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Application of Lowry Protein Determination to Influenza Vaccine

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Abstract □ The Lowry protein test has been successfully applied to the determination of microgram amounts of protein in influenza vaccine. Because of the tendency of the virus to aggregate, additional techniques such as sonoration were needed. Precision among replicates was good; the greatest difference of a single value from the average was 1.7%.

Keyphrases □ Influenza vaccine—protein determination □ Lowry protein test—analytic method □ Folin reagent—color formation □ Colorimetric analysis—spectrophotometer

The precise determination of protein concentration was considered essential as part of the control measures applied to influenza vaccine.[1] The Lowry (1) modification of the Folin-Ciocalteu (2) test was selected as being one of the most sensitive methods.

Experience has indicated that the Lowry test should not be applied to a new protein with its environment without research and/or development pertaining to the new application. In general, the procedure involved precipitation of protein from the vaccine sample with trichloroacetic acid, centrifugation, and application of the Lowry test to the pellet.

METHODS AND MATERIALS

Vaccine used in this work contained 0.85% w/v sodium chloride and 0.2% w/v gelatin. The same lot of concentrated vaccine was used for all determinations except the study of precipitation at different concentrations of trichloroacetic acid.

The standard protein for the Lowry test was crystallized bovine plasma albumin.[2] For each series of test samples a solution was made up of 10 mg. in 200 ml. aqueous 0.85% sodium chloride. This solution is stable at 6° for at least 1 month and is used for the Lowry test without previous precipitation.

The Lowry reagents are those originally described in the literature and are analytical reagent grade. They are combined to give Solutions A, B, and C according to the method of Oyama and Eagle (4).

The Folin reagent[3] is diluted 1 part with 1 part deionized or distilled water just before use. The original concentrate should be stored at about 6°.

Trichloroacetic acid (TCA), 30% w/v, is kept as a stock solution in a dark bottle at about 6°. This solution is stable for at least 1 month under these conditions. As needed for treatment of the test samples, a 10% w/v and a 5% w/v solution were made up from the stock solution.

Before pipeting out test samples, it is important to sonorate[4] the entire vaccine solution for a minimum of 1 min.

For each test, 2 ml. of sample solution was pipeted into a 13-ml. conical centrifuge tube having a flathead glass stopper. To each tube is added 2 ml. 10% w/v TCA plus 1 ml. 5% w/v TCA. The 5-ml. volume is used to give good dissolution of gelatin while still keeping the volume small enough to allow mixing in the centrifuge tube. These tests should be carried out in triplicate. The suspension briefly is mixed with a mixer.[5] The suspension is centrifuged[6] for 30 min. at 3,000 r.p.m. using a bucket-type rotor of 6-in. radius. The supernatant is discarded. TCA, 5 ml. of 5%, is added, and the pellet is dispersed by brief mixing with the mixer. After a second centrifugation and decantation, the drop of remaining supernatant is removed from the lip of the tube with absorbent tissue.

The Lowry test is performed on the precipitate. Using a total volume of 10 ml. of Reagent C for each tube, 5 ml. of the reagent was pipeted into the tube and sonorated to dissolve. The extract was decanted into a separate test tube and the extraction was repeated with a second 5 ml. of the reagent. The extracts were combined and mixed with the mixer.

Depending upon the expected concentration of protein, as indicated by experience, aliquots of the extract are taken for completion of the Lowry test. In the author's experience the volume has ranged from 1 to 2.5 ml. When needed, fresh Reagent C is added to a total volume of 2.5 ml. A dilution correction is used later. One milliliter of deionized or distilled water is now added and mixed with the mixer. For the vaccine protein tests, the remainder of the Lowry test is continued as for the protein standards and reagent blanks after Reagent C has been added to them.

For the protein standards, 1 ml. of the stock albumin solution (50 mcg. albumin) is placed in each of three tubes.

For reagent blanks, 1 ml. of deionized water is placed in each of two tubes. (Only one is needed; the second is a precautionary measure.)

To the standards and blanks 2.5 ml. Reagent C is added and mixed with the mixer.

The following applies to all solutions. Let stand in Reagent C for 10 min. or slightly longer. Jet in 0.25 ml. of diluted Folin reagent

[1] As produced at Eli Lilly and Co., Indianapolis, Ind.
[2] Armour Pharmaceutical Co., Chicago, Ill.
[3] Hartman-Leddon Co., Philadelphia, Pa., and Fisher Scientific Co., Chicago, Ill.
[4] Sonogen Automatic Cleaner, Branson Instruments, Inc., Stamford, Conn.
[5] Vortex-Genie, Scientific Industries, Inc., Springfield, Mass.
[6] Size 2, model V, International Equipment Co., Boston, Mass.

Table I—TCA Preparation of Influenza Vaccine Protein[a]

TCA (w/v), %	Absorbance, 750 mμ Vaccine[b]	Absorbance, 750 mμ Gelatin	Gelatin A/ Total A, %
2.5	0.869	0.133	15.3
5.0	0.890	0.121	13.6
7.5	0.975	0.319	32.7
10.0	1.59	0.918	57.7

[a] Each tabulated value is an average of two test values. [b] The absorbance values tabulated as vaccine include, of course, some gelatin.

with immediate mixing with a mixer. It is convenient to use a 0.25-ml. syringe[7] fitted with a plastic tip from a wash bottle; Folin reagent corrodes a metal needle. The reaction mixture is allowed to stand 1 to 1.5 hr.

The absorbance of each test sample and albumin standard is determined against a reagent blank at 750 mμ in a spectrophotometer.[8] If the test sample has too high or low a value to be read on the standard curve (see below), that determination should be repeated with an appropriate volume of test solution.

A standard curve was made from dilutions of a solution of albumin, 1.5 mg. in 10 ml. of aqueous 0.85% sodium chloride. The curve included determinations of total albumin from 4.5 to 150 mcg.

Absorbance values ranged from 0.021 to 0.603. The plot has a slight gradual curvature. The largest standard error, $S\bar{x}$, for any point on the curve was 0.001.

In calculating the amount of protein per milliliter of sample, the average absorbance of the test sample (in triplicate) is applied to the standard curve and a value in micrograms protein (uncorrected) is found. This value is corrected in two ways. First, the value is corrected according to the average absorbance of the tubes containing standard albumin.

$$A \frac{\text{standard curve}}{\text{standard tubes}} \times \frac{\text{mcg. protein for test sample from standard curve}}{} = \text{mcg. protein, corrected}$$

where A is absorbance at 750 mμ.

Second, the mcg. protein/ml. is determined according to the sample volume, and aliquot of Reagent C which was used.

$$\text{corrected mcg. protein/ml.} = \frac{10 \text{ ml.}}{\text{aliquot} \times 2 \text{ volume in ml.}}$$[9]

RESULTS AND DISCUSSION

As mentioned previously, it was of interest to follow precipitation of vaccine protein at various concentrations of TCA. Since gelatin is included in the vaccine formulation, its precipitation was followed also. The vaccine protein was precipitated from a preparation containing gelatin while gelatin was precipitated from a vaccine diluent which did not contain virus protein. The Lowry test was used on the centrifuged pellet. One-milliliter samples were used in all cases. Table I presents these data.

The sudden increase in absorbancy at 7.5% TCA seems to be due mainly to precipitation of gelatin. For this reason a concentration of 5% TCA was chosen.

The precision of the method *per se*, as well as precision between laboratories[10] is shown for the same vaccine preparation in Table II.

The higher absorbance values for Series 5 and 6 are due to use of new albumin and Folin reagents. The albumin standards reduced these values to give the corrected protein values which are tabulated.

Calculations from this table will show that the greatest percent difference of any test sample from the average is 1.7%.

This protein determination is not, of course, the only physical test or necessarily the final physical test to be applied to the finished vaccine. It is a step in the direction of developing reliable test methods which at present are offset to some extent by the variability of certain tests for biological potency. With the development of precise determinations of vaccine potency, this test can give reliable values for specific activity of vaccine, *i.e.*, biological units per unit weight of protein.

Table II—Lowry Protein Test of Influenza Vaccine

Series	Absorbance, 750 mμ	Protein, mcg./ml. Corrected Values
	Laboratory M756	
1	0.307, 0.305, 0.300	332
2	0.311, 0.308, 0.305	338
3	0.307, 0.307, 0.302	334
4	0.307, 0.304, 0.302	332
	Laboratory M752	
5	0.392, 0.394, 0.395	327
6	0.422, 0.442, 0.418	331
Average		332

It should be pointed out that this protein test is arbitrary in the sense that only total precipitated protein is measured and while a small amount of gelatin may be included under the test conditions, this total protein can be precisely determined and related to other assays.

A logical interpretation of the preliminary data suggests that most, if not all, of the high molecular weight virus protein (or virus *per se*) and a minimum of the highly water-soluble gelatin are precipitated. Also to be considered are the possible interactions of proteins during precipitation and dissolution as well as a possible difference in response to the Lowry reagents.

Table III indicates assay results on a representative series of manufactured lots of a single strain of purified, concentrated virus vaccine.

Table III—Representative Protein Determinations on Single Strain of Influenza Virus Vaccine

Vaccine Lot	Protein, mg./ml.
1	0.880
2	0.940
3	0.411
4	0.697
5	0.704
6	0.699
7	0.841

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This work followed earlier approaches to the application of the Van Slyke (3) method of protein precipitation and the Lowry method of protein determination to evaluation of purified virus preparations that were carried out in the Lilly Laboratories by Dr. John Ross.

[7] Becton, Dickinson and Co., Rutherford, N. J.
[8] Beckman model DU, Beckman Instruments Inc., Fullerton, Calif.
[9] The value 2 is used since 2-ml. samples are used.
[10] At Eli Lilly and Co.