DATA REPORT

PARTICULATE PROTEIN MEASUREMENT

IN OCEANOGRAPHIC SAMPLES BY DYE BINDING

BY

FRANK W. SETCHELL

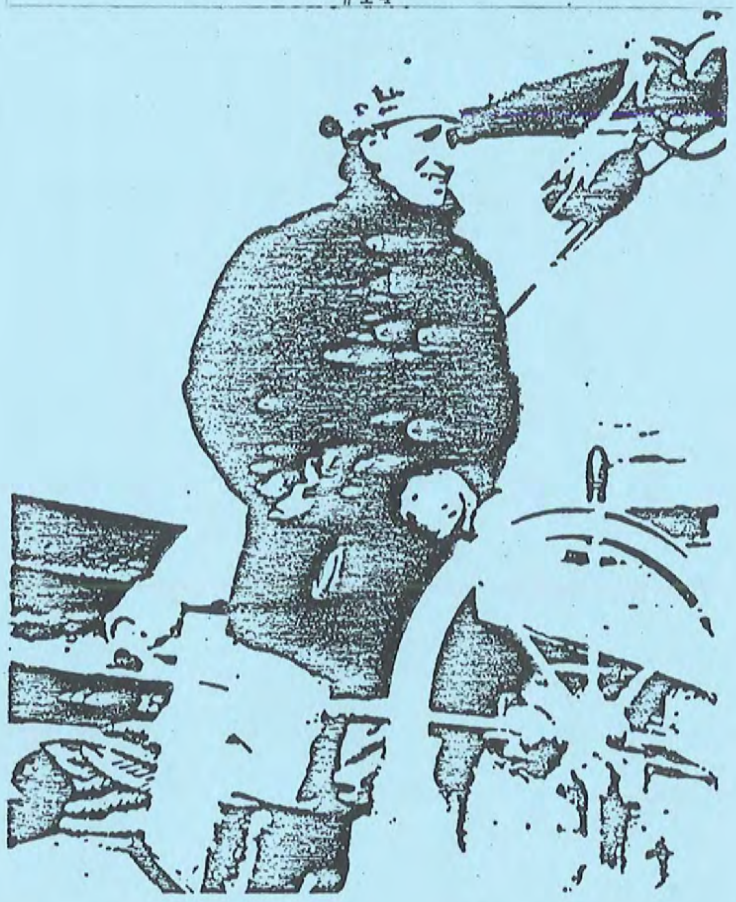
BIGELOW LABORATORY FOR

OCEAN SCIENCES

WEST BOOTHBAY HARBOR

MAINE, 04575, U.S.A.

Technical Report
#14



Henry Bigelow

BIGELOW LABORATORY
for
OCEAN SCIENCES

Abstract

Particulate protein measurements were made at seven stations in the Gulf of Maine using a new dye binding method. Measurements were made on cell-free homogenates. Protein concentration ranged from 2 to 212 $\mu g \cdot l^{-1}$ (0.02 to 0.68 $\mu g\text{-at } N \cdot l^{-1}$) and averaged 58 $\mu g \cdot l^{-1}$. ETS activity and chlorophyll were significantly correlated with protein concentrations.

INTRODUCTION

Particulate matter in the sea is of interest to oceanographers for it represents both living material (plankton) and non-living material (detritus) which may act as a food source for bacteria, zooplankton, fish or, if settled out, the benthos. Much of the organic fraction of the particulate is proteinaceous including the living organism (Riley, 1970). Furthermore, the vast majority of particulate nitrogen found in the sea is in the form of protein or peptide (Packard and Dortch, 1975).

The microplankton consists not only of phytoplankton but also bacteria and microzooplankton. Therefore, while chlorophyll-a specific respiratory electron transport system (ETS) activity or respriation may be useful and quite appropriate in euphotic zone measurements (Setchell and Packard, 1979) in deeper water or for zooplankton or bacterial studies another measure of biomass is required. Garfield et al., (1979) have utilized particulate protein as a basis for normalizing ETS activity in deep sea samples. The fluorescamine method of protein analysis (Packard and Dortch, 1975) used by Garfield et al., (1979) is extremely sensitive. It suffers, however, from difficulties in the blanks as well as being incompatable with the ETS homogenate buffer reagents and thus requires that a separate sample be filtered.

In seeking a method of protein analysis which can be used on the homogenates used for ETS measurements or other biochemical assays, several factors must be considered: 1. a broad range of sensitivity allowing analysis of extremely low concentrations in deep sea samples as well as high concentrations found in phytoplankton or zooplankton samples;

2. relative insensitivity to reagents in the ETS homogenate; 3. simplicity such that it can be used on many samples with a minimum of manipulation; 4. speed, so that samples can be processed and the results obtained within a few hours in order to facilitate interaction with colleagues at sea, and; 5. compatibility with shipboard operations, including reagents which are relatively mild and are without noxious or toxic vapors.

In this paper I describe the application of the Coomassie Brilliant Blue G-250 dye-binding assay of Bradford (1976) to the particulate matter in seawater in the form of ETS assay homogenates. Field data from the Gulf of Maine for protein and its relationship to ETS activity. Particulate nitrogen and chlorophyll are also discussed.

Acknowledgements

My thanks to F.D. King and T.T. Packard for helpful discussions during the experimental phase and in preparation of this paper. Additionally, I thank R.C. Dugdale and G.S. Grunseich for the opportunity to participate in the cruises, the Captain and Crew of R/V CHALLENGE for their cooperation, D. Dombrowski who prepared the manuscript, J. Rollins who executed the figures, G.S. Grunseich generoulsy provided the particulate nitrogen data. This work is Contribution No. 79-023 from Bigelow Laboratory for Ocean Sciences and was supported by Contract No. N00014-76-C-0271 from the Office of Naval Research to T.T. Packard.

Materials and Methods

Seawater samples were taken at stations in the Gulf of Maine during a series of day cruises aboard the R/V CHALLANGE (Fig. 1.). Water was obtained either from a vertical profiling submersible pumping system (integrated interval samples) or from 30 l Niskin bottles closed at discrete depths. Subsamples of two or four l were filtered and assayed for ETS activity. A portion of the cleared supernatant of the centrifuged homogenate was promptly frozen and returned to the laboratory for protein analysis. A complete summary of these data will be found in Jones and Setchell (1980). Chlorophyll was assayed by the technique of Yentsch and Menzel (1963), though no correction for phaeopigments was made.

Additionally, fine determination of particulate nitrogen were made (Grunsiech, unpublished data) on samples from the same batch or protein measurements were made. A batch pyrolysis method was used ( ).

Protein was assayed using a modification of the Coomassie Brilliant Blue dye-binding method, originally described by Bradford (1976), with bovine γ-globulin as standard. My modifications are similar to those described by Spector (1978) in which the volume of dye reagent to protein sample is varied depending upon the level of sensitivity needed. For simplicity and consistency, I chose to use commercially prepared dye reagent and protein standard (Bio-Rad Laboratories, Richmond, CA., U.S.A.). The dye reagent was diluted to the concentration used by Bradford (1976) and Spector (1978); 1 part concentrated commercial dye: 4 parts dilluted water.

For high concentration of protein 0.1 ml of standard or sample was reacted with 2.0 ml diluted dye reagent; for low concentration 0.4 ml of sample or standard was reacted with 2.0 ml dye reagent. Absorbance of the samples were measured at 595 nm on a Beckman ACTA-CII dual beam spectrophotometer using 1 cm cuvettes against a reagent blank consisting of ETS grinding buffer and dye reagents in the same proportion as the assay mixtures.

The Coomassie Blue dye binding assay is relatively insensitive to many common reagents which seriously interfere with protein determination by other methods (Bradford, 1976), including the non-ionic detergent Triton X-100^{R} present in the ETS homogenate buffer. However, in the high sensitivity assay the ratio of 0.4 ml sample to 2.0 ml dye raises the concentration of Triton X-100^{R} to the point where serious interference occurs. Holloway (1973) has shown that Triton X-100 can be removed with neutral styrene divinyl benzene beads (Bio beads, SM-2, Bio-Rad Laboratories, Richmond, CA, U.S.A.) in either a batch or column method. I chose the batch method because of the fact that several samples are usually to be run at once and to avoid the dilution inherent in column methods which would seriously reduce assay sensitivity so important in oligotrophic or deep sea samples. Approximately 0.5 gm of moist pretreated beads (Holloway, 1973) are added to the cleared ETS homogenates (or standards). The vials are then agitated on a vortex mixer with a shaker head attachment. The vigorous agitation provided and the relatively low level of detergent ($_{ca}$0.2%v/v) require only about 20 minutes to sufficiently lower the detergent concentration to the pount at which foaming is no longer noted. The assay can then be run.

I have found that the assay standard curve for the high concentration assay is linear between approximately 40 and 950 µg protein per ml of sample (i.e. 4 and 95 µg·0.1 ml^{-1} in the reaction mixture). The low concentration assay after pretreatment to remove the detergent is linear between approximately 1.5 and 35 µg protein · ml^{-1} sample. (Fig. 2A and B).

Alternatively, one may achieve the excellent sensitivity of the low concentration assay by using the same ratio of sample or standard to dye reagent as in the high concentration assay (o.1ml:2ml) and measuring the difference in absorbance between 465nm (the absorbance maximum of the unreacted dye) and 595nm (the absorbance maximum of the bound dye). The spectrophotometer is zeroed at 465nm with sample and reagent blank in the machine. The absorbance at 595nm is then used to calculate protein concentration (Beardon, 1978). Fig. 2C shows a typical standard curve for high sensitivity derived from this method. This simplification obviates the need for pretreatment and will be utilized in the future.

Calculation of protein concentration (in µg · l^{-1} γ-globulin equivalents) is from the linear regression equation of protein standard against absorbance at 595nm (vs. reagent blank), with correction for the volume of the ETS homogenate and the volume of seawater sampled:

$$\text{Protein} = \frac{(m^{A}_{595} + b) \times HV)}{FV}$$

Where m = slope of the regression equation $^{A}_{595}$ = the sample absorbance (vs. reagent bland) b = intercept, HV = homogenate volume and FV µ volume of seawater filtered.

It is important to note that, as with most standard curves, those for the protein assay show the protein on the x axis and absorbance on the y axis. For calculating the protein content of a sample from the absorbance of the dye-protein solution, the correct equation is the regression of protein on absorbance (x on y) (Simpson et al., 1960) not the more conventional regression of absorbance on protein (y on x). Though the difference would be small with the strong correlation observed, unless the correlation is perfect (r = 1.00. . .0) there is a finite difference between the two regressions which will affect the calculations. Since the effort is the same to calculate either regression, and the correlation co-effecient is identical, it is prudent to eliminate this inviting but avoidable systematic error.

Additionally, Chiappelli et al., (1979) have shown that standard curves can be constructed either by preparing a series of dilutions of the protein standard or by simply diluting the assay mixture with dye reagent once the color has developed. I have chosen to use the serial dilution of protein, rather than the dye reagent method. A single error in the preparation of the first standard for the dye dilution method can invalidate an entire day's work. Such a dilution error in the former method generally will be seen as an unusual outlying point which can be re-run easily.

Intercalibration of Dye Binding assay with the Lawry Assay

In order to document the usefulness and comparability of protein measurements made with the Coomassie Blue assay with those made by

another standard method, a series of measurements were made with my Coomassie Blue method and the Lawry technique (Lawry et al., 1953). Four 1 seawater samples were obtained from the Bigelow Laboratory dock (W. Boothbay Harbor, Maine, U.S.A.), filtered and prepared first on the field sampler. Protein was estimated by the two methods using bovine 8-globulin or standard. Results are shown in Fig.

Results

The partuculate protein measured at 7 stations and several depths ranged from 2.0 to 212. μg $\cdot l^{-1}$ (0.02 to 2.43 μg-at N $\cdot l^{-1}$) with a mean of 58.1 μg $\cdot l^{-1}$ (0.68 μg- N $\cdot l^{-1}$) ETS activity and total chlorophyll pigments ranged from 0.34 to 9.94 $\mu l O_2 h^{-1}$. -1 and 0.31 to 4.59 μg $\cdot$ l^{-1} respectively. (Table 1).

ETS activity and protein concentration were significantly correlated, (Fig. 3) though only approximately 58% (r^2 = 0.58) of the variations in ETS activity is accounted for by variations in protein concentration. Protein N (calculated from protein) was, similarly, strongly correlated with total chlorophyll pigments (Fig. 4) with approximately 71% (r^2 = 0.71) of variations in protein N explainable by variations in chlorophyll. The dispersion in the ETS to protein relationship is likely a result of the non-living component of the particulate matter. The variation in the chlolophyll and protein relationship is partially due to chlorophyll per cell variations with light intensity and other environmental factors, no correction for phaeopigments, and zooplankton and bacteria included in the protein measurement.

Though only five determination of particulate nitrogen were made on samples for which protein data were available, nevertheless, they are highly correlated with an intercept consistent with a significant proportion on non-protein cellular nitrogen (Fig).

Discussion

THE PROTEIN ASSAY

The Coomassie brilliant blue protein assay has been tested and modified for specific purposes by numerous workers since its introduction. Recently, Kochert (1978) has described a version for phycological work in the laboratory. However, no reports have been found in which the assay has been applied to marine particulate matter in field studies.

My modified version of Bradford's (1976) protein assay meets the criteria set forth in the introduction. In addition, the method has proved to have excellent precision (Table). Though less sensitive than fluorometric protein analysis, the dye binding assay possess a significant advantage. The fluorogenic reagents available for protein measurement, fluorescamine and o-phthalaldehyde, both react with primary amines, (Udenfriend et al., 1973, Roth, 1971) which includes proteins, small molecular weight peptides and other substances of biological importance found in cell-free homogenates. Gradford (1976 and personal communication) has observed that Coomassie brilliant blue specifically binds protein but not small peptides, not is there evidence that combines with other biologically important material. Thus, while the dye binding assay is less sensitive than the fluorometric methods, one may specifically measure protein with several times the sensitivity of the Lowry (Lowry et al., 1953) method (Bradford 1976) without non-protein artifacts.

Since the introduction of the method by Bradford (1976) controversies concerning it have arisen in the literature. The first of these has to do with the choice of protein standard. Both product literature (Bio-Rad Laboratories, 1979) and the scientific literature (Pollard, et al., 1978) point out that the best standard is a purified solution of the protein under study. This desirable approach is not available to persons working on complex biological mixtures such as plankton homogenates.

The second controversy appears to be a corollary of the first. There are significant protein to protein variations in both the standard Lowry method and the Coomassie Blue method, though I have found that bovine γ-globulin, used in this study, and α-casein used by Packard and Dortch (1975) and Garfield et al,, (1979) with the flourecamine assay yeild identical standard curves with Coomassie Blue.

Of particular note in the unusually high absorbance produced by Coomassie Blue when bound to bovine serum albumin (BSA) commonly used on a standard for the Lowry assay, and certain other proteins (Cytochrome-C myoglobin, hemoglobin). To use BSA or a standard for the Coomassie Blue assay in cases where the material to be analyzed is not known to have similar high absorbance when found is to systematically underestimate protein content in those samples. Indeed, Chiappelli et al., (1979) have observed just such a systematic difference in apparent protein content in their comparison study of the Lowry and Coomassie Blue assays. Conversily, Pollard et al., (1978) found that bovine adrenal subcellular fractions were more like BSA 8-globulin in their reactivity to Coomassie Brilliant Blue.

Notwithstanding the differences, I have found our results comparable with protein measurements made by other workers using other methods and standards (see below). Other workers have found the Coomassie Blue method quite useful on materials as diverse as adrenal gland subcellular fraction (Pollard et al., 1978) and potato tuber extracts (Snyder and Desborough, 1978). Chiapelli et al., (1979) summarize their feeling, and mine: "Despite (the objection due to protein-protein difference). We decided to investigate the possibility that with a complex mixture of protein, such as encountered in many experimental situations, differences in dye binding among individual proteins might be minimized in summation....." (emphasis theirs).

Intercalibration

The results of the intercalibration study demonstrate that the modified Coomassie Blue assay described here is an effective means of quantifying protein in marine particulate matter.

In conclusion, I suggest that the Coomassie Blue technique described here is quite useful for oceanographic work, particularly when large numbers of samples are to be run. Requiring only a single reagent addition and but 3-5 minutes for color development, it is amenable to automation or semi-automation, and may be of great potential use for survey work as well as providing an estimate of biomass in ETS and other cell free homohenates.

Field Data

Though the data suite from the Gulf of Maine presented here is small and the purpose of this communication is, largely, to demonstrate the usefulness of the described assay for oceanographic work, nevertheless it is possible to draw some conclusions about the protein or an indication of biomass. All of the commonly used measures of plankton biomass have inherent limitations. The variation in ETS/chl-a observed by Setchell and Packard (1978, 1979) are not unlike the ETS/protein variation seen by Garfield et al., (1979) or the ETS/protein and Chl/protein variation reported here.

Dortch (1980 and submitted ms.) has noted that total cellular nitrogen and cellular protein are also quite variable in their relative properties. One may deduct that the practice of considering phytoplankton particulate nitrogen and particulate protein interchangeable is not defensible.

Further, in studies in which nitrogen surfaces was varied in three marine diatoms, Dorch (submitted) observed that protein, as measured by the Lowry method, was less variable per unit cell volume, than was protein determined by the fluorescomine technique. This latter method measures not only protein but other primary as well. Bohlen, et al (197) cautions that protein as measured by both the Lowry method and fluorescomine may systematically overestimate actual protein content when appropriate corrective measures are taken. Since Coomassie Blue selectively binds to protein and not small peptide or amines this error is avoided. Even though variation in measurements normalized to protein are still to be expected owing to species-species difference, detection, physiological state, etc. and analytical method which minimized any one of these while not exagerating another is helpful.

References

Beardon, J.C. Jr., (1978). Quantitation of submicrogram quantities of protein by an improved protein-dye binding assay. Biochem. Biophys. Acta., 533: 525-529.

Bio-Rad Laboratroies, (1979). Bio-Rad protein assay instruction manual. (1979).

Bradford, M.M., (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein using the principle of protein-dye binding. Anal. Biochem,, 72: 248-254.

Chiapelli, F., Vasil, A., and Haggerty, D.F., (1979). The protein concentration of crude cell and tissue extracts as estimated by the method of dye-binding: Comparison with the Lowry method. Anal. Biochem., 94: 160-165.

Garfield, C., Packard, T.T., and Codispoti, L.M. (1979). Particulate protein in the Peru Upwelling System. Deep Sea Res., 26: 623-639.

Grunseich, G.S., R.C. Dugdale, N.F. Breitner and J.J. MacIsaac. (1980). Sample conversion, mass spectrometry and calculation for ^{15}N analysis of phytoplankton nutrient uptake. C.U.E.A. Tech. Rep. no. 44, pp 89.

Kenner, R.A., Ahmed, S.I., (1975). Measurements of Electron Transport activities in marine phytoplankton. Mar. Biol., 33: 119-127.

Lowry, O.H., Rosebrough, N.J., Farr, A.L., and Randall, R.J., (1951). Protein measurement with the folin phenol reagent. J. Mar. Res. 33: 265-275.

Owens, T.G., King, F.D., (1975). The measurement of respiratory electron transport system activity in marine zooplankton. Mar. Biol., 30: 27-36.

Packard, T.T., (1971). The measurement of respiratory electron transport activity in marine phytoplankton. J. Mar. Res., 29: 235-244.

Packard, T.T., Dortch, Q., (1975). Particulate protein-nitrogen in North Atlantic surface waters. Mar. Biol., 33: 347-354.

Pollard, H.B., Menard, R., Brandt, H.A., Pazoles, C.J., Crenty, C.E., and Roma, A., (1978). Application of Bradford's protein assay to adrenal gland subcellular fraction. Anal. Biochem., 86; 761-763.

Riley, G.A., (1970). Particulate matter in seawater. Adv. Mar. Biol. 8: 1-118.

Setchell, F.W., and Packard, T.T., (1979). Phytoplankton respiration in the Peru Upwelling. J. Plankton. Res., 1: 343-354.

Synder, J.C., and Desborough, S.L., (1978). Rapid estimation of popato tuber total protein with Coomassie Brilliant Blue G-250. Theo. Appl. Genet., 52: 135-139.

Spector, T., (1978). Refinement of the Coomassie Blue method of protein quantitation. Anal. Biochem., 86: 142-146.

Yentsch, C.S,, Menzel, D.W., (1963). A method for the determination of phytoplankton chlorophyll and phaeophytin by fluorenscence. Deep Sea Res., 10: 221-231.