

DOCUMENT CONTROL DATA - R & D

(Security classification of title, body of abstract and indexing annotation must be entered when the overall report is classified)

1. ORIGINATING ACTIVITY (Corporate author) Naval Research Laboratory Washington, D. C. 20390		2a. REPORT SECURITY CLASSIFICATION Unclassified	
		2b. GROUP	
3. REPORT TITLE THE USE OF CARBOCYANINE DYE TO DETECT TRACE AMOUNTS OF PROTEIN IN BIOLOGICAL MEDIA			
4. DESCRIPTIVE NOTES (Type of report and inclusive dates) Final report on one phase of the problem; work is continuing on other phases.			
5. AUTHOR(S) (First name, middle initial, last name) Beverly J. Michajluk, Lt(jg), USNR, and George I. Loeb			
6. REPORT DATE December 21, 1970		7a. TOTAL NO. OF PAGES 16	7b. NO. OF REFS 7
8a. CONTRACT OR GRANT NO. NRL Problem G04-01		9a. ORIGINATOR'S REPORT NUMBER(S) NRL Report 7196	
b. PROJECT NO. RR 104-03-41-5503			
c.		9b. OTHER REPORT NO(S) (Any other numbers that may be assigned this report)	
d.			
10. DISTRIBUTION STATEMENT This document has been approved for public release and sale; its distribution is unlimited			
11. SUPPLEMENTARY NOTES		12. SPONSORING MILITARY ACTIVITY Department of the Navy (Office of Naval Research), Arlington, Va. 22217	
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14. KEY WORDS	LINK A		LINK B		LINK C	
	ROLE	WT	ROLE	WT	ROLE	WT
Biopolymer Detection Dye-Biopolymer Interaction Marine Polymer Detection						

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ABSTRACT

To test its applicability as a detector of biopolymers, a carbocyanine dye was allowed to react with proteins, polypeptides, polysaccharides, lipids, and supernatants from marine cultures, and changes in the visible spectra were observed. β -Lactoglobulin and gelatin have thus far proved satisfactory as standards; the smallest detectable concentration was 2 ppm.

Since different spectral features become prominent when the type of polymer or the state of the polymer is varied, it may be possible to classify marine polymers in very dilute solution by spectral means. Evidence has been found for considering a peak at 605 nm as characteristic of a denatured protein.

PROBLEM STATUS

This is a final report on one phase of the problem; work on other aspects of the problem continues.

AUTHORIZATION

NRL Problem G04-01
Project RR 104-03-41-5503

Manuscript submitted September 10, 1970.

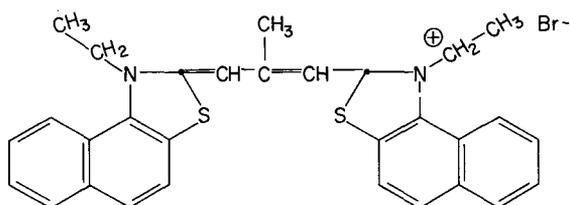
THE USE OF A CARBOCYANINE DYE TO DETECT TRACE AMOUNTS OF PROTEIN IN BIOLOGICAL MEDIA

INTRODUCTION

Metachromasia, or the identification of biological material by color changes in an adsorbed dye, has long been used by histologists to identify biochemical constituents in cells. When the dye interacts with a solution of macromolecules, its spectral characteristics may change, resulting in a color change detectable by the eye.

Sheppard (1942) discovered that trace amounts of protein favor the aggregation of certain carbocyanine dyes. Kay et al (1964) have suggested that 4,5,4',5'-dibenzo-3,3'-diethyl-9-methyl-thiocarbocyanine bromide (ϕ_2E_2MCB), shown in Fig. 1, might be promising as a material for detecting protein; only trace amounts of the polymer are necessary to cause spectral changes in the visible region.

Fig. 1 - 4,5,4',5' - Dibenzo-3,3' - diethyl - 9 - methyl - thiocarbocyanine bromide (ϕ_2E_2MCB)



Kay and his associates have found six absorbance maxima in the dye spectra and postulated that they correspond to six possible types of dye aggregates. Arranged in the order of increasing aggregation, the maxima are 570, 535, 510, 450, 555, and 650 nm. The lowest degree of aggregation, i. e. , monomeric dye (peak at 570 nm), is prominent in organic solvents, in low dye concentrations, and at high temperatures. The 650-nm band, or "J band," represents the greatest degree of aggregation, large enough to be sedimented by centrifugation. The 555-nm band, when present, may be associated with the J-band maximum. Dimers of dye are indicated by the 535-nm band, while bands at 450 nm and 510 nm are characteristic of intermediate species.

Since the optical density of $2 \times 10^{-5}M$ ϕ_2E_2MCB in water is a maximum at 510 nm, it should be possible to either increase or decrease the aggregation; thus, reaction with an anionic species may change the absorbance peaks (Kay et al. , 1964). We are studying the dye-polymer system to evaluate its feasibility for use as a detector of marine biopolymers by testing ϕ_2E_2MCB with proteins, polypeptides, polysaccharides, lipids, and supernatant media from marine cultures.

EXPERIMENTAL PROCEDURES

Dye Preparation

The dye used in this investigation, may also be called 1-ethyl-2 [3-(1-ethylnaphtho [1, 2d] thiazolin-2-ylidene)-2-methylpropenyl] -naphthol [1, 2d] thiazolium bromide, and

was obtained from Eastman Organic Chemicals, Rochester, New York. Because dye solutions fade when exposed extensively to bright light, and absorb to glass, we found it necessary to keep them in the dark and to coat the inside of glassware and spectrophotometer cells with a silicone preparation (Siliclad).

Kay et al. (1964) report storing 4×10^{-5} M aqueous solutions in glass containers for 37 days at 22° C without a major decline in absorbance. However, we found the direct preparation of stable aqueous solutions difficult. After testing several organic reagents, dimethyl sulfoxide (DMSO) was found to be a satisfactory solvent. DMSO (Baker analyzed reagent) was distilled and stored at 4° C. To prepare dye stock solutions, 0.022 g of the dye powder per ml was dissolved in DMSO that had been brought to room temperature. Stirring 15 to 30 minutes totally dissolved the powder. Solutions were then stored (frozen) at 4° C. Four ml of this stock solution was diluted to 1 liter with 10^{-3} M tris* buffer (pH 8.8) to give a 16×10^{-5} M dye solution. Solutions were stirred for 1/2 hour; they could then be stored 2 to 3 weeks at 20° C in actinic flasks. The final concentration was calculated from the optical density at 510 nm, using data from Kay et al. (1964):

$$E_{510}^{1\%} = 1.10 \times 10^3 .$$

These same authors reported that maximum polyanion detection efficiency was obtained at a pH of 7 to 9, with 4×10^{-5} M dye at a temperature of 20° to 35° C.

Protein Preparation

β -Lactoglobulin (β -L) was obtained from Pentex Inc., Kankakee, Ill; bovine serum albumin (BSA) was an Armour Pharmaceutical product; and gelatin (U.S.P. grade) was purchased from Fisher Scientific Co., Fairlawn, N. J. Poly-L-aspartic acid (PLA, average molecular weight = 20,000) and poly-L-glutamic acid (PLG, average molecular weight = 60,000) were purchased from Mann Research Laboratories and Pilot Chemicals, Inc., respectively. Biological-culture liquids were gifts of Mrs. Marcia Loeb of the University of Maryland Zoology Department and D. F. Wilson and P. J. Hannan of NRL.

Stock solutions of BSA and gelatin were 1% in 10^{-3} M tris buffer (pH 8.8); β -lactoglobulin was 1% in 10^{-2} M KCl; and PLA and PLG were 1% in 10^{-3} M tris buffer. (Dropwise addition of concentrated NH_4OH was required to dissolve the polypeptides. Solutions were immediately back titrated to pH 8.8 with 0.1 N HCl). Preparations of BSA and gelatin were dialyzed against 10^{-3} M tris buffer. However, because of the high purity of the β -lactoglobulin preparation and the small molecular size of the polypeptides, these were used as received. Supernatants from copepod seawater cultures, chlorella, and phaeodactylum cultures were filtered with either Whatman 42 filter paper or a Gelman filter (22- μ pore size), so as to remove solid culture particles, and then dialyzed against 10^{-2} M KCl at 4° C. The brine shrimp culture (*Artemia salina*) was first filtered with Whatman 1 paper. Media from *Artemia*, *Chrysaora quincirrh*a (Chesapeake Bay sea-nettle), and lobster (*Homarus sp.*) cultures were centrifuged at 15,000 G for 10 minutes. The supernatant liquids were then dialyzed against 10^{-2} M KCl at 4° C to obtain a known salt environment. Seawater, both before and after foaming (Wallace and Wilson, 1969), was dialyzed against 0.01M KCl. After dialysis, samples were used without further dilution.

*Tris-hydroxymethylaminomethane.

Concentrations of BSA

$$E_{279}^{1\%} = 6.7 \text{ (Foster and Sterman, 1956)}$$

and β -L

$$E_{280}^{1\%} = 9.4 \text{ (Tanford et al., 1960)}$$

were determined by uv absorption. Dry-weight determinations were used for gelatin, PLA, and PLG. Protein concentrations of the biological culture media were not determined.

The pure protein and polypeptide preparations were diluted with tris buffer (10^{-3} M, pH 8.8) to obtain dilution series from 0.6 to 9 ppm. All dilutions were made with Hamilton syringes fitted with Chaney adaptors. No dilution was made after dialysis of the marine culture media.

All preparations except the temperature study group were warmed to room temperature. Portions of the samples were mixed with equal volumes of 4×10^{-5} M dye solution in silicone-coated test tubes. The samples were then placed in a dark box until maximum color was produced. Reaction times to attain absorbance readings were established as 1 hr for BSA, gelatin, PLA, PLG and all biological cultures and 3 hours for β -L, in agreement with Kay et al. (1964). Spectral absorption curves in the 400- to 700-nm range were obtained using a Cary 14 spectrophotometer* and 1.0-cm siliconed glass cells. Reference cells contained 2×10^{-5} M dye in 10^{-3} M tris buffer unless otherwise stated. Therefore, the resulting spectra were difference spectra providing high sensitivity. Position on the absorbance scale was adjusted with a neutral density filter.

RESULTS

Dye Properties

Although the DMSO stock solution shows a blue color, there is no shift in the dye absorption peak from the red-purple found in water solution when DMSO stock solution is diluted to 4×10^{-5} M with the tris buffer (Fig. 2).

Fading of the dye solution color in room light is a function of concentration. Rapid loss of color results if the concentration of the dye is less than 1×10^{-6} M. More concentrated solutions (4×10^{-5} to 4×10^{-4} M) do not fade seriously in short exposure to room light.

The dye in DMSO and tris buffer was stored at 20°C in actinic flasks in a dark cabinet. Test samples must also be placed in the dark while awaiting maximum color formation. Slight fading may occur during manipulation before the samples are stored, but it is not significant.

Time studies have shown that dye absorbance decreases with time even in the closed cell compartment of the spectrophotometer (Fig. 3). Hence, we found it necessary to correct for fading in the cell compartment by taking periodic reference spectra of the blank solution. The decrease in reference color may be compensated if a rigid time schedule is established when adding dye and taking the spectrum.

*We are indebted to Dr. Victor Ritz of NRL for the use of this instrument.

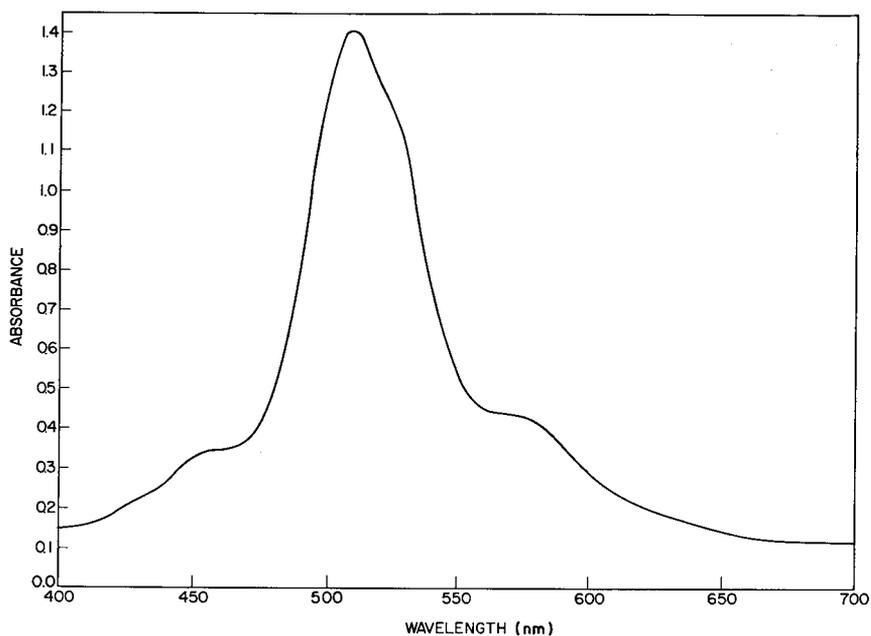


Fig. 2 - Spectrum of the experimental dye (ϕ_2E_2MCB). The dye concentration was $2 \times 10^{-5}M$ in $10^{-3}M$ tris buffer, pH 8.8. The curve was taken using $0.001M$ tris buffer as the reference in 1-cm glass cells.

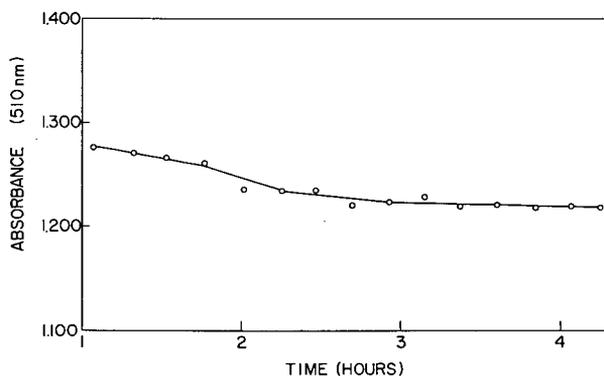


Fig. 3 - Decrease in absorbance of dye at 510 nm. The dye was $2 \times 10^{-5}M$ in $10^{-3}M$ tris buffer, pH 8.8, using $10^{-3}M$ tris buffer as the reference in 1-cm glass cells.

Cells were rinsed with acetone to remove residual adsorbed dye and then washed with a commercial detergent (Tide). Copious rinsing with distilled water is of utmost importance, since organic material remaining inside the cell influences the spectra.

Results obtained from proteins are summarized in Table 1.

Table 1
Sensitivity of Detection of Trace Amounts of Protein in Dye

Protein	Concentration (ppm)	Wavelength Maxima (nm)	Smallest Detectable Concentration (ppm)
β -Lactoglobulin	8	625	2
	2	590	
Bovine serum albumin	2-8	480	2
Gelatin	8	605	2
	2	620	
Poly-L-aspartic acid	50	605	0.6
	0.6	620	
Poly-L-glutamic acid	50	605	0.6
	0.6	620	

Note: The smallest detectable concentration of protein was determined by diluting the protein with 0.001M tris buffer and using a final dye concentration of 2×10^{-5} M. All measurements were made in 1.0-cm glass cells.

β -Lactoglobulin (β -L)

Dye and β -L (2-8 ppm) result in a color change from pink to purple, the extent of change depending on the protein concentration. Below 2 ppm the color is that of dye alone. A purple color is produced when the protein concentration lies between 2 ppm and 8 ppm. Kay et al. (1964), using 20-ppm β -L in 2×10^{-5} M dye and 2×10^{-5} M dye as reference, obtained absorption maxima at 646, 560, and 480 nm. Our difference spectra with β -L of 8 ppm or less showed a 625-nm peak which shifted to 590 nm as the β -L concentration decreased to 2 ppm. Although a slight shoulder appeared, no maximum at 560 nm was seen (Fig. 4). However, a well-defined valley appeared at 510 nm, indicating the binding of dye molecules. This binding reduces absorbance at the wavelength of the unbound dye maximum.

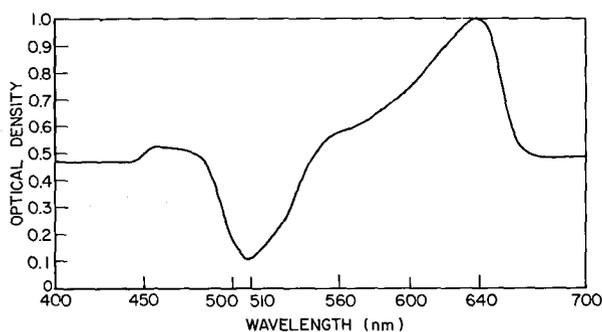


Fig. 4 - Spectrum of β -lactoglobulin (9 ppm). Both the protein and dye were buffered with 10^{-3} M tris, pH 8.8. The reference was 2×10^{-5} M dye in 1-cm glass cells, and $dA/dc = 0.054 \pm 0.0009$.

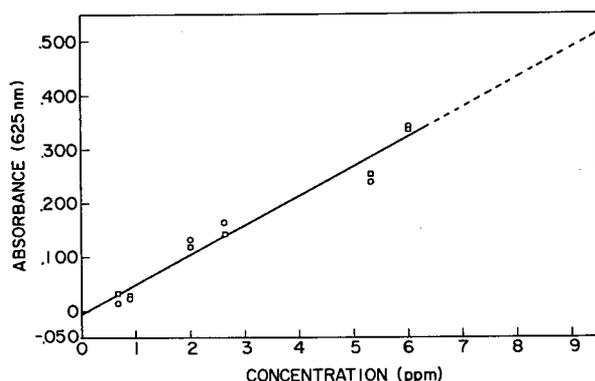


Fig. 5 - Relationship between absorbance (625 nm) and β -L concentration. Tris (10^{-3} M, ph 8.8) was used as buffer. The reference was 2×10^{-5} M dye in 1-cm glass cells. Two data runs are indicated by the circles and squares.

Absorbance at 625 nm was linear with concentration (Fig. 5). An estimation of the slope by the least-squares method puts dA/dc (c in parts per million protein) at 0.054, with a standard deviation of ± 0.0009 . The reproducible value suggests the possibility of using β -lactoglobulin as a standard for the method, since it is commercially available in high purity at reasonable cost.

Bovine Serum Albumin (BSA)

Dye and BSA between 2 and 8 ppm do not result in a visible color change, but difference spectra showed the expected wavelength maximum at 480 nm (Kay et al. 1964) (Fig. 6). At concentrations less than 2-ppm BSA, the 480-nm peak disappears. Although there appears to be an approximately inverse relation (Fig. 7) between free dye optical density at 510 nm and BSA concentration (1-5.5 ppm), indicating binding, the results have not as yet been reproducible within the (2- to 8-ppm) range for this peak or the 480-nm peak.

Gelatin

Dye and gelatin (2-8 ppm) yield a blue color visibly different from that caused by β -L. The wavelength maximum occurs between 615-645 nm with only one peak (Fig. 8). No maxima or minima are found in the difference spectrum at less than 615 nm with concentrations less than 3 ppm. At higher concentrations there are suggestions of small peaks at 500 and 450 nm.

A linear relationship was found to exist between absorbance and concentration in the 2 to 9 ppm range: $dA/dc = 0.090 \pm 0.008$ (Fig. 9). The smallest detectable amount of

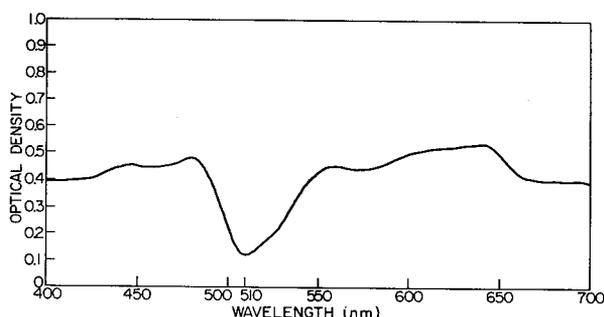


Fig. 6 - Spectrum of bovine serum albumin (16 ppm). The protein and dye were buffered with 10^{-3} M tris, pH 8.8. The reference was 2×10^{-5} M dye in 1.0-cm glass cells.

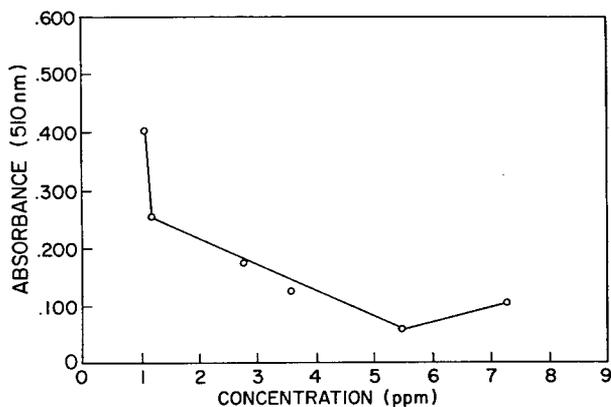


Fig. 7 - Relationship between absorbance (510 nm) and BSA concentration. Both the protein and dye were buffered with 10^{-3} M tris, pH 8.8. The reference was 2×10^{-5} M dye in 1.0-cm glass cells.

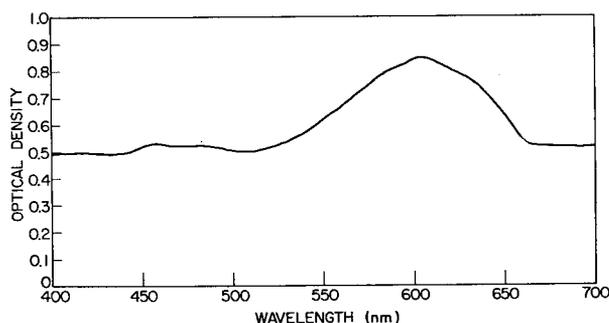
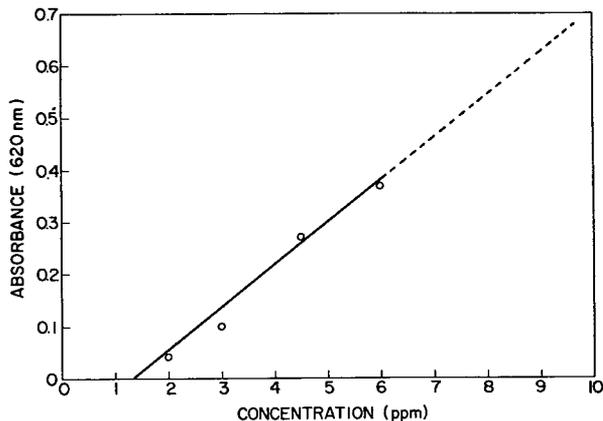


Fig. 8 - Spectral curve of gelatin (7 ppm). The protein and dye were buffered with 10^{-3} M tris, pH 8.8. The reference was 2×10^{-5} M dye in 1.0-cm glass cells.

Fig. 9 - Relationship between absorbance (620 nm) and gelatin concentration. The protein and dye were buffered with 10^{-3} M tris, pH 8.8. The reference was 2×10^{-5} M dye in 1.0-cm glass cells and $dA/dc = 0.090 \pm 0.0081$.



gelatin was 2 ppm. At concentrations less than 3 ppm, the visible color of the dye-protein solution approaches that of the unbound dye solutions.

Polypeptides

The two polypeptides investigated were poly-L-glutamic acid (PLG) and poly-L-aspartic acid (PLA). These materials are polymers of amino acids which have carboxylic acid side chains on every unit and differ only in their side chains, i. e., PLA contains one methylene unit and PGA contains two methylene units. As might be expected, the properties of these polymers are very similar.

When treated with the dye, these polymers produce a broad region of optical density between 540 and 640 nm and, indicative of binding of the dye, a decreased optical density at 510 nm, the wavelength of maximum absorption of the dye in the absence of polymer. The solution appears deep purple to the eye at polymer concentrations above 0.6 ppm.

The ratio of optical density at 550 nm to that at 600 or higher nm decreases with decreasing polymer concentration so that 620 nm is the most sensitive wavelength for detection of PLG at low concentration. Spectra of dye solutions containing a low concentration of PLG and PLA are shown in Figs. 10 and 11, respectively. When the optical density at 620 nm is plotted against PLG concentration (Fig. 12), the sharp decline at concentrations lower than 2 ppm is evident. Below 0.6 ppm detection of the band at 620 nm could not be consistently achieved. Similar results are obtained for PLA. At higher concentrations, a maximum at 605 nm is found; its possible significance will be discussed below. No one wavelength showed a linear relationship between optical density and concentration over an extended concentration range. A minor peak was observed in the 475-nm region.

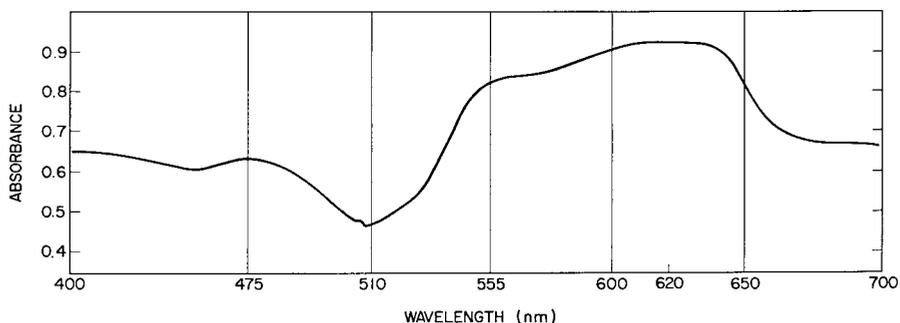


Fig. 10 - Spectrum of poly-L-glutamic acid (0.6 ppm). Both the polypeptide and dye were buffered with 10^{-3} M tris, pH 8.8. The reference was 2×10^{-5} M dye in 1.0-cm glass cells.

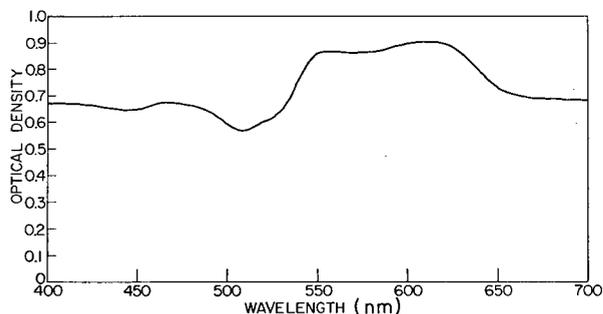
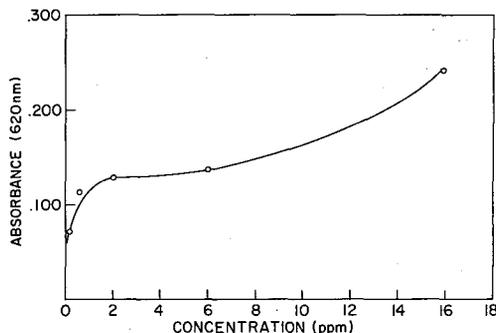


Fig. 11 - Spectrum of poly-L-aspartic acid (0.6 ppm). Both the polypeptide and dye were buffered with 10^{-3} M tris, pH 8.8. The reference was 2×10^{-5} M dye in 1.0-cm glass cells.

Fig. 12 - Relationship between absorbance (620 nm) and PLG concentration. The protein and dye were buffered with 10^{-3} M tris, pH 8.8. The reference was 2×10^{-5} M dye in 1.0-cm glass cells.



Biological Cultures

The results obtained for biological cultures are summarized in Table 2. The supernatant from the strongly aerated lobster aquarium gave a maximum at 605 nm. *Chlorella*, *Chrysaera quincirra* (Chesapeake Bay sea-nettle), and *Artemia salina* (brine shrimp) supernatants showed peaks at 625, 655, and 627 nm, respectively. Dialyzed seawater, used as a reference, did not change the absorption spectrum of the dye.

Table 2
Spectral Properties of Culture Media Treated With Dye

Culture	Medium	Wavelength Maxima (nm) at Three Temperatures		
		10° C	25° C	30° C
Natural microbiological population	Seawater	—	637	—
Phaeodactylum	Guillard's*	—	607	—
Chlorella	Burk's† medium	—	625	—
Homarus (lobster)	Artificial seawater	607	605	606
<i>Chrysaera quincirra</i> (jellyfish)	Artificial seawater	610	655	606
<i>Artemia salina</i> (brine shrimp)	NaCl	590	627	605
Foamate	Seawater	—	605	—

*Hannan, et al. (1963).

†Guillard and Ryther (1962).

Note: Cultures were dialyzed against 10^{-2} M KCl and later buffered with 10^{-3} M tris (pH 8.8). Dye (4×10^{-5} M) was added to cultures at room temperature. Spectral difference curves were taken using 2×10^{-5} M dye as reference and 1.0-cm glass cells. Cultures at 10°C and 30°C were kept at that temperature for 1/2 hr. and then put into the cell compartment of the spectrophotometer.

Cooling the supernatants from lobster, jellyfish, and brine shrimp to 10° through 14° C causes an absorption peak to appear some 15 to 35 nm nearer the short wavelength end of the spectrum. Such a shift suggests the formation of another series of dye aggregates on the macromolecule not so strongly polymerized as at room temperature (Kay et al., 1964).

The biological cultures heated to 30° C for 1/2 hour resulted in peaks at 605 to 606 nm. Collected foam from seawater* was condensed and dialyzed. This material, stored frozen so as to prevent the growth of microorganisms, yielded an absorption peak between 605 to 606 nm when treated with dye. Again, unfoamed seawater dialyzed against 10⁻² M KCl showed no color change when tested with the dye.

Other Materials

Ficol, dextran, and Triton X-100, uncharged polymers, were also tested but induced no color change in the dye. Stearic acid and lauric acid, representative of the charged lipids, produced yellow or orange color when treated with dye. Such a reaction indicates the possibility of a wider range of use for this method. However, only preliminary experiments with lipids have been done to date.

DISCUSSION

Complexes between the cationic dye φ_2E_2 MCB and a polymer chain are formed if there is a net negative charge on the polymer chain. Proteins are usually negatively charged at the experimental pH(8.8) as their acidic groups have been converted to the acid anion (COO⁻), and the amino groups are uncharged. A small number of proteins whose isoelectric point is high, such as bovine pancreatic ribonuclease, may escape detection (Kay et al., 1964). However, it is expected that in the neighborhood of living organisms, a significant amount of negatively charged polymers will be present and can be detected.

Bovine serum albumin, β -lactoglobulin, and gelatin, three commercially available protein preparations, and two synthetic polypeptides, poly-L-aspartic acid and poly-L-glutamic acid, have been used to confirm the results of earlier investigators of this method of detecting trace amounts of protein. We have confirmed the sensitivity of their method for these materials in general, although differences were found. As observed by the previous workers, different proteins interact with φ_2E_2 MCB to give different absorption spectra in the visible range. The positions of the absorption maxima reflect the extent of interaction of the dye molecules complexed with the polymer. The synthetic polypeptides consist exclusively of acidic amino-acid monomer units. BSA, on the other hand, typical of natural proteins, has approximately 15% acidic amino-acid residues distributed along its polymer chain. Thus, the polymers in which every residue bears a negative charge are detected at 0.6 ppm, while the proteins are detected at 2 ppm.

In the case of the synthetic polymers, our results differ from those of Kay et al., in that we observed absorption maxima at 475 and 540 to 630 nm, while Kay found one very intense peak at 530 nm, which is characteristic of dimer formation. Differences in molecular-weight distribution and pH of the test solution might account for the apparently higher degree of aggregation found in this study. Further, we observed difference spectra which accentuate small differences in the spectra.

*Kindly donated by G. T. Wallace, Jr.

The maximum at 605 nm is of great interest since we have found it in those cases where we have reason to believe that denatured or random-coil polypeptide structures may exist. These are in foamates from seawater and lobster habitats (where surface denaturation would occur), in heated media from phaeodactylum, sea nettle, and brine shrimp cultures, and in solutions of the synthetic polypeptides PLA and PLG, which normally exist in random-coil forms at neutral or basic pH. Therefore, we feel that this peak may be of general use in evaluating the denaturation of proteins by various agents, although the question deserves further study. At low concentrations, this absorption shifts to a broad maximum at 620 nm.

The effects of cooling are difficult to interpret at this time. However, Kay et al. (1964) suggest that the spectral changes at low temperatures indicate that dye aggregates induced by inorganic salts are favored.

To establish a reference standard for field work, one must take into account the stability of both dye and protein. β -L requires nearly a 3-hour equilibration time but has excellent reproducibility. The equilibration time for gelatin is much shorter, but it is not commercially available in as high purity and therefore may not be as consistent when different lots are compared.

In conclusion, it appears that the tested dye has possibilities as an investigative tool in field oceanography. Preparation of the dye is simple and once in solution provides no storage problems for 2 weeks. The method itself requires only the adding of dye to the desalted protein sample and awaiting maximum color development. With this dye, not only is it possible to determine the presence of biopolymers but also to achieve a preliminary classification. In addition, with highly negative substances detection is possible down to 0.6 ppm (0.00006%).

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