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ORIGINAL ARTICLE

Protein Measurement with the Folin Phenol Reagent

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Summary

1. A study is presented of the measurement of proteins with the Folin phenol reagent after alkaline copper treatment. The basic reactions have certain peculiarities which need to be taken into consideration in using this reagent.
2. Directions are given for measurement of proteins in solution and proteins which have been precipitated with acid, etc. A micro procedure is also described for the measurement of as little as 0.2 γ of protein.
3. The differences in the amount of color obtained with a number of proteins is recorded. Interfering substances are listed.

Introduction

Since 1922 when Wu proposed the use of the Folin phenol reagent for the measurement of proteins (l), a number of modified analytical procedures utilizing this reagent have been reported for the determination of proteins in serum (2-G), in antigen-antibody precipitates (7-9), and in insulin (10).

Although the reagent would seem to be recommended by its great sensitivity and the simplicity of procedure possible with its use, it has not found great favor for general biochemical purposes.

In the belief that this reagent, nevertheless, has considerable merit for certain application, but that its peculiarities and limitations need to be understood for its fullest exploitation, it has been studied with regard to effects of variations in pH, time of reaction, and concentration of reactants, permissible levels of reagents commonly used in handling proteins, and interfering substances. Procedures are described for measuring protein in solution or after precipitation with acids or other agents, and for the determination of as little as 0.2 γ of protein.

Methods

Reagents

Reagent A, 2 per cent Na_2CO_3 in 0.10 N NaOH. Reagent B, 0.5 per cent $CuSO_4.5H_2O$ in 1 per cent sodium or potassium tartrate. Reagent C, alkaline copper solution. Mix 50 ml. of Reagent A with 1 ml. of Reagent B. Discard after 1 day. Reagent D, carbonate-copper solution, is the same as Reagent C except for omission of NaOH. Reagent E, diluted Folin reagent. Titrate Folin-Ciocalteu phenol reagent ((II), Eimer and Amend, Fisher Scientific Company, New York) with NaOH to a phenolphthalein end-point. On the basis of this titration dilute the Folin reagent (about 2-fold) to make it 1 N in acid. Working standards may be prepared from human serum diluted 100- to 1000-fold (approximately 700 to 70 λ per ml.). These in turn may be checked against a standard solution of crystalline bovine albumin (Armour and Company, Chicago); 1 γ is the equivalent of 0.97 γ of serum protein (see below). Dilute solutions of bovine albumin have not proved satisfactory for working standards because of a marked tendency to undergo surface denaturation.

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Procedure for Proteins in Solution or Readily Soluble in Dilute Alkali

(Directions are given for a final volume of 1.1 to 1.3 ml., but any multiple or fraction of the volumes given may be employed as desired[1].)

To a sample of 5 to 100 γ of protein in 0.2 ml. or less in a 3 to 10 ml. test-tube, 1 ml. of Reagent C is added. Mix well and allow to stand for 10 minutes or longer at room temperature. 0.10 ml. of Reagent E is added very rapidly and mixed within a second or two (see below). After 30 minutes or longer, the sample is read in a calorimeter or spectrophotometer. For the range 5 to 25 γ of protein per ml. of final volume, it is desirable to make readings at or near λ = 750 mμ, the absorption peak. For stronger solutions, the readings may be kept in a workable range by reading near λ = 500 m/l (**Fig. 2**). Calculate from a standard curve, and, if necessary, make appropriate correction for differences between the color value of the working standard and the particular proteins being measured (see below).

It is unnecessary to bring all the samples and standards to the same volume before the addition of the alkaline copper reagent, provided corrections are made for small differences in final volume. The critical volumes are those of the alkaline copper and Folin reagents.

If the protein is present in an already very dilute solution (less than 25 γ per ml.), 0.5 ml. may be mixed with 0.5 ml. of an exactly double strength Reagent C and otherwise treated as above.

Insoluble Proteins, etc.

Many protein precipitates, *e.g.* tungstate precipitates, will dissolve readily in the alkaline copper reagent. However, after proteins have been precipitated with trichloroacetic or perchloric acid, for example, they will dissolve rather poorly in the 0.1 N alkali of this reagent. They become even harder to dissolve if subsequently extracted with fat solvents, and still more so if dried at 100°.

It is not possible to cover all cases, but the following may be helpful in measuring the protein of acid precipitates. If the amount of protein is not great, so that it is spread rather thinly, it will usually dissolve in 3 hour or so in 1 N NaOH at room temperature. Therefore, one may add, for example, 0.1 ml. of 1 N NaOH to 5 to 100 γ of precipitated protein.

After $\frac{1}{2}$ hour or more, 1 ml. of Reagent D (no NaOH) is added, followed after 10 minutes by 0.1 ml. of diluted Folin Reagent E as usual.

With larger samples, or very stubborn precipitates, it may be necessary to heat for 10 minutes or more at 100° in 1 N alkali. Although this may lower the readings, they will be reproducible and can be measured with similarly treated standards.[2]

Microanalysis

With a Beckman spectrophotometer adapted to 0.05 ml. volume (12), as little as 0.2 γ of protein may be measured with reasonable precision. Aside from reducing the volumes of sample and reagents, the only necessary change is to use sufficiently slender tubes for the reaction. If the tubes are too large in diameter, low values will result. The following is illustrative of a procedure in which it is desired to precipitate the protein in order, for example, to measure an acid-soluble constituent of the same specimen. In this example, it is assumed that the sample volume is negligible. Otherwise a smaller volume of more concentrated trichloroacetic acid would be used.

To the sample containing 0.2 to 3 γ of protein in a tube of 3 mm. inner diameter and 4 cm. long,[3] are added 10 μl. of 5 per cent trichloroacetic acid.[4] After being mixed and centrifuged, 8 μl. of the supernatant fluid are removed. To the precipitate are added 5 μl. of 8 N NaOH. The sample is thoroughly mixed by tapping or "buzzing,"[5] and is covered with a rubber cap or Parafilm. After 30 minutes, 50 μl. of Reagent D are added and the sample is mixed by "buzzing." After 10 minutes or more, 5 μl. of diluted Folin Reagent E are added with *immediate* "buzzing," and the samples are read after 30 minutes. Standards are perhaps best prepared by precipitating 5 μl. of 5, 10, 20, etc., mg. per cent solutions of serum protein with 5 μl. of 10 per cent trichloroacetic acid, with subsequent treatment as for the other samples.

Experimental

There are two distinct steps which lead to the final color with protein: (a) reaction with copper in alkali, and (b) reduction of the phosphomolybdic-phosphotungstic reagent by the copper-treated protein.

Reaction with Copper in Alkaline Solution

The salient features of this reaction follow. (1) The color obtained in the absence of copper is probably attributable entirely to the tyrosine and tryptophan content (16, 17), and this is not greatly increased by alkaline pretreatment ((4-6) and **Table 1**). (2) In the presence of copper, alkaline treatment of proteins results in a 3- to 15-fold increase in color, but, in contrast, the presence of copper has only a small effect on the color obtained with free tyrosine and tryptophan (Herriott (17, 18) and **Table 1**). (3) The reaction with copper, although not instantaneous, is nearly complete in 5 or 10 minutes at room temperature under the prescribed conditions. Heating to 100” or increasing the concentration of alkali accelerates the reaction with copper without changing the final color. (4) Pretreatment with alkali alone does not alter the subsequent reaction with copper in alkaline solution. Even pretreatment for an hour at 60° with 2 N NaOH, or for 5 minutes at 100° with 1 N NaOH, when followed by the usual copper treatment, has almost no effect on subsequent color. Prolonged heating with strong alkali will, however, decrease the final color.[2]

Although the alkaline copper reaction and the biuret reaction appear to be related, they are not strictly proportional, nor, with different proteins, is the amount of biuret color directly proportional to the increment caused by copper in the color with the Folin reagent (**Table 1**).

Table 1 Extinction Coefficients of Proteins Variously Treated

The equivalent extinction coefficient ε^{N}_{750} (or 550) is defined as the optical density at λ= 750 (or 550) mμ with 1 atom of N per liter. Nitrogen was measured by the Kjeldahl procedure of Miller and Houghton (24). The biurct color was developed with the reagents of Weichselbaum (25). Source of proteins, crystalline trypsin, crystalline chymotrypsin, and crystalline bovine albumin, Armour and Company, Chicago; cytochrome c, Sigma Chemical Company, St. Louis; crystalline zinc insulin, Eli Lilly and Company, Indianapolis; gelatin, Difco Laboratories, Inc., Detroit; L-tyrosine, Eastman Kodalr Company, Rochester.

Protein	ε^{N}_{750}				
	Copper absent		Alkaline† copper treatment	Increment with Cu	Biuret color
	No pretreatment	Alkali* treatment			
Trypsin	733	910	3,600	2690	26.3
Insulin	989	998	3,000	2002	24.4
Chymotrypsin	278	425	2,930	2505	25.8
Cytochrome c	703	738	2,495	1757	‡
Human serum	320	365	2,120	1755	21.4
Bovine serum albumin	312	358	2,050	1692	21.8
Gelatin	79	78	1,145	1067	18.0
Tyrosine	13,700	13,850	15,100	1250	

* 30 minutes in 0.1 N NaOH at room temperature before addition of Folin reagent.

† Regular treatment as described under the procedure.

‡ Not valid because of the color of the cytochrome c.

Table 2 Color Increments from Small Amounts of Copper

Serum protein concentration, 12.1 γ per ml. *K* and chromogenic-bound copper (Cu-protein) calculated from *K* = Cu × protein/Cu-protein = (Cu(tota1 protein - Cu-protein))/Cu-protein = (Cu(maximum ΔO. D.* - Δ O. D.))/ Δ O. D. (assuming that chromogenic copper bound to protein is proportioned to Δ O. D.).

Total Cu	O. D.* at 750 mμ	Δ O. D.	Δ O. D., per cent of maximum Δ	*K*	Cu-protein† (calculated)
10^{-6} *M*				× 10^{-6}	
0	78				
8	166	88	42	2.9	0.05
20	237	159	76	3.0	0.10
40	267	189	91	2.8	0.12
2000	286	208	100		0.13

* Optical density.

† Moles per 117 gm. of protein, *i.e*. per amino acid residue.

A very small amount of copper is sufficient to give nearly maximum final color (**Table 2**). The action does not appear to be catalytic. Assuming the simple relationship copper + protein ⇔ copper-protein obtains, the data with low copper concentrations may be utilized to calculate an apparent dissociation constant of 3 × 10^{-6} mole per liter with a maximum of 1 mole of chromogenic protein-bound copper per 7 or 8 amino acid residues (**Table 2**).

Mehl, Pacovska, and Winder (19) conclude with Rising and Yang (20) that in the biuret reaction approximately 1 atom of copper is bound for each 4 amino acid residues, and Mehl *et al*. calculated dissociation constants for the reaction copper + protein ⇔ copper-protein averaging 10 times larger than the one reported herein for the formation of Folin-reactive material. Thus, of the total possible sites for copper combination, it would appear that only about half produce significant reduction of the Folin reagent, and that furthermore this fraction has a considerably greater affinity for copper than the rest.

Reduction of Folin Reagent

Three main points may be made as follows: (1) When the Folin reagent is added to the copper-treated protein, maximum color results if the reduction occurs at about pH 10. (2) At this pH the reagent is only reactive for a short time (16). It is for this reason that even a few seconds delay in complete mixing will lessen the amount of color (**Fig. 1**). The decrease in reactivity of the reagent appears to be a function of the disappearance of the original yellow color of the phosphomolybdate (half time of 8 seconds (**Fig. 1**)) and is presumably due to dissociation of the phosphate from the molybdate. Surprisingly, the color with protein continues to develop for a number of minutes after the reagent itself has become unreactive to freshly added protein (**Fig. 1**). Possibly the primary reduction product rearranges, since the absorption spectrum changes in shape between 3 minutes and 30 minutes (**Fig. 2**). (3) During the 1st minute or so after the addition of the Folin reagent, extra acid is liberated (**Fig. l**), which also may result from the dissociation of the phosphomolybdate. Thergfore, for maximum color, the solution must to neutralize the excess phosphoric acid, and Na&03, to buffer the mixture near pH 10, gives more color than any amount of either reagent alone (**Fig. 3**).

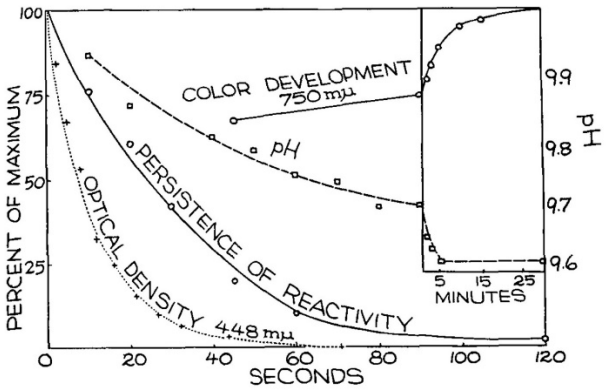

Fig. 1 "Persistence of reactivity" was measured by adding Folin reagent to protein-free alkali; after the given times, copper-treated protein was added in a small volume, and the color at 750 mμ was measured 30 minutes later. "Color development" refers to a sample of serum protein treated in the regular manner. The points on "optical density 448 mμ" are observed (no protein present); the curve is theoretical for a monomolecular reaction with a half time of 8 seconds.

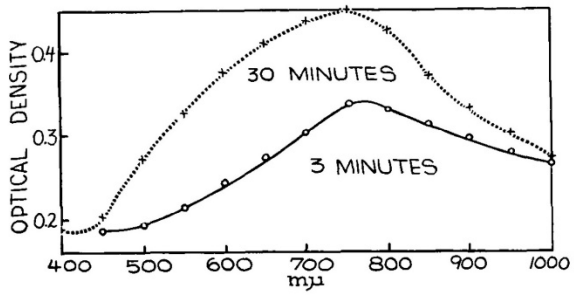

Fig. 2 Absorption spectra 3 and 30 minutes after the addition of Folin reagent to a solution containing 23.3 γ of serum protein per ml.

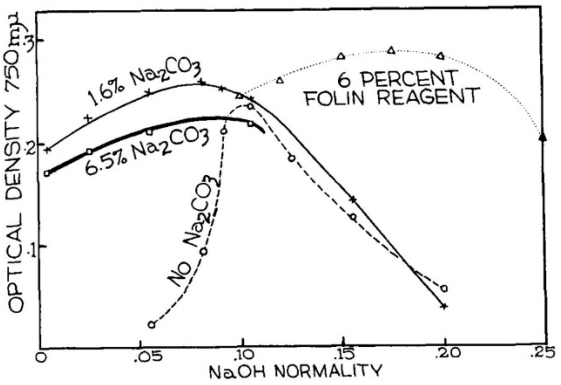

Fig. 3 Effect of alkali concentration on final color development. NaOH concentration is calculated before addition of 0.1 volume of diluted Folin reagent. Except as noted, final concentration of Folin reagent 3 per cent and Na_2CO_3 1.6 per cent. All samples (copper-treated protein) were identical in composition until a few seconds before addition of Folin reagent (see the text). Final protein concentration 12 γ per ml.

Extinction Coefficients and Proportionality

Different pure proteins give different extinction coefficients with the Folin reagent (**Table 1**). The extremes were observed with trypsin and gelatin which differed by a factor of 3 in chromogenicity. It will be seen that without copper much greater differences occur. The variation in chromogenicity must be kept in mind, but it is much less marked with mixtures of proteins as found in various tissues (**Table 3**), and for many purposes is not a serious drawback. The relation of color to protein concentration is not quite linear (**Table 4**).

Specificity and Interfering Substances

Few substances encountered in biological work cause serious interference. Only a little color was obtained with either acid extracts or the lipides extracted from five different tissues (**Table 3**). Consequently measurements on non-extracted whole tissue would be in error by only 3 to 6 per cent, whereas values based on N determination would be overestimated by 15 to 20 per cent.

Uric acid (16), guanine, and xanthine (21, 22) react with the Folin reagent. Guanine gives about 50 per cent

more color than serum protein, weight for weight. The color is not enhanced by copper. Curiously, guanosine does not react appreciably. Hypoxanthine gives no color if purified (21). No more than a trace of color was obtained with adenine, adenosine, cytosine, cytidine, uracil, thymine, or thymidine (see also Funk and Macallum (22)).

Neither color nor interference with protein color development was observed with the following substances at the given *final* concentrations: urea (0.5 per cent), guanidine (0.5 per cent), sodium tungstate (0.5 per cent), sodium sulfate (1 per cent), sodium nitrate (1 per cent), perchloric acid (0.5 per cent neutralized), trichloroacetic acid (0.5 per cent neutralized), ethyl alcohol (5 per cent), ether (5 per cent), acetone (0.5 per cent), zinc sulfate (0.1 per cent), barium hydroxide (0.1 per cent). Most phenols, except nitrophenols, reduce the reagent (16); therefore thymol and to a lesser degree sulfosalicylic acid interfere, whereas picric acid up to 0.1 per cent is permissible. Glycine (0.5 per cent) decreases the color with protein by 50 per cent. Hydrazine over 0.5 mg. per cent increases the blank.

Ammonium sulfate greater than 0.15 per cent final concentration decreases color development. This is partly due to a decrease in alkalinity, and up to 0.25 per cent or so can be tolerated if an equivalent amount of extra alkali is added to the sample. Extra copper does not seem to help.

Microanalysis

With final volumes less than 0.1 ml., the amount of color is proportionately less than on the macro scale, especially if the reaction is carried out in wide tubes. Extensive testing did not definitely identify the cause of the decreased color. Neither oxygen, carbon dioxide, nor glass surface seemed to be involved. The critical step is the period of standing with alkali and copper.

Table 3 Apparent Protein Content of Whole Tissues (Rabbit) and Tissue Extracts Calculated from Kjeldahl N and from Folin Color

The tissues were homogenized and precipitated with 5 per cent trichloroacetic acid (TCA), and the lipides removed by successive extraction with 0.1 N potassium acetate in ethanol, ethanol, and isopropyl ether. (The purpose of the acetate is to neutralize the acid and prevent solution of some protein in the ethanol.) The N was determined as in **Table 1**. The extinction coefficients were calculated from the N and color of the extracted precipitates.

Material analyzed		Extracted ppt.	TCA extract	Lipide extracted	Whole tissue	$\frac{N}{\varepsilon_{750}}$ *
		Per cent	*Per cent*	*Per cent*	*Per cent*	
Brain	Based on N × 6.25	9.5	1.08	1.05	11.8	1960
	" " Folin color	9.5	0.15	0.28	9.8	
Kidney	" " N × 6.25	13.9			15.9	1865
	" " Folin color	13.9	0.30	0.21	14.5	
Liver	" " N × 6.25	17.1			19.9	1875
	" " Folin color	17.1	0.49	0.28	18.0	
Skeletal muscle	" " N × 6.25	17.8			20.1	1942
	" " Folin color	17.8	0.15	0.09	18.8	
Heart	" " N × 6.25	13.0			15.3	1975
	" " Folin color	13.0	0.20	0.17	13.4†	

* See Table 1.

† By summation; other values are direct determinations.

Table 4 Measurement of Small Amounts of Protein from Rabbit Brain

Finn1 volume 0.082 ml.

Optical* density at 750 mμ	$E^{1\%}_{1cm.}$ at 750 mμ	Protein		Optical* density at 750 mμ	$E^{1\%}_{1cm.}$ at 750 mμ	Protein	
		Found	Present			Found	Present
		γ	γ			γ	γ
0.038		0.13	0.16	0.280	229	0.98	1.00
0.044		0.15	0.16	0.292	236	1.03	1.00
0.040		0.14	0.16	0.283	233	0.99	1.00
0.089	221	0.33	0.33	0.365	227	1.30	1.32
0.095	236	0.35	0.33	0.367	228	1.31	1.32
0.091	226	0.34	0.33	0.365	227	1.32	1.32
0.184	228	0.65	0.66	0.441	219	1.60	1.66
0.191	236	0.67	0.66	0.443	220	1.62	1.66
0.191	236	0.67	0.66	0.444	221	1.61	1.66

* Corrected for blank.

The practical solution to this interesting difficulty seems to be to use slender tubes and to run standards under the same conditions. **Table 4** illustrates the reproducibility of protein measurements on small brain samples. Rabbit brain was homogenized and diluted 200- to 2000-fold. Aliquots of 3.6 μ1. were analyzed for protein at a final volume of 0.082 ml. The amount of protein present was calculated from macro analyses. It is seen that the error is usually not over 0.02 γ.

Discussion

The measurement of protein with copper and the Folin reagent has certain advantages. (1) It is as sensitive as with Nessler's reagent, yet requires no digestion. (2) It is 10 or 20 times more sensitive than measurement of the ultraviolet absorption at λ = 280 mμ and is much more specific and much less liable to disturbance by turbidities. (3) It is several fold more sensitive than the ninhydrin reaction (23) and is somewhat simpler, as well as much easier to adapt for small scale analyses. Free amino acids give much more color than proteins with the ninhydrin reaction, whereas the reverse is true with the Folin reagent. (4) It is 100 times more sensitive than the biuret reaction.

There are two major disadvantages of the Folin reaction. (a) The amount of color varies with different proteins. In this regard it is less constant than the biuret reaction, but more constant than the absorption at λ = 280 mμ. (b) The color is not strictly proportional to concentration. From a consideration of the advantages and disadvantages, the reasonable applications of the copper-Folin reaction would seem to include (1) measurement of protein during enzyme fractionations, etc., (2) measurement of mixed tissue proteins, particularly when absolute values are not needed, (3) measurement of very small absolute amounts of protein, or highly diluted protein (*e.g.* spinal fluid) or protein mixed with colored substances or other nitrogen-containing substances, and (4) analyses of large numbers of similar protein samples, such as antigen-antibody precipitates.

Note

1. For example, with the Klett calorimeter, transfer 25 to 500 γ of protein in not over 1 ml. volume to a calorimeter tube. Add water if necessary to make 1 ml. Add 5 ml. of Reagent C, and, after 10 minutes, 0.5 ml. of Reagent E. Readings are taken after 30 minutes with the No. 66 filter. If the readings are too high, substitute the No. 54 filter for sample, standards, and blanks.
2. Bovine serum albumin is especially difficult to redissolve after precipitation. Several 40 mg. samples were precipitated with trichloroacetic acid, washed with

alcohol and isopropyl ether, and dried. These samples dissolved very slowly in 2 ml. of 1 N NaOH. However, after standing overnight, the protein appeared to be nearly all dissolved and aliquots gave readings 97 per cent of those obtained with non-precipitated samples. Other samples were heated for 30 minutes at 100° in 1 N NaOH. These samples dissolved and the solution turned slightly yellow. The final readings checked well but were only 82 per cent of those obtained with non-precipitated samples. Possibly, heating at lower temperature with the 1 N NaOH would have sufficed, although heating with weaker alkali would not have been effective, judging from other experience. The use of stronger alkali than 1 N did not appear to be an improvement.

3. These tubes are cleaned by rinsing with dilute NaOH, boiling in half concentrated HNO_3, and rinsing several times in redistilled water. Filling or emptying of a beaker full of tubes (tall form of beaker without lip) is accomplished by slow centrifugation for a few seconds. For emptying, the tubes are transferred upside down to a second beaker with a false bottom of stainless steel screen. With the slow centrifugation required beakers will not be broken.
4. Suitable micro pipettes are the Lang-Levy variety (13, 14). For entering these narrow tubes the bent tip must be especially short and slender.
5. The tube is held at an angle against a rapidly rotating flattened rod or nail. Any high speed hand tool mounted in a clamp is satisfactory. The contents of the tube will mix violently without spilling (15). A similar effect may be had with a commercial rubber-tipped massage vibrator.

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