FURTHER STUDIES ON THE NEW COOMASSIE BRILLIANT BLUE G-250 PROTEIN ASSAY

by

JOHN DANIEL STOOPS

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Approved by:

Delbert D. Mueller

Delbert D. Mueller, Ph.D.
Major Professor

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TABLE OF CONTENTS

Page

Acknowledgment ii

List of Figures and Tables iii

Introduction 1

Materials 8

Preparation and Procedure 9

Results 12

Discussion 39

Recommendation 44

Bibliography. 45

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LIST OF FIGURES AND TABLES

Figure Page

1. Structure of Orange G 4

2. Structure of Coomassie Brilliant Blue G-250 6

3. Effect of pH on Absorption Spectra of 200 mg/L Dye 13

4. Effect of pH on 30 μg Lysozyme with Different Dye Concentrations. 16

5. Absorption Spectrum of 30 μg of Lysozyme with 300 mg/L Dye. . 17

6-7. Study of Color Development on Fresh Dye Reagents. 18

8-9. Study of Color Development on 19 Day Old Dye Reagents 19

10. Assay Stability Study 22

11. Lysozyme Standard Curves with Different Dye Concentrations . 23

12. Standard Curves of Different Proteins 27

13. Standard Curves of Trypsin and Modified Trypsin 28

14. Structure of Poly (ε-aminomethacrylyl-L-lysine) and Polyvinylpolypyrrolidone. 30

15. Standard Curve of Poly (ε-aminomethacrylyl-L-lysine) with 200 mg/L Dye. 31

16. Absorption Spectrum of Polyvinvypolypyrrolidone with 200 mg/L Dye. 32

17. Absorption Spectra of Dextran Sulfate with 200 mg/L Dye . . . 33

18. Absorption Spectrum of Tri-Lysine with 200 mg/L Dye 34

19. Assay Sensitivity versus Positive Charge Density on the Protein 37

Table | Page

I. Effect of pH on Different Dye Concentrations 14

II. Reagent Stability Study 21

III. Linear Regression of Standard Curve of Lysozyme Using Different Concentration of Dye 24

IV. Effects of a Few Amino Acids on the Coomassie Brilliant Blue G-250 Protein Assay Method 25

V. Effects of some Polymers on the Coomassie Brilliant Blue G-250 Protein Assay Method. 35

VI. The Effect of Positive Charge Density on Assay 36

Introduction

The determination of protein in a sample is very important to the researcher. A satisfactory assay is one that is sensitive yet free of interferences from substances commonly present in the extraction medium or in the material from which the protein is isolated.

Over the years different assays have been introduced. Among the more common ones are the Kjeldahl nitrogen determination, Biuret, Lowry, and the Udy dye binding method. This thesis is concerned with a new dye binding method which used coomassie brilliant blue G-250 which was introduced by Bradford (1976). Before discussing this technique, however, the well established methods will be discussed in regard to their relative advantages and disadvantages.

The Kjeldahl nitrogen determination (Chibnall et al, 1943; Harrow et al, 1968) has been used for many years and is the procedure by which all other protein assays are usually compared. This method involves digestion of the protein in concentrated sulfuric acid which produces ammonium sulfate from any nitrogen in the sample. In a protein the ammonia can arise from nitrogen in the peptide linkage, the N-terminal end, or any amino acid residue containing nitrogen in its side chain. The ammonia is freed by making the solution alkaline with NaOH and is distilled into standard HCl. Normally the ammonia is determined by titration of the excess HCl with standard NaOH. Alternatively, ammonia can be determined by a colorimetric procedure. The protein is calculated by multipling the N found by a number which depends on the amino acid composition of the protein. This procedure can not distinguish between protein N and non-protein N and the digestion must be complete or else low N values will be obtained. The time needed for this procedure is over two hours.

The biuret procedure (Gornall et al, 1949; Bailey, 1967; Harrow et al, 1962) determines protein by the color resulting from a complex between cupric ion and the peptide linkages in the protein. One disadvantage is that ammonium salts interfer with the reaction by forming cupric complexes. This assay takes 30 minutes for development time and can be used to determine between 1-10 mg of protein. There is a microbiuret procedure, known as the Benedict's reagent, which can be used to assay for 0.1-2.0 mg of protein.

The Lowry (Chou and Goldstein, 1960; Lowry et al, 1951; Harrow et al, 1962) which uses the Folin-Ciocalteu reagent, is currently the most sensitive protein assay used since it allows 1-100 μg of protein to be assayed. However there are many substances which interfer with this assay such as, EDTA, ethanol, glycerol, $MgCl_2$, mercaptoethanol, KCl, phenol, sucrose, NaCl, sodium dodecyl sulfate, Triton X-100 and Tris.[1] Unfortunately, many of these are commonly employed in cellular extraction media. The assay depends on the reduction of the phosphomolybdates and phosphotungstates in the reagent by tyrosine and tryptophan residues in the protein as well as some chelation between Cu^{2+} and peptide bonds, which results in a blue color and is measured spectrophotometrically. This method has some other disadvantages in that the color is not strictly proportional to concentration and the color varies with different proteins. It is however 100-fold more sensitive than the biuret, requires no digestion of protein and can be completed in about 40 minutes.

Finally, an assay which is widely used and depends upon dye binding was introduced by Udy (1954). The dye used is orange G which has the

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[1]This information was obtained from Bio-Radiations Bulletin 25, September 1977.

structure shown in Fig. 1. The dye binds to guanidyl, imidazole and amino groups of the protein at pH 2.2. Protein is determined by measuring the amount of free dye remaining after removal of the protein-dye complex.

Bradford (1976) showed that another dye could be used for quantitative protein assay in solution. Diezel et al (1972) originally introduced this dye for protein staining on polyacrylamide gels in the place of coomassie brilliant blue R-250, with generally improved results. The dye reagent as described by Bradford contains 100 mg of coomassie brilliant blue G-250 dissolved in 50 ml 95% ethanol to which 100 ml 85% (w/v) phosphoric is added. This solution is diluted to a final volume of one liter. The main advantages of the coomassie brilliant blue G-250 dye method is that it is as sensitive as the Lowry method yet is not interfered with by many of the common buffer ingredients as is the Lowry method. Furthermore, the assay only takes 20 minutes for development. A protein assay kit was introduced by Bio-Rad Laboratories which used Bradford's method in 1976. Furthermore in May 1976, Bradford applied for a patent on this dye reagent.[2]

Coomassie brilliant blue G-250 is a dye of the class triphenylmethane with the Colour Index Constitution number 42655. It is used to dye wool, nylon and silk fibers. It is also known as xylene brilliant cyanin G. In the Colour Index its generic name is C.I. acid blue 90. This dye was first synthesized in 1913 by M. Weiler along with its close chemical relative coomassie brilliant blue R-250. The dye is only slightly soluble in cold but soluble in hot water, both producing a bright blue colored solution. It is also soluble in ethanol giving a similar blue color. On

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[2] Chemical Abstracts Vol 87 (1977) p 195.

Fig. 1. Structure of orange G. This compound is used in the Udy dye-binding assay.

dissolving the dye in concentrated H_2SO_4 it is bright red which changes to orange-red on dilution. In an aqueous solution of NaOH it is violet in color. As can be seen coomassie brilliant blue G-250 exists in solution in at least two major color forms - red and blue. From our studies it is blue on binding with protein or at pH values of 1.1 or greater in the ethanolic - H_2PO_4 solution. The red color predominates at pH values of less than 1.0 when no protein is present. Clearly the color developed by coomassie brilliant blue G-250 depends on the pH of its solution.

The Colour Index defines acid dyes as water soluble anionic chromophores that are applied to nitrogenous fibers from acid or neutral baths. Attachment to the fiber is attributed at least partly to ionic bond formation between anionic groups in the dyes and cationic groups in the fiber. De St. Groth et al (1963) describes the interaction of coomassie brilliant blue R-250 with proteins as follows "in slightly acid media, the dye anion is electrostatically attracted to the $-NH_3+$ groups of the protein, and within this primary combination van der Waals forces hold the reactants together. The dye-protein complex is firm, although fully reversible by dilution under appropriate conditions of pH."

The color of the dye and its behavior in solution can be partially understood through its structure (Fig. 2). In the triarylmethane class the chromophore is the quinonoid grouping of >C=Ar=N (as in Baeyer's fushsonimine) or >C=Ar=0 (as in Baeyer's fushsone) where Ar is an aromatic ring. The chromogen is completed by adding two aryl groups to the methane carbon. The dye molecule is extended by the introduction of two or three auxochromes to the aromatic rings, usually para to the methane carbon. Finally acids are generated from the parent compounds by introduction of

Fig. 2. Structure of coomassie brilliant blue G-250.

sulfonic acid groups (Colour Index Vol. 4). These groups also make the dye more water soluble. The amino groups in this class of dyes are protonated in acid medium which results in a color change. In general they are soluble in alcohol (Srámek, 1977).

After making an assay solution as described by Bradford, but using Eastman Kodak Company instead of Sigma Chemical Company's coomassie brilliant blue G-250 dye, no blue color developed as expected when lysozyme solutions were added. This was surprising since the Eastman Kodak Company product was purchased because it was listed as 98% pure while the Sigma Chemical Company dye was only about 60% pure. After some experimentation it was discovered that if a drop of 1N NaOH were added, a blue color developed in the protein sample but not the blank. A blue color also developed when the sample was heated in a hot water bath but reverted upon cooling.

Consequently, it was decided to investigate the potential of this dye more fully than it had been dealt with in Bradford's report. An area that Bradford had studied was how protein effected the absorption spectrum of coomassie brilliant blue G-250 between 700 to 400 nm. Some areas that were apparently not investigated are: i) how pH effects the absorption spectrum, ii) the optimum amount of NaOH to add for maximum differential absorption in the presence of protien, iii) stability of the dye reagent, iv) what concentration of the dye gives the greatest sensitivity, and v) what characteristic of proteins produce the color development.

Materials

Compounds used in these studies were obtained from (Sigma Chemical Company) bovine serum albumin, bicine, and polyvinylpolypyrrolidone; (Worthington Biochemical Corporation) trypsin, lysozyme, and chymotrypsinogen; (Nutritional Biochemical Corporation) tetraglycine; (Aldrich Chemical Company, Inc.) phenylglyoxal monohydrate; while L-lysine dihydrochloride, L-phenylalanine, acetyl-L-histidine, and trilysine were from a variety of places. Poly-(ε-aminomethacrylyl-L-lysine) was from a previous synthesis in our laboratory (Kakuda et al, 1971). The Bio-Rad Protein assay kit was from Bio-Rad Laboratories. Phosphoric acid (85% w/v) was from Mallinckrodt Chemical Works or Fisher Scientific Company. Coomassie brilliant blue G-250 was from Eastman Kodak Company, Lot number C7B.

Instruments included a Cary 14R recording spectrometer, a Beckman Century SS pH meter with an Ingold combination pH electrode, a Matheson Scientific Company Super-mixer, and an electric timer from Precision Scientific Company.

Preparation and Procedure

Preparation of Dyes

The Bio-Rad diluted dye reagent was prepared as instructed by diluting 1 part dye reagent concentrate to 4 parts distilled water. Besides the Bio-Rad reagent four other dye reagents were made plus the one suggested by Bradford (100 mg/L). These additional reagents contained 200, 300, 400, or 500 mg coomassie brilliant blue G-250 dissolved in 50 mL absolute ethanol plus 100 mL 85% phosphoric acid which was diluted to one liter with distilled water.

Protein and Amino Acid Standard Solution

Standard stock solutions were made using between 1.0 and 2.0 mg/mL of protein or amino acid dissolved in 100 mM bicine buffer pH 8.2.

Assay

Usually 5 mL of reagent was added to varying amounts of protein contained in 0.1 mL of solution. However, when NaOH had been added to the reagent a volume of 5.3 mL was used. The absorptinn at 595 nm was read in a 1-cm standard cuvette after an appropriate development time. It was blanked against the reagent.

Absorption Spectra of Dye at Various pH Values

Spectra of the 100, 200, and 300 mg/L dye solutions as well as the Bio-Rad dye reagent were obtained after addition of 0, 0.1, 0.2, 0.3 mL of 3N NaOH or 0.2, 0.25, 0.3 mL of 6N NaOH to 5 mL of the respective reagents along with enough water to make a total volume of 5.3 mL. Absorption spectra of these solutions were recorded between 700 and 400 nm

using 1 cm cells blanked against distilled water. The pH was measured with an Ingold model 8735 3 mm O.D. combination electrode connected to a Beckman Century SS pH meter.

Effect of pH on Absorbance of Dye-Lysozyme Complex

With the 100, 200, and 300 mg/L dye and the Bio-Rad dye reagent either 0, 0.1, 0.2, 0.3 mL of 3N NaOH or 0.2, 0.25, 0.3 mL of 6N NaOH was added per 5 mL of dye and enough water to make the total addition 0.3 mL. 5.3 mL of the reagent was then added to 30 µg of lysozyme in 0.1 mL bicine buffer. The absorbance at 595 nm was measured against the appropriate dye blank and pH measured as before.

Stability of the Dye Reagent

The 100, 200, 300 mg/L dye and Bio-Rad reagent were prepared by adding the following quantities to 5 mL of each dye solution: Bio-Rad, 0.3 mL of water; 300 mg/L dye, 0.25 mL 3N NaOH and 0.05 mL water; the 200 mg/L and 100 mg/L, 0.3 mL 3N NaOH. This was done to give what was considered the optimum state of the dye as had been established from the absorbance versus pH studies. Both the absorption spectra and rate of color development were observed on alternate days for a total of 10 days and finally again when the dye was 19 days old. Absorption spectra of the dye reagents were obtained between 700-400 nm as described above. The color development of each protein-dye complex was followed at 595 nm for 30 minutes. This was done using 20 and 40 µg of lysozyme in 0.1 mL bicine buffer added to 5.3 mL of the dye reagent which was rapidly mixed and immediately put into a 1 cm cell and the absorbance recorded against a blank of the dye reagent without protein. The timing was started when the

reagent was fully added to the protein solution, which required approximately 20-30 seconds for addition from a pipet. Beginning with the second day and alternate days thereafter for 10 days, duplicate assays at 595 nm for 20 and 40 μg of lysozyme were obtained after 20 minutes of development time to estimate the reproducibility of the method and to better define the ageing process of the dye. The pH of each reagent and its protein complex were measured as before.

Standard Curves

Standard curves were made using various amounts of the proteins in a total sample size of 0.1 mL. To this was added 5.0 mL if no NaOH was present or 5.3 mL if NaOH was present in the dye reagent. Duplicate or triplicate readings were taken of the various quantities of proteins. Between 2-200 μg of protein were used for the standard curve. All samples were allowed to develop for 20 minutes before being read.

Standard Curve Stability

A quantity of 200 mg/L dye reagent was prepared with 0.3 mL 3N NaOH per 5 mL of dye reagent added directly to the stock solution. A standard curve was made using between 7.5-150 μg of lysozyme on the first day and again when the dye was one and two weeks old.

Assay of Substances Besides Protein Affecting Color Development

These assays were done the same way as with protein. The substance of interest was dissolved in 100 mM bicine pH 8.2, an aliquot was put into a test tube, diluted to a total volume of 0.1 mL, then 5.3 mL of 200 mg/L dye reagent with NaOH was added, and after 20 minutes for development a differential absorbance was read at 595 nm.

Results

It was observed that addition of sodium hydroxide changed the color of the dye reagent solution. Therefore, the absorption spectrum of the dye was observed for different pH values against a distilled water blank. In the absence of any added base the visible spectrum of the dye shows a maximum in the 460 nm range, a valley in the 585-590 region and another band at 650 nm. As the pH is raised with NaOH the intensity of the short wave length band decreases, the valley starts to blue shift, and the absorbance at 650 nm increases in intensity (Fig. 3). As more and more NaOH is added the 650 nm band develops a shoulder on the low wavelength side. This occurs at about pH 1.1 for the 200 mg/L dye solution. Furthermore, when this pH is reached the 650 nm band is now the major absorbance and the short wavelength band begins to blue shift. It shifts by 10-15 nm over the total pH range observed. The high wavelength absorbance also starts to blue shift as the shoulder develops at 595 nm. Over the course of the pH changes, two isosbestic points are noted. The first one is noticed during the time the short wavelength and 650 nm bands are decreasing and increasing, respectively. The second one is observed while the high wavelength absorbance is blue shifting. The first one, depending upon what dye concentration is being studied, falls in the 540-555 nm region and the second is in the 523-526 nm region (Table I).

The effect of pH on the differential absorbance of the protein-dye complex versus dye solution also was investigated using the dye solution without protein as the blank. In all cases the absorption of the sample over the blank increased with pH to a certain point and then rapidly decreased. The pH at which a maximum is observed depends on the dye

Fig. 3. Effect of pH on absorption spectra of the 200 mg/L Dye.

A. 0 mL NaOH, pH 0.868

B. 0.1 mL 3N NaOH, pH 0.923

C. 0.2 mL 3N NaOH, pH 1.002

D. 0.3 mL 3N NaOH, pH 1.041

E. 0.2 mL 6N NaOH, pH 1.151

F. 0.25 mL 6N NaOH, pH 1.215

G. 0.3 mL 6N NaOH, pH 1.269

(Between 670-550 nm the absorbance scale for solutions E-G is 1-2 instead of 0-1)

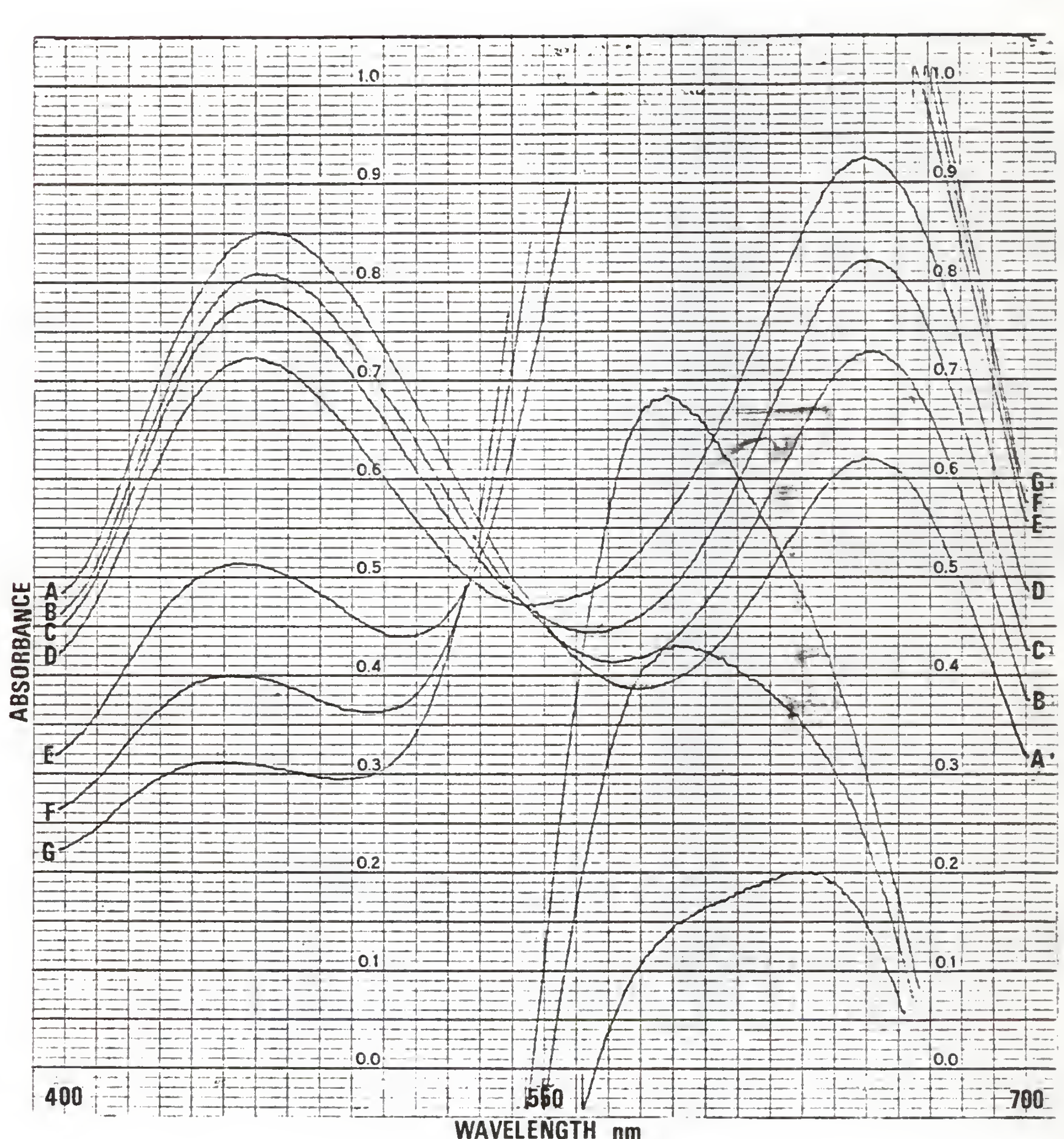

ABSORBANCE
WAVELENGTH nm
400
550
700
1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
A
B
C
D
E
F
G

Table I. Effect of pH on Different Dye Concentrations.

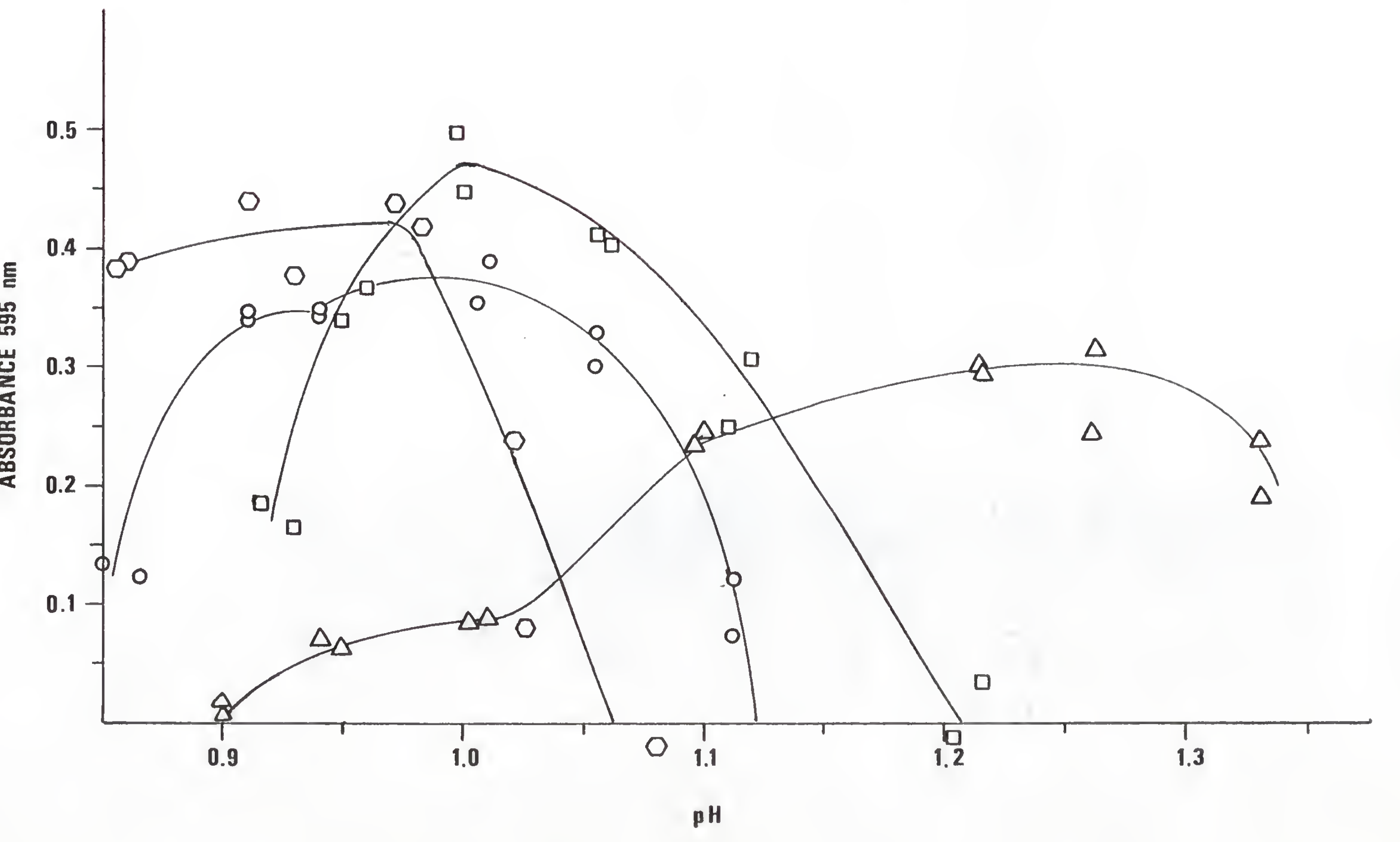
pH	High Wavelength Band nm	High Wavelength Band A	595 nm	Isosbestic point nm	Valley nm	Valley A	Low Wavelength Band nm	Low Wavelength Band A
				100 mg/L				
0.886	650	0.258	0.180	555	585	0.178	463	0.449
0.909	650	0.322	0.213	555	580	0.205	463	0.442
0.945	650	0.356	0.228	555	575	0.210	461	0.431
1.007	650	0.411	0.254	555	565	0.224	461	0.406
1.063	650	0.443	0.276	555	555	0.232	457	0.385
1.135	645	0.512	0.401	526	520	0.243	455	0.307
1.205	628	0.590	0.572	526	500	0.210	455	0.234
				200 mg/L				
0.868	650	0.620	0.402	--	580	0.388	463	0.850
0.923	652	0.728	0.452	544	571	0.412	461	0.808
1.002	652	0.821	0.509	544	566	0.444	460	0.781
1.041	650	0.923	0.591	544	544	0.471	458	0.723
1.151	630	1.200	1.155	525	506	0.440	455	0.513
1.215	591	1.430	1.429	525	497	0.363	453	0.399
1.269	588	1.682	1.672	525	486	0.295	449	0.311
				300 mg/L				
0.881	650	0.783	0.569	549	590	0.563	465	1.345
0.912	650	0.926	0.675	549	580	0.652	465	1.255
0.940	69	1.038	0.739	549	572	0.689	464	1.198
1.003	647	1.326	1.075	--	528	0.765	464	0.990
1.050	610	1.710	1.709	523	509	0.704	464	0.795
1.131	Off Scale			523	493	0.577	460	0.605
1.237	Off Scale			523	490	0.540	460	0.560
				Bio-Rad				
0.848	648	0.694	0.549	540	585	0.542	465	1.145
0.888	648	0.790	0.609	540	577	0.590	465	1.109
0.950	648	0.945	0.739	540	555	0.670	462	1.033
1.010	643	1.127	0.918	540	530	0.678	462	0.900
1.053	638	1.363	1.239	523	511	0.585	460	0.690
1.088	595	1.760	1.761	523	498	0.494	458	0.540
1.157		Off Scale		523	485	0.390	453	0.401

concentration and varied from 1.25 for the 100 mg/L dye solution to 0.97 for the 300 mg/L preparation (Fig. 4). The spectrum of the protein-dye complex versus distilled water (Fig. 5) also was recorded using 30 μg of lysozyme. It can be seen in Fig. 5 that addition of protein to the dye solution mimics pH increases in that it also produces the shoulder at 595 nm which results in the difference absorbance at this wavelength when blanked against dye reagent. It should be noted that in Fig. 5 no NaOH has been added.

Development time for a maximal differential absorbance of the dye-protein complex was investigated using four concentrations of dye with 20 and 40 μg of lysozyme. There was no apparent correlation between concentration of dye and development time for maximum absorption. The 100 mg/L solution required at least 30 min to attain maximum absorption while the absorption for the assay using 200 mg/L decreased over the entire 30 min observation period. The 300 mg/L and Bio-Rad reagents behaved more like the 100 mg/L preparations in that a maximum was reached after mixing. Furthermore, both the 300 mg/L and Bio-Rad dye reagent took longer to reach a maximum as the dye aged. Bio-Rad required at least 10 min in all cases. In some cases after reaching the maximum the absorption would decrease again within 30 min and in others would remain constant (Fig. 6-9). Within the 20 to 40 μg range no effects on development time from protein concentration were apparent.

The shelf-life of ready to use dye reagent was investigated. The 300 mg/L and Bio-Rad reagent had noticeable precipitation on the 9th day after preparation. To the contrary, no precipitation was discernible in the 100 and 200 mg/L solutions during the 19 days of the experiment. The

Fig. 4. Effect of pH on 30 μg lysozyme with different dye concentrations. A 30 μg sample of lysozyme was assayed with four dye concentrations with various amounts of NaOH, either 0, 0.1, 0.2, 0.3 mL 3N NaOH or 0.2, 0.25, 0.3 mL 6N NaOH. △ 100 mg/L dye, □ 200 mg/L dye, ○ 300 mg/L dye and ⬡ Bio-Rad Protein Assay reagent.

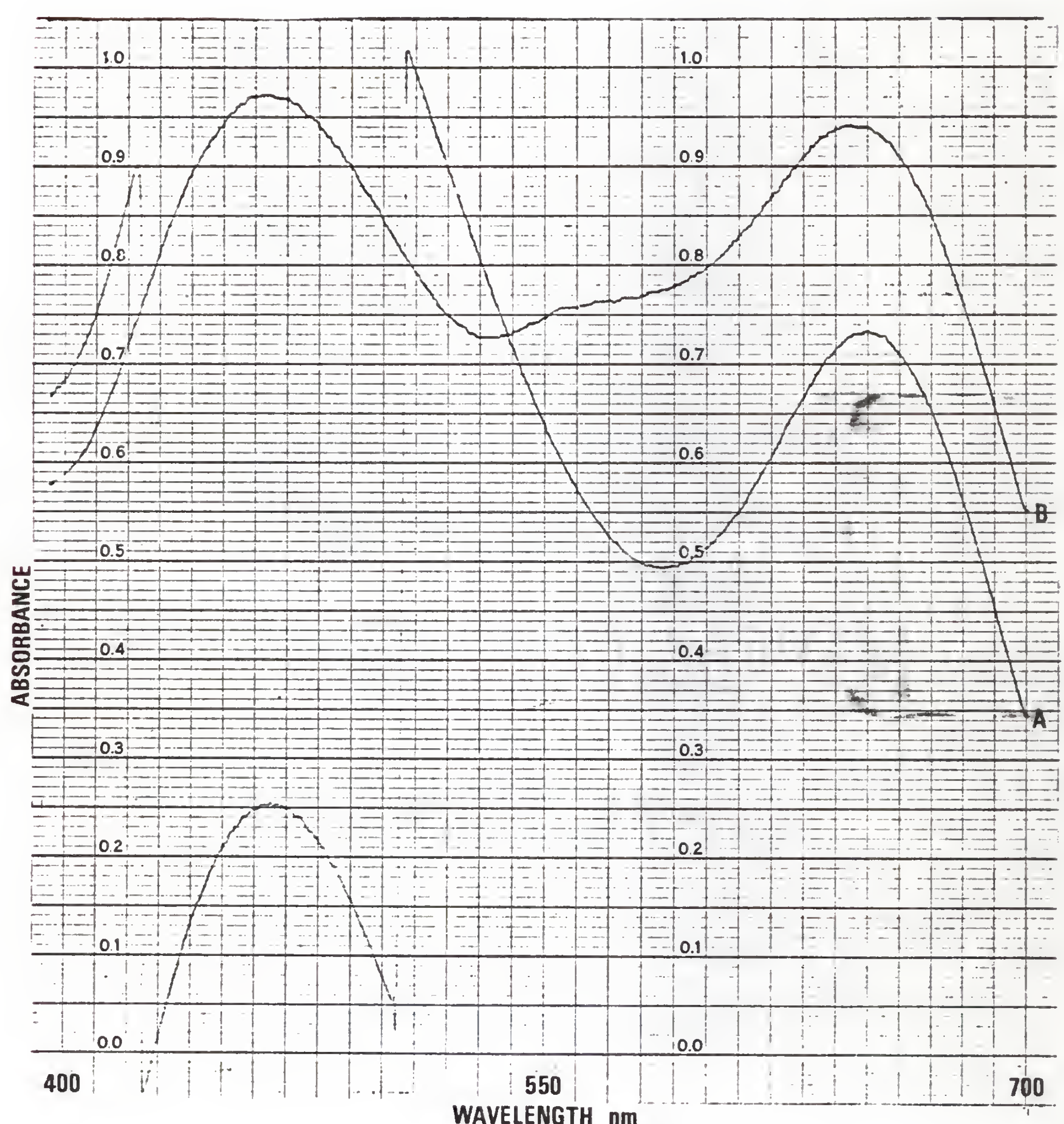


ABSORBANCE 595 nm
0.5
0.4
0.3
0.2
0.1
0.9
1.0
1.1
1.2
1.3
pH

Fig. 5. Absorption spectrum of 30 μg lysozyme with 300 mg/L dye.

A. 5 mL reagent with 0.3 mL H_2O plus 0.10 mL bicine pH 0.870

B. 5 mL reagent with 0.3 mL H_2O plus 0.07 mL bicine and 0.03 mL 1 mg/mL lysozyme, pH 0.855.



1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
ABSORBANCE
B
A
400
550
700
WAVELENGTH nm

Figs. 6 & 7. Study of color development on fresh dye reagents. Protein samples were ○ 20 and □ 40 μg of lysozyme in 0.1 mL.

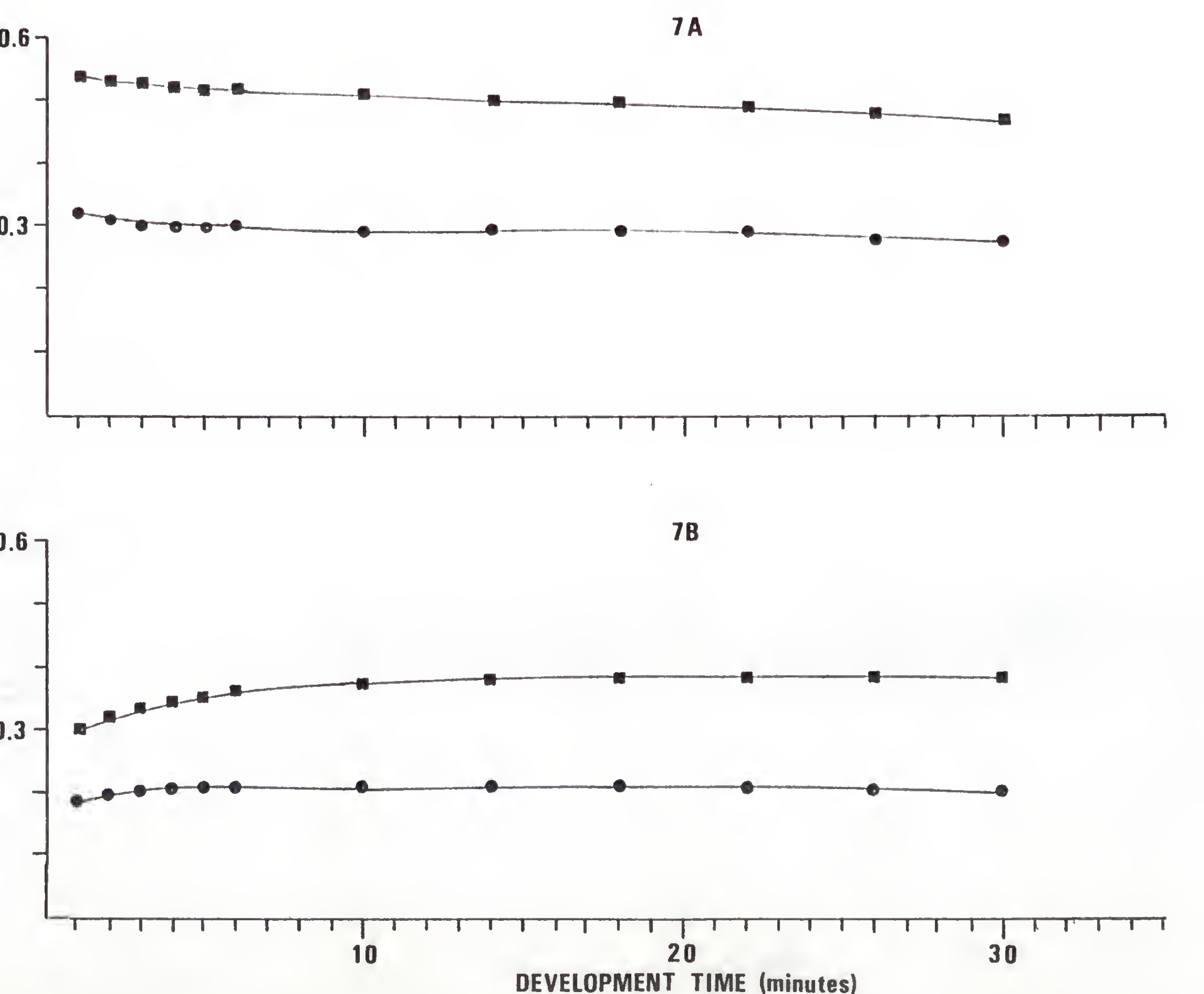
6A. 100 mg/L dye reagent with 0.3 mL 3N NaOH per 5 mL reagent.

6B. 200 mg/L dye reagent with 0.3 mL 3N NaOH per 5 mL reagent.

7A. 300 mg/L dye reagent with 0.25 mL 3N NaOH per 5 mL reagent.

7B. Bio-Rad reagent with 0.3 mL H_2O per 5 mL reagent.

6A
6B
0.6
0.3
ABSORBANCE 595 nm
10
20
30
DEVELOPMENT TIME (minutes)


7A
7B
0.6
0.3
ABSORBANCE 595 nm
10
20
30
DEVELOPMENT TIME (minutes)

Figs. 8 & 9. Study of color development on 19 day old dye reagents. Protein samples were ○ 20 and □ 40 μg of lysozyme in 0.1 mL.

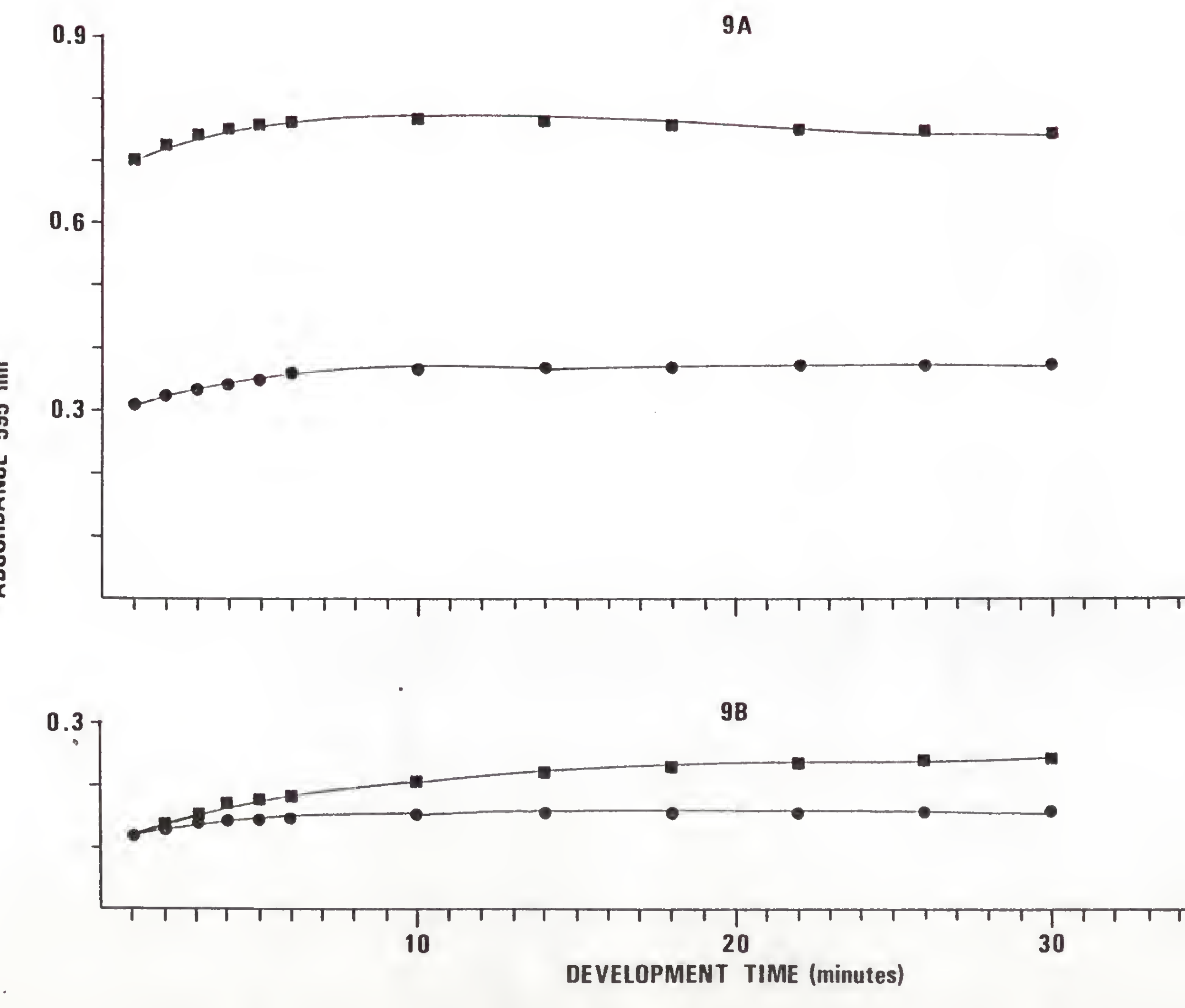
8A. 100 mg/L dye reagent with 0.3 mL 3N NaOH per 5 mL reagent.

8B. 200 mg/L dye reagent with 0.3 mL 3N NaOH per 5 mL reagent.

9A. 300 mg/L dye reagent with 0.25 mL 3N NaOH per 5 mL reagent.

9B. Bio-Rad reagent with 0.3 mL H_2O per 5 mL reagent.

8A
8B
0.6
0.3
10
20
30
DEVELOPMENT TIME (minutes)
ABSORBANCE 595 nm


9A
0.9
0.6
0.3
ABSORBANCE 595 nm
9B
0.3
10
20
30
DEVELOPMENT TIME (minutes)

100 mg/L dye was stable giving the same readings each day observed. The 200 mg/L and Bio-Rad gave random variation during the experiment time. The 300 mg/L increased in absorption as the dye aged (Fig. 10 and Table II).

The differential absorption of the dye-protein complex, however, is strongly dependent upon dye concentration. For this set of experiments the pH of each dye solution was adjusted to maximize ΔA at 595 nm. A standard curve ranging from 15 to 150 μg of lysozyme was prepared for each dye concentration 1 day after preparation. The standard curve for the 100 mg/L dye bends over rapidly with increasing concentration. With higher dye concentration the tendency for decreased sensitivity with high protein concentrations was much less pronounced (Fig. 11). The sensitivity of the 200 and 300 mg/L were quite similar, while on the other hand the 400 and 500 mg/L gave similar results. The ΔA (595 nm) measured for 75 μg of lysozyme are 0.41, 0.72, 0.63, 1.02 and 1.10 for 100 mg/L through 500 mg/L of dye, respectively. Linear regression analyses were performed on each curve from 0-75 μg/mL of protein and again for other ranges of protein concentrations. These results are summarized in Table III.

In addition, Bradford and Bio-Rad Laboratories found that fewer common biochemicals interfered with this method than with the Lowry method. Most notable among those severly affecting the Lowry method are Tris and the amino acids. Consequently, several amino acids also were examined by our procedure. These included L-(+)-lysinedihydrochloride, L-phenylalanine, and acetyl-L-histidine. No substantial interferences were detected except that all gave a slight, random negative differential absorbance (Table IV).

The sensitivity of the assay depends on the type of protein used. Standard curves were made for bovine serum albumin, lysozyme, chymotrypsinogen

Table II. Reagent Stability Study

Development time: 20 minutes

20 µg Lysozyme

Age of Dye Reagent (days)	100 mg/L	200 mg/L	300 mg/L	Bio-Rad
1	0.176	0.256	0.382	0.153
	0.160	0.256	0.327	0.159
3	0.170	0.253	0.400	0.180
	0.162	0.255	0.330	0.151
6	0.150	0.312	0.386	0.203
	0.146	0.283	0.370	0.123
8	0.160	0.276	0.431	0.186
	0.150	0.308	0.371	0.179
10	0.171	0.297	0.498	0.198
	0.158	0.273	0.399	0.189
		40 µg Lysozyme		
1	0.221	0.400	0.510	0.332
	0.223	0.455	0.523	0.353
3	0.233	0.419	0.510	0.283
	0.224	0.467	0.567	0.298
6	0.211	0.522	0.548	0.275
	0.204	0.528	0.585	0.272
8	0.213	0.468	0.582	0.330
	0.232	0.489	0.674	0.312
10	0.218	0.430	0.592	0.281
	0.224	0.484	0.931	0.292

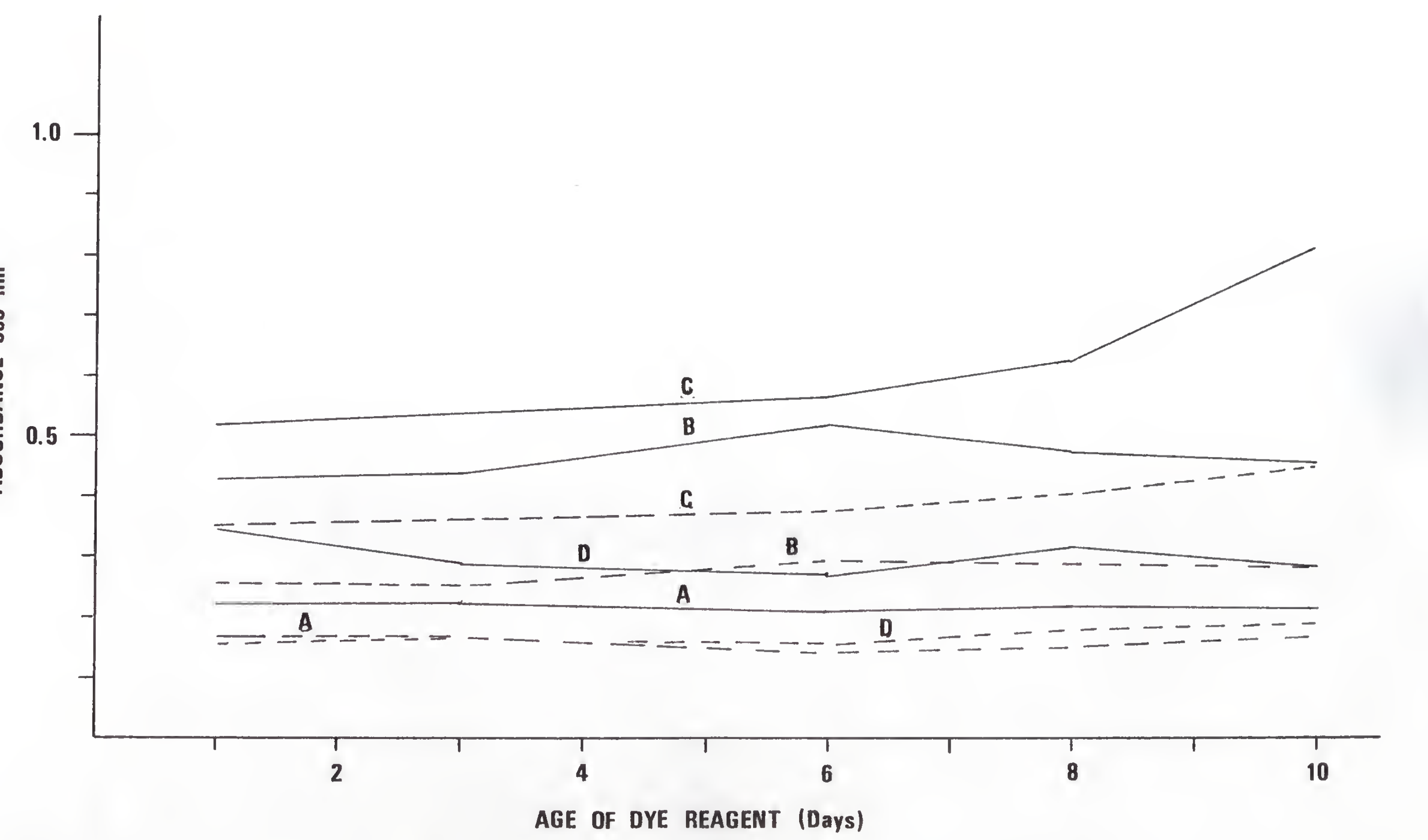
Fig. 10. Assay stability study. Samples contained ---- 20 and —— 40 μg of lysozyme in 0.1 mL.

A. 100 mg/L dye reagent with 0.3 mL 3N NaOH per 5 mL reagent.

B. 200 mg/L dye reagent with 0.3 mL 3N NaOH per 5 mL reagent.

C. 300 mg/L dye reagent with 0.25 mL 3N NaOH per 5 mL reagent.

D. Bio-Rad protein assay with 0.3 mL H_2O per 5 mL reagent.



ABSORBANCE 595 nm
1.0
0.5
C
B
C
D
B
A
A
D
2
4
6
8
10
AGE OF DYE REAGENT (Days)

Fig. 11. Lysozyme standard curves with different dye concentrations with between 15-150 µg of lysozyme in 0.1 mL.

△ 100 mg/L dye reagent with 0.3 mL 3N NaOH per 5 mL reagent, pH 1.105; □ 200 mg/L dye reagent with 0.3 mL 3N NaOH per 5 mL reagent, pH 1.108; ⬡ 300 mg/L dye reagent with 0.25 mL 3N NaOH per 5 mL reagent, pH 1.061; ◇ 400 mg/L dye reagent with 0.3 mL H_2O per 5 mL reagent, pH 0.940; and ○ 500 mg/L dye reagent with 0.3 mL H_2O per 5 mL reagent, pH 0.915.

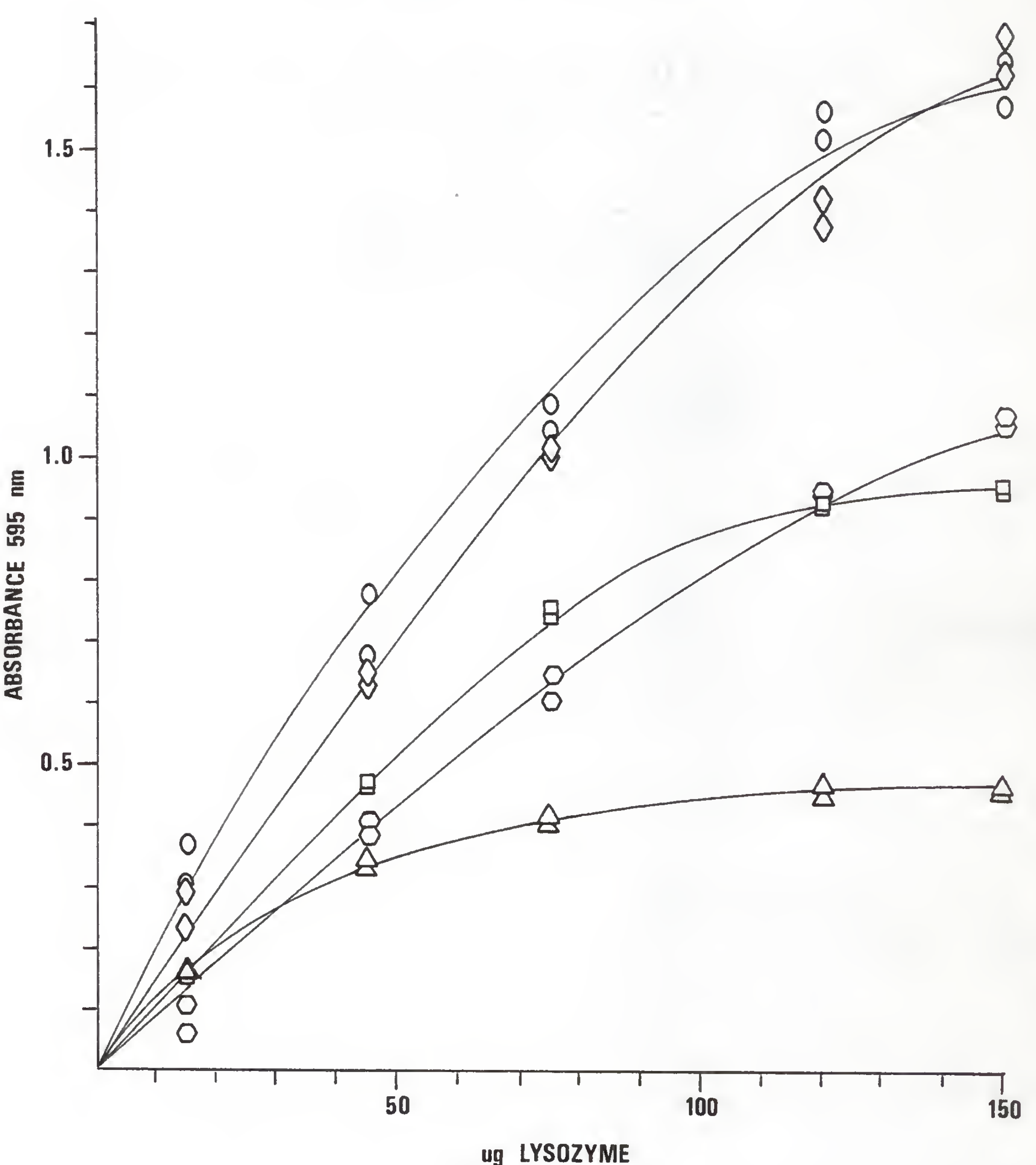

1.5
1.0
0.5
ABSORBANCE 595 nm
50
100
150
ug LYSOZYME

Table III Linear Regression of Standard Curve of Lysozyme Using Different Concentration of Dye.

	100 mg/L	200 mg/L	300 mg/L	400 mg/L	500 mg/L
			15 - 75 μg Lysozyme Range		
Y-intercept	0.129	0.014	-0.041	0.083	0.162
Slope	0.004	0.010	0.009	0.012	0.012
R	0.816	0.996	0.986	0.996	0.982
			15 - 150 μg Lysozyme Range		
Y-intercept	0.214	0.178	0.031	0.171	0.270
Slope	0.002	0.006	0.007	0.010	0.010
R	0.746	0.901	0.979	0.989	0.963

Table IV. Effects of a Few Amino Acids on the Coomassie Brilliant Blue G-250 Protein Assay Method.

μg A.A.	Δ Absorbance (595 nm)
L(+)-Lysine dihydrochloride	
20	-0.040
20	-0.030
60	-0.020
60	-0.040
100	-0.010
100	-0.016
L-Phenylalanine	
50	-0.036
50	-0.015
100	-0.019
100	-0.015
Acetyl-L-Histidine	
50	-0.020
50	-0.005
100	-0.025
100	-0.018

and trypsin using the 100 mg/L dye reagent and are shown in Fig. 12, which clearly illustrate the differences in sensitivity. Both Bradford (1976) and Bio-Rad Laboratories also noted differences among the proteins which they examined (about 5 and 20 proteins, respectively.)

Why should proteins give different analyses? One possibility suggested by the structure of the dye molecule is the amount of positive charge carried by the protein at pH 1.0. Previously it had been noticed (Reisner et al., 1975) that lysine-rich, arginine-poor histones on polyacrylamide gels are not visualized when stained with coomassie brilliant blue G-250 while arginine-rich histones were. This data suggests, of course, that it is not just positive charge but rather a preferential interaction with arginine residues which produce the required spectral changes. Analysis of trypsin by the coomassie brilliant blue G-250 also appeared to fit this pattern since its sensitivity is among the lowest of the proteins assayed and it has only two arginine residues per molecule. Therefore, it was decided to test this hypothesis more directly by chemically modifying these residues in trypsin using the phenylglyoxal method of Delarco and Liener (1973). Furthermore, the extent of reaction could be estimated from the fact that when one arginine residue is modified the enzymatic activity is reduced by 50% and when both have reacted it is zero. The reaction performed according to the procedure of Delarco and Liener gave a product which had only 12% the activity of native trypsin from which it was concluded that about 1.8 of the 2 residues had been modified. This modified trypsin, however gave greater sensitivity which would imply that coomassie brilliant blue G-250 dye does not interact preferentially with arginine residues and other factors must be involved (Fig. 13).

Fig. 12. Standard curves of different proteins. Each curve was made using between 2-10 μg of the respective protein. ⬡ Bovine serum albumin, □ lysozyme, △ chymotrypsinogen, and ○ trypsin. 5 ml dye reagent (100 mg/L), 0.1 ml protein, and 0.3 ml 3N NaOH pH 1.1. The line for each protein was calculated using a least square fit on the respective data.

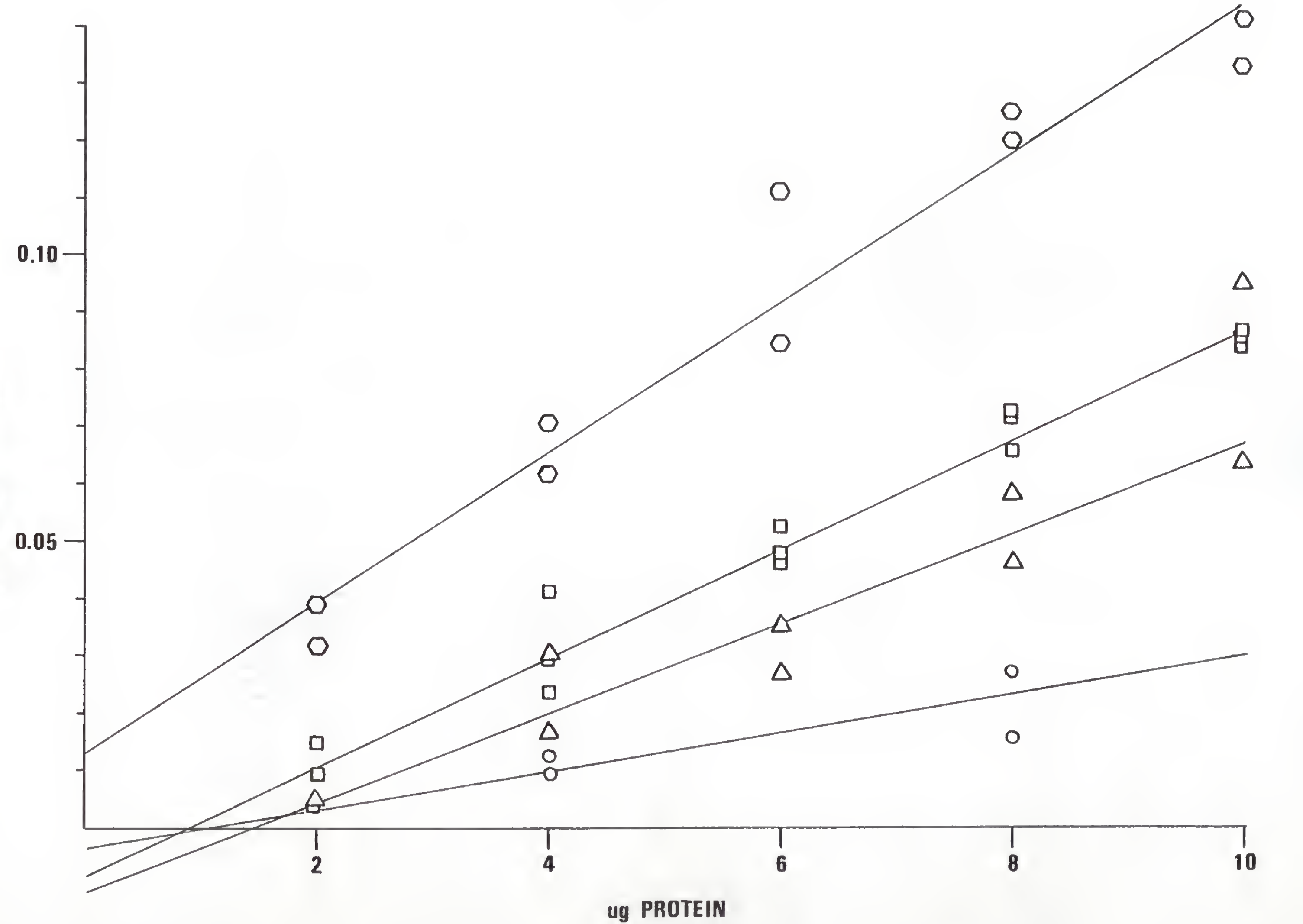
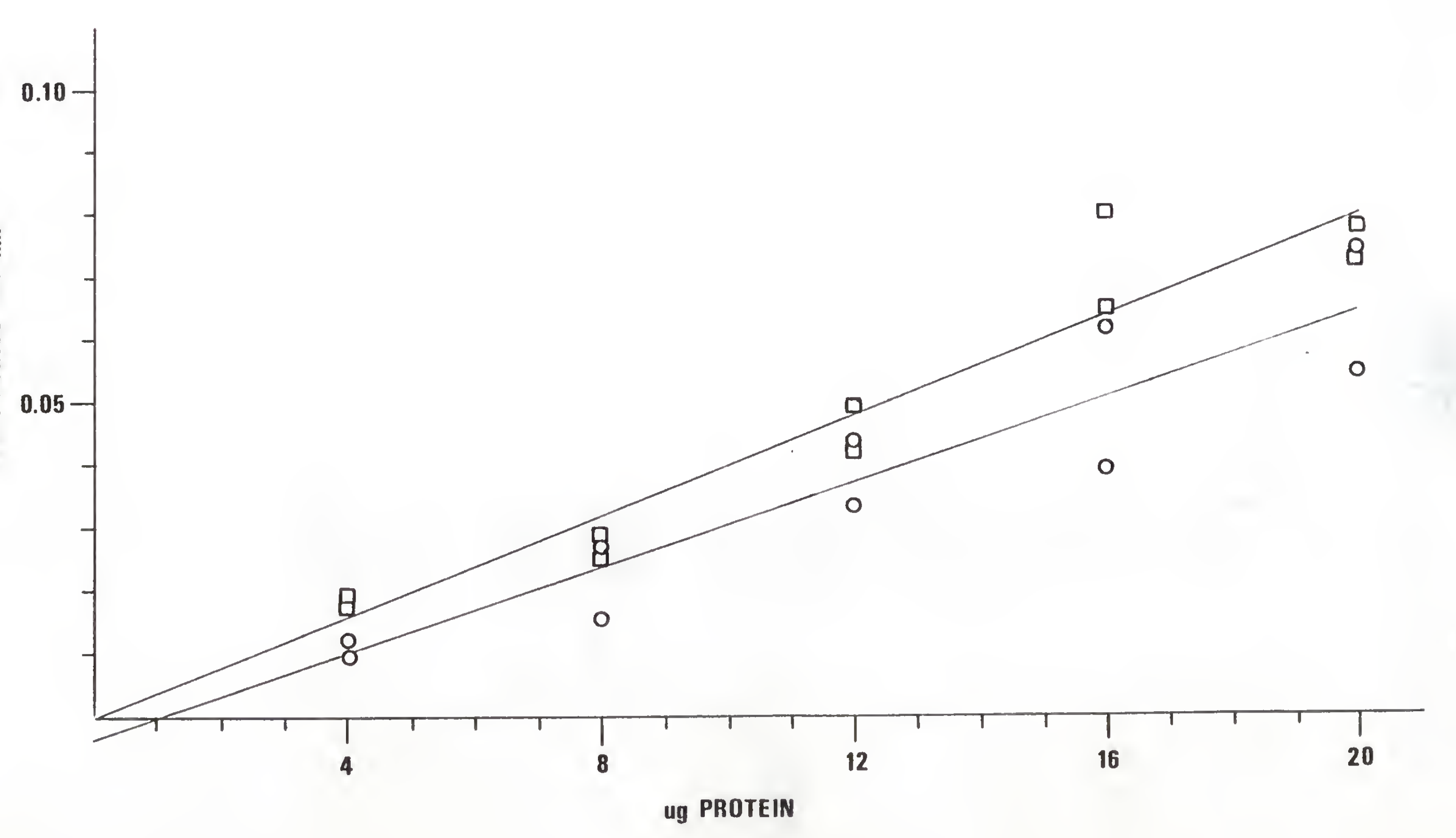

ABSORBANCE 595 nm
0.10
0.05
ug PROTEIN
2
4
6
8
10

Fig. 13. Standard curves of trypsin and modified trypsin. Each curve was made using between 4-20 μg of the respective protein. ○ Trypsin and □ modified trypsin. 5 mL dye reagent (100 mg/L), 0.1 mL protein and 0.3 mL 3N NaOH pH 1.1.


ABSORBANCE 595 nm
0.10
0.05
ug PROTEIN
4
8
12
16
20

Another possible factor might be the density of positive charge on the surface of the protein. Therefore, the sensitivity of the reagent toward PMAL [Poly-(ε -aminomethacrylyl-L-lysine)] was investigated. This linear polymer has a very high positive charge density at pH 1.0 since the carboxyl group has a pKa of 2.42 (Fig. 14). Indeed the reaction with the G-250 dye proved to be very sensitive (Fig. 15) and gives a linear response between 2.5-40 μg but little additional change at higher polymer concentration using the 200 mg/L dye reagent (Table V).

The possible role of amide groups in producing a ΔA (595) also was investigated using PVPP [polyvinylpolypyrrolidone] as a model polymeric amide. This linear polymer has a cyclic cis amide group in the side chains and in the presence of dye the visible spectrum shows an increase at 460 nm while the 650 nm absorbance decreases (Table V and Fig. 16). Therefore, it probably is not the amide group alone in a protein which produces a 595 nm shoulder. Finally, a negatively charge polymer was assayed (dextran sulfate) which resulted in a slight positive differential absorbance. This absorbance, however, decreased as the concentration wss increased (Fig. 17). Therefore, it appears that positive charge is essential for the production of ΔA (595).

However, does the reaction depend only on positive charges or are there other forces involved in the association? Consequently, L-lysyl-L-lysyl-L-lysine a highly positive charged tripeptide was assayed to see if a macromolecular environment was necessary to produce the shoulder at 595 nm. This tripeptide actually gave a small decrease in absorption at 595 nm and the spectrum of the dye-trilysine solution against distilled

Fig. 14. Structure of poly- (ϵ -aminomethacrylyl-L-lysine) and polyvinylpolypyrrolidone.

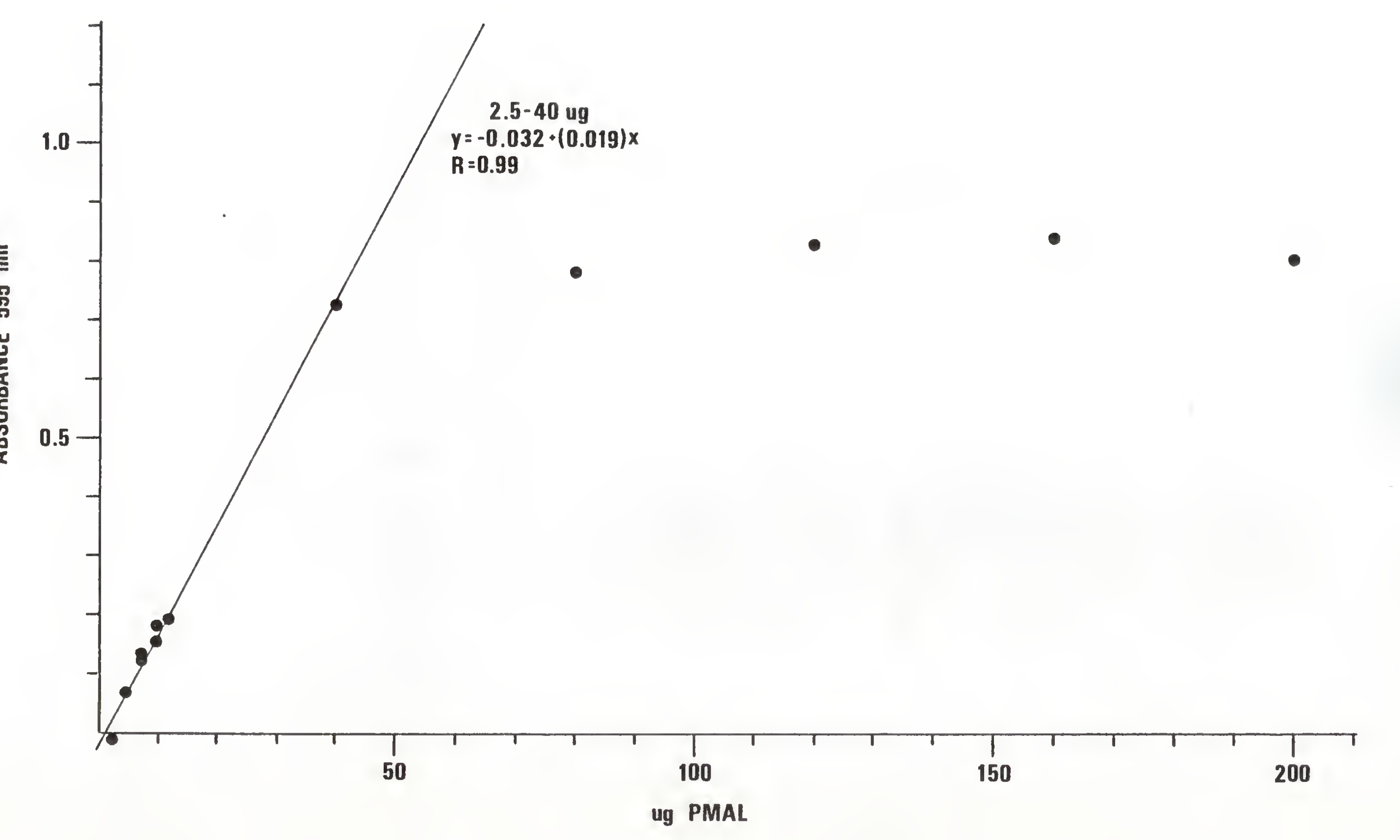
A. Poly-(ϵ -aminomethacrylyl-L-lysine).

B. Polyvinvypolypyrrolidone.

A

B

Fig. 15. Standard curve of Poly (ε-aminomethacrylyl-L-lysine) with 200 mg/L dye. Between 2.5-200 μg were used in 0.1 mL with 5 mL dye reagent, and 0.3 mL 3N NaOH.



2.5-40 ug
y=-0.032+(0.019)x
R=0.99
ABSORBANCE 595 nm
1.0
0.5
50
100
150
200
ug PMAL

Fig. 16. Absorption spectrum of polyvinylpolypyrrolidone with 200 mg/L.

A. 200 mg/L dye with 0.3 ml 3N NaOH per 5 mL reagent.

B. 200 µg PVPP in 0.1 mL with 5 mL 200 mg/L dye reagent with 0.3 mL 3N NaOH per 5 mL reagent.

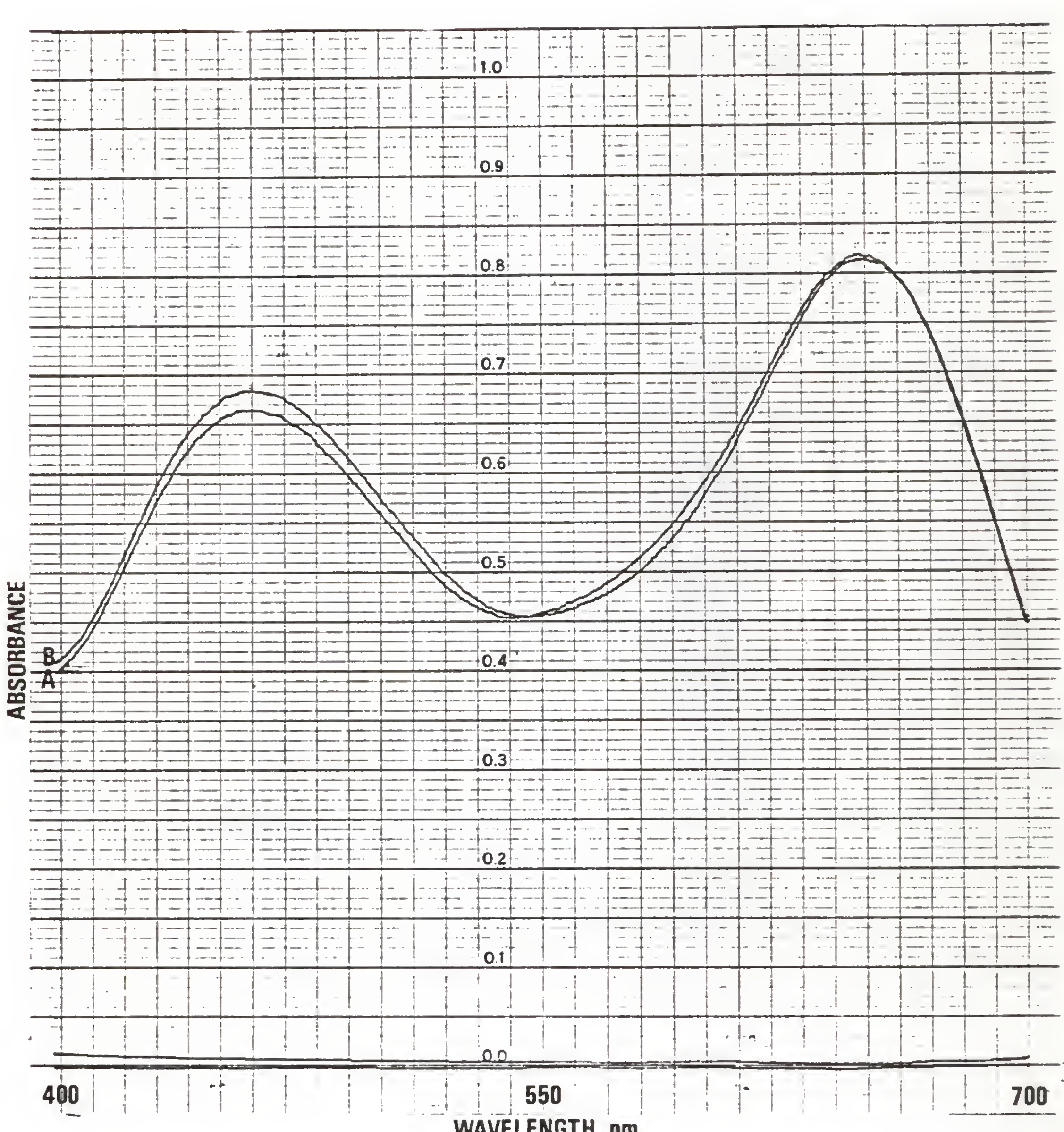

1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
ABSORBANCE
B
A
400
550
700
WAVELENGTH nm

Fig. 17. Absorption spectra of dextran sulfate with the 200 mg/L reagent.

A. 0.3 mL 3N NaOH per 5 mL reagent.

B. 50 μg dextran sulfate in 0.1 mL with 5 mL reagent with 0.3 mL 3N NaOH per 5 mL reagent.

C. 200 μg dextran sulfate in 0.1 mL with 5 mL reagent with 0.3 mL 3N NaOH per 5 mL reagent.

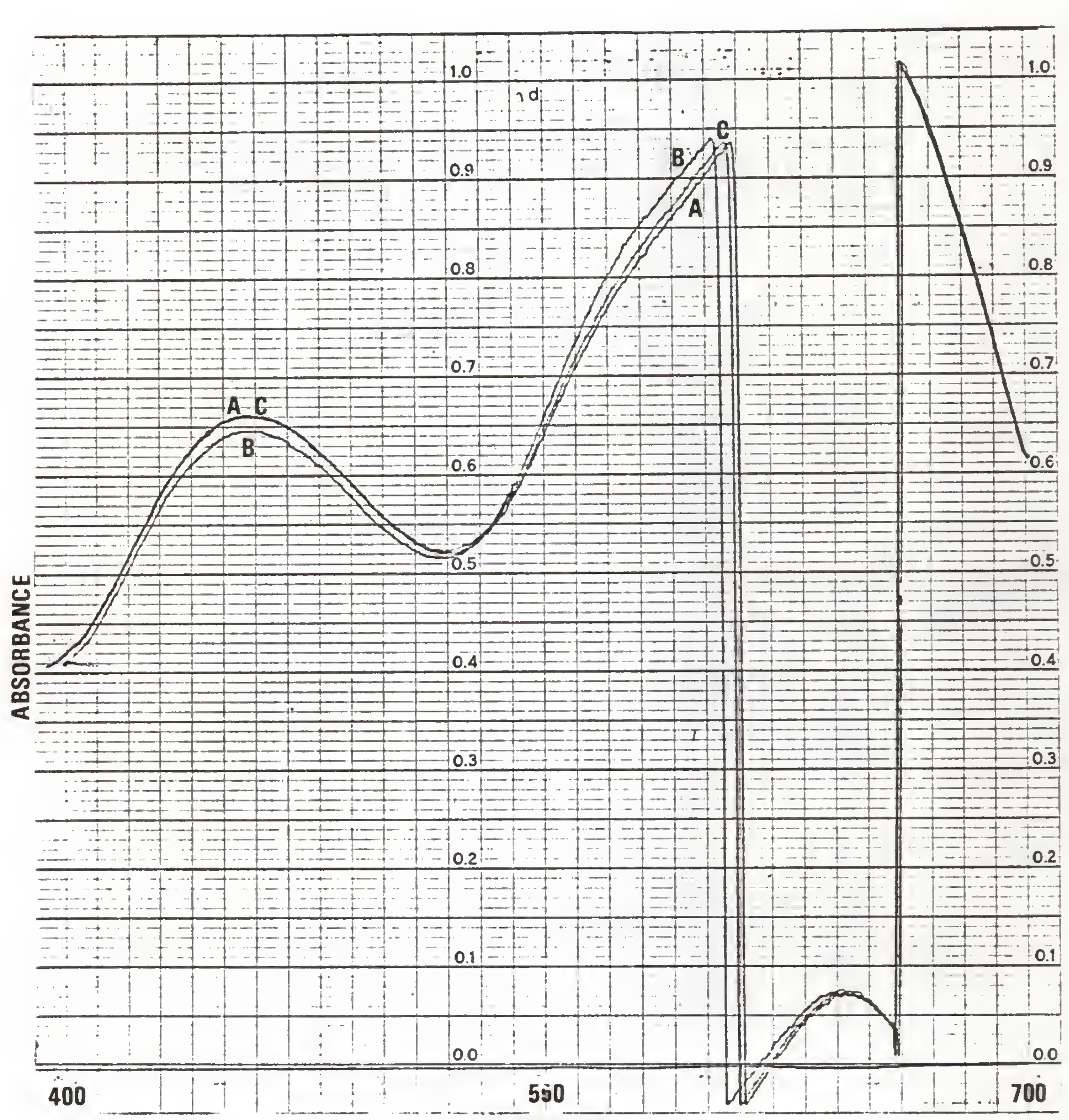

ABSORBANCE
WAVELENGTH nm
400
550
700
0.0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
0.8
0.9
1.0
A
B
C

Fig. 18. Absorption spectrum of trilysine with 200 mg/L dye.

A. 0.3 mL 3N NaOH per 5 ml reagent.

B. 100 µg trilysine in 0.1 mL with 5 mL dye reagent with 0.3 mL 3N NaOH per 5 mL reagent.

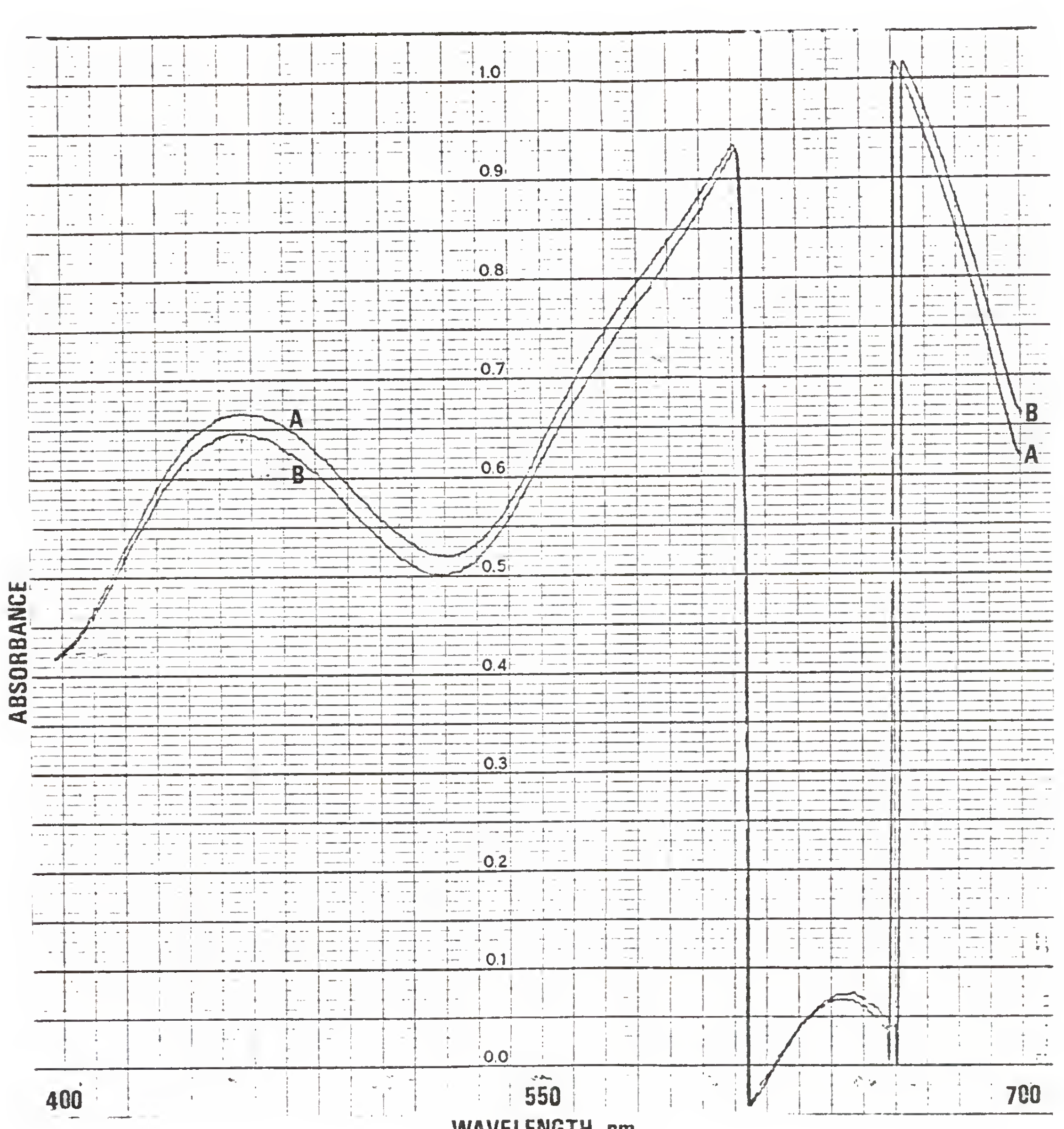

1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
A
B
B
A
ABSORBANCE
400
550
700
WAVELENGTH nm

Table V. Effects of some Polymers on the Coomassie Brilliant Blue G-250 Protein Assay Method.

μg PVPP	Δ Absorbance
Polyvinylpolypyrrolidone	
40	negative
80	0.015
120	-0.008
160	-0.001
180	-0.027

μg Dextran	Δ Absorbance
Dextran Sulfate	
50	0.040
100	0.010
200	0.004

μg PMAL	Δ Absorbance
Poly (ε-Aminomethacrylyl-L-lysine)	
2.5	-0.007
2.5	-0.012
5.0	0.069
5.0	0.069
7.5	0.132
7.5	0.123
10.0	0.157
10.0	0.180
40	0.729
80	0.785
120	0.830
160	0.840
200	0.801

Table VI. The Effect of Charge Density on Assay.

(Sensitivity Data from Bio-Rad Laboratories Technical Bulletin No. 1051. The number of positive charges per μg of protein was calculated by finding the number of basic amino acids in the protein, lysine, arginine, and histidine, and was multiplied by how many moles are present in a μg of protein).

Protein 10 mg/ml	Number positive charges per μg protein $\times 10^{10}$	Lowry mg/ml	Bio-Rad mg/ml
Alcohol Dehydrogenase	12.31	5.0	7.8
α-Amylase	9.11	6.0	8.3
BSA	13.8	8.4	21.1
Carbonic Anhydrase	12.68	8.9	13.0
Catalase	11.0	6.3	9.7
Cytochrome C	19.80	11.3	25.3
β-Galactosidase	8.69	9.9	7.9
Hemoglobin	15.94	8.3	19.9
Histones	19.5	9.2	15.8
Lysozyme	12.58	12.6	9.9
Myoglobin	20.35	7.9	20.7
Pepsin	4.57	12.4	4.1
Ribonuclease	13.15	15.9	5.3
Trypsin	8.34	15.5	4.9
Trypsin inhibitor	11.2	10.3	6.1
Transferrin	11.3	9.0	12.6

Linear Regression

	Lowry	Bio-Rad
Y-intercept	11.42	-3.83
Slope	-0.13	1.24
R	0.03	0.67

Fig. 19. Assay sensitive versus positive charge density on the protein. Sensitivity data from Table VI (Bio-Rad Laboratories Technical Bulletin 1051) versus the positive charge on the protein which was calculated from known amino acid analyses.

cytochrome C
bovine serum albumin
myoglobin
hemoglobin
histones
carbonic anhydrase
transferrin
lysozyme
catalase
α-amylase
alcohol dehydrogenase
β-galactosidase
trypsin inhibitor
ribonuclease
trypsin
pepsin
20
10
5
10
15
20
ABSORBANCE/1 mg/mL
(POSITIVE CHARGES/ug) x 10^{10}

water showed a decreased 455 nm band as well. On the other hand there was a small increase at 645 nm (Fig. 18).

Discussion

The change of absorption spectrum of the dye with NaOH no doubt is due to deprotonation of one of the ionizable amine groups in this dye (Perrin 1965). There are three forms of the dye compound. First, that which is present at very low pH values, where the maximum absorption band is at 460 nm, which probably represents the protonated form. At higher pH values (0.8 to 1.0), this form is in equilibrium with a second form as indicated by the isosbestic point in the 540-555 nm region for the four dye concentrations. During this time the bands at 460 and 650 nm are decreasing and increasing, respectively. A third form appears in the pH range of 1.05 to 1.15, depending upon the dye concentration. The new isosbestic point is shifted to the 523-526 nm, since a shoulder is developing near 595 nm on the short wavelength side of the 650 nm band. The long wavelength band is also going through a blue shift in this pH interval.

The absorption spectrum of dye with protein has the same form as that of the dye when the third form is present. However, it should be noted that this occurs at pH values where the third form would not be present from neutralization effects alone. In other words, the third form of the dye can be produced either by raising the pH or by interaction with a protein. It is unlikely, therefore, that the third form results from a further neutralization of the dye molecule. It appears more reasonable that an aggregated state is involved, which in one case involves dye molecules alone and in the other between dye-protein complexes producing a similiar electronic transition. With this in mind the data illustrated in Fig. 4 can be more readily understood. Whenever the reagent pH is too high there is a decrease in ΔA [595] compared to what is seen

at optimum pH. This occurs because there is enough NaOH present to produce the blue shift and shoulder in the dye reagent itself by neutralization alone. Before this point was reached most of the ΔA (595) had resulted from the shoulder at this wavelength produced by the protein-dye complex.

The time involved in color development is important to see how quickly this protein determination can be done at maximum sensitivity. The time study shows that the absorbance of the dye-protein complex changes slightly over the 30 min of observation. After reaching maximum value, the absorbance could either stay the same up to 30 min or decrease depending on the concentration of dye reagent used. In general there was no correlation between time to reach a maximum absorption and dye or protein concentration. For example the maximum absorbance for Bio-Rad or 100 mg/L is not reached until nearly 30 min. These results can be compared to the statement of Bradford (1976) in which a 5 to 20 min reaction period was recommended. If this procedure were followed, it appears from the data in Figs. 6 to 9 that the time of reading would have to be selected for maximum precision. We did this in our assays as well, but choose 20 min to achieve greater sensitivity and more uniformity between dye concentrations. A twenty minute development period allows about 10 samples to be assayed in 40 minutes.

The dye reagent stability was found to be dependent on the concentration of the dye present in the solution. The dye reagent was the most stable at 100 mg/L of coomassie brilliant blue G-250. So from this, if the dye was needed for a long period of time, the 100 mg/L would be the most appropriate, which is also the concentration described by Bradford

(1976). However, for more sensitivity higher concentrations of dye could be used. The Bio-Rad seems to be between 200 and 300 mg/L of dye with something else present comparing its absorption spectrum to those at 200 and 300 mg/L (Table I).

The standard curves of the proteins, lysozyme, BSA, trypsin, and chymotrypsinogen are linear at low concentration but at higher concentrations bend down, indicating that the assay may be dye limited. The standard curve for lysozyme was found to depend upon the concentration of dye used. The higher the dye concentration up to at least 500 mg/L, the greater the differential absorbance and the lesser the tendency for a negative deviation from Beer's law at higher protein concentrations. Apparently the interaction which occurs between the dye and protein is dye limited. This is shown through the standard curves made for lysozyme with different dye concentration (Fig. 11). What is interesting, however, is that the 200 and 300 mg/L solution gave similar standard curves while 400 and 500 mg/L also give similar curves but different from the 200 and 300 mg/L. A phenomenon such as this might result from a competition for a dye molecule by both protein and dye-dye aggregates whose magnitude varies with total concentration.

It had been realized from experiments in other laboratories (De St. Groth et al, 1963 and Colour Index 1971) that the dye interacts with cationic proteins and synthetic polymers much better than the neutral or anionic macromolecules. This is expected from what is known about acid dyes. There apparently is ionic bond formation between the anionic groups of the dye and cationic groups in the polymer, similiar to the binding of orange G in the Udy assay (Fig. 1). As seen in Fig. 2 coomassie brilliant

blue G-250 also has two sulfonic acid groups which probably take part in ionic bond formation between dye and polymer.

Further studies were undertaken, therefore to see if positive charges alone or whether additional factors were involved in producing the 595 nm shoulder. The polymers chosen were polyvinylpolypyrrolidone, and uncharged polymer (Fig. 14B), dextran sulfate, a negatively charged polymer and poly-(ϵ -aminomethacryly-L-lysine), a highly positively charged polymer at pH 1. As can be seen in Fig. 16 and Table VI, PVPP gave a negative absorbance reading at 595 nm indicating that this polymer does not form a complex with the dye or that the complex formed does not give the 595 nm shoulder. From the absorption spectrum of PVPP-dye mixture it is also apparent that the short wavelength band has increased relative to that which would normally be seen for the dye alone at this pH. Furthermore, there is only randomness in the absorbance versus polymer concentration data indicating no complex formation. Low concentration of dextran sulfate gives slightly positive absorbance but as the concentration increases the differential absorbance decreased, possibly indicating that this anionic polymer is causing disruption of dye-dye aggregation most likely through electrostatic forces. It was noted that PMAL (Fig. 15) gave a linear response between 2.5-40 μg with the 200 mg/L dye reagent but very little increase at higher concentrations (up to 200 μg). This seems to indicate that at 40 μg the interaction of dye-polymer is complete for this concentration of dye. The high positive charge density of this polymer seems to be the reason for the color, since as already mentioned a highly negative or uncharged polymer gave little differential absorbance. To produce a ΔA (595), however, the substance must be macromolecular as shown by the

absence of a differential absorbance at this wavelength with trilysine.

A study of positive charge/μg protein versus sensitivity expressed as 1000 μg/ml of protein was made using results given in Bio-Rad Laboratories Technical Bulletin 1051. From the study it was seen that there is a greater correlation between charge density and ΔA (595) (Fig. 19 and Table VI) for this method than the Lowry method. Fig. 19 shows data for sixteen proteins. Clearly a strong correlation exists between net positive charge and sensitivity. Linear regression gave a 0.67 correlation coefficient between these variables (Table VI). Table VI also lists the results obtained by Bio-Rad Laboratories for the same proteins using the Lowry method. Linear regression for this data gives a correlation coefficient of 0.03 indicating the Lowry procedure is independent of positive charge.

Finally, it can be concluded that other factors are involved besides positive charge density. This was illustrated by the results for trypsin and modified trypsin. The modification decreased the number of basic amino acids by at most two from the twenty which are present in trypsin. However, there was an increase of at most four hydrophobic groups since two phenylglyoxal molecules react with each arginine residue (Takahashi, 1968). Therefore, this might explain the increase of sensitive for modified trypsin by increasing hydrophobic interactions even though the net positive charge was reduced by two per molecule.

Recommendations

The pH of the dye reagent needs to be such that the optimum ΔA (595) can be obtained for the dye concentration used. The pH should be recorded as part of the procedure of the assay. If a strong alkaline buffering system is used, the buffer should be present in the blank at the same concentration as that of the sample.

The development time should be the same within a few minutes for all samples assayed. As mentioned a twenty min development time was used, which allows 10 samples to be assayed in forth min at two min intervals.

If the dye reagent is to be used over several days, a concentration of 100 mg/L of coomassie brilliant blue G-250 is advisable. However for sensitivity purposes a concentration of 400 mg/L can be used, but may not remain as stable.

On making standard curves the protein of interest should be used or one which has close to the same number of positive charges per μg of protein.

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FURTHER STUDIES ON THE NEW COOMASSIE BRILLIANT BLUE G-250 PROTEIN ASSAY

by

JOHN DANIEL STOOPS

B. S. Kansas State University, 1975

AN ABSTRACT OF A MASTER'S THESIS

submitted in partial fulfillment of the

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MASTER OF SCIENCE

Graduate Biochemistry Group

KANSAS STATE UNIVERSITY
Manhattan, Kansas

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Color development with the coomassie brilliant blue G-250 protein assay method introduced by M. M. Bradford in 1976 was found to vary with the source of dye. Investigation into those effects showed the response of the dye to depend critically on the pH of the reagent solution. At pH values above 1.1 the visible spectrum of the dye is very similar to that obtained at pH value 0.8 when substantial protein is present. Consequently it was possible to optimize the sensitivity of the reagent toward lysozyme by appropriate adjustment of pH. The pH value giving optimal response, however, varied with the concentration of dye used in preparation of the reagent. Furthermore the sensitivity of the assay to a fixed protein level increases with increasing dye concentration. Dye concentrations of 200 mg per liter or greater appear to have reduced stability compared to the three weeks or better shelf-life of the 100 mg per liter solution. The absorbance at 595 nm also depends on the protein assayed. Studies into the nature of this variation using small molecules and polymeric model compounds show that the sensitivity depends directly on the net positive charge on the macromolecule. Even with postively charged compounds, however, appropriate color development requires a macromolecular environment suggesting that other factors besides ionic interactions are involved.