A QUANTITATIVE DETERMINATION OF THE OSMIUM TETROXIDE–LIPOPROTEIN INTERACTION

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The investigation of the fine structure of biological material by means of the electron microscope has depended, to a very large extent, on the deposition of osmium compounds within the tissue during treatment with a buffered osmium tetroxide solution, and the value of such treatment in the fixing of structure and enhancement of contrast is well accepted. Since electron microscopic observations have depended so heavily on this material, it is of importance to know the physico-chemical parameters that are concerned in the interaction between osmium tetroxide and the tissue components. Successful interpretation of the electron micrographs is only possible through knowledge of these parameters.

Several qualitative studies have been made of the reaction of osmium tetroxide with biological materials. Altman in 1894 (1) showed that osmium tetroxide would blacken oleic acid and olein, but that the saturated fatty acids, palmitic and stearic acid, and their triglycerides were not affected. Bahr in 1954 (2) extended the observations of blackening by osmium tetroxide to some 250 biologically important compounds, and showed that osmium tetroxide would react with unsaturated lipids and proteins but not with saturated hydrocarbons, carbohydrates, or nucleic acids.

The importance of the unsaturated fatty acids in binding osmium was further emphasized by Wigglesworth (3) in 1957 who felt that osmium tetroxide staining presented an histology based on lipids as compared to normal protein and nucleic acid histology. He also presented an hypothesis that there was a polymerization of the unsaturated lipids by osmium cross-linking of ethylenic double bonds. This insoluble complex of lipids and osmium is particularly liable to occur in layers of oriented lipid. Again in 1959 Hess pointed out the essentially lipid nature of osmium binding sites (4).

In a histochemical study Adams (5) in 1960 found that destruction of the reducing groups: —SH, —NH_2, —CHO, —CHOH—, CHOH—, and indolyl did not diminish osmiophilia as measured by blackening of the tissue. Saturating the lipid, however, led to complete extinction of the osmiophilia. He also noted that tissue proteins did not react with OsO_4, and postulated that such groups as —SH behave differently in a rigid system, such as protein, than when they are free.

However, in interpreting the structure of lipoid films, Stoeckenius (6) has come to the conclusion that the dark area seen in OsO_4-fixed lipid layers corresponds to the layers of hydrophilic groups (carboxyl groups) rather than to the double bond sites in the fatty acid chain. A similar conclusion was reached by Trurnit and Schidlovsky (7), but the bound osmium was not detectable either by electron microscopy or spectrophotometrically. It would seem that further quantitative information on the uptake of osmium by biological lipoprotein molecules would be of value in the study of the osmium textroxide staining process.

Several methods for the analysis of osmium have been developed, including chemical (8, 9) and x-ray fluorescence (10, 11). The method of x-ray fluorescence was used in this study because of its accuracy, non-destructiveness, and ability to detect osmium without chemical extraction from the biological sample. Also, this method is independent of the chemical state of the osmium atoms.

The biological material used for interaction with osmium tetroxide was isolated classes of serum

lipoproteins. The chemistry of these lipoproteins is well known in terms of amount of protein and lipid, biochemical nature of lipid present (triglyceride, phospholipid, cholesterol ester, etc.), and fatty acids present in these lipids. They have been studied with the electron microscope, and their dimensions have been determined (12–14). They can be centrifugally separated into fractions representing several lipid protein ratios, and they present the material to be reacted with osmium tetroxide in the macromolecular form of a naturally occurring complex with varying lipid protein proportion.

METHODS

For this study, three human serum lipoprotein classes, centrifugally isolated human albumin, bovine serum albumin, and bovine hemoglobin, were used. Low density (S_f20-10^5), intermediate density (S_f0-20), and high density (HDL) lipoprotein classes were isolated from the normal non-fasting serum of three individuals. The ultracentrifugal techniques were essentially as previously described (15, 16). Lipoprotein concentrations were determined by analytic ultracentrifugation (17) for the high density lipoprotein classes, and were quantitated by refractometry (18) for the low density lipoproteins. The concentration of the isolated lipoprotein fractions ranged from 0.8 to 2.9 per cent lipoprotein. One-half milliliter of lipoprotein solution was placed in 2.36 ml of buffered (phosphate buffer, pH 7.4) 1 per cent osmium tetroxide, and kept at room temperature for ½ hour. One-tenth milliliter was then removed, placed on a filter paper disk, dried sufficiently for the excess osmium tetroxide to evaporate, and the osmium

TABLE I
Osmium Uptake

	Sample	Weight of lipoprotein, protein, or salt	Weight of Os over bkg.	Sample	Average
		μg	μg	μg Os/μg	
Low density	L1	274	261	0.95	
	L2	393	400	1.02	*1.05*
	L3	194	231	1.19	
Intermediate density	I1	403	400	0.99	
	I2	350	250	0.71	*0.86*
	I3	425	37[illegible]	0.87	
High density	H1	244	154	0.63	
	H2	173	79	0.46	*0.56*
	H3	227	133	0.59	
Salt background	SL1	152	<20	<0.13	
	SL2	157	<20	<0.13	*<0·13*
	SL3	154	<20	<0.13	
	SI1	1,610	<20	<0.012	
	SI2	1,610	20	0.012	*≤0·012*
	SI3	1,750	<20	<0.012	
	SH1	4,520	30	0.006	
	SH2	4,470	28	0.006	*0.007*
	SH3	4,520	38	0.008	
Bovine serum albumin	BSA1	528	<20	<0.04	*<0.04*
	BSA2	528	<20	<0.04	
Bovine hemoglobin	Hb1	530	44	0.083	*0.098*
	Hb2	530	59	0.113	
Human albumin	HSA	160	5	0.047	*0.047*

content assayed by the x-ray fluorescence. All samples were prepared for osmium analysis by evaporation of aliquots of solutions onto a specific region of identical filter paper disks. The disks were supported lucite holders of dimensions standard for the Siemens x-ray fluorescence apparatus utilized. Analyses for osmium were made on the $L\beta_1$, line (λ = 1.197 A) with the following operating parameters: Tungsten tube, 50 kilovolts, 38 milliamperes, LiF monochromator, scintillation detector. Yields were 31 counts/min/μg osmium, Standard Counting Error = 4 μgOs (10). Three standards were prepared from an osmium chloride solution and contained 28, 77, and 164 μg of osmium. Control samples of the ultracentrifugal salt background were obtained for each lipoprotein sample by taking the second milliliter from the centrifuge tube after pipetting off the top milliliter containing the lipoprotein. These salt backgrounds were reacted with the buffered OsO_4 solution in the same way as the lipoprotein samples and served as the control for each lipoprotein sample. Samples of the three lipoprotein classes following reaction with the buffered OsO_4 were also extracted with chloroform-methanol patterned after Sperry and Brand (19). The resultant lipid extracts were transmethylated (20) and analyzed by gas-liquid chromatography (21). Additional confirmation of identity of the extracted fatty acids was provided by re-analyses by gas-liquid chromatography of the hydrogenated methyl ester fractions.

RESULTS

Table I shows the osmium content of the lipoprotein classes, albumin, and hemoglobin, after reaction with buffered osmium textroxide for ½ hour. The results are given in terms of weight of lipoprotein present in the sample, the amount of osmium in the sample, and the ratio microgram osmium per microgram lipoprotein. The very low

TABLE II

Physico-Chemical Data on Serum Lipoproteins

Lipoprotein class	Molecular weight (mean)	Size range	Protein	Lipid	Fatty acid double bond per molecule
		A	*per cent*	*per cent*	
S_f20–10^6	1×10^7	300–10,000	12	88	21,000
S_f0–20	3×10^6	200–300	23	77	5,200
HDL_{2+3}	220,000	78–100	50	50	280
Albumin	70,000		99.7	0.3	0.5

TABLE III

Fatty Acid Composition (Mass Per Cent Methyl Ester) of Extract from Osmium Tetroxide–Fixed Lipoproteins

Fatty acid	S_f20–10^5	S_f0–20	Serum fatty acid values (22)		
			CSE	TG	PL
14:0	2.1	4.1			
15:0	1.5	1.3			
16:0	60.4	58.6	10.4	30.9	33.9
16:1	3.3	1.7	3.3	3.9	1.1
17:0	2.2	1.4			
18:0	17.4	17.2	1.2	4.6	14.1
18:1	6.2	2.1	17.2	37.7	11.3
18:2	0.9	0.5	54.5	15.5	21.2
20:0	0.6	0.6			
Misc. remainder	15.8	16.8	13.4	7.4	18.4
	per cent	*per cent*	*per cent*	*per cent*	*per cent*
Identified as saturated	84.2	83.3	~16.6	~40.3	~53.1

CSE, Cholesterol Ester; TG, Triglyceride; PL, Phospholipid.

density S_f20-10^5 class shows the greatest osmium uptake followed by the intermediate density S_f20-10^5 and the high density HDL.

Table II shows the physico-chemical data for these lipoprotein classes as determined in previously reported investigations (16). Also shown is an estimate of the number of double bonds contained in fatty acid in each molecule. This estimation is based on fatty acid composition as determined by gas chromatography (22). The amount of osmium found in lipoprotein is proportional to the amount of lipid present: 1.05 μgOs/μg lipoprotein for the low density class, 0.86 μgOs/μg lipoprotein for the intermediate density class, and 0.56 μgOs/μg lipoprotein for the high density lipoprotein. Only small amounts of osmium were found in the protein samples: 0.098 μgOs/μg protein for hemoglobin, 0.047 μgOs/μg protein for human serum albumin, and the amount of osmium found in the sample of bovine serum albumin was at or below the background level.

Table III shows the fatty acid composition of the extractable lipids following treatment with osmium tetroxide. For comparison, the fatty acid compositions of total serum glycerides cholesterol esters, and phospholipids are also presented. The fatty acid composition of unfixed S_f20-10^5 and S_f0-20 lipoproteins would be expected to reflect their respective chemical composition. Thus, fatty acid composition of S_f20-10^5 and S_f0-20 would tend to resemble that of serum glycerides and cholesterol esters, respectively. Although recovery was limited from the fixed lipoproteins, the fatty acids extracted indicate that the unsaturated fatty acids are preferentially involved in the fixation process. Also, it may be inferred that significant amounts of saturated fatty acids apparently are not altered chemically despite equivalent exposure to osmium tetroxide.

DISCUSSION

The results indicate that nearly all of the osmium reaction occurs with lipid rather than protein. As the percentage protein in the molecule increased, the osmium uptake decreased, and the pure proteins used bound very little osmium. We have observed, however, that if the reaction time is prolonged (4 days) a large amount of osmium (0.4 μgOs/μg protein) is bound by albumin. This is in agreement with the observations of Adams (5) who found that, after 18 hours, albumin was not stained by OsO_4, but that after 3 days the solution was dark brown, and after 5 days it was black.

A knowledge of the position of the osmium atoms within the lipid molecule would be of great help in the interpretation of electron micrographs of osmium-fixed material. One of two sites seems possible: the hydrophilic carboxyl group (6) or the hydrophobic double bonds of the fatty acid chains (5). A combination of both sites is also possible (6).

Riemersma (27) has suggested that reduction may occur at double bonds but that the reduced osmium is deposited at the polar ends of lipid molecules. If this hypothesis of osmium migration proves to be correct, it would be of considerable importance in the interpretation of electron micrographs.

The many observations that OsO_4 does not stain saturated lipids would seem to indicate that the fatty acid double bond is the most likely reaction site. Our observations here on the nature of the extractable lipids from OsO_4-fixed lipoproteins would support this view.

It is hoped that studies now in progress on the amount of osmium bound by saturated and unsaturated lipids will help us to determine the actual binding sites of osmium in the lipid molecule.

The role of protein in the binding of osmium is rather difficult to assess. If albumin and hemoglobin can be assumed to be representative of the protein of lipoprotein, then the mass of osmium bound to the protein moiety would be quite small. However, in the investigations of Bahr, (23–26), the protein components of tissue were thought to bind osmium as readily as did lipid. It is possible that the physical state of the biological material is of importance here and that tissue lipids and proteins in bulk behave differently from those in macromolecular form.

Bahr also calculated a theoretical capacity of tissue lipid to bind osmium and stated that this value should be 0.47 gmOs/gm fat (23). This amount is less than half that measured in the low density lipoprotein class (88 per cent lipid) and would seem to be considerably less than the amount of osmium actually bound to lipid.

SUMMARY

A quantitative measurement of the uptake of osmium by lipoprotein has been made using an x-ray fluorescence technique to analyze for osmium. The results indicate that osmium tetroxide

reacts to a large extent with the lipid component. The possible binding sites are discussed.

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