Ca^{2+} gradients are not small, Ca^{2+} obeys a nondiffusive transport equation. The transport equation comes in two pieces. The first term is the usual type of diffusion term but with a diffusion coefficient dependent on Ca^{2+}. This is the same expression that was derived by Irving et al. However, the second term in the transport equation is a nonlinear function of the Ca^{2+} gradient and can be significant in regions where the gradient is large.

This elegant result has a number of important consequences. Most importantly, it shows that the effects of buffers cannot, in general, be modeled by a reduction in the diffusion coefficient of Ca^{2+}. A small amount of mobile buffer can have a disproportionately large effect on the transport equation. A number of ways in which this result affects the interpretation of experimental data spring to mind. For instance, there has been some controversy in the literature as to the identity of the diffusing messenger that propagates the Ca^{2+} waves observed in *Xenopus*, and calculation of the effective diffusion coefficient of Ca^{2+} has played a central role in these arguments. However, such effective diffusion coefficients cannot always be defined and, therefore, such arguments are at best unreliable. Other examples are discussed by Wagner and Keizer.

Although the work of Wagner and Keizer has advanced our understanding of the effects of buffers, many questions remain unanswered. How will buffers affect the existence of waves? Can mobile buffers cause the breakdown of wave propagation, or will they have little effect? How will the wave speed be affected? Intuitively, one expects that mobile buffers will have a tremendous influence on the speed of propagating waves, but this remains to be quantified. What effect will buffers have on wave profiles? It has already been shown that the relationship between the space constant of the wave front, the speed of the wave, and the diffusion coefficient of Ca^{2+} is profoundly affected by buffers (Sneyd and Kalachev, 1994), but can one make more explicit predictions? The new transport equation will play a pivotal role in the study of such theoretical questions. The incorporation of buffering terms into a single transport equation will, one hopes, simplify the analysis of wave propagation in buffered systems. Instead of having to deal with multiple diffusion equations, theoreticians, instead, can study the behavior of a single equation, with all the simplifications this implies.

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Resonance Raman Microspectroscopy in Biology

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The development of resonance Raman spectroscopy as a spectroscopic and analytical tool has occurred rapidly over the past twenty years. Progress in implementing this technique has been driven by the molecular level insight it provides, by the experimental versatility with which it can be implemented, and by advances in laser, detector, and spectrograph technology. The article in this issue of *Biophysical Journal* by Salmaso et al. demonstrates many of these advances in a Raman microspectroscopic characterization of mammalian peroxidases. This class of enzyme has attracted considerable recent interest because of their critical roles in the antimicrobial defense systems in higher animals.

The insight into molecular process available from resonance Raman spectroscopy derives from the fact that it provides vibrational data under conditions of resonance with optical (electronic) transitions. Thus, it ties the inherently high information content of vibrational spectroscopy to the dissective capabilities of selective optical excitation and is ideally suited to modern high resolution laser technology. The underlying principles of both its vibrational and electronic aspects are well understood. This sound theoretical basis, coupled with the fact that it can be carried out over a broad temperature range with both pulsed and continuous wave lasers, extends its range beyond static structural characterization to kinetic and dynamic applications (e.g., Riordan and Vallee, 1993).

Laser excitation is bright and easy to manipulate optically, which provides considerable flexibility in sample geometry and physical state. All that is necessary is to bring laser light to a focus on a sample positioned at the focus of the spectrometer collection optics. This situation minimizes the requirements for sample volume, which can be considerably less than 1 μl, and allows solid, liquid, and gaseous samples to be used. The sustained progress in laser technology over the past three decades has provided continuously tunable excitation frequencies from the infrared to the vacuum ultraviolet region; moreover, with modern mode-locking methods, temporal resolution to the subpicosecond regime is routinely available. Only uncertainty broadening, which becomes appreciable in the femtosecond region, now limits the time resolution of a Raman measurement.

These developments in laser technology have been matched by improvements in detector technology,

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spectrometer design, and laser light rejection. Multielement detectors, initially vidicons and photodiode arrays, and more recently charge coupled devices (CCDs), have provided the hardware necessary to monitor the complete spectral distribution of Raman scattered light with both the spectral resolution and high quantum yield necessary. Moreover, the most recent generation of back-thinned CCD detectors has extended the useful spectral range accessible to Raman spectroscopy well into the near infrared, as experiments with the photosynthetic bacterial reaction center have demonstrated (Palaniappan et al, 1993). Holographic notch filters, which attenuate elastically scattered laser photons effectively without inordinately decreasing the intensity of inelastically scattered Stokes photons, have been coupled with compact, efficient spectrographs to provide high resolution and high throughput. These have eliminated the need for complex, double, or triple monochromators which, because of the necessarily high number of optical components, have poor optical throughput.

Salmaso and co-workers have capitalized on a number of these advances in their continuing development of a confocal Raman microscope (Puppels et al., 1990). In their article in this issue, the Dutch group has applied the technology to the study of the immunologically important enzyme, eosinophil peroxidase (EPO), at the level of a single living cell. EPO is a member of a larger class of heme-containing mammalian peroxidases that includes lactoperoxidase, intestinal peroxidase, and myeloperoxidase (MPO). At least two of these proteins, EPO and MPO, are critical to antiparasitic defenses, because these enzymes catalyze the formation of cytotoxic hypohalous acids from hydrogen peroxide and halide ions after phagocytosis of the invading microbe. EPO has also been implicated in the anti-tumor activity of interleukin 4. The optical and Raman spectroscopic properties of these proteins are unusual, as might be expected from the unorthodox chemistry they catalyze, and they have generated considerable debate in the literature. The origin of the striking red-shift in the optical spectrum of MPO, for example, continues to be debated, despite the fact that a high resolution crystal structure now exists (Zeng and Fenna, 1992). Recent Raman and biochemical data suggest that charged amino acids in the active site play a significant role in altering the electronic and vibrational properties of the heme chromophore in this peroxidase (Floris et al., 1994).

Within this context, the work of Salmaso et al. is of considerable interest. As they demonstrate, Raman microspectroscopy of human eosinophilic granulocytes allows in situ measurements to be made with high spectral resolution. In the current implementation of their microscope techniques, they have developed methods for variable laser wavelength excitation, which provides access to excitation profile characterization, and for depolarization measurements, which is critical in assigning observed vibrations to chromophore normal modes. By using these methods, they provide a detailed interpretation of the vibrational properties of EPO, both in situ and in isolated form. They refine earlier assignments of the Raman spectral characteristics so that a clear picture of the active site in the resting enzyme is now available. As with the well characterized plant peroxidases, such as horseradish peroxidase, the heme chromophore is iron protoporphyrin IX, which assumes a six-coordinate, high-spin configuration in the ferric resting enzyme, both in granulocytes and in its isolated form. Similar to the situation in MPO, local protein effects appear to modulate the properties of the chromophore significantly. Salmaso et al. localize these interactions to the protoheme vinyl groups, which can relate critically to activity through redox and conformational mechanisms. Their low frequency data suggest that active site tailoring through the peripheral substituents is a general mechanism in the mammalian peroxidases for implementing cytotoxic product generation.

The present work systematizes the interpretation of the vibrational data available on resting mammalian peroxidases. Extension of the Raman approach to outstanding questions is now feasible. Key among these are the following: 1) Can axial ligand assignments in the resting enzyme be confirmed by isotope substitution?; 2) Is the unusually high iron-histidine vibrational frequency in reduced plant peroxidases also a characteristic of the mammalian peroxidases?; 3) What is the detailed nature of the local protein/heme peripheral substituent interaction and how does it relate to activity; 4) Can the microspectroscopic technique be extended to resolving mechanistic issues in real time on single cells?; 5) How do the mammalian peroxidases protect themselves from the corrosive products of their catalysis? Raman methods will undoubtedly continue to play a large role in addressing these issues.

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Cross-Talk Between Membranes

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In this issue of *Biophysical Journal*, Lückhoff and Clapham (1994) describe

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