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Klems, J.H.

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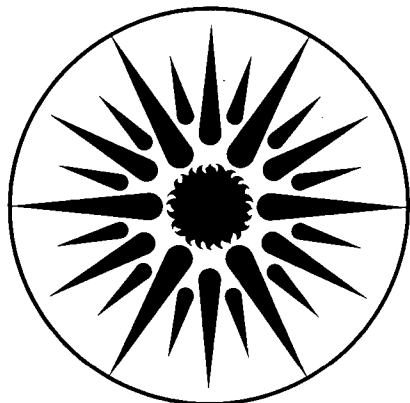
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J.H. Klems
Windows and Daylighting Group
Applied Science Division
Lawrence Berkeley Laboratory
1 Cyclotron Road
Berkeley, California 94720

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J. H. Klems
Applied Science Division
Lawrence Berkeley Laboratory
1 Cyclotron Road
Berkeley, CA 94720

ABSTRACT

A new pump-probe technique, called XIFAME, is proposed as a contrast mechanism for X-ray microscopy. The technique consists of using a laser to produce holes in the valence levels of selected molecules; these holes then permit inverse fluorescent absorption transitions to occur. The method would permit the selective mapping of simple molecular structures and oxidation states of light elements. Cross section estimates are made, and it is argued that the technique is potentially more sensitive than imaging techniques based on the atomic absorption edge. It is also argued that XIFAME has advantages over XANES for spectroscopic measurements of very dilute molecular structures.

INTRODUCTION

X-ray microscopy, with a current state-of-the-art spatial resolution[1,2] of 40-75 nm, might seem a poor competitor for biological studies when compared with x-ray diffraction, electron microscopy, scanning tunneling microscopy, or atomic force microscopy, all of which are capable of atomic resolution. However, each of the high-resolution techniques

has distinct limitations: x-ray diffraction requires crystalline samples; electron microscopy requires elaborate preparation including deposition on a conductive substrate and application of a conductive coating; both scanning tunneling and atomic force microscopy currently require binding of samples on a substrate, and in any case remain techniques for imaging surfaces because of their dependence on an intrusive probe. Thus, the promise of synchrotron-radiation-based X-ray microscopy for studying cell structures in a relatively undisturbed state is an advantage which outweighs its resolution limitations for certain types of studies.

A second advantage is the possibility of mapping the location of specific chemical elements within a cell or cell structure by comparing images taken above and below an absorption edge for that element. This differential absorption technique is already in use for macroscopic imaging[3] and its extension to microscopy seems straightforward. It would allow the study of elements which are both biologically important and present in low concentration in cells, such as iron, magnesium and sulfur. An alternative technique using a scanning microprobe and analysis of the fluorescent X-ray emission is also under development.[4, 5, 6] Since both structure and chemistry are important determinants of cell function; the ability to study them simultaneously represents a unique advantage.

However, most of the chemical reactions in a cell involve compounds of carbon, oxygen, nitrogen and hydrogen, without any characteristic trace element. Elemental mapping is of no help in these cases. For elements such as sulfur determination of the ionization state is important for understanding the chemical reactions occurring. Extending X-ray microscopy to include the mapping of chemical compounds and ionization states would represent a significant advance. In this work I suggest a mechanism by which it might be accomplished.

A CHEMICALLY-SENSITIVE X-RAY CONTRAST MECHANISM

The normal sequence of events involved in X-ray absorption is shown in Figure 1. An initial X-ray absorption (1) excites an inner shell electron to the continuum, leaving the atom an ion with a K-shell hole. (For simplicity we consider carbon-like atoms, where there is only one inner shell.) As indicated, transitions to states where the electron is loosely bound to the ion or rescattered by neighboring atoms are also possible. These other final states produce the near-edge structure in the absorption cross section; they are typically within a few eV of the ionic final state, and will not concern us in this discussion. Next, the system drops to a much lower energy state by fluorescent emission (2) of an X-ray with an electron from one of the valence states dropping into the inner shell hole. This

is the X-ray fluorescent transition. The ion is left with an unoccupied valence electron state, and eventually returns to the ground state through a second fluorescent transition (3), which is typically in the visible or ultra-violet.

It should be possible to reverse the sequence of events (2) and (3) in a two-beam, "pump-probe" experiment. An intense laser or synchrotron radiation beam (pump) tuned to transition (3) could produce a population of atoms or ions with a valence hole; these in turn could absorb an incident X-ray beam (probe) tuned to one of the transitions (2). This is the process that I call X-ray Inverse Fluoresence Allowed by Molecular Excitation (XIFAME). Normally, of course, the inverse-fluorescent X-ray transition is forbidden because the upper valence state is filled.

The energy of the probe transition (2) is primarily determined by the atomic levels involved, with shifts due to molecular binding effects on the order of a few percent. The pump transition (3), however, is determined by the valence electron energies, i.e., the molecular state. By using the probe beam to form an image in an X-ray microscope or holographic apparatus and by tuning the pump beam to specific molecular absorption peaks, one could selectively map particular chromophores. For example, aromatic carbon rings in the amino acids tryptophan, tyrosine and phenylalanine cause strong UV absorption bands in the 205-230 and 250-300 nm regions.[7] By pumping these absorption transitions and probing at (approximately) the carbon K_α energy an X-ray map of these amino-acids could be produced. One would in effect be doing UV microscopy (wavelength-limited resolution ~300 nm) with X-ray resolution (current practical limit 50 nm; wavelength-limited resolution ~5nm). Because the probe beam energy lies below the carbon absorption edge, there would be little absorption due to atoms not selected by the pump beam; this would enhance contrast and limit X-ray damage to the specimen.

The combination of an atom-specific probe and a molecular-selective pump gives XIFAME additional potential for molecular selectivity. For example, the UV absorption bands of tryptophan, phenylalanine and tyrosine overlap, so that these three would be indistinguishable in the mapping suggested above using a carbon K_α probe. However, only the tryptophan chromophore contains nitrogen. Using a nitrogen K_α probe would image only the tryptophan. Similarly, the sulfur-containing structures cysteine, cystine and methionine have weak absorption in the 190-230 nm region which is easily masked by that of other protein components. If, however, a sulfur K_β or L probe were used these could be distinguished from the background, and their differing UV absorptions[8] at 205, 215 and 250 nm might be used to distinguish between them.

While the terms, such as K_α, characterizing atomic X-ray fluorescent transitions have been used in the above discussion, the energies characterizing the inverse fluorescent transitions will vary somewhat with molecular structure. Thus XIFAME spectroscopy must determine the correct probe energies prior to imaging applications. This study may have interest in its own right. It is well known[9] that the energy of an X-ray fluorescent emission peak for an element in a compound depends on the chemical structure. This is a combination of two effects: an electrostatic shift of the inner shell electron energy due to rearrangement of the valence electrons[10] and a shift of the valence electron energy due to chemical bonding. For light elements this can result in a shift of several eV for the K emission peak from atoms in differing oxidation states[11]; shifts of this magnitude should be resolvable in synchrotron radiation experiments. Thus, XIFAME would provide a direct method of mapping oxidation states as well as simple chemical structures.

ESTIMATE OF XIFAME CROSS SECTION

An estimate of the XIFAME absorption cross section in an isolated carbon atom with a valence hole is simplified by the fact that it is the inverse process to fluorescent X-ray emission. Standard formulas[12] can then be used to relate the absorption cross section to the radiative and total level widths for the excited carbon atom:

$$\sigma(E) = \frac{\pi}{2E_0^2}(\hbar c)^2 \frac{(\hbar\Gamma_{RAD})(\hbar\Gamma_{TOT})}{(E-E_0)^2 + (\hbar\Gamma_{TOT})^2} \qquad (1)$$

and taking published values[13] gives a value at the transition energy E_0 (277 eV for carbon) of 4.3×10^{-17} cm^2 (4.3×10^4 Kilobarns). In Figure 2 this cross section is compared with the photoabsorption cross section[14] in carbon. As can be seen from the figure, the XIFAME cross section is large compared to the latter. Since the natural line width (0.6 eV) is comparable to or greater than the expected experimental resolution of a synchrotron radiation beam, it can be seen that the XIFAME transition should be readily separable from the carbon edge.

Next, let us apply this atomic cross section to the estimate of a relevant molecular cross section. We choose benzene as a simple example because of its obvious relevance to the aromatic structures in the amino acids discussed above. Since the molecular structure is only a small perturbation on the primarily atomic X-ray transition, one can express the cross section as an atomic process where the final state is the projection of the molecular valence-hole state onto the atomic 2p state:

$$\sigma_B(E) = \sigma_X(E;E_M)\sum\left|\left\langle\Psi_M\middle|\psi_{2p}\right\rangle\right|^2 \qquad (2)$$

where σ_B is the benzene cross section, σ_X represents the atomic cross section of equation (1) evaluated at $E_0 = E_M$, the molecular transition energy, and the summation represents a sum over final states and an average over initial states. Calculation of the true molecular state Ψ_M would be a complicated task quite irrelevant to our present purpose of producing a crude rate estimate. Instead, we use a simple molecular-orbital model, following Hertzberg,[15] and assume that the vacancy is in the e_{1g} state (D_{6h} symmetry group), since this is the state that would be reached by a single-photon absorption from the ground state. Since this state is twofold-degenerate in this simple model, we must average over two initial states and sum over six possible final states, corresponding to a K-shell hole in each of the six carbon atoms of the ring. The result is then

$$\sum\left|\left\langle\Psi_M\middle|\psi_{2p}\right\rangle\right|^2 = 1, \qquad (3)$$

which is equivalent to the statement that the π orbitals in the molecule are formed of p atomic wave functions. The molecular XIFAME cross section is therefore approximately equal to the atomic cross section, evaluated at the energy corresponding to an X-ray transition to the molecular valence state.

This argument can readily be extended to phenol and indole, the chromophores in tyrosine and tryptophan, respectively. The electron in a molecular π state is completely delocalized and can be considered a free particle[16] shared among the N atoms participating in the bond. The probability for the electron to be found at any one of the atoms is $1/N$; hence the XIFAME cross section per atom is σ_X/N, and that for the entire molecule is σ_X. Thus, for a single valence hole, the XIFAME cross section will be given by σ_X for any chromophore, provided only that the valence hole is in a π state.

For σ bonds involving carbon, s-p hybridization would result in a reduction of the cross section, perhaps by a factor of 2 to 4. For oxygen, nitrogen and sulfur one would expect the σ bonds to be primarily atomic p-wave in character, since these are the unfilled atomic orbitals. When nitrogen and sulfur occur in high oxidation states one would expect some s-p hybridization in the σ states with a possible reduction of the XIFAME cross-section similar to carbon σ bonds.

ESTIMATE OF XIFAME SENSITIVITY

While the above cross section estimates are quite encouraging, they are only one element of a successful technique based on XIFAME. The other elements are the number of molecules in which it is possible to produce a valence hole for the necessary length of time, the magnitude of the concentration of the molecule that one wishes to map, and the presence of interfering background processes. While the diversity of possible XIFAME-based studies prevents a categorical evaluation of any of these quantities, in this section I shall demonstrate by example that the set of feasible applications of the technique is not empty.

Let N_0 denote the number of molecules of interest per unit volume, N denote the number (per unit volume) with a valence hole state, σ_f the cross section for production of a hole state, τ the lifetime of the valence hole state, and I_0 the incident photon flux of the pump beam. Then, since the Franck-Condon principle prevents stimulated de-excitation of the excited state by the pumping radiation, the equilibrium value of N is given by

$$\left(\frac{N}{N_0}\right) = \frac{I_0\sigma_f\tau}{I_0\sigma_f\tau+1}. \tag{4}$$

Thus the population will consist essentially of molecules with a valence hole provided I_0 is large compared with $1/(\sigma_f\tau)$.

Fluorescent lifetimes of tryptophan, tyrosine and phenylalanine are in the range 1-6 ns (depending on the chemical environment), while the fact that fluorescent quantum yields are 20% or less indicates that the rate-determining processes are non-radiative[17] One would expect that non-fluorescent amino acids, such as cysteine, for which fluorescent lifetime data is not available, would have a comparable lifetime. Accordingly, let us take 3 ns as a reasonable estimate for τ. The critical photon flux $1/(\sigma_f\tau)$ then varies over the range $4\times10^{23}\,\text{cm}^{-2}\text{s}^{-1}$ (tryptophan at 205 nm) to $4-6\times10^{25}\,\text{cm}^{-2}\text{s}^{-1}$ (cysteine at 195 nm; tyrosine at 275 nm). These flux levels are achievable over volumes greater than a cell size with commercially available excimer lasers; accordingly, in subsequent estimates we can assume that $N/N_0 \approx 1$. While these are quite high photon fluxes, they need to be maintained only for the very short duration of the probe beam, which in a synchrotron is of the order of tens

of picoseconds. Sample heating and damage could thus be limited by matching the laser pulse to the time structure of the X-ray beam.

In an experiment which attempted to image a particular molecular species by the XIFAME technique, the signal-to-background ratio would be given by

$$\frac{S}{B} = \frac{\left(\dfrac{N}{N_0}\right) N_x \sigma_x}{\sum_i N_i \sigma_i},$$ (5)

where N_x is the number of chromophores per unit volume and N_i, σ_i are the number density and photoabsorption cross section, respectively, of each of the atomic species present in the cell. Let us consider the case of imaging the aromatic amino acids (tyrosine, tryptophan and phenylalanine) in a generic bacterial cell[18] with the amino acid composition of E. Coli.[19] For a dried cell the value of S/B is 4. This means that the average concentration of aromatic amino acids in the cell is easily detectable above the atomic background; imaging should therefore have quite good contrast. For a cell in an aqueous environment the situation is more marginal: S/B is 0.6. In these estimates the computation was done assuming the XIFAME peak lies at the energy of the elemental carbon K_α line, although in reality the energy would be determined by the molecular state. Since the cross sections vary only slowly with energy this assumption should have little effect on the value of S/B.

We can put this estimate into perspective by comparing imaging or mapping by the XIFAME technique to elemental mapping by moving alternately above and below the atomic absorption edge. In this comparison we can take as a figure of merit the ratio $\sigma_x/\Delta\sigma$, where $\Delta\sigma$ is the magnitude of the cross section change at the the absorption edge. For the carbon edge this ratio is approximately 40, which means that XIFAME is a considerably more sensitive technique.

A more interesting situation for elemental mapping is the case of sulfur, for which mapping based on either the sulfur K or L absorption edge has been proposed.[20] In this case the ratio $\sigma_x/\Delta\sigma$ is about 7 for the L edge region and 300 for the K edge (using sulfur K_β as the XIFAME transition); hence, XIFAME is inherently the more sensitive technique for mapping the common sulfur-containing structures cysteine, cystine and methionine.

SPECTROSCOPIC APPLICATIONS OF XIFAME

The iron-sulfur structures in mitochondrial electron-transfer chain proteins provide an example in which XIFAME has potential advantages for non-imaging spectroscopic studies. The chemical state of the iron atoms in these structures can be studied by electron spin resonance techniques, but no comparable method presently exists for sulfur. The study of sulfur biochemistry using XANES (X-ray Absorption Near Edge Structure) has been proposed.[20] Differences in the ionization state of sulfur in compounds may shift the absorption edge by up to 13 eV,[21] with the edge appearing at lower energy for compounds in which the sulfur is in a lower oxidation state. Measurements of the near-edge spectra of methionine and glutathione show differences (both energy shifts and shape changes) between the reduced and oxidized forms of these compounds.[22] XANES studies would utilize these differences to determine the oxidation states of sulfur from accurately-measured near-edge spectra.

It is instructive to compare XANES and XIFAME in a hypothetical experiment to determine the sulfur oxidation states in an iron-sulfur structure under a given set of conditions. Assume that the iron-sulfur structure is contained in a protein of molecular weight 20,000, and that the protein can be concentrated to 1 millimolar in solution. We can then estimate the signal-to-background ratio for a XANES experiment, if we assume the atomic composition of the protein (exclusive of the iron-sulfur structure) is not too different from that of E. Coli. We then find that the XANES signal-to-background ratio in solution is 5×10^{-4}, with the background coming predominantly from oxygen in water molecules. If the experiment could be done using dried protein, then the signal-to-background ratio would improve to 3×10^{-3}. For XIFAME the corresponding signal-to-background ratio is 0.13 for protein in aqueous solution and 0.8 for dried protein. Clearly XIFAME has a large advantage, although both techniques have a problem with separating out the signal from the ambient absorption.

These estimates are based on an evaluation of σ_X for the sulfur K_β transition, using published values of the level widths from Ref. 13. These estimates give essentially an upper limit to the molecular cross section because of eqn. 3. While one would expect sulfur p-states to be strongly represented in the molecular state of an iron-sulfur structure, it is possible that there is hybridization of the states, which would reduce the XIFAME cross section. In fact, studies of K_β emission in simple sulfur compounds by Perera and LaVilla[23, 24] do show that the relative intensities of peaks corresponding to different valence states change with the chemical environment of the sulfur. It would therefore be

unwise to conclude categorically from the above signal-to-background estimates that XIFAME is the better technique, although it does appear likely to be more sensitive.

It is fair to say that separation of signal from background would be a serious problem for either technique in our hypothetical experiment. In fact, in all of the XANES work cited, a prominent peak occurs which Perera and LaVilla interpret as a transition to the first unoccupied valence molecular state. In the K_β emission measurements the several observed peaks are transitions from occupied valence molecular states, and these are the transitions which would occur in XIFAME. In Refs. 23 and 24 the compounds studied all have sulfur in similar ionization states; however, the observed shift with sulfur oxidation state of the prominant absorption peak in Refs. 21 and 22 would imply, assuming the interpretation of Perera and LaVilla is correct, that the XIFAME peaks would shift by a comparable amount. Ref. 11, which finds a 2 eV shift as sulfur goes from a +3 to a +6 formal oxidation state supports this interpretation. Thus, in either technique the different oxidation states should be resolvable.

The key advantage to XIFAME in this situation is that the pump laser beam gives an extra degree of freedom which can be manipulated to reduce the background. With the laser beam off the XIFAME absorption spectrum would disappear; the background spectrum, however, would remain unchanged. A laser on/laser off differential measurement would thus provide a very powerful method of separating a small signal from a large background. There is no comparable possibility with XANES.

CONCLUSIONS

Using the pump-probe technique XIFAME, mapping the aromatic amino acids in bacterial cells is within the range of current technology. By extension, any visible or UV chromophore with a predominantly π-bond character can be detected, and the technique is intrinsically more sensitive than elemental mapping based on cross section changes at absorption edges. There are special possibilities for improving signal to background that make the technique of interest for non-imaging spectroscopic studies of structures present at low concentrations. Although XIFAME is at present an untried technique, these potential applications make it well worth exploring.

ACKNOWLEDGEMENTS

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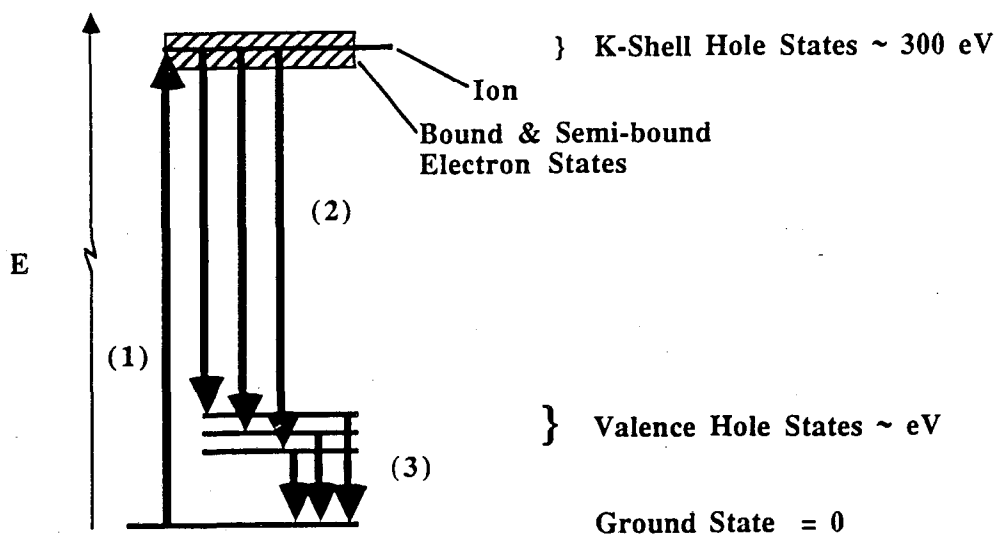24. R.C.C. Perera and R.E. LaVilla, J. Chem. Phys. **84** (8), 4228 (1986).

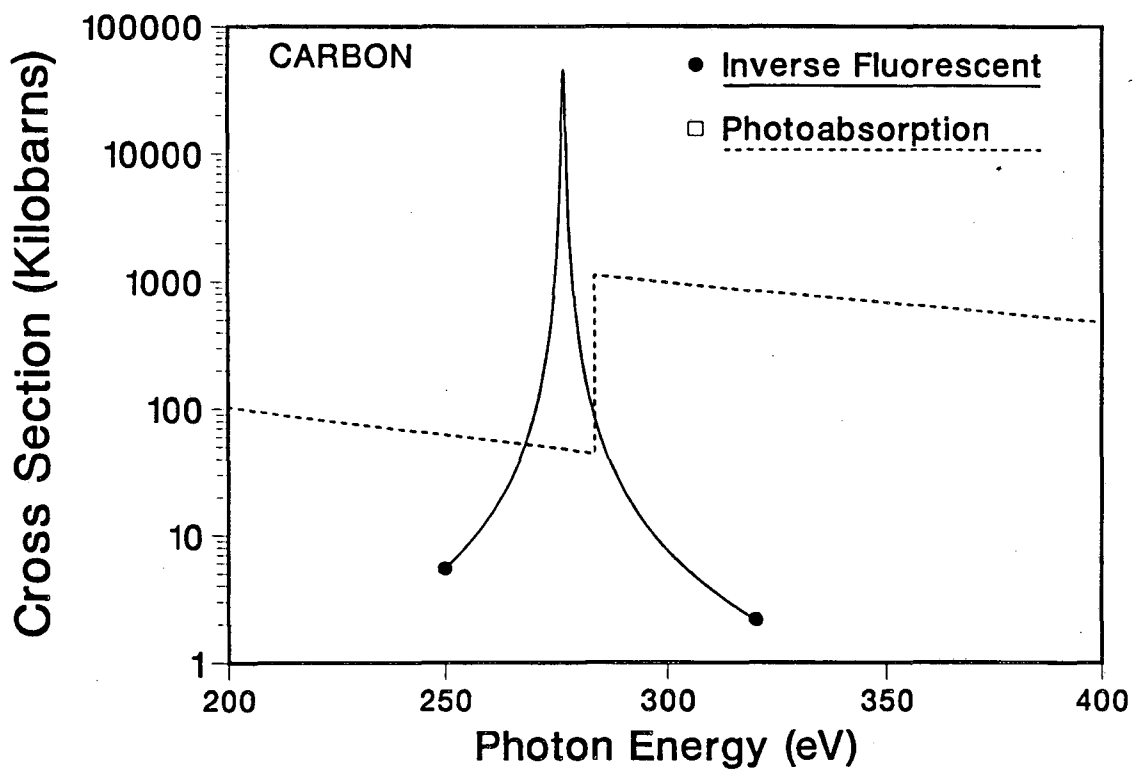FIG. 1. X-ray absorption and fluorescent emission transitions.

FIG. 2 Calculated inverse fluorescent cross section compared with normal photoabsorption.

LAWRENCE BERKELEY LABORATORY
UNIVERSITY OF CALIFORNIA
INFORMATION RESOURCES DEPARTMENT
BERKELEY, CALIFORNIA 94720