paired breeding, inflate state averages to abnormal levels (*11*).

Wintering mallard ducks occupy a relatively low trophic level compared to that of other avian species. In addition, seasonal migrations were under way at the time collections were made, and individuals in transit from less contaminated locations were undoubtedly included in our sample. Consequently, the presence of high concentrations of residue in the mallards underscores the seriousness of the contamination problem at Wheeler. The elevated mean DDTR concentration in muscle of wintering crows (Table 1) shows the potential for greater effects higher in the food web of the refuge. Piscivorous birds would be expected to suffer the most, and the depauperate state of this component of the local fauna is notable. According to refuge personnel (*3*), thousands of double-crested cormorants (*Phalacrocorax auritus*) used to winter at Wheeler but are now rarely seen; bald eagles (*Haliaeetus leucocephalus*) have not bred in the refuge since the late 1940's; and a mixed-species heron rookery (250 to 300 nests) showed a precipitous population decline in 1950–1951, and all reproductive activity there has since ceased (*12*). Several endangered species inhabit the refuge, including gray bats (*Myotis grisecens*), Indiana bats (*Myotis sodalis*), and transient bald eagles, but the impact of the DDT on these animals is not known.

In addition to the reproductive effects implied by the DDT residue data, direct avian mortality due to DDT poisoning also seems possible. Earthworms collected from beneath the litter of a bottomland hardwood forest 70 m northwest of the confluence of the drainage ditch and Huntsville Spring Branch had a dry weight DDTR concentration of 326 ppm (including 224 ppm DDT), which approaches or exceeds concentrations in earthworms that have been associated with lethality in wild populations of birds of the genus *Turdus* (*13*). The samples of earthworms collected in different habitats 0.1 and 0.3 km from the plant site contained DDTR at 74.2 and 5.2 ppm (dry weight), respectively. These concentrations do not imply lethality, but approach or exceed dietary levels that were shown to impair reproduction in experimental studies of several bird species (*14*).

To our knowledge, these data constitute the first documentation of gross pollution with industrial DDT in terrestrial and freshwater ecosystems. Past studies have reported extensive contamination of marine life off Southern California by DDT that was discharged directly into the ocean by a Los Angeles pesticide manufacturer (*15*). Very little was known about the effects of DDT on ecosystems at the time these disposal practices were initiated. Just as little is known about many chemicals in wastes currently being discharged into the environment. These examples demonstrate the environmental consequences of inadequate waste disposal practices. The persistence of harmful residues nearly a decade after the cessation of DDT manufacture near Wheeler emphasizes the need for safeguards and well-planned programs for disposing of all toxic chemical wastes.

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Calcium Regulation During Stimulus-Secretion Coupling: Continuous Measurement of Intracellular Calcium Activities

Abstract. *Accurate measurements of intracellular calcium activities in salivary gland epithelial cells of the insect* Phormia regina *were obtained with microelectrodes in which* N,N′-*di(11-ethoxycarbonyl)undecyl*-N,N′-*4,5-tetramethyl-3,6-dioxaoctane diacid diamide was incorporated in a liquid membrane system. When calibrated in solutions approximating the ionic concentration of the cell interior, these microelectrodes gave rapid stable responses that were linear functions of the logarithm of calcium activities and were not affected by potassium, sodium, and magnesium. Continuous monitoring of calcium activities during serotonin-induced saliva release provided direct evidence of hormonal influence on transmembrane calcium movement and spontaneous regulation of intracellular calcium by stimulated cells.*

Sensitive intracellular measurements of ionized Ca^{2+} are essential for understanding the mechanisms of Ca^{2+} regulation in cells and for the elucidation of cellular events such as contraction, excitation, secretion, and hormone action. The most successful procedures currently employed for measuring Ca^{2+} in biological systems make use of the photoluminescent protein aequorin and metallochrome absorbance indicators (*1*). Although these methods are extremely sensitive and can be used to measure the Ca^{2+} content of subcellular fractions as well as of intact cells, satisfactory calibration of the indicator signal over a wide range of Ca^{2+} concentrations is difficult because of changes in nonspecific absorption. This difficulty can be offset to some extent by using different

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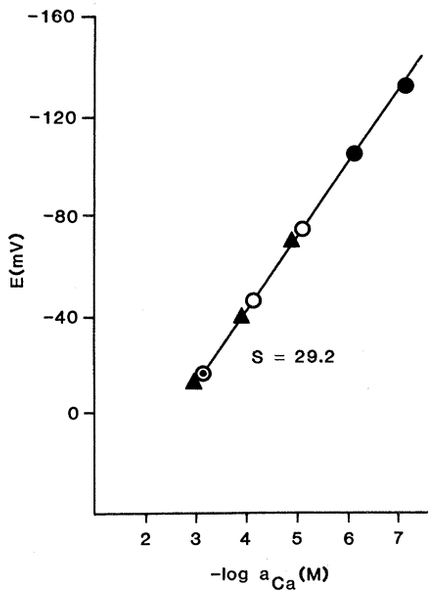

Fig. 1. Steady-state electrical potentials (with respect to a grounded reference electrode in the test solution) registered by a Ca^{2+}-selective liquid ion-exchanger microelectrode in (▲) pure $CaCl_2$ solutions; (○) saline solutions approximating the cytosol ($CaCl_2$ + 140 m*M* KCl + 10 m*M* NaCl + 2 m*M* $MgCl_2$); (●) similar saline solutions buffered with EDTA; and (⊙) Ringer solution. The line was fitted to the data by least-squares analysis.

indicators when Ca^{2+} concentrations vary widely. The use of Ca^{2+}-selective microelectrodes (*2*) offers the possibility of accurately measuring intracellular Ca^{2+} electrode potentials and of continuously monitoring this parameter during cellular activity. When the neutral lipophilic carrier molecule *N,N'*-di(11-ethoxycarbonyl)undecyl-*N,N'*,4,5-tetramethyl-3,6-dioxaoctane diacid diamide was incorporated in a liquid membrane system, outstanding Ca^{2+} selectivity was obtained (*3*). Our recent success in using a similar neutral carrier to develop a Na^+-selective ion-exchanger microelectrode that was essentially free from interference by K^+ (*4*) prompted us to use the above carrier as the calcium-selective component in a liquid ion-exchanger microelectrode.

For accurate measurement of intracellular Ca^{2+} activity (a^i_{Ca}), Ca^{2+}-selective microelectrodes must meet a number of criteria. These include (i) sufficiently small tip size to prevent undue cell damage; (ii) sensitivity to Ca^{2+} concentrations of the order of $10^{-7}M$ and a high degree of specificity in the presence of K^+, Na^+, or Mg^{2+}; and (iii) a response time rapid enough to follow biologically induced changes in Ca^{2+}.

We used the following procedure to construct Ca^{2+}-selective microelectrodes that satisfied the above conditions. A 10 percent (weight to volume) solution of *N,N'*-di(11-ethoxycarbonyl)undecyl-*N,N'*,4,5-tetramethyl-3,6-dioxaoctane diacid diamide in 3-nitro-*o*-xylene was used as the membrane phase in liquid ion-exchanger microelectrodes prepared by conventional methods (*5*, *6*). To obtain a system whose ion-exchange kinetics were compatible with a sufficiently fast microelectrode response, we incorporated the lipophilic anion dibromosalicylate [added as 1 percent (weight to volume) of calcium 3,5-dibromosalicylic acid] in this solution. The apparent response time of these electrodes in our experiments was of the order of 1 second. Parallel experiments in our laboratory with other kinds of liquid ion-exchanger microelectrodes indicate that the response time we measured is greatly affected by internal capacitances in the recording circuit. The intrinsic response time of the microelectrodes is probably much less than 1 second.

Leak conductance between the hydrophobic solution of ion-selective ligand and the inner surface of the glass micropipette can be reduced by making the glass hydrophobic. This was accomplished by silanizing the free hydroxyl groups (*6*, *7*), both those covalently bound to silicon and those bound by van der Waals forces to the glass surface.

Calcium microelectrode potentials (E_{Ca}) were recorded in 2×10^{-3}, 2×10^{-4}, and 2×10^{-5} $CaCl_2$ solutions containing 140 m*M* KCl, 10 m*M* NaCl, and 2 m*M* $MgCl_2$. Activity coefficients of Ca^{2+} in these solutions were calculated from the data of Butler (*8*). Concentrations of K^+ and Na^+ were chosen to approximate those reported for epithelial cells in *Calliphora* salivary glands (*9*). In addition, two solutions were used in which Ca^{2+} was buffered to a calculated *p*Ca of 6 and 7, respectively, with the calcium-disodium and disodium salts of EDTA. Potassium chloride was added to these solutions to bring them to virtually the same ionic strength (0.157) as the other calibrating solutions.

A representative calibration is shown in Fig. 1. It is apparent that the microelectrode response to Ca^{2+} was a linear function of log a_{Ca} between 10^{-3} and $10^{-7}M$. With 13 microelectrodes the average slope was 27.7 ± 0.9 [standard error of the mean (S.E.M.)] mV per decade change in a_{Ca} ($r > .995$ in every instance). This did not differ significantly ($P > .05$) from its theoretical value (29.4 mV). In addition, Fig. 1 includes the electrode potential in the Ringer solution used in the experiments described below. This solution contained 123 m*M* NaCl, 25 m*M* KCl, 2 m*M* $MgCl_2$, 2 m*M* $CaCl_2$, and 10 m*M* glucose (*10*). Despite

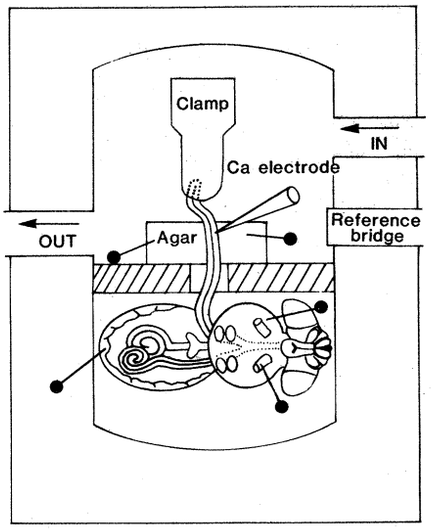

Fig. 2. Plexiglas perfusion chamber used in these experiments. The chamber was lined with black silicone rubber and was separated into two compartments by a wall. The fly was dissected in one compartment. The free end of the gland was drawn through a narrow channel in the wall into the other (perfusion) compartment and was held in place over a block of agar-Ringer gel by a fine capillary clamp. The perfusion solution flowed continuously through the compartment. The reference electrode was an agar bridge in contact with the serosal fluid and connected to ground through a calomel half-cell.

the difference in composition, the electrode potential in this solution corresponded exactly to that registered in the calibrating solution containing the same amount of Ca^{2+} and having the same ionic strength.

Because Mg^{2+} complexes strongly with EDTA, we did not add Mg^{2+} to calibrating solutions buffered to *p*Ca 6 or 7, but assessed possible effects of Mg^{2+}on E_{Ca} at these levels indirectly. We measured E_{Ca} in solutions containing 2×10^{-3}, 2×10^{-4}, and $2 \times 10^{-5}M$ $CaCl_2$, either alone or together with 100 m*M* $MgCl_2$. When the difference in mean activity coefficients was taken into account, E_{Ca} in pure $CaCl_2$ solutions fell on the regression line calculated for mixtures containing K^+, Na^+, and Mg^{2+} (Fig. 1), an indication that, aside from their effect on ionic strength, these ions did not affect E_{Ca}. When solutions containing $CaCl_2$ plus 100 m*M* $MgCl_2$ were used, the lines relating E_{Ca} to the logarithm of the Ca^{2+} concentration were parallel to those for pure $CaCl_2$, but E_{Ca} was 5 to 7 mV more negative. We were unable to find precise values for Ca^{2+} activity coefficients under these conditions, but obtained an approximate value of 0.5 from Butler's analysis (*8*). Using this value and the mean value for the slopes obtained with our microelectrodes (27.7

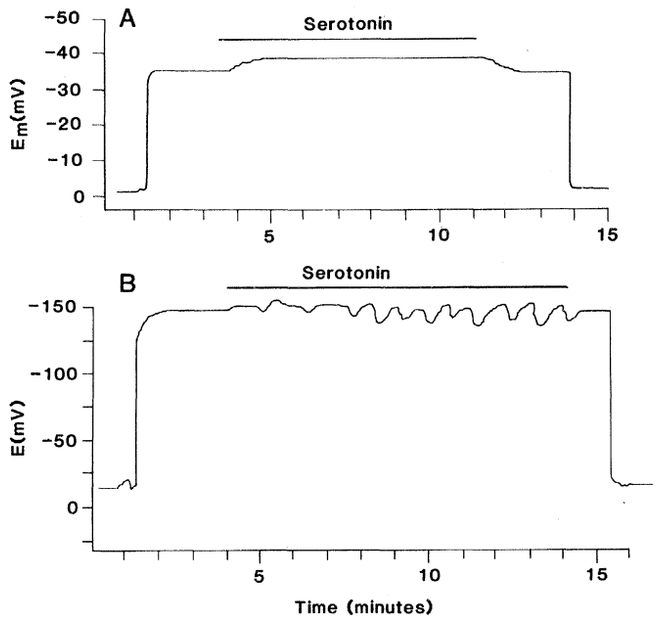

Fig. 3. (A) Serosal impalement of a salivary epithelial cell with an open-tip microelectrode. Serotonin ($10^{-8}M$) was added to the serosal medium during the period indicated by the bar. The stepped increments and decrements in E_m during addition and withdrawal of serotonin are artifacts due to the recorder used to obtain this tracing. (B) A similar impalement of an epithelial cell with a Ca^{2+}-selective microelectrode.

mV), and assuming that $MgCl_2$ acts only to increase the ionic strength of the calibrating solution, we calculated that incorporation of $MgCl_2$ in solutions containing Ca^{2+} ions in the concentration range 10^{-3} to $10^{-5}M$ should change E_{Ca} by -7 to -8 mV. Thus, these experiments did not uncover any specific effect of Mg^{2+} on E_{Ca}. We therefore consider it unlikely that Mg^{2+} would interfere significantly with the response of these microelectrodes to intracellular Ca^{2+}.

The salivary glands of the blowfly, *Phormia regina*, are paired simple tubular glands that extend the length of the fly. We used the abdominal portion, which is the secretory region and is approximately 1 cm long. It is composed of a homogeneous population of epithelial cells 25 to 30 μm in diameter surrounding a central lumen with a diameter of 30 to 40 μm. The serosal surface is essentially free from muscle, nerve, vascular, and connective tissue and is covered by a simple basement lamina. This facilitated isolation of the gland and cell impalement. Studies with a related species, *Calliphora* (*11*), showed that serotonin ($10^{-8}M$) increases the rate of fluid secretion in these glands. These studies provide indirect evidence that calcium may be important in initiating this secretory process. We therefore chose the salivary gland of *P. regina* as a model to evaluate our microelectrodes. Since the effectiveness of the electrodes depends on their ability to monitor experimentally induced changes in Ca^{2+} levels, we decided to study the changes that occur in intracellular Ca^{2+} levels when the serosal surface of these glands is stimulated with serotonin.

Glands were mounted at 23°C under open-circuit conditions in a specially designed perfusion chamber (Fig. 2) and perfused with oxygenated Ringer solution (*10*) at *p*H 7.1. Cells were impaled through their serosal surface with Ca^{2+}-selective and open-tip microelectrodes. The perfusion system, including the preparation of open-tip microelectrodes, and recording methods, and so forth, are described in (*5*). Glass micropipettes for open-tip and Ca^{2+}-selective microelectrodes were pulled under identical conditions.

Calcium-selective microelectrodes were calibrated in $CaCl_2$ solutions containing 140 mM KCl, 10 mM NaCl, and 2 mM $MgCl_2$ before and after each experiment. If the slope of the plot of E_{Ca} versus log a_{Ca} (S_{Ca}) changed by more than ± 1 mV between calibrations, or if the microelectrode potential in the serosal medium changed by more than ± 2 mV during an experiment, the results were discarded. The equation $a^{i}_{Ca}/a^{o}_{Ca} = 2.303 \exp[(\Delta E - \bar{E}_m)/S_{Ca}]$ was used to calculate a^{i}_{Ca} from 17 recordings in tissues from seven different insects. Here a^{i}_{Ca} is the intracellular calcium activity and a^{o}_{Ca} is the calcium activity of the external medium, ΔE is the difference between the steady-state electrode potentials in the cell interior and the serosal medium, S_{Ca} is obtained with the Ca^{2+} microelectrode used to measure each value of ΔE, and $\bar{E}_m$ is the average serosal membrane potential [-34.5 ± 1 (S.E.M.) mV], found in 26 impalements with tissues from ten different flies.

The average intracellular calcium activity in these experiments was (4.7 ± 0.6) $\times 10^{-7}M$ ($N = 17$; range, 1.1 to 9.7 $\times 10^{-7}M$). This value can be directly related to the ionic concentration, (8.7 ± 1.2) $\times 10^{-7}M$ Ca^{2+} in these experiments, because the calibration was performed with standards having the same ionic strength (*12*). These measurements are in fairly good agreement with values of intracellular calcium in different tissues obtained by other investigators using less direct methods (*1*).

The sensitivity of the electrodes to small changes in intracellular Ca^{2+} activity was evaluated by studying the Ca^{2+} potential of activated salivary glands. In *Calliphora*, activation of the salivary gland by $10^{-8}M$ serotonin is accompanied by an increase in net $^{45}Ca^{2+}$ efflux from the cells (*11*). We examined the effect of $10^{-8}M$ serotonin on E_m and E_{Ca}. In 19 experiments with open-tip microelectrodes, serotonin produced a rapid hyperpolarization (mean, 5.1 ± 0.4 mV) of E_m. This hyperpolarization continued until the serotonin was removed. One of these experiments is shown in Fig. 3A.

When the cells were impaled with a Ca^{2+}-selective electrode, there was a rapid negative deflection of E_{Ca}. This reached a steady state approximately 1 minute after impalement. Figure 3B illustrates one of nine experiments, with three different glands, in which cells were impaled with a Ca^{2+} electrode and then stimulated at the serosal surface with serotonin. In this experiment, E_{Ca} was -140 mV. This corresponds to a free Ca^{2+} concentration of $2.3 \times 10^{-7}M$. When the cells were exposed to serotonin, spontaneous oscillations in E_{Ca} occurred. These oscillatory deflections represent a depolarization of E_{Ca} followed by a spontaneous return to a hyperpolarized base potential. This can be inferred from the fact that the difference between the steady-state value of E_{Ca} before exposure to serotonin and the maximal negative value of E_{Ca} developed after exposure to this agent (mean, 5.0 ± 0.5 mV; $N = 9$) did not differ significantly ($P > .8$) from the hyperpolarization of E_m found under identical conditions. The amplitude of these oscillatory deflections in all experiments ranged from 5 to 16 mV, with a mean value of 9.8 ± 0.6 mV ($N = 56$ in nine experiments). The first few oscillations were always of a relatively low magnitude (5 to 7 mV). In the experiment illustrated, the deflections ranged from 5 to 16 mV with a mean value of 11.1 ± 1 mV ($N = 11$). This mean corresponds to a change in free intracellular Ca^{2+} concentration of $3.7 \times 10^{-7}M$ (2.3 to 6.0 $\times 10^{-7}M$). The maximum change was 6.7 $\times 10^{-7}M$ (2.3 to 9.0 $\times 10^{-7}M$). Because of

the limited frequency response of the recording system, the tracings we obtained may not quantitatively reflect the kinetics of the cellular response to serotonin, but these experiments provide direct evidence of an increase in the intracellular free Ca^{2+} concentration in response to serotonin. They also demonstrate the ability of the cells to buffer increases in free cytoplasmic Ca^{2+} that occur as the result of hormonal stimulation. Whether the cellular Ca^{2+} buffering system involved in the response illustrated by Fig. 3B is mitochondrial or extramitochondrial (*13*) remains to be established.

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Initiation of Sulfate Activation: A Variation in C_4 Photosynthesis Plants

Abstract. *In leaves of plants with C_4 photosynthesis, sulfur assimilation is initiated in bundle sheath cells whereas carbon and nitrogen assimilation are initiated in mesophyll cells. The activation of sulfate by adenosine triphosphate sulfurylase in leaves of C_4 plants occurs in chloroplasts of bundle sheath cells and is effected by two isozymes of approximately equal activities that accounted for 95 to 100 percent of the total leaf activity.*

The assimilatory sulfate reduction pathway in higher plants is thought to be localized within the chloroplast (*1*). However, two distinct types of chloroplasts are present within leaves of plants that assimilate carbon via the C_4 pathway of photosynthesis (*2*). Not only are these chloroplasts localized in two different C_4 leaf cell types, but each is characterized by its own complement of enzymes. The chloroplasts of the mesophyll cells lack ribulose-1,5-bisphosphate (RuBP) carboxylase (*2, 3*) while the chloroplasts of the bundle sheath cells lack nitrite reductase (*4*). Thus, in C_4 plants, the enzymes catalyzing early assimilatory steps of carbon and nitrogen metabolism are compartmentalized within specific cells and chloroplasts. We also have found that the initial enzyme in sulfur assimilation, adenosine triphosphate (ATP) sulfurylase, is compartmentalized. Approximately 95 percent of the activity can be localized in the bundle sheath cell chloroplast. In the 18 C_4 species examined, only trace amounts of ATP sulfurylase activity is in the mesophyll cell. Our findings indicate that in C_4 plants the first step in leaf sulfur assimilation proceeds largely in the bundle sheath chloroplast.

Table 1. Intercellular localization of ATP sulfurylase in the leaves of various plants. Mesophyll protoplasts and bundle sheath strands were obtained from 0.5- to 1.0-mm leaf slices of 2- to 4-week-old seedlings. Incubation was in 0.5*M* sorbitol, 1 m*M* $CaCl_2$. 2 percent (weight to volume) cellulysin, 0.3 percent (weight to volume) pectinase, 0.1 percent bovine serum albumin (weight to volume) and 5 m*M* MES (4-morpholineethane sulfuric acid), *p*H 5.25. In some species 0.25 percent Rohament P (Rohm, G.m.b.H., West Germany) was included. Incubation time at 30°C varied from 1 to 3 hours. Mesophyll protoplasts were harvested in a medium containing 0.5*M* sorbitol, 1 m*M* $CaCl_2$, and 5 m*M* Hepes, *p*H 7.0; they were purified by a method similar to that of method 2 of Edwards *et al.* (*9*). Protein extracts were made in 100 m*M* tris (*p*H 8.2), 2 m*M* β-mercaptoethanol, 1 m*M* EDTA, and 1 percent PVP-40 (polyvinylpyrrolidone). Activity of ATP sulfurylase was determined by a bioluminescence assay (*7*). N.D., not detectable.

Organism	Activities*		
	Whole leaf	Mesophyll protoplasts	Bundle sheath strands
	C_4 plants		
NADP⁺-malate enzyme type			
Bothriochloa caucasica	29.3	1.6	54.3
Cymbopogon martini	22.6	2.3	88.8
Digitaria sanguinalis	42.2	6.0	91.9
Echinochloa colonum	23.6	3.8	56.0
Echinochloa crus-galli	18.4	1.4	72.6
Euchlaena mexicana	24.1	2.4	64.4
Pennisetum americanum	79.1	0.7	183.8
Sorghum bicolor	22.4	1.9	93.8
Zea mays	5.3	0.4	16.9
NAD⁺-malate enzyme type			
Chloris distichophylla	26.6	N.D.	36.8
Eleucine indica	16.2	0.8	21.5
Panicum bergii	24.4	0.8	39.7
Panicum miliaceum	10.9	N.D.	15.5
PEP-carboxykinase type			
Brachiaria erucaeformis	20.0	3.2	64.3
Chloris gayana	28.7	1.1	53.2
Panicum maximum	35.5	2.5	37.8
Panicum molle	33.0	4.1	67.2
Urochloa mosambicensis	51.2	0.5	162.9
	C_3 plants		
Avena sativa	26.7	N.D.	N.D.
Triticum aestivum	41.9	N.D.	N.D.
	CAM† plant		
Kalanchoë daigremontiana	11.2	N.D.	N.D.

*Micromoles of ATP produced per milligram of chlorophyll protein per hour. †Crassulacean acid metabolism.

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