsic properties of the proteins. Racusen measured the stoichiometry of Amido Black binding by proteins (18) and found nearly identical molar ratios of sorbed dye to basic amino acids, *viz.* 0.5 molecule of Amido Black/cationic site. The interaction was believed to be electrostatic.

The minimal size of peptide capable of interaction is somewhere between penta- and nonapeptides. These conclusions agree with the data of Righetti and Chillemi (10), who detected slight Coomassie G-dependent aggregation of octapeptides, no interaction with nonapeptides, and pronounced interaction with tetradecapeptides.

We agree with van Kley and Hale (4) that caution should be exercised in using the Coomassie method. Pending development of a better reagent, the best that can be done is to choose the most appropriate standard for protein determination. The amino acid compositions of more than 200 proteins (19) indicate that in most of them the basic amino acid content ranges from 10–17 mol % with a sharp maximum at 13 mol %. The commonly used standard, bovine serum albumin, has basic amino acid content of 16.5 mol % and gives underestimated values of mass for most of the proteins listed by Reeck (19). The optimal choice for the Coomassie G method seems to be egg-white lysozyme, with basic amino acid content of 13.2 mol %.

[3] The abbreviations used are: SDS, sodium dodecyl sulfate; DMSO, dimethyl sulfoxide; TEMED, *N,N,N′,N′*-tetramethylethylenediamine.

REFERENCES

1. Bradford, M. M. (1976) *Anal. Biochem.* **72,** 248–254
2. Read, S. M., and Northcote, D. H. (1981) *Anal. Biochem.* **116,** 53–64
3. Pierce, J., and Suelter, C. H. (1977) *Anal. Biochem.* **81,** 478–480
4. Van Kley, H., and Hale, S. M. (1977) *Anal. Biochem.* **81,** 485–487
5. Geyl, D., Bock, A., and Isono, K. (1981) *Mol. Gen. Genet.* **181,** 309–312
6. Nørby, J. G., Ottolenghi, P., and Jensen, J. (1980) *Anal. Biochem.* **102,** 318–320
7. Fazekas de St. Groth, S., Webster, R. G., and Datyner, A. (1963) *Biochim. Biophys. Acta* **71,** 377–391
8. Dayhoff, M. O. (1969) *Atlas of Protein Sequence and Structure,* Vol. 4, Section D, National Biochemical Research Foundation, Silver Spring, MD
9. Waley, S. G., and Watson, J. (1953) *Biochem. J.* **55,** 328–337
10. Righetti, P. G., and Chillemi, F. (1978) *J. Chromatogr.* **157,** 243–251
11. Subramanian, S. (1982) *Arch. Biochem. Biophys.* **216,** 116–125
12. Putnam, F. W., and Neurath, H. (1945) *J. Biol. Chem.* **195,** 195–209
13. Foster, J. F., and Aoki, K. (1958) *J. Am. Chem. Soc.* **80,** 5215–5219
14. Aoki, K. (1958) *J. Am. Chem. Soc.* **80,** 4904–4909
15. Deker, R. V., and Foster, J. F. (1966) *Biochemistry* **5,** 1242–1254
16. Reynolds, J. A., Herbert, S., Polet, H., and Steinhardt, J. (1967) *Biochemistry* **6,** 937–947
17. Weber, K., and Osborn, M. (1966) *J. Biol. Chem.* **244,** 4406–4412
18. Racusen, D. (1973) *Anal. Biochem.* **52,** 96–101
19. Reeck, G. (1976) in *CRC Handbook of Biochemistry and Molecular Biology, Proteins* (Fasman, G. D., ed) 3rd Ed., Vol. III, pp. 504–511, CRC Press, Cleveland, OH
20. Haschmeyer, R. H., and Haschemeyer, A. E. V. (1983) *Proteins,* pp. 254–294, Wiley-Interscience, New York

WHY DOES COOMASSIE BRILLIANT BLUE R INTERACT DIFFERENTLY WITH DIFFERENT PROTEINS?

(A partial answer).

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MATERIALS AND METHODS

Materials - Coomassie Brilliant Blue G-250 minimum purity 91% and Coomassie Brilliant Blue R-250 purity 80% were obtained from Serva as "Serva Blau G" and "Serva Blau R", respectively: DMSO, acrylamide, TEMED, ammonium persulfate and urea were purchased from Merck; Bis-Tris from Sigma; EDTA from Fluka. The following proteins and peptides were purchased from Sigma: Cytochrome c, RNAse A, egg-white lysozyme. Trypsin, pepsin, pepsinogen were from Worthington. Gelatin was from Difco. The following peptides and polypeptides were also purchased from Sigma: L-carnosine, bradykinin (triacetate salt), vasopressin, oxytocin, gramicidin S, insulin A Chain (oxidized form), insulin B Chain (oxidized form), poly-(L-histidine) and poly (L-arginine). Poly (L-ornithine) and poly (L-glumatic) acid were obtained from the Weizmann Institute.

Polyacrylamide gel electrophoresis and staining - Disc gel electrophoresis was carried out in 6 mm tubes (5). Urea solution was passed through a mixed-bed ion exchanger. Staining of the gel was carried out with 0.05% Coomassie R solution in 14% w/v trichloracetic acid. The dye solution was freshly prepared from 1% w/v ethanolic solution of Coomassie R and trichloroacetic acid. The volume ratio between the dye solution and the gel was kept constant - 4 ml of Coomassie R solution to 1 ml of gel. Staining took place in screw-cap test tubes kept in a horizontal position and midly shaken at 37°C for 18 h. Destaining was carried out with 7% v/v acetic + 5% v/v methanol at room temperature.

DMSO extraction of Coomassie R - A segment of the gel 12 mm in length (vol 0.3 ml), containing the band of stained protein was cut out and immersed in 0.7 ml DMSO. Extraction was carried out at 37°C for 18 h with mild shaking. The concentration of the eluted Coomassie R was determined spectrophotometrically at 595 nm in a Zeiss spectrophotometer Model M4 QIII. The background value of about 0.010-0.020 was determined for every gel and subtracted from the readings.

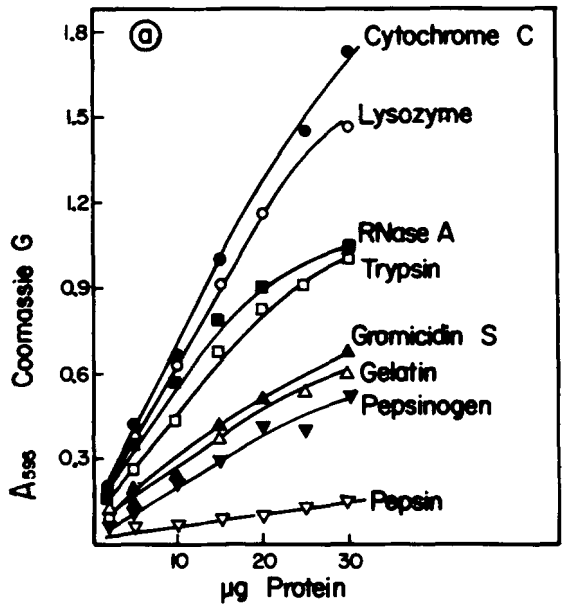

Fig. 1a. Response of proteins to Coomassie G. 1 ml Coomassie G reagent solution #1 (2) was added to protein in 0.1 ml. The reagent contains 0.1% w/v Serva Blue G in 1.6 M phosphoric acid and 0.8 M ethanol, and is filtered twice through Whatman filter paper #1 and #3. Absorbance was recorded on a Zeiss spectrophotometer at 595 nm.

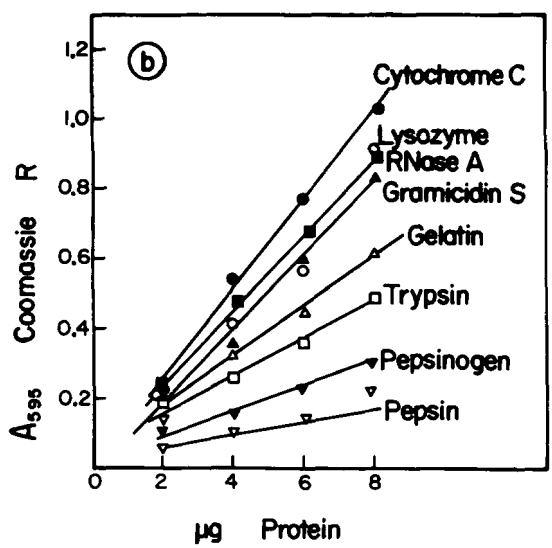

Fig. 1b. DMSO extraction of Coomassie R bound by various proteins. Proteins were subjected to electrophoresis on polyacrylamide gels in 6 mm tubes. Staining with Coomassie R, destaining and DMSO extraction were as described under Materials and Methods. Absorbance readings are shown.

TABLE I. INTERACTION OF COOMASSIE R WITH DIFFERENT PROTEINS.

	POSITIVE CHARGES / molecule	COOMASSIE R / molecule	COOMASSIE R / charge	COOMASSIE R / 10.000 M_r	ASSOCIATION CONSTANT K_D (μM^{-1})
Gramicidin S	2	4.4	2.2	35.3	0.009
Pepsin	5	11	2.2	3.6	0.013
Pepsinogen	18	26	1.4	6.4	0.027
Trypsin	20	29	1.4	19.2	0.012
RNase	19	47	2.5	34.7	0.008
Cytochrome C	27	45	1.7	33.8	0.012
Lysozyme	18	48	2.7	27.9	0.028

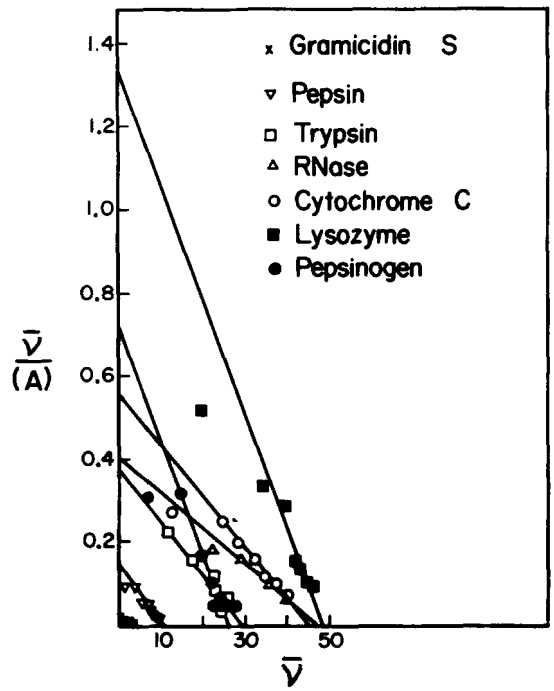

Fig. 2. Scatchard analysis of the binding of Coomassie R to proteins. Each protein in a constant amount was subjected to electrophoresis in polyacrylamide gel in 1 ml volume, and stained in 4 ml of Coomassie R solution in 14% w/v trichloroacetic acid at decreasing concentrations of dye: 0.5, 0.4, 0.3, 0.2, 0.15, 0.05 and 0.025 mg/ml in screw-cap test tubes, shaken mildly at 37°C for 24 h. Destaining and dye extraction steps are as described under Materials and Methods. (A) - concentration of the free dye after staining. A - DMSO extracted bound dye. $\bar{\nu}$ - ratio of A to the protein. All values are expressed as pmol. On plotting $\bar{\nu}/(A)$ against $\bar{\nu}$, a linear dependence was obtained (except for one or two of the more diluted dye solutions). Extrapolation gave the number of dye molecules (n) bound per protein molecule and the product ($k_o n$) where k_o is the association constant for the dye and the protein (20).

TABLE II. INTERACTION OF COOMASSIE G WITH DIFFERENT PEPTIDES

	AMINO-ACID RESIDUES / molecule	POSITIVE CHARGES / molecule	A_{595} COOMASSIE G BOUND / 10 μg polypeptide
L-Carnosine	2	2	0.003
Oxytocin	9	1	0.100
Vasopressin	9	2	0.060
Bradykinin	9	3	0.060
Gramicidin S	10	2	0.260
Insulin A chain	19	1	0.012
Insulin B chain	30	5	0.430

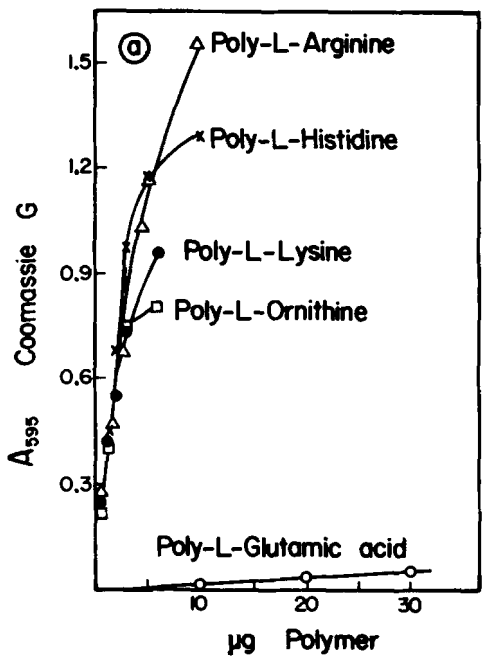

Fig. 3a Response of homopolymers to Coomassie G. Reaction conditions as described in Fig. 1a.

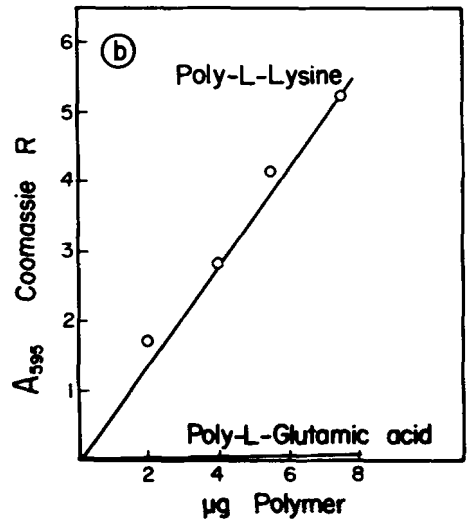

Fig. 3b. DMSO extraction of Coomassie R bound by two homopolymers. Reaction conditions are as described in Fig. 1b. The Coomassie R bound to 100 μg poly-(L-glutamic acid) gave an absorbance value of 0.052. Absorbance in the range of 2-8 μg poly-(L-glutamic acid) in this experiment is an interpolation from the 100 μg value.

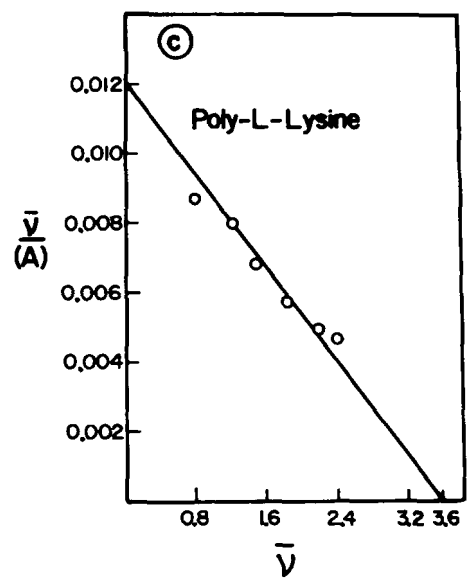

Fig. 3c. Scatchard plot for poly-(L-lysine). Experimental details are as described in Fig.2 The abscissa depicts the number of dye molecules per lysine residue.