Anal. Chem. 1994, *66*, 1183–1188

Elimination of the Acetaminophen Interference in an Implantable Glucose Sensor

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Acetaminophen has been one of the most serious electrochemical interferences to oxidase-based amperometric biosensors that measure H_2O_2. A study was carried out to investigate various polymer materials for their selectivity as the sensor inner membrane. A composite membrane of cellulose acetate and Nafion was found to eliminate acetaminophen and other electrochemical interferences effectively while at the same time maintaining reasonable diffusivity for hydrogen peroxide. The excellent in vivo performance of the sensor was attributed not only to significantly reduced steady-state sensitivity to acetaminophen but also to very slow acetaminophen response. These features, combined with rapid acetaminophen clearance pharmacokinetics, led to the decreased response as demonstrated in the rat.

Hydrogen oxide-based amperometric sensors have found wide application in HPLC detection, flow injection analysis, and immunoassays because they utilize direct measurement of H_2O_2, the product of a common enzymatic reaction, and provide convenient, sensitive methods for numerous analytes. This approach also has wide application in the development of implantable oxidase-based biosensors because it facilitates simple sensor configuration and miniaturization. The detection of H_2O_2, however, has an inherent problem. The amperometric measurement of H_2O_2 requires an applied potential of 600–700 mV (vs Ag/AgCl). Many endogenous reducing species such as ascorbate and urate and some drugs such as acetaminophen (paracetamol) will also be oxidized at the electrode causing an error.

The seriousness of the interference depends on the application requirements. An HPLC detector, for example, is less likely to suffer from major interferences because components are separated prior to the electrochemical detection. An implantable glucose sensor, on the other hand, is prone to all potential effects from such species since physical separation of the analyte, glucose, and its complex matrix is impossible. For an enzyme-based glucose sensor, its biospecificity is defined by the enzyme while the electrochemical selectivity between H_2O_2 and other electroactive interferences has to be dealt with in a way which is compatible with sensor function.

When evaluating the level of interference to an implanted biosensor, an unchanged background imposes no serious problem as long as the sensor can be calibrated in vivo. In such a case the constant background is attributed to I_0 and can be corrected.[1] In a needle-type miniature glucose sensor developed in our laboratory, the major contribution of the endogenous interferences can be eliminated by an inner cellulose acetate membrane.[2] In vivo measurements in rats and dogs showed no fluctuation in background current.[3,4] The interference from acetaminophen, however, cannot be overlooked. Early studies on a similar sensor have emphasized this problem.[5] It could cause up to 40% bias in the sensor output at 5 mM glucose level in vitro[2] and as much as 80–200% when the sensor is implanted in rat subcutaneous tissue (this study). To develop a reliable glucose sensor of clinical interest, it is important that the device be free from additional restrictions such as the ban on using this ordinary analgesic. Acetaminophen, in fact, is the most serious electrochemical interference that has been so far encountered in the course of sensor development. It is therefore chosen as the representative of electrochemical interferents in this study, and the sensor specificity is evaluated against this interferant.

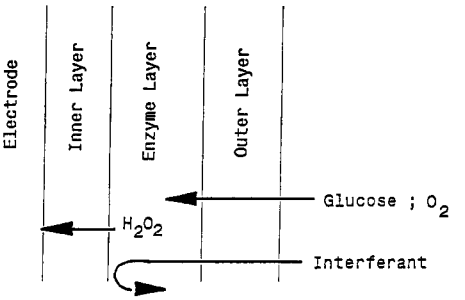

Figure 1. Schematic of sensor membranes.

Figure 1 shows the schematic of a typical glucose sensor membrane configuration in which at least three layers on the electrode surface are necessary. The inner layer serves as the support for enzyme immobilization and also as the selective membrane for H_2O_2. The second layer is the enzyme layer where glucose is specifically converted to the electroactive species, H_2O_2. The third and outermost layer must have the property of allowing maximum passage of oxygen and retarded passage of glucose to ensure an overall glucose diffusion controlled process. This membrane must also be biocompatible for in vivo applications.

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There are generally three approaches to eliminate interferences from an electrochemical biosensor. The first is to incorporate an active component such as an enzyme, redox reagent, or ligand in the outer layer of the sensor to destroy the in-coming interferants before they reach the electrode.[6] The second is to use a passive membrane that possesses a pore size and chemical selectivity that retard the interferant. The third is to incorporate a "mediator" to shuttle electrons between the reduced enzyme and the electrode such that the required applied potential is reduced. The interferences may not be electroactive at low potentials but may still be electrocatalytically oxidized. The first method has seen some in vitro application but so far no in vivo application. The third approach, although often effective for a short periods of time, frequently results in long-term instability. The mediator may also not be competitive with environmental oxygen for oxidation of the reduced enzyme. A reliable mediator for implantable sensors is not yet available. The second approach, being somewhat simpler, is the objective of this study.

For the outer membrane, its major task is to control glucose flux, impose a minimum barrier to oxygen, and generate minimum tissue response. It is difficult in practice to find a polymer that allows reasonable glucose diffusion but which at the same time blocks all the other interferants. Thus a combination of membranes with different characteristics must be employed.

To effectively evaluate the impact of acetaminophen on an implanted sensor, the therapeutic level and the pharmacokinetics of the drug in the tissue has to be established. Previous studies have confirmed that oral or intravenous administration of acetaminophen in normal human subjects produces maximum plasma concentration in 20–50 min, and the peak plasma concentration rests in the range of 0.1–0.2 mM when the maximum therapeutic dose (1000 mg) is introduced.[7-9] An obvious difference between oral intake and intravenous or intraperitoneal injection is that the peak appears at longer times for oral administration, with consequently different pharmacokinetics. Experiments were performed to establish the protocols by intraperitoneal infusion with adequate timing which simulate oral ingestion of acetaminophen. A flow injection analysis measurement of plasma acetaminophen is also introduced for *in situ* monitoring.

EXPERIMENTAL SECTION

Reagents. Cellulose acetate (CA) (39.8 acetyl content) and Nafion (5% solution in low aliphatic alcohols) were purchased from Aldrich. Polyurethane (PU) (SG 85A) was from Thermedics Inc. (Woburn, MA). The Eastman-Kodak AQ 55-D polymer (an anionic amorphous polyester containing sulfonate groups and having a glass transition temperature of 55 °C) was a gift from Prof. R. N. Adams (University of Kansas). Ascorbic acid, acetaminophen, 1,3-diaminobenzene (DAB), and 1,3-dihydroxybenzene (resorcinol) were all purchased from Aldrich as analytical grade.

All the in vitro measurements were conducted in pH 7.4 phosphate buffer of 0.1 M containing 0.15 M NaCl and 0.1 g/L NaN_3 prepared from deionized distilled water. A 5% CA solution was prepared by dissolving CA in 2:1 acetone–ethanol; 5% PU was made in 98% tetrahydrofuran (THF) and 2% dimethylformamide (DMF) with stirring over 15 h at room temperature. The 28% AQ 55-D solution was diluted to 5% with 1:1 acetone–cyclohexane for use. The deposition of electropolymerized DAB/resorcinol was performed according to Geise et al.[10] All electrodes used were prepared from the Teflon-coated Pt–Ir wire obtained from Medwire Corp. (Mount Vernon, NY) with a wire diameter of 0.17 mm and overall diameter of 0.25 mm. Details of the sensor preparation have been reported elsewhere.[2] The composite membrane as the inner layer of the sensor was prepared by alternative deposition of CA and Nafion on the bare Pt of the sensor cavity. The CA was deposited by dipping the sensor in the 5% CA solution followed by drying at room temperature for 5 min. This step was then repeated. A three-turn wire loop (diameter 3 mm) was constructed from 0.7-mm-o.d. Cu wire. The inside of the loop was filled with the 5% Nafion solution. The sensor cavity was then passed through the loop horizontally first in one direction and then the other. The sensor was dried at room temperature for 10 min, and this step was repeated two more times. The above cycle of CA and Nafion deposition was repeated two additional times, and the sensor was then stored in clean environment at room temperature before enzyme immobilization.

Procedure. The in vitro evaluation of the sensor characteristics was carried out with a single-compartment cell immersed in a 37 °C water bath (Fisher, Isotemp Model 900). A chloridized Ag coil served as the reference/counter electrode. Glucose (0.5 M), 0.05 M ascorbic acid, acetaminophen, and hydrogen peroxide were injected into the cell with a micropipet, and the sensors were poised at 600-mV constant potential while the current was measured with BAS LC 4A amperometric detectors (BioAnalytical Systems, Inc., West Lafayette, IN) and recorded with Kipp and Zonen BD40 chart recorders.

The measurement of plasma acetaminophen was performed by flow injection analysis. An EG&G Princeton Applied Research Model 400 EC detector, an ISCO Model 314 syringe pump (Lincoln, NE), and a Rheodyne manual injection valve Model 7125 (Cotati, CA) with a 20-μL sample loop were configured in a flow system. The detector cell assembly was the same as the one used for lactate FIA detection[11] except that a wire electrode coated with CA and PU was used as the acetaminophen-sensing electrode polarized at 700 mV vs Ag/AgCl. An injection volume of 60 μL was found necessary to obtain reproducible results when the flow rate was 0.15 mL/min.

The rat was anesthetized by halothane inhalation. A gas flow of oxygen and air mixture was regulated by flow meters (Cole Parmer, Chicago, IL), and a shunt led a controlled part of the gas flow to bubble through a halothane tube. The ratio of oxygen, air, and halothane could all be freely regulated to control the degree of anesthesia at various stages of the experiment. The ratio of oxygen to air in the gas mixture was

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about 1:2 at the beginning of the anesthesia and was gradually increased to 1:1 for prolonged experiment. The flow of halothane must be also regulated accordingly (usually decreased) to maintain normal breathing rate. The sensors were implanted in the subcutaneous tissue on the back of the rat and polarized with the amperometric detectors. After the sensor output stabilized, about 1.5 mL of blood was obtained from the tail vein by cutting the tip of the tail and was then heparinized and centrifuged. The plasma was divided into samples, one of which was spiked with 0.2 mM acetaminophen while the other served as a blank. The detector electrode was immediately calibrated with the standard acetaminophen-containing plasma (acetaminophen adsorbs to proteins if long standing is allowed), and the background was obtained from the corresponding plasma blank. The rat was then infused with acetaminophen saline solution, and blood was sampled at 5-min intervals followed by measurement of the plasma acetaminophen concentration.

The detector sensitivity was recalibrated again with the same procedure after 3 h when the plasma acetaminophen had decreased to an undetectable level.

The protocol for the in vivo experiments with rats has been described elsewhere[3,12] and has been approved by the Institutional Animal Care and Use Committee.

RESULTS AND DISCUSSION

Study of Sensor Inner Membrane Characteristics. The glucose sensors developed in our laboratory have an average sensitivity of 2.3 ± 1.1 nA/mM glucose in vitro. The response of 0.2 mM acetaminophen (the maximum plasma therapeutic level) at a bare Pt–Ir electrode of the same surface area is about 1800 nA at a 600-mV applied potential (vs Ag/AgCl). To reduce the interference of acetaminophen to less than 10% at the 5 mM glucose level, 99.9% elimination is necessary. The response of an unmodified sensor to 0.2 mM acetaminophen is in the range of 20–40 nA (compared to 1800 nA). This means that the unmodified cellulose acetate, enzyme, and polyurethane layers have a combined effect of 98–99% discrimination against acetaminophen. However, when the total response to 5 mM glucose is only 10–20 nA, this 99% reduction is far from sufficient.

To effectively evaluate the membranes selectivity, the response to H_2O_2, ascorbic acid, and acetaminophen on bare and membrane-coated wire electrodes was measured. Figure 2 shows the response patterns of the three species on electrodes coated with cellulose acetate (CA), AQ 55-D, Nafion, and electrodeposited DAB/resorcinol.[10] An instantaneous current increase upon addition of an electroactive species indicates that the given membrane does not have a discriminating effect on the test species. For example, CA is effective with ascorbate but not with acetaminophen and peroxide. AQ 55-D is permeable to all three species while the electropolymerized DAB/resorcinol membrane is more effective on acetaminophen. Nafion, as the H_2O_2-selective membrane, has the most desired feature, eliminating both ascorbate and acetaminophen to a substantial extent.

The sensitivity of the membrane-covered electrodes was monitored for a period of 40 days. The electrodes were kept in pH 7.4 buffer at room temperature during this period and measured for their response to ascorbate, acetaminophen, and hydrogen peroxide daily. The characteristics of CA, AQ 55-D, and Nafion are essentially unchanged over this period, indicating that these membranes do not have time-dependent features. The electropolymerized DAB/resorcinol, however, starts to show significant response to acetaminophen 2 or 3 days after preparation, and the response increases gradually to reach a maximum in about 20 days when the response stabilizes at 15% of that on a bare electrode. This corresponds, in fact, to a complete loss of its acetaminophen-discriminating characteristics because a majority of the electrode-modifying membranes can reduce the response of an active species 5–20% simply by providing a diffusional barrier at the electrode surface (see Table 1 for relevant numbers). Although reports have shown that significant elimination could be achieved in flow injection analysis,[10] it is nevertheless not applicable to in vivo sensors where comparison between a coated and a bare electrode is often misleading. The level of interference to a glucose sensor of 2–3 nA/mM sensitivity is accordingly intolerable. A thorough study was conducted by varying the conditions of electropolymerization such as concentration, the ratio of the two components, electrolysis mode (potential scan, constant potential, and constant current), temperature, and total charge control. None in our hands led to better stability

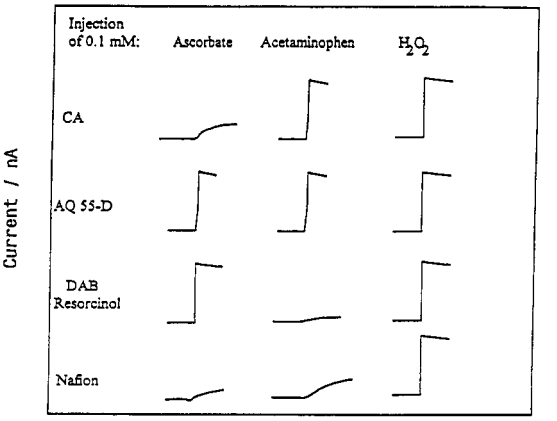

Figure 2. Response patterns of three electroactive species on membrane-coated electrodes.

Table 1. Effect of Multilayer Coating of Different Polymers[a]

membrane	no. of coatings	sensitivity, nA/0.1 mM ascorbate	acetaminophen	H_2O_2
none	0	400	900	350
CA	1	200	330	340
	2	26	130	210
	3	5	110	90
	4	0	87	46
AQ55-D	1	190	320	330
	2	200	360	330
Nafion	1	10	97	260
	2	10	70	210
	3	9	63	190
	4	7	42	120
DAB/resorcinol	day 1	190	0	300
	day 10	190	35	300
	day 20	190	120	300
CA/Nafion	1	0	7	65
	2	0	5	49
	3	0	2	35

[a] Current values are the average of three replications.

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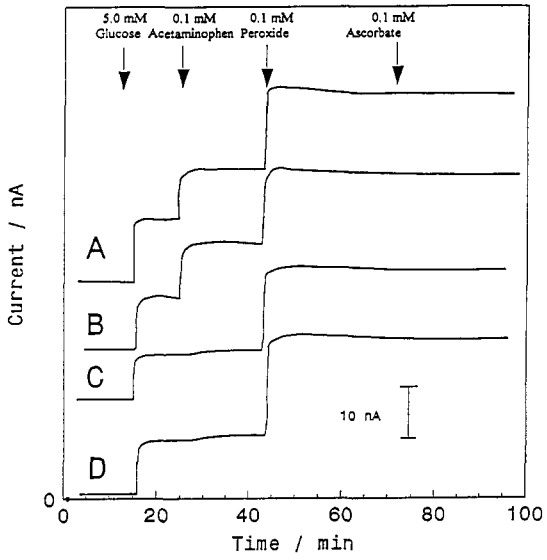

Figure 3. In vitro test of sensor membrane selectivity on electrochemical interference.

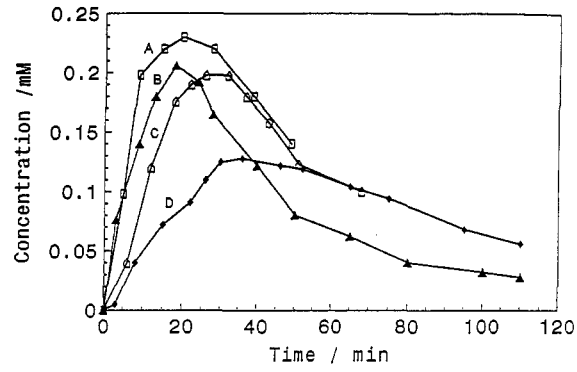

Figure 4. Rat plasma acetaminophen pharmacokinetics: (a) quick injection, 37.1 mg/kg; (B) quick injection, 22.7 mg/kg; (C) 15-min infusion, 26.6 mg/kg; (D) 25-min infusion, 18.1 mg/kg.

than that shown in Table 1. This method was therefore discarded.

It is demonstrated in Figure 2 that chemical species have different response times on different membrane-coated electrodes. An instantaneous response pattern (e.g. acetaminophen on CA) means that the electrode activity cannot be totally eliminated with this membrane by merely increasing its thickness. A slow response with reduced magnitude, on the other hand, indicates that an increase in the thickness can further block this species effectively.

Table 1 shows the effectiveness of membrane thickness (assuming that the thickness is proportional to the number of layers) on inhibition of the three species. The increase in the thickness was varied by consecutive deposition of polymer layers on the wire electrodes. The effect of thickness on the electropolymerized DAB was a separate study, and no difference was observed (as mentioned above). Since the AQ 55-D polymer did not show any promising properties in this application, it was thereafter rejected. The remaining two candidates are cellulose acetate and Nafion. The first strategy is to increase number of layers to obtain the goal. From the results of Table 1 it appears that this strategy works, but not completely. Up to three coatings of CA and Nafion do not result in the desired discrimination against acetaminophen. Further increase up to six or seven coatings does not give proportional improvement with thickness. Considering the increase in response time due to the membrane thickness, this approach became counterproductive. A favorable effect was achieved when a combined film of cellulose acetate and Nafion was coated (shown as the last row in Table 1). One layer of the combined film eliminates ascorbate completely, and three layers eliminate acetaminophen to below the 5-nA level. This membrane, as the inner layer of the glucose sensor, will also be able to provide the required characteristics for hydrogen peroxide detection. Figure 3 shows the response patterns of four glucose sensors. The measurement was carried out in pH 7.4 phosphate buffer. Glucose (5.0 mM), acetaminophen (0.1 mM), H_2O_2 (0.1 mM), and ascorbic acid (0.1 mM) were injected after the baseline was stabilized. All the sensors have the configuration of Figure 1 except that sensors A and B have only a cellulose acetate inner membrane while sensors C and D have a combined inner membrane of cellulose acetate and Nafion. The improvement is clearly demonstrated.

Study of in Vivo Characteristics. There are numerous documented studies of acetaminophen pharmacokinetics in humans and rats.[7-9,13,14] It is generally observed that plasma concentration is approximated by a two-compartment model. The duration of the plasma peak is related to the doses administered. Higher doses yield a broader peak, and the decrease begins at a later time, i.e. the peak concentration is preserved for a longer period.

To establish a kinetic model in rats in order to simulate the timing and concentration in humans, intraperitoneal infusion was used to administer a 0.05 mM acetaminophen solution. The aim was to simulate the oral intake of the analgesic. Since oral administration in human results in slow intake and the peak comes around 30–50 min (varying with the forms of the drug), the ip infusion in rats has to be carried out by controlling the infusion rate. In doing so, the overall dose was controlled and compared with the normal human dosage, 16–20 mg/kg of body weight (BW) (1000 mg/50–60 kg). Figure 4 shows the kinetic curves of plasma acetaminophen at different infusion rates and doses. Quick injection results in the early appearance of the plasma peak (10–20 min) and higher peak concentration (curves A and B) while slow infusion leads to a broader peak but lower peak concentration (curves C and D). Considering that the use of the analgesic for humans is oral and the peak time is longer than 20 min, an infusion time of 15–20 min is appropriate to produce a reasonable kinetic profile. The total dose shown in curves A–C are all well above normal dosage. Curve D approximates the normal level, and its peak concentration is near 0.13 mM.

It is evident from the above discussion that the described composite Nafion membrane imposes two response characteristics on the sensor. First it reduces the static (steady-state) response to acetaminophen. This means that acetaminophen can still pass through the membrane, but the resulting flux at the electrode surface is significantly reduced. Second, the time required to reach the steady state is greatly increased. If this time is long with respect to the transient pharmacokinetic

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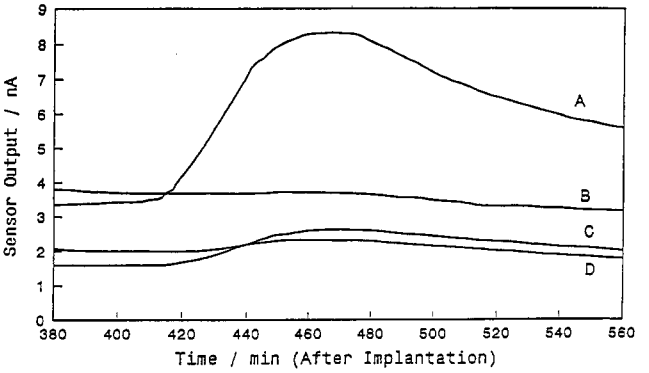

Figure 5. In vivo acetaminophen response on sensors of different in vitro sensitivity. In vitro sensitivity to acetaminophen: (A) 120 nA/mM; (B) 11 nA/mM; (C) 38 nA/mM; (D) 15 nA/mM. In vitro response time to 0.2 mM acetaminophen: (A) 3 min; (B) 22 min; (C) 5 min; (D) 18 min.

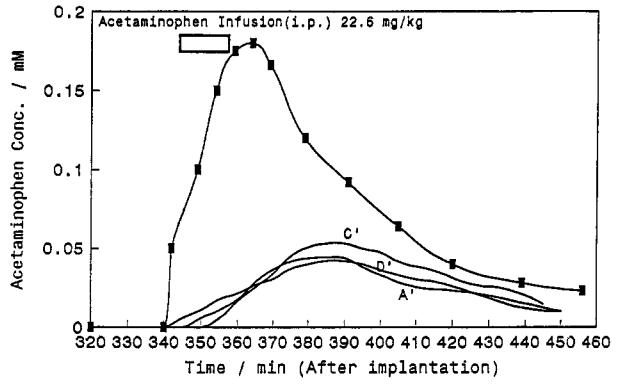

Figure 6. Acetaminophen pharmacokinetics in rat plasma and tissue. Tissue curves are calculated for sensor output based on their in vitro sensitivities. Curves A′, C′, and D′ correspond to sensors A, C, and D in Figure 5.

variation of acetaminophen, then it is possible that the resulting *dynamic* response of the sensor would reflect negligible levels of acetaminophen. This is the result of low steady-state sensitivity to acetaminophen and slow dynamic response.

To test the above concept, a series of four sensors were prepared. The sensors contain no glucose oxidase: the enzyme layer is replaced with bovine serum albumin (BSA). The influence of acetaminophen pharmacokinetics is shown in Figure 5. Sensor A has an in vitro sensitivity of 120 nA/mM, and sensors B, C, and D 11, 38, and 15 nA/mM, respectively. The in vitro response times (time required to reach 95% of steady-state response) are respectively 3, 22, 3, and 18 min upon addition of 0.2 mM acetaminophen. Sensors A and C contain no Nafion membrane where B and D are constructed with inner composite membrane of CA/Nafion. As noted in Figure 5, the non-Nafion sensors having relatively high in vitro sensitivity to acetaminophen also show a subcutaneous tissue response as well. Sensor B shows no response at all whereas sensor D shows a slight response. Although the static (in vitro) measurements are important in predicting sensor behavior in vivo, they can give an overly pessimistic picture.

A notable lag time between the plasma and tissue peak times is observed for all the experiments (Figure 6). Typically 20–30 min is very common, and the tissue peak always occurs around the beginning of the elimination phase corresponding to a plasma concentration that is much lower than its peak value. This is consistent with normal two-compartment

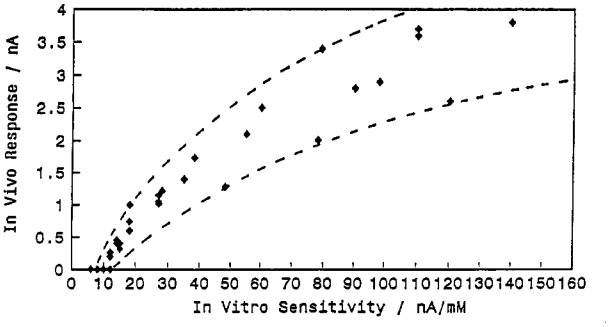

Figure 7. Correlation between sensor in vitro sensitivity and in vivo peak current due to acetaminophen.

pharmacokinetics, which is significantly different from that of glucose administration. The lag time for a direct quick injection (ip) of glucose is between 5 and 15 min, depending on the dose administered, the age of the animal, and the state of anesthesia. For slower glucose infusion, the lag time is even much shorter and is often negligible. This long lag time for acetaminophen suggests that the peak concentration in subcutaneous tissue is lower than that in plasma because the corresponding plasma concentration has dropped to 50–60% of its peak value when the tissue concentration reaches the peak. Presumably the tissue concentration should not be substantially higher than the plasma value at equilibrium (the volume of distribution for acetaminophen is 1 L/kg in humans[15]).

It is possible to predict the dynamic response from the in vitro sensitivity. Large numbers of glucose sensors fabricated with varied sensitivities to acetaminophen were tested in rats. The results are shown in Figure 7. The data shown are the correlation between the actual sensor in vivo bias (peak current in sensor output) caused by infusion (ip) of a therapeutic dose of acetaminophen and the corresponding sensitivity to acetaminophen in vitro. The data scattering is believed to be mainly due to the variation in dosage and rat body weight as well as individual metabolism. It is, however, clear that the maximum in vivo acetaminophen interference can be predicted from the sensor's in vitro characteristics. In this study sensors that have in vitro acetaminophen sensitivity of 10 nA/mM or less all show minimal response. The correlation between in vivo response and in vitro sensitivity in Figure 7 curves off in the low-sensitivity range and passes through the zero interference level at 10 nA/mM sensitivity. This is because the sensitivity decrease is always accompanied by an increase in response time. For a sensor with acetaminophen sensitivity of 40 nA/mM, for example, the response time is less than 5 min. But for a sensor of 10 nA/mM response to acetaminophen, the response time can be as long as 25–30 min. The effectiveness of this process is aided by the fact that the change in acetaminophen concentration is only momentary. This is illustrated in Figure 8 in which a normal glucose sensor C(a) (CA inner membrane) and an improved sensor C(c) (CA/Nafion inner membrane) are implanted in a rat along with an acetaminophen sensor 8C(b). Figure 8A,B shows the time-dependence plasma concentrations of acetaminophen and glucose during the experiment. The sensors with CA inner

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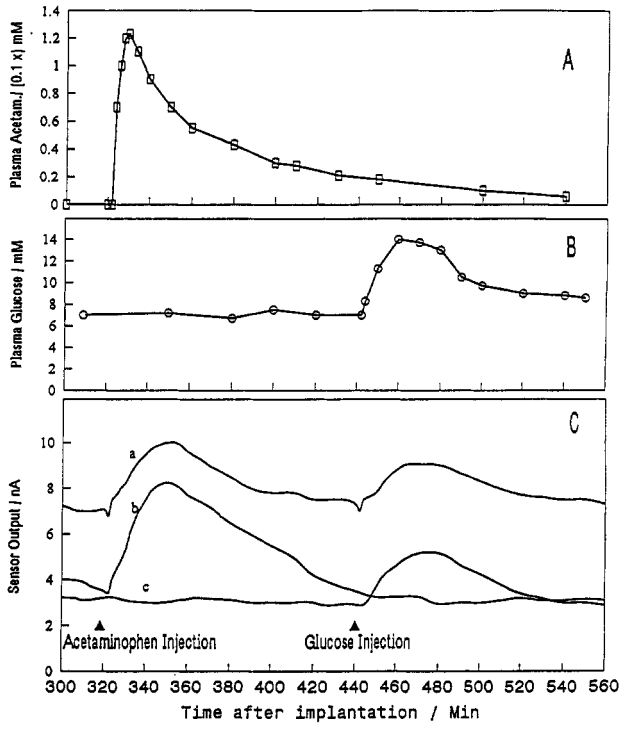

Figure 8. In vivo performance of glucose sensors of different inner membranes: (A) plasma acetaminophen; (B) plasma glucose. Curves in Figure 8C: (a) CA membrane glucose sensor; (b) CA membrane acetaminophen sensor; (c) Nafion/CA membrane glucose sensor.

membranes (Figure 8C(a) and (b)) show serious interference from acetaminophen while the sensor with the CA/Nafion membrane (Figure 8C(c)) is interference free and also functions more reliably for glucose monitoring. The CA/Nafion membrane shows another significant improvement over the CA membrane alone. The in vivo background current has decreased from 3-6 to 0.5–1.5 nA in rat subcutaneous tissue, which means endogenous electroactive species other than acetaminophen are also excluded. This improvement increases the signal/background ratio and therefore makes the in vivo calibration more accurate.

In conclusion we have established a new approach for sensor inner membrane fabrication and have effectively eliminated the interference from acetaminophen, one of the most serious electrochemical interferences for implantable biosensors. The correlation between the in vitro response to acetaminophen and the in vivo bias level was also established. The mechanism of this passive membrane strategy involves reducing the diffusivity of acetaminophen in the inner membrane and increasing the response time. Establishing the tissue pharmacokinetics at the therapeutic dose is also important for evaluating the potential interferences at a practical level. This result provides a useful tool for future sensor evaluation. The improvement in sensor performance is also reflected in its in vivo background decrease, indicating that the sensor's specificity is also improved. We have recently demonstrated in humans that ingestion of acetaminophen has no effect on in vivo sensor response.[16]

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ACKNOWLEDGMENT

This work was supported in part by the National Institutes of Health, Grant DK30718, and the Center for Bioanalytical Research, University of Kansas.

Received for review July 21, 1993. Accepted January 10, 1994.•

• Abstract published in *Advance ACS Abstracts,* February 15, 1994.