Use of 2-(4-Hydroxybenzeneazo) Benzoic Acid for Protein Determination

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The compound, 2-(4-hydroxybenzeneazo) benzoic acid, also known as HBABA, was evaluated for its ability to detect cell protein. While this test may be suitable for large protein concentrations, it is not sensitive enough for the stated purpose with concentration below 1.0 mg.

THE CANCER Chemotherapy National Service Center describes a technique used for the determination of cell growth or inhibition in its program using the cell culture system as a primary screen for the detection of antitumor agents (1, 2). Their protocol is based upon the Folin-Ciocalteu method for the measurement of cell protein as modified by Eagle and Oyama (3). This involves the colorimetric determination of cell protein as an indirect measurement of cell growth.

In 1954, Rutstein *et al.* suggested that blood or serum protein levels could be determined quantitatively by interaction of the protein with the anionic dye, 2-(4-hydroxybenzeneazo) benzoic acid (4). Their method was reported to be more accurate and more rapid than electrophoretic methods (5).

In this research, an attempt was made to adapt Rutstein's procedure to the determination of cell growth along the lines of the CCNSC protocol.

EXPERIMENTAL

Rutstein's original standard curve was duplicated by using the procedure he described (4). Since this work was done within the range of normal concentrations of human serum albumin (approximately 3.5 to 4.5 mg.%), the aliquots of serum needed were small but the original quantity of reagents was large. The total volume of the reaction mixture was 25 ml. The range of detection of the standard curve was from 0 to 14 mg. The curve was found to be linear up to a level of 8 mg.

In this study, using the CCNSC protocol (1, 2), cell protein concentration of HeLa cell cultures was found to be in the range of 0.005 to 0.1 mg. Initially, to determine whether HBABA would detect protein at these levels, a series of protein concentrations within these ranges were prepared. Armour's protein standard solution[1] was used as the protein source. Because of the low quantity of protein which the authors desired to detect, a modification of the volume of reagents used was required.

To determine if the dye would detect cellular protein levels, a series of protein concentrations ranging from 0.005 to 0.1 mg. was prepared. The procedure followed was the same as that described by Rutstein (4) for serum albumin determinations, but the volume of the reactants was adjusted as follows.

A sufficient quantity of acetate buffer was added to the specified quantity of bovine albumin to give a total volume of 2 ml. To this was added 1 ml. of normal saline followed by 4 ml. of the HBABA solution. Ten minutes were allowed for completion of the reaction and development of color. Per cent transmittance and absorbance was read in a colorimeter at 520 mμ. The final solution volume was maintained at 7.0 ml., and pH adjusted to 6.2.

TABLE I.—STANDARD CURVE FOR HBABA[a]

Protein Concn.	Transmittance, %[b]	Absorbance
0.0	93.8	0.0278
1.0	92.3	0.0348
2.0	91.6	0.0381
3.0	89.1	0.0501
4.0	87.4	0.0585
5.0	85.5	0.0680
6.0	84.4	0.0736
7.0	83.2	0.0799
8.0	82.4	0.0841
9.0	81.5	0.0888
10.0	81.1	0.0910

[a] 2-(4-Hydroxybenzeneazo) benzoic acid. [b] Each reading is the average of three tests.

In the determination of cell protein levels, the following procedure was applied.

Two milliliters of a suspension containing 100,000 cells/ml. was centrifuged at 2500 × g for 10 min. The supernatant liquid was removed from the packed cells by decantation. To the cells was added 2 ml. of a 0.05% trypsin solution and 5 drops of 0.01 *N* hydrochloric acid, for the purpose of lysing the cells. Two milliliters of acetate buffer solution was added to the lysate and the mixture shaken vigorously. Finally, the HBABA solution was added, 10 min. allowed for color development, and the per cent transmittance and absorbance read in a suitable colorimeter at 520 mμ.

RESULTS AND CONCLUSIONS

Using this modified procedure, it was determined that HBABA could be used to detect protein concentrations above 1.0 mg. However, this dye does not give consistent or reproducible results below a concentration of 0.3 mg. (Table I).

Similar results were found when this procedure was used to determine the protein concentration of cell cultures standardized to contain 100,000 cells/ml. of media.

It is to be concluded that while this assay may be adequate enough to be used in the clinical evaluation of serum protein levels, it is not sensitive enough to apply to the determination of cellular protein concentrations.

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Received March 4, 1966, from the College of Pharmacy, St. John's University, Jamaica, N. Y. 11432.
Accepted for publication September 19, 1966.
[1] Protein Standard Solution supplied by Armour Pharmaceutical Co., Kankakee, Ill.