ACTA CHEMICA SCANDINAVICA 10 (1956) 1432—1439

A Procedure for the Determination of Proteins and Similar Compounds in Large Numbers of Fractions

BENGT v. HOFSTEN

Institute of Biochemistry, University of Uppsala, Uppsala, Sweden

Fraction collector work gives rise to large numbers of samples, and the chemical analysis of these necessitates methods which are sensitive but as simple as possible. Some of the current methods used when nitrogenous biological material is fractionated have been experimentally evaluated, and their relative merits are discussed.

A relatively simple procedure using a ninhydrin reaction is proposed. In this method a small sample from every fraction is first hydrolyzed with HCl at 120°, after which the acid is removed *in vacuo*. The ninhydrin reaction is then performed on a suitable aliquot of the residue. Useful technical arrangements and precautions are described, and the advantages and limitations of the method are discussed.

Fractionation work using chromatography, electrophoresis or counter current distribution gives rise to a great number of fractions, the analysis of which requires considerable time. The determination of biological or chemical activity in these fractions is often the main problem, but the activity is usually wanted as specific activity. In the case of fractionation of nitrogenous compounds, the specific activity is often expressed as activity per mg dry weight or nitrogen. Dry weight determinations of proteins is no easy matter and requires a comparatively large amount of material. In certain cases the simple method of Johnson [1] can be used to obtain an estimate of total organic matter. In this method, conc. H_2SO_4 and sodium dichromate of known strength reacts with the organic compounds in the sample. The sensitivity of the method is high but the accuracy is rather low, especially when interfering metals are present.

The determination of nitrogen according to Kjeldahl and conversion to protein by multiplication with a certain factor has long been the standard method for protein determinations. Besides the variable nitrogen content of different proteins, all other nitrogen containing substances interfere. Many different methods of precipitating the protein before the determination have been proposed, but it is often difficult to get a quantitative precipitate without co-precipitation [2]. Dialysis or ultrafiltration has been used extensively, but

some proteins tend to become adsorbed to the membranes, and neither precipitation nor dialysis is suitable for compounds of intermediate size such as peptides and certain enzymes.

Ordinary digestion of the sample and distillation of ammonia according to Kjeldahl is not practical with large numbers of fractions and, in addition, samples containing less than 0.1 mg N are difficult to analyse. A hypobromite titration is useful and easy to perform even on a few micrograms of nitrogen, provided small but usually constant errors can be tolerated. As modified by Harvey [3], the titration can be carried out directly in the digestion flasks or tubes without distillation, which is a definite advantage. It is thus possible to perform a large number of determinations in a comparatively short time, provided the approximate nitrogen content is known so that a suitable amount of hypobromite can be added. The method needs to be standardized on samples of known nitrogen content, and its pH-sensitivity is sometimes irritating. Boissonas and Haselbach [4] have used a sulfuric acid digestion followed by neutralization and a ninhydrin reaction to determine the nitrogen content in series of fractions. This elegant method is claimed to give good values in the 1—20 μg range, and it is certainly valuable when the total nitrogen content of fractions is wanted. For accurate determination of small quantities of nitrogen, the diffusion method of Conway [5] combined with the sealed-tube digestion of Greenbaum *et al.*[6] is certainly more exact.

When the available amount of protein is comparatively large, turbidimetric determinations may be useful. A procedure using trichloroacetic acid is very rapid and has been much used by Smith in his work on proteolytic enzymes [7] and by Stadtman *et al.*[8]. More than 20 μg protein per ml is needed for a determination and the precision of the method is rather low because of the inherent difficulties of all turbidimetric determinations.

The method of Greif [9] modified by Nayyar and Glick [10] using bromosulfalein has been developed particularly for use in histochemistry. The acid bromosulfalein reagent is used to precipitate the protein, and the residual concentration of the dye is measured at an alkaline pH. The method is useful down to very small amounts of protein and the specificity is high. In fractionation work, it should be useful in distinguishing between proteins and low molecular weight material, but as it involves a precipitation followed by a centrifugation on a micro-scale, it is of limited use in the analysis of large numbers of fractions.

The much used method of Lowry *et al.*[11], in which a combination of the biuret and Folin reactions is used, has the advantage of being rather specific for proteins. It is definitely the easiest method in many cases, and has a quite high accuracy if it is calibrated with the protein determined. The Lowry method is rather sensitive to the pH of the analyzed solution, and in many cases the only possibility of overcoming this is a preliminary precipitation. The tyrosine content of the sample influences the colour yield, but this disadvantage is balanced by the sensitivity of the method compared to a simple biuret reaction.

For a preliminary localization of protein material in fractions, the absorption of proteins at 280 mμ is often made use of routinely because of the simplicity and relative sensitivity of the method. The reviews by Dannenberg [12] and Beaven and Holiday [13] are useful when ultraviolet absorption data of proteins

are compared. Because of the abundance of ultraviolet absorbing substances in biological materials, it is often difficult to use the absorbance at 280 mμ for strictly quantitative purposes, and some buffers containing aromatic substances nessesitate other methods.

Ninhydrin reacts directly with proteins and peptides, and this has been utilized by Kunkel and Ward [14] for the quantitative determination of serological precipitates. As it is the free amino groups that are responsible for the reaction, different proteins give variable amounts of colour, and a slight contamination of ammonium salts will produce relatively large errors. Moreover, certain peptide linkages seem to be broken up by the comparatively violent reaction involved in the ninhydrin procedure. These factors make the use of a direct ninhydrin reaction of proteins uncertain and unsuitable for material of unknown behaviour.

The purpose of this study is to investigate the possibility of using the ninhydrin reaction of Moore and Stein [15] on aliquots from large numbers of fractions after hydrolysis and subsequent evaporation to dryness over sodium hydroxide. Upon acid hydrolysis, proteins and peptides are broken down chiefly into amino acids and ammonium salts. As the reaction of ninhydrin with these substances, with the exception of proline and hydroxyproline, is practically quantitative, the colour yield is an estimate of the nitrogen content of the samples. It is true that some nucleic acid components may be converted to ninhydrin-reacting compounds during the hydrolysis but ringbound nitrogen does not interfere. The main disadvantage of the method is the high sensitivity for ammonia, but this can be corrected for. The procedure of Boissonas [16] employing a solution of 1 % KOH in methanol to volatilize ammonia without decomposition of the amino acids is relatively simple, and the difference in ninhydrin colour obtained before and after such a treatment is an estimate of ammonium salts, provided the small amounts formed during hydrolysis can be neglected.

DESCRIPTION OF PROCEDURE USING HYDROLYSIS AND NINHYDRIN REACTION

The following procedure is suitable for fractions containing more than about 3 μg of nitrogen (in the form of proteins, peptides, amino acids etc.) per ml, but smaller amounts can also be determined by slight modifications, as will be outlined later.

One-tenth ml from every fraction is pipettes into small ampoules or tubes preferably with the aid of constriction pipets of the Lang-Levy type. Simple, inexpensive 2 or 3 ml ampoules of the sort * shown in Fig. 1 have been found suitable and easy to open and seal. With the aid of a semiautomatic syringe ** shown in the same figure, 0.5 ml of distilled constant-boiling hydrochloric acid is added, after which the ampoules are sealed over a narrow flame. Hydrolysis is carried out in a thermostated oven at 120°C for 10—24 h. The ampoules are then cooled below room temperature and opened with a sharp file. They are put in a desiccator with sodium hydroxide and dried *in vacuo* overnight. 2 ml

* Manufactured by Johnsen & Jorgensen Flint Glass LTD, London.
** Available through KELAB, Stockholm, Sweden.

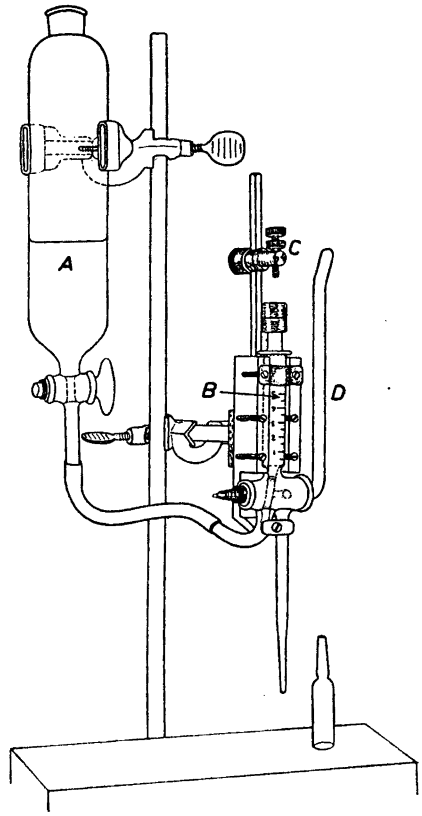

Fig. 1. Semiautomatic syringe used to deliver reagents into ampoules. The solution in A is forced into the all-glass syringe B by means of a slight air pressure. The volume to be delivered is set by the screw C and the plunger, which is filled with mercury, empties the syringe when the handle D of the stopcock is turned.

of water is added to each ampoule with a semi-automatic syringe, and 1 ml of the resulting solution is taken for ninhydrin analysis. This is performed according to Moore and Stein [15] using 1 ml ninhydrin solution prepared by dissolving 2.0 g ninhydrin and 0.3 g hydrindantin in 75 ml methyl cellusolve, adding 25 ml 4 N sodium acetate buffer pH 5.5 immediately before use. The reaction is carried out in test tubes immersed for 15 minutes in a boiling water bath, after which the tubes are immediately cooled and diluted with 3 ml 50:50 ethanol-water. When the tubes have been thoroughly shaken to oxidize any residual hydrindantin, they are read at 570 mμ against blanks from ampoules containing buffer, that have been subjected to the same procedure as the samples to be analysed. It is of special importance to use well cleaned tubes for the reaction, and the use of semi-automatic syringes for the addition of ninhydrin solution and dilution is of great convenience.

When extinction values that are too high are obtained with a final volume of 5 ml, the contents of the tubes can be further diluted with 5 or 10 ml ethanol:water without deviation from the linear relationship between the amount of nitrogen and colour. If the hydrolyzed residue in the ampoules is dissolved in 2 ml of water, of which 1 ml is taken for the ninhydrin reaction, the upper limit of such a determination is about 10 μg N taken from the fractions. However, as the hydrolysis of much larger amounts of protein is complete under the conditions described, as much as 1 mg N can be determined simply by diluting a portion of the 1 ml remaining in the ampoules. Table 1 shows that it is possible to determine very wide range of protein concentrations in this way without significant loss in precision. As test sample, a commercial protein standard, Pro-Sol,

Table 1. Quantitative ninhydrin determination of different amounts of hydrolyzed protein. The extinction values are given for equal amounts of hydrolyzed protein.

μg of protein taken	% of hydrolyzed residue taken for the ninhydrin analysis	extinction at 570 mμ
5 550	0.5	0.38
1 110	0.5	0.39
504	2.5	0.38
111	10	0.41
55.5	50	0.39
11.1	50	0.37
5.55	50	0.39
2.78	50	0.39

distributed by the Standard Scientific Supply Corp., New York, has been used. This standard is a partly purified preparation of bovine serum albumin and has been used throughout this investigation.

It has been found that the concentration of hydrochloric acid during hydrolysis is not critical, as long as it is at least 3 M. When comparatively large and unequal volumes are to be hydrolyzed, samples can be reduced in volume before the addition of HCl, but this is seldom necessary.

If very small amounts of material are available, the following modifications can be made. The hydrolyzed residue in the ampoules is dissolved in only a few tenths of a ml, an aliquot of which is taken for analysis with ninhydrin with a final volume of 1 ml. In this way as little as 0.1 μg of protein N can be determined with comparatively high precision. It is possible to perform the ninhydrin reaction directly in the ampoules, but it is then more difficult to oxidize the residual hydrindantin. For approximate determinations, the ninhydrin colour can then be read in the ampoules, as these vary comparatively little in width.

No detailed investigation of the time necessary for the complete hydrolysis of different proteins has been made; however, it seems advisable to try different times before using the method on samples of unknown behaviour. Since a few hours is sufficient in most cases, and prolonged hydrolysis does not seem to change the colour yield with ninhydrin very much, 24 hours has been used routinely at this laboratory. Fig. 2 shows the influence of different times of hydrolysis of Pro-Sol. As the ninhydrin reaction gives slightly different amounts of colour depending on the reagents and the exact procedure em-

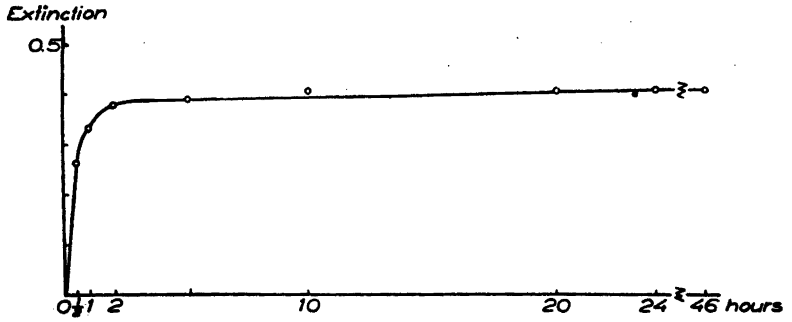

Fig. 2. The relationship between colour yield and time of hydrolysis at 120°.

Table 2. Precision of method. 3.53 µg of Pro-Sol nitrogen pipetted into each of eight ampoules. After hydrolysis at 120° and solution of the dry residue in 2 ml of water, 1 ml was taken for a ninhydrin reaction with a final volume of 5 ml.

	Extinction at 570 mµ minus blank reading			
Samples	0.409	0.410	0.420	0.419
	0.410	0.411	0.420	0.423
Mean value		0.415		
Standard deviation of mean		0.006		

ployed at a laboratory, it is necessary to calibrate the method with proteins of known nitrogen content if accurate quantitative data are wanted. It is then possible to convert the colour to protein nitrogen, but in fractionation work it is often sufficient to compare the colour yields of the different fractions directly. Table 2 illustrates the precision of the method when the same amount of protein is hydrolyzed in a series of ampoules. When other proteins have been tested, they have given similar values, but no detailed comparison between different proteins has been made as yet.

To keep blank readings as low as possible, it is necessary to use ammonia-free reagents and to clean all glassware well. The hydrochloric acid used for hydrolysis is best distilled in Pyrex and kept in a bottle provided with an arrangement to protect it from ammonia in the atmosphere. Water is obtained

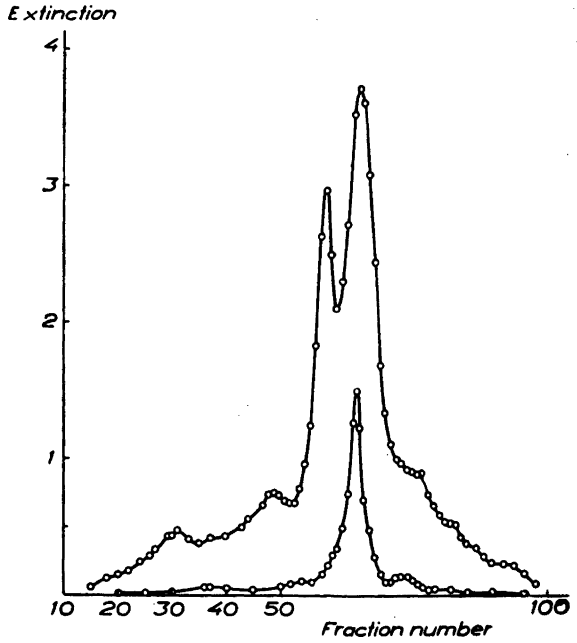

Fig. 3. Electropherogram of an extract from hog neurohypophysis. Lower curve: ninhydrin colour without hydrolysis. Upper curve: ninhydrin colour after hydrolysis with HCl at 120°. The extinction values are given for equal sample size in estimating the two curves.

Table 3. Comparison of some methods of protein determination. The approximate extinction values are given for a concentration of 1 μg of Pro-Sol protein nitrogen per ml measured in a 1 cm cell.

Method	Wavelength used mμ	Extinction
Ultraviolet absorption	280	0.0046
Turbidity with trichloroacetic acid at a final concentration of 3 %	420	0.0082
Direct ninhydrin reaction	570	0.092
Method according to Lowry *et al.*[11]	750	0.124
Hydrolysis and ninhydrin reaction	570	1.17

by distillation from an acid medium in a Pyrex still. The ampoules are sealed when delivered from the manufacturer and therefore do not contribute to high blanks, but the tubes used for the ninhydrin reactions must be kept free from contamination. If fractions can not be analysed soon after collection, they should be stored in a box provided with an acid adsorbent. If blank readings higher than 0.1 on the optical density scale are obtained, the reagents should be tested for impurities.

As an example of the usefulness of the method, Fig. 3 shows an electropherogram of an extract from hog neurohypophysis *. Electrophoresis was carried out in an apparatus described by Porath [17] with a buffer composed of 0.1 M pyridine adjusted to pH 5.0 with acetic acid. The fractions eluted from the column had a volume of approximately 10 ml each, of which 0.25 ml was taken for the ninhydrin reaction without hydrolysis. 0.2 ml was pipetted into ampoules and hydrolyzed for 24 hours with 0.5 ml 6.4 N HCl at 120°C. After removal of HCl, 2 ml of water was added, and 1 ml of the resulting solution taken for the ninhydrin reaction. The extract is a very complicated mixture of amino acids and peptides, and no attempt is made in this connection to discuss its different components. From the shapes of the two curves, it is possible to draw certain conclusions about the contents of the different fractions, because a high ratio between the colour yield of hydrolyzed and unhydrolyzed material indicates the presence of peptide-bound nitrogen. In this special case, it would have been impossible to use either optical density readings at 280 mμ or nitrogen determinations because of the pyridine content of the buffer. Pyridine does not react with ninhydrin and has the advantage of being volatile, so that the fractions can be lyophilized.

Table 3 has been arranged to allow a comparison of some of the colorimetric methods discussed. The values have been obtained from determinations made on the previously mentioned protein standard, Pro-Sol. In order to facilitate a comparison of the sensitivity of the methods, the extinction has been given for 1 μg of protein nitrogen per ml solution measured at the specified wave length.

The author wishes to thank Professor A. Tiselius and colleagues at the Institute for helpful discussions. The technical assistance of Mrs. B. Malmström and Mrs. I. Stark is gratefully acknowledged. This investigation was supported by grants from the *Swedish Natural Science Research Council.*

* Data from experiments by H. Kiessling.

REFERENCES

1. Johnson, M. J. *J. Biol. Chem.* **181** (1949) 707.
2. Gorbach, G., Dedic, G. and Philippi, E. H. *Hoppe-Seylers' Z. physiol. Chem.* **301** (1955) 185.
3. Harvey, H. W. *Analyst* **76** (1951) 657.
4. Boissonas, R. A. and Haselbach, C. H. *Helv. Chim. Acta* **36** (1953) 576.
5. Conway, E. J. *Microdiffusion analysis and volumetric error*, Crosby Lockhood & Son Ltd., 3. Ed. 1950.
6. Grunbaum, B. W., Kirk, P. L., Green, L. G. and Koch, C. W. *Anal. Chem.* **27** (1955) 384.
7. Smith, E. L. *Private communication.*
8. Stadtman, E. R., Novelli, G. D. and Lipman, F. *J. Biol. Chem.* **191** (1951) 365.
9. Greif, R. L. *Proc. Soc. Exptl. Biol. Med.* **75** (1950) 813.
10. Nayyar, S. N. and Glick, D. *J. Histo. Cyto. Chem.* **2** (1954) 282.
11. Lowry, O. H., Rosebrough, N. J., Farr, A. L. and Randall, R. J. *J. Biol. Chem.* **193** (1951) 265.
12. Dannenberg, H. *Angew. Chem.* **63** (1951) 208.
13. Beaven, G. H. and Holiday, E. R. *Advances in Protein Chem.* **7** (1952) 320.
14. Kunkel, H. G. and Ward, S. M. *J. Biol. Chem.* **182** (1950) 597.
15. Moore, S. and Stein, W. H. *J. Biol. Chem.* **211** (1954) 907.
16. Boissonas, R. A. *Helv. Chim. Acta* **33** (1950) 1975.
17. Porath, J. *Acta Chem. Scand.* **8** (1954) 1813.

Received July 3, 1956.