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preoxidation by a factor of 2500 and the glucose/interferant current ratio is increased 10^3 fold. Undesired electroreduction of hydrogen peroxide can result when HRP is also "wired" to the electrode. Such unwanted "wiring" is prevented by incorporating an electrically insulating barrier layer between the wired GO film and the HRP film. The hydrogen peroxide necessary for elimination of interferants can be added externally, or, when this is not possible, it can be generated *in situ* by means of a coupled enzyme reaction.

Elimination of Electrooxidizable Interferant-Produced Currents in Amperometric Biosensors

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ABSTRACT

Electrooxidizable ascorbate, urate and acetaminophen, that interfere with amperometric glucose assays are completely and rapidly oxidized by hydrogen peroxide in a multilayer electrode. The multilayer electrode is composed of an immobilized, but not electrically "wired", horseradish peroxidase (HRP) film coated onto a film of electrically "wired" glucose oxidase (GOX). The "wired" enzyme is connected by a redox epoxy network to a vitreous carbon electrode. The current from the electrooxidizable interferants is decreased by their peroxidase-catalyzed preoxidation by a factor of 2500 and the glucose/interferant current ratio is increased 10^3 fold. Undesired electroreduction of hydrogen peroxide can result when HRP is also "wired" to the electrode. Such unwanted "wiring" is prevented by incorporating an electrically insulating barrier layer between the wired GO film and the HRP film. The hydrogen peroxide necessary for elimination of interferants can be added externally, or, when this is not possible, it can be generated *in situ* by means of a coupled enzyme reaction.

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## INTRODUCTION

Electrooxidizable biological fluid constituents, such as ascorbate, urate and acetaminophen interfere with the amperometric assay of glucose. Interference is not restricted to a particular type of glucose sensing anode. It is encountered in electrodes diffusionally mediated by O_2/H_2O_2 or ferrocene derivatives and in electrodes with redox polymers that relay electrons from $FADH_2$ centers of glucose oxidase to electrodes.[1-5] In the latter, interferants are electrooxidized both at the electrode surface and by the redox polymer that is directly electrically connected to the electrode.

In hydrogen-peroxide type electrodes the selectivity can be improved by electrode-coatings that increase the overpotential of interferant electrooxidation, but not that of H_2O_2 oxidation;[6] by membranes that exclude the interferants by size or charge; and by preoxidation of interferants at a separate flow-through electrode. Yacynych et al [7,8] showed that electropolymerized diaminobenzene and resorcinol form on electrodes charge and size excluding films that are H_2O_2 permeable, but effectively exclude interferants in H_2O_2 type glucose sensors. Using low (100) molecular weight cutoff membranes, Guilbault et al excluded from surfaces interferants other than acetaminophen.[9] Lobel and Rishpon[10] and Beh et al[11] used cellulose acetate as an exclusion membrane. Harrison et al[12] and Bindra and Wilson[13] used Nafion-type, sulfonated perfluorinated polymers for exclusion of anionic interferants, while Gorton et al used other sulfonated polymers for their exclusion.[14] Ikeda et al preelectrooxidized ascorbate and urate on a gold minigrid,[15] while Yao et al,[16] as well as Okawa et al,[17] used flow-through electrolytic oxidation columns. Yao[18] used an oxidizing cupric complex containing precolumn. Ascorbate was also eliminated through its ascorbate oxidase catalyzed oxidation by Nagy et al[19] and by Wang et al.[20]

Here we discuss horseradish peroxidase (HRP) catalyzed preoxidation of interferants. After HRP is oxidized by H_2O_2 it rapidly oxidizes both neutral and charged interferants. It does not oxidize, however, either glucose or lactate at an appreciable rate. Peroxidases are well known catalysts for oxidation of hydrogen donors (HA) by H_2O_2, accepting electrons from phenols, amino acids, benzidines, ascorbate, urate and NADH.[21,22] The HRP catalyzed reaction is:

$$2HA + H_2O_2 \xrightarrow{\quad HRP \quad} 2A + 2H_2O \qquad (1)$$
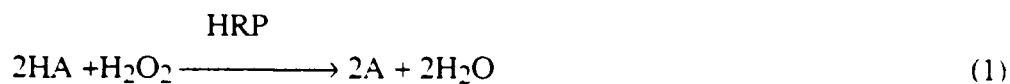
In an earlier communication[23] we showed that HRP films on glucose electrodes eliminate interferant-electrooxidation caused currents. Here we describe processes for forming the HRP-based interferant-eliminating films; analyze the parameters affecting the activity of these films; consider their unwanted interaction with glucose sensing layers, based on electrical connection of glucose oxidase redox centers to electrodes through electron-relaying redox polymer networks; describe methods of internal generation of H_2O_2 within HRP films; discuss the interfering peroxide electroreduction reaction;[24-28] and describe methods for making electrodes that are more selective for glucose.

Experimental Section.

Materials: The glucose-sensing electrodes were made with our earlier described glucose oxidase "wiring" crosslinkable polymer, abbreviated as POs-EA. The polymer has a polyvinyl pyridine backbone with part of the pyridines complexed to $[Os(bpy)_2Cl]^{+/2+}$ and with part of the pyridines quaternized with ethylamine.[29] Polyvinyl imidazole (PVI) was prepared according to a published procedure.[30] Glucose oxidase, GO, (EC 1.1.3.4, type X, 128 units/mg), horseradish peroxidase, HRP, (EC 1.11.1.7,

type I, 85 units/mg or type VI, 260 units/mg) and catalase (EC 1.11.1.6, 2600 units/mg) were purchased from Sigma. Lactate oxidase, LOD, (from pediococcus sp., 33 units/mg) was supplied by Finnsugar. Polyethylene glycol diglycidyl ether (MW 400) was purchased from Polysciences. All other chemicals were of the purest available commercial grade. Glutaraldehyde stock solutions were prepared using "grade I" solutions and kept frozen. Fresh stock solutions of ascorbate, urate and acetaminophen were prepared daily, because of their gradual decomposition. Stock solutions of glucose were allowed to mutarotate at room temperature for 24 hours before use and kept refrigerated. Stock solutions of hydrogen peroxide were prepared by diluting a 30% solution. The H_2O_2 solutions were normalized with potassium permanganate.[31]

The working electrodes were 3 mm diameter glassy carbon rods (Atomergic V-10 grade) sealed in teflon or glass tubes with epoxy resin. The electrodes were pretreated by sequentially polishing with 5.0, 1.0 and 0.3 µm alumina powder on a cloth felt, sonicated, rinsed with deionized water and dried under nitrogen.

Glucose sensing electrodes were prepared using a solution containing 2 mg/ml POs-EA, 0.12 mg/ml polyethylene glycol diglycidyl ether and 1 mg/ml glucose oxidase in a 5 mM pH 7.8 HEPES buffer solution. The polished electrode was coated with a 2 µl droplet of this mixture. The coating was cured for 48 hours under vacuum at about 10 torr.

To make the otherwise large pore size glucose sensing redox polymer network impermeable to peroxidase, the electrode with the sensing layer was further crosslinked by dipping in a 0.25% glutaraldehyde solution for 5 seconds, then rinsed with a phosphate buffer solution. The barrier layer between the sensing and interferant eliminating layers was made of a solution containing 10 mg/ml PVI and 2 mg/ml ethylene glycol diglycidyl ether. A 2 µl droplet of this solution was applied to the glucose sensing layer and was cured for 48 hours under vacuum.

Electrodes were made with HRP films immobilized (a) directly on the polished electrode surface, (b) on the sensing layer covered electrode after making the sensing layer impermeable to HRP with glutaraldehyde (c) on the electrode surface, coated first with the sensing, then the barrier layer. The HRP was immobilized by one of two methods, one using glutaraldehyde,[32] the other involving $NaIO_4$ oxidation.[33] When glutaraldehyde was used, 5 µl of an HRP (type I) solution (100 mg/ml in a 0.1 M pH 7.3 phosphate buffer containing 5 mg/ml glutaraldehyde) was applied and left to air-dry and cure for 2 hours. When the periodate method was used, 50 µl of a 12 mg/ml $NaIO_4$ solution was added to 100 µl of an HRP (type VI) solution (20 mg/ml in 0.1 M sodium bicarbonate) and incubated in the dark, at room temperature, for 2 hours. After incubation, 10 µl of the oxidized enzyme solution was spread on the surface of the electrode and allowed to dry and cure for 2 hours.

LOD containing HRP layers were prepared by co-immobilizing LOD with HRP using the above glutaraldehyde method except that the HRP solution now contained also 100mg/ml LOD.

Measurements. Electrochemical experiments were performed with a Princeton Applied Research model 273 potentiostat/galvanostat. Rotating disk electrode experiments were performed with a Pine Instruments AFMSRX rotator with an MSRS speed controller. The three-electrode cell contained 0.1M NaCl buffered with phosphate (0.1 M, pH 7.2) coated as needed. Saturated calomel reference (SCE) and platinum wire auxiliary electrodes were used. All measurements were under air and at room temperature. The working electrode was rotated at 1000 RPM unless otherwise specified. In the constant potential experiments, the working electrode was poised at 0.4V (SCE).

RESULTS AND DISCUSSION

HRP Catalysis of Oxidation of Interferants by H_2O_2

Figure 1 shows cyclic voltammograms of the HRP film coated glassy carbon electrode in solutions with $10^{-3}M$ acetaminophen (a), urate (b) or ascorbate (c) but no H_2O_2, and also in a $10^{-3}M$ urate and $10^{-3}M$ H_2O_2 containing solution. The electrooxidation of urate, as well as that of the other interferants, (not shown) is completely suppressed: the interferant electrooxidation currents reversibly reappear/disappear in solutions without/with H_2O_2. In controls, where the HRP is replaced by albumin in the immobilized film the electrooxidation currents do not decrease upon addition of H_2O_2. In experiments with HRP coated electrodes, decomposition of the peroxide by a small amount of catalase causes reappearance of the interferant electrooxidation waves as does addition of azide, a peroxidase inhibitor. We conclude from these experiments that elimination of the interferants requires both HRP and peroxide.

Because our purpose was to improve the selectivity of glucose electrodes made by wiring glucose oxidase to a 3-dimensional POs-EA bound redox epoxy[34] most of our measurements were at 0.4V (SCE), the plateau potential of these electrodes. Figure 2 shows the steady-state current of the HRP-film coated electrode poised at this potential. Injection of ascorbate ("A") causes the appearance of an oxidation current that disappears upon addition of excess H_2O_2. Further ascorbate additions have no effect on the current as long as the peroxide is not exhausted. The experiment of Figure 2 was performed with an electrode heavily loaded with HRP. In this electrode interferant elimination was not controlled by the amount of enzyme, but by the ratio H_2O_2 to interferant. At low HRP loadings, the elimination reaction is controlled by the HRP activity of the film and/or by mass transport of the reactants.

To examine the effect of HRP loading, a series of electrodes with increasing HRP loadings was prepared. The loadings were chosen to be sufficiently light to allow only partial elimination of the ascorbate even in the presence of excess H_2O_2. Figure 3 shows that an electrode loaded with only $60U/cm^2$ HRP does not completely eliminate interference by $0.1mM$ ascorbate. Results obtained for a series of electrodes with HRP loadings between 0 and $180U/cm^{-2}$ are summarized in Table I. Loading of $180U/cm^{-2}$ is, as seen, necessary to completely eliminate interference by $0.1mM$ ascorbate. From Table I it is also evident that as the HRP layer thickness increases the electrooxidation current of ascorbate decreases even in the absence of H_2O_2 for the obvious reason that transport of ascorbate to the electrode is reduced. As expected, when the elimination is HRP-loading limited, increasing the hydrogen peroxide concentration does not decrease the interferant electrooxidation current. On the contrary, because HRP is inhibited by excessive H_2O_2 concentrations, the interferant oxidation current increases rather than decreases at high H_2O_2 concentrations. As a consequence, and as seen in Figure 3, when the electrode is HRP-limited, injection of a large excess of H_2O_2 causes first a drop, then an increase, in the interference current. This increase results from the buildup of the H_2O_2 concentration to an inhibiting level after the ascorbate has been depleted.

The extent of the ascorbate elimination is mass transfer dependent in HRP-loading limited ($15 U/cm^{-2}$) electrodes (Table II). Increase in electrode rotation rate now results in an increase in the ascorbate oxidation current both in the absence and in the presence of H_2O_2. At higher rotation rates the electrode, limited by its enzyme loading, copes only with a decreasing fraction of the arriving ascorbate.

As seen in Figures 2 and 3 the ascorbate electrooxidation current appears noisy, even though our system is not. The noise suggests that the electrooxidation of ascorbate

might be chaotic or oscillatory[35]. Elimination of ascorbate by peroxide causes disappearance of the "noise".

Figure 4 shows a family of "titration" curves of ascorbate by H_2O_2. Elimination of interference by increasing concentrations of ascorbate requires increasing concentrations of peroxide. Because complete elimination of ascorbate at concentrations exceeding 8mM requires H_2O_2 concentrations above HRP inhibiting levels. it cannot be accomplished using HRP, though non-inhibited peroxidases are likely to eliminate concentrated interferants. Interference by ascorbate concentrations below 8mM is completely eliminated with HRP films. Because only the interferant/H_2O_2 ratio in the solution is known. and the critical interferant/H_2O_2 ratio in the film is not. we observe variations between electrodes having different mass transfer characteristics.

In order to determine the upper limit of the interferant elimination capacity of HRP films, an electrode with a high loading of enzyme was prepared. In this electrode addition of H_2O_2 decreased the ascorbate electrooxidation current by a factor of >2500.

Processes for HRP Immobilization in Films.

With the objective of simultaneously optimizing specific activity, permeability and mechanical ruggedness, while not harming the underlying glucose-sensing layer. we evaluated several processes for making HRP films. After a preliminary survey we narrowed the immobilization processes to (a) crosslinking with glutaraldehyde: (b) periodate oxidation of HRP oligosaccharides to aldehydes and coupling with polyacrylamide hydrazide:[36,37] and (c) self-crosslinking of the periodate oxidized HRP through formation of Schiff bases between the HRP-oligosaccharide-derived aldehydes and HRP-amines, such as lysyl amines. The second and third method yielded smoother and better films. Glutaraldehyde crosslinking. though widely practiced. causes a greater loss in enzyme activity than periodate oxidation to HRP polyaldehyde. followed by formation of

either polyacrylhydrazones or lysyl-Schiff bases. The films made of the HRP polyaldehyde adhered well to both uncoated and variously polymer coated electrodes and, unlike the glutaraldehyde films, did not crack during their drying and curing. The periodate process yielded the best films for HRP type VI; while with the glutaraldehyde process, the best results were obtained for HRP type I. The difference might be associated with differences in the amount or distribution of oligosaccharides in the various HRPs.

Interaction between the glucose-sensing and the interferant eliminating layers.

Figure 5 shows cyclic voltammograms for an electrode modified with a glucose-sensing film consisting of a three-dimensional redox epoxy gel, formed of POs-EA and glucose oxidase upon crosslinking with PEGDE.[34] It is seen that in the 0.4-0.5V (SCE) plateau and under the conditions of the experiment the current increment associated with 5mM glucose can be exceeded by that associated with 0.5mM ascorbate. This is also the case for acetaminophen and for urate. Evidently electrooxidation of the three interferants takes place not only at the carbon surface, but also in the electron-relaying glucose sensing layer. While the rate of electrooxidation of glucose can be limited by the rate of electron transfer between enzyme $FADH_2$ centers and that a fraction of the osmium centers that are in their proximity, electrooxidation of the interferants proceeds at any of the osmium centers, wherefore its rate can be high. This, combined with the higher concentration of anionic interferants in the polycationic polymer[38] aggravates the problem of interference. Fortunately the interference is completely eliminated in HRP-film coated electrodes, as will be seen.

When an arbitrary HRP film is coated on the glucose sensing electrode and the current associated with electrooxidation of ascorbate is measured (Figure 6), addition of

H_2O_2 not only suppresses the anodic ascorbate current, but reverses it, producing a cathodic current. Thus, interference through electrooxidation of ascorbate is now replaced by interference through electroreduction of H_2O_2. The H_2O_2 electroreduction current is seen also in the absence of ascorbate, but not in the absence of either the HRP or a redox-polymer network. Furthermore, HRP-film mediated electroreduction of H_2O_2 takes place whether the HRP and the redox epoxy films are mixed or separated, as long as the two contact each other. Even loosely adsorbed HRP on the redox polymer produces a cathodic H_2O_2 electroreduction current. Dipping of the POs-EA redox epoxy -modified electrode in an HRP solution for a few minutes and rinsing also produces an electrode showing a cathodic current. We thus conclude that the HRP molecule (MW 40,000) can permeate into pores of the redox epoxy network when these are sufficiently large.

Permeation of the HRP into POs-EA is suggested by the following comparative experiments. Two five-electrode batches of thick-layer POs-EA electrodes were prepared a) with HRP and b) with bovine serum albumin (BSA). The BSA electrodes were then dipped in an HRP containing solution for 3 hours. The peroxide response of the two batches became similar. In a control experiment two thick layer POs-EA electrodes were prepared a) with GO and b) with BSA. The BSA electrode was dipped in a GO containing solution for 24 hours. The glucose response of the BSA electrode was still an order of magnitude smaller than that of the GO electrode. The difference between the first and second set of experiments is explained by the difference in the size of HRP and GO. GO being a larger enzyme (MW 160,000) permeates less into the POs-EA network when the BSA electrode is dipped in the GO containing solution. It is adsorbed only on the outer surface of the POs-EA layer and is thus poorly "wired". HRP, being a relatively small enzyme (MW 40,000), permeates into the POs-EA network when the BSA electrode is dipped in a HRP-containing solution. The enzyme penetrates into the POs-EA film and is well "wired". The permeability of POs-EA films to HRP was also seen in experiments

where the usual diepoxide crosslinker PEGDGE. with nine ethylene glycol units, was replaced by a short analog, MEGDGE. *mono*ethyleneglycol diglycidyl ether. Similar electrodes were prepared with POs-EA, BSA and either MEGDGE or PEGDGE. The electrode: were cured, exposed to HRP and then poised at 400 mV in the presence of H_2O_2. The cathodic H_2O_2 reduction current of the electrode with the long crosslinker was five times higher than that of the short crosslinker. These results suggest that the more tightly crosslinked redox gel obtained with the short crosslinker is one less permeable to the HRP.[11]

Figure 7 shows cyclic voltammograms of the POs-EA redox epoxy modified electrode coated with an electrically contacting HRP film in the absence of hydrogen peroxide (a) and in its presence (b). H_2O_2 electroreduction commences at +0.45V (SCE) and reaches its plateau already at a potential as positive as +0.3V (SCE). This obviously electrocatalytic reduction of H_2O_2 involves multiple steps, summarized by reactions 2 and 3.

$$2Os^{2+} + H_2O_2 + 2H^+ \xrightarrow{\text{HRP}} 2Os^{3+} + 2H_2O \tag{2}$$

$$Os^{3+} + e^- \longrightarrow Os^{2+} \tag{3}$$
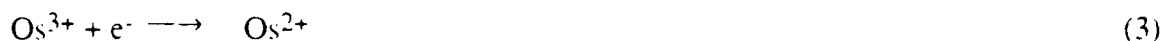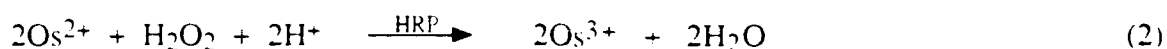
Diffusional redox-couple mediation of the reduction of hydrogen peroxide by peroxidases is well established and forms the basis of peroxide reducing electrodes.[39,40] Here the redox polymer network catalyzed electroreduction interferes with the assay glucose, lowering the glucose electrooxidation current.

We overcome the electroreduction of H_2O_2 by either electrically insulating the HRP film from the GO-redox polymer film or by poising the electrode (and thus the electrically connected network of the glucose sensing film) at a sufficiently oxidizing

potential, where H_2O_2 is no longer catalytically reduced. This potential is near 0.5V(SCE) (Figure 7). Because 0.5V (SCE) is also near the threshold for electrooxidation of H_2O_2, electrical insulation by a barrier film is preferred for the most accurate glucose assays of biological fluids. In less demanding assays, poising the electrodes at 0.5V (SCE) is, however, simple and adequate.

An electrode with an insulating barrier layer is shown schematically in Figure 8. Ideally, the insulating barrier layer will fully cover the surface of the sensing redox matrix, but will not be thick enough to substantially slow glucose transport to the sensing layer. Insulating films made of cellulose acetate, Nafion and polypyrrole formed respectively by spin coating, casting, or electropolymerization were tested. These did not perform well: the H_2O_2 reduction current was only partially suppressed and the glucose current was considerably diminished. A good barrier layer was, however, prepared by cross-linking polyvinyl imidazole with ethylene glycol diglycidyl ether. The resulting gel effectively prevented electrical contact between the sensing layer and did not excessively reduce the catalytic glucose electrooxidation current.

Another method of avoiding the electrical contact between the glucose sensing layer and HRP film involves making the redox polymer containing sensing redox polymer containing layer impermeable to HRP. When a POs-EA/GO/PEGDGE electrode was additionally crosslinked with glutaraldehyde, the electrode, when poised at 0.4V (SCE), did not show a cathodic current in the presence of H_2O_2 after exposure to HRP. Evidently direct electrical communication between the redox polymer and HRP requires proximity of segments of the two. Exposure of the Os redox polymer to glutaraldehyde crosslinks the matrix sufficiently to prevent diffusion of the 40 kilodalton HRP into the 3-dimensional epoxy matrix.[29]

Interferant - insensitive glucose electrodes.

The just described results show the feasibility of three types of interferant eliminating electrodes that do not electroreduce H_2O_2: (a) Two-layer electrodes poised at 0.4V (SCE) with the sensing layer crosslinked and made HRP-impermeable by glutaraldehyde; (b) Two-layer electrodes poised at 0.5V (SCE); and (c) Three-layer electrodes with a PVI based electrically insulating barrier. The glucose response of a two-layer electrode of the second type is shown in Figure 9. In the presence of H_2O_2 no ascorbate related current is seen while the risetime is fast. Figure 10 shows results obtained with an electrode of the first type, having a glutaraldehyde treated sensing layer, coated with an HRP film. In the presence of H_2O_2 only glucose is electrooxidized and the measured current is dependent only on its concentration. In the absence of H_2O_2 both glucose and ascorbate are electrooxidized and the measured current reflects the combined electrooxidation of glucose and ascorbate. Similar results were obtained for urate and acetaminophen.

Figure 11a shows the glucose response of a two-layer electrode in the presence of all three interferants (ascorbate, urate and acetaminophen) at their physiological levels. Comparison of this response curve with that obtained in the absence of interferants (Figure 11b) shows that the electrode is totally insensitive to these common electrooxidizable interferants. The presence or absence of H_2O_2 does not affect the glucose current itself proving that glucose is not oxidized at an appreciable rate by H_2O_2 in the HRP film. Figure 11c shows the glucose response of the Fig. 11a electrode before it was coated with the peroxidase layer. The HRP layer slows the diffusion and decreases the current response. As a result, the apparent Michaelis constant[34] of the electrode (K_m) increases from 18 mM for the uncoated electrode to 25 mM for the coated one. Optimization of the thickness of the HRP layer thus involves a compromise between completeness of interferant elimination, requiring thicker films, and fast response, requiring thinner ones.

Stability.

The interferant elimination capability of HRP coated electrodes remains unchanged for two months of repeated use when the electrode is stored in air at room temperature between the measurements. The stability is attributed to the initial excess HRP activity of the films. Because the initial activity is higher than essential for eliminating the interferants, the HRP films retain adequate activity even after their partial deactivation.

Built-In Sources of H_2O_2.

While addition of H_2O_2 is feasible in clinical and laboratory analyzers, it is inappropriate for either disposable sensors or in sensors operating *in vivo*. These require a built-in source of peroxide.

Hydrogen peroxide is the oxidation product of oxidase-catalyzed reactions of molecular oxygen and substrates, e.g. lactate. Thus, lactate oxidase (LOD) mediates the oxidation of lactate by oxygen, the products being pyruvate and H_2O_2 (eq. 4). The *in situ* generated H_2O_2 is adequate for eliminating interferants. At the concentration of lactate in physiological fluids (0.6-1.8 mM)[41] enough H_2O_2 is produced to oxidize all the interferants at their physiological concentrations.

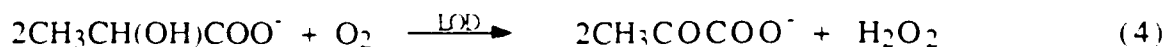$$2CH_3CH(OH)COO^- + O_2 \xrightarrow{LOD} 2CH_3COCOO^- + H_2O_2 \qquad (4)$$

Figure 12 shows the results obtained with an electrode similar to the one used in the experiment of Figure 10, but having LOD co-immobilized with HRP in its outer film. Successive additions of lactate ("L") eliminate equivalent amounts of ascorbate ("A"). When this electrode was used in a solution containing physiological levels of the interferants, (0.1mM ascorbate, 0.5mM urate, 0.1mM acetaminophen), as well as

physiological levels of lactate (0.5mM) and glucose, the measured current was proportional to the glucose concentration only, i.e. was that measured in the absence of any of the interferants (Figure 13).

The simultaneous presence of several enzymes and of different reaction pathways employing common reagents creates - at least in principle - the possibility of new interfering or competing reactions. Lactate oxidase has been wired by the osmium-based redox polymer and its films used in determining lactate concentration.[42] Thus, the direct electrooxidation of lactate must be prevented. i.e. the barrier layer must also isolate the LOD from the electron relays. The earlier discussed physical isolation methods are adequate for this purpose. No lactate associated currents are observed in electrodes that do not electroreduce H_2O_2. A secondary cause of interference can be the change in oxygen partial pressure, through the oxygen being consumed by lactate (eq. 4) and the Os(III) centers and O_2 competing for glucose oxidase- $FADH_2$ electrons (eq. 5). We find, however, that the redox epoxy wired glucose oxidase electrode is insensitive to changes in oxygen partial pressure or lactate concentration. Raising the lactate concentration to 1.5 mM changes the electrocatalytic oxidation current of glucose (5.0 mM) by less than 2%.

$$GO\text{-}FADH_2 \ + \ O_2 \quad \longrightarrow \quad GO\text{-}FADH \ + \ H_2O_2 \tag{5}$$

As seen in Figure 14 the initially generated H_2O_2 is not decomposed by external catalase, a common enzyme in physiological samples. The figure shows that the LOD - HRP film coated electrode rejects ascorbate either upon injecting H_2O_2 (a) or lactate (b). But the subsequent addition of catalase (100 units) affects the ascorbate rejection only when H_2O_2 is externally added (a), not when it is internally generated (b). When ascorbate is rejected by adding lactate, the addition of catalase does not cause a change in the current. However, when ascorbate is eliminated by adding H_2O_2, addition of catalase results in a

gradual recovery of the oxidation current, until it eventually stabilizes at the level observed prior to the addition of H_2O_2. The slow current increase results from the gradual decrease in H_2O_2 concentration in the bulk of the solution (eq. 6). With the HRP-LOD film the oxidation of interferants is not impaired by external catalase, because the H_2O_2 is generated and utilized in the bienzyme layer itself.

$$2 H_2O_2 \quad \underline{\quad CATALASE \quad} \quad O_2 + 2H_2O \qquad (6)$$
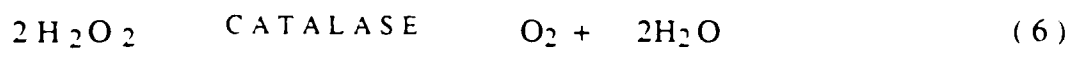
CONCLUSIONS

Horseradish peroxidase (HRP) based interferant eliminating layers provide practically absolute selectivity to amperometric glucose sensors. In the presence of the enzyme, H_2O_2 preoxidizes all the common electrooxidizable interferants. Figure 15 summarizes the relevant reactions in multilayered electrodes discussed in this work. In the glucose electrode, electrons from the substrate are transferred to the glucose oxidase (GOX) and are relayed, via non-diffusing osmium redox centers, to the electrode. The analyzed solution contains electrooxidizable ascorbate, urate and acetaminophen. These species may be oxidized directly at the electrode surface or through electron relays of the enzyme-wiring redox polymer network. Their electrooxidation increases the anodic current. To prevent their interference, the sensing layer is coated with a layer of immobilized peroxidase, that catalyzes the oxidation of the interferants by H_2O_2. Such oxidation destroys the interferants prior to their reaching the wired glucose oxidase, where they would otherwise be electrooxidized. The H_2O_2 can be either externally supplied or produced in situ through an oxidase catalyzed O_2 oxidation of a second solute such as lactate. Electrical contact between the HRP and the osmium redox centers must and can readily be prevented, for example by inserting an electrically insulating barrier film

between the glucose sensing layer and the HRP layer by further crosslinking the sensing layer, or by operating the electrode at 0.5V (SCE).

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TABLE I- Effect of HRP loading on the effectiveness of elimination of ascorbate

HRP (U.cm^{-2})	I_A (μA)	I_{AP}(μA)	% elimination
0	3.48	3.48	0
15	1.67	1.39	16
30	1.22	0.78	23
45	0.89	0.47	44
60	0.74	0.34	52
90	0.66	0.16	74
150	0.55	0.08	86
180	0.48	0.00	100

I_A - current measured for 0.1 mM ascorbate. I_{AP} - current measured for 0.1 mM ascorbate and 0.2 mM H_2O_2.

TABLE II Effect of rotation rate on the effectiveness of elimination of ascorbate.

ω (RPM)	I_A(μA)	I_{AP}(μA)	% elimination
0	0.27	0.09	68
10	0.52	0.32	39
20	0.65	0.42	36
50	0.86	0.60	30
70	0.95	0.67	29
100	1.04	0.76	27
200	1.23	0.94	23
500	1.47	1.20	18
700	1.56	1.30	17
1000	1.67	1.39	16
2000	1.89	1.59	15
4000	1.98	1.73	13

I_A - current measured for 0.1 mM ascorbate. I_{AP} - current measured for 0.1 mM ascorbate and 0.2 mM H_2O_2.

FIGURE CAPTIONS

Figure 1

Cyclic voltammograms of an HRP coated glassy carbon electrode in 1.0mM interferant containing solution in the absence of H_2O_2 (a) acetaminophen; (b) urate; (c) ascorbate ; (d) in the presence of 1.0mM H_2O_2 and urate. Scan rate 10 mV/s.

Figure 2

Elimination of the ascorbate electrooxidation current in an HRP - coated glassy carbon electrode. "A" denotes injection of ascorbate, each injection increasing its concentration by 0.1 mM; H_2O_2 denotes injection of hydrogen peroxide, each injection increasing its concentration by 0.2 mM. Glassy carbon electrode 0.4 V (SCE).

Figure 3

Partial elimination of ascorbate with a lightly loaded (60 U/cm^2) HRP electrode. "A" denotes injection of ascorbate (0.1 mM final concentration); "H_2O_2" denotes injection of H_2O_2 (0.2 mM final concentration). Glassy carbon electrode. 0.4V (SCE).

Figure 4

Titration of ascorbate by hydrogen peroxide using a HRP coated electrode (250 U/cm^2). Initial ascorbate concentrations : a) 0.1 mM; b) 0.2 mM; c) 0.3 mM; d) 0.5 mM; e) 1.0 mM; f) 2.0 mM; g) 4.0 mM; h) 8.0 mM; i) 20.0 mM; j) 40.0 mM.

Figure 5

Cyclic voltammograms (a) in phosphate buffer; (b) with added 5.0 mM glucose; (c) with 5.0 mM glucose and 0.5 mM ascorbate. POs-EA/GO electrode; scan rate 2 mV/s.

Figure 6

Current reversal upon adding H_2O_2 in an electrode having a POs-EA/GO sensing layer coated with an HRP layer. "A" injection of ascorbate (0.1 mM final concentration); "H_2O_2" injection of drogen peroxide (0.2 mM final concentration). 0.4V (SCE).

Figure 7

Reduction of hydrogen peroxide on a two layers electrode having a POs-EA/GO sensing layer coated with an HRP layer. Cyclic voltammograms (a) in a phosphate buffer solution; (b) with added H_2O_2 (0.1 mM). Scan rate 2 mV/s.

Figure 8

Three layers electrode for interferant elimination. A barrier layer electrically insulates the glucose sensing layer from the interferant eliminating layer.

Figure 9

Elimination of interferants in a two layers electrode. "A" injection of ascorbate (0.1 mM final concentration); "P" injection of hydrogen peroxide (0.2 mM final concentration); "G" injection of glucose increasing its concentration in 1.0 mM steps. 0.5V (SCE).

Figure 10

Effect of adding hydrogen peroxide on the current of a three layers electrode: additions of glucose 5mM final concentration "G" and ascorbate 0.1mM final concentration "A" (a) in the presence and (b) in the absence of H_2O_2 ("P"). 0.4V (SCE).

Figure 11

Dependence of the electrocatalytic glucose oxidation current density on glucose concentration: (a) bilayer electrode in the presence of hydrogen peroxide (0.5 mM) and physiological levels of interferants: [ascorbate]=0.1 mM, [urate]=0.5 mM, [acetaminophen]=0.1 mM; (b) bilayer electrode, no interferants; (c) uncoated electrode, no interferants. The insert shows the dependence for a wider concentration range. 0.5V (SCE).

Figure 12

Elimination of ascorbate through *in situ* production of lactate in a three layer electrode having an interferant eliminating layer with both HRP and LOD. "A" denotes an injection of ascorbate to increase its concentration by 0.1 mM steps; "L" denotes an injection of lactate to increase its concentration by 0.1 mM. 0.4V (SCE).

Figure 13

Dependence of the electrocatalytic oxidation current density on glucose concentration in an HRP/LOD - coated electrode : (a) (□) in the presence of lactate (0.5 mM) and physiological levels of three interferants: [ascorbate] = 0.1 mM, [urate] = 0.5 mM, [acetaminophen] = 0.1 mM; (b) (•) in the absence of interferants.

Figure 14

Effect of catalase on the elimination of ascorbate by an interferant eliminating layer containing HRP co-immobilized with LOD, (a) externally added H_2O_2 (b) internally generated H_2O_2 through lactate oxidase catalyzed oxidation of lactate by O_2.

Figure 15

Scheme of reactions in the multilayered enzyme electrode.