THE REACTIVITY AND STAINING OF TISSUE PROTEINS WITH PHOSPHOTUNGSTIC ACID

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ABSTRACT

After aldehyde-fixation, treatment with phosphotungstic acid (PTA) in aqueous acidic medium was shown to produce an intense electron-opaque stain with minimal distortion of organelles. Mitochondrial matrix, cisternae of the endoplasmic reticulum, and the Z-band of muscle were densely stained, whereas membranes stood out in negative contrast. Staining of glycogen or lipid was not apparent. Under certain conditions the stain density reflected the concentration of protein based on the quantitative reaction of PTA with the positively charged groups, although the stoichiometry of the reaction between PTA and protein varied with the kind of protein. The staining conditions established should provide a base for the use of the method in quantitative electron microscopy, particularly on thin sections.

INTRODUCTION

Quantitative electron microscopy has been used to determine the mass of whole particles such as viruses and mitochondria (1, 11) by measurement of optical densities of the photographic images. Since, however, in sections of the thicknesses used in electron microscopy, most biological substances give negligible contrast, it is necessary to develop a specific and stoichiometric stain which will increase electron opacity sufficiently for measurement of the mass of a substance such as protein. Aside from questions of stoichiometry, osmium tetroxide, uranyl, and lead salt stains do not qualify because they lack specificity.

Phosphotungstic acid (PTA) has long been used as a protein precipitant (19), and Hall et al. (9, 10) introduced it for positive staining of whole muscle and collagen fibrils. From these and other studies (5–7, 11–13, 18), it was concluded that PTA acted as an anionic stain for the positively charged groups of protein. PTA has been used sporadically as a positive stain in tissue sections, usually as an adjunct to other stains after osmium tetroxide fixation (2, 7). Huxley (12) used PTA in absolute alcohol to stain OsO_4-fixed muscle tissue, and stated that its use in aqueous media had a deleterious effect on tissue structure. With PTA treatment after OsO_4 fixation, Watson (20) obtained weak and variable staining. The purpose of this study was to establish conditions for the stoichiometric reaction of PTA with tissue proteins, and to develop a method in which PTA in aqueous solution served as a single and reliable stain for aldehyde-fixed tissue, with a view to its potential use in quantitative electron microscopy.

MATERIALS AND METHODS

Specificity of the PTA Reaction

PRECIPITATION AND TEST FOR SOLUBLE COMPLEX FORMATION: 10 volumes of aqueous 5% PTA were added to 1 volume each of the following: arginine (1%), leucine (1%), poly-L-lysine

(1%), poly-L-glutamic acid (1%), oleic acid, DNA (1% in 0.15 M NaCl), and aqueous solutions of glycogen (1%), bovine nasal septum chondromucoprotein (1%), highly purified hyaluronic acid (0.5%), chondroitin sulfate A (0.5%) histamine (0.2%) and serotonin (0.2%). To 1 volume each of cholesterol (0.1% in absolute ethanol), lecithin (0.1% in ethanol), cephalin (0.1% in chloroform) and sphingomyelin (0.1% in ethanol), cephalin (0.1% in chloroform) and sphingomyelin (0.1% in chloroform), 10 volumes of 1% PTA in the respective solvents were added. Solutions containing the substances that gave no precipitate with PTA, i.e. glycogen, DNA, and chondroitin sulfate A, were dialysed exhaustively against 0.01 N HCl to test for the presence of soluble PTA complexes.

DEAMINATION: To 10 ml of 1% bovine plasma albumin successive 35-ml volumes of HNO_2 (14) were added until gas formation ceased. The yellow precipitate formed was washed, dried, and treated with PTA solution as before, washed extensively with 0.01 N HCl (see below), and PTA was determined by the hydroquinone method (13). Another 10 ml portion of the albumin solution was treated with 10 volumes of 5% PTA, the precipitate was washed with 0.01 N HCl and then reacted with HNO_2 as above.

Stoichiometry of the Reaction between PTA and Selected Proteins

PREPARATION OF PROTEIN: One volume of bovine plasma albumin (Armour) was fixed for 24 hr with 10 volumes of 4% glutaraldehyde in 0.067 M potassium phosphate buffer, pH 7.4, dialysed against running tap water for 1 wk (until the water, concentrated 50 times, became Schiff-negative), dried, and ground in a mortar to a fine powder. Unfixed rat skeletal muscle was finely minced, acetone-extracted, dried, and ground to a fine powder. A portion was fixed as described for albumin and washed three times, 1 hr each time in 0.2 M buffered sucrose, dried, extracted, and ground as before. Bovine collagen was extracted from calf skin with 0.5 M acetic acid (8) and freeze-dried. A portion was shredded finely, fixed, washed as before, and finely shredded again.

REACTION WITH PTA: The solutions of unfixed and fixed albumin were precipitated with 10 times their volume of 5% PTA (pH 2), and the precipitates were centrifuged and dried in a 37°C incubator for 1 day. The other powdered preparations were treated with excess 5% PTA for 2 hr, and the resulting precipitates were dried as before. Samples of approximately 50-mg were washed 12 times (5 min each) with 25-ml volumes of 0.01 N HCl (pH 2), and dried to constant weight *in vacuo* over silica gel at room temperature.

DETERMINATION OF PROTEIN: Samples of PTA-protein precipitate of approximately 10-mg were analyzed for nitrogen (Kjeldahl) and protein was calculated (6.25 × nitrogen).

COLORIMETRIC DETERMINATION OF PTA: The procedure employed by Kuhn et al. (13) was used. 10-mg samples of precipitate were individually extracted with 5 ml of 0.067 M phosphate buffer, pH 7.4 (all bound PTA appeared to go into solution since subsequent treatment with 1 N NaOH caused no detectable increase of PTA). 10 ml of 10% hydroquinone in concentrated H_2SO_4 were added to 1 ml of each extract, the volume was adjusted to 25 ml with concentrated H_2SO_4, and the absorbance was read after 2 hr at 520 mμ in a Beckman, Model DU, spectrophotometer (Beckman Instruments, Inc., Fullerton, Calif.). PTA standards 0.25 and 0.50 mg ($P_2O_5 \cdot 24\ WO_3 \cdot x\ H_2O$ analytical reagent, dried in a desiccator over silica gel) per ml phosphate buffer were included in each run.

GRAVIMETRIC DETERMINATION OF PTA: 50-mg samples of fixed, PTA-stained, acid-washed skeletal muscle precipitates were each placed in a medium-porosity, sintered glass microfilter funnel (Gooch type) and dried to constant weight. Precipitates were washed extensively with 0.067 M phosphate buffer to remove PTA, and finally dried to constant weight in the funnels. The resulting weight loss represented the mass of PTA bound to protein at low pH. The protein in each residue was determined by Kjeldahl analysis.

EFFECT OF PH: Acid-washed precipitates of albumin were individually washed further with one of the following: 0.1 M citrate buffer, pH 2.2; 0.1 M acetate buffer, pH 5.5; distilled water; 0.1 M phosphate buffer, pH 7.4, or 1 N NaOH, pH 13.

BINDING OF PTA TO DIAZOTIZABLE GROUPS: Trinitrobenzene sulfonic acid (TNBS) was used to estimate amino groups in the proteins (17). 5-ml samples of 0.5% bovine plasma albumin were brought to pH 2.0 with HCl. 0.5, 1.0, 1.5, and 2.0 ml volumes of 1% PTA were added to respective samples of albumin, and the volume of each was adjusted to 10 ml with water, final pH 2.0. The precipitates were centrifuged and each supernatant was assayed for remaining amino groups: 0.2 ml of supernatant was added to 0.4 ml of TNBS and 0.2 ml of 4% $NaHCO_3$; after 2 hr 5 ml of water was added and the absorbance was measured immediately at 340 mμ. In the original method, HCl was added to stabilize the color but it was omitted here to avoid precipitation of protein. It was found that Beer's law applied, nevertheless. L-leucine was employed as the standard for the reaction.

Staining Tissue with PTA for Electron Microscopy

A variety of tissues, including rat liver and cardiac muscle and human red blood cells, was fixed over-

night in 1% glutaraldehyde in 0.067 M potassium phosphate buffer, pH 7.4, with added sucrose to give a final osmolality of 300 ± 10 milliosmols. After three washings, 1 hr each, in phosphate-buffered 7.5% sucrose, small blocks were stained for 3 hr in 5% PTA in 6.25% Na_2SO_4, then washed three times, 1 hr each, in 2% ammonium acetate (formic acid added to pH 2.0). They were then dehydrated in graded ethanols with added formic acid (pH 2.0), rinsed briefly in propylene oxide, and embedded in Epon-Araldite (15). Sections with silver-gray to gray interference colors were cut onto 0.01 N HCl on a Huxley ultramicrotome, and examined at 50 or 75 kv in a Hitachi HU-11A electron microscope with a 50 μ objective aperture. Similar sections were washed on droplets of 0.067 M potassium phosphate buffer (pH 7.4) or 0.1 N NaOH (pH 13) to remove PTA before examination.

RESULTS

Specificity of the Reaction

Phosphotungstic acid precipited albumin, poly-L-lysine, arginine, histidine, histamine, and serotonin, but failed to precipitate polyglutamic acid, glycogen, chondromucoprotein, chondroitin sulfate A, the cerebral lipids, oleic acid, or DNA from solution. The tests for formation of soluble PTA complexes by the dialysis experiments revealed that none could be detected, except in the case of glycogen which always bound a trace of PTA. More extensive dialysis lessened, but did not entirely abolish, the positive test with glycogen.

Deamination of protein decreased its binding of PTA by 85%. After protein had reacted with PTA, nitrogen evolution from HNO_2 treatment did not occur.

Raising the pH of the washing solution led to removal of the stain. When precipitates of PTA-albumin were treated with buffer at pH 5.5 or higher, they dissolved. No loss of PTA was evident after washing with buffer at pH 2.2, and only small losses were noted after brief washing with distilled water, but the pH of the unbuffered water was lowered by contact with the precipitate.

Stoichiometry of the Reaction Between PTA and Selected Proteins

In Table I it can be seen that values for the stoichiometric binding of PTA to different proteins varied with the nature of the latter, and that glutaraldehyde fixation depressed the uptake significantly. For a given protein preparation the uptake was constant and reproducible, and two different, fixed rat muscle preparations showed similar binding of PTA. Both gravimetric and colorimetric determination of PTA uptake by the fixed muscle gave comparable results. Glutaraldehyde fixation diminished the difference in the uptake by different proteins.

Uptake increased when the ratio of PTA to protein in solution increased. In the presence of excess albumin, the PTA to protein ratio was less (1.16) than that which resulted from the presence of excess PTA, i.e. when all albumin was precipitated. A maximum was reached when the ratio of PTA to protein in solution was 2:1 or more by weight, and the resulting ratio in the precipitate became 1.54.

Electron Microscopy

Sections showed, over-all, good contrast and delineation of subcellular structures (Figs. 1, 2). The matrix of mitochondria and the cisternae of the endoplasmic reticulum (Fig. 3) and the Z-band of muscle were very densely stained, and the outlines of the membranes were seen in negative contrast. Other structures, such as microbodies, desmosomes, and myofibrils, stained with moderate intensity. Cell membranes either were not discernible, or were demonstrable only as negative images against their positively stained surroundings, as in the case of the mitochondria. Neither glycogen nor lipid vacuoles were stained. When stained tissue sections were washed with buffer at pH 7.4, the increase in contrast over that of unstained tissue was lost.

DISCUSSION

To produce the least detectable increase in contrast in sections 700 A thick, the mass per unit volume must be increased by about 7% under the common operating conditions of the electron microscope (4). The uptake of PTA by glutaraldehyde-fixed proteins (Table I) produced measurable contrast as seen in the electron micrographs. The preservation of fine structure was good when aqueous PTA was used in a solution of proper osmolarity, and the staining results depicted were considerably different from those reported heretofore (2, 12, 20) in which osmium tetroxide was used as fixative. Membranes, in general, were not stained, and possibly were removed during dehydration. Glycogen also was not stained and possibly was extracted by the acid solution during

TABLE I

Uptake of Phosphotungstic Acid by Proteins

Protein sample	PTA-protein precipitate	Protein	PTA	Protein / Precipitate	PTA / Precipitate	PTA / Protein
	mg	*mg*	*mg*			
Bovine plasma albumin, unfixed	10.0	4.2	6.4	0.42	0.64	
	10.0	4.3	6.5	0.43	0.65	
	10.0		6.5		0.65	
	10.1	3.9		0.39		
	9.7	4.0	6.4	0.42	0.66	
Mean				0.42	0.65	1.54
Bovine plasma albumin, fixed	9.7	3.6		0.38		
	9.9	3.6		0.37		
	9.8	3.6	5.2	0.37	0.54	
	10.1		5.4		0.54	
	10.1		5.6		0.56	
Mean				0.37	0.55	0.67
Bovine collagen, unfixed	10.2	4.6	6.3	0.45	0.61	
	9.7	4.7	6.0	0.48	0.62	
	10.0	4.6	6.3	0.46	0.63	
	10.1	4.6		0.45		
	9.9		6.1		0.62	
Mean				0.46	0.62	1.35
Bovine collagen, fixed	9.9	5.2	4.8	0.53	0.48	
	10.2	5.5	5.4	0.54	0.52	
	8.8	4.5		0.52		
	9.0	4.7		0.52		
	9.7		4.9		0.51	
	10.0		5.0		0.50	
Mean				0.53	0.50	0.95
Rat skeletal muscle, unfixed	10.0	4.5		0.45		
	10.1	4.4		0.44		
	10.0	4.4	5.4	0.44	0.54	
	9.8		5.5		0.56	
	9.8		5.6		0.57	
Mean				0.44	0.56	1.24
Rat skeletal muscle, fixed	9.7	5.3		0.55		
	9.8	5.3		0.54		
	9.9	5.2	3.7	0.53	0.38	
	9.9		3.7		0.38	
	10.0		3.5		0.35	
Mean				0.54	0.37	0.69
Rat skeletal muscle fixed (PTA determined gravimetrically)	50.1		18.5		0.37	
	50.0		18.8		0.38	
	50.2		19.2		0.38	
Mean				0.54	0.38	0.70

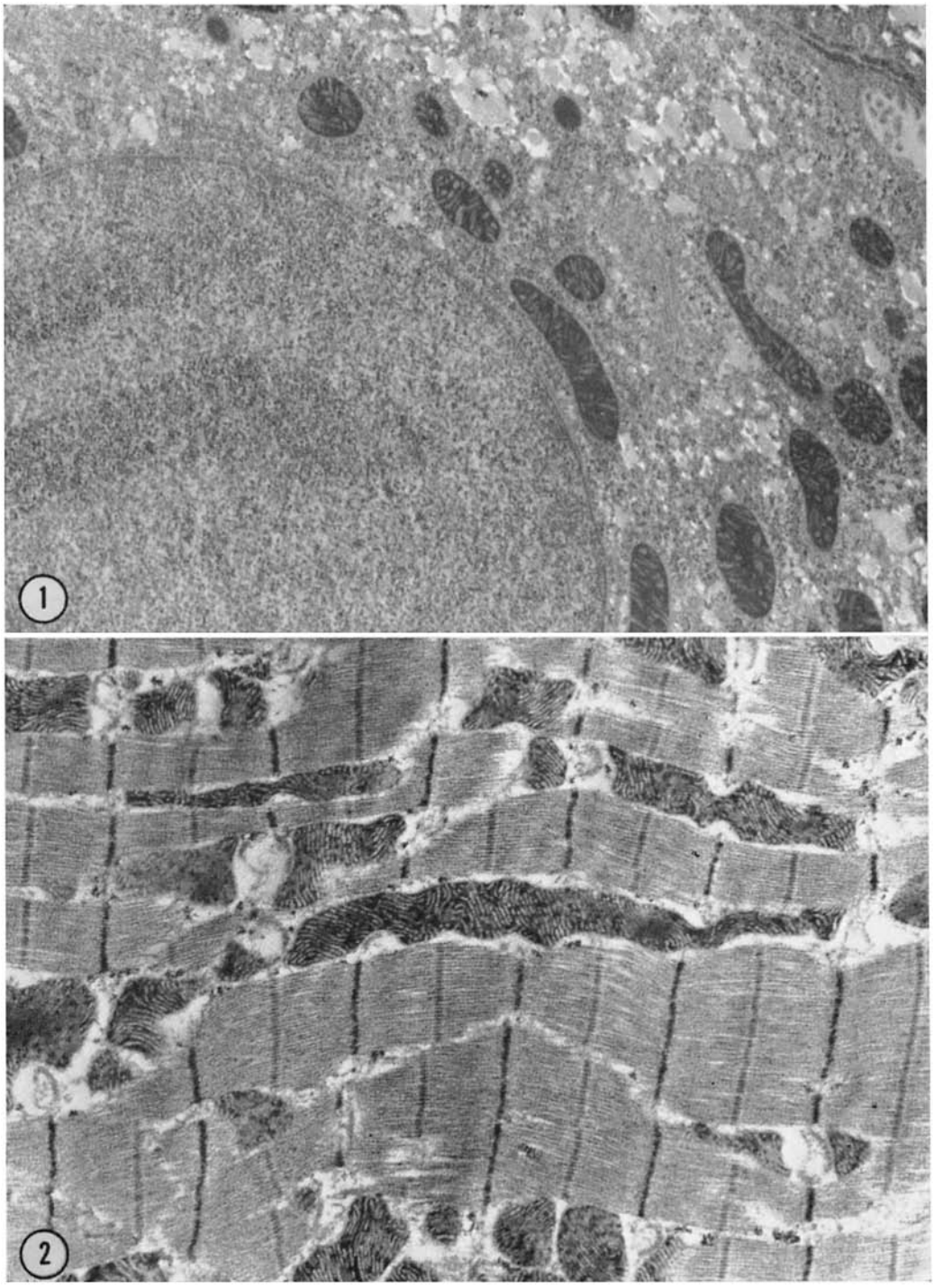

Figure 1 Rat liver parenchymal cell, fixed in glutaraldehyde, and stained with 5% aqueous phosphotungstic acid before dehydration in acid alcohol. There is uniform general staining with marked staining of mitochondrial matrix and desmosomes. × 15,000.

FIGURE 2 Rat heart muscle cell, fixed and stained as for Fig. 1. The Z-band stains as densely as the mitochondrial matrix. × 15,000.

the preparative procedure (16). The pronounced staining of mitochondrial matrix and cisternae of the membrane systems contrasts with that found in the usual preparations in which osmium-stained membranes stand out against a pale matrix or cisterna.

The specificity of this reaction is somewhat limited because staining of positively charged groups extends to nonprotein substances such as certain basic amino acids and peptides and the biological amines, epinephrine, serotonin, and histamine. However, the aldehyde fixative can be expected to dissolve significant amounts of these water-soluble substances.

The stoichiometric relationship between the PTA stain and a protein varies with the amino acid composition of the latter, and it would have to be determined for each protein studied, e.g. red cell hemoglobin or mitochondrial matrix protein. Differences in the stoichiometry would be expected to have little influence when specific proteins are to be stained, as in the case of antigen markers in immunological reactions.

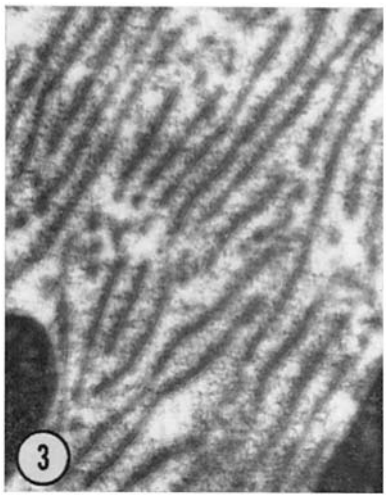

FIGURE 3 Rat liver parenchymal cell. The cisternae of the endoplasmic reticulum stain densely, and the membranes stand out in "negative" contrast. × 35,000.

The density imparted to the mitochondrial matrix and cisternae of the endoplasmic reticulum was greater than that given by the usual glutaraldehyde-OsO_4 fixation and uranyl-lead staining, and suggests that more protein was retained by the aldehyde fixation-PTA staining procedure than by the usual procedure. In a recent publication, Bernhard and Leduc (3) reported fixation of tissue in aldehyde, freeze-sectioning of it without plastic embedding, and treatment with neutral PTA solution to give *negative* staining. The micrographs resembled those in this report; but the dense *negative* staining of the mitochondrial matrix indicated low protein concentration, whereas our dense positive staining demonstrated high concentration. A possible explanation for this discrepancy may be that the treatment of the frozen sections with PTA at neutral pH caused some loss of protein in the negative staining procedure.

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