

## A RECEPTOR-BASED CAPACITIVE BIOSENSOR

The receptor-based capacitive biosensor combines the most recent advances in microelectronic technology with molecular biology and receptor biochemistry. Receptor-based biosensors have the potential for generic detection of chemical agents and toxins on the battlefield and are the best countermeasure to the threat of biotechnologically engineered agents. In addition, important spin-offs will be realized in medical diagnostics, controlled drug administration, environmental monitoring, and industrial process control.

### INTRODUCTION

The production and use of chemical agents in armed conflicts and their potential for use in low-intensity conflicts pose a threat to the security of U.S. forces. The threat is compounded by recent advances in biotechnology, which have blurred the distinction between traditional chemical agents and biological organisms and toxins. Toxins currently used as exotic neurobiological probes in laboratory research may soon be produced in large quantities by advanced fermentation methods and engineered to have precise physicochemical and pharmacologic properties that would make them useful to a military adversary.

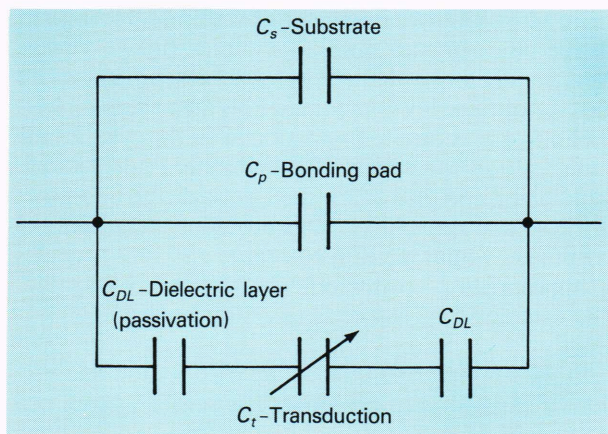
The detection of chemicals and toxins in combat, in the environment, or in body fluids has required analytical procedures that, although sensitive, are time-consuming and expensive. Further, these procedures require some knowledge of the identity of the species to be detected. Clearly, a new detection strategy is desirable. The lethal or incapacitating effects of many chemicals and toxins, the pharmacologic effects of drugs, and the action of viruses result from molecular recognition of these agents by physiological targets known generically as receptors. Receptors are macromolecular binding proteins that are vital for cellular function and can recognize and respond to agents at extremely low concentrations. Threat potential and pharmacologic potency are direct correlates of receptor affinity, making receptors, by nature, extremely efficient detectors. Receptor-based assays are used routinely in the laboratory, but their use as detectors has only lately been proposed.

Recent advances in microelectronics have resulted in the development of sensitive, inexpensive microsensors that operate in real time; however, they also must be made for specific applications, and they lack the recognition capability required to detect and identify physiologically significant compounds in a multicomponent mixture. Several types of microsensors have been designed, including capacitive sensors, fiber-optic waveguides, chemical field-effect transistors, and piezoelectric crystals. All rely on transduction of either an optical signal based on fluorescence or bioluminescence,<sup>1</sup> an electronic signal resulting from a change in the sensor's die-

lectric properties or electrical field,<sup>2</sup> or a chemomechanical signal resulting from alteration of a protein's mechanical properties during binding.<sup>3</sup>

Biosensors are devices that combine biological materials with electronic microsensors, a merger that results in sensitivity to biological parameters. Biosensors have been designed to detect proteins, toxins, living cells, and specific chemical compounds, but their applications are limited because selectivity and sensitivity depend on molecular recognition, which is often inadequate. Flexibility can be introduced by the addition of receptors, biological molecules capable of specific molecular recognition. The biological recognition site permits a receptor-based biosensor to detect a compound within a complex mixture of ions and chemicals in air, water, or multicomponent liquids.<sup>4</sup> This is directly analogous to the operations of antibodies, enzymes, and receptors as they exist in situ, awash in a complex environment of ions, hormones, transmitters, and other biological materials, yet responding only to their appropriate ligands. For example, a biosensor that used an antibody as its recognition element would be expected to detect the antigen or hapten (the part of an antigen that can be recognized by an antibody but that is not antigenic per se) to which that antibody was raised; a biosensor that used the acetylcholine (ACh) receptor (AChR) would be considerably broader in its response, signaling the presence of any of the large number of toxins and ligands that bind to it. Immobilization of an antibody or enzyme on a sensor generally would confer its specificity on the sensor; conversely, a receptor with a more generic recognition capability would confer like qualities on the biosensor.

One promising microsensor development is the capacitive sensor, which has been described for measuring humidity,<sup>5</sup> gases,<sup>6</sup> and the extent of polymeric cure.<sup>7</sup> An equivalent circuit model of the capacitive sensor was developed by partitioning the total capacitance into component capacitances according to dielectric composition, as shown in Fig. 1. Significant for the development of capacitive biosensors are the variable capacitances caused by the biochemical layer and the aqueous film. The expected change in biosensor capacitance can be computed



**Figure 1**—A simplified equivalent circuit model of the capacitive sensor.

by a capacitance cell model in which the dielectric constant of the biochemical layer is varied. For example, when antibody protein binds to the appropriate hapten immobilized on the surface of the biosensor, water is displaced, causing a change in the dielectric properties of the biochemical layer. These binding reactions are in dynamic equilibrium; therefore, free hapten, which is being analyzed or measured (i.e., the analyte), will cause displacement of the surface-bound antibody in relation to the analyte concentration.

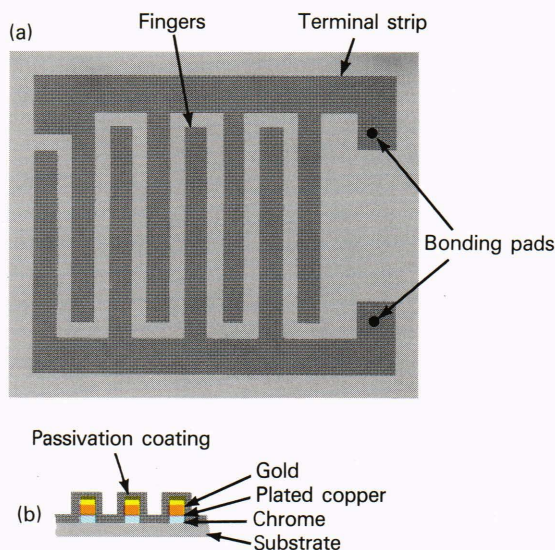
The focus of our research is to couple biological recognition sites (receptors) with a capacitive microsensor. The resulting biosensor would combine the sensitive and selective recognition capabilities of receptors with the signal transduction and amplification characteristics of electronic microsensors. The specific question we address is whether the biosensor can detect receptor-specific ligands in real time and at physiologically relevant concentrations. Implicit in the question is whether this detection capability is correlated with the in-vivo potencies of the receptor ligands.

## METHODS

### Planar Capacitive Sensor

The planar capacitive sensor used in these studies, which was invented and patented by A. L. Newman,<sup>8</sup> has a substrate composed of silicon coated with 1  $\mu\text{m}$  of silicon dioxide ( $\text{SiO}_2$ ). The interdigitated capacitor was formed on the substrate by depositing a thin layer of aluminum etched to a photographically defined pattern. The metallization pattern consisted of 93 interdigitated fingers, which were 1.2 cm long, 1  $\mu\text{m}$  high, 50  $\mu\text{m}$  wide, and spaced about 50  $\mu\text{m}$  apart. The entire pattern was covered with a 150-nm-thick passivating layer consisting of plasma-deposited silicon oxynitride. A thin layer of sputtered  $\text{SiO}_2$  was then deposited to provide a surface for the final layer of 3-aminopropyl-triethoxy silane, the free amino group used for attachment to organic or biochemical groups through standard coupling chemistries.

Figure 2 shows the interdigitated and cross-sectional structure of the device. For antibody experiments, the hapten was immobilized on the surface and the antibody



**Figure 2**—Schematic (a) and cross-sectional (b) views of the planar capacitive sensor.

was bound to the hapten. For receptor experiments, two radically different strategies were used. In the first receptor configuration, a calmodulin “tether” was attached to the derivatized silane surface, and the receptor was then electrostatically coupled to the tether. This is analogous to the antibody/hapten configuration in that bulk displacement of the antibody or receptor occurs in the presence of a competing antigen or ligand. Thus a material with a low dielectric constant (e.g., protein) is replaced by a material with a high dielectric constant (e.g., water), causing a change in capacitance that can be measured if the surface is configured as a planar capacitor. In the second receptor configuration, a lipid bilayer impregnated with receptor was deposited on the capacitive sensor surface. The interaction of receptor with ligands alters the conformation of the receptor protein, and the movement of charges is detected by the sensor.

Initial work with antibody-modulated biosensors used the T-2 mycotoxin as the analyte. The silicon surface was silanized by reacting the surface silanol groups with gamma-aminopropyltriethoxysilane to introduce a gamma-amino group onto the surface. T-2 hemisuccinate was then synthesized by heating T-2 in the presence of pyridine and succinic anhydride, and the T-2 hemisuccinate was conjugated to the gamma-amino function in the presence of a water-soluble carbodiimide.

Typical experiments involved measuring the capacitance changes that occur when T-2-specific antibodies are added to a solution bathing the capacitor and when free T-2 is added. When 1  $\mu\text{g}/\text{ml}$  T-2-specific antibody was added, the capacitance, measured at 1 kHz with a General Radio 1657 LCR DigiBridge, dropped from 2180 to 2120 pF. When 1  $\mu\text{g}/\text{ml}$  T-2 was added, the capacitance returned to 2180 pF within 2 min.<sup>9</sup>

### Immobilization of the Protein

Calcium magnesium adenosine triphosphatase ( $\text{Ca}^{++}\text{Mg}^{++}\text{ATPase}$ ) was purified from commercially

available cow brain and pharmacologically characterized.<sup>10,11</sup> The kinetic data were consistent with it being a single synaptic membrane protein composed of the ATPase and either a diltiazem binding site or a change in conformation of the associated  $\text{Ca}^{++}$  channel following diltiazem administration.

$\text{Ca}^{++}\text{Mg}^{++}$ ATPase was coupled to gamma-aminopropyltriethoxysilane-derivatized,  $\text{SiO}_2$ -coated surfaces using succinylated calmodulin in the presence of carbodiimide as the linking molecule. The reaction was carried out for 2 h at 5°C, and the sensors were washed four times with 0.01 M Tris buffer containing 16 vol.% glycerol and 0.04 vol.% Triton X-100 to remove unreacted reagents. Adenosine triphosphate hydrolysis by the immobilized protein was then determined to ensure that it was still active. Thus the surface of the biosensor was used in an affinity-chromatographic fashion, resulting in noncovalent association of the ATPase with the surface-linked calmodulin molecule. This is an ideal situation because the calmodulin molecule (mol. wt. =  $16 \times 10^3$ ) is much smaller than the ATPase moiety (mol. wt. =  $140 \times 10^3$ ), such that removal of the ATPase portion results in significant changes in surface capacitance.

Toxin challenge to the sensor was carried out with purified Mojave toxin from a rattlesnake, *Crotalus scutulatus scutulatus*. The sensor was immersed in buffer in a flow chamber, and toxin was added to a final concentration of 50 nM. In the control situations, either 300  $\mu\text{g}$  calmodulin or buffer alone was added to the chamber. Capacitance changes were measured with the DigiBridge.

#### Immobilization of Nicotinic AChR

AChR was purified from the electric organ of a fish, *Torpedo nobiliana*. To form the interface of the receptor with the capacitive sensor, a special AChR preparation was made. A mixture of 1-alpha-phosphatidylcholine and cholesterol in chloroform (75:25), stored under nitrogen, was evaporated to dryness to form a thin film on the inside of a round-bottom flask. Cholate buffer was added such that the final lipid concentration was 25 mg/ml, and the contents of the flask were sonicated to clarity. The lipids were dialyzed, and the final product was designated "liposome preparation" and stored at -80°C.

The general method for forming planar lipid bilayers on a small aperture (e.g., Ref. 12) is as follows. Flat sheets of planar bilayer form on a flat surface of the capacitive sensor from monolayers in the same way they form on glass. The bilayer consists of two monolayers formed in succession. Phospholipids (1 mM in *n*-hexane) are spread on the surface of a Ringer's buffer supplemented with 4 mM calcium chloride. The equilibrium surface pressure at the air/liquid interface drops from approximately  $72 \times 10^{-5}$  to below  $35 \times 10^{-5}$  N/cm when a monolayer of lipid forms on the aqueous surface. A capacitive sensor, already immersed in the aqueous buffer, is slowly withdrawn (2 cm/min) by means of hydraulic lift, causing the deposition of a monolayer of phospholipids on the sensor surface. The sensor is immersed in another trough containing phospholipid vesicles, forming the second monolayer. Since the vesicles in the second immersion are enriched in AChR protein,

the result is a bilayer impregnated with receptor layered on the interdigitated surface of the capacitive sensor. Phosphatidylserine and phosphatidylethanolamine are used to form the lower monolayer because the two phospholipids are known to predominate on the cytoplasmic (inner) side of the native cell membrane. Phosphatidylcholine and cholesterol are used to form the liposomes in the second monolayer. This procedure simulates the asymmetry found in cell membranes and thus provides an appropriate microenvironment for receptor function. The completed biosensor is stored in buffer until it is tested.

In the present experiments, we used a modified version of this two-step protocol. The sensor was dipped in 2% soybean azolectins in *n*-hexane for 1 min, air-dried for 1 min, and then lowered into phosphate-buffered saline (PBS) solution with a thin film of AChR-impregnated liposomes spread on the surface. The downstroke ended when the sensor cleared the liposome layer, and the direction was reversed at the same speed (0.5 cm/min).

Capacitive sensors varied in their basal capacitance response when placed into the buffered saline solutions, the major determinant being the composition of the sensor's surface coating. Sensors covered with a borophosphosilica glass (BPSG) were superior and more stable than sensors covered with 100- or 500-nm-thick  $\text{SiO}_2$  films, as shown in Table 1. Capacitive sensors were reusable for up to six experiments if washed in warm detergent solution, rinsed under running water, and dried in air.

## RESULTS

### $\text{Ca}^{++}\text{Mg}^{++}$ ATPase Biosensor

Approximately 50 to 55 ng of calmodulin, representing about 5% of the starting total, were immobilized to each sensor surface. These results are in good agreement with theoretical calculations of 25 to 75 ng, depending on whether the calmodulin orients itself in a "lying down" or "standing" configuration on the derivatized surface. Steric considerations are suggested, since the  $\text{Ca}^{++}\text{Mg}^{++}$ ATPase shows decreased ATPase activity as the amount of calmodulin is increased from 250 to 2500 ng.

Assay conditions and capacitance after toxin challenge are summarized in Table 2. A comparison of the sensors coated with receptors and challenged with either calmodulin or Mojave toxin shows a 34% increase in capacitance following addition of the toxin. Washing the sensors with buffer containing  $\text{Ca}^{++}\text{Mg}^{++}$ ATPase results in a return to baseline capacitance, and the sensors are responsive to further toxin challenge.

### AChR Biosensor

The AChR biosensor displayed pharmacologic specificity. When exposed separately to seven neurotransmitters, it responded with increased capacitance readings to ACh and did not respond to glutamate, gamma-aminobutyric acid (GABA), norepinephrine, histamine, 5-hydroxytryptamine, or dopamine (Fig. 3). The biosensor also differentiated between agonists (compounds that bind to a receptor and mimic the actions of the natural

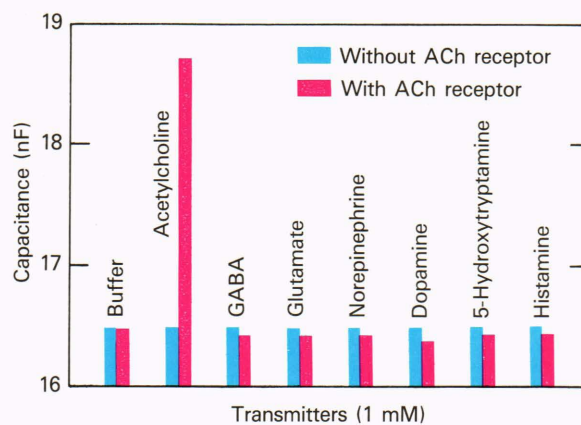
**Table 1**—Capacitance response of three types of sensors in different media.

Medium	Capacitance in Different Media (pF)		
	Type I 500-nm SiO <sub>2</sub> film	Type II 100-nm SiO <sub>2</sub>	Type III BPSG
Air	920	845	894
<i>n</i> -hexane	1,030	986	995
2% phospholipids in <i>n</i> -hexane	1,140	1,171	1,174
2% phospholipid liposomes (small unilamellar vesicles) in water	87,976	7,457	4,434
Water	4,300	7,590	4,122
Buffer (PBS)	> 100,000	11,207	4,480
10-mM ACh in PBS	> 100,000	14,188	9,731
10-mM <i>d</i> -TC in PBS	95,040	8,627	4,390
Lipid liposomes, 1- mg/ml Ca <sup>++</sup> -free Ringer's solution from 2% cholate	71,725	7,516	4,437
AChR-liposomes in 2% cholate ex- tract with 1-mg/ml lipids [azolectin + cholesterol (6:1)]	73,229	6,817	4,415

**Table 2**—Response of the Ca<sup>++</sup>Mg<sup>++</sup>ATPase capacitive sensors (all plates contained immobilized Ca<sup>++</sup>Mg<sup>++</sup>ATPase Ca<sup>++</sup> channel complex).

Assay Condition	Baseline Capacitance (pF)	Challenge Capacitance (pF)
No additions	28,382	—
Buffer + 50 μl Ca <sup>++</sup> (4 μM)	10,538	10,168
Buffer + 50 μl Ca <sup>++</sup> (4 μM) + calmodulin (300 μg)	5,821	5,692
Buffer + 50 μl Ca <sup>++</sup> (4 μM) + Mojave toxin (180 μg)	6,763	7,642

ligand for that receptor) and antagonists (compounds that bind to a receptor and block the action of the natural ligand for that receptor); agonists such as ACh and carbamylcholine increased capacitance, but antagonists such as *d*-tubocurarine (*d*-TC) and the alpha-neurotoxin of cobra (*Naja naja*) venom did not. Further, when the biosensor was pretreated with the reversible antagonist *d*-TC and then slowly perfused with ACh containing buffer, it recovered its response to ACh. This did not occur when the irreversible antagonist *Naja* toxin was

**Figure 3**—Response of the AChR biosensor to neurotransmitters.

used as the pretreatment, as shown in Fig. 4. The sensor's sensitivity to agonists was dose dependent, and detection limits were on the order of 1 nmol ACh/ml (1 μM ACh), as shown in Fig. 5. Storage in buffer for several hours after receptor immobilization did not reduce the sensitivity of the biosensor to ACh.

Control biosensors made of immobilized lipids only, without AChR protein, did not respond to any of the test drugs and were used to establish baseline capacitance measurements for each sensor. Biosensors with immobilized AChR and acetylcholinesterase displayed sensitivity to both receptor-active drugs and anticholinesterases. The organophosphate diisopropyl fluorophosphate (DFP) inhibited acetylcholinesterase irreversibly, and the high concentration of unhydrolyzed ACh stimulated the AChR and increased capacitance (Table 3).

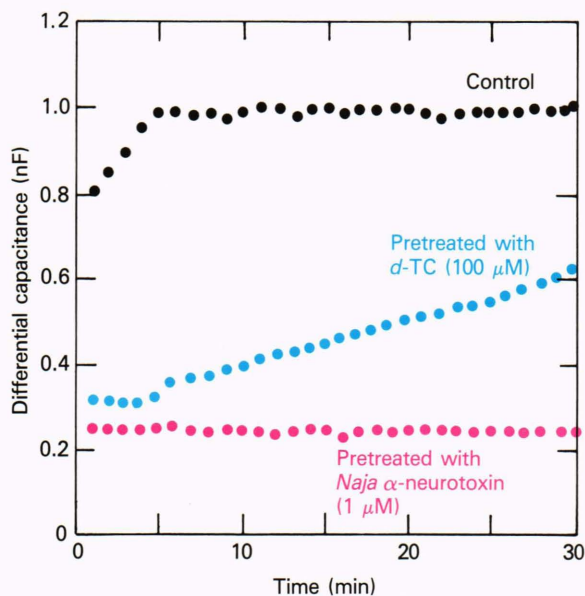
Finally, biosensors constructed from protein extracted from rat brain, which is multisynaptic (i.e., contains numerous receptor types), responded positively to all seven transmitter substances, as predicted.

## DISCUSSION

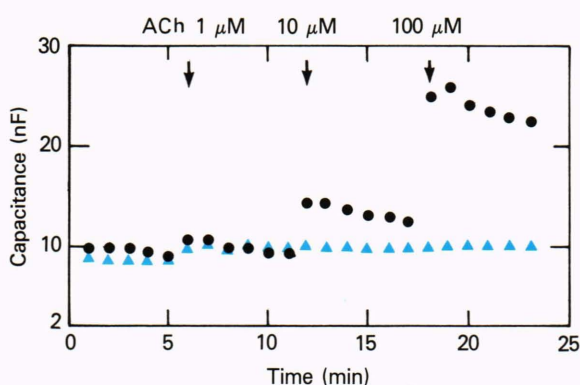
The data given here represent proof of the concept of a receptor-based capacitive biosensor. Significantly, a responsive system can be made using two qualitatively different receptor types and two different immobilization approaches. However, the actual events at the surface of the biosensor that lead to transduction of a signal from the receptor binding event remain speculative.

In the Ca<sup>++</sup> channel studies, two mutually exclusive hypotheses may be considered. First, the binding of the toxin to the receptor may displace the receptor from the calmodulin, either by direct competition or by inducing a conformational change in the receptor. Alternatively, the receptor may remain attached to the calmodulin and the conformational changes that follow binding may alter the configuration of charges at the surface. Resolution of this issue will require deposition studies using either radiolabeled or fluorescently tagged receptors.

Experiments using AChR embedded in a lipid bilayer suggest that the salient event is the opening of the channel caused by agonist binding and subsequent ion transloca-



**Figure 4**—Response of the AChR sensor to reversible (*d*-TC) and irreversible (*Naja* alpha-neurotoxin) antagonists.



**Figure 5**—Dose-dependent response of the AChR sensor to ACh (filled circles). Triangles = control.

**Table 3**—Response of Type II and Type III biosensors to ACh and *d*-TC in the presence and absence of DFP.

	Type II		Type III	
	–DFP	+DFP	–DFP	+DFP
PBS	20,153 (± 490)	20,404 (± 1503)	23,428 (± 1561)	24,573 (± 3)
PBS + 10-nM ACh	24,225 (± 290)	24,562 (± 440)	25,573 (± 3273)	30,242 (± 580)
ACh	4,102	4,158	2,145	5,669
PBS + 10-mM <i>d</i> -TC	18,268	19,015	17,041	—

tion. This is indicated by the ability of receptor antagonists to block ion channel opening, a situation that leads to problems in designing a biosensor that is responsive to both agonists and antagonists as well as compounds that

interact with the allosteric sites in the ion channel. (Allosteric sites are binding sites on a receptor, other than the “active site,” which binds the natural ligand. These binding sites may be associated with an ion channel coupled to the receptor and may modulate the binding of ligands to the active site.) Specifically, it will be necessary to design a system with some intrinsic level of receptor activity such that an agonist or antagonist will trigger a signal by, respectively, increasing or decreasing ion translocation.

While these studies support the concept of a receptor-based biosensor, they remain preliminary laboratory demonstrations with at least two major, albeit implicit, unresolved issues: receptors must be produced in quantities sufficient for practical applications and they must be immobilized and stabilized in an interfacial system compatible with microsensors.

Production of large (i.e., gram) quantities of purified receptor proteins requires cloning the genes that encode for the subunits and reassembling them into functional receptors in an expression vector that can be scaled up to production levels. (Expression vectors are cell lines in cultures, used to produce or express proteins for which a cloned gene codes.) Several such systems are promising candidates.<sup>13–16</sup> Alternatively, artificial receptors might be produced using macrocyclic compounds to which receptor-active site fragments are attached<sup>17</sup> or by raising antiidiotypic antibodies to the receptor.<sup>18</sup> (Antiidiotypic antibodies are antibodies raised against another antibody to mimic the antigenic site to which the original antibody was raised.) Such artificial technology sacrifices the ability of the native receptor to recognize classes of compounds, but may be useful in limited applications and may enhance biosensor stability. A discussion of immobilization (e.g., lipid–protein interactions, biomaterials, surface chemistry, biomedical polymers) is beyond the scope of this article; however, several excellent texts are available on the subject.<sup>19,20</sup>

If the foregoing technical issues can be resolved, receptor-based biosensors will be able to meet battlefield detection requirements, but military applications represent only a small fraction of their potential use. The development of flexible receptor- and enzyme-based biosensors will have profound implications for basic research, medicine, industry, and environmental health. Specific applications include microbioassays for mutagens;<sup>21</sup> chemical analyses for toxins, drugs, and chemicals in biological fluids;<sup>22</sup> process control and fermentation;<sup>23</sup> industrial monitoring of the workplace and of toxic waste dumps;<sup>24</sup> veterinary medicine; and toxicological screening.<sup>25</sup> Despite powerful economic incentives to develop biosensor technology, the lack of reliable biosensors for many industrial applications has precluded automation, resulting in increased costs.

The development of a biosensor that uses biological recognition sites to detect compounds of physiological significance represents a qualitative advance that could lead to true generic sensing. Long-term applications are limited only by the imagination. Ultimately, it will be possible to design intelligent drug-administration devices that use the biosensor to monitor serum levels of drugs and

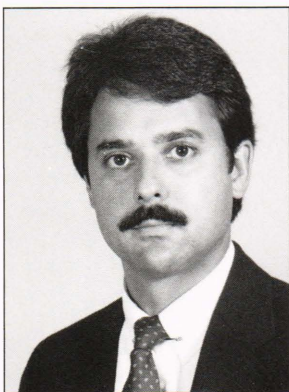
hormones and dispense drugs in appropriate amounts, and to build biosensors that detect carcinogenic markers while they are still in the precancerous state.

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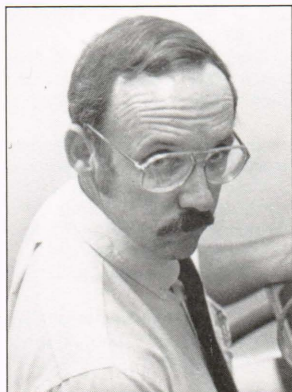
## THE AUTHORS



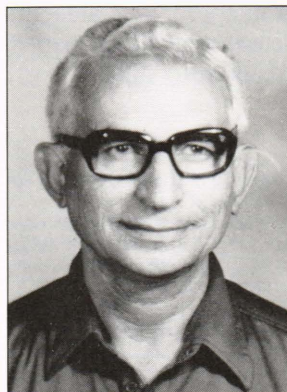
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