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PERSISTENT FREE RADICAL ESR SIGNALS IN MARINE BIVALVE TISSUES

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ABSTRACT

Freeze-dried homogenates of the oyster *Crassostrea rhizophorae* collected from waters in Puerto Rico near urban and industrial sites as well as at relatively pristine locations yielded electron spin resonance (ESR) spectra characteristic of free radicals as well as spectral components of transition metal ions, dominated by manganese. The magnitudes of these ESR signals and the concentrations of trace elements (determined by X-ray fluorescence) varied considerably among oyster samples, masking any potential correlation with polluted waters. Laboratory studies were initiated to identify the factors controlling the magnitudes of the tissue free radical ESR signals. Another mollusc, *Mytilus californianus* collected at the Bodega Marine laboratory in northern California, was fractionated into gonads and remaining tissue. Freeze-dried homogenates of both fractions exhibited ESR signals that increased gradually with time (over a period of days at room temperature). ESR signals were observed in freeze-dried perchloric acid (PCA) precipitates of the homogenates. ESR signals were also observed in delipidated PCA precipitates and in chloroform extracts of these precipitates. Acid hydrolysis to degrade proteins to amino acids produced a residue, which yielded much larger ESR free radical signals after freeze-drying (by at least one order of magnitude) than had been observed in unhydrolyzed samples. Rehydration of these samples did not diminish the magnitudes of the ESR signals significantly. Thus a major pathway for generating *post-mortem* ESR signals in biological tissues may be proteolytic activity. Freshly thawed homogenates of *Crassostrea rhizophorae* also exhibited ESR signals. By equilibrating the homogenates with defined gas mixtures, the signals were observed to increase under oxygen and decrease under nitrogen. The effect of varying the oxygen tension was especially marked in homogenates that had been supplemented with ascorbic acid, suggesting that the concentration of antioxidants in mollusc tissues may exert a strong modulating influence on ESR signals. A laboratory model of copper stress in *Crassostrea rhizophorae* was developed to study the effect of this transition metal on tissue free radicals. Preliminary results suggested that sublethal copper exposure had little effect on tissue free radicals, except possibly for a signal enhancement in an oyster fraction that was enriched in kidney granules. Since kidney granules are known to accumulate heavy metals in mussels and probably other marine bivalves, this signal enhancement may prove to be an indicator of free radical processes associated with heavy metal deposition in molluscs.

INTRODUCTION

As government agencies throughout the world have begun to confront the task of cleaning up contamination of the environment they have come to the realization that they lack the means to assess whether or not specific efforts to attain environmental restoration will be adequate. Clearly the world's environment cannot be restored to its pre-industrial state; the task at hand is to define "acceptable risk," which will provide criteria for assessing remediation strategies. In the consideration of acceptable risk, one must include not only immediate human health and animal survival issues, but must also consider the integrity of ecosystems and long-term risks to animal and plant communities, including subtle biological effects that may be cumulative chronologically or in terms of various bioaccumulation processes. Dramatic bioconcentration of toxic agents that can occur as these agents move through the food chain[1] provide one example of the enormous challenge confronting the researcher and regulator grappling with the problems of risk definition and amelioration.

Among the diverse approaches being explored for assessing risk, studies addressing fundamental toxicity mechanisms at the biochemical, subcellular and organismal level have been highly productive and continue to produce important new insights that guide the evaluation of the potential effects of environmental pollutants on biological communities. One of the most exciting areas of recent research has been free radical biochemistry, which has shown that destructive free radicals are ubiquitous in the biological world, being implicated in virtually all normal and abnormal physiological processes. This has led to a great surge of interest in developing new markers of free radical damage, with the expectation that these markers would facilitate progress in understanding, and possibly intervening in, a variety of important human health problems, including the major killer diseases, the toxicology of xenobiotics and human aging.

It has long been known that persistent free radicals, detectable as electron spin resonance (ESR) signals are associated with wet and dry biological tissues.[2] The magnitudes of the signals correlate with the *in vivo* metabolic activity of the tissue. Some free radical signals arise subsequent to tissue isolation, e.g., during lyophilization procedures.[3] Although it has been inferred that some of the radical signals arise from semiquinones whose concentrations depend on the interaction of tissue quinonoids with reductants and oxidants, and that complexes of ascorbate with proteins are among these species,[4] many other contributions to the observed ESR

signals can readily be suggested. Among the possible additional free radical sources are non-porous polymeric structures, in which free radical centers would be unable to react with each other or with redox-active molecules in the intracellular milieu by virtue of their immobilization and sequestration.

Electron paramagnetic resonance (EPR) is used as an analytical tool for paramagnetic metal ion complexes, which exhibit a larger range of magnetic moments than do unpaired electrons in free radicals. EPR spectra can give useful information about the coordination chemistry of transition metal ions. Since heavy metals generally exert their biological effects as organic complexes; the the characterization of such complexes by EPR is important in studies of metal toxicity mechanisms. In their seminal work, S.G. George et al[5] used low temperature EPR to show that metal-containing granules isolated from the kidneys of *Mytilus edulis* contain a variety of ESR signals, including a number of distinct transition metal ion signals and a free radical signal.[5] A substantial fraction of the granules (14-19% of the dry weight) remained insoluble after acid hydrolysis, but the EPR spectrum of this fraction was not reported. This report of large free radical signals in lysosomal residual bodies prompted us to consider the possibility that the indigestible pigmented polymers that accumulate in some lysosomes (e.g., age pigments) might comprise major free radical centers in these and, perhaps other, cells. This study was undertaken, in part, to characterize the free radicals associated with acid-indigestible tissue residues, using marine mussels and oysters as convenient sources of tissues containing high accumulations of such indigestible material. However, the main rationale for our work was to further develop techniques for measuring tissue free radicals by ESR with the goal of implementing this methodology as a routine risk assessment tool.

MATERIALS AND METHODS

Chemicals: All chemicals were from Sigma Chemical Co.

ESR Spectroscopy: First derivative ESR spectra (100 kHz modulation) were recorded at ambient temperature (22°C) on a Varian E109-E spectrometer (X-band). Instrument settings, unless indicated otherwise were: modulation amplitude 2.5 G, scan range 100 mT, magnetic field centered at 343 mT, microwave power 10 mW.

Oyster tissue homogenization: Freshly killed *Crassostrea rhizophorae*, or tissue fractions thereof, were homogenized in ice-cold de-ionized water with a tissue grinder (Tekmar).

Mussel tissue preparations: *Mytilus californianus*: collected from Bodega Bay, CA were dissected to separate the gonad from the rest of the body tissue, and the two fractions were then lyophilized and pulverized into 1 μm particles with a ball mill (Braun). A portion of the lyophilized tissue was subsequently extracted twice with 4 vol of 5% perchloric acid,[6] followed by lipid extraction with 20 volumes of methanol:CH_2Cl_2:cyclohexane (1:2:2) and acid digestion in 6N HCl at 110°C for 38 hrs. The residues from the acid digestion were washed with distilled water to remove acid and filtered through Centriprep (Amicon) filter cartriges with 10 kD molecular mass cutoff. All samples were lyophilized before ESR analysis.

Preparation of wet homogenates for ESR: Freshly thawed homogenates were either placed directly into glass capillaries (75 μl), which were then sealed (Seal-Ease, Clay Adams, Parsippany, N.J.) or gas-permeable tubing or mixed with ascorbate in 1.5 ml plastic centrifuge tubes (Eppendorf). The procedure for placing homogenates into gas-permeable tubing consisted of filling the conical plastic tip of a microliter pipettor (Pipetman, Rainin Instrument Co.) from the large end, packing the homogenates into the narrow end of the pipette tip by centrifugation and using the pipettor to push the thick slurry into a segment of gas-permeable tubing that had been tightly fitted over the pipette tip. Kinetics of gas equilibration with aqueous samples in gas-permeable tubing were determined by ESR oximetry.[7]

Trace element determinations: XRF analyses were conducted as published.[8] Freeze-dried oyster tissue homogenates were pulverized and 2.54-cm diameter pellets were pressed at 15,000 psi and weighed. Typically, this procedure yielded pellets of 30 mg/cm^2 and 0.03 cm in thickness.

RESULTS

Trace element compositions of oysters: Oysters were collected from four sources in Puerto Rico, judged to be pristine or polluted to varying degrees, and analyzed by XRF (Table). The sites were: Jobanes (samples 1 & 2), which served as a pristine control, the less pristine yet still relatively unpolluted Parguera channels (samples 3 & 4), Tallaboa (samples 5 & 6), which is located near large petrochemical plants and is likely to be the most polluted environment and the waters adjoining the town of Parguera (samples 7 & 8), which are exposed to untreated sewage (mostly of residential origin). Concentrations of the elements are presented as ug/g of dry weight, except Zn*, which is ug/0.01g of dry weight.

The trace element composition of the oysters indicates that there is little correlation of the analyzed elements with the locations from which the oysters were collected. In particular, the oysters collected from the waters adjoining the petrochemical complex have at most marginal increases in some of the elements (Zn, Br, Sr). Although the data could have been improved by

increasing the number of oysters, it was of greater interest to pursue other markers of pollutant exposure, with the expectation that better correlations with collection sites would be found. This led to the biophysical studies that are reported in the following section.

TRACE ELEMENT ANALYSES OF OYSTER HOMOGENATES

Sample#	1	2	3	4	5	6	7	8
Element								
Cr	25±6	6±4	10±5	<16	14±9	<14	9±8	19±9
Mn	19±4	14±3	35±4	9±7	33±6	41±5	20±6	16±6
Fe	560±20	180±10	460±20	220±20	370±20	270±10	290±20	290±20
Ni	32±3	2±1	5±1	6±3	9±2	4±1	4±1	5±1
Cu	350±20	67±3	190±10	220±20	350±20	420±20	370±20	280±20
Zn*	45±2	15±1	21±1	22±2	59±3	84±3	91±4	21±1
As	17±2	21±2	32±2	21±2	12±2	15±2	22±2	23±2
Se	3.1±.4	1.6±.4	5.3±.4	4.3±1.2	4.3±.6	4.2±.4	4.2±.3	2.2±.4
Br	310±10	210±10	280±10	360±40	360±40	520±20	640±30	610±30
Rb	<6	<9	<9	<6	<6	<6	<6	<6
Sr	49±2	38±2	55±2	54±5	105±6	92±5	114±7	97±6
Pb	5±2	<2	<2	<6	<4	<2	<3	<4
Ag	<3	<3	2±1	<7	8±3	5±2	<3	2±2
Cd	<3	<3	<3	<9	<4	<3	<4	<5
Sn	12±2	6±2	18±3	8±5	9±3	<3	10±3	7±4
Sb	<3	<4	<4	<12	<6	<4	<5	<6

ELECTRON SPIN RESONANCE RESULTS

Freeze-dried oysters collected from waters in Puerto Rico: ESR spectra of freeze-dried tissue homogenates derived from oysters collected at three locations in Puerto Rico are shown in Fig 1. All but the spectrum at the bottom of the figure are dominated by the six line ESR spectrum of manganese. Additional manganese lines are clearly evident in the ESR spectra of the Boqueron samples, which show two additional broader lines between each pair of the narrow principal lines. All of the spectra also exhibit a very broad line just left of center in the figure, which is most clearly evident in the spectrum at the bottom of the figure. This broad feature is due mainly to copper and iron complexes. In terms of developing a new biomarker of damage, the most promising spectral component is the sharp free radical signal, which is identified by

arrows. This feature is seen in all of the samples, although its intensity varies considerably even among oysters collected from a given water body. Potentially this feature provides a damage marker, which can be correlated with toxicity. In reviewing these results, we noted that the largest free radical signal (third spectrum from the top) was observed in an oyster from the most polluted source. However, since only one of four oysters samples produced a large signal, and since only a few oysters were to be found near the petrochemical complexes, we shifted our attention to experiments designed to produce large ESR signals in oysters under laboratory conditions. The experimental design was predicated on the hypothesis that copper-mediated free radical processes would lead to the accumulation of polymeric material in oysters which would be associated with large persistent ESR free radical signals.

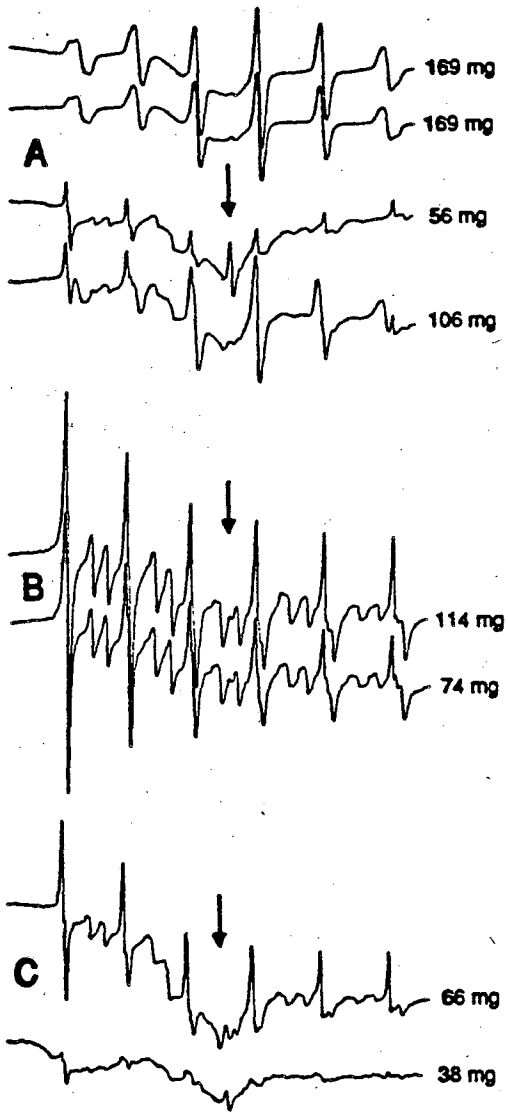

Fig 1. ESR spectra of freeze-dried oyster homogenates. The arrow at a g-value of 2.00 shows the position of the free radical signal. Weights refer to the freeze-dried tissue in the ESR tube. Guayanilla (A) is located near petrochemical complexes, Boqueron (B) is a town whose adjoining waters are exposed to sewage, and Vieques (C) is an island whose waters were judged to be unpolluted.

Effects of sublethal concentrations of copper on oysters in the laboratory: Oysters were placed in synthetic seawater supplemented with various sublethal copper concentrations. ESR spectra of freeze-dried homogenates of these oysters all exhibited free radicals signals with substantial variability between oysters that had been treated in the same manner (Fig 2). As had been observed with oysters collected in the field, the variability of ESR signals among different oysters was greater than any systematic differences due to the effects of copper. Therefore, it was clear that more control of the factors responsible for this variability had to be achieved before meaningful comparisons with different degrees of copper (or environmental) stress could be undertaken.

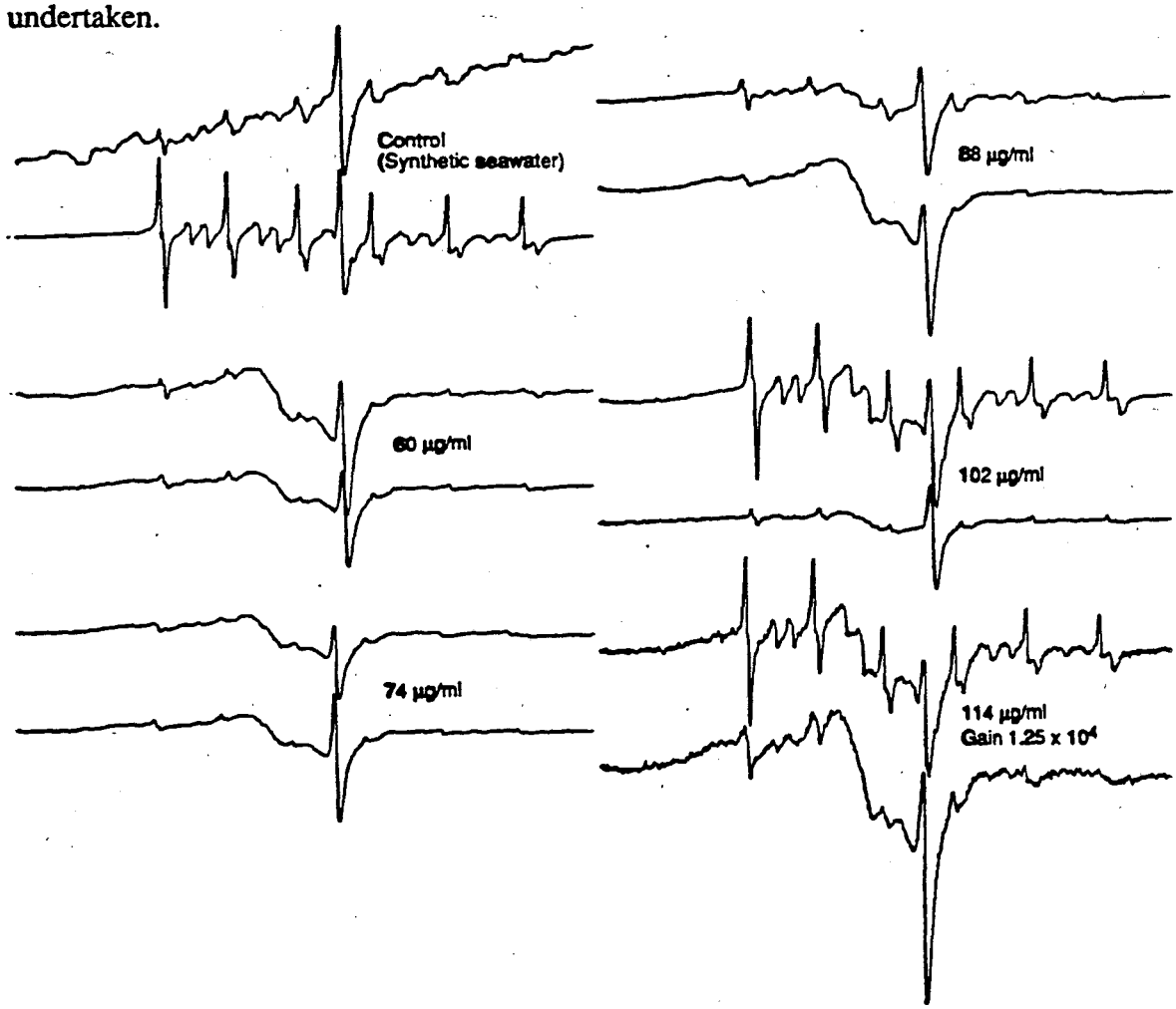

Fig 2. ESR spectra of freeze-dried homogenates of oysters exposed to sublethal copper concentrations in the laboratory. Synthetic seawater was supplemented with various amounts of $CuSO_4$, shown in the figure as ug/ml. The instrument gain was 5 x 10^3, unless indicated otherwise.

Freeze-dried mussels collected from waters in California: To seek an understanding of the variability in the ESR signals of bivalves, another organism, easily available for routine analyses in Berkeley, was chosen. Consistent with the report of George, et. a., mussel tissues also produced ESR signals, and in freeze-dried material large free radical signals were readily observable at room temperature, facilitating routine data analyses. Freeze-dried *Mytilus californianus* homogenates were stored in quartz tubes at room temperature and examined periodically by ESR, which showed that the magnitudes of the signals increased gradually with time of incubation. The extent of this signal increase after prolonged storage can be considerable as illustrated by the two spectra at the top of Fig 3.

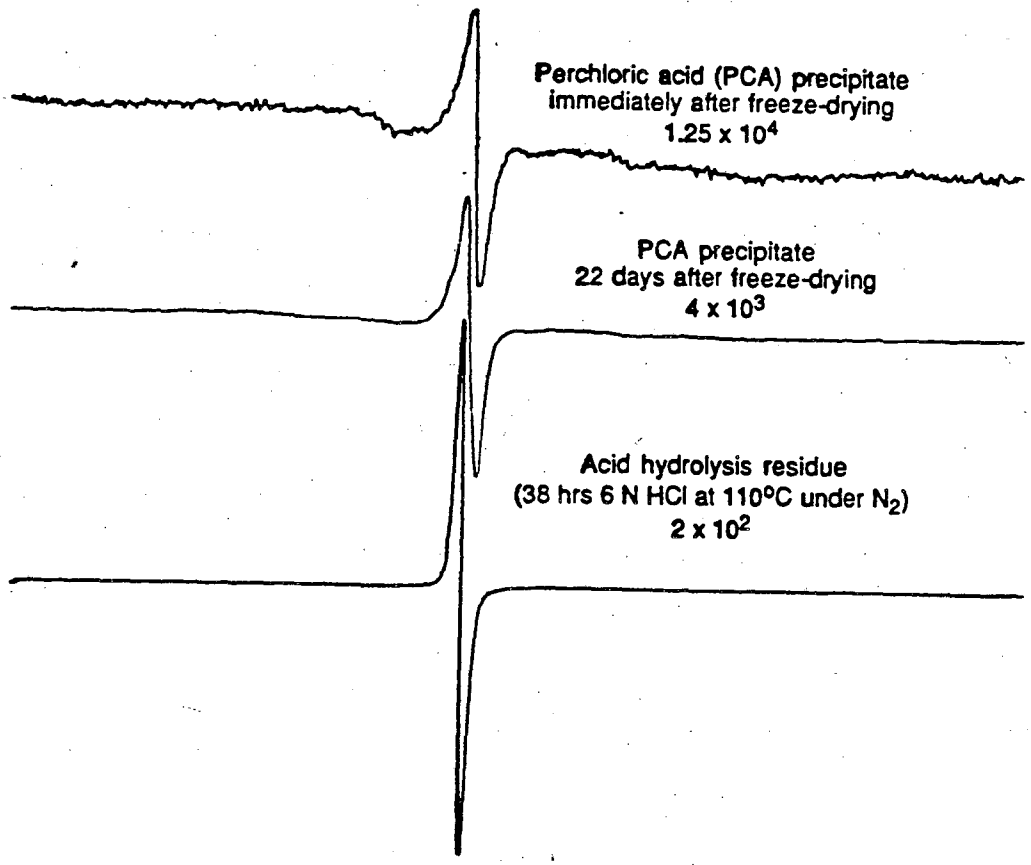

Fig 3. Effect of room temperature storage and acid hydrolysis/freeze-drying on ESR spectra of freeze-dried *Mytilus californianus* (gonad-free fraction). Numbers refer to instrument gains.

To examine the possibility that a large contribution to the ESR signal could arise from residual polymeric materials resistant to proteolysis, mussel homogenates were acid hydrolyzed under conditions sufficient to fully hydrolyze normal proteins to their amino acids. When freeze-dried residues of this acid hydrolyses were analyzed by ESR, the magnitudes of the free radical signals were substantially larger than those observed in unhydrolyzed samples (Fig 3).

Studies of acid-precipitated and solvent-extracted freeze-dried oyster tissue: Perchloric acid treatment of tissue homogenates precipitates proteins, other macromolecules and macromolecular assemblies like membranes, while low-molecular weight solutes remain in solution. Freeze-dried preparations of the resulting precipitate exhibit ESR signals (Fig 4). Extraction of the freeze-dried precipitate with chloroform/methanol, which removes hydrophobic species (primarily membrane lipids and non-polar lipoprotein complexes) yields a significantly diminished ESR signal. The lipid extract, exhibited relatively weak ESR signals, due to the smaller sample volumes of the chloroform solutions.

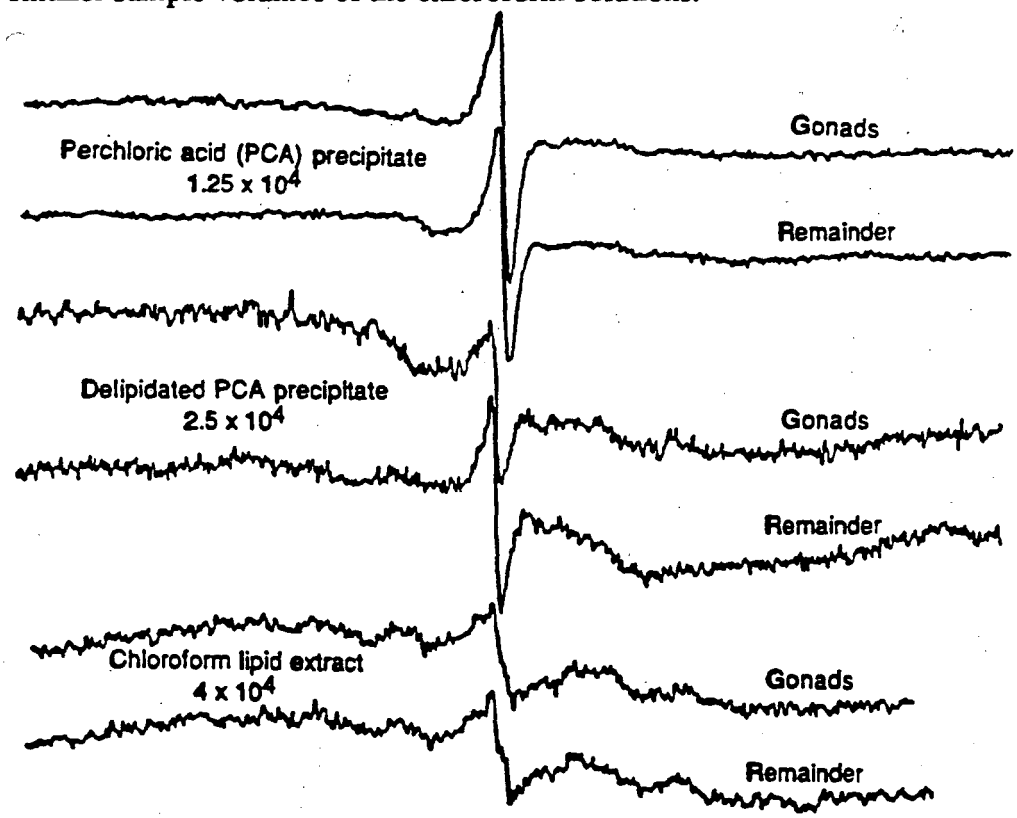

Fig 4. ESR spectra of *Mytilus californianus* extracts. The sample volume of the chloroform extract (1 mm diameter glass capillary) was considerably smaller than the volumes of the freeze-dried material (5 mm NMR tubes).

Studies of freshly thawed oyster homogenates equilibrated with known gas mixtures: Although wet tissue samples do not afford the same sensitivity as freeze-dried tissue (because of the necessity to work with small sample volumes), ESR signals were readily detected in oyster tissue fractions that had been freshly thawed (Fig 5). The signal intensities of four fractions were of comparable magnitudes and did not correlate with copper exposure (sublethal conditions). However, a fifth fraction, enriched in kidney granules, exhibited a significantly larger signal (relative to the broad underlying transition metal ion spectrum) than was seen in the other samples. The signal overlaps with a three-line ESR signal of a nitroxyl radical that was inadvertently introduced into this sample (Fig 5). The presence of this contaminant in the sample was turned to advantage, using it as a reference standard with which the stability of the endogenous ESR signal could be compared. For purposes of the subsequent discussion, we shall refer to the smaller two satellite lines as a doublet, since only two of the nitroxyl lines were resolved and suitable for measurement.

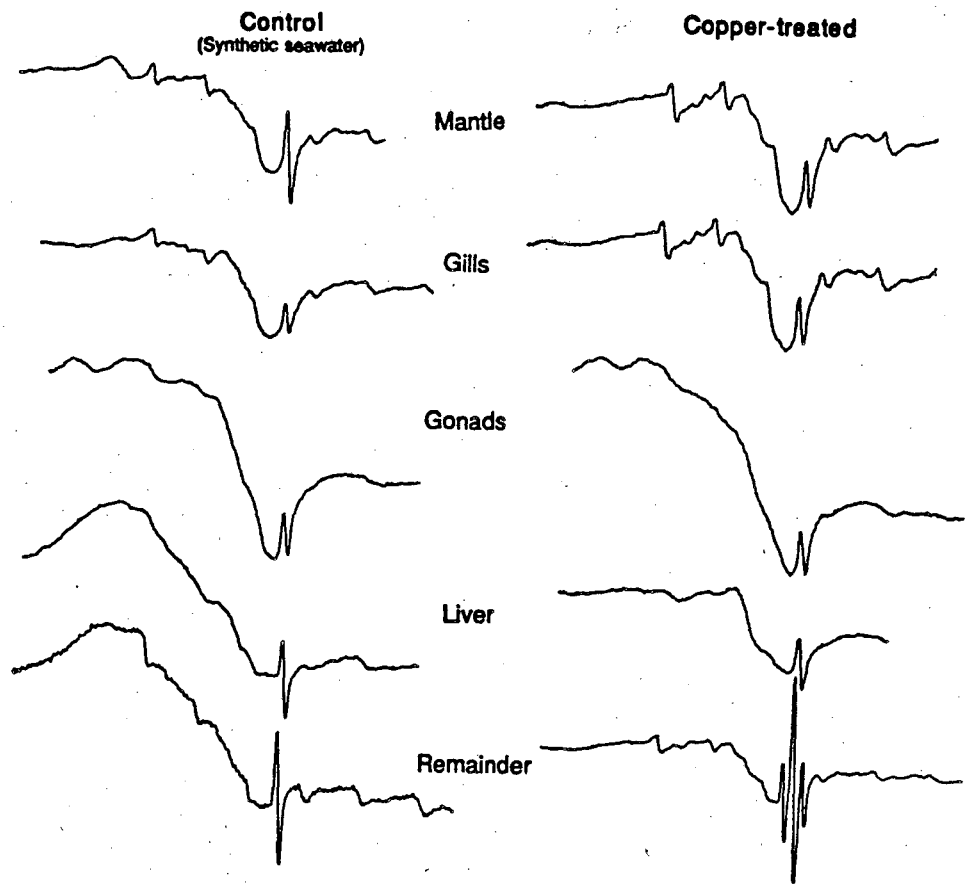

Fig 5. ESR spectra of freeze-thawed homogenates of oysters exposed to sublethal concentrations of $CuSO_4$ in the laboratory. The modulation amplitude was 1.0 mT.

Signal decay kinetics: Although highly persistent, the endogenous ESR signal eventually decayed and this would clearly have to be considered in any effort to develop the ESR assay as a quantitative tool. Moreover, we expected to find that this signal decay would be affected by the oxygen tension. Therefore the kinetics of the signal decay were studied in samples exposed to controlled gas mixtures in gas-permeable tubing. Under both aerobic and anaerobic conditions, the rate of decay of the ESR doublet was faster than that of the singlet and the former had become undetectable after a significant singlet signal remained (Fig 6). The signal intensity was only slightly affected by oxygen tension, with small increases occuring under oxygen exposure and small decreases (relative to the overall downward trend of line heights) occurring under nitrogen.

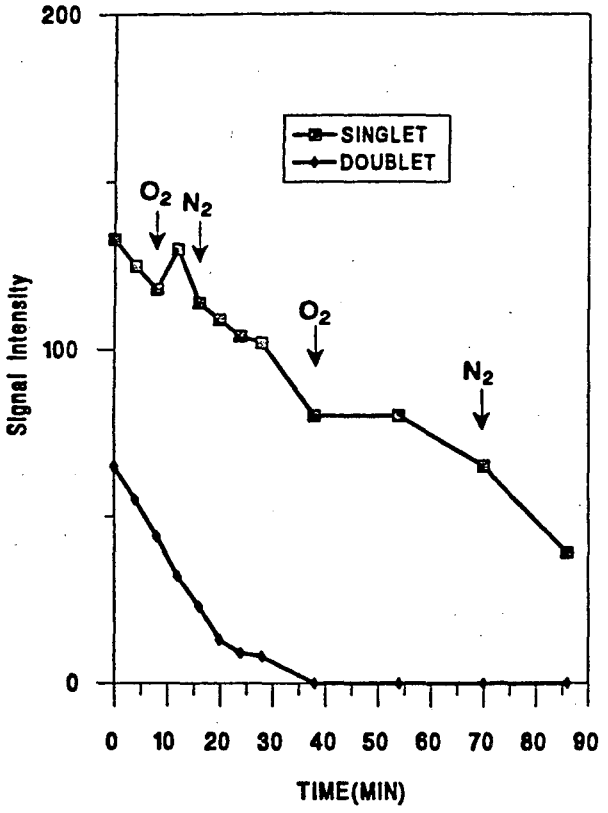

Fig 6. ESR signal decay in freeze-thawed homogenates of copper-treated oysters. The sample, in gas-permeable tubing, was exposed to different gas streams, initiated at the times indicated by the arrows.

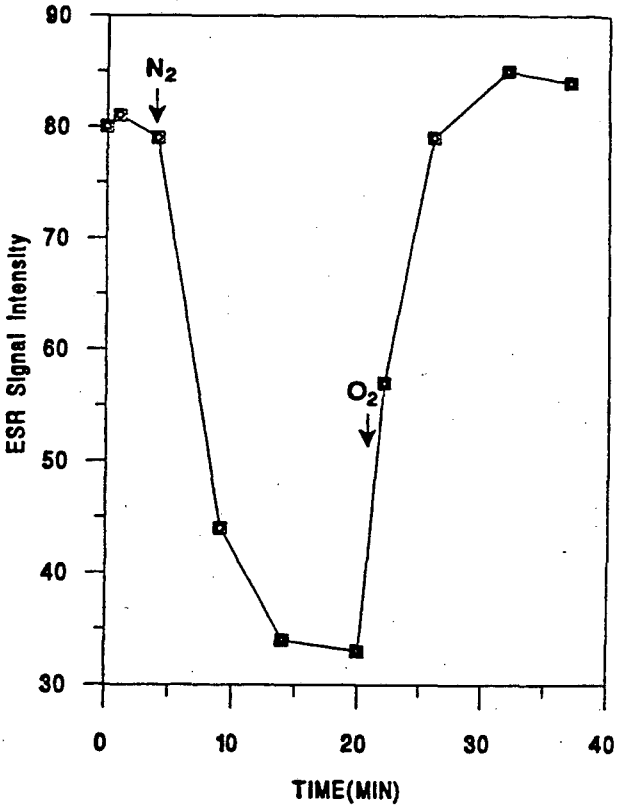

Fig 7. ESR signal modulation by oxygen tension in ascorbate-supplemented oyster homogenates. The kidney-enriched fraction (see spectrum of Control "Remainder" in Fig 5) was supplemented with 4 mM ascorbic acid and analyzed in gas-permeable tubing.

Free radical signal intensity dependence on oxygen concentration in ascorbate-supplemented oyster homogenates: To study the effect of a reductant well-known to reduce free radicals,[9] oyster homogenates were treated with 4 mM ascorbic acid and placed in gas-permeable tubing. The free radical ESR signal was measured as a function of the time of exposure while the sample was exposed to either oxygen or nitrogen (Fig 7). The signal intensity increased under oxygen and decreased under nitrogen to a much greater extent than had been observed in homogates that had not been treated with ascorbate (Fig 6 vs. fig 7). The kinetics of the change in signal intensity were comparable to those of gas equilibration with the samples as determined in separate experiments by ESR oximetry (data not shown).

DISCUSSION

Studies of oysters collected in the field: Neither trace element composition nor ESR spectroscopy of transition metal and free radical signals correlated among the majority of oysters collected from clean or pollutant-exposed environments. Differences among animals from a given source were generally greater than between groups of animals collected at different sites. The failure to obtain trace element profiles that could be correlated with environmental contamination suggests that this analytical tool may not provide a suitable indicator of pollutant exposure of Puerto Rican waters, except possibly for statistical studies involving large numbers of animals. However, in our field collection efforts, we had considerable difficulty in finding any oysters in the waters exposed to petrochemical process waters, suggesting that adequate numbers of oysters for such analyses may not be available. Similarly, the ESR signals of both transition metal ions and free radicals exhibited considerable variability among specimens that were exposed to the same environments, which precluded the possibility of establishing a correlation with source contamination. In principle, the free radical signals, unlike trace element profiles, could correlate with a large variety of environmental stresses, including exposure to organic molecular species. However, only one of four oyster samples from the petrochemical area exhibited a free radical ESR signal that was markedly different from samples collected elsewhere (Fig 1), suggesting again that large numbers of oysters would probably be required to establish significant relationships between ESR signals and exposure to water contaminants.

Effect of laboratory copper stress: In tissue homogenates and cell lysates, copper ions are potent free radical catalysts, causing autoxidation of a variety of biological reductants and promoting the formation of hydroxyl radicals from hydrogen peroxide.[10] Copper toxicity in

marine organisms is a well-established phenomenon and conditions for imposing defined copper stress in the laboratory have been described.[11] In light of the involvement of copper in many free radical processes *in vitro*, we examined the effect of copper stress on the ESR signals of oyster homogenates. The lack of a clearcut correlation of copper exposure with ESR signals (Fig 2) does not rule out free radical signal accumulation as a toxicity indicator, since even the laboratory conditions elicited considerable ESR signal variability among oysters that had been treated similarly (oysters were maintained in the fish tanks for relatively brief periods subsequent to being collected from their natural environments). Nevertheless, it appeared quite likely that further progress would be greatly facilitated by an understanding of the underlying causes of the variability of ESR signals among oysters and this was the goal of the studies described below.

Effects of tissue processing: To pursue the goal of understanding the fundamental factors affecting the reproducibility of ESR signals in tissues, another marine bivalve, more readily available for routine analyses, was chosen. Examination of a large number of samples revealed that the magnitudes of the free radical ESR signals of freeze-dried tissue invariably increased with time, sometimes to a considerable extent. Thus, one parameter that must be controlled to obtain reproducible data when working with freeze-dried tissue is the elapsed time between sample preparation and ESR analysis.

To test the possibility that a measurable contribution to the ESR signal arises from unpaired electrons that are sequestered within polymerized biological material similar to lipofuscin, we attempted to isolate ESR signals arising from residual material by examining tissues that had been subjected to extensive acid hydrolysis (which would decompose normal proteins but not extensively crosslinked lipoprotein to amino acids). Acid-hydrolyzed oyster tissue did in fact produce ESR free radical signals in freeze-dried residual material, but the magnitudes of these signals exceeded by far the signals that had been observed in comparable amounts of freeze-dried material that had not been hydrolyzed. Thus, acid hydrolysis clearly amplifies the processes that form free radicals (at least those that occur in freeze-dried biological materials; however, we should note that rehydration of the freeze-dried material with 0.14 M sodium phosphate buffer, pH 7.4, did not suppress the magnitudes of these ESR signals). These observations suggests that other hydrolytic processes, notably those that are activated subsquent to cell death, may exert a stimulating effect on ESR signal intensities, at least in freeze-dried tissue. One possible mechanism that could be involved would be the liberation of transition

metal ions from metallo-enzymes like heme proteins, which would catalyze free radical chain reactions, driven by the autoxidation of cellular reductants like ascorbic acid. This possibility will be tested with protease inhibitors in planned experiments.

Analyses of tissue fractions: The detection of large ESR signals in freeze-dried PCA precipitates implies that these signals are not dependent on low molecular weight reductants like ascorbate or oxidants like hydrogen peroxide. The substantial decrease in the signals after delipidation (Fig 4) suggests that hydrophobic lipoprotein-derived materials contribute substantially to the ESR signals that are observed in freeze-dried tissues. Importantly, the ESR signals detected in chloroform extracts indicate that at least some PCA-precipitatable material is hydrophobic, whose free radical content can be quantified, in principle, subsequent to solvent extraction. Work is in progress to implement novel ESR techniques for such quantification.

Free radicals in freeze-thawed oyster fractions: To circumvent the artifacts associated with freeze-drying and aging, frozen oyster homogenates were thawed just prior to ESR experiments to study free radical signals in wet tissue samples. Since free radical signals were comparable in all organs examined, and there were no detectable differences between control and copper stressed oysters in four of the fractions, copper-mediated free radical processes appear not the dominate in the formation and/or accumulation of persistent free radical signals detectable in these oyster fractions (at least in sublethal copper stress). The increased magnitude of the ESR singlet observed in the "Remainder" fraction of the copper stressed oysters may indicate that copper-mediated reactions play a role in the formation of a distinct ESR signal that is specific to this fraction. The previous characterization of kidney granules highly enriched in heavy metals and exhibiting free radical ESR signals may be related to this observation. Further experiments are in progress to evaluate the specificity of this free radical signal for the kidney-enriched fraction and how its magnitude is related to the severity of copper stress. These experiments are also extending the range of copper exposures to include lethal conditions.

Decay of free radical signals: Although the radicals observed in wet tissue are highly persistent, they eventually decay. The decay of the nitroxyl radical is faster than that of the singlet, consistent with the idea that part of the endogenous ESR signal is associated with free radicals that are imbedded in residual bodies such as found in the kidney granules, where they would be stabilized by immobilization . The slight increase of the signal intensity under oxygen and decrease under nitrogen suggests that the bulk of the ESR signal observed in these wet

tissues is not due to an ongoing free radical generating process that depends on the presence of oxygen such as an autoxidation of quinones leading to superoxide radicals, or a process dependent on a steady-state concentration of hydrogen peroxide.

Ascorbate effects: Many free radicals are rapidly reduced by ascorbic acid and this can often be demonstrated experimentally by detecting the ESR signal of the ascorbyl radical. For example, in a liposome oxidation model system containing a-tocopherol and ascorbic acid, one can demonstrate that initially only the ascorbyl radical ESR signal is detectable and that only after all the ascorbate has been oxidized can the tocopheroxyl radical be detected.[12] The radical signal that was detected in ascorbate-supplemented oyster homogenates was indistinguishable from the signal observed in control samples, and the characteristic doublet of the ascorbyl radical could not be detected (data not shown). However, the presence of ascorbate substantially altered the oxygen sensitivity of the endogenous ESR signal (Fig 7). This suggests that the tissue radicals can be generated by processes involving both easily autoxidizable reducing agents and oxygen and that such processes can be detected in terms of the oxygen sensitivity of the ESR signal in gas-permeable tubing.

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