Modern Methods in Protein Chemistry

Review Articles

Volume 2

Editor H. Tschesche

W DE G

de Gruyter

Modern Methods in Protein Chemistry

Volume 2

Review Articles

including those from an
International Conference held in
Bielefeld, F.R. of Germany, June 1-2, 1984

Editor
Harald Tschesche

Walter de Gruyter · Berlin · New York 1985

Editor

Harald Tschesche, Dr. rer. nat.
Professor für Biochemie
Lehrstuhl für Biochemie
Fakultät für Chemie
Universität Bielefeld
D-4800 Bielefeld, F.R. of Germany

CIP-Kurztitelaufnahme der Deutschen Bibliothek

Modern methods in protein chemistry: review articles.
Vol. 2 Review articles including those from an international conference held in Bielefeld, FR of Germany, June 1 – 2, 1984.
– Berlin; New York: de Gruyter, 1985.
ISBN 3-11-010180-7 (Berlin . . .)
ISBN 0-89925-079-3 (New York)

ISBN 3 11 010180 7 Walter de Gruyter · Berlin · New York
ISBN 0-89925-079-3 Walter de Gruyter, Inc., New York

Printing: Gerike GmbH, Berlin. – Binding: Dieter Mikolai, Berlin. – Printed in Germany.

Modern Methods in Protein Chemistry
Review Articles Volume 2

PREFACE

This book is a continuation of the series of articles published in 1983 in a previous volume by de Gruyter Publishers*. It is again the intention of the editor to attempt a survey of the present status in the different fields of analytical methods available to the protein chemist for analysing and characterizing a distinct protein. The editor hopes that the book will satisfy the current need of all those involved in the aims of protein chemistry to keep up with the rapid development and perfection of all analytical and preparative methods. The articles review recent methodological progress, present the most advanced applications and give a number of key references. The references in particular should enable the reader to orient himself among the literature and adapt the method to his own particular problem.

Most of the papers were presented at the Conference on "Modern Methods in Protein Chemistry" held at the Center of interdisciplinary Research (ZiF) in Bielefeld, June 1-2, 1984. This meeting was kindly supported by the Gesellschaft für Biologische Chemie and organized for the study group of chemical protein analysis. Further support was provided by: the Center of interdisciplinary Research, Applied Biosystems GmbH, Bayer AG, Behringwerke AG, Boehringer Mannheim GmbH, Ciba-Geigy, Degussa Pharma Gruppe, Diamalt AG, Du Pont de Nemours GmbH, Hoechst AG, LKB Instrument GmbH and E. Merck.

The increasing demand for micro and submicro scale analytical procedures already observed at the 1982 meeting in Damp/Kiel was fully confirmed. This trend is reflected again in many of the articles in this volume. It is the hope of the authors and the editor that the present articles will serve to extend the present knowledge and methodological experience of the reader, thereby stimulating scientific progress and saving unnecessary time and effort.

Bielefeld, May 1985 Harald Tschesche

*Modern Methods in Protein Chemistry. Review Articles. Editor: H. Tschesche 1983. X, 464 pages. Hardcover. ISBN 3 11 009514 9

CONTENTS

MODERN MICROMETHODS

Volker Neuhoff
Max-Planck-Institute for Experimental Medicine
D-3400 Göttingen

INTRODUCTION

Micromethods do not seem to be widely applied in neurochemical analysis, perhaps because of a widespread prejudice that micromethods are only useful for "microminded", technically perfect experimenters. In addition, there may be worries about the reproducibility of micro techniques. A microgel, however, with many clearly separated and well-defined bands, each representing some nanograms of protein (see Fig. 10), is rather impressive when seen for the first time, especially when it is realized that the time taken for separation, staining, and destaining is only about one-tenth of the time necessary for the equivalent procedure using a macroscale method. A saving of experimental time is inherent in most of the micromethods described in some detail in this chapter. Such micromethods are also reproducible if they are performed correctly. The use of micromethods is therefore to be recommended even when the amount of material to be analyzed is not limited. Furthermore, now that it is harder to get sufficient funds for relevant research, micromethods are useful because the equipment needed for the analysis is not expensive.

The correct procedure for a micromethod is no more difficult to learn than is any other method. The time needed to learn any method is strongly dependent on the handiness and experience of the experimenter, and this is the same for learning a micro- or a corresponding macromethod. Obviously,

---

Reproduced with permission from Neuhoff, V.: Selected Micromethods for use in Neurochemistry, in: Handbook of Neurochemistry, 2nd Edition, Vol. 2 (A. Lajtha Ed.). Plenum Publishing Corporation New York, 1982 pp. 349-395.

Modern Methods in Protein Chemistry, Volume 2

every experimenter will take some time to be satisfied with the application of a micromethod and to clearly under stand its critical features and limitations. But these are points that apply to every method. Generally, there are no arguments against micromethods, and it is surprising that in biochemical research, where so often only small sample volumes are available, the application of micromethods is not more widespread. On the other hand, it is not surprising that progress in microscale methods is mostly connected with neurochemical applications. For example, the first application of polyacrylamide gel electrophoresis on the microscale was carried out in 1964 when Pun and Lombrozo (1) fractionated brain proteins. Since then, microscale methods for determination of RNA (2,3) and RNA base composition (4,5), measurement of enzyme activity and substrates of single neurons (6-12), mass determination (13,14), micro-flame photometry (15,16), picoliter volumes (17), microchromatography on thin-layer plates for dansyl amino acids (18-21), phospholipids (22-24), isoenzymes (25), glycoproteins, (26) and sugars (27), as well as other auxiliary methods such as microhomogenization (28) have mostly come from neurochemical laboratories.

This chapter, because of the limited space available, will neither review all micromethods possible nor describe techniques for preparation of minute amounts of defined tissue material or single nerve cells (see ref. 29). The methods selected for description are those with which the author has personal experience and therefore the necessary critical competence to describe. Very seldom are micromethods developments of their own. Usually they are deduced from macro procedures adapted to a micro scale, thereby more or less automatically increasing the sensitivity by a factor of between 10 and 1000. Most normal methods can be rather simply converted to micro versions, although one has to accept that every method will have its special merits and disadvantages.

PROTEIN DETERMINATION WITH MICROLITER VOLUMES

The quantitative determination of protein is a common prerequisite for many biochemical analyses. The different methods currently available often have a drawback in that agents used for preparation of biological tissues, e.g., sodium dodecyl sulfate (SDS), Triton X-100, NP 40, mercaptoethanol,

urea, interfere with the analysis. In such cases, a protein precipitation step is often required, which is not only time consuming but also uncertain with respect to quantification. The volume routinely used for protein determinations is often rather large, and for microdeterminations, special and often cumbersome modifications are necessary. The method described here avoids all of these disadvantages and furthermore allows quantitative determination of small amounts of protein, even if the volume used (in the range between 1 and 5 µl) is unknown.

The method is, in principle, a spot analysis, a type of analytical method first introduced in 1859 by H. Schiff. Such methods had their peak approximately 40-50 years ago but are nowadays almost forgotten. However, spot methods still have very many advantages (30). One major point is the fact that by spotting sample solutions onto a suitable layer, the contents will be concentrated over a very small area. During fixation, staining, and washing out of the excess stain, all interfering components not firmly bound to the sample being analyzed, will be almost completely washed out. With this protein determination procedure, as described recently (31), it is possible to determine the protein concentration in a microliter volume when neither the volume nor the protein content in that volume are known.

Performance of the Protein Determination

Cellulose acetate strips commonly used for electrophoresis are used in this procedure (see Appendix, Scheme 1 for details). Prior to use, the acetate strips are stored in a moist chamber to facilitate the application of the sample. If a series of determinations is to be performed, the sample number may be indicated simply by cutting the corners of the acetate layer for the first five samples or by combining cut corners with arrowlike cuts on the sides. The number of strips that are stained and destained together is not critical. Sample application, staining, and destaining are done at room temperature, and commercially available 0.5-, 1-, 2-, or 5-µl capillaries are used. Cleaning of the capillaries (32) prior to use is recommended. The capillaries are filled by capillary attraction simply by being dipped into the solution to be analyzed. Complete filling has to be confirmed, and for routine determinations, 2-µl capillaries have been found

to be the best. The standard deviation for a given protein con centration depends on the capillary volume as follows: 0.5 µl, 6.5%; 1 µl, 2.5%; 2 µl, 1.5%; 5 µl, 2.4%; 10µl, 7.5%. In most instances, immediately after sample application, the acetate strip is transferred to a petri dish containing the stain dissolved in methanol/acetic acid. Prefixation of a protein spot in methanol/acetic acid before staining results in a loss of 70-80% of the dye-binding capacity. In contrast, drying at room temperature or with a hot air stream has no influence on quantitative staining, thereby allowing for repeated sample application in the case of very dilute protein solutions.

Evaluation with Densitometry

If protein spots on acetate paper are round, any densitometer can be used for evaluation of Amido black-stained spots after the sample-containing strip is made transparent (31). Four typical densitograms of spots, each representing 2 µl of bovine serum albumin (2 µl of a solution containing 1 mg protein/ml), are shown in Fig. 1. If a sample is repeatedly measured ten times (31) and is repositioned in the equipment for measurement each time, the coefficient of variation is 0.9%. The integral of the area recorded is equivalent to the amount of protein in the spot (compare Fig. 2a). In cases in which it is difficult to obtain an ideal round spot during sample application, e.g., if the sample contains high concentrations of sucrose, glycerol, urea, etc. use of Hoechst 2495 (a fluorescent benzoxanthene derivative) is recommended for staining followed by fluorometric evaluation after elution of the stain (see below) or spot fluorometry in situ (33). For optical reasons, evaluation by densitometry across the diameter is not possible with irregular spots, and complete scanning of the spots with a

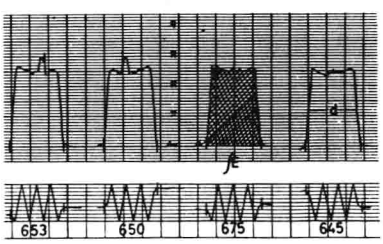

Fig. 1. Densitograms of four spots stained with amido black and representing 2 µg protein each. The densitograms were obtained with a Zeiss Gel Scanner (ZK4) and an adapter for micro-gel evaluation (123). ∫E, integrated absorption; d, spot diameter.

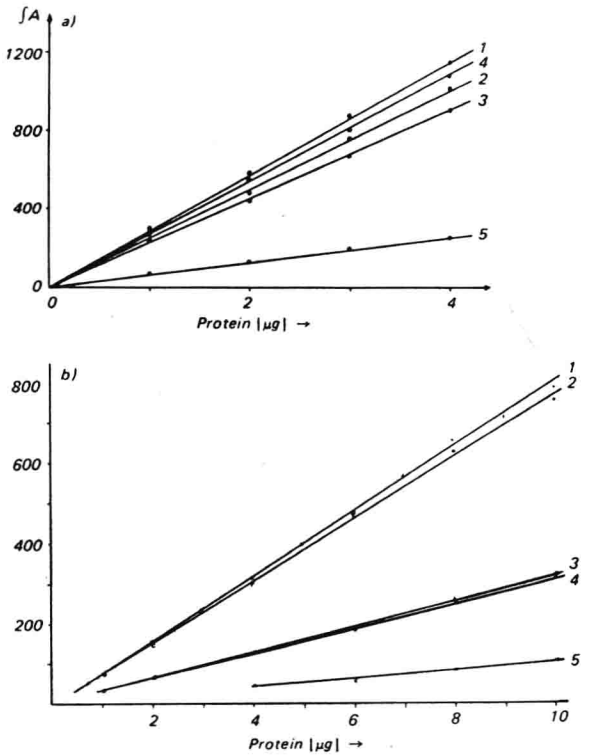

Fig. 2. Calibration curves for different proteins obtained by densitometric evaluation after amido black staining (a) and staining with Hoechst 2495 and spot fluorometry *in situ* (b). The curves are calculated according to $y = b \cdot x^a$ ($\log y = \log b + a \cdot \log x$) r, correlation coefficient; n, number of spots evaluated per curve; abscissae, µg protein; ordinate in a, integrated absorption; in b, arbitrary units. (1) Bovine serum albumin: (a) $r = 0.9989$, $n = 15$; (b) $r = 0.9928$, $n = 41$. (2) Human serum albumin: (a) $r = 0.9975$, $n = 15$; (b) $r = 0.9946$, $n = 24$. (3) Transferrin: (a) $r = 0.9989$ $n = 15$; (b) $r = 0.9725$, $n = 23$. (4) Globulin: (a) $r = 0.9985$, $n = 15$; (b) $r = 0.995$, $n = 24$. (5) Bovine myelin: (a) $r = 0.9935$, $n = 15$; (b) $r = 0.9306$, $n = 14$. Note not only the differences in stainability of the different proteins but also the differences between the two stains.

scanning microscope is prohibitively expensive and not acceptable as a routine method. Therefore, the evaluation is performed with a simple spot fluorometer (33) whereby the fluorescence of the whole spot is measured as an integral and is therefore independent of the form of the spot.

Photometric and Fluorometric Evaluation

If none of the abovementioned instruments is available for evaluation of stained spots, quantitative determination can easily be performed with a

normal spectrophotometer for amido black staining after complete solubilization of the acetate strip containing the stained spot in a suitable volume of dioxane, N,N-dimethylformamide, or dimethylsulfoxide. Instead of a photometer, a fluorometer can be used after staining of the protein spots with Hoechst 2495 and solubilizing with the same three solvents; alternatively, elution of the chromophor Hoechst 2495 from the protein spot with 1% NH_4OH followed by fluorometry is possible.

Fluorescence is measured, in arbitrary units, between the peak maximum and the corresponding blank value. If the value is calculated according to the equation

FI = arbitrary units (sample - blank) x volume/amplification

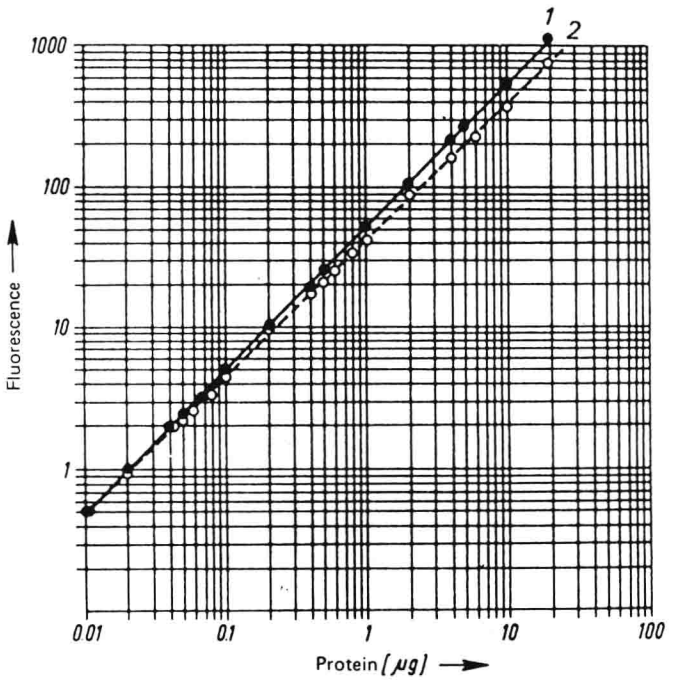

Fig. 3. Calibration curves for bovine serum albumin obtained after staining with Hoechst 2495 and either (1) elution of the stain in 15% NH_4OH or (2) solution of the acetate strip containing the sample in dimethylsulfoxide. Abscissa, µg protein; ordinate, arbitrary units. Fluorescence was measured with a Turner spectrophotometer with an excitation wavelength of 430 nm (for dimethylsulfoxide) or 425 nm (for NH_4OH) and an emission wavelength of 475 nm. The measured volumes were 2 ml for 0.01–2 µg and 5 or 15 ml for the larger amounts. The curves are calculated according to $y = b \cdot x^n$. Curve 1: $\log y = \log 51.8 + 1.01 \log x$; $r = 0.9994$; $n = 36$. Curve 2: $\log y = \log 41.9 + 0.97 \log x$; $r = 0.9995$; $n = 34$.

complete independence of volume and amplification of the instrument are reached, and a calibration curve over a wide concentration range can be prepared as shown in Fig. 3. If measurements are to be performed in the lowest concentration range possible (0.01-0.1 mg protein/ml), the precautions for fluorometric measurements described previously (34) should be followed. In this case, the lowest measuring value should be approximately five times that of the blank.

The calibration curves shown in Fig. 2 are obtained with different sample volumes in the range between 0.5 and 5 µl. Further, the dye binding of different proteins (bovine and human serum albumin, γ-globulin, transferrin, and myelin) with amido black (a) and Hoechst 2495 (b) is shown in Fig. 2. As expected, the slope for each protein is different, but there is no essential difference between the two stains. The very low binding of the myelin preparation is because of its low protein content (approximately 20%) (35). Since drying of a spot has no influence on the dye-binding capacity, several sample volumes can be applied one after another and dried between applications. The calibration curves shown in Fig. 3 were prepared in this way. Protein solutions containing 0.01, 0.1, 1.0, and 10 mg/ml were spotted repeatedly using 1-µl, 2-µl, or 5-µl capillaries. Spots were then

Table I
Compounds Tested for Their Reaction with Amido Black 10B and Hoechst 2495

Compound	Reaction with	
	Amido black	Hoechst 2495
Urea	Negative	Negative
Glucose	Negative	Negative
Sucrose	Negative	Negative
Ammonium sulfate	Negative	Negative
Chloral hydrate	Negative	Negative
Dodecyl sulfate	Negative	Negative
Triton X-100	Negative	Negative
NP 40	Negative	Negative
Mercaptoethanol	Negative	Negative
Desoxycholate	Negative	Negative
tRNA (2 mg/ml)	Negative	Positive
sRNA	Negative	—
Cholesterol	Negative	Negative
Egg kephalin (saturated solution)	Positive	Negative
Egg lecithin	Negative	Negative
Ampholine	Positive	Positive

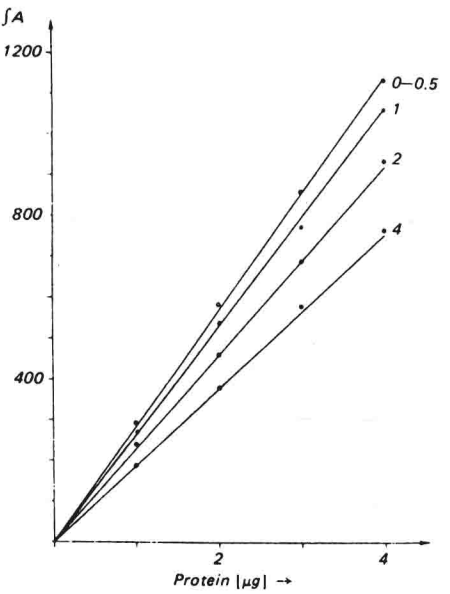

Fig. 4. Relationship between protein concentration and the integrated absorption after amido black staining for different concentrations of dodecyl sulfate. Abscissa, µg protein; ordinate, integrated absorption. Each curve was calculated according to $y = b \cdot x^a$ on the basis of 12 evaluated spots. The correlation coefficients are 0.9995, 0.9985, 0.9985, and 0.9979 for 0–0.5%, 1%, 2%, and 4% dodecyl sulfate present in the sample, respectively. Bovine serum albumin was the protein standard. Note that 0.5% dodecyl sulfate has no influence on the stainability with amido black; staining with Hoechst 2495 is also not affected by dodecyl sulfate (results not shown in this Figure).

stained with Hoechst 2495 and, after destaining, were solubilized in 2 ml dimethylsulfoxide (Fig. 3, curve 2) or eluted in 2 ml 1% NH_4OH and evaluated fluorometrically (Fig. 3, curve 1).

Interference in the Method

Compounds tested for their influence on the staining procedure are listed in Table I. High concentrations of egg cephalin (saturated solution) can be stained with amido black but not with Hoechst 2495. High concentrations of tRNA (2 mg/ml) are weakly stained with Hoechst 2495 but not with amido black. None of the listed compounds in a protein solution has any influence on the quantitative staining. The influence of dodecyl sulfate on amido black binding to protein is shown in Fig. 4. The presence of 0.5% dodecyl sulfate has no effect, but 1%, 2%, and 4% dodecyl sulfate decreases the staining by 6.2%, 17,4%, and 33,0%, respectively, in comparison with the same protein concentration (bovine serum albumin) without dodecyl sulfate.

A stoichiometric relationship between protein concentration and staining

can be observed. This is demonstrated in the calibration curves in Fig. 4. This stoichiometry, on the other hand, may be use ful for the determination of an unknown dodecyl sulfate concentration. The presence of mercaptoethanol in a sample solution has no further effect. In contrast to amido black, the binding of Hoechst 2495 to all of the proteins studied so far is not influenced by dodecyl sulfate (0.5-2%) or dodecyl sulfate plus mercaptoethanol (1% each). Thus, turbid protein solutions containing unsolubilized particles or even protein sediments can easily be converted to clear solutions suitable for protein determination with Hoechst 2495 by the addition of a suitable volume of 4% SDS/conc. NH_4OH (1:1 v/v), e.g., 100 µl protein solution plus 10 or 20 µl SDS/NH_4OH.

Table II
Differences for Known Amounts of Different Proteins and Brain Extracts Measured According to Lowry and with Spot Analysis: Values Are Related to the Corresponding Standard Calibration Curve Prepared with Bovine Serum Albumin

Protein	Method using amido black[31]	Method of Lowry *et al.*[36]
Bovine serum albumin	0	0
Human serum albumin	−14.9%	−15.6%
γ-Globulin	− 3.3%	+15.6%
Transferrin	−19.6%	−14.9%
Water-soluble proteins from brain tissue	1.72 mg/ml (1.4% S.D.)	2.12 mg/ml (2.1% S.D.)
Brain total particulate fraction in 1% SDS	2.12 mg/ml (1.72% S.D.)	2.62 mg/ml (2.9% S.D.)

When solutions with a known concentration (1 mg/ml) of bovine serum albumin, human serum albumin, γ-globulin, and transferrin are assayed according to Lowry et al. (36) or with the amido black method described here, and the known amount of protein (by weight) is related to the appropriate calibration curve using bovine serum albumin as a standard, differences are observed for both methods (as shown in Table II). For human serum albumin and transferrin, the negative difference is of the same order, but for γ-globulin, there is a negative difference of 3.3% for the amido black method and a positive difference of 15.6% after determination according to the method of Lowry et al. (36) Similar types of differences are also observed for protein determinations of biological material (Table II).