MEASUREMENT OF PROTEIN CONCENTRATION BY QUANTITATIVE ELECTRON MICROSCOPY

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ABSTRACT

The method of quantitative electron microscopy was applied to the measurement of protein concentration in thin sections. The human erythrocyte was selected as a model because of its apparently uniform protein concentration. Phosphotungstic acid (PTA) in aqueous solution was used as a reversible stain for protein, and PTA-stained Dowex resin spheres were embedded along with the red cells as standards for measurement of section thickness. The mass of stain removed from a given area of sectioned red cell by buffer (pH 7.4) was measured by quantitative electron microscopy. From the stoichiometry of the reaction between PTA and red cell protein established in this study, the amount of protein present in the measured area was calculated. From this amount of protein and the measured thickness, the concentration of protein was calculated and expressed as g/100 ml, for comparison with the clinical laboratory value for hemoglobin. Groups of red cells from the same sample were measured on 3 different days and their mean values (g/100 ml $\pm$ SD) were 29 $\pm$ 3.9, 30 $\pm$ 2.7, and 33 $\pm$ 4.6, compared to the clinical laboratory value of 32.1 g/100 ml packed cells, after correction for volume change and protein loss during fixation.

INTRODUCTION

This investigation was concerned with the measurement of protein concentration in thin sections by quantitative electron microscopy, as an extension of previous studies of PTA as a quantitative stain for protein (5) and of the use of PTA-stained Dowex resin spheres (Dow Chemical Co., Midland Mich.) to measure thickness within sections (6). Silvester and Burge (7) employed quantitative electron microscopy to measure the per cent uptake of two relatively weak stains, triiodobenzoyl chloride and triiodophenyl isocyanate, by the proteins of sperm cell membranes. On the basis of this study, Burge (2) later concluded that partial loss of mass of the embedded biological material during electron microscopy would lead to significant error in its measurement. Bahr et al. (1) determined the effects of the electron beam on gelatin films and found that loss of mass could be as high as 25%.

To minimize the error due to these effects on the measurement of protein in thin sections, the mass of a dense stain (PTA) removable from the protein was determined. In this way any loss of mass per unit area due to loss of protein during electron microscopy would be a small fraction of the total loss in mass per unit area due to removal of the stain. Accurate measurement requires that the stain not sublime appreciably and that it be removed completely. As an empirical test of the measurement, the human red blood cell was selected as a model whose protein concentration was relatively uniform throughout the cell. The protein concentration of individual cells was estimated by quantitative electron microscopy, and the hemoglobin concentration of populations was estimated by standard clinical laboratory methods. The method of quantitative electron microscopy was advantageous in demonstrating the distribu-

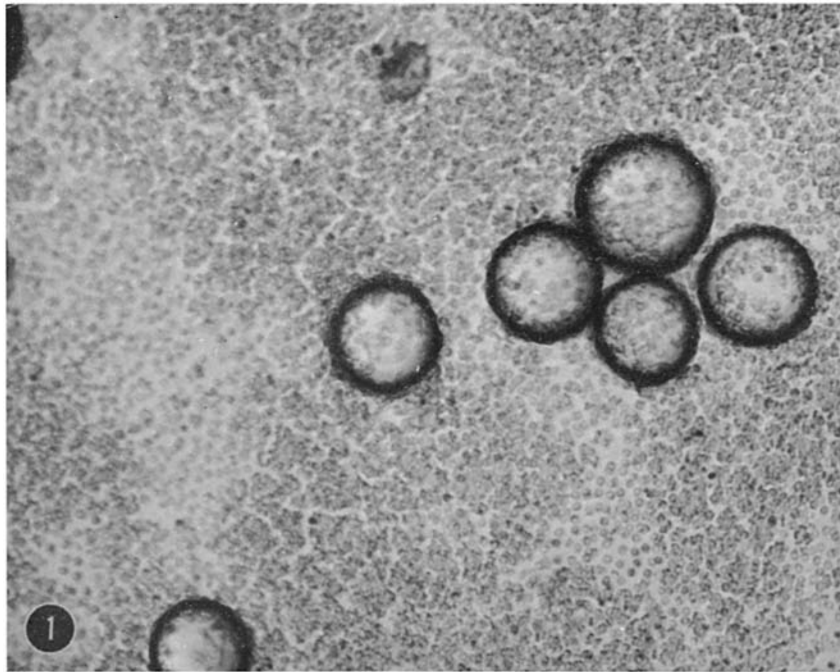

FIGURE 1 Human red blood cells and PTA-Dowex resin spheres after embedding. × 150.

tion of protein concentrations among individual cells and provided a statistical definition of the population.

MATERIALS AND METHODS

Preparation of Human Red Blood Cells and Dowex-PTA

Blood was obtained by venipuncture with use of a Vacutainer tube (Becton, Dickinson & Co., Rutherford, N. J.) containing 2 mg of ethylenediaminetetraacetate per ml blood. The cells were washed three times in 0.15 M NaCl, by using 25 volumes per unit volume of blood each time. The washed red cells were added slowly to 20 volumes of 1% paraformaldehyde in 0.1 M potassium phosphate buffer (pH 7.4) and were left to fix at 4°C for 24 hr. They were washed twice, 20 min each time, in 20 volumes of 7.5% sucrose in 0.1 M potassium phosphate buffer, left overnight in the buffered sucrose, and washed twice again as before. They were added slowly to 20 volumes of 5% PTA in 2% ammonium acetate, stained for 2 hr, and washed three times, one hr each time, in 2% ammonium acetate brought to pH 2 with concentrated HCl. The cells were then placed in small flat pans of aluminum foil and dried *in vacuo* over silica gel at 23°C. Dowex resin AG 1 × 2 (Dow Chemical Co., Midland, Mich.) was stained with 5% PTA as described previously (6). The stained resin was added to the dried red cells and the pan was filled with Epon-Araldite (mixture 3 of Mollenhauer [3]). The suspension was left standing at room temperature (23°C) for 3 days and then at 60°C for 2 days. The appearance of the embedded spheres and cells is shown in Fig. 1. Sections (Fig. 2) showing dark silver-gray interference colors were cut with a diamond knife onto 0.01 N HCl, by using a Huxley ultramicrotome set at a low speed of traverse.

Change in Red Cell Volume on Embedding

The diameters of 200 unfixed cells in 0.15 M NaCl and of 200 embedded (Fig. 1) red cells were measured by using a Cooke-AEI image-splitting eyepiece (Frank Cooke, North Brookfield, Mass.) The degree of volume change was taken as the ratio of the cubes of the diameters of fixed to unfixed cells.

Loss of Protein During Fixation

Washed red cells from 1 ml of blood were suspended in a final volume of 15 ml of 0.9% NaCl. Two 1-ml volumes of the suspension were used for protein estimation by Kjeldahl analysis. 5 ml of the suspension were placed in each of two centrifuge tubes, and to each tube 5 ml of 2% paraformaldehyde in 0.2 M potassium phosphate buffer (pH 7.4) were added. Each tube was agitated every 30 min for 2 hr and was

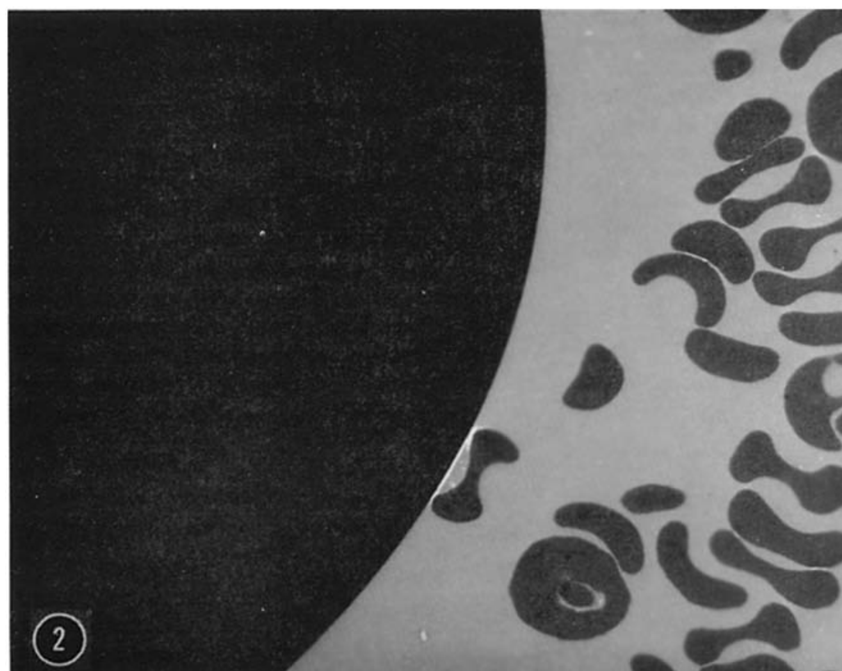

FIGURE 2 Section of PTA-Dowex resin sphere and adjoining red cells. Note uniformity of staining. × 2400.

left standing at 4°C for a total fixation time of 24 hr. The red cells were then packed tightly by centrifugation. The supernatant was removed carefully with a pipette, and its volume was measured. 2 ml of each supernatant were used for estimations of Kjeldahl nitrogen, and protein was taken as 6.25 × nitrogen.

Stoichiometry of PTA-Red Cell Protein Reaction

Red cell protein was obtained from washed, type A, human red blood cells by freeze-thawing and centrifugation to remove the cell ghosts. It was fixed in 25 times its volume of 1% paraformaldehyde in 0.1 M potassium phosphate buffer. Further treatment and measurement of its reaction with PTA were done according to the method described previously for fixed bovine plasma albumin (5).

Determination of Section Thickness and Protein Concentration

From the use of polystyrene latex spheres (approximately 1260 A diameter) as weight standards, a straight line regression was obtained for transmittance of the negative photographic images as a function of mass of the spheres (4) (Fig. 3). Section thickness was derived from the use of sectioned PTA-Dowex as already described (6). Magnification was approximately 14,700 times, measured on a diffraction grating replica cross-ruled at 21,600 lines per cm. An area 2 × 5 mm on the photographic image of the red cell was scanned in a Jarrell-Ash recording microdensitometer (Jarrell-Ash Co., Waltham, Mass.), and the area under the square-wave generated (Fig. 3) was measured; the value was converted to mass by the factor derived from the polystyrene latex spheres. The sections were placed on droplets of 0.1 M phosphate buffer (pH 7.4) overnight and micrographs of the red cells were again made and scanned in the densitometer. The difference in mass of the stained and destained protein was taken as the mass of stain taken up in the red cell area measured. The thickness in this area was taken as equivalent to that of the immediately adjacent PTA-Dowex (Fig. 2).

The mass of stain present in the defined area (0.046 μ^2) of the red cell was measured by quantitative electron microscopy. From the known stoichiometry of PTA and fixed red cell protein, the mass of protein present in the stated area was calculated. From the area and thickness the volume was calculated, and the value for protein concentration was expressed as grams of protein per 100 ml, for comparison with results on unfixed samples of the same blood obtained by standard clinical laboratory methods for measurement (triplicate) of hematocrit and hemoglobin.

RESULTS

Red Cell Hemoglobin Concentration

The average hemoglobin concentration was 14.8 g per 100 ml of blood and the average hematocrit value was 42 ml per 100 ml. From these data the concentration was calculated to be 35.2 g per 100 ml of packed red cells.

Stoichiometry of the Red Cell Protein-PTA Reaction

The fixed red cell protein bound 1.7 times its weight of PTA. If the density of fixed protein is taken as 1.32 (7), the calculated density of the stained protein becomes 3.56. The transmittance of the stained red cell in electron micrographs was always nearly equal to that of the PTA-Dowex, whose density was calculated to be 3.36 (Figs. 2, 3).

Change in Red Cell Volume after Embedding

The red cells appeared to retain their shape as biconcave discs quite well, although some sickling was evident (Figs. 1, 2). The average diameter and SD of the fresh cells was 7.27 ± 0.52 μ, and that of the fixed embedded cells 7.37 ± 0.30 μ. From the ratio of the diameters cubed, the volume of embedded to unembedded cells was calculated to be 1.04. After correction for volume change during embedding, the hemoglobin concentration was calculated to be 33.8 g per 100 ml of packed cells.

Loss of Protein During Fixation

Two 1-ml volumes of a suspension of fresh red cells contained 10.75 and 10.80 mg protein. After

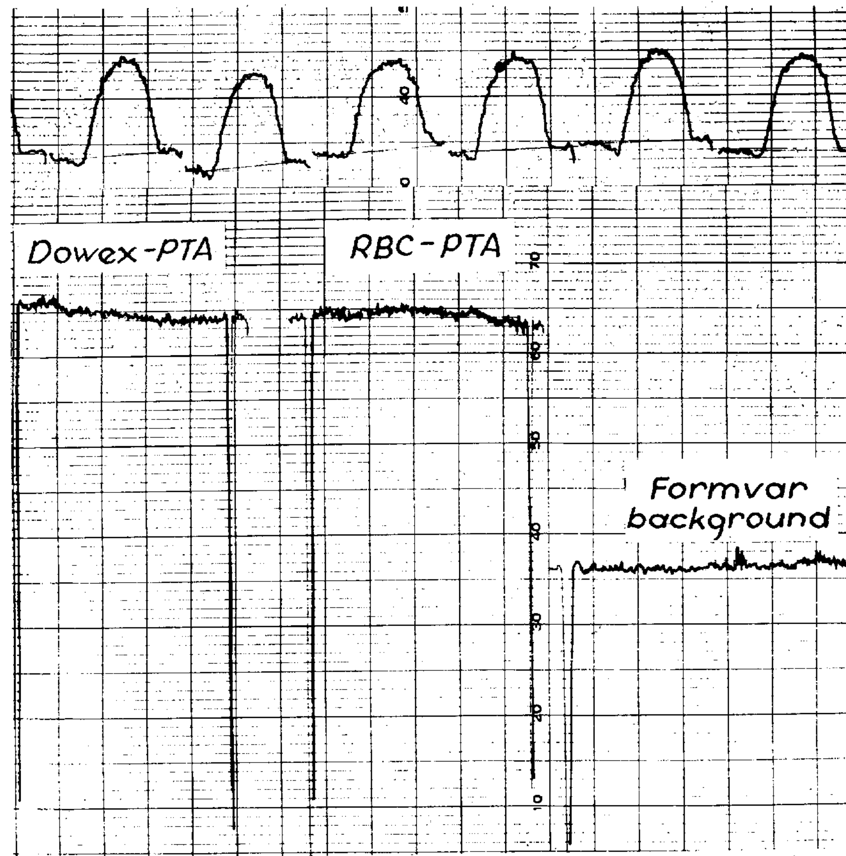

FIGURE 3 Above: densitometric tracings of electron micrographs (negatives) of whole polystyrene spheres used as mass standards. Below: tracings of background film and sectioned PTA-Dowex resin sphere and red cell.

TABLE I

Quantitative Electron Microscopic Measurement of Section Thickness and Red Blood Cell Protein Concentration

Section thickness	Protein concentration	Section thickness	Protein concentration
(*A*)	(*g/100 ml*)	(*A*)	(*g/100 ml*)
660	34	376	32
409	24	534	29
392	28	321	30
530	26	321	34
611	28	542	30
621	32	530	30
603	31	530	45
607	24	538	34
586	35	538	32
734	29	293	32
	—	565	27
	Mean 29 ± 3.9	565	39
		877	26
546	27	293	29
492	29	412	14
518	27	412	25
528	33	356	30
632	28	356	27
458	35	356	23
590	30	396	31
597	28	483	38
656	32	550	27
610	31	573	29
	—	676	37
	Mean 30 ± 2.7	676	31
		526	27
			—
			Mean 33 ± 4.6

fixation the two supernatant solutions contained 0.55 and 0.52 mg of protein, respectively. The mean loss was 5.1% and, after correction for it and volume change on embedding, the hemoglobin concentration was calculated to be 32.1 g per 100 ml of packed cells.

Red Cell Protein Concentration from Quantitative Electron Microscopy

The mean protein concentrations found for the three groups of red cells (Table I) are 94, 91, and 103%, respectively, of the hemoglobin concentration which was calculated from the clinical laboratory data corrected for volume change on embedding and protein loss during fixation.

DISCUSSION

The similarity between the value for red cell hemoglobin concentration derived from clinical laboratory methods and the value for protein obtained by quantitative electron microscopy indicates that the latter can give valid measurements of protein concentration in ultrathin sections under certain conditions. The method is limited for the present to fixed, embedded material, and it is important to consider changes in mass of the materials being studied during the course of tissue preparation and irradiation in the electron microscope. Use of a staining method, which increases mass and thus minimizes error due to the effects of the electron beam on the materials, has the limitation that staining intensity depends on the concentration of reactive groups, and this, in turn, varies with the type of protein or other material stained. This feature is of special importance if normal and pathologically altered tissues are to be compared, since a difference in relative composition of protein may involve changes in concentration of groups. The method might be expected to yield valid results in those instances in which a well-defined marker is to be added to the tissue, as in the case of antigen-antibody reactions.

The great advantage of the method stems from its high resolution and great sensitivity. As a result, selected areas within cells or organelles can be measured and a range of individual values can be demonstrated which, ordinarily, cannot be obtained by biochemical procedures requiring large populations of cells or organelles.

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