MEASUREMENT OF THICKNESS WITHIN SECTIONS BY QUANTITATIVE ELECTRON MICROSCOPY

LLOYD SILVERMAN, BERIT SCHREINER, and DAVID GLICK

From the Division of Histochemistry, Department of Pathology, Stanford University Medical School, Palo Alto, California 94304. Dr. Schreiner's present address is the Zoological Laboratory, University of Oslo, Norway

ABSTRACT

To apply the method of quantitative electron microscopy to the measurement of mass in thin sections, the thickness of the section at or very near the structure to be studied must be known. Dowex anion exchange resin AG 1 × 2, stained with phosphotungstic acid (PTA) at pH 6.4, was used as a thickness standard which could be embedded and sectioned. The sectioned PTA-Dowex appeared uniformly stained and exhibited suitable electron opacity. The stoichiometry of the reaction between PTA and the Dowex resin was measured by three independent methods based on gravimetric, colorimetric, and nitrogen determinations whose results showed close agreement. From the PTA uptake, the density of the stained spheres was calculated. Mass of a defined area of PTA-Dowex was measured by quantitative electron microscopy, and from this mass and density, the volume and then the thickness were calculated. The values for thickness were compared to those obtained by interference microscopy on the embedding medium alone in the same sections.

INTRODUCTION

Several principles have been applied to measurement of the thickness of sections used in electron microscopy (15). Porter and Blum (9) and Sjöstrand (14) used a shadowing technique to measure thickness at the edge of sections, but later the method was found to give widely varying results along a given edge (16). Peachey (8) correlated interference colors with thickness given by ellipsometry, and found that each color represented a range of about 300 A, too great for quantitative use. Williams and Meek (16) used a radioactivity method with S^{35} to derive average thickness for sections, but by interferometry and microdensitometry they demonstrated that within a single section the thicknesses varied 10–25%. The latter finding was in accord with that of Cosslett (2), who concluded that a satisfactory method was still not available at the time.

Quantitative electron microscopy, on which the measurement of thickness in this study was based, was developed by Bahr and Zeitler (1), Lenz (6), Hall (4), and others for determining the mass of whole biological particles such as red blood cells, viruses, and mitochondria (1); it has also been used to measure antigen-antibody reactions on influenza virus (11). When the method is applied to thin sections, two major problems are encountered. First, nonmineralized tissues are not significantly electron opaque at these thicknesses and require staining for identification and measurement. Second, section thickness must be measured very near the body whose mass is to be deter-

mined, to avoid errors due to variation in thickness within the section. This necessitates embedding a thickness-standard very near the site of the determination. Silvester and Burge (13) have used spermatozoa heads as a standard to measure *relative* thickness by quantitative electron microscopy. The thickness of the embedding resin near the object can be measured similarly, but such resins provide little electron opacity and, in addition, are known to sublime significantly in the electron beam (10).

For measurement of *absolute* section thickness, it seemed desirable to develop a readily available standard which would have suitable electron opacity, chemical stability, resistance to the electron beam, and a known density. For this purpose, spheres of a loosely cross-linked anion exchange resin, Dowex AG 1 × 2 (Dow Chemical Co., Midland, Mich.), stained with phosphotungstic acid (PTA), were used (12). The stoichiometry of the staining reaction was examined by three different methods, because of the possibility that each might be influenced by physical or chemical changes induced in the Dowex by steps in the methods.

MATERIALS AND METHODS

Reaction of Dowex with PTA

Dowex AG 1 × 2 (Biorad, 15–50 μ diameter) was washed free of contaminating heavy metals by four alternate washes in 2 N NaOH and HCl as recommended by the manufacturer.

5% aqueous PTA was brought to pH 6.4 by addition of 2.5 M KOH. Other pH values proved unsatisfactory either because the Dowex was damaged (pH 2) or because it was insufficiently stained (pH 9 and above). The Dowex was placed in a chromatography column and the PTA solution was added until chloride ion was no longer detected in the effluent by silver nitrate. The Dowex-PTA was washed exhaustively with distilled water to remove unbound PTA.

Gravimetric Determination of PTA-Dowex Stoichiometry

The determination was carried out in two ways. First, samples of PTA-Dowex, approximately 50 mg each, were weighed into separate medium-porosity, sintered glass microfilter funnels. The samples were washed 12 times with 25-ml portions of 2 N NaOH to remove the PTA bound to the Dowex, three times with 25-ml portions of distilled water to remove the NaOH, and finally five times with 25-ml volumes of 0.01 N HCl to restore the original chloride form of the

Dowex. The resin was then dried to constant weight at 37°C *in vacuo* over silica gel. The weight loss was taken as the mass of PTA removed from the complex.

In the second method, samples of approximately 50-mg of Dowex were weighed into individual filter funnels and treated with an excess of the PTA solution. The PTA-Dowex formed was washed three times with 25-ml portions of distilled water and dried to constant weight as before. The increase in weight gave the amount of PTA taken up by the Dowex.

Colorimetric Determination of PTA-Dowex Stoichiometry

50-mg samples of PTA-Dowex were extracted individually with 25 ml of 2 N NaOH. 0.5 to 1.0 ml aliquots of the extract were added to 10 ml of 10% hydroquinone in concentrated H_2SO_4. The absorbance of the reddish-brown solution was measured at 520 mμ in a Beckman DU spectrophotometer (Beckman Instruments, Inc., Fullerton, Calif.). Dowex alone was used as control, and PTA standards were included with every run. This procedure followed the method used by Kuhn et al. (5).

Determination of PTA-Dowex Stoichiometry by Kjeldahl Measurement of Nitrogen

Samples of washed Dowex and PTA-Dowex, approximately 5 mg each, were weighed into separate aluminum foil cups, 3 mg weight; the cups with sample were placed in digestion flasks, 2 ml of concentrated H_2SO_4 were added to each, and the cups were heated until the solutions were clear. $(NH_4)_2SO_4$ standards with and without aluminum cups were used. Nitrogen concentrations of unstained and stained Dowex were determined by the Kjeldahl technique, and the amounts of Dowex in the samples of PTA-Dowex were calculated.

Determination of Section Thickness by Quantitative Electron Microscopy

Samples of the PTA-Dowex, washed and dried as described for the chemical studies, were embedded in Epon-Araldite (mixture 3 of Mollenhauer (7)) in flat aluminum foil pans. They were let stand for 2 days at each of the temperatures, 23°, 37°, and 60°C. The embedded spheres were cut with a diamond knife on a Huxley ultramicrotome set at a low speed of traverse. Single sections were floated onto water and placed on Formvar-coated grids (Fig. 1). The procedure for quantitative electron microscopy was carried out as described previously (11). Spheres of polystyrene latex (Dow Chemical Co.), about 0.088 μ diameter, were sprayed onto coated grids and photographed at a magnification of 18,000 times with a Hitachi HU-11A electron microscope, operated at 75 kv, with a
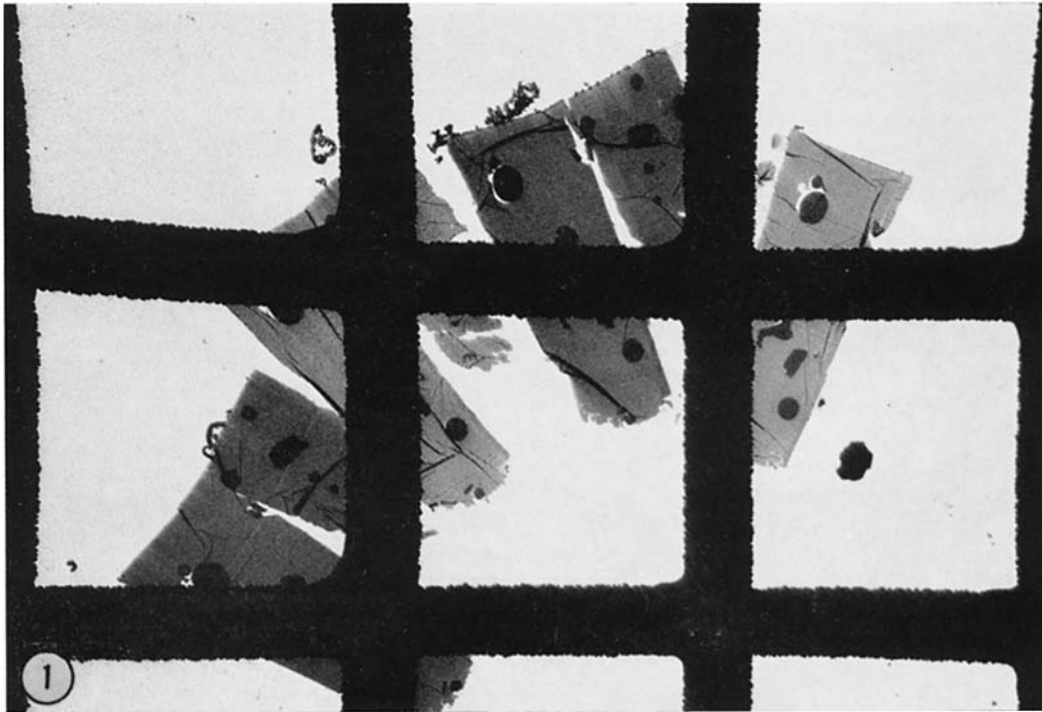
FIGURE 1 Sections of PTA-Dowex embedded in Epon-Araldite. The electron opacity of the embedded spheres is seen to vary with the section thickness. × 150.

50μ objective aperture and liquid nitrogen in the anticontamination chamber. The photographic negative images were scanned with a Jarrell-Ash recording microdensitometer (Jarrell-Ash Co., Waltham, Mass.), and the areas under the resulting curves were measured by planimetry. A straight line relationship was obtained by the method of least squares between the areas and the masses of the spheres (calculated from their measured diameters and known density). Sections of embedded PTA-Dowex were photographed under the same conditions, and the optical transmission of a standard area was measured. The mass of PTA-Dowex in this area was then calculated. The density of the PTA-Dowex (3.36) was calculated from the density of the Dowex, and the measured uptake of PTA. From mass and density, the volume was obtained and then from this volume and the area measured in the PTA-Dowex section the thickness was calculated.

Measurement of Section Thickness by Interference Microscopy

The refractive index of the embedding medium, measured on an Abbe refractometer (Abbe Engineering Co., N.Y.), was 1.518. The thicknesses of 10 sections were determined by interference microscopy after measurement by quantitative electron microscopy; the measurements of another 10 sections were carried out in the reverse order. The Leitz interference microscope with separate object and control beams (E. Leitz Inc., Burlingame, Calif.) made it possible to examine sections on copper grids. The vernier method of measurement was employed with monochromatic light at 5460 A.

RESULTS

Results of the gravimetric, colorimetric, and Kjeldahl determinations of the uptake of PTA by Dowex, listed in Tables I–III, were in good agreement. Dowex took up about 2.2 times its weight of PTA, and the complex had a calculated density of 3 36.

The data in Table IV show that in most cases measurements of section thickness by electron or interference microscopy were in good agreement. However, larger discrepancies (20% or more) were found in certain instances. In these cases, it was observed that the same section showed considerable variation in thickness in different areas, and it could not be certain that the measurements were made in the same area by both methods. This

TABLE I

Gravimetric Determination of PTA Uptake by Dowex AG 1 × 2 at pH 6.4

PTA-Dowex	Dowex	PTA (calc.)	% PTA uptake
mg	*mg*	*mg*	
52.7	16.3	36.4	223
50.6	15.9	34.7	218
68.3	21.6	46.7	216
50.7	15.8	34.9	222
50.8	16.0	34.8	218
			Mean 220

TABLE II

Colorimetric Determination of PTA Uptake by Dowex at pH 6.4

PTA-Dowex	PTA	Dowex (calc.)	% PTA uptake
mg	*mg*	*mg*	
50.5	34.5	16.0	216
50.4	34.5	15.9	218
51.0	35.5	15.5	229
50.2	35.5	14.7	242
50.2	34.0	16.2	210
50.2	35.0	15.2	230
			Mean 222

TABLE III

Kjeldahl-Nitrogen Determination of PTA Uptake by Dowex AG 1 × 2 at pH 6.4

PTA-Dowex	Nitrogen	Dowex (17.3 × N)	PTA (calc.)	% PTA uptake
mg	*mg*	*mg*	*mg*	
5.40	0.09	1.56	3.84	246
6.50	0.10	1.73	4.77	276
5.31	0.09	1.56	3.75	240
6.30	0.12	2.08	4.22	203
5.92	0.11	1.90	4.02	212
5.20	0.10	1.73	3.47	201
5.12	0.09	1.56	3.56	228
4.93	0.09	1.56	3.37	216
5.15	0.09	1.56	3.59	230
				Mean 228

result emphasizes the desirability of having a method of measuring the thickness very near the object whose mass is to be determined.

TABLE IV

Comparison of Measurements of Section Thickness (A) by Quantitative Electron and Interference Microscopy

	Section No.	Electron Microscopy	Interference Microscopy	EM/IM
EM followed by IM	1	365	374	0.98
	2	399	616	0.65
	3	508	484	1.05
	4	643	594	1.08
	5	387	396	0.98
	6	619	593	1.04
	7	476	506	0.94
	8	745	840	0.87
	9	382	396	0.96
	10	781	812	0.96
IM followed by EM	1	464	484	0.96
	2	484	535	0.90
	3	546	559	0.98
	4	721	865	0.83
	5	617	847	0.73
	6	558	581	0.96
	7	514	532	0.97
	8	590	488	1.21
	9	510	389	1.31
	10	462	490	0.94

DISCUSSION

Dowex anion exchange resin stained with PTA provided a standard whose electron opacity was particularly well suited for the measurement of section thickness in the range 300–1000A. With an electron microscope operated at lower voltage (e.g. 50 kv or less), such standards should be useful for still thinner sections (to about 25 A) (2).

The major advantage of this method over interference microscopy for measurement of section thickness is that it permits measurements very close to the object to be studied, and so obviates error due to variations in thickness within the section.

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