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THE CHEMICAL NATURE OF OSMIUM TETROXIDE FIXATION AND STAINING OF MEMBRANES BY X-RAY PHOTOELECTRON SPECTROSCOPY

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SUMMARY

X-ray photoelectron spectroscopy was used to determine the oxidation states of osmium compounds present in erythrocyte ghost preparations and related systems treated with osmium tetroxide. Osmium tetroxide and cholesterol, codeposited at −100 °C, began to react at −70 °C, and Os(VI) was formed. Similarly, Os(VI) was detected for the known cholesterol-osmate ester prepared and purified chemically. However, osmium tetroxide applied in phosphate buffer (pH 7.2) gave rise to large proportions of Os(IV) and Os(III) species in addition to Os(VI) compounds. Egg phosphatidylcholine likewise produced a mixture of Os(VI), Os(IV), and Os(III), but dipalmitoyl phosphatidylcholine failed to give significant amounts of osmium containing products under identical conditions. Glutaraldehyde gave a mixture of compounds with the same osmium oxidation states when allowed to react with aqueous osmium tetroxide. Unfixed and glutaraldehyde-fixed erythrocyte ghosts also produced mixtures of Os(VI), Os(IV) and Os(III) under conditions identical to those of normal tissue processing. Additionally, the mixture of adducts initially formed by treatment with osmium tetroxide was further reduced by dehydration of the tissue with ethanol, resulting in a final mixture which was 50–60 % Os(III).

The results support a scheme for the reaction of osmium tetroxide with tissues in which the initial reaction site is the double bonds of unsaturated lipids to form Os(VI) derivatives. Subsequent hydrolysis and further reduction yield complexes of Os(IV) and Os(III). A mixture of these three states is present in membrane specimens during microscopic observation. Os(VI) and Os(IV) could be present as osmate esters and osmium dioxide, respectively; Os(III) could be present as an oxo- or amino complex(es). The photoelectron spectrum of intact erythrocyte ghosts can be synthesized from the spectra of phospholipid and cholesterol only, suggesting the predominance of the reaction with lipids in the fixation process.

Abbreviations: ESCA, Electron Spectroscopy for Chemical Analysis, also known as X-ray Photoelectron Spectroscopy.

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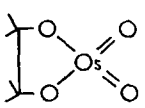
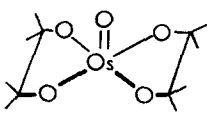
INTRODUCTION

Although osmium tetroxide has been extensively used as a fixative and stain from the earliest applications of electron microscopy to biological samples [1], the mechanism of its interactions with various cellular substrates and the interpretation of resulting micrographs is poorly understood [2–12]. Proposals regarding the mechanism of fixation have centered predominantly upon the interaction of osmium tetroxide with unsaturated lipids to form cyclic esters of Os(VI) containing a *cis*-diol linkage [3, 9, 11–16]. In addition to the familiar monoester A, the fixative properties have been attributed to the formation of diesters such as B [14] or dimeric monoesters between two unsaturated lipids such as C [9].



A



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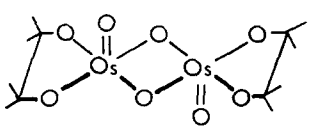

C

Scheme I

Concerning "electron staining," there is widespread agreement that the density observed in electron micrographs corresponds to the distribution of osmium in the sample. Along with the Os(VI) esters, osmium is said to be present in the form of "osmium black," defined as $OsO_2 \cdot nH_2O$ [4, 5, 7, 14–17] or coordination polymers of Os(IV) [18]. Thus, in both fixation and staining, osmium presumably exists as compounds of only Os(VI) and/or Os(IV). One other hypothesis arguing that osmium tetroxide [Os(VIII)] may be hydrogen bonded to substrates [8] has since been discounted [10].

Most hypotheses regarding the mechanism of fixation and staining by osmium tetroxide have been largely based upon the study of model chemical compounds and have not generally focused on the analyses of specimens after treatments normally used prior to examination by electron microscopy. Since these treatments involve exposure to aqueous environments of varying pH, as well as reducing agents such as glutaraldehyde and ethanol, it is essential to determine if the osmate esters have sufficient stability to retain their structure and whether lower-valent osmium compounds in fact are formed. In aqueous solution in the presence of air, Os(VI) has been shown to undergo a pH-dependent disproportionation to Os(VIII) and Os(IV) [19, 20], and, in one case, the intermediacy of Os(V) was proposed [21]. Polarographic evidence further suggests that Os(VI), Os(IV), and Os(III) are produced from

Os(VIII) under sufficiently reducing conditions and at appropriate pH [22, 23]. In order to gain further insight into the mechanisms of osmium tetroxide fixation and staining and better assess the ultrastructural interpretations based thereon, we have undertaken the present investigation. It involves the use of a spectroscopic technique that allows the direct determination of the oxidation state of osmium in typical electron microscopy specimens and related systems under conditions that closely resemble those of the electron microscope, i.e., sample irradiation and high vacuum. Knowing the oxidation state of the osmium in the specimen, inferences may be made regarding the genesis and structure of the osmium compounds themselves.

X-ray photoelectron spectroscopy

When a substance is subjected to ionizing radiation, some of the absorbed energy is reemitted as photoelectrons whose kinetic energies equal the differences between their binding energies and the energy of the incident radiation. The binding energy of a given electron is uniquely determined by its particular electronic environment, the element, atomic level, and oxidation state. Thus, by measuring the energy distribution of the ejected electrons, one can determine the elements in a sample as well as their oxidation states. When X-ray excitation is used, the technique is commonly called Electron Spectroscopy for Chemical Analysis (ESCA) or X-ray Photoelectron Spectroscopy (XPS).

The linear variance of electron binding energy with oxidation state is a general feature of ESCA spectroscopy, and has been demonstrated for a large number of elements [24]. Thus, it has become accepted practice to construct a plot of binding energy vs. oxidation state, using standard compounds of well-defined electronic structure, and to infer the oxidation state of unknown samples by reference to the calibration curve. This procedure is successful particularly if it is recognized that integral oxidation states can occasionally be too rigid a formalism to accurately reflect the actual electron distribution of a given atom.

Important practical considerations for ESCA include its nondestructive nature, its small sampling area (approx. two square millimeters) and shallow sampling depth (< 100 Å), and the small sample quantity required ($<$ one microgram). In addition, the analysis is carried out under a high vacuum ($< 10^{-5}$ Torr), and thus the sample must have a low vapor pressure; gases and liquids are usually frozen on a variable temperature probe. Samples can exhibit "radiation damage," although this is normally noticeable only after long irradiation times (hours). Finally, electrically insulating samples can acquire a positive surface charge due to the ejection of electrons; the resulting potential can retard the electrons and lead to large apparent binding energy shifts. This "charging effect" can be prevented by bathing the sample in the flux from an auxiliary electron gun ("flood gun"). A detailed account of the theory and practice of ESCA is provided by Siegbahn et al. [24], and Lucchesi and Lester [25] give a succinct summary of the theory and descriptions of commercially available ESCA instruments.

METHODS AND MATERIALS

The following compounds were obtained commercially, and the identity and purity of each was verified by appropriate methods, i.e., infrared spectroscopy, ultra-

violet-visible spectroscopy, and thin-layer chromatography: $K_4Os(CN)_6 \cdot 6H_2O$ (K and K Laboratories), $OsCl_3$ (Englehard Industries), K_2OsCl_6 (Alpha Inorganics), $K_2OsO_2(OH)_4$ (listed as $K_2OsO_4 \cdot 2H_2O$ by Alpha Inorganics), OsO_2 (K and K Laboratories), OsO_4 (Stevens Metallurgical Corp.), glutaraldehyde (Fisher), cholesterol (Sigma), egg phosphatidylcholine (Koch-Light), and dipalmitoyl phosphatidylcholine (Analabs). Additional osmium compounds were synthesized according to literature procedures: $OsO_4 \cdot$ pyridine [26, 27], $Os_2O_6 \cdot (\text{pyridine})_4$ [26, 27], $Os[SC(NH_2)_2]_6Cl_3$ [28]. Osmate ester bis(pyridine) adducts were prepared from the following olefins: cyclohexene [26], methyloleate [3, 14, 29], and cholesterol [12, 27]. Erythrocyte ghosts were prepared from rat whole blood by the method of Dodge, et al. [30].

ESCA spectra were obtained on a Hewlett-Packard 5950A ESCA Spectrometer System using Al K_α X-radiation. The sample solutions were applied to gold-plated sample plates, and the Au $4f_{\frac{7}{2}}$ emission at 83.0 eV was used to calibrate the spectra. Unless otherwise noted, the spectra were accumulated at ambient temperature, in a scanning mode at 2 eV/s over a 90–40 eV window, using a flood gun, and with an X-ray beam power of 800 W. The precision was 0.2 eV, and the digital data processor was set at 256 or 64 data channels, respectively, for 50 or 10 eV windows. The scanning time in minutes and the maximum number of counts above background are given in parentheses at the end of the description of each experiment. In the spectra, the x-axis corresponds to the electron binding energy and the y-axis to the total electron count. Absolute comparisons of peak heights between two different samples are not valid due to differences in sample application, counting time, etc. However, the ratios of various peaks in a particular spectrum are significant and can be directly compared with those in other spectra.

Computer-simulated spectra were generated by a PDP-10/11 minicomputer using an iterative curve fitting routine in which four identical doublets were summed, each of whose binding energies and amplitudes were independently variable. Each component of the doublet was a Gaussian curve; the splitting (2.7 eV), peak width at half maximum (1.4 eV), and height ratio (0.8 : 1 $4f_{\frac{5}{2}} : 4f_{\frac{7}{2}}$) were determined from the experimental spectra of the standards. The best fit of computed to experimental spectra was determined empirically. Spectra were not reproduced by mixing solutions of standards in appropriate ratios because disproportionations often occurred.

Standards Os(VIII)

An evacuated flask equipped with a teflon needle valve and an outlet tube was filled to approx. 25 Torr pressure with osmium tetroxide vapor from a vacuum manifold. Care was taken to exclude water from the system. This flask was mounted on an accessory port of the spectrometer so that the stream of vapor could be directed onto a cooled (−100 °C) sample plate. The initial pressure inside the sample preparation chamber was $5 \cdot 10^{-5}$ Torr. A film of osmium tetroxide sufficiently thick to produce interference colors was condensed on the plate upon briefly opening the valve. The spectrum of osmium tetroxide was recorded at −100 °C. Temperature had no apparent effect on the electron emission per se. An X-ray beam power of 1200 W was used (13 min; 2500 counts).

Five μl of a 2 mM solution of $OsO_4 \cdot$ pyridine in cyclohexane was applied to a sample plate, and the solvent was allowed to evaporate. The sample was cooled to −170 °C under 1 atm of dry nitrogen in the spectrometer sample preparation cham-

ber. The chamber then was evacuated, and the spectrum was recorded at −170 °C (13; 19 700).

Os(VI)-Os(II)

Sample plates were prepared by applying 5 μl of a freshly prepared 2 mM solution of the osmium compound in an appropriate solvent. The solvent was evaporated, and the spectrum then was obtained under the standard conditions. This information is summarized in Table I along with the binding energy data.

Reactions of osmium tetroxide with lipids

Cholesterol was coated on a sample plate by applying 5 μl of a 1 mg/ml methanol solution and evaporating the solvent. The cholesterol carbon 1*s* emission at 283 eV could be seen, and there was no signal in the 65–45 eV region. The reaction with solid osmium tetroxide was carried out by condensing vapor on the cholesterol layer at −100 °C as described above. With the beam power at 1200 W, a spectrum was accumulated at −100 °C (6; 6390). Spectra were also recorded after the temperature had been raised to −70 °C (7; 2500) and to 22 °C (14; 1940).

A cholesterol-coated plate was also treated with a drop of 2 % osmium tetroxide in 0.3 M phosphate buffer (pH 7.2) for 2 h at 4 °C. The sample plate then was rinsed with distilled water, and a spectrum of the product was recorded (114; 8300). The spectra of the reaction products of osmium tetroxide with egg phosphatidylcholine (28; 11 000) and dipalmitoyl phosphatidylcholine (28; 3000) were obtained in the same way.

Reaction of osmium tetroxide with glutaraldehyde

Equal volumes of 50 % aqueous glutaraldehyde and 4 % aqueous osmium tetroxide solutions were mixed (mol ratio 30 : 1) and held overnight at 4 °C. 5 μl of the resulting black suspension was applied to a sample plate, and the ESCA spectrum was obtained (60; 1080).

Reaction of osmium tetroxide with erythrocyte ghosts

A drop of a suspension of rat erythrocyte ghosts in 7 mM phosphate buffer (pH 7.2) was applied to a sample plate, dried in a stream of nitrogen and scanned in the spectrometer for 165 min. There was no discernible peak in the 65—45 eV region except for the Na $2s_{\frac{1}{2}}$ singlet at 61 eV. That ghosts in fact were present was indicated by the observation of a N $1s_{\frac{1}{2}}$ singlet at 398 eV. Likewise, no emission was observed from 65 to 45 eV after fixation of the ghosts in 4 % glutaraldehyde at 4 °C for 1 h.

A phosphate-buffered (pH 7.2) ghost suspension was fixed in a 2 % osmium tetroxide solution at 4 °C for 1 h. The ghosts then were washed thoroughly several times by centrifugation and resuspension in fresh buffer, and the ESCA spectrum was obtained (60; 440). Another spectrum was obtained from a suspension of ghosts prefixed in 4 % phosphate-buffered glutaraldehyde at 4 °C for 1 h before being refixed with osmium tetroxide as above (60; 460). A portion of the washed, glutaraldehyde-fixed, and osmium tetroxide-refixed ghosts was scanned (64; 1250) after dehydration by immersion of the pellet for approx. 15 min in each of the following: 25, 50, 70, 80, 90, 95, and 100 % ethanol. A portion of the ethanol-dehydrated ghosts was embedded in Epon resin and sectioned. The sections were picked up on copper electron micro-

scopy grids, and the grids were mounted on ESCA sample plates with double-sided Scotch tape. This assembly was fitted with a gold-plated mask so that only the sections and grid were exposed. The only emission observed in the region of interest was a singlet at 56.4 eV (180; 1360). The same peak was obtained from Epon alone.

RESULTS

Osmium standards

The variation in the binding energies of Os 4*f* electrons was observed in compounds containing osmium in well-defined oxidation states. Two peaks are generally observed in the emission of *f*-electrons; these arise from *f*-orbital interactions with the electron spin and correspond to differing energies associated with total angular momenta of $\frac{7}{2}$ and $\frac{5}{2}$. As shown in Fig. 1, doublets in fact were obtained. Fig. 2 shows that the variation of binding energy with oxidation state is linear. The numerical data are given in Table I.

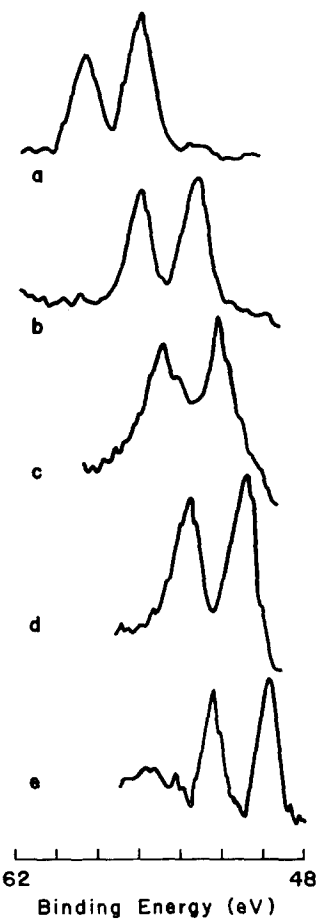

Fig. 1. ESCA spectra of the 4*f* electrons of osmium in various oxidation states: (a) Os(VIII), OsO_4; (b) Os(VI), $K_2OsO_2(OH)_4$; (c) Os(IV), K_2OsCl_6; (d) Os(III), $OsCl_3$; and (e) Os(II), $K_4Os(CN)_6$.

TABLE I

ESCA OF OSMIUM STANDARDS

Sample	Solvent	Os oxidation state	Mean Os $4f_{\frac{7}{2}}$ binding energy (eV)
$K_4Os(CN)_6$	water	II	49.7
$OsCl_3$	water	III	51.0
$Os[SC(NH_2)_2]_6Cl_3$	methanol	III	50.4
K_2OsCl_6	water	IV	52.3
OsO_2	*	IV	51.8
$K_2OsO_2(OH)_4$	water	VI	53.5
$Os_2O_6 \cdot pyridine_4$	water	VI	53.3
Methyl oleate $\cdot OsO_4 \cdot pyridine_2$	acetone	VI	53.8
Cyclohexene $\cdot OsO_4 \cdot pyridine_2$	acetone	VI	53.2
OsO_4	**	VIII	56.3
$OsO_4 \cdot$ pyridine	Cyclohexane	VIII	55.9

* A small piece of double-sided Scotch tape was affixed to the sample plate and covered with OsO_2 powder.

** Solid phase at −170 °C.

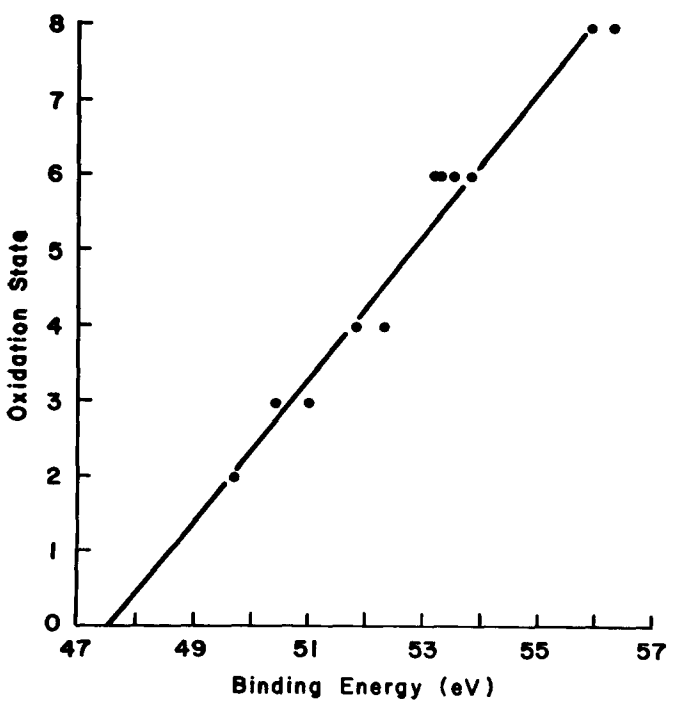

Fig. 2. Plot of Os $4f_{\frac{7}{2}}$ electron binding energies versus osmium oxidation states.

Reaction of osmium tetroxide with lipids

When the sample plate coated with layers of cholesterol and osmium tetroxide at −100 °C was examined, a doublet due to the Os(VIII) 4*f* electrons was observed (cf. Figs. 1a and 3a). There was but a trace of the lower half ($4f_{\frac{7}{2}}$) of an Os(VI) doublet [the upper half ($4f_{\frac{5}{2}}$) coincides with Os(VIII) $4f_{\frac{7}{2}}$]. Upon warming the sample to −70 °C, a triplet was observed (Fig. 3b), which is attributed to overlapping doublets of Os(VIII) and Os(VI) of roughly equal intensity. Upon reaching 22 °C, the signal due to Os(VIII) had disappeared, leaving essentially an Os(VI) doublet (cf. Figs. 1b and 3c).

When a cholesterol-coated plate was exposed to phosphate-buffered aqueous osmium tetroxide, a more complex mixture of osmium-containing products was obtained (cf. Figs. 3c and 4a). The calculated spectrum that best approximated the experimental spectrum was derived from a mixture of 18 % Os(VI), 37 % Os(IV) and 45 % Os(III) (cf. Figs. 4a and 4b). Likewise, the egg phosphatidylcholine reaction products gave a spectrum derived from a mixture of 56 % Os(VI), 24 % Os(IV), and 20 % Os(III) (cf. Figs. 4c and 4d). The adducts of dipalmitoyl phosphatidylcholine and osmium tetroxide gave a weak, broad, unresolved emission in the 50–60 eV region.

In addition, the spectra obtained from cholesterol and egg phosphatidylcholine were corrected for differences in scanning times, and their weighted sum was determined graphically. The spectra were proportioned according to the weight ratio of

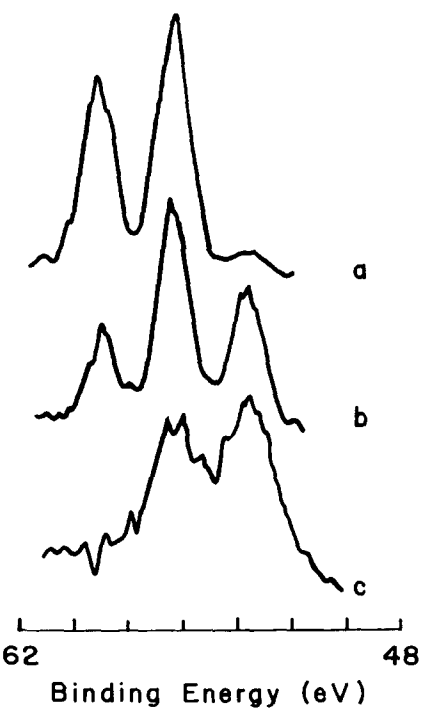

Fig. 3. ESCA spectra of cholesterol coated with a film of osmium tetroxide at (a) −100 °C, (b) −70 °C, and (c) 22 °C.

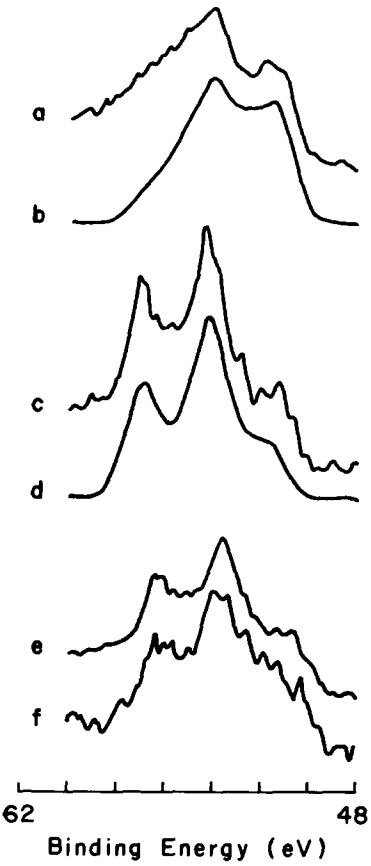

Fig. 4. ESCA spectra of the products of the reaction of phosphate buffered osmium tetroxide with lipids: (a) cholesterol; (b) calculated spectrum of 18 % Os(VI), 37 % Os(IV), and 45 % Os(III); (c) egg phosphatidylcholine; (d) calculated spectrum of 56 % Os(VI), 24 % Os(IV), and 20 % Os(III); (e) weighted sum of (a) and (c); (f) osmium tetroxide-fixed erythrocyte ghosts.

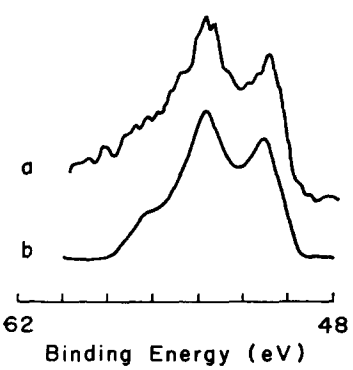

Fig. 5. The reaction of glutaraldehyde with unbuffered, aqueous osmium tetroxide solution: (a) ESCA spectrum of the reaction products, and (b) calculated spectrum of 21 % Os(VI), 29 % Os(IV), and 50 % Os(III).

cholesterol to phospholipid in rat erythrocytes, viz. 1/2.7 [31]. This calculated spectrum is illustrated along with that obtained from osmium tetroxide-treated rat erythrocyte ghosts in Figs. 4e and 4f.

Reaction of osmium tetroxide with glutaraldehyde

These reagents in aqueous solution were combined and allowed to react to completion. As with the buffered osmium tetroxide-cholesterol system, the presence of a mixture of Os(VI), Os(IV), and Os(III) was indicated (Fig. 5). The spectrum calculated for 21 % Os(VI), 29 % Os(IV), and 50 % Os(III) is an excellent fit for the observed spectrum of the osmium-glutaraldehyde adducts. No oxidation states less than III were observed.

Reaction of osmium tetroxide with erythrocyte ghosts

Suspensions of rat erythrocyte ghosts were examined after osmium fixation alone and after each stage of a typical processing for electron microscopy, i.e., after glutaraldehyde fixation, osmium tetroxide refixation, and ethanol dehydration. The ESCA spectra are presented in Fig. 6. The spectrum obtained after osmium tetroxide fixation alone was not substantially altered by prefixation of the ghosts with glutaraldehyde (cf. Figs. 6a and 6c). The calculated spectra (Figs. 6b and 6d) also indicate that the mixture of osmium oxidation states is approximately the same in each case: 40, 33 and 27 % vs. 42 % Os(VI); 37 % Os(IV) and 21 % Os(III), respectively. Subsequent ethanol dehydration, however, greatly enriched the amount of Os(III) observed (Fig. 6e). The calculated spectrum indicated that the observed spectrum was

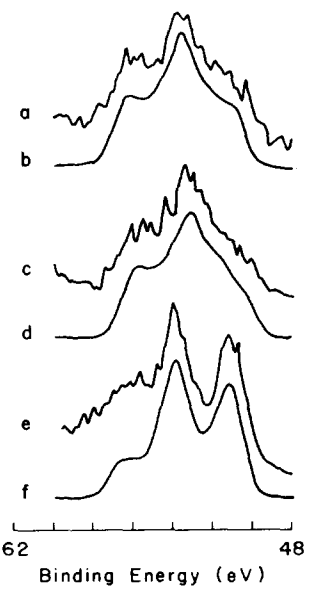

Fig. 6. ESCA spectra of the products of reaction of osmium tetroxide with erythrocyte ghosts: (a) osmium tetroxide-fixed only; (b) calculated spectrum of 40 % Os(VI), 33 % Os(IV), and 27 % Os(III); (c) glutaraldehyde-fixed and osmium tetroxide-refixed; (d) calculated spectrum of 42 % Os(VI), 37 % Os(IV), and 21 % Os(III); (e) fixed as in (c) and then dehydrated with ethanol; and (f) calculated spectrum of 23 % Os(VI), 20 % Os(IV), and 57 % Os (III).

derived from a mixture of 23 % Os(VI), 20 % Os(IV), and 57 % Os(III).

No Os 4*f* emissions were observed from sections of Epon-embedded, fixed and dehydrated ghosts, although examination in the electron microscope revealed a high concentration of stained membranes. The singlet observed at 56.4 eV, which was also observed with pure Epon, is attributed to titanium, which has a $3s_{\frac{1}{2}}$ emission at approx. 59 eV [24]. There was no other emission in the region of interest.

DISCUSSION

Application of ESCA to osmium compounds

Previous attempts to elucidate the nature of osmium tetroxide-membrane interactions have usually involved the study of model compounds prepared by standard chemical methods. All of these investigations have been hindered by the general instability and intractability of organo-osmium compounds. This has resulted in many reported but poorly characterized species. The bis(pyridine) adduct of cholesterol osmate ester is a case in point. This compound partially decomposed on repeated preparative thin-layer and column-adsorption chromatography; likewise, a satisfactory recrystallization system was not found. Thus, this compound was not obtained completely pure, and its ESCA spectrum always exhibited evidence of osmium in oxidation states other than VI. (Note that the adduct itself contains a potential reducing function, the C_3-hydroxyl group.)

Additionally, all of the well characterized osmium adducts of biological interest contain Os(VI); that is, they are osmate esters. Therefore, inorganic complexes of osmium were chosen as primary standards, since these include all possible oxidation states and are among the best characterized osmium species available. Two well characterized osmate esters of olefins have also been included as standards, and it can be seen that their observed binding energies are in agreement with those of Os(VI) inorganic standards. Binding energies of all of the standards cluster around discrete energy levels associated with each particular oxidation state. This is to be anticipated since all of the standards, including the Os(VI)-esters, have highly electronegative ligands, and thus simple, integral oxidation numbers adequately describe their electronic structures. This approach should be adequate for the products of membrane fixation as well, since the primary ligands involved in bio-osmium complexes will assuredly be oxygen and nitrogen. For transition metal complexes with certain ligands, e.g., those with substantial covalent or π-donor character, a more refined interpretation of ESCA binding energies may be required. In this case, results such as noninteger oxidation states are possible. Nevertheless, such data are still significant in terms of effective reduction of the metal center. Ligands which can introduce these complications are not found in biological systems, with the possible but unlikely exception of protein sulfur. Cook et al. [32] discuss more fully the applicability of the simple oxidation state approach to transition metal complexes, and provide some examples of the variations which can occur with platinum, a third-row transition metal like osmium.

The validity of using a formal oxidation state approach is experimentally supported by the quality of the least-squares line fit shown in Fig. 2. Since the relationship is linear (correlation coefficient 0.983), the binding energy for an oxidation state for which a stable complex is not readily available can be predicted. Conversely,

the oxidation state of osmium in an experimental sample can be determined by measuring the appropriate electron binding energies.

Since the linewidths and splittings of the Os 4*f* doublets are of the same order of magnitude as the differences in binding energies of adjacent oxidation states, assignment of oxidation states and ratios for mixtures was often difficult. This situation was greatly ameliorated by computer simulation of spectra. Not only did this procedure facilitate the analysis of oxidation states and their respective proportions in mixtures of osmium products, it also greatly increased the reliability and precision of these assignments. Regarding the spectra presented here, the fit of a calculated spectrum of a mixture of three oxidation states to an experimental spectrum is essentially unique. Even minor changes in the ratios of constituents are readily apparent (cf. Figs. 4b vs. 5b, and Figs. 6b vs. 6d). The presence of Os(IV) in synthetic spectra which included equal parts of Os(VI) and Os(III) was detectable at a level of approx. 10 %, and was unmistakable at 20 %. Changes in the other components, i.e., Os(VI) and Os(III), were distinguishable at a level of approx. 2.5 %, and this would also be the case for oxidation states VIII, VII, II and 0.

An acceptable simulation of all spectra of mixtures in this paper could be achieved with combination of states VI, IV and III only. Os(V) was omitted from these calculations, as there is no evidence for its presence (but see below), and excellent correspondence was obtained without its inclusion. We estimate the compositional assignments to be accurate to ±5 %.

ESCA spectroscopy of model systems and membranes

The results obtained from the solid OsO_4-cholesterol system indicate that the two components coexist essentially without reaction at −100 °C. Upon warming to room temperature, however, all of the osmium tetroxide sublimed or reacted, leaving only Os(VI). The observation of Os(VI) is, of course, consistent with the expected formation of a cholesterol mono- or diester, and the low temperature at which the reaction is observed is indicative of a very low energy of activation. Similarly, primarily Os(VI) is observed for the pyridine-complexed osmate ester of cholesterol prepared and purified by chemical methods. Under conditions which approximate those of a normal tissue processing, however, the result is strikingly different; large amounts of Os(III) and Os(IV) appear in the reaction products of a cholesterol film with phosphate-buffered osmium tetroxide. Computer simulation of the spectrum indicates that the mixture is 18 % Os(VI), 37 % Os(IV) and 45 % Os(III) (Figs. 4a and 4b). Likewise, egg phosphatidylcholine gave a mixture of the same oxidation states, but in different proportions (56, 24 and 20 %, respectively, Figs. 4c and 4d), while dipalmitoyl phosphatidylcholine gave essentially no reaction. In addition, the sum of the spectra of the two unsaturated lipids, weighted to the ratio of phospholipid/cholesterol found in rat erythrocytes, is a good approximation to that of osmium tetroxide-fixed erythrocyte ghosts (Figs. 4e and 4f).

Glutaraldehyde is both a potent reductant and a common fixative for electron microscopy specimens. The reaction of glutaraldehyde with osmium tetroxide has been investigated previously, but the product, "osmium black", was not chemically characterized [33]. When osmium tetroxide was allowed to react to completion with a large excess of glutaraldehyde, the ESCA results demonstrated that this product too is a mixture of Os(VI), Os(IV), and Os(III) (21, 29, and 50 %, respectively, Fig. 5), and that no Os(II) or Os(0) was formed. The reducing ability of glutaraldehyde

dramatizes the desirability of completely removing excess aldehyde before continuing a tissue preparation. That such removal can in fact be accomplished is indicated by the similarity of the spectra of osmium tetroxide-fixed erythrocyte ghosts with and without glutaraldehyde prefixation (Figs. 6a and 6c).

Erythrocyte ghosts are a convenient and much-used model system for biological membranes, and thus were chosen as a substrate for ESCA investigation. Phosphate-buffered osmium tetroxide solution reacted with unfixed and glutaraldehyde-fixed erythrocyte membranes to yield, as above, a mixture of Os(VI), Os(IV) and Os(III) (approx. 40, 35 and 25 %, respectively, Figs. 6a–6d).

However, subsequent ethanol dehydration of the osmium-fixed membranes resulted in a 2-fold increase in the proportion of Os(III). This enrichment was apparently due to a reduction or disproportionation of high-valent osmium rather than a selective extraction process. This tentative conclusion is based on indirect evidence such as the rate of accumulation of the spectra and the physical appearance of the samples. Ethanol has previously been mentioned as a reducing agent for osmium under similar circumstances [7, 16], and the process of dehydration itself has been shown to alter the observed periodicity of myelin membranes [34].

If erythrocyte ghosts are reacted with osmium tetroxide in distilled water, instead of buffer, a spectrum (not shown) similar to those above, e.g., Fig. 6a, is obtained. This spectrum does, however, show less evidence for lower oxidation state components. This result, along with others described above, suggests that the final product mix is affected by common reagents of routine fixation processes, viz., glutaraldehyde, buffer salts, pH and ethanol. While these effects have not as yet been investigated in detail, ESCA offers a convenient and powerful method of doing so.

No Os 4*f* electron emission was observed from Epon-embedded sections of osmium-fixed ghosts; this may be due to the surface-sampling nature of ESCA. X-ray photoelectrons have sufficient energy to escape from only the top few percent of a typical electron microscopy section. Although the concentration of ghosts was sufficiently high to easily be seen by transmission electron microscopy, there apparently was not sufficient osmium-containing material near enough to the surface to have been detected by ESCA. Furthermore, Epon contains a small amount of titanium catalyst, and its $3s_{\frac{1}{2}}$ emission at 56.4 eV may have obscured a weaker Os 4*f* signal.

As stated earlier, ESCA experiments on mixtures such as those encountered here can identify particular elements and their oxidation state(s), but not necessarily the stoichiometry of the components. Nevertheless, it is desirable to briefly speculate on the possible nature of the observed osmium species. Likely candidates for Os(VI) include osmate esters, osmate salts $[M^{2+}OsO_2(OH)_4]$, and oxides (OsO_3); nitrogen ligands could occupy some sites in the coordination shell [35]. Os(IV) could well be present as hydrated OsO_2; a variety of other Os(IV) adducts containing nitrogen and oxygen ligands are known [35]. There is some evidence for Os(III) hydroxy complexes, and Os(acetylacetone)$_3$ as well as a number of Os(III) complexes with amines and heterocyclic bases have been prepared [35]; Os(III) could be present as such species. No unequivocal evidence for Os(V) was obtained in these experiments.* In addition, Os(V) was not required to adequately simulate the

* The presence of Os(V) in the Os(VI) standards could account in part for the low binding energy values and broad lines sometimes observed for Os(VI) (Fig. 2). Behrman and co-workers give indirect evidence that osmate esters and potassium osmate disproportionate in dilute acid or water to OsO_4 and an Os(V) oxide that is further reduced to $OsO_2 \cdot nH_2O$.

experimental spectra, and it is less attractive than other oxidation states on chemical grounds. Thus, Os(V) was assumed to be present only as a minor component (0–5 %). Regardless of the composition of the particular species in fixed membranes, it is clear that simple osmate esters can be but a minor component.

Regarding the possible presence of osmium tetroxide in these preparations, either present originally or formed by oxidation of lower-valent osmium, sublimation would quickly occur under the pressures and temperatures normally found in the ESCA spectrometer. Thus, no conclusions about its presence or absence can be drawn from the experiments carried out at ambient temperature. However, similar conditions are encountered in an electron microscope, and osmium tetroxide, as such, is also unlikely to be found there.

The results described here suggest some conclusions concerning the nature of osmium fixation. It is apparent that studies of model compounds prepared under simplified conditions can provide only an incomplete picture of the total process. If osmium adducts are allowed to form under conditions usually employed in specimen preparation, a complex mixture of products is formed, containing substantial amounts of low-valent osmium complexes in addition to the osmate esters usually postulated. This is equally true for whole membranes and for isolated lipid components of membranes. On the other hand, the present studies on the products of osmium fixation of membrane components in situ have provided some insights. The low energy of activation of the reaction of osmium tetroxide with cholesterol (and by implication, with other unsaturated substrates), the lack of any reaction with dipalmitoyl phosphatidylcholine and other saturated compounds, and the fact that the membrane ESCA spectrum can be reproduced from the spectra of unsaturated lipids only, provide direct support for the previous contention [3, 5, 7, 14, 16, 36] that unsaturated lipids are the predominant substrates for OsO_4 in membranes. This does not preclude, however, the reaction of OsO_4 with other membrane components. In particular, the corollary experiment with proteins only cannot reasonably be performed since undenatured membrane proteins are properly required, and current methods do not allow the isolation of these free from lipids. Previous studies on mitochondria [37] and myelin [38] have shown that the unit membrane structure is preserved even if > 95 % of the lipids have been extracted prior to osmium tetroxide fixation. The reaction with residual lipid may account for these results, or less favored reactions with proteins or carbohydrates may have occurred, given the deficiency of lipid.

Additionally, the results obtained with cholesterol and osmium tetroxide in solid and aqueous phases, the reducing effect of alcohol, and, in general, the existence of half or more of the osmium in oxidation states IV and III indicate that hydrolysis and reduction of initially-formed osmate esters are important processes in fixation and staining. With respect to fixation, it should be noted that immobilization due to formation of osmate diesters, B, [14] or dimeric monoesters, C, [9] are not inconsistent with these results. However, Os(VI) accounts for only approx. 40 % of the osmium in fixed membranes before dehydration and approx. 20 % afterwards. Thus, this mechanism of crosslinking membrane components appears to be less important than might previously have been supposed.

Finally, insofar as electron staining of membranes with osmium is concerned, the essential problem is to reconcile the "railroad track" pattern with a spacing of approx. 8 nm with the lipid bilayer model of biomembranes. To this end, various

authors have proposed the movement of osmium to the hydrophilic interface by bending of the lipid osmate esters [3, 17] and the migration and subsequent deposition of osmium oxides [39]. Although bending of lipid osmate esters is again not inconsistent with the ESCA results, the preponderance of Os(IV) and Os(III) in fixed membrane favors the migration hypothesis. In fact, the finding of substantial amounts of lower-valent (presumably polar) osmium adducts in stained membranes makes the proposition that the final osmium products reside at and beyond the lipid water interface very attractive. Nevertheless, there is no conclusive evidence, either in this work or elsewhere, that migration of osmium species with respect to some fixed reference point such as the plane of the lipid head groups actually occurs. This would be desirable to examine by another method.

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REFERENCES

1 Pease, D. C. (1964) Histological Techniques for Electron Microscopy, 2nd edn, pp. 34–49, Academic Press, New York
2 Stoeckenius, W. (1962) in The Interpretation of Ultrastructure (Harris, R. J. C., ed.), pp. 349–367, Academic Press, New York
3 Stoeckenius, W. and Mahr, S. C. (1965) Lab. Invest. 14, 458–469
4 Adams, C. M. W., Abdulla, Y. H. and Bayliss, O. B. (1967) Histochemie 9, 68–77
5 Korn, E. D. (1968) J. Gen. Physiol. 52, 257s–278s
6 Stoeckenius, W. and Engelman, D. M. (1969) J. Cell Biol. 42, 613–646
7 Riemersma, J. C. (1970) in Some Biological Techniques in Electron Microscopy (Parsons, D. F., ed.), pp. 70–87, Academic Press, New York
8 Litman, R. B. and Barrnett, R. J. (1972) J. Ultrastruct. Res. 38, 63–86
9 Collin, R., Griffith, W. P., Phillips, F. L. and Skapski, A. C. (1973) Biochim. Biophys. Acta 320, 745–747
10 Collin, R. J. and Griffith, W. P. (1974) J. Histochem. Cytochem. 22, 992–996
11 Collin, R., Griffith, W. P., Phillips, F. L. and Skapski, A. C. (1974) Biochim. Biophys. Acta 354, 152–154
12 Collin, R. J., Jones, J. and Griffith, W. P. (1974) J. Chem. Soc. Dalton, 1094–1097
13 Korn, E. D. (1966) Biochim. Biophys. Acta 116, 325–335
14 Korn, E. D. (1967) J. Cell Biol. 34, 627–638
15 Dreher, K. D., Schulman, J. H., Anderson, O. R. and Roels, O. A. (1967) J. Ultrastruct. Res. 19, 586–599
16 Riemersma, J. C. (1968) Biochim. Biophys. Acta 152, 718–727
17 Riemersma, J. C. (1963) J. Histochem. Cytochem. 11, 436–442
18 Hanker, J. S., Kasler, F., Bloom, M. G., Copeland, J. S. and Seligman, A. M. (1967) Science 156, 1737–1738
19 Cairns, J. F. and Roberts, H. L. (1968) J. Chem. Soc. (C), 640–642
20 Perichon, J. S., Palous, S. and Buvet, R. (1963) Bull. Soc. Chim. 982–988
21 Subbaraman, L. R., Subbaraman, J. and Behrman, E. J. (1972) Inorg. Chem. 11, 2621–2627
22 Meites, L. (1957) J. Am. Chem. Soc. 79, 4631–4637
23 Cover, R. E. and Meites, L. (1961) J. Am. Chem. Soc. 83, 4706–4710

24 Siegbahn, K., Nordling, C., Fahlman, A., Nordberg, R., Hamrin, K., Hedman, J., Johnson, G. Bergmark, T., Karlson, S. E., Lindgren, I. and Lindberg, B. (1968) ESCA: Atomic, Molecular and Solid State Structure by Means of Electron Spectroscopy, Technical Report No. AFML-TR-68-189 (Air Force Materials Laboratory, Wright-Patterson AFB, Ohio)
25 Lucchesi, C. A. and Lester, J. E. (1973) J. Chem. Ed. 50, A205–A224, A269–A285
26 Criegee, R., Marchand, B. and Wannowius, H. (1942) Ann. 550, 99–133
27 Griffith, W. P. and Rosetti, R. (1972) J. Chem. Soc. Dalton, 1449–1453
28 Sauerbrunn, R. D. and Sandell, E. B. (1953) J. Am. Chem. Soc. 75, 3554–3556
29 Korn, E. D. (1966) Biochim. Biophys. Acta 116, 317–324
30 Dodge, J. T., Mitchell, C. and Hanahan, D. J. (1963) Arch. Biochem. Biophys. 100, 119–130
31 Nelson, G. J. (1967) Biochim. Biophys. Acta 144, 221–232
32 Cook, C. D., Wan, K. Y., Gelius, U., Hamrin, K., Johansson, G., Olsson, E., Siegbahn, H., Nordling, C. and Siegbahn, K. (1971) J. Am. Chem. Soc. 93, 1904–1909
33 Hopwood, D. (1970) Histochemie 24, 50–64
34 Moretz, R. C., Akers, C. K. and Parsons, D. F. (1969) Biochim. Biophys. Acta 193, 1–11
35 Griffith, W. P. (1968) The Chemistry of the Rarer Platinum Metals, Chapter 3, Interscience Publishers, New York
36 Jost, P., Brooks, U. J. and Griffith, O. H. (1973) J. Mol. Biol. 66, 313–318
37 Fleischer, S., Fleischer, B. and Stoeckenius, W. (1967) J. Cell Biol. 32, 193–208
38 Napolitano, L., Lebaron, F. and Scaletti, J. (1967) J. Cell Biol. 34, 817–826
39 Robertson, J. D. (1966) in Principles of Biomolecular Organization, (Wolstenholme, G. E. W. and O'Connor, M., eds.), pp. 357–408, Little, Brown and Co., Boston